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MECHANISMS OF LEUKOTRIENE FORMATION: HEMOGLOBIN-CATALYZED TRANSFORMATION OF 15-HPETE INTO 8,15-DiHETE and 14,15-DiHETE ISOMERS

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Summary. Four isomers of 8,15-diHETE as well as 14,15-diHETEs are isolated and characterized after exposure of 15-HPETE to hemoglobin. It is found that 83% of the C-8 oxygen atoms in 8(R),15(S)-diHETE and 8(S),15(S)-diHETE, and 41% of the C-8 oxygen atoms in 8(R),15(S)-11Z-diHETE and 8(S),15(S)-11Z-diHETE are derived from $H_2{}^{18}O$. These results suggest that hemoglobin catalyzes the transformation of 15-HPETE into these products via a free radical process, possibly involving the intermediacy of 14,15-LTA. Intact human leukocytes contain a distinct enzyme system for catalyzing the conversion of 15-HPETE into 14,15-LTA. This enzyme activity is inhibited by ETYA and is rapidly denatured upon homogenization of the intact leukocytes.

The leukocytes not only contain relatively large amounts of 5- and 15-lipoxygenases but also enzyme systems that transform 5-HPETE and 15-HPETE into LTA (1) and 14(15)-LTA (2-4), respectively. Although these reactive allylic epoxides are important precursors to a diverse group of mediators of inflammation and hypersensitivity (5), the mechanism of their formation in biological systems remains unclear. Herein, we report our investigation on the transformations of 15-HPETE catalyzed by human leukocytes and by hemoglobin.

Materials and Methods

Arachidonic acid (99%), hemoglobin (type 1, bovine, 75% methemoglobin), methemoglobin (Grade 1, bovine) and glutathione were products of Sigma. 4-Hydroxy-2,2,6,6-tetramethyl piperidinooxy (HTMP) and tert-butyl hydroperoxide (t-BHP) were purchased from Aldrich Chemicals Co. $^{18}O_2$ (97%) and $H_2{}^{18}O$ (97%) were supplied by KOR Isotopes. ETYA was kindly provided by Dr. W. E. Scott, Hoffmann-LaRoche, Inc., Nutley, N.J. Human leukocyte concentrates from American Red Cross, Madison, WI, were used for preparation of leukocytes suspension (4). 15-HPETE (7) and $H^{18}O_2$-15-HPETE (17) were prepared by published procedures. Methods used for GC-MS analyses of samples were described (4).

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[1]Abbreviations: HPLC, high pressure liquid chromatography (SP, straight phase; RP, reverse phase); LT, leukotriene; LTA, leukotriene A; 14,15-LTA, (S)-trans-14,15-oxido-5,8,Z-10,12E-icosatetraenoic acid; 5-HPETE, 5(S)-hydroperoxy-6E-8,11,14Z-icosatetraenoic acid; 15-HPETE, 15(S)-hydroperoxy-5,8,11Z,13E-icosatetraenoic acid; ETYA, 5,8,11,14-icosatetraynoic acid; 15-HETE, 15(S)-hydroxy-5,8,11Z,13E-icosatetraenoic acid; 8(R),15(S)-11Z-diHETE, 8(R),15(S)-5Z,9E,11Z,13E-icosatetraenoic acid; 8(S),15(S)-11Z-diHETE, 8(S),15(S)-5Z,9E,11Z,13E-icosatetraenoic acid; 8(R),15(S)-diHETE, 8(R),15(S)-5Z,9,11,13E-icosatetraenoic acid; 8(S),15(S)-diHETE, 8(S),15(S)-5Z,9,11,13E-icosatetraenoic acid; 5,15-diHETE, 5(S),15(S)-dihydroxy-6,8,11,13-icosatetraenoic acid.

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Preparation of 10,000 g supernatant of human leukocytes. A human leukocyte suspension (2×10^8 cells/ml) in 0.1 M phosphate buffer, pH 7.0, was sonicated at 4°C with Branson sonicator (70 watts, 40 sec), and then centrifuged (10,000 g, 15 min).

Transformation of 15-HPETE by human leukocytes. The human leukocyte suspension (50 ml, $1.8\text{-}2.5 \times 10^7$/ml) in 6.5 mM phosphate buffer (4), was incubated with 15-HPETE (30 µg/ml) at 37°C. Separately, the leukocytes were preincubated for 10 min with ETYA. After 15 min, the reaction was quenched by the addition of ethanol (3 volumes). Incubation of 15-HPETE (30 µg/ml) with the leukocyte extract (5 ml) was similarly carried out in 50 ml of the same buffer (4) at 37°C for 15 min, and the reaction was terminated by adding 3 volumes of ethanol. After evaporation of ethanol, the aqueous layer was acidified (pH 3.2).

Transformation of 15-HPETE by hemoglobin (Hb). Hb (15 mg) was incubated with 15-HPETE (30 µg/ml) in 50 ml of 0.1 M phosphate buffer, pH 7.0, at 37°C. In a separate experiment, Hb was preincubated for 10 min with ETYA. After 15 min incubation, the reaction was terminated by acidification (pH 3.2). Incubation of $H^{18}O_2$-15-HPETE (90 µg/ml) with Hb (15 mg) was similarly carried out in the same buffer. In the incubation with $H_2{}^{18}O$, 15-HPETE (2 mg) was added to 2 ml of 0.1 M phosphate buffered $H_2{}^{18}O$ (pH 7.0) containing 10 mg of Hb. After 15 min, the reactions were terminated by acidification (pH 3.2). For the $^{18}O_2$ gas experiment, a 500 ml three necked-round bottom flask containing 20 mg Hb in 70 ml 0.1 M phosphate buffer, pH 7.0, was evacuated and flushed with N_2 7 times (17). Following the introduction of 100 ml $^{18}O_2$ by a vacuum line, the system was adjusted to 1 atmosphere with N_2 and warmed up to 37°C. The reaction was initiated by injecting 15-HPETE (10 mg) into the system and terminated by acidifying (pH 3.2) after 15 min incubation.

Incubation of 15-HETE with hemoglobin and t-BHP. 15-HETE (60 µg/ml) was added to 30 ml of 0.1 M phosphate buffer, pH 7.0, containing 1 mM t-BHP and hemoglobin (15 mg). The reaction was allowed to continue for 15 min at 37°C, and then terminated by acidification (pH 3.2).

Cis → trans isomerization of diHETEs. The reaction mixture contained: 2.5 µmoles of GSH, 6.25 µmoles of LiOH, and 0.3 µmoles of diHETEs in 0.5 ml of dimethoxyethane/water (4:1, v/v). After 4 hrs at 25°C, the products were acidified (pH 3.2). In a separate experiment, 0.5 µmole of HTMP was added to the reaction mixture.

Purification. The acidified samples were extracted with ethyl ether (3 times), and chromatographed onto HPLC, as described before (4).

Results

Transformation of 15-HPETE by human leukocytes. Previous studies have shown that intact human leukocytes converted 15-HPETE mainly into 14,15-diHETEs and some 8,15-diHETEs and 5,15-diHETE (2-4). To gain an insight into the mechanism of this transformation, we examined the inhibition pattern of LT formation by ETYA. Figure 1 shows

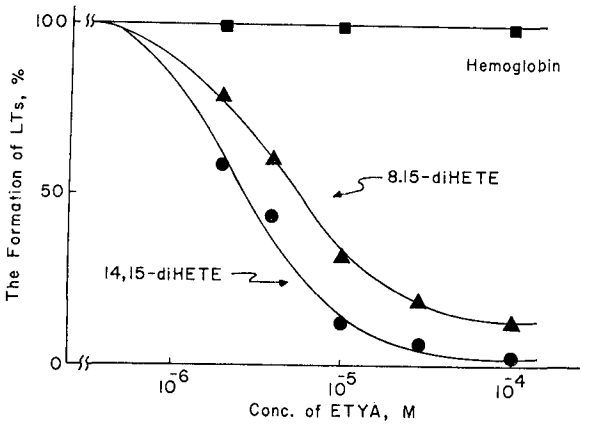


Fig. 1. The effect of ETYA on LT formation from (S)-15-HPETE. Total LT formed by HB (■—■); 8,15-diHETEs (▲—▲) and 14,15-diHETEs (●—●) formed by leukocytes.

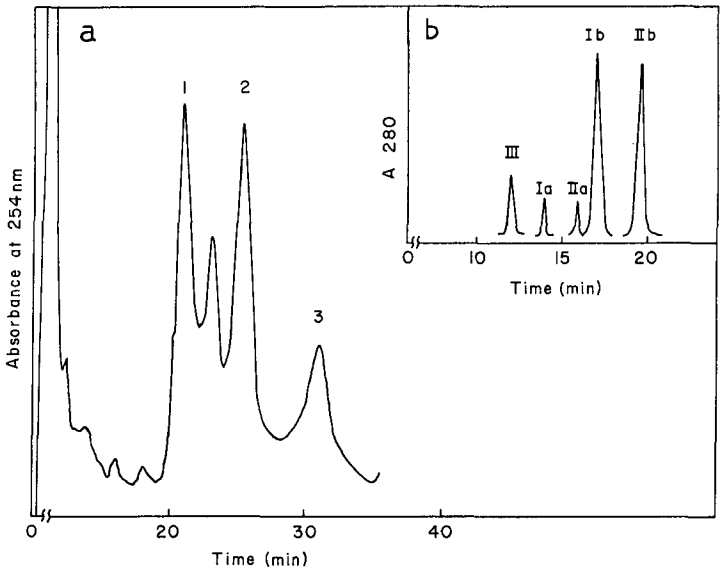


Fig. 2. a) RP-HPLC profile of diHETE products from the incubation of (S)-15-HPETE with 10,000 g supernatant of leukocytes. Radial-Pak 8C18O 5 μ column (0.8 x 10 cm, Waters) with C_{18} precolumn. Mobile phase: methanol-water (60:40), pH 5.4 (0.05% HAC + NH_4OH); flow rate, 2 ml/min.
b) SP-HPLC profile of diHETEs methyl esters. Radial-Pak 8S10 5 μ column (0.8 x 10 cm, Waters) with silica gel precolumn. Mobile phase: hexane-2-propanol-HAC (95:5:0.1); flow rate, 1.5 ml/min.

that while the formation of 14,15-diHETEs was completely inhibited by ETYA ($[I]_{50}$ = 2.5 μM), the formation of 8,15-diHETEs was found to be less susceptible to ETYA inhibition. Even at 10^{-4} M ETYA, the formation of 8,15-diHETEs was not completely suppressed. This observation suggested that LT formation from 15-HPETE may be catalyzed by ETYA-sensitive and insensitive components.

When 15-HPETE was incubated with the 10,000 g supernatant fraction of human leukocytes, the product profile as revealed by HPLC analysis (Fig. 2) was found to differ markedly from that of intact leukocytes (4). 8,15-DiHETEs were the major products of this incubation and the amount of 14,15-diHETEs and 5,15-diHETE decreased markedly. This cell-free system accounted for approximately 60% of the LTs forming activity of the corresponding amount of intact leukocytes. Moreover, the total LT formation by this cell extract was less susceptible to ETYA inhibition ($[I]_{50}$ = 10^{-5} M) than the intact cell system.

Hemoglobin-catalyzed transformation of 15-HPETE. In the course of purifying human leukocytes for our studies, we noticed that these preparations were always contaminated with a small quantity of hemoglobin, which was suspected to contribute to the conversion of 15-HPETE into LTs. When 15-HPETE was exposed to bovine hemoglobin, a mixture of diHETEs possessing the characteristic conjugated triene chromophores were formed. The HPLC profile of the products (Fig. 3) was virtually identical to that obtained with the 10,000 g supernatant fraction (Fig. 2), except the amount of 14,15-diHETEs formed was somewhat lower and no 5,15-diHETE was detected. The yield of Hb-

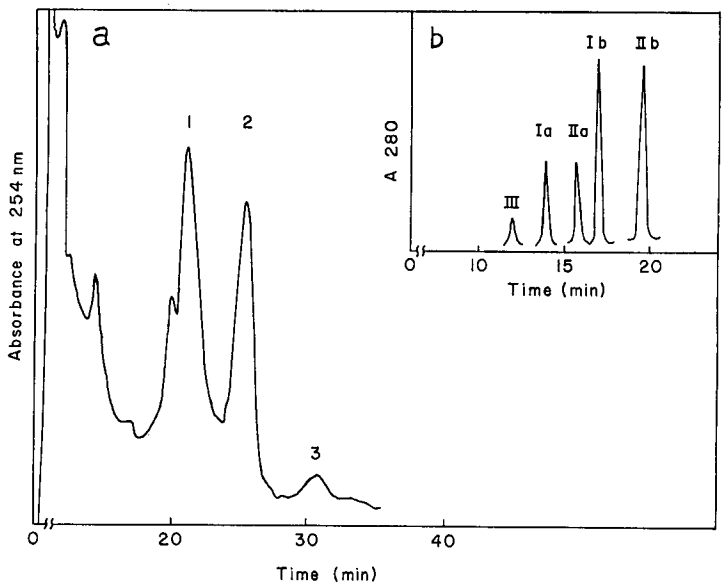


Fig. 3. a) RP-HPLC profile (same as Fig. 2a) of diHETEs from the incubation of (S)-15-HPETE with bovine hemoglobin.
b) Same as Fig. 2b.

catalyzed LT formation was around 1% as compared to 2% for intact human leukocytes. Moreover, Hb-catalyzed LT formation was not inhibited by ETYA (Fig. 1). A similar product profile was obtained using methemoglobin.

Peaks 1, 2 and 3 (Fig. 3a) were separately treated with diazomethane and then purified by SP-HPLC. Peak 1 (retention time 21 min) was resolved into two peaks, Ia and Ib with retention times of 14 and 17 min, respectively. Likewise peak 2 (retention time 25.4 min) was separated into IIa (15.9 min) and IIb (19.6 min). The UV spectra of the minor peaks Ia and IIa exhibited maxima at 268.5 nm with higher shoulders at 259 nm than at 279 nm whereas the major peaks Ib and IIb exhibited maxima at 269 nm with shoulders of equal heights at 259 nm and 280 nm. All four samples were trimethylsilylated and then subjected to GC-MS spectrometric analyses. All four derivatives gave identical mass spectra with prominent ions at 494(M^+), 479 (M^+-15), 463 (M^+-31), 423 [M^+-$\cdot(CH_2)_4CH_3$], 404 (M^+-Me_3SiOH), 353 [M^+-$\cdot CH_2CH{=}CH(CH_2)_3CO_2CH_3$], 321 [$M^+$-$Me_3SiO\dot{C}H(CH_2)_4CH_3$], 263 [$M^+$-(141+ 90)], 243 [$Me_3SiO\dot{C}H(CH_2)(CH)_2(CH_2)_3CO_2CH_3$ and M^+-(90 + 90 + 71)] and 173 [$Me_3SiOCH(CH_2)_4CH_3$], in good agreement with the fragmentation pattern of trimethylsilyl-8,15-diHETE methyl ester (6).

On SP-HPLC, peak Ia comigrated with an authentic sample of 8(S),15(S)-dihydroxy-5Z,9E,11Z,13E-icosatetraenoic acid methyl ester (7). Treatment of 8(S),15(S)-dihydroxy 5Z,9E,11Z,13E-icosatetraenoic acid with LiOH and glutathione afforded a product whose methyl ester comigrated with IIb. Since thiyl radicals (RS•) are known to catalyze the cis → trans isomerization of double bonds (8), it follows that the structure of IIb is 8(S),15(S)-dihydroxy-5Z,9E,11E,13E-icosatetraenoic acid methyl ester. This structural assignment is consistent with the observation that HTMP (10^{-3} M) inhibited this radical

catalyzed isomerization (>60%). Moreover, this radical catalyzed isomerization of Ia into IIb was also observed during the incubation of 15-HPETE with Hb.

Compound Ib was identified as 8(R),15(S)-dihydroxy-5Z,9E,11E,13E-icosatetraenoic acid methyl ester, for it has the same retention time on SP-HPLC as an authentic specimen, prepared by acid hydrolysis of 14,15-LTA methyl ester (9). Because radical catalyzed isomerization of IIa (Fig. 3b) afforded Ib, it is reasonable to deduce the structure of IIa as 8(R),15(S)-dihydroxy-5Z,9E,11Z,13E-icosatetraenoic acid methyl ester.

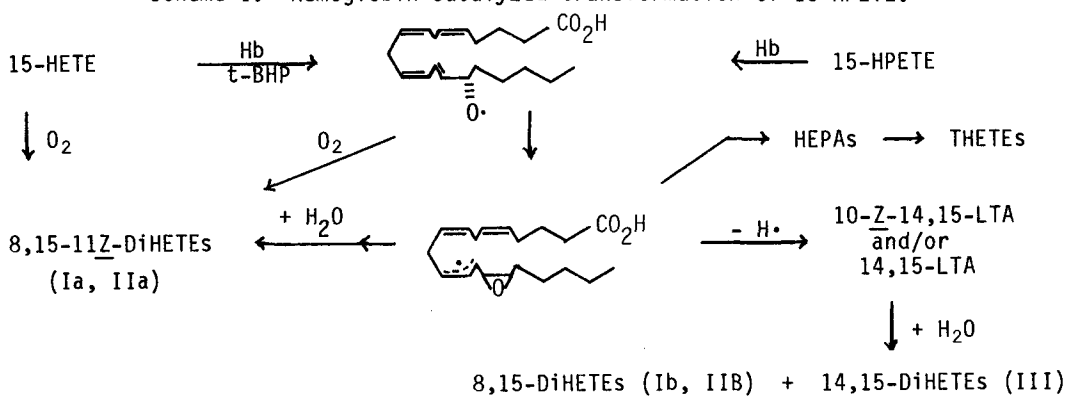
Hydrolysis of 14,15-LTA methyl ester is known to give 14,15-diHETE methyl ester and two 8,15-diHETE methyl ester isomers (4). Peak 3 (retention time 31 min, Fig. 3a) showed a UV maximum at 272 nm with shoulders at 262 and 282 nm. Its methyl ester derivative comigrated with an authentic sample of synthetic 14,15-diHETE methyl ester on SP-HPLC; mass spectrum of its trimethylsilyl ether derivative gave prominent ions at m/e 479 (M^+-15), 394 (M^+-100), 321 [M^+-$Me_3SiO\dot{C}H(CH_2)_4CH_3$] and 173. These results support the view that peak III (Fig. 3b) corresponds to 14,15(S)-dihydroxy-5Z,8Z,10E,12E-icosatetraenoic acid methyl ester.

$H_2{}^{18}O$ and $^{18}O_2$ incorporation studies. After incubating 15-HPETE with Hb in $H_2{}^{18}O$, the resulting four LTs were converted into their methyl esters and purified as before. GC-MS analyses of their trimethylsilyl ether derivatives revealed that significant amount of ^{18}O isotope was incorporated into the hydroxyl group at C-8 of all these four derivatives, which showed characteristic ions at m/e 496 (M^+), 481 (M^+-15), 425 [M^+-$\cdot(CH_2)_4CH_3$], 406 (M^+-Me_3SiOH), 355 [M^+-$\cdot CH_2$-CH=CH$(CH_2)_3CO_2Me$], 323 [M^+-$Me_3SiO\dot{C}H(CH_2)_4CH_3$], 265 [$M^+$-(141 + 90)], 245 [$Me_3Si{}^{18}O\dot{C}H(CH_2)(CH)_2(CH_2)_3CO_2CH_3$], 173 [$Me_3SiOCH(CH_2)_4CH_3$]. From the intensity of the relevant ions, it is estimated that approximately 41% of ^{18}O was incorporated into the C-8 hydroxyl of Ia and IIa and 83% into Ib and IIb.

When 15-HPETE was exposed to Hb under an atmosphere of $^{18}O_2$, it was found that about 9% of ^{18}O resided in the C-8 hydroxyl of Ia and IIa but no significant quantity of ^{18}O isotope was detected in Ib and IIb.

No ^{18}O isotope was noted in Ia, IIa, Ib, and IIb after exposure of ^{18}O-labelled 15-HPETE(R-^{18}O-^{18}OH) to Hb.

Scheme I. Hemoglobin-catalyzed transformation of 15-HPETE.



Discussion

It is well documented that hemoproteins and Fe^{II} can catalyze the homolytic fission of hydroperoxides of unsaturated fatty acids to generate a variety of hydroxy-epoxy (HEPA) and trihydroxy unsaturated acids (THETE) (10,11). For example, the major products after exposure of 15-HPETE to Hb were identified to be 13-hydroxy-14,15-epoxy 5,8,11-icosatrienoic acid, 11,12,15-trihydroxy-5,8,13-icosatrienoic and 11,14,15-trihydroxy-5,8,12-icosatrienoic acids (12). Our present investigations show that at least four additional minor compounds are present in the product accounting for approximately 1 percent of the total. These compounds were characterized as isomers of 8,15-diHETEs and 14,15-diHETEs.

The origin of the oxygen atoms at C-8 of Ia, IIa, Ib and IIb was established in experiments with ^{18}O-enriched water, atmospheric $^{18}O_2$ and $H^{18}O_2$-labelled 15-HPETE. It was shown that 83% of the oxygen atom at C-8 of Ib and IIb was derived from $H_2{}^{18}O$ and 17% of Ib and IIb were formed without incorporation of OH from the solvent.

This observation suggested that 15-HPETE was converted into an unstable intermediate, such as 14,15-LTA and/or 10Z-14,15-LTA, via a free radical process. Hydrolysis of 14,15-LTA is expected to result in the incorporation of $H_2{}^{18}O$ into C-8 positions of these two 8,15-diHETEs (Ib, IIb). This assumption is supported by the isolation of 14,15(S)-dihydroxy-5Z,8Z,10E,12E-icosatetraenoic acid (peak 3, Fig. 3a) from the incubation and that hydrolysis of 14,15-LTA methyl ester afforded Ib, IIb and III. The remaining 17% of unlabelled Ib and IIb may be derived via the radical catalyzed isomerization of *cis* to *trans* double bonds, e.g., Ia → IIb and IIa → Ib. In the case of Ia and IIa, 41% of the C-8 oxygen atoms were found to originate from $H_2{}^{18}O$. Although one may envisage that they may be correspondingly derived from the hydrolysis of the unstable 10Z-14,15-LTA (9), this hypothesis appears unlikely because hydrolysis of 10Z-14,15-LTA methyl ester likewise afforded only Ib, IIb and III.

Approximately 9% of the C-8 oxygen atoms in Ia and IIa was derived from $^{18}O_2$ gas but none of the atmospheric oxygen appeared in Ib and IIb. This result suggested that Ia and IIa are partially formed via a quasi-lipoxygenase type reaction, which was recently observed (13). In fact, when 15-HETE was incubated with Hb in the presence of t-BHP (14), the predominant products formed after methylation corresponded to Ia and IIa. Although at this stage one is unable to rationalize the origin of the remaining 50% of C-8 oxygen atoms in Ia and IIa, it is possible that this portion could arise via autooxidation of 15-HETE during work-up. The results of our present investigations seem to suggest that there seems to be two possible mechanisms for the formation of 14,15-LTA from 15-HPETE in biological systems. One of the enzyme systems is inhibited by ETYA and appeared to be rather unstable. This activity was rapidly destroyed upon homogenation of the leukocytes. A second ETYA insensitive system proceeds via a free radical process catalyzed by hemoproteins such as Hb. The initial step probably entails the homolytic cleavage of the 15-hydroperoxide to generate an

alkoxy radical which may then rearrange to a carbon centered radical (15,16). The fate of the latter radical is not fully understood but one may envisage the generation of 14,15-LTA via the loss of an H· equivalent (e.g., this could occur via a stepwise loss of an electron and a proton). Also, it may directly generate the 8,15-11Z-diHETEs via some yet undefined process.

In view of the ubiquitous distribution of hemoproteins in tissues, the physiological significance of this free radical pathway of LTA formation from the respective hydroperoxides cannot be overlooked. Although the yield of this transformation is low (0.5-1%), this level of conversion is comparable to the level of the ETYA sensitive process occurring in intact leukocytes.

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