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ScienceDirect
Life Sciences 82 (2008) 290–300
Life Sciences
www.elsevier.com/locate/lifescie

Induction of adiponectin by natural and synthetic phenolamides in mouse and human preadipocytes and its enhancement by docosahexaenoic acid

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Received 7 July 2007; accepted 28 November 2007

Abstract

Adiponectin, the adipose-derived cytokine, plays an important role in preventing metabolic syndromes. To develop new adiponectin inducers, eight species of ferulic esters and amides, and five related compounds were synthesized and tested on the stimulation of adiponectin production in mouse 3T3-L1 and normal human preadipocytes. The ferulamides with an aromatic ring in the *N*-substituent are very active in inducing adiponectin as compared with the known active compounds, curcumin, [6]-gingerol, and capsaicin, and furthermore the activities of these ferulamides are remarkably stronger than those of the corresponding esters or the straight chain octylamide. The most active compound, *N*-(2-phenylethyl)ferulamide (**7**), was found to activate the PPAR (peroxisome proliferator-activated receptor) γ-RXR (retinoid X receptor) α heterodimeric complex in the PPRE (PPAR-responsive element)-driven luciferase reporter assay. The adiponectin production by **7** is synergistically enhanced by coaddition of a PPARγ-specific agonist, pioglitazone (PGZ), or another PPARγ agonist, docosahexaenoic acid (DHA), in cultured preadipocytes. The compound **7** alone did not show a statistically significant effect on the plasma adiponectin level in KK-A^y/Ta mice, while 1% **7** in the diets significantly lowered the blood glucose and triglyceride levels and 0.3% **7** mixed with DHA oil in the diets significantly increased the adiponectin level as compared with the control. These results suggest that the present ferulamides would be useful lead compounds in developing more potent agents for treatment of metabolic syndromes through promoting the endogenous adiponectin production, and that such an activity is possibly enhanced by the coadministration with DHA.

Keywords: Adiponectin; Phenolic compounds; Ferulic acid; Amide; Docosahexaenoic acid; Thiazolidinedione; 3T3-L1; Preadipocyte; Diabetes

Introduction

Metabolic syndromes such as type 2 diabetes and atherosclerosis are closely related to decrease in the plasma concentration of adiponectin, an adipocyte-derived cytokine (Fang and Sweeney, 2006). The pathophysiologically low adiponectin level in the blood of diabetes patients is improved by thiazolidinedione antidiabetics such as pioglitazone (PGZ) and rosiglitazone (Eguchi et al., 2007), but these drugs have some side effects (Nesto et al., 2004). Several new synthetic drugs (e.g., telmisartan (Moriuchi et al., 2007), bezafibrate (Hiuge et al., 2007), and rimonabant (Hollander, 2007)) have been found to function as adiponectin inducers, while a few natural products such as anthocyanin (Tsuda et al., 2004), xanthohumol (Nozawa, 2005), polymethoxylated flavones (Li et al., 2006), catechins (Cho et al., 2007), and sulfatide (Bruun et al., 2007) have been reported to enhance adiponectin production as safer agents for treating or preventing the metabolic syndromes. This variety of compounds may reflect the complex signaling cascade in the adipogenesis and the regulation of adiponectin gene expression involving many transcription factors (Rosen et al., 2000; Seo et al., 2004; Fox et al., 2006). Recently, we found that the curry pigment, curcumin (**1**), and the pungent components in ginger and red pepper, [6]-gingerol (**2**) and capsaicin (**3**), stimulated adiponectin production in cultured mouse and human preadipocytes (Yamazaki and Kawano, 2005; Yamazaki et al., 2006a,b). The up-regulation of adiponectin expression by capsaicin was also reported by other investigators (Hsu and Yen, 2007). These phenolic compounds have the common structural feature of a guaiacol unit being bound to a hydrophobic moiety through a hydrophilic linker (Fig. 1). Following

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doi:10.1016/j.lfs.2007.11.016

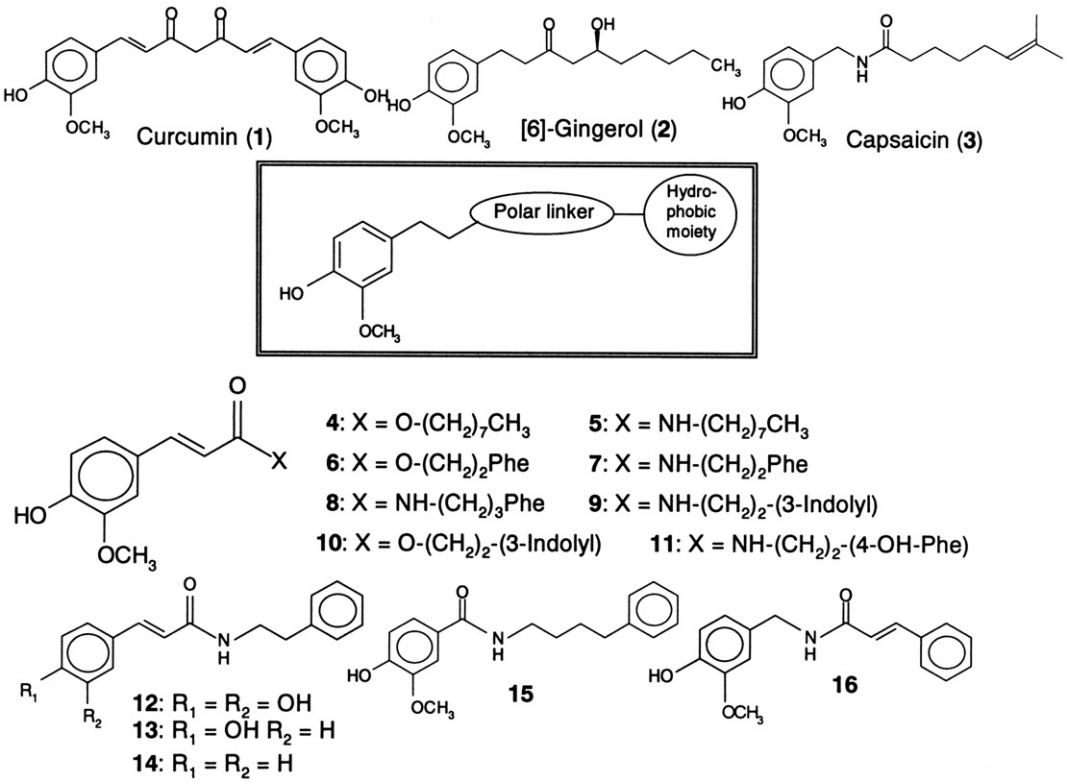

Fig. 1. Structures of the compounds.

this molecular architecture, we tried to synthesize a stronger adiponectin inducer.

Materials and methods

Chemicals

PGZ and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) were purchased from Alexis Biochemicals (San Diego, CA) and Cayman Chemical Company (Ann Arbor, MI), respectively. Curcumin was obtained from Nagara Science Ltd. (Gifu, Japan) and [6]-gingerol and capsaicin were from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Synthesis

N-(2-Phenylethyl)ferulamide (**7**) was synthesized as follows. To an ice-cold solution of 25 ml β-phenethylamine and 50 ml pyridine in 100 ml toluene was added dropwise a 350 ml toluene solution containing 30 g *O*-acetylferuloyl chloride prepared from ferulic acid by the previous method (Hatfield et al., 1991). The reaction mixture was stirred at room temperature overnight, and divided into two portions. Each portion was mixed with 500 ml ethyl acetate and washed successively with 150 and 100 ml water, 150 and 100 ml 10% citric acid, 150 and 100 ml 10% sodium bicarbonate, and 100 and 50 ml saturated sodium chloride solutions. The organic layers were combined, dried over sodium sulfate, and then evaporated to give an oil, which was crystallized from ethyl acetate to afford 32.6 g *O*-acetyl-*N*-(2-phenylethyl) ferulamide. This product was dissolved in 500 ml ethanol, and then mixed with 12 ml hydrazine hydrate. After standing at room temperature for 1 h, the solution was added with 12 ml acetic acid. The solvent was removed by a rotary evaporator and the residue was mixed with 500 ml ethyl acetate and 100 ml water in a separatory funnel. The organic layer was washed twice with 200 ml saturated brine, dried over sodium sulfate, and then evaporated to give an oil, which was crystallized from benzene. One half amount of the crude product was recrystallized from ethanol to give 10.6 g **7** as needles (81% yield), mp 153–154 °C, MS *m/z* 297.1355 (M^+) (calcd. $C_{18}H_{19}NO_3$=297.1364), ^{1}H NMR ($CDCl_3$) δ: 2.889 (t, 2, J=7 Hz, CH_2), 3.664 (dt, 2, J=7 and 6 Hz, CH_2), 3.903 (s, 3, OCH_3), 5.597 (t, 1, J=6 Hz, N*H*), 5.896 (s, 1, O*H*), 6.173 (d, 1, J=15.5 Hz, C*H*═CH), 6.894 (d, 1, J=8 Hz, H-5), 6.968 (d, 1, J=2 Hz, H-2), 7.041 (dd, 1, J=8 and 2 Hz, H-6), 7.2–7.4 (m, 5, phenyl), and 7.539 (d, 1, J=15.5 Hz, CH═C*H*).

The other ferulic derivatives, except for **11** were synthesized by the same method as described above with the corresponding alcohol or amine, and isolated by crystallization or preparative TLC to show the following properties: 1-octyl ferulate (**4**) — oil, MS *m/z* 306.1833 (M^+) (calcd. $C_{18}H_{26}O_4$=306.1830), ^{1}H NMR ($CDCl_3$) δ: 0.886 (t, 3, J=7 Hz, CH_3), 1.2–1.5 (10, $(CH_2)_5$), 1.699 (qt, 2, J=7 Hz, CCH_2C), 3.928 (s, 3, OCH_3), 4.189 (t, 2, J=7 Hz, CH_2O), 5.874 (s, 1, O*H*), 6.294 (d, 1, J=16 Hz, C*H*═CH), 6.916 (d, 1, J=8 Hz, H-5), 7.035 (d, 1, J=2 Hz, H-2), 7.076 (dd, 1, J=8 and 2 Hz, H-6), and 7.691 (d, 1, J=16 Hz, CH═C*H*); *N*-(1-octyl) ferulamide (**5**) — oil, MS *m/z* 305.2029 (M^+) (calcd. $C_{18}H_{27}NO_3$=305.1989), ^{1}H NMR ($CDCl_3$) δ: 0.887 (t, 3, J=7 Hz, CH_3), 1.2–1.5 (10, $(CH_2)_5$), 1.580 (qt, 2, J=7 Hz, CCH_2C), 3.393 (t, 2,

J=7 Hz, CH_2N), 3.932 (s, 3, OCH_3), 5.589 (br. s, 1, NH), 5.871 (br. s, 1, OH), 6.255 (d, 1, J=15 Hz, CH═CH), 6.918 (d, 1, J=8 Hz, H-5), 7.007 (d, 1, J=2 Hz, H-2), 7.073 (dd, 1, J=8 and 2 Hz, H-6), and 7.555 (d, 1, J=15 Hz, CH═CH); β-phenethyl ferulate (**6**) — oil, MS m/z 298.1194 (M^+) (calcd. $C_{18}H_{18}O_4$=298.1204), ^{1}H NMR ($CDCl_3$) δ: 3.018 (t, 2, J=7 Hz, CH_2), 3.924 (s, 3, OCH_3), 4.419 (dt, 2, J=7 and 6 Hz, CH_2), 5.896 (br. s, 1, OH), 6.270 (d, 1, J=15.9 Hz, CH═CH), 6.912 (d, 1, J=8 Hz, H-5), 7.017 (d, 1, J=2 Hz, H-2), 7.062 (dd, 1, J=8 and 2 Hz, H-6), 7.2–7.4 (m, 5, phenyl), and 7.598 (d, 1, J=15.9 Hz, CH═CH); N-(3-phenylpropyl)ferulamide (**8**) — fine needles from benzene/hexane/acetone, mp 75–78 °C, MS m/z 311.1510 (M^+) (calcd. $C_{19}H_{21}NO_3$=311.1520), ^{1}H NMR ($CDCl_3$) δ: 1.869 (q, 2, J=7 Hz, CH_2), 2.681 (t, 2, J=7 Hz, CH_2), 3.338 (dt, 2, J=7 and 6 Hz, CH_2), 3.903 (s, 3, OCH_3), 6.501 (d, 1, J=16 Hz, CH═CH), 6.836 (d, 1, J=8 Hz, H-5), 7.048 (dd, 1, J=8 and 2 Hz, H-6), 7.161 (d, 1, J=2 Hz, H-2), 7.13–7.305 (m, 6, phenyl and NH), 7.445 (d, 1, J=16 Hz, CH═CH), and 7.985 (br. s, 1, OH); N-[2-(3-indolyl) ethyl]ferulamide (**9**) — fine needles from ethanol, mp 163–165 °C (lit. mp 163–165 °C (Vecchietti et al., 1979)), MS m/z 336.1424 (M^+) (calcd. $C_{20}H_{20}N_2O_3$=336.1473); ^{1}H NMR ($CDCl_3$+10% MeOH-d_4) δ: 3.047 (t, 2, J=7 Hz, CH_2), 3.722 (t, 2, J=7 Hz, CH_2), 3.892 (s, 3, OCH_3), 6.161 (d, 1, J=15.7 Hz, CH═CH), 6.859 (d, 1, J=8 Hz, H-5), 6.965 (d, 1, J=2 Hz, H-2), 7.012 (dd, 1, J=8 and 2 Hz, H-6), 7.079 (s, 1, H-2′), 7.121 (m, 1, H-5′ or 6′), 7.205 (m, 1, H-6′ or 5′), 7.395 (m, 1, H-7′ or 4′), 7.486 (d, 1, J=15.7 Hz, CH═CH), and 7.628 (m, 1, H-4′ or 7′); 2-(3-indolyl) ethyl ferulate (**10**) — fine needles from benzene, mp 73–74 °C, MS m/z 337.1314 (M^+) (calcd. $C_{20}H_{19}NO_4$=337.1313); ^{1}H NMR ($CDCl_3$) δ: 3.185 (dt, 2, J=7 and 1 Hz, CH_2), 3.930 (s, 3, OCH_3), 4.496 (t, 2, J=7 Hz, CH_2O), 5.862 (br. s, 1, OH), 6.302 (d, 1, J=16 Hz, CH═CH), 6.920 (d, 1, J=8 Hz, H-5), 7.018 (d, 1, J=2 Hz, H-2), 7.069 (dd, 1, J=8 and 2 Hz, H-6), 7.097 (d, 1, J=2 Hz, H-2′), 7.147 (ddd, 1, J=8, 8, and 1 Hz, H-5′ or 6′), 7.218 (ddd, 1, J=8, 8, and 1 Hz, H-6′ or 5′), 7.385 (dd, 1, J=8 and 1 Hz, H-7′ or 4′), 7.611 (d, 1, J=16 Hz, CH═CH), 7.685 (dd, 1, J=8 and 1 Hz, H-4′ or 7′), and 8.038 (br. s, 1, NH).

The following reference compounds (**12**, **14**, and **15**) were analogously prepared using the corresponding acids (3,4-di-O-acetylcaffeic acid, cinnamic acid, or 4-O-acetylvanillic acid) and phenylethylamine or phenylbutylamine: N-(2-phenylethyl)caffeamide (**12**) — fine needles from ethanol/benzene/hexane, mp 154–155 °C, MS m/z 283.1202 (M^+) (calcd. $C_{17}H_{17}NO_3$=283.1207), ^{1}H NMR (acetone-d_6) δ: 2.847 (t, 2, J=7 Hz, CH_2), 3.534 (dt, 2, J=8 and 7 Hz, CH_2), 6.420 (d, 1, J=16 Hz, CH═CH), 6.827 (d, 1, J=8 Hz, H-5), 6.943 (dd, 1, J=8 and 2 Hz, H-6), 7.060 (d, 1, J=2 Hz, H-2), 7.16–7.32 (m, 6, phenyl+NH), 7.404 (d, 1, J=16 Hz, CH═CH), and 8.216 (br. s, 2, OH×2); N-(2-phenylethyl)cinnamamide (**14**) — needles from benzene/ethyl acetate, mp 122–123 °C, MS m/z 251.1306 (M^+) (calcd. $C_{17}H_{17}NO$=251.1309), ^{1}H NMR ($CDCl_3$) δ: 2.927 (t, 2, J=7 Hz, CH_2), 3.699 (dt, 2, J=7 and 6 Hz, CH_2), 5.667 (br. s, 1, OH), 6.347 (d, 1, J=16 Hz, CH═CH), 7.244–7.592 (m, 10, phenyl×2), and 7.650 (d, 1, J=16 Hz, CH═CH); N-(4-phenylbutyl)-2-hydroxy-3-methoxybenzamide (**15**) — needles from benzene/ethyl acetate/hexane, mp 142–143 °C, MS m/z 299.1520 (M^+) (calcd. $C_{18}H_{21}NO_3$=299.1520), ^{1}H NMR (acetone-d_6) δ: 1.644 (m, 4, (CH_2)$_2$), 2.652 (t, 2, J=7 Hz, CH_2), 3.399 (dt, 2, J=6 and 7 Hz, CH_2), 3.862 (s, 3, CH_3), 6.836 (d, 1, J=8 Hz, H-5), 7.122–7.287 (m, 5, phenyl), 7.406 (dd, 1, J=8 and 2 Hz, H-6), 7.561 (d, 1, J=2 Hz, H-2), 7.589 (br. s, 1, NH), and 8.075 (br. s, 1, OH).

N-[2-(4-Hydroxyphenyl)ethyl]ferulamide (**11**) was synthesized by the coupling of ferulic acid with tyramine in t-BuOH using dicyclohexylcarbodiimide (DCC), purified by preparative TLC, and crystallized from acetone/chloroform as needles, mp 142–143 °C (lit. mp 144.5–145 °C (Yoshihara et al., 1981)), MS m/z: 313.1324 (M^+) (calcd. $C_{18}H_{19}NO_4$=313.1313); ^{1}H NMR ($CDCl_3$+10% MeOH-d_4) δ: 2.762 (t, 2, J=7 Hz, CH_2), 3.546 (t, 2, J=7 Hz, CH_2), 3.866 (s, 3, OCH_3), 6.197 (d, 1, J=15.4 Hz, CH═CH), 6.771 (d, 2, J=8 Hz, H-2′ and 6′), 6.839 (d, 1, J=8 Hz, H-5), 6.962 (d, 1, J=2 Hz, H-2), 6.997 (m, 1, H-6), 7.027 (d, 2, J=8 Hz, H-3′ and 5′), and 7.474 (d, 1, J=15.4 Hz, CH═CH). N-(2-Phenylethyl)-4-hydroxycinnamamide (**13**) was also prepared by the DCC-mediated coupling of p-coumaric acid with 2-phenylethylamine — fine needles from benzene/hexane, mp 118–121 °C, MS m/z: 267.1236 (M^+) (calcd. $C_{17}H_{17}NO_2$=267.1258), ^{1}H NMR ($CDCl_3$+10% MeOH-d_4) δ: 2.869 (t, 2, J=7 Hz, CH_2), 3.162 (t, 2, J=7 Hz, CH_2), 6.163 (d, 1, J=16 Hz, CH═CH), 6.799 (d, 2, J=8 Hz, H-2′ and 6′), 7.195–7.357 (m, 5, Phenyl), 7.343 (d, 2, J=8 Hz, H-3′ and 5′), and 7.506 (d, 1, J=16 Hz, CH═CH).

N-[(4-Hydroxy-3-methoxyphenyl)methyl]cinnamamide (**16**) was synthesized as follows. Vanillylamine hydrochloride (10 g) was dissolved in 25 ml 5 N NaOH and mixed with 150 ml water. To the ice-cold mixture was slowly added a solution of 11 g cinnamic chloride in 2 ml toluene under vigorous stirring. After stirring at room temperature for 2 h, the mixture was neutralized with 6 N HCl and then extracted with 800 ml ethyl acetate. The organic layer was washed with 10% $NaHCO_3$ and concentrated by an evaporator. The residue was dissolved in 500 ml ethanol and mixed with 10 ml hydrazine monohydrate. The mixture was left at room temperature for 2 h, supplemented with 10.2 ml acetic acid, and then concentrated by an evaporator. The residue was dissolved in 900 ml ethyl acetate and the solution was usually worked up to give 15.6 g compound **16** upon evaporation. Some part of the crude product was crystallized from benzene as needles, mp 145–147 °C, MS m/z: 283.1150 (M^+) (calcd. $C_{17}H_{17}NO_3$=283.1207), ^{1}H NMR ($CDCl_3$) δ: 3.771 (s, 3, CH_3), 4.367 (d, 2, J=7 Hz, CH_2), 5.704 (br. s, 1, OH), 6.151 (t, 1, J=6 Hz, NH), 6.426 (d, 1, J=16 Hz, CH═CH), 6.791 (dd, 1, J=8 and 2 Hz, H-6), 6.825 (d, 1, J=2 Hz, H-2), 7.322–7.486 (m, 5, phenyl), 7.653 (d, 1, J=16 Hz, CH═CH), and 8.861 (d, 1, J=8 Hz, H-5).

Cell culture

Mouse 3T3-L1 cell line was obtained from Health Science Research Resources Bank (Sennan-shi, Japan), and cultured in DMEM (Sigma) containing 10% fetal bovine serum (FBS). Cells were precultured for 3 days, collected with an EDTA-trypsin solution, and resuspended in the medium at the rate of 6×10^4 cells/ml. The suspension was divided into wells of 96-well plates (for ELISA) and 12- or 24-well plates (for PCR) by 0.2 ml and 1.5 or 2 ml, respectively. The plates were precoated

Table 1
Adiponectin productive activity in cultured preadipocytes

Compounds	3T3-L1 cells		Human preadipocytes	
	E_{max} (relative to PGZ)	D_{50} (μM)	E_{max} (relative to PGZ)	D_{50} (μM)
PGZ	1	0.5	1	nd [a]
Curcumin	<0.1	nd	0.04	42
Gingerol	0.8	68	0.01	76
Capsaicin	<0.1	nd	<0.01	nd
4	<0.1	nd	–	–
5	0.2	5	–	–
6	0.2	67	0.04	75
7	16.4	86	0.81	77
8	5.4	72	–	–
9	11.0	59	0.35	77
10	0.3	28	–	–
11	9.2	118	>0.23 [b, a]	>180
12	0.1	nd	–	–
13	<0.1	nd	–	–
14	0.6	49	–	–
15	1.8	54	–	–
16	1.3	77	–	–

The dose-production dependency was studied in quadruplicate wells with 0.1, 0.3, 0.5, 0.7, 1, 2, and 4 μM PGZ, 1, 2, 4, 10, 20, 30, 50, 70, and 100 μM curcumin, 1, 3, 10, 30, 50, 70, 100, and 150 μM compound **4**, **5**, and **12**, 0.1, 0.3, 1, 3, 10, 30, 50, 70, 100, and 200 μM compound **10**, and 10, 30, 50, 70, 100, 150, 200, and 300 μM other compounds. E_{max} (maximal adiponectin production) and D_{50} (half-maximal dose) were determined from the plots, and the maximal adiponectin production was given as relative to the maximum by PGZ (92 or 62 ng/ml at 0.7 or 0.9 μM) in the case of 3T3-L1 cells or to the amount by 4 μM PGZ (6 or 15 ng/ml) in the case of human preadipocytes. Values are given as the mean of two separate experiments. nd, not determined. –, not tested.

[a] Plot did not reach the maximum at the largest concentration tested.

[b] Value at 300 μM.

with a collagen coating solution (Toyobo). After 3 days incubation, the medium was changed to a fresh medium (0.2, 1.5, or 2 ml/well) containing 0.1–4 μM PGZ or 10–50 μM DHA, if necessary. Ethanol solutions (2–6.7 μl) containing 0.1–30 mM test compounds or the corresponding volume of ethanol (as control) were added to the culture and the plates were incubated for 10–14 days before harvesting the medium and cells. The stock solution of PGZ (10 mM) was prepared in dimethyl sulfoxide (DMSO) and further diluted with ethanol before addition to the culture.

Human subcutaneous preadipocytes were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) and cultured according to the supplier's manual with PBM (Cambrex) as the basal medium. The precultured cells were suspended in PBM containing 2 mM glutamine and 10% FBS at the rate of 4.7×10^4 cells/ml. The cell suspensions were added by 0.2 ml into each well of 96-well plates precoated with the collagen solution. After 3 days incubation, the medium was changed to a fresh one (0.2 ml/well) containing 0.01 mg/ml insulin, 0.1 μM dexamethasone, and if necessary, 10–50 μM DHA. Samples were added in the same way as described above, and the plates were incubated for 14 days before collecting the medium. Adiponectin in the medium was determined by ELISA (mouse/rat or human adiponectin ELISA Kit, Ohtuska Pharmaceuticals, Tokyo, Japan). In addition, accumulation of lipid droplets in many cells (40–50% or more for the potent compounds such as **7** at their optimum concentrations) was found in 3T3-L1 cells as well as in human preadipocytes by microscopic examination just before the recovery of medium.

RT-PCR

Cells in the 12- or 24-well plates were washed with Ca- and Mg-free Dulbecco's phosphate-buffered saline and treated with a phenol reagent (Isogen, Wako Pure Chemicals, 0.3 or 0.2 ml/well). The extracted RNA was purified from proteins and DNA, and precipitated with 2-propanol according to the manufacturer's instructions. The total RNA was converted to cDNA by a reverse transcriptase system (Invitrogen). Quantitative PCR was performed in a Corbett Rotor-Gene 2000 Real-Time Cycler using the cDNA, QuantiTect SYBR Green PCR system (Quiagen), and the following primers: 5′-AAGGACAAGGCCGTTCTCT-3′ and 5′-TATGGGTAGTTGCAGTCAGTTGG-3′ for mouse adiponectin, and 5′-GACGAGGCCCAGAGCAAGAGA-3′ and 5′-TAGAT GGGCACAGTGTGGGTGA-3′ for β-actin. Cycling conditions were 94 °C — 1 min, 64 °C — 1 min, and 72 °C — 1 min for adiponectin, and 94 °C — 30 s, 62 °C — 30 s, and 72 °C — 30 s for β-actin.

Reporter plasmids and PPARγ activation assay

A firefly luciferase reporter plasmid with 3 copies of a PPAR-responsive element (PPRE) in rat acyl-CoA oxidase (ACO-PPREx3-Luc) was generated, as described previously (Heim et al., 2002). To generate protein expression vectors containing a full-length human PPAR (peroxisome proliferator-

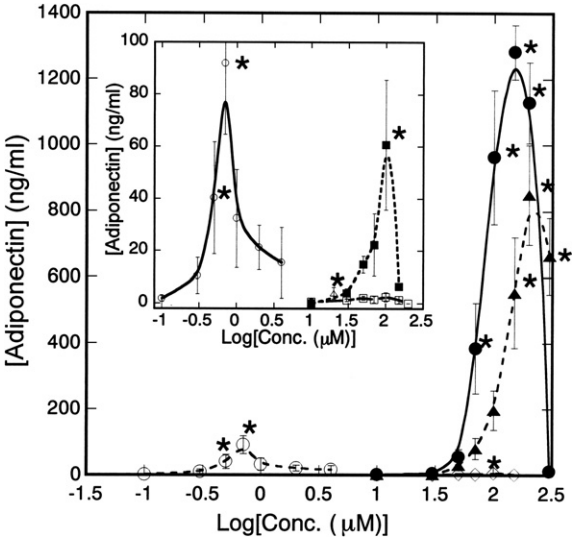

Fig. 2. Adiponectin production in mouse 3T3-L1 cells cultured with the ferulamides and ferulates. Cells (1.2×10^4/well containing 0.2 ml medium in 96-well plates) were incubated for 14 days without or with 0.1, 0.3, 0.5, 0.7, 1, 2, or 4 μM PGZ (open circle), 10, 20, or 30 μM curcumin (open triangle), or 10, 30, 50, 70, 100, 150, 200, or 300 μM capsaicin (open square), [6]-gingerol (closed square), **6** (open diamond), **7** (closed circle), or **11** (closed triangle), and adiponectin concentration in the medium was determined by ELISA. Data shown are the means ± S.D. of quadruplicate samples in a representative experiment that was repeated twice with comparable results.*represents $p < 0.05$ vs. control by Dunnett's *t*-test for each compound.

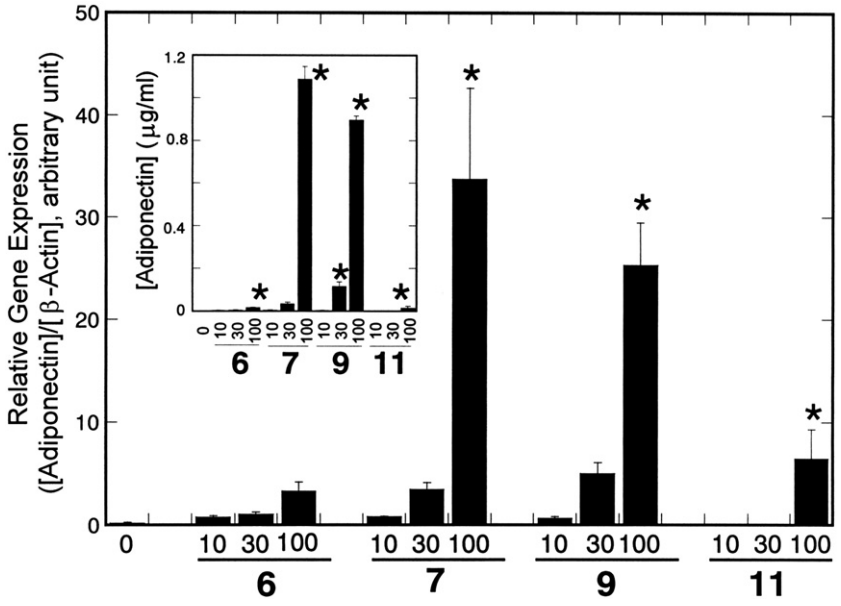

Fig. 3. Adiponectin gene expression in mouse 3T3-L1 cells. Cells (9.4×10^4 in each well containing 2 ml medium in 12-well plates) were incubated for 10 days without or with 10, 30 or 100 μM **6**, **7**, **9**, or **11**, and then treated with Isogen for RNA extraction. The medium was also collected for adiponectin determination by ELISA (see inset). Ratio of the expressed adiponectin gene vs. β-actin gene was determined by quantitative RT-PCR. Data shown are the means±S.D. ($n=3$). *represents $p<0.05$ vs. control by Dunnett's *t*-test for each compound.

activated receptor) γ1 or RXR (retinoid X receptor) α (pcDNA-PPARγ1, pcDNA-RXRα), the corresponding cDNAs from human cDNA libraries were amplified by PCR and subcloned into the pcDNA3.1(+) mammalian expression plasmid (Invitrogen). CV-1 cells were cultured in 24-well plates and transfected with ACO-PPREx3-Luc, pcDNA-PPARγ1 and pcDNA-RXRα, using LipofectAMINE and PLUS reagents (Invitrogen). Twenty four hours after the transfection, the cells were treated with test compounds (dissolved in DMSO) for another 24 h. The luciferase activity in the cells was determined using Dual-Luciferase Reporter Assay System (Promega).

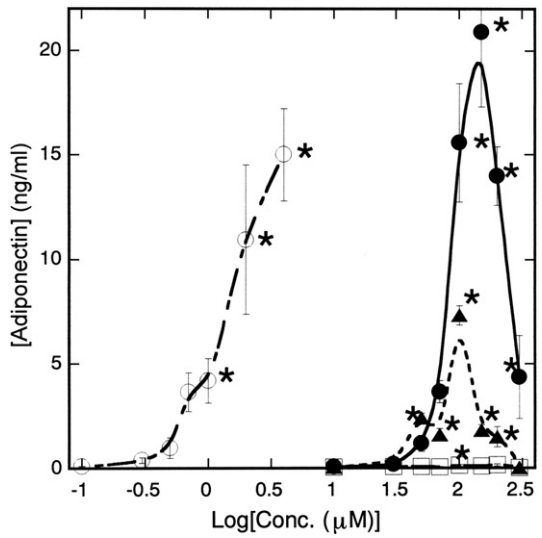

Fig. 4. Adiponectin production in human preadipocytes cultured with the present ferulamides. Human preadipocytes (9.4×10^3 in each well containing 0.2 ml medium in 96-well plates) were cultured in quadruplicate wells for 14 days without or with 0.1, 0.3, 0.5, 0.7, 1, 2, or 4 μM PGZ (open circle) or 10, 30, 50, 70, 100, 150, 200, or 300 μM capsaicin (open square), **7** (closed circle), or **9** (closed triangle). Adiponectin in the medium was determined by ELISA. Data shown are the means±S.D. ($n=4$) in a representative experiment that was repeated twice with comparable results.*represents $p<0.05$ vs. control by Dunnett's *t*-test for each compound.

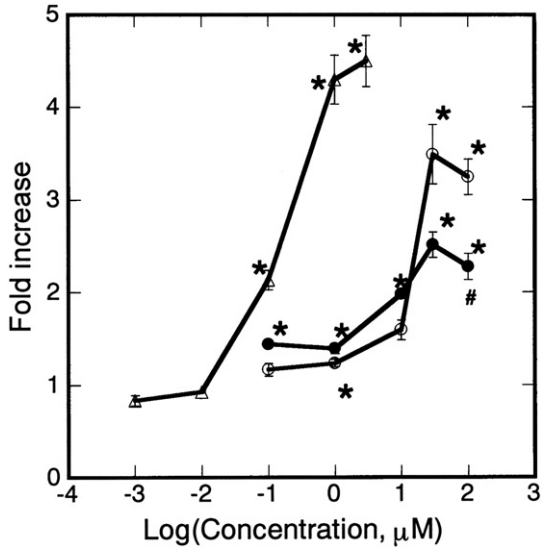

Fig. 5. Activation of PPARγ/RXRα heterodimers by the ferulamide **7**. Dose–response curves of **7** for luciferase activity in CV-1 cells in the absence (open circle) or presence (closed circle) of 3 μM GW9662 and that of rosiglitazone (triangle, as reference) are shown. The cells were transfected with a PPRE-driven luciferase gene and cDNAs encoding PPARγ and RXRα. The values were normalized to the mean luciferase activity in the absence of test compounds, and expressed as means±S.E.M. ($n=4$). *represents $p<0.05$ vs. control by Dunnett's *t*-test, and # represents $p<0.05$ vs. reaction of **7** without GW9662 by Dunnett's T3 test for the control, control+GW9662, 100 μM **7**, and 100 μM **7**+GW9662.

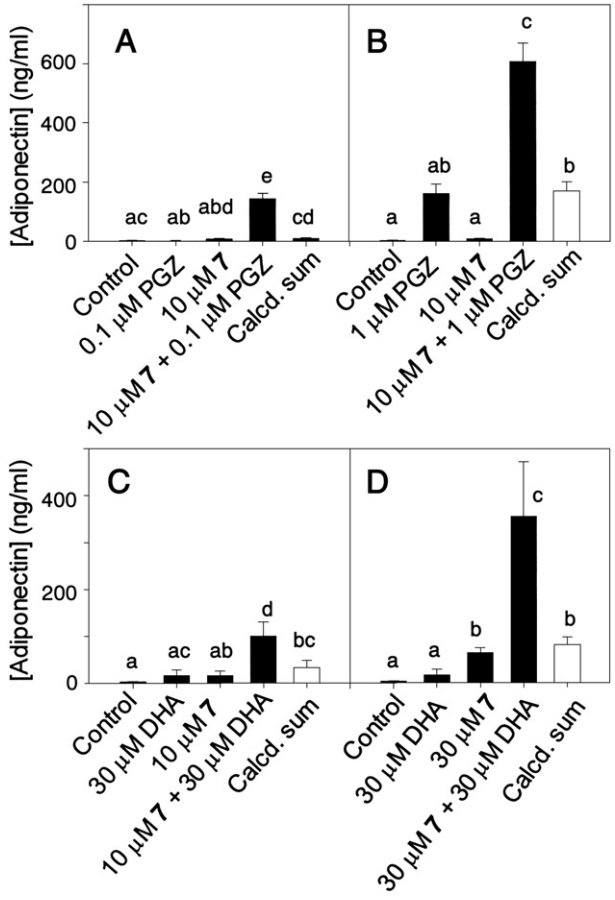

Fig. 6. Synergistic enhancement of adiponectin production by coaddition of the ferulamide **7** with PGZ or DHA in 3T3-L1 cells. Cells were incubated for 11 days (A and B) or 14 days (C and D) in triplicate wells in the same way as described before without or with 10 μM **7** and/or 0, 0.1, or 1 μM PGZ (A and B), or without or with 10 or 30 μM **7** and/or 30 μM DHA (C and D) and adiponectin in the medium was determined by ELISA. Data shown are the means±S.D. (n=3–9). Means without a common letter differ, $p<0.05$. The calculated sum values (Calcd. sum) were obtained from the data for PGZ, DHA, or **7** alone. The amplification rates (combination data/calculated sum) are 11.6, 3.6, 3.0, and 4.4 for the data of A, B, C, and D, respectively.

Animal studies

The experimental protocol was approved by the Institutional Animal Care and Use Committee of AIST. Thirteen male KK-A^y/Ta Jc1 mice (5 weeks old) were purchased from Nippon Clea (Tokyo, Japan) and housed in individual cages in a temperature, humidity, and light-controlled room (22–23 °C, 50%, and 12 h light/dark cycle). Diets containing 0 or 1% ferulamide **7** were prepared by mixing 120 g of a normal chow (CE-2, Nippon Clea) with 6 ml ethanol containing 0 or 1.2 g **7** and an autoclaved solution of 2.4 g starch in 48 ml water. Each mixture was firmly packed in a dish (15 cm in diameter) and dried at room temperature for a few days. The mice took these cakes and autoclaved tap water ad libitum. The body weight and food intake of each mouse was measured every weekday. On day 34, blood was taken from the tail end of the mice anesthetized with ethyl ether in a container and blood glucose was directly measured with a glucose sensor (Sanwa Kagaku Kenkyusho, Nagoya, Japan). Aliquots (9 μl) of the blood were mixed with 50 mg/ml heparin solution (3 μl each) and the blood cells were removed by centrifugation (10,000 rpm, 10 min). The plasma was analyzed by SDS-PAGE (1 μg protein/lane) under the condition of Laemmli with 10–20% density gradient gels. Western blot was performed with Hybond-P membranes and adiponectin was detected with mouse anti-adiponectin, mouse monoclonal antibody (Chemicon, MAB3608) and rabbit anti-mouse Ig G_1-HRP conjugate (Zymed, 61-0120), and ECL (Plus) detection kit (Amersham Biosciences). Serum albumin in the fresh gels (cut out after electrophoresis) was detected by Coomassie brilliant blue staining. The band intensities were digitized with a scanner and a computer program (Gel Pro Analyzer, Bio-Rad Laboratories) to calculate the relative adiponectin level against serum albumin. Triglyceride in the plasma was determined with an enzymatic assay kit (Triglyceride E-Test Wako). In the experiment with DHA, diets were prepared by mixing 250 g low fat diet (A-13251, Nippon Clea) with 14 ml corn oil or 2.7 ml corn oil plus 11.3 ml DHA enriched oil [DHA oil; DHA70, Nippon Kagaku Shiryo, Hakodate, Japan; DHA and eicosapentaenoic acid (EPA) were contained in 70% and 4% of the total fatty acids, respectively] and 3.75 ml ethanol containing 0 or 0.75 g ferulamide **7**. The mixed powder was divided into screw-capped small tubes (32 g each), in which the air was replaced with nitrogen using a vacuum apparatus. The tubes were tightly stoppered and stored in a refrigerator until use. The diets were changed every day (8 g for one mouse a day). The residual diet in the feed box was collected and weighed for calculating the intake. Blood was taken on day 31 and 37, and treated in the same way as described above, but the plasma adiponectin level and glucose concentration were determined with the ELISA and an enzymatic assay kit (Glucose C-II test Wako), respectively.

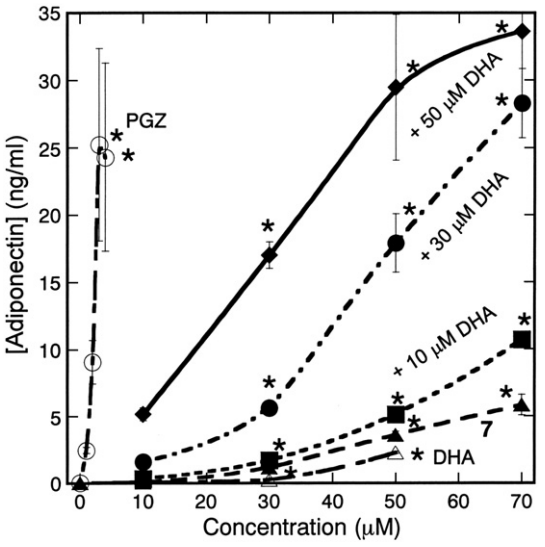

Fig. 7. Synergistic enhancement of adiponectin production by coaddition of ferulamide **7** with DHA in human preadipocytes. Cells (9.6×10^3/well containing 0.2 ml medium in 96-well plates) were incubated in triplicate wells for 14 days without or with 10, 30, 50, or 70 μM ferulamide **7** and/or 10, 30, or 50 μM DHA, or 1, 2, 3, or 4 μM PGZ. Data shown are the means±S.D. (n=3) in a representative experiment that was repeated twice with comparable results. *represents $p<0.05$ vs. control by Dunnett's t-test for each compound or for each group of the same DHA concentration.

Statistical analysis

All experimental measurements are expressed as means± standard deviation (S.D.). Statistical comparisons between two groups were evaluated by two-tailed Student's *t*-test and those among more than three groups were done by one-way ANOVA followed by Dunnett's *t*-test, Tukey's test, or Dunnett's T3 test using SPSS 15.0 J software, unless otherwise stated. Differences with $p<0.05$ were considered to be significant.

Results

We chose ferulic acid as the synthon for the guaiacol unit and coupled it with some alcohols and amines to form 3 species of ferulic esters (**4**, **6**, and **10**) and 5 species of ferulamides (**5**, **7**, **8**, **9**, and **11**) (Fig. 1). In addition, three analogs lacking a guaiacol unit (**12**, **13**, and **14**) and two guaiacol derivatives of no ferulamide skeleton (**15** and **16**) were synthesized for comparison. These compounds and curcumin, [6]-gingerol, capsaicin, and PGZ were tested on adiponectin production at different concentrations in mouse 3T3-L1 cells by determining adiponectin in the culture medium by ELISA. From the dose-adiponectin production plots, the intrinsic activity or maximal efficacy (E_{max}) and potency (D_{50}) were determined. The results are summarized in Table 1 and the plots for the typical compounds are shown in Fig. 2. Curcumin and capsaicin showed a marginal E_{max} and [6]-gingerol showed only an E_{max} comparable to that for PGZ (cf. inset of Fig. 2). In contrast to these known compounds, four ferulamides (**7**, **8**, **9**, and **11**) showed 5 to 16 times stronger activity than PGZ. The phenethylferulamide **7** was the most active among them. These ferulamides were designed to have a hydrophobic aromatic ring in the *N*-substituent. Another ferulamide with a long alkyl group (**5**) was less active than PGZ. The ferulates **4**, **6**, and **10** and analogs of **7** with no guaiacol functional groups (**12**, **13**, and **14**) were all less active than PGZ. The amides **15** and **16**, which were guaiacol derivatives but different from ferulamides, were slightly more

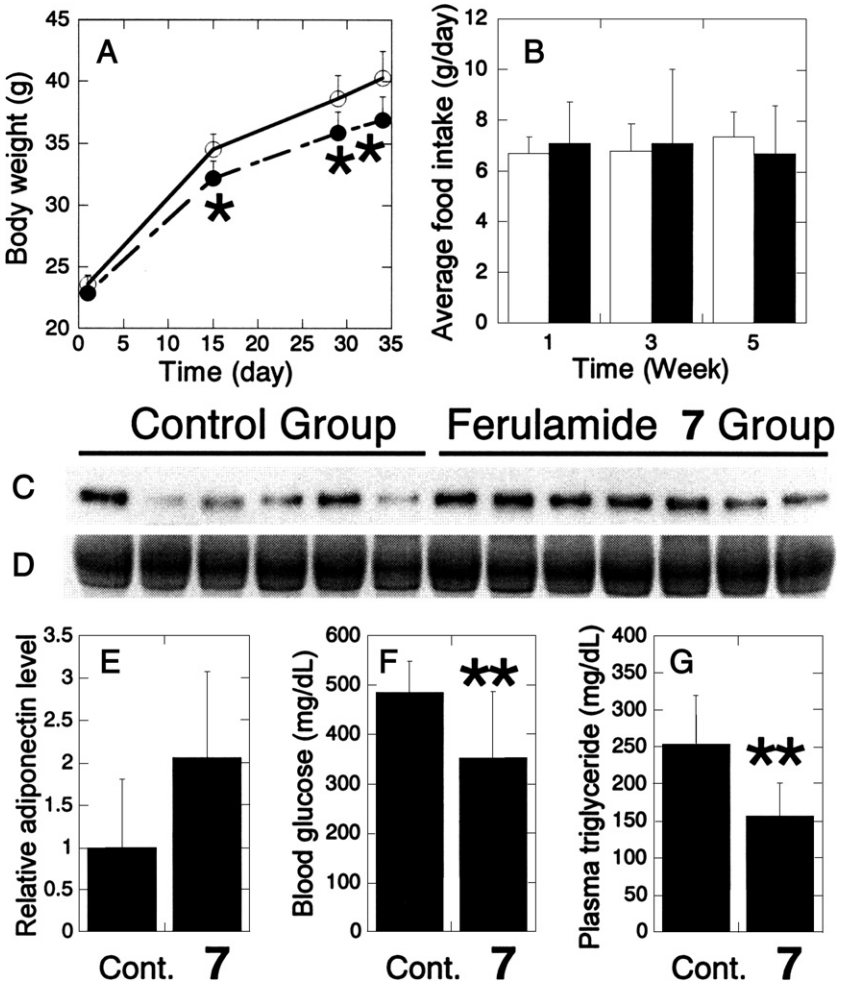

Fig. 8. Experiment with KK-A^y/Ta Jc1 mice fed diets without or with ferulamide **7** (1%). Number of mice was 6 in the control group and 7 in the **7**-fed group. (A) Average body weight of control (open circle) and **7**-fed group (closed circle); (B) average daily food intake of the control (open bar) and **7**-fed group (closed bar) in the 1st, 3rd, and 5th week; (C) Western blots for adiponectin in the plasma collected on day 34 from the control mice (6 bands on the left side) and the **7**-fed mice (7 bands on the right side); (D) Coomassie brilliant blue-stained serum albumin bands in the same polyacrylamide gel as electrophoresed for the adiponectin detection; (E) plasma adiponectin level calculated from relative intensities of the bands in panels C and D; (F) blood glucose concentration; and (G) plasma triglyceride concentration. Data shown are the means±S.D. ($n=6$ or 7 for A and E, $n=42$ or 49 for B, $n=11$ or 14 for F, and $n=18$ or 21 for G). *and **represent $p<0.05$ and $p<0.01$ vs. control, respectively.

active than the non guaiacol analogs (**12**, **13**, and **14**). These facts indicate that compounds having a guaiacol group linked with an aromatic ring via an alkylamide chain such as ferulamide **7** are promising as the strong adiponectin inducer. The stimulation of adiponectin gene expression by the ferulamides was confirmed by quantitative RT-PCR (Fig. 3). The patterns of gene expression and protein production of adiponectin with different compounds and concentrations were nearly parallel with each other (see the inset).

The ferulamides **7**, **9**, and **11** and the ferulate **6** were further studied on the adiponectin production in human preadipocytes (Fig. 4 and Table 1). The maximal efficacy (E_{max}) of curcumin, [6]-gingerol, and capsaicin was less than 5% of the adiponectin amount produced by 4 μM PGZ, but the E_{max} of ferulamide **7** approximated to the value by PGZ. Thus, the activity of adiponectin induction in human cells by the natural guaiacol derivatives such as curcumin was also markedly improved by adopting the ferulamide structure. The activities of the ferulamides **7** and **9** were stronger than that of the ferulate **6**, and the long alkyl amide **5** was only slightly active (data not shown), being consistent with the result in 3T3-L1 cells.

Gene expression of adiponectin is mainly controlled by a transcription factor PPARγ1 in a heterodimeric complex with another transcription factor RXRα (Iwaki et al., 2003). To examine the functional significance of PPARγ in the adiponectin induction by the ferulamides, we studied the effect of **7** on the luciferase activity in CV-1 cells transfected with a PPRE-driven luciferase gene and cDNAs encoding PPARγ and RXRα. The ferulamide **7** increased the luciferase activity of the CV-1 cells in a dose-dependent manner, suggesting that this compound stimulated transcriptional activity of PPARγ/RXRα heterodimers (Fig. 5). However, a competitive PPARγ antagonist GW9662 (2-chloro-5-nitro-*N*-phenylbenzamide) (Schopfer et al., 2005) did not clearly shift the dose-response curve to the high concentration side, though the maximal response to **7** was attenuated in the presence of GW9662. On the other hand, the adiponectin production by **7** in 3T3-L1 cells was increased by simultaneous addition of the PPARγ-specific ligand, PGZ, to a higher level than the sum of the responses to either compound alone, especially at their low concentrations (Fig. 6A, B). A similar effect of co-addition was found with another PPARγ agonist DHA (Krey et al., 1997) in 3T3-L1 cells (Fig. 6C, D) as well as in human preadipocytes (Fig. 7).

Finally we studied the biological activity of the ferulamide **7** in vivo. Diabetic male KK-A^y/Ta mice were fed a diet containing 1% **7** for 5 weeks. The adiponectin level in the **7**-fed mice was not significantly different from that of the control mice, while the

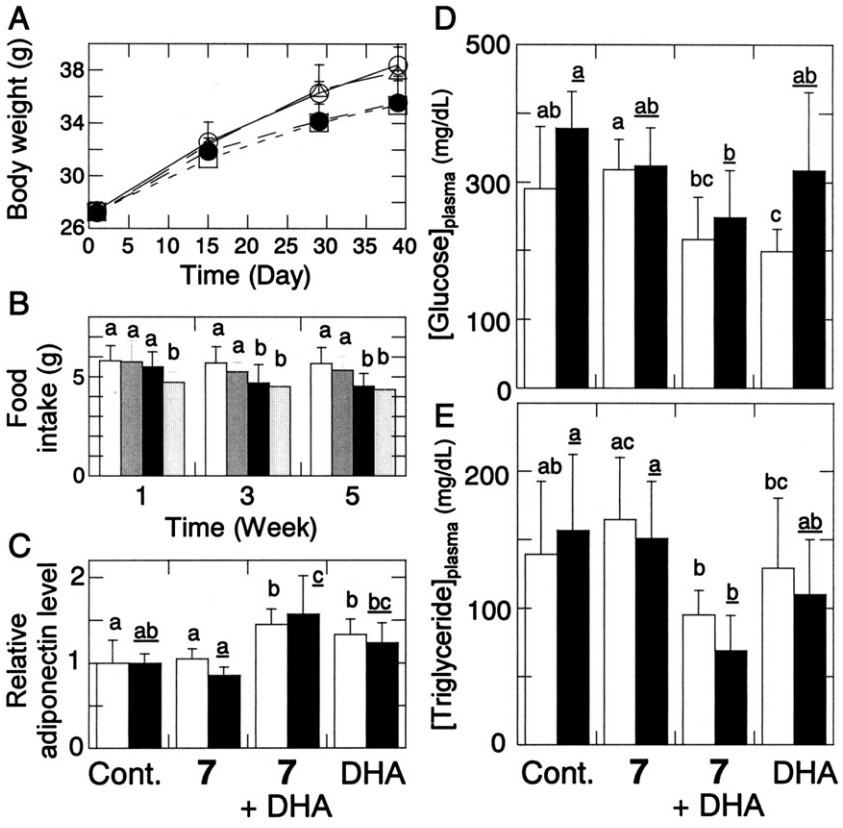

Fig. 9. Experiment with KK-A^y/Ta Jc1 mice fed diets containing ferulamide **7** (0.3%) and/or DHA oil (4%), or none of them. Number of mice was 4 in each group. (A) Average body weight of control (open circle), **7**-fed group (triangle), **7** and DHA oil-fed group (closed circle), and DHA oil-fed group (square); (B) average daily food intake of the control (open bar), **7**-fed group (grey bar), **7** and DHA oil-fed group (closed bar), and DHA oil-fed group (light grey bar) in the 1st, 3rd, and 5th week; (C) relative adiponectin level in plasma; (D) plasma glucose concentration; and (E) plasma triglyceride concentration. The blood was taken on day 31 (open bar) and day 37 (closed bar). Data shown are the means±S.D. (n=4 for A, n=28 for B, n=8–16 for C, and n=12 for D and E). Means without a common letter differ, $p<0.05$, but letters without underlines are applied to comparison of the day-31 samples and underlined letters are to day-37 samples.

blood glucose and plasma triglyceride concentrations were 27–38% lower in the **7**-fed group than in the control group (Fig. 8). The animal experiment was repeated with DHA oil and **7**, but the latter was used at a reduced rate (0.3%). Ferulamide **7** alone at this rate showed little effect on the plasma adiponectin level, but the level increased when DHA oil was supplemented (Fig. 9C). Multiple comparisons indicate that the adiponectin level of **7**+DHA group was significantly different from those of the control and the **7** alone group in both day-31 and day-37 samples. The adiponectin level of the DHA alone group was not significantly different from either that of the **7**+DHA group or that of the control group in the day-37 samples. The plasma glucose and triglyceride levels were lowered by the coadministration of **7** and DHA oil. From a statistical analysis by multiple comparison, the glucose and triglyceride levels of the **7**+DHA group were significantly different from those of the control and the **7** alone group (but with some exceptions). However, no significant difference was detected between the 7+DHA group and the DHA alone group. In addition, the mice fed the diets containing 1% **7**, **7**+DHA oil, or DHA oil alone showed 2–9% smaller body weight than the control in the 2nd–5th weeks, but the body weight of any mice increased constantly during the experimental period (Figs. 8A and 9A).

Discussion

The present results validated our initial molecular design for new adiponectin inducers (Fig. 1) based on the structures of curcumin, [6]-gingerol, and capsaicin, with which we had found stimulation of adiponectin induction. We decided to connect the guaiacol group with the hydrophobic moiety using an ester or amide bond for synthetic convenience, and hence we employed ferulic acid as the synthon. We prepared 8 species of ferulic derivatives and several related analogs to study the structure–activity relationship. Among the synthesized compounds, *N*-arylalkylferulamides (**7**–**9** and **11**) showed the strong activity (E_{max}) in 3T3-L1 cells. The strongest compound was phenethylferulamide **7**, which completely fulfilled our design. The fact that analogs **12**–**14** were remarkably less active than the above ferulamides indicates the importance of 4-hydroxy-3-methoxy functional groups for the present activity. This relation is supported by the moderate activity of two guaiacol derivatives **15** and **16** that lack the ferulamide skeleton. The *p*-hydroxyphenethyl derivative **11** showed weaker activity (smaller E_{max} and larger D_{50}) than the phenethyl derivative **7**, implying that the hydrophobicity of the *N*-substituent is another important point for the present activity. A comparison between the data of **5** and **7** shows that a long alkyl group in the *N*-substituent reduces the efficacy (E_{max}) but increases the potency (D_{50}) as compared with the aromatic ring. The compound **16** is an aryl analog of capsaicin, and their E_{max} values are in accord with the above consideration. As the linker between the guaiacol unit and the hydrophobic moiety, the amide linkage gave markedly more potent compounds than the ester linkage as shown by the pairs of **6** and **7** or **9** and **10**. This effect might be related to the stronger polarity of the amide bond as compared with the ester bond. Thus we reached the potent compounds **7**–**9** and **11** according to our initial hypothesis. These ferulamides were 20–200 times more potent and at least 10 times more potent than curcumin, [6]-gingerol, or capsaicin in 3T3-L1 cells and in human preadipocytes, respectively, in the view of efficacy. Further improvement of the activity needs understanding of the structure–activity relationship on the level of molecular interaction between the compounds and the binding domain of their receptors.

Initially, we expected that the ferulamide derivatives would stimulate the adiponectin expression through binding to the transcription factor PPARγ since the PPARγ-specific ligands thiazolidinediones induced adiponectin (Eguchi et al., 2007; Iwaki et al., 2003), and since curcumin, with which we had found the adiponectin inductive activity, was reported to activate PPARγ (Nishiyama et al., 2005). The activation of the PPARγ–RXRα heterodimers by the ferulamide **7** was clear from the reporter assay, but the experiment using GW9662 did not confirm that it was performed only through binding to PPARγ. The synergistic enhancement of adiponectin production by the combination of **7** with PGZ suggests that **7** might activate the heterodimeric partner RXRα. In fact, many species of RXR-specific ligands have been reported to promote adipogenesis either singly or in combination with PPARγ ligands (Sato et al., 2001; Sewter et al., 2002; Leibowitz et al., 2006). However, the true mechanism by which **7** induces adiponectin remains to be elucidated in the future.

The present compounds were prepared by chemical synthesis, but they are also natural products. The ferulamide **7** is reportedly contained in a garden plant, petunia (Jassey et al., 1982), and the indolylferulamide **9** is present in safflower seeds (Sato et al., 1985), sweet corn kernels (Ehmann, 1974), and bark of a tropical tree (Vecchietti et al., 1979). *p*-Hydroxyphenethylferulamide **11** is contained in pepper root (Yoshihara et al., 1981) and beet (Kujala et al., 2002), and as an anti-inflammatory constituent in some herbs (Yokozawa et al., 2001). In addition, several ferulamides including **5** and **11** were shown to stimulate insulin secretion in cultured pancreatic cells (Nomura et al., 2003). However, the adiponectin-inducing activity has not been reported for these ferulamides (except for some parts of our studies disclosed in a patent application (Yamazaki et al., 2005)).

The activities of these compounds are considerably high as compared with those of the known active compounds (i.e., curcumin, [6]-gingerol, and capsaicin) that are currently used in food or traditional medicine. Although the in vivo activity in increasing adiponectin level was not significant, the effectiveness in decreasing blood glucose and triglyceride levels was exhibited by the ferulamide **7** in diabetic mice (Fig. 8F, G). These facts seemed to imply the usefulness of **7**. However, the concentration required to induce adiponectin in the cultured human preadipocytes was over 10-fold higher than that of PGZ, and the induced amount of adiponectin by 30 μM **7** was very smaller (approximately 1/20) than the amount by 3–4 μM PGZ (Fig. 7). This fact made us apprehensive about whether the adiponectin-inducing activity of these ferulamides was still too weak to be applied in human health care. We noted the enhancement of adiponectin production by combining **7** with PGZ, but PGZ is a toxic pharmaceutical with side effects. Then, we carried out the experiment using DHA with the expectation that application of the foodstuff DHA instead of PGZ would safely

strengthen the activities of the ferulamides. The result is promising as the combination of 30 μM **7** with 50 μM DHA amplified the adiponectin amount to more than 15 times that given by 30 μM **7** alone, resulting in approaching a level of the value by 3 μM PGZ (Fig. 7). In the animal experiment, the group fed **7**+DHA oil showed the tendency that the adiponectin level increased and the glucose and triglyceride levels decreased as compared with other groups (Fig. 9C, D, E). However, the significant difference was only detected against the control or the **7** alone group. The **7**+DHA group was not significantly different from the DHA alone group in any case. DHA oil itself was reported to increase plasma adiponectin level in mice (Flachs et al., 2006). The result of the multiple comparisons suggests that the effect of the combination of **7** with DHA on blood glucose and triglyceride levels should be mainly due to DHA in this animal experiment. Nevertheless, the contribution of **7** would not be completely excluded, since the adiponectin level in day-37 samples (Fig. 9C, closed bars) showed no significant difference between the DHA group and the control in contrast to that between the **7**+DHA group and the control. Optimization of the dose and ratio of **7** to DHA in the diets would make their combination effect more clear. On the other hand, further improvement of the efficacy of the ferulamide is undoubtedly required, because the present dose of **7** (0.4 to 1.4 g/kg/day as estimated from the content and average food intake) is too high even as a supplement to health food. If these problems are solved, the prominent plasma triglyceride-lowering effect by the combination of DHA with a ferulamide such as **7** will have a practical importance for treatment of dyslipidemia. DHA is known to improve the hypertriglyceridemic condition by the regulation of lipoprotein metabolism (Lombardo et al., 2007), while the ferulamide (or the DHA partner) would promote the effect of DHA on the level involving different transcription factors (Duval et al., 2007; Chu et al., 2006; Zhang et al., 2006). In addition, the combination with other compounds will result in reducing the necessary amount of DHA so that the bad smell of the oxidation products from DHA oil is diminished.

We used DHA as a PPARγ agonist in this experiment, but it has been reported that DHA also activates RXRα (Lengqvist et al., 2004). Moreover, DHA alters the expressions of many genes that may affect the adipogenic signaling (Takahashi et al., 2002). It therefore cannot be concluded that the effect of the combination is only due to the specific activation of the two transcription factors respectively by the ferulamide and DHA.

Conclusion

The natural and synthetic ferulamides having a hydrophobic aromatic ring in the *N*-substituent show markedly stronger adiponectin-inducing activities in vitro than the known active compounds, curcumin, [6]-gingerol, or capsaicin, and these activities are further enhanced by coaddition with DHA. The most active ferulamide **7** can suppress the blood glucose and triglyceride levels in diabetic mice. The present ferulamides are useful lead compounds in developing more potent agents, natural or synthetic, for treatment of metabolic syndromes through promoting the endogenous adiponectin production, and that such an activity is possibly enhanced by the coadministration with DHA.

Acknowledgments

We thank Drs. Yoji Sato and Hiromi Yoshida (National Institute of Health Sciences) for conducting the PPRE-driven reporter assay of compound **7**.

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