

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

Guohong Liu, Mirta Grifman, James Macdonald, Peter Moller¹, Flossie Wong-Staal and Qi-Xiang Li

Immusol Inc., 10790 Roselle Street, San Diego, California 92121, USA

¹Department of Pathology, University of Ulm, Albert-Einstein-Allee 11, D-89081 Ulm, Germany

(Correspondence should be addressed to Q-X Li; Email: li@immusol.com)

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.

Journal of Endocrinology (2007) **194**, 569–578

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 (TNF α), 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 $\mu\text{g}/\text{ml}$; 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, Shklyav *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 PPAR γ 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 PPAR γ 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 μ M dexamethasone, 1 μ g/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 μ M dexamethasone, and 1 μ g/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 μ M, 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}-CCCAACATGCCCATTC-GCTTACCA-BHQ3'; forward primer: 5'^{GTGTGGGAT-TGGAGACTTACGTTAC3'}; reverse primer: 5'^{TAGT-GGTTTTGCTGATTGTAGAAGATC3'}. The sequences for ap2 gene are: probe: 5'^{FAM}-TCATGAAAGGCGT-CACTTCCACGAGA-BHQ3'; forward primer: 5'^{AT-GATAAACTGGTGGTGAATGC3'}; reverse primer:

5'CGTCCCTGGCTTATGCTCTC3'. Sequences for LPL gene are: probe: 5'-FAM-CATACATTCCTGTTA-CCGTCCAGCCAT-BHQ-3'; forward primer: 5'-CAG-CAAAACCTTCATGGTGAT-3'; reverse primer: 5'-CAAGTTTGGCACCCAACCTC-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 PPAR γ -binding assay

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

PPAR γ -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-PPAR γ -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 \pm 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 (Wabitsch *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 PPAR γ . 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 PPAR γ 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

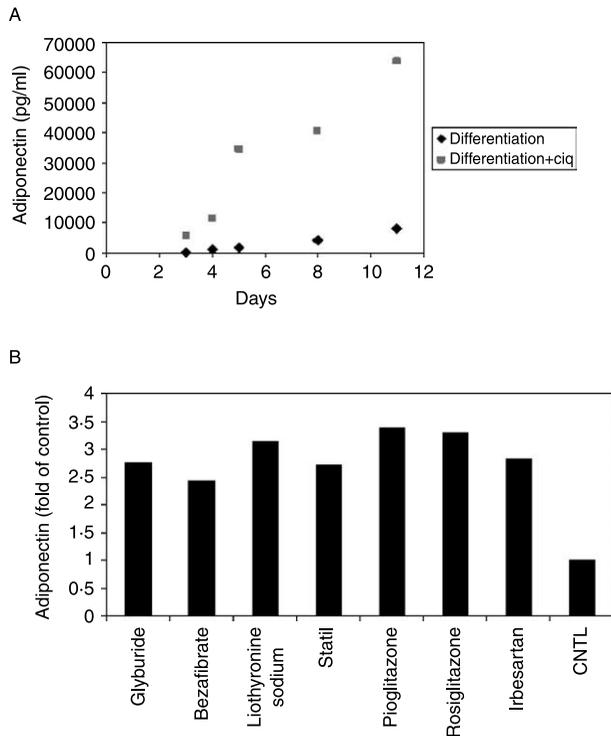


Figure 1 ELISA-based screening to identify adiponectin up-regulating compounds. (A) Human adipocyte cell line LiSa cells were differentiated with or without a PPAR γ agonist, ciglitazone. Adiponectin levels in the conditioned medium were measured by ELISA. (B) Compound library screening identified several PPAR regulators that are known to up-regulate adiponectin levels in adipocytes.

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

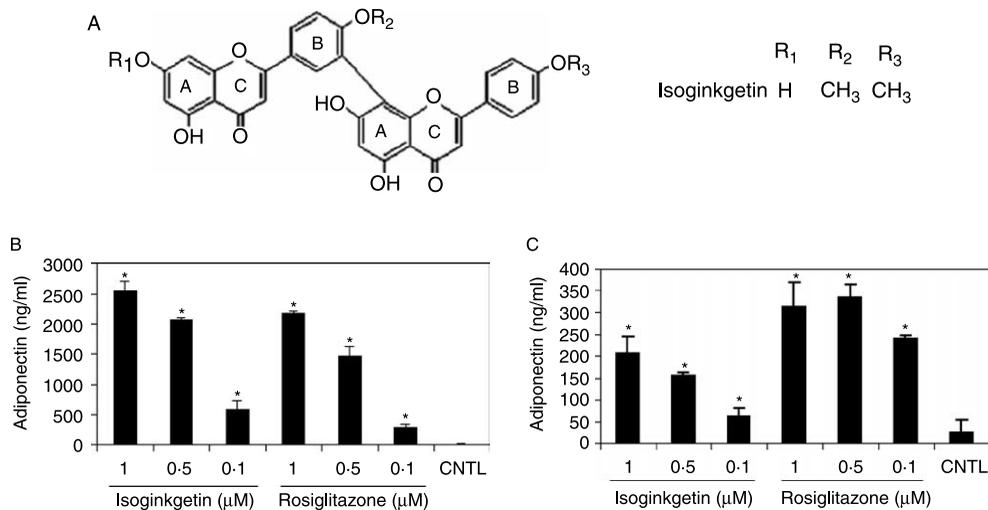


Figure 2 Effect of isoginkgetin on adiponectin production in human adipocyte LiSa cells and mouse adipocyte 3T3-L1 cells. Structure of isoginkgetin, which belongs to the biflavone compound family (A). Isoginkgetin up-regulated adiponectin secretion in LiSa cells (B) and 3T3-L1 cells (C). Different concentrations of isoginkgetin or rosiglitazone were incubated with LiSa or 3T3-L1 cells for 4 days before the assay of adiponectin level.

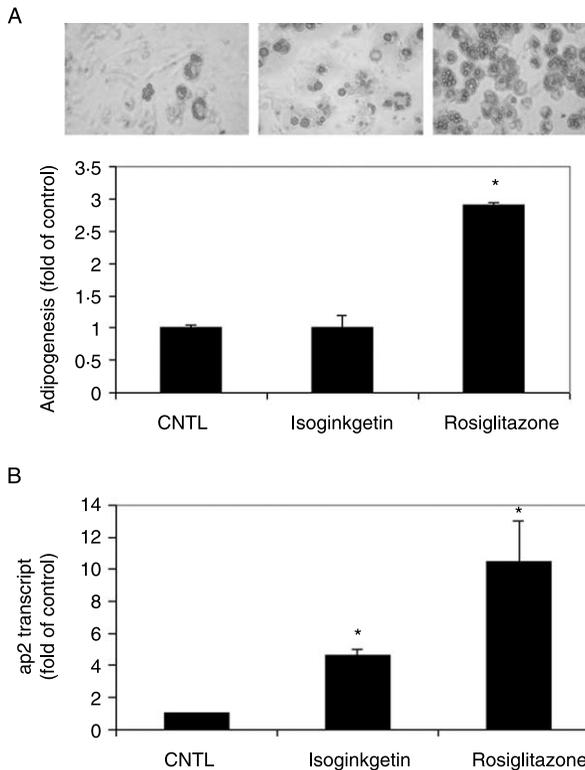


Figure 3 Effect of isoginkgetin on adipogenesis. (A) Effect of isoginkgetin and rosiglitazone on lipid droplets accumulation in 3T3-L1 cells. Compounds or DMSO control were incubated with 3T3-L1 cells for 3 days. The lipid droplets accumulation in the cells were stained with oil-red O (upper), and quantitatively measured (lower). (B) Effect of isoginkgetin and rosiglitazone on ap2 transcripts. Either 1 μ M isoginkgetin or 1 μ M rosiglitazone was incubated with LiSa cells for 3 days. The cellular ap2 mRNA levels were measured by TaqMan real-time RT-PCR.

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 PPAR γ 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

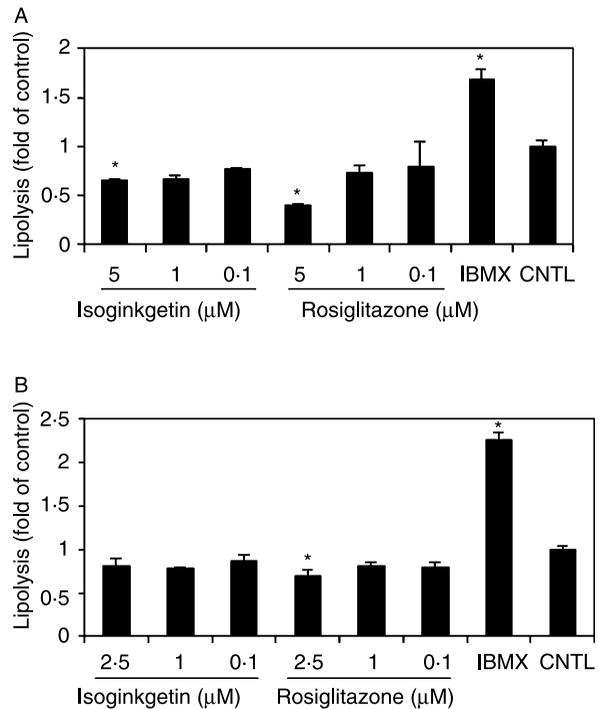


Figure 4 Effect of isoginkgetin and rosiglitazone on lipolysis in (A) LiSa cells and (B) 3T3-L1 cells. Differentiated cells were starved in serum-free medium for 4 days followed by incubation with indicated compounds for 6 h. Glycerol levels in the medium were measured and designated as lipolysis level. Cells treated with DMSO only were used as control (CNTL). Known lipolysis stimulator IBMX was used as positive control.

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 PPAR γ agonist

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

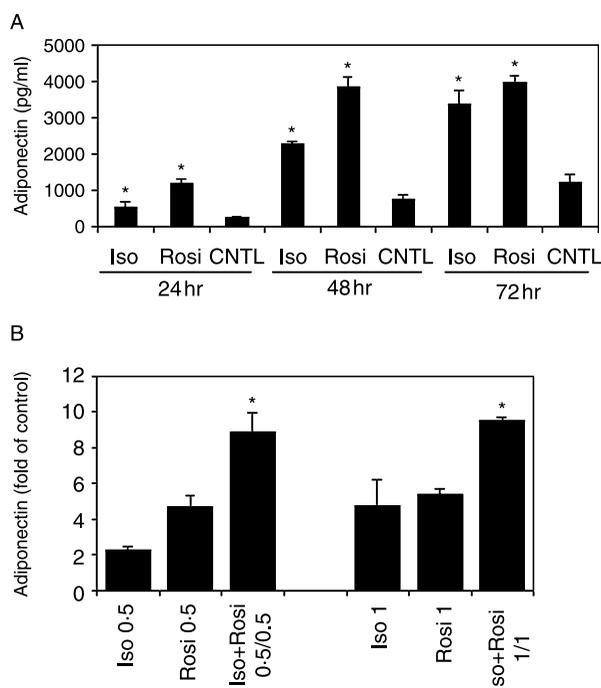


Figure 5 Time course and combination effect on adiponectin production induced by isoginkgetin and rosiglitazone. (A) Time courses of adiponectin production mediated by isoginkgetin and rosiglitazone in LiSa cells. Either 1 μ M isoginkgetin or 1 μ M rosiglitazone was incubated with 6-day-differentiated 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 μ M or 1 μ M) or in combination (0.5 μ M/0.5 μ M, 1 μ M/1 μ M) to the differentiated LiSa cells for 4 days. Medium adiponectin levels were determined by ELISA.

one half of the affinity of rosiglitazone binding to PPAR γ (EC_{50} of 0.25 μ M; Liu *et al.* 2005). In a chimeric hPPAR γ -GAL4 transactivation assay in COS-1 cells, isoginkgetin weakly activated PPAR γ with only 43% maximal activity at 10 μ M (Table 1), while rosiglitazone acted as a potent PPAR γ agonist with EC_{50} of 0.02 μ M (Liu *et al.* 2005). According to the data on these two experiments, isoginkgetin is a poor PPAR γ agonist despite its ability to bind to PPAR γ . Considering that isoginkgetin enhanced extracellular adiponectin production (EC_{50} of 0.1–0.5 μ M) at concentrations much lower (20- to 100-folds less) than that required for PPAR γ activation, it is unlikely that activation of PPAR γ by isoginkgetin contributed significantly to the observed elevated adiponectin production.

To confirm the above hypothesis, we examined whether the weak PPAR γ agonist activity of isoginkgetin plays any role in its effect on adiponectin secretion. A PPAR γ antagonist bisphenol A diglycidyl ether (BADGE) was used to treat LiSa cells. A volume of 500 μ M BADGE blocked the effect of rosiglitazone on medium and intracellular adiponectin production, but did not inhibit the action of

isoginkgetin, indicating that PPAR γ activity is not required for the up-regulation of adiponectin by isoginkgetin (Fig. 7A and B). These observations further confirmed that the two compounds have distinct mechanisms.

The AMPK is activated by isoginkgetin, and the AMPK pathway is apparently involved in adiponectin production

AMPK is one of the signaling molecules that were suggested to be involved in the anti-diabetic effect of rosiglitazone (Fryer *et al.* 2002a). In adipocytes, AMPK activity is crucial for fatty acid oxidation. To examine whether isoginkgetin also targets AMPK activity, the levels of phosphorylated AMPK at Thr 172 were assessed by western blot. AMPK activity appears to be significantly reduced upon cell differentiation (Fig. 8A). Isoginkgetin and rosiglitazone both enhanced the phosphorylation of AMPK in differentiated LiSa cells compared with dimethyl sulphoxide (DMSO)-treated cells (Fig. 8A). Thus, it seems one of the possible signaling molecules involved in isoginkgetin action is AMPK.

On the other hand, the observed phosphorylation of AMPK might also be induced by the increased adiponectin level in the medium because adiponectin itself can increase AMPK activity in liver, skeletal muscle cells, and adipocytes (Tomas *et al.* 2002, Yamauchi *et al.* 2002, Wu *et al.* 2003) and AdipoR1 expression was detected in LiSa cells (data not shown). To examine whether the AMPK activation is involved in adiponectin production, LiSa cells were treated with AMPK inhibitor, compound C. Compound C has been a well-studied AMPK inhibitor decreasing the level of phospho-AMPK (Zhou *et al.* 2001, Fryer *et al.* 2002b, Inoki *et al.* 2003, Lee *et al.* 2003). As shown in Fig. 8B, adiponectin production was significantly blocked by compound C (20 μ M), suggesting a direct involvement of AMPK in the isoginkgetin, as well as rosiglitazone, -induced adiponectin production. Since the AMPK inhibitor overrides the isoginkgetin effect, it likely functions downstream of the molecular target of isoginkgetin in this novel adiponectin regulation pathway.

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 PPAR γ agonists (Maeda *et al.* 2001, Combs *et al.* 2002). The PPAR γ agonists, such as the drug Avandia (rosiglitazone), not only ameliorate insulin resistance but also cause weight gain

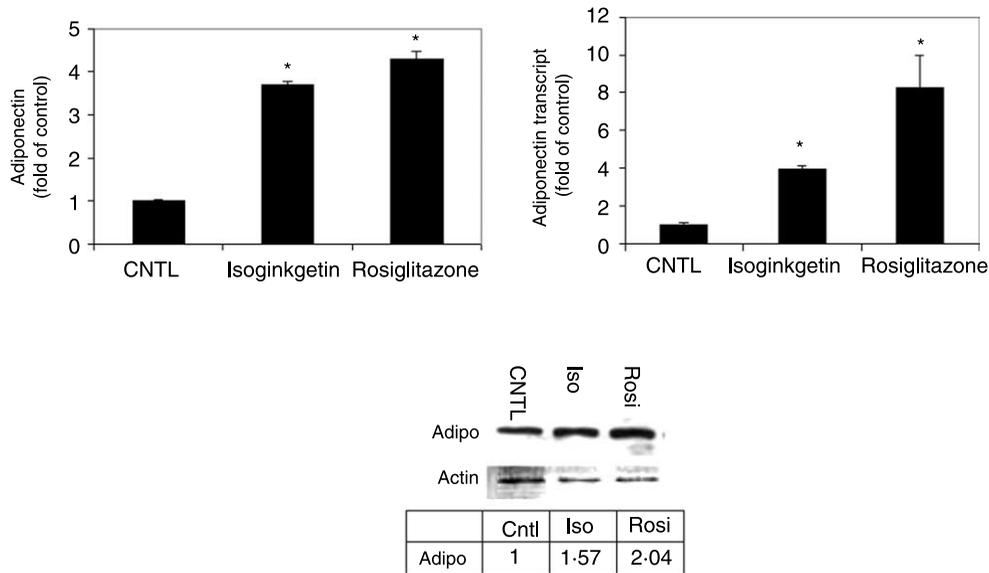


Figure 6 Isoginkgetin induces elevation in adiponectin transcript level. Either 1 μ M IMS2545 or 1 μ M rosiglitazone was incubated with differentiated LiSa cells for 4 days. The secreted adiponectin levels in the medium (A), the intracellular adiponectin mRNA levels (B), and the intracellular adiponectin protein levels (C) were measured. Non-treated LiSa cells were used as control (CNTL).

which further increases the risk of type II diabetes and limits its application for diabetes. PPAR α 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 PPAR γ 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'Agli & Bosisio (2002). This could be due to the different experimental settings, such as incubation time. In addition, Dell'Agli observed a bi-phase effect of isoginkgetin on lipolysis, an enhancement of lipolysis was only observed at 0.03–0.3 μ M 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 PPAR γ 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 PPAR γ 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 PPAR γ -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

	Isoginkgetin	Rosiglitazone
EC ₅₀ (μ M)		
PPAR γ binding	0.125	0.25
PPAR γ transcriptional activity	~10	0.02
Adiponectin production	0.1–0.5	0.1–0.5

PPAR γ binding and PPAR γ 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 LiSa cells treated with the compound.

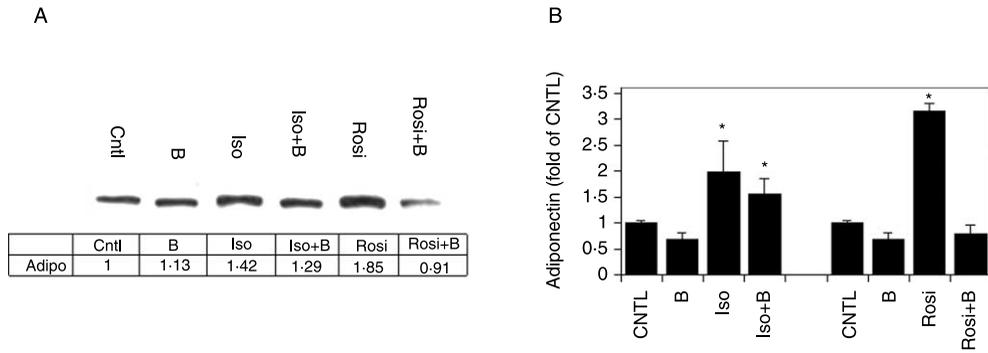


Figure 7 The action of isoginkgetin is independent of PPAR γ activities. (A and B) PPAR γ activity is not required for isoginkgetin-regulated adiponectin secretion. Differentiated LiSa cells were treated with 1 μ M isoginkgetin (Iso) or 1 μ M rosiglitazone (Rosi) in the presence or absence of 500 μ M 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).

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). PPAR γ 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 phospho-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 PPAR γ agonist activity can also be explored to identify more active compounds *in vivo*. Currently, insulin-sensitizing drugs mainly include PPAR γ 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

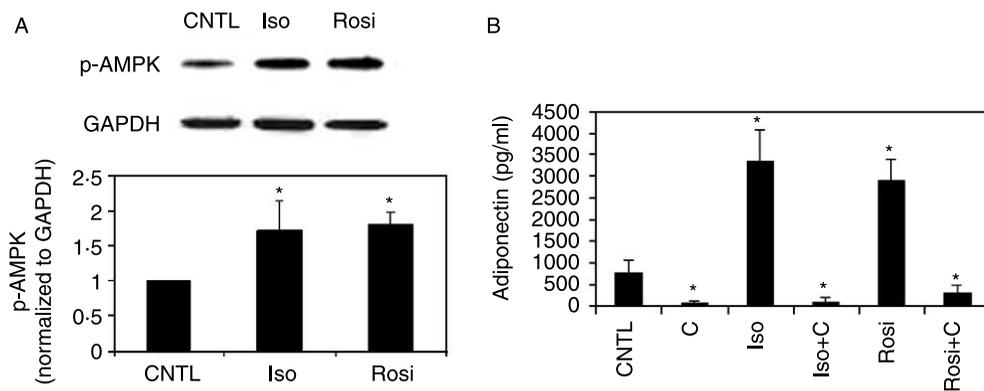


Figure 8 AMPK is involved in adiponectin production. (A) Isoginkgetin up-regulates phospho-AMPK expression. Whole cell lysates from undifferentiated LiSa cells (un-diff), and differentiated LiSa cells treated with DMSO vector control (CNTL), 1 μ M isoginkgetin (Iso), or 1 μ M rosiglitazone (Rosi) were 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 μ M isoginkgetin (Iso) or 2.5 μ M rosiglitazone (Rosi), in the presence or absence of 20 μ M 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.

Acknowledgements

The authors would like to thank Dr Xiuyuan Hu for useful suggestions and Ben Keilly, Rebecca Lynn, Reeti Desai and Jeremy To for technical assistance. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Funding

This work was funded wholly by the Immusol R&D budget.

References

- Adams AD, Yuen W, Hu Z, Santini C, Jones AB, MacNaul KL, Berger JP, Doebber TW & Moller DE 2003 Amphipathic 3-phenyl-7-propylbenzoxazoles; human pPAR gamma, delta and alpha agonists. *Bioorganic and Medicinal Chemistry Letters* **13** 931–935.
- Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K *et al.* 1999 Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochemical and Biophysical Research Communication* **257** 79–83.
- Berger J, Leibowitz MD, Doebber TW, Elbrecht A, Zhang B, Zhou G, Biswas C, Cullinan CA, Hayes NS, Li Y *et al.* 1999 Novel peroxisome proliferator-activated receptor (PPAR) gamma and PPARdelta ligands produce distinct biological effects. *Journal of Biological Chemistry* **274** 6718–6725.
- Bogan JS & Lodish HF 1999 Two compartments for insulin-stimulated exocytosis in 3T3-L1 adipocytes defined by endogenous ACRP30 and GLUT4. *Journal of Cell Biology* **146** 609–620.
- Combs TP, Wagner JA, Berger J, Doebber T, Wang WJ, Zhang BB, Tanen M, Berg AH, O'Rahilly S, Savage DB *et al.* 2002 Induction of adipocyte complement-related protein of 30 kilodaltons by PPARgamma agonists: a potential mechanism of insulin sensitization. *Endocrinology* **143** 998–1007.
- DeFeudis FV, Papadopoulos V & Drieu K 2003 *Ginkgo biloba* extracts and cancer: a research area in its infancy. *Fundamental and Clinical Pharmacology* **17** 405–417.
- Dell'Agli M & Bosio E 2002 Biflavones of *Ginkgo biloba* stimulate lipolysis in 3T3-L1 adipocytes. *Planta Medica* **68** 76–79.
- Fasshauer M, Kralisch S, Klier M, Lossner U, Bluher M, Klein J & Paschke R 2003 Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. *Biochemical and Biophysical Research Communication* **301** 1045–1050.
- Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE & Lodish HF 2001 Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *PNAS* **98** 2005–2010.
- Fryer LG, Parbu-Patel A & Carling D 2002a The Anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. *Journal of Biological Chemistry* **277** 25226–25232.
- Fryer LG, Parbu-Patel A & Carling D 2002b Protein kinase inhibitors block the stimulation of the AMP-activated protein kinase by 5-amino-4-imidazolecarboxamide riboside. *FEBS Letters* **531** 189–192.
- Inoki K, Zhu T & Guan KL 2003 TSC2 mediates cellular energy response to control cell growth and survival. *Cell* **115** 577–590.
- Ke N, Albers A, Claassen G, Yu DH, Chatterton JE, Hu X, Meyhack B, Wong-Staal F & Li QX 2004 One-week 96-well soft agar growth assay for cancer target validation. *Biotechniques* **36** 826–828, 830, 832–823.
- Lee M, Hwang JT, Lee HJ, Jung SN, Kang I, Chi SG, Kim SS & Ha J 2003 AMP-activated protein kinase activity is critical for hypoxia-inducible factor-1 transcriptional activity and its target gene expression under hypoxic conditions in DU145 cells. *Journal of Biological Chemistry* **278** 39653–39661.
- Liu K, Xu L, Berger JP, MacNaul KL, Zhou G, Doebber TW, Forrest MJ, Moller DE & Jones AB 2005 Discovery of a novel series of peroxisome proliferator-activated receptor alpha/gamma dual agonists for the treatment of type 2 diabetes and dyslipidemia. *Journal of Medicinal Chemistry* **48** 2262–2265.
- Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N *et al.* 2001 PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes* **50** 2094–2099.
- Maeda N, Shimomura I, Kishida K, Nishizawa H, Matsuda M, Nagaretani H, Furuyama N, Kondo H, Takahashi M, Arita Y *et al.* 2002 Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. *Nature Medicine* **8** 731–737.
- Mayerson AB, Hundal RS, Dufour S, Lebon V, Befroy D, Cline GW, Enocksson S, Inzucchi SE, Shulman GI & Petersen KF 2002 The effects of rosiglitazone on insulin sensitivity, lipolysis, and hepatic and skeletal muscle triglyceride content in patients with type 2 diabetes. *Diabetes* **51** 797–802.
- McTernan PG, Harte AL, Anderson LA, Green A, Smith SA, Holder JC, Barnett AH, Eggo MC & Kumar S 2002 Insulin and rosiglitazone regulation of lipolysis and lipogenesis in human adipose tissue *in vitro*. *Diabetes* **51** 1493–1498.
- Mora S & Pessin JE 2002 An adipocentric view of signaling and intracellular trafficking. *Diabetes Metabolism Research and Reviews* **18** 345–356.
- Qi Y, Takahashi N, Hileman SM, Patel HR, Berg AH, Pajvani UB, Scherer PE & Ahima RS 2004 Adiponectin acts in the brain to decrease body weight. *Nature Medicine* **10** 524–529.
- Saha AK, Avilucea PR, Ye JM, Assifi MM, Kraegen EW & Ruderman NB 2004 Pioglitazone treatment activates AMP-activated protein kinase in rat liver and adipose tissue *in vivo*. *Biochemical and Biophysical Research Communication* **314** 580–585.
- Shklyav S, Aslanidi G, Tennant M, Prima V, Kohlbrenner E, Kroutov V, Campbell-Thompson M, Crawford J, Shek EW, Scarpace PJ *et al.* 2003 Sustained peripheral expression of transgene adiponectin offsets the development of diet-induced obesity in rats. *PNAS* **100** 14217–14222.
- Son JK, Son MJ, Lee E, Moon TC, Son KH, Kim CH, Kim HP, Kang SS & Chang HW 2005 Ginkgetin, a Biflavone from Ginkgo biloba leaves, inhibits cyclooxygenases-2 and 5-lipoxygenase in mouse bone marrow-derived mast cells. *Biological and Pharmaceutical Bulletin* **28** 2181–2184.
- Stefan N, Vozarova B, Funahashi T, Matsuzawa Y, Weyer C, Lindsay RS, Youngren JF, Havel PJ, Pratley RE, Bogardus C *et al.* 2002 Plasma adiponectin concentration is associated with skeletal muscle insulin receptor tyrosine phosphorylation, and low plasma concentration precedes a decrease in whole-body insulin sensitivity in humans. *Diabetes* **51** 1884–1888.
- Tomas E, Tsao TS, Saha AK, Murrey HE, Zhang Cc C, Itani SI, Lodish HF & Ruderman NB 2002 Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation. *PNAS* **99** 16309–16313.
- Wabitsch M, Bruderlein S, Melzner I, Braun M, Mechttersheimer G & Moller P 2000 LiSa-2, a novel human liposarcoma cell line with a high capacity for terminal adipose differentiation. *International Journal of Cancer* **88** 889–894.
- Wu X, Motoshima H, Mahadev K, Stalker TJ, Scalia R & Goldstein BJ 2003 Involvement of AMP-activated protein kinase in glucose uptake stimulated by the globular domain of adiponectin in primary rat adipocytes. *Diabetes* **52** 1355–1363.
- Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K *et al.* 2002 Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nature Medicine* **8** 1288–1295.

- Yamauchi T, Kamon J, Waki H, Imai Y, Shimozawa N, Hioki K, Uchida S, Ito Y, Takakuwa K, Matsui J *et al.* 2003a Globular adiponectin protected ob/ob mice from diabetes and ApoE-deficient mice from atherosclerosis. *Journal of Biological Chemistry* **278** 2461–2468.
- Yamauchi T, Kamon J, Ito Y, Tsuchida A, Yokomizo T, Kita S, Sugiyama T, Miyagishi M, Hara K, Tsunoda M *et al.* 2003b Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. *Nature* **423** 762–769.
- Yoshikawa T, Naito Y & Kondo M 1999 *Ginkgo biloba* leaf extract: review of biological actions and clinical applications. *Antioxidants and Redox Signaling* **1** 469–480.
- Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N *et al.* 2001 Role of AMP-activated protein kinase in mechanism of metformin action. *Journal of Clinical Investigation* **108** 1167–1174.

Received in final form 6 June 2007

Accepted 10 June 2007

Made available online as an Accepted Preprint

21 June 2007