Proteomic and functional characterization of endogenous adiponectin purified from fetal bovine serum

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Adiponectin is a plasma protein exclusively secreted from fat tissue. Many recent pharmacological studies suggest that recombinant adiponectin has multiple therapeutic potentials for obesity-related metabolic disorders, including type 2 diabetes, dyslipidemia, insulin resistance and atherosclerosis. However, the physiological relevance of these findings remains to be further established. In the present study, we have purified endogenous adiponectin from fetal bovine serum and characterized its post-translational modifications and physiological functions in animal models. Endogenous bovine serum adiponectin consists predominantly of full-length proteins that form multiple oligomeric complexes, including trimers, hexamers and higher molecular species. Two-dimensional gel electrophoresis revealed that bovine serum adiponectin exists as multiple post-translationally modified isoforms with distinct molecular weight and isolectric point. Further analysis using mass spectrometry and Edman degradation sequencing demonstrated that five conserved lysine residues (Lys 28, 60, 63, 72 and 96) within the collagenous domain of bovine adiponectin are hydroxylated and glycosylated by a glucosylα(1–2)galactosyl group. Injection of endogenous bovine adiponectin into C57 mice potently decreased circulating glucose levels and enhanced lipid clearance after a high fat meal. Chronic administration of this protein for a period of two weeks significantly increased insulin sensitivity and glucose tolerance, and depleted hepatic lipid accumulation in high-fat fed mice. These results provide direct evidence that endogenous bovine adiponectin is a physiological hormone that can regulate lipid and glucose metabolism.

Keywords: Adipokines / Adiponectin / Insulin resistance / Metabolic syndrome / Obesity / Type 2 diabetes

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1 Introduction

Adiponectin (also called ACRP30, GBP28 and aPM1) is a secretory protein that is specifically expressed in adipose tissue [1]. The protein belongs to the soluble defense collagen superfamily that contains a modular design comprising an *N*-terminal collagen-repeat domain and a *C*-terminal, characteristic complement factor C1q-like globular head domain. Growing evidence indicates that abnormality of the adiponectin gene and decreased production of adiponectin from adipose tissue might be one of the key factors that link obesity with insulin resistance, type 2 diabetes, dyslipidemia and cardiovascular disease [2]. The adiponectin gene is located within chromosome 3q27 [3, 4], a susceptibility locus for type 2 diabetes and other metabolic syndromes. Several recent reports suggest that genetic variations of the adiponectin gene may predispose humans to insulin resistance and type 2 diabetes [5, 6]. A strong positive correlation between adiponectin levels and insulin sensitivity has been found in both humans and experimental animals. Many clinical

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Abbreviations: FBSAd, fetal bovine serum adiponectin; **FFA**, free fatty acid; **HF**, high-fat; **HisAd**, His-tagged full-length adiponectin; **ipGTT**, intraperitoneal glucose tolerance test; **ITT**, insulin tolerance test; **TG**, triglyceride

studies have found that plasma adiponectin levels are significantly decreased in obese individuals and patients with insulin resistance, type 2 diabetes and coronary artery disease [7–9]. A prospective study on Pima Indians indicated that a decline in plasma adiponectin concentrations preceded the decrease in insulin sensitivity [10].

Many recent pharmacological studies have demonstrated that adiponectin is an insulin-sensitizing hormone with direct antidiabetic, anti-inflammatory and anti-atherogenic functions. Acute *in vivo* administration of a truncated globular domain of adiponectin decreases postprandial plasma free fatty acid following a high fat meal, and chronic administration of this protein causes sustained weight loss in mice without affecting food intake [11]. Injection of recombinant full-length adiponectin produced from mammalian cells abolishes hyperglycemia in several diabetic animal models, including obese ob/ob, nonobese diabetic and streptozotocin-treated mice [12]. Infusion of adiponectin into conscious mice significantly decreases glucose production, possibly through downregulation of the hepatic expression of the gluconeogenic enzymes. Furthermore, chronic treatment of adiponectin could restore insulin sensitivity in both high-fat (HF) fed and lipoatrophic mice, by increasing β-oxidation of fatty acid in muscle, and thus decreasing muscular triglyceride content [13]. We have recently found that chronic administration of recombinant full-length adiponectin generated from mammalian cells can alleviate alcoholic and nonalcoholic fatty liver diseases associated with obese ob/ob mice, and can also ameliorate dyslipidemia and insulin resistance in these animal models [14]. In addition, recombinant adiponectin has been shown to prevent foam cell formation [15] and to inhibit different growth factor-stimulated proliferation of smooth muscle cells [16].

Despite these promising findings, the molecular and structural basis that underlies the multiple biological functions of adiponectin remains largely elusive. It is still controversial as to which form of adiponectin is physiologically active. Several groups have found that a truncated globular domain of adiponectin generated from *Escherichia coli* can potently increase insulin sensitivity, enhance lipid clearance and stimulate muscular fatty acid oxidation, while full-length bacterially generated adiponectin has little such activities [11, 13, 17]. However, there is no convincing evidence showing the existence of a truncated globular domain of adiponectin in the circulation. In contrast, other studies [12, 18] have shown that full-length adiponectin expressed in mammalian cells has insulin-sensitizing activities and can rapidly decrease circulating blood glucose levels in several diabetic animal models. We have recently demonstrated that adiponectin secreted from 3T3-L1 adipocytes is post-translationally modified into multiple isoforms, and several lysines (Lys 68, 71, 80 and 104) within its collagenous domain of mouse adiponectin are hydroxylated and glycosylated [19]. Our *ex vivo* functional analysis revealed that adiponectin produced from mammalian cells is much more potent than the bacterially generated full-length protein in enhancing the ability of insulin to inhibit hepatic glucose production, suggesting that post-translational modification might play a role.

All the aforementioned studies have been using different sources of recombinant adiponectin, which might be structurally and functionally different from endogenous adiponectin. In this study, we have purified endogenous adiponectin of large quantity and high purity from fetal bovine serum. Our results revealed that the preponderance of bovine serum adiponectin was full-length proteins that were post-translationally modified into multiple glycoforms. Several conserved lysine and proline residues within the collagenous domain of endogenous adiponectin were found to be hydroxylated and some hydroxylysines subsequently glycosylated. Furthermore, we found that endogenous adiponectin purified from the circulation is a physiologically active hormone that can directly regulate glucose and lipid metabolism as well as insulin sensitivity.

2 Materials and methods

2.1 Reagents

PEG and CHCA were purchased from Sigma (St. Louis, MO, USA). The TEV protease, prokaryotic expression vector pPROEX HTb and fetal bovine serum were from Invitrogen (Carlsbad, CA, USA). Immun-Blot kit for glycoprotein detection was from Bio-Rad (Hercules, CA, USA). Trypsin was from Roche (Mannhein, Germany). All the consumables for 2-DE, Sepharose fast flow beads and the ECL detection system were the products of Amersham Biosciences (Uppsla, Sweden). The VIVASPIN concentrators were purchased from Vivascience (Gloucs, UK). All calibration standards for MS were from Applied Biosystems (Foster City, CA, USA). Ni-NTA column was purchased from Merck (Darmstadt, Germany).

2.2 Purification of adiponectin from fetal bovine serum

Fetal bovine serum was incubated with 10% w/v PEG6000 with shaking at 4°C overnight. The samples were then centrifuged at 15 000 × *g* for 30 min at 4°C. The pellet was dissolved in 10 mL of 0.1 M sodium phosphate buffer (pH 6.5) with 0.8 M NaCl and filtered through a 0.2 μm filter paper. The filtered samples were loaded onto a Zn^{2+}-chelating fast flow sepharose affinity column and eluted

with 12 column volumes of the elution buffer containing 0.02 M sodium phosphate (pH 7.4), 0.15 M NaCl and 0.02 M EDTA. The fractions containing adiponectin were detected by Western blotting, pooled and concentrated to ~100 μL using a VIVASPIN concentrator. The sample was then centrifuged at 15 000 × *g* for 5 min to remove any particulate material and separated using a HiPrep™ 16/60 Sephacryl S-300 High Resolution column (Amersham Biosciences) on BioCAD™ Workstation Perfusion Chromatography system (Applied Biosystems) according to the manufacture's instructions. The fractions containing adiponectin were concentrated and further purified using the Econo-Pac® High Q cartridge (Bio-Rad). Elution was performed using the gradient from 50 to 500 mM NaCl with 20 mM HEPES (pH 8.0) over 50 min at the flow rate of 2.0 mL/min. The eluted fractions containing adiponectin were pooled and stored at −80°C until further use.

2.3 Expression and purification of His-tagged recombinant adiponectin from *E. coli*

A vector pPRO-His-Ad that encodes full-length adiponectin with a 6 × His tag at its *N*-terminus was used for expression and purification of full-length adiponectin from BL 21 cells [19]. After induction with 1 mM of isopropyl β-thiogalactopyranoside, His-tagged full-length adiponectin (HisAd) was purified from the bacterial lysates using Ni-NTA agarose column according to the manufacturer's instructions. Following purification, the *N*-terminal tag was removed by cleavage with recombinant TEV protease. The purity of the protein was confirmed by SDS-PAGE and HPLC.

2.4 2-DE, Western blotting and carbohydrate detection

15 μg of the purified bovine adiponectin was separated by 2-DE and stained with silver as we described previously [20]. Western blot analysis of adiponectin was performed using a rabbit anti-adiponectin polyclonal antibody [19]. The glycoproteins were detected using Immun-Blot kit (Bio-Rad) following the manufacturer's instructions.

2.5 In-gel trypsin digestion and RP-HPLC separation

Protein spots of interest separated by 2-DE or SDS-PAGE were excised and subjected to in-gel trypsin digestion as described previously [21]. The extracted tryptic peptide mixtures were fractionated by RP-HPLC on a Jupiter 5μ $C1_8$ column (250 × 2.00 mm, Phenomenex (Torrance, CA, USA). The pre-warmed column (37°C) was washed for 7 min with water containing 0.1% v/v TFA followed by elution using a 50 min linear gradient from 8% to 36% v/v ACN in water at a flow rate of 200 μL/min. Each fraction was collected manually and subjected to further *N*-terminal amino acid sequencing or MS analysis.

2.6 MS analysis

For MALDI-TOF MS analysis, 0.5 μL of the tryptic peptide mixtures or RP-HPLC separated peptides was mixed with an equal amount of CHCA matrix (10 mg/mL in 60% v/v ACN/0.3% v/v TFA), spotted onto the sample plates and air-dried. Refelectron MS analyses were performed on a Voyager DE PRO Biospectrometry Workstation (Applied Biosystems) using a pulsed laser beam (nitrogen laser, λ = 337 nm). All ion spectra were recorded in the positive mode with the accelerating voltage of 20.0 kV. The spectrometer was externally calibrated using Cal Mix 2 standard mixture.

MS/MS experiments were performed using QSTAR XL quadrupole orthogonal acceleration time-of-flight mass spectrometer equipped with an oMALDI ion source (Applied Biosystems). The instrument was controlled using the Analyst® QS software and was operated in the positive mode. The pulse rate was set to 30 Hz and the power level is 8% v/v. Argon was used as the collision gas at a recorded pressure of 60 psi. The collision energy was optimized for different ions by ramping up the energy over the *m/z* range proportional to one-tenth of the *m/z* value of the precursor ion.

2.7 ^{1}H NMR spectroscopy

Samples were exchanged twice in D_2O (99.9atom%, Cambridge Isotope Laboratories (Andover, MA, USA) with intermediate lyophilization. Finally, the material was dissolved in 0.6 mL D_2O. 1-D resolution enhanced 500-MHz ^{1}H NMR spectra were recorded on a Bruker DRX-500 spectrometer at a probe temperature of 300 K. Chemical shifts are expressed in ppm by reference to internal acetone (δ 2.225) [22].

2.8 Establishment of the high fat-induced mouse model of insulin resistance and adiponectin treatment

All the experimental protocols were approved by the Institutional Animal Ethics Committee. C57BL/6J mice (male, 21 days) were fed with either standard chow or high fat food as we previously described [20]. The body weight and food intake were monitored every three days.

After seven weeks of feeding with high fat food, these mice develop insulin resistance as judged by intraperitoneal glucose tolerance test (ipGTT), insulin tolerance test (ITT) and elevated levels of insulin.

For the chronic treatment of adiponectin, the protein was continuously delivered into the mice using ALZET osmotic pumps (Cuppertinu, CA, USA) as we described previously [14]. The pumps were filled with protein solutions according to the manufacture's instructions and incubated in the saline solution at 37°C for 4 h. During implantation, the animals underwent a short halothane (2–5% v/v with 2 L/min oxygen) anaesthetic and a 0.5 cm incision was made on the lower back of the animal. A small subcutaneous pocket was made in a rostral (head) direction and the pump was inserted to administer either vehicle (saline) or adiponectin for 2 weeks at a constant rate of 30 μg/day.

2.9 Lipid clearance

Mice were fasted for 3 h and then fed with a high-fat meal (6 g butter, 6 g olive oil, 10 g non-fat dry milk, 10 g sucrose dissolved in 12 mL distilled water) by intragastric gavage (250 μL/animal). Immediately after the high-fat meal, different sources of adiponectin (40 μg/g body weight) or saline were intraperitoneally injected into the mice. Blood samples were collected at different times for the measurement of triglyceride and nonesterified free fatty acid.

2.10 ipGTT and ITT

For ipGTT, fasted mice (16 h) were given a glucose load by intraperitoneal injection (1 g glucose/kg body weight). The levels of blood glucose were measured at different time intervals. For ITT, mice were fasted for 4 h, and were then intraperitoneally injected with insulin at the dosage of 1 U/kg body weight. Blood glucose levels were monitored at different times after insulin treatment.

2.11 Measurement of glucose, triglyceride and free fatty acid concentrations

Blood glucose from tail veins was measured using Accu-Chek Comfort Glucometer (Roche Diagnostics). The levels of plasma free fatty acid (FFA) and triglycerides (TG) were determined using a Roche FFA kit and a Triglyceride GPO reagent (Pointe Scientific Lincoln Park, MI, USA), respectively. Hepatic content of triglycerides was quantified as we previously described [14].

2.12 Statistical analysis

Experiments were reproduced in at least two independent experiments. The results were presented as means of at least triplicate determinations ± SD. Significance was determined by Student's *t* test or one-way ANOVA. In all statistical comparisons, a *p* value of less than 0.05 was considered statistically significant.

3 Results

3.1 Endogenous adiponectin purified from fetal bovine serum is post-translationally modified into multiple glycosylated isoforms

To obtain a sufficient amount of endogenous circulating adiponectin for further characterization, fetal bovine serum was chosen as a source for purification. To this end, adiponectin was precipitated by PEG6000, and subsequently purified using Zn^{2+}-chelating chromatography, gel-filtration and ion exchange chromatography. The result in Fig. 1A shows that adiponectin was effectively precipitated by PEG6000 and dramatically enriched by Zn^{2+}-chelating chromatography. Further separation of the enriched adiponectin using gel-filtration chromatography revealed that this protein was mainly eluted within three major fractions with M_r of 690, 460 and 230×10^3, which represent the different oligomeric complexes of this protein (Fig. 1B). SDS-PAGE analysis of protein samples purified from each step revealed that adiponectin was gradually enriched, and there was only one major protein band with an M_r of ~30 000 after purification with ion-exchange chromatography (Fig. 1C). Carbohydrate detection confirmed that this purified endogenous adiponectin is highly glycosylated (Fig. 2A). By contrast, the recombinant adiponectin purified from *E. coli* is not glycosylated. *N*-terminal sequencing analysis generated a sequence of "EDNMEDPPL", which matches the fragment immediately following the predicted secretory signal peptide. This result further confirms that the purified adiponectin exists as an uncleaved full-length form. 2-DE analysis of the purified bovine endogenous adiponectin revealed that this protein existed as multiple isoforms with slightly different M_r and p*I* values, a pattern similar to that observed from 3T3-L1 adipocytes (Fig. 2B) [19].

3.2 Several conserved lysine residues within the collagenous domain of adiponectin are hydroxylated and glycosylated

Our previous study demonstrated that several lysine residues of mouse adiponectin secreted from 3T3-L1 adipocytes are hydroxylated and subsequently glycosylated by

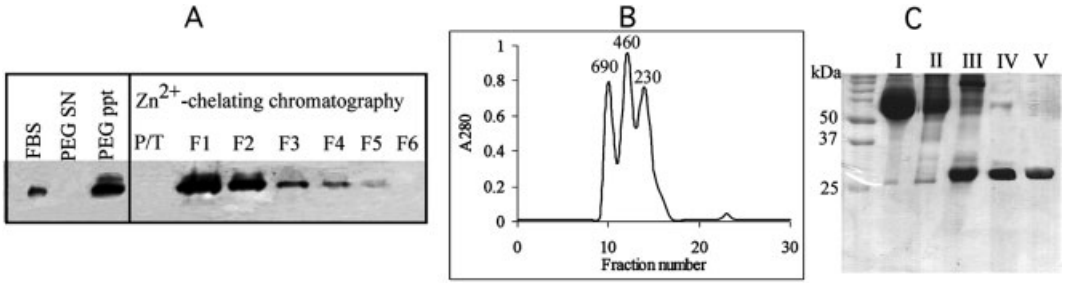


Figure 1. Purification of endogenous adiponectin from fetal bovine serum. (A) Bovine serum adiponectin was enriched by PEG precipitation and Zn^{2+}-chelating chromatography. A, 20 μg aliquot of protein from total fetal bovine serum (FBS), supernatant recovered after PEG precipitation (PEG SN) and PEG pellets (PEG ppt) were separated on a 12% SDS-PAGE gel and subjected to Western blotting analysis using rabbit anti-adiponectin polyclonal antibody (left). The PEG precipitated proteins were re-dissolved, loaded onto a Zn^{2+}-chelating column and the retained proteins eluted with the elution buffer. 10 μL of protein from passing through (P/T) or elutes from different fractions were analyzed by Western blotting (right). (B) Gel-filtration purification of proteins enriched by Zn^{2+}-chelating chromatography. Adiponectin is distributed in three fractions with apparent molecular masses of 690, 460 and 230 kDa as detected by Western blotting analysis. (C) SDS-PAGE analysis of protein enriched following each step of adiponectin purification. 20 μg of protein from fetal bovine serum (I), or 5 μg of protein from PEG precipitation (II), eluates of Zn^{2+}-chelating chromatography (III), gel-filtration (IV) or ion-exchange chromatography (V) were separated on a 12% SDS-PAGE gel, and visualized by CBB R250 staining.

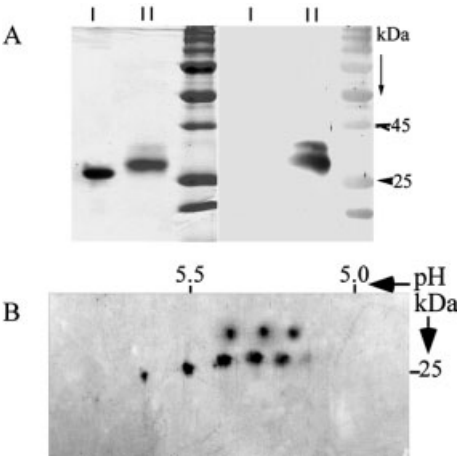
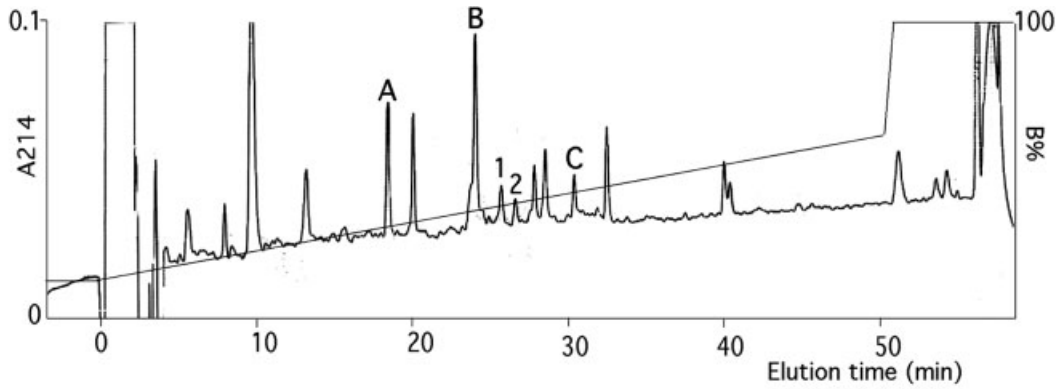


Figure 2. Endogenous circulating bovine adiponectin is glycosylated and exists as multiple isoforms. (A) 2 μg of adiponectin purified from fetal bovine serum (I) or recombinant adiponectin purified from *E. coli* (II) were separated on a 12% SDS-PAGE gel, and subjected to CBB staining (left) and carbohydrate detection using Immun-Blot kit (right). In (B) the purified bovine adiponectin was separated by analytical 2-DE and visualized by silver staining.

a glucosylgalactosyl group [19]. To explore the physiological relevance of this finding, we investigated whether similar modifications also occur in endogenous adiponectin directly purified from the circulation. To this end, the purified bovine circulating adiponectin was trypsin-digested and the tryptic peptide mixtures were fractionated by RP-HPLC (Fig. 3). Each peak fraction was collected and subjected to MALDI-TOF MS analysis, Edman protein sequencing or MS/MS analysis using QSTAR XL quadruple-TOF mass spectrometer. The observed mass of each peptide was then compared with its theoretical mass in order to identify the modified peptides. This analysis detected three major peptide fragments (A, B and C) that are modified, as the observed masses are different from their theoretically predicted masses (Fig. 3). During amino acid sequencing, we noticed that all the lysine residues on these three peptides were not detectable. In addition, these lysine residues are resistant to the protease cleavage by trypsin, which specifically cleaves the peptide bond *C*-terminal of lysine and arginine residues. This evidence collectively suggested that several lysine residues within these three tryptic fragments are modified. For peptide A, the difference between the experimentally observed mass (1695 Da) and its theoretical mass (1355 Da) is 340 Da, which exactly equals to the mass of a glucosylgalactosyl hydroxyl group, suggesting Lys 96 within this fragment is hydroxylated and glycosylated. The mass difference for peptide B is 1037 Da, which is equivalent to three glucosylgalactosyl hydroxyl groups plus an additional hydroxyl group. The *C*-terminal fragment of peptide B (from amino acid 64 to 87) has also been found during RP-HPLC separation (peak 2, Fig. 3). Amino acid sequencing and MALDI-TOF analysis re-



Peptide	Sequences	Positions	Observed mass (Da)	Theoretical mass (Da)	Mass difference (Da)
A	KGEPGEAAYVYR	96-107	1695.7605	1354.8517	340.9088
B	DGTPGEKGEKGDP GLLGPKGETGDV GMTGAEGPR	54-87	4302.9018	3265.5521	1037.3497 (3×340+17)
C	EDNMEDPPLPKGA CAGWMAGIPGHP GHNGTPGR	18-50	3829.2201	3438.1241	391.0960 (340+3×17)
1	GACAGWMAGIPG HPGHNGTPGR	29-50	2223.7156	2172.6696	51.0460 (3×17)
2	GDPGLLGPKGETG DVGMTGAEGPR	64-87	2624.3565	2267.0852	357.2713 (340+17)

Figure 3. RP-HPLC, amino acid sequencing and mass spectrometric analysis of the tryptic peptide mixtures of endogenous adiponectin from fetal bovine serum. The protein was digested by trypsin and fractionated by RP-HPLC. Each fraction was collected and analyzed by amino acid sequencing and MS using either MALDI-TOF or QSTAR XL quandrupole-TOF. The bottom table shows the amino acid sequences of the modified peptides and their experimentally observed and theoretical masses.

vealed that the difference between the experimentally observed mass and that of theoretical mass for this peptide is 357 Da, which equals to one glucosylgalactosyl hydroxyl group plus an additional hydroxyl group. These results suggested that the three glucosylgalactosyl hydroxyl groups might be attached on the three lysine residues, Lys 60, 63 and 72 of bovine adiponectin respectively and the extra hydroxyl group might occur on Pro 86 [19]. The mass difference for peptide C is 391 Da, which matches to one glucosylgalactosyl hydroxyl group plus another three hydroxyl groups. The *C*-terminal fragment of peptide C starting from amino acid 29 to amino acid 50 was also found by RP-HPLC analysis (Peak 1, Fig. 3). The mass difference for this peptide fragment is 51 Da that equals to three hydroxyl groups, suggesting that the three proline residues (39, 42 and 48) within this fragment are hydroxylated. Hydroxylation of these three proline residues was also confirmed by amino acid analysis (data not shown). The glucosylgalactosyl hydroxyl group might be attached on Lys 28 of peptide C. MS/MS analysis using Q-STAR® pulsar hybrid mass spectrometer further confirmed the glycan loss of these peptides (data not shown).

We subsequently performed large-scale RP-HPLC purifications of peptide A and B for NMR analysis to confirm the carbohydrate residues attached on these two peptides. The resulting ^{1}H-NMR spectra demonstrated that in both peptides there are two carbohydrate anomeric signals, corresponding to α-glucose (δ 5.40; $J_{1,2}$ 3.6 Hz) and β-galactose (δ 4.65; $J_{1,2}$ 7.7 Hz) presented at the ratio of 1:1, indicating the presence of a GlucosylGalactosyl group. The presence of this carbohydrate group was further confirmed by comparison of the ^{1}H-NMR spectra to that of GlucosylGalactosyl Hydroxylysine [Glcα(1-2) Galα(1-Hyl] isolated from urine (data not shown).

In summary, these results collectively indicate that four proline residues (39, 42, 48 and 86) within the collagenous domain of bovine circulating adiponectin are hydroxylated, and that the five lysine residues (Lys28, 60, 63, 72 and 96) within this region are hydroxylated and subsequently glycosylated. The attached carbohydrate groups are glucosylα(1–2)galactosyl groups. Notably, the four lysines (60, 63, 72 and 96) in peptide A and B are equivalent to lysine 68, 71, 80 and 104 of murine adiponectin from 3T3-L1 adipocytes, all of which have been shown to be hydroxylated and glycosylated in our previous study [19].

3.3 Endogenous adiponectin can acutely decrease circulating glucose and enhance lipid clearance

To explore the physiological functions of endogenous adiponectin, we investigated its effects on glucose and lipid metabolism in mice. To this end, full-length adiponectin from fetal bovine serum or *E. coli* was intraperitoneally injected into C57BL/6J mice at a dose of 40 μg/g body weight, and blood glucose levels were measured at different time points after treatment. As shown in Fig. 4A, adiponectin purified from fetal bovine serum potently decreased blood glucose concentrations as early as 1.5 h after injection. The potency of adiponectin purified from *E. coli* was significantly lower than that from fetal bovine serum. These results demonstrate that endogenous adiponectin is a functionally active hormone that can decrease blood glucose levels *in vivo*.

To evaluate the effects of adiponectin on lipid metabolism, C57BL/6J mice were fed with HF meal by intragastric gavage, and then immediately injected (interpertoneal) with the adiponectin protein derived from fetal bovine serum or *E. coli*. Analysis of FFAs and TGs in the serum samples revealed that endogenous adiponectin purified from fetal bovine serum significantly inhibited high fat diet-induced rise in circulating FFAs. This inhibitory effect of adiponectin was observed by 1.5 h after injection (Fig. 4B). Endogenous bovine adiponectin also significantly decreased TG levels, although this effect only occurred at 3 h after injection (Fig. 4C). On the other hand, the lipid clearance ability of bacterially generated full-length adiponectin is significantly lower than that of endogenous full-length adiponectin from fetal bovine serum.

3.4 Endogenous adiponectin can increase insulin sensitivity and deplete hepatic lipid accumulation in high-fat fed mice

We next tested the chronic effects of the endogenous adiponectin in high-fat fed mouse models with insulin resistance. Five weeks after being fed with a high-fat diet, endogenous adiponectin from fetal bovine serum or full-length adiponectin purified from *E. coli* were introduced into the mice using Alzet Osmotic pumps for a period of two weeks. This analysis showed that the mice fed with the high-fat diet for seven weeks developed severe insulin resistance and glucose intolerance (Fig. 5). Chronic treatment of endogenous adiponectin significantly increased insulin sensitivity and enhanced glucose tolerance of the

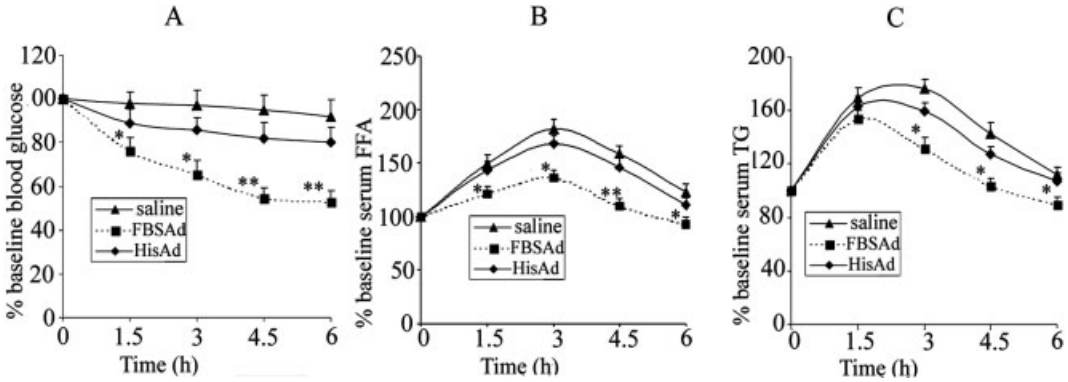


Figure 4. The acute effects of adiponectin on blood glucose and lipid clearance following an oral lipid challenge. (A) C57BL/6J mice were injected (intra peritoneal) with adiponectin purified from fetal bovine serum (FBSAd) or *E. coli* (HisAd) at a dose of 40 μg/g body weight. The levels of blood glucose were measured at different times after the injection. In (B) and (C), mice were fed with high-fat meal by intragastric gavage (vol.= 1% of body weight), and immediately injected with saline, adiponectin purified from FBS (FBSAd) or adiponectin derived from *E. coli* (HisAd). At different times after treatment, circulating levels of FFAs and TGs were measured respectively. The results are expressed as means $\pm$ SD (n = 6). *, $p < 0.05$ and **, $p < 0.01$ *versus* HisAd treatment or saline.

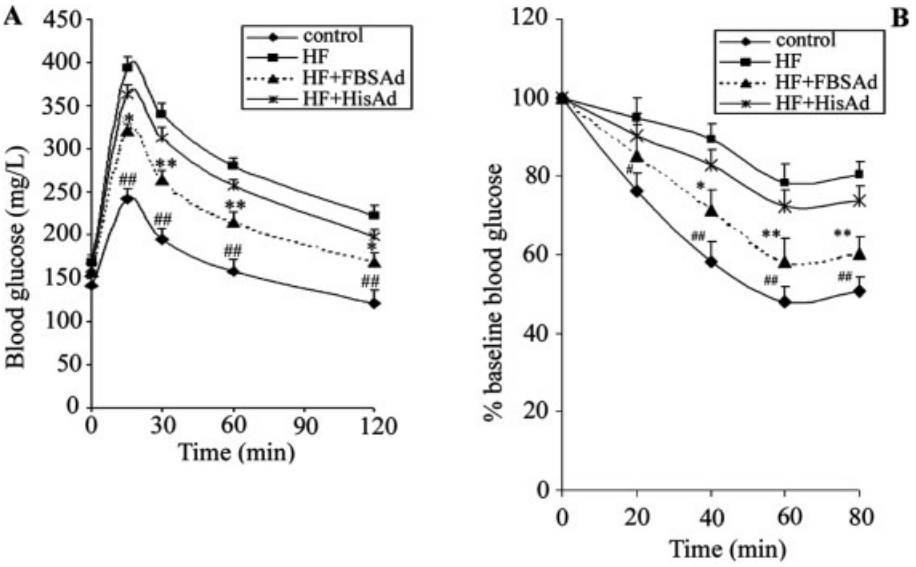


Figure 5. Chronic effects of adiponectin on insulin sensitivity and glucose tolerance in high-fat fed mice. C57BL/6J mice were fed with control diet, or high-fat diet treated with saline (HF), adiponectin purified from FBS (HF+FBSAd) or *E. coli* (HF+HisAd) as described in the text. Two weeks after adiponectin treatment, the mice were subjected to ipGTT (A) and ITT (B) *, $p < 0.05$ and **, $p < 0.01$ HF mice treated with FBSAd *versus* the HF or HF+HisAd group. #, $p < 0.05$ and ##, $p < 0.01$ control diet group *versus* HF diet group (n = 5–7).

high-fat fed mice (Fig. 5). In addition, this treatment also dramatically decreased hepatic lipid accumulation induced by high-fat feeding (Fig. 6). On the other hand, the potency of bacterially generated full-length adiponectin to increase insulin sensitivity and to reduce hepatic lipid contents was significantly attenuated compared to that of endogenous adiponectin purified from fetal bovine serum.

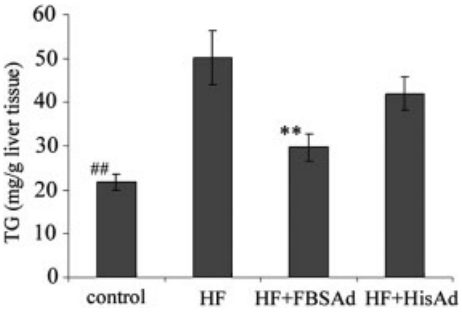


Figure 6. Chronic effects of adiponectin on high-fat diet-induced hepatic lipid accumulation. Liver specimens were taken from control, or high-fat fed mice treated with saline (HF), FBSAd (HF+FBSAd), or HisAd (HF+HisAd) for the last two weeks of feeding period, and the hepatic lipid was determined as described in the method. *, $p < 0.05$ and **, $p < 0.01$ HF mice treated with FBSAd *versus* those treated with HisAd. #, $p < 0.05$ and ##, $p < 0.01$ control diet group *versus* HF diet group (n = 5–7).

4 Discussion

It is increasingly recognized that adiponectin plays an important role in the regulation of lipid and glucose metabolism [1]. However, the physical and biochemical properties of endogenous adiponectin are still poorly characterized. In this study, we have applied a multi-dimensional chromatographic strategy to purify endogenous adiponectin from fetal bovine serum to investigate its post-translational modifications and to evaluate its potential physiological functions. Our results demonstrated that this purification protocol allows us to produce a sufficient amount of endogenous adiponectin with high purity. Gel filtration analysis revealed that endogenous adiponectin forms different oligomers with the M_r of 230, 460 and 690 $\times 10^3$, respectively, which may represent trimers, hexamers and higher molecular weight species of adiponectin [23]. These results are consistent with several previous studies that demonstrated that circulating adiponectin in both human and mouse serum forms several oligomeric species [24–26].

Many research groups have been using different sources of recombinant adiponectin for its functional assessments. While these studies consistently demonstrated the multiple metabolic roles of adiponectin with pharmacological interest, the physiological relevance of these findings is still uncertain. Studies from Dr Scherer's group and ourselves have been using full-length recombinant adiponectin generated from mammalian cells [12, 14, 18, 19]. Acute injection of this form of recombinant adiponectin can decrease hyperglycemia by inhibiting hepatic glucose production. Chronic administration of this protein can alleviate alcoholic and nonalcoholic fatty liver diseases associated with obesity. Our more recent study has found that long-term treatment with this form of recombinant adiponectin can also ameliorate HIV protease inhibitors induced dyslipidemia, by enhancing fatty acid oxidation and inhibiting lipogenesis in the liver tissue [27].

In contrast with our studies, several other research groups have primarily focused on the pharmacological activities of the truncated globular domain of adiponectin generated from *E. coli* [11, 13]. These studies have found that chronic administration of this fragment can potently reduce high-fat induced body weight gains and enhance insulin sensitivity by increasing muscular fatty acid β-oxidation, and acute administration of this globular fragment can accelerate lipid clearance following high-fat meal. Notably, bacterially generated full-length adiponectin has none or little of such activities. Although the pharmacological functions of globular domain of adiponectin are now well recognized, this fragment obviously lacks the structural integrity of plasma adiponectin that predominantly exists as full-length oligomeric complexes. There is no convincing evidence demonstrating the presence of the truncated globular domain of adiponectin in the circulation. In fact, studies from Dr Scherer's group and ourselves have not been able to detect any degraded products of adiponectin in either human or mouse serum [19, 24].

In this study, we have evaluated the *in vivo* physiological properties of endogenous full-length adiponectin directly purified from the circulation. We found that the endogenous adiponectin can acutely decrease glucose levels and enhance plasma lipid clearance following high-fat meal. In addition, chronic treatment with this form of adiponectin can significantly improve insulin sensitivity and glucose tolerance, and can also decrease the hepatic lipid accumulation induced by high-fat diet. These results provide direct evidence that circulating full-length adiponectin is a physiological hormone that can regulate systematic lipid and glucose metabolism. Notably, we found that both chronic and acute metabolic effects of endogenous full-length adiponectin are much more potent than those of bacterially generated full-length adiponectin, suggesting that post-translational modifications or oligomerization, but not proteolytic cleavage, is important for the physiological functions of adiponectin. Our results are consistent with a very recent transgenic study, which demonstrated that a life-long elevation of circulating adiponectin by two to three-fold increases lipid clearance and hepatic insulin sensitivity [24]. It is worthy to note that the authors of this study were unable to detect any degraded adiponectin product in their transgenic mice. However, our present results do not exclude the presence or the physiological role of globular adiponectin. It is still possible that globular adiponectin can be produced locally by proteolytic cleavage, or that plasma globular adiponectin might not be abundant enough to be detected by our analytical procedures.

Our previous study on 3T3-L1 adipocytes has shown that adiponectin secreted from this cell line are post-translationally modified into multiple isoforms, and several conserved lysine residues within the collagenous domain are modified by hydroxyl glucosylgalactosyl groups [19]. An *ex vivo* functional analysis indicated that this modification might be critical for the activities of adiponectin, at least with respect to its ability to inhibit hepatic glucose production. Consistent with these *in vitro* findings, our present study demonstrated that endogenous adiponectin in the circulation exists as multiple glycoforms, and similar modifications [consisting of Glcα(1–2)Galβ(1-)] also occur on the five conserved lysine residues within the collageneous domain of this protein. In addition, we have found that several proline residues within this domain are modified by hydroxylation. These data collectively implicate that the collageous domain of adiponectin and the post-translational modifications within this domain are physiologically important for the metabolic functions of this hormone. The critical role of the collagenous domain of adiponectin was also suggested by a recent genetic study on the Japanese and French populations, which showed that mutations within this region are closely correlated with insulin resistance and type 2 diabetes [28].

The mechanisms by which the aforementioned post-translational modifications regulate the metabolic functions of adiponectin remain to be defined. It is possible that these modifications are critical for the stabilization of the collagen-like stalk and the formation of high order oligomeric complexes required for the full metabolic activities of endogenous adiponectin. In line with this speculation, a recent study suggests that both endogenous mouse adiponectin and adiponectin expressed in HEK293 cells can form higher order oligomeric structures with molecular masses of ~690 kDa, but adiponectin purified from *E. coli* cannot form this complex [23]. More recently, it has been shown that different oligomers of adiponectin activate different signaling pathways and exert distinct biological functions [29]. It is also likely that the hydroxyl and glucosygalactosyl groups are directly involved in the interaction of this hormone with its receptors. These possibilities are currently under investigation in our laboratory.

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5 References

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