Prostaglandin E_2-like activity of 20:3*n*-9 platelet lipoxygenase end-product

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Received 12 November 1984

5,8,11-Icosatrienoic acid (20:3*n*-9), a fatty acid associated with platelet hyperactivity, was oxygenated by platelet lipoxygenase. The end-product of this pathway was purified by high-performance liquid chromatography (HPLC) and characterized as 12-hydroxy-5,8,10-icosatrienoic acid [12-OH-20:3(5,8,10)] by capillary gas-liquid mass spectrometry. When tested upon platelet aggregation, 12-OH-20:3(5,8,10) exhibited a biphasic effect. At low concentrations (below 5×10^{-7} M) it potentiated aggregation but inhibited it at higher levels, a pattern similar to that obtained with prostaglandin E_2. However, since the amounts of 12-OH-20:3(5,8,10) generated under thrombin stimulation are in the range of concentrations with potentiating effects, it seems that the 12-OH derivative is responsible for the hyperaggrebility of 20:3*n*-9-rich platelets.

Human platelet *Lipoxygenase-5,8,11-icosatrienoic acid* *Platelet aggregation*

1. INTRODUCTION

High saturated fat diets, with or without linoleic acid deficiency, promote the accumulation of 20:3*n*-9 in blood lipids and platelets [1–3]. An increase of 20:3*n*-9 in platelet phospholipids resulting from saturated fat feeding has been associated with the enhancement of certain platelet functions, especially platelet aggregation induced by thrombin both in animals [2,3] and man [4]. We have reported that enriching human platelets with 20:30*n*-9 in vitro markedly enhances the response of platelets to aggregation through the lipoxygenase pathway [5]. On the other hand, we have also found that several lipoxygenase products, particularly from arachidonic acid, inhibit stereospecifically PGH_2-induced platelet aggregation [6]. Here, the platelet lipoxygenase end-product of 20:3*n*-9 was purified, identified by mass spectrometry and its biologic effect tested on platelet aggregation.

Abbreviations: PG, prostaglandin; 20:3*n*-9, 5,8,11-icosatrienoic acid; U46619, 9-methano analogue of PGH_2; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; GC-MS, gas-liquid chromatography-mass spectrometry; TMS, trimethylsilyl; BSTFA, bis-silyl trifluoroacetamide

2. MATERIALS AND METHODS

Human platelets, isolated from their plasma as described [7], were homogenized by sonication. The homogenate was centrifuged for 20 min at 13000 × *g* to pellet most of the granules and mitochondria. The supernatant used as a platelet lipoxygenase source was incubated for 20 min with 10^{-4} M [^{14}C]20:3*n*-9 (1.6 Ci/mol) chemically synthesized [8]. After acidic (pH 3) extraction with diethyl ether, the lipids were separated by TLC using a mixture of hexane-diethyl ether-acetic acid (60:40:1, v/v) as eluent. The radiolabelled substance migrating like 12- or 15-monohydroxy-icosaenoic acids was scraped from the plate, ex-

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00145793/85/$3.30

tracted with diethyl ether and further purified by reversed phase HPLC [6].

Mass spectra of the 20:3*n*-9 monohydroxy derivative and its hydrogenated form were performed with an LKB 2085 capillary gas-liquid chromatography-mass spectrometer (GC-MS). These substances were analysed as methyl esters (after treatment with an ethereal solution saturated with diazomethane for 20 min, at room temperature) and trimethylsilyl ethers (after treatment with BSTFA-hexane, 50:50, v/v, for 1 h at 60°C). The hydrogenated form of the OH-20:3 was obtained by hydrogen bubbling in a methanolic solution of the derivative in the presence of platinum(IV). Fragmentation of the molecules was performed by electron impact (20 eV) and the ions detected with an accelerating voltage of 3.5 kV and a trap current of 50 μA [9].

Platelet aggregation was performed on normal human platelets isolated from their plasma [7]. The aggregating agents, thrombin or the 9-methano analogue of PGH_2 (U46619) which is a thromboxane A_2 mimetic, and 12-OH-20:3(5,8,10) diluted in ethanol (0.5%) were simultaneously added to platelets. Platelet aggregation was then measured according to Born's technique [10]. The percentage of aggregation was determined after a 5 min incubation period in comparison to the control (ethanol alone).

3. RESULTS AND DISCUSSION

The monohydroxy derivative obtained by incubating 20:3*n*-9 with a platelet subfraction containing the lipoxygenase (microsomes + cytosol) was pre-purified by TLC where it co-chromatographed with 12- or 15-hydroxyicosatetraenoic acid (12- or 15-HETE). The hydroxy derivative of 20:3*n*-9 was further purified by reverse-phase (rp)-HPLC. Fig.1 gives an example of this purification. The retention time of the monohydroxy derivative of 20:3*n*-9 was slightly longer than that of 20:3*n*-6 (12-OH-8,10,14-20:3) indicating that it is slightly less polar. The hydroxy derivative of 20:3*n*-9 was then purified on a semi-preparative scale for further studies. The UV spectrum of the compound then purified (not shown) presented a maximal absorbance at 235 nm. Spectra with a similar shape have been described for other monohydroxy derivatives of long-chain polyunsaturated fatty

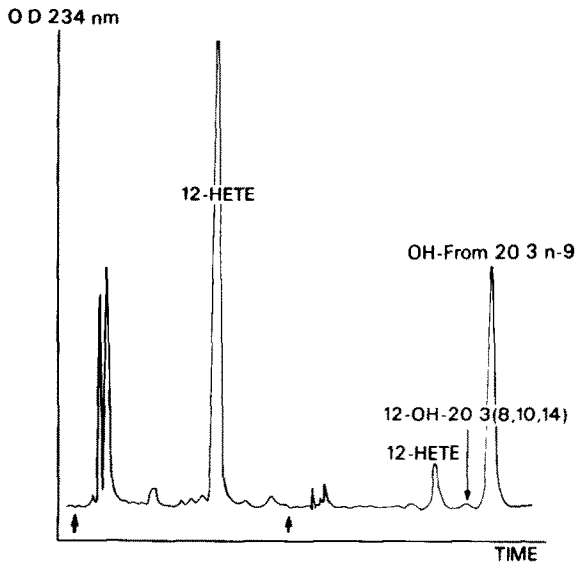

Fig.1. HPLC profile of the 20:3(*n*-9) monohydroxy derivative *vs* that of 12-HETE and 12-OH-20:3(8,10,14) on a 25 cm, 1/4 inch column packed with nucleosil C_{18}, 5 μm. The mobile phase [methanol-water (acidified to pH 3 with acetic acid), 76:24, v/v] was used at 1.0 ml/min. UV detection performed at 234 nm.

acids. They are characterized by the presence of a *cis-trans* conjugated double bond with a hydroxyl or hydroperoxyl group in position α of the *trans* double bond [11,12]. The carbon position of the hydroxy derivative of 20:3*n*-9 was investigated by capillary GC-MS [9]. The mass spectrum of the methyl ester, trimethylsilyl ether derivative (fig.2, left) shows a main peak at *m/z* 267 (fragment from C_8 to C_{20}, containing the OTMS) and a second peak at *m/z* 295 (fragment from C_1 to C_{12}, including the OTMS). After hydrogenation of the latter derivative as in other studies [9], the mass spectrum (fig.2, right) shows two main fragments at *m/z* 215 and 301 corresponding to C_{12}–C_{20} and C_1–C_{12}, respectively (both containing the OTMS group). These spectra confirm that the hydroxyl group of the monohydroxy derivative produced from 20:3*n*-9 by human platelets is in fact at the 12 position.

Purified by semi-preparative rp-HPLC, 12-OH-20:3(5,8,10) was quantified by both its specific radioactivity (according to that of the precursor) and its absorbance at 235 nm, using the same

molar coefficient of absorption (30000) as for other monohydroxy derivatives from icosaenoic acids [12–14]. According to its concentration, 12-OH-20:3(5,8,10) exerted a biphasic effect on platelet aggregation induced either by thrombin or U46619 (fig.3). We have reported that under similar experimental conditions, PGH_2-induced aggregation is affected by lipoxygenase derivatives of other fatty acids in a stereospecific manner [6]. However, whereas the other active derivatives inhibited the aggregation at any concentration, 12-OH-20:3(5,8,10) potentiates aggregation at concentrations below 0.5×10^{-6} M as shown here. In fact, the pattern of 12-OH-20:3(5,8,10) activity on aggregation appears to be very similar to that observed with PGE_2 tested under similar conditions (fig.3). PGE_2 is known to exert a biphasic effect on ADP-induced aggregation [15,16].

We have shown that 20:3*n*-9 is able to potentiate platelet aggregation through a mechanism involving the lipoxygenase pathway [5]. In the same study we found that 20:3*n*-9-rich platelets may generate 2.1 nmol (per 10^9 platelets) of lipoxygenase end-product when triggered with 0.1 U/ml

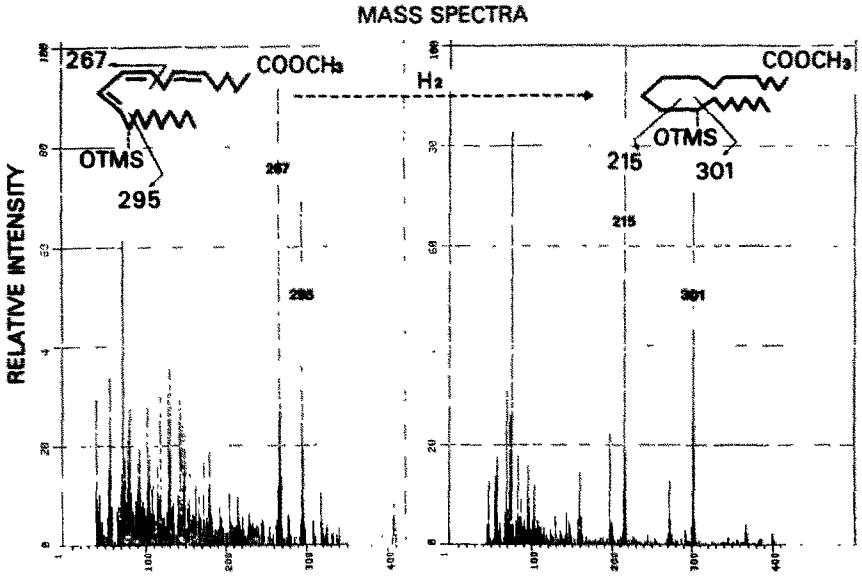

Fig.2. Mass spectra of 20:3(*n*-9) monohydroxy derivative (left) and its hydrogenated form (right). These substances were analysed as methyl esters, trimethylsilyl ethers. The fragments, especially from the hydrogenated derivative (m/z = 215 and 301), clearly indicate that 20:3(*n*-9) oxygenation was done on C_{12}.

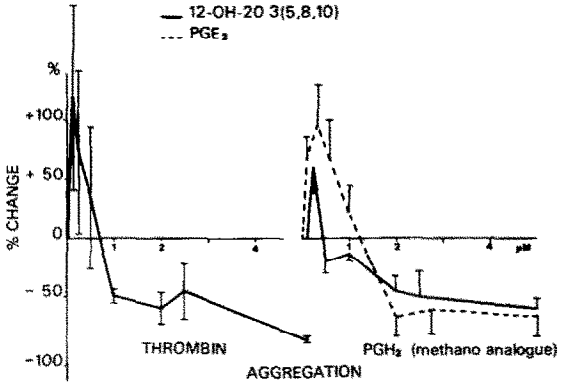

Fig.3. Influence of 12-OH-20:3(5,8,10) at different concentrations on aggregation induced by thrombin (0.015 U/ml) or the 9-methano analogue of PGH_2. In comparison, the effect of PGE_2 on aggregation induced by the 9-methano analogue of PGH_2 is also shown. Results are expressed as the mean ± SE of the change (%) in reactivity in the presence (D) or absence (C) of the molecule as a percentage of the control (C), 100 × (D − C)/C. Each point represents the pooled results of 4–15 experiments.

thrombin for 4 min. We extrapolate that this amount corresponds to approx. 0.7 nmol/ml platelets at physiological concentrations. Under these conditions, the concentration of 12-OH-20:3(5,8,10) formed under thrombin stimulation would be lower than 0.5×10^{-6} M at the early stage of aggregation where the monohydroxy derivative would act. The physiological activity of 12-OH-20:3(5,8,10) would then be a potentiating effect on platelet aggregation.

In conclusion, it seems that the marked increase in platelet aggregation associated with saturated fat feeding and elevation of 20:3*n*-9 in platelet phospholipids, might be due to the formation of a 12-lipoxygenase derivative produced during platelet activation. Moreover, these results confirm that the lipoxygenase product metabolites might also regulate platelet functions in addition to prostaglandins.

ACKNOWLEDGEMENTS

This work was supported by the Institut de la Santé et de la Recherche Médicale (CRL 825043). We thank Dr J.E. Pike, Upjohn Co., Kalamazoo, USA, for kindly supplying the 9-methano analogue of PGH_2 used in this study.

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