



The dipeptide H-Trp-Glu-OH (WE) shows agonistic activity to peroxisome proliferator-activated protein- α and reduces hepatic lipid accumulation in lipid-loaded H4IIE cells



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ABSTRACT

Dipeptides digested from dietary proteins can be directly absorbed by the intestine and delivered to the circulatory system. However, the dipeptides' metabolic roles and biological activities are largely unknown. Lipid-loaded H4IIE cells stimulated with H-Trp-Glu-OH (WE) exhibited reduced lipid accumulation, of which the effect was abolished by peroxisome proliferator-activated receptor (PPAR) α gene knock down. A luciferase assay showed that the WE dipeptide induced PPAR α transactivation in a dose-dependent manner. Surface plasmon resonance and time-resolved fluorescence resonance energy transfer analyses demonstrated that WE interacts directly with the PPAR α ligand binding domain (K_D , 120 μ M; EC_{50} , 83 μ M). Cells stimulated with WE induced PPAR α and its responsive genes and increased cellular fatty acid uptake. In conclusion, WE reduces hepatic lipid accumulation in lipid-loaded hepatocytes via the activation of PPAR α by a direct interaction.

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Dipeptides are one of the major products resulting from the digestion of dietary proteins, and these peptides have significant nutritional value. Because the uptake rate of dipeptides is known to be higher than that of free amino acids, it has been suggested that dipeptides may be efficiently utilized for both anabolic and catabolic metabolism or may have specific bioactivities. Pharmacokinetic studies demonstrated that dipeptide uptake is independent from that of free amino acid uptake in patients with genetically impaired transport of a specific amino acid.¹ The rapid absorption of dipeptides is mediated by peptide transporters in the small intestine and activated G-cells in the stomach.² The human peptide transporters 1 (hPepT1) and 2 (hPepT2) are known to take up di- and tripeptides specifically but not peptides with four or more amino acids^{2–4} in a proton-dependent manner.^{3,4} In addition, the tripeptide histidine transporter (PHT1),^{4,5} which is expressed in Caco-2 cells, has been proposed to absorb dipeptides in the

intestine.⁵ Although dipeptide uptake and delivery mechanisms have been elucidated, the biological functions of the dipeptides are largely unknown.

Due to the high bioavailability and effective biological activities of dipeptides, the number of biomedical applications of dipeptides increases each year. Evidence suggests that several dipeptides have hypotensive activity. For example, the tryptophan-containing dipeptides Ile-Trp and Lys-Trp, which are extracted from fish, seaweed, and mushrooms, reduce blood pressure.⁶ The digestion-resistant dipeptides Val-Tyr, Val-Trp, Ile-Tyr and Ile-Trp derived from sardines also show antihypertensive effects⁷ via the inhibition of the angiotensin I converting enzyme;^{6,7} thus the extracts and hydrolysates from sardine muscles, including bioactive dipeptides, were approved as health foods in Japan. Additionally, soy peptides have been intensively studied due to their hypolipidemic and anti-obesogenic activities.⁶ Several other dipeptides have also been suggested to have biological functions, including Lys-Glu (antitumor activity), Leu-Ile (neuroprotective activity), and Tyr-Gly (lymphocyte proliferation activity), which provide new insights into the investigation of biomedical applications for dipeptides.^{8,9}

Peroxisome proliferator-activated proteins (PPARs) are ligand-activated nuclear receptors that regulate the transcription of genes

Abbreviations: WE, H-Trp-Glu-OH; PPARs, peroxisome proliferator-activated proteins; LBD, ligand-binding domain; SPR, surface plasmon resonance; TR-FRET, time-resolved fluorescence resonance energy transfer; FATP4, fatty-acid transport protein 4; ACS, acyl-CoA synthetase; CPT1, carnitine palmitoyltransferase 1; ACOX, acyl-CoA oxidase.

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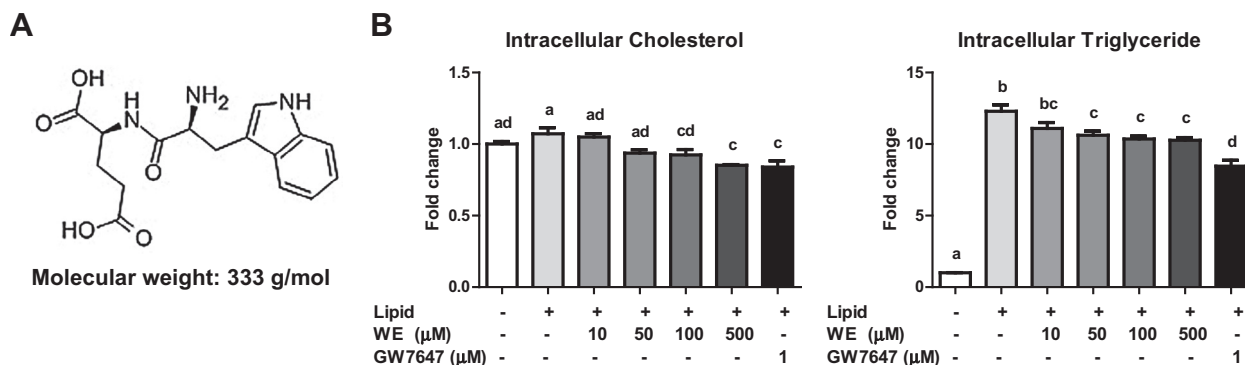


Figure 1. WE reduces the intracellular cholesterol and TG concentrations in lipid-loaded hepatocytes. (A) The chemical structure of WE. (B) The intracellular cholesterol and TG concentrations. Lipid-loaded H4IIE cells were stimulated with various concentrations of WE (10, 50, 100, and 500 μM) or GW7647 (1 μM) for 24 h. The intracellular cholesterol and TGs were extracted from the cells, and the concentrations of cholesterol and TGs were measured with enzymatic assays. The data are presented as the mean ± SEM, and significant differences were calculated using a one-way ANOVA test. Different letters indicate significant differences between the groups.

involved in lipid and glucose metabolism and cell development and differentiation.^{10,11} The three subtypes of PPAR, alpha (α), gamma (γ), and beta/delta (β/δ), share similar structures and functions in several organs.¹¹ The three-dimensional structures of the three subtypes of PPAR showed that they share a similar mechanism of activation; however, compared to PPARγ, PPARα contained a bulkier ligand-binding pocket.¹² Additionally, the natural

pigment astaxanthin has been reported as an agonist of PPARα and an antagonist of PPARγ.¹³

PPARα is mainly expressed in the liver and regulates hepatic lipid metabolism via the constitutive transcription of genes involved in fatty acid uptake, utilization, and peroxisomal and mitochondrial fatty acid β-oxidation.¹⁴ The activation of PPARα is primarily induced by synthetic ligands, including the fibrate drugs

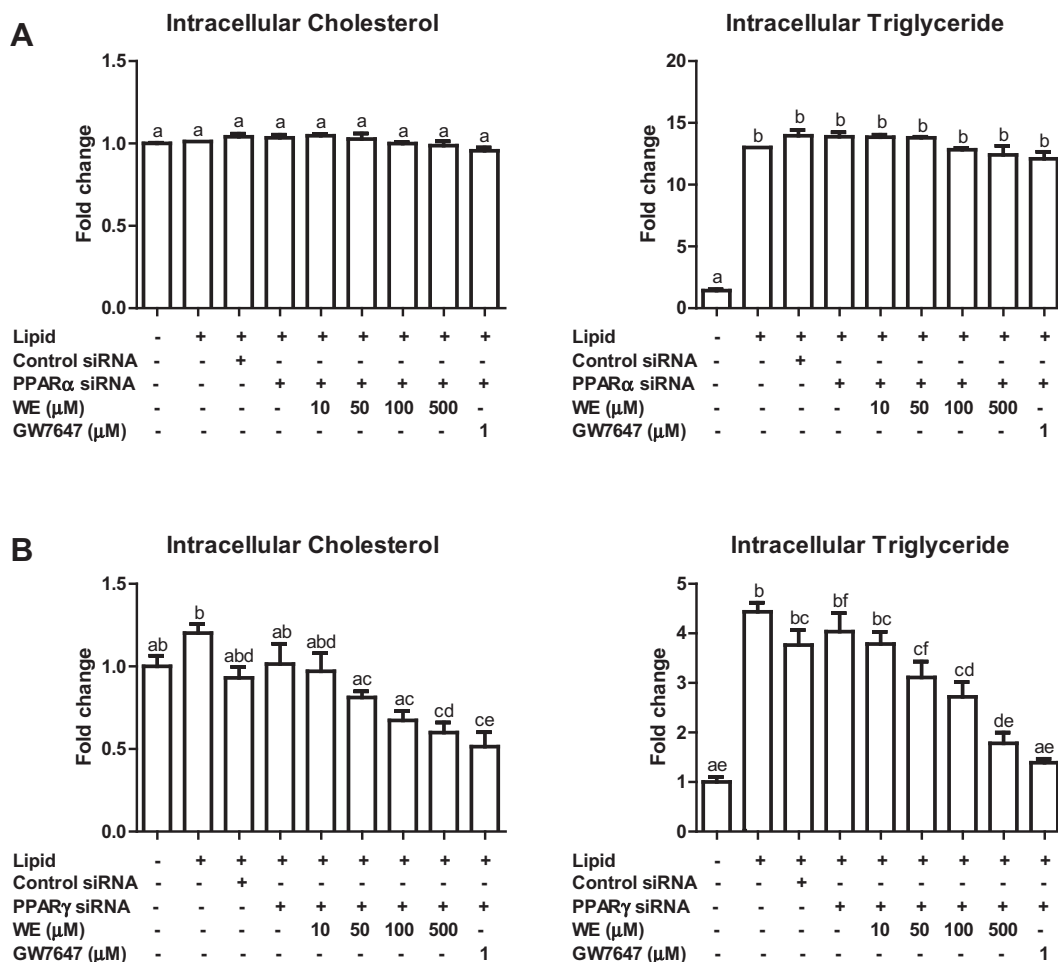


Figure 2. Hypolipidemic effects of WE dipeptide is PPARα-dependent but by PPARγ. (A) Intracellular cholesterol (A, left) and TG (A, right) concentrations of PPARα siRNA transfected H4IIE cells. (B) Intracellular cholesterol (B, left) and TG (B, right) concentrations of PPARγ siRNA transfected H4IIE cells. H4IIE rat hepatocytes were transfected with either PPARα or PPARγ siRNA then lipid loaded. Cells were then stimulated with WE (10, 50, 100, or 500 μM) or GW7647 (1 μM) for 24 h.¹⁸ The data are presented as the mean ± SEM, and significant differences were calculated using a one-way ANOVA test. Different letters indicate significant differences between the groups.

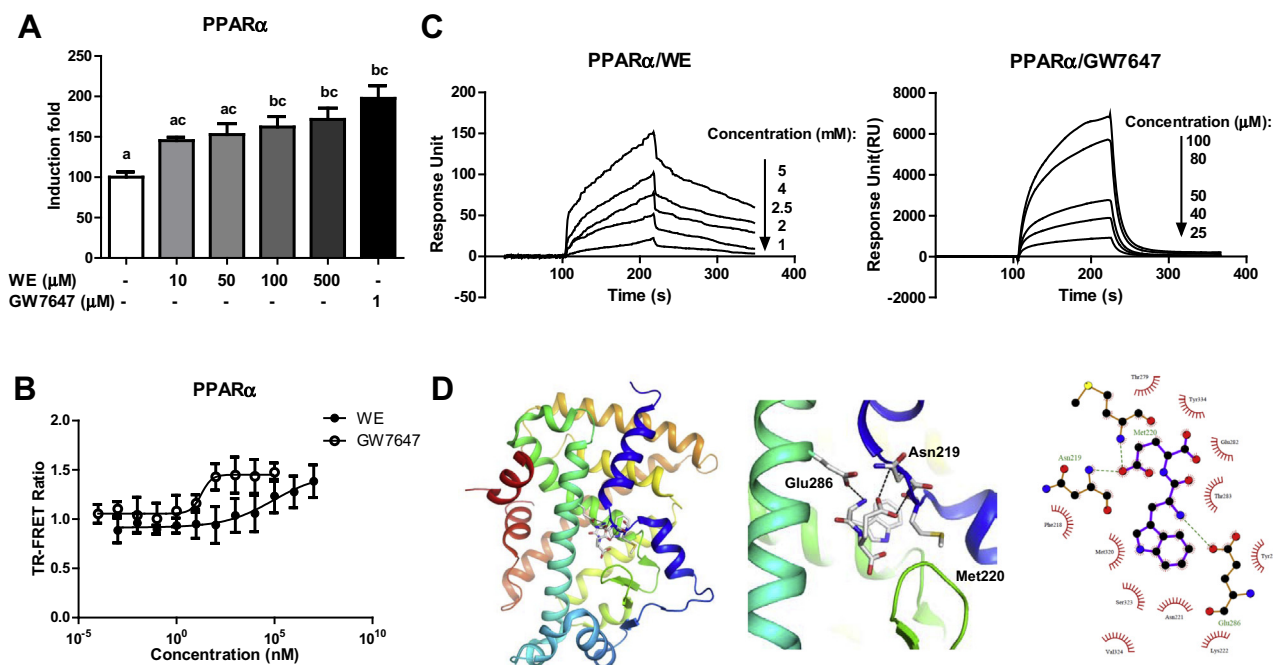


Figure 3. WE induces the transactivation of PPAR α and directly binds to and activates PPAR α . (A) Luciferase assay. The pSG5-PPAR α and pCMV-3xPPRE-Luc constructs were co-transfected into CHO-K1 cells, and the cells were treated with WE (10, 50, 100, or 500 μ M) or GW7647 (1 μ M) for 24 h. WE and GW7647 activate PPAR α transactivation. (B) WE (10^{-3} – 10^7 nM) or GW7647 (10^{-4} – 10^5 nM) were incubated with the PPAR α LBD protein and the co-activator (PGC1 α), and the treatment of WE and GW7647 induced the co-activator activity of the PPAR α LBD as measured by a TR-FRET co-activator assay. (C) WE (C, left) and GW7647 (C, right) directly bind the PPAR α LBD protein as measured by a SPR assay. (D) Molecular docking of WE to the PPAR α LBD. The data are presented as the mean \pm SEM, and significant differences were calculated using a one-way ANOVA test. Different letters indicate significant differences between the groups.

used to treat hyperlipidemia, and endogenous ligands, including fatty acids and fatty acid-derived compounds. The administration of PPAR α agonists ameliorates hepatic steatosis by enhancing mitochondrial fatty acid oxidation in mice.¹⁵ Conversely, PPAR α knockout mice have been shown to have a lower capacity for constitutive mitochondrial fatty acid oxidation compared with wild-type mice,¹⁶ leading to a higher susceptibility to hepatic steatosis when fed a high-fat diet.¹⁷ Therefore, the activation of PPAR α may be used to prevent and treat hepatic steatosis. In a previous activity screen, we found that the H-Trp-Glu-OH (WE, molecular weight = 334 g/mol, Figure 1A, Pepton, Deajeon, Korea) dipeptide reduced the intracellular cholesterol and triglyceride levels in lipid-accumulated hepatocytes; therefore, we further investigated the mechanism that regulates PPAR α .

WE reduces the cellular lipid concentration in lipid-loaded hepatocytes via activation of PPAR α : We examined the potential hypolipidemic activities of the WE dipeptide in vitro in lipid-loaded H4IIE cells stimulated with WE.^{18–20} The results showed that WE reduced the intracellular cholesterol significantly in a dose-dependent manner and that the levels decreased by 14% and 21% (at 100 and 500 μ M WE, respectively) compared with the unstimulated control (Fig. 1B). The intracellular triglyceride (TG) concentrations were significantly reduced by 14% and 16% in cells stimulated with 100 and 500 μ M WE compared with the unstimulated control (Fig. 1B). To investigate whether the hypolipidemic effects of WE is PPAR α specific, we knocked down the expressions of PPAR α using siRNA. Since WE was reported as a PPAR γ antagonist, PPAR γ knock-down experiment was performed as well. In cells transfected with by PPAR α siRNA, intracellular cholesterol and TG concentrations were not reduced with increasing concentrations of WE (Fig. 2A). However, intracellular cholesterol and TG concentrations were significantly by WE stimulation dose-dependent manner in cells transfected with PPAR γ siRNA (Fig. 2B). These demonstrated that hypolipidemic activity of WE in cultured hepatocytes is achieved by PPAR α activation and PPAR γ does not play a role.

WE is a PPAR α ligand: Hepatic steatosis is mainly caused by TG accumulation in the liver, which is induced by increased hepatic fatty acid synthesis and/or decreased β -oxidation.²¹ PPAR α regulates the expression of several genes involved in hepatic fatty acid metabolism, and we hypothesized that the dipeptide WE functions as a PPAR α agonist that reduces intracellular lipid concentrations in hepatocytes. In the reporter gene assay performed as described previously,^{22,23} WE stimulated the transactivation of PPAR α by 53% and 62% at 100 and 500 μ M, respectively, whereas the PPAR α agonist GW7647 (1 μ M) induced PPAR α transactivation by 98% (Fig. 3A). The agonistic activities of WE and PPAR α were examined with TR-FRET analyses using the PPAR α co-activator PGC1 α as described previously²³ (Fig. 3B). WE activated PPAR α with a half maximal effective concentration (EC₅₀) of 83.4 μ M (Table 1). Direct binding of WE to the PPAR α LBD protein was further tested by a SPR-Biacore assay as described previously²³ (Fig. 3C). WE directly associated with the PPAR α LBD protein, and the K_D of this interaction was 120 μ M; GW7647 also bound the PPAR α LBD protein (with a K_D value of 96.4 nM) (Table 1). The binding modeling assay of WE to PPAR α protein²⁴ showed that WE bound to PPAR α through hydrogen bonding via its hydroxyl and amino groups either Glu286, Asn219 or Met220 (Fig. 3D). These results indicate that WE directly binds to PPAR α and induces conformational changes to recruit a coactivator peptide, thus increasing PPAR α transactivation.

The PPAR subtypes share a similar structure, allowing several compounds to bind two or three subtypes of PPARs as dual- or

Table 1

The equilibrium dissociation constants (K_D values) measured by SPR and the half-maximal effective concentrations (EC₅₀ values) of WE and GW7647 to PPAR α as measured by a TR-FRET assay

	WE	GW7647
K_D value (SPR)	120 μ M	96.4 nM
EC ₅₀ value (TR-FRET assay)	83.4 μ M	18.6 nM

pan-agonists. On the other hand, due to the structural differences between the PPAR subtypes, some compounds have PPAR α agonistic and PPAR γ antagonistic effects simultaneously, including the natural compound astaxanthin.¹³ Our results showed that the reported PPAR γ antagonist WE²⁵ directly bound to PPAR α and moderately induced its activity. PPAR α activation induces hepatic peroxisomal proliferation and hepatomegaly;²⁶ thus activation of PPAR α by WE stimulation may regulate hepatic lipid metabolism and prevent or treat hepatic steatosis. Numerous natural and synthetic compounds have been identified as PPAR α agonists with EC₅₀ values in the range of several hundred nM to several μ M. However, these compounds are often difficult to administer orally and have low bioavailability. In contrast, dietary dipeptides are much more efficiently absorbed.⁴ The transport of dipeptides across epithelial membranes by specific dipeptide transporters has been described,^{2–4} and the plasma concentrations of Trp and Glu were reported as 50 and 30 μ M, respectively, in fasting humans.²⁷ Thus, although WE has a relatively high EC₅₀ (83.4 μ M), daily intake of this protein could have a beneficial effect via the activation of PPAR α . Furthermore, because di- and tripeptide transporters such as hPepT1 and hPepT2 specifically recognize molecules that have similar structures to di- and tripeptides,² PPAR ligands could be developed based on the structure of WE that may exhibit increased bioavailability. It has been reported that the WE dipeptide is a PPAR γ antagonist with an IC₅₀ of 8.67 μ M,²⁵ and activation of PPAR γ is reported to increase lipogenesis in organs via induces the transcriptional response genes involved in fatty acid synthesis, thus as an antagonist of PPAR γ , WE may reduce lipid concentrations via inhibits lipogenesis. However our results show that PPAR γ knockdown did not affect hypolipidemic activity of WE dipeptide. This indicates that antagonistic effect of PPAR γ does not play a role in the reduction of lipid accumulation in hepatocytes by WE. Three subtypes of PPARs show distinctive tissue distributions and PPAR α is a major subtype in hepatocytes. Although WE could bind to PPAR γ with higher affinity than PPAR α according to the published data,²⁵ hypolipidemic effects of WE on hepatocytes are operated by PPAR α due to low expression of PPAR γ .

WE regulates the transcription of PPAR α -responsive genes involved in hepatic lipid metabolism: Next, we investigated the expression of PPAR α -responsive genes upon stimulation with the WE dipeptide as described previously.^{28,29} Stimulation with 100 and 500 μ M WE induced PPAR α transcription by 1.8- and 1.9-fold, respectively, in hepatocytes (Fig. 4). Moreover, the expression of a PPAR α -responsive gene involved in fatty acid uptake, fatty-acid transport protein 4 (FATP4), was increased by 1.6- and 1.7-fold at 100 and 500 μ M WE, respectively, compared to the unstimulated control. The expression of acyl-CoA synthetase (ACS) was induced by 1.4- and 1.5-fold by 100 and 500 μ M WE treatment, respectively.

FATP4 is activated by PPAR α and is a key contributor to fatty acid transmembrane-related processes in the liver.³⁰ Knockout mutations in FATPs in mouse hepatocytes significantly decrease the rate of fatty acid uptake.^{31,32} When the fatty acids enter cells, they are rapidly activated by conversion to fatty acyl-CoAs via the activation of ACS by FATPs. Therefore, the activation of FATP4 and ACS suggests that the increased fatty acid uptake in WE-treated H4IIE cells may occur via the up-regulation of PPAR α -responsive genes.

Furthermore, the PPAR α -responsive genes that are associated with fatty acid β -oxidation were activated by WE stimulation in H4IIE cells. WE (500 μ M) increased the expression of carnitine palmitoyltransferase 1 (CPT1) and acyl-CoA oxidase (ACOX) by 1.6 and 1.4-fold, respectively (Fig. 3). CPT-1 transfers fatty acids into the mitochondrial matrix and is the major regulator of hepatic mitochondrial β -oxidation.^{21,33} Patients with hepatic steatosis exhibit significantly reduced CPT-1 expression.³⁴ ACOX is an important gene involved in peroxisomal β -oxidation, which catalyzes the conversion of acyl-CoA to trans-2,3-dehydroacyl-CoA in the liver, and mice lacking ACOX showed massive accumulation of very-long-chain fatty acids in hepatocytes and severe microvesicular steatosis.³⁵ Thus, the activation of CPT-1 and ACOX in hepatocytes by WE may improve hepatic steatosis via an increase mitochondrial β -oxidation.

WE increases the fatty acid uptake of H4IIE cells: PPAR α regulates the expression of genes involved in the influx of fatty acids from

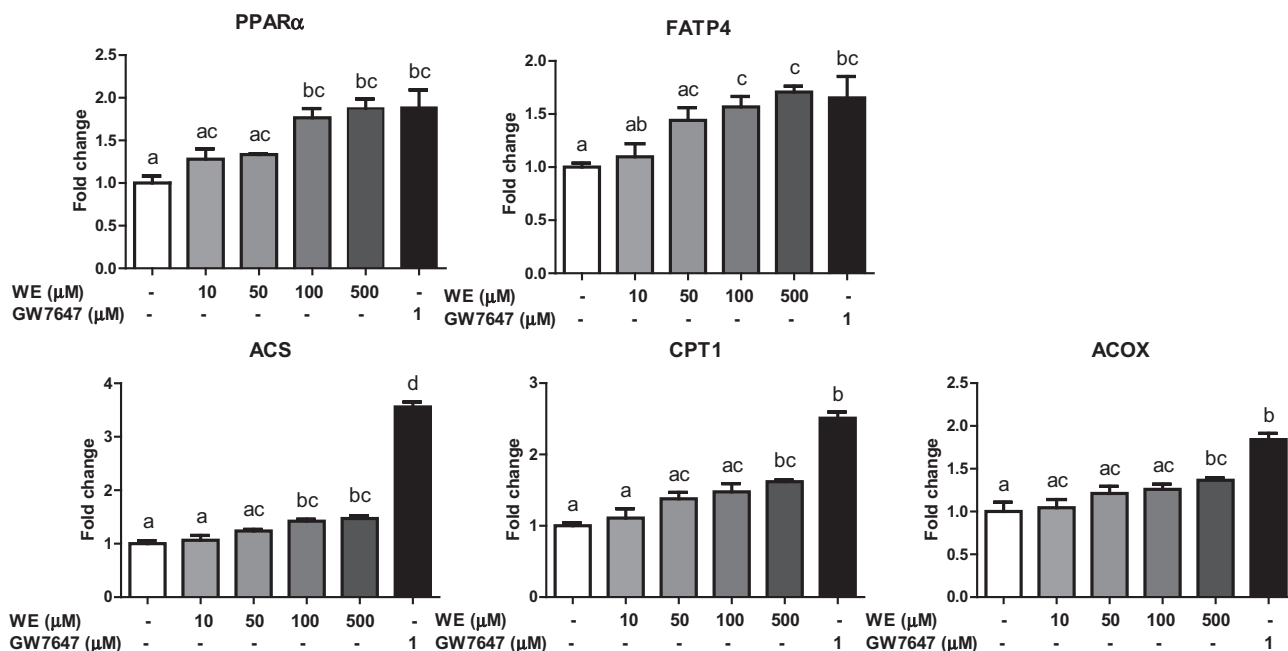


Figure 4. WE induces the transcription of PPAR α and the expression of PPAR α -responsive genes in hepatocytes. The H4IIE cells were stimulated by various concentrations of WE (10, 50, 100, or 500 μ M) or GW7647 (1 μ M) for 24 h, and the expression of PPAR α and PPAR α -responsive genes was investigated by qPCR. FATP4, fatty-acid transport protein 4; ACS, acyl-CoA synthetase; CPT1, carnitine palmitoyltransferase 1; ACOX, acyl-CoA oxidase. The data are presented as the mean \pm SEM, and significant differences were calculated using a one-way ANOVA test. Different letters indicate significant differences between the groups.

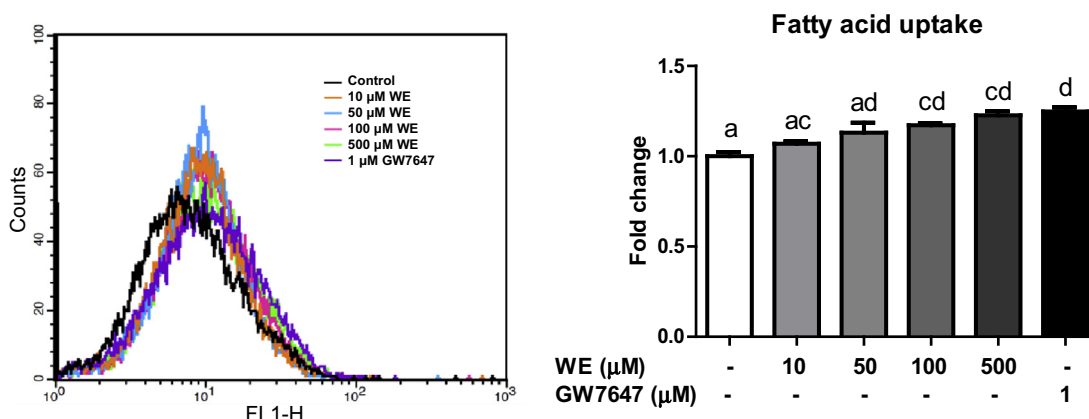


Figure 5. WE increases the fatty acid uptake of hepatocytes. The H4IIE cells were stimulated by various concentrations of WE (10, 50, 100, or 500 μM) or GW7647 (1 μM) for 24 h. The intracellular fluorescence-labeled fatty acids were measured to investigate the fatty acid uptake. The data are presented as the mean ± SEM, and significant differences were calculated using a one-way ANOVA test. Different letters indicate significant differences between the groups.

blood.³⁶ Therefore, we investigated the fatty acid uptake of H4IIE when the cells were treated with various concentrations of WE as described previously³⁷ using fluorescence-labeled fatty acids (C1-BODIPY 500/510 C1, C12; Invitrogen, Carlsbad, CA, USA) and flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA). The fatty acid uptake of WE-stimulated hepatocytes was measured by FACS. WE stimulation increased the fatty acid uptake of the H4IIE cells by 1.2-fold for 100 and 500 μM WE, as did GW7647 at 1 μM (Fig. 5). Our results suggest that the effect of WE on fatty acid uptake in hepatocytes occurs via direct binding and activation of WE to PPAR α .

Experiments in animal models and humans have demonstrated that soy peptides are bioactive and have hypolipidemic and anti-obesity activities that reduce body weight, total plasma cholesterol, and triacylglycerol concentrations.³⁸ However, the active peptide(s) have not been identified. The supplementation of black soy peptides in the diets of overweight/obese humans induced significant reductions in body weight, body mass index (BMI), body fat percentage and body fat mass as well as in leptin levels.³⁹ Here we suggest that WE may be a key hypolipidemic compound in soy-bean peptides.

In conclusion, we identified a direct interaction between the dipeptide WE and PPAR α that induced the agonistic activity of PPAR α . We also demonstrated that WE accumulation reduced the intracellular cholesterol and TG levels significantly in lipid-loaded H4IIE cells. The hypolipidemic effect of WE was achieved through the activation of PPAR α and PPAR α -responsive genes involved in hepatic fatty acid uptake and fatty acid β -oxidation; hepatic fatty acid uptake increased, as well. Dipeptides have greater uptake and absorption efficiencies than do synthesized and natural PPAR α ligands, and considering the association of hepatic steatosis with fatty acid uptake and utilization, the intake of food containing proteins that could be digested to form WE may prevent and improve hepatic steatosis. Additionally, the structure of WE could serve as a basis for the development of more effective PPAR α ligands.

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