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Biochemical Pharmacology, Vol. 42, No. 4, pp. 959–962, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Inhibitory potencies of fish oil hydroxy fatty acids on cellular lipoxygenases and platelet aggregation

(*Received* 5 *August* 1989; *accepted* 1 *April* 1991)

Recent epidemiological studies suggest that the low incidence of thrombosis in Greenland Eskimos may be due to the large amounts of two polyunsaturated fatty acids (PUFAs)* in their diet, i.e. eicosapentaenoic acid [EPA, 20:5 (n-3)] and docosahexaenoic acid [DHA, 22:6 (n-3)] [1]. Most mammalian cells can lipoxygenate these PUFAs and some of the biological effects of these PUFAs are probably due to the effects of their metabolites. Platelets have been reported to convert EPA and DHA to 12-hydroxyeicosapentaenoic acid (12-HEPE) and 14-hydroxydocosahexaenoic acid (14-HDHE), respectively, whereas leukocytic 5- and 15-lipoxygenases (LOs) produce 5-HEPE and 15-HEPE and 7-HDHE and 17-HDHE, respectively [2–5]. Previous studies have shown that different hydroxyeicosatetraenoic acids (HETEs), derived from arachidonic acid (AA), can modulate cellular LOs and certain platelet functions [6, 7]. The present study examined the relative potencies of isomeric HEPEs and HDHEs on (a) exogenous AA metabolism in human and rat platelets and in human polymorphonuclear (PMN) leukocytes, and (b) aggregation of platelets from these species.

Materials and Methods

5(*S*)-HEPE, 12(*S*)-HEPE and 20:5 (n-3) were purchased from BioMol Research Laboratories, Inc. (Plymouth Meeting, PA). 14(*S*)-HDHE, 15(*S*)-HETE, 15(*S*)-HEPE, and 17(*S*)-HDHE were prepared as previously described [3, 8–10]. 22:6 (n-3) was obtained from Nuchek Prep, Inc. (Elysian, MN) and collagen from the Chrono-Log Corp. (Havertown, PA). Young adult male Wistar rats, approximately 300 g, were fed diets for 7 days. The corn oil (CO) diet contained 5% (w/w) CO plus basal diet (all other necessary nutrients; Teklad, Madison, WI), whereas the menhaden oil (MO)-supplemented diet contained 4% MO (Zapata-Haynie Corp., Reedville, VA) plus 1% CO by weight [11]. Human PMN leukocytes and platelets and rat platelets were isolated as previously described [6, 12, 13].

The effects of hydroxy fatty acids (HOFAs) on [^{14}C]AA metabolism in PMN leukocytes (2×10^7/mL) and platelets (5×10^7/mL) were determined by previously described methodologies [6, 13]. Aggregation studies were performed with gel-filtered platelets that had been recalcified with 1 mM $CaCl_2$ following centrifugation [12].

Results and Discussion

Human platelets (5×10^7/mL) metabolized exogenously added [^{14}C]AA (16 μM) to [^{14}C]12-HETE (29 ± 3.8%, N = 10), [^{14}C]12-hydroxyheptadecatrienoic acid (6.5 ± 0.63%, N = 9), and [^{14}C]thromboxane B_2 ([^{14}C]TXB_2) (2.6 ± 0.32%, N = 9) upon product separation and analysis by TLC. The identity of these metabolites was confirmed by comparison of HPLC retention times of authentic standards. When platelets were pretreated with various HEPEs, HDHEs or 15-HETE followed by the addition of [^{14}C]AA substrate, several of these HOFAs inhibited the platelet 12-LO as shown by decreased formation of [^{14}C]-12-HETE (Table 1). 15-HEPE was the most effective inhibitory HOFA tested, followed by 15-HETE and 17-HDHE, whereas the HOFAs produced by the 5- and 12-LOs did not appreciably inhibit the human platelet 12-LO at the concentrations tested (up to 50 μM). The relative inhibitory potencies of 15-HEPE and 17-HDHE contrast with the results reported by Mitchell *et al.* [9]. One possible explanation is that intact platelets, rather than a 105,000 *g* platelet supernatant, were used in the present studies. 17-HDHE, 15-HEPE and 15-HETE also inhibited the platelet cyclooxygenase pathway (measured as [^{14}C]TXB_2 formation) but this pathway was 3- to 10-fold less sensitive to the inhibitory effects of these acids than the 12-LO pathway. When the effects of these fatty acids were tested on the human PMN 5-LO, it was found that this enzyme was also less sensitive to inhibition than the 12-lipoyxgenase and that the order of inhibitory potencies was 15-HETE > 17-HDHE > 15-HEPE. Since the 5-LO pathway leads to leukotriene production [14], these findings indicate that fish oil HOFAs are less potent inhibitors of leukotriene production than the corresponding HOFA derived from

* Abbreviations: AA, arachidonic acid; CO, corn oil; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDHE, hydroxydocosahexaenoic acid; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; HOFA, hydroxy fatty acid; RP-HPLC, reverse-phase high pressure liquid chromatography; LO, lipoxygenase; MO, menhaden oil; PGE_2, prostaglandin E_2; PMN, polymorphonuclear; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography; and TXB_2, thromboxane B_2.

Table 1. Relative inhibitory potencies (I_{50}) of various fish oil hydroxy fatty acids and 15-HETE on arachidonic acid metabolism by the human platelet 12-lipoxygenase and cyclooxygenase and the PMN 5-lipoxygenase

	I_{50} (μM)					
Hydroxy fatty acid	Platelet 12-Lipoxygenase		Platelet Cyclooxygenase		PMN 5-Lipoxygenase	
15-HEPE	2.1 ± 0.79*	(5)	34 ± 8.7	(7)	36 ± 3.2†	(4)
15-HETE	4.2 ± 0.90	(8)	50 ± 14	(6)	6.9 ± 1.1	(12)
17-HDHE	7.0 ± 1.8	(7)	24 ± 7.9	(6)	20 ± 3.7†	(3)
5-HEPE	>70	(2)	>70	(2)	>50	(3)
12-HEPE	>50	(2)	>50	(2)	>50	(2)
14-HDHE	>50	(3)	>70	(3)	>50	(4)

Results are the means ± SEM of the I_{50} values of the HOFAs (range tested: 1–70 μM) on the platelet 12-LO and cyclooxygenase and the PMN 5-LO as measured by the metabolism of [^{14}C]AA (16 μM) to [^{14}C]12-HETE, [^{14}C]TXB_2 and [^{14}C]5-HETE, respectively. The numbers in parentheses represent the number of separate experiments. Product formation was measured by TLC [6].

*,† Statistically different from the I_{50} of 15-HETE: * $P < 0.05$, and † $P < 0.001$.

AA. These results also confirm previous suggestions that the active sites of the 5- and 12-LOs are probably different since there are different structural requirements in the HOFA for its optimum interaction at the catalytic site of these enzymes [15]. In general, it appears that the 15-LO metabolites are the most potent inhibitors of the 5- and 12-LOs.

To determine whether dietary incorporation of EPA and DHA into platelet lipids could affect the inhibitory effectiveness of the fish oil HOFAs on the platelet oxygenases, rats were fed diets supplemented with either CO or MO. After 7 days on these diets, the animals were killed, and platelets were isolated. The concentration of n-6 PUFAs in platelet lipids (20:4 and 22:4) decreased in the MO-supplemented diet (from 20 and 3.0% to 14 and 0.74%, respectively), whereas the levels of N-3 PUFAs (20:5, 22:5 and 22:6) in these lipids increased with this diet (from 0.3, 0.47 and 0.51% to 8.1, 2.8 and 1.2%, respectively). These results correlate with observations by others [16]. When the metabolite profile obtained from rat platelets and exogenously added [^{14}C]AA was examined by RP-HPLC, it was found that the two major AA metabolites were [^{14}C]12-HETE and [^{14}C]prostaglandin E_2 ([^{14}C]PGE_2. In platelets from CO-supplemented rats, these metabolites accounted for 22.8 ± 3.7% and 3.9 ± 0.30%, respectively, of the recovered radioactivity (N = 8), and these values did not differ significantly from the values obtained with cells from MO-supplemented rats [21.8 ± 3.6 and 3.3 ± 0.32% (N = 8), respectively]. [^{14}C]TXB_2 appeared to be a minor metabolite since it was only detected in two out of six different experiments where it represented one-sixth of the amount of PGE_2 formed. Next, the effects of the more potent HOFAs on the rat platelet 12-LO were examined. With platelets from rats fed the CO-supplemented diet, the order of inhibitory potencies was 15-HEPE ≈ 15-HETE > 17-HDHE (Table 2). Platelets from MO-fed rats were more refractory and only 15-HEPE was able to inhibit the 12-LO with an I_{50} of less than 100 μM. The 12-LO in rat platelets was at least 10-fold less sensitive to HOFA inhibition than the corresponding enzyme in humans. None of these HOFAs ($I_{50} > 80$ μM) inhibited [^{14}C]PGE_2 production in platelets obtained from rats on either diet.

The effects of these five fish oil HOFAs and 15-HETE on platelet aggregation induced by submaximal concentrations of collagen were also examined (Table 3). 15-HETE and 14-HDHE were the strongest inhibitors (I_{50} = 0.6 to 0.7 μM) of platelet aggregation from human donors on a normal diet, followed in decreasing order of potencies by 17-HDHE > 12-HEPE ≈ 15-HEPE > 5-HEPE. When the collagen-induced aggregation of platelets from rats fed diets supplemented with either CO or MO was examined, the results from three of four experiments showed that platelets from MO-fed rats exhibited a reduced tendency to aggregate. Platelets from rats on a MO diet required more collagen (0.8 ± 0.08 μg/mL, N = 4) to produce a 50% change in light transmission than cells from rats on a CO diet (0.6 ± 0.05 g/mL, N = 4). A possible explanation for these results is that CO-derived platelets produce more TXA_2 than MO-derived platelets [16]. Indirect evidence to support this hypothesis is our observation that collagen (3 μg/mL) induced a 4-fold greater release of AA from CO-derived platelets (567 ± 314 ng/3 × 10^8 cells, N = 3) than from MO-derived platelets (133 ± 29 ng/3 × 10^8 cells, N = 3). However, platelets from animals on either diet showed about the

Table 2. Relative inhibitory activities (I_{50}) of two fish oil hydroxy fatty acids and 15-HETE on arachidonic acid metabolism by platelet 12-lipoxygenase from rats fed either a corn oil- or a menhaden oil-supplemented diet

	Inhibitory potency on platelet 12-lipoxygenase from rats fed:			
	Corn oil diet		Menhaden oil diet	
Hydroxy fatty acid	I_{50} (μM)			
15-HEPE	47 ± 15	(4)	73 ± 20	(4)
15-HETE	55 ± 13	(4)	>100	(4)
17-HDHE	>100	(3)	>100	(3)

Results are the means ± SEM of the I_{50} values of the HOFAs on platelet 12-LO as measured by the formation of [^{14}C]12-HETE from [^{14}C]AA using a protocol similar to that described in the legend of Table 1. The number of experiments is given in parentheses. Platelets were obtained from rats fed either a CO- or a MO-supplemented diet.

Table 3. Relative antiaggregatory potencies (I_{50}) of various fish oil hydroxy fatty acids and 15-HETE on collagen-induced aggregation in human and rat platelets

Hydroxy fatty acid	Antiaggregatory potency on platelets from Human on normal diet		Rats on Corn oil diet		Rats on Menhaden oil diet	
	I_{50} (μM)					
15-HETE	0.7 ± 0.15	(3)	0.6 ± 0.16	(4)	0.3 ± 0.08	(4)
14-HDHE	0.6 ± 0.32	(3)	0.6 ± 0.12	(3)	0.4 ± 0.09	(3)
17-HDHE	3.4 ± 0.66*	(3)	1.6 ± 0.18†	(4)	1.3 ± 0.15†	(4)
12-HEPE	5.8 ± 0.75‡	(3)	3.3 ± 0.29†	(3)	3.0 ± 0.26†	(3)
15-HEPE	6.2 ± 1.3*	(3)	2.7 ± 0.25†	(3)	2.5 ± 0.24†	(4)
5-HEPE	11.3 ± 1.6‡	(3)	5.4 ± 0.20†	(3)	5.3 ± 0.44†	(3)

Results are the means ± SEM of the I_{50} values of the HOFAs on platelet aggregation induced by an amount of collagen to produce a submaximal response of approximately 50% change in light transmission. The number of experiments is given in parentheses. The collagen dose was 0.2 μg/mL (N = 3) for the human platelet studies, 0.6 ± 0.05 μg/mL (N = 4) for platelets from the CO-fed rats and 0.8 ± 0.08 μg/mL (N = 4) for platelets from MO-fed rats. Aggregation studies were conducted as described in Ref. 12. A pooled batch of platelets from two rats was used in each rat aggregation experiment. Each HOFA was added in ethanol (final concentration 0.5%) 1 min prior to the addition of collagen.

*–‡ Statistically different from the I_{50} of 15-HETE: * $P < 0.02$, † $P < 0.001$, and ‡ $P < 0.01$.

same sensitivity to inhibition by these HOFAs. Since collagen-induced platelet aggregation in both humans and rats seems to involve TXA_2 formation [17], it is not surprising that the magnitude and order of antiaggregatory potencies of these HOFAs on human and rat platelets are quite similar. The approximately 20- to 100-fold differences in the antiaggregatory effectiveness and the rat 12-LO inhibitory potencies of these HOFAs and similar differences observed with human platelets suggest that this antiaggregatory action does not involve the 12-LO pathway but could be due to interference with the prostaglandin endoperoxide/TXA_2 receptor site [18, 19], platelet phospholipase A_2 [20] or adenylate cyclase.

In summary, the presence of dietary EPA and/or DHA can lead to the formation of certain hydroxylated metabolites which are capable of (1) regulating both platelet and PMN lipoxygenases, and (2) inhibiting platelet aggregation.

Acknowledgements—This work was supported by a grant from the National Institutes of Health, 5R01 HL39168. The authors wish to thank Dr. J. P. Church and Mr. T. C. Simon for technical assistance.

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