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12-Hydroperoxyeicosatetraenoic Acid Inhibits Main Platelet Functions by Activation of Soluble Guanylate Cyclase

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SUMMARY

12-Hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) as well as several other fatty acid hydroperoxides are potent inhibitors of platelet activation. 12-HPETE but not 12-hydroxy-5,8,10,14-eicosatetraenoic acid blocks the U46619- and the thrombin-triggered aggregation of aspirin-treated platelets, dose dependently. 12-HPETE suppresses thromboxane production by inhibiting platelet cyclooxygenase and stimulates its own production by increasing lipoxygenase activity, although this effect does not explain the inhibitory activity of 12-HPETE during the initial phase of cell activation. The inhibitory effect is related to altered calcium homeostasis during platelet activation. 12-HPETE inhibits calcium release from intracellular stores and modifies the influx of extracellular calcium. The inhibitory effect on calcium mobilization is explained by activation of soluble guanylate cyclase. These inhibitory properties are shared by sodium nitroprusside, a compound known to activate soluble guanylate cyclase. Fatty acid hydroperoxides, especially 12-HPETE, produce a rapid and dose-dependent activation of soluble guanylate cyclase, using intact human platelets as a detection system. Activation of the enzyme shows a position isomer specificity, with 12-HPETE being the most potent activator. The generation of the labile lipoxygenase product 12-HPETE during platelet activation may modulate platelet reactivity by increasing cyclic GMP. This pathway may contribute to a physiological feedback mechanism to limit the size of a growing platelet plug.

Platelet aggregation is under strict regulatory control, to ensure rapid vessel closure in case of an injury but otherwise not to disturb normal blood flow. Certain agents that are known to increase either cGMP or cAMP cause negative modulation of the aggregatory process (1–3). Physiologically, this is mediated by the two endothelium-derived regulators prostacyclin (PGI_2) (4) and endothelium-derived relaxation factor (5–8). Activation of the soluble form of guanylate cyclase is achieved by a variety of vasodilators, which have been collectively called "nitrogen oxide-containing vasodilators" or "nitrovasodilators," substances containing or releasing the free radical nitric oxide (9–11). Alternatively, soluble guanylate cyclase can also be activated by unsaturated fatty acids and fatty acid peroxides (12–14). Previous investigations (13, 14) examined the effect of fatty acid peroxides on soluble guanylate cyclase activity in the absence of reducing agents. Intact platelets, however, contain glutathione in millimolar concentrations (15), as well as glutathione peroxidase, and, therefore, hydroperoxides may be immediately reduced at the cellular level.

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The importance of decreasing platelet aggregability in patients with coronary artery diseases has been recognized, and in view also of their smooth muscle-relaxing properties the mechanism of cGMP-increasing agents has become of prime interest. It is known that cGMP-elevating substances and membrane-permeable analogs of cGMP block or at least reduce the initial cytosolic calcium increase caused by the different platelet activators (16–19). This effect of cyclic GMP on calcium mobilization also explains the observed inhibitory effect on the phospholipase A_2/arachidonic acid pathway (20). Cyclic GMP-elevating agents reduce the level of 1,2-diacylglycerol formation and subsequent protein kinase C-mediated protein phosphorylation (21, 22), which is consistent with inhibition of PI breakdown (PI response). cGMP also inhibits the activation-associated phosphorylation of myosin light chain (23) and causes phosphorylation of specific platelet proteins, with a 50-kDa protein being the most prominent, although its function remains unknown (24).

It has been reported that 12-HPETE inhibits platelet aggregation (25, 26), although the mechanism remains unknown. In this study we report on the effect of hydroperoxyeicosatetraenoic acids (namely 12-HPETE) on the activity of soluble gua-

ABBREVIATIONS: PGI_2, prostaglandin I_2; 12-HPETE, 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; TxB_2, thromboxane B_2; U46619, (15*S*)-hydroxy-11,9-[epoxymethano]prosta-5,13-dienoic acid; PGG_2, prostaglandin G_2; PGH_2, prostaglandin H_2; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PI, phosphatidylinositol; IP_3, inositol-1,4,5-trisphosphate; tBuBHQ, 2,5-di(*tert*-butyl)-1,4-benzohydroquinone; HHT, 10-hydroxyheptadecatrienoic acid.

nylate cyclase in intact human platelets. We present evidence that activation of soluble guanylate cyclase by fatty acid peroxides accounts for the inhibitory effects on different platelet functions. The sites of action of 12-HPETE on arachidonic acid metabolism and calcium movements will be defined.

Experimental Procedures

Materials

U46619 was purchased from Paesel (Frankfurt, FRG), thrombin was bought from Hoffmann-La Roche (Basel, Switzerland), and arachidonic acid was delivered by Larodan (Malmö, Sweden). Radiochemicals were obtained from Du Pont (Dreieich, FRG). Prostacyclin, sodium nitroprusside, BW 755C, dipyridamole, and aspirin were purchased from Sigma Chemie (Deisenhofen, FRG). All other materials were as previously described (27, 28) or were obtained in the highest grade of purity available from local commercial sources.

Methods

Preparation of platelet-rich plasma and washed human platelets. Preparation of platelet-rich plasma and suspensions of washed human platelets was mainly carried out as previously outlined (28). In order to inhibit platelet cyclooxygenase, platelet-rich plasma was incubated with 1 mM aspirin (dissolved in ethanol) for 30 min before the washing procedure was started. Platelet cyclooxygenase and lipoxygenase were inhibited by preincubation with 100 μM BW 755C for 5 min before each individual experiment was started. Aggregation studies were also performed according to the Ref. 28.

Calcium measurements. Calcium measurements in intact platelets were carried out as stated in Ref. 28. External calcium was adjusted by the addition of 1 mM $CaCl_2$ or by the addition of EGTA.

Calcium influx into platelets was measured using the manganese technique described by Sage *et al.* (29). This method has previously been employed to show that stimulation of receptor-mediated calcium entry will result in the concomitant stimulation of Mn^{2+} influx and is based on the observation that Mn^{2+} binds to fura-2 and quenches its fluorescence. The use of two excitation wavelengths, 335 nm and 362 nm, allows monitoring at a calcium-sensitive and a calcium-insensitive wavelength, respectively, simultaneously. The use of the excitation at 362 nm thus allows the selective study of Mn^{2+} entry without interference in the signal caused by changes in $[Ca^{2+}]_i$.

Arachidonic acid metabolism. Metabolism of exogenous [1-^{14}C] arachidonic acid (1.7–4.5 × 10^5 dpm/assay) was assayed using 3–5 × 10^5 platelets/μl. Incubations were stopped after 90 sec with chloroform/methanol and metabolites were extracted and spotted on silica gel 60 plates in order to separate the metabolites. Substances were localized by autoradiography and quantitated by liquid scintillation counting, as outlined in Ref. 28.

Determination of cyclic GMP. Washed human platelets (0.5 ml) were incubated with the different agents for the times indicated (normally 90 sec) and then the incubations were stopped by addition of 0.25 ml of 10% trichloroacetic acid. After centrifugation at 10,000 × g for 5 min, 100–400 μl of an ether extract were assayed as described by Ortmann (30). Hydroperoxyeicosatetraenoic acids and the cGMP phosphodiesterase inhibitor dipyridamole (100 μM) were normally preincubated for 2 min.

Hydroperoxyeicosatetraenoic acid and endoperoxide biosynthesis. Prostaglandin endoperoxides PGG_2 and PGH_2 were generated by incubation of arachidonic acid with sheep seminal vesicle microsomes, according to the procedure published by Hecker *et al.* (27). Fatty acid hydroperoxides were generated either by incubation of platelet cytosol or soybean lipoxidase with arachidonic acid, according to the methods in Ref. 27, or by photo-oxidation of arachidonic acid, according to the method of Porter *et al.* (31).

Statistical methods. Results are expressed as mean values ± standard deviation of a number of determinations in individual experiments from different blood donors. In the case of calcium entry measurements, only one typical trace of a minimum of five different experiments is shown. Results in all experiments presented were of similar magnitude to those shown, and variablity did not exceed 10% of the effect observed.

Results

Effect of 12-HPETE on human platelet aggregation. When 12-HPETE was preincubated with aspirin-treated washed human platelets, a dose-dependent inhibition of U46619-stimulated aggregation was observed (Fig. 1). From the sigmoidal dose-response curve, an IC_{50} of 2.1 ± 0.6 μM (mean ± standard deviation from four different experiments) was estimated. Use of several other platelet agonists also revealed the potent inhibitory activity of 12-HPETE on platelet activation.

Using aspirin-treated platelets and thrombin (0.1 unit/ml) as an agonist, qualitatively the same results were obtained, except that somewhat higher concentrations of 12-HPETE were required for inhibition (IC_{50} = 12 ± 4 μM, mean ± standard deviation from three different experiments). With higher concentrations of agonists, the dose-response curve shifted to the right, indicating that the inhibitory action of 12-HPETE counteracted the strength of the PI response elicited by the agonists.

The time of preincubation was also varied, and this showed that by 5 sec after 12-HPETE addition the inhibitory effect appeared. Because 12-HETE was completely ineffective in the same concentration range, it can be assumed that the oxidizing power of the hydroperoxide was essential for inhibition of platelet aggregation. Concerning the positional specificity of the various HPETEs, 5- and 15-HPETE were less efficient than the 12-HPETE isomer. Experiments showed about 50% inhibition of thrombin (0.1 unit/ml)-induced aggregation by 10 μM 12-HPETE, whereas 15-HPETE was inactive under the same conditions. It has already been reported that the 11-, 9-, and 8-HPETE isomers were also less active than 12-HPETE (25).

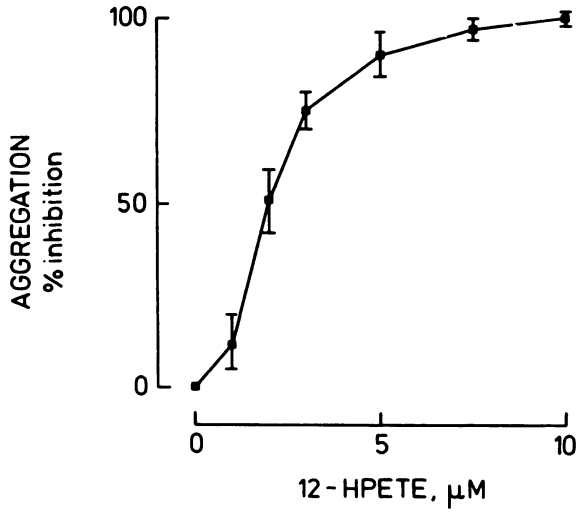

Fig. 1. Inhibition of the aggregation of aspirin-treated human platelets by 12-HPETE. Aspirin-treated washed human platelets were incubated for 2 min with the indicated concentrations of 12-HPETE before stimulation with 0.2 μM U46619. Inhibition was calculated compared with values for controls incubated with ethanol instead of hydroperoxide. Preparation of platelet suspensions and aggregation studies were carried out as described in Experimental Procedures.

Influence of 12-HPETE on platelet eicosanoid metabolism. All previous experiments were carried out in the presence of aspirin in order to eliminate any interference with an inhibition of thromboxane A_2 formation by 12-HPETE, as reported (25). Although an effect of 12-HPETE on the different eicosanoid pathways seemed unlikely under our conditions, it was worthwhile to confirm these findings with [^{14}C]arachidonate.

Because a time dependency of this interaction was not known, we preincubated 12-HPETE at 0, 10, 30, 60, and 120 sec and found a decline in cyclooxygenase pathway activity (TxB_2 and HHT) but a compensatory rise in 12-lipoxygenase activity (12-HETE), with also a slight increase in the steady state concentration of free arachidonate (Fig. 2). Interestingly, without a preincubation time (0 sec; simultaneous addition of labeled arachidonate and 12-HPETE), no marked effects on the eicosanoid metabolism were evident, although we observed complete inhibition of the aggregatory response. This confirms that a decrease of the cyclooxygenase pathway may partially contribute to the inhibition by 12-HPETE but that another major mechanism must be involved.

In order to definitely exclude the action of 12-HPETE on the cyclooxygenase/thromboxane pathway as the underlying inhibitory mechanism, we measured platelet metabolite formation using two different 12-HPETE concentrations and only one fixed preincubation time. For comparison, two other inhibitors of platelet aggregation, sodium nitroprusside and prostacyclin, were investigated in parallel (Table 1).

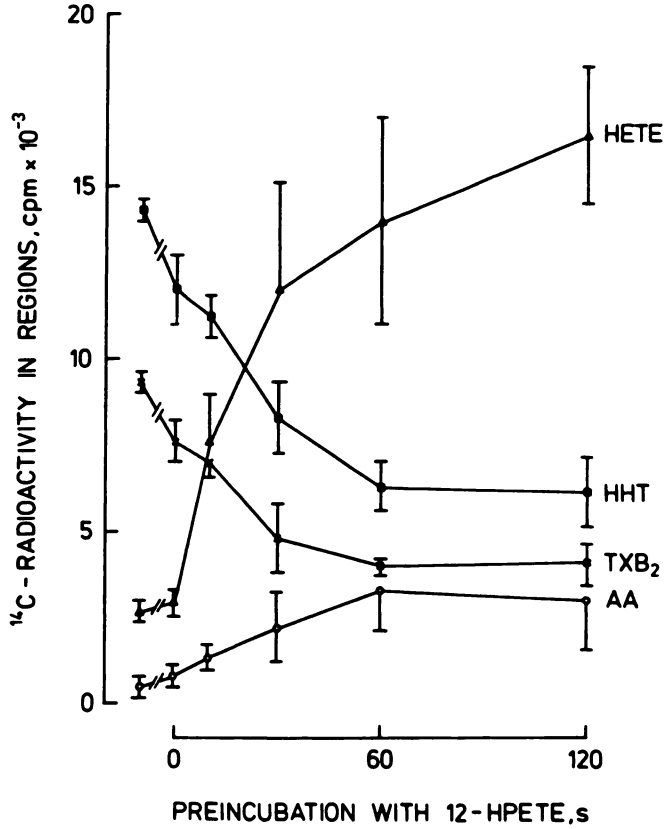

Fig. 2. Influence of the preincubation period with 12-HPETE on the metabolism of [1-^{14}C]arachidonic acid by platelets. Suspensions of washed human platelets were incubated without or, for different times (preincubation with 12-HPETE varied from 0 to 120 sec), with 10 μM 12-HPETE, before addition of labeled [1-^{14}C]arachidonic acid. Metabolites from three different experiments were extracted, separated, and counted as outlined in Experimental Procedures. Results are expressed as cpm corresponding to each individual metabolite (mean ± standard deviation). *AA*, free arachidonic acid.

It was found that neither prostacyclin, sodium nitroprusside nor 2.5 μM 12-HPETE inhibited the formation of TxB_2 significantly, although all three compounds prevent aggregation. Only at the higher concentration of 12-HPETE did the described effects on the cyclooxygenase and lipoxygenase pathway become obvious. Therefore, inhibition of platelet cyclooxygenase alone cannot explain the antiaggregatory effect of 12-HPETE. These data encouraged us to search for a different mechanism to explain the inhibitory effect of 12-HPETE.

Interference of 12-HPETE with platelet calcium movements. In order to gain further insight into the action of 12-HPETE, we investigated its effect on cytosolic calcium increase and calcium movements across the platelet cell membrane during the PI response caused by the different agonists. In aspirin-treated platelets, 12-HPETE, in a concentration-dependent fashion, inhibited the cytosolic calcium increase in fura-2-loaded thrombocytes, after stimulation with 0.2 μM U46619 (Fig. 3). The IC_{50} value under these conditions was 3.2 ± 0.7 μM (mean ± standard deviation, four experiments). These experiments were carried out in the presence of 100 μM BW 755C, in order to simultaneously block platelet cyclooxygenase and lipoxygenase, and with the addition of 1 mM EGTA to the incubation buffer. Therefore, the inhibition should represent a blocking effect on the calcium release from the dense tubular system, which comprises the IP_3-releasable calcium store in human platelets. However, in platelets incubated with 1 mM external calcium, 12-HPETE caused a similar dose-dependent inhibition of the cytosolic calcium increase. The IC_{50} for the arachidonic acid (2.5 μM)-induced cytosolic calcium increase was determined to be 2.6 ± 0.5 μM 12-HPETE (mean ± standard deviation, three experiments). By measurement of the inhibitory effect of 12-HPETE on the calcium increase in aspirin-treated platelets in the presence of 1 mM EGTA challenged with thrombin (0.08 unit/ml), the inhibitory constant was found to be 16 ± 5 μM (mean ± standard deviation, three experiments).

Cytosolic calcium increases are usually linked to platelet activation and are attributed to a calcium release from the dense tubular system, as well as to an extracellular calcium influx. In a first approach, we observed an inhibitory effect of 12-HPETE on the receptor-mediated calcium release from intracellular stores. We then measured the effect of 12-HPETE on the receptor-gated calcium influx by monitoring divalent cation entry into fura-2-loaded cells, in the presence of 500 μM extracellular Mn^{2+}. Fig. 4 shows the effect of 10 μM 12-HPETE and 10 μM sodium nitroprusside on the fluorescence-quenching properties of Mn^{2+} after the addition of 2.5 μM arachidonic acid (Fig. 4A) or 0.18 μM U46619 (Fig. 4B).

Monitoring of the fluorescence at the calcium-sensitive wavelength (335 nm) indicated a fluorescence increase after the addition of arachidonic acid or U46619, followed by subsequent fluorescence quenching due to Mn^{2+} influx. Sodium nitroprusside, a compound known to stimulate the soluble form of guanylate cyclase, reduced the transient cytosolic calcium increase in the same manner as 12-HPETE, after addition of one of the agonists. Examination of the fura-2 fluorescence at the calcium-insensitive but still Mn^{2+}-sensitive wavelength (362 nm) revealed that sodium nitroprusside and 12-HPETE dra-

TABLE 1

Effects of different platelet inhibitors on [^{14}C]arachidonic acid metabolism and aggregation of intact human platelets

Washed human platelets were incubated for 2 min with the indicated concentrations of 12-HPETE, prostacyclin (PGI_2), or sodium nitroprusside (SNP) before exogenous [1-^{14}C]arachidonic acid was added. Metabolites were quantitated as outlined in Experimental Procedures, and results are expressed as mean ± standard deviation of cpm in the region of each spot, from three individual experiments. Numbers in parentheses express the results as percentage values, compared with the controls, which were set as 100%. Aggregation was measured as detailed in Experimental Procedures.

Additions	Metabolite			Aggregation
	TXB_2	HHT	12-HETE	
Control	4899 ± 575	9361 ± 617	2315 ± 82	+
12-HPETE (2.5 μM)	4632 ± 996 (95)	6967 ± 543 (74)	2853 ± 39 (123)	–
12-HPETE (10 μM)	654 ± 102 (13)	1086 ± 169 (12)	9607 ± 685 (415)	–
PGI_2 (100 ng/ml)	4850 ± 250 (99)	7522 ± 518 (80)	1784 ± 19 (77)	–
SNP (10 μM)	4810 ± 605 (98)	7310 ± 705 (78)	2229 ± 280 (96)	–

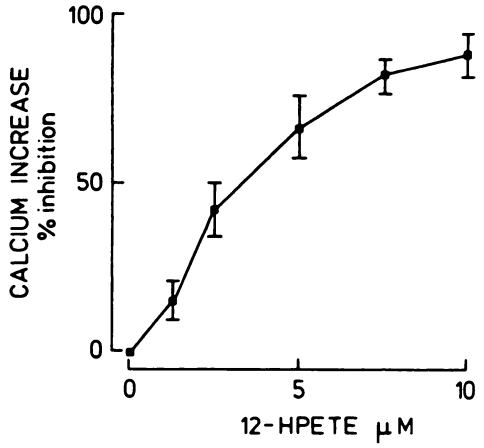

Fig. 3. Inhibition of a cytosolic calcium increase by 12-HPETE in fura-2-loaded platelets. Measurement and calculation of cytoslic free calcium was carried out as detailed in Experimental Procedures (mean ± standard deviation, four experiments). Inhibition was calculated compared with control values obtained in the absence of 12-HPETE. Experiments were carried out in the presence of 1 mM EGTA and 0.2 μM U46619 as an agonist.

matically altered the rate of fura-2 fluorescence quenching, compared with controls. The addition of 10 μM ionomycin showed no substantial further effect on the fluorescence quenching, and the addition of 2 mM external Ni^{2+} totally blocked the influx of Mn^{2+} (data not shown). Therefore, the opening of receptor-operated channels is inhibited by the action of sodium nitroprusside and 12-HPETE. We observed no differences in the potency of 12-HPETE concerning calcium influx and calcium mobilization from the intracellular stores. Thus, nitroprusside and 12-HPETE inhibit the arachidonic acid- and the U46619-mediated Ca^{2+} influx in a similar fashion.

Platelet cyclic nucleotide levels. Although several reports described an activation of soluble guanylate cyclase by fatty acid and fatty acid hydroperoxides, it is still not known whether activation of the enzyme also takes place in intact cells. Table 2 shows the effect of different lipoxygenase and cyclooxygenase metabolites on the cGMP level in intact human platelets. All additions were for 2 min.

In examination of the effect of the arachidonic acid metabolites at two different concentrations, it became obvious that 12-HPETE was the most efficient stimulus for platelet guanylate cyclase. It was also seen that 15-HPETE was more active than 5-HPETE. Both cyclooxygenase metabolites (PGG_2 and PGH_2) were nearly inactive. These results clearly indicate that activation of soluble guanylate cyclase may occur under physiological conditions, using intact cells instead of crude or purified enzyme preparations.

Fig. 5 illustrates that activation of soluble guanylate cyclase is due to the hydroperoxy- and not the hydroxy-fatty acid. Addition of 12-HETE to intact human platelets did not result in activation of soluble guanylate cyclase. In contrast to the hydroxy-fatty acid, the corresponding hydroperoxy-fatty acid (12-HPETE) produced a dose-dependent activation of the enzyme. Half-maximal stimulation was seen with concentrations around 5 μM. At concentrations above 20 μM 12-HPETE, no significant further activation was evident. Because the intracellular level of cGMP is controlled not only by activation of soluble guanylate cyclase, one also has to take the action of cGMP-degrading enzymes into consideration. In order to evaluate the contribution of cGMP-specific phosphodiesterases to the increase of cGMP induced by 12-HPETE, we performed the radioimmunoassay in the presence of 100 μM dipyridamole, a cGMP phosphodiesterase inhibitor (Fig. 6). Dipyridamole is considered cGMP specific, because destruction of cGMP is inhibited more than 30 times better than that of cAMP.

In the presence of dipyridamole, the elevation of the cGMP level was nearly linear with respect to the concentration of 12-HPETE. It was also obvious that, compared with the assay without the cGMP phosphodiesterase inhibitor, 12-HPETE produced a significantly higher degree of cGMP accumulation. At a concentration of 10 μM 12-HPETE with dipyridamole, we measured roughly twice the amount of cGMP, compared with the effect of 12-HPETE alone. After addition of 100 μM dipyridamole alone for 2 min, the cGMP level was already elevated 2.4-fold. Therefore, the 100% value in Fig. 6 represents an elevated cGMP level, compared with the 100% value in Fig. 5.

When we were carrying out aggregation studies, we noticed that 12-HPETE inhibited the aggregatory response quite quickly, without any significant time delay. Because of these results, we performed the time course experiment described in Fig. 7. After addition of 20 μM 15-HPETE to washed platelets, it was seen that the increase in the cGMP level reached the maximum after only 30 sec of incubation. Additional experiments using 12-HPETE showed a significant cGMP increase after only 5 sec. Another time course experiment (Fig. 8) describes the relationship between aggregation of human platelets challenged with 1 unit/ml thrombin and the activation of soluble guanylate cyclase.

Thrombin-induced aggregation was accompanied by a progressive elevation in the level of cGMP. The increase in cGMP was proportional to the concentration of thrombin (data not shown). Interestingly, the cGMP level slowly increased for 3

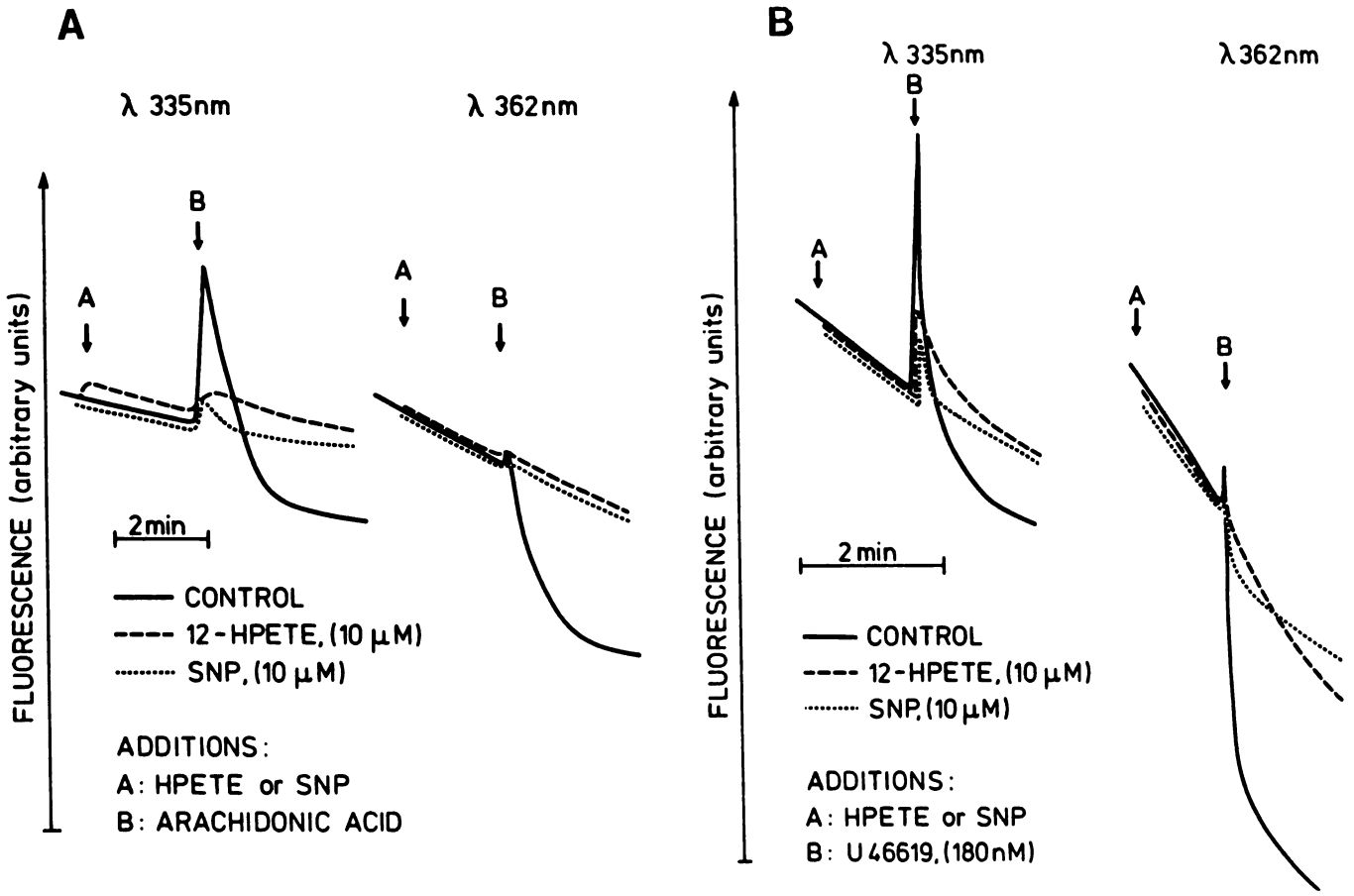

Fig. 4. Modulation of calcium movements by 12-HPETE and sodium nitroprusside. A, Platelets stimulated with 2.5 μM arachidonic acid. B, Platelets stimulated with 0.18 μM U46619. The fluorescence emission (≥450 nm) of fura-2-loaded platelets was recorded simultaneously at the two excitation wavelengths, 335 nm and 362 nm, in the presence of 500 μM manganese chloride, as described in Experimental Procedures. At the indicated time points (*arrows*), 12-HPETE or sodium nitroprusside (*SNP*) (*point A*) or the individual agonist (*point B*) was added.

TABLE 2

cGMP level in human platelets and its modulation by arachidonic acid metabolites

Washed human platelets were incubated for 2 min with arachidonic acid or its metabolites, and cGMP was determined as described in Experimental Procedures (mean ± standard deviation, three experiments).

Additions	cGMP	
	10 μM	20 μM
	pmol/5 × 10^6 *platelets*	
Control	0.078 ± 0.006	
12-HPETE	0.176 ± 0.012	0.253 ± 0.005
15-HPETE	0.122 ± 0.004	0.162 ± 0.002
5-HPETE	0.105 ± 0.006	0.131 ± 0.011
PGG_2	0.091 ± 0.006	0.094 ± 0.002
PGH_2	0.073 ± 0.010	0.103 ± 0.003
C20:4	0.094 ± 0.011	0.116 ± 0.002

min, although the aggregatory response was already maximal after a period of 30 sec. This experiment demonstrates that, in the sequence of events taking place during the activation of platelets, soluble guanylate cyclase is activated.

In order to demonstrate a correlation between the reduction of intracellular calcium levels and inhibition of aggregation, we performed the following experiment (Fig. 9). Fura-2-loaded platelets were preincubated without or with the indicated concentrations of sodium nitroprusside for 2 min before stimulation of cells with 2 μM arachidonic acid. The level of intracel-

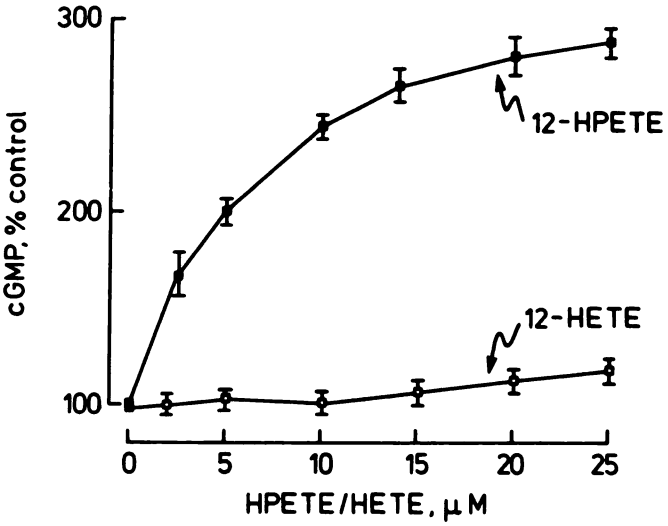

Fig. 5. Dose-dependent effect of 12-HPETE and 12-HETE on platelet cGMP level. Washed human platelets (4–6 × 10^8 platelets/ml) were incubated with 12-HPETE or 12-HETE for 2 min at the concentrations indicated, and cGMP was determined by radioimmunoassay, as detailed in Experimental Procedures. Results are expressed as a cGMP increase compared with control incubations (0.081 ± 0.006 pmol of cGMP/ml of platelets; mean ± standard deviation, three experiments), which received the corresponding solvent.

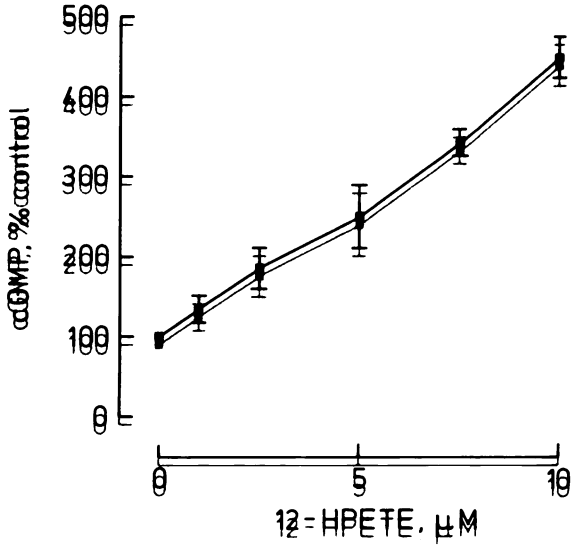

Fig. 6. Dose-dependent effect of 12-HPETE in the presence of dipyridamole on platelet cGMP level. Platelets were incubated with 100 μM dipyridamole for 2 min before 12-HPETE at the indicated concentrations was added for additional 2 min. Determination of cGMP and other details are as in Fig. 5.

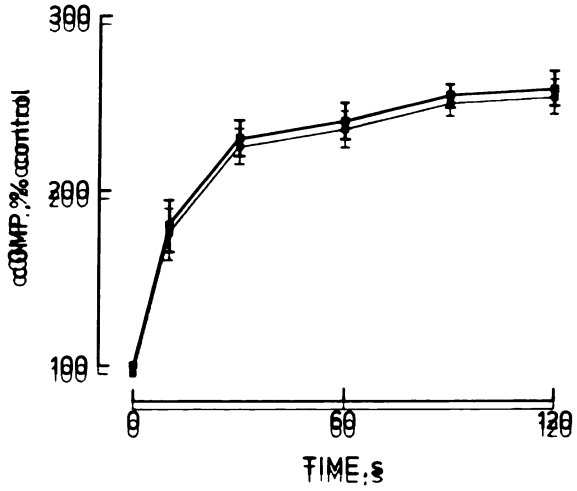

Fig. 7. Time-dependent elevation of platelet cGMP caused by 15-HPETE. Incubations after the addition of 20 μM 15-HPETE were for the different times indicated. Reactions were stopped and cGMP was determined. For other details, see Fig. 5.

of sodium nitroprusside gradually decreased the peak level of calcium increase after platelet activation, compared with the calcium signal observed in the absence of nitroprusside. Only if the calcium level was kept below a certain threshold level was aggregation also inhibited.

Discussion

Cyclooxygenase activity and aggregation of human platelets. The observations documented in this report confirmed the results of Siegel *et al.* (25), Aharony *et al.* (26), and Hammarström *et al.* (32) that 12-HPETE inhibits aggregation as well as TxB_2 formation of human platelets. 12-HPETE suppresses prostaglandin and thromboxane production by inhibiting platelet cyclooxygenase, and this could limit the size of the normal hemostatic platelet plug. The inhibitory action of 12-HPETE may follow the reported self-inactivation mech-

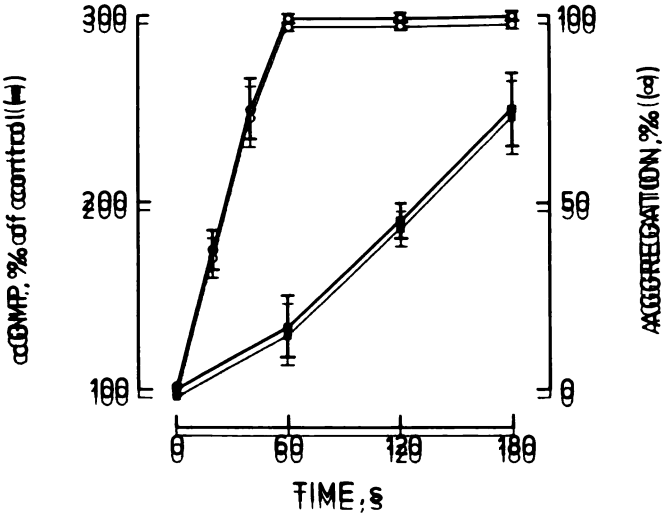

Fig. 8. Time course of aggregation and changes in cGMP after exposure of platelets to thrombin. Washed human platelets were stimulated with 1 unit/ml thrombin and aggregation as well as changes in the cellular cGMP level were measured at different times, as described in Experimental Procedures. The maximal change in light transmission caused by thrombin is set as 100%. The data are means ± standard deviations of three replicate experiments. Details of the cGMP determinations are analogous to the conditions of Fig. 5.

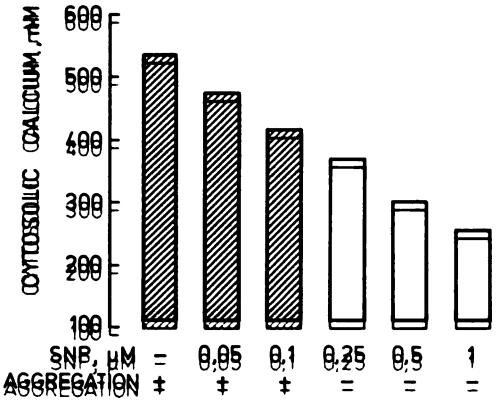

Fig. 9. Correlation between intracellular calcium and aggregation. Fura-2-loaded platelets were incubated with the indicated concentrations of sodium nitroprusside (SNP) for 2 min before challenge with 2 μM arachidonic acid. Aggregation and intracellular calcium were monitored. The resting intracellular calcium was around 100 nM, and only the peak increase in intracellular calcium is given. Aggregation: ±, 100% aggregation; =, no visible formation of platelet aggregates. Equivalent results were obtained in three different experiments.

anism of cyclooxygenase activity in the presence of peroxides (33).

Our data document that the inhibitory effect of 12-HPETE on platelet cyclooxygenase alone cannot explain the inhibitory activity of hydroperoxy-fatty acids during platelet activation. This is demonstrated by using aspirin-treated thrombocytes, mimicking a situation in which the self-amplification through endogenously formed thromboxane A_2 is no longer necessary to induce aggregation. This was concluded from the effect of 12-HPETE in aspirin-treated platelets, employing U46619 or thrombin as an agonist. Another indication that the effect on cyclooxygenase cannot be the only inhibitory mechanism on platelet aggregation became obvious when we measured inhibition of platelet aggregation without significantly impaired HHT and TxB_2 formation (see Table 1). Furthermore, the

inhibitory effect of 12-HPETE on platelet aggregation was observed without delay, whereas inhibition of cyclooxygenase was clearly a time-dependent process.

Cytosolic calcium homeostasis in human platelets. It is generally accepted that platelet activation is associated with altered calcium homeostasis. A cytosolic calcium increase is the consequence of IP_3-induced calcium release from internal stores and/or receptor-mediated extracellular calcium entry.

Our experiments suggest that 12-HPETE inhibits U46619-mediated calcium mobilization from internal stores, as well as calcium influx. 12-HPETE impaired the U46619-stimulated quenching of fura-2 fluorescence by extracellular Mn^{2+}, confirming inhibition of agonist-stimulated divalent cation influx. However, these results should be interpreted with great caution. Inhibition of calcium influx may only be secondary to the previous inhibition of calcium release from internal stores and all subsequent calcium-activated processes, a situation matching the proposed model of Putney and co-workers (34), in which calcium entry is only initiated by depletion of the intracellular IP_3-sensitive Ca^{2+} pool. Further experiments using tBuBHQ to release Ca^{2+} from intracellular stores (35) were carried out in fura-2-loaded platelets in the presence of extracelular Mn^{2+}. tBuBHQ in the absence of extracellular calcium transiently increased intracellular calcium, suggesting both the release of Ca^{2+} from intracellular stores and the entry of Ca^{2+} from the extracellular space (data not shown). When tBuBHQ promoted fura-2 quenching, 12-HPETE was able to inhibit the Mn^{2+} influx, either by directly inhibiting calcium influx or by removing Ca^{2+} from the cytoplasmic space. More experiments are necessary to clarify the action of hydroperoxy-fatty acids on receptor-operated calcium channels.

The use of different platelet agonists revealed that the inhibitory activity of 12-HPETE concerning platelet calcium homeostasis is a general phenomenon and not restricted to a particular agonist/receptor interaction. Therefore, the effect on calcium movements may contribute to the underlying inhibitory mechanism of fatty acid hydroperoxides during the initial phase of platelet activation. This especially applies if a functional cyclooxygenase is not required for aggregation, as in the presence of a nonsteroidal antiinflammatory drug. Alternative inhibitory mechanisms for cGMP action probably also contribute to the antiaggregatory activity, such as an inhibition of the PI response (22, 28) or inhibition of arachidonic acid release (20).

Nitroprusside in the same concentration range has been previously shown to inhibit both Ca^{2+} influx and Ca^{2+} mobilization during platelet responses to thrombin (17) or platelet-activating factor (16). This is consistent with the situation seen here using U46619, in which 12-HPETE inhibits calcium mobilization and calcium influx equipotently. Morgan and Newby (19) also demonstrated increased cGMP levels induced by nitroprusside and inhibition of ADP-mediated Ca^{2+} influx and Ca^{2+} mobilization in human platelets.

Calcium mobilization is a prerequisite for platelet activation, although a cytosolic calcium increase does not automatically lead to aggregation (36). There seems to be a certain threshold level for calcium mobilization and subsequent aggregation. However, a calcium increase above the threshold level is not automatically associated with aggregation, as seen in Fig. 9 or in the paper of Pollock and Rink (36). On the other hand, the data presented in Fig. 9 point to a correlation between the reduction of calcium levels and the inhibition of aggregation. Interestingly, the reduction in cytosolic calcium results in a complete inhibition of the aggregatory response, although calcium release is not completely blocked. Therefore, minor changes in platelet calcium homeostasis may affect the balance between proaggregatory and antiaggregatory pathways, although a strict correlation does not exist. This observation supports our hypothesis that reduced intracellular calcium levels may contribute to but are not the only cause of the antiaggregatory effect of hydroperoxides. In a highly regulated process like platelet aggregation, with self amplification as well as feedback mechanisms, calcium probably is not the only parameter controlling these processes, and cGMP may affect other changes as well.

Furthermore, the interpretation of our calcium data is strenghtened by several experiments carried out to correlate the effect of cGMP and smooth muscle relaxation (37–39). cGMP is an important mediator of smooth muscle relaxation, and in these systems cGMP-dependent protein kinases appear to be the mediators of the reduction in Ca^{2+} levels upon elevation of intracellular cGMP. These results also indicate a correlation between increased cGMP levels and an altered calcium homeostasis.

Soluble guanylate cyclase and cGMP. There are a limited number of reports on peroxide activation of soluble guanylate cyclase (see Ref. 40 for review; see also literature cited therein). Whereas this applied for the isolated enzyme in the absence of reducing agents, we show here that fatty acid hydroperoxides increase cGMP levels in whole platelets. The stimulation shows isomeric specificity, with 12-HPETE being the most efficient isomer. This is consistent with data of Siegel *et al.* (25) on the efficiency of fatty acid hydroperoxides in the inhibition of platelet aggregation, where 12-HPETE also was the preferred position isomer. In contrast to the rather slow inhibition of cyclooxygenase, the increase of cGMP is observed with no apparent time delay. The additional effects of a cGMP phosphodiesterase inhibitor rule out the possibility that 12-HPETE elevates cGMP only indirectly, by inhibition of platelet phosphodiesterase. This demonstrates that the actual cGMP concentrations are underestimated because of the action of phosphodiesterases.

The rapid response of the cGMP levels could indicate a direct action of the hydroperoxides rather than an indirect one by oxidation of other cellular components. The various sulfhydryls of guanylate cyclase and the oxidative self-activation of the enzyme make an oxidative modification at SH groups likely.

Recently, it has been demonstrated by Radomski *et al.* (41) that human platelets contain an NO synthase that is activated when platelets are stimulated. Consequently, the generation of NO should modulate platelet reactivity by increasing cGMP. We also demonstrated that platelet aggregation induced by thrombin was accompanied by an increased level of cGMP. Therefore, the contribution of the NO synthase pathway and the intermediate formation of 12-HPETE, with the concomitent activation of soluble guanylate cyclase, remains to be established. It is possible that both processes act in the same direction to limit the size of a growing platelet plug and in this way represent an inhibitory feedback mechanism. Attention should now be focused on the molecular mechanisms leading to soluble guanylate cyclase activation.

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