Isoginkgetin enhances adiponectin secretion from differentiated adiposarcoma cells via a novel pathway involving AMP-activated protein kinase

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Abstract

Adiponectin is an anti-diabetic hormone secreted by adipocytes. Circulating adiponectin levels are lower in obese and type II diabetic patients than in healthy people. Weight loss or thiazolidinedione treatment increases plasma adiponectin levels. Animal models and human studies suggest that elevated adiponectin levels increase insulin sensitivity. We screened a library of drug-like compounds and natural products for novel agents enhancing adiponectin production. We identified isoginkgetin, a compound derived from the leaves of Ginkgo biloba, to up-regulate adiponectin secretion with potency comparable to that of rosiglitazone, a known modulator of adiponectin production. However, unlike rosiglitazone, peroxisome proliferators-activated receptor γ activity seems not required for the action of isoginkgetin, and isoginkgetin has only a slight effect on adipogenesis, which makes it an attractive candidate for anti-diabetic treatment. Further investigation revealed that both isoginkgetin and rosiglitazone activate AMP-activated protein kinase (AMPK) in adipocytes. Our findings suggest a novel mechanism for the elevation of adiponectin by isoginkgetin, which is different from that of rosiglitazone. Furthermore, this novel mechanism for adiponectin regulation involving AMPK can potentially facilitate new understanding of metabolic diseases and identification of new targets, as well as agents that increase plasma adiponectin levels.

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Introduction

Obesity is a well-recognized risk factor for the development of type II diabetes and cardiovascular diseases. The extra fat stored in adipocytes, liver, and muscle cells disturb the response of these cells to insulin, leading to insulin resistance, a hallmark of type II diabetes. Recent studies revealed that adipocytes are not only storage receptacles but are also responsible for communication with other organs to maintain metabolic balance by secreting hormones and cytokines such as leptin, tumour necrosis factor α (TNFα), and adiponectin (Mora & Pessin 2002). Adiponectin is a recently discovered protein of 30 kDa exclusively synthesized and secreted from adipocytes, which circulates at high levels (5–30 μg/ml; Arita et al. 1999). The concentration of adiponectin is inversely correlated to the body mass index, and the plasma levels of glucose, insulin, and triglyceride. Animal studies indicate that adiponectin has anti-diabetic and anti-atherogenic activities. Adiponectin knockout mice showed severe insulin resistance and diabetes (Maeda et al. 2002). Transgenic expression of globular domain of adiponectin ameliorated insulin resistance in ob/ob mice and reduced atherosclerosis in apoE–deficient mice (Yamauchi et al. 2003a). Administration of adiponectin in diet-induced obesity mice increased insulin sensitivity and reduced body weight (Fruebis et al. 2001, Shklyaev et al. 2003). In humans, insulin-resistant states such as obesity and type II diabetes are associated with lower levels of plasma adiponectin. Weight loss and therapy with thiazolidinediones, the drugs that enhance insulin sensitivity through stimulation of peroxisome proliferators–activated receptor-γ (PPAR-γ), increase adiponectin levels (Combs et al. 2002). All these studies indicate that adiponectin might be an important therapeutic molecule for metabolic diseases.

The site and mechanism of the putative anti-diabetic action of adiponectin are currently being investigated. Most studies have focused on liver and skeletal muscle cells where increased insulin sensitivity was observed upon adiponectin treatment. Adiponectin may also act on brain since intracerebroventricular injection of adiponectin reduced body weight in mice (Qi et al. 2004). The adiponectin actions are believed to be mediated by the two adiponectin receptors on the surface of the effector cells, adipoR1 and adipoR2, which are abundantly expressed in skeletal muscle and liver respectively (Yamauchi et al. 2003b). In skeletal myocytes, adiponectin activates 5′-AMP-activated protein kinase (AMPK), and thus stimulates fatty acid oxidation and glucose uptake (Yamauchi et al. 2002). In liver, adiponectin regulates molecules involved in gluconeogenesis (Yamauchi et al. 2002). The activation of
the insulin receptor in skeletal muscle was also found to be regulated by adiponectin (Stefan et al. 2002).

So far, the regulation of adiponectin production is not fully understood. Several hormones or cytokines have been found to influence adiponectin levels. While insulin enhances adiponectin production by cells (Bogan & Lodish 1999), TNF-α and interleukin (IL)-6 suppress it (Fasshauer et al. 2003). β-Adrenergic stimulation inhibits adiponectin gene expression, indicating a possible role of the adiponectin reduction in catecholamine-induced insulin resistance (Fasshauer et al. 2003). Rosiglitazone, a PPARγ agonist, ameliorates insulin resistance at least partially by elevating adiponectin level in vivo. However, rosiglitazone has side effects such as promoting adipogenesis, causing body weight gain and recently shown to increase cardiovascular risk. Therefore, a small molecule that enhances adiponectin production, but is not adipogenic, will be of important therapeutic value.

In this study, we screened a compound library for agents up-regulating adiponectin production, and identified isoginkgetin. Isoginkgetin is a natural compound isolated from the leaf extracts of Ginkgo biloba. It belongs to biflavones, which are composed of a large number of natural compounds and exhibit multiple biological activities including antioxidant, anti-inflammatory, and anti-tumor activities (Yoshikawa et al. 1999, DeFeudis et al. 2003). Ginkgetin, an isomer of isoginkgetin, has been showed to possess anti-inflammatory activities via the inhibition of cyclooxygenase (COX)-2 and 5-LOX (lipoxygenase) in vitro and inhibition of rat adjuvant-induced arthritis in vivo (Son et al. 2005). However, whether isoginkgetin has a potential anti-diabetic property has not been investigated. In human adipocyte LiSa cells and mouse adipocyte 3T3-L1 cells, isoginkgetin stimulates adiponectin secretion with potency comparable to that of rosiglitazone, but only weakly enhances adipogenesis. The mechanisms of isoginkgetin and rosiglitazone appear different for their distinct dependency on PPARγ activity and the kinetics of their actions. Our findings suggest a possible novel pathway for adiponectin up-regulation mediated by isoginkgetin. These in vitro results suggest the possibility of using isoginkgetin to improve insulin sensitivity via adiponectin up-regulation. Additional in-depth mechanism studies of isoginkgetin may shed light on the regulatory machinery of adiponectin secretion and provide novel strategies for developing adiponectin up-regulating compounds.

Materials and Methods

Reagents

Isoginkgetin was purchased from Gaia Chemical Corp. (Gaylordsville, CT, USA). Rosiglitazone was purchased from Tocris (Ellisville, MO, USA). Cell culture reagents were obtained from Invitrogen. Adiponectin antibody was purchased from Affinity BioReagents (Golden, CO, USA). Phospho-AMPK antibody was purchased from Cell Signaling (Beverly, MA, USA). Compound C was obtained from Calbiochem (Darmstadt, Germany). All other reagents and chemicals were obtained from Sigma. Spectrum compound library was purchased from Microsource Discovery Systems Inc. (Gaylordsville, CT, USA).

Cell cultures and differentiation

3T3-L1 mouse fibroblasts were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). The early passages of the cells were used in this study. The human adipocyte LiSa-2 cell line was generated and characterized by Dr Peter Moller (Wabitsch et al. 2000). Both 3T3-L1 cells and LiSa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplied with 10% fetal bovine serum in incubator with 5% CO2 at 37 °C. After the cells reached confluence, differentiation was induced by incubating cells in DMEM with 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone, 1 µg/ml insulin, and 10% bovine growth serum. Isoginkgetin, rosiglitazone, or vehicle control DMSO was added either in differentiated LiSa cells or in 3T3-L1 cells, and incubated for indicated time. The conditioned medium was collected for further analysis.

ELISA measurement of adiponectin levels

Adiponectin concentration in conditioned medium was measured using DuoSet human adiponectin ELISA (R&D system, Minneapolis, MN, USA) and mouse adiponectin ELISA kit (B-Bridge International Inc., Sunnyvale, CA, USA) respectively.

Compound library screening

LiSa cells were cultured in 96-well plates. Differentiation was induced by adding 0.5 mM IBMX, 1 µM dexamethasone, and 1 µg/ml insulin to the culture medium. Three days post-differentiation, compounds from the library were added to the medium at a final concentration of 5 µM, and incubated for 4 days followed by ELISA measurements of adiponectin levels in the conditioned medium.

TaqMan real-time RT-PCR analysis

TaqMan real-time RT-PCR was performed as previously described (Ke et al. 2004). The dual-labeled fluorescent probe and PCR primer sequences for adiponectin-coding region are: probe: 5′FAM-CCCCAACATGCCCATTC-GCTTTTACCA-BHQ3′; forward primer: 5′GTGTGGGAT-TGGAGACTTACGTTAC3′; reverse primer: 5′TAGT-GTTTTGTGATTGAAAGATC3′. The sequences for ap2 gene are: probe: 5′FAM-TCATGAAAGCCGT-CACTTCACCGAGA-BHQ3′; forward primer: 5′AT-GATAAACTGTTGCCGAAATGC3′; reverse primer:
5'CAGCTCCATGGCTATGCTC3'. Sequences for LPL gene are: probe: 5'-FAM-CATAGATTGTTACCATGGTGAT-CCTCAGCCCAT-BHQ-3'; forward primer: 5'-CAG-CAAAACCTTCATGGTGAT-3'; reverse primer: 5'-CAAGTTTTGGCACCCAACTC-3'.

**Western blot analysis**

Cells were lysed in cell lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 1 mM NaF, 0.5 mM Na3VO3, 0.1 U/ml aprotinin, 10 U/ml leupeptin, 4 μg/ml pepstatin A). The lysates were centrifuged at 15 000 g for 10 min, and the supernatants were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane. After blocking the membrane in PBS containing 5% nonfat milk (Bio-Rad Inc.), the proteins were blotted by indicated antibodies. After washing with PBS containing 0.1% Tween-20, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody. The blots were developed with the ECL kit (Amersham).

**Oil-red O staining**

The measurement of the lipid accumulation in 3T3-L1 cells was performed using Adipogenesis Assay Kit (Millipore, Billerica, MA, USA). Briefly, 3T3-L1 cells were cultured in 96-well plate. After differentiation and treatment with compounds, the cells were stained with oil red O solution. Quantitative results were obtained by adding dye extraction solution to the cells and measuring the absorbance from each well.

**Lipolysis measurement**

LiSa cells or 3T3-L1 cells were differentiated for 9 days, followed by incubating the cells in serum-free medium for 4 days. Compounds were then added to the cells and incubated for 6 h. Glycerol levels in the medium were measured according to the manufacturer's instruction (Zen-Bio Inc., Research Triangle Park, NC, USA).

**Human PPARγ-binding assay**

The assay was performed as previously described (Adams et al. 2003). Briefly, Human PPARγ receptor was expressed as glutathione-s-transferase (GST) fusion proteins in Escherichia coli. After purification, the GST–PPARγ protein was used to establish a scintillation proximity assay-based receptor binding assay.

**PPARγ –GAL4 chimeric transactivation assay**

The assay was performed as previously described (Berger et al. 1999). Briefly, COS-1 cells were seeded in 96-well cell culture plates. After 24 h, pcDNA3–PPARγ–GAL4 (galactose inducible transcription factor 4) expression vector, pUAS(5X)-tk-luc reporter vector, and pCMV-lacZ as an internal control were transfected. The cells were then incubated for ~48 h in medium containing test compound. Cell lysates were produced and luciferase activity in cell extracts was determined.

**Statistical analysis**

The indicated data are presented as means ± S.D. Statistical comparisons were performed between experimental groups and control group using Student t-test, with P<0.05 (*) designated as significant. All the data presented are based on triplicate experiments (n = 3) unless otherwise stated.

**Results**

**Isoginkgetin enhances adiponectin production in adipocytes**

We were interested in identifying small molecules that up-regulate adiponectin production by adipocytes. We set up an adiponectin ELISA-based screen in 96-well plates to test a compound library composed of known drugs and natural products. A human liposarcoma cell line, LiSa, capable of differentiation under chemically defined culture conditions (Wäbisch et al. 2000) was used in the screening. After induction, LiSa cells express high levels of genes that are specific for differentiated adipocytes, such as PPARγ. The induced cells also functionally resemble differentiated adipocytes in response to insulin and lipolytic agents, rendering suitable as a human adipocyte model for drug screening. We first tested adiponectin production by LiSa cells after differentiation via measuring the secreted adiponectin in the culture medium using the ELISA. Several known PPARγ agonists in the library were among the hits identified during the screening, validating the screening strategy (Fig. 1).

One novel hit was isoginkgetin, a natural bioflavone compound derived from the leaves of G. biloba (Fig. 2A). Isoginkgetin enhanced adiponectin production in a dose-dependent manner in both LiSa cells (Fig. 2B) and mouse adipocyte 3T3-L1 cells (Fig. 2C). The potency (EC50 0.1–0.5 μM) of the up-regulation of adiponectin production by isoginkgetin was comparable to that of rosiglitazone, an anti-diabetic drug. However, unlike rosiglitazone, isoginkgetin did not increase the level of leptin, another well-known adipocyte-produced hormone (data not shown), indicating the specific effects of isoginkgetin on adiponectin production.

**Isoginkgetin does not enhance adipogenesis**

Although rosiglitazone is marketed as an insulin sensitizer, it causes weight gain due to its adipogenesis-enhancing property, rendering it undesirable. To determine whether isoginkgetin also promotes adipogenesis, oil-red O staining was used to measure lipid droplets accumulation in 3T3-L1 cells (Fig. 3A). While rosiglitazone greatly potentiated
adipogenesis, isoginkgetin only slightly induced lipid accumulation when compared with the vehicle control. Ap2, an adipocyte specific gene, has been used as a marker for adipogenesis since its expression increases upon differentiation. Taqman real-time RT-PCR analysis showed that ap2 expression was up-regulated by isoginkgetin treatment to much lesser levels than rosiglitazone treatment (Fig. 3B). Another marker of adipogenesis, lipoprotein lipase (LPL), was also tested similarly by Taqman analysis, and no effect on LPL expression was observed by isoginkgetin treatment (data not shown). Therefore, the effects of isoginkgetin on adipogenesis are significantly less than those of rosiglitazone.

Besides adipogenesis, lipolysis is another metabolic effect to be considered when studying compounds targeting adipocytes, since enhanced lipolysis causes elevated free fatty acid level in blood and increases the risk for insulin resistance. Contrary to a previous study reporting isoginkgetin-induced increase in lipolysis in 3T3-L1 cells (Dell’Agli & Bosisio 2002), we observed a slight decrease in lipolysis in isoginkgetin-treated LiSa cells (Fig. 4A) and 3T3-L1 cells (Fig. 4B). Rosiglitazone also showed similar effects as isoginkgetin on lipolysis (Fig. 4), consistent with others’ findings (Mayerson et al. 2002, McTernan et al. 2002). Thus, elevating plasma fatty acid level to further worsen insulin resistance should not be a concern for in vivo application of isoginkgetin.

Mechanisms of action of isoginkgetin and rosiglitazone seem to be different

We further investigated the mechanism of action of isoginkgetin by comparing its effects with those of adipogenesis, isoginkgetin only slightly induced lipid accumulation when compared with the vehicle control. Ap2, an adipocyte specific gene, has been used as a marker for adipogenesis since its expression increases upon differentiation. Taqman real-time RT-PCR analysis showed that ap2 expression was up-regulated by isoginkgetin treatment to much lesser levels than rosiglitazone treatment (Fig. 3B). Another marker of adipogenesis, lipoprotein lipase (LPL), was also tested similarly by Taqman analysis, and no effect on LPL expression was observed by isoginkgetin treatment (data not shown). Therefore, the effects of isoginkgetin on adipogenesis are significantly less than those of rosiglitazone.

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rosiglitazone. First, we examined the kinetics of adiponectin induction by the two compounds. Interestingly, while rosiglitazone increased adiponectin level as early as 24 h, reaching its maximal effect in 48 h, isoginkgetin acts with slower kinetics, sustaining the increase of extracellular adiponectin level during the course of 72 h (Fig. 5A). This apparent distinction in the effect kinetics suggests potential different mechanisms of actions between these two compounds. Second, when we combined these two compounds in treatment, an additive effect on adiponectin production was observed (Fig. 5B).

It has been reported that PPARγ agonists elevate both adiponectin expression and secretion (Maeda et al. 2001). We were curious about how isoginkgetin promotes adiponectin production. Taqman real-time RT-PCR and western blot analysis were performed to measure adiponectin transcript and protein levels in LiSa cells (Fig. 6). Though isoginkgetin stimulated medium adiponectin level to a similar extent as rosiglitazone based on ELISA (Fig. 6A), the increase in adiponectin transcripts (Fig. 6B) and intracellular adiponectin protein levels (Fig. 6C) induced by isoginkgetin was significantly lower than that induced by rosiglitazone. These data suggest that the mechanisms by which isoginkgetin and rosiglitazone regulate adiponectin production at the transcript, protein and the secretory levels might be different. It appears that the regulation on adiponectin production by isoginkgetin can be attributed, at least partially, to transcriptional modification. The difference between adiponectin gene transcript and protein expression could be due to post-translational modification. However, the difference between the protein expression and medium adiponectin level suggests that isoginkgetin also regulates adiponectin secretion.

**Isoginkgetin is a weak PPARγ agonist**

Since it is known that PPARγ activation can increase adiponectin levels and our data suggest that isoginkgetin also up-regulates adiponectin production, we tested whether isoginkgetin is also a PPARγ agonist. Our data by human PPARγ-binding assay demonstrated that isoginkgetin binds to PPARγ with EC50 of 0.125 μM (Table 1), approximately...
Isoginkgetin enhances adiponectin secretion

Figure 5 Time course and combination effect on adiponectin production induced by isoginkgetin and rosiglitazone. (A) Time courses of adiponectin production mediated by isoginkgetin and rosiglitazone in LiSa cells. Either 1 μM isoginkgetin or 1 μM rosiglitazone was incubated with 6-day-differentiated LiSa cells for indicated time, and medium adiponectin levels were assayed. (B) Additive effect of isoginkgetin and rosiglitazone on adiponectin level. Isoginkgetin (Iso) and rosiglitazone (Rosi) were applied either alone (0.5 μM or 1 μM) or in combination (0.5 μM/0.5 μM, 1 μM/1 μM) to the differentiated LiSa cells for 4 days. Medium adiponectin levels were determined by ELISA.

Discussion

The recent discovery of adiponectin and its role in metabolic syndrome has pointed to a new direction of therapeutics. The fact that adiponectin levels are inversely correlated with the degree of obesity and insulin resistance suggests the benefit of lifting plasma adiponectin levels. The feasibility of this approach has been demonstrated in various animal models (Shklyaev et al. 2003, Yamauchi et al. 2003a). We are interested in exploring approaches based on up-regulation of adiponectin using small molecules. At present, the only available small molecules with this property are the synthetic PPARγ agonists (Maeda et al. 2001, Combs et al. 2002). The PPARγ agonists, such as the drug Avandia (rosiglitazone), not only ameliorate insulin resistance but also cause weight gain...
which further increases the risk of type II diabetes and limits its application for diabetes. PPARα agonists are also being investigated for this application due to their weight loss effect in rodents (Liu et al. 2005), but the potency of these agonists are generally lower than PPARγ agonists.

In this study, we report a natural compound, isoginkgetin, up-regulating adiponectin production with potency comparable to that of rosiglitazone, but devoid of the potent adipogenesis-promoting effect of rosiglitazone. Isoginkgetin is one of the major active constituents in G. biloba extract, which has been used as an ancient Chinese remedy for its multiple biological activities, including anti-oxidant, anti-inflammatory, and anti-tumor activities (Yoshikawa et al. 1999, DeFeudis et al. 2003). The cellular signaling induced by this compound is largely unknown. This is the first study to reveal the potential insulin-sensitizing effects of isoginkgetin based on its regulation of adiponectin. Our observation of the effect of isoginkgetin on lipolysis is different from the results reported by Dell’Aglì & Bosio (2002). This could be due to the different experimental settings, such as incubation time. In addition, Dell’Aglì observed a bi-phase effect of isoginkgetin on lipolysis, an enhancement of lipolysis was only observed at 0.03–0.3 μM isoginkgetin, and the abolishment of this effect is not explainable by cytotoxicity. Therefore, more detailed studies under different experimental conditions need to be carried out to address this distinction.

The mechanism of isoginkgetin action is apparently distinct from that of rosiglitazone for a number of reasons. First, inhibition of PPARγ activity did not affect the effect of isoginkgetin, whereas the activity of rosiglitazone was significantly diminished. Second, isoginkgetin and rosiglitazone exhibited differential stimulation kinetics. Third, results on the adiponectin mRNA and protein levels suggest the differential regulation of adiponectin synthesis and secretion by these two compounds. Fourth, although both compounds have comparable effects on adiponectin production, they have differential effects on adipogenesis and PPARγ activation.

Regulation of adiponectin production seems complex and has not been fully understood. Hormones and cytokines, including insulin, TNF-α, and interleukin-6, exhibited different effects on adiponectin production in vitro, while the signaling pathways mediating these regulations are largely unknown. The results that isoginkgetin potentiates adiponectin secretion in a likely PPARγ-independent manner suggest new types of targets and agents that can be explored for new therapy.

**Table 1** Comparison of the activities of isoginkgetin and rosiglitazone

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<th>Isoginkgetin</th>
<th>Rosiglitazone</th>
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<tr>
<td><strong>EC50 (μM)</strong></td>
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<tr>
<td>PPARγ binding</td>
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<td>0.25</td>
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<tr>
<td>PPARγ transcriptional activity</td>
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<td>0.02</td>
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<tr>
<td>Adiponectin production</td>
<td>0.1–0.5</td>
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PPARγ binding and PPARγ transcriptional activities were measured according to the methods described in Materials and Methods. The EC50 of adiponectin production was measured by ELISA using the conditioned medium from LiSα cells treated with the compound.

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**Figure 6** Isoginkgetin induces elevation in adiponectin transcript level. Either 1 μM IMS2545 or 1 μM rosiglitazone was incubated with differentiated LiSα cells for 4 days. The secreted adiponectin levels in the medium (A), the intracellular adiponectin mRNA levels (B), and the intracellular adiponectin protein levels (C) were measured. Non-treated LiSα cells were used as control (CNTL).
AMPK is a key regulator for energy metabolism in vivo. Activation of AMPK is involved in fat oxidation via the inhibition of downstream acetyl CoA carboxylase (ACC; Tomas et al. 2002). PPARγ agonists activate AMPK and inhibit ACC both in vitro and in vivo in skeletal muscle (Saha et al. 2004). Our data suggest that AMPK or genes upstream of the AMPK pathway maybe effectors of isoginkgetin action. Further experiments, for example introducing the kinase-dead AMPK into adipocytes to investigate its effect on isoginkgetin-regulated adiponectin production, would be helpful to confirm the involvement of AMPK. Whether isoginkgetin activates AMPK by increasing the ratio of AMP:ATP, as rosiglitazone does (Fryer et al. 2002a), needs to be further investigated. An interesting finding that inhibition of AMPK-reduced basal adiponectin production in differentiated adipocytes suggests the general role of AMPK in the regulation of adiponectin. Whatever the mechanism, the activation of AMPK and inhibition of phosphor-ACC by isoginkgetin will be beneficial for lipid regulation. Moreover, AMPK pathway can be further explored for adiponectin up-regulation.

In conclusion, isoginkgetin is a promising candidate compound for the treatment of insulin resistance based on our in vitro studies. Further in vivo studies are warranted. Analogs of isoginkgetin possessing higher potency in adiponectin production and lower PPARγ agonist activity can also be explored to identify more active compounds in vivo. Currently, insulin-sensitizing drugs mainly include PPARγ agonists and compounds that target the insulin signaling pathway. This study presents for the first time a potential new strategy for the discovery of insulin sensitizers.

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**Figure 7** The action of isoginkgetin is independent of PPARγ activities. (A and B) PPARγ activity is not required for isoginkgetin-regulated adiponectin secretion. Differentiated LiSa cells were treated with 1 µM isoginkgetin (Iso) or 1 µM rosiglitazone (Rosi) in the presence or absence of 500 µM BADGE. Four days later, intracellular adiponectin levels were analyzed by western blot (A). Adiponectin levels in the conditioned medium were analyzed by ELISA (B). Quantitative analysis of intracellular adiponectin expression was indicated in the table below the blot (A).

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<th>Adipo</th>
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<th>Iso</th>
<th>Iso+B</th>
<th>Rosi</th>
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<tr>
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<td>1.42</td>
<td>1.29</td>
<td>1.85</td>
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</table>

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**Figure 8** AMPK is involved in adiponectin production. (A) Isoginkgetin up-regulates phospho-AMPK expression. Whole cell lysates from undifferentiated LiSa cells (un-diff), and differentiated LiSa cells treated with DMSO vector control (Cntl), 1 µM isoginkgetin (Iso), or 1 µM rosiglitazone (Rosi) were blotted with antibodies that recognizes Thr-172-phosphorylated AMPK. GAPDH blotting demonstrated equal protein loading. (B) AMPK inhibitor Compound C inhibited isoginkgetin- or rosiglitazone-induced adiponectin secretion. Differentiated LiSa cells were incubated with 2.5 µM isoginkgetin (Iso) or 2.5 µM rosiglitazone (Rosi), in the presence or absence of 20 µM Compound C (C) for 4 days. Adiponectin levels in the medium were measured by ELISA. Differentiated LiSa cells without any compound treatment were used as control (Cntl).
by screening adiponectin production. Moreover, the critical genes involved in isoginkgetin action may provide novel targets for anti-diabetic therapy.

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References


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