Monohydroxylated fatty acid substrate specificity of human leukocyte 5-lipoxygenase and ω -hydroxylase

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Various monohydroxylated fatty acids were synthesized from eicosapolyenoic acids, namely arachidonic (20:4 ω -6), timnodonic (20:5 ω -3), dihomogammalinolenic (20:3 ω -6) and mead (20:3 ω -9) acids. 12-Hydroxy derivatives, as well as 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), were produced with platelets as the enzyme source, and 15-hydroxy derivatives were produced by soya bean lipoxygenase treatment. Each monohydroxylated fatty acid was incubated with human leukocytes in the presence or absence of the calcium ionophore A23187, and dihydroxylated products were analysed by h.p.l.c. 12-Hydroxy derivatives of 20:4 ω -6, 20:5 ω -3 and 20:3 ω -9 were similarly oxygenated by both the 5-lipoxygenase and the ω -hydroxylase. As expected, the 12-hydroxy derivative of 20:3 ω -6 was not a substrate for 5-lipoxygenase, but surprisingly, ω -6 oxygenated products, like 15-OH-20:4 or HHT, were not converted by the enzyme, although being potential substrates because of the presence of two double bonds at C-5 and C-8. ω -6 oxygenated derivatives were also poorly converted by leukotriene B₄ ω -hydroxylase, a cytochrome P-450-dependent enzyme. It is concluded that both leukocyte 5-lipoxygenase and ω -hydroxylase exhibit a substrate specificity towards monohydroxylated fatty acids with respect to their double bonds and/or the carbon position of the alcohol function.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) may be metabolized by lipoxygenases (EC 1.13.11.12) to their respective hydroperoxide products (Hamberg & Samuelsson, 1967; Needleman, 1986).

In blood platelets, the lipoxygenase specifically oxygenates C-12 of eicosapolyenoic acids (Nugteren, 1975). Although part of the hydroperoxides produced may be degraded into hydroxyepoxy compounds and into subsequent trihydroxy derivatives via a non-enzymic path (Jones *et al.*, 1978), most of the hydroperoxide intermediate is reduced into the corresponding hydroxy derivative (Needleman, 1986) by a glutathione-dependent peroxidase (Bryant & Bailey, 1980). Leukocytes differ from platelets in this respect in providing a 5-hydroperoxy derivative from arachidonic acid which is actively transformed into a 5,6-epoxy compound, leukotriene A₄ (LTA₄) (Borgeat & Samuelsson, 1979a,b; Samuelsson, 1983).

In addition, the cyclo-oxygenase pathway may also produce monohydroxy derivatives from prostanoid precursors, namely 12-hydroxyheptadecatrienoic acids, resulting from the breakage of the cyclopentane ring of prostaglandin cyclic endoperoxides, e.g. 12-hydroxyheptadecatrienoic acid (HHT) from prostaglandin H₂ (Samuelsson *et al.*, 1978).

During platelet-leukocyte interactions, the 12-hydroxy derivative of arachidonic acid (12-HETE) may become a substrate of the 5-lipoxygenase in stimulated leukocytes to provide the double lipoxygenase products 5S,12S-diHETE (Borgeat *et al.*, 1981; Marcus *et al.*, 1982). On

the other hand, it has also been reported that 12-HETE like LTB₄, can be further oxygenated by cytochrome P-450 ω -hydroxylase of unstimulated leukocytes to provide 12S,20-diHETE (Marcus *et al.*, 1984, 1987). The later hydroxylase activity has been recently characterized as a mixed mono-oxygenase requiring NADPH as a cofactor and has been localized in the leukocyte microsomal fraction (Marcus *et al.*, 1987).

The present work was conducted to determine the substrate specificity of both leukocyte 5-lipoxygenase and ω -hydroxylase towards various monohydroxylated fatty acids differing in the position of the hydroxyl group and/or the number and the position of double bonds. We conclude that the position of the hydroxyl group from the methyl end might be crucial for the double oxygenation.

MATERIALS AND METHODS

Reagents

Unlabelled fatty acids, arachidonic (20:4 ω -6), dihomogammalinolenic (20:3 ω -6) and timnodonic (20:5 ω -3) acids, were obtained from Sigma. Mead acid (20:3 ω -9) was a generous gift from Professor H. Sprecher. [1-¹⁴C]5,8,11,14-20:4, [1-¹⁴C]5,8,11,14,17-20:5 and [2-¹⁴C]8,11,14-20:3 were purchased from Amersham International. Dextran 250 was provided by Sigma and the calcium ionophore A23187 by Boehringer-Mannheim. Silica gel G plates were purchased from Merck, and Nucleosil C₁₈ 3 μ m was obtained from Macherey-Nagel, Düren, Germany. Organic solvents

Abbreviations used: HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; (di)HETE, (di)hydroxy-5,8,11,14-eicosatetraenoic acid; LT, leukotriene; PUFA, polyunsaturated fatty acid.

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(analytical grade) were provided by SDS, Peypin, France.

Preparation of cell suspensions

Platelets were prepared according to a technique previously described (Lagarde *et al.*, 1979). Briefly, blood was collected onto acid citrate dextrose as anti-coagulant from normal donors who had not taken any drug for at least 10 days before the venipuncture. After centrifugation at 200 *g* for 15 min, platelet-rich plasma was removed and acidified to pH 6.4 before centrifugation at 900 *g* for 10 min. The pellets were then resuspended in a Tyrode/Hepes buffer, pH 7.35.

Dextran 250 (0.5 vol. of 3 % in 0.9 % NaCl) was added to the remaining blood obtained after removing platelet-rich plasma. Gravity sedimentation of red cells was allowed to occur for 90 min at room temperature and the upper phase, containing leukocytes, was centrifuged for 15 min at 80 *g*. The pellet (around 2×10^6 cells) was resuspended into 1 ml of distilled water for 15 s and isotonicity was recovered by adding 1 ml of double-strength Tyrode/Hepes. This suspension was centrifuged for 10 min at 60 *g* and leukocytes were resuspended in Tyrode/Hepes containing 2 mM-calcium.

Preparation of the hydroxylated fatty acids

12-Hydroxylated fatty acids from 20:3 ω -6, 20:3 ω -9, 20:4 ω -6 and 20:5 ω -3 were produced from platelets which were incubated separately with 100 μ M of the corresponding substrate including 14 C-labelled tracer doses for 30 min at 37 °C. After acidic lipid extraction with 10 vol. of diethyl ether, the hydroxylated fatty acids were separated by t.l.c. using the solvent mixture hexane/diethyl ether/acetic acid (60:40:1, by vol.). They were scraped off at R_f 0.13 and extracted with diethyl ether. HHT (12-OH-17:3) was produced similarly from 20:4 ω -6.

15-Hydroxy derivatives from 20:3 ω -6 and 20:4 ω -6 were produced by a soya bean lipoxygenase treatment according to the method of Funk *et al.* (1976).

Incubations of hydroxylated fatty acids with leukocytes

Each hydroxy fatty acid [HHT, 12-OH-20:4, 12-OH-20:3 (ω -6 and ω -9), 12-OH-20:5, 15-OH-20:4

and 15-OH-20:3 (ω -6)] was incubated separately at 5×10^{-5} M with 1 ml of leukocyte suspension (3×10^7 /ml) at 37 °C in the presence or absence of 10^{-6} M-ionophore A23187. Incubations were terminated by acidic lipid extraction with 10 vol. of diethyl ether containing 2 nmol of prostaglandin B₁ or B₂ as an internal standard. The organic phase was immediately removed to avoid any lactonization of 5-hydroxy derivatives, and evaporated under N₂.

Chromatographic separations

Lipid extracts were separated on silica gel G plates using the mixture hexane/diethyl ether/acetic acid (25:75:1, by vol.) as the eluent. The radioactive dihydroxylated compounds were detected with a Berthold radiometer, scraped off together with prostaglandins B, extracted with diethyl ether and evaporated under nitrogen before h.p.l.c. analysis.

Dihydroxy derivatives were then separated by reversed phase h.p.l.c. This was carried out with a 3.2 mm i.d., 15 cm length column packed with Nucleosil C₁₈ 3 μ m. The eluent was methanol/water (pH 3 with acetic acid), 7:3 (v/v) in most cases and 67:33 (v/v) when prostaglandin B₂ was the internal standard instead of prostaglandin B₁. This solvent was pumped at a flow rate of 0.7 ml/min. Compounds eluted were detected simultaneously at 234 nm and 270 nm, and were quantified according to their respective maximal u.v. absorbance (234 nm, $\epsilon = 3 \times 10^4$ M⁻¹·cm⁻¹ for 12*S*,20-dihydroxy derivatives; 270 nm, $\epsilon = 2.8 \times 10^4$ and 5×10^4 M⁻¹·cm⁻¹ for prostaglandins B and 5*S*,12*S*-dihydroxy derivatives, respectively) by using a Hewlett Packard diode array detector model 1040A.

RESULTS

After 10 min incubation of leukocytes with 5×10^{-5} M of various monohydroxylated PUFAs in the presence of 10^{-6} M-ionophore A23187, dihydroxy derivatives could be separated by t.l.c. and further analysed by h.p.l.c. (Fig. 1). The most prominent peak obtained from 12-HETE was 5*S*,12*S*-diHETE, having the same retention time as that of standard LTB₄, as reported previously (Borgeat *et al.*, 1982; Marcus *et al.*, 1982), but a different u.v. spectrum (5*S*,12*S*-diHETE, $\lambda_{\max}$. 259, 268, 279 nm;

Fig. 1. Reversed phase h.p.l.c. of dihydroxylated fatty acids produced by leukocytes (3×10^7 cells) incubated with 5×10^{-5} M-12-HETE in the presence of 10^{-6} M-calcium ionophore A23187 for 10 min

Dihydroxylated fatty acids and prostaglandin B₁ (PGB₁), the internal standard, were separated with a Nucleosil C₁₈ 3 μ m column and eluted by methanol/water (pH 3 with acetic acid) 7:3, v/v.

Fig. 2. U.v. spectra of 12S,20-diHETE (—), 5S,12S-diHETE (---) and LTB₄ (·····)

Spectra were obtained directly from the corresponding eluted compounds with a Hewlett-Packard diode array detector.

Fig. 3. Reverse phase h.p.l.c. of dihydroxylated fatty acids produced by leukocytes (3×10^7 cells) incubated with 5×10^{-5} M-12-HETE in the absence of calcium ionophore A23187 for 30 min

The analytical conditions were as in Fig. 1.

LTB₄, λ_{max} 261, 271, 282 nm) (Fig. 2). Two small peaks of slightly more polar compounds (Fig. 1) (shorter retention times) could be observed, presumably corresponding to minor isomers, as described previously (Borgeat *et al.*, 1982; Croset & Lagarde, 1983). Under these conditions, small amounts of another dihydroxylated product, slightly more polar than prostaglandin, appeared with a λ_{max} of 235 nm (Fig. 1). This product was most prominent when 12-HETE was incubated with leukocytes in the absence of calcium ionophore (30 min), whereas no 5S,12S-diHETE could be detected (Fig. 3). This product has been assumed to be 12S,20-diHETE as described by Marcus *et al.* (1984, 1987).

When 12-HETE was replaced by the 12-lipoxygenase product of 5,8,11-20:3 (20:3 ω -9) a similar pattern could be observed, but the corresponding products appeared less polar (Fig. 4). The u.v. spectra of these products were superimposable on those of 5S,12S-diHETE and 12S,20-diHETE respectively. Fig. 4 also shows small quantities of 5S,12S-diHETE, possibly formed from 12-HETE, as a contaminant of the initial

12-OH-5,8,10-20:3. In contrast to what was observed for 5S,12S-diHETE, very few more polar isomers of 5S,12S-diOH-20:3 (ω -9) were detected.

Again, when 12-lipoxygenase product of 5,8,11,14,17-20:5 (20:5 ω -3) was used instead of 12-HETE, similar amounts of more polar compounds, assumed to be 5S,12S-diOH-20:5 and 12S,20-diOH-20:5, were produced (results not shown). Their u.v. spectra were also superimposable on those of 5S,12S-diHETE and 12S,20-diHETE, respectively.

On a quantitative basis, the 5-lipoxygenase and the ω -lipoxygenase products of 12-HETE and 12-OH-20:3 (ω -9) were determined by using prostaglandin B₁ as an internal standard. For measuring the corresponding products from 12-OH-20:5, the more polar prostaglandin B₂ was used instead. The molar absorbancies (ϵ) were assumed to be 28000, 50000 and 30000 M⁻¹·cm⁻¹ for all prostaglandins B, 5S,12S-dihydroxy and 12S,20-dihydroxy derivatives, respectively. The results appearing in Table 1 show that 12-HETE, 12-OH-20:3 (ω -9) and 12-OH-20:5 were similarly transformed by both 5-

Fig. 4. Reverse phase h.p.l.c. of dihydroxylated fatty acids obtained from 12-OH-20:3 (ω -9) as described in Fig. 1.

Table 1. Formation of dihydroxy derivatives from various monohydroxylated fatty acids

The first two columns report those produced in the presence of 10^{-6} M calcium ionophore A23187 for 10 min; the third column reports those produced in the absence of ionophore for 30 min. Each compound was quantified by using a molar absorbance equal to that of the corresponding product from 12-HETE. Results are means $\pm$ S.E.M. Abbreviations: N.D., not detected; * different from 12-HETE ($P < 0.02$); † different from 12-OH-20:3 (ω -6) ($P < 0.02$).

Substrates	Dihydroxy derivative formation (nmol/ 10^8 leukocytes)		
	In presence of ionophore A23187		In absence of ionophore A23187
	5-Lipoxygenase products	ω -Hydroxylase products	ω -Hydroxylase products
12-HETE	24.3 ± 3.0 ($n = 8$)	2.4 ± 1.1 ($n = 8$)	15.8 ± 3.1 ($n = 8$)
12-OH-20:3 (ω -9)	25.6 ± 7.3 ($n = 5$)	1.4 ± 0.9 ($n = 5$)	21.5 ± 9.6 ($n = 5$)
12-OH-20:5 (ω -3)	20.6 ± 8.4 ($n = 5$)	0.8 ± 0.2 ($n = 5$)	13.2 ± 6.5 ($n = 4$)
12-OH-20:3 (ω -6)	N.D.	N.D.	$35.4 \pm 5.2^*$ ($n = 4$)
15-OH-20:4 (ω -6)	N.D.	N.D.	0.8 ± 0.6 ($n = 4$)
15-OH-20:3 (ω -6)	N.D.	N.D.	$3.4 \pm 2.2^{*\dagger}$ ($n = 4$)
12-OH-17:3 (HHT)	N.D.	N.D.	N.D.

lipoxygenase and ω -hydroxylase. Whereas no 5-lipoxygenase product could be detected in the absence of ionophore, a very small amount of ω -hydroxylase products was measured in ionophore-activated leukocytes.

As expected, because of the absence of a double bond at C-5, 12-OH-8,10,14-20:3 was not a substrate for the leukocyte 5-lipoxygenase (Table 1). Conversely, the product was efficiently oxygenated by the ω -hydroxylase. The conversion was even higher ($P < 0.02$) than that of 12-HETE. This contrasts with the quasi-absence of both 5-lipoxygenation and ω -hydroxylation of ω -6 oxygenated derivatives, like 15-OH-20:4, 15-OH-20:3 (ω -6) and HHT (Table 1). The absence of 5-lipoxygenation of 15-OH-20:4 and HHT, having a *cis* double bond at C-5, was rather unexpected.

DISCUSSION

The double oxygenation of PUFAs in a co-operative process between platelets and leukocytes is a good example of cell-cell interactions. This has been well investigated with arachidonic acid (Borgeat *et al.*, 1981, 1982; Marcus *et al.*, 1982, 1984, 1987). No data are available concerning other PUFAs of nutritional value, and such a study may reveal the substrate specificity of enzymes involved in the process.

From our work one can notice that 12-lipoxygenase products of polyeicosaenoic acids, having their first double bond at position 5 [12-HETE, its analogues 12-OH-20:3 (ω -9) and 12-OH-20:5], were similarly oxygenated by both leukocyte 5-lipoxygenase and

ω -hydroxylase. Surprisingly, 15-HETE (15-OH-5,8,11,13-20:4), which has also the potential to be a substrate for the 5-lipoxygenase, was not converted by this enzyme, at least under our conditions. Indeed, 15-HETE has been described as an inhibitor of leukocyte 5-lipoxygenase (Vanderhoeck *et al.*, 1982), and only a weak conversion has been found when leukocytes were stimulated by very high concentrations (38 μ M) of the calcium ionophore A23187 (Maas *et al.*, 1982). HHT (12-OH-5,8,10-17:3), was also not converted by the leukocyte 5-lipoxygenase. Although no explanation can be provided for this fact, we may speculate that since HHT (12-OH-5,8,10-17:3) has a *trans* double bond at C-8, instead of a *cis* double bond in other potential substrates of 5-lipoxygenase, this would inhibit the proton extraction at C-7, which is a crucial step in the action of 5-lipoxygenase. This does not exclude that HHT, being a ω -6 oxygenated product, would also inhibit 5-lipoxygenase, as reported for various analogues and homologues of 15-HETE towards 12-lipoxygenase (Mitchell *et al.*, 1984).

The ω -oxygenation of 12-HETE by leukocytes has been described as a cytochrome *P*-450-dependent process (Marcus *et al.*, 1984, 1987). This occurs with efficiency in unstimulated cells. Our results show that the conversion of 12-lipoxygenase products by this hydroxylase was much more active in unstimulated than in calcium-ionophore-stimulated leukocytes. The 5-lipoxygenation being a fast process in stimulated leukocytes, this is likely to reflect a decreased substrate availability for the ω -hydroxylation. Our results also reveal that the position of the hydroxyl group may be important for the ω -oxygenation. In particular, ω -6 oxygenated products, including 15-HETE, 15-OH-20:3 (ω -6) and 12-HHT, were very poor substrates of the ω -hydroxylase, in agreement with a recent report concerning 15-HETE (Soberman *et al.*, 1987). This may reveal a steric hindrance induced by the hydroxyl group located closer to the ω end, thus inhibiting the action of the enzyme.

We conclude from the data that leukocyte 5-lipoxygenase and ω -hydroxylase exhibit a certain substrate specificity towards monohydroxy derivatives of PUFAs. The position and the geometry of the double bonds as well as the position of the hydroxyl group appear to be limiting. These data are particularly relevant for the metabolism of 12-HETE and HHT during platelet-leukocyte interactions. Both compounds being formed in approximately equal amounts from endogenous 20:4 ω -6 in activated platelets, the former will be potentially further oxygenated by leukocyte 5-lipoxygenase and/or ω -hydroxylase whilst the latter will be virtually not metabolized. This also indicates that 15-HETE, mainly produced by leukocytes themselves, will not be metabolized via these pathways. The leukocyte oxygenation of other hydroxylated fatty acids used in the present

work might have some biological significance in drastic nutritional modifications involving the corresponding fatty acid precursors.

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