Characterization and biological effects of di-hydroxylated compounds deriving from the lipoxygenation of ALA

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Abstract We have recently described a di-hydroxylated compound called protectin DX (PDX) which derives from docosahexaenoic acid (DHA) by double lipoxygenation. PDX exhibits anti-aggregatory and anti-inflammatory properties, that are also exhibited by similar molecules, called poxytrins, which possess the same E,Z,E conjugated triene geometry, and are synthesized from other polyunsaturated fatty acids with 22 or 20 carbons. Here we present new biological activities of di-hydroxylated metabolites deriving from α-linolenic acid (18:3n-3) treated by soybean 15-lipoxygenase (sLOX). We show that 18:3n-3 is converted by sLOX into mainly 13(S)-OH-18:3 after reduction of the hydroperoxide product. But surprisingly, and in contrast to DHA which is metabolized into only one di-hydroxylated compound, 18:3n-3 leads to four di-hydroxylated fatty acid isomers. We report here the complete characterization of these compounds using high field NMR and GC-MS techniques, and some of their biological activities. These compounds are: 9(R),16(S)-dihydroxy-10E,12E,14E-octadecatrienoic acid, 9(S),16(S)-dihydroxy-10E,12E,14E-octadecatrienoic acid, 9(S),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic acid, and 9(R),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic acid. They can also be synthesized by the human recombinant 15-lipoxygenase (type 2). Their inhibitory effect on blood platelet anti-inflammatory properties were compared with those already reported for PDX.—Liu, M., P. Chen, E. Véricel, M. Lelli, L. Béguin, M. Lagarde, and M. Guichardant. Characterization and biological effects of di-hydroxylated compounds deriving from the lipoxygenation of ALA. J. Lipid Res. 2013. 54: 2083–2094.

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N-3 polyunsaturated fatty acids (PUFAs) have been well studied during the last decades because they regulate in vivo many physiological functions. It is assumed that long-chain n-3 PUFAs have beneficial effects on cardiovascular and coronary artery diseases (1, 2), rheumatoid arthritis (3–5), blood pressure control and heart hypertension (6, 7), and they might prevent multiple sclerosis and Alzheimer’s disease (8–11). More recently, new lipid mediators deriving from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been described. Among them, resolvins are oxygenated molecules that derive from EPA and DHA (12, 13) and are separated into two series: E-series (from EPA) and D-series (from DHA) (14). In addition to the D-series resolvins, protectin D1 (PD1) is produced from DHA via a mechanism involving an epoxide intermediate (15), as well as maresin which can be produced by macrophages (16). PD1 has been described as a potent anti-inflammatory agent without an anti-aggregatory effect (15, 17). In contrast, protectin DX (PDX), an isomer of PD1, which derives from DHA via a double lipoxygenation, reveals inhibitory effects on blood platelet aggregation. PDX inhibits platelet cyclooxygenase (COX)-1 and antagonizes the thromboxane A2-induced aggregation (18). We found that di-hydroxylated compounds called poxytrins, synthesized from C20 and C22 PUFAs via the soybean 15-lipoxygenase (sLOX) and having the same E,Z,E conjugated triene motif, including PDX, are all anti-aggregatory agents (19). On the other hand, very few data are available concerning the oxygenated metabolism of α-linolenic acid (ALA) (18:3n-3). Investigations relating to ALA are particularly relevant because...
this fatty acid is taken in large amounts (1–2 g/day in human adults) as a nutrient and hardly accumulates in plasma, suggesting an active metabolism. Moreover, its conversion into EPA and DHA is relatively limited. A conversion of ALA into its long-chain product DHA was found to be around 1% in men (20) and 2% in young women (21).

Formation of 9,16-dihydroxyoctadecatrienoic acid (9,16-di-HOTE) isomers from ALA by sLOX-2 have already been reported first in 1984 by Feiters et al. (22) without establishing the double bond configuration or the stereochemistry of the asymmetric carbons. Further studies presented by Sok and Kim (23, 24) reported four isomers, but again their characterization was not fully established, especially that of the all trans isomers. In 1991 Grechkin et al. (25) showed that potato tuber lipoxygenase (LOX) generated mainly 9(S)-hydroperoxy-octadeca-10E,12Z,15Z-trienoic acid [9(S)-HpOTE] which is different from the sLOX which produces mainly 13(S)-hydroperoxy-9Z,11E,15Z-octadecatetraenoic acid [13(S)-HpOTE]. Also, two compounds after reduction by NaBH₄ were characterized as 9,16-di-HOTE (10E,12Z,14E) except for the stereochemistry of the carbon 16 which remains undetermined and trans isomers were not detected. Moreover, the biological properties of the 9,16-di-HOTE isomers have not been investigated yet. Because ALA is the most abundant n-3 PUFAs in human diet, it is relevant to investigate more thoroughly its oxygenated metabolism, especially by 15-LOX that is expressed in blood leukocytes and most endothelial cells, and compare the biological activity of the metabolites to that of PDX, the main 15-LOX product of DHA.

MATERIALS AND METHODS

Materials

ALA, sLOX (E.C. 1.13.11.12, Type I-B, 131,000 units/mg), platinum oxide (PtO₂), N,N,N,N,N-trimethyl-N,N-difluoroacetamide, COX-1, and COX-2 (E.C. 1.14.99.1) were from Sigma-Aldrich. Human recombinant 15-LOX-2 (0.23 units/mg), prostaglandin D₂ (PGD₂)-δ₁ and prostaglandin E₂ (PGE₂)-δ₁ were from Cayman. 9(S)-hydroxy-10E,12Z,15Z-octadecatetraenoic acid [9(S)-HOTE], 9(S)-HpOTE, and 13(S)-hydroxy-9Z,11E,15Z-octadecatetraenoic acid [13(S)-HOTE] were from Interchim. Organic solvents were from Carlo-Erba. All chemicals used were reagent grade or the highest quality available.

Biosynthesis of 9,16-dihydroxy-ALA derivatives

ALA was incubated with sLOX (type I-B) in sodium-borate buffer. Synthesized hydroperoxides were reduced by sodium borohydride (NaBH₄) as previously described (18). After acidification, mono- and di-hydroxylated fatty acids were extracted on a C18 solid-phase cartridge as previously described (18). Commercial 9(S)-HOTE, 9(S)-HpOTE, and the homemade racemic 9(±)-HOTE chemically produced by oxygen treatment of ALA and isolated by reverse-phase high-performance liquid chromatography (RP-HPLC) were further treated by sLOX in order to generate 9,16-di-HOTEs with defined stereochemistry.

Purification of mono- and di-hydroxylated fatty acids

Mono- and di-hydroxylated fatty acids were analyzed by RP-HPLC on a Waters XBridge C18 column (4.6 × 250 mm, 3.5 μm) by using a linear solvent gradient: solvent A was a mixture of acetonitrile/water acidified to pH 3 (10/90, v/v), and solvent B was acetonitrile. The flow was set at 1 ml/min. Mono- and di-hydroxylated fatty acids were detected with a diode array detector at 235 nm and 270 nm, respectively, and collected separately.

GC-MS analysis of mono- and di-hydroxylated fatty acids

Mono- and di-hydroxylated fatty acids isolated by RP-HPLC were hydrogenated using PtO₂ as a catalyst, and then derivatized into methyl esters and TMS ethers. Samples were then analyzed by GC-MS using the electron impact ionization (EI) mode in order to localize the hydroxyl groups on the fatty chain (18).

NMR of 9,16-di-HOTEs

The NMR experiments were performed on a Bruker-Biospin NMR spectrometer operating at 23.4 Tesla (1,000 MHz of Proton Larmor frequency) and equipped with a Cryoprobe. All the spectra were acquired in a CDCl₃ solution, working at 298 K and referencing the chemical shifts to tetramethylsilane as an internal standard. Standard sequences were used for 1D, DQF COSY (26–28), and J-resolved (29–31) spectra. The H pulse lengths of 9.0 μs, 1 s, and 5 s were used as recycle delay for J-resolved and COSY spectra. A weak selective decoupling of 270 Hz of power during the acquisition time (900 ms) was used in 1D selective homo-decoupling experiments to simplify some coupling patterns. 2D DQF COSY was acquired with 8 scans and 2,048 × 1,024 real points (F₂ × F₁) with acquisition times of 114 ms and 57 ms for the direct and indirect dimensions. J-resolved 2D was acquired with 16 scans and 8,192 × 256 real points (F₂ × F₁) in the direct dimension and 1,280 ms in the indirect J-coupling dimension. COSY spectra were processed with a 4,096 × 4,096 point matrix using square cosine and square sine window function for the direct and indirect dimensions. J-resolved 2D spectra were processed with an 8,192 × 2,048 point matrix with square cosine window function for both the dimensions.

Incubation of ALA, 9(S)-HOTE, and 9(±)-HOTE with human recombinant 15-LOX-2

ALA, 9(S)-HOTE, and 9(±)-HOTE were incubated separately with human recombinant 15-LOX-2 in Tris-HCl buffer as previously described (32, 33). Resulting hydroperoxides were reduced by NaBH₄. Lipids were extracted after acidification to pH 3 with acetic acid and di-hydroxylated metabolites were purified as described above.

Preparation of cell suspensions

Platelet suspensions. Venous blood from healthy volunteers who had not taken any medication for at least one week was collected at the local blood bank (Etablissement Français du Sang). Blood was drawn onto anticoagulant and centrifuged at 200 g for 15 min at 20°C to obtain the platelet-rich plasma upper phase, and platelets were isolated as described previously (34). Briefly, platelet-rich plasma was acidified to pH 6.4 with 0.15 M citric acid and immediately centrifuged at 900 g for 10 min, and platelet pellets were suspended into Tyrode-HEPES buffer (140 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 12 mM NaHCO₃, 0.41 mM NaH₂PO₄, 5 mM HEPES, and 5.5 mM glucose, pH 7.35).

Leukocyte suspensions. Leukocytes were isolated from blood obtained from Etablissement Français du Sang, as previously described (35). Dextran (2% final concentration) was added to the lower phase obtained after the first blood centrifugation at 200 g. Erythrocytes were then sedimented under gravity for 45 min at 15°C. The erythrocyte-depleted supernatant containing leukocytes
was centrifuged at 400 g for 10 min. The resulting leukocyte-rich pellets were resuspended into saline buffer, and layered over a Ficoll-Paque Plus (density 1.077). After centrifugation, the fraction above the erythrocyte layer was removed and centrifuged, and resuspended in water for 20 s to lyse the contaminant erythrocytes. Osmotic pressure was immediately restored with NaCl, followed by centrifugation, which provided a white pellet consisting of whole leukocytes that was resuspended into Tyrode-HEPES buffer. The morphological examination and trypan blue exclusion tests were performed to determine the cell count and purity, and evaluate the viability of neutrophils.

**Measurement of platelet aggregation and incubation with human leukocytes**

Blood platelet aggregation was determined by the turbidimetric method of Born (36) using a dual aggregometer (ChronoLog, Havertown, PA). To measure changes in the light transmission rate, 400 μl of platelet suspension was heated at 37°C for 1 min with stirring, incubated in the presence or absence of di-hydroxylated compounds (added in 1 μl of ethanol) for one more minute, and then with collagen as a platelet aggregatory agent for 4 min at 37°C. Concentrations of collagen were adjusted to obtain nearly 75% aggregations for control platelets.

Isolated human leukocytes, suspended in a Tyrode-HEPES buffer containing 2 mM Ca²⁺ were preincubated in the presence or absence of 1 μM of different 9,16-di-HOTE isomers for 3 min at 37°C, and triggered with 1 μM ionophore A23187 plus 10 μM arachidonic acid for 10 min. The reaction was stopped by acidifying the media to pH 3 with acetic acid. The oxygenated metabolites were then extracted as previously described (18).

**GC-MS measurement of purified COX-1 and COX-2 products**

Commercial COX-1 (40 units) and COX-2 (40 units) were preincubated for 15 min in a 0.1 M Tris-HCl buffer pH 8.1 with or without 1 μM of different di-hydroxylated metabolites and incubated for 10 min with 10 μM arachidonic acid used as a substrate. Albumin (45 g/l) was added at the end of incubation to facilitate the formation of PGD₂ and PGE₂ from PGH₂ (37). After acidification to pH 3 with acetic acid, PGE₂ and PGD₂ were extracted by 10 vol of diethyl ether stabilized by 10% ethanol. Deuterated PGD₂ and PGE₂ used as internal standards were added for the quantification. Prostaglandins were derivatized into methoxime, pentafluorobenzyl esters, and TMS and further analyzed by negative-ion chemical ionization GC-MS. PGD₂ and PGE₂ were measured using the SIM mode. Selected ions corresponding to [M-181]⁻ (loss of the pentafluorobenzyl group): m/z 524 for both derivatized PGD₂ and PGE₂ and m/z 528 for their corresponding deuterated internal standards (38, 39) were measured.

**Statistical analysis**

All results are expressed as means ± standard error of the mean (SEM) or means ± standard deviation (SD). P < 0.05 was set as the level of significance as determined by two-tailed Student’s t-tests. One-way ANOVA analysis was used to assess differences between groups.

![Fig. 1. Typical RP-HPLC chromatogram of ALA metabolites](image-url)

ALA was incubated with sLOX, and further treated by NaBH₄ to reduce hydroperoxides into hydroxylated derivatives. The lipid extract was then analyzed by RP-HPLC. Mono-hydroxylated fatty acids (A) and di-hydroxylated fatty acids (B) were detected at λ = 235 nm and 270 nm, respectively. On the right side of the chromatograms, the UV spectra acquired on the top of chromatographic peaks are reported. mAU, milli absorbance units.
Localization by GC-MS of hydroxyl groups on the fatty chain

Mono- and di-hydroxylated compounds from ALA mentioned above were hydrogenated and derivatized as methyl esters and TMS ethers as described in Materials and Methods and analyzed by GC-MS using the EI mode.

The EI mass spectrum of the hydrogenated mono-hydroxylated ALA derivative (Fig. 2A) shows characteristic ions at: m/z 371 (M-15) loss of CH₃, m/z 315 (M-71) which corresponds to the loss of CH₂(CH₂)₃CH₃, and m/z 173 [TMS-O-CH-(CH₂)₄CH₃]. Such a fragmentation pattern clearly indicates the presence of the hydroxyl group on carbon 13 of the fatty chain. So ALA was mainly converted into 13(S)-HpOTE, further reduced by NaBH₄ into hydroxylated product for analytical purposes.

All four hydrogenated di-hydroxylated compounds from ALA exhibited the same mass spectra (Fig. 2B). Their fragmentation pattern shows the following characteristic ions: at m/z 459 (M-15, loss of CH₃); at m/z 445 (M-29, loss of CH₂CH₃); at m/z 317 (M-157, loss of CH₂(CH₂)₆COOCH₃); at m/z 259 [TMS-O-CH(CH₂)₇COOCH₃]; at m/z 131 (TMS-O-CH(CH₂)CH₃); and at m/z 73 (TMS) which indicates an oxygenation at carbons 9 and 16.

**Fig. 2.** Electron impact mass spectrum of the Me-TMS derivative of the hydrogenated HOTE (A) and hydrogenated di-HOTE (B) synthesized from ALA by sLOX followed by reduction of hydroperoxides into hydroxylated derivatives by NaBH₄ treatment.
Determination of carbon stereochemistry of di-hydroxylated metabolites synthesized from ALA

Commercial 9(S)-HOTE and racemic 9(±)-HOTE, prepared as described in Materials and Methods, were incubated separately with sLOX as described with ALA, and further reduction by NaBH₄, 9(S)-HOTE was metabolized into a main compound eluted at 33.4 min (Fig. 3A). This peak was attributed to 9(S),16(S)-di-HOTE because sLOX produces almost exclusively the S enantiomer via a double dioxygenation mechanism (40), although soybean LOX generates almost exclusively 15(S)-HpOTE from ALA (40) as it was already shown (Fig. 1A). On the other hand, the 9(R),16(S)-di-HOTE peak, eluting at 33.7 min, was also formed (Fig. 3B) when racemic 9(±)-HOTE was incubated with sLOX, compared with 9(S)-HOTE. The two diastereoisomers 9(S),16(S)-di-HOTE and 9(R),16(S)-di-HOTE eluted at 33.4 min and 33.7 min (Fig. 3B), respectively, exhibited the same intensity in agreement with the equivalent amounts of 9(S)- and 9(R)-HOTE present in the racemic mixture. The latter chromatogram (Fig. 3B) is similar to that presented in Fig. 1B, suggesting that the two peaks (retention time 33.2 and 33.5 min, Fig. 1B) observed with ALA as a substrate correspond to the two diastereoisomers 9(S),16(S)-di-HOTE and 9(R),16(S)-di-HOTE, respectively.

This assignment was also confirmed by the fact that chromatograms of di-hydroxylated isomers from 9(S)-HpOTE and 9(S)-HOTE were almost identical, but different from those with ALA as a substrate (Fig. 4). As a matter of fact, another major E/Z,product (retention time 33.5 min, see also Fig. 1B) is likely 9(R),16(S)-di-HOTE. In addition, the same compound appeared when the racemic 9(±)-HOTE was incubated with sLOX (Fig. 3B, retention time 33.7 min).

However, we may assume that the two all trans diastereoisomers synthesized from ALA, as this will be confirmed by NMR below (retention time 32.1 and 32.6 min) (Fig. 1B), were likely to be all trans 9(R),16(S)-di-HOTE and 9(S),16(S)-di-HOTE, respectively, according to the separation of 8(R),15(S)- and 8(S),15(S)-all trans-dihETEs reported in (41, 42). Such an elution order has also been established for the separation of 5(S),12(R)- and 5(S),12(S)-all trans LTB₄ and 5(S),12(R)- and 5(S),12(S)-all trans LTB₄ diastereoisomers (43–45).

NMR determination of the conjugated triene double bond geometry

A NMR investigation was performed to determine the E/Z configuration in the triene moiety. A change in the E/Z configuration significantly modifies the ¹H₋¹H J-couplings among the protons bound to the sp² carbons in C=C double bonds, assuming values around ~17 Hz for E configuration and around ~10 Hz for Z configuration (46). Furthermore, molecules like 9(S),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic acid (33.2 min, Fig. 1B) and 9(R),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic acid...
shift. Nevertheless, because the determination of the configuration in the $E,Z,E$ molecules is demonstrated above, we can safely assign by exclusion the configuration of the $E,E,E$ molecules on the basis of the marked differences among the spectra of $E,E,E$ and $E,Z,E$.

**Metabolism of ALA by human recombinant 15-LOX-2**

This experiment was done to check whether the human recombinant 15-LOX type 2 is also able to synthesize such metabolites. For this purpose ALA, 9(S)-HOTE, and 9(±)-HOTE were separately incubated with this enzyme. We found that human recombinant 15-LOX-2 is able to convert ALA into 13-HOTE as previously described with the sLOX (result not shown). However, ALA was less markedly metabolized into di-hydroxylated fatty acids, the main ones being all trans 9,16-di-HOTE isomers. Interestingly enough, the mono-hydroxylated derivatives 9(S)-HOTE and 9(R)-HOTE were well converted into 9(S),16(S)-$E,Z,E$-di-HOTE and 9(R),16(S)-$E,Z,E$-di-HOTE, respectively (result not shown) by the human recombinant LOX.

**Inhibitory effect of 9,16-di-HOTEs on COX-1 and anti-aggregatory effects**

The inhibition of sheep COX-1 by different 9,16-di-HOTE isomers was tested at 1 μM by measuring both PGD$_2$ and PGE$_2$ as described in Materials and Methods. Results reported in Table 1 show that 1 μM of 9(S),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic and 9(R),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic acids decreased significantly the synthesis of both PGD$_2$ and PGE$_2$ by around 28% and 38%, respectively. Such results are in agreement with previous data showing that poxytrins, characterized by a $E,Z,E$
Fig. 5. 1D spectra and J-couplings for 9(S),16(S)-dihydroxy-octadeca-10E,12E,14E-trienoic acid (A) and 9(R),16(S)-dihydroxy-10E,12E,14E-octadecatrienoic acid (B). Resonance assignment and J-couplings were determined from the analysis of a DQF COSY, and 2D J-resolved spectra (data not shown). Spectra were acquired with eight scans and 2 s of recycle delay, the acquisition time was 1.8 s. Spectra were processed with exponential line broadening of 0.3 Hz as window function.
Fig. 6. 1D spectra and J-couplings for 9(S),16(S)-dihydroxy-octadeca-10E,12Z,14E-trienoic acid (A) and 9(R),16(S)-dihydroxy-octadeca-10E,12Z,14E-trienoic acid (B). Resonance assignments and J-couplings were determined from the analysis of a DQF COSY and 2D J-resolved spectra (data not shown). Spectra were acquired with eight scans and 2 s of recycle delay, the acquisition time was 1.8 s. Spectra were processed with exponential line broadening of 0.3 Hz as window function.
Di-hydroxylated metabolites from ALA 2091

triene geometry, are inhibitors of platelet COX-1 (19), whereas the all trans isomers are inactive.

Such results are in agreement with data relating to platelet aggregation induced by collagen. Platelet aggregation was significantly decreased by 64% and 65% in presence of 1 μM 9(S),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic acid and 9(R),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic acid, respectively (Fig. 7). In addition, the aggregation started to be reversed from 2 min following the agonist addition. As previously shown (19), all trans isomers had no effect on platelet aggregation.

Anti-inflammatory effect of 9,16-di-HOTEs

After showing that 9,16-dihydroxy-10E,12Z,14E-octadecatrienoic acid isomers possess anti-aggregatory properties as those previously described for poxytrins (19), it was important to know whether such di-hydroxylated compounds issued from ALA may exhibit anti-inflammatory properties. We then investigated the effects of 9,16-di-HOTEs on COX-2 as well as the 5-LOX pathway.

**Effect of 9,16-di-HOTEs on human recombinant COX-2.**

The inhibition of human recombinant COX-2 by 1 μM of 9,16-di-HOTE isomers was assessed by measuring PGD<sub>2</sub> and PGE<sub>2</sub> as described above. Results reported in Table 1 show that 9(S),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic and 9(R),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic acids significantly inhibited COX-2 activity by 9% and 21%, respectively.

**TABLE 1. Effect of 9,16-di-HOTEs on cyclooxygenase-1 and -2**

|         | Control | 9(S),16(S)-di-HOTE | 9(R),16(S)-di-HOTE |
|---------|---------|-------------------|-------------------|
| COX-1   | 134.9 ± 15.8 | 96.8 ± 14.0<sup>a</sup> | 83.2 ± 9.0<sup>b</sup> |
| (PGD<sub>2</sub> + PGE<sub>2</sub>) |         |                   |                   |
| COX-2   | 136.1 ± 11.0 | 124.5 ± 8.8<sup>a</sup> | 107.4 ± 7.2<sup>b</sup> |
| (PGD<sub>2</sub> + PGE<sub>2</sub>) |         |                   |                   |

Purified COX-1 and COX-2 were incubated with or without 1 μM of different 9,16-di-HOTE isomers and with 10 μM arachidonic acid. PGD<sub>2</sub> and PGE<sub>2</sub> were measured by GC-MS. Results expressed in ng of PGD<sub>2</sub> and PGE<sub>2</sub> represent the mean ± SEM of four determinations. Only the inhibitory effects of E,Z,E isomers are shown.

<sup>a</sup>P < 0.05.

<sup>b</sup>P < 0.01 versus control.

**Effect of 9,16-di-HOTEs on the metabolism of arachidonic acid by the human polymorphonuclear leukocyte 5-LOX.**

The inhibition of the human 5-LOX pathway by 1 μM of 9,16-di-HOTE isomers was assessed by measuring the following metabolites: 5(S)-HETE, LTB<sub>4</sub> and its two all trans isomers. As reported in Table 2, only the 9(R),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic acid isomer significantly decreased the formation of leukotrienes and 5(S)-HETE by around 10% and 20%, respectively. All other isomers did not inhibit such a production (results not shown). Such data suggest that the inhibition concerns only 5-LOX but not leukotriene A<sub>4</sub> hydroxylase. Taken together, these data suggest that the 9(R),16(S)-dihydroxy-10E,12Z,14E-octadecatrienoic acid isomer may have a potential anti-inflammatory effect.

**DISCUSSION**

ALA is an essential fatty acid present in vegetable oils, and as such available in human diet. It is the precursor of EPA and DHA, but its conversion into EPA and DHA is rather limiting with around 10% to the former and a further 10% to the latter, which means only 1% conversion of ALA to DHA (47, 48). ALA is also rather well oxidized (49, 50), that could partially explain its low accumulation in plasma and tissues. In addition, we describe here a pathway which could explain some beneficial effects of ALA in the vascular system at large by reducing both the
TABLE 2. Effect of 9,16-di-HOTEs on human leukocyte 5-LOX

|                | Control | 9(R),16(S)-di-HOTE E.Z.E |
|----------------|---------|--------------------------|
|                 |          |                          |
| LTB<sub>4</sub>| 9.57 ± 0.66 | 8.78 ± 0.78*              |
| 5-HETE         | 0.89 ± 0.03 | 0.71 ± 0.05*              |
| 5-LOX products | 10.46 ± 0.68 | 9.49 ± 0.81*              |

Isolated human leukocytes were incubated in the presence or absence of 1 μM of different 9,16-di-HOTE isomers at 37°C and triggered with 1 μM of ionophore A23187 and 10 μM of arachidonic acid. Oxygenated metabolites were then extracted and quantified by RP-HPLC. Results expressed in nmol of total LTB<sub>4</sub> (LTB<sub>4</sub> + LTB<sub>1</sub>) isomers and 5-HETE represent the mean ± SEM of five determinations. Only the inhibitory effect of 9(R),16(S)-di-HOTE is shown.

*P< 0.05, versus control.

production of pro-inflammatory eicosanoids PGD<sub>2</sub>/PGE<sub>2</sub> and LTB<sub>4</sub> (51–53). We recently showed that a new di-hydroxylated metabolite from DHA called PDX, which is an isomer of the anti-inflammatory PD1, exhibits anti-aggregatory properties, both by inhibiting collagen-induced prostaglandin production, including thromboxane A<sub>2</sub>, and thromboxane A<sub>2</sub>-induced aggregation (19). In the present paper we report the full characterization of the lipoxygenation of ALA, and the anti-inflammatory and anti-aggregatory effects of two LOX end-products. The structure of the four LOX metabolites that we herein describe have already been partially reported (23, 24), but the proposed stereochemistry structures of the 9(S),16(R)-di-HOTE do not fit with our experimental data. On the other hand, their biological properties have not been investigated.

Our data show that ALA is mainly converted into its 13-oxygenated derivative 13-HOTE by 15/16LOX. However, formation of a small amount of the 9-oxygenated product 9-HOTE could also be observed. In contrast to DHA, which is converted into only one main di-hydroxylated compound (PDX) via a double lipoxygenation, four di-hydroxylated compounds were observed from ALA following incubation with 15-LOX, characterized as 9,16-di-HOTEs. The stereochemistry of these di-hydroxylated compounds was assessed by incubating 9(S)-HOTE and racemic 9(±)-HOTE separately with sLOX (see the above results). The UV spectra of the two main di-hydroxylated products are superimposed to that of PDX, which possesses an E,Z,E conjugated triene geometry. The diastereoisomers are likely produced via a dioxygenation mechanism as previously described for PDX (18) and these two compounds are likely to have an E,Z,E conjugated triene geometry. Such a geometry was further confirmed by high field NMR which allowed us to determine precisely the <sup>1</sup>H-<sup>1</sup>H J-couplings among the protons bound to the σ<sup>2</sup> carbons in C=C double bonds. The geometry of the conjugated trienes of two additional but less abundant (around half the amount of the E,Z,E 9(S),16(S)-di-HOTE and 9(R),16(S)-di-HOTE) di-hydroxylated products was supposed to be E,E,E because their UV spectra were superimposed to that of all trans LTB<sub>4</sub>, which has such an E,E,E conjugated triene geometry. This all trans geometry was also confirmed by NMR to determine the <sup>1</sup>H-<sup>1</sup>H J-couplings among the protons bound to the σ<sup>2</sup> carbons in C=C double bonds. Based on data from the literature, we may suggest that the stereochemistry of these two minor products (Fig. 1B) is 9(R),16(S)- and 9(S),16(S)-all trans-di-HOTE, in order of increasing retention time, respectively. Such a configuration is also in agreement with the 5(S),12(R)- and 5(S),12(S)-all trans LTB<sub>4</sub> and the 5(S),12(R)- and 5(S),12(S)-all trans LTB<sub>4</sub> diastereomers already described (43–45). We conclude that the four di-hydroxylated compounds issued from ALA treated by sLOX are 9(R),16(S)-di-hydroxy-10E,12E,14E-octadecatrienoic acid, 9(S),16(S)-di-hydroxy-10E,12Z,14E-octadecatrienoic acid, 9(S),16(S)-di-hydroxy-10E,12Z,14E-octadecatrienoic acid, and 9(R),16(S)-di-hydroxy-10E,12Z,14E-octadecatrienoic acid, in order of increasing retention time, respectively. Such data suggest that there are two different mechanisms explaining the formation of these 9,16-di-HOTE isomers. The E,Z,E compounds 9(S),16(S)-di-HOTE and 9(R),16(S)-di-HOTE would result from a dioxygenation mechanism as previously shown for PDX (18), whereas the all trans 9,16-di-HOTE isomers might derive from unknown intermediates.

We also investigated the metabolism of ALA incubated with human recombinant 15-LOX-2. Interestingly, 9(S)-HOTE or 9(R)-HOTE were both well converted into the corresponding 9,16-di-HOTEs whereas ALA was less efficiently metabolized into those di-hydroxylated fatty acids than by sLOX. Such a result might be biologically relevant because 9(S)-HOTE can be produced through cyclooxygenases, and 9(R)-HOTE can be produced via aspirinated COX-2, both in an aborted cyclooxygenation process (54).

The present study also shows some biological effects of the E,Z,E di-hydroxylated 9,16-di-HOTE isomers. We observed that 9(S),16(S)-di-hydroxy-10E,12Z,14E-octadecatrienoic acid and 9(R),16(S)-di-hydroxy-10E,12Z,14E-octadecatrienoic acids decreased significantly prostaglandin synthesized by recombinant COX-1. Moreover, these E,Z,E isomers inhibit platelet aggregation triggered by collagen both by decreasing the extent of aggregation and making it partially reversible (Fig. 7). However, these E,Z,E di-hydroxylated ALA metabolites appear to be less potent inhibitors than PDX under the same conditions, although the anti-aggregatory effects observed are in agreement with our previous data showing that epoxytrins, characterized by an E,Z,E conjugated triene, are inhibitors of COX-1 (19).

COX-2 is an inducible enzyme becoming abundant at sites of inflammation, and is upregulated in various carcinomas, having a central role in tumorigenesis (55). The inhibition of COX-2 is a major mode of action to reduce inflammation (56). In our study, we found that 9(S),16(S)-di-hydroxy-10E,12Z,14E-octadecatrienoic and 9(R),16(S)-di-hydroxy-10E,12Z,14E-octadecatrienoic acids exhibit an inhibitory effect on the human recombinant COX-2.

Moreover, 9(R),16(S)-di-hydroxy-10E,12Z,14E-octadecatrienoic acid also decreased significantly the formation of LTB<sub>4</sub> and 5(S)-HETE from arachidonic acid with human polymorphonuclear leukocytes, indicating the inhibition of 5-LOX.

It is interesting to note that in all the tests evaluated, the E,Z,E 9(R),16(S) isomer was a more potent inhibitor than the E,Z,E 9(S),16(S) isomer. This reinforces the interest of the 15-lipoxygenation of 9(R)-HOTE produced by aspirinated COX-2 (54) to synergize with aspirin in atherothrombogenesis.
In conclusion, we observe from the present study that four di-hydroxylated compounds are produced from ALA treated by sLOX. They are 9(R),16(S)-dihydroxy-10E,12E,14Z-octa
decatetraenoic acid, 9(S),16(S)-dihydroxy-10E,12E,14Z-octa
decatetraenoic acid, 9(S),16(S)-dihydroxy-10E,12Z,14E-octa
decatetraenoic acid, and 9(R),16(S)-dihydroxy-10E,12Z,14E-octa
decatetraenoic acid. Interestingly, 9,16-dihydroxy-10E,12Z,14E-octadecatetraenoic acid isomers exhibit anti-aggregatory properties as other poxytrins (19). In addition, 9(R),16(S)-dihydroxy-10E,12Z,14E-octadecatetraenoic acid, which can be produced from 9(R)-HOTe by aspirin-induced COX-2, inhibits both cyclooxygenases and the 5-LOX pathway and appears to be a potential anti-inflammatory compound in vitro.

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