

Hydroxyeicosapentaenoic acids from the Pacific krill show high ligand activities for PPARs^S

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Abstract PPARs regulate the expression of genes for energy metabolism in a ligand-dependent manner. PPARs can influence fatty acid oxidation, the level of circulating triglycerides, glucose uptake and insulin sensitivity. Here, we demonstrate that 5-hydroxyeicosapentaenoic acid (HEPE), 8-HEPE, 9-HEPE, 12-HEPE and 18-HEPE (hydroxylation products of EPA) obtained from methanol extracts of Pacific krill (*Euphausia pacifica*) can act as PPAR ligands. Two of these products, 8-HEPE and 9-HEPE, enhanced the transcription levels of GAL4-PPARs to a significantly greater extent than 5-HEPE, 12-HEPE, 18-HEPE, EPA, and EPA ethyl-ester. 8-HEPE also activated significantly higher transcription of GAL4-PPAR α , GAL4-PPAR γ , and GAL4-PPAR δ than EPA at concentrations greater than 4, 64, and 64 μ M, respectively. We also demonstrated that 8-HEPE increased the expression levels of genes regulated by PPARs in FaO, 3T3-F442A, and C2C12 cells. Furthermore, 8-HEPE enhanced adipogenesis and glucose uptake. By contrast, at the same concentrations, EPA showed weak or little effect, indicating that 8-HEPE was the more potent inducer of physiological effects.—Yamada, H., E. Oshiro, S. Kikuchi, M. Hakozaiki, H. Takahashi, and K-i. Kimura. **Hydroxyeicosapentaenoic acids from the Pacific krill show high ligand activities for PPARs.** *J. Lipid Res.* 2014. 55: 895–904.

Supplementary key words peroxisome proliferator-activated receptor • fatty acid oxidation • adipogenesis • glucose uptake

PPARs are members of a nuclear receptor superfamily that play critical roles in the regulation of storage and catabolism of lipids (1). They contribute to these regulation processes by activating gene expression in a ligand-dependent manner that involves recognition of and binding to peroxisome proliferator response elements composed of TGACCT-related direct repeats separated by one nucleotide (2, 3). PPARs form heterodimers with peroxisome proliferator response elements via the retinoid-X receptor,

which is a receptor for 9-*cis*-retinoic acid (4, 5). Three types of PPAR have been identified, namely α , γ , and δ . PPAR α is expressed at high levels in the liver where it promotes fatty acid oxidation, ketogenesis, lipid transport, and gluconeogenesis (6, 7). PPAR α responds to the concentration of fatty acids in the liver and enhances fatty acid breakdown by upregulating genes encoding β -oxidation enzymes (8–10). PPAR γ is highly expressed in adipose tissue, where it serves as an essential regulator for adipocyte differentiation. PPAR γ promotes lipid storage in mature adipocytes by increasing the expression of several key genes (11, 12). PPAR δ (also known as PPAR β) is widely expressed, but with relatively higher levels in the brain, colon, and skin (13–15); PPAR δ is the predominant PPAR isoform in skeletal muscle. Studies using transgenic mice showed that targeted expression of activated PPAR δ increases the numbers of oxidative type I muscle fibers, which enhance whole-body insulin sensitivity and exercise endurance capacity (16).

The identification of unsaturated fatty acids as PPAR ligands provided firm evidence that the direct interaction of nuclear receptors with these fatty acids is required for some PPAR-dependent transcription activity (4, 8, 17–19). Unsaturated fatty acids can bind to all three types of PPAR, with PPAR α exhibiting the highest affinity for concentrations equivalent to circulating blood levels (8, 20). In contrast, the long-chain fatty acid, erucic acid (C22:1), is a weak ligand that appears to have more affinity for PPAR δ (21). Overall, saturated fatty acids are poor PPAR ligands compared with unsaturated fatty acids (4, 8, 19). Eicosanoids, a class of fatty acids, are mainly derived from arachidonic acid (AA) either via the lipoxygenase pathway,

Abbreviations: AA, arachidonic acid; Angptl4, angiopoietin-like protein 4; Cpt, carnitine palmitoyltransferase; CYP4A, cytochrome P450 CYP4A enzyme; EPA-Et, EPA ethyl-ester; Ehhadh, enoyl-CoA hydratase/3-hydroxyacyl CoA; Fabp, fatty acid-binding protein; HEPE, hydroxyeicosapentaenoic acid.

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leading to the formation of leukotrienes and hydroxyeicosatetraenoic acids (HETEs), or via the cyclooxygenase pathway that produces prostaglandins and thromboxanes. They can act as activators for different PPARs; for example, the prostaglandin D2 derivative, 15-deoxy- Δ 12,14-PGJ2, is a ligand for PPAR γ (17, 22); 8-HETE, a compound associated with phorbol ester-induced inflammation, is a ligand for PPAR α (8, 19); and leukotriene B4, a chemotactic inflammation mediator, binds to PPAR α (19, 23).

Increasing levels of triglycerides are associated with obesity and are indicators of progressive development of insulin resistance, hypertension, and hyperlipidemia (24, 25). Thus, normalization of triglyceride levels has been investigated as a possible means of prevention for these conditions. It is well-known that dietary PUFAs can ameliorate some of the deleterious effects of these disorders (26, 27). This property may reflect both activation of PPAR-dependent β - and ω -oxidation pathways (28), as well as PUFA-dependent suppression of lipogenic and glycolytic enzymes (29). Krill are a source of PUFAs, as their oil is rich in EPA and docosahexaenoic acid. In a similar manner to fish oil, krill oil has been shown to have anti-inflammatory effects and to lower the levels of triglycerides and cholesterol in the plasma (30, 31). Although lipids are major bioactive compounds in krill oil, we found in a previous study that a water-soluble extract from Pacific krill could suppress weight gain in mice caused by a high fat diet. The levels of triglycerides in the livers of mice given a water-soluble extract from Pacific krill decreased (32).

Here, we investigated krill extracts and found that hydroxyeicosapentaenoic acids (HEPEs) (5-, 8-, 9-, 12-, and 18-HEPE) displayed PPAR ligand activities. Interestingly, the PPAR ligand activities of 8-HEPE and 9-HEPE were significantly greater than EPA. These results indicate that hydroxylation of EPA at the C-8 or C-9 position increased ligand activity for PPARs.

MATERIAL AND METHODS

Reagents

HEPEs (purity >98%) and EPA (purity >98%) were purchased from Cayman Chemical (Ann Arbor, MI). EPA ethyl-ester (EPA-Et) (purity >99%), the PPAR agonists GW7647 and GW1929, and the PPAR α antagonist MK-886 were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-Deoxy-D-glucose, the PPAR γ antagonist GSK0660, and the PPAR δ antagonist T0070907 were purchased from Sigma-Aldrich (St. Louis, MO). The PPAR agonist GW501516 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Extraction and isolation from Pacific krill

We used dried Pacific krill purchased from Kawashu (Iwate, Japan). The krill were powdered and then extracted with methanol under reflux for 4 h. The methanol extract was diluted with distilled water (1:4 by volume) and subjected to column chromatography using Diaion HP-20 (Mitsubishi Chemical, Tokyo, Japan). A fraction was eluted with 80% methanol from the HP-20. This fraction was diluted with distilled water (1:3 by volume) and applied to an InertSep C18 (GL Science Inc., Tokyo, Japan). The

fraction was eluted with 100% methanol. HEPEs were separated on an InertSustain ODS-3 column (20.0 mm diameter \times 250 mm; GL Science Inc.) with isocratic elution of acetonitrile/water/formic acid (55/45/0.1) at a flow rate of 15 ml/min and detection at 235 nm (Fig. 2A). The fraction that contained compounds 3 and 4 was subjected to recycle-HPLC, and peaks 3 and 4 were separated on an InertSustain ODS-3 column (20.0 mm diameter \times 250 mm; GL Science Inc.) with isocratic elution of acetonitrile/water/formic acid (60/40/0.1) at a flow rate of 15 ml/min and detection at 235 nm (Fig. 2B).

Identification of HEPEs

The compounds purified from Pacific krill were identified as 5-, 8-, 9-, 12-, and 18-HEPE by LC-TOFMS (Agilent Technologies, Palo Alto, CA) analysis. The HEPEs were separated on an InertSustain ODS-3 column (2.0 mm diameter \times 250 mm; GL Science Inc.) with isocratic elution of acetonitrile/water/formic acid (40/60/0.5) at a flow rate of 0.3 ml/min. Physicochemical properties, such as monoisotopic mass, molecular formula, UV spectrum, and retention time in the HPLC, were identical with reference standard compounds (supplementary Tables II, III; supplementary Fig. III).

Cell culture

3T3-F442A (DS Pharma Biomedical Co., Ltd., Osaka, Japan), NIH-3T3, and C2C12 cells (RIKEN BioResource Center, Ibaraki, Japan) were cultured in DMEM containing 10% FBS (Invitrogen, Carlsbad, CA) and antibiotic antimycotic solution (Sigma-Aldrich). FaO cells (DS Pharma Biomedical Co.) were cultured in Ham's F12 containing 10% FBS and antibiotic antimycotic solution. Myogenesis was induced in C2C12 cells using differentiation medium (DMEM with 2% horse serum).

Luciferase reporter assay

The ligand-dependent transcriptional activities of PPARs were assayed using luciferase reporter systems. The ligand binding domains of mouse PPARs (PPAR α , amino acids 201–468; PPAR γ , amino acids 238–506; PPAR δ , amino acids 171–441) were cloned into the pFN26A (BIND) hRluc-neo Flexi Vector (Promega, Tokyo, Japan). NIH-3T3 cells were seeded in 24-well culture plates (1×10^5 cells/well) and cultured overnight. pFN26A vector carrying an inserted PPAR ligand binding domain and the pGL4.35 vector (Promega) were cotransfected using Lipofectamine 2000 (Invitrogen) into NIH-3T3 cells; the transfected cells were cultured for 36 h. The cells were cultured with HEPE, EPA, EPA-Et, GW7647, GW1929, or GW501516 for 24 h and then harvested using passive lysis buffer (Promega). Renilla and firefly luciferase activities were determined using a dual luciferase assay system (Promega).

Quantitative real-time PCR

FaO, C2C12, and 3T3-F442A cells were seeded in 24-well culture plates (1×10^5 cells/well) and cultured overnight. 8-HEPE, EPA, GW7647, GW1929, or GW501516 was then added to the culture for 6 (FaO and C2C12) or 24 h (3T3-F442A). Total RNAs were extracted with an RNeasy kit (QIAGEN, Tokyo, Japan) and used to synthesize cDNAs using a PrimeScript RT reagent kit (Takara, Shiga, Japan); all kits were used according to the manufacturers' recommendations. Quantitative real-time PCR was performed with the gene-specific primers listed in supplementary Table I and Fast SYBR Green master mix (Applied Biosystems, Foster City, CA).

Triglyceride staining and quantification

3T3-F442A cells were seeded in 24-well culture plates (1×10^5 cells/well) and cultured overnight. 8-HEPE or EPA was then added to the culture for 7 days at which time the cells were

stained with Oil Red O. Stained cells were examined using a Nikon microscope ($\times 400$). An adipogenesis assay kit (Bio Vision, Mountain View, CA) was used to measure triglyceride content.

2-Deoxyglucose uptake measurement

C2C12 cells were seeded in 24-well culture plates (2×10^4 cells/well) and cultured overnight. The medium was then replaced with DMEM containing 2% horse serum and antibiotic antimycotic solution and the cells were cultured for a further 5 days. Following this treatment to induce myogenesis, they were cultured in DMEM containing 8-HEPE, EPA, or GW501516 for 6 h. A 2-deoxyglucose uptake measurement kit (Cosmo Bio, Tokyo, Japan) was used to quantify glucose uptake into the C2C12 cells.

Statistical analysis

Statistically significant differences between the experimental groups were identified using one-way ANOVA and Tukey's post hoc tests. Data are shown as means \pm SD.

RESULTS

Activation of transcription of GAL4-PPARs by Pacific krill extracts

To examine methanol extracts from Pacific krill for possible PPAR ligand activity, we created fusion proteins in which the yeast GAL4 DNA-binding domain was linked to the ligand-binding domain of PPAR α , PPAR γ , or PPAR δ (GAL4-PPAR α , GAL4-PPAR γ , or GAL4-PPAR δ). When a reporter containing a GAL4 upstream activating sequence is utilized, these chimeric receptors allow PPAR activity to be assayed independently of endogenous receptors. We confirmed the specificity of the GAL4-PPAR α , GAL4-PPAR γ , and GAL4-PPAR δ reporter systems using PPAR agonists (supplementary Fig. 1). Using this reporter approach, we found that Pacific krill extracts activated transcription of GAL4-PPAR α , GAL4-PPAR γ , and GAL4-PPAR δ in a dose-dependent manner (Fig. 1).

HEPEs activated transcription of GAL4-PPARs

We purified the compounds in the Pacific krill extracts that activated transcription of GAL4-PPAR α using an activity-guided fractionation method (Fig. 2; supplementary Fig. II). LC-TOFMS identified the compounds in the active fractions as HEPEs (supplementary Tables II, III; supplementary Fig. III). Transcription of GAL4-PPAR α and GAL4-PPAR γ was activated by 5-, 8-, 9-, 12-, and 18-HEPE (Fig. 3A, B), while transcription of GAL4-PPAR δ was activated by 5-, 8-, 9-, and 12-HEPE (Fig. 3C). Surprisingly, 8- and 9-HEPE showed significantly greater transcriptional activation of GAL4-PPARs than 5-, 12-, 18-HEPE, EPA, and EPA-Et. 8-HEPE is known to be able to activate PPAR α (8); however, no reports have previously been made on whether it could activate PPAR γ or PPAR δ , nor has its activity for PPARs been compared with EPA. We therefore compared the relative PPAR ligand activities of 8-HEPE and EPA at a range of concentrations (4, 16, 64, and 128 μ M; Table 1). With regard to GAL4-PPAR α , 8-HEPE had a significantly greater effect on transcription activation at all concentrations tested (Table 1). For GAL4-PPAR γ and GAL4-PPAR δ , 8-HEPE showed a significantly greater effect at the highest tested concentrations (64 and 128 μ M; Table 1). Forman, Chen, and Evans (8) showed that 8-HETE also activates PPAR α . Here, we compared the relative activities of 8-HEPE and 8-HETE as ligands for PPARs. We found that AA, 8-HETE, and 8-HEPE activated transcription of GAL4-PPAR α and GAL4-PPAR γ at a concentration of 5 μ M (Fig. 4A, B), while 8-HEPE activated transcription of GAL4-PPAR δ (Fig. 4C). We also found that 8-HEPE showed a greater effect than 8-HETE on the transcription levels of GAL4-PPAR α and GAL4-PPAR δ .

Here, we demonstrate that HEPEs from Pacific krill can act as PPAR α activators (Fig. 2; supplementary Figs. II, III). Moreover, through use of Diaion HP-20 column chromatography to increase concentrations, we found that their activities varied in a concentration-dependent manner

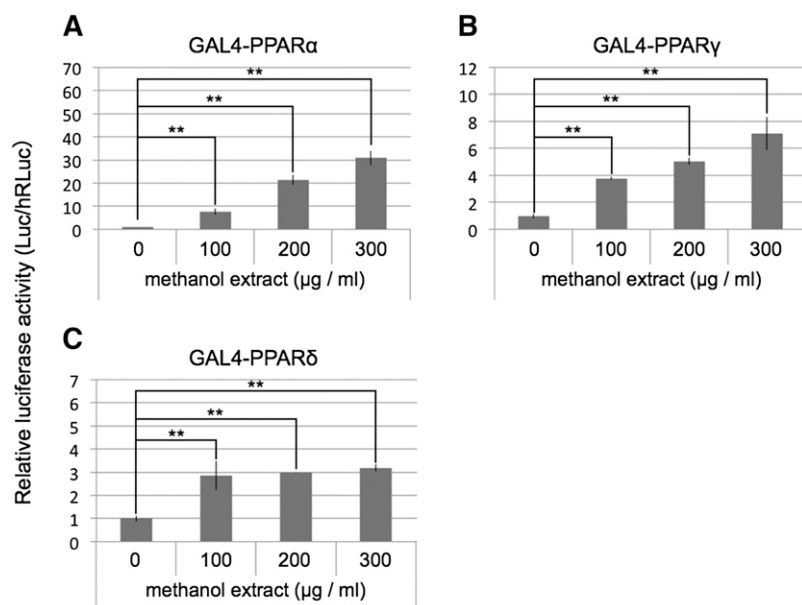


Fig. 1. GAL4-PPAR assays of a methanol extract from Pacific krill. NIH-3T3 cells were transfected with reporter plasmids and cultured with the extract for 24 h. Then, firefly luciferase (Luc) activities were measured, and the values were normalized against Renilla luciferase (hRLuc) activity. Plotted values represent the mean \pm SD from four independent cultures. ** $P < 0.01$.

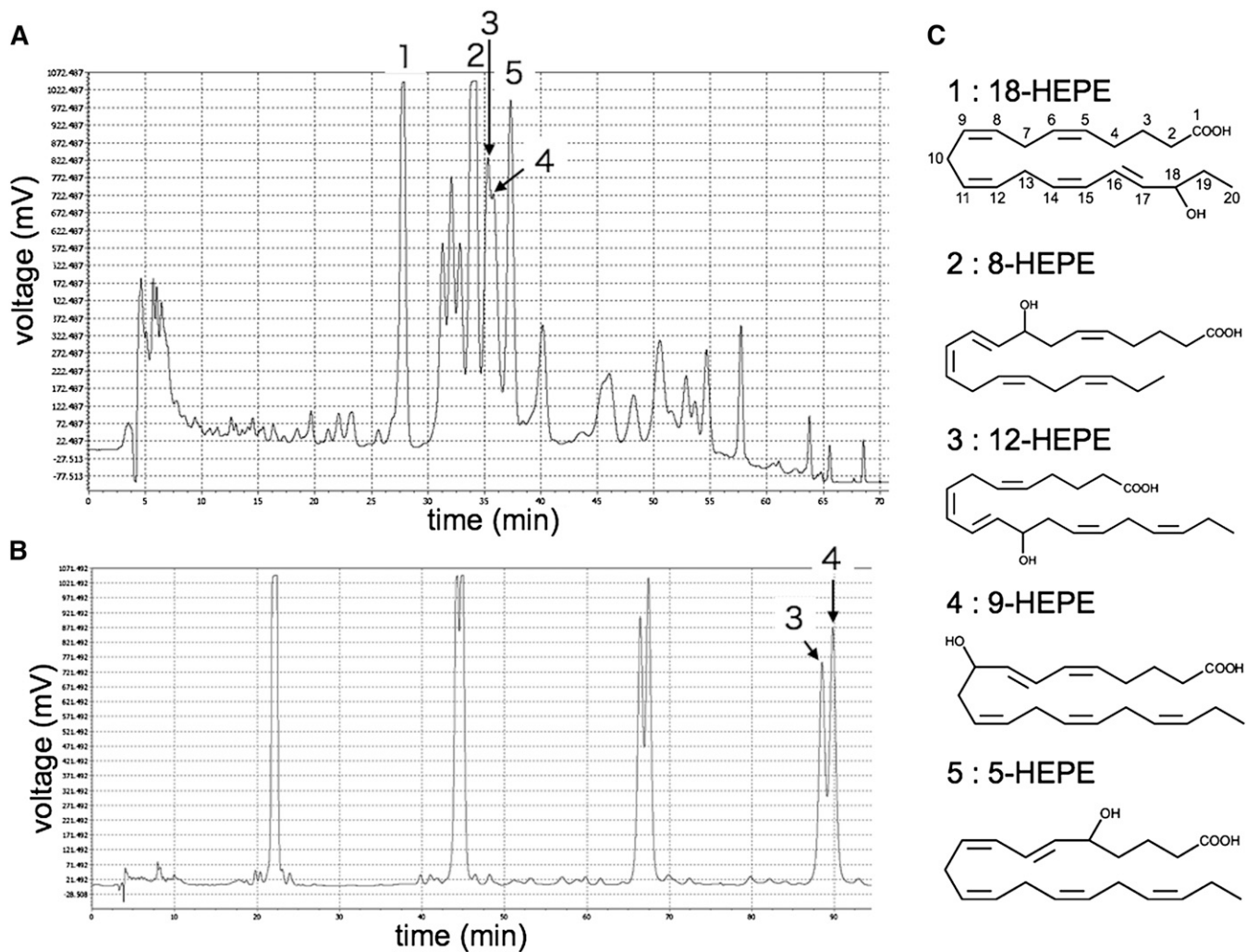


Fig. 2. HPLC chromatograms. A: Extract purified using HP-20 and InertSep C18 (20 mg). Five compounds that activate GAL4-PPAR α transcription are present. B: Chromatogram from the recycle-HPLC separation of compounds 3 and 4. LC-TOFMS identified the following compounds: 1, 18-HEPE; 2, 8-HEPE; 3, 12-HEPE; 4, 9-HEPE; and 5, 5-HEPE. C: The structures of the different HEPEs. The numbers on 18-HEPE indicate carbon positions.

(supplementary Fig. IIB). Measurements of HEPEs and EPA indicated that HP-20 chromatography was sufficient to concentrate HEPEs (supplementary Table IV) and that InertSep C18 chromatography removed EPA. Methanol extract of Pacific krill contained 0.72 $\mu\text{g}/\text{mg}$ 8-HEPE, 0.24 $\mu\text{g}/\text{mg}$ 9-HEPE, and 7.1 $\mu\text{g}/\text{mg}$ EPA (supplementary Table IV). A cocktail that contained the same relative amounts of 8-HEPE and 9-HEPE, as in the methanol extract, showed about 60% of the activity of the methanol extract. However, addition of EPA to the cocktail did not increase activity (supplementary Fig. IV). These results indicate that 8-HEPE and 9-HEPE are the major PPAR α activators in Pacific krill extracts.

8-HEPE increases expression of genes related to fatty acid oxidation in mitochondrial and peroxisomal pathways

PPAR α regulates the expression of genes encoding enzymes and proteins responsible for fatty acid oxidation. To examine the activity of 8-HEPE as a PPAR α ligand, we investigated whether 8-HEPE increased the expression of genes

regulated by PPAR α . Previous studies showed that Wy14,643, an activator of PPAR α , upregulated expression of liver fatty acid-binding protein (*Fabp1*), enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase (*Ehhadh*), cytochrome P450 CYP4A enzyme (*Cyp4a1*) and carnitine palmitoyltransferase (*Cpt1a*, *Cpt2*) in mouse liver cells (10) and in the rat hepatoma cell line, FaO (33). We cultured FaO cells with EPA, 8-HEPE, or GW7647, a PPAR α -specific agonist, for 6 h. Expression of *Fabp1*, *Ehhadh*, and *Cpt1a* was increased by 8-HEPE, but not by EPA; expression of *Cyp4a1* and *Cpt2* was increased by EPA and 8-HEPE. Overall, 8-HEPE showed a greater effect on gene expression levels than EPA (Fig. 5). MK-886, a PPAR α -specific antagonist, inhibited 8-HEPE-induced expression of *Fabp1*, *Ehhadh*, and *Cpt2* (Fig. 5).

8-HEPE induces adipogenesis in mouse preadipocyte cells

PPAR γ plays a central role in regulation of gene expression and differentiation in adipocytes (11). We therefore examined whether 8-HEPE could act as a PPAR γ ligand in

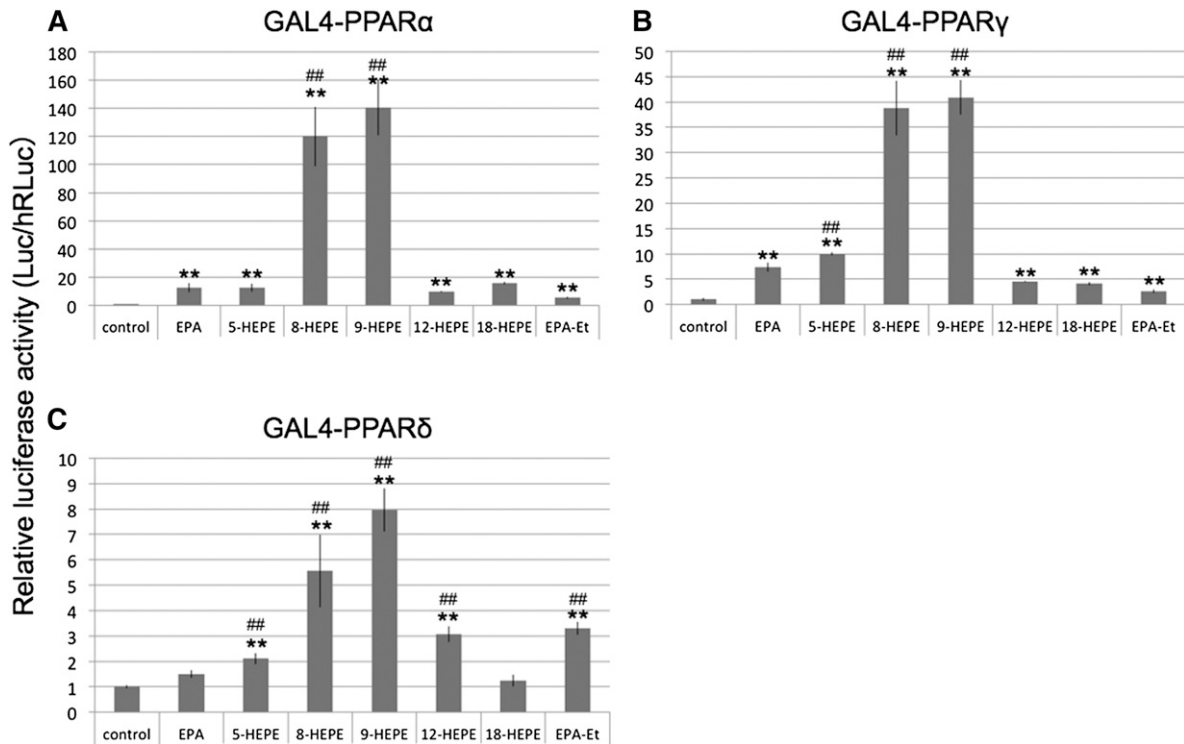


Fig. 3. GAL4-PPAR assays of HEPES. NIH-3T3 cells were transfected with reporter plasmids and cultured with 128 μ M EPA, EPA-Et, or HEPES for 24 h. Then firefly luciferase (Luc) activities were measured, and the values were normalized against Renilla luciferase (hRLuc) activity. Plotted values represent the mean \pm SD from four independent cultures. Significant differences from the control are indicated by $**P < 0.01$. Significant differences from EPA are indicated by $##P < 0.01$.

these cells. We cultured 3T3-F442A cells, a mouse preadipocyte cell line, with EPA, 8-HEPE, or GW1929, a PPAR γ -specific agonist, for 24 h. We found that expression of *Fabp4* and *Pparg* was increased by 8-HEPE, but not by EPA (Fig. 6A, B). Both 8-HEPE and GW1929 increased the level of *Fabp4* expression (Fig. 6A), but only 8-HEPE had an effect on *Pparg* expression (Fig. 6B). T0070907, a PPAR γ -specific antagonist, inhibited 8-HEPE-induced expression of *Fabp4* (Fig. 6A).

Next, we cultured 3T3-F442A cells with 50 μ M EPA or 8-HEPE, or 1 μ M GW1929 for 7 days, and measured triglyceride accumulation to evaluate adipocyte differentiation. Compared with the control or EPA-treated cells, a greater level of triglyceride accumulation was observed in 8-HEPE-treated cells (Fig. 6C–E).

8-HEPE increases *Angptl4* expression and enhances glucose uptake in mouse myoblast cells

PPAR δ is the predominant isoform found in skeletal muscle; the agonist GW501516, which is PPAR δ -specific, increases the expression of angiopoietin-like protein 4 (*Angptl4*) (34, 35) and enhances glucose uptake (36) in C2C12 cells, a mouse myoblast cell line. *Angptl4* is one of the major targets of PPAR δ , and *Angptl4* is the gene that is most highly induced by long-chain fatty acids in human myotubes (34). Here, we examined the relative level of *Angptl4* expression by real-time PCR. We cultured C2C12 cells with EPA 8-HEPE or GW501516 for 6 h and found that expression of *Angptl4* was increased to a greater extent by 8-HEPE than EPA (Fig. 7A). GSK0660, a PPAR δ -specific antagonist, inhibited 8-HEPE-induced expression of *Angptl4* (Fig. 7A).

TABLE 1. Activities of EPA and 8-HEPE in the induction of GAL4-PPARs transcription

Compound	GAL4-PPAR α	GAL4-PPAR γ	GAL4-PPAR δ
EPA			
4 μ M	1.20 \pm 0.18	1.58 \pm 0.23	1.39 \pm 0.39
16 μ M	2.80 \pm 0.92 ^a	2.23 \pm 0.32 ^a	1.42 \pm 0.08 ^a
64 μ M	7.10 \pm 1.32 ^a	4.37 \pm 0.47 ^a	1.43 \pm 0.23
128 μ M	12.65 \pm 3.20 ^a	7.35 \pm 0.88 ^a	1.50 \pm 0.15 ^a
8-HEPE			
4 μ M	7.28 \pm 0.87 ^{a,b}	1.49 \pm 0.28	0.90 \pm 0.09
16 μ M	31.69 \pm 3.50 ^{a,b}	2.53 \pm 0.35 ^a	1.48 \pm 0.11 ^a
64 μ M	95.60 \pm 10.13 ^{a,b}	6.02 \pm 0.42 ^{a,b}	1.90 \pm 0.08 ^{a,b}
128 μ M	119.90 \pm 21.13 ^{a,b}	38.78 \pm 5.40 ^{a,b}	5.55 \pm 1.43 ^{a,b}

Values represent the mean \pm SD from four independent cultures.

^aSignificant differences from the control are indicated by $P < 0.01$.

^bSignificant differences from EPA are indicated by $P < 0.01$.

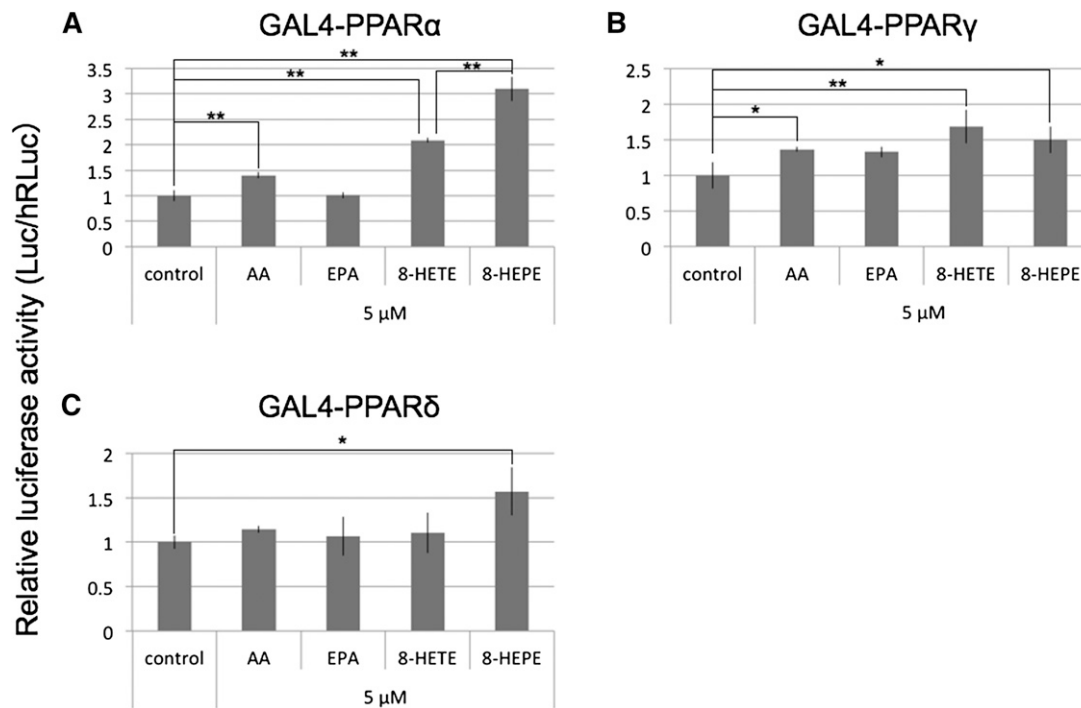


Fig. 4. GAL4-PPAR assays of AA, EPA, 8-HETE, and 8-HEPE. NIH-3T3 cells were transfected with reporter plasmids and cultured with 5 μ M AA, EPA, 8-HETE, or 8-HEPE for 24 h. Then firefly luciferase (Luc) activities were measured, and the values were normalized against Renilla luciferase (hRLuc) activity. Plotted values represent the mean \pm SD from four independent cultures. 8-HETE and 8-HEPE standards were used. * P < 0.05, ** P < 0.01.

We then measured glucose uptake in C2C12 cells that had been induced to undergo myotube differentiation for 5 days. 8-HEPE enhanced the uptake of glucose in these cells (Fig. 6B); the level of 8-HEPE-enhanced glucose

uptake was higher, albeit nonsignificantly, than that induced by EPA (Fig. 6B). These results indicate that 8-HEPE acts as a PPAR δ ligand. Overall, our analyses show that although 8-HEPE has a greater activity than EPA in upregulating

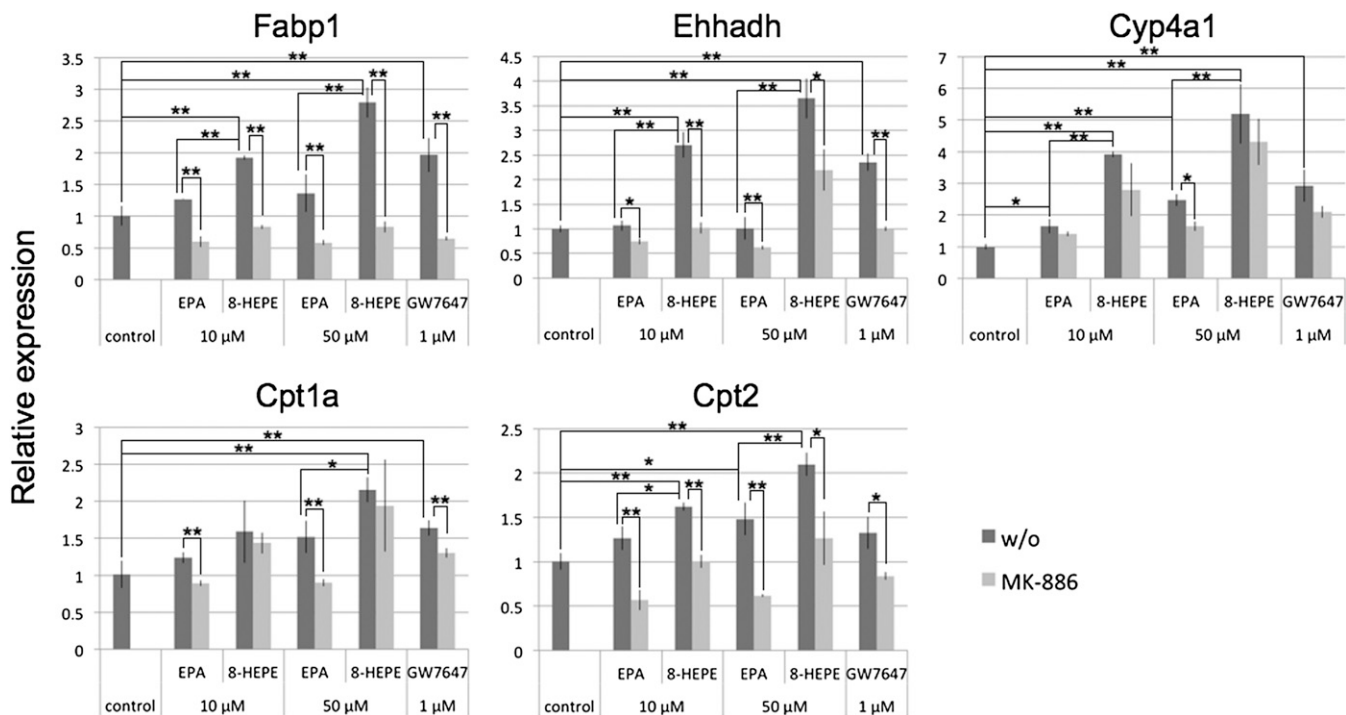


Fig. 5. Gene expression after 8-HEPE activation of PPAR α in FaO cells. The cells were cultured with EPA, 8-HEPE, or GW7647 for 6 h. The PPAR α antagonist MK-886 (50 μ M) was added to the media at the same time as EPA, 8-HEPE, or GW7647. Gene expression levels were measured by real-time PCR and normalized against expression of *Actb*. Plotted values represent the mean \pm SD from three independent cultures. 8-HEPE purified from Pacific krill was used. * P < 0.05, ** P < 0.01. w/o, without.

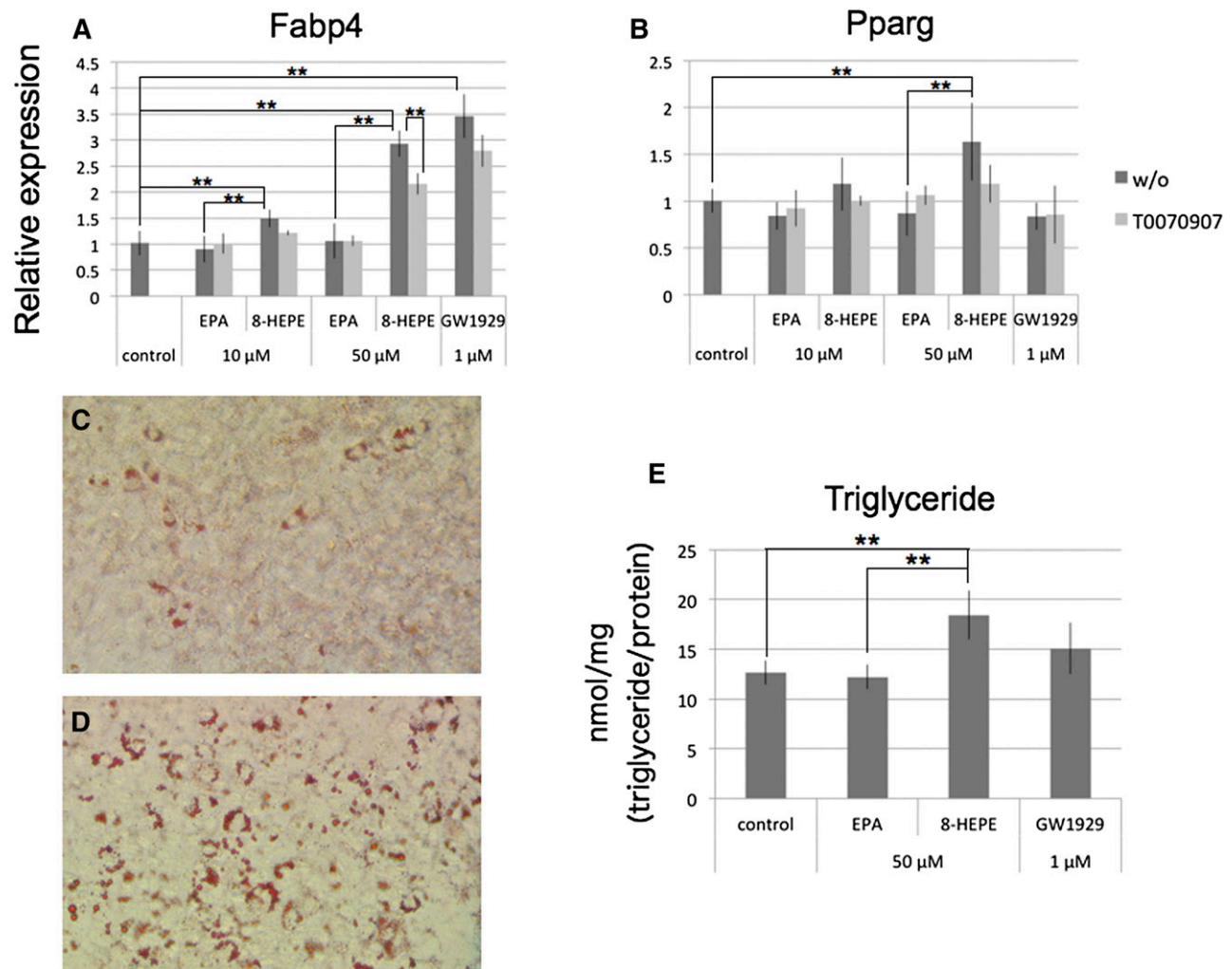


Fig. 6. The effect of 8-HEPE treatment on adipogenesis in 3T3-F442A cells. A, B: 3T3-F442A cells were cultured with EPA, 8-HEPE, or GW1929 for 24 h. The PPAR γ antagonist T0070907 (5 μ M) was added to the media at the same time as EPA, 8-HEPE, or GW1929. Expression of *Fabp4* and *Pparg* was measured by real-time PCR and normalized against expression of *Rplp0*. C–E: 3T3-F442A cells were cultured with 50 μ M of EPA or 8-HEPE, or 1 μ M GW1929 for 7 days. Cells cultured with EPA (C) or 8-HEPE (D) were stained by Oil Red O. E: Triglyceride contents were measured and normalized against protein concentration. Plotted values represent the mean \pm SD from four independent cultures. 8-HEPE purified from Pacific krill was used. ** $P < 0.01$. w/o, without.

Angptl4 expression, its effect on glucose uptake is comparable to EPA.

DISCUSSION

Our analyses here demonstrate that 8-HEPE and 9-HEPE are PPAR ligands and that their activities are greater than those of 5-HEPE, 12-HEPE, 18-HEPE, EPA, and EPA-Et (Fig. 3). These results indicate that the position of hydroxylation on EPA is correlated with the strength of ligand activity for PPARs and that hydroxylation at the C-8 or C-9 positions produces more effective ligands. A similar phenomenon has been found in HETEs in which the position of hydroxylation influences ligand activity for PPAR α (20). It was also reported that 8-HETE has a significantly greater ability to activate PPAR α compared with 5-HETE, 11-HETE, 12-HETE, and 15-HETE (20). This report and the present results show that hydroxylation at the C-8 position is

important for activation of PPAR α in both HETEs and HEPEs. However, 8-HETE and 8-HEPE activities with regard to PPAR γ and PPAR δ have not previously been identified (8, 20). The likely reason is that the concentrations of 8-HETE and 8-HEPE tested in the experiments were too low to activate PPAR γ or PPAR δ . For example, earlier studies used 3 μ M 8-HETE and 1 μ M 8-HEPE (8, 20), whereas, in the present study, activation of PPAR γ and PPAR δ was observed at concentrations greater than 5 μ M 8-HEPE (Fig. 4). For PPAR α and PPAR δ , 8-HEPE showed a greater effect than 8-HETE, while both 8-HETE and 8-HEPE were similarly active with regard to PPAR γ (Fig. 4). Previous studies did not compare the relative ligand activity of 8-HEPE and EPA for PPARs. As shown here, 8-HEPE had a greater ability than EPA to activate PPAR α , PPAR γ , and PPAR δ at concentrations of more than 4 μ M, 64 μ M, and 64 μ M, respectively (Table 1). The concentration of total fatty acids in serum is about 700 μ M (37), while serum concentrations of specific fatty acids that are abundant in

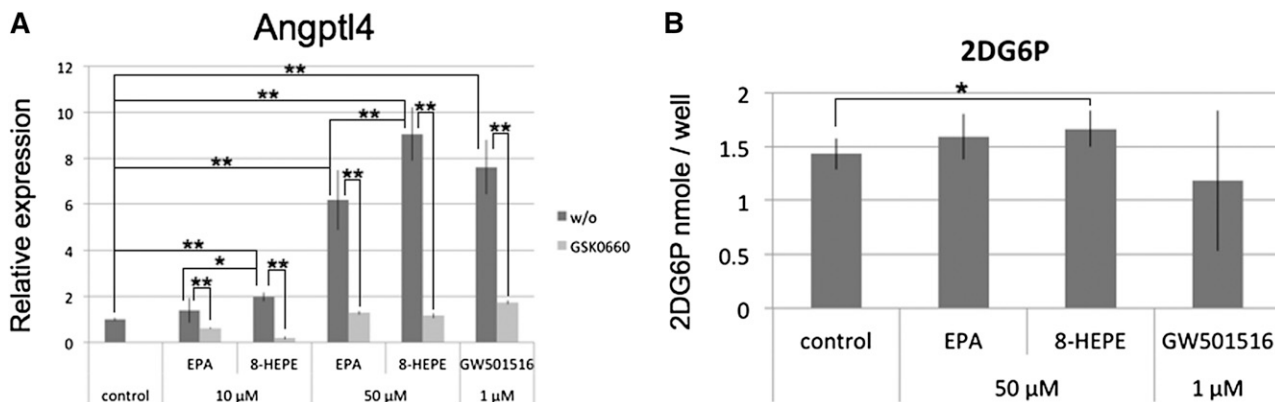


Fig. 7. The effect of 8-HEPE treatment on *Angptl4* expression and glucose uptake in C2C12 cells. A: C2C12 cells were cultured with EPA, 8-HEPE, or GW501516 for 6 h. The PPAR δ antagonist GSK0660 (25 μ M) was added to the media at the same time as EPA, 8-HEPE, or GW501516. Expression of *Angptl4* was measured by real-time PCR and normalized against expression of *Actb*. B: C2C12 cells, in which myogenesis had been induced, were cultured with 50 μ M of EPA or 8-HEPE, or 1 μ M GW501516 for 6 h. Glucose uptake was measured in the cells using 2-deoxyglucose. 8-HEPE purified from Pacific krill was used. * P < 0.05, ** P < 0.01. 2DG6P, 2-deoxyglucose-6-phosphate. w/o, without.

the diet, such as linoleic acid and AA, generally fall into the range 25–30 μ M (8). Physiologically, most free fatty acids in serum are bound and buffered with albumin. The concentration of unbound free fatty acids is in the nanomolar range (38).

PPARs regulate the expression of numerous genes related to energy metabolism, and the activation of PPARs can have beneficial effects in obesity and diabetes (1, 10, 39–41). PPAR α activators regulate obesity by increasing hepatic fatty acid oxidation and decreasing the levels of the circulating triglycerides responsible for adipose cell hypertrophy and hyperplasia (42–44); PPAR γ activators induce adipocyte gene expression and improve insulin sensitivity (11, 45); and, PPAR δ activators induce fatty acid oxidation in skeletal muscle (46) and activate glucose transport in myotubes (36). Our analyses of the effects of 8-HEPE in FaO, 3T3-F442A, and C2C12 cells showed that it induced expression of genes known to be regulated by PPAR α (Fig. 5), PPAR γ (Fig. 6A), and PPAR δ (Fig. 7A); this 8-HEPE-induced upregulation of gene expression was suppressed by PPAR antagonists. 8-HEPE also induced adipogenesis (Fig. 6B–E) and enhanced glucose uptake (Fig. 7B). These results indicate that 8-HEPE acts as a ligand for intrinsic PPAR α , PPAR γ , and PPAR δ .

Eicosanoids are a family of biologically active oxygenated derivatives of C20 PUFAs. Two pathways are principally involved in the production of eicosanoids. The lipoxygenase pathway transforms PUFAs into lipoxins, leukotrienes, and monohydroxy fatty acids, whereas the cyclooxygenase pathway produces prostaglandins and thromboxanes. Production of HEPES has been observed in platelets (47), macrophages (48, 49), and lung cells (50); and Tomio et al. (51) showed that 12-HEPE and 15-HEPE are decreased in 12/15-lipoxygenase knockout mice. In general, the physiological effects of HEPES are unclear; however, it has been suggested that 12-HEPE and 15-HEPE have a suppressive effect on the development of endometriotic lesions (51). There have been comparatively few investigations of the biochemical or physiological

properties of HEPES, including 8-HEPE. It was shown that *Escherichia coli* hemolysin induced production of 8-HEPE in rabbit macrophages (48). However, no enzyme with the capability of transforming EPA into 8-HEPE has been identified to date. In 8-HETE, 8(S)-lipoxygenase can transform AA into 8(S)-HETE (52), a compound that can stereoselectively activate PPAR α (8, 20). Future studies will be need to address the questions of 8-HEPE stereoselectivity and the identity of the enzyme that can transform EPA into 8-HEPE. Our study indicates that 8-HEPE and 9-HEPE induce fatty acid oxidation, adipogenesis, and glucose uptake via activation of PPARs in vivo. These metabolic responses are very interesting areas of research for the regulation of obesity and diabetes. The content of 8-HEPE in krill is more than 10 times higher than in the Japanese pilchard (*Sardinops melanostictus*) or the Pacific saury (*Cololabis saira*), species that are generally regarded as possessing high EPA contents (supplementary Table V). Our study shows that Pacific krill will be a valuable source of HEPES for addressing the physiological functions of HEPES. **FIG**

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