Hydroxy Monounsaturated Fatty Acids as Agonists for Peroxisome Proliferator-Activated Receptors

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Received November 12, 2009; accepted February 5, 2010; published online February 8, 2010

The physiological and pathological role of oxidized polyunsaturated fatty acids (PUFAs) has been extensively studied, whereas those of hydroxy monounsaturated fatty acids (MUFA)s are not well understood. This study demonstrated that 11-hydroxy-(9Z)-octadecenoic acid (9Z)-11-HOE, which was isolated from adlay seeds (Coix lacryma-jobi L. var. ma-yuen STAF.), can activate peroxisome proliferator-activated receptor (PPAR)α, δ and γ in luciferase reporter assays more efficiently than (9Z)-octadecenoic acid (oleic acid), and to the same degree as linoleic acid. (9Z)-11-HOE increased the mRNA levels of UCP2 and CD36 in C2C12 myotubes and THP-1 cells, respectively, and these effects were blocked by the PPARδ- and γ-specific antagonists GSK0660 and T0070907, respectively. Evaluation of the structure–activity relationship between hydroxy MUFA and PPAR activation revealed that (9E)-11-HOE, the geometrical isomer of (9Z)-11-HOE, activated PPARs more potently than (9Z)-11-HOE, and that PPAR activation by hydroxyl MUFA was not markedly influenced by the position of the hydroxy group or the double bond, although PPARδ seemed to possess ligand specificity different to that of PPARγ or γ. Additionally, the finding that 11-hydroxy octadecanoic acid, the hydrogenated product of (9E)-11-HOE, was also capable of activating PPARs to a similar extent as (9E)-11-HOE indicates that the double bond in hydroxy MUFA is not essential for PPAR activation. In conclusion, (9Z)-11-HOE derived from adlay seeds and hydroxy MUFA with a chain length of 16 or 18 acted as PPAR agonists. Hydroxylation of MUFA may change these compounds from silent PPAR ligands to active PPAR agonists.

Key words hydroxyl fatty acid; peroxisome proliferator-activated receptor; oleic acid; palmitoleic acid; metabolic syndrome

The metabolic syndrome is characterized by an excess accumulation of visceral fat, followed by insulin resistance, diabetes, hyperlipidemia and hypertension, ultimately leading to a high risk for cardiovascular complications. Fatty acids are ubiquitous biological molecules that are used as metabolic fuels, essential components of cellular membranes, covalent regulators of signaling molecules, and precursors of signaling molecules. However, excessive accumulation of fatty acids, particularly saturated fatty acids (SFAs), in the liver, muscle and pancreas cause a toxic response known as lipotoxicity,1,2 which compromises insulin signal transduction or induces cell death through reactive oxygen species, diacylglycerol and ceramide production.3–4) This process also suppresses glycogen synthesis in the liver, glucose uptake in skeletal muscle and insulin secretion in pancreatic β-cells. In addition, SFAs such as palmitic and stearic acids are reported to activate macrophages in adipose tissue via Toll-like receptor 4 (TLR4), followed by the acceleration of a vicious inflammatory circle between macrophages and adipocytes. This finally results in altered adipokine production and increased release of free fatty acids to cause insulin resistance in non-adipose tissues.5) In contrast, polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFA)s show preventive effects against lipotoxicity, although the mechanisms by which unsaturated fatty acids (UFAs) exert these effects are varied. The preventive effect of PUFAs, particularly ω-3 fatty acids, are mainly ascribed to the inhibition of lipogenesis and reduced tissue and plasma triglyceride levels by suppressing sterol regulatory element-binding protein (SREBP)-1c expression,6,7) activating adenosine 5′-monophosphate (AMP)-activated protein kinase (AMPK),8) and stimulating β-oxidation through PPARα activation.9,10) PUFAs, such as eicosapentaenoic and docosahexaenoic acids, preferentially suppress SFA-induced nuclear factor kappa B (NF-xB) activation and cyclooxygenase (COX)-2 expression by blocking a common signaling pathway derived from TLR-4.11,12) These results strongly suggest that PUFAs may be capable of inhibiting SFA-induced TLR-4 activation, thus suppressing the vicious inflammatory cycle in adipose tissue, and maintaining insulin sensitivity in peripheral tissues. Recent intriguing findings demonstrate that lipoxins, resolvins, protectins and 13-hydroxyoctadecadienoic acid (13-HODE), intracellular oxygenation products generated from arachidonic, eicosapentaenoic, docosahexaenoic, and linoleic acids, respectively, play critical roles in the resolution of acute inflammation.13–15)

The beneficial effects of MUFA,s, particularly oleic and palmitoleic acids, which are very abundant in foods, have been extensively studied. MUFA-enriched diets have been reported to improve lipoprotein profiles and glycemic control in patients with type 2 diabetes, and may reduce the risk of coronary heart diseases.16,17) Palmitic acid is known to produce ceramide or diacylglycerol, which causes serine phosphorylation of downstream insulin signaling molecules (i.e., insulin receptor substrate 2 and Akt) through the activation of various kinases (i.e., c-Jun NH2-terminal kinase (JNK), protein kinase Cδ, 1Kb kinase), to induce insulin resistance.18) In contrast, oleic acid and palmitoleic acid can prevent palmitate-induced insulin resistance and inflammation in skeletal muscle cells, adipocytes, and hepatocytes.18–20) Note of palmitoleic acid, which is derived from adipose tissue, was recently reported to act as an insulin-sensitizing...
hormone and improves glucose metabolism. However, it was recently reported that stearoyl-CoA desaturase-1 deficiency, which leads to a decrease in oleic acid and palmitoleic acid levels, can protect mice from obesity and hepatic steatosis. Thus, the effects and underlying mechanisms of MUFAs are still not fully known and appear to be controversial.

Considering the beneficial effects of UFAs, we should take into account the knowledge that fatty acids are endogenous agonists of all peroxisome proliferator-activated receptors (PPARs). PPARs are nuclear hormone receptors that function as ligand-activated transcription factors. Among the PPAR family, consisting PPARα, δ and γ, PPARα is the most promiscuous of the PPARs, interacting with both SFAs and UFAs; PPARδ interacts with SFAs and UFAs, albeit less efficiently than PPARα; and PPARγ shows the most restricted fatty acid binding profile, interacting most efficiently with PUFAs and only weakly with MUFAs and diunsaturated fatty acids. Some metabolites of UFAs have also been identified as natural ligands for PPARs. Oxidized eicosapentaenoic acid and 9- and 13-hydroxyoctadecadienoic acid (HODE) can activate PPARα to alleviate inflammation and 9- and 13-HODE can also activate PPARγ to block NF-κB activation. Oxidized docosahexaenoic acid also activates PPARγ more potently than native docosahexaenoic acid and their EC50 values are smaller than or comparable to that of pioglitazone. In addition, some linoleic acid metabolites biosynthesized in plants such as corn and rice were reported to exhibit anti-inflammatory effects. Taken together, the oxygenation of fatty acids not only facilitates their catabolism, but also plays an important role in the generation of novel signaling molecules.

In this context, the biological activity of oxygenation products of MUFAs with a chain length of 18 or 16, which are abundant UFAs in foods, has not been well studied. Therefore, we performed this study to examine the biological activity of hydroxy MUFAs to shed light on the beneficial effects of UFAs, although MUFAs are thought to be resistant to oxidation. Here, we report that 11-hydroxy-(9Z)-octadecenoic acid (9Z-11-HOE) is a better PPAR agonist than the non-oxidized fatty acids tested in this study, and that the hydroxylation of MUFAs likely changes them from silent PPAR ligands to active PPAR agonists.

MATERIALS AND METHODS

Reagents The PPARγ antagonist T0070907 and the PPARα agonist WY14643 were purchased from Cayman Chemicals (Michigan, U.S.A.). The PPARγ agonist rosiglitazone and the PPARδ agonist GW501516 were from Alexis Biochemicals (San Diego, CA, U.S.A.). The PPARα antagonist MK886, the PPARδ antagonist GSK0660, linoleic acid, oleic acid, stearic acid, vaccenic acid, elaidic acid and petroselinic acid were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Palmitoleic acid was purchased from Nu-Chek Prep. Inc. (Elysian, MH, U.S.A.).

Cell Culture Mouse C2C12 myoblasts, human HEK293 embryonic kidney cells and human monocyte-like THP-1 cells were obtained from RIKEN Cell Bank (Ibaraki, Japan). HEK293 cells were maintained in minimum essential medium (MEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Cell Culture Biosciences Inc., Tokyo, Japan), 1% non-essential amino acids (Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich). C2C12 myoblasts were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. THP-1 cells were maintained in RPMI1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. To induce differentiation into macrophage-like cells, THP-1 cells were plated at 5×105 cells/well in 12-well culture plates, and incubated for 72 h with 200 nm 12-O-tetradecanoyl-phorbol-13-acetate (Sigma-Aldrich). When cells had differentiated into myotubes, they were treated with 50 μM of various fatty acids or (9Z)-11-HOE in the absence or presence of antagonists for PPARα (MK886, 20 μM), δ (GSK0660, 1 μM) or γ (T0070907, 1 μM) for 8 h.

Preparation of Rat Primary-Cultured Hepatocytes Hepatic parenchymal cells were isolated from male Sprague-Dawley rats (200 g) (Crea Japan Inc., Tokyo, Japan) by Seglen’s two-step collagenase perfusion method. Cell viability was determined by the 0.25% trypan blue dye exclusion method, and cells with viability exceeding 91.0±1.0% were used in this study. Hepatocytes were seeded into collagen-coated six-well culture dishes at 1×106 cells/well in William’s E medium (Invitrogen, CA, U.S.A.). (pH 7.4), supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μM insulin and 1 μM dexamethasone. After incubation for 4 h at 37 °C in 5% CO2 in air, the medium was replaced with the hormone-supplemented medium, and incubated for a further 24 h. The cells were washed twice with phosphate-buffered saline (PBS) and then incubated in William’s E medium containing test samples without FBS for 48 h.

Differentiation of C2C12 Myoblasts into Myotubes C2C12 myoblasts were cultured in DMEM supplemented with 10% FBS in 5% CO2 in air. For the differentiation experiment, 2 d after the C2C12 myoblasts (1.5×105 cells/well) had reached confluence in 12-well culture plates, the media was switched to the differentiation medium (DMEM supplemented with 1% horse serum (Invitrogen, CA, U.S.A.)). Four days later, when cells had differentiated into myotubes, the cells were treated with 50 μM of various fatty acids or (9Z)-11-HOE in the absence or presence of antagonists for PPARα (MK886, 20 μM), δ (GSK0660, 1 μM) or γ (T0070907, 1 μM) for 8 h.

Preparation of Hydroxy Monounsaturated and Saturated Fatty Acids Hydroxy MUFAs were prepared by hydroxylation with selenium dioxide tert-butylhydroperoxide (Wako Pure Chemical Industries, Osaka, Japan). Briefly, in a round-bottomed flask equipped with a reflux condenser, 19.1 mg SeO2 (Sigma-Aldrich), 75 μl tert-butylhydroperoxide and 0.5 ml dichloromethane were mixed at room temperature and stirred for 0.5 h. Then, 80 mg of MUFA was added to the reaction mixture and was stirred for 24 h at room temperature. The reaction mixture was extracted with ethyl acetate, and the products were purified by silica gel column chromatography, followed by HPLC (YMC J’shere ODS-H80, 10 mm×250 mm; 70% acetonitrile). The structures of the resulting hydroxy MUFAs were determined by 1H-NMR and EI-MS, and the position of the hydroxyl groups was de-
termed by Ei-MS analysis of the TMS ether for each sample. To prepare hydroxy saturated fatty acid, a solution of (9E)-11-HOE (5 mg) in methanol (1 ml) was treated with 5% palladium on carbon under a stream of hydrogen gas for 1 h. Filtration of the reaction mixture and concentration of the filtrates gave the hydrogenated compound.30 The structure of the resulting hydroxy saturated fatty acid was analyzed by 1H-NMR.

Luciferase Reporter Gene Assay  
PPARα, δ and γ transcription activity was determined by luciferase reporter assays as follows. HEK293 cells (1.5 × 10^5 cells/well) were seeded in 24-well culture plates and maintained in minimum essential medium (MEM) containing 10% FBS and 10 ml/l of non-essential amino acids at 37°C under 5% CO2 in air. The cells were transfected by a standard calcium-phosphate precipitation method with 1—100 ng of the pCMX-mPPARα, δ or γ expression vector, 100—300 ng pPRE×3-tk-Luc reporter plasmid and 10 ng of the pCMX-β-gal expression vector. After transfection, the cells were thoroughly washed with fresh medium and incubated in the presence of the test compounds at the concentrations indicated in the figures or the figure legends. After incubation for an additional 48 h, the cells were harvested to determine the luciferase and β-galactosidase activities. Luciferase activity was normalized relative to the activity of an internal β-galactosidase control and was expressed as the relative luciferase activity. The luciferase activity was determined in triplicate experiments.

RNA Preparations and Quantitative Real-Time PCR  
Total RNA was isolated using TRizol reagent (Invitrogen Corp., CA, U.S.A.). For real-time RT-PCR, 1 μg of RNA was DNase-treated and reverse-transcribed using ReverTra Ace and oligo(dT) nucleotides (Toyobo Biochemicals, Tokyo Japan) according to the manufacturer’s instructions. Quantitative real-time PCR was performed with a Thermal Cycler Dice TP800 system (Takara) using SYBR Premix Ex Taq II (Takara) with cycles of 95 °C for 5 s and 60 or 62 °C for 30 s. The cycle threshold value, which was determined using a second derivative, was used to normalize the expression of the indicated genes using β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene. The primer sequences used in this study were as follows: rat acetyl-CoA oxidase (ACO), forward primer 5′-CTTCTTGTGCTTGTCCCTCTCTCC-3′ and reverse primer 5′-GCGGTCTACCGCTCTGTA-3′; rat GAPDH, forward primer 5′-GCATGGCCCTTCCGTGTTCC-3′ and reverse primer 5′-GGGTTGGTCAGGGTTTTCTTACTC-3′; mouse uncoupling protein 2 (UCP2), forward primer 5′-GGGCCCTTTCCGCGCAGG-3′ and reverse primer 5′-GCCACAGCCAGATTGAGAAC-3′; mouse β-actin, forward primer 5′-TGTCCTGCTGATCC-3′ and reverse primer 5′-AGGACGCTTGGAGACAAC-3′ and reverse primer 5′-GCCACAGCCAGATTGAGAAC-3′; human β-actin, forward primer 5′-GGGCCCTTTCCGCGCAGG-3′ and reverse primer 5′-GCCACAGCCAGATTGAGAAC-3′.

Statistical Analysis  
Data were expressed as means±S.D. Data were evaluated for statistical significance by one-way ANOVA, followed by Bonferroni’s t-test. A value of p<0.05 was considered to be statistically significant.

RESULTS

PPAR Agonist Activity of (9Z)-11-HOE

We previously isolated six hydroxy MUFAs from adlay seeds and determined their structures during PPARγ ligand screening of 88 crude drugs.34 As fatty acids and their metabolites are endogenous agonists of PPARRs, we first selected a hydroxylated derivative of oleic acid, 11-hydroxy-(9Z)-octadecenoic acid ((9Z)-11-HOE), the structure of which is shown in Table 1, and examined whether it can activate PPARα, δ and γ in luciferase reporter assays. As shown in Fig. 1, (9Z)-11-HOE exhibited agonist activity for all three subtypes of PPARRs, but was a slightly better agonist for PPARγ and δ than for PPARα, although more potent activity was seen for PPARγ at higher concentrations. The activity of (9Z)-11-HOE was then compared with stearic (octadecenoic acid; OA), oleic ((9Z)-octadecenoic acid; (9Z)-OE), and linoleic acids ((9Z,12Z)-octadecadienoic acid; (9Z,12Z)-ODE) (Fig. 2). While an SFA, stearic acid, and an MUFA, oleic acid, showed only marginal activity for PPARα, δ and γ at the concentrations used in this study, a diunsaturated fatty acid, linoleic acid, exhibited more potent activity for PPARα and γ than stearic and oleic acid, as previously reported.35 (9Z)-11-HOE activated PPARα to a similar extent as linoleic acid and more potently than stearic acid and oleic acid (Fig. 2A). (9Z)-11-HOE also activated PPAR δ and γ more potently than stearic, oleic and linoleic acids (Figs. 2B, C). These results indicate that (9Z)-11-HOE serves as a PPAR agonist with higher affinity than stearic and oleic acids.

Induction of PPAR Target Genes by (9Z)-11-HOE

To evaluate the activity of (9Z)-11-HOE as a PPARα agonist, we first examined whether or not (9Z)-11-HOE increased the mRNA levels of a PPARα target gene, ACO, in rat primary-cultured hepatocytes by quantitative real-time RT-PCR. Although data were not shown, (9Z)-11-HOE failed to increase the mRNA levels of ACO, regardless of the activation of PPARα in the luciferase reporter assay. Similarly, no increase

Fig. 1. Activation of PPARα, δ and γ by (9Z)-11-HOE in the Luciferase Reporter Assay

HEK293 cells were co-transfected with the respective nuclear receptor expression vectors (pCMX-mPPARs) and pPRE×3-tk-Luc with pCMV-β-gal, as described in Materials and Methods. After transfection, the cells were treated with increasing concentrations of (9Z)-11-HOE for 48 h. The activity was normalized using β-gal and expressed as fold-change relative to that of vehicle-treated cells. Open circles, PPARα; closed circles, PPARδ; open square, PPARγ. Values are means±S.D. of three determinants from a representative of three independent experiments, which showed similar results.
in mRNA levels of PPARα target genes, cytochrome P450 (CYP4A1) and mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMGCS) occurred in rat primary-cultured hepatocytes following treatment with (9Z)-11-HOE (data not shown). Therefore, we assessed the activity of (9Z)-11-HOE as a PPARδ agonist. We found that (9Z)-11-HOE increased the mRNA levels of a PPARδ target gene, UCP2, in C2C12 myotubes in a dose-dependent manner, and that the activity was significantly greater than that of stearic, oleic or linoleic acids and was completely blocked by a PPARδ antagonist, GSK0660 (Fig. 3).

To further assess the activity of (9Z)-11-HOE as a PPARγ...
agonist, we tested whether (9Z)-11-HOE increased the mRNA levels of a PPARγ target gene, CD36, in THP-1 cells by quantitative real-time RT-PCR. As shown in Fig. 4, (9Z)-11-HOE increased the mRNA levels of CD36 in a dose-dependent manner, while the activity was significantly greater than that of stearic, oleic and linoleic acids. This effect was completely abrogated by the PPARγ antagonist T0070907. Furthermore, (9Z)-11-HOE enhanced 3T3-L1 preadipocyte differentiation to stimulate lipid accumulation like the PPARγ agonist rosiglitazone, although data were not shown. These results indicate that (9Z)-11-HOE can activate PPARδ and γ in cells to induce the transcriptional activation of UCP2 and CD36.

Effect of Hydroxylation and Configuration of the Double Bond on PPAR Activation by Hydroxy MUFAs

The hypothesis that hydroxylation of MUFAs could change their PPAR agonist activity beyond that of the original natural MUFAs, the natural or synthetic hydroxy MUFAs listed in Table 1 were screened for their PPAR agonist activity by luciferase reporter assays for all three subtypes of PPARs. The MUFAs tested in this study, i.e., petroselinic acid ((6Z)-OE), elaidic acid ((9E)-OE), vaccenic acid ((11Z)-OE) and palmitoleic acid ((9Z)-HE), failed to show any agonist activity for PPARs (data not shown). Oleic acid ((9Z)-OE) also failed to show any activity for all three subtypes of PPARs, while its hydroxylated product, (9Z)-11-HOE, activated PPARs more potently than oleic acid (Fig. 5). When the activity of (9Z)-11-HOE was compared with that of its geometrical isomer (9E)-11-HOE, the latter was more potent than the former for the activation of all three subtypes of PPARs.

Activation of PPARs by Various Hydroxy MUFAs with Chain Lengths of 18 or 16

We next examined the difference in activity among hydroxy MUFAs with a hydroxy group and double bond at different positions. All four hydroxy MUFAs with a chain length of 18 tested in the study showed comparable activation of PPARs, with relatively small differences in their activity for each PPAR, as evi-
denced by the findings that (11E)-10-HOE was more potent for PPARα and γ than the other MUFAs tested and that (11E)-13-HOE possessed more potent activity for PPARδ than the other MUFAs tested (Fig. 6A). We further assessed the PP AR activation by hydroxy MUFAs with a chain length of 16. (9E)-8-HHE and (9E)-11-HHE showed similar activities in terms of the activation of PP ARs and exhibited the comparable activity to (9E)-11-HOE for PPARα and γ. However, in the activation of PPARδ, both (9E)-8-HHE and (9E)-11-HHE were less potent than (9E)-11-HOE (Fig. 6B).

**Activation of PPARs by Hydroxy SFA and MUFA**

Finally, we tested whether hydroxy SFAs possess potent PPAR agonist activity as hydroxy MUFAs. For all three subtypes of PP ARs, 11-HOA, which was the hydroxy SFA produced by reducing (9E)-11-HOE, was found to activate PP ARs more potently than stearic acid and to a similar extent as (9E)-11-HOE (Fig. 7).

**DISCUSSION**

In the present study, we first selected (9Z)-11-HOE as a hydroxy MUFA to evaluate the potency of PPAR activation and the effect of hydroxylation of MUFA on PPAR activation. Because (9Z)-11-HOE possesses the same geometrical conformation as oleic acid, this test only determined the effect of hydroxylation. The results clearly demonstrated that (9Z)-11-HOE activates all three subtypes of PPARs in PPAR luciferase reporter assays. In C2C12 myotubes, (9Z)-11-HOE upregulated mRNA expression of UCP-2, a PPARδ target gene, in a dose-dependent manner, confirming its activity as a PPARδ agonist. In THP-1 cells, (9Z)-11-HOE increased mRNA expression of CD36, a PPARγ target gene, also in a dose-dependent manner, confirming its activity as a PPARγ agonist. However, (9Z)-11-HOE failed to increase the mRNA
expression of the PPARα target genes ACO, CYP4A1 and mHMGCS in rat primary hepatocytes. It was reported that oleic acid is a ligand for PPARα at the concentration as high as 100—200 μM, much higher than the concentration used in the present study.35,36 However, it is unable to activate PPARα target genes in rat primary hepatocytes because of its prompt incorporation into triacylglycerol, phospholipids or cholesteryl ester following its uptake into hepatocytes.37,38 That may also be true for (9Z)-11-HOE. (9Z)-11-HOE has a structure very similar to oleic acid and may be incorporated into triacylglycerol, phospholipids or cholesteryl ester in hepatocytes as rapidly as oleic acid, resulting in its inability to activate PPARα target genes. In fact, hydroxy polyunsaturated fatty acids, such 12(5)-hydroxyeicosatetraenoic acid, 15-hydroxyeicosatetraenoic acid and 13-hydroxyoctadecadienoic acid are also known to be incorporated into cellular phospholipids.39,40 In contrast, as endogenous lipid synthesis is less active in C2C12 and THP-1 cells relative to hepatocytes, (9Z)-11H0E should be available as an agonist for PPARs.

To further investigate whether hydroxy MUFAs have PPAR agonist activity, the activation of PPARs was tested in PPAR luciferase reporter assays. The most intriguing finding in the present study is that hydroxyl MUFAs were more potent PPAR agonists compared with the original MUFAs such as oleic, elaidic and palmitoleic acids, which are innately weak agonists for PPARs. (9Z)-11-HOE exhibits greater activity than oleic acid ((9Z)-OE), but possesses less potent activity than (9E)-11-HOE, the geometrical isomer of (9Z)-11-HOE, for all three subtypes of PPARs. Thus, although hydroxylation of oleic acid increased the activity of these fatty acids in PPAR reporter assays, the study of the structure–activity relationship findings suggest that hydroxy MUFAs are equally recognized by all three subtypes of PPARs. In addition, hydroxy MUFAs with a chain length of 16 showed weaker activity for PPARδ than PPARα and γ, although the activities for PPARα and γ were similar between hydroxy MUFAs with chain lengths of 18 and 16. The structure–activity relationship findings suggest that hydroxy MUFAs are equally recognized by all three subtypes of PPARs, although PPARδ appears to possess different ligand specificity compared with PPARα and γ. A large number of PPAR ligands possess a carboxylic acid molecule at one end and a hydrophobic tail at the other end. Several polyunsaturated lipids with α,β-unsaturated ketones, such as 15-deoxy-d12,14-prostaglandin J2, are known to activate receptor-mediated transcription by covalently binding to a unique cysteine in the ligand-binding domain of PPARγ.27,41 In the case of hydroxyl MUFAs without α,β-unsaturated ketones, a hydroxy group on the molecules might contribute a hydrogen bond to an amino acid in the ligand-binding pocket to stabilize the PPAR–ligand complex, resulting in potent activation of PPARs. Additionally, 11-H0A, a hydroxy SFA, activated PPAR as potently as (9E)-11-HOE, suggesting that the existence of a double bond in hydroxy fatty acids is not essential to activate PPARs. However, as evaluation of the structure–activity relationship between hydroxy SFAs and PPAR activation remains to be determined, we cannot ignore the implication of the double bond in PPAR activation by hydroxy MUFAs. Taken together, the finding that hydroxy MUFAs were PPAR agonist more potent than natural MUFAs supports a novel role of MUFAs in cell physiology or pathology, and paves the way for the development of novel PPAR agonists.

Inappropriately high levels of saturated fatty acids in tissues, cells and plasma are prone to cause obesity and insulin resistance, followed by hypertension, hyperlipidemia, and cardiovascular complications. In contrast, an oleic acid-enriched diet is known to be effective in reducing coronary risk by improving lipoprotein profiles and glycemic control.16,17 Similarly, palmitoleic acid was recently reported to enhance muscle insulin action and suppress hepatosteatosis by acting as a lipokine.21 However, the precise mechanism underlying the beneficial effects of these fatty acids are not yet fully understood. Therefore, it is conceivable that these beneficial effects might partly result from the generation of hydroxy MUFAs. This idea seems plausible, because oleic and palmitoleic acids are more abundant in cells or foods than linoleic, linolenic and other PUFAs. Therefore, studies are needed to investigate whether hydroxy MUFAs exist or are generated in physiological or pathological conditions, and whether the beneficial effects of oleic and palmitoleic acids on lipid and glucose metabolism are partly explained by the generation of hydroxy MUFAs.

Although the intracellular generation or existence of such hydroxyl MUFAs remains to be determined in mammalian cells unlike adlay seeds and bacteria,34,42,43 it is possible that hydroxy MUFAs generated by β-oxidation in mitochondria or peroxisomes play an important role in some physiological or pathological conditions, and that exogenous hydroxy MUFAs inhibit acyl-CoA oxidase, which is involved in β-oxidation, to indirectly increase accumulation of acyl-CoA leading to PPAR activation.44,45 In addition, PPARs are known to form heterodimers with retinoid X receptor (RXR), which are activated by either the ligand for PPARs or RXRs. When we therefore examined whether hydroxy MUFAs were able to activate RXRα using RXR luciferase reporter assay, hydroxy MUFAs did not exhibit agonist activity for RXRα (RXRα agonist bexarotene (1 μM): 6.02±0.56, (9Z)-11-HOE: 1.06±0.06, (9E)-11-HOE: 0.89±0.04, (11E)-13-HOE: 0.78±0.06, (11E)-10-HOE: 1.14±0.11, (6E)-8-HOE: 1.47±0.17; hydroxy MUFAs were used at the concentration of 50 μM; data represent relative luciferase activity compared with vehicle control). These results indicate that hydroxy MUFAs do not activate PPAR target genes via RXR activation.

Despite the proven benefits of targeting PPARs, safety concerns have recently led to late stage development failures of various PPAR agonists including novel specific PPARγ agonists and dual PPARα/γ agonists. The class side effects of thiazolidinediones include body weight gain, hemodilution, peripheral edema, mild anemia and a possible increased risk for congestive heart failure, which limits their clinical use.46 On the other hand, PPARα ligands belonging to the fibrate class are generally considered to be safe drugs with few side effects. Nevertheless, some adverse effects might be related to PPARα activation in humans including rare cases of myopathy and rhabdomyolysis, increased in creatinine and
homocysteine levels, lithogenicity and gastrointestinal complaints.\(^{47}\) PPAR\(\alpha\) agonists are also reported to stimulate proliferation and survival of cancer cells in vitro and promote tumor growth in mice, although the role of RAR\(\alpha\) in carcinogenesis is still controversial.\(^{48,49}\) Although the development of some PPAR\(\gamma\) and PPAR\(\alpha/\gamma\) dual agonists has been discontinued,\(^{47}\) the reason was always compound specific. This encourages us to explore novel PPAR agonists without adverse side effects reported to date and with preserved efficacy, in addition to distinct structures. Furthermore, given the efficacy of agonists for all three subtypes of PPARs, pan PPAR agonists should be superior therapeutic agents for treating aspects of the metabolic syndrome including insulin resistance and type 2 diabetes compared with selective PPAR\(\gamma\) agonists and PPAR\(\alpha/\gamma\) dual agonists, and compared with each PPAR agonist, generating comparable insulin sensitization and glucose-lowering profiles, and providing favorable lipid profiles without the significant adverse effects associated with PPAR agonists.\(^{50}\) In this respect, hydroxy MUFAs should be considered as candidates for such novel pan PPAR agonists and are expected to provide beneficial effects on various aspects of the metabolic syndrome.

In conclusion, this study showed that the hydroxy products of MUFAs were potent PPAR agonists and activated all three PPARs more efficiently than original MUFAs.

Acknowledgements This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (19500704).

REFERENCES