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Persicaria hydropiper (L.) Spach and its Flavonoid Components, Isoquercitrin and Isorhamnetin, Activate the Wnt/β-catenin Pathway and Inhibit Adipocyte Differentiation of 3T3-L1 Cells

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Obesity, which is related to metabolic syndrome and is associated with liver disease, represents an epidemic problem demanding effective therapeutic strategies. Evidence shows that the Wnt/β-catenin pathway is closely associated with obesity and that small molecules regulating the Wnt/β-catenin pathway can potentially control adipogenesis related to obesity. Eleven plant extracts activating the Wnt/β-catenin pathway were screened by using HEK 293-TOP cells retaining the Wnt/β-catenin signaling reporter gene. An extract of *Persicaria hydropiper* (L.) Spach was found to activate Wnt/β-catenin signaling. *P. hydropiper* is grown worldwide in temperate climates and is found widely in Southeast Asia. The *P. hydropiper* extract inhibited the differentiation of adipocyte 3T3-L1 cells. Isoquercitrin and isorhamnetin, constituents of *P. hydropiper*, also activated Wnt/β-catenin signaling and suppressed the differentiation of 3T3-L1 cells. These results indicate that isoquercitrin in *P. hydropiper* suppresses the adipogenesis of 3T3-L1 cells via the inhibition of Wnt/β-catenin signaling. *P. hydropiper* and isoquercitrin may therefore be potential therapeutic agents for obesity and its associated disorders. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: flavonoid; 3T3-L1 preadipocyte; isoquercitrin; Wnt/β-catenin pathway.

INTRODUCTION

Obesity is an energy balance disorder in which nutrient intake chronically exceeds energy expenditure, resulting in excessive white adipose tissue accumulation. Obesity is also associated with a decrease of health-related quality of life and increase of medical expenditure. An understanding of molecular and cellular events regulating adipogenesis is crucial for designing rational therapies for the prevention and treatment of obesity and for treatment of metabolic syndromes (Fruhbeck *et al.*, 2001).

The Wnt/β-catenin signaling pathway mediates key cell–cell signaling events during embryogenesis, is required for adult tissue maintenance and is mandatory in adipogenesis (Liu and Farmer, 2004). Wnt ligands are secreted proteins that act through autocrine and paracrine mechanisms to influence the differentiation of many different cell types (Logan and Nusse, 2004). Although Wnt family proteins can inhibit preadipocyte differentiation through both β-catenin-dependent and β-catenin-independent mechanisms (Kennell and MacDougald, 2005), current genetic evidence supports β-catenin as a particularly crucial regulator of adipogenesis (Arango *et al.*, 2005). In the canonical Wnt signaling pathway, β-catenin plays a central role as a transcriptional coactivator. Upon the binding of Wnt ligands to the frizzled receptors and low density lipoprotein receptor related protein (LRP) coreceptors, cytoplasmic β-catenin is hypo-phosphorylated, stabilized and translocated into the nucleus for activation of target genes by functioning with the T cell factor/lymphoid-enhancing factor (TCF/LEF) family of transcription factors (Gregorieff and Clevers, 2005).

Pharmacological agents that activate canonical Wnt signaling and stabilize free cytosolic β-catenin block adipocyte differentiation (Ross *et al.*, 2000). Conversely, the inhibition of Wnt signaling in preadipocytes stimulates differentiation (Bennett *et al.*, 2002; Li *et al.*, 2007). These results suggest that preadipocytes produce endogenous Wnt proteins that repress adipogenesis (Prestwich and MacDougald, 2007). Wnt10b inhibits adipogenesis by inhibition of expression of CCAAT/enhancer binding protein-α (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPARγ) which activate the genes involving adipogenesis (Ross *et al.*, 2000; Liu and Farmer, 2004).

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This study investigated the role of Wnt/β-catenin signaling in the adipocyte differentiation of 3T3-L1 cells by monitoring the status of Wnt/β-catenin signaling and factors involving the differentiation of adipocytes. The importance of Wnt/β-catenin signaling in adipocyte differentiation was confirmed by inhibiting the adipocyte differentiation of 3T3-L1 cells by LiCl, the GSK3β inhibitor which activates the Wnt/β-catenin signaling. To identify natural sources that inhibit adipocyte differentiation, 11 plant origin natural products were screened for their ability to activate the Wnt/β-catenin signaling by using Wnt/β-catenin signaling reporter cells. It was shown that the *Persicaria hydropiper* (*P. hydropiper*) extract retained compounds that strongly activate Wnt/β-catenin signaling. The role of *P. hydropiper* extract in the activation of the Wnt/β-catenin signaling was further confirmed by the induction and nuclear localization of β-catenin. The *P. hydropiper* extract induced the differentiation of 3T3-L1 cells, indicating the role of the Wnt/β-catenin activator in the adipocyte differentiation. As *P. hydropiper* possesses a strong peppery taste, it is used as a spice by the Chinese and Malays to flavor some of their traditional dishes. The *P. hydropiper* extract contains flavonoid compounds that show promising pharmacological activities (e.g. powerful antioxidant activity that can protect the human body from free radicals) (Sánchez-Gallego *et al.*, 2010). Because flavonoids are major components of *P. hydropiper* (Yang *et al.*, 2011), the study tested whether the flavonoids in *P. hydropiper*, such as isoquercitrin and isorhamnetin, function in the activation of the Wnt/β-catenin signaling and inhibit adipogenesis. It was identified that isoquercitrin and isorhamnetin flavonoids specifically activate the Wnt/β-catenin signaling and inhibit the adipocyte differentiation of 3T3-L1 preadipocytes. Overall, the Wnt/β-catenin activators, *P. hydropiper* extract and flavonoid such as isoquercitrin and isorhamnetin, can potentially be used for the development of pharmaceutical and nutraceutical products.

MATERIALS AND METHODS

Preparation of plant extracts. The following 11 plant extracts including *Persicaria hydropiper* (L.) Spach were purchased from the Plant Extract Bank (http://extract.pdrc.re.kr; Daejeon, Korea): *Limonium tetragonum* (whole plant), *Oenanthe javanica* (whole plant), *Typha orientalis* (flowers), *Lactuca sativa* (whole plant), *Opuntia ficus-indica var. saboten* (fruits), *Persicaria hydropiper* (L.) Spach (whole plant), *Ginkgo biloba* (leaves), *Geranium nepalense* subsp. *Thunbergii* (whole plant), *Achillea sibirica* (whole plant), *Sterculiae Scaphigerae* (seed) *Semen* and *Cartami Flos* (whole plant). The extracts were obtained from 30–40 g of crude sample mixed with 200 mL of methanol using a solvent extraction equipment (ASE300 Accelerated Solvent Extracor, Dionex Corporation, USA) at 50 °C, 1500 psi for 20 min and dried (Modul spin 40, Biotron Corporation, Canada) at 40 °C for 24 h. *Sterculiae Scaphigerae Semen* and *Cartami Flos* (whole plant) extracts were obtained from 100 g of crude sample mixed with 2 L of water using a boiling water extraction equipment (Herb Extractor, Daewoong Pharmaceutical Co., Korea) during 2.5 h and lyophilized for 12 h.

Cell culture. The 3T3-L1 preadipocytes (Zhang *et al.*, 2009), which were obtained from Dr Jae Woo Kim at Yonsei University College of Medicine, were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, 110 mg/L pyruvate supplemented with heat inactivated 10% (v/v) calf serum (Gibco, CA, USA), 100 μg/mL penicillin, 100 μg/mL streptomycin in a CO_2 incubator at 37 °C. To induce adipocyte differentiation, 3T3-L1 cells were cultured with DMEM plus 10% (v/v) heat inactivated fetal bovine serum (FBS) (Gibco) containing 520 μM isobutylmethylxanthine, 1 μM dexamethasone and 167 nM insulin. After 2 days, the medium was then changed, and 167 μM of insulin in distilled water was added in a 1:1000 dilution. On day 4, the medium was replaced with DMEM containing 10% FBS, and changed with fresh identical medium every 2 days. To measure the antidifferentiation effects of each drug, 3T3-L1 preadipocytes were induced to differentiate in the presence of different concentrations of LiCl, plant extracts or flavonoid (isoquercitrin or isorhamnetin).

Luciferase assay. HEK 293-TOP cells (3×10^4) were seeded into 96-well plates and incubated in medium with 10% FBS for 1 day. The cells were then treated with plant extract (1 μg/mL) or flavonoids (Sigma, St Louis, MO) (50 μM) for 24 h. Total cell lysates were extracted with 25 μL 1x reporter lysis buffer (Promega, Madison, WI) per each well and luciferase activities were measured by adding 25 μL luciferin (USB, Cleveland, OH) per well using a microplate luminometer (BMG Labtech, Offenburg, Germany).

Oil Red O staining. The dye solution was prepared as follows: 150 mg Oil Red O was dissolved in 30 mL of isopropanol. Then, the precipitate was removed by filtration and the supernatant was stored at room temperature after 20 mL of bidistilled water was added. Adipocyte cell layers were washed with PBS, fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, stained with the Oil Red O dye solution for 1 h, and then washed with distilled water. The cells were checked by a bright-field optical microscope (Nikon TE-200U, Tokyo, Japan).

Immunoblot analysis. The cells were rinsed once with ice-cold phosphate buffered saline (PBS) and harvested by scraping in 120 μL of RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA). They were then incubated on ice for 20 min, and centrifuged at 12000 rpm for 30 min. The supernatant was then transferred to a fresh tube. Protein concentrations were determined using a Bio-Rad protein assay kit. The lysates containing 20 μg of protein were separated by 8–12% sodium dodecyl sulfate (SDS) polyacrylamide gel (acrylamide: bis-acrylamide at a ratio of 29:1). Immunoblot analysis was performed, as described previously (Kim *et al.*, 2009) using anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000), -PPARγ (Abcam, Cambridge, UK, 1:1000), -C/EBPα (Cell signaling, Co., Danvers, MAP) or -β-actin (Santa Cruz Biotechnology, 1:5000) antibody followed by HRP-conjugated secondary antibodies at a dilution of 1:5000. The blots were developed by enhanced

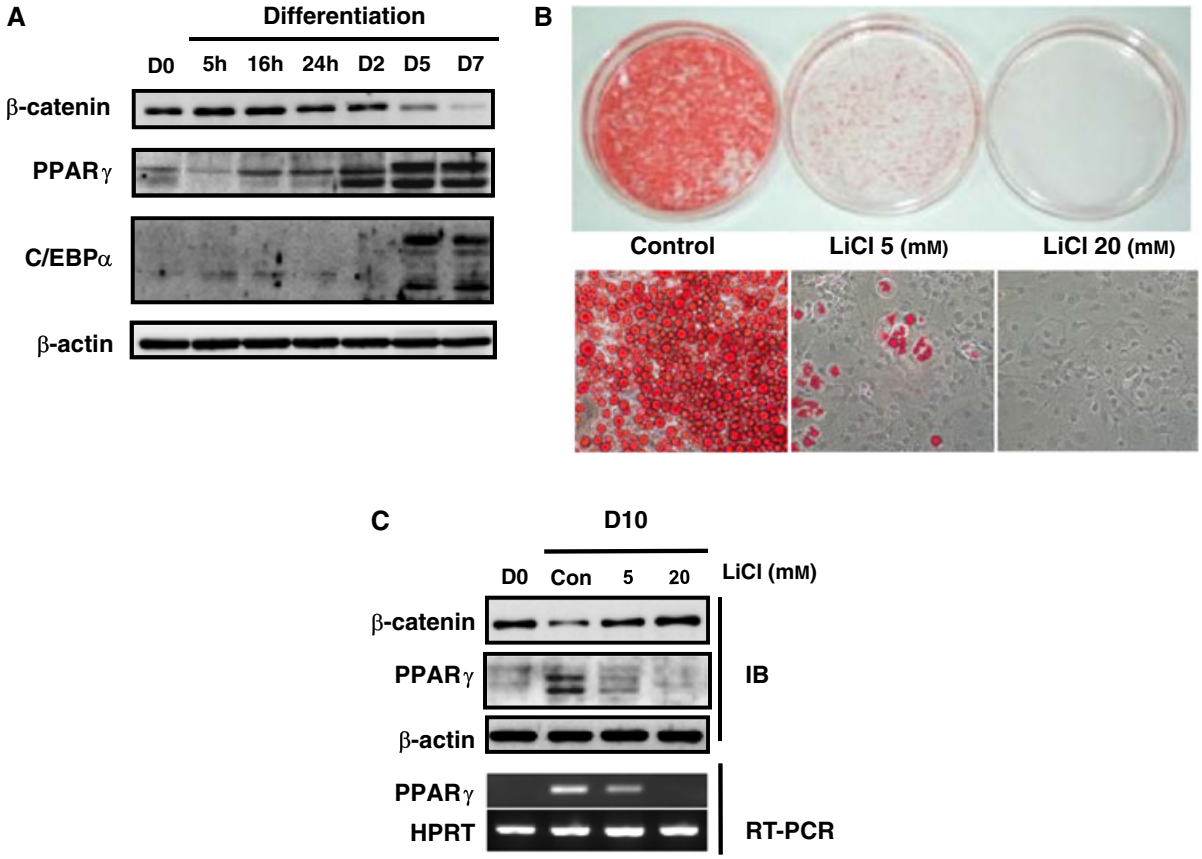
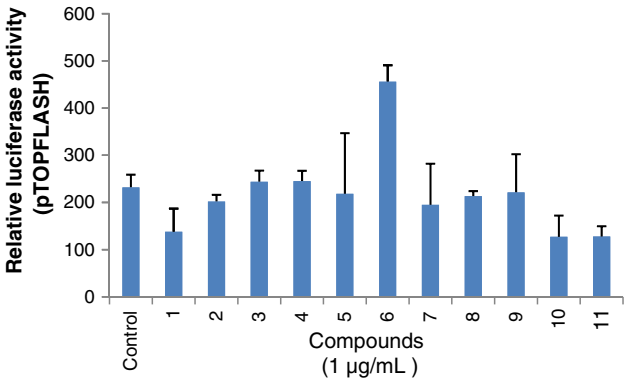


Figure 1. Wnt/β-catenin signaling involves the adipocyte differentiation of 3T3-L1 preadipocyte cells. (A) 3T3-L1 preadipocyte cells were grown in DMEM, and induced to differentiation to 7 days as described in the Materials and Methods. Total cell lysates were analysed by immunoblotting analyses with anti-β-catenin, -PPARγ, -C/EBPα or β-actin antibodies. (B) 3T3-L1 cells were induced to differentiation for 7 days with or without 5 or 20 mM LiCl. Intracellular lipids of 3T3-L1 cells were visualized by Oil Red O staining as described in the Materials and Methods. (C) 3T3-L1 preadipocyte cells were grown in DMEM, and differentiated for 10 days with or without 5 or 20 mM LiCl. Total cell lysate were analysed by immunoblotting or by RT-PCR analysis as described in the Materials and Methods. Original magnification: B, ×200.

chemiluminescence (Amersham Bioscience, Uppsala, Sweden).

RT-PCR. Total RNA was prepared using a TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. M-MLV reverse transcriptase (Invitrogen) was utilized to synthesize cDNA from the mRNA. PCRs were performed with Taq DNA polymerase at 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min, using a System 2700 (Applied Biosystems, Foster City, CA). The sequences of the primers used were as follows: mouse *HPRT*: Fwd 5′-CCTGCTGGATTACATTAAAGCGCT-3′ Rev 5′-GTCAAGGGCATATCCAACAACAAA -3′; *PPARγ*: Fwd 5′-TATGGGTGAAACTCTGGGAG -3 Rev 5′-GCTGGAGAAATCAACTGTGG-3′; The PCR products were resolved by 2% agarose gel electrophoresis and photographed using a LAS 3000 luminescent image analyser (Fujifilm, Tokyo, Japan).

Immunocytochemistry. The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed with PBS. For permeabilization, the cells were treated with 0.1% Triton X-100 for 15 min at room temperature. Then the cells were blocked with 5% BSA and 1% normal goat serum in PBS for 30 min at room temperature and successively incubated with mouse anti-β-catenin antibody (BD transduction laboratory, Lexington, KY, 1:100) overnight at 4 °C. The



1; *Limonium tetragonum*
2; *Oenanthe javanica*
3; *Typha orientalis*
4; *Lactuca sativa*
5; *Opuntia ficus-indica var. saboten*
6; *Persicaria hydropiper*
7; *Ginkgo biloba*
8; *Geranium nepalense subsp. thunbergii*
9; *Achillea sibirica*
10; *Sterculiae Scaphigerae Semen*
11; *Cartami Flos*

Figure 2. Screening of 11 plant extracts using the Wnt reporter system. Effect of different plant extracts on the Wnt/β-catenin pathway reporter activity. HEK293 cells containing pTOPFlash reporter gene in its chromosome were cultured and treated with different plant extracts at 1 μg/mL concentration, and cellular extracts were prepared after 24 h as described in the Materials and Methods.

cells were rinsed with PBS and then incubated with Alexa Fluor 488-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR, 1:400) or Alexa Fluor 555-conjugated goat anti-mouse antibody (Molecular Probes, 1:400) for 1 h at room temperature, counterstained with 4'-6-diamidino-2-phenylindole (DAPI) (Boehringer Mannheim, Mannheim, Germany, 1:5000) and examined under a confocal microscope, LSM510 META (Carl Zeiss, Gottingen, Germany).

RESULTS

The Wnt/β-catenin signaling pathway involves the adipocyte differentiation of 3T3-L1 cells

To confirm the role of Wnt/β-catenin signaling in adipocyte differentiation, the β-catenin levels during the adipocyte differentiation of 3T3-L1 cells were tested. The β-catenin level was high in the early differentiation period up to 2 differentiation days; however, its level was critically low at days 5 and 7 after initiation of differentiation when the differentiation was critical as revealed by the significant up-regulation of PPARγ and C/EBPα (Fig. 1A). The differentiation of cells monitored by Oil Red O staining was totally abolished by the treatment with 20 mM of LiCl, the GSK3β inhibitor activating the Wnt/β-catenin signaling (Fig. 1B). The role of Wnt/β-catenin signaling in antidifferentiation was indicated by the critical inhibition of PPARγ induction by LiCl treatment in 3T3-L1 cells.

P. hydropiper extract activates the Wnt/β-catenin signaling pathway

To identify natural plant products, which activate the Wnt/β-catenin signaling, 11 plant extracts were screened (*Limonium tetragonum, Oenanthe javanica, Typha orientalis, Lactuca sativa, Opuntia ficus-indica var. saboten, Persicaria hydropiper, Ginkgo biloba, Geranium nepalense* subsp. *Thunbergii, Achillea sibirica, Sterculiae Scaphigerae Semen* and *Cartami Flos*), all of which are known to contain flavonoids. The luciferase activity of HEK 293-TOP cells specifically increased the reporter activity approximately 2-fold by treatment with 1 μg/mL of *P. hydropiper* extract compared with the non-treated control (Fig. 2). Activation of the Wnt/β-catenin pathway by *P. hydropiper* extract was further confirmed by immunocytochemical analyses showing increased nuclear localization of β-catenin in 3T3-L1 cells (Fig. 3A). Here, β-catenin was similarly increased and nuclear localized as shown by the treatment with LiCl, which was used as the positive control. The activation of the Wnt/β-catenin signaling by *P. hydropiper* extract is specific, and did not

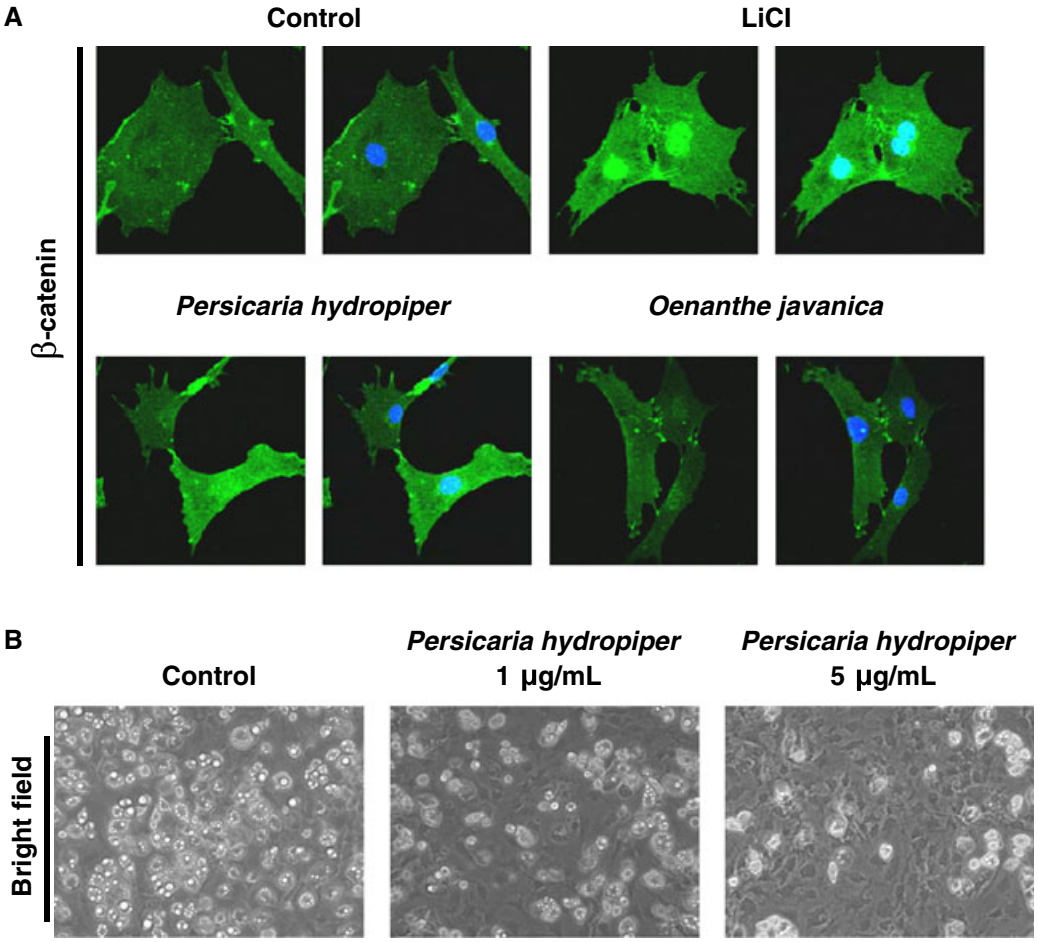


Figure 3. *P. hydropiper* extract activates the Wnt/β-catenin signaling pathway. (A) 3T3-L1 cells were stimulated with 20 mM LiCl, or 1 μg/mL of the *P. hydropiper* or *O. javanica* extract. Cells were subjected to immunocytochemical analysis with the anti-β-catenin antibody. Nuclei were counterstained by DAPI. (B) 3T3-L1 cells were treated with *P. hydropiper* extract (1 μg/mL or 5 μg/mL), and the morphologies of the cells were photographed 7 days after the induction of differentiation with a bright-field optical microscope. Original magnification: A, ×400; B, ×200.

occur after treatment with the *Oenanthe javanica* extract. The role of *P. hydropiper* extract in the differentiation of 3T3-L1 cells was confirmed by its inhibitory effect on adipocyte differentiation (Fig. 3B).

Isoquercitrin and isorhamnetin activate the Wnt/β-catenin signaling

The isorhamnetin and isoquercitrin are flavonoids which are known to be present in *P. hydropiper*. Therefore, it was checked whether these flavonoids can activate the Wnt/β-catenin pathway and adipocyte differentiation. It was shown that 50 μM isoquercitrin increased the Wnt/β-catenin signaling reporter activity by approximately 4 fold (Fig. 4A). An identical concentration of isorhamnetin also increased the Wnt/β-catenin signaling activity; however, the degree of activation was half that induced by isoquercitrin (Fig. 4A). To further investigate the isoquercitrin and isorhamnetin in the activation of Wnt/β-catenin signaling, the status of β-catenin was also monitored by immunocytochemical analysis (Fig. 5). β-Catenin was critically increased and its nuclear localization was prominently increased after treatment with isoquercitrin or isorhamnetin (Fig. 5; left panel). β-Catenin, however, did not significantly change after treatment with rutin and quercetin (Fig. 5). Overall, isoquercitrin and isorhamnetin specifically activate the Wnt/β-catenin signaling in 3T3-L1 cells. The study further investigated the role of the flavonoids in the inhibition of adipocyte differentiation of 3T3-L1 cells. The adipocyte differentiation was reduced critically by either isoquercitrin or isorhamnetin. Rutin, which did not activate the Wnt/β-catenin signaling did not affect adipocyte differentiation. However, quercetin, which also did not activate the Wnt/β-catenin signaling, still inhibited adipocyte differentiation (Fig 5; right panel Fig 6).

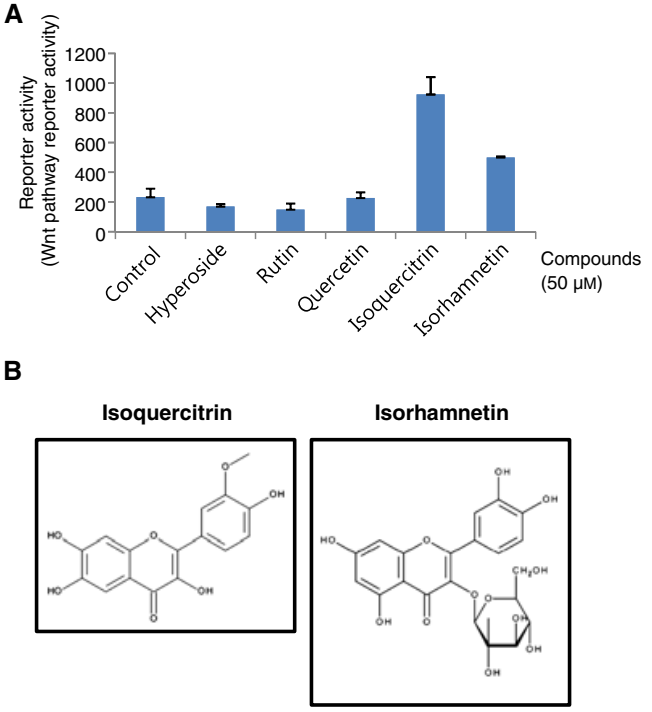


Figure 4. Screening of the flavonoids in *P. hydropiper* using the Wnt reporter system. (A) An effect of flavonoids on the activity of Wnt/β-catenin signaling reporter activity. HEK293 cells containing pTOPFlash reporter were cultured, and treated with flavonoids (each 50 μM). Luciferase activities were measured as described in Fig. 2. (B) Structures of isoquercitrin and isorhamnetin.

DISCUSSION

The Wnt/β-catenin pathway plays important physiological roles including embryonic development and cellular proliferation. There are growing studies indicating that the Wnt/β-catenin pathway plays a role in adipose cell communication and the subsequent regulation of adipogenesis (Ross *et al.*, 2000; Kennell and MacDougald, 2005; Prestwich and MacDougald, 2007). The Wnt/β-catenin signaling plays a role in the suppression of the differentiation of preadipocyte cells, which was indicated by a concomitant decrement and increment of β-catenin and PPARγ/C/EBP, respectively, during the adipocyte differentiation of 3T3-L1 cells. The role of Wnt/β-catenin signaling in the suppression of adipocyte differentiation was further indicated by the reduced induction of PPARγ by the treatment of LiCl, an activator of the Wnt/β-catenin signaling, during adipocyte differentiation. Therefore, the Wnt/β-catenin pathway could be an attractive target for the development of drugs to treat obesity (Christodoulides *et al.*, 2009). Recently, the clinical importance of herbal medicine has received considerable attention, and flavonoids, which are often found in herbal ingredients and foods, are known to possess many biological properties. This study investigated whether natural products of plant origin can regulate the Wnt/β-catenin signaling and adipogenesis. *P. hydropiper*, one of the herbal plants used for the spicy taste in Chinese traditional dishes, was identified as an activator of the Wnt/β-catenin signaling. The *P. hydropiper* extract subsequently inhibited the adipocyte differentiation of 3T3-L1 cells. It was further shown that active flavonoids in *P. hydropiper*, such as isoquercitrin, retain functions for the activation of Wnt/β-catenin signaling and the suppression of adipogenesis. The activation of Wnt/β-catenin signaling by isoquercitrin is specific, as shown by the induction of β-catenin which did not occur by using other flavonoids such as rutin or quercetin. The inhibition of adipocyte differentiation still occurred after treatment with quercetin, although this did not occur after treatment with rutin. These results indicate that adipocyte differentiation by quercetin may have occurred independently of the Wnt/β-catenin signaling. Quercetin affects adipogenesis and apoptosis by modulation of adenosine monophosphate-activated protein kinase (AMPK) and mitogen activated protein kinase (MAPK) pathways in 3T3-L1 cells (Ahn *et al.*, 2008; Hsu and Yen, 2006).

Our study indicates that isoquercitrin in *P. hydropiper* may inhibit adipogenesis via the activation of Wnt/β-catenin signaling. Our results also agree with other reports on the mechanism for the antiobesity effects of flavonoids such as isorhamnetin and quercetin (Lee *et al.*, 2008; Ahn *et al.*, 2008), and silibinin and polyphenol such as epigallocatechin-3-gallate, genestin, resveratrol (Rayalam *et al.*, 2008; Andersen *et al.*, 2010), curcumin (Ahn *et al.*, 2010), isoflavone (Kim *et al.*, 2010) and baicalin (Lee *et al.*, 2009). The role of different flavonoids in the regulation of Wnt/β-catenin signaling was revealed by the increment of protein levels of β-catenin by isorhamnetin, and the subsequent induction

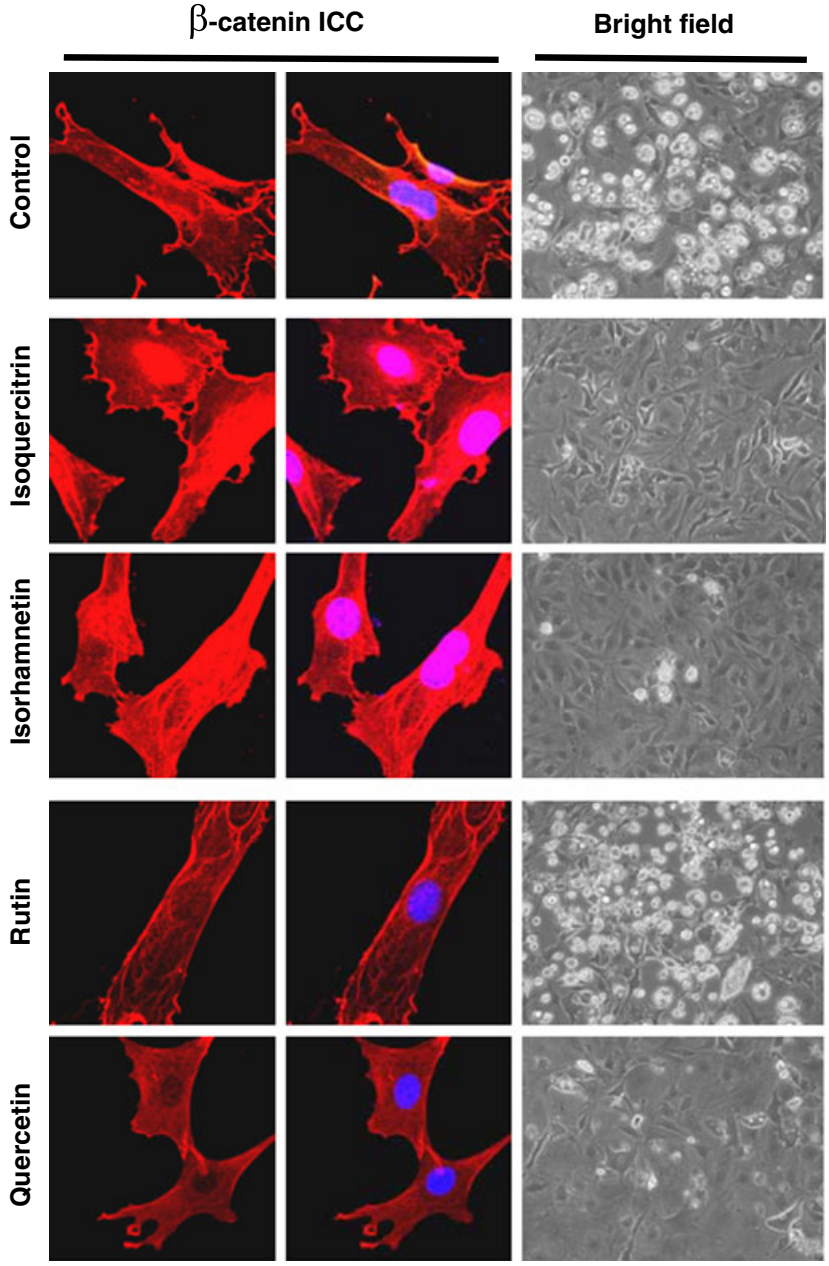


Figure 5. The flavonoids, isoquercitrin and isorhamnetin, activate Wnt/β-catenin signaling. Effects of quercetin and its derivatives on activation of the Wnt/β-catenin pathway and adipocyte differentiation in 3T3-L1 cells. Left panel, 3T3-L1 cells were grown and different flavonoids were treated at a concentration of 50 μM. Cells were subjected to immunocytochemical analyses by using anti-β-catenin antibody. Nuclei were counter stained by DAPI. Cell images were captured as in Fig. 3B. Right panel, 3T3-L1 cells were treated with different flavonoids at a concentration of 50 μM, and the morphology of the cells was photographed as described in Fig. 3B. Original magnification: Left panel, ×400; Right panel, ×200.

of β-catenin target genes: c-myc, cyclin D1 and PPARδ (Lee *et al.*, 2010). Isorhamnetin also reduced the mRNA expression levels of C/EBPα and PPARγ, which are known to be inhibited by c-myc or by cyclin D1 and PPARδ, respectively (Lee *et al.*, 2010). Isorhamnetin inhibits the adipogenic differentiation of human mesenchymal stem cells (hAMSCs) via the stabilization of β-catenin (Lee *et al.*, 2010). Baicalin not only up-regulates the antiadipogenic regulators, C/EBPγ, C/EBP homologous protein and Kruppel-like factor (KLF)2, but also down-regulates the proadipogenic regulator, KLF15 (Lee *et al.*, 2009). The overall effects of baicalin on these upstream regulators of adipogenesis were antiadipogenic, resulting in the inactivation of downstream genes and the inhibition of cellular fat accumulation (Lee *et al.*, 2009). The identification of *P. hydropiper* and its flavonoid components as the negative adipogenesis factors indicate that these can potentially be used for the development of nutraceutical or therapeutic drugs for the treatment of obesity.

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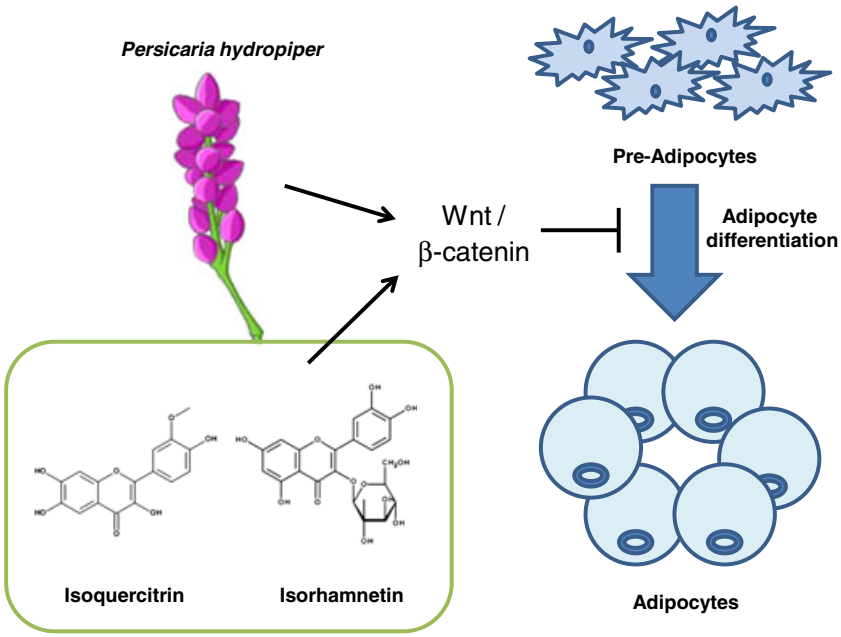

Figure 6. A model for inhibition of the differentiation of preadipocytes by isquercitrin/isorhamnetin/*P. hydropiper* extract. *Persicaria hydropiper* (L.) Spach and its flavonoid components, isoquercitrin and isorhamnetin, inhibit the adipocyte differentiation via the activation of Wnt/β-catenin signaling.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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