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Curcuminoids and Sesquiterpenoids in Turmeric (*Curcuma longa* L.) Suppress an Increase in Blood Glucose Level in Type 2 Diabetic KK-A^y Mice

TOZO NISHIYAMA,[||] TATSUMASA MAE,*[,||] HIDEYUKI KISHIDA,[†] MISUZU TSUKAGAWA,[||] YOSHIHIRO MIMAKI,[‡] MINPEI KURODA,[‡] YUTAKA SASHIDA,[‡] KAZUMA TAKAHASHI,[§] TERUO KAWADA,[#] KAKU NAKAGAWA,[||] AND MIKIO KITAHARA[||]

Functional Foods Development Division, and Life Science Research Laboratories, Life Science RD Center, Kaneka Corporation, Takasago, Hyogo 676-8688, Japan, Laboratory of Medicinal Plant Science, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Hachioji 192-0392, Japan, Department of Molecular Metabolism and Diabetes, Department of Internal Medicine, Tohoku University School of Medicine, Sendai 980-8574, Japan, and Laboratory of Molecular Function of Food, Division of Food Science and Biotechnology, Graduate School of Agriculture (Uji Campus), Kyoto University, Kyoto 611-0011, Japan

Turmeric, the rhizome of *Curcuma longa* L., has a wide range of effects on human health. The chemistry includes curcuminoids and sesquiterpenoids as components, which are known to have antioxidative, anticarcinogenic, and antiinflammatory activities. In this study, we investigated the effects of three turmeric extracts on blood glucose levels in type 2 diabetic KK-A^y mice (6 weeks old, $n =$ 5/group). These turmeric extracts were obtained by ethanol extraction (E-ext) to yield both curcuminoids and sesquiterpenoids, hexane extraction (H-ext) to yield sesquiterpenoids, and ethanol extraction from hexane−extraction residue (HE-ext) to yield curcuminoids. The control group was fed a basal diet, while the other groups were fed a diet containing 0.1 or 0.5 g of H-ext or HE-ext/100 g of diet or 0.2 or 1.0 g of E-ext/100 g of diet for 4 weeks. Although blood glucose levels in the control group significantly increased ($P < 0.01$) after 4 weeks, feeding of 0.2 or 1.0 g of E-ext, 0.5 g of H-ext, and 0.5 g of HE-ext/100 g of diet suppressed the significant increase in blood glucose levels. Furthermore, E-ext stimulated human adipocyte differentiation, and these turmeric extracts had human peroxisome proliferator-activated receptor-γ (PPAR-γ) ligand-binding activity in a GAL4-PPAR-γ chimera assay. Also, curcumin, demethoxycurcumin, bisdemethoxycurcumin, and ar-turmerone had PPAR-γ ligand-binding activity. These results indicate that both curcuminoids and sesquiterpenoids in turmeric exhibit hypoglycemic effects via PPAR-γ activation as one of the mechanisms, and suggest that E-ext including curcuminoids and sesquiterpenoids has the additive or synergistic effects of both components.

KEYWORDS: ***Curcuma longa* L.; turmeric extract; curcuminoid; sesquiterpenoid; type 2 diabetes; KK-A^y mice; PPAR-γ ligand**

INTRODUCTION

Turmeric is the rhizome of *Curcuma longa* L., and is widely used as a spice, coloring, flavoring, and traditional medicine. Turmeric and its extract have various beneficial effects on human health (*1*). Curcuminoids, such as curcumin, demethoxycurcumin, and bisdemethoxycurcumin, are yellowish turmeric pigments, and have antioxidative (*2*, *3*), anticarcinogenic (*4*, *5*), antiinflammatory (*6*), antihepatotoxic (*7*), and hypocholesterolemic (*8*) activities. Sesquiterpenoids, such as ar-turmerone, α-turmerone, β-turmerone, and curlone, are components of turmeric essential oil, and have antioxidative (*9*) and mosquitocidal (*10*) activities and inhibit the production of prostaglandin E_2 and NO (*11*, *12*).

Type 2 diabetes, obesity/abdominal obesity, hypertension, and dyslipidemia are closely linked to insulin resistance; clustering of these risk factors in the same individual has been called the metabolic syndrome, which is a major public health problem (*13*, *14*). A crucial role in the development of the metabolic

* To whom correspondence should be addressed. Tel: +81-6-6226-5402; fax: +81-6-6226-5059; e-mail: Tatsumasa.Mae@kn.kaneka.co.jp.
[||] Functional Foods Development Division, Kaneka Corporation.
[†] Life Science Research Laboratories, Kaneka Corporation.
[‡] Tokyo University of Pharmacy and Life Science.
[§] Tohoku University School of Medicine.
[#] Kyoto University.

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syndrome is played by adipocytes, which are highly specialized cells involved in energy regulation and homeostasis. Adipocyte differentiation is a tightly controlled process in which determinant genes such as those of peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer binding protein-α lead to programmed adipocyte differentiation (*15*, *16*).

PPARs belong to the superfamily of nuclear receptors (*17*). Among PPARs, PPAR-γ is the predominant molecular target for insulin-sensitizing thiazolidinedione drugs such as troglitazone, pioglitazone, and rosiglitazone, which have been approved for use in type 2 diabetic patients (*18*, *19*).

In the present study, we investigated the effects of three turmeric extracts on blood glucose levels in type 2 diabetic KK-A^y mice. Furthermore, to ascertain their mechanism of action, we tested these extracts in adipocyte differentiation and GAL4-PPAR-γ chimera assays.

MATERIALS AND METHODS

Reagents. Curcumin tech., a reagent of Sigma Chemical Co. (St. Louis, MO), including curcumin, demethoxycurcumin, and bisdemethoxycurcumin was used as a standard of curcuminoids mixture. Other chemicals, solvents, and reagents were commercially available from Nacalai Tesque (Kyoto, Japan), Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Invitrogen Corp. (Carlsbad, CA).

Preparation of Turmeric Extracts. Turmeric ethanolic extract (E-ext) and hexanic extract (H-ext) were obtained from finely powdered turmeric (*Curcuma longa* L., harvested in India) by extraction twice with five volumes of ethanol and hexane, respectively, and filtration and evaporation of the solvent. An ethanolic extract from the residue of hexane extraction (HE-ext) was also obtained using the same procedure.

Purification of Curcuminoids and Ar-turmerone. E-ext was applied to porous ion-exchange resin DIAION HP-20 column chromatography, and eluted sequentially with 30, 50, and 80% (v/v) methanol/water, methanol, ethanol, and ethyl acetate. From the methanol fraction, curcumin, demethoxycurcumin, and bisdemethoxycurcumin were purified by repeating silica gel chromatography with elution with hexane/acetone (2:1, 3:2, and 4:3, v/v) and chloroform/acetone (99:1 and 19:1, v/v). An essential oil fraction was obtained from E-ext by silica gel chromatography with elution with ethyl acetate/hexane (1:9, v/v), and then ar-turmerone was purified by repeating silica gel chromatography with elution with acetonitrile/water (65:35, v/v). Their structures were identified by comparison of NMR spectral data with published values (*20*−*22*).

TLC and HPLC Analysis. The TLC plate used was a Silica gel 60 F_{254} (MERCK, Darmstadt, Germany) and was developed with a solvent system of chloroform/methanol (9:1, v/v). Spots were detected by spraying with sulfuric acid/ethanol (5:95, v/v) and heating at 350 °C for a few minutes.

HPLC analysis was performed using a Wakosil-II 5C18HG column, 4.6 × 250 mm (Wako Pure Chemical Industries, Ltd.) at 30 °C. The mobile phase was a gradient of acetonitrile (solvent A) and 2 mL/L phosphoric acid/water (solvent B) at a flow rate of 0.7 mL/min with 50% A for 0−10 min, linear increase from 50 to 80% A for 10−15 min, 80% A for 15−35 min, and 50% A for 35−50 min. Peaks were detected at wavelengths of 426 nm for curcuminoids and 240 nm for sesquiterpenoids. The injection volume of a sample was 20 μL in ethanol solution.

Animal Experiment. Female genetically diabetic KK-A^y/Ta mice (Clea Japan, Inc., Tokyo, Japan) were housed in an animal laboratory with a controlled environment at 20−24 °C temperature, 45−65% humidity, and a 12-h (7:30−19:30) light/dark cycle. The mice were at 6 weeks of age divided into seven groups of five mice each by body weight and blood glucose level. The control group was fed a basal diet, and treatment groups were fed a diet containing E-ext (0.2 or 1.0 g/100 g of diet), H-ext (0.1 or 0.5 g/100 g of diet) or HE-ext (0.1 or 0.5 g/100 g of diet). The basal diet (Oriental Yeast, Co., Ltd., Tokyo, Japan) consisted of 200 g/kg casein, 499.48 g/kg corn starch, 100 g/kg sucrose, 100 g/kg soybean oil, 50 g/kg cellulose powder, 35 g/kg AIN-93 mineral mixture (*23*), 10 g/kg AIN-93 vitamin mixture (*23*), 2.5 g/kg choline bitartrate, 0.02 g/kg *tert*-butylhydroquinone, and 3 g/kg L-cystine. Its energy ratio was fat 22%, carbohydrate 58.5%, and protein 19.5%, and total energy was 17 MJ/kg diet. Mice consumed these diets and water ad libitum for 4 weeks. Blood samples were obtained from the tail vein at a fixed time (8:30−9:00), and blood glucose levels were determined using a blood glucose level monitor Glutest Ace (Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan) before and after 4-week feeding.

Human Adipocyte Differentiation. Effects on human adipocyte differentiation were examined at Zen-Bio, Inc. (Research Triangle Park, NC). The test measured the accumulation of lipid droplets in the cells, which occurred during the differentiation from preadipocytes to adipocytes. Human preadipocytes (Zen-Bio, Inc.) were isolated from subcutaneous adipose tissue from elective surgery in healthy donors following the ethical approval. The primary human subcutaneous preadipocytes were plated at 1.35×10^4 cells/well on 96-well plates, and cultured with DMEM/Ham's F-12 nutrient medium (1:1, v/v) containing 15 mmol/L HEPES buffer, 30 mL/L fetal bovine serum (FBS), 33 μmol/L biotin, 17 μmol/L pantothenate, 100 nmol/L human insulin, 0.25 mmol/L 3-isobutyl-1-methylxanthine, 1 μmol/L dexamethasone alone or supplemented with a sample for 3 days. The cells received three additional feedings with the above medium not containing 3-isobutyl-1-methylxanthine, alone or supplemented with a sample for 11 days. Medium alone was used as a control, and rosiglitazone maleate at 1 μmol/L as a positive control. The cells were washed with PBS and lysed. Accumulated triglyceride was then measured as glycerol using Infinity reagent (Sigma Chemical Co.), which contained a microbial lipase. The degree of adipocyte differentiation was determined as the amount of glycerol liberated from accumulated triglyceride.

PPAR-γ Ligand-Binding Assay. PPAR-γ ligand-binding activity was assayed by the method described in our previous report (*24*). Briefly, a plasmid expressing a fusion protein of GAL4 DNA-binding domain and human PPAR-γ ligand-binding domain and a reporter plasmid including the luciferase gene were transfected into CV-1 cells cultured in 96-well plates. The medium was changed to DMEM-supplemented 100 mL/L charcoal-treated FBS and sample, and the cells were cultured for 24 h. Sample dissolved in dimethyl sulfoxide (DMSO) was added to the medium at a final concentration of 1 mL/L of DMSO. The cells were washed with PBS containing Ca^{2+} and Mg^{2+}, and luciferase activities were measured. PPAR-γ ligand-binding activity of a sample was calculated as the ratio of luciferase activity of a sample to that of the solvent control.

Statistical Analysis. Data were analyzed using the SAS/STAT software computerized statistical analysis program (SAS Institute, Carey, NC). When significant differences were detected by one-way ANOVA, Turkey's multiple comparison test was applied. Differences were considered significant at $P < 0.05$. Values in the text are mean ± SEM.

RESULTS

Components in Turmeric Extracts. Yields of E-ext, H-ext, and HE-ext from turmeric were 12.2, 7.2, and 6.3 g/100 g of turmeric, respectively. Some spots were detected by TLC analysis of these extracts. Curcumin ($R_f = 0.60$), demethoxycurcumin (0.48), bisdemethoxycurcumin (0.39), and ar-turmerone (0.79) were used as standards. H-ext yielded one major spot with the same characteristics as that of ar-turmerone. HE-ext yielded three major spots corresponding to curcumin, demethoxycurcumin, and bisdemethoxycurcumin. E-ext yielded four major spots corresponding to ar-turmerone, curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Moreover, concentrations of curcuminoids and sesquiterpenoids in these extracts were measured by HPLC analysis. Concentrations of curcumin (retention time = 15.9 min), demethoxycurcumin (14.4 min), and bisdemethoxycurcumin (13.0 min) were 12.4, 3.2, and 2.6 g/100 g in E-ext, respectively, and 28.2, 6.0, and 3.9 g/100 g in HE-ext, respectively (**Table 1**). However

Table 1. Concentrations (g/100 g of extract) of Curcuminoids and Sesquiterpenoids in Turmeric Extracts[a]

	E-ext	H-ext	HE-ext
curcuminoids			
curcumin	12.4	trace	28.2
demethoxycurcumin	3.2	ND[b]	6.0
bisdemethoxycurcumin	2.6	ND[b]	3.9
subtotal	18.3	NA[b]	38.1
sesquiterpenoids			
ar-turmerone	6.7	10.8	1.4
others[c]	29.5	46.6	2.3
subtotal	36.3	57.4	3.7

[a] Concentration was determined by HPLC analysis. [b] ND, not detected. NA, not available. [c] Concentration of other sesquiterpenoids such as α-turmerone, β-turmerone, and curlone was expediently calculated using ar-turmerone as a standard compound.

Table 2. Calculated Intakes (mg/kg of body weight/day) of Turmeric Extracts and Their Components, Curcumin and Ar-turmerone, in KK-A^y Mice Fed E-ext, H-ext or HE-ext during 4 Weeks of Feeding[a]

	turmeric extract	curcumin	ar-turmerone
E-ext			
0.2 g/100 g of diet	259	32	17
1.0 g/100 g of diet	1495	185	100
H-ext			
0.1 g/100 g of diet	138	NA[b]	15
0.5 g/100 g of diet	620	NA[b]	67
HE-ext			
0.1 g/100 g of diet	137	39	2
0.5 g/100 g of diet	626	177	9

[a] Intake of turmeric extract was calculated from total food intake and mean body weight of the mice. Intakes of curcumin and ar-turmerone were calculated from the intake of turmeric extract and the concentration data shown in Table 1. [b] NA, not available.

curcuminoids were hardly detected in H-ext. Ar-turmerone was found in a concentration of 6.7 g/100 g in E-ext, 10.8 g/100 g in H-ext, and 1.4 g/100 g in HE-ext (**Table 1**). Other sesquiterpenoids such as α-turmerone, β-turmerone, and curlone were detected in these extracts, but these concentrations were expediently calculated using ar-turmerone as a standard since none of these compounds were commercially available. Thus, TLC and HPLC analysis showed that major components in turmeric extracts were sesquiterpenoids for H-ext, curcuminoids for HE-ext, while E-ext contained a mixture of all of these compounds.

Animal Experiment. Body weight gain of KK-A^y mice during 4 weeks of feeding in the control group was 15.6 ± 0.73 g/4 weeks, and those in the treatment groups were 15.4 ± 0.92 and 12.8 ± 1.15 g/4 weeks at 0.2 and 1.0 g of E-ext/100 g of diet, 17.8 ± 0.55 and 16.0 ± 0.79 g/4 weeks at 0.1 and 0.5 g of H-ext/100 g of diet, and 16.2 ± 1.71 and 17.8 ± 0.75 g/4 weeks at 0.1 and 0.5 g of HE-ext/100 g of diet; thus, the body weight gains in the groups fed diets containing turmeric extracts were similar to that in the control group. Intakes of turmeric extracts in the treatment groups were calculated from total food intake and mean body weight of the mice. The doses were 259 and 1495 mg/kg of body weight/day at 0.2 and 1.0 g of E-ext/100 g of diet, 138 and 620 mg/kg of body weight/day at 0.1 and 0.5 g of H-ext/100 g of diet, and 137 and 626 mg/kg of body weight/day at 0.1 and 0.5 g of HE-ext/100 g of diet (**Table 2**). On the basis of these intakes of turmeric extracts and the concentration data of components (shown in **Table 1**), intakes of curcumin and ar-turmerone in the treatment groups were calculated. The intakes of curcumin in the mice fed HE-ext at 0.1 and 0.5 g/100 g of diet were 39 and 177 mg/kg of body weight/day, and those of ar-turmerone in the mice fed H-ext at 0.1 and 0.5 g/100 g of diet were 15 and 67 mg/kg of body weight/day (**Table 2**). It was indicated that the intakes of curcuminoids in the HE-ext groups or sesquiterpenoids in the H-ext groups were almost similar to those of curcuminoids and sesquiterpenoids in the E-ext groups.

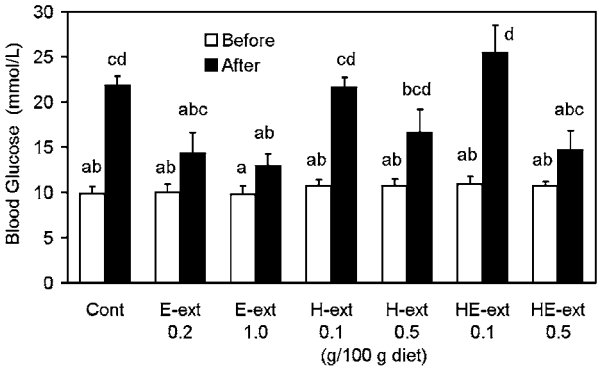

Figure 1. Blood glucose level in KK-A^y mice fed diet alone (Cont) or diet containing E-ext at 0.2 or 1.0 g/100 g of diet, H-ext at 0.1 or 0.5 g/100 g of diet, or HE-ext at 0.1 or 0.5 g/100 g of diet before and after 4 weeks of feeding. Values are means ± SEM, $n = 5$. Means without a common letter differ, $P < 0.05$.

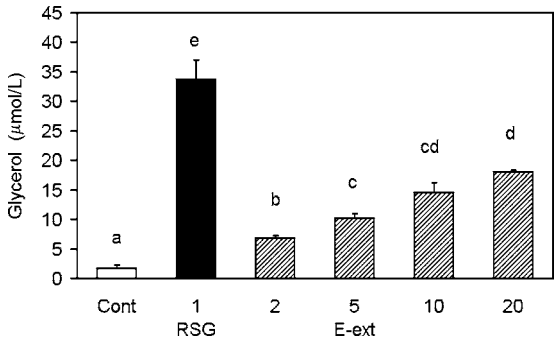

Figure 2. Effect of turmeric ethanolic extract (E-ext) at 2, 5, 10, and 20 mg/L on human adipocyte differentiation. Medium alone was used as a control (Cont), and rosiglitazone (RSG) at 1 μmol/L as a positive control. Accumulated triglyceride was determined as glycerol concentration. Values are means ± SD, $n = 3$. Means without a common letter differ, $P < 0.05$.

Compared with before feeding, blood glucose levels in the control group were significantly ($P < 0.01$) increased (**Figure 1**), indicating hyperglycemia after 4 weeks of feeding. However, blood glucose levels in the E-ext groups remained the same as before feeding. In the H-ext and HE-ext groups, blood glucose levels were significantly ($P < 0.01$) increased at the lower dose (0.1 g/100 g of diet), but remained unchanged at the higher dose (0.5 g/100 g of diet). Feeding of E-ext and the higher doses of H-ext and HE-ext thus suppressed the significant increase in blood glucose level in type 2 diabetic KK-A^y mice.

In Vitro Experiments. When human preadipocytes were cultured with E-ext, glycerol liberated from accumulated triglyceride increased ($P < 0.05$) in a dose-dependent manner (**Figure 2**), indicating that E-ext stimulated adipocyte differentiation. The potency of E-ext was weaker ($P < 0.05$) than that of rosiglitazone, a potent PPAR-γ agonist.

E-ext, H-ext, and HE-ext had human PPAR-γ ligand-binding activity in a GAL4-PPAR-γ chimera assay (**Figure 3A**). Among these extracts, the highest activity was observed for E-ext. Curcumin, demethoxycurcumin, bisdemethoxycurcumin, and ar-

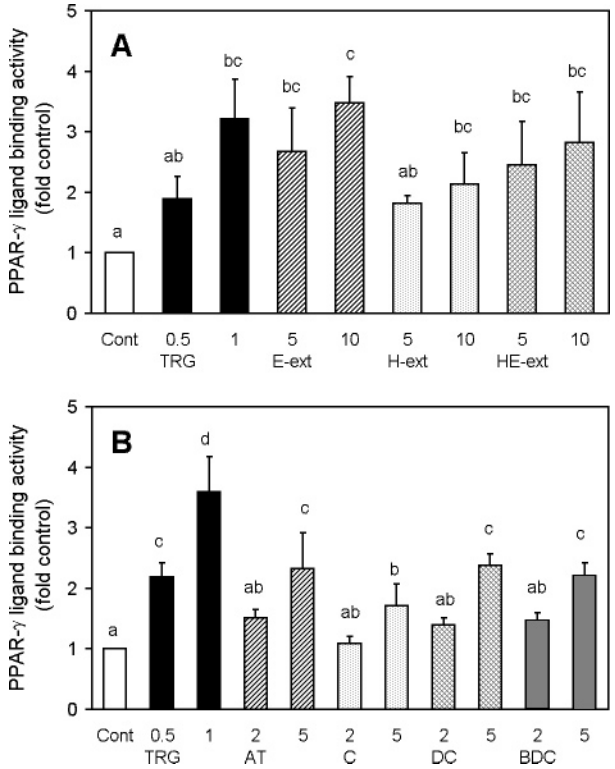

Figure 3. PPAR-γ ligand-binding activity of turmeric extracts at 5 and 10 mg/L (A) and compounds at 2 and 5 mg/L (B) in a GAL4-PPAR-γ chimera assay system: ethanol extract (E-ext), hexane extract (H-ext), ethanol extract from hexane–extraction residue (HE-ext), ar-turmerone (AT; 18.5 and 46.2 μmol/L), curcumin (C; 10.9 and 27.2 μmol/L), demethoxycurcumin (DC; 11.8 and 29.6 μmol/L) and bisdemethoxycurcumin (BDC; 13.0 and 32.5 μmol/L). DMSO at 1 mL/L was used as a solvent control (Cont), and troglitazone (TRG) at 0.5 and 1 μmol/L (= 0.22 and 0.44 mg/L) as a positive control. Values are means ± SD, $n = 3$ experiments. Means with different letters differ, $P < 0.05$.

turmerone, the major components in turmeric extracts, also had this activity (**Figure 3B**).

DISCUSSION

Curcuminoids and sesquiterpenoids are contained in turmeric and its extract as components. Arun et al. reported that turmeric and curcuminoids are effective in controlling blood glucose level and enzymes of glucose metabolism in alloxan-induced diabetic rats, and suggested that curcumin had a better hypoglycemic effect than turmeric (*25*, *26*). On the other hand, very little has been determined concerning the effects of sesquiterpenoids on diabetes. To investigate the effects of these components, we prepared three turmeric extracts using ethanol and hexane as solvents: H-ext containing sesquiterpenoids, HE-ext containing curcuminoids, and E-ext containing both components. TLC and HPLC analysis and extraction yields of these turmeric extracts suggested that E-ext was almost corresponding to a half-and-half mixture of H-ext and HE-ext.

This study showed that H-ext and HE-ext each suppressed an increase in blood glucose levels in type 2 diabetic KK-A^y mice, implying that both sesquiterpenoids and curcuminoids have hypoglycemic effects. E-ext at 0.2 g/100 g of diet suppressed an increase of blood glucose, but neither H-ext nor HE-ext at 0.1 g/100 g of diet did. This result indicated that the hypoglycemic effect of the combination of sesquiterpenoids and curcuminoids was stronger than the individual effects of the two. Hence, E-ext containing both turmeric components had stronger hypoglycemic effects on type 2 diabetes than either curcuminoids or sesquiterpenoids alone.

Curcumin activates PPAR-γ in rat hepatic stellate cells activated by oxidative stress (*27*). PPAR-γ agonists, such as troglitazone, pioglitazone, and rosiglitazone, are known to stimulate adipocyte differentiation, and to have hypoglycemic effects. In general, PPAR-γ agonists exhibit comparable potency and efficacy in assays using either the GAL4-PPAR-γ chimera or the full-length receptor (*28*). To investigate the mechanism of hypoglycemic effect of turmeric extracts, we conducted adipocyte differentiation and GAL4-PPAR-γ chimera assays. E-ext accelerated triglyceride accumulation in adipocytes in a dose−response fashion. Three turmeric extracts and their major components, curcumin, demethoxycurcumin, bisdemethoxycurcumin, and ar-turmerone, exhibited PPAR-γ ligand-binding activity in a GAL4-PPAR-γ chimera assay. E-ext had the highest PPAR-γ ligand-binding activity of the three extracts and four purified components. These results suggested that curcumin, demethoxycurcumin, bisdemethoxycurcumin, and ar-turmerone are natural PPAR-γ agonists, and that adipocyte differentiation is mediated through activation of PPAR-γ. Furthermore, these results implied that turmeric extracts exhibited hypoglycemic effects via PPAR-γ activation as one of the mechanisms.

In conclusion, our results indicated that turmeric ethanolic extract containing both curcuminoids and sesquiterpenoids was more strongly hypoglycemic than either curcuminoids or sesquiterpenoids, and suggested that turmeric components exhibited additive or synergistic effects.

ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; E-ext, turmeric ethanolic extract; FBS, fetal bovine serum; H-ext, turmeric hexanic extract; HE-ext, turmeric ethanolic extract from hexane−extraction residue; PPAR-γ, peroxisome proliferator-activated receptor-γ.

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