Journal of Biomedical Science (2006) 13:763–772
DOI 10.1007/s11373-006-9109-3

Fractionation and identification of 9*c*, 11*t*, 13*t*-conjugated linolenic acid as an activator of PPARα in bitter gourd (*Momordica charantia* L.)

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Received 12 April 2006; accepted 25 July 2006
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Key words: PPARα, 9*cis*, 11*trans*, 13*trans*-conjugated linolenic acid, wild bitter gourd, transactivation assay, Acyl CoA Oxidase

Summary

Bitter gourd (*Momordica charantia* L.) is a common vegetable in Asia that has been used in traditional medicine for the treatment of Diabetes. PPARs are ligand-dependent transcription factors that belong to the steroid hormone nuclear receptor family and control lipid and glucose homeostasis in the body. We previously reported that the ethyl acetate (EA) extract of bitter gourd activated peroxisome proliferator receptors (PPARs) α and γ. To identify the active compound that activated PPARα, wild bitter gourd EA extract was partitioned between *n*-hexane and 90% methanol/10% H_2O, and the *n*-hexane soluble fraction was further separated by silica gel column chromatography and finally by preparative HPLC. A transactivation assay employing a clone of CHOK1 cells stably transfected with a $(UAS)_4$-tk-alkaline phosphatase reporter and a chimeric receptor of GAL4-rPPARα LBD was used to track the active component. Based on Mass, NMR, and IR spectroscopy, 9*cis*, 11*trans*, 13*trans*-conjugated linolenic acid (9*c*, 11*t*, 13*t*-CLN) was identified as a PPARα activator in wild bitter gourd. The isolated 9*c*, 11*t*, 13*t*-CLN rich fraction also significantly induced acyl CoA oxidase (ACO) activity in a peroxisome proliferator-responsive murine hepatoma cell line, H4IIEC3, implying that 9*c*, 11*t*, 13*t*-CLN was able to act on a natural PPARα signaling pathway as well. The content of 9*c*, 11*t*, 13*t*-CLN was estimated to be about 7.1 g/kg of our dried wild bitter gourd sample. The concentration of 9*c*, 11*t*, 13*t*-CLN and activation activity in the hydrolyzed EA extract of the seeds was higher than that of the flesh. The potential health benefits of 9*c*, 11*t*, 13*t*-CLN through the PPARα regulated mechanism are worthy to be further characterized in *in vivo* studies.

Abbreviations: ACO – Acyl CoA oxidase; AP – Alkaline phosphatase; CLA – Conjugated linoleic acid; CLN – Conjugated linolenic acid; LBD – Ligand-binding domain; PPAR – Peroxisome proliferators activated receptor; PP – Peroxisome proliferators; PPRE – Peroxisome proliferators responsive elements; RXR – Retinoic X receptor; TLC – Thin layer chromatography; TZD – Thiazolidinedione

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Introduction

Bitter gourd is a common vegetable in Asian countries and has also been recognized as being of medicinal value in India and in Traditional Chinese medicine. The most noteworthy health benefit of bitter gourd are the hypoglycemic potential demonstrated in normal [36] and diabetic [2, 7, 27, 31] rats as well as in human subjects with type II diabetes mellitus [21] and its hypolipidemic effect [3, 15, 26, 29]. The mechanism for the hypoglycemic effect of bitter gourd is still unclear, although it has been shown to inhibit glucose absorption [23], promote hepatic glucose utilization [31], possess an insulin-like polypeptide [18], and even to increase the insulin-positive cell number in the pancreas [1].

The peroxisome proliferator activated receptors (PPARs) are a highly conserved set of ligand-activated transcription factors in the nuclear hormone receptor superfamily [9]. Upon activation by a ligand, PPAR heterodimerizes with retinoid X receptor (RXR) and promotes the transcription of its target genes through binding to a peroxisome proliferator responsive element (PPRE) [9]. The target genes of PPAR are mainly a homogeneous group of genes that participate in aspects of lipid catabolism such as fatty acid uptake and binding, fatty acid oxidation in microsome, mitochondria and peroxisome as well as lipoprotein assembly and transport. PPARs thus play a key role in the control of lipid and glucose homeostasis through genomic action [9].

Three subtypes of this receptor exist, namely PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3), which display tissue-selective expression pattern and distinct biological functions. PPARα is predominantly expressed in liver, heart, kidney and skeletal muscle where it controls fatty acid catabolism. PPARγ is highly expressed in brown and white adipose tissue where it triggers cellular differentiation, promotes lipid storage and modulates the action of insulin. Recent report further demonstrated a role of PPAR in regulating glucose homeostasis, cellular differentiation and apoptosis, cancer development as well as in the control of the inflammatory response [9].

In humans, lipid homeostasis is a delicate balance between dietary intake, *de novo* synthesis, and catabolism. Obesity, insulin resistance, and hypertension are resulted from these lipid disorders, which together are known as the metabolic syndrome [11]. PPARs are regarded as important targets for the treatment of metabolic syndrome [5, 14].

Being the ligand/activator of PPARα, fibrate-type hypolipidemic drugs can induce the expression of genes that participate in aspects of lipid catabolism such as fatty acid uptake and binding, fatty acid oxidation (in microsomes, peroxisomes, and mitochondria), and lipoprotein assembly and transport [28]. PPARγ has been implicated as the primary receptor modulating the insulin sensitizing activity of the TZDs (Thiazolidinediones) class of anti-diabetic drugs [22], and it is well known for its role in adipogenesis at the cellular level [9]. Therefore, substantial attempts are being made to develop new therapeutic agents for hyperlipidemia, insulin resistance, or atherosclerosis based on their activity toward PPARs [5]. For example, PPAR α/γ dual agonists were developed as effective therapeutic agents for the treatment of insulin resistance and hypertriglyceridemia in the metabolic syndrome [5].

PPAR ligands/activators of food/diet origin may provide health benefit without the toxicity concern in the drug development, as long as the food/diet is consumed in a reasonable amount on a regular basis. A number of PPARs activators that are of food/diet origin have been identified. Conjugated linoleic acids [25] and phytanic acid [12] are well-known examples of which health benefits have been demonstrated. The hypolipidemic effect of fish oil [8] has been attributed, at least in part, to the activation of PPARα.

In our previous study, it was found that the ethyl acetate (EA) extract of bitter gourd could activate not only PPARα but also PPARγ [6], and the EA extract up-regulated the expression of PPARα target genes in H4IIEC3 cells and in C57BL/6J mice (manuscript in preparation). These observations implied that bitter gourd, with the characteristic of PPAR α/γ dual agonist, may have potential health benefit for ameliorating hyperglycemic and hyperlipidemic conditions [13, 15, 16, 24]. In this study, we fractionated and identified the active compound that can activate PPARα in wild bitter gourd and estimated the content and distribution of the active compound in wild bitter gourd.

Materials and methods

Materials

Eleven different varieties of fresh wild bitter gourds, total 113 kg, were provided by Hualien District Agricultural Research and Extension Station, Council of Agricultural, Executive Yuan, Taiwan. The whole fruit of wild bitter gourds were cut into small pieces, freeze-dried, and grounded to give 7.7 kg of raw material.

Cell culture

The stable expression of Gal4-rPPARα LBD chimeric receptor and $(UAS)_4$-tk-AP reporter gene in CHO-K1 cells, clone 77, constructed by Chao and Huang [6] was used to screen the activators in wild bitter gourd. Cells of Clone 77 were grown at 37°C in the presence of 5% CO_2 in Ham's F-12 medium supplemented with 10% fetal bovine serum (Gibco BRL, Rockville, Md., USA).

Transactivation experiments and reporter gene assay

For the transactivation experiments, the tested samples, extract and fractions isolated from the wild bitter gourd, were dissolved in a minimal amount of ethanol or dimethyl sulfoxide (DMSO). They were then diluted to appropriate concentrations with Ham's F-12 medium containing 10% serum replacement (TCM, Celox, St. Paul, Minn., USA) immediately before use. The procedure of the transactivation assay has previously been described [6]. Briefly, stably transfected cells were seeded in 96-well plates and incubated with medium containing Wy-14643 or the tested samples or vehicles (DMSO or ethanol) for 48 h. Culture medium was then collected and assayed for AP activity using CSPDR (Tropix, Applied Biosystems, Foster City, Calif., USA) as the substrate and Sapphire-II as the enhancer. Chemiluminescence was measured in a luminometer (Wallac 1420 Victor2 multiple label counter; Perkin Elmer, Turku, Finland). The viability of treated cells was checked by the MTT (Sigma, St. Louis, Mo., USA) assay. Data reported were confined to those for which treatment did not significantly change the cell viability. Folds of activation were calculated by taking the AP activity of vehicle-treated cells as 1.

Fractionation and chemical identification

The 7.7 kg dried powder of the wild bitter gourd was extracted with EA (80 l) at room temperature for 2 days and repeated once. The pooled extract was evaporated in a rotary evaporator (Buchi, Essen, Germany) to remove the solvent and yielded a residue, EA extract (EAE), which was suspended in *n*-hexane (1 l), and this was then partitioned with 1 l of 90% methanol/10% H_2O for 3 times. The resulted two fractions were evaporated, respectively, and yielded *n*-hexane extract (HE) and 90% methanol/10% H_2O extract ($MeOH/H_2OE$). The HE afforded a black syrup (234.85 g), which was subsequently chromatographed over Silica gel with a *n*-hexane/EA gradient solvent system. The eluted fractions showing similar thin layer chromatography (TLC) and ^1H NMR patterns were gathered, evaporated, and weighed; the evaporated crude compounds were screened for the activation activity upon PPARα by the transactivation assay.

Preparative HPLC (Merck LichroCART 250-10 Cat. 1.50179 Lichrosorb Si 60 (7 μm)) was used for further purification of the active compound. IR, NMR, and Mass spectroscopy were used to elucidate the chemical structure of the eluted fractions and, finally, the active compound. IR spectra were recorded on a Perkin-Elmer 983G spectrophotometer, samples were dissolved in CCl_4 for the measuring ^1H and ^{13}C NMR spectra using a Varian Unity Plus 400 spectrometer, and EIMS were obtained on a JEOL JMS-HX 300 mass spectrometer.

Hydrolysis reaction

The triglyceride fraction separated from the HE by silica gel column chromatography was saponified by dissolving in 1 N NaOH solvent composed of $MeOH/H_2O = 4/1$ and reacted at 60 °C. After the reaction was completed, the reactant was acidified to pH < 2 and then partitioned with *n*-hexane. The *n*-hexane extract was evaporated and subjected to ^1H NMR spectroscopy and transactivation assay to track the compounds with the activation activity of PPARα.

Treatment of the H4IIEC3 cell line with active fraction and ACO activity assay

H4IIEC3 cells (American Type Culture Collection) are Reuber rat hepatoma cells that offer a good in vitro model system of the induction of peroxisomes and peroxisomal β-oxidation enzymes by peroxisome proliferators (PP). The procedure of the treatment of H4IIEC3 cells has previously been described [6]. H4IIEC3 cells were maintained at 37 °C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 20% horse serum (Gibco) and 5% fetal bovine serum (Gibco). At near confluence, cells were seeded at a density of 60,000 cells/ml into 10-cm dishes and grown for 24 h in the same medium under 5% CO_2 at 37 °C. The isolated 9 *cis*, 11 *trans*, 13 *trans*-conjugated linolenic acid (9*c*,11*t*,13*t*-CLN) rich fraction with the activation activity of PPARα in the transactivation assay was dissolved in a minimal amount of absolute ethanol and diluted with the medium to a concentration of 45, 90, or 180 μM, respectively, and used to treat these cells. Blank dishes received vehicle (medium containing 0.1% absolute ethanol) only. Cells treated with medium containing 50 μM Wy-14643 served as positive control. After 72 h of treatment, cells were harvested for analysis of ACO activity. Harvested cell pellets were homogenized, and the postnuclear supernatant fraction was isolated and analyzed for ACO activity according to the method of Small et al. [30] using palmitoyl-CoA as the substrate. Cell extracts were incubated in a mixture containing substrate, aminotriazole, horseradish peroxidase and 2′,7′-dichlorofluoresin (Leuco-DCF) diacetate. The hydrogen peroxide produced in the ACO reaction convert Leuco-DCF to DCF catalyzed by horseradish peroxidase. The changes in the absorbance at 502 nm from DCF was used to calculate ACO activity.

Data analysis

Data reported were expressed as the mean ± standard deviation of triplicate wells, and were representative of at least three separate experiments with similar results. The significance of difference between each treatment was analyzed by oneway ANOVA (Analysis of Variance) and Duncan's Multiple Range Test or Student's *t* test using SAS (SAS 8.1, Cary, N.C., USA) software.

Results

Fractionation and identification of the active compound

The EAE extracted from the dried powder of wild bitter gourd (7.7 kg) was partitioned, and HE and MeOH/H_2OE were obtained. In the transactivation assay, HE significantly activated PPARα at the concentrations of 100 μg/ml and 150 μg/ml, whereas MeOH/H_2OE did not (Figure 1).

In a small-scale experiment, 1.01 g of HE was first separated by silica gel chromatography with an *n*-hexane/EA gradient solvent system, and 62 fractions were eluted. Fractions showing similar TLC and ^{1}H NMR patterns were pooled to obtain 8 fractions and each was tested for the PPARα activation by the transactivation assay. As shown in Figure 2, the fraction 45~57 showed significant activation on PPARα at the concentration of 50 μg/ml. According to the ^{1}H NMR spectra, the fraction 45~57 was mainly composed of various fatty acids, including an unusual fatty acid with a unique ^{1}H NMR pattern. On the other hand, the fraction 20~24 was composed of triglyceride and

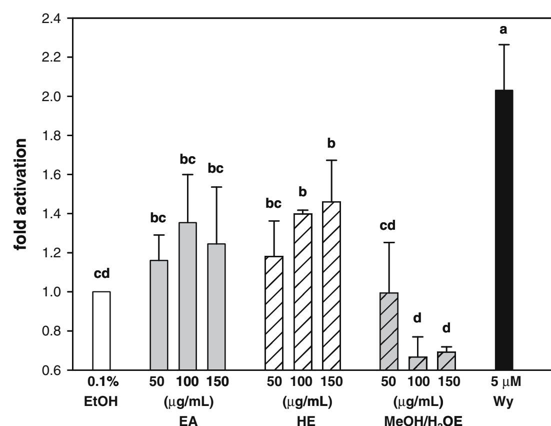

Figure 1. Ligand dependent transactivation of GAL4-rPPARα ligand binding domain by partitioned fractions of ethyl acetate extract (EAE) of wild bitter gourd whole fruit. The EAE was partitioned between *n*-hexane (HE) and 90% MeOH/10% H_2O (MeOH/H_2OE). Values are means ± SD of triplicate wells in a representative experiment. Data shown are representative of at least three separate experiments. Wy-14643 (5 μM) is the positive control and 0.1% ethanol (EtOH) is the vehicle. Multiple comparison among all values shown in the figures were analyzed statistically by oneway ANOVA and Duncan's Mutiple Range Test. Values not sharing the same letters are significantly different from one another ($p < 0.05$).

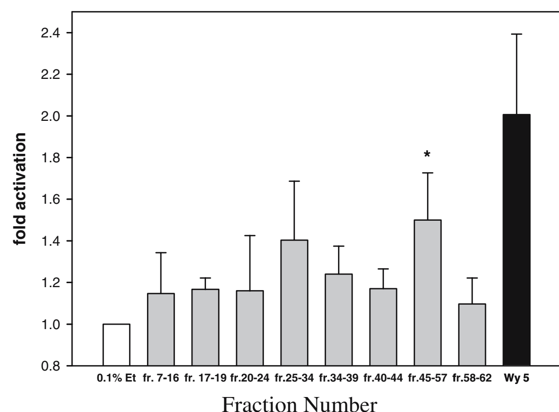

Figure 2. Ligand dependent transactivation of GAL4-rPPARα ligand binding domain by fractions eluted from silica gel chromatography. *n*-Hexane soluble fraction partitioned from wild bitter gourd ethyl acetate extract was separated by silica gel chromatography. Eluted fractions were tested by the transactivation assay at a concentration of 50 μg/ml. Values are means ± SD of triplicate wells in a representative experiment. Data shown are representative of three separate experiments. Wy-14643 (5 μM) is the positive control and 0.1% ethanol (Et) is the vehicle. *Indicated significantly different between the vehicle and the fraction 45–57 by Student's *t* test ($p < 0.05$).

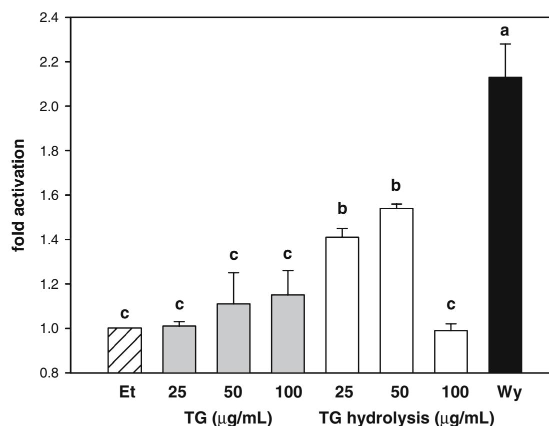

Figure 3. Ligand dependent transactivation of GAL4-rPPARα ligand binding domain by unhydrolyzed and hydrolyzed TG fraction obtained from silica gel chromatography (fraction 27–30, see text). Values are means ± SD of triplicate wells in a representative experiment. Data shown are representative of three separate experiments. Wy-14643 (5 μM) is the positive control and 0.1% ethanol (EtOH) is the vehicle. Multiple comparison among all values shown in the figures were analyzed statistically by oneway ANOVA and Duncan's Mutiple Range Test. Values not sharing the same letters are significantly different from one another ($p < 0.05$).

had the highest yield (52.05%) among the eluted fractions. Interestingly, the unique ^{1}H NMR pattern shown in spectra of the fraction 45∼57 was also observed in the spectra of fraction 20∼24, implying that the unusual fatty acid might also existed in the triglyceride in the fraction 20∼24 in an esterified form.

The fractionation procedure was repeated with a large amount of HE (205.92 g) with 195 fractions eluted. The eluted fractions 17∼60 were identified as triglyceride (119.06 g). Among these, fractions 27∼30 (41.76 g) were pooled and hydrolyzed to obtain free fatty acids. At concentrations of 25 and 50 μg/ml, the hydrolysate showed significant activation on PPARα, whereas the unhydrolyzed fraction 27∼30 did not (Figure 3).

The triglyceride hydrolysate was further separated by preparative HPLC, and three crude fractions were obtained. Based on IR and NMR spectra, the first two fractions were identified as mixtures of ordinary fatty acids. The third fraction, however, contained mainly the unusual fatty acid. This unusual fatty acid was identified as 9*c*, 11*t*, 13*t*-CLN. The ^{1}H-NMR spectrum of 9*c*, 11*t*, 13*t*-CLN (400 MHz CCl_4) was: δ0.85 (3H, t, J = 6.3 Hz, H-18); 1.23 (12H, H-4, −5, −6, −7, −16, −17); 1.61 (2H, quint, J = 6.3 Hz, H-3); 2.05–2.02 (4H, m, H-7, −15); 2.32 (2H, t, J=7.6 Hz, H-2); 5.37 (dt, J = 11.0 Hz, J = 7.5 Hz, H-9); 5.68 (dt, J = 14.0 Hz, J = 7.0 Hz, H-14); 5.96 (t, J = 11.0 Hz, H-10); 6.08 (dd, J = 14.0 Hz, J = 11.0 Hz, H-13); 6.14 (dd, J = 14.0 Hz, J = 11.0 Hz, H-12); 6.36 (dd, J = 14.0 Hz, J = 11.0 Hz, H-11). The UV λmax (C_6H_{12}) spectrum of 9*c*, 11*t*, 13*t*-CLN was 262, 272, 284 nm. The IR spectrum of 9*c*, 11*t*, 13*t*-CLN was 3200–2500, 1790, 917 (–COOH), 1428, 1408, 1273, 1000, 970, 751, 724. Based on the mass spectra, this third fraction was composed of 77% 9*c*, 11*t*, 13*t*-CLN, 11% stearic acid, and 12% palmitic acid. As shown in Figure 4, at the concentration of 180 μM, the isolated 9*c*, 11*t*, 13*t*-CLN rich fraction was found to activate PPARα to an extent that was equivalent to the positive control, 5 μM Wy-14643 ($p > 0.05$), and its EC_{50} was about 30.35 μM.

Comparison of the activity between the isolated 9c, 11t, 13t-CLN-rich fraction and some fatty acids

To compare the activation activities of PPARα among the isolated 9*c*, 11*t*, 13*t*-CLN-rich fraction and various common fatty acids, Clone 77 cells

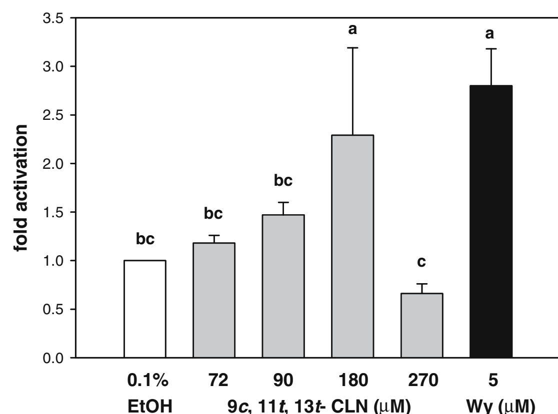

Figure 4. Ligand dependent transactivation of GAL4-rPPARα ligand binding domain by the isolated 9*c*, 11*t*, 13*t*-CLN-rich fraction. Values are means ± SD of triplicate wells in a representative experiment. Data shown are representative of three separate experiments. Wy-14643 (5 μM) is the positive control and 0.1% ethanol (EtOH) is the vehicle. Multiple comparison among all values shown in the figures were analyzed statistically by oneway ANOVA and Duncan's Mutiple Range Test. Values not sharing the same letters are significantly different from one another ($p < 0.05$).

were treated with the isolated 9*c*, 11*t*, 13*t*-CLN-rich fraction, palmitic acid, oleic acid, linoleic acid, linolenic acid, and 9*c*, 11*t*-CLA. As shown in Figure 5, all of the tested fatty acids showed significant activation on PPARα except palmitic acid, whereas 9*c*, 11*t*-CLA and the isolated 9*c*, 11*t*, 13*t*-CLN-rich fraction activated PPARα to a higher extent than the remainings.

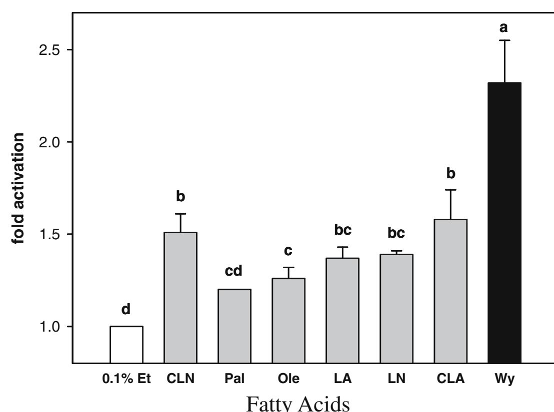

Figure 5. Ligand dependent transactivation of GAL4-rPPARα ligand binding domain by various fatty acids (180 μM). Pal, Palmitic Acid, Ole, Oleic Acid, LA, Linoleic Acid, LN, Linolenic Acid, CLN, the isolated 9c, 11t, 13t-CLN Rich Fraction, CLA, 9c, 11t-Conjugated Linoleic Acid. Values are means ± SD of triplicate wells in a representative experiment. Data shown are representative of three separate experiments. Wy-14643 (5 μM) is the positive control and 0.1% ethanol (Et) is the vehicle. Multiple comparison among all values shown in the figures were analyzed statistically by oneway ANOVA and Duncan's Mutiple Range Test. Values not sharing the same letters are significantly different from one another ($p < 0.05$).

Activity of the isolated 9c, 11t, 13t-CLN-rich fraction on the nature PPARα in H4IIEC3 cell line

To test whether the isolated 9*c*, 11*t*, 13*t*-CLN-rich fraction can act on natural PPARα, H4IIEC3 cells were treated for 72 h. Cells treated with 180 μM of the isolated 9*c*, 11*t*, 13*t*-CLN-rich fraction showed significantly high activity of ACO that was about 4.1-fold ($p < 0.05$) that of vehicle-treated cells and about 1.7-fold that of the positive control, 50 μM Wy-14643 treated cells (Figure 6).

The content and distribution of the active compound in wild bitter gourd

Based on NMR and Mass spectra, there was about 28.1% 9*c*, 11*t*, 13*t*-CLN in the triglyceride fraction (the fraction 20∼24), and about 29% in the free fatty acid fraction (the fraction 45∼57) eluted from the HE fraction in the first small scale experiment. In addition, the yield of the triglyceride fraction

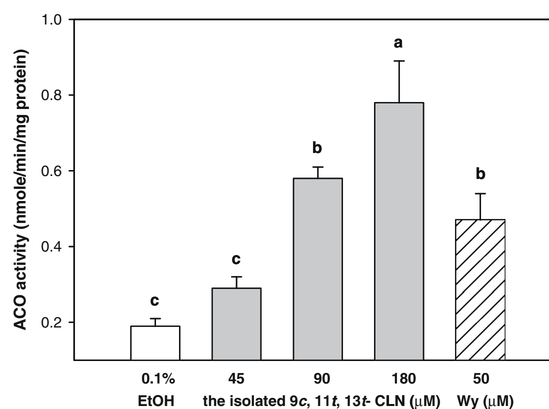

Figure 6. The induction of acyl CoA oxidase activity by the isolated 9*c*, 11*t*, 13*t*-CLN-rich fraction and Wy-14643 in H4||EC3 cell line treated for 72 h. Values are means ± SD of triplicate experiments. Data shown are representative of three separate experiments. Wy-14643 (50 μM) is the positive control and 0.1% ethanol (EtOH) is the vehicle. Multiple comparison among all values shown in the figures were analyzed statistically by oneway ANOVA and Duncan's Mutiple Range Test. Values not sharing the same letters are significantly different from one another ($p < 0.05$).

was 56.68% of the total HE, and that of the free fatty acids fraction was 14.05%. Based on these data, the content of 9*c*, 11*t*, 13*t*-CLN in the EAE was estimated to be about 19.3%, and the content of 9*c*, 11*t*, 13*t*-CLN in wild bitter gourd was estimated to be about 7.1 g/kg dried wild bitter gourd and about 0.42 g/kg fresh wild bitter gourd.

The seeds and the flesh were separated from fresh wild bitter gourd, and the hydrolyzed EA extracts of the two parts were prepared, respectively. As Suzuki et al. reported that 9*c*, 11*t*, 13*t*-CLN showed absorption maxima at 268.8 nm [32], we monitored the UV spectrum and the absorption at 268.8 nm to estimate the concentration of 9*c*, 11*t*, 13*t*-CLN in the EA extract of seeds and flesh. The results showed that 9*c*, 11*t*, 13*t*-CLN of the EA extract of seeds (28.7 g/100 g EA extract) was higher than that of flesh (7.6 g/100 g), agreed with significant higher activation of PPARα in the hydrolyzed seed EA extract than in the hydrolyzed flesh EA extract. However, because the proportion of the seeds was only about 2% (wt/wt) of the whole bitter gourd fruit, the distribution of 9*c*, 11*t*, 13*t*-CLN in the seeds was nearly 30% that of the whole fruit. Besides, we estimated the amount of 9*c*, 11*t*, 13*t*-CLN in seven varieties of wild bitter gourd by UV absorption at 268.8 nm. The average content was about 22.42 $\pm$ 5.22% in EA extract, and the average activation activity of PPARα at the concentration of 50 μg/ml was 69 $\pm$ 18% that of the positive control, 5 μM of WY-14643.

Discussion

Bitter gourd is an important cultivated vegetable crop in Asian countries and is recognized as being of medicinal value in Indian and Chinese traditional medicine. Indeed, the juice or whole powder of bitter gourd fruit has been reported to have various medicinal properties, including anticarcinogenic [20], hypoglycemic [13, 16, 24, 36], and hypotriglyceridemic [15] effects. These effects are closely related to the role of PPAR in regulating lipid and glucose homeostasis, cellular differentiation and apoptosis, and cancer development as well as in controlling the inflammatory response [17]. However, the mechanism and active compounds responsible for these biological effects of bitter gourd have not been fully elucidated. Based on our previous observation [6] that the EA extract of wild bitter gourd could significantly activate PPARα, this study continued to search for the active compound showing this transcriptional activity in wild bitter gourd.

In the separation procedure, EAE and the partitioned HE, but not the methanol/H_2O soluble fraction, showed significant activation on PPARα. This indicated that the PPARα activator in wild bitter gourd was relatively hydrophobic, agreed with the fact that the ligand binding structure of PPARα was more accessible to hydrophobic molecules [37].

In the first small scale experiment, 8 fractions showing diverse TLC and ^{1}H NMR patterns were obtained from the silica gel chromatography of the HE. All of the 8 fractions tended to activate PPARα to some extent, whereas only the fractions 45~57 showed significantly higher activation of PPARα than the vehicle control. The NMR spectra of the 8 fractions were not alike. The only similarity was that various long chain fatty acids, including oleic acid, linoleic acid, and linolenic acid, were scattered from the first to the final fraction. Since these fatty acids are known to activate PPARα, this may explain the mild activity of the 8 fractions in activating PPARα. Based on ^{1}H NMR spectra, the fraction 45~57 was composed of ordinary fatty acids and an unusual fatty acid with unique structure. We then hypothesized that the activators of PPARα in wild bitter gourd might be the unusual fatty acid with unique structure. Unfortunately, the yield of the fraction 45~57 was too low to be purified further. Given that the unique spectra pattern of the unusual fatty acid found in the fraction 45~57 was observed in the eluted triglyceride fraction 20~24, this latter fraction did not activate PPARα at all. This result agreed with that free fatty acids but not those esterified in triglycerides could activate PPARα.

After repeated chromatography and preparative HPLC, we identified the fatty acid with unique structure, 9*c*, 11*t*, 13*t*-CLN, and the 9*c*, 11*t*, 13*t*-CLN-rich fraction could activate PPARα to an extent that was comparable to the positive control, Wy-14643 ($p > 0.05$). 9*c*, 11*t*, 13*t*-CLN has been reported to be a form of fatty acids distributed in bitter gourd and mainly in seeds [32, 33].

The cell based ligand dependent transactivation assay used in this study to track the active compound in PPARα activation has the limitation that it cannot reveal the in vivo biological activity.

For example, it is not known whether the 9*c*, 11*t*, 13*t*-CLN esterified in the TG of bitter melon could be digested, absorbed and transported to the cellular location where endogenous PPARα existed. However, several in vivo studies using 9*c*, 11*t*, 13*t*-CLN or bitter melon oil rich in this fatty acids have been reported. In addition to increase the antioxidant activity of blood and liver lipid in rats [10], 9*c*, 11*t*, 13*t*-CLN has also been reported to modulate the body fat and triglyceride metabolism in a manner that is different from CLA [19]. Although CLN increased serum and liver triglyceride level, it reduced the weight of perirenal and epididymal adipose tissue and enhanced the activity of β-oxidation in liver mitochondria and peroxisomes to a higher extent than CLA [19]. Noguchi et al. fed rats with bitter gourd oil (BGO) diets for 4 weeks and found occurrence of *c*9, *t*11-conjugated linoleic acid (*c*9,*t*11-CLA) in the liver of rats [26], and the conversion of *c*9,*t*11, *t*13-CLN to CLA by an enzymatic biohydrogenation in rat tissue was confirmed later [34, 35]. Since CLA has been reported to have benefits such as antiatherogenic and antidiabetic effects [4], it is therefore worth investigating if 9*c*, 11*t*, 13*t*-CLN per se or after transforming to *c*9,*t*11-CLA *in vivo* that may have the beneficial effect on metabolic syndrome.

ACO is known to be one of PPARα target genes, since the PPAR-specific binding sequence (PPRE) has been detected in the promoter region of ACO. Administration of a PPARα ligand will up-regulate the expression of ACO gene, which in turn will increase peroxisomal β-oxidation. This regulation is known as the PPARα signaling pathway. In this study, the isolated 9*c*, 11*t*, 13*t*-CLN rich fraction induced a significant increase of ACO activity in the H4IIEC3 cells, implying that 9*c*, 11*t*, 13*t*-CLN might up-regulate target genes of PPARα in this cell line, as did the positive control, WY-14643. As a chimeric receptor was used in the transactivation assay, the results observed in H4IIEC3 cells demonstrated that 9*c*, 11*t*, 13*t*-CLN could work on natural PPARα and trigger the signaling pathway as well. However, results from our transactivation assay and H4IIEC3 cell experiment could not reveal whether 9*c*, 11*t*, 13*t*-CLN per se or its metabolite(s) may act as a ligand of PPARα. A binding experiment is required to clarify this point. Furthermore, *in vivo* studies are also needed to confirm the physiological and metabolical effect of 9*c*, 11*t*, 13*t*-CLN.

The isolated 9*c*, 11*t*, 13*t*-CLN seemed to have a higher stability in the form of triglyceride than in the free form of fatty acid. The form of triglyceride was stable under room temperature for one month; however, the free form must be frozen under -20 °C and filled with nitrogen to prevent from oxidation.

It has been reported that there are three isomers of CLN in bitter gourd and each showed different UV absorption maxima. 9*c*, 11*t*, 13*t*-CLN was reported to have absorption maxima at 268.8 nm, and only exist in the seeds of bitter gourd [32]. We followed this procedure and estimated the relative content of 9*c*, 11*t*, 13*t*-CLN in hydrolyzed EA extracts of the seeds and flesh from wild bitter gourd, respectively, based on the UV absorption. The concentration of 9*c*, 11*t*, 13*t*-CLN was higher in the EA extract of seeds than in that of flesh. Moreover, the hydrolyzed EA extract of the seeds also showed higher activation of PPARα than that of the flesh implying 9*c*, 11*t*, 13*t*-CLN was the active compound in wild bitter gourd.

In conclusion, a PPARα activator in the ethyl acetate extract of wild bitter gourd was identified to be 9*c*, 11*t*, 13*t*-CLN. The EC_{50} of it in our transactivation assay was about 30.35 μM. The potential health benefits of 9*c*, 11*t*, 13*t*-CLN through the PPARα regulated mechanism await to be further characterized in *in vivo* studies.

Acknowledgements

This study was financially supported by a grant (NSC93-2317-B-002-010) from the National Science Council, Taiwan. We thank Mr. Jong-Ho Chuan of Hualien District Agricultural Research and Extension Station, Council of Agricultural, Executive Yuan, Taiwan, for providing wild bitter gourd samples and Mr. Yong-Yu Liao of Da-Chun Company for assistance with freeze-drying of samples.

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