L-ASCORBIC ACID-INDUCED CHANGES IN ENDOPLASMIC RETICULUM INCREASES ADIPONECTIN OLIGOMERIZATION

By

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Abstract

Adiponectin is a hormone secreted from adipose tissue that plays a role in maintaining insulin sensitivity (1). The molecular mechanisms by which adiponectin promotes insulin sensitivity, however, remain uncertain. Specifically, the insulin-sensitizing action of each adiponectin oligomer has not been firmly established. Adiponectin exists in three distinct oligomerization states: a trimer, a hexamer, and a "high molecular weight" species (HMW). The trimeric species is the basic structural unit of adiponectin; HMW adiponectin is an oligomer of trimers and is stabilized by disulfide bonds (2). Increased insulin sensitivity as a result of dieting, exercise, and thiazolidinedione treatment is most closely associated with the HMW form of adiponectin, as opposed to the hexameric or trimeric forms (3). Furthermore, low circulating levels of HMW adiponectin is a biomarker for type 2 diabetes mellitus (4). It is therefore important to understand how adiponectin oligomer formation is regulated in the endoplasmic reticulum (ER). Imbalances in the redox state of the ER lead to ER stress, which causes protein misfolding (5). It has been shown that ER stress is onset by obesity and results in insulin resistance (6). Chaperone proteins such as ErоI and PDI provide the oxidizing equivalents necessary for proper protein folding (5). L-ascorbic acid is also thought to play a role in the oxidative folding of proteins in the endoplasmic reticulum, specifically in the formation of disulfide bonds (7). In effort to understand the effect of ER stress on the adiponectin folding pathway, we have investigated adiponectin oligomerization under various redox states in the endoplasmic reticulum. We have found that ascorbate is effective in increasing HMW adiponectin oligomerization.

Introduction

Adiponectin is a serum protein secreted from adipocytes that plays a role in maintaining insulin sensitivity by regulating lipid and glucose metabolism. Secretion of adiponectin decreases in diabetic or obese humans and animals (1). The ability of adiponectin to impart insulin sensitivity has been demonstrated by transgenic overexpression and knockout rodent models (8). However, the molecular mechanisms by which adiponectin exerts its metabolic action are not completely understood. The signaling properties of adiponectin are complex because adiponectin homo-oligomerizes into three major isoforms: trimer, hexamer, and high molecular weight (HMW), each of which have distinct biological activity (2). Although these oligomers all seem to be important to the physiological function of adiponectin, insulin sensitivity is most closely correlated with increased HMW concentration of adiponectin than with the hexameric or the trimeric isoforms, which indicates that the HMW oligomer is the active form of adiponectin (3). It is therefore important to understand the regulatory mechanisms of HMW adiponectin oligomerization.

Disulfide bonds are required for the assembly of hexameric and HMW adiponectin (2). A number of groups have been demonstrated that a conserved cysteine (C22) is the site of disulfide bond formation. A C22A mutation in mouse models prevents adiponectin oligomerization beyond the trimeric species (9). Disulfide bond formation and rearrangement occurs in the endoplasmic reticulum (ER) (5). Improper disulfide bond formation is observed in adipose and liver tissue in obese rats and mice (6). Decreased HMW adiponectin formation may be caused by improper disulfide formation in the ER.

It has been shown that higher molecular adiponectin oligomers are retained in the endoplasmic reticulum upon folding by a thiol-mediated linkage to the ER chaperone ERp44 (10). Cysteine 22 of mature mouse adiponectin is involved in the covalent interaction with ERp44 because a C22A mutant of adiponectin lacks this stabilization. Adiponectin is released from the endoplasmic reticulum by the ER oxidoreductase, EroI, via a mechanism that is not yet fully understood. EroI is a membrane-associated flavoprotein which provides oxidizing equivalents in the ER lumen and thus is responsible for setting the ER oxidation state (5).The catalytic cycle of Ero1 involves protein disulfide isomerase (PDI), which shuttles electrons to EroI. The electrons are then passed from the cysteine active site on the flexible loop of Ero1 to FAD cofactor. The terminal electron acceptor is molecular oxygen, yielding hydrogen peroxide and activated (oxidized) Ero1 (5). It is thought that Ero1 prevents potentially dangerous fluctuations in the ER redox environment via modulation of by a homeostatic feedback system in the ER (5). Imbalances in the redox state of the ER leads to ER stress, has been shown to link obesity to insulin resistance (6).

L-ascorbic acid is thought to play a role in the oxidative folding of proteins in the endoplasmic reticulum, specifically in the formation of disulfide bonds in the ER (7). The lumen endoplasmic reticulum is an oxidizing environment, which is necessary for proper formation of disulfide bonds. It is therefore surprising that the lumen of the ER contains high concentration of ascorbate, a reducing agent (7). However, facilitated diffusion of the oxidized form of ascorbate, dehydroascorbate (DHA), has been observed in rat liver microsomes. It is thought that DHA uptake is mediated by the glucosetransporter T3

subunit of the glucose-6-phosphatase system (7). Local ascorbate oxidation has also been observed in microsomal vesicles (7). Ascorbate accumulation in the ER lumen is likely attributed to the reduction of DHA after it is localized to the ER, although the mechanism of this process remains unclear.

In order to understand the effect of disulfide bond formation on HMW adiponectin assembly, we have investigated adiponectin oligomerization under various redox states in the endoplasmic reticulum. Modulation of the ER redox environment was achieved by addition of L-ascorbic acid.

Methods and Materials

Reagents – DMEM formulation: The powder was purchased from Cambrex. Sodium Pyruvate, Sodium Biocarbonate, Glutamine, and Penicillin/Streptomycin were purchased from HyClone. Insulin, Dexamethasone, 3-Isobutyl-1-methylxanthine were purchased from Sigma.

Adiponectin Oligomer Analysis – Adiponectin samples were run on a non-denaturing NuPage 7% Tris-Acetate gel (Invitrogen). For immunoblotting, samples were transferred to a nitrocellulose membrane (Whatman) and blocked in milk in TBST. The membranes were probed with rabbit anti-adiponectin primary antibody (Genset) and reacted with goat anti-rabbit HRP.

3T3-L1 differentiation – Fibroblasts were propagated and maintained to confluency in Dubelco's Modified Eagle's Medium (DMEM) with 10% FBS and penicillin and streptomycin(MM). Post-confluency, fibroblasts were differentiated by incubation with DM1 (10ug/ml Insulin, 1uM dexamethasone (DEX), and 0.5mM 3-Isobutyl-1-methylxanthine (IBMX) in MM) media for 2 days, followed by incubation with DM2 (5ug/mL in MM) for 2 days. After DM2, adipocytes were maintained in MM until mature.

HEK-293T Transfection – HEK293T cells were transfected as described previously (2).

Gel Filtration – Conditioned media collected from transfected HEK-293T were applied to a Superdex 200 (GE Healthcare) equilibrated. Fractions were analyzed as described above for adiponectin oligomer composition.

Ero1 oxidation state – Adipocytes were differentiated as described above. 150mg/L Ascorbate was added to Opti-MEM (Invitrogen) and incubated at 37°C for 4 hours. To assess reduced and oxidized Ero1, 5mM Dithiothreitol (DTT) and 5mM diamide (DIA), were incubated at 37°C for 45 minutes. After completion of incubation, media was removed, washed twice with chilled 1x PBS, pH 7.3, and lysed with 1x RIPA buffer with Mini Complete Protease Inhibitors (Roche) + 5mM AMS to alkylate free thiols. Post lysis, proteins were precipitated with 10% w/v Trichloroacetic acid (TCA). Precipitate was pelleted and washed three times with 70% Acetone. Pellet was resuspended in 5mM Hepes, 5% SDS, pH 7.2 + 8mM AMS. Samples were incubated on ice for 20 min, and 37°C for 30 min to allow thiol alkylation. Prior to running samples on a 10% Bis-Tris gel (Invitrogen), samples were diluted with 4x LDS sample buffer (Invitrogen). For a subset of samples, 10x reducing reagent (Invitrogen) was added. Samples were run on a 10% Bis-Tris gel and transferred to a nitrocellulose membrane (Whatman). The membrane was blocked in PBS:Odyssey Blocking Buffer (Licor) and probed with rabbit α-Ero1 antibody (Novus Biologicals). Goat anti-rabbit IR680 secondary antibody (Licor) was used to detect Ero1. The membrane was scanned with the Odyssey infrared imaging system at 700nm (Licor)

Thapsigargin – Differentiated 3T3-L1 adipocytes were treated with 300nM thapsigargin for 14 hours at 37°C. Conditioned media was collected and adipocytes were lysed with RIPA buffer supplemented with complete

Human serum - Patients received the usual care for their diabetes with instruction on a healthy diabetic diet by a dietician. Blood glucose monitoring was encouraged. The Hemoglobin A1C target was $\leq$ 7.0. Pioglitazone and matching placebos were prepared

by Takeda Pharmaceuticals and were given as a single daily dose of 30mg/day, or placebo, each morning. If after 8 weeks the hemoglobin A1C was > 7.0 or the fasting plasma glucose $\geq$ 100 mg/dL, the dose of pioglitazone was increased to 45 mg/day which occurred in all but one participant. If individuals had an increase in the hemoglobin A1C > 12% or an increase in the fasting plasma glucose > 240 mg/dL they were to be treated with sulfonylureas or insulin, but this was not necessary in this study. Subjects on sulfonylureas or metformin who experienced hypoglycemia had the dose of these medications reduced or the medication discontinued. Patients visited the clinic weekly for 4 weeks and each month thereafter. During these visits blood pressure was measured and a blood sample was collected for adiponectin oligomerization distribution. Adiponectin oligomerization was assessed as described above.

Results

Validation of native gel immunoblot analysis. We assemsed whether adiponectin oligomers can be separated on native gels because the gel filtration is not amenable to large numbers of samples. Gel filtration has been previously used to isolate adiponectin trimer, hexamer, and high molecular weight species (2). *Figure 1* is a native gel immunoblot of adiponectin oligomers separated by gel filtration chromatography. The blot was probed with an antibody raised against the globular domain of mouse adiponectin. Trimer, hexamer, and HMW oligomers are clearly distinguished by native gel analysis. The fractions with highest concentration of each oligomer correspond to the peaks previously determined for the three adiponectin oligomers in gel filtration chromatography. A comparison of various adiponectin samples is shown in *Figure 2A*. Purified calf HMW adiponectin is an octadecamer and has a migration pattern that is similar to that of the highest molecular weight species in the human, mouse, calf, and fetal calf serum. Additional protein bands in the serum samples have migration patterns that correspond to those of the purified calf hexamer and trimer adiponectin. Purified calf trimer, hexamer, and octadecamer were used as standards to determine the molecular weight of the other high molecular weight species (greater than hexamer) in the serum samples. Using linear regression analysis, we determined that the two high molecular weight species are dodecamers and nonamers respectively. These results collectively establish the efficiency of native gel immunoblotting for analyzing adiponectin oligomerization.

ER stress inhibits HMW adiponectin oligomerization. Thapsigargin is an ER-stress inducer that acts by tightly binding to Ca^{2+} ATPases, thereby preventing the entry of calcium ions into the endoplasmic reticulum. In order to determine whether ER stress affects adiponectin oligomerization, 3T3-L1 adipocytes were treated with 300nM thapsigargin for 14 hours. As shown in *Figure* 2, the cells treated with thapsigargin secreted adiponectin primarily in trimeric form. Because the cell lysate of the thapsigargin-treated sample does not contain adiponectin, it appears that adiponectin is secreted from the cell before it can oligomerize.

Oxidizing and reducing agents differentially affect adiponectin oligomerization. To determine the dependence of adiponectin oligomerization on ceullar redox conditions, we treated differentiated 3T3-L1 adipocytes with 3μM 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 100mg/L ascorbate. DPPH is a stable free radical and ascorbic acid is thought to assist in oxidative protein folding in the endoplasmic reticulum. A native gel immunoblot of conditioned media probed with anti-adiponectin antibody (*Figure 3*) indicates that DPPH significantly reduced the concentration of secreted HMW adiponectin, while ascorbate had the opposite effect on HMW adiponectin concentration. The concentration of hexamer remained constant among treated and control samples.

Ascorbic acid increases HMW adiponectin oligomerization. Increased concentration of HMW adiponectin was observed with ascorbate treatment of 3T3-L1 adipocytes for 18 hours. *Figure 4A* shows that the HMW adiponectin oligomerization positively correlated with ascorbate concentration in the media. Optimal HMW adiponectin formation is achieved at 100mg/L ascorbate. The total intracellular adiponectin concentration

remained constant (*Figure 4B*), as shown by a denaturing gel immunoblot analysis of the corresponding lysates to the samples shown in *Figure 4A*.

Increased adiponectin HMW oligomerization is not due to post-translational modifications. To examine the role of post-translational modifications on adiponectin oligomerization, we determined whether hydroxylation and glycosylation are sufficient for HMW formation, as has been previously proposed (11, 12, 13). Ascorbate acts as a cofactor for prolyl and lysyl hydroxylase, which are involved in the hydroxylation of proline and lysine residues, respectively, in collagenous domains. Ascorbate-induced increase of HMW asiponectin was not likely due to increased hydroxylation affinity of propyl and lysyl hydroxylase for ascorbate. Using amino acid analysis, we found that hexamer and HMW oligomers of adiponectin from 293T HEK cells are equally hydroxylated on proline and lysine residues (see *Table 1*). This data indicates that hydroxylation of adiponectin is not the sole factor contributing to increased oligomerization. Thus, ascorbic acid is not likely increasing oligomerization via its role as a cofactor to prolyl and lysyl hydroxylase. The degree of glycosylation of hexamer and HMW adiponectin was compared using native gel analysis. A plot of log of monomers versus distance migrated (*Figure 5B*) indicate that the hexamer and HMW bands (labeled, *Figure 5A*) treated with and without sialidase travel the same distance relative to each other. Sialidase treatment does not appear to effect the migration pattern of either hexamer or HMW preferentially. Taken together, these results provide evidence that hydroxylation and glycosylation alone cannot explain the structural differences between hexamer and HMW adiponectin.

Ascorbate alters the redox state of EroI in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with ascorbate, dithiothreitol (a reducing reagent), and diamide (an oxidant). After cell lysis, 4-acetamido-4'malemidylstibene-2,2'disulfonic acid (AMS) was added to the lysate. AMS reacts with reduced cysteines and prevents the formation of disulfide bonds. It also adds a molecular weight of 1kD to the molecule with which it reacts. Therefore, the oxidization state of a sample can be visualized by gel electrophoresis; a more reduced sample will have a greater molecular weight than a more oxidized sample and thus will not migrate as far down the gel. The adipocytes treated with dithiothreitol contained reduced EroI, while the adipocytes treated with diamide contained oxidized EroI. EroI in adipocytes treated with ascorbate is more reduced than the control sample.

Discussion

We have shown that assembly of HMW adiponectin is dependent on normal ER function. Thapsigargin, an ER stress inducer, is extremely effective in inhibiting the formation of HMW adiponectin, as virtually no adiponectin appears in the cell lysate. Adiponectin exists in the conditioned media, however, primarily as trimer. These data show that adiponectin is likely secreted from the ER before proper disulfide bond formation can occur.

ER stress caused by abnormal redox conditions impairs adiponectin oligomerization. We have shown that a reducing reagent, DPPH, potently decreases HMW adiponectin formation. Conversely, oxidizing agents such as ascorbic acid promote adiponectin oligomerization. In order to produce this effect, ascorbic acid must exist in high concentration. The optimal ascorbate concentration for enhanced adiponectin oligomerization is 100mg/L.

Ascorbic acid is important for hydroxylation and subsequent glycosylation of collagenous domains because of its function as a cofactor to both prolyl and lysyl hydroxylase. It has been shown that post-translational modifications are required for HMW formation. It can be argued that ascorbate is increasing adiponectin oligomerization via its function as a cofactor to prolyl/ lysyl hydroxylase. However, it is not likely that increased adiponectin oligomerization results from increased hydroxylase activity. The concentration of ascorbate required to increase HMW adiponectin assembly is 20-fold greater than that needed to activate prolyl/lysyl hydroxylase (14). Also, posttranslational modifications, such as hydroxylation and O-linked glycosylation, are not sufficient for HMW oligomerization. We have shown via amino acid analysis that

hexamer and HMW adiponectin contain about the same number of hydroxyproline and hydroxylysine residues. These data provide evidence against the proposition that post-translational modifications alone dictate the formation adiponectin oligomers.

We have demonstrated that ascorbate has an effect on the redox regulatory mechanism of EroI, an oxidative folding protein. To our knowledge, ascorbate is the only known substance capable of changing the EroI redox state. The addition of ascorbate reduces EroI and causes a shift in its migration pattern of due to its reaction with AMS. EroI is activate in its oxidized form; reduced EroI indicates decreased activity. Therefore, we propose that excess ascorbate is fulfilling the role of EroI, thereby down-regulating its activity. This provides evidence that ascorabte assists in oxidative protein folding.

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Figure Legends

Figure 1. Native gel immunoblot of adiponectin oligomers separated with gel filtration. Human serum adiponectin was purified and passed over Superdex 200 resin. Fractions containing HMW, hexamer, and trimer adiponectin were collected, analyzed by native gel electrophoresis, and probed with α-adiponectin antibody (Genset Corporation).

Figure 2. Native gel immunoblot of adiponectin from various sources. (A) Native gel α-adiponectin immunoblot of 1: human plasma, 2: purified calf HMW adiponectin, 3: purified calf hexamer adiponectin, 4: mouse serum, 5: 3T3-L1 conditioned media, 6: calf serum, and 7: fetal calf serum. (B) Plot of log(monomer) versus migration distance (cm). Linear regression analysis was used to determine the oligomeric state of HMW adiponectin species.

Figure 3. The effect of thapsigargin-induced ER stress on adiponectin oligomerization. (A) 3T3-L1 adipocytes were treated with 300nM thapsigargin for 14 hours. The conditioned media of treated and control adipocytes was analyzed by native gel α-adiponectin immunoblot. (B) Native gel α-adiponectin immunoblot of total adiponectin in the samples shown in (A).

Figure 4. The effect of oxidizing and reducing agents on adiponectin oligomerization. Differentiated 3T3-L1 adipocytes were treated with 3μM 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 100mg/L ascorbate. The conditioned media of treated and control cells was analyzed by a native gel immunoblot probed with α-adiponectin.

Figure 5. HMW adiponectin oligomerization increases with increasing ascorbate concentration. (A) 3T3-L1 adipocytes were treated with 1, 10, 100, 200 mg/L ascorbate for 18 hrs. Conditioned media was analyzed by a native gel immunoblot probed with α-adiponectin antibody. (B) A denaturing gel immunoblot (α-adiponectin) of the corresponding lysates to the samples shown (A), which indicate total intracellular adiponectin concentration.

Figure 6. Sialidase treatment of human serum adiponectin. (A) Human serum adiponectin was treated with sialidase. A plot of the natural log of monomer standards versus migration distance for (B) sialidase-treated adiponectin and (C) untreated adiponectin.

Figure 7. Ascorbate treatment changes the oxidation state of ErоI, adiponectin chaperone. Adipocytes (3T3-L1) were treated with 150mg/L ascorbate for 4 hours, 5mM dithiothreitol for 45 minutes, or 5mM diamide for 45 minutes. An aliquot of the treated and control cell lystates were reduced with dithiothreitol. The reduced and non-reduced cell lysate samples were analyze by denatured immunoblot analysis probing with α-Ero1.

Figure 1

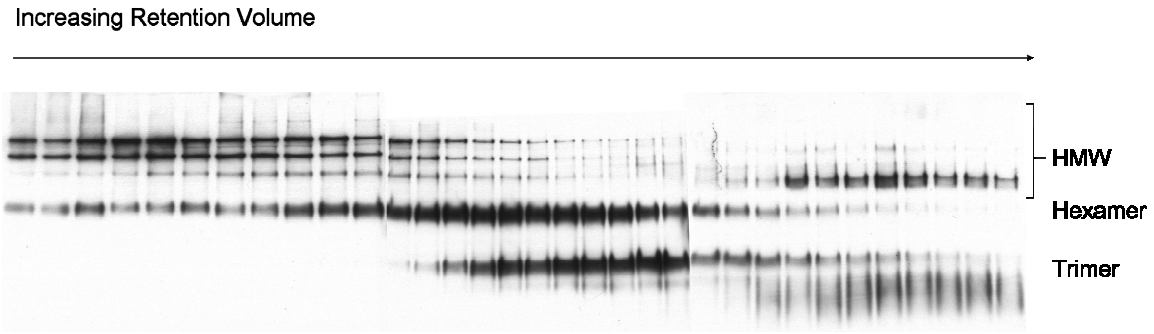


Figure 2

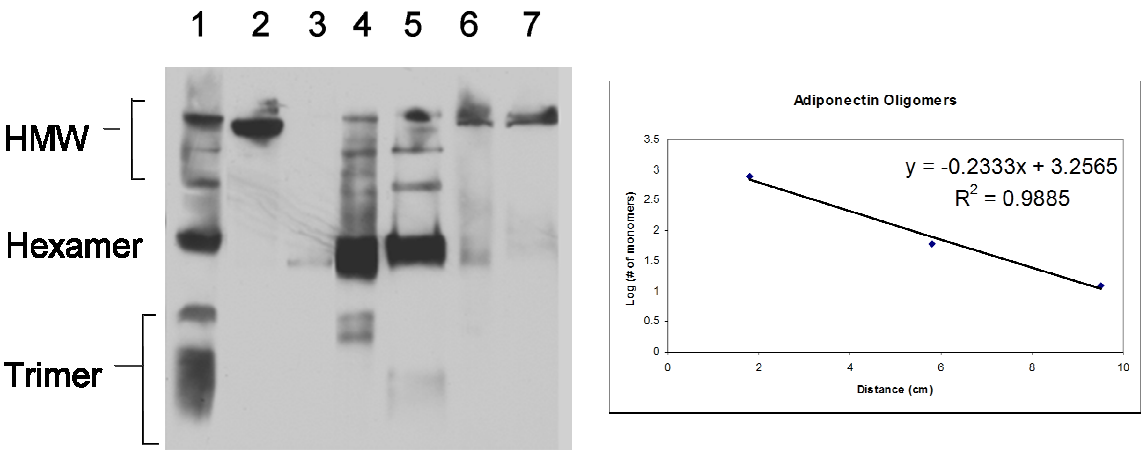

1 2 3 4 5 6 7
HMW
Hexamer
Trimer
Adiponectin Oligomers
y = -0.2333x + 3.2565
R2 = 0.9885
Log (# of monomers)
Distance (cm)
0
0.5
1
1.5
2
2.5
3
3.5
0
2
4
6
8
10

Figure 3

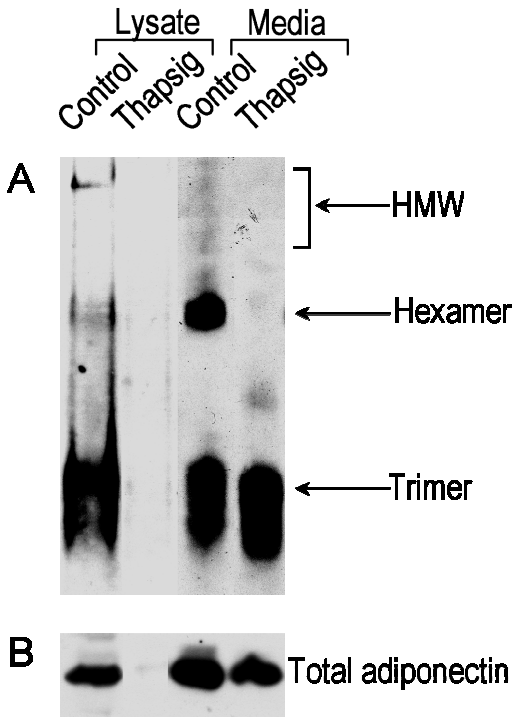

Lysate
Media
Control
Thapsig
Control
Thapsig
A
HMW
Hexamer
Trimer
B
Total adiponectin

Figure 4

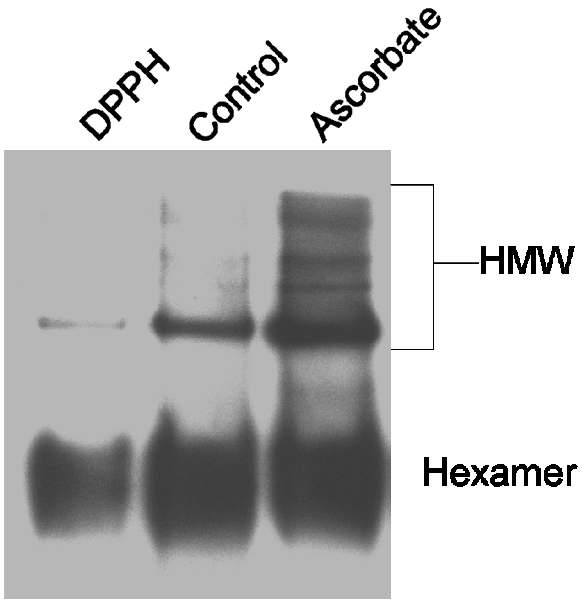


Figure 5

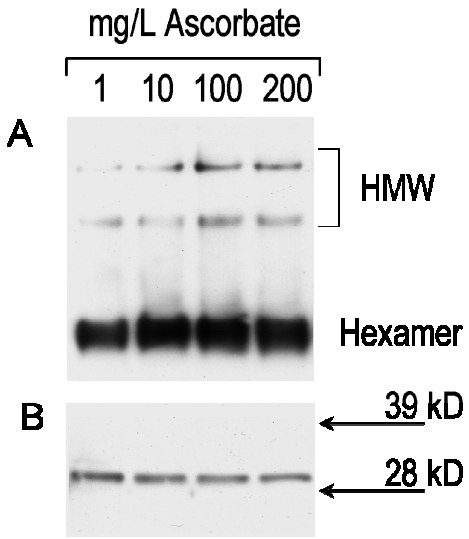


Table 1

Table 1: Extent of hydroxylation in proline and lysine residues of HMW and hexameric adiponectin produced from 293T cells.

		HMW		Hexamer	
	Predicted Residues	Experimental Residues	% Error	Experimental Residues	% Error
Proline	16	11.2	12.5	12.3	0.0
Hydroxyproline		3.4		3.6	
Lysine	13	10.2	0.0	11.3	7.7
Hydroxylysine		2.8		3.4	
		% Hydroxylated		% Hydroxylated	
Proline		23.3		22.6	
Lysine		21.6		22.8	

100µg of purified protein were hydrolyzed in 6N HCl/0.1% phenol at 110°C for 24 hrs. Amino acids were separated by IEX in Hitachi L8800 analyzer.

Figure 6

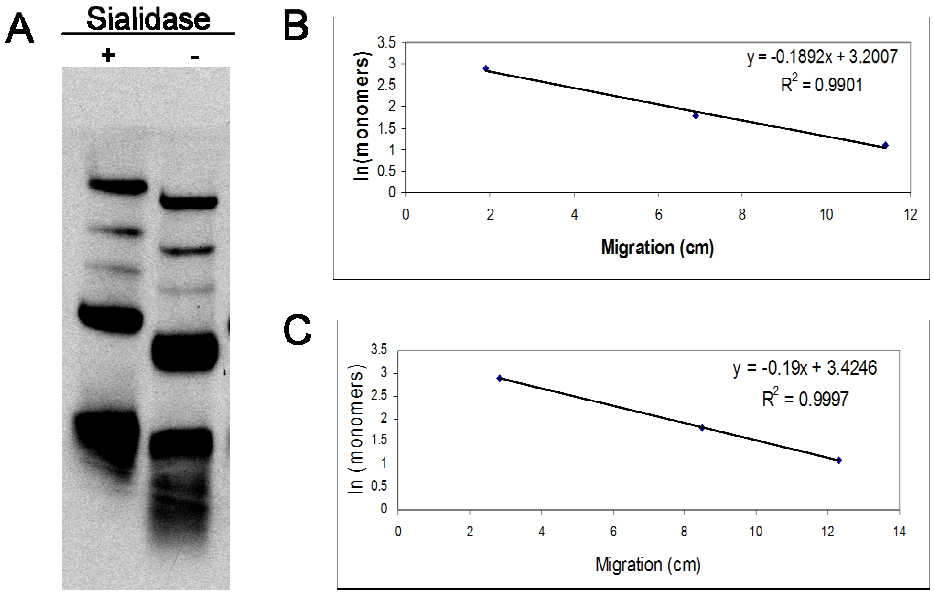
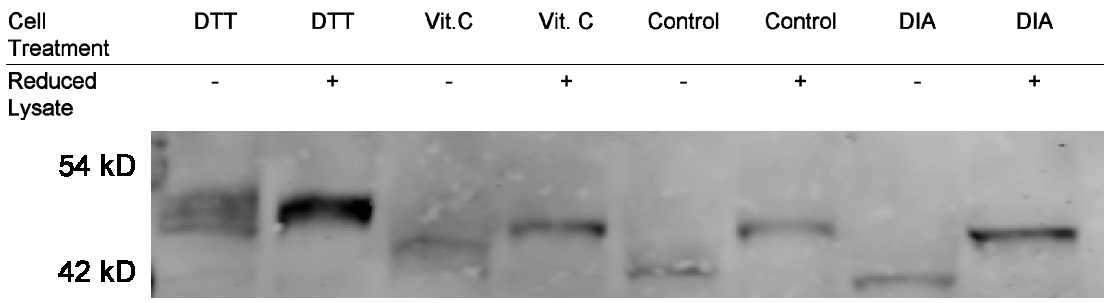

A
Sialidase
+
-
B
ln(monomers)
y = -0.1892x + 3.2007
R2 = 0.9901
Migration (cm)
C
ln (monomers)
y = -0.19x + 3.4246
R2 = 0.9997
Migration (cm)

Figure 7


Cell Treatment
DTT
DTT
Vit.C
Vit. C
Control
Control
DIA
DIA
Reduced Lysate
-
+
-
+
-
+
-
+
54 kD
42 kD