Journal of Ethnopharmacology 133 (2011) 803–809

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Cornus kousa F.Buerger ex Miquel increases glucose uptake through activation of peroxisome proliferator-activated receptor γ and insulin sensitization

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ARTICLE INFO

Article history:
Received 16 March 2010
Received in revised form 7 September 2010
Accepted 2 November 2010
Available online 9 November 2010

Keywords:
Peroxisome proliferator-activated receptor γ (PPARγ)
Cornus kousa F.Buerger ex Miquel
Oriental wild plants
Type 2 diabetes
Insulin resistance

ABSTRACT

Aim of the study: Cornus kousa F.Buerger ex Miquel, an oriental medicinal plant, has been traditionally used for the treatment of hyperglycemia, but its molecular mechanism remains unknown. The goal of this study was to investigate the peroxisome proliferator-activated receptor γ (PPARγ) ligand-binding activity of *Cornus kousa* and to determine the effects of *Cornus kousa* on insulin sensitization in 3T3-L1 cells for the treatment of type 2 diabetes.

Materials and methods: PPARγ luciferase transactivation assay was used to evaluate the PPARγ ligand-binding activity of *Cornus kousa* leaf extract. Western blot analysis, oil Red O staining, and glucose uptake assay were performed to evaluate PPARγ agonistic activity and insulin sensitizing effects of *Cornus kousa* leaf extract (CKE) in 3T3-L1 cells.

Results: CKE increased PPARγ ligand-binding activity in a dose-dependent manner. In addition, CKE enhanced adipogenesis and the expression of PPARγ target proteins, including glucose transporter 4 (GLUT4) and adiponectin, as well as proteins involved in adipogenesis, including PPARγ and CCAAT/enhancer binding protein α (C/EBPα) in 3T3-L1 adipocytes. Furthermore, CKE led to significant induction of glucose uptake and stimulated insulin signaling, but not to activation of AMP-activated protein kinase (AMPK) signaling. The enhanced glucose uptake by CKE were abolished by treatment with bisphenol a diglycidyl ether (BADGE), a PPARγ antagonist, or LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K), but not by compound C, an AMPK inhibitor.

Conclusion: Consistent with the high PPARγ ligand-binding activity, CKE increased glucose uptake through PPARγ activation and insulin signaling. These results suggest that CKE could have pharmacological effects for the treatment of hyperglycemia and type 2 diabetes.

1. Introduction

Type 2 diabetes mellitus is a serious risk factor for coronary artery disease, peripheral vascular disease, and death due to hyperglycemia. It is a metabolic syndrome characterized by high glucose levels caused by defects in insulin action (Hill et al., 2009). The symptoms are mainly caused by lowered insulin sensitivity of metabolically active cells, such as fat, muscle, and liver cells, rather than by impaired insulin production (Fadini et al., 2005). Insulin resistance in fat cells is characterized by increased hydrolysis of stored triglycerides, which produces free fatty acids in the blood to attenuate insulin sensitivity (McGrray, 2001). Therefore, it is crucial to prevent excessive production of free fatty acids from adipocytes, and to increase sensitivity to insulin action.

PPARs are members of the nuclear receptor superfamily. Three isoforms of PPARs exist, called α, δ, and γ. Among them, PPARγ is a main target for the treatment of various diseases, including diabetes, atherosclerosis, cancer, and inflammation (Vamecq and Latruffe, 1999; Kersten et al., 2000). PPARγ, which is mainly expressed in adipose tissue, regulates glucose metabolism and its activation increases insulin sensitivity. It has been reported that the *in vitro* binding ability of specific ligands to PPARγ closely correlates with their *in vivo* potency as insulin sensitizing molecules (Rangwala and Lazar, 2004). In addition, mice harboring a mutant PPARγ gene that increases PPARγ activity are protected from obesity-associated insulin resistance (Rangwala et al., 2003). Thiazolidinediones (TZDs) are a class of synthetic PPARγ ligands that improve insulin resistance in target tissues, and are used for the treatment of type 2 diabetes (Olefsky, 2000).

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doi:10.1016/j.jep.2010.11.007

Medicinal plants have been widely used for the treatment of various metabolic disorders, such as type 2 diabetes, hyperlipidemia, obesity, and atherosclerosis, by modulating nuclear receptor signaling (Huang et al., 2005; Rau et al., 2006). A few natural compounds have been identified as PPARγ agonists and shown to improve insulin resistance (Jaradat et al., 2002; Han et al., 2006; Takahashi et al., 2009). These studies revealed that natural pharmacologic plants have PPARγ agonistic activity, and significantly improve type 2 diabetes.

Cornus kousa F. Buerger ex Miquel is a tree distributed in eastern Asia. Traditionally, eastern Asian has eaten the fruits and has drunk fruit wine and tea made from the leaves. In addition, the fruits and leaves of *Cornus kousa* have been medicinally used as edible materials for the treatment of diarrhea, immune diseases, hemorraging, and hyperglycemia in eastern Asian traditional medicine (Vareed et al., 2006; Lee et al., 2008). *Cornus kousa* leaves have been reported to contain isoquercitrin, tannin, and phenolics, such as gallic acid and chlorogenic acid, which is a potent hypoglycemic agent (Ryu and Yook, 1971; Prabhakar and Doble, 2009; Kim et al., 2010). *Cornus kousa* also showed inhibitory effects on lipid peroxidation, inflammation, and tumor cell proliferation, as well as an antioxidant effect (Vareed et al., 2007; Babu et al., 2009). However, the PPARγ agonistic activity of *Cornus kousa* and the molecular mechanism underlying its hypoglycemic activity remain unexplored. In an effort to discover natural ligands of PPARγ, *Cornus kousa* leaf extract (CKE) was investigated in 3T3-L1 cells for PPARγ agonistic activity and insulin sensitizing effects.

2. Materials and methods

2.1. Plant material and chemical reagents

Cornus kousa leaves were collected in Korea, and identified by Dr. Nam-In Baek, Department of Oriental Medicinal Materials and Processing, Kyunghee University (Yongin, Korea). A specimen voucher has been deposited in the Department of Biotechnology, Yonsei University (Seoul, Korea). For this study, the dried *Cornus kousa* leaves (100 g) were extracted using accelerated solvent extraction system (ASE-300, Dionex) with 100% ethanol at 50 °C. The yield of extract was 15.0% and the extract was dissolved in DMSO at a concentration of 10 mg/ml to prepare the stock solution. 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) was purchased from Invitrogen. Bisphenol a diglycidyl ether (BADGE), troglitazone (Tro), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and insulin were purchased from Sigma (St. Louis, MO). Antibodies that recognize phosphorylated acetyl-CoA carboxylase (ACC) Ser79, AMP-activated protein kinase (AMPK) Thr172, p38 mitogen-activated protein kinase (MAPK) Thr180/Tyr182, 3′-phosphoinositide dependent kinase-1 (PDK-1) Ser241, insulin receptor substrate-1 (IRS-1) Tyr1222, and Akt (Ser473) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies, which recognize PDK-1, IRS-1, Akt, glucose transporter 4 (GLUT4), Adiponectin, and PPARgamma, and LY294002 were also purchased from Cell Signaling Technology. Antibodies that recognize α-tubulin and CCAAT/enhancer binding protein α (C/EBPα) were purchased from Calbiochem (San Diego, CA) and Santa Cruz Biotechnology (Santa Cruz), respectively.

2.2. Cell culture and differentiation

COS-7 cells and 3T3-L1 pre-adipocytes were obtained from American Type Culture Collection (ATCC, Manassas, VA), and grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 10% bovine calf serum (Welgene; Daegu, Korea), respectively, and were supplemented with penicillin (120 units/ml), streptomycin (75 μg/ml), gentamycin (160 μg/ml), and amphotericin B (3 μg/ml) in an atmosphere of 5% CO_2 at 37 °C. For differentiation of 3T3-L1 pre-adipocytes into adipocytes, the cells were grown in 24-well plates until 2 days post-confluence and then induced with differentiation medium (combination of 0.5 mM IBMX, 0.2 μM DEX, and 1.7 μM insulin in DMEM medium with 10% FBS) along with various concentrations of CKE. Two days after induction, the differentiation medium was replaced with medium containing 1.7 μM insulin with CKE. The medium was subsequently replaced again with fresh culture medium (DMEM with 10% FBS and 1.7 μM insulin) with CKE after 2 days. The extent of differentiation was measured by oil Red O staining to detect the appearance of multi-nucleated cells. The final concentration of DMSO to the cells was 0.1% in the medium when testing the various concentration of *Cornus kousa* extract.

2.3. Cell viability assay

Cell viability was determined by MTT assay. Transfected COS-7 cells and 3T3-L1 pre-adipocytes were treated with CKE (0.1–100 μg/ml) for 24 h and 48 h, respectively, and MTT solution (1 mg/ml) was added. After 4 h incubation, the supernatant was removed, and DMSO was added. The absorbance was determined at 570 nm with an ELISA reader.

2.4. Gal4/PPARγ transactivation assay

The PPARγ ligand-binding activity of CKE was measured using a Gal4/PPARγ transactivation assay system, as previously described (Han et al., 2006). The ligand binding domain of hPPARγ was subcloned in-frame into the pFA-CMV vector (Stratagene, La Jolla, CA) to create pFA-hPPARγ. Cells of the host cell line typically used in this assay, COS-7, were plated in a 24-well culture plate at a density of 4×10^6 cells/well and incubated for 5 h before transfection. Each expression plasmid, including pFA-hPPARγ, pFR-β-galactosidase (Stratagene), and Gal4-driven luciferase reporter plasmid (pFR-luc) was co-transfected into COS-7 cells using Plus reagent and Lipofectamine reagent (Invitrogen Carlsbad, CA). After 4 h of transfection, the medium containing transfection reagent and plasmids was changed to fresh DMEM containing 10% FBS and the cells were incubated for 16 h for stabilization. The cells were then treated with various concentrations of CKE and were incubated for an additional 24 h. Treated cells were wash with cold phosphate-buffered saline (PBS) twice, lysed with 60 μl/well lysis reagent (Promega, Madison, WI), and incubated 4 °C for 10 min. The luciferase assay substrate (Promega, Madison, WI) was then added. Relative light units (RLU) were measured using a MicroLumatPlus LB 96V luminometer (Berthold, Wildbad, Germany). The β-galactosidase activity of the cell lysates was also measured. The luciferase activity was normalized to the β-galactosidase activity for each treatment.

2.5. Oil Red O staining

On day 6 of the induced adipocyte differentiation, the adipocytes were stained with oil Red O dye. The cells were fixed with 10% formaldehyde and then stained with oil Red O. Lipid droplets in adipocytes were stained red. After elution of stained cells by 100% pure isopropanol, oil Red O was quantified by measuring the optical absorbance at 490 nm.

2.6. Glucose uptake assay

The glucose uptake assays were performed as previously described (Alonso-Castro et al., 2008). Briefly, cells were pre-