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The Dipeptide H-Trp-Arg-OH (WR) Is a PPARα Agonist and Reduces Hepatic Lipid Accumulation in Lipid-Loaded H4IIE Cells

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Abstract Dipeptides absorbed by the intestinal epithelium are delivered to circulation, but their metabolic roles are not yet clearly understood. We investigated the biological activities of a dietary dipeptide, H-Trp-Arg-OH (WR), on the regulation of peroxisome proliferator-activated receptor (PPAR) α activity. Reporter gene assays revealed that WR dose-dependently induced PPARα transactivation. Surface plasmon resonance experiments demonstrated that WR interacts directly with the PPARα ligand binding domain, and time-resolved fluorescence energy transfer analyses revealed recruitment of a co-activator peptide, fluorescein-PGC1α, to PPARα, confirming the direct binding of WR to PPARα and occurrence of conformational changes. WR induced cellular fatty acid uptake and the expression of PPARα response genes in fatty acid oxidation, thus reducing intracellular triglyceride accumulation in lipid-loaded hepatocytes. In conclusion, the dietary dipeptide WR activates PPARα and reduces hepatic lipid accumulation in lipid-loaded hepatocytes.

Keywords Dipeptide · H-Trp-Arg-OH (WR) · Lipid metabolism · PPARα

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Introduction

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors belonging to the steroid/thyroid hormone receptor superfamily [1], and activation of PPARs by ligands regulates the transcriptions of response genes involved in the metabolism of lipid and glucose and in cell development and differentiation [2]. There are three subtypes of PPAR, alpha (α), γ, and beta/delta (β/δ), which have different tissue distribution and physiological functions, whereas the three subtypes share similar structures and functions [3]. As the first identified isoform, PPARα regulates the constitutive transcription of genes involved in fatty acid-metabolizing enzymes and genes involved in fatty acid oxidation occurring in mitochondria, peroxisomes, and microsomes in the liver [4]. The administration of PPARα agonists, such as fibrates, has been reported for the treatment of metabolic syndrome, especially those associated with cardiovascular complications, and ameliorates hepatic steatosis by enhancing mitochondrial fatty acid oxidation in rodent models [5, 6]. Conversely, knockout of PPARα results in massive lipid accumulation in the liver, severe hypoketonemia, hypoglycemia, hypothermia, and increased free fatty acid levels in plasma [7], in addition to inducing a lower capacity for constitutive mitochondrial fatty acid oxidation [8], which leads to a higher susceptibility to hepatic steatosis under dietary high-fat overload [9]. Furthermore, studies reveal that PPARα regulates lipid homeostasis by increasing fatty acid β-oxidation and providing energy to cells while cutting the long-chain fatty acids, thereby preventing lipid accumulation and toxicity in cells [10]. In addition, the activation of PPARα stimulates the cellular uptake of fatty acids by upregulating the response gene expression of fatty acid transport proteins (FATPs) [11]. However, the activation of PPARα with synthetic agonists, fibrates for instance, both increases hepatic fatty acid oxidation and also increases lipogenesis via the regulation of genes involved in this process, which has encouraged researchers to try to identify natural compounds in daily consumed food with moderate PPARα agonistic effects.

Dietary proteins are digested into amino acids and small peptides and are removed from the stomach and small intestine. Using 20 amino acid building blocks, 400 types of dipeptides are produced from dietary sources, including H-Trp-Arg-OH (WR). Tryptophan and arginine are essential and semiessential dietary amino acids, respectively [12]. Both tryptophan and arginine are abundant in a wide variety of foods, including dairy products, meat, seafood, and some plants [13, 14]. Therefore, the WR dipeptide can be produced from dietary proteins by the actions of digestive proteases and peptidases in the GI tract. The metabolic and biological roles of individual amino acids have been intensively investigated; however, the biological functions of di- or tripeptides are not largely unknown, although their production and uptake during digestion was demonstrated nearly three decades ago.

The intestinal uptake of di- and tripeptides is mediated by brush border membrane proteins, peptide transporters [15, 16]. Cloned transporters catalyze the active transport of intact di- and tripeptides and utilize a transmembrane electrochemical H^+ gradient as the driving force. The characteristic of H^+ coupling makes PEPT 1 and PEPT 2 unique among the transporters thus far identified in mammalian cells. In addition, the peptide transporters have immediate pharmacologic relevance because a number of peptide-like drugs are recognized as substrates by these transporters. Recently, in cultured cell lines of intestinal and renal origin that express PEPT 1 and PEPT 2 have been identified. These cell lines are likely to facilitate studies on the regulatory aspects of the peptide transporters.

The applications of dipeptides have attracted interest from academic and industrial researchers. Some exemplary applications of dipeptides are the development of potential drugs or active compounds in natural food supplements and functional foods. It has been demonstrated that some dipeptides, such as Val-Tyr, Ile-Tyr, Ile-Trp, and Lys-Trp, which

are extracted from fish meat, seaweed, or mushrooms, display antihypertensive activities. Lys-Glu has been demonstrated to possess antitumor activity, and Tyr-Gly enhanced proliferation of peripheral blood lymphocytes [17]. These activities indicate that dipeptides potentially have various biological activities. In addition, dipeptides are very appealing for drug discovery and development because of their cost-effectiveness, the possibility of oral administration, and the simplicity of molecular structural and quantitative structure-activity studies.

To investigate the potential biological activities of WR dipeptide, we examined the potential interaction of WR with PPARα and the related hypolipidemic activities in vitro.

Materials and Methods

Materials and Reagents

The dipeptide H-Trp-Arg-OH (WR, molecular weight is 360.19 g/mol; Fig. 1a) was synthesized by Peptron (Deajeon, Korea), and GW7647 was purchased from the Cayman Chemical Company (Ann Arbor, Michigan, USA). All cell culture reagents were purchased from Hyclone (Logan, UT, USA), and total RNA extraction reagents (RNAiso Plus) and the real-time polymerase chain reaction (PCR) premix (SYBR® Premix Ex Taq™) were purchased from Takara (Otsu, Japan), and the Oligo $(dT)_{15}$ primer was purchased from Promega (Madison, WI, USA).

Cell Culture and Lipid Content Measurement

H4IIE rat hepatocytes, obtained from the Korean Cell Line Bank (Seoul, Korea), were maintained in MEM/EBSS (Hyclone, Logan, UT, USA) containing 20 % heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1 % penicillin/streptomycin (PEST; Welgene Inc., Seoul, Korea), at 37 °C in a humidified atmosphere of 5 % CO_2. After culturing in 6-well culture plates for 24 h, the H4IIE cells were treated with free fatty acids (palmitic acid and oleic acid, 400 μM each) and 0.5 % bovine serum albumin (BSA; Bovogen Biologicals, Melbourne, Australia) for 24 h, and then the cells were treated with WR (50, 100, 500, or 1000 μM) or 1 μM GW7647 for a further 24 h (0.01 % DMSO as a vehicle). The H4IIE cells were washed twice with 1 ml of PBS, and then intracellular lipid was extracted as described previously [18]. The intracellular total cholesterol and triglyceride concentrations were quantified with a Cobas C111 automatic analyzer (Roche Diagnostic Systems Inc., Indianapolis, IN, USA) as described previously [19]. Cellular lipid concentrations were normalized with cellular protein concentrations, as measured with a BCA protein assay (Pierce Biotechnology, Rockford, USA).

Transfection and Luciferase Assay

CHO-K1 cells were cultured in DMEM-F/12 (Hyclone, Logan, UT, USA) medium in 24-well plates at a density of 2×10^5/well, and then pSG5-PPAR alpha (Addgene, MA, USA) and pCMV-3xPPRE-Luc were co-transfected using Hilymax (Dojindo, MD, USA), according to the manufacturer's instructions for 24 h. Cells were treated with WR (50, 100, 500, or 1000 μM) for 24 h, and the luciferase activity was quantified using a firefly luciferase assay kit (Biotium, Hayward, CA, USA) as described previously [20].

Purification of PPARα Ligand Binding Domain Proteins

The human PPARα ligand binding domain (LBD) (amino acid residues 280–468) was cloned into an expression vector pET-32a-c(+) (Novagen, Darmstadt, Germany), and then the recombinant plasmid was transformed into a *Escherichia coli* Rosetta (DE3) strain as described previously [21]. In brief, the PPARα LBD protein expression was induced by adding 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Bio Basic Inc. Ontario, Canada) at 18 °C for 16 h. The cells were harvested by centrifuging at 13,000 rpm and disrupted by sonication, and then the supernatant was collected for purification. The lysate of cells was loaded onto a HiTrap™ Chelating HP Column (GE Healthcare, Giles, Bucks HP8 4SP, UK), and the PPARα LBD protein was gradually eluted by a gradient elution program (from 8 to 70 % of elution buffer) using a Bio-Rad BioLogic LP system (Hercules, CA, USA). The purified PPARα LBD protein was concentrated and stored at −80 °C for further use.

Surface Plasmon Resonance

Binding analyses of PPARα LBD protein to WR or GW7647 were performed using a Biacore 2000 instrument (GE Healthcare, Uppsala, Sweden) as described previously [20]. The PPARα LBD protein was immobilized on a CM5 sensor chip (GE Healthcare, Uppsala, Sweden) via a standard primary amine coupling reaction as 8000–12,000 resonance units (RUs); WR (2.5–10 mM) or GW7647 (25–100 μM) were automatically injected into flow cells. WR or GW7647 bound the PPARα LBD protein, resulting in an increase in the surface plasmon resonance (SPR) signal (expressed as RUs) identified as the association ("on rate", ka) phase, and then only the running buffer (1× PBS) without sample was injected, which dissociated the bound complex consisting of the sample and PPARα LBD protein, thus resulting in a decrease in the SPR signal (RU) that was described as the dissociation ("off rate", kd) phase. The ka and kd were used to fit a 1:1 Langmuir binding-fitting model to calculate the equilibrium dissociation constants ("binding constant", K_Ds) of WR and GW7647 to PPARα LBD protein using the BIAevaluation software version 3.1 (GE Healthcare, Uppsala, Sweden).

Time-Resolved Fluorescence Resonance Energy Transfer Assays

The activation effect of WR and GW7647 for PPARα LBD protein was measured by a LanthaScreen™ time-resolved fluorescence resonance energy transfer (TR-FRET) co-activator assay (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Serial concentrations of WR (10^{-3}–10^{6} nM) or GW7647 (10^{-4}–10^{5} nM) were added to PPARα LBD protein with a fluorescein-PGC1α co-activator peptide (1 % DMSO as a vehicle). The solutions were then incubated at room temperature for 2 h. The 520/495 TR-FRET ratio was measured by a Spectra Max instrument with an excitation filter at 340 nm and an emission filter at 495 and 520 nm. Binding curves were generated by plotting the emission ratio vs. the log [ligand], and the EC_{50} values were calculated by fitting the equation for a sigmoidal dose response (varying slope) using the GraphPad™ Prism® 5.0.

Fatty Acid Uptake Assay

H4IIE cells were cultured in 12-well plates at a density of approximately 5×10^{5} cells/well for 24 h and treated with WR (50, 100, 500, and 1000 μM) or GW7647 (1 μM) for a further 24 h (0.01 % DMSO as a vehicle). The fatty acid uptake of the treated hepatocytes was measured by using a fluorescence-labeled fatty acid (C1-BODIPY 500/510 C1, C12; Invitrogen, Carlsbad,

CA, USA). The intracellular fatty acid of the H4IIE cells was assessed with flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA) and analyzed using a CellQuest Pro software (BD Biosciences, San Jose, CA, USA).

Quantitative Real-Time PCR

H4IIE cells were cultured in six-well plates at a density of 10^6/well for 24 h and treated with WR (50, 100, 500, and 1000 μM) or GW7647 (1 μM) for a further 24 h (1 % DMSO as a vehicle). Then, the total RNA was extracted as described before [22]. The cDNA synthesis was performed using M-MLV Reverse Transcriptase, oligo-dT, and dNTPs (Mbiotech, Korea). The gene expression, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference gene, was measured by qPCR reactions run on a Bio-Rad iQ5 iCycler system (Bio-Rad, Hercules, CA, USA) using RealMasterMix SYBR ROX reagent (5 PRIME, Hamburg, Germany).

Statistical Analysis

The data are presented as the mean+SEM. The significant differences between the experimental treated groups and control group were calculated by a one-way ANOVA. $P<0.05$ was considered statistically significant.

Results and Discussion

WR is an Agonistic Ligand for PPARα

Previous luciferase-based PPARα activity screening revealed that the peptide fraction from fermented brown rice extracts displays strong agonistic activity and reduced cellular triglyceride (TG) accumulation in lipid-loaded hepatocytes (data not showed). Based on the literature search, we performed a luciferase-based activity screen with several synthetic dipeptides and found that WR induces PPARα transactivation.

In the luciferase reporter gene assay, 1 mM WR (Fig. 1a) induced PPARα transactivation by 51 %, whereas the synthetic PPARα agonist GW7647 (1 μM) induced the transactivation of PPARα by 59 % (Fig. 1b) compared with the control. To investigate the direct reaction of WR to PPARα, we performed a SPR-Biacore assay and demonstrated that WR and the PPARα agonist GW7647 bound PPARα LBD protein directly (Fig. 1c). In addition, the K_D value of WR to PPARα calculated by the BIAevaluation system was 604 μM, whereas GW7647 associated with PPARα LBD protein has a K_D value of 32.4 nM (Table 1). After binding PPARα directly, WR induced a PPARα agonistic effect as measured by induction of the activity of PPARα co-activator PGC1α by serial concentrations of WR using TR-FRET analyses (Fig. 1d). The half-maximal effective concentration (EC_{50}) value of WR with PPARα was 437 μM, whereas with EC_{50} value of GW7647 with PPARα was 5.9 nM (Table 1). The activation of PPARα is primarily induced through ligand binding, and both synthetic ligands include the fibrate drugs used to treat hyperlipidemia, and endogenous ligands include fatty acids and fatty acid-derived compounds could induce the ligand activity of PPARα [3]. However, most synthetic PPARα ligands with potent activity, fenofibrate and bezafibrate for instance, were reported to have side effects such as myopathy, rhabdomyolysis, homocysteinemia, creatininemia, and lithogenicity [23], which allows us to search for alternative natural compounds with moderate agonistic activity but no side effects.

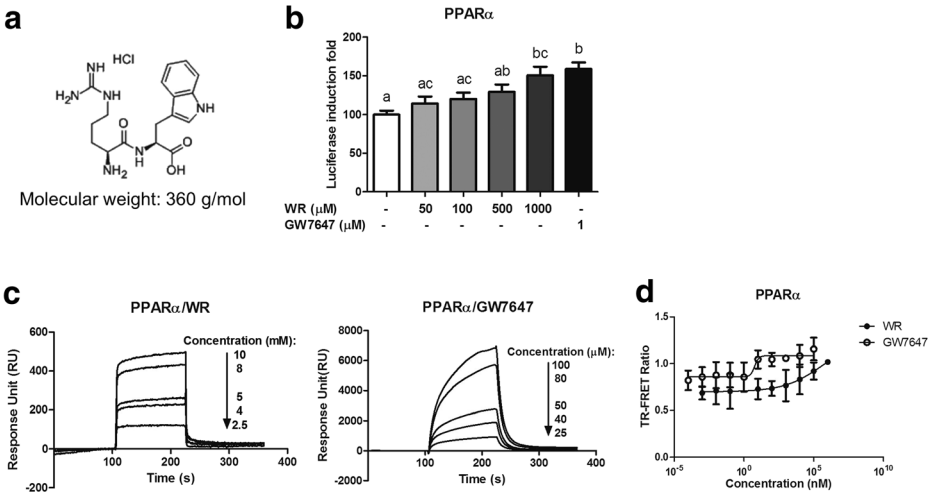

Fig. 1 WR induces the transactivation of PPARα and directly binds to PPARα to agonistically activate PPARα. **a** Chemical structure of WR. **b** Reporter gene assay. After co-transfection of pSG5-PPARα and pCMV-3xPPRE-Luc, the CHO-K1 cells were treated with serial concentration of WR (50, 100, 500, and 1000 μM) and GW7647 (1 μM) for 24 h. WR and GW7647 induces PPARα transactivation. **c** SPR analysis. The direct binding of WR (**c** *left*) and GW7647 (**c** *right*) to PPARα LBD protein was measured by SPR assay. **d** TR-FRET assay. WR and GW7647 induces co-activator activity (PGC1α) of PPARα LBD performed by TR-FRET co-activator assay. *WR* H-Trp-Arg-OH, *PPAR* peroxisome proliferator-activated receptors, *SPR* surface plasmon resonance, *TR-FRET* time-resolved fluorescence resonance energy transfer, *PGC1α* peroxisome proliferator-activated receptor gamma co-activator 1 alpha, *LBD* ligand binding domain. The data are presented as the mean±SEM, and a one-way ANOVA were used to calculate the significant differences. Significant differences between any two groups are identified by different *letters*

WR Reduces Cellular Lipid Content in Lipid-Loaded H4IIE Cells

The results suggested that WR was an agonist of PPARα, which plays a critical role in lipid metabolism in the uptake, transport, and oxidation of fatty acids [24, 25]. Thus, we next investigated the hypolipidemic activity of WR in lipid-loaded hepatocytes. The results demonstrated that the intracellular TG levels in H4IIE cells were reduced in cells stimulated with WR (1 mM) by 1.5-fold compared with controls. The PPARα agonist GW7647 reduced intracellular TG concentrations by 4.1-fold (Fig. 2b). Intracellular cholesterol concentrations were marginally affected in cells treated with WR (Fig. 2a). These results confirm that activation of PPARα by WR improves the lipid accumulation in hepatocytes, especially the reduction of TG levels in lipid-loaded H4IIE cells.

Table 1 The equilibrium dissociation constants (K_Ds) measured by SPR and half-maximal effective concentration (EC_{50}) of WR and GW7647 to PPARα measured by TR-FRET assay

PPARα	WR	GW7647
K_D (SPR)	604 μM	32.4 nM
EC_{50} (TR-FRET assay)	437 μM	5.9 nM

SPR surface plasmon resonance, *WR* H-Trp-Arg-OH, *PPAR* peroxisome proliferator-activated receptors, *TR-FRET* time-resolved fluorescence resonance energy transfer

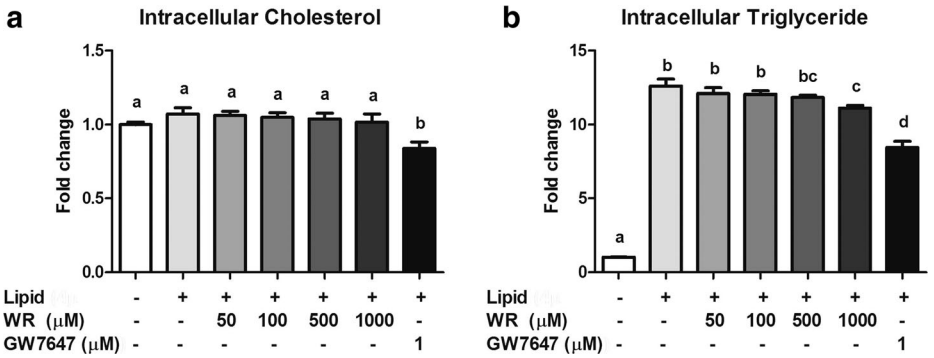

Fig. 2 WR reduces the intracellular TG levels in lipid-stimulated hepatocytes. The hyperlipidemia of H4IIE cells were induced by lipid-loading, and the cells were stimulated by serial concentrations of WR (50, 100, 500, and 1000 μM) and GW7647 (1 μM) for 24 h. The intracellular cholesterol (**a**) and TG (**b**) were measured by a Cobas C111 automatic analyzer. *WR* H-Trp-Arg-OH, *TG* triglyceride. The data are presented as the mean±SEM, and a one-way ANOVA were used to calculate the significant differences. Significant differences between any two groups are identified by different letters

WR Stimulates Accelerated Fatty Acid Uptake of H4IIE Cells

PPARα is reported to reduce plasma TG levels by inducing fatty acid uptake and subsequent beta-oxidation. Thus, we investigated cellular fatty acid uptake in H4IIE cells stimulated with WR with FACS analysis using a fluorescence-labeled fatty acid described in the method. The cellular fatty acid uptake was increased by 1.2-fold in cells stimulated with WR compared with controls (Fig. 3). The WR activity was weaker than the synthetic agonist GW7647, but WR produced a dose-dependent increase of cellular fatty acid uptake. This demonstrated that the activation of PPARα induced cellular fatty acid uptake for oxidation and thus may reduce lipid accumulation in lipid-loaded hepatocytes.

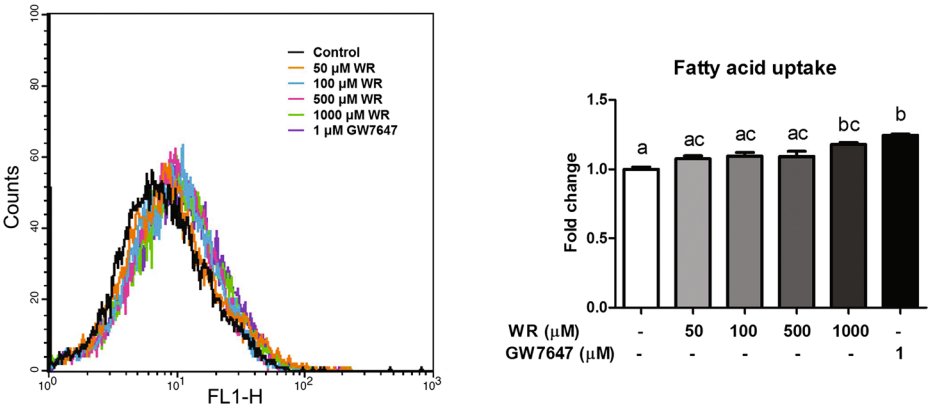

Fig. 3 WR stimulation increases the fatty acid uptake of H4IIE cells. After being treated with serial concentrations of WR (50, 100, 500, and 1000 μM) and GW7647 (1 μM) for 24 h, the fatty acid uptake of H4IIE cells was performed by measuring the intracellular fluorescence-labeled fatty acid level. *WR* H-Trp-Arg-OH. The data are presented as the mean±SEM, and a one-way ANOVA were used to calculate the significant differences. Significant differences between any two groups are identified by different *letters*

WR Regulates the Gene Expression of PPARα and Its Responsive Genes in Hepatic Lipid Metabolism

PPARα is the key transcription nuclear receptor in hepatic lipid metabolism that regulates the expression of several genes involved in fatty acid uptake and oxidation of fatty acids [25, 26]. WR stimulation induced PPARα gene expression by 1.5- (500 μM) and 1.7-fold (1000 μM), whereas the PPARα agonist GW7647 (1 μM) activated gene expression by 1.8-fold in H4IIE cells (Fig. 4). Moreover, the PPARα-responsive genes involved in fatty acid uptake, such as fatty acid transport protein 4 (FATP4) and acyl-CoA synthetase (ACS), were upregulated (Fig. 4). The gene expression of FATP4 and ACS were increased by 1.5- and 1.8-fold at 1000 μM, respectively, whereas the agonist GW7647 (1 μM) increased FATP and ACS expression by 1.5- and 4.1-fold, respectively. FATPs and ACS facilitate fatty acid transport through the cell membrane and their esterification, respectively, and thus prevents fatty acid influx [27]. In addition, the agonists of PPARα, such as fibrate stimulation, have been reported to induce mRNA expression of FATPs and ACS in rodent livers [28], which may explain the mechanism underlying WR's increase of fatty acid uptake in hepatocytes.

PPARα mediates genes associated with fatty acid β-oxidation as well, and the qPCR results demonstrated that WR stimulation in the H4IIE cells upregulated gene expression involved in hepatic β-oxidation as well (Fig. 4). WR (1000 μM) activated carnitine palmitoyltransferase 1 (CPT1) by 1.7-fold and GW7647 (1 μM) induced gene expression of CPT1 by 2.1-fold. CPT1 is the key regulator of the mitochondrial gateway for fatty acid uptake of mitochondrial matrix and thus regulates hepatic mitochondrial β-oxidation [1, 29]. Therefore, upregulation of CPT1 by WR stimulation may improve hepatic lipid metabolism via direct activation of PPARα. Furthermore, another PPARα target gene, acyl-CoA oxidase, (ACOX) which is involved in peroxisomal β-oxidation by catalyzing acyl-CoA to trans-2,3-dehydroacyl-CoA in the liver

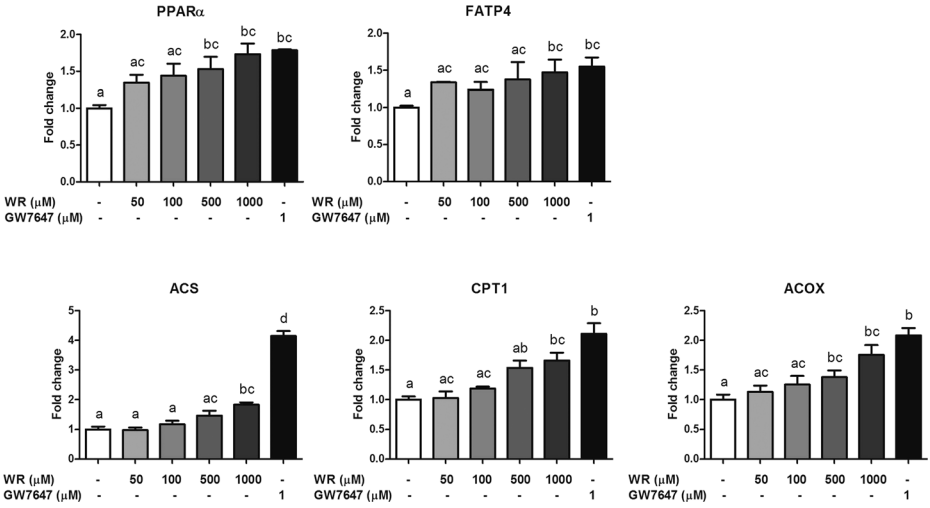

Fig. 4 WR induces the gene expression of PPARα and its target genes in hepatocytes. After being treated with a serial concentration of WR (50, 100, 500, and 1000 μM) and GW7647 (1 μM) for 24 h, the gene expression of PPARα and its responsive genes were measured by qPCR. *WR* H-Trp-Arg-OH, *PPAR* peroxisome proliferator-activated receptors, *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±SEM, and a one-way ANOVA was used to calculate the significant differences. Significant differences between any two groups are identified by different *letters*

[30], was activated by WR stimulation by 1.4- and 1.8-fold for 500 and 1000 μM, respectively, whereas GW7647 (1 μM) induced ACOX expression by 2.1-fold. The gene expression results suggest that WR stimulation upregulated the PPARα response genes involved in fatty acid uptake and β-oxidation, which thus improved the hepatic lipid metabolism in H4IIE cells.

In conclusion, our research suggests that the dipeptide WR bound with and induced the activation of PPARα directly and reduced the intracellular TG level in lipid-stimulated hepatocytes. The hypolipidemic effects of WR were achieved by increased hepatic fatty acid uptake and upregulated expression of genes involved in hepatic fatty acid uptake and β-oxidation. Therefore, the intake of food-containing proteins, including dipeptides such as WR, may have beneficial effects for human lipid metabolism.

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