



Isorhamnetin-induced anti-adipogenesis is mediated by stabilization of β -catenin protein

Jongsung Lee^{a,b}, Jienny Lee^a, Eunsun Jung^a, Wangtaek Hwang^a, Yeong-Shik Kim^b, Deokhoon Park^{a,*}

^a Biospectrum Life Science Institute, 101-701 SK Ventium, 522 Dangjung Dong, Gunpo City, 435-833 Gyunggi Do, Republic of Korea

^b Natural Products Research Institute, College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea

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ABSTRACT

Aims: Previous studies have shown that isorhamnetin has anti-adipogenic effects in mouse 3T3-L1 cells. This study was conducted to elucidate the inhibitory mechanisms of isorhamnetin during adipogenic differentiation of human adipose tissue-derived stem cells (hAMSCs).

Main methods: The effect of isorhamnetin on adipogenic differentiation of hAMSCs was quantified by Oil Red O staining and a triglyceride assay. In addition, real-time PCR and Western blot were used to determine the expression of adipogenesis-related genes.

Key findings: Isorhamnetin inhibited the adipocyte differentiation of hAMSCs. Additionally, when the effects of Wnt antagonists that promote adipogenesis were evaluated, isorhamnetin was found to down-regulate the mRNA levels of sFRP1 and Dkk1, but had no effect on the mRNA levels of sFRP2, sFRP3, sFRP4 and Dkk3. Isorhamnetin also inhibited the expression of Wnt receptor and co-receptor genes. Furthermore, isorhamnetin increased the protein levels of β -catenin, an effector molecule of Wnt signaling, but had no effect on the mRNA levels of β -catenin. The phosphorylation level of GSK 3 β was also increased by isorhamnetin. These results were confirmed by the fact that the expression of c-myc, cyclin D1 and PPAR δ , which are target genes of β -catenin, was upregulated by isorhamnetin. Moreover, isorhamnetin reduced the mRNA expression levels of C/EBP α and PPAR γ , which are known to be inhibited by c-myc or by cyclin D1 and PPAR δ , respectively.

Significance: Our results indicate that isorhamnetin inhibits the adipogenic differentiation of hAMSCs and that its mechanisms are mediated by the stabilization of β -catenin.

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Introduction

Increases in the number of fat cells are observed in cases of severe human obesity (Kuczmarski et al. 1994), and obesity is closely associated with a number of pathological disorders including non-insulin (INS)-dependent diabetes, hypertension, hyperlipidemia and cardiovascular diseases (Alessi et al. 2004). Therefore, obesity is becoming an increasing threat in both industrialized and developing countries.

Mesenchymal stem cells (MSCs) are believed to be multipotent cells that possess the ability to differentiate into cells of multiple tissue lineages, including chondrocytes, osteocytes, adipocytes, myocytes and neuronal cells (Prockop 1997; Pittenger et al. 1999; Deans and Moseley 2000; Woodbury et al. 2000). The results of several recent studies have shown that human adipose tissue-derived stem cells are multipotent and capable of undergoing in vitro differentiation into adipocytes as well as other mesenchymal cell

lineages (Halvorsen et al. 2001; Sen et al. 2001). Differentiation of mesenchymal stem cells (MSCs) into adipocytes is regulated by a variety of endocrine and paracrine factors, although the manner in which these factors function remains poorly understood. Recently, several studies have suggested that Wnt is one of the principal factors involved in differentiation (Ross et al. 2000; Bennett et al. 2003; Kanazawa et al. 2005). Wnt signaling is controlled by soluble extracellular antagonists, including secreted Frizzled-related proteins (sFRPs), Wnt inhibitory factor-1 (WIF-1), Cerberus and Dickkopfs (Dkk) (Logan and Nusse 2004).

Flavonoids have received considerable attention due to their biological and physiological importance. Isorhamnetin, which is a flavonoid found in seabuckthorn, has been reported to have antioxidant activity, the ability to increase the resistance of human LDL to oxidation induced by Cu²⁺, radical scavenging activity and anti-tumor activity (Yan et al. 2002; Janisch et al. 2004). In previous studies, our group demonstrated the anti-adipogenesis effect of isorhamnetin in murine 3T3-L1 cells (Lee et al. 2009). However, the inhibitory mechanisms of isorhamnetin on adipogenesis have not been studied to date. In this study, we investigated the effect of isorhamnetin on the adipogenic differentiation of human adipose tissue-derived stem cells (hAMSCs)

* Corresponding author. Tel.: +82 31 436 2090; fax: +82 31 436 0605.
E-mail address: pdh@biospectrum.com (D. Park).

and its mechanisms of action. We found that isorhamnetin inhibits the adipogenic differentiation of hAMSCs via the stabilization of β -catenin.

Materials and methods

Reagents

Isorhamnetin, isobutylmethylxanthine, dexamethasone, anti- β -actin monoclonal Ab and insulin were purchased from Sigma-Aldrich (St. Louis, MO, USA). TRIzol reagent, random primers, Moloney murine leukemia virus reverse transcriptase, MesenPro RS™, STEM PRO® and adipocyte differentiation media were obtained from Invitrogen (Carlsbad, CA, USA). Anti-phospho-GSK 3 β , anti-GSK 3 β , anti-histone β -catenin were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The pTOPFlash and pFOPFlash plasmids were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Dkk-1 recombinant mouse protein was purchased from assay designs (Ann Arbor, MI, USA).

Human adipose tissue-derived mesenchymal stem cell culture and stimulation

Human adipose tissue-derived mesenchymal stem cells (hAMSCs) (Invitrogen, Carlsbad, CA, USA) were seeded in 6-cm diameter dishes at a density of 15×10^4 cells/well. The cells were grown in MesenPro RS™ media (Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5% CO₂ humidified atmosphere. To induce differentiation, 2-day post confluent hAMSCs (day 0) were incubated for 21 days with STEM PRO® adipocyte differentiation media (Invitrogen, Carlsbad, CA, USA). To examine the effects of isorhamnetin on the adipocyte differentiation of hAMSCs, 2-day post confluent hAMSCs were stimulated with STEM PRO® adipocyte differentiation media (Invitrogen, Carlsbad, CA, USA) in the presence or absence of the indicated concentrations of isorhamnetin for 21 days. Isorhamnetin was reconstituted in DMSO and stored at -20 °C until use.

3T3-L1 cell culture and stimulation

3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were seeded in 6-cm diameter dishes at a density of 15×10^4 cells/well. The cells were grown in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS at 37 °C in a 5% CO₂ humidified atmosphere. To induce differentiation, 2-day post confluent 3T3-L1 preadipocytes (day 0) were incubated for 3 days with differentiation medium [0.5 mM isobutylmethylxanthine, 0.25 μ M dexamethasone and 1 μ g/ml insulin in phenol red-free DMEM supplemented with 10% charcoal-stripped FBS]. The preadipocytes were then maintained in and re-fed every 3 days with maintenance medium [phenol red-free DMEM supplemented with 10% charcoal-stripped FBS and 1 μ g/ml insulin]. To examine the effects of isorhamnetin on adipocyte differentiation, 2-day post confluent 3T3-L1 preadipocytes were stimulated with differentiation medium in the presence or absence of the indicated concentrations of isorhamnetin for 3 days. The medium was then replaced with maintenance media in the presence or absence of the indicated concentrations of isorhamnetin every 3 days until the end of the experiment on day 9.

Oil Red O staining

Human adipose tissue-derived stem cells that were treated as described above were washed with PBS and then fixed with 10% formalin for 30 min. Next, the cells were washed twice with distilled water, after which they were stained for at least 1 h at room temperature in freshly diluted Oil Red O solution (six parts Oil Red O stock solution and four parts H₂O; Oil Red O stock solution is 0.5% Oil Red

O in isopropanol). The results were confirmed by three independent experiments.

Triglyceride assay

Human adipose tissue-derived mesenchymal stem cells that were treated as described above were washed with PBS and then harvested into 25 mM Tris buffer (pH 7.5) containing 1 mM EDTA. The cells were then sonicated to homogenize the cell suspension, after which they were assayed for total triglycerides using a triglyceride assay kit (Cayman Chemical, Ann Arbor, MI, USA). The protein content of the homogenates was also determined using a protein assay kit (Pierce, Rockford, IL, USA). The results were confirmed by three independent experiments.

RNA preparation and real-time quantitative RT-PCR

Total cellular RNA was extracted from the hAMSCs 6 h, 1 day or 2 weeks after inducing differentiation or from the 3T3-L1 cells 1 day or 6 days after inducing differentiation using TRIzol reagent according to the manufacturer's instructions. The cDNA was synthesized from 1 μ g of total RNA in a reaction mixture with a final volume of 20 μ l using random primers and Moloney murine leukemia virus reverse transcriptase. Real-time quantitative RT-PCR analyses of the genes described in Tables 1 and 2 were then conducted as previously described using the Mx3005P Real-time PCR System (Stratagene, La Jolla, CA, USA) (Lee et al. 2009). The results were confirmed by four independent experiments.

Western blot analysis

Cultured cells treated with the indicated concentrations of isorhamnetin were harvested, after which the nuclear extracts were prepared using nuclear and cytoplasmic extraction reagent (Pierce, Rockford, IL, USA). Alternatively, cultured cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM EGTA, 10 mM β -mercaptoethanol) containing 5 μ g/ml leupeptin, 5 μ g/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride and then disrupted by sonication. The protein concentrations were then determined using a Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA, USA). Western blotting was

Table 1
Sequences of human primers used for real-time PCR.

Gene name	Forward primer	Reverse primer
18S	CGCCGCTAGAGGTGAAATCT	CATTCTTGGCAAATGCTTTCCG
PPAR γ	TTCAGAAATGCCTTGCACTG	CCAACAGCTTCTCCTTCTCG
C/EBP α	TGGACAAGAACAGCAACGAG	TTGTCACTGGTCAGCTCCAG
C/EBP β	ACTTCTACTACGAGGCGGACTG	GAGAAGAGGTCGGAGAGGAAGT
C/EBP δ	GACTCAGCAACGACCCATACC	TGCTCAGTCTTTTCTCTTAT
LRP5	GCCCTACATCATTCCAGGAA	GGGTGGATAGGGGTCTGAGT
LRP6	CCCATGCACCTGGTTCTACT	CCAAGCCACAGGGATACAGT
FZD1	GTGAGCCGACCAAGGTGTAT	AGCACAGCACTGACCAAATG
FZD4	TGGGCATTTTTTCGGTATTC	TGCCCCAACAAAGACATA
FZD6	CGATAGCAGCCTGCAATA	ACGGTGCAAGCCTATTTTG
FZD7	CGCCTCTGTCTCTACCTC	CCATGAGCTTCTCCAGCTTC
sFRP4	TCGCCCATCAAGATGTTCT	ATCATCTTGAAAGCCCACTC
Dkk1	CCTTGGATGGGTATTCCAGA	CCTGAGGCACAGTCTGATGA
Dkk3	CCATCCATGTGACCCGAAATTCAC	TCCAGCAGTGCAGCGCGGCAGC
sFRP1	GGTCATGCAGTCTCTGGCT	TCCTCAGTGCAAACCTCGCTG
sFRP2	ACCGAGGAAGCTCCAAAGGTAT	TCATCTCTCACAGGTGCACTG
sFRP3	CTCATCAAGTACCGCCACTCGTG	CGGAAATAGTCTTCTGTAGCTC
β -Catenin	GCCGGCTATTGTAGAAGCTG	GAGTCCCAAGGAGACCTTCC
c-Myc	AGCGACTCTGAGGAGGAACA	TCCGCTCTTGACATTTCTCT
PPAR δ	GCAGGCTCTAGAATTCATC	GTGCAGCCTTAGTACATGTC
Cyclin D1	CGATGCCAACCTCTCAACGAC	CCAGCATCCAGGTGGCGACC

Table 2
Sequences of mouse primers used for real-time PCR.

Gene name	Forward primer	Reverse primer
18S	CGCCGCTAGAGGTGAAATCT	CATTCTTGGCAAATGCTTTTCG
LRP5	ACCCGCTGGACAAGTTCATC	TCTGGGCTCAGGCTTTGG
LRP6	ACCTCAATGCGATTGTTC	GGTGCAAGAAGCCTCTGC
FZD1	CAGCAGTACAACGGCGAAC	GTCCTCTGATTCGTGTGGC
FZD4	CGTCTGCCTAGATGCAATCA	CTGCAGCATGCCTAATGAGA
FZD6	TCTCCAGGTGATCTCTGTTT	TGTGGGCTGTCTCTCTCT
FZD7	TGGCCAAATGGTGATTGT	CCATCCTCTCATGGTGCTT
Dkk1	GGTGACACCTGACCTTCTT	GAGGGGAAATTGAGGAAAGC
sFRP1	GCCACAACGTGGGCTACAA	ACCTCTGCCATGGTCTCTGTG

conducted as previously described (Qian et al. 2006). The results were verified by repeating the experiments three times.

Luciferase reporter assay

To assay for activation of β -catenin/TCF target genes, 3T3-L1 preadipocytes were transfected with TOPFlash or FOPFlash reporter along with 1 μ g of Renilla luciferase expression vector driven by a thymidine kinase promoter (Promega, Madison, WI, USA) (internal standard) using Superfect transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. After 24 h, the cells were incubated with DMEM containing the indicated concentrations of isorhamnetin for 14 h. The luciferase activity of the cells was then determined. Briefly, the cells were harvested, lysed and centrifuged. Next, the supernatants were assayed for luciferase activity using a Dual Luciferase Assay system (Promega, WI, USA) and a LB953 luminometer (Berthold, Germany). The luciferase activity was expressed as a ratio of the TOPFlash-dependent firefly luciferase activity to the control thymidine kinase Renilla luciferase activity (% control). The results were confirmed by four independent transfections.

Statistical analysis

All data are expressed as the means \pm SD. Differences between the control and the treated group were evaluated by a Student's *t*-test. For all analyses, a $p < 0.05$ was considered to be statistically significant.

Results

Isorhamnetin inhibits adipogenic differentiation of hAMSCs

We first examined the effects of isorhamnetin on adipogenic differentiation of hAMSCs. Oil Red O staining revealed that the lipid accumulation in isorhamnetin treated cells was significantly lower than in control cells (Fig. 1A). To further characterize the effects of isorhamnetin on adipogenic differentiation, the cellular triglyceride content was measured. Treatment with isorhamnetin inhibited the triglyceride accumulation on day 21 after full differentiation had occurred ($p < 0.05$) (Fig. 1B). These results are consistent with our previous results demonstrating the anti-adipogenic effect of isorhamnetin in mouse 3T3-L1 cells.

Effects of isorhamnetin on the expression of Wnt antagonists during adipogenic differentiation of hAMSCs

It is well known that canonical Wnt signaling inhibits adipogenesis by stabilizing β -catenin, but that Wnt antagonists promote adipogenesis through the degradation of β -catenin. Therefore, we examined the effects of isorhamnetin on the expression of Wnt antagonists during adipogenic differentiation. Among the Wnt antagonists, the mRNA levels of sFRP1 and Dkk1 were down-regulated by isorhamnetin (Fig. 1C). However, isorhamnetin had no effect on the mRNA levels of sFRP2, 3 and 4 and Dkk3 (data not shown). These data were

confirmed in mouse 3T3-L1 cells (Table 3). These results suggest that sFRP1 and Dkk1 are involved in the isorhamnetin-induced anti-adipogenesis that occurs in hAMSCs.

Effects of isorhamnetin on the expression of Wnt receptors during adipogenic differentiation of hAMSCs

Several studies have shown that Wnt antagonists utilize the connections of receptors such as FZD1, FZD4, FZD6, FZD7, LRP5 and LRP6 to inhibit Wnt signaling (Heller et al. 2003; Logan and Nusse 2004). Therefore, we evaluated the effects of isorhamnetin on the expression of these receptors during adipogenic differentiation of hAMSCs. We found that the expression of all of the tested Wnt receptor genes was reduced by treatment with isorhamnetin (Fig. 2). In addition, we found the same results in mouse 3T3-L1 cells (Table 3). These findings suggest that these receptors are involved in isorhamnetin-induced anti-adipogenic effects.

Involvement of β -catenin in isorhamnetin-induced anti-adipogenesis in hAMSCs

The results described above demonstrated that isorhamnetin inhibits adipogenesis and reduces the expression of Wnt antagonists (Dkk1 and sFRP1) and Wnt receptors. Therefore, we conducted real-time quantitative PCR and Western blot analysis for β -catenin, a downstream effector of canonical Wnt signaling, to determine if Wnt signaling is affected by isorhamnetin. As shown in Fig. 3A and B, while the mRNA expression of levels of β -catenin was not altered by isorhamnetin, the protein levels increased significantly in response to treatment with isorhamnetin. Nuclear translocation also increased upon treatment with isorhamnetin (Fig. 3C). In addition, isorhamnetin increased the level of phosphorylated GSK 3 β protein (Fig. 3D). These findings suggest that isorhamnetin-induced stabilization of β -catenin may contribute to the anti-adipogenic actions of isorhamnetin.

Effects of isorhamnetin on Wnt/ β -catenin signaling in 3T3-L1 preadipocytes

We found that isorhamnetin induces anti-adipogenesis through stabilization of β -catenin. However, it is important to determine if these effects of isorhamnetin are a direct cause of the changes in Wnt signaling. Therefore, we conducted luciferase reporter assays using pTOPFlash, a TCF-responsive gene in 3T3-L1 preadipocytes, without treating the cells with a differentiation-inducing cocktail. As shown in Fig. 4A, isorhamnetin up-regulated β -catenin response transcription in a concentration-dependent manner, whereas it did not affect FOPFlash reporter activity in 3T3-L1 preadipocytes. Moreover, isorhamnetin-induced reporter activation was inhibited upon treatment with Dkk-1 protein (Fig. 4B). These results indicate that isorhamnetin targets the Wnt/ β -catenin signaling pathway.

Effects of isorhamnetin on the expression of β -catenin target genes during adipogenic differentiation of hAMSCs

It is well known that c-myc, cyclin D1 and PPAR δ are β -catenin target genes. Therefore, to confirm the isorhamnetin-induced stabilization of β -catenin, the expression of c-myc, cyclin D1 and PPAR δ was assessed during adipogenic differentiation. The mRNA levels of c-myc, cyclin D1 and PPAR δ were significantly higher in cells that were treated with isorhamnetin during adipogenic differentiation when compared with the control cells (Fig. 5A), indicating that isorhamnetin increases the mRNA levels of c-myc, cyclin D1 and PPAR δ through the stabilization of β -catenin.

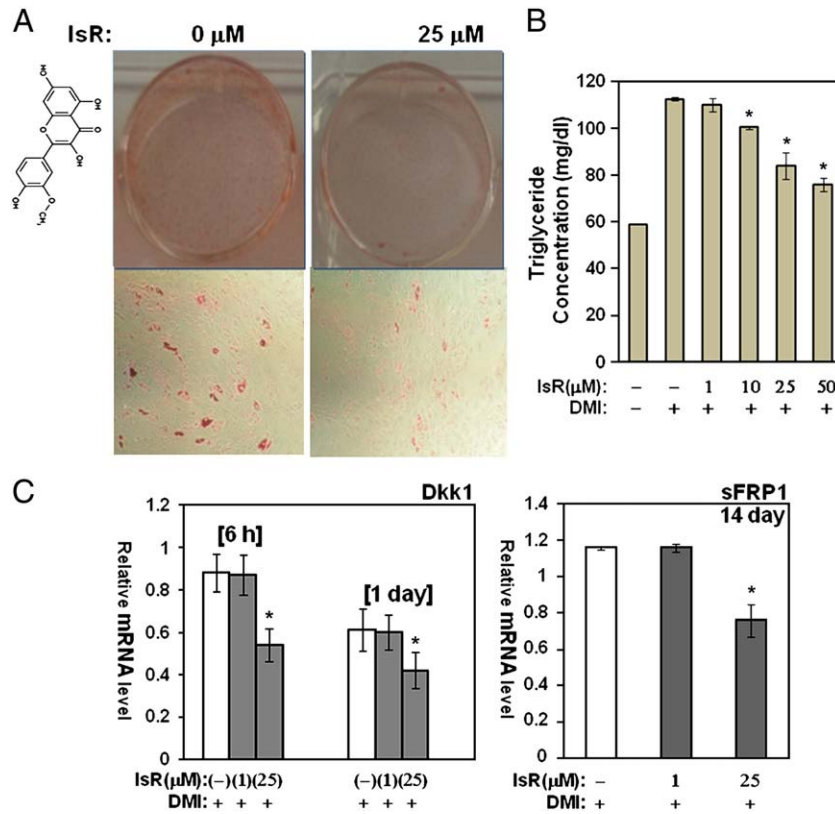


Fig. 1. Effects of isorhamnetin on adipogenic differentiation of hAMSCs and the expression of Wnt antagonists during adipogenic differentiation of hAMSCs. Two-day post confluent hAMSCs (day 0) were treated with the indicated concentrations of isorhamnetin every 3 days for 21 days. (A) Intracellular lipids were stained with Oil Red O. (B) The triglyceride content was measured using a Triglyceride assay kit (Cayman Chemical, Ann Arbor, MI). Data are expressed as the means ± S.D. The results were confirmed by three independent experiments. *, $p < 0.05$ vs. controls. (C) Two-day post confluent hAMSCs (day 0) were treated with the indicated concentrations of isorhamnetin every 3 days for 2 weeks. At 6 h, 1 day or 2 weeks after differentiation was induced, the total RNA was isolated and the mRNA levels of the indicated genes were measured by real-time quantitative RT-PCR. The results are expressed relative to untreated cells after normalization to 18S rRNA. Data are expressed as the means ± S.D. *, $p < 0.05$ vs. controls. The results were confirmed by four independent experiments. IsR: isorhamnetin, DMI: differentiation media.

Effects of isorhamnetin on the expression of early and late adipogenesis-related genes during adipogenic differentiation of hAMSCs

It has been reported that the expression of c-myc prevents adipogenesis by inhibiting the expression of C/EBPα (Freytag and Geddes 1992). In addition, it has been suggested that the enhanced expression of both cyclin D1 and PPARδ may contribute to the inhibition of adipogenesis by suppressing basal PPARγ activity in preadipocytes (Fu et al. 2005; Shi et al. 2002). Therefore, we investigated the involvement of isorhamnetin-induced expression of β-catenin target genes in the expression of the late adipogenic differentiation stage markers, C/EBPα and PPARγ. As expected, the mRNA levels of C/EBPα

and PPARγ were significantly reduced in cells that were treated with isorhamnetin during adipogenic differentiation when compared with the control cells (Fig. 5B), indicating that isorhamnetin inhibits adipogenesis through the β-catenin/β-target genes, C/EBPα and PPARγ. However, the mRNA levels of C/EBPβ and C/EBPδ, which are early adipogenic differentiation stage markers and the upstream regulators of both PPARγ and C/EBPα, were not reduced by isorhamnetin (Fig. 5B).

Discussion

The results of this study demonstrated that isorhamnetin inhibits adipogenic differentiation of human adipose-derived mesenchymal stem cells (hAMSCs), and that its inhibitory mechanisms are mediated by the stabilization of β-catenin. Namely, the stabilized β-catenin increases the expression of β-catenin target genes such as c-myc, cyclin D1 and PPARδ, consequently repressing the expression of PPARγ and C/EBPα, which are major adipogenic differentiation factors.

Canonical Wnt signaling is negatively regulated by Dkks and sFRPs. These proteins either bind directly to Wnts (sFRPs) or FZD receptors (sFRPs) or interact with LRPs (Dkks). Inhibitors that bind to Wnts or FZD receptors have the ability to block all Wnt-mediated pathways (Jones and Jomary 2002), whereas Dkks only suppress the canonical pathway (Zorn 2001). While Wnt/β-catenin signaling plays a role in the early stages of adipogenesis, the expression patterns of Wnt/β-catenin signaling components vary (Park et al. 2008). Specifically, mRNA expression of Dkk1, FZD1 and FZD7 is upregulated transiently during the early stages of adipogenesis for up to 48 h. However, mRNA expression

Table 3

Expression of genes which are involved in Wnt signaling in 3T3-L1 cells during adipogenesis.

Gene name (incubation time)	No treatment	Isorhamnetin (25 μM)
LRP5 (6 days)	1 ± 0.04	0.72 ± 0.08*
LRP6 (6 days)	1 ± 0.06	0.65 ± 0.1*
FZD1 (1 day)	1 ± 0.04	0.73 ± 0.09*
FZD4 (6 days)	1 ± 0.09	0.81 ± 0.05*
FZD6 (6 days)	1 ± 0.03	0.83 ± 0.04*
FZD7 (1 day)	1 ± 0.05	0.75 ± 0.07*
Dkk1 (1 day)	1 ± 0.04	0.70 ± 0.09*
sFRP1 (6 days)	1 ± 0.05	0.68 ± 0.05*

3T3-L1 cells were differentiated in vitro in the presence or absence of isorhamnetin and total RNA was extracted at the time points indicated. mRNA levels of the indicated genes were determined by real-time PCR. Results are expressed as fold difference relative to the untreated control. Rel. expr., * $p < 0.05$.

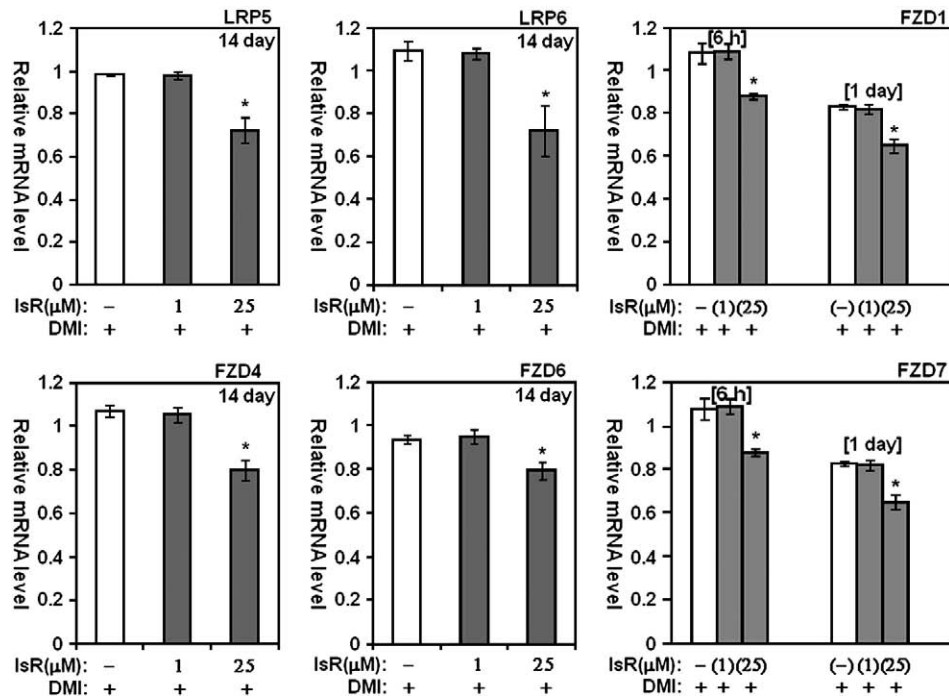


Fig. 2. Effects of isorhamnetin on the expression of Wnt receptors during adipogenic differentiation of hAMSCs. Two-day post confluent hAMSCs (day 0) were treated with the indicated concentrations of isorhamnetin every 3 days for 2 weeks. At 6 h, 1 day or 2 weeks after differentiation was induced, the total RNA was isolated and the mRNA levels of the indicated genes were measured by real-time quantitative RT-PCR. The results are expressed relative to untreated cells after normalization to 18S rRNA. Data are expressed as the means \pm S.D. *, $p < 0.05$ vs. controls. The results were confirmed by four independent experiments. IsR: isorhamnetin, DMI: differentiation media.

of sFRP4, FZD4 and LRP5 increases gradually during adipogenesis in hAMSCs. In addition, sFRP1, sFRP2, sFRP3 and Dkk3 did not produce any significant differences during the adipogenic induction period. Based on

the expression patterns of each component of Wnt/ β -catenin signaling, different time points were introduced in this study. Among the Wnt antagonists evaluated in this study, Dkk1 and sFRP1 were down-

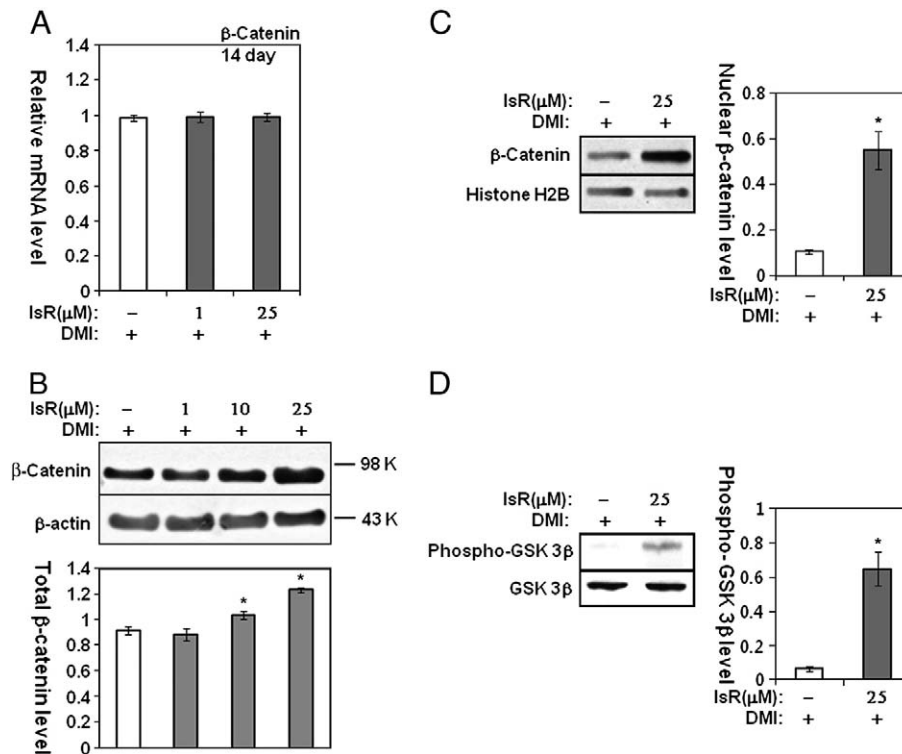


Fig. 3. Effects of isorhamnetin on the level of β -catenin mRNA and its protein during adipogenic differentiation of hAMSCs. Two-day post confluent hAMSCs (day 0) were treated with the indicated concentrations of isorhamnetin every 3 days for 2 weeks. At 2 weeks after differentiation was induced, (A) the mRNA levels of the β -catenin gene were measured by real-time quantitative RT-PCR. The results are expressed relative to untreated cells after normalization to 18S rRNA. Data are expressed as the means \pm S.D. *, $p < 0.05$ vs. controls. The results were confirmed by four independent experiments. The total lysates (B and D) and nuclear extracts (C) were analyzed by Western blot using the indicated antibodies. The results were verified by repeating the experiments three times. IsR: isorhamnetin, DMI: differentiation media.

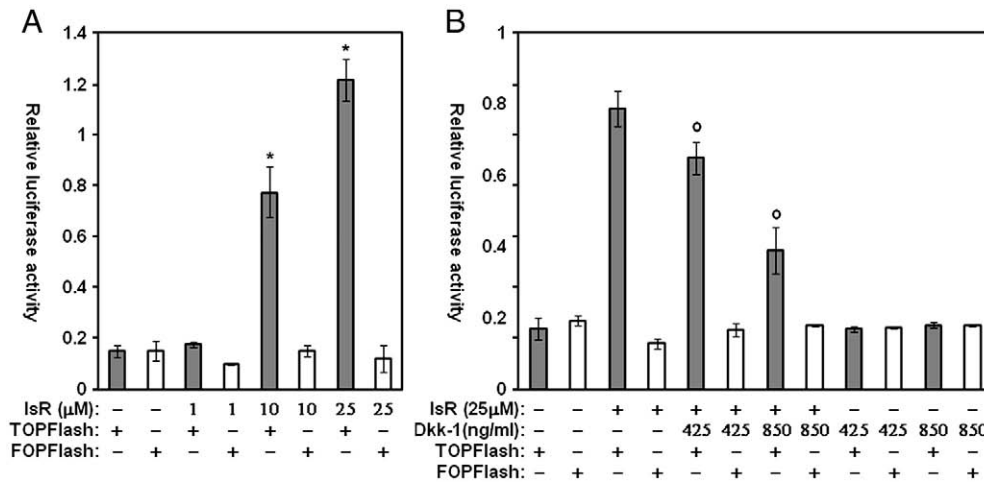


Fig. 4. Effects of isorhamnetin on the Wnt/β-catenin signaling pathway in 3T3-L1 preadipocytes. (A and B) 3T3-L1 preadipocytes were transfected with TOPFlash plasmid and incubated in either the presence or absence of Dkk-1 along with the indicated concentrations of isorhamnetin for 14 h, after which the luciferase activity was determined. Data are expressed as the means ± S.D. *, $p < 0.05$ vs. controls. ^o, $p < 0.05$ vs. isorhamnetin treated controls. The results were confirmed by four independent transfections. IsR: isorhamnetin.

regulated in response to treatment with isorhamnetin during hAMSC adipogenesis. In addition, Wnt receptor genes such as LRP5, LRP6, FZD1, FZD4, FZD6 and FZD7 were all found to be down-regulated by isorhamnetin. These results suggest that regulation of the Dkk1, sFRP1 and Wnt receptors may contribute to isorhamnetin-induced anti-adipogenesis. However, further work is necessary to elucidate the relationship between isorhamnetin-induced anti-adipogenesis and downregulation of Wnt antagonists and Wnt receptors.

A β-catenin/TCF4(TCF2)-dependent pathway was recently found to mediate tumor necrosis factor-α-induced inhibition of adipogenesis (Cawthorn et al. 2007). In that study, even though TNF-α inhibited the

expression of C/EBPα and PPARγ, the expression of C/EBPβ was not altered (Cawthorn et al. 2007). In the present study, we found that the effects of isorhamnetin were similar to the previously reported effects of TNF-α in that it increased the stabilization of β-catenin with no increase in the mRNA level and that it enhanced the mRNA levels of the β-catenin target genes, c-myc, cyclin D1 and PPARδ. Furthermore, isorhamnetin inhibited the expression of C/EBPα and PPARγ, which are the target genes of c-myc, cyclin D1 and PPARδ. These results suggest that the anti-adipogenic mechanisms of isorhamnetin may be mediated by Wnt signaling through the stabilization of β-catenin, specifically the β-catenin-dependent pathway.

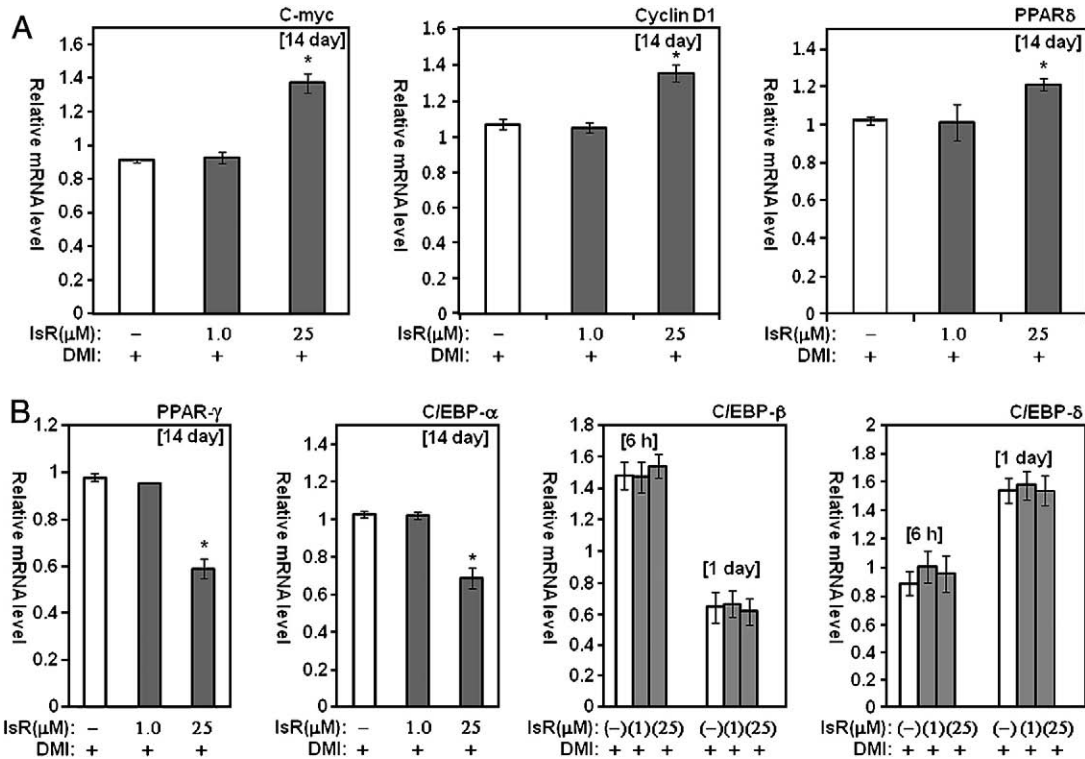


Fig. 5. Effects of isorhamnetin on the expression of β-catenin target genes and adipogenic differentiation stage markers during adipogenic differentiation of hAMSCs. Two-day post confluent hAMSCs (day 0) were treated with the indicated concentrations of isorhamnetin every 3 days for 2 weeks. At 6 h, 1 day or 2 weeks after differentiation was induced, the mRNA levels of the β-catenin target genes (A) and the adipogenic differentiation stage markers (B) were measured by real-time quantitative RT-PCR. The results are expressed relative to untreated cells after normalization to 18S rRNA. Data are expressed as the means ± S.D. *, $p < 0.05$ vs. controls. The results were confirmed by four independent experiments. IsR: isorhamnetin, DMI: differentiation media.

A large body of literature indicates that substantial progress has been made concerning our knowledge of bioactive components in plant foods and their links to obesity. Polyphenols constitute a group of plant metabolites (Bravo 1998) that are widely found in fruits, vegetables, cereals, legumes and wine (Aherne and O'Brien 2002). A number of studies have been conducted to investigate the antiobesity effects of polyphenols such as apigenin and luteolin (Han et al. 2003), kaempferol (Yu et al. 2006), myricetin and quercetin (Kwon et al. 2007), genistein and diadzein (Kim et al. 2006; Naaz et al. 2003; Dang and Lowik 2004), cyaniding (Tsuda et al. 2005), grape seed proanthocyanidin extract (GSPE) (Preuss et al. 2000), xanthohumol (Nakagawa et al. 2005) and epigallocatechin gallate (EGCG) (Wolfram et al. 2006). Among these compounds, genistein, EGCG, quercetin and capsaicin have been shown to inhibit adipocyte differentiation through the activation of AMP-activated protein kinase (Hwang et al. 2005; Ahn et al. 2008). Upregulation of *insig-1* and *insig-2* by silibinin exhibits anti-adipogenic effects (Ka et al. 2009). Solfacone and baicalein also inhibit adipocyte differentiation by enhancing the expression of heme-oxygenase-1 (HO-1) and COX-2, respectively (Tanaka et al. 2009; Cha et al. 2006). However, to the best of our knowledge, there have been no reports of isorhamnetin, flavonoids or antioxidants inhibiting adipogenesis through the stabilization of β -catenin.

C/EBP β and C/EBP δ are known to act as early markers of adipogenic differentiation in hAMSCs. In addition, they activate the expression of both PPAR γ and C/EBP α , which are major adipogenic factors. In this study, we found that isorhamnetin had no effect on the mRNA levels of C/EBP β and C/EBP δ . These results suggest that isorhamnetin operates downstream of C/EBP β and C/EBP δ and upstream of PPAR γ and C/EBP α . Specifically, isorhamnetin-induced β -catenin signaling operates downstream of C/EBP β and C/EBP δ , consequently inhibiting adipogenic differentiation.

In summary, we investigated the effects of isorhamnetin on Wnt signaling during adipogenic differentiation of hAMSCs. The results revealed that isorhamnetin inhibits adipogenic differentiation of hAMSCs and suggest that these effects may be mediated by the β -catenin-dependent pathway.

Conclusion

Isorhamnetin showed anti-adipogenic effects in human adipose tissue-derived mesenchymal stem cells. Further, its mechanisms of action were found to be mediated by the stabilization of β -catenin, which is involved in Wnt signaling.

Conflict of interest statement

We authors declare that there are no conflicts of interest.

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