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Essential roles of insulin, AMPK signaling and lysyl and prolyl hydroxylases in the biosynthesis and multimerization of adiponectin

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Abstract

Post-translational modifications (PTMs) of the adiponectin molecule are essential for its full bioactivity, and defects in PTMs leading to its defective production and multimerization have been linked to the mechanisms of insulin resistance, obesity, and type-2 diabetes. Here we observed that, in differentiated 3T3-L1 adipocytes, decreased insulin signaling caused by blocking of insulin receptors (InsR) with an anti-InsR blocking antibody, increased rates of adiponectin secretion, whereas concomitant elevations in insulin levels counteracted this effect. Adenosine monophosphate-activated protein kinase (AMPK) signaling regulated adiponectin production by modulating the expression of adiponectin receptors, the secretion of adiponectin, and eventually the expression of adiponectin itself. We found that lysyl hydroxylases (LHs) and prolyl hydroxylases (PHs) were expressed in white-adipose tissue of *ob/ob* mice, wherein LH3 levels were increased compared with controls. In differentiated 3T3-L1 adipocytes, both non-specific inhibition of LHs and PHs by dipyridyl, and specific inhibition of LHs by minoxidil and of P4H with ethyl-3,4-dihydroxybenzoate, caused significant suppression of adiponectin production, more particularly of the higher-order isoforms. Transient gene knock-down of LH3 (*Plod3*) caused a suppressive effect, especially on the high molecular-weight (HMW) isoforms. These data indicate that PHs and LHs are both required for physiological adiponectin production and in particular are essential for the formation/secretion of the HMW isoforms.

Highlights:

- Decreased insulin signaling stimulates adiponectin production, whereas insulin itself exerts powerful suppressive effects on adiponectin production.
- Adiponectin negatively regulates its production through binding to its receptors and activating the AMPK signaling pathway.
- Both lysyl and prolyl hydroxylases are required for adiponectin production and multimerization, for which both the hydroxylation/glycosylation of lysyl and hydroxylation of prolyl residues are essential for the formation and secretion of the higher-order adiponectin isoforms.

Key words

Adiponectin; Adipose tissue; Biosynthesis; Hormones; Molecular mechanisms; Post-translational modification.

1. Introduction

Levels of adiponectin, the most abundant adipokine present in human serum, have been inversely correlated with insulin resistance, obesity, and type-2 diabetes in many studies (Arita, et al. 1999; Hotta, et al. 2000). Weight loss, caloric restriction, or thiazolidinedione (TZD) treatment increase adiponectin levels, and such increase associates with increased insulin sensitivity (Bobbert, et al. 2005; Combs, et al. 2003; Li, et al. 2007; Maeda, et al. 2001). Therefore, the adiponectin pathway may be a promising candidate for the development of drugs to treat type-2 diabetes and other obesity-related diseases in which insulin resistance plays a significant pathogenic role. Adiponectin enhances insulin sensitivity in both skeletal muscle and liver (Yamauchi, et al. 2002). Its insulin sensitizing effect is due primarily to its ability to activate AMPK (Wu, et al. 2003; Yamauchi et al. 2002) via two distinct receptors: Adipor1 and Adipor2 (Yamauchi, et al. 2003; Yamauchi, et al. 2007). Impaired adiponectin signaling caused by decreased expression of the hormone itself or of its receptors, leads to insulin resistance (Iwabu, et al. 2010; Kadowaki, et al. 2006; Yamauchi et al. 2007).

Current knowledge of the regulation of adiponectin expression and secretion is far from complete. Adiponectin synthesis is said to be regulated at several levels through multiple mechanisms (Halberg, et al. 2009; Liu and Liu 2010; Wang, et al. 2008). Though the relationship between plasma insulin and adiponectin levels has been studied extensively, the exact role of insulin in adiponectin biosynthesis and secretion remains uncertain and the reported effects of insulin on adiponectin regulation are equivocal. While insulin has been reported to increase adiponectin mRNA expression in mature human adipocytes (Hajri, et al. 2011) and in a brown-adipocyte cell line (Viengchareun, et al. 2002), insulin reportedly decreased adiponectin mRNA expression in 3T3-L1 adipocytes (Fasshauer, et al. 2002); intensifying glycemic control with insulin also reportedly lowered adiponectin in type-2 diabetic patients (Abi Khalil, et al. 2011). On the other hand, adiponectin secretion was reportedly enhanced in adipocytes from mice with fat-specific disruption of the *Insr* gene (Bluher, et al. 2002), and in patients with a genetically-defective InsR when compared with healthy controls (Semple, et al. 2006).

Adiponectin is secreted from adipocytes into the circulation as three classes of oligomers: the low molecular-weight (LMW, trimers), middle molecular-weight (MMW, hexamers), and HMW (12-18 monomers) isoforms (Pajvani, et al. 2003; Tsao, et al. 2002; Waki, et al. 2003). Different adiponectin oligomers elicit distinct biological responses in different target tissues: furthermore, it has been reported that the HMW isoforms are more metabolically active and closely associated with peripheral insulin sensitivity (Pajvani et al. 2003; Pajvani, et al. 2004; Wang et al. 2008). The proportions of the oligomers change according to metabolic status and in various disease states. In patients with type-2 diabetes, the serum levels of HMW adiponectin are selectively decreased (Basu, et al. 2007). Impaired multimerization of adiponectin reportedly occurs when levels are decreased as in obesity and insulin resistance (Pajvani et al. 2004; Waki et al. 2003). Therefore, oligomerization represents a key mechanism that regulates several activities of adiponectin.

However, until now, factors contributing to adiponectin oligomerization have remained obscure. Structurally, adiponectin belongs to the complement C1q-like protein family, whose members consist of an NH_2-terminal signal peptide, a short hyper-variable region, a collagenous domain composed of sequential Gly-*X*-*Y* repeats, and a COOH-terminal globular-head domain homologous to that of C1q itself (Hu, et al. 1996; Scherer, et al. 1995). A number of conserved prolyl residues exist within the collagenous domain, which can undergo hydroxylation. Several conserved lysyl residues are also present within both the collagenous and variable domains and these can undergo hydroxylation with subsequent glycosylation (Wang, et al. 2006; Wang, et al. 2004). Our laboratory has previously shown that, in order for adiponectin to display full bioactivity, it requires PTMs of several lysyl residues in its collagenous domain, which are hydroxylated and glycosylated in the mature protein. Defective lysyl hydroxylation and glycosylation impairs adiponectin production and multimerization into the HMW isoforms that are required for expression of its full bioactivity (Wang, et al. 2006).

Here, we aimed to further characterize the processes and pathways in white adipocytes that are reportedly responsible for the posttranslational modification and multimerization of adiponectin, with emphasis on factors that may also regulate the production of these PTMs and the biosynthesis of biologically active adiponectin.

2. Materials and methods

This study was approved by the University of Auckland Animal Ethics Committee. Its performance was consistent with principles described in the Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals 2011), and the ARRIVE guidelines for the reporting of animal research (Kilkenny, et al. 2010).

Chemical reagents were of analytical grade and obtained from Sigma, unless otherwise stated.

2.1. *Mice*- *Ob/ob* mice and age-matched C57/BL littermates (*non-obese controls*) controls were obtained from the Animal Physiology Unit at the School of Biological Sciences, University of Auckland. *Ob/ob* mice become obese through genetically-mediated deficiency of the hormone leptin. They were weaned at 3-weeks of age, and their bodyweight and blood glucose values monitored weekly thereafter until the end of the relevant observation periods. Glucose tolerance tests were performed by intra-peritoneal (i.p.) injection of 2 mg glucose/g-bodyweight into non-anesthetised mice after 12-hours' fasting: thereafter blood samples were taken from the tail-vein at time points of 0, 15, 30, 60 and 120 min. On a separate experimental day, mice were also tested for insulin sensitivity by i.p. injection of 0.5 mU soluble human insulin (Actrapid®; Novo Nordisk, Copenhagen, Denmark)/g-bodyweight and measurement of blood glucose (Advantage II; Roche, Basle, Switzerland). Animals were housed throughout in a temperature-controlled unit with a fixed 12-h light/dark cycle, and had *ad libitum* access to food (Teklad Rodent Chow 2018; Harlan Laboratories, IN) and tap-water. At either 10-weeks or 27-weeks of age, animals were anesthetized and blood collected by cardiac puncture. Serum levels of insulin, leptin, amylin and adiponectin were measured by using commercial ELISA kits (ALPCO Diagnostics, Salem, NH; or Millipore, St. Charles, MO). Epididymal white adipose tissue (WAT) was collected and stored either in Allprotect tissue reagent (QIAGEN Gmbh, Hilden, Germany) or at -80°C as required.

2.2. *Differentiation of 3T3-L1 cells*- Mouse-derived 3T3-L1 pre-adipocytes were maintained and differentiated as previously described (Wang, et al. 2002). Briefly, 3T3-L1 pre-adipocytes were maintained as sub-confluent cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. For differentiation, 2-days post-confluence, cells were incubated in differentiation medium (DMEM containing 10% fetal bovine serum (FBS), 0.25 µM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 µg/ml insulin) for 2 days. This was followed by incubation with fresh DMEM containing 10% FBS and 1 µg/ml insulin for another 2 days. Then cells were grown in DMEM containing 10% FBS only and medium was replaced every 2-3 days until lipid droplets were evident in most cells (8~11 days post differentiation).

2.3. *Effect of insulin and/or glucose on adiponectin production in the presence/absence of insulin-receptor antibody*- Eight to eleven days after differentiation, 3T3-L1 adipocytes were washed with phosphate-buffered saline (PBS) and incubated in serum-free DMEM containing 0.2% bovine serum albumin (BSA, low fatty-acid content) for 6-h. Then, the medium was changed to fresh DMEM containing 0.2% BSA (low fatty-acid content) with different concentrations of glucose (5 or 25 mM) and/or insulin (1 or 100 ng/ml) in the presence or absence of insulin-receptor antibody (Abcam, U.K.) for 24-h. The cells and media were then collected for further analysis.

2.4. *Effect of blocking and stimulating the AMPK signaling pathway on adiponectin production*- Fully-differentiated 3T3-L1 adipocytes were washed with PBS and incubated in serum-free DMEM containing 0.2% BSA (low fatty-acid content) with the AMPK inhibitor, compound C (CAS 866405-64-3; 50 μM) or the AMPK stimulator, AICAR (500 μM) for 4-h. The cells and media were collected after 4-h incubation for further analysis.

2.5. *Effect on adiponectin production of inhibition of prolyl hydroxylation and lysyl hydroxylation/glycosylation*- Fully-differentiated 3T3-L1 adipocytes were washed with PBS and incubated in serum-free DMEM containing 0.2% BSA (low fatty acid content) for 6-h. Media were changed to fresh DMEM containing: 0.2% BSA (low fatty acid content) and in addition rosiglitazone (an insulin sensitizer, 1 μM); and/or GW-9662 (a PPARγ antagonist, 1.5 μM); dipyridyl (a non-specific hydroxylase inhibitor, 1000 μM); ethyl-3,4-dihydroxybenzoate (a prolyl-4-hydroxylase inhibitor, 400 μM); or minoxidil (a lysyl hydroxylase inhibitor, 1000 μM), respectively. After 24-h incubation, media were changed to fresh DMEM containing the different treatments, and incubated for a further 24-h. Cells and media were then collected for analysis.

2.6. *Small interfering RNA (siRNA) construction and transfection*- Small interfering RNAs directed against lysyl hydroxylase 3 (*Plod3*) were commercially constructed by using the Silencer® select siRNA (Ambion Life Technologies, CA). Eight to eleven days after initiation of differentiation, 3T3-L1 adipocytes were re-plated in 24-well plates at a density of ~50×10^3 cells/well, and incubated overnight in DMEM containing 10% FBS. On the following day, cells were washed with PBS and transfected with siRNA (40 nM) by using DeliverX™ and DeliverX Plus siRNA transfection kit (Panomics, Fremont, CA) according to the manufacturer's instructions. A scrambled siRNA was used as a negative control and a siRNA against *Gapdh* was used as a positive control. Cells for RNA extraction were collected in RLT buffer (RNeasy® micro kits; QIAGEN, Valencia, CA) 24-h after transfection and stored at -80 °C until processing. The cells used for protein extraction were incubated in fresh serum-free DMEM containing 0.2% BSA (low fatty-acid content) for another 24-h; cells and media were then collected, processed and stored at -80 °C for further analysis.

2.7. *Reverse transcription and quantitative real time PCR*- Total RNA was purified from 3T3-L1 cell pellets by utilizing RNeasy® Micro Kit (QIAGEN), or from murine adipose tissue by using an RNeasy® Lipid Tissue Mini Kit (QIAGEN) according to the manufacturer's instructions. Complementary DNA was synthesized by using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Life Technologies, Auckland, New Zealand). Quantification of cDNA was performed by using a Quant-iT™ OliGreen ssDNA assay kit (Life Technologies). Amplification of each target cDNA was performed by using a Roche PCR Reagent Kit (Roche, IN) and quantified by using the Roche 4800 detection system according to the manufacturer's instructions. A combination of reference genes used were specifically determined for different type of samples by using geNorm (Vandesompele, et al. 2002) and Normfinder (Andersen, et al. 2004). Oligonucleotide primer pairs for specific genes employed are listed in Table 1.

2.8. *Protein extraction and Western Blotting*- Proteins from 3T3-L1 cells or murine tissue were extracted as described previously (Zhang, et al. 2008; Zhang, et al. 2013). Briefly, the cells or tissues were lysed in ice-cold lysis buffer (50 mmol/l Tris-HCl, pH 8; 150 mmol/l NaCl; 1% NP-40; 0.5% sodium deoxycholate; and 0.1% sodium dodecylsulfate) with a proteinase inhibitor cocktail (Roche). The cells were vortexed for 30-s and centrifuged at 4 °C × 13,000 *g* for 20-min, while homogenized tissue (TissueLyser, QIAGEN) was centrifuged for 1-h. Supernatants were collected and stored at -80 °C until use. Protein concentration in the supernatant was determined by bicinchoninic acid protein assay (Pierce, Rockford, IL). For adiponectin detection, non-reduced and non-heat-denatured protein samples were then separated by SDS-PAGE (Life Technologies) and transferred to PVDF or nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). For other proteins as indicated, reduced and heat-denatured samples were used. Powdered milk (5% (w/v)) was used to block nonspecific binding sites prior to incubation with primary antibody directed against each specific protein as indicated (antibodies

for adiponectin (ab3455), Glt25d2 and PDI from Abcam, UK; antibody for lysyl hydroxylase 3 from Santa Cruz Biotechnology, US). After incubation with secondary antibody (IgG–horseradish peroxidase conjugate, GE Healthcare, Buckinghamshire, UK), proteins were visualized by using ECL Prime Western Blotting detection reagents (GE Healthcare) according to the manufacturer's instructions. An LAS 4,000 image reader (Fuji Photo Film, Tokyo, Japan) was used to detect signals and take images of the membranes. Images were analyzed using Multi Gauge software (Fuji Photo Film).

2.9. *Statistical analysis*- Data are expressed as mean ± SE. Differences between means were analyzed using Student's *t*-test, or analysis of variance (ANOVA) with appropriate *post-hoc* tests. Statistical difference was considered significant at $P < 0.05$. Normality and equality of variance of samples were tested by applying the Shapiro-Wilk test or Spearman rank correlation, respectively. If either of these two tests failed, then Kruskal-Wallis one-way ANOVA with Dunn's multiple comparisons test was used for the statistical analysis; otherwise, one-way ANOVA with the *post-hoc* Holm-Sidak method was used for further statistical analysis.

3. Results

3.1. *Phenotypic characterization of ob/ob mice* Both 10- and 27-week-old *ob/ob* mice had increased bodyweight compared with age-matched controls (Table 2). Blood glucose values were elevated in 10-week *ob/ob* mice whereas 27-week mice had normal glucose levels, consistent with prior reports. Both age groups of *ob/ob* mice showed abnormal glucose tolerance and insulin sensitivity (Figure 1), and both had elevated serum insulin levels (Table 2). Interestingly, while 10-week *ob/ob* mice had increased serum adiponectin levels and correspondingly increased HMW-adiponectin levels compared with age-matched controls, 27-week *ob/ob* mice showed levels of adiponectin comparable with both age-matched controls and 10-week *ob/ob* mice. However, the HMW/total adiponectin ratio was increased in 27-week *ob/ob* mice compared with 10-week *ob/ob* mice (Table 2). Compared with 10-week control mice, 27-week control mice (Table 2) showed slight but significant elevations in serum insulin levels, accompanied by relatively impaired insulin sensitivity (Figure 1). In these 27-week controls, the increased HMW adiponectin, which was reflected by the increased HMW/total ratio, might contribute to the maintenance of normal blood glucose levels in spite of insulin resistance.

Although circulating levels of adiponectin were increased in 10-week *ob/ob* mice, total adiponectin protein levels in their white-adipose tissue were not significantly different from control values: however, their HMW-adiponectin expression was decreased, whereas levels of MMW- and LMW-isoforms were increased compared with age-matched controls (Figure 2). Taken together with the unchanged composition of the circulating adiponectin isoforms, we interpret these data to indicate that overall adiponectin production was up-regulated in the 10-week *ob/ob* mice: however, the extent of increase in the HMW isoforms was lower than that of the MMW and LMW isoforms. In these mice, secretion of the HMW isoforms could be greater than that of the other two isoforms, leading to the relative increase in plasma levels: alternatively, the clearance from the plasma of the HWM isoforms could be lower than those of the other two isoforms.

3.2. *Effects of hyperinsulinemia, hyperglycemia and/or impaired insulin signaling on adiponectin production* To determine the effects of hyperinsulinemia, hyperglycemia and/or decreased insulin signaling on adiponectin production, fully-differentiated 3T3-L1 adipocytes were incubated with different concentrations of glucose and insulin in the presence or absence of an insulin-receptor antibody that binds to the α-subunit of the receptor, thereby impairing the binding and downstream signaling of insulin (Figure 3). Under the condition of normal glucose (5 mM) and normal insulin (1 ng/ml) concentrations in the presence of the insulin-receptor antibody, 3T3-L1 adipocytes secreted increased amounts of adiponectin compared with those released in the absence of this antibody. High concentrations of glucose

(25 mM) further stimulated the increase in extracellular adiponectin: however, increased concentrations of insulin (100 ng/ml) counterbalanced this effect (Figure 3B). These results are consistent with reports that insulin decreased adiponectin expression in 3T3-L1 adipocytes (Fasshauer et al. 2002); and that mice with adipose tissue-specific disruption of the *Insr* gene (Bluher et al. 2002) and patients with a genetically-defective InsR (Semple et al. 2006), had increased circulating levels of adiponectin compared with matched controls. These results are also consistent with the observations in our *ob/ob* mice that the presence of insulin resistance and elevated insulin levels was accompanied by elevated/or unchanged serum levels of adiponectin compared with control values (Table 2 & Figure 1).

3.3. *Observations on the role of adiponectin receptors in the regulation of adiponectin production* In contrast to their increased or unchanged serum levels of adiponectin, the mRNA levels of adiponectin in the white-adipose tissue were decreased in both age groups of *ob/ob* mice compared with age-matched controls (Figure 4), consistent with posttranscriptional regulation. We also observed the abundant expression of adiponectin receptors (*Adipor1* & *Adipor2*) in WAT, and that *ob/ob* mice showed decreased expression levels compared with age-matched controls, particularly at 27 weeks (Figure 4). This finding could reflect an auto-regulatory mechanism in response to increased circulating concentrations of adiponectin.

Adiponectin and its receptors reportedly exert their actions mainly through activation of the AMPK pathway (Kadowaki et al. 2006; Yamauchi et al. 2003). Here, we used an AMPK inhibitor, compound C (Zhou, et al. 2001), and an AMPK stimulator, AICAR (Yamauchi, et al. 2002), to probe the AMPK signaling pathway and thus determined effects on adiponectin production in fully-differentiated 3T3-L1 adipocytes. We found that compound C significantly suppressed extracellular adiponectin levels by down-regulating all three isoforms (Figure 5A). AICAR stimulated extracellular adiponectin levels and specifically decreased the MMW/total ratio and increased the LMW/total ratio inside the cell (Figure 5B). However, at the mRNA expression level, compound C up-regulated the expression of adiponectin and its receptors whereas AICAR down-regulated the expression of adiponectin and adiponectin receptor 2 (Figure 5C). This finding appears consistent with the action of a pathway through which adiponectin can regulate its own production in WAT, at least in part via the AMPK pathway. Moreover, this regulation may act at several levels by modulating the expression of its receptors, the secretion of adiponectin, and eventually the expression of adiponectin itself. For example, this finding is consistent with the down-regulation of adiponectin receptors in the WAT of *ob/ob* mice, which had elevated circulating adiponectin.

3.4. *Expression of several proteins that regulate adiponectin in murine WAT* A family of lysyl hydroxylases (EC 1.14.11.4), comprising lysyl hydroxylase (LH)-1, -2a, -2b, and -3, is said to be responsible for catalysing the hydroxylation of protein-bound lysyl residues to yield hydroxylysyl residues, which can then undergo subsequent glycosylation (Passoja, et al. 1998). Here, we confirmed the expression of these lysyl hydroxylases (*Plod1, 2a, 2b & 3*) in mouse WAT (Figure 6). We also confirmed the expression therein of prolyl hydroxylases (*P4ha1* and *P4ha2*), which are responsible for prolyl hydroxylation (Gorres and Raines 2010): inhibition of these has been reported to lower collagen production (Majamaa, et al. 1987; Sasaki, et al. 1987). Glycosyltransferase 25-domain-containing protein 1, (*Glt25d1*) and 25-domain-containing protein 2 (*Glt25d2*) are proteins that can catalyse hydroxylysyl glycosylation (Schegg, et al. 2009) as reported for collagen biosynthesis: here we found that these proteins are also expressed in WAT.

We observed that expression levels of mRNAs corresponding to LH1 (*Plod1*) and LH3 (*Plod3*) were both altered in *ob/ob* mice, in the same direction as the changes in serum levels of adiponectin and the HMW isoforms (Figure 6). Western blots showed that 10-week *ob/ob* mice had markedly increased expression of LH3 protein (Figure 7A) compared with matched controls. Levels of protein disulfide-isomerase (PDI), the β-subunit of P4H (Kivirikko and Myllyharju 1998), were not altered significantly in the 10-week *ob/ob* mice; nor were levels of Glt25d2 protein (Figures 7B&7C).

The levels of mRNAs corresponding to additional endoplasmic reticulum (ER)-resident proteins that are reportedly associated with adiponectin retention and HMW formation/secretion (Cortini and Sitia 2010; Qiang, et al. 2007; Wolf 2008), were also changed in *ob/ob* mice, for example, ER-resident protein 44 (*Erp44)* and endoplasmic oxidoreductase 1-like protein α (*Ero1l*), both of which interact with PDI and are said to play roles in disulfide bond formation during adiponectin multimerization. The protein expression level of Erp44 tended to be decreased in *ob/ob* mice (Figure 7D, $P = 0.09$). This decrease may account for the relatively deceased HMW formation compared with the other two isoforms in these mice but facilitate the release of HMW adiponectin from the ER which therefore accounts for the increased secretion of HMW observed (Table 2 & Figure 2). We also studied another ER-resident protein, DsbA-L (disulfide bond A oxidoreductase-like protein), which was down-regulated in both age groups of *ob/ob* mice compared with matched controls (Figure 6). Increased expression of DsbA-L promotes adiponectin multimerization whereas suppression of this protein lowers both production and secretion of adiponectin (Liu, et al. 2012; Liu, et al. 2008). PPARγ is also thought to regulate mRNA levels, and processing and secretion of adiponectin (Astapova and Leff 2012): however, here corresponding mRNA levels were not changed in younger *ob/ob* mice whereas they were lowered in older *ob/ob* mice when compared with matched controls.

3.5. *Effects of inhibition of prolyl and lysyl hydroxylases on adiponectin production and multimerization*
To further characterize the function of enzyme systems that might modulate adiponectin and promote formation of HMW isoforms, we used specific and nonspecific inhibitors to inhibit the function of lysyl hydroxylases and/or prolyl hydroxylases to observe their subsequent effect in fully-differentiated 3T3-L1 adipocytes. Rosiglitazone is a representative TZD that reportedly acts to stimulate production of adiponectin, especially the HMW isoforms, via activation of PPARγ (Phillips, et al. 2003; Phillips, et al. 2009). Here, we used rosiglitazone to up-regulate the requirement for hydroxylation activity which corresponds to increased adiponectin production (that is, increased requirement for posttranslational modification), in order to accentuate the effect of decreased hydroxylase activity on adiponectin production and multimerization. Our results are consistent with other reports (Maeda et al. 2001; Phillips et al. 2009): rosiglitazone-treated 3T3-L1 adipocytes showed increased expression of adiponectin at both mRNA and protein levels, with a marked increase in the HMW isoforms (Figure 8): this effect was totally abolished by the PPARγ antagonist, GW9662 (Figure 8).

Dipyridyl, a non-specific hydroxylase inhibitor (Chvapil, et al. 1967; Switzer and Summer 1973), strongly suppressed the production of adiponectin (Figure 9), especially the HMW and MMW isoforms (Figure 9) which was reflected in the significantly decreased extracellular adiponectin level with decreased HMW/MMW-to-total adiponectin ratios and increased LMW ratio. Although rosiglitazone stimulated production of adiponectin, especially the HMW isoforms, dipyridyl totally abolished this effect (Figures 9).

Ethyl-3,4-dihydroxybenzoate, a specific inhibitor of prolyl-4-hydroxylase (Majamaa et al. 1987; Sasaki et al. 1987), had effects comparable with those of dipyridyl, as reflected in the significantly decreased extracellular and intracellular levels of adiponectin (Figure 9). Although they both showed decreased extracellular levels of the HMW isoforms, there was an obvious difference between the effects of these two inhibitors on the intracellular composition of different adiponectin isoforms (Figure 9). Whereas ethyl-3,4-dihydroxy-benzoate-treated adipocytes showed an increased intracellular HMW ratio, dipyridyl-treated cells showed the converse (Figure 9). Taken together, these results are consistent with the requirement for prolyl-4-hydroxylase and lysyl hydroxylases in adiponectin production, and support the importance of both prolyl and lysyl hydroxylation for adiponectin multimerization. The difference between the effects of dipyridyl and ethyl-3,4-dihydroxybenzoate may suggest that lysyl hydroxylases are critical for HMW-isoform formation; however, blocking the activity of prolyl-4-hydroxylase may result in misfolded HMW adiponectin whose secretion is thus impaired.

Minoxidil, a specific inhibitor for all three lysyl hydroxylases (Murad and Pinnell 1987; Zuurmond, et al. 2005), had no significant effect on total adiponectin production when administered alone (Figure 9), whereas it did increase HMW-isoform production (Figure 9). However, co-administration of minoxidil totally abolished the effect of rosiglitazone, as indicated by decreased extracellular and intracellular adiponectin levels without changes in the proportion of isoforms (Figures 9). This effect is opposite to that observed in cells treated with rosiglitazone in the absence of minoxidil, which showed increased adiponectin production with significant increases in the HMW isoforms (Figure 8). We further determined the expression of LH isoforms at the mRNA level after minoxidil treatment: we found that that minoxidil at 1000 μM supressed the expression of LH isoforms to an extent that *Plod1* (LH1) reduced to ~50% of control value, *Plod2a* (LH2a) & *Plod3* (LH3) to ~75%, with no change of *Plod2b* (LH2b).

3.6. *Effect of transient knock-down of mRNA species corresponding to lysyl hydroxylase 3 (Plod3) on adiponectin production and multimerization* Three lysyl hydroxylases are expressed in white adipocytes, but lysyl hydroxylase 3 (*Plod3*) is the only one that possesses both hydroxylation and glycosylation activities (Heikkinen, et al. 2000). We therefore transiently knocked-down mRNA corresponding to *Plod3* in fully-differentiated 3T3-L1 adipocytes by using siRNA to measure the effect on adiponectin production and multimerization. To verify the specificity and efficiency of transfection, a scrambled siRNA was included as a negative control, and a siRNA for *Gapdh* as a positive control: the scrambled siRNA showed no significant effect on the mRNA expression of *Plod3* whereas *Gapdh* siRNA suppressed expression of *Gapdh* to 25% of its normal levels (data not shown).

We found that *Plod3* mRNA levels were ~30% of control values in treated 3T3-L1 adipocytes whereas rosiglitazone had no effect on *Plod3* mRNA levels (Figure 10). The knock-down of *Plod3* had no effect on the expression of *Plod1&2* (data not shown). Although the mRNA expression level of adiponectin was unchanged in these treated cells (Figure 10), adiponectin protein level was significantly decreased, with decreases in both HMW and MMW isoforms but a corresponding increase in the LMW isoform (Figure 10). Moreover, although rosiglitazone treatment increased the levels of adiponectin mRNA (Figure 10), the protein-level effects of rosiglitazone on adiponectin were totally abolished by *Plod3* knock-down (Figures 8&10). These results emphasize that LH3 is critical to adiponectin production, especially of the higher-order isoforms.

4. Discussion

Adiponectin is a major secretory product of the adipocyte, and numerous studies have linked low plasma adiponectin levels to obesity, insulin resistance, inflammation, and an increased risk of coronary artery disease (Antoniades, et al. 2009; Arita et al. 1999; Hu et al. 1996; Trujillo and Scherer 2005). Defects in the multimerization of adiponectin occur in type-2 diabetes, and replenishment of adiponectin in animal models elicits reversal of diabetic condition, at least in part by stimulating fatty acid oxidation and decreasing triglyceride content in liver and muscle (Basu et al. 2007; Waki et al. 2003). Until today, the regulatory mechanisms underlying adiponectin expression, multimerization and secretion remain incompletely understood. Here we have described a number of factors contributing to adiponectin expression and multimerization, with emphasis on the effects of insulin and of impaired insulin signaling; the self-regulation by adiponectin via the AMPK signaling pathway; and the roles of lysyl/prolyl hydroxylases.

Our *ob/ob* mice displayed decreased adiponectin mRNA but elevated protein level of adiponectin when compared with age-matched controls (Table 2, Figures 2&4). These results are consistent with other reports (Haluzik, et al. 2004; Purushotham, et al. 2007; Ueno, et al. 2004) and also indicate that adiponectin biogenesis is regulated at the posttranscriptional level. That insulin resistance appeared in our

ob/ob mice is reflected by their decreased insulin sensitivity, and significantly increased circulating insulin in the presence of hyperglycemia (Figure 1 & Table 2): this may result from the lack of leptin (Silha, et al. 2003), since leptin replacement has been reported to attenuate insulin resistance and blood glucose in *ob/ob* mice in vivo (Wendel, et al. 2008).

As we mentioned before, although the relationship between plasma insulin and adiponectin levels has been studied extensively, the reported effects of insulin on adiponectin regulation are inconsistent. Here, we hypothesized that impaired insulin signaling could modify the biogenesis of adiponectin. We found elevated extracellular levels of adiponectin after incubating differentiated 3T3-L1 cells with an InsR-blocking antibody at 5 mM glucose and 1 ng/ml insulin, when compared with control values (no antibody) (Figure 3). A high glucose concentration (25 mM) further stimulated this increase in extracellular adiponectin: however, increased insulin (100 ng/ml) reversed this effect (Figure 3). These results support the idea that insulin can suppress adiponectin levels through receptor-mediated signaling in WAT. This view is consistent with observations that patients carrying mutant *Insr* genes encoding functionally-impaired InsR (Semple et al. 2006), patients with anti-InsR autoantibodies (Semple, et al. 2007), and mice with fat-specific disruption of the *Insr* gene (Bluher et al. 2002), all present with very high levels of adiponectin. These findings are also consistent with further observations: namely that replenishment of leptin in *ob/ob* mice, which alleviated insulin resistance, also suppressed adiponectin levels (Ueno et al. 2004); and that intensified glycemic control with insulin therapy lowered adiponectin levels in type-2 diabetic patients (Abi Khalil et al. 2011). These results could explain the elevated/unchanged circulating adiponectin levels in the *ob/ob* mice studied here, which had insulin resistance.

Here, *Adipor1* and *Adipor2* mRNA levels were both decreased in WAT from *ob/ob* mice (Figure 4): this could reflect a feedback response to the elevated circulating level of adiponectin (Table 2) and thereby tend to down-regulate adiponectin production. These findings are consistent with those from a transgenic mouse model with transient overexpression of exogenous adiponectin, wherein lowering of adiponectin expression at both mRNA and protein levels was observed together with down-regulated expression of adiponectin receptors (Bauche, et al. 2006).

Since adiponectin and its receptors elicit their biological effects mainly through the AMPK pathway (Kadowaki et al. 2006; Yamauchi et al. 2003), and information about the involvement of the AMPK signaling pathway in adiponectin regulation in adipocytes is somewhat limited, here, we treated 3T3-L1 adipocytes with the AMPK inhibitor, compound C, or the AMPK stimulator, AICAR, to probe the AMPK signaling pathway. Our results (Figure 5A) show that 4-h treatment with compound C was sufficient to lower extracellular adiponectin, whereas this treatment had no significant effect on intracellular hormone levels. Furthermore, treatment with AICAR increased extracellular adiponectin levels but had no effect on its intracellular level (Figure 5B). At the mRNA level, compound C up-regulated the expression of adiponectin and its receptors whereas AICAR down-regulated the expression of adiponectin and adiponectin receptor 2 (Figure 5C). These findings may indicate that increased AMPK pathway signaling down-regulates the expression of adiponectin and its receptors and suppresses the production/secretion of adiponectin, whereas decreased AMPK pathway signaling up-regulates the expression of adiponectin and its receptors and stimulates the production/secretion of adiponectin. These findings support the view that adiponectin itself can control its own production via an autocrine negative-feedback loop through binding to its receptors in adipocytes. This is meaningful when considering that high adiponectin levels can also lead to or cause energy wasting, and attenuate macrophage activity (Van Berendoncks, et al. 2012; Yokota, et al. 2003; Yokota, et al. 2000): these effects may be disadvantageous for survival under conditions of calorie deprivation, infection or inflammation.

Previous studies have indicated that adiponectin becomes increased following treatment with TZDs, which act as synthetic PPARγ ligands (Iwaki, et al. 2003; Phillips, et al. 2003; Phillips, et al. 2009). Here, we observed that, in 3T3-L1 adipocytes, rosiglitazone stimulated adiponectin expression at both mRNA

and protein levels, with significant increase of the HMW isoforms, whereas the PPARγ antagonist, GW-9662, totally abolished this effect (Figure 8). However, several studies in humans have shown little or no change in the mRNA level of adiponectin in response to TZDs, whereas all studies reported two- to three-fold increases in plasma adiponectin (Rasouli, et al. 2006; Tiikkainen, et al. 2004), suggesting that much of the regulation of adiponectin occurs post-transcriptionally. Therefore, the regulation of adiponectin by PPAR γ agonists in adipocytes might depend, at least in part, on the system studied.

Impaired multimerization of adiponectin has been reported to be associated with lowered levels of total adiponectin, for example in obesity and insulin resistance (Pajvani et al. 2004; Waki et al. 2003). However, until now, the mechanisms underlying this oligomerization remain incompletely understood. Our laboratory has reported that the full bioactivity of adiponectin requires post-translational modification of several lysyl residues in its collagenous domain, which can be hydroxylated and glycosylated in the mature protein (Wang et al. 2006). The enzymes that catalyse the hydroxylation of lysyl residues in collagens and more than 15 other proteins using X-Lys-Gly-sequences as substrates are lysyl hydroxylases, which are lumen-oriented membrane proteins localized in the ER (Kivirikko and Myllyla 1982; Myllyharju and Kivirikko 2001; Passoja et al. 1998). Here we confirmed the expression of LHs in the WAT of mice: moreover, the expression of LH1 and LH3 was changed in *ob/ob* mice in the same direction as changes in its circulating adiponectin levels (Figure 6). In 10-week *ob/ob* mice, the WAT expression of LH3 was dramatically increased compared with age-matched controls (Figure 7A): this increase may be consistent with the increased production of adiponectin in these *ob/ob* mice and facilitate secretion of the HMW isoforms.

From studies of collagen, it is proposed that hydroxylysyl residues have two important functions: they are essential for the stability of physiological intermolecular collagen cross-links, and their hydroxyl groups serve as potential attachment sites for carbohydrate units, either the monosaccharide galactose or the disaccharide glucosyl-galactose (Gelse, et al. 2003). The functions of the hydroxylysyl-linked carbohydrate units in adiponectin are not fully understood and relevant structural studies are lacking: in the related case of the fibril-forming collagens, they can influence the lateral packing of collagen molecules into fibrils and modulate the fibril diameters (Notbohm, et al. 1999). Mutations in LH1 lead to the clinical phenotype of Ehlers–Danlos syndrome type VI (Yeowell and Walker 2000), which is biochemically characterised by a deficiency of triple-helical hydroxylysyl residues in collagen (Eyre, et al. 2002). Surprisingly, in these patients, there was no compositional (hydroxylysyl/hydroxyprolyl ratio) or functional alteration of C1q, a structural homologue of adiponectin that contains a collagen-like sequence of ~80 amino acid residues (Hanauske-Abel and Rohm 1980). Those authors suggest that there may be an isoform of LH that is specific for collagen and C1q. An almost complete lack of hydroxylated lysyl residues in the collagen telopeptides is seen in Bruck syndrome, where LH2 is mutated (Bank, et al. 1999; Ha-Vinh, et al. 2004; van der Slot, et al. 2003). These connective tissue disorders indicate that the different lysyl hydroxylases have substrate specificity, with LH1 catalysing modifications of lysyl residues in the helical collagenous domains (Pasquali, et al. 1997), and LH2b acting on telopeptide lysyl residues (Mercer, et al. 2003; Pornprasertsuk, et al. 2004). LH3 is different from the other two lysyl hydroxylases in that it also displays glucosyl and galactosyl transferase activities, thereby contributing to the glycosylation of hydroxylysyl residues in the collagen molecule (Heikkinen et al. 2000). The embryonic lethality of LH3 knock-out mice confirms that this lysyl hydroxylase provides essential functions in the body (Rautavuoma, et al. 2004).

Minoxidil (1000 μM) reportedly suppresses levels of LH mRNAs in fibroblasts (Zuurmond et al. 2005), its most profound effect being on LH1 expression. It can almost completely inhibit LH1 mRNA levels: however it affects the mRNA levels of LH2b and LH3 to a much lesser extent, to ~35% of control values (Zuurmond et al. 2005). However, our results showed that, in fully-differentiated 3T3-L1 adipocytes, minoxidil at 1000 μM exerted a suppressive effect on the mRNA expression levels of LHs to the extent that *Plod1*was reduced to ~50% of control value, *Plod2a* & *Plod3* to ~75%, whereas *Plod2b*

was unchanged. Minoxidil alone had no effect on the production of adiponectin, but increased the levels of the HMW isoforms both inside and outside cells, consistent with increased production and secretion. On the other hand, the MMW-isoform was decreased. Moreover, addition of rosiglitazone had no further stimulatory effect (Figure 9). Our observations could therefore be consistent with the hypothesis that LHs may be present in excess amounts even under normal conditions which is supported by the observation that rosiglitazone alone stimulated adiponectin expression without modifying the expression of LHs (Figure 8); or there may be a LH isoform specific for adiponectin with LH2 and/or LH3 responsible for hydroxylation of its lysyl residues; or there may exist a compensatory mechanism such that the activity of specific LH isoforms increase in the presence of minoxidil, which match the demand for hydroxylation and glycosylation. However, to clearly understand the effect of minoxidil on the mRNA expression of LHs in adipocytes and their effect(s) on adiponectin production, studies of dose-response and enzyme activity would be necessary to provide more information. Our most recent data (not shown) showed that minoxidil at 2 mM did not exert a suppressive effect on the mRNA expression of LHs but stimulated the expression of LH2b with increased formation of HMW adiponectin. This finding provides another pathway that furthers our understanding of the specificity of LHs for adiponectin production, especially of its higher order isoforms. Here, our observations confirm the involvement of LHs in adiponectin production, especially the formation of the HMW isoforms (Figure 9). This involvement was further accentuated by rosiglitazone treatment.

The involvement of LHs in adiponectin production is further supported by another observation concerning the transient knock-down of the mRNA corresponding to LH3 (*Plod3*), the only one of the three LHs that possesses both hydroxylation and glycosylation activities (Heikkinen et al. 2000). Transfected 3T3-L1 adipocytes showed significant decreases in adiponectin production (Figure 10B) with lowering of both HMW and MMW isoforms but increase of the LMW isoforms. In these transfected cells, rosiglitazone treatment significantly increased adiponectin mRNA levels (Figure 10A), but had no effect on its protein expression (Figure 10B). These results indicate that LH3 is important for adiponectin production and formation of the higher-order isoforms as well as its secretion. However, our current results cannot differentiate between the hydroxylation and glycosylation activities of LH3 and their respective effects on adiponectin production and multimerization; also, we cannot rule out other proteins which may also contribute to the glycosylation of the lysyl residues in adiponectin, for example, Glt25d, whose expression was also altered in *ob/ob* mice (Figure 6). Therefore, future studies could address these questions by more detailed biochemical investigations.

In a recent report (Ruotsalainen, et al. 2012) where the authors expressed recombinant mouse adiponectin in $LH3^{-/-}$ knockout mouse embryonic fibroblasts (MEFs), there was reportedly increased expression of recombinant mouse adiponectin in those $LH3^{-/-}$ knockout MEFs compared with wild-type cells and there was a total lack of production of HMW and MMW isoforms in the culture medium. However, the $LH3^{-/-}$ knockout cells had serious defects in secretion of the recombinant protein. Since the authors didn't report the cellular distribution of isoforms, the effect of $LH3^{-/-}$ knockout on adiponectin isoforms formation cannot be fully elucidated from the study reported. In addition, these authors reported decreased serum levels but normal adipose tissue levels of adiponectin in their LH3 mutant mice (wherein lysyl hydroxylase activity as inactivated by a point mutation) compared with matched wild-type controls. The authors suggested that, without LH3-catalyzed lysyl hydroxylase activity, the adiponectin produced is insufficiently modified and thus inefficiently secreted. However, the increased HMW/total adiponectin ratio in the serum of their LH3 mutant mice might also suggest that the mutation facilitating HMW isoform formation or secretion, which could be determined in light of the as-yet unreported isoform distribution in their adipose tissue.

In our current study, the use of the non-specific hydroxylase inhibitor (dipyridyl, 1000 μM) to inhibit both prolyl and lysyl hydroxylation (Chvapil et al. 1967; Switzer and Summer 1973) dramatically decreased adiponectin production, namely that of the HMW and MMW isoforms, whereas it increased the

LMW isoforms (Figures 9). This observation provides further support for the importance of prolyl/lysyl hydroxylation and the requirement for one or more lysyl hydroxylase and prolyl hydroxylases for adiponectin formation, especially for the higher-order isoforms. On the other hand, ethyl-3,4-dihydroxybenoate (400 μM), a specific inhibitor of prolyl-4-hydroxylase (Sasaki et al. 1987), exerted a similar effect as dipyridyl (Figures 9). However, there was a clear difference between the effects of these two inhibitors on the intracellular composition of different isoforms. Whereas ethyl-3,4-dihydroxybenzoate-treated adipocytes showed increased intracellular HMW ratios, dipyridyl-treated cells showed decreased ratios (Figure 9). This difference could provide further support for the view that LHs are critical for HMW formation, but that the activity of prolyl-4-hydroxylase(s) may be required for proper HMW formation and secretion. We interpret our results to indicate the essential role of prolyl hydroxylases/prolyl hydroxylation for adiponectin production and secretion, especially for its HMW-isoform secretion. Since these findings are original, further studies by investigation of the effect of mutagenesis of specific prolyl residues on adiponectin production would provide useful information to further our understanding of this aspect.

In conclusion, adiponectin biogenesis is a complex process that is regulated at several levels in white adipocytes. Whereas decreased insulin signaling stimulates adiponectin production, insulin itself exerts powerful suppressive effects on adiponectin production. Adiponectin negatively regulates its production through binding to its receptors and activating the AMPK signaling pathway. In addition, both lysyl and prolyl hydroxylases (namely lysyl hydroxylase 3 and prolyl 4 hydroxylase) are required for adiponectin production and multimerization, for which both the hydroxylation/glycosylation of lysyl and hydroxylation of prolyl residues are essential for the formation and secretion of the higher-order adiponectin isoforms.

Disclosure statement

The authors declare that there is no duality of interest associated with this manuscript.

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Figure 1

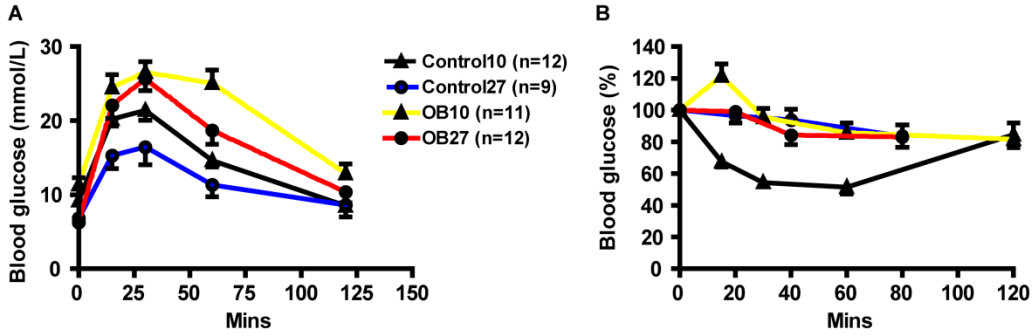


Figure 2

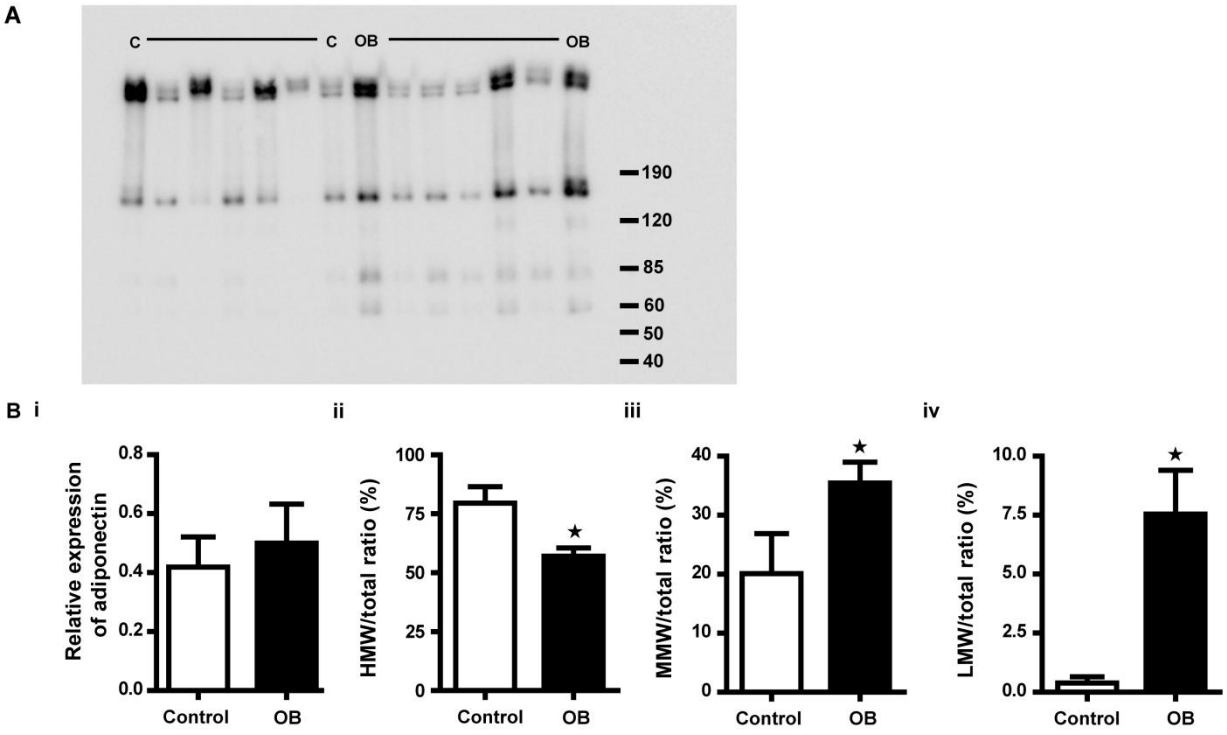


Figure 3

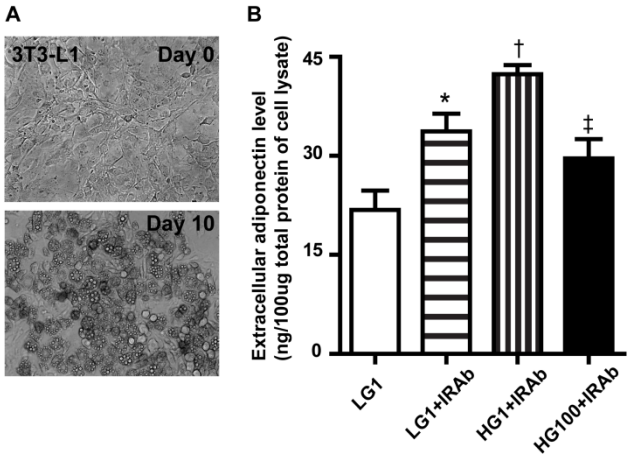
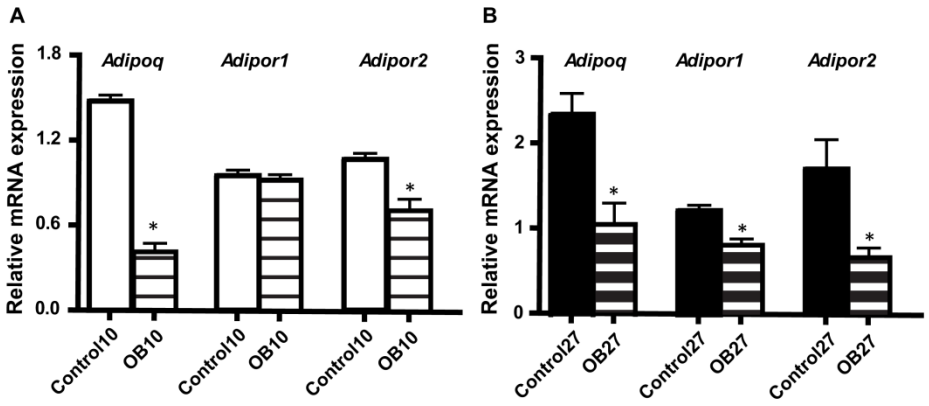


Figure 4



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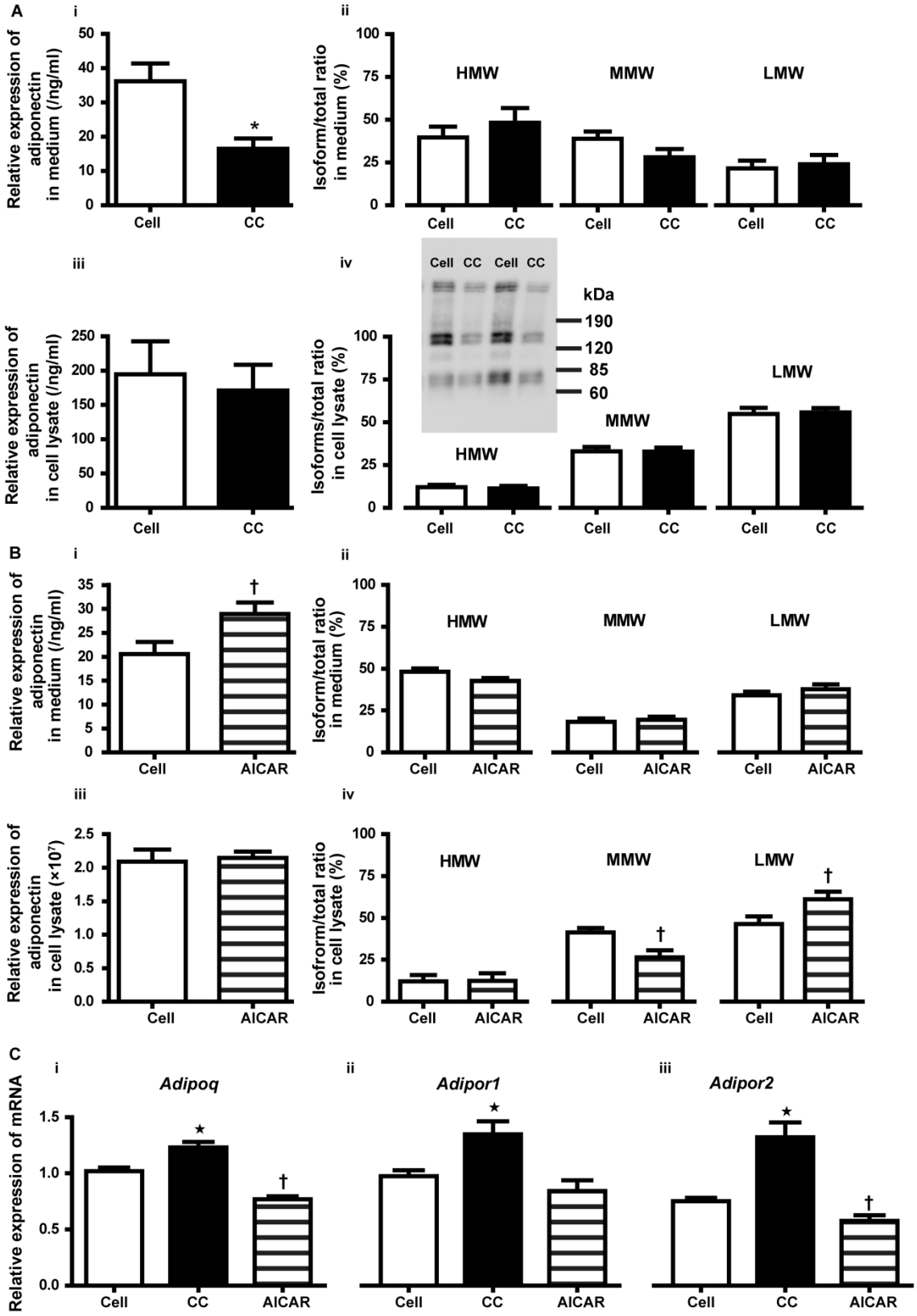

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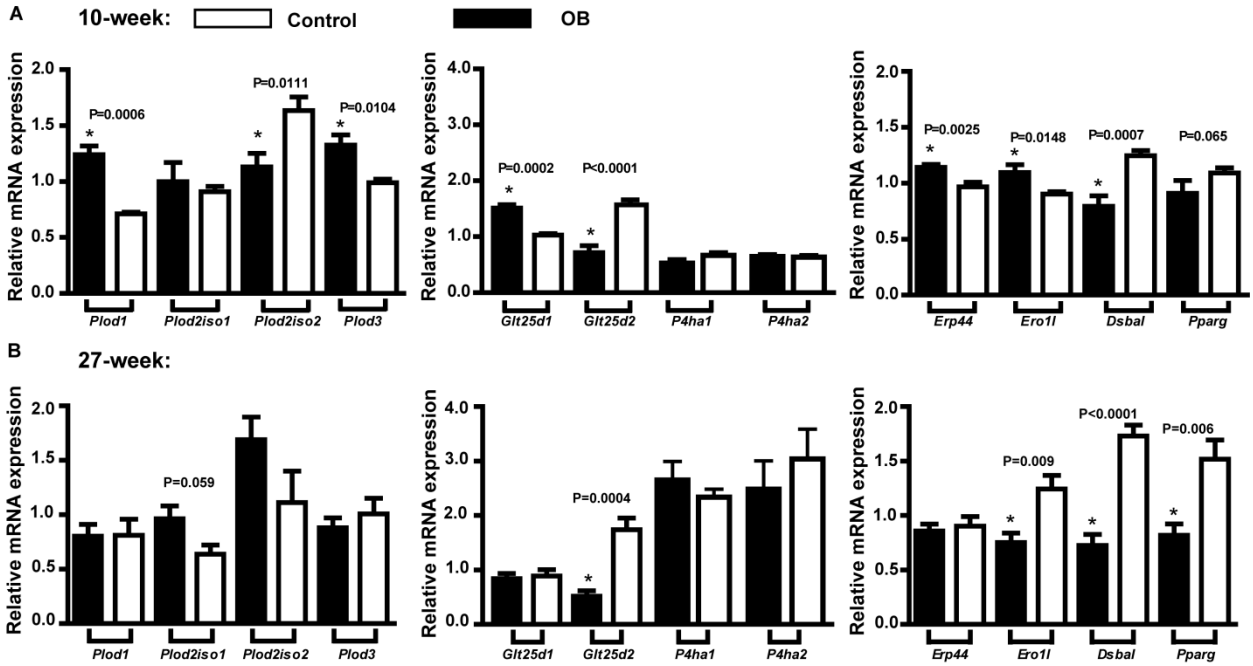

Figure 7

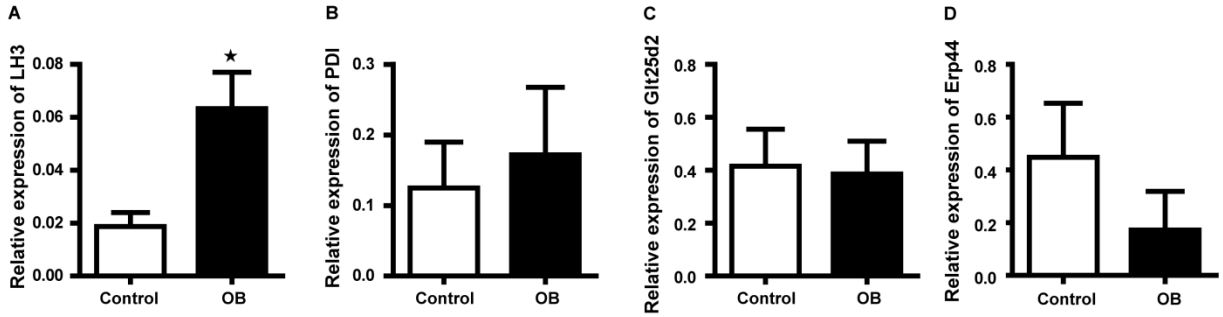

Figure 8

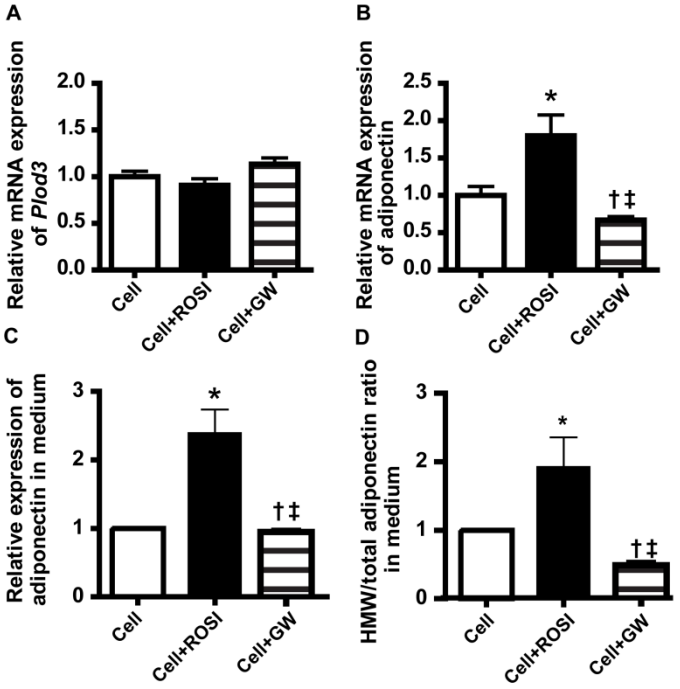

Figure 9

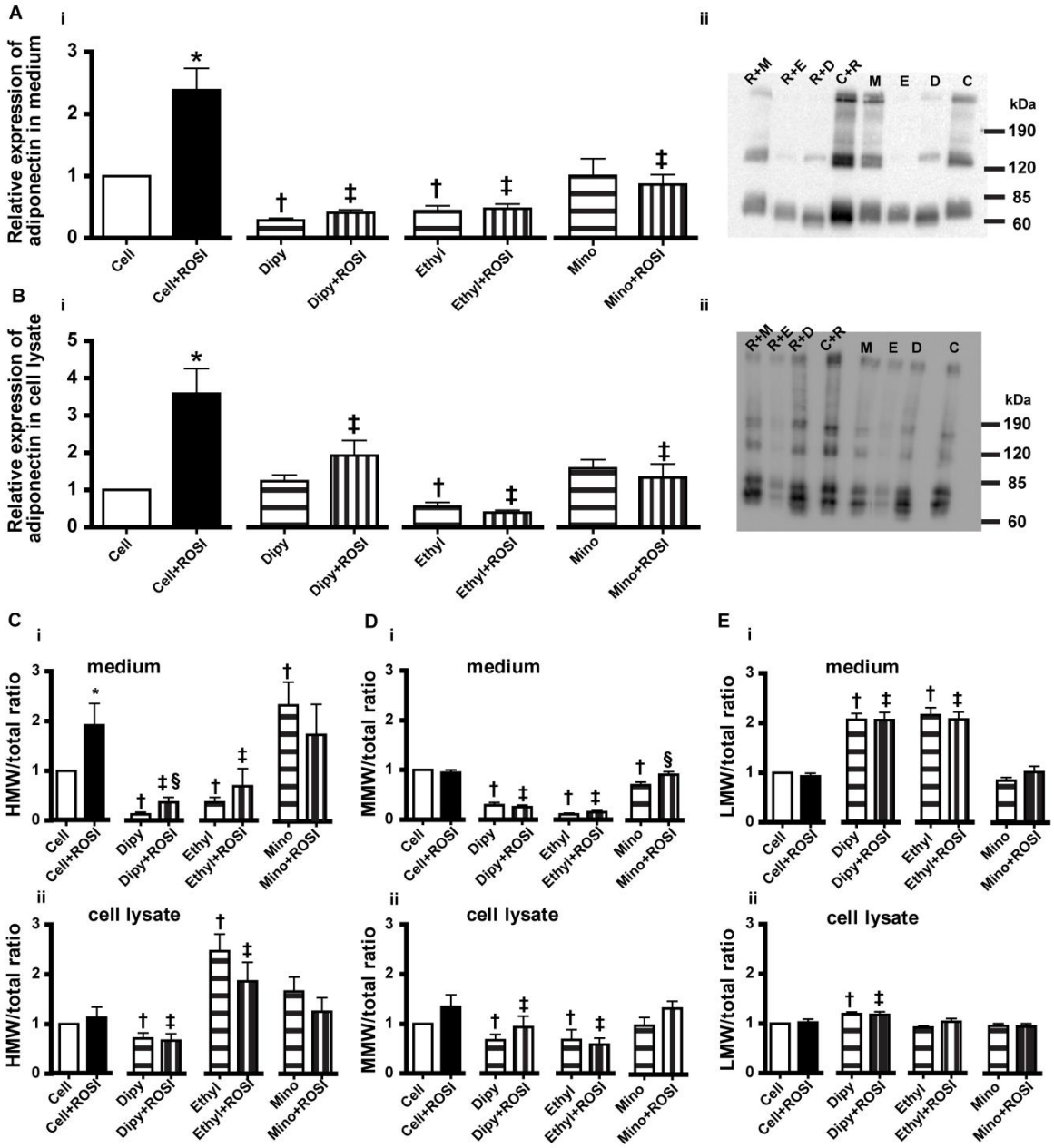

Figure 10

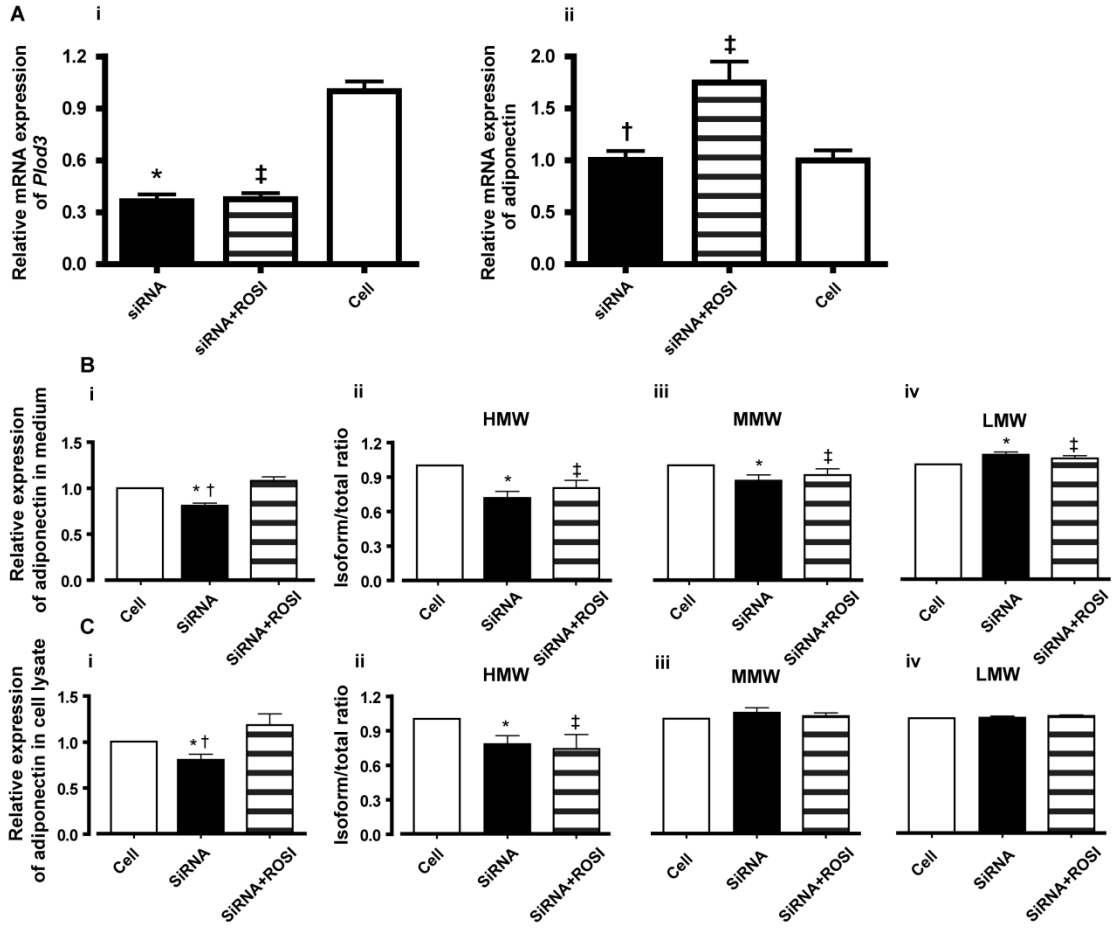

Figure 1 Glucose tolerance test (A) and insulin tolerance test (B) in *ob/ob* mice and age-matched controls. Both age group *ob/ob* mice (OB10: 10-week, n = 11; OB27: 27-week, n = 12) showed abnormal glucose tolerance and insulin tolerance compared with corresponding age-matched controls. Compared with 10-week controls (Control10, n = 12), 27- week control mice (Control27, n = 9) also showed diminished insulin tolerance. Data represent means ± SE, repeated-measures two-way ANOVA, $P < 0.05$.

Figure 2 Protein expression level of adiponectin in WAT from 10-week *ob/ob* mice (OB, n = 7) and age-matched controls (n = 7). (A) shows a representative western blot image. *Ob/ob* mice showed no significant change in the total expression of adiponectin (Bi), but had decreased HMW/total ratio (Bii) and increased MMW/total (Biii) and LMW/total ratio (Biv). Data represent means ± SE, Student's *t*-test, $*P < 0.05$, Control vs OB.

Figure 3 Differentiated 3T3-L1 adipocytes were incubated with different concentrations of insulin and glucose in the presence or absence of an insulin receptor blocking antibody. (A) Images show the differentiation of 3T3-L1 pre-adipocytes (Day 0) to adipocytes (Day 10). (B) Extracellular adiponectin levels were measured by using commercial ELISA kits and results were normalized by total protein level of the cell lysate. Under normal glucose (5 mM) and insulin (1 ng/ml) concentrations (LG1), the presence of insulin receptor antibody (IRAb) increased extracellular adiponectin levels. High glucose (25 mM, HG1) further increased these levels; however, high insulin levels (100 ng/ml, HG100) reversed this effect. Data represent means ± SE, one-way ANOVA with *post-hoc* test, n = 9 replictes/group, $P < 0.05$, *LG1 vs LG1+IRAb; †LG1 vs HG1+IRAb; ‡HG1+IRAb vs HG100+IRAb.

Figure 4 Messenger RNA expression levels of adiponectin (*Adipoq*) and its receptors (*Adipor1* & *Adipor2*) in murine WAT. (A) 10-week *ob/ob* mice (OB10, n = 8) showed decreased levels of adiponectin compared with age-matched controls (Control10, n = 8) with decreased expression of adiponectin receptor 2 (*Adipor2*) but no change in receptor 1 (*Adipor1*). (B) 27-week *ob/ob* mice (OB27, n = 6) showed decreased expression levels of adiponectin with decreased expression level of both receptors compared with controls (Control27, n = 5). Data represent means ± SE, Student's *t*-test, $*P < 0.05$, Control vs OB.

Figure 5 Differentiated 3T3-L1 adipocytes were treated with the AMPK inhibitor (compound C, CC) and AMPK stimulator (AICAR). Western blots showed that CC significantly suppressed extracellular adiponectin levels (Ai) but had no effect on the proportion of its isoforms (ii) compared with controls. Neither the intracellular level of adiponectin (Aiii) nor the proportion of the isoforms (Aiv) showed changes. The inserted image shows a representative western blot of adiponectin in the medium. AICAR exerted a stimulatory effect on the extracellular level of adiponectin (Bi), but no effect on the proportion of its isoforms (Bii). It had no effect on the intracellular level of adiponectin (Biii), but decreased the MMW/total ratio and increased the LMW/total ratio (Biv). The mRNA expression levels of adiponectin (*Adipoq*) and its receptors (*Adipor1& 2*) were up-regulated by CC treatment, but down-regulated by AICAR treatment (C). Densitometry results were normalized to the total protein levels of the cell lysates (ng/ml). Data represent means ± SE, n = 9 replicates/group, unpaired Student's *t*-tests, $P < 0.05$, *Cell vs CC, †Cell vs AICAR.

Figure 6 Messenger RNA expression of a number of proteins in WAT from (A) 10-week *ob/ob* (OB) mice and matched controls (n = 8/group) and (B) 27-week *ob/ob* mice (n = 6) and matched controls (n = 5). Data represent means ± SE, Student's *t*-test, $*P < 0.05$, Control vs OB.

Figure 7 Protein expression levels of (A) lysyl hydroxylase 3 (LH3), (B) protein disulfide-isomerase (PDI), (C) glycosyltransferase 25-domain containing protein 2 (Glt25d2) and (D) ER-resident protein 44 (Erp44) in white adipose tissue from 10-weeks *ob/ob* (OB) mice (n = 7/group) and age-matched controls (n = 7). Data represent means ± SE, Student's *t*-test, **P* < 0.05, Control vs OB.

Figure 8 Rosiglitazone (ROSI) had no effect on the mRNA expression of *Plod3* (A), but increased the expression of *Adipoq* mRNA (B, n = 9 replicates/group). Western blots showed that it also increased adiponectin expression at the protein level (C, n = 17 replicates/group), especially of HMW isoforms (D) in 3T3-L1 adipocytes. However, the PPARγ antagonist, GW-9662 (GW), totally abolished this effect. Data are means ± SE: one-way ANOVA with appropriate *post-hoc* tests, *P* < 0.05, *Cell vs Cell+ROSI; †Cell vs Cell+GW; ‡ Cell+ROSI vs Cell+GW.

Figure 9 A non-specific hydroxylase inhibitor (dipyridyl, Dipy), and specific inhibitors for prolyl 4 hydroxylase (ethyl-3,4-dihydroxybenoate, Ethyl) and for lysyl hydroxylases (minoxidil, Mino) suppressed (Ai) extracellular and (Bi) intracellular adiponectin levels in 3T3-L1 adipocytes. Inserted images show representative western blots of (Aii) extracellular and (Bii) intracellular adiponectin isoforms. (C) Extracellular (i) and intracellular (ii) HMW isoforms to total adiponectin ratios; (D) Extracellular (i) and intracellular (ii) MMW isoforms to total adiponectin ratios; (E) Extracellular (i) and intracellular (ii) LMW isoforms to total adiponectin ratios. Data are means ± SE, n = 14 replicates/group: one-way ANOVA with appropriate *post-hoc* tests, *P* < 0.05, *Cell (C) vs Cell+ROSI (C+R); †Cell vs Dipy (D) or Ethyl (E) or Mino (M); ‡ Cell+ROSI vs Dipy+ROSI (R+D) or Ethyl+ROSI (R+E) or Mino+ROSI (R+M). §Dipy vs Dipy+ROSI or Mino vs Mino+ROSI.

Figure 10 Transient knock-down of gene expression of *Plod3* (siRNA) in 3T3-L1 adipocytes (Ai) showed no effect on adiponectin mRNA levels (Aii). In addition, it had no effect on the action of rosiglitazone to increase the expression of adiponectin at the mRNA level. n = 9 replicates/group. Western blots showed that transient knock-down of *Plod3* significantly suppressed adiponectin production (Bi&Ci) with decreased HMW (Bii&Cii) and MMW (Biii&Ciii) isoforms but increased LMW isoforms (Biv&Civ). In addition, the knock-down of *Plod3* totally abolished the effect of rosiglitazone to increase adiponectin production at the protein level. n = 17 replicates/group. Data represent means ± SE, one-way ANOVA with appropriate *post-hoc* tests, *P* < 0.05, *siRNA vs Cell; †siRNA vs siRNA+ROSI; ‡Cell vs siRNA+ROSI.

TABLE 1: Oligonucleotide primer pairs for specific genes employed

Genes	Primers	
	Forward (5'→3')	Reverse (5'→3')
Adiponectin (*Adipoq*)	GCACTCCCTGGTCTCCACGACTC	TGTGAAGCCCCCATACCAAATGTGA
Adiponectin receptor 1 (*Adipor1*)	ACATCTGGACACATCTGCTTGGTTTT	ATTTGGTCTCAGCATCGTCAAGATTCC
Adiponectin receptor 2 (*Adipor2*)	ACAAGAATCCGTGGAGCTCAGCA	ATGGTAAGTTGACCCCAAATCCTGTC
Lysyl hydroxylase 1 (*Plod1*)	CGCCAAGCTAGAGGACAACC	GGTCCTCCATCCACACTCCA
Lysyl hydroxylase 2 (*Plod2*) isoform 1	GACTCCCCTACTCCGGAAACA	AGCAGTTGATATCAGCCGTCCA
Lysyl hydroxylase 2 (*Plod2*) isoform 2	ATGCTAGAGATATGGGTGTGTTTATGT	GCAGTTGATATCAGCCGTCCA
Lysyl hydroxylase 3 (*Plod3*)	AGGGCTGCGGGAGAAACTCA	TCCGGATGCGCACACGATTC
Endoplasmic oxidoreductase 1-like protein α (*Ero1l*)	AGGCCTTGCTTAAGCTCCTACA	GGCCCATGACTCAAACCCTTAAATA
ER resident protein 44 (*Erp44*)	TATCATGCAAGCGGAAGGTGGCA	CACAGGGAGCAGGCCGATTCT
Peroxisome proliferator-activated receptor gamma (*Pparg*)	AGGGAGTTCCTCAAAAACCTGCGGA	CCTGGGCGGTCTCCACTGAGAAT
Disulfide bond A oxidoreductase-like protein (*Dsbal*)	ATGGATGCGTGTATGGTCTCGAGATG	CAGGCTGCATCCGTGTTCTCAA
Glycosyltransferase 25-domain-containing 1 (*Glt25d1*)	TACGGCTTCCTGCCCGT	CATGAAGACCTCATCGAAGCCCATC
Glycosyltransferase 25-domain-containing 2 (*Glt25d2*)	ACAGGATGCTGCGCACACTCT	TGTGTTGAGTGCCTTCCCATCCA
Prolyl-4-hydroxylase alpha 1 (*P4ha1*)	TGGGCATCCTGTAAACGCAT	AAGCCATCCGACATGTCCTT
Prolyl-4-hydroxylase alpha 2 (*P4ha2*)	AGCTATTGCAGGTCGCAAAC	CGCTTGAAAGCATCTTGCTCA
Prolyl-4-hydroxylase alpha 3 (*P4ha3*)	AGGAGCCACGGCTTTTATCT	CCACCAAAACAGTGCAGCAT

TABLE 2 Characteristics of *ob/ob* (OB) mice and their age-matched controls

	Control10	OB10	Control27	OB27
Body weight (g)	26.1 ± 0.4 (12)	49 ± 1 (11)*	41 ± 1 (13)†	66 ± 1 (11)‡§
Blood glucose (mmol/L)	8.4 ± 0.3 (12)	16.9 ± 0.2 (11)*	6.9 ± 0.2 (10) †	7.5 ± 0.7 (11)‡
Serum insulin (ng/ml)	1.1 ± 0.2 (10)	58 ± 10 (10)*	4.2 ± 1.7 (10)†	47 ± 10 (10)§
Serum amylin (ng/ml)	0.4 ± 0.1 (8)	1 ± 0.1 (8)*	0.15 ± 0.04 (8)†	0.5 ± 0.1 (8)‡§
Serum leptin (ng/ml)	6.1 ± 0.9 (8)	undetectable	28.3 ± 1.7 (8)†	undetectable
Serum adiponectin (µg/ml)	17.1 ± 0.8 (10)	24.5 ± 2.1 (10)*	21.5 ± 1.1 (10)†	24.1 ± 1.3 (10)
Serum HMW adiponectin (µg/ml)	2.6 ± 0.3 (10)	3.6 ± 0.5 (10)*	4.5 ± 0.4 (10)†	4.7 ± 0.5 (10)
Serum HMW/total adiponectin ratio	0.15 ± 0.01 (10)	0.15 ± 0.01 (10)	0.21 ± 0.01 (10)†	0.19 ± 0.01 (10)‡

Data are mean ± SE. Numbers of animals are shown in parentheses. Student's *t*-test, $P < 0.05$: * OB10 vs Control10; †Control27 vs Control10; ‡ OB27 vs OB10, § OB27 vs Control27.

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