POSTTRANSLATIONAL MODIFICATIONS ON THE FOUR CONSERVED LYSINE RESIDUES WITHIN THE COLLAGENOUS DOMAIN OF ADIPONECTIN ARE REQUIRED FOR THE FORMATION OF ITS HIGH-MOLECULAR-WEIGHT OLIGOMERIC COMPLEX

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Adiponectin is a multifunctional adipokine that circulates as several oligomeric complexes in blood stream. However, the molecular basis that regulates the production of the adiponectin oligomers remains largely elusive. We have previously shown that several conserved lysine residues (68, 71, 80, and 104) within the collagenous domain of adiponectin are modified by hydroxylation and glycosylation (Wang Y et al, JBC, 2002, 277:19521-9). Here we investigated the potential roles of these posttranslational modifications in oligomeric complex formation of adiponectin. Gel filtration chromatography revealed that adiponectin produced from mammalian cells formed trimer, hexamer, and high molecular weight (HMW) oligomeric complexes. These three oligomeric forms were differentially glycosylated, with the HMW oligomer having the highest carbohydrate contents. Disruption of hydroxylation and glycosylation by substitution of the four conserved lysines with arginines selectively abrogated the intracellular assembly of the HMW oligomers in vitro as well as in vivo. In type 2 diabetic patients, both the ratios of HMW/total adiponectin and the degree of adiponectin glycosylation were significantly decreased compared to healthy controls. Functional studies on adiponectin-null mice revealed that abrogation of the lysine hydroxylation/glycosylation markedly decreased the ability of adiponectin to stimulate the phosphorylation of AMP-activated protein kinase in the liver tissue. Chronic treatment of db/db diabetic mice with wild type adiponectin alleviated hyperglycemia, hypertriglyceridemia, hepatic steatosis and insulin resistance, whereas full-length adiponectin without proper posttranslational modifications and HMW oligomers showed substantially decreased activities. Taken together, these data suggest that hydroxylation and glycosylation on the lysine residues within its collagenous domain are critically involved in regulating the formation of its HMW oligomeric complex, and consequently contribute to the insulin-sensitizing activity of adiponectin in hepatocytes.

INTRODUCTION

Adiponectin, a hormone synthesized by adipocytes, is an abundant serum adipokine with potent insulin sensitizing activity (1-3). Unlike most other adipokines, plasma levels of adiponectin are significantly decreased in obese individuals and patients with insulin resistance, Type 2 Diabetes Mellitus (T2DM) and cardiovascular diseases (4-7). Elevation of circulating adiponectin by either transgenic overexpression or direct supplementation with recombinant adiponectin can alleviate many metabolic abnormalities associated with various insulin resistant and/or diabetic animal models (8-12). Globular domain of adiponectin decreases postprandial blood glucose, enhances lipid clearance and increases insulin sensitivity by enhancing fatty acid β-oxidation in skeletal muscle (8). On the other hand, full-length adiponectin generated from mammalian cells enhances the sensitivity of insulin to inhibit hepatic glucose
production by suppressing the expression of several key enzymes involved in gluconeogenesis, such as phosphoenolpyruvate carboxylase and glucose-6-phosphatase (10).

Besides its metabolic effects, adiponectin also possesses anti-atherogenic, anti-inflammatory and hepato-protective functions. This adipokine can prevent atherosclerotic lesion formation in apolipoprotein E-deficient mice, possibly by decreasing endothelial expression of inflammatory adhesion molecules, blocking foam cell formation and inhibiting proliferation of aortic smooth muscle cells (11,13-16). It can also alleviate both alcoholic- and non-alcoholic steatohepatitis, as well as liver injuries induced by toxins such as carbon tetrachloride and lipopolysaccharides (17-20). More recently, adiponectin has been proposed to play a protective role against cardiac hypertrophy and ischemia/reperfusion induced myocardial infarction through the activation of AMP-activated protein kinase (AMPK) in myocardium (21-23).

Adiponectin structurally belongs to the complement C1q-like protein family that consists of an NH2-terminal collagenous domain comprising of 22 Gly-X-Y repeats and a COOH-terminal globular head domain (3,24,25). In the circulation, adiponectin is predominantly present as several characteristic oligomeric complexes (26-29). The basic building block of the adiponectin complex is trimer or low molecular weight (LMW) oligomer, which is formed via hydrophobic interactions within its globular domain. Two trimers self associate to form a disulfide-linked hexamer or middle molecular weight (MMW) oligomer, which further assembles into a bouquet-like higher molecular weight (HMW) multimeric complex that consists of 12-18 monomers. Different oligomeric forms of adiponectin might activate different signaling pathways and exert distinct functions on its target tissues (16,27,30-32). The globular domain of adiponectin, which can only form trimers, is more potent than the other two oligomers in inducing AMPK activation and fatty acid β oxidation in skeletal muscles (33). On the other hand, the HMW complex is the most active form of adiponectin in suppressing hepatic glucose production (26,30). Furthermore, only the HMW adiponectin, but not the trimeric and hexameric form, can protect endothelial cells from apoptosis (34).

It has recently been proposed that the ratios of HMW/total adiponectin, but not the absolute amounts of adiponectin, are closely correlated with insulin sensitivity in humans and rodents. The percentage composition of the HMW form in db/db diabetic mice is much lower than its wild type littersmates, despite having similar levels of total adiponectin in the circulation (29,30). Clinical studies have also demonstrated that patients with T2DM and coronary heart disease are associated with a selective reduction in the HMW oligomeric adiponectin (35,36). On the other hand, weight reduction or treatment with the insulin-sensitizing drug rosiglitazone preferentially elevates the HMW form of adiponectin without significant effects on the other two oligomeric complexes (30,37). Interestingly, two rare missense mutations (G84R and G90S) identified in T2DM patients can form trimer and hexamer, but lack the capacity to form the HMW complex (36). These data collectively suggest that impaired formation of the HMW adiponectin might be causally associated with insulin resistance and T2DM.

The mechanism that regulates the formation and distribution of the adiponectin oligomeric complexes remains largely obscure at this stage. We have previously shown that adiponectin is modified at the posttranslational level during its secretion from adipocytes (38). Several conserved lysine residues (68, 71, 80 and 104) within the collagenous domain of murine adiponectin are hydroxylated and glycosylated by a glucosyl (1-2)galactosyl group, which might in turn contribute to its insulin-sensitizing activity in hepatocytes (28,38). In this study, we investigated the potential roles of these posttranslational modifications in regulating the oligomeric complex formation of adiponectin in cell culture systems, animal models and human subjects.

**EXPERIMENTAL PROCEDURES**

**Materials**—Minoxidil, dexamethasone, 3-isobutyl-1-methylxanthine, FLAG peptide and anti-FLAG M2 affinity gel were purchased from Sigma. Dulbecco's Modified Eagle's Medium (DMEM) and Lipofectamine transfection reagent were from Invitrogen. The QuickChange site-directed mutagenesis kit was from Stratagene. Adeno-X Expression System and the Immun-Blot kit for glycoprotein detection were from BD Biosciences Clontech and Bio-Rad Inc respectively. The antibodies against total AMP-activated protein kinase (AMPK) α and phospho-AMPKα (Thr172) were obtained from Cell Signaling. The Complete Protease Inhibitor Cocktail Tablets were from Roche Diagnostics.

**Site-directed mutagenesis**—The expression vector pcDNA-Ad-F, which encodes the full-length murine adiponectin with a FLAG epitope tag at its COOH terminus (38), was used as a template to construct the vectors encoding the adiponectin variants in which the four lysines (residues 68, 71,
expression vectors encoding various FLAG-tagged adiponectin variants with one, two, three or all four lysine residues substituted with arginines were constructed by sequential mutation of each site. All these mutations were confirmed by DNA sequencing.

Expression and purification of recombinant murine adiponectin and its variants—The vectors for expression of wild type adiponectin or its various variants were transfected into HEK293 cells using Lipofectamine reagent according to the manufacturer’s instructions. The transfected cells were incubated in serum free DMEM media containing 0.2 % Vitamin C and 0.2 % BSA for 48 hr. The FLAG-tagged recombinant proteins were purified using the anti-FLAG M2 monoclonal antibody affinity gel as we previously described (38). The cells were solubilized with a lysis buffer containing 1 % Triton X-100, 25 mM HEPES, 5 mM EDTA, 100 mM NaCl in the presence of the protease inhibitor cocktails and stored at -80°C until use.

Construction and production of recombinant adenoviruses encoding wild type or mutated adiponectin—The adenovirus expression vectors that encode FLAG-tagged adiponectin and its variants were generated by using the Adeno-X Expression System, as we previously described (39). The recombinant virus was packaged and amplified in HEK293 cells and purified by CsCl density gradient centrifugation. The titers of the recombinant virus were determined by plaque assay.

Quantification of different oligomeric forms of adiponectin using gel filtration chromatography and ELISA—Serum samples, conditioned culture medium or cell lysates were diluted with PBS to a final volume of 1 ml, loaded onto an AKTA explorer fast protein chromatography system, fractionated through a Hiloald 16/60 Superdex 200 column (GE Healthcare), and eluted with PBS at the flow rate of 1 ml/min. Each 1-ml fraction was collected and subjected to ELISA analysis for murine adiponectin to determine the concentrations of each oligomeric form of adiponectin, as we described elsewhere (29).

Western Blotting analysis—For detection of different oligomeric adiponectin complexes, mouse serum samples, cell lysates or conditioned culture medium were incubated with a non-reducing sample buffer (1% SDS, 5% glycerol, and 10 mM of Tris-HCl, pH 6.8) at room temperature for 10 min, separated by a 4–20% gradient SDS-PAGE, transferred to polyvinylidene difluoride membranes, and immunoblotted with affinity purified rabbit anti-mouse adiponectin IgG antibody as we previously described (29).

For detection of total or phosphorylated AMPK, 50 µg protein homogenates from liver or gastrocnemius muscle were separated by 10% SDS-PAGE, and probed with anti-total AMPK or phospho-AMPK as described elsewhere (33).

For glycoprotein detection, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes and glycoproteins were detected using the commercial Immun-Blot kit according to the manufacturer’s instructions (38).

Pulse-chase experiment—HEK293 cells were transfected with expression vectors that encode wild type adiponectin or its variants. 24 hr after transfection, cells were starved in methionine- and cysteine-free DMEM for 1 hr, and then replaced with the same fresh medium plus 50 µCi/ml of [35S]methionine and [35S]cysteine (Redivue Pro-mix L-[35S], GE Healthcare) for 2 hr. The labeling medium was then replaced with cold DMEM plus a 20-fold excess of methionine and cysteine for different time period. Both culture medium and cells were harvested for immunoprecipitation using a rabbit anti mouse adiponectin antibody (29,38). Cells were washed with PBS, solubilized in an IP buffer (25 mM HEPES pH 7.5, with 5 mM EDTA and EGTA, 100 mM NaCl, 10% glycerol, 1% Triton X-100, plus protease inhibitor cocktails), and the cell debris was removed by centrifugation (13,000 x g, 5 min). All the cell lysates were adjusted to an equal protein concentration (0.5 µg/µl) with IP buffer. 500 µl of cell lysates was preincubated with 50 µl of protein A/G beads (GE Healthcare) for 1 hr to remove nonspecific bindings. The supernatants were incubated with 10 µg of the rabbit anti-mouse adiponectin IgG overnight at 4 °C with shaking, and 50 µl of protein A/G beads was then added into the supernatant for another 1 h. The beads were precipitated and washed with IP buffer three times, and the immunoprecipitated complexes were eluted by incubation with 100 µl SDS-PAGE loading buffer. The eluted samples were separated by 12% SDS-PAGE and then analyzed by phosphorimaging (BAS 2000; Fujifilm). The radioactivity associated with each band was quantified as we previously described (29).

Lysyl hydroxylase inhibitor study in rat primary adipocytes—White fat precursor cells were isolated from the epididymal fat pad of 6-week-old male Wistar rats, and differentiated into mature adipocytes as described previously (29). Ten days
after differentiation, ~80% of the cells became mature adipocytes filled with lipid droplets, as assessed by Oil Red O staining. The cells were treated with either vehicle (0.1% ethanol) or the lysyl hydroxylase inhibitor minoxidil at indicated concentrations for 2 days in the presence of 10% FBS. The cells were then replaced with a serum-free medium containing 0.2% BSA and the inhibitor for another 24 hr. Both cells and the conditioned media were collected for analysis of adiponectin oligomeric complexes as above.

Animal studies—C57BL/6J adiponectin knockout mice (40) were housed under controlled light/dark cycle (12/12 hr) and temperature conditions, with free access to water and a standard mouse diet. 8-10 week old male mice weighing 20–25 g were administered with 5 × 10^7 plaque-forming units (p.f.u) of various recombinant adenovirus via tail vein injection. At different time points, small drops of blood samples were taken from the tails for analyzing the composition of different oligomeric forms of adiponectin. For the acute studies, the animals were fasted overnight and intravenously injected with various forms of recombinant adiponectin (2 mg/kg body weight). After 10 min, mice were sacrificed, blood was collected by cardiac puncture, and tissues were quickly dissected and snap frozen in liquid nitrogen for further analyses.

Male C57BKS db/db mice (10- to 11-wk-old, from the Jackson Laboratory) were used to evaluate the chronic effect of various forms of adiponectin. The protein was continuously delivered into the mice using ALZET osmotic pumps (DURECT Corp., Cupertino, California, USA) as we described previously (18,19). The pumps were filled with various protein solutions or saline as control. Mice were anesthetized and a ~0.5 cm incision was made at the lower back of the animals. A small subcutaneous pocket was made to insert the pumps that deliver the protein solutions at a constant rate for a period of two weeks. All the experimental protocols were approved by the Animal Ethics Committee at the University of Hong Kong.

Measurement of blood parameters, intraperitoneal glucose tolerance test (ipGTT) and insulin tolerance test (ITT)—Fasting (16 hr) plasma glucose levels and triglyceride (TG) concentrations were measured by using a Glucose (GO) assay kit (Sigma) and Triglyceride GPO reagent (Pointe Scientific Inc., Lincoln Park, Michigan, USA) respectively. Fasting serum insulin concentrations were quantified by using the commercial ELISA kits from Mercodia AB (Uppsala, Sweden). Serum total cholesterol free fatty acids (FFAs) was analyzed by using the kits from Wako (Richmond, VA, USA) and Roche Diagnostics respectively. TG contents in liver and gastrocnemius muscle were analyzed as described elsewhere (18,39).

For ipGTT, overnight fasted mice (16 hr) were given a glucose load by intraperitoneal injection (1 g glucose/kg body weight). For ITT, mice were starved for 6 hr, and then intraperitoneally injected with insulin (1U/kg body weight). Plasma glucose levels were measured at different time points as indicated.

Gene expression analysis—Total RNA were isolated from the collected mice tissues using the RNeasy kit (Qiagen). Subsequently, mRNA was reverse transcribed into cDNA using the oligo-dT primer (Roche). mRNA expression of mouse adiponectin was determined by PCR using the forward primer: 5’-CACCCAAGGGAACTTGTGCAG-3’ and the reverse primer: 5’-GGTCGTAGGTTGAAGAGAACGG-3’. The forward and reverse primers for beta-actin were 5’-GGACTCCTATGTGGGTGACGAGG-3’ and 5’-GGGAGAGCATAGCCCTCGTAGAT-3’ respectively.

Studies on human subjects—Serum samples from 12 male patients with T2DM and 12 age-matched male healthy controls were selected from our previous studies (39). The study protocol for these human samples was approved by the Ethics Committee of the Medical Faculty, University of Hong Kong. The clinical characteristics of these subjects are described in Table 2. Plasma levels of total adiponectin and the ratios of HMW versus total adiponectin were determined by an in-house human adiponectin ELISA method and gel filtration chromatography, as we previously described (29). To determine the carbohydrate contents of endogenous human adiponectin, 1 ml of serum samples were depleted of albumin and immunoglobulin G using the ProteoExtract Albumin/IgG Removal Kit (Calbiochem, San Diego, USA). The remaining supernatant was then incubated with 100 µl of the Sepharose beads coupled with mouse non-immune IgG to remove non-specific bindings. The clarified supernatant was subsequently incubated with 100 µl of the Sepharose beads coupled with mouse monoclonal anti-human adiponectin IgG (29) at 4°C overnight.

The beads were washed with TBS and the bound protein complexes eluted with 0.1 M glycine HCl (pH 2.5). The eluted fractions were concentrated and the adiponectin concentrations were quantified as above.
Results

The three oligomeric complexes of adiponectin produced from mammalian cells are differentially glycosylated—Our previous study demonstrated that both endogenous adiponectin and recombinant adiponectin produced from mammalian cells is posttranslationally modified by hydroxylation and glycosylation (38). To investigate the roles of these posttranslational modifications on oligomeric complex formation of adiponectin, we generated recombinant adiponectin from both prokaryotic and HEK293 cells. Gel filtration analysis revealed that adiponectin produced from HEK293 cells formed HMW, MMW, and LMW oligomeric complexes, which were eluted at 45.5–49, 52.5–56.5, and 58.5–62 min respectively (Figure 1A). A non-heating and non-reducing SDS-PAGE analysis resolved this recombinant protein into three distinct bands with apparent molecular masses of >250 kDa, ~180 kDa and ~90 kDa, which is equivalent to the HMW, MMW and LMW oligomeric forms respectively (Figure 1B). On the other hand, adiponectin derived from E. coli, which is not glycosylated, formed only LMW and MMW oligomers, but lacked the capacity to further assemble into the HMW oligomeric complexes. Carbohydrate detection revealed that the three oligomeric forms of adiponectin derived from mammalian cells were differentially glycosylated (Figure 2). The carbohydrate contents of the HMW adiponectin were significantly higher than those of the MMW and LMW oligomeric complexes. These results suggest that the four hydroxylysines and their attached glycosides might be important for efficient secretion of adiponectin.

Substitution of the four lysine residues with arginines causes decreased secretion of adiponectin and impaired intracellular assembly of its HMW complexes—We have previously reported that glycosylation of adiponectin occurs primarily on the four hydroxylysine residues (68, 71, 80 and 104) within its collagen-like domain (28,38). Compared to wild type adiponectin, the adiponectin mutant with the four lysines replaced by arginines (ADN-K/R) is much less effective in enhancing insulin’s ability to suppress hepatic glucose production (38). Here, we investigated whether the posttranslational modifications on these four lysines of adiponectin play any roles in its secretion and/or oligomeric complex formation in HEK293 cells transfected with different expression vectors. Quantitative ELISA analysis revealed that the concentration of wild type adiponectin released into the culture medium was much higher than that of the mutant ADN-K/R (Figure 3A), although the steady state adiponectin mRNA levels were comparable (data not shown). Pulse-chase experiment with 35S demonstrated that the secretion rate of wild type adiponectin was much faster than that of the mutant ADN-K/R (Figure 3B and 3C). At 15 hr after chasing, the majority of wild type adiponectin synthesized during a 2-hr pulse period was released into the media, whereas ~40% of the mutant ADN-K/R was still retained inside the cells. These results suggest that the four hydroxylysines and their attached glycosides might be important for efficient secretion of adiponectin.

Non-heating and non-reducing gel electrophoresis and Western Blotting analysis showed that wild type adiponectin formed all the three oligomers (Figure 4). The HMW, MMW and LMW oligomeric complexes accounted for ~37%, ~42% and ~19% respectively inside the cells as well as in the conditioned culture medium. On the other hand, both intracellular and extracellular ADN-K/R was present predominantly as the LMW and MMW oligomeric forms, whereas the HMW oligomers were barely detectable. Notably, ADN-K/R oligomers migrated slightly faster than those of wild type adiponectin, suggesting the loss of the carbohydrate moieties. These data demonstrate that posttranslational modifications on these four conserved lysine residues are critical for intracellular assembly of the HMW oligomeric adiponectin complexes.

To further determine the contribution of the posttranslational modifications on each of the four lysine residues to the secretion of adiponectin and its oligomeric complex formation, we generated a series of vectors for expression of the adiponectin variants with one, two or three of the lysines substituted by arginines. These expression vectors were transiently transfected into HEK293 cells for assessing the capacity of these adiponectin variants to form the HMW oligomeric complex. Quantitative ELISA analysis revealed that adiponectin released...
into conditioned medium was progressively decreased with sequential substitution of the four lysines replaced by arginines (data not shown). Gel filtration analysis showed that single mutation on each of the four lysines (ADN-68, ADN-71, ADN-80 and ADN-104) only caused a slight, but not significant decrease in the formation of the HMW oligomeric adiponectin (Figure 5). On the other hand, the ability of the double mutants (ADN-68/71 and ADN-80/104) and triple mutants (ADN-68/71/80 and ADN-68/71/104) to assemble into the HMW oligomeric complex was significantly attenuated. Notably, the percentage composition of the HMW oligomeric form versus total adiponectin was gradually decreased following sequential mutation of one, two, three and all the four lysine residues, suggesting that the hydroxylysines and the attached glycosides might function in a cooperative manner to facilitate the formation of the HMW oligomeric adiponectin.

Inhibition of lysyl hydroxylases by minoxidil decreases the formation of the HMW adiponectin oligomers in rat adipocytes—A family of lysyl hydroxylases (LH, EC 1.14.11.4), consisting of LH1, LH2a, LH2b and LH3, is responsible for catalyzing conversion of lysine into hydroxylysine, which provide attachment sites for further glycosylation (41). Minoxidil has been shown to inhibit the activity of LH by decreasing the gene expression of all the three isoenzymes (42,43). We therefore tested the effect of minoxidil on the oligomeric complex formation of endogenous adiponectin in primary rat adipocytes. In line with our previous report, we found that the HMW oligomer accounted for ~35% of total adiponectin in the conditioned medium of adipocytes, which was significantly lower than that inside the cells (Figure 6), suggesting that secretion of the HMW oligomer from adipocytes is relatively slower than the other two oligomeric forms (29). Incubation of mature rat adipocytes with 500 µM minoxidil for a period of 72 hr led to a ~70% reduction of lysyl hydroxylase activity (data not shown). The percentage composition of the HMW complex versus total adiponectin in both cell lysates and conditioned medium of adipocytes were substantially decreased following treatment with this compound. Treatment with 500 µM minoxidil also decreased the percentage composition of the HMW/total adiponectin in HEK293 cells transfected with a vector that encodes wild type adiponectin (data not shown).

Disruption of hydroxylation and glycosylation on the four lysine residues impairs the production of the HMW oligomeric adiponectin in vivo—To further evaluate the effect of lysine hydroxylation/glycosylation on oligomeric complex formation in vivo, we generated recombinant adenoviruses that encode wild type adiponectin or its variant ADN-K/R, in which the four lysines were replaced by arginines. The recombinant adenovirus was introduced into adiponectin-null mice via tail vein injection. RT PCR analysis revealed that the adiponectin gene was primarily expressed in the liver tissue following injection with the recombinant adenoviruses. Despite having a similar mRNA expression level in the liver tissue, the protein concentrations of wild type adiponectin in both liver tissue and in the circulation were substantially higher than those of the adiponectin variant ADN-K/R (Figure 7), suggesting that lysine hydroxylation and glycosylation might be involved in the stabilization of adiponectin in vivo. In serum as well as the liver lysates, the predominant oligomeric form of wild type adiponectin is the HMW oligomer, which account for ~65% of total adiponectin (Figure 8). On the contrary, the HMW oligomeric complex only accounted for ~9% of the total adiponectin variant ADN-K/R, suggesting that hydroxylation/glycosylation on lysine residues is critical for the formation of the HMW adiponectin oligomers in vivo.

Hydroxylation/glycosylation and formation of the HMW complexes are critically involved in adiponectin-induced activation of AMP-activated protein kinase in liver, but not in skeletal muscle—Adiponectin exerts its multiple metabolic effects primarily via inducing phosphorylation and activation of AMPK in liver and skeletal muscle (33). To investigate the potential roles of lysine hydroxylation/glycosylation and subsequent formation of the HMW oligomers in regulating the metabolic activities of adiponectin, we next supplemented the adiponectin-null mice with various forms of adiponectin with varying degrees of the posttranslational modifications. Intravenous injection of different forms of the recombinant adiponectin into the adiponectin-null mice at the dosage of 2 mg/kg body weight raised plasma levels of adiponectin from 0 to ~7-10 µg/ml within 5 min. In the liver tissue, wild type adiponectin produced from HEK 293 cells, which contains properly hydroxylated/glycosylated lysines and also forms the HMW oligomeric complexes, potently induced phosphorylation of AMP-activated protein kinase (AMPK) (Figure 9). Disruption of lysine
hydroxylation/glycosylation by either substitution of the four lysines with arginines, or by the lysyl hydroxylase inhibitor minoxidil, significantly attenuated the activities of adiponectin on phosphorylation of AMPK. Bacterially generated full-length or globular adiponectin, both of which lack the posttranslational modifications and cannot form the HMW oligomeric complex, had no obvious effects. In skeletal muscle, globular adiponectin potently induced AMPK phosphorylation, whereas the same dosage of full-length adiponectin produced from different sources exhibited little activity.

Lysine hydroxylation/glycosylation and HMW oligomeric complexes are important for the anti-diabetic activities of full-length adiponectin in db/db diabetic mice—We next investigated the chronic effects of various forms of adiponectin in db/db mice, an established obese/diabetic mouse model with insulin resistance. To this end, mice were surgically implanted with an ALZET osmotic pump, which delivered various forms of adiponectin (1.5 mg/kg body weight. day) or physiological saline at a constant rate. Delivery of various forms of adiponectin protein at this dosage caused approximately 2.0- to 2.5- folds elevation of the circulating adiponectin levels throughout the 2-week treatment period. These treatments had no obvious effect on food intake, body weight gains, and serum levels of insulin and total cholesterol (Table 2). Wild type adiponectin produced from HEK293 cells, which possesses properly hydroxylated/glycosylated lysines and also forms the HMW oligomeric complexes, significantly decreased blood glucose levels and serum TG concentrations, and also caused a drastic reduction of TG accumulation in the liver tissue, and a modest decrease of TG contents in skeletal muscle (Table 2). These data further support several previous reports showing that globular adiponectin increases insulin sensitivity primarily through reduction of muscular lipid accumulation, while full-length adiponectin with proper posttranslational modifications and HMW oligomeric complexes exerts its beneficial metabolic effects mainly via its hepatic actions (16,30,31).

Both the ratio of HMW/total adiponectin and the degree of adiponectin glycosylation are decreased in T2DM patients—To explore the clinical relevance of the above findings, we next investigated the relationship between the posttranslational modifications and the oligomeric complex formations in 12 T2DM patients and 12 age-/sex-matched healthy controls. The clinical characteristics of these subjects are summarized in Table 3. Compared to the healthy controls, plasma levels of total adiponectin as well as the percentage composition of HMW versus total adiponectin were significantly decreased (Figure 11). Furthermore, the carbohydrate contents of adiponectin in T2DM patients were also significantly lower than that in healthy controls. There is a strong positive correlation between the ratios of HMW/total adiponectin and the carbohydrate contents of adiponectin ($r=0.213$, $p<0.05$). These results indicate that decreased glycosylation of hydroxylsines might be causally linked to the impaired formation of the HMW adiponectin oligomers in certain pathological conditions.

DISCUSSION
Regulation of oligomeric complex formation is increasingly recognized to be an important mechanism that modulates the pleiotropic biological functions of adiponectin. Several recent studies have demonstrated that the ratio of HMW/total adiponectin is more closely associated with glucose tolerance and insulin sensitivity than the plasma levels of total adiponectin (30,36,37). The ratio of HMW/total adiponectin is decreased in obese subjects and patients with T2DM and coronary heart disease, and this decrease is reversed following moderate weight reduction and treatment with thiazolidinediones (30,35,44). We and others have found that the sexual dimorphism of adiponectin in both humans and rodents is primarily attributed to the difference in the HMW adiponectin, with females having significantly higher levels of...
this oligomer than males (26,29). While these data support the role of the HMW adiponectin as an endogenous insulin sensitizer, the molecular basis that mediates the formation of the HMW oligomeric complexes at the posttranslational level remains poorly understood.

Several previous studies have demonstrated the necessity of disulfide bonds mediated by Cys39 in the formation of hexameric and HMW adiponectin, but have not shown whether these disulfide bonds were sufficient for the high order structural formation of adiponectin. In this study, we provide both in vivo and in vitro evidence to support the notion that posttranslational modifications, specifically the hydroxylation and further glycosylation on several lysine residues within the collagenous domain, are required for intracellular assembly of the HMW adiponectin oligomers. Firstly, bacterially generated full-length adiponectin, which lacks posttranslational modifications, could not form the HMW oligomers (Figure 1). Secondly, ablation of hydroxylation and glycosylation by substitution of the four lysines (68, 71, 80 and 104) with arginines impeded the intracellular assembly of the HMW adiponectin in both cell culture system and in mice (Figures 4 and 8). In addition, treatment with minoxidil, a lysyl hydroxylase inhibitor, also resulted in a marked reduction of the HMW adiponectin (Figure 6). Consistent with our results, a recent study on lysyl hydroxylase 3 null mice showed that ablation of this enzyme led to a total absence of hydroxyllysine and its attached carbohydrates in type IV collagen, and impaired high order structure formation of this protein (43). Notably, in addition to its lysyl hydroxylase activity, lysyl hydroxylase 3 also possesses relatively low levels of glucosyltransferase and galactosyltransferase activities, suggesting that this enzyme alone is sufficient for catalyzing all the three steps including lysine hydroxylation and its further attachment with a glucosyla (1-2)galactosyl group (45). Further study is warranted to investigate whether this enzyme is involved in regulating the HMW oligomeric complex formation of adiponectin via catalyzing lysine hydroxylation and glycosylation of adiponectin in adipocytes.

The intracellular assembly and secretion of adiponectin oligomeric complexes are a complex process that might vary in different cell types. The ratio of HMW/total adiponectin inside rat primary adipocytes is ~65%, which is significantly higher than that expressed in HEK293 cells (~35%). On the other hand, the percentage compositions of HMW/total adiponectin in the culture medium of adipocytes are much lower than those in the cell lysates, suggesting that the HMW adiponectin is selectively retained inside the cells. We have recently reported that the secretion of HMW adiponectin from adipocytes is much slower than the other two oligomeric complexes, and that testosterone treatment further decreases the secretion of this oligomeric form (29). Acute injection of insulin and glucose has been shown to selectively decrease the circulating levels of HMW adiponectin in mice, although the underlying mechanism remains to be determined (30). In the present study, we show that the decreased ratio of HMW/total adiponectin in type 2 diabetic patients is closely associated with the reduced degree of adiponectin glycosylation (Figure 11), implying that impaired posttranslational modifications of adiponectin might be causally linked with T2DM by disrupting the high order structure formation of this adipokine.

Several previous studies from Dr. Scherer’s group suggest the HMW oligomer of adiponectin to be the major active form responsible for its insulin sensitizing effect in hepatocytes (3,10,46). The HMW adiponectin oligomers can decrease blood glucose levels by activation of AMPK, which in turn inhibit hepatic glucose production by decreasing the expression of key gluconeogenic genes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (10,30,33). Consistent with these reports, our present study also demonstrated that acute injection of wild-type adiponectin produced from mammalian cells could induce phosphorylation of AMPK in the liver tissue (Figure 9). Chronic treatment of db/db diabetic mice with wild type adiponectin led to a significant decrease in blood glucose and serum TG concentrations, and also improved glucose tolerance and insulin sensitivity (Table 2 and Figure 10). Notably, these changes were associated with a marked reduction of hepatic TG accumulation. All these beneficial metabolic effect of full-length adiponectin were significantly attenuated with the decrease in the composition of the HMW oligomers, and was abolished with the depletion of the HMW oligomers by ablation of lysine hydroxylation and glycosylation. Therefore, the magnitude of AMPK phosphorylation in the liver tissue and the metabolic effects of adiponectin in db/db diabetic mice appear to be correlated well with the composition of its HMW oligomers. In contrast to their actions in the liver, none of the full-length adiponectin, regardless of the composition of the HMW oligomers, had any obvious effect on AMPK phosphorylation in skeletal muscle. These findings are in agreement
with recent clinical reports showing that plasma levels of the HMW oligomers of adiponectin are strongly correlated with hepatic insulin sensitivity, but less relevant with the insulin sensitivity in muscle (44,47,48). In line with previous reports (32,33), our results also demonstrated that globular adiponectin, which forms exclusively as trimers, had potent effects in activation of AMPK and in reduction of lipid accumulation in skeletal muscle, which was associated with a significant alleviation of hypertriglyceridemia and insulin resistance. Nevertheless, globular adiponectin lacks ability to activate AMPK in the liver and to decrease fasting blood glucose in db/db diabetic mice (Figure 9 and Table 2). Taken together, these results support the notion that globular adiponectin and full-length adiponectin exert distinct metabolic actions in different target tissues (16,30,31).

In summary, this study provide the evidence demonstrating that hydroxylation and glycosylation on several conserved lysine residues within the collagenous domain of adiponectin are required for the formation of its HMW oligomeric complexes, which in turn confer the insulin sensitizing effects of this adipokine in hepatocytes. In addition, decreased levels of the HMW adiponectin and reduced carbohydrate contents of this protein were concurrently observed in patients with T2DM, suggesting that these events might be causally linked with this disease. Further investigation is needed to identify the precise enzyme that mediates lysine hydroxylation and glycosylation of adiponectin in adipocytes, and to elucidate the underlying mechanisms that regulate these events.

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FOOTNOTES

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The abbreviations used are: HMW, high molecular weight; MMW, middle molecular weight; LMW, low molecular weight; ADN-K/R, adiponectin mutant with the four lysines mutated to arginines; ADN-68, ADN-71, ADN-80 and ADN-104, adiponectin mutants with amino acid 68, 71, 80 or 104 replaced by arginines respectively; AMPK, AMP-activated protein kinase; T2DM, type 2 diabetes mellitus.

FIGURE LEGENDS

Figure 1. Oligomeric complex distribution of recombinant adiponectin expressed in E. coli and HEK293 cells. The protein samples were prepared as described under “Experimental Procedures”. 20 µg of recombinant adiponectin from E. coli or HEK293 cells was fractionated by gel filtration chromatography (A), or separated by a non-reducing, non-heating SDS-PAGE and stained with Commassie Brilliant Blue R250 (B).

Figure 2. Detection of the carbohydrate contents for the three oligomeric complexes of adiponectin produced from HEK293 cells. Recombinant adiponectin produced from HEK293 cells was fractionated by gel filtration as in Figure 1, and the major fractions corresponding to the HMW, MMW and LMW oligomeric complexes of adiponectin were collected, pooled and concentrated as we described elsewhere (29). 2 µg of each oligomeric form was separated by SDS-PAGE, and the carbohydrate content of each oligomeric form of adiponectin was analyzed by glycoprotein detection kit as described in “Experimental Procedures”. *, P < 0.05 versus MMW and LMW (n=4).

Figure 3. Substitution of the four lysine residues with arginines leads to decreased secretion efficiency of adiponectin. HEK293 cells were transfected with the same amount of vectors that encode either wild type (ADN-WT) or the mutant (ADN-K/R) adiponectin. At 24 hr after transfection, adiponectin concentrations in the extracellular medium were quantified by ELISA (A). Alternatively, the transfected cells were pulse-labeled with 35S methionine for 2 hr, and chased in a cold medium for various periods as indicated. 35S-labelled adiponectin in both conditioned medium and in cell lysates
was immunoprecipitated with anti-mouse adiponectin antibody, separated by SDS-PAGE and visualized by phosphorimaging (B). The graph in panel C shows the percentage of adiponectin secreted into the conditioned culture medium at different time points after chasing. ** P<0.01 versus ADN-WT.

Figure 4. Comparison of the oligomeric complex distribution between wild type adiponectin (ADN-WT) and the adiponectin variant (ADN-K/R) expressed in HEK293 cells. HEK 293 cells were transfected with the vectors that encode either ADN-WT or ADN-K/R. 48 hr after transfection, both cell lysates and conditioned media were harvested and subjected to ELISA analysis for total mouse adiponectin. 200 ng of total adiponectin from cell lysate or conditioned medium was separated by a non-reducing and non-heating SDS-PAGE and immuno-blotted with anti-mouse adiponectin antibody (A). In panel B, conditioned medium was fractionated, and the fractions corresponding to the three oligomeric forms of adiponectin were pooled. The percentage composition of each oligomeric form versus total was determined as described under “Experimental Procedures”. *, P < 0.01 versus ADN-WT (n=4). Note that the percentage compositions of each oligomeric form in cell lysates are similar with those in the conditioned medium (data not shown).

Figure 5. Sequential substitution of the four lysine residues with arginines attenuates the formation of adiponectin HMW complexes in a progressive manner. HEK293 cells were transiently transfected with various expression vectors that express wild type adiponectin (WT-ADN) or adiponectin variants with one, two, three or four lysines replaced by arginines as indicated. At 48 hr after transfection, adiponectin concentrations in the conditioned medium were quantified by ELISA. 2 µg of wild type adiponectin or its different variants were subjected to gel filtration separation and the percentage composition of HMW complexes versus total adiponectin was determined as in Figure 4. * P < 0.05 and **, P < 0.01 versus WT-ADN (n=4).

Figure 6. Effects of minoxidil on oligomeric complex formation of adiponectin in rat adipocytes. Differentiated rat adipocytes were pre-treated without or with 500 µM minoxidil in DMEM plus 10% fetal bovine serum for 48 hr, and were then grown in the same condition for another 24 hr, except that serum was replaced by 0.5% BSA. Cell lysates and conditioned medium containing 200 ng adiponectin were separated by 4-20 % SDS-PAGE, and probed with anti-mouse adiponectin antibody (A). In panel B, cell lysates and conditioned medium from adipocytes were fractionated by gel filtration chromatography, and the percentage composition of each oligomeric complex of adiponectin was determined as in Figure 4.

Figure 7. Adenovirus-mediated expression of wild type adiponectin (ADN-WT) and its variant (ADN-K/R) in adiponectin KO mice. 7 days after intravenous injection with 5 x 10³ p.f.u. of the recombinant adenovirus encoding luciferase (control), ADN-WT or ADN-K/R, mice were sacrificed and total RNA extracted from the liver tissue was subjected to RT PCR analysis for mRNA expression analysis of the murine adiponectin or actin gene (A). Total adiponectin concentrations in the liver (B) and in the circulation (C) were measured by an ELISA method. *, P < 0.01 versus ADN-WT (n=5).

Figure 8 Ablation of lysine hydroxylation and glycosylation impairs the formation of the HMW adiponectin in mice. Liver homogenates or serum samples from mice infected with the recombinant adenovirus for expression of wild type adiponectin or the variant ADN-K/R were prepared as in Figure 7. Each sample containing 200 ng of total adiponectin was separated by SDS-PAGE in a non-heating and non-reducing condition and immunoblotted with anti-mouse adiponectin antibody (A). In panel B, liver homogenates or serum samples were fractionated by gel filtration, and the percentage composition of the HMW oligomer versus total adiponectin was determined as in Figure 4. *, P < 0.01 versus control (n=4).

Figure 9 Differential effects of different forms of adiponectin on phosphorylation of AMPK in liver and gastrocnemius muscle. Various forms of recombinant adiponectin were intravenously injected into C57BL/6J adiponectin knockout mice at the dosage of 2 mg/kg body weight. 10 min
after injection, mice were sacrificed to dissect liver (A) and gastrocnemius muscle (B). 50 µg of protein homogenates from these tissues were separated by SDS-PAGE, and immuno-blotted with anti-total AMPK or phospho-AMPK as indicated. AND-WT, wild type adiponectin produced in HEK293 cells; ADN-K/R: the adiponectin variant with the four lysines replaced by arginines, produced in HEK293 cells; ADN-WT + minoxidil: wild type adiponectin produced in HEK293 cells in the presence of 500 µM minoxidil; fAd-E. coli: full-length adiponectin expressed in E. coli; gAd-E. coli: globular head of adiponectin produced in E. coli. Representative immunoblots are shown for each quantitative analysis performed in experiments repeated for at least three times. *P < 0.05 compared with control.

**Figure 10** Chronic effects of various forms of adiponectin on glucose tolerance and insulin sensitivity in db/db diabetic mice. ipGTT and ITT were conducted at day 11 and day 13 after various treatments. In ITT study, Plasma glucose levels were normalized to those at t=0 min in each treatment group. ADN-WT, wild type adiponectin produced in HEK293 cells; ADN-K/R: the adiponectin variant with the four lysines replaced by arginines, produced in HEK293 cells; fAd-E. coli: full-length adiponectin expressed in E. coli; gAd-E. coli: globular head of adiponectin produced in E. coli. *P <0.05 versus saline-treated group (n=4-5).

**Figure 11** The carbohydrate contents of adiponectin and the ratios of HMW/total adiponectin in T2DM patients and healthy individuals. 2 µg of human adiponectin purified from each serum sample by affinity chromatography was subjected to SDS-PAGE and carbohydrate estimation analysis as described under “Experimental Procedures”. Representative gels are shown in A. B, Quantitative analysis of carbohydrate contents. C, serum samples from these subjects were fractionated by gel filtration chromatography and each fraction was analyzed using an in house ELISA method for human adiponectin to determine the ratio of the HMW oligomers as described. *P < 0.01 versus healthy controls.
| Mutation site | Sequences (5’-3’) |
|---------------|------------------|
| 68            | Forward: GGCACCTCTGGAGAGCGGGGAGAGAAAGG  
                Reverse: CCTTTTCTTCCCCGCTTCCAGGAGTGCC |
| 71            | Forward: GGAGAGAAGGGAGAGCGAGGAGATGCAGGTC  
                Reverse: GACCTGCATCTCCTCGCTCTCCCTTCTCTCC |
| 68/71         | Forward: GCACTCCTGGAGAGCGAGGAGCGAGGAGATGCAGG  
                Reverse: CCTGCATCTCCTCGCTCTCCCTTCTCTCC |
| 80            | Forward: GGTCTTTCTGTGCTCTCGGAGAGCAGCAGAG  
                Reverse: CTGCTGTCTACCCCGAGGACCAAGAAGACC |
| 104           | Forward: CCCCTGGCAGGCGAGGAGAGCCTGGAG  
                Reverse: CTCCAGGCTCTCCTGCCAGGGG |

Table 1. The primer sequences used for Site-directed Mutagenesis
Table 2. Metabolic parameters after treatment with different forms of adiponectin or saline (as control) for 2 weeks

|                     | Saline  | WT-ADN  | ADN-K/R | fAd-E.Coli | gAd-E.Coli |
|---------------------|---------|---------|---------|------------|------------|
| Plasma glucose (mg/dL) | 276.5 ± 15.2 | 229.3 ± 10.9* | 253.7 ± 12.4 | 268.3 ± 14.6 | 259.6 ± 12.1 |
| Plasma insulin (ng/ml) | 12.6 ± 0.5 | 11.5 ± 0.4 | 12.1 ± 0.6 | 13.0 ± 0.7 | 12.8 ± 0.6 |
| Body weight (g)      | 42.2 ± 2.3 | 43.4 ± 2.7 | 41.9 ± 3.1 | 43.0 ± 2.4 | 40.8 ± 2.9 |
| Average food intake (g) | 6.6 ± 0.3 | 6.8 ± 0.2 | 6.5 ± 0.4 | 6.3 ± 0.3 | 6.7 ± 0.4 |
| Serum TG (mg/dL)     | 221.3 ± 13.7 | 157.9 ± 8.8** | 195.7 ± 9.6 | 186.2 ± 11.3 | 134.6 ± 7.1** |
| TG contents in liver (mg/g tissue) | 18.7 ± 0.8 | 11.6 ± 0.4** | 16.9 ± 0.7 | 17.2 ± 0.6 | 15.3 ± 0.5* |
| TG contents in muscle (mg/g tissue) | 10.8 ± 0.4 | 8.5 ± 0.3* | 9.7 ± 0.5 | 9.3 ± 0.4 | 6.7 ± 0.3** |
| Serum total cholesterol (mg/dL) | 143.6 ± 8.2 | 149.4 ± 6.9 | 138.6 ± 8.4 | 152.5 ± 9.1 | 140.7 ± 7.9 |
| Serum FFAs (mmol/L)  | 1.07 ± 0.06 | 0.74 ± 0.03* | 0.96 ± 0.04 | 0.91 ± 0.05 | 0.69 ± 0.02* |

1 wild type adiponectin produced in HEK293 cells; 2 the mutant ADN-K/R produced in HEK293 cells; 3 full-length adiponectin produced in E.Coli; 4 globular adiponectin produced in E.Coli; * P<0.05, ** P<0.01 versus saline treated db/db diabetic group. Note that there was no significant difference in body weight among each group prior to the treatments. Fasted values in C57BKS db/+ lean mice: plasma glucose = 112.7 ± 5.4 mg/dL; plasma insulin = 1.2 ± 0.1 ng/ml; serum TG = 82.3 ± 3.7 mg/dL; serum total cholesterol = 76.2 ± 5.3 mg/dL; serum FFAs = 0.47 ± 0.02 mmol/dL; TG in liver = 6.8 ± 0.4 mg/g tissue; TG in muscle = 3.3 ± 0.2 mg/g tissue.
|                                | Healthy control (n=12) | T2DM (n=12)  |
|--------------------------------|------------------------|--------------|
| Age (years)                    | 44 ± 2                 | 46 ± 2       |
| Body mass index (kg/m²)        | 21.9 ± 0.3             | 36.2 ± 1.3*  |
| Fasting glucose (mM)           | 5.23 ± 0.06            | 11.74 ± 0.83*|
| Serum levels of total adiponectin (µg/ml) | 8.47 ± 1.26          | 5.21 ± 0.64* |

*, P<0.01 versus healthy control
Figure 1

A

B
Figure 3

Panel A: Bar graph showing adiponectin concentration in extracellular medium (μg/mg protein) for ADN-WT and ADN-K/R.

Panel B: Western blot images for ADN-WT and ADN-K/R in cell lysates and conditioned medium at chase times 0, 5, 10, and 15.

Panel C: Graph plotting % of adiponectin released into extracellular medium against chase time (hr) for ADN-WT and ADN-K/R.
Figure 4

A

| Cell lysate | Culture medium |
|-------------|----------------|
| ADN-WT      | ADN-K/R        |
| ADN-WT      | ADN-K/R        |

B

% of total adiponectin

- HMW
- MMW
- LMW

ADN-WT
ADN-K/R

*
Figure 7

A

| Control | ADN-WT | ADN-K/R |
|---------|--------|---------|

Adiponectin

β-actin

B

Adiponectin levels (mg/g liver protein)

Control ADN-WT ADN-K/R

C

Serum adiponectin (μg/ml)

Control ADN-WT ADN-K/R
Figure 8

A

| Liver homogenates | Serum |
|-------------------|-------|
| ADN-WT            | ADN-CR|
| ADN-WT            | ADN-CR|

B

![Bar graph showing comparison of HMW/total adiponectin (%)](image)

- ADN-WT
- ADN-K/R
- ADN-WT
- ADN-K/R

Liver homogenates

Serum
Figure 9

A

|          | control | ADN-WT | ADN-K/R | ADN-WT+ minoxidil | fAd-E.coli | gAd-E.coli |
|----------|---------|--------|---------|--------------------|------------|------------|
| pAMPK    |         |        |         |                   |            |            |
| AMPKα    |         |        |         |                   |            |            |

B

|          | control | ADN-WT | ADN-K/R | ADN-WT+ minoxidil | fAd-E.coli | gAd-E.coli |
|----------|---------|--------|---------|--------------------|------------|------------|
| pAMPK    |         |        |         |                   |            |            |
| AMPKα    |         |        |         |                   |            |            |

AMPK phosphorylation (fold of control)

* indicates significant difference from control.
Figure 10

ipGTT

ITT

Plasma glucose (mg/dL)

Saline
ADN-WT
ADN-K/R
fAd-E.Coli
gAd-E.Coli

Time (min)

Plasma glucose (% of change)

Saline
ADN-WT
ADN-K/R
fAd-E.Coli
gAd-E.Coli

Time (min)
Figure 11

A

B

C

Carbohydrate contents (fold of control)

% of HMW/total asparagin

Healthy control

T2DM

Healthy control

T2DM

Healthy control

T2DM

*
Posttranslational modifications on the four conserved lysine residues within the collagenous domain of adiponectin are required for the formation of its high-molecular-weight oligomeric complex

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