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Double dioxygenation by mouse 8*S*-lipoxygenase: Specific formation of a potent peroxisome proliferator-activated receptor α agonist

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Abstract

Mouse 8*S*-lipoxygenase (8-LOX) metabolizes arachidonic acid (AA) specifically to 8*S*-hydroperoxyeicosatetraenoic acid (8*S*-HPETE), which will be readily reduced under physiological circumstances to 8*S*-hydroxyeicosatetraenoic acid (8*S*-HETE), a natural agonist of peroxisome proliferator-activated receptor α (PPARα). Here, we investigated whether 8-LOX could further oxygenate AA and whether the products could activate PPARs. The purified recombinant 8-LOX converted AA exclusively to 8*S*-HPETE and then to (8*S*,15*S*)-dihydroperoxy-5*Z*,9*E*,11*Z*,13*E*-eicosatetraenoic acid (8*S*,15*S*-diHPETE). The k_{cat}/K_m values for 8*S*-HPETE and AA were 3.3×10^3 and 2.7×10^4 M^{-1} s^{-1}, respectively. 8-LOX also dioxygenated 8*S*-HETE and 15*S*-H(P)ETE specifically to the corresponding 8*S*,15*S*-disubstituted derivatives. By contrast, 15-LOX-2, a human homologue of 8-LOX, produced 8*S*,15*S*-diH(P)ETE from 8*S*-H(P)ETE but not from AA nor 15*S*-H(P)ETE. 8*S*,15*S*-diHETE activated PPARα more strongly than 8*S*-HETE did. The present results suggest that 8*S*,15*S*-diH(P)ETE as well as 8*S*-H(P)ETE would contribute to the physiological function of 8-LOX and also that 8-LOX can function as a potential 15-LOX.

Keywords: 8*S*-Lipoxygenase; 15*S*-Lipoxygenase-2; Arachidonic acid; Multifunction; Bacterial expression; Kinetics; DiHPETE; PPARα; ^{1}H NMR

Lipoxygenases (LOXs) are enzymes that add molecular oxygen to polyunsaturated fatty acids (PUFAs) to produce fatty acid hydroperoxides, which are further metabolized to various lipid mediators [1]. LOXs are widespread both in the plant and animal kingdom [1]. Plant LOXs mainly react with linoleic and linolenic acids to initiate an octadecanoid pathway which produces various oxylipins including jasmonic acid, one of the most ubiquitous plant hormones [1,2]. Animal LOXs mainly react with arachidonic acid to produce hydroperoxyeicosatetraenoic acids (HPETEs), which are further converted to various eicosanoides including inflammatory leukotrienes and antiinflammatory lipoxins [1,3,4].

Based on the positional specificity, mammalian LOXs are classified into 5-, 8-, 12-, and 15-LOXs [1]. 12- and 15-LOXs are further classified into four and two isozymes, respectively [1]. Most of these LOXs are conserved throughout mammals, suggesting that mammals conserve common LOX functions. Strange exceptions of this tendency are 8-LOX and 15-LOX-2. 8-LOX is detected only in mice [1,5], while 15-LOX-2 in other mammals [1,6,7]. Recent cloning revealed that 8-LOX and 15-LOX-2 share 78% homology in the primary structure, although they specifically produce different products, 8*S*-HPETE and 15*S*-HPETE, respectively [5,6,8]. Whether 8-LOX and 15-LOX-2 share common

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physiological function and why mice conserve 8-LOX rather than the more general 15-LOX-2 remain to be elucidated.

Some LOXs catalyze not only the initial dioxygenation of the original PUFA substrate, but also further reaction including epoxide formation in leukotriene synthesis and secondary dioxygenation in lipoxin formation [1,3,4]. In this study, we examined whether 8-LOX can further react with the first reaction product, and found that, after producing 8*S*-HPETE from arachidonic, 8-LOX further converts 8*S*-HPETE specifically to (8*S*,15*S*)-dihydroperoxy-5*Z*,9*E*,11*Z*,13*E*-eicosatetraenoic acid (8*S*,15*S*-diHPETE) by itself. We also confirmed that 8*S*,15*S*-diHETE, as well as 8*S*-HETE [9], activated peroxisome proliferator-activated receptor α (PPARα).

Materials and methods

Materials. Complementary DNA of mouse 8-LOX and human 15-LOX-2 was obtained by screening of mouse skin cDNA library [5] and by PCR cloning from cDNAs prepared from mRNA of human hair root [6], respectively. Arachidonic acid was purchased from Cayman (Ann Arbor, MI) and purified by RP-HPLC before use. (8*S*,15*S*)-Dihydroperoxy-5*Z*,9*E*,11*Z*,13*E*-eicosatetraenoic acid (8*S*,15*S*-diHPETE) standard was prepared from arachidonic acid and soybean LOX (Biozyme Laboratories, Gwent, UK) [10]. Standards of HETEs were prepared by vitamin E-controlled auto-oxidation of arachidonic acid [11]. Ni–NTA–agarose was purchased from Qiagen (Tokyo, Japan). All solvents used for HPLC were of HPLC grade. Monkey CV1 kidney cells were purchased from American Type Culture Collection.

Construction of bacterial expression plasmids. Mouse 8-LOX was expressed with or without His-tag at the N-terminal. The additional His-tag sequence was added to the N-terminal of 8-LOX by re-amplifying its cDNA by PCR using an upstream primer containing a *Nde*I site at the 5′ end (5′-CCATATGGCGAAATGCAGGGTGAGA-3′) and a downstream primer containing an *Eco*RI site at the 5′ end (5′-GGAATTCATGTTAGATGGAGACACTGTTCTC-3′). After being cloned into pCR2.1 cloning vector (Invitrogen, Tokyo, Japan), a PCR-error free clone was selected by DNA sequencing and transferred into a pET28 bacterial expression vector (Novagen, Madison, WI) using the *Nde*I and *Eco*RI sites. The resulting recombinant protein will contain additional 20 amino acids (MGSSHHHHHHSSGLVPRGSH) at the N-terminal. In order to express 8-LOX without a His-tag, the 8-LOX cDNA was re-amplified by PCR using an upstream primer containing a *Nco*I site at the 5′ end (5′-CCATATGGCGAAATGC AGGGTGAGA-3′) and the same downstream primer above mentioned. The resulting correct clone was transferred to pET28 vector using *Nco*I and *Eco*RI sites. Human 15-LOX-2 in pcDNA3 mammalian expression vector (Invitrogen) [6] was transferred to pET28c vector using *Bam*HI and *Eco*RI sites, resulting in a construct with additional 35 amino acids (MGSSHHHHHHSSGLVPRGSHMASM TGGQQMGRGSS) at the N-terminal.

Bacterial expression of the recombinant enzymes. Each bacterial expression plasmid was transformed into the BL21 (DE3) strain of *Escherichia coli* (Novagen). The transformed cells were selected on LB plates containing kanamycin (30 μg/ml) and then cultured in 600 ml of 2× YT medium containing the same antibiotic (30 μg/ml) at 37 °C. When the OD_{600} value of the culture became 0.6–1.0 (typically this took about 5–6 h), IPTG was added so that its final concentration was 0.1 mM. The cells were further grown at 20 °C for 20 h. This expression period could be prolonged until 40 h with a slight increase in the enzyme yield. After harvested by centrifugation at 5000 rpm for 10 min at 4 °C, the cells were suspended in small amount of lysis buffer (50 mM sodium-phosphate buffer, pH 8.0, containing 10 mM imidazole, and 300 mM NaCl) and homogenized by sonication on ice. The cell-free homogenate was centrifuged at 10,000*g* for 20 min at 4 °C, and the supernatant was further centrifuged at 100,000*g* for 1 h at 4 °C. The resulting soluble fractions were mixed with 0.3 ml of 50% Ni–NTA–agarose slurry (Qiagen, Tokyo, Japan) for 1 h at 4 °C and then poured into an empty open column. After non-binding components were eluted with 4 ml washing buffer (50 mM sodium-phosphate buffer, pH 8.0, containing 20 mM imidazole, 300 mM NaCl, and 20% glycerol), the His-tagged protein was eluted with 2 ml elution buffer (50 mM sodium-phosphate buffer, pH 8.0, containing 250 mM imidazole, 300 mM NaCl, and 20% glycerol). The purified protein was desalted using a Hi-Trap desalting column (Amersham-Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with 50 mM sodium-phosphate buffer, pH 8.0, containing 20% glycerol, and stored at −80 °C until use. The recombinant enzyme without His-tag was also expressed in the same method and then partially purified as 30–40% ammonium sulfate precipitates of the soluble fraction derived from the cell free homogenate in the lysis buffer without imidazole. The His-tag free enzyme was also desalted before use. The protein concentration was assayed by the Bradford method (Bio-Rad, Tokyo, Japan).

Enzyme assay and the analyses of the products. The enzyme preparations were incubated with arachidonic acid in 50 mM sodium-phosphate buffer, pH 7.4, at 25 °C. The final concentration of EtOH used as a solvent of lipids was 1% of the total reaction mixture. The reaction was monitored using UV-1600 spectrophotometer (Shimadzu, Kyoto, Japan) by spectrophotometric scanning of the reaction mixture every 5 min or by kinetic measurement. The reaction products were extracted by the method of Bligh and Dyer [12] or by solid extraction using Sep-pack C18 cartridge columns (Waters, Tokyo, Japan). Reduction of HPETEs to HETEs was performed with triphenylphosphine (TPP). Ethereal diazomethane was used for methylation of free acids. Reversed-phase (RP)-HPLC was performed using TSK-GEL ODS-80TS (5 μm, 0.46 × 15 cm, TOSOH, Tokyo, Japan) or an AM-3E2 (5 μm, 0.3 × 15 cm, YMC, Kyoto, Japan) column for analyses, and an AM-322 column (5 μm, 1 × 15 cm, YMC) for preparation. Straight-phase (SP)-HPLC was performed using YMC Pack SIL columns (5 μm, 0.46 × 15 cm and 1 × 15 cm for analyses and preparation, respectively, YMC). For chiral-phase (CP)-HPLC, a Chiralcel OD column (0.46 × 25 cm, DAICEL, Tokyo, Japan) was used. The HPLC system was composed of LC-9A high performance pomp, SPD-6AV UV–Vis spectrophotometric detector, and C-R6A chromatopac (Shimadzu, Kyoto, Japan).

GC–MS analyses. The methyl esters of the samples (300 μg) were dissolved in 5 μl of dry pyridine and reacted with 30 μl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (Tokyo Kasei Kogyo, Tokyo, Japan) at room temperature for 2 h. GC–MS was performed using M-80B GC–MS spectrometer (Hitachi, Tokyo, Japan). The trimethylsilylated methyl ester derivatives were separated by an OV-17 column (10 m × 0.32 mm) at 220 °C using helium as a carrier gas at a flow rate of 30 ml/min. The injector and the interface were both at 250 °C. The chemical ionization mass spectra were obtained using *iso*-butane as a reaction gas.

NMR analyses. The methyl esters of the samples (1 mg) were dissolved in 0.5 ml of deuterio-chloroform containing 0.05% (v/v) trimethylsilane (Wako Pure Chemical Industries, Osaka, Japan). The NMR spectra were measured using JNM-A400 NMR spectrometer (JEOL, Tokyo, Japan). The methyl ester of 8*S*,15*S*-diHETE from arachidonic acid by 8-LOX: ^{1}H NMR δ (400 MHz, $CDCl_3$) ppm: 0.892 (3H, t, $J = 6.8$ Hz, H-20), 1.28–1.35 (6H, m, H-17, 18, 19), 1.54 (2H, m, H-16), 1.528 (1H, d, $J = 4.3$ Hz, OH), 1.709 (2H, quintet, $J = 7.4$ Hz, H-3), 1.777 (1H, d, $J = 4.2$ Hz, OH), 2.110 (2H, br quartet, $J = 7.2$ Hz, H-4), 2.326 (2H, t, $J = 7.4$ Hz, H-2), 2.33 (2H, m, H-7), 3.670 (3H, s, OCH_3), 4.197 (1H, d quartet, $J = 6.4$ (q), 4.2 (d) Hz, H-8 or H-15), 4.258 (1H, quintet, $J = 5.8$ Hz, H8 or H-15), 5.454 (1H, dtt, $J = 10.7$ (d), 7.2 (t), 1.2 (t) Hz, H-5 or H-6), 5.540 (1H, dtt, $J = 10.7$ (d), 7.2 (t),

1.2 (t) Hz, H-5 or H-6), 5.746 (1H, dd, $J = 15.1$, 7.1 Hz, H-9 or H-14), 5.763 (1H, dd, $J = 15.0$, 6.5 Hz, H-9 or H-14), 5.978 (1H, ddd, $J = 10.9$, 9.3, 1.2 Hz, H-11 or H-12), 6.001 (1H, ddd, $J = 10.7$, 9.7, 1.2 Hz, H-11 or H-12), 6.692 (1H, ddd-like, $J = 15.1$, 10.3, 1.2 Hz, H-10 or H-13), 6.721 (1H, ddd-like, $J = 15.1$, 10.3, 1.2 Hz, H-10 or H-13). The shape of the ^{1}H NMR signals derived from H-8 and H-15 was highly dependent on the experimental condition due to their spin coupling with the geminal hydroxy groups. Some signal pairs (H-5 and H-6, H-8 and H-15, H-9 and H-14, H-10 and H-13, and H-11 and H-12) were not assigned completely because of their highly symmetric indistinguishable signals.

Luciferase assay. Monkey CV1 kidney cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 200 μM ascorbic acid, and 10 mg/ml penicillin and streptomycin, at 37 °C in 5% CO_2. The luciferase assay was performed as previously described [13] by an advanced high-sensitive system developed from the dual luciferase system (Promega, WI, USA) using co-expression of coactivator cAMP-response element binding protein (CREB) binding protein (CBP). Ligand-binding domains (LBDs) of PPARs were expressed as a part of fusion proteins containing the GAL4 DNA-binding domain (DBD, residues 1–147) constructed using the pM mammalian expression vector (Clontech, CA, USA). The PPAR-LBD expression plasmids were pM-PPARα (residues 204–505 of the human PPARα), pM-PPARδ (residues 139–440 of the human PPARδ), and pM-PPARγ (residues 167–467 of the human PPARγ). Briefly, the CV1 cells were transfected with p4xUASg-tk-luc (a reporter plasmid), one of the PPAR-LBD expression plasmids, pCMX-CBP (an expression plasmid for CBP) and pRL-CMV (an internal control for normalization of the transfection efficiency), using Lipofectamine (Invitrogen) according to the manufacturer's specifications. After cultured for 24 h in maintenance medium, the transfected cells were culturing for additional 24 h in a medium containing test compound or thiazolidinedione, T174 [14], as a positive control (provided by Tanabe Seiyaku, Osaka, Japan). Luciferase activity was determined by the dual luciferase system according to the manufacturer's specifications (Promega).

DNA sequencing. PCR products and expression plasmids in pET28 vectors were all sequenced using Dye terminator cycle sequencing FS ready reaction kit (Applied Biosystems, Tokyo, Japan) and ABI PRISM 310 genetic analyzer (Applied Biosystems).

Results

Bacterial expression, purification, and characterization of 8S-lipoxygenase

The recombinant 8-LOX was expressed in *E. coli*. Although 8-LOX activity and protein were scarcely detected in the initial expression trials using conventional LB medium, the 2× YT medium significantly improved the expression so that the expressed 8-LOX was readily recognized even in a cell-free homogenate by SDS–PAGE (Fig. 1A, lane 1). Most of the expressed enzyme was recovered in the soluble fraction (Fig. 1A, lane 2), from which the 8-LOX was purified to almost homogeneity by a single step of Ni–NTA affinity chromatography (Fig. 1A, lane 4). A significant amount of 8-LOX was also detected in the membrane fraction (Fig. 1A, lane 3), although the total protein was one-twentieth of the soluble fraction (data not shown). The typical recovery of purified 8-LOX was more than 6 mg from a 200-ml culture. Spectrophotometric scanning of the reaction of the purified 8-LOX and arachidonic acid revealed an increase in the absorption at 237 nm until 30 min (Fig. 1B), where 8*S*-HPETE was the only product (Fig. 1C), indicating that the added His-tag does not affect the positional and stereo specificity of 8-LOX.

Specific secondary reaction of 8S-lipoxygenase

After reaching the maximum value, the absorption at 237 nm gradually declined (Fig. 1B, inset), suggesting further metabolism of 8*S*-HPETE because the UV spectrum of 8*S*-HPETE did not change at least for 3 h when it was incubated in the reaction buffer without enzyme (data not

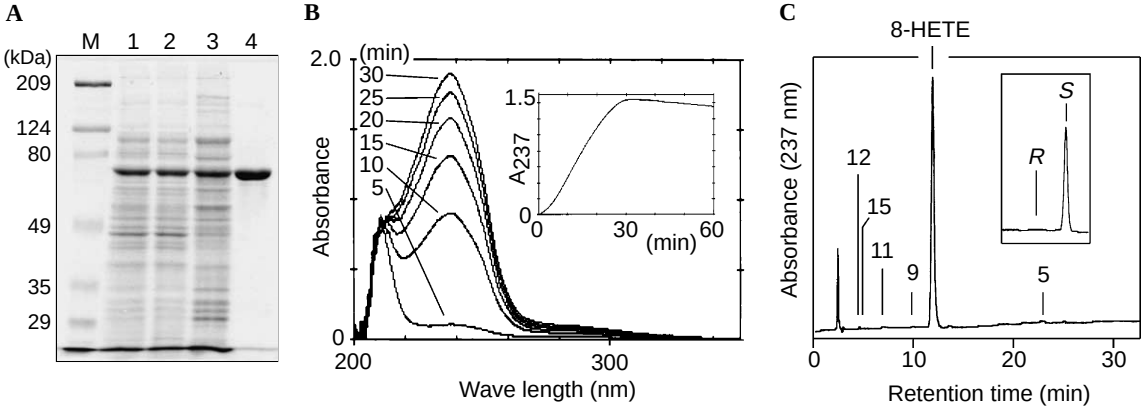

Fig. 1. Bacterial expression, purification, and 8*S*-HPETE formation of 8-LOX. SDS–PAGE stained with Coomassie brilliant blue (A) contains cell free extract (lane 1), soluble fraction (lane 2), membrane fraction (lane 3), and purified 8-LOX (lane 4). The amount of protein loaded was 30 μg in lanes 1–3 and 10 μg in lane 4. Reaction of the purified 8-LOX (260 μg) and 100 μM of arachidonic acid at 37 °C in 0.5 ml of 50 mM sodium-phosphate buffer, pH 7.4, was monitored by spectrophotometric scanning by every 5 min for 30 min (B) and by kinetic measurement of the absorbance at 237 nm for 1 h (B, inset). (C) The HPETE products were extracted at 30 min using a Sep-Pak C18 cartridge column, reduced with TPP, separated by RP-HPLC, and then analyzed by SP-HPLC using a solvent system of *n*-hexane/isopropanol/glacial acetic acid (100:2:0.1, by volume, 1 ml/min). (C, inset) The detected 8-HETE was methylated and analyzed by CP-HPLC using a solvent of *n*-hexane/isopropanol (100:2, by volume, 1 ml/min).

shown). Usage of twice amount of 8-LOX clearly revealed that all the arachidonic acid was rapidly converted to 8*S*-HPETE within 5 min and then further to compounds exhibiting a novel absorption at 270 nm (Fig. 2A). RP-HPLC analyses of the reaction products with detection at various wavelengths revealed the presence of one main polar product (compound I) (Fig. 2B). Further RP- and SP-HPLC analyses confirmed that the compound I was the only main product (Figs. 2C and E). Partially purified 8-LOX without His-tag also gave completely same product (Fig. 2D), confirming that the formation of compound I should be an intrinsic feature of 8-LOX.

Identification of the secondary reaction product of 8S-lipoxygenase

Compound I was reduced with TPP before purification to avoid any decomposition due to unstable hydroperoxy group. The UV spectrum of compound I exhibited absorption peaks at 259.7, 268.9, and 279.3 nm with relative intensities of 1.1:1.4:1.0 (Fig. 3A), indicating the presence of a conjugated triene chromophore with *cis-trans-cis* rather than all-*trans* geometry [15]. The GC-CIMS analysis of compound I revealed an apparent molecular ion at *m/z* 495 (M+H), as well as other informative fragment ions at *m/z* 479 (M+H-15, loss of $^{\cdot}CH_3$), 405 (M+H-90, loss of $HOSiMe_3$), 315 (M+H-180, loss of $2 \times HOSiMe_3$), 243 ($Me_3SiO^+{=}CHCH_2CH{=}CH(CH_2)_3CO_2CH_3$), 173 (base peak, $Me_3SiO^+{=}CH(CH_2)_4CH_3$),

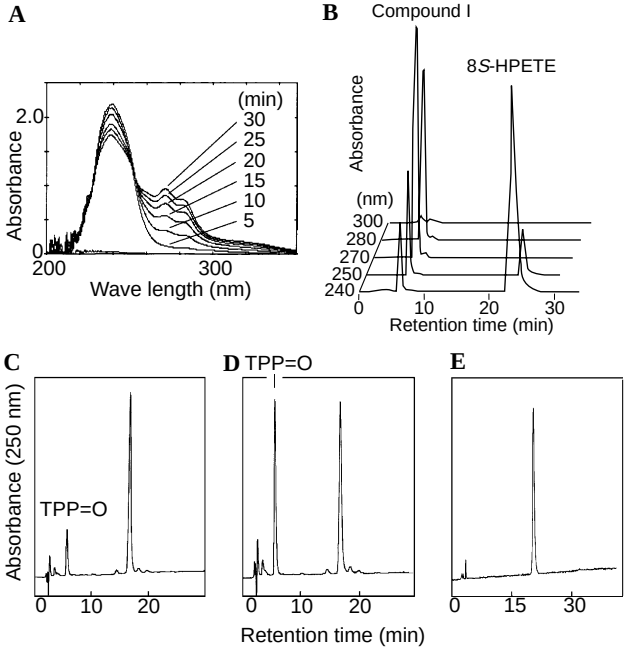

Fig. 2. Formation of a conjugated triene compound by 8-LOX. (A) Reaction using twice the amount of 8-LOX (520 μg) was monitored by spectrophotometric scanning as in Fig. 1B. (B) The extracted reaction products were analyzed by RP-HPLC using a TSK-GEL ODS-80TS column (5 μm, 4.6 × 150 mm) and a solvent system of methanol/water/glacial acetic acid (75:25:0.01, by volume, 1 ml/min). The eluates were detected at various wavelengths indicated. (C) Compound I in (B) was purified, reduced with TPP, and analyzed by RP-HPLC using methanol/water/glacial acetic acid (68:32:0.01, by volume, 1 ml/min). (D) The arachidonic acid metabolites by the partially purified 8-LOX without His-tag were analyzed the same way as in (C). (E) The main peak in (C) was collected, methylated, and analyzed by SP-HPLC using a solvent system of *n*-hexane/isopropanol (100:3, by volume, 1 ml/min) and the eluates were detected by the absorbance at 250 nm. TPP=O, TPP oxide.

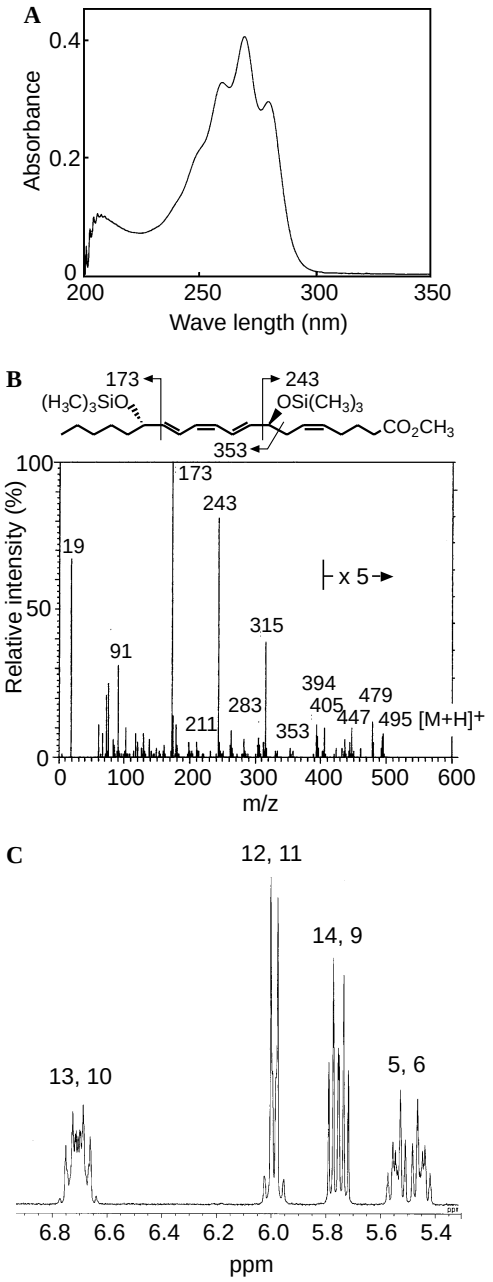

Fig. 3. UV (A), GC-CIMS (B), and ^{1}H NMR (C) spectrum of the conjugated triene compound by 8-LOX. For UV and ^{1}H NMR spectrum, methyl ester of the purified compound was used. For GC-CIMS analysis, the methyl ester was further modified to a TMS ether derivative.

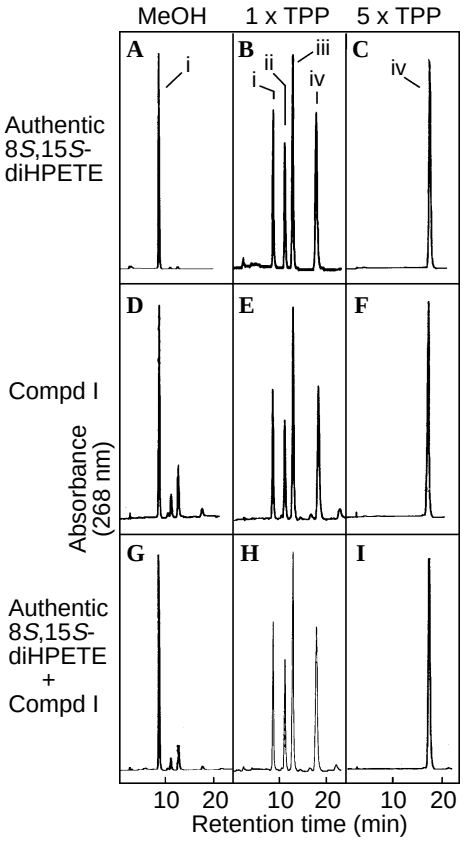

Fig. 4. Identification of the triene compound formed by 8-LOX. Both the authentic 8*S*,15*S*-diHPETE prepared using soybean LOX-1 and the triene compound formed by 8-LOX were methylated, reduced with TPP, and then analyzed by SP-HPLC using a solvent system of *n*-hexane/isopropanol (100:3, by volume, 1 ml/min). The amount of TPP was 5 and 1 equivalent for full and partial reduction, respectively. Methanol was added as non-reduction control. (A–C) Authentic 8*S*,15*S*-diHPETE. (D–F) 8-LOX product. (G–I) Co-chromatography of the soybean LOX-1 and 8-LOX products. The labels of compounds derived from authentic 8*S*,15*S*-diHPETE; i, 8*S*,15*S*-diHPETE; ii, (8*S*-OOH,15*S*-OH)-diH(P)ETE; iii, (8*S*-OH,15*S*-OOH)-diH(P)ETE; iv, 8*S*,15*S*-diHETE.

confirming that compound I is a 8,15-dioxygenated derivative of arachidonic acid (Fig. 3B) [15]. The strong signals at *m*/*z* 173 and 243 also supported the triene structure of 9-*trans*, 11-*cis*, 13-*trans* rather than 9,11,13-all-*trans* [15].

The structure of compound I, especially the *trans*-*cis*-*trans* geometry of the conjugated triene chromophore, was confirmed further by ^{1}H NMR spectroscopy. Both compound I and the authentic 8*S*,15*S*-diHPETE were methylated with ethereal CH_2N_2 and reduced with TPP before ^{1}H NMR analyses. The ^{1}H NMR spectrum of compound I (Materials and methods) was indistinguishable from that of the authentic 8*S*,15*S*-diHPETE and also from that previously reported [16]. Although characteristic signals of olefine protons overlapped due to the highly symmetric structure of the conjugated triene chromophore, significant couplings were evaluated (Fig. 3C). The H-9 and H-14 signals of almost identical splitting pattern exhibited a large coupling constant value of 15.1 Hz, indicating the *trans*-geometry of their double bonds. Besides the corresponding large coupling constant value of 15.1 Hz, the H-10 and H-13 signals exhibited another large coupling constant value of 10.3 Hz, suggesting that the geometry of the double bonds between C-10 and C-11 and between C-12 and C-13 should also be *trans*. On the other hand, the coupling constant values of 9.2 Hz exhibited by the H-11 and H-12 signals indicate the *cis* geometry of the double bond between C-11 and C-12. All these ^{1}H NMR data support the 9-*trans*, 11-*cis*, and 13-*trans* geometry of the compound I.

In order to confirm whether compound I was dihydroperoxide, compound I was carefully compared with the authentic 8*S*,15*S*-diHPETE. The methyl esters of the authentic 8*S*,15*S*-diHPETE and its reduced form, 8*S*,15*S*-diHETE-Me, eluted at 8.6 and 18.2 min, respectively, in SP-HPLC (Figs. 4A and C). Partial reduction of 8*S*,15*S*-diHPETE-Me with an equivalent amount of TPP yielded additional peaks between 8*S*,15*S*-diHPETE and 8*S*,15*S*-diHETE due to the derivatives with only one of the two hydroperoxy groups reduced to hydroxy (Fig. 4B). Compound I and all its fully or partially reduced derivatives eluted exactly at the same time as the authentic 8*S*,15*S*-diHPETE and its derivatives, respectively (Figs. 4D–I), confirming that compound I should be dihydroperoxide.

Based on all the results, the compound I formed from arachidonic acid by 8-LOX was identified to be (8*S*, 15*S*)-dihydroperoxy-5*Z*,9*E*,11*Z*,13*E*-eicosatetraenoic acid (8*S*,15*S*-diHPETE).

Substrate specificity of the second dioxygenation by 8S-lipoxygenase

The formation of 8*S*,15*S*-diHPETE indicates that 8-LOX can exhibit a 15-LOX activity. The reaction efficiency of the second dioxygenation was estimated using 8*S*-HPETE, 8*S*-HETE, 15*S*-HPETE, and 15*S*-HETE as well as arachidonic acid. Actually, 8-LOX readily reacted with each substrate and formed specifically one main product, 8*S*,15*S*-diH(P)ETE, as observed in Fig. 2. Kinetic parameters against each substrate were determined by Lineweaver–Burk plot (Table 1). 8*S*-HPETE and 8*S*-HETE exhibited Michaelis constant values comparable to that of arachidonic acid, while 15*S*-HPETE and 15*S*-HETE showed much higher values (Table 1). On the other hand, V_{max} against 15*S*-HPETE was by far larger than that of others including arachidonic acid (Table 1). The overall reaction efficiency estimated by k_{cat}/K_m value was arachidonic acid > 8*S*-HPETE = 15*S*-HPETE > 8*S*-HETE = 15*S*-HETE (Table 1).

8S,15S-diHPETE formation by 15S-lipoxygenase-2

The specific formation of 8*S*,15*S*-diHPETE from arachidonic acid by 8-LOX led us to examine whether

Table 1
Kinetic parameters of 8-LOX

Substrate	K_m (μM)	V_{max} (μM min^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Arachidonic acid	1.2	4.3×10^{-1}	3.2×10^{-2}	2.7×10^{4}
8*S*-HPETE	2.1	9.2×10^{-2}	7.0×10^{-3}	3.3×10^{3}
8*S*-HETE	5.7	4.6×10^{-2}	3.5×10^{-3}	6.1×10^{2}
15*S*-HPETE	39	2.0	1.5×10^{-1}	3.9×10^{3}
15*S*-HETE	15	1.3×10^{-1}	1.0×10^{-2}	7.0×10^{2}

15-LOX-2, a human homologue of 8-LOX, could also produce 8*S*,15*S*-diHPETE from arachidonic acid. The purified 15-LOX-2 converted arachidonic acid specifically to 15*S*-HPETE as reported previously [6], but did not produce 8*S*,15*S*-diHPETE from arachidonic acid or 15*S*-H(P)ETE, even when used in large amount (data not shown). On the other hand, 15-LOX-2 readily reacted with 8*S*-HPETE or 8*S*-HETE with a specific activity comparable to that of 15*S*-HPETE formation from arachidonic acid, yielding 8*S*,15*S*-diH(P)ETE as the sole product (data not shown). Therefore, 15-LOX-2 reacted only at the available 15*S*-position and never exhibited any "8-LOX" activity.

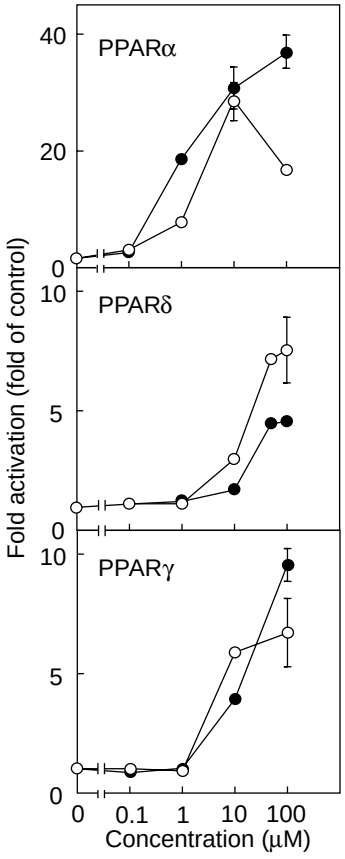

Fig. 5. PPAR agonist activity of 8*S*-HETE and 8*S*,15*S*-diHETE. PPAR agonist activity was determined basically by Dual-Luciferase Reporter Gene Assay system [13]. The CV1 cells were transfected with p4xUASg-tk-luc, pRL-CMV, and pCMX-CBP as well as pM-hPPARα, pM-hPPARβ or pM-hPPARγ for 24 h, and then treated with each test compound of various concentrations for another 24 h; open circles, 8*S*-HETE; closed circles, 8*S*,15*S*-diHETE. The relative luciferase activities were determined as fold-induction to that of the vehicle control. The values are means ± SEM of 3–4 assays.

PPAR agonist activity of 8S-HETE and 8S,15S-diHETE

As PPARs can be activated by eicosanoids including 8*S*-HETE [9,17], we examined whether 8*S*,15*S*-diHETE also activates PPARs. As previously reported [9], 8*S*-HETE significantly activated PPARα even at 1 μM, while it also activated both PPARδ and PPARγ at a higher concentration (Fig. 5). 8*S*,15*S*-diHETE activated PPARα even more stronger than 8*S*-HETE, and also PPARδ and PPARγ in higher concentration (Fig. 5), confirming that 8*S*,15*S*-diHETE, as well as 8*S*-HETE, can be a PPARα selective agonist.

Discussion

8-LOX has so far been detected only in mice [1,5], while its homologue 15-LOX-2 seems more common in mammals including human and bovine [6,7]. Therefore, one will wonder whether 8-LOX is physiologically equivalent to 15-LOX-2 and what is the physiological role of 8-LOX. In order to approach such questions, we are investigating any clue based on the enzymological properties of 8-LOX as well as 15-LOX-2.

For the enzymological study of 8-LOX, we first had to establish an effective preparation system. During cloning, we experienced severe difficulties in expression of this enzyme, while 15-LOX-2 was easily expressed in all the protocol [5,6]. Qiao et al.[18] expressed 8-LOX in COS-7 cells and *E. coli*, while the expression level seemed to be modest as deduced from a purification ratio as high as 380-fold. Again in this study, we experienced severe difficulty in the bacterial expression of 8-LOX. Although the expression was almost negligible in a LB medium, the pET28/8-LOX construct produced a high amount of enzyme in the 2× YT medium (Fig. 1). This expression system made it possible to purify 8-LOX and analyze its reaction properties in detail.

In this study, we tried to evaluate the multifunction of 8-LOX, especially focusing on its possible "15-LOX" activity, because 8-LOX shares 78% homology with human 15-LOX-2 [5] and their positional specificity

can be exchangeable by switching two critical amino acids [19]. We confirmed that the expressed 8-LOX converted arachidonic acid to pure 8*S*-HPETE and then further to 8*S*,15*S*-diHPETE specifically (Figs. 2 and 3). The specificity and efficiency of 8*S*,15*S*-diHPETE synthesis could make this reaction functional in vivo (Fig. 3 and Table 1), proposing a possible 8-LOX pathway (Fig. 6), where a portion of the produced 8*S*-HPETE would be further metabolized to 8*S*,15*S*-diHPETE. On the other hand, 8-LOX also produces 8*S*,15*S*-diHPETE from 15*S*-HPETE possibly provided by 12-LOX [10,11,15]. Both 8*S*-HPETE and 8*S*,15*S*-diHPETE will be reduced by endogenous reductase to 8*S*-HETE and 8*S*,15*S*-diHETE, respectively, and exhibit their physiological functions. 8-LOX is the first LOX that produces 8*S*,15*S*-diHPETE specifically by itself. Binding of arachidonic acid to 8-LOX is strictly controlled only in one direction, while the 8*S*-hydro(pero)xy group in 8*S*-H(P)ETE will facilitate its re-binding in the other direction. The possible bi-directional binding of substrate in the substrate-binding site of 8-LOX indicates that 8-LOX can function as a "15-LOX" under certain circumstances.

In contrast to the potential "15-LOX" activity of 8-LOX, we never detected "8-LOX" activity of 15-LOX-2 because 15-LOX-2 never produced 8*S*,15*S*-diHPETE from arachidonic acid or 8*S*-H(P)ETE, although 15-LOX-2 metabolized 8*S*-H(P)ETE quite efficiently to 8*S*,15*S*-diH(P)ETE. Therefore, 15-LOX-2 reacted specifically only at the available 15*S*-position of substrates. The physiological importance of 8*S*,15*S*-diH(P)ETE formation by 15-LOX-2 is unclear because there is no enzyme detected in humans to produce 8*S*-H(P)ETE efficiently.

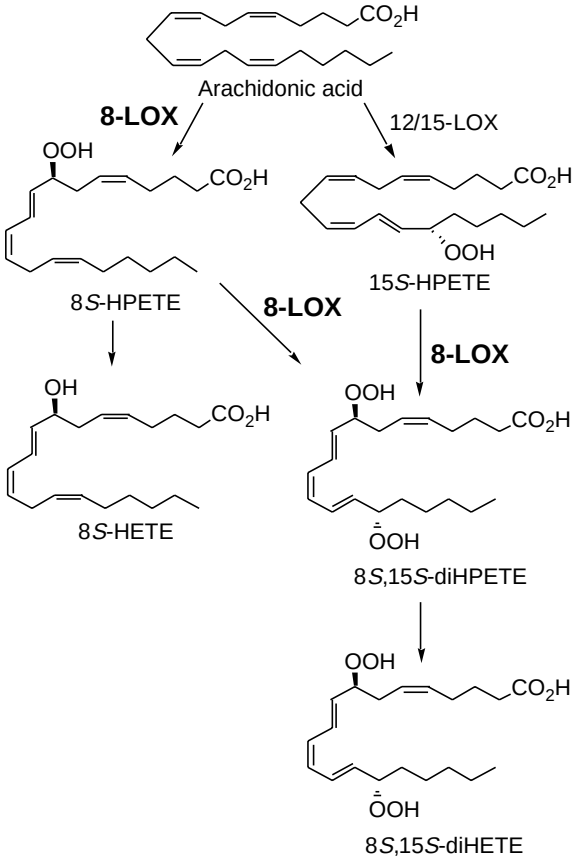

Fig. 6. Arachidonic acid metabolism by 8-LOX.

In mammalian systems, 8*S*,15*S*-diHETE was detected as a minor component in the arachidonic acid metabolites by human eosinophils [15] and porcine leukocytes [20]. The first report of the physiological function of 8*S*,15*S*-diHETE described its chemotactic activity for human polymorphonuclear leukocytes [10], although there are also contradicting results [21]. On the other hand, 8*S*,15*S*-diHETE exhibits weak chemotactic activity for human and guinea pig eosinophils [22,23]. Another physiological activity of 8*S*,15*S*-diHETE was reported as an antagonist against the hyperalgesia due to 8*R*,15*S*-diHETE [24]. Although these results suggest the presence of specific receptor for 8*S*,15*S*-diHETE, a detailed physiological activity of 8*S*,15*S*-diHETE remains to be elucidated.

As possible cellular targets of 8*S*,15*S*-diHETE, PPARs are examined here. Actually, 8*S*,15*S*-diHETE activated PPARα stronger than 8*S*-HETE. This new potent natural ligand of PPARα would, together with 8*S*-HETE, strengthen the control of gene expression by PPARα. Activation of PPARα is important especially in skin. PPARα activators induce differentiation and inhibit proliferation of keratinocytes in vitro and in vivo [25,26], and, furthermore, partially inhibit tumor promotion in mouse skin [27]. Actually, transgenic mice overexpressing 8-LOX exhibit highly differentiated skin phenotype [28] and even resistance against tumor promotion by TPA [29]. It is likely that 8*S*,15*S*-diHETE as well as 8*S*-HETE contribute to the physiological function of 8-LOX via PPARα activation.

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