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INHIBITION BY LIPOXYGENASE PRODUCTS OF TXA_2-LIKE RESPONSES OF PLATELETS AND VASCULAR SMOOTH MUSCLE

14-HYDROXY FROM 22:6N-3 IS MORE POTENT THAN 12-HETE

MARTINE CROSET,* ANGELO SALA,† GIANCARLO FOLCO‡ and MICHEL LAGARDE*§

*INSERM U63, Institut Pasteur, Faculté de Médecine Alexis Carrel, Lyon, France, and CNRS UA 273 Dijon, France, †Institute of Pharmacological Sciences, University of Milan, 20133 Milan, and ‡Institute of Pharmacology and Pharmacognosy, University of Parma, 43100 Parma, Italy

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Abstract—Lipoxygenase products, which are formed in great amounts in platelets during their activation, have been prepared from arachidonic acid (20:4n-6), the main polyunsaturated fatty acid (PUFA) esterified in platelet phospholipids, and from two major PUFAs of fish fat, eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids. These compounds have been synthesized using platelet suspension as enzymic source, purified by high performance liquid chromatography, and their structure were checked by gas chromatography–mass spectrometry. Their effects were investigated *in vitro* upon human platelet aggregation induced by 11,9-epoxy-methano-analogue of PGH_2 (U-46619) and upon thromboxane A_2-induced vasoconstriction of rabbit aorta. All hydroxylated fatty acids inhibited U-46619-induced aggregation in a concentration-dependent fashion. Compounds issued from 22:6n-3 were the most potent inhibitors and their IC_{50} differed significantly from that of 12-hydroxy-eicosatetraenoic acid (12-HETE). Among them, 14-hydroxy-docosahexaenoic acid (14-OH-22:6) was the most effective anti-aggregating molecule (IC_{50}: 0.45 μM). 10 μM 12-HETE and 14-OH-22:6 inhibited 60% and 75% of smooth muscle contraction induced by TXA_2-like material, respectively. At 1 μM, solely 14-OH-22:6 had an inhibitory effect on andrenaline-, angiotensine- or histamine-induced contraction.

Since thromboxane receptors in platelets and vascular smooth muscle cells present strong similarities, it is concluded that hydroxylated fatty acids can antagonize prostanoid action probably by interfering with their receptor sites.

In blood platelets, polyunsaturated fatty acids (PUFA), mainly arachidonic acid (20:4n-6), are almost exclusively esterified in membrane phospholipds, and may be liberated and metabolized through the cyclooxygenase and/or the lipoxygenase pathway, subsequently to stimulation by aggregating agents like thrombin and collagen. 20:4n-6 is metabolized into pro-aggregatory prostanoids (PGG_2/H_2) and thromboxane A_2 (TXA_2) via the cyclooxygenase pathway [1], and into 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) further reduced into 12-hydroxy derivative, 12-HETE, by a glutathione-dependent peroxidase [2]. Whereas the biological activity of cyclooxygenase products has been well documented, that of lipoxygenase ones has not been fully elucidated [3–6]. These products, however, may be formed in greater amounts than the cyclooxygenase metabolites because of the self-inactivation of cyclooxygenase [7] and the autocatalysis of lipoxygenase [8]. Moreover, the cyclooxygenase has a relatively strict requirement for substrate recognition. Besides 20:4n-6, it oxygenates solely dihomo-gamma-linolenic (20:3n-6), eicosapentaenoic (20:5n-3), adrenic (22:4n-6) and 4,7,10,13,16-docosapentaenoic (22:5n-6) acids [9–11]. In contrast, the lipoxygenase can oxygenate a wide variety of PUFAs since it only requires a 1,4-*cis*,*cis*-pentadiene structure from carbon 8 [12]. Even two monohydroxy derivatives may be formed from 7,10,13,16,19-docosapentaenoic (22:5n-3) and docosahexaenoic (22:6n-3) acids [11, 13, 14]. This makes the lipoxygenase pathway an efficient one for the oxygenation of most PUFAs which may arise in certain nutritional states [15]. In addition to the n-9 lipoxygenase of platelets, n-6 lipoxygenase producing 15-HETE and 13-hydroxy-9,11-octadecadienoic acid (13-HODE) has been described in the endothelium [16, 17] and leukocytes [18, 19]. 15-HETE has also been shown to be a major hydroxy product in atherosclerotic lesions of the rabbit aorta [20].

Several studies have reported that lipoxygenase products may modulate platelet functions. First, 13-hydroperoxy-9,11-octadecadienoic acid (13-HPODE) was shown able to inhibit platelet aggregation [21]. Although the use of dual inhibitors of cyclooxygenase and lipoxygenase led to conclude that lipoxygenase products of 20:4n-6 may potentiate the aggregation [22, 23], 12- and 15-hydroperoxy derivatives of 20:4n-6 have been reported as inhibitors of platelet cyclooxygenase [24, 25] and platelet aggregation [24–26]. Hydroxylated fatty acids issued from 20:4n-6, 20:3n-6, and 20:5n-3 inhibit prostanoid-induced platelet aggregation when exogenously added [24–27]. Besides, arachidonic acid-induced platelet aggregation has been found higher in patients with myeloproliferative disorders accompanied with a lipoxygenase deficiency [28].

§ To whom correspondence should be addressed.

Finally, in contrast to 13-HODE, 12-HETE appears to promote platelet adhesion to intact endothelium [29].

In this work, we have compared the anti-thromboxane activity of 12-HETE and lipoxygenase products of the major fatty acids of fish fat [15], 20:5n-3 and 22:6n-3, in relation to their effect on arachidonic acid metabolism. We have also tested the effect of lipoxygenase products on thromboxane-induced vasoconstriction of rabbit aorta in view of the observation that platelets and vessel wall possess similar receptors for thromboxane [30].

MATERIALS AND METHODS

Reagents. [^{14}C]-arachidonic acid (55 Ci/mole) was provided from Amersham International, U.K., and [^{14}C]-20:5n-3 and -22:6n-3 (>50 Ci/mole) were purchased from New England Nuclear (Boston, MA). Unlabelled fatty acids were obtained from Sigma (St. Louis, MO), and 22:6n-3 was purified by thin-layer chromatography before use. Nucleosil C_{18} 5 μm for high performance liquid chromatography (HPLC) was purchased from Macherey-Nagel (Düren, F.R.G.). Lichrosorb 5 μm and Silica gel plates were obtained from Merck (Darmstadt, F.R.G.). The 11,9-epoxy-methano-analogue of PGH_2 (U-46619) was a gift from Dr. J. E. Pike, Upjohn, Kalamazoo, MI.

Preparation of platelet suspensions. Venous blood from donors who had not taken any drug for at least ten days before sampling, was collected in siliconized vials containing 1:10 volume acidic citrate–dextrose (ACD) (0.8% citric acid, 2.2% tri-sodium citrate, 2.45% dextrose, pH 4.5). After centrifugation for 15 min at 100 *g*, platelet-rich plasma (PRP) was removed, acidified to pH 6.4 with citric acid 0.15 M, and immediately centrifuged at 900 *g* for 10 min [31]. Platelets were then resuspended into a Tyrode-HEPES buffer pH 7.35 (NaCl 136 mM, $NaHCO_3$ 12 mM, NaH_2PO_4 0.41 mM, KCl 2.7 mM, $MgCl_2$ 1 mM, HEPES 5 mM and glucose 5.5 mM) and adjusted to 3.10^8/ml before use.

Synthesis of hydroxy-polyunsaturated fatty acid isomers. Platelet suspensions, used as a source of 12-lipoxygenase, were incubated for 20 min at 37° with either [^{14}C]-22:6n-3 (100 μM, 0.02 nci/nmole) to prepare 14-hydroxy-4,7,10,12,16,19-docosahexaenoic acid (14-OH-22:6) and 11-hydroxy-4,7,9,13,16,19-docosahexaenoic acid (11-OH-22:6) or [^{14}C]-20:4n-6 (100 μM, 0.02 nCi/nmole) to prepare 12-HETE. 12-Hydroxy-5,8,10,14,17-eicosapentaenoic acid (12-OH-20:5) was obtained by incubating platelet suspensions with 20:5n-3 (40 μM, 0.02 nCi/nmole) in the presence of 20:4n-6 (10 μM) in order to stimulate the formation of the hydroxylated compound from 20:5n-3 [32]. Reactions were terminated by acidification to pH 3 and the fatty acid derivatives were extracted three times with an equal volume of diethyl ether in the presence of butylated hydroxytoluene (50 μM) as an antioxidant. 17-Hydroxy-4,7,10,13,15,19-docosahexaenoic acid (17-OH-22:6) was prepared by soybean lipoxygenase treatment of 22:6n-3 followed by reduction of the hydroperoxide intermediate with $NaBH_4$ [33]. All the synthesized lipoxygenase products were assumed to be of the *S*-configuration.

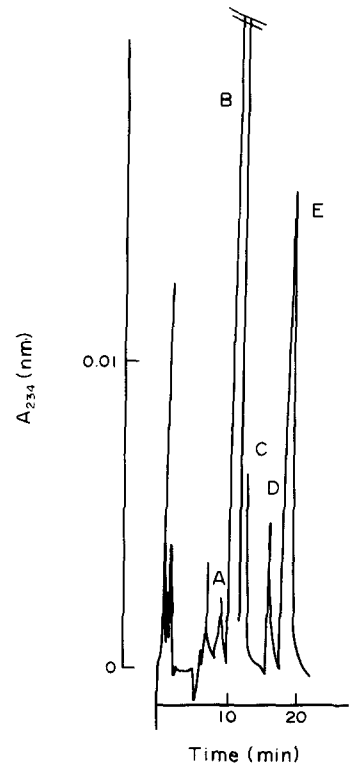

Fig. 1. Normal phase HPLC separation of hydroxylated fatty acids produced from 22:6n-3 by human platelets. After purification by TLC, the compounds were injected as free fatty acids onto a Lichrosorb 5 μm column (4.6/250 mm) and resolved using hexane/isopropanol/acetic acid (100/0.5/0.1, v/v) as mobile phase with a flow rate of 2 ml/min. A, C and D are non-enzymatically formed hydroxylated fatty acids; B and E represent 14-OH-22:6 and 11-OH-22:6, respectively.

Purification and characterization of hydroxylated PUFA isomers. Each oxygenated derivative was separated from the remaining substrate by thin-layer chromatography using the solvent mixture: hexane/diethyl ether/acetic acid (60/40/1, v/v). The hydroxylated compounds localized on the plate by their radioactivity were extracted from the gel three times with diethyl ether. Further purification was achieved by straight phase HPLC using a Lichrosorb 5 μm column (4.6/250 mm). The autoxidation of 22:6n-3 has been described very active [34], and we have observed the tendency to form minor autoxidized products during biological synthesis. These products eluted very closely to the two major enzyme-derived products. A complete resolution was achieved by straight phase HPLC using the solvent mixture: hexane/isopropranol/acetic acid (100/0.5/0.1, v/v) (Fig. 1). To separate 14-OH-22:6 from the contaminating 12-HETE, a slightly more polar mobile phase was necessary. The purity of the recovered compounds was greater than 95%, based on a second analysis by reverse phase HPLC using a nucleosil C_{18} 5 μm column. The structure of hydroxylated compounds from 22:6n-3 was confirmed by gas chromatography–mass spectrometry using a Nermag R10-10 quadripole interfaced in a

gas chromatography equipped with a SE 54-coated capillary column. Purified hydroxy compounds were derivatized as methyl ester, trimethylsilylether derivatives and catalytically hydrogenated in the presence of PtO_2 as previously described [13, 35]. Briefly, the ME–TMS compounds were dissolved in methanol, small amounts of PtO_2 was added (around 1 mg/ml methanol) and hydrogen gas was gently bubbling for 5 min at room temperature. Methanol solution was then separated from the catalyst. Electron impact mass spectra of these derivatives gave two main fragments corresponding to the breakage on both sides of the OTMS, including this group [35]. 14-OH-22:6 exhibited major ions at *m/z* 329 and 215, 11-OH-22:6 at 257 and 287 and 17-OH-22:6 at 371 and 173.

Each compound exhibited a lambda max at 235 nm, when analyzed in ethanol, which is consistent with conjugated double bonds in the *cis–trans* configuration [36]. The various monohydroxy derivatives were assumed to have the same molar extinction coefficiency and their quantification was done by using a coefficiency of 30,000 at 235 nm. They were stored at −20° in ethanol under nitrogen.

Platelet aggregation measurements. Aggregation tests were performed according to the turbidimetric method of Born [37]. The cell suspension was prewarmed for 2 min and the aggregation induced by adding U-46619. Each hydroxy compound was added in ethanol (final concentration 0.25%) simultaneously to the aggregating agent, and the optical signal recorded for 4 min. For each hydroxy compound the concentration inhibiting 50% of the control aggregation (IC_{50}) was determined.

Oxygenated metabolism of [^{14}C]-20:4*n*-6 *by platelets.* Intact platelets (0.4 ml) were incubated for 4 min at 37° with 2 μl of ethanol containing 10 μM [^{14}C]-20:4n-6 in the presence of appropriate hydroxylated fatty acids (10 μM). Incubations were terminated by the addition of 3 vol. of ethanol and total lipids were extracted twice with chloroform in the presence of butylated hydroxy toluene (BHT). The resulting organic phase was reduced under vacuum and lipid residues were submitted to thin layer chromatography on silica gel. A first elution with the mixture hexane/diethylether/acetic acid (60/40/1, v/v) allowed the separation of 12-HETE ($R_f = 0.29$) from 12-hydroxy-heptadecatrienoic acid (HHT) ($R_f = 0.20$). A second elution with the mixture diethyl ether/methanol/acetic acid (90/1/2, v/v) separated TXB_2 ($R_f = 0.28$) from other compounds. After each run a quantitative radiochromatogram was performed. Total radioactivity was considered as representing the initial amount of the labelled substrate. The integrated peaks, then calculated as percentage of total radioactivity, could be quantified in nanomoles of products [32].

Superfusion experiments of rabbit aorta. Guinea-pigs of either sex were killed by exsanguination and their heart and lungs removed: most of the heart was cut away and the pulmonary artery and trachea were cannulated. The lungs were then suspended in a chamber and perfused through the pulmonary artery with Krebs solution (millimolar composition: NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 5, $NaHCO_3$ 25), gassed with 95% O_2 and 5% CO_2, at a flow rate of 10 ml/min [38]. The pulmonary effluent superfused two spirally cut strips of thoracic aorta of rabbit (RbA), set up for isotonic recording and stabilized for a period of 2 hr with a counterweight of 2 g. The vascular strips were treated with a mixture of receptor antagonists as suggested by Gilmore *et al.*, and with indomethacin (3 μM) [39] in order to increase the specificity of the tissues to TXA_2 and to prevent endogenous prostaglandin generation. The lower aortic strip was continuously superfused with absolute ethanol (Carlo Erba, Milan) at a constant flow of 0.1 ml/min (final ethanol concentration 1%) and the lung was challenged with histamine (2.5 and 5 μg as bolus injection) at constant time. The substances under examination, dissolved in ethanol, were superfused on the lower aortic strip only, at a flow rate of 0.1 ml/min, giving a final concentration of 1–10 μM, for a period of 3–6 min, before challenging the lung with a bolus injection of histamine.

Statistics. All the results were compared with the paired *t*-test.

RESULTS

Effects of hydroxylated PUFA isomers on platelet aggregation and 20:4n-6 metabolism

On each batch of platelets used, we first determined the concentration of 11,9-epoxy-methano-analogue of PGH_2 inducing 50% of aggregation and then used the double of this concentration to obtain submaximal aggregation. The range of concentration obtained varied from 0.5 μg/ml to 0.15 μg/ml according to different batches of platelets. Each hydroxylated compound tested exhibited a dose-dependent inhibitory effect upon aggregation, as examplified by 12-HETE or 14-OH-22:6 (Fig. 2). Determinations of IC_{50} for each compound showed significant differences in their potencies to inhibit U-46619-induced aggregation (Fig. 3). 12-HETE and 12-OH-20:5 were the least potent inhibitors with a comparable IC_{50} of 2.94 μM and 2.87 μM, respectively. All the three isomers issued from 22:6n-3 exhibited

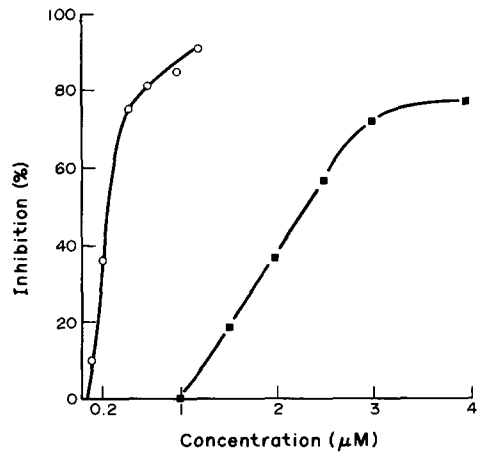

Fig. 2. Concentration-dependent inhibition of U-46619-induced platelet aggregation by 12-HETE (■——■) and 14-OH-22:6 (○——○). The hydroxylated fatty acids were added simultaneously with the aggregating agent. Data are mean of three determinations.

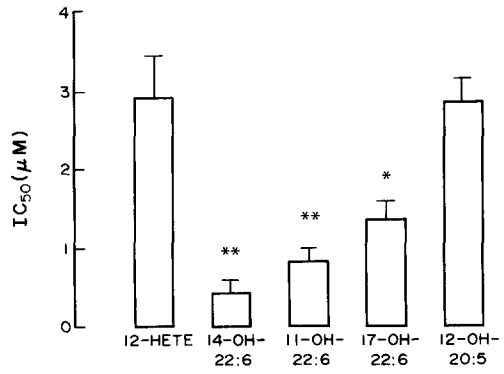

Fig. 3. Inhibitory activity of hydroxylated fatty acids on platelet aggregation induced by 11,9-epoxy-methano-analogue of PGH_2. The concentration of hydroxylated fatty acids from 22:6n-3 and 20:5n-3 inhibiting 50% of a submaximal aggregation (IC_{50}) was compared to IC_{50} of 12-HETE. Results are expressed as mean ± SE of seven determinations on different platelet batches (*$P < 0.01$; **$P < 0.001$).

a significantly lower IC_{50} than 12-HETE and among them, 14-OH-22:6 was the most potent inhibitor of aggregation ($IC_{50} = 0.45\ \mu M$). It was significantly more potent than 11-OH-22:6 ($P < 0.001$) and 17-OH-22:6 ($P < 0.001$). The IC_{50} of these compounds were strongly dependent on U-46619 concentration used to induce the aggregation. When 50% aggregation was induced, IC_{50} for 12-HETE and 14-OH-22:6 were $0.76 \pm 0.30\ \mu M$ and $0.125 \pm 0.06\ \mu M$ respectively (N = 4).

The effect of these compounds on exogenous 20:4n-6 metabolism were also investigated. At variance with 22:6n-3 itself, which inhibited cyclooxygenase product formation from 20:4n-6, none of the 22:6n-3 hydroxyated metabolites nor 12-HETE could interfere with 20:4n-6 metabolism, except for a tendency of 17-OH-22:6 to inhibit 12-lipoxygenase (Table 1).

Effects of 12-HETE and 14-OH-22:6 on thromboxane-induced contraction of rabbit aorta

The injection of histamine into the isolated guinea-pig lungs promoted a dose-dependent formation and release of TXA_2-like material as indicated by the contraction of both rabbit aortas. The superfusion of the lower aorta with 0.1% ethanol resulted in a slight increase of the basal tone but did not change its responsiveness to TXA_2-like activity released by the challenged lung. The contraction of the two aortic strips were, in fact, invariably well matched. The pretreatment of the lower aortic strip with 14-OH-22:6 (10 μM), caused a marked reduction of the smooth muscle contraction, as compared to the contraction that was still taking place in the upper aortic strip (Fig. 4). The average percentage decrease of the contractile response in four experiments was 75 ± 5% and the TXA_2 receptor antagonism was quickly reversible upon interruption of the treatment. The effect was dose-dependent, since a lower concentration (1 μM), showed an inhibition of only 10 ± 2%. We could observe a similar trend by pretreating the aortic strips with 12-HETE (Fig. 4). At a concentration of 10 μM the average percentage inhibition in three experiments was 60 ± 4.5%, but the lower concentration utilized (1 μM) failed to cause any inhibition of the aortic strip contractions. The TXA_2 receptor antagonism by 12-HETE was also quickly reversible upon interruption of the treatment. The activity of the lipoxygenase product of 22:6n-3 and 12-HETE seems to be specific for TXA_2, because the smooth muscle contractions elicited by bolus injection of agonists such as noradrenaline (200 ng), angiotensin II (500 ng) and histamine (1 μg), were left unchanged by both substances, as observed in separate experiments.

DISCUSSION

An inhibitory effect of 12-HPETE and its positional isomer, 15-HPETE, on thromboxane synthesis and platelet aggregation has been described earlier [24–26]. The inhibition of thromboxane formation was, however, not completely responsible for the inhibition of platelet aggregation [24, 25]. On the other hand, a reduced aggregation was observed when high concentrations of exogenous 20:4n-6 were used, in relation to an increase of the ratio 12-HETE/TXB_2 [24], suggesting that lipoxygenase products of 20:4n-6 may inhibit the thromboxane-induced aggregation. Moreover, 12- and 15-HETE, which do not affect cyclooxygenase activity at concentrations tested, inhibit thromboxane-induced aggregation [24, 27]. In this work, the effect of various hydroxylated fatty acids deriving from the main fatty acids of fish fat, 20:5n-3 and 22:6n-3, upon U-46619-induced aggregation, was compared to 12-HETE. Their effect on the oxygenated metabolism of exogenous 20:4n-6 was also studied. The aggregation was dose-dependently inhibited. It is known that U-46619 binds to thromboxane A_2 receptor on platelet membrane [40] and activates secondarily phospholipase C, then stimulating the release of exogenous 20:4n-6 and its oxygenation into pro-aggregatory prostanoids [41]. The compounds that we have tested might have inhibited an eventual

Table 1. Effect of hydroxylated fatty acids on arachidonic acid metabolism by human platelets

	Control	12-HETE	14-OH-22:6	11-OH-22:6	17-OH-22:6	22:6n-3
HHT	1.47 ± 0.27	1.38 ± 0.19	1.58 ± 0.18	1.32 ± 0.38	1.23 ± 0.24	*0.97 ± 0.41
TXB_2	1.09 ± 0.25	1.21 ± 0.18	1.31 ± 0.27	1.04 ± 0.37	0.98 ± 0.41	**0.70 ± 0.32
HETE	0.80 ± 0.15	0.82 ± 0.10	0.90 ± 0.14	0.93 ± 0.19	0.46 ± 0.32	1.04 ± 0.33

10 μM [^{14}C]-20:4n-6 were incubated with platelet suspension alone (control) or in the presence of hydroxylated fatty acids or 10 μM 22:6n-6. The metabolites were extracted and quantified by radiochromatography.

Results, expressed in nanomoles/10^9 platelets, are mean ± SD of 3 or 4 determinations (*$P < 0.05$; **$P < 0.02$).

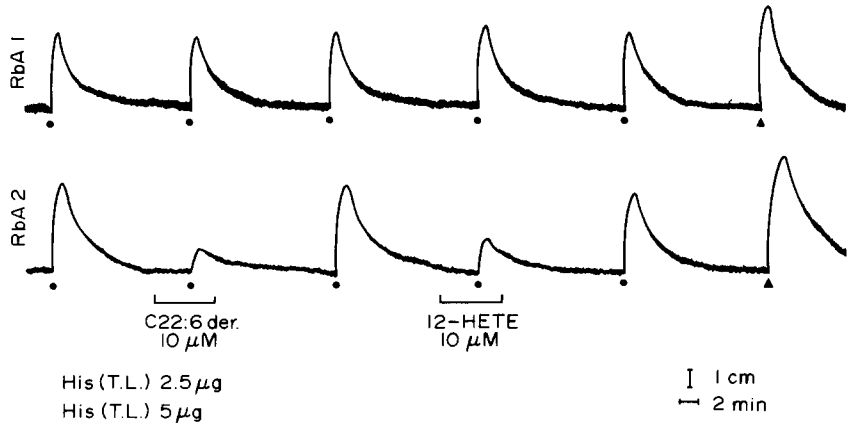

Fig. 4. Bioassay of TXA_2-like activity on a pair of superfused rabbit aortic strips (RBA). The upper RBA (RBA 1) was superfused with the effluent of the perfused guinea-pig lung. The lower rabbit aorta (RBA 2) was also superfused with 1% ethanol or alternatively with 14-OH-22:6 (10 μM) or 12-HETE (10 μM).

secondary aggregation due to the formation of endogenous thromboxane, by interfering with the metabolism of 20:4n-6, as it has been described for their fatty acid precursors, 20:5n-3 [42] and 22:6n-3 [43]. However, none of the hydroxy derivatives tested was able to alter the oxygenation of exogenous 20:4n-6, except 17-OH-22:6 which decreased 12-HETE formation as described previously [44]. Moreover, when we tested the effects of 14-OH-22:6 and 12-HETE against low concentrations of U-46619 (0.02–0.05 μg/ml), which are described to be independent of endogenous 20:4n-6 release, they still inhibited aggregation and the IC_{50} ratio obtained for both compounds was equal to that observed when aggregation was induced by higher concentrations of U-46619. In this regard, when the agonist concentration was doubled, the IC_{50} for each hydroxy derivative was multiplied by four. This might reflect the contribution of endogenous PGH_2/TXA_2 formation at higher agonist concentration. We conclude that the mono-hydroxylated fatty acids act at the pro-aggregatory prostanoids receptor site rather than by inhibiting cyclooxygenase/thromboxane synthase activities. A recent report has described the purification from calf platelets of a haemoprotein, different from cyclooxygenase, which can bind U-46619 [45]. The protein can also bind 20:4n-6 and 20:3n-6, both PUFAs that have been shown to inhibit U-46619-induced platelet aggregation [46]. The interaction of 20:4n-6 with the haemoprotein might partly explain the well known inhibition of platelet aggregation when induced by high concentrations of 20:4n-6. The more potent inhibitory effect of hydroxy compounds, as compared to their fatty acids precursors (more than tenfold), would be linked to the alcohol function of the derivatives. This would confer them more structural analogy with PGH_2/TXA_2. In this respect, the *S*-configuration of monohydroxy derivatives, as well as the position of the hydroxyl group, appears to be essential [27]. Similarly, 14-OH-22:6 was more active than its 11- and 17-positional isomers, suggesting that it is better structurally related to PGH_2/TXA_2.

Some results argue for strong similarities between thromboxane receptors in platelets and vascular smooth muscle cells [30] and we have examined the effect of 12-HETE and 14-OH-22:6 upon TXA_2-induced contraction of rabbit aorta strips. TXA_2-like material was produced by perfusion of isolated guinea-pig lung with histamine. Contraction of aorta induced by the perfusate was almost completely abolished by pre-treating the aorta with lipoxygenase products. Both 12-HETE and 14-OH-22:6 were inhibiting, the latter being more potent than the former, similarly to that observed towards platelet aggregation. Besides, their anti-TXA_2 specificity was attested by absence of effect on noradrenaline-, angiotensin-, or histamine-induced contraction. These results argue again for a relatively specific anti-TXA_2 activity of lipoxygenase products.

Taking into account the relatively high potency of lipoxygenase products of 22:6n-3 as TXA_2 antagonists, it is concluded that these products may largely contribute to the inhibition by 22:6n-3 of arachidonic acid-induced platelet aggregation [43] and coronary constriction [47].

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