Glycosylation of human adiponectin affects its conformation and stability

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Abstract

The collagenous region of adiponectin is glycosylated *in vitro* with glucosylgalactosyl moieties on four conserved lysines. We investigated the glycosylation of human adiponectin *in vivo*. Sugar vicinyl hydroxides on adiponectin were oxidized with 10 or 1 mM metaperiodate, and the result analyzed by two-dimensional electrophoresis and immunoblotting. Only 10 mM metaperiodate caused significant changes in electrophoretic mobility and an altered susceptibility to proteinase K digestion. Such treatment also increased the susceptibility of hexamers and high molecular weight (HMW) isoforms to dissociation by SDS. By contrast, untreated low molecular weight (LMW) isoforms were readily dissociated by low concentrations of SDS. Reduced HMW isoforms were able to partially reassemble following the removal of dithiothreitol, and this process was unaffected by metaperiodate. The presence of sialic acid was detected by Maackia amurensis Lectin II blotting, and by oxidation with 1 mM metaperiodate, followed by detection with Emerald Green 300 fluorescent dye. Quantitation of sugars on affinity-purified adiponectin from nine human plasmas showed that dimers of HMW isoforms contained a 1.3-fold greater amount of total sugar than LMW isoforms. However, both contained similar amounts of sialic acid. We conclude that glucosylgalactosyl residues contribute to the conformation of HMW human plasma adiponectin. In addition, the HMW isoform contains greater amounts of glucosylgalactosyl residues than the LMW isoform, and these sugars are important in determining its stability *in vivo*.

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Introduction

Adiponectin is an adipocyte-specific protein related in structure to the complement component C1q. As with C1q, it self-associates into trimers, as well as higher molecular weight isoforms. Depending upon the assay system employed, adiponectin has been shown to have insulin sensitizing, anti-atherogenic, and anti-inflammatory properties, and it interacts with a range of other proteins, including growth factors (Wang *et al.* 2005), and with lipopolysaccharides (Peake *et al.* 2006). The activity of the protein may depend upon the molecular structure, with the high molecular weight (HMW) isoforms being associated with insulin sensitivity in man (Pajvani *et al.* 2004). In addition, mutations of adiponectin have been reported in diabetic patients in whom the protein shows impaired multimerization (Waki *et al.* 2003). The basic subunit consists of a collagenous N-terminal domain, and a C-terminal globular head; these subunits associate to form a trimer, the low molecular weight (LMW) complex. Adiponectin may also form sulfhydryl-dependent hexamers (medium molecular weight; MMW) and HMW isoforms containing nine dimers (Suzuki *et al.* 2007). These are stable *in vitro* and *in vivo* (Peake *et al.* 2005), and do not interconvert. However, the mechanisms which

determine the production of these different forms of the molecule remain unclear.

A number of studies have investigated the role of post-translational modifications in the assembly of the HMW isoform. The collagenous region of adiponectin produced *in vitro* contains four conserved lysines that are both hydroxylated and glycosylated with a glucosylgalactosyl moiety (Wang *et al.* 2002, 2004, Richards *et al.* 2006). Also present in bovine and mouse plasma adiponectin is sialic acid, possibly on O-linked glycans (Sato *et al.* 2001). However, the function of this modification has not been determined.

Mutant forms of recombinant human adiponectin expressed in a variety of mammalian cell lines have shown the importance of the glucosylgalactosyl moiety. When the modified lysines are replaced with arginine residues, the molecule has impaired function and is unable to form the HMW isoforms associated with insulin-sensitizing activity (Wang *et al.* 2006b). In this study, we investigated the role of sugars in native adiponectin derived from human plasma, since it is recognized that the choice of expression vector or culture conditions can affect the glycosylation of recombinant proteins (Skibeli *et al.* 2001, Brooks 2004). Its role in maintaining the structure and conformation of the protein was determined by the gentle conversion of

vicinyl hydroxides on sugars to aldehyde by metaperiodate. Destruction of glucosylgalactosyl residues, but not of sialic acid, led to an increased susceptibility to protease digestion and a weaker interaction between the subunits comprising the HMW and MMW isoforms. In addition, we examined the quantity of sugars present in LMW and HMW isoforms isolated from human plasma and showed that the HMW isoforms were glycosylated to a greater degree than LMW isoforms. This correlated with resistance to disruption by SDS of the HMW multimer.

Materials and methods

Superose column chromatography

Adiponectin from the plasma of a healthy male donor was resolved on an FPLC Superose 12 column (Pharmacia) equilibrated with PBS (Peake *et al.* 2005). Adiponectin levels in fractions were measured by commercial ELISA (R&D, Minneapolis, MN, USA).

The ability of the isoforms of plasma adiponectin to reassociate following reduction to the trimer by dithiothreitol (DTT) was determined by pooling fractions 4 and 5 (HMW) and 10 and 11 (LMW) following FPLC analysis. These samples were made to 0.1 M DTT and incubated for 60 min at 37 °C, and then transferred into PBS by passage down a G25 column. After incubation for 60 min at 37 °C, followed by overnight incubation at 4 °C, samples were reanalyzed by fast protein liquid chromatography (FPLC). In some experiments, samples were also reacted in the dark for 30 min with 10 mM metaperiodate before overnight incubation and reanalysis by FPLC.

Electrophoretic analysis of adiponectin in plasma

Plasma proteins were analyzed by native electrophoresis (Peake *et al.* 2005). In selected experiments, individual lanes were excised and analyzed in the second dimension (2D) by native electrophoresis, or equilibrated at room temperature with non-reducing SDS-PAGE buffer before 2D analysis. In other experiments, strips were incubated with 10 mM metaperiodate for 30 min before 2D separation. Blots were probed with a monoclonal (BD Biosciences, Sydney, NSW, Australia) or rabbit polyclonal (Sigma) antibody to adiponectin. These were detected with an affinity-purified, peroxidase-conjugated antibody to globulin (Bio-Rad) followed by chemiluminescence. Films were scanned and analyzed by Bio-Rad Quantity One software.

Enzyme digestion

To investigate any effect on protein structure induced by the destruction of sugars containing vicinyl

hydroxides, adiponectin was incubated for 30 min in the dark with either 0.1 M acetate buffer (pH 5.5) or with the same buffer containing 10 mM sodium metaperiodate. Samples were then buffer exchanged into PBS by passage down a G25 column, and incubated for 1 h at 37 °C with dilutions of 20 mg/ml proteinase K (Sigma) in PBS. The resulting fragments were analyzed by non-reducing SDS analysis and blotting.

Detection of glycosylation

Adiponectin from nine normal human plasmas (five males) was separated by FPLC analysis as above into HMW and LMW isoforms (Peake *et al.* 2005). Adiponectin was immunoprecipitated with a monoclonal antibody (R&D), and a polyclonal rabbit anti-mouse antibody (Dako, Glostrup, Denmark) conjugated to Sephadex (Pharmacia). The Sephadex beads were washed six times in cold PBS containing 1% NP40 and 0.1% SDS, before analysis by non-reducing SDS electrophoresis. The amount of adiponectin was determined by analysis of western blots probed with monoclonal anti-human adiponectin (BD). Total glycoprotein was detected by staining of gels with ProQ Emerald Green 300, following manufacturer's instructions (Molecular Probes, Invitrogen). Sialic acid residues were detected in an identical fashion, except that the gel was incubated in 1 mM metaperiodate for 30 min, as described in the ECL glycoprotein detection system (Amersham Life Science).

Lectin chromatography

To investigate the presence of sialic acid, HMW and LMW fractions of adiponectin were immunoprecipitated before analysis by non-reducing SDS electrophoresis and western blotting. Blots were probed with biotinylated 1 µg/ml Maackia amurensis Lectin II (Vector Labs, Burlingame, CA, USA) in Tris buffer (pH 7.5) containing 0.2 M NaCl. Lectin was detected by an avidin-peroxidase conjugate and chemiluminescence. Blots were reprobed with a monoclonal antibody to confirm that lectin was bound to adiponectin.

Statistical analysis

Electrophoretic analyses were performed a minimum of three times; results for representative experiments are shown. Data shown on the quantity of sugars in the fractions of individual plasmas and the amount of adiponectin in FPLC fractions represent the mean of duplicate analyses. Comparisons between groups were performed using paired and unpaired *t*-tests or the Wilcoxon signed-rank test. *P* values <0.05 were considered statistically significant.

Results

1D gel electrophoresis

As previously described (Peake *et al.* 2005), plasma adiponectin was resolved by native gel electrophoresis into four major bands (Fig. 1A); there were two HMW bands, as well as MMW, and LMW bands. A minor band was encountered in some samples, between the HMW and MMW isoforms. Incubation with 10 mM metaperiodate led to an altered electrophoretic pattern, with an increased number of bands, most of which moved further than usual in the gel (Fig. 1A). Incubation with 1 mM metaperiodate did not have any significant effect.

Figure 1 (A) A western blot, following native gel electrophoresis of plasma adiponectin. This shows the effect on adiponectin of incubation with acetate buffer (lane 1), acetate buffer containing 10 mM metaperiodate (lane 2), and 1 mM metaperiodate (lane 3). Two HMW bands are shown, as well as the MMW and LMW bands. (B and C) Western blots showing the separation of plasma adiponectin by native electrophoresis are shown as horizontal inserts at the top of 1B and 1C. Below these inserts is a western blot showing the separation of adiponectin by 2D (native gel/native gel) electrophoresis. Plasma was separated in the second dimension by native electrophoresis following incubation of gel strips from the first dimension with either acetate buffer (1B), or buffer containing 10 mM metaperiodate (1C). (D) A western blot following non-reducing SDS gel electrophoresis showing the effect on plasma adiponectin of incubation with acetate buffer (lane 1), or acetate buffer containing 10 (lane 2) or 1 mM (lane 3) metaperiodate. Molecular weight markers (kDa) are shown at left. (E and F) A western blot following 2D (native gel/non-reducing SDS gel) separation of plasma adiponectin. Plasma was separated in the first dimension by native electrophoresis, followed by incubation of gel strips with either acetate buffer (1E), or buffer containing 10 mM metaperiodate (1F). These were separated in the second dimension by non-reducing gel electrophoresis in 0.4% SDS. The same molecular weight markers as in 1D are shown at left.

In non-reducing SDS gels, the pattern seen was unaffected by incubation with 1 mM metaperiodate (Fig. 1D). However, incubation with 10 mM metaperiodate led to a different pattern of bands, with a loss of many bands of high MW. There was an increased representation of the dimer of ~ 65 kDa (Fig. 1D).

2D gel electrophoresis

We sought to clarify these results by 2D electrophoresis. Adiponectin was separated in the first dimension by native electrophoresis, followed by incubation of the gel with either buffer, or buffer containing 10 mM metaperiodate, and subsequent 2D analysis by either native (Fig. 1B and C) or non-reducing SDS electrophoresis (Fig. 1E and F).

Native/native 2D gels

Adiponectin incubated with buffer alone was unaffected in 2D analysis (Fig. 1B). However, incubation with 10 mM metaperiodate produced species derived from HMW, MMW, and LMW isoforms that had greater electrophoretic mobility than the native isoform (Fig. 1C).

Native/non-reducing SDS 2D gels

Non-reducing analyses of native gel strips resolved the HMW isoforms into several bands >170 kDa, but not into the dimer of ~ 65 kDa. The LMW fraction was resolved into three bands, including the dimer (Fig. 1E). However, incubation with metaperiodate led to a different pattern for both the HMW and MMW isoforms (Fig. 1F). In both cases, there was extensive formation of the dimer, while the amount of dimer present in the LMW fraction was not significantly affected. Metaperiodate had little effect on the amount of dimer derived from LMW adiponectin, the proportion of which remained 0.4–0.5 under a range of SDS concentrations (Fig. 2). By contrast, both HMW and MMW isoforms were far more susceptible to dissociation by SDS after incubation with metaperiodate than with acetate buffer alone. Incubation with SDS at 37°C rather than room temperature did not have a significant additional effect (not shown).

Reassociation of HMW and LMW fractions after reduction with DTT

Size-sorting analysis by FPLC showed that reduction converted all HMW adiponectin to the LMW trimer. However, on removal of DTT, the trimers derived from this fraction were able to substantially reassemble into species of HMW (Fig. 3). However, it was clear from Fig. 3, and from other analyses on native gels, that they

Figure 2 Effect of incubation with metaperiodate or acetate buffer on the quantity of adiponectin dimer in HMW, MMW, and LMW isoforms from plasma adiponectin. Plasma was separated by 2D (native gel/non-reducing SDS gel) electrophoresis, as shown in Fig. 1E and F. Before separation in the second dimension, gel strips were incubated in varying concentrations of SDS.

had not fully reassembled to the size of the HMW isoform present in plasma. On the other hand, LMW isoforms remained a similar size after removal of DTT.

When reduced fractions of adiponectin were additionally oxidized with metaperiodate, the same results were seen. There were no significant changes in the apparent sizes of HMW or LMW isoforms following oxidation with metaperiodate alone (not shown).

Enzymatic digestion

To further investigate the influence of glycosylation on the structure of adiponectin, the effect of incubation with metaperiodate on the susceptibility of adiponectin to enzymatic digestion by proteinase K was evaluated (Fig. 4A). Adiponectin was present as a dominant band of ~65 kDa consistent with the dimer, along with another band of ~24 kDa. On incubation with increasing concentrations of proteinase K, there were clear differences between adiponectin samples that had or had not been preincubated with 10 mM metaperiodate. Samples treated with 10 mM metaperiodate showed the appearance of a major breakdown product of ~35 kDa not seen in samples preincubated in buffer alone. Similarly, there was an increased susceptibility of metaperiodate-treated adiponectin to digestion by 0.05 mg/ml enzyme in terms of the formation of a band of ~20 kDa, and in terms of the amount of adiponectin remaining as a dimer. Plasmas similarly treated after incubation with 1 mM

metaperiodate showed no differences to those treated with buffer alone (not shown).

Glycosylation of adiponectin

The presence of sialic acid on human adiponectin was strongly suggested by the ability of Maackia amurensis Lectin II to bind to affinity-purified HMW and LMW adiponectin (Fig. 4B). This was confirmed by the staining of polyacrylamide gels with ProQ Emerald 300 stain after oxidation with 1 mM metaperiodate (Fig. 5C) rather than 44 mM metaperiodate employed to detect total glycoprotein (Fig. 5B).

Recombinant adiponectin was of slightly higher MW than affinity-purified human adiponectin (Fig. 5A), and equivalent amounts of this protein, as determined by analysis of antigenic blots and gels stained with ProQ Emerald 300, showed an increased degree of glycosylation. Both HMW and LMW fractions were glycosylated, and both contained vicinyl hydroxides able to be oxidized by 1 mM metaperiodate.

Quantitative analysis of the intensity of Emerald 300 staining (Fig. 5B and C) with reference to the intensity of antigenically detected adiponectin (Fig. 5A) showed that staining intensity was proportional to the quantity of adiponectin present, and that both HMW and LMW fractions contained lesser amounts of total sugar and sialic acid than the recombinant molecule. There were no significant differences between repeated assays of

Figure 3 FPLC size-sorting analyses of HMW and LMW fractions of adiponectin derived from human plasma. These HMW and LMW fractions were incubated with PBS (open symbols) or DTT (closed symbols). This was then removed before further incubation with PBS. These samples were then reanalyzed by size-sorting FPLC. Data from each analysis were normalized to the highest concentration of adiponectin detected in the individual fractions of each FPLC analysis (y axis). The x axis shows individual fractions from the FPLC column.

the same plasmas, and the s.e.m. of repeated observations was $\pm 11\%$.

A paired-comparison analysis in nine individual normal plasmas revealed that the total amount of glycosylation in the HMW fractions was significantly greater than that in the LMW fractions ($P=0.01$), although there was no significant difference between levels of sialic acid in these fractions (Fig. 6A). The ratio of the levels of sugars in the HMW to the LMW fraction for these plasmas (Fig. 6B) was 1.32 ± 0.10 for total sugars, which was significantly greater than the expected 'null' value of 1.0 ($P=0.01$). There were no significant differences in this ratio for the amount of sialic acid (1.18 ± 0.12).

Discussion

Different isoforms of adiponectin activate different signaling pathways and have differing effects in a range of tissues (Tsao *et al.* 2002, 2003, Wang *et al.* 2005). The data in this report emphasize the importance of glycosylation to the structure and conformation of these isoforms in human plasma. The distinction between recombinant forms of the molecule and those found in the plasma is important, since it is recognized that the choice of expression vector or culture conditions can affect the glycosylation of recombinant proteins (Brooks 2004). We showed recombinant human adiponectin to be of higher molecular weight, and more heavily glycosylated, than that present in human plasma.

The glucosylgalactosyl-modified lysines on the collagenous portion of adiponectin detected *in vitro* are presumed to be those present *in vivo* (Wang *et al.* 2002, 2004, Richards *et al.* 2006). Sialic acid has also been detected, but only in adiponectin purified from bovine and mouse plasma (Sato *et al.* 2001). Our data identify that sialic acid is present in equal amounts on HMW and LMW isoforms derived from human plasma. The analysis of total glycosylation revealed that dimers of the HMW isoform had 1.3 times the glycosylation present in the LMW isoform. Since this figure would include both glucosylgalactosyl and sialic acid residues, the true ratio of glucosylgalactosyl residues in HMW versus LMW isoforms is likely to be much greater than 1.3. This conclusion is supported by a study of unseparated plasma adiponectin in type 2 diabetic patients, in whom glycosylation of adiponectin was reduced in comparison with adiponectin from healthy controls. This may well have reflected a parallel fall in the proportion of the HMW isoforms (Wang *et al.* 2006b), shown by our results to have a greater amount of sugar than LMW isoforms.

Selective oxidation of sialic acid with 1 mM metaperiodate allowed Emerald 300 dye binding. However, only incubation with 10 mM metaperiodate had any effect on adiponectin's electrophoretic mobility, or upon the bands yielded on digestion with proteinase K. This effect was likely to be related to the conformation of the protein, rather than any effect on charge. Metaperiodate oxidizes vicinyl hydroxyl groups on sugars to form aldehydes, and while 1 mM metaperiodate was sufficient to oxidize sialic acid in readily detectable

Figure 4 (A) A western blot following non-reducing SDS gel electrophoresis showing the effect of incubation with 10 mM metaperiodate on the cleavage of adiponectin by proteinase K. Plasmas were incubated in buffer containing metaperiodate, or buffer alone. Following this treatment, samples were re-equilibrated with PBS, and then incubated for 60 min at 37 °C with proteinase K. The cleavage pattern generated by proteinase K on samples preincubated with metaperiodate (odd lane numbers), or buffer alone (even lane numbers) is shown. Proteinase K was used at concentrations of 0 (lanes 1 and 2), 1.30 (lanes 3 and 4), 0.44 (lanes 5 and 6), 0.15 (lanes 7 and 8), 0.05 (lanes 9 and 10), or 0.0015 mg/ml enzyme (lanes 11 and 12). Molecular weight markers are shown at left. (B) A western blot following non-reducing SDS gel electrophoresis showing the binding of Maackia amurensis Lectin II, which has specificity for sialic acid, to affinity-purified HMW (lane 1) and LMW (lane 2) fractions of adiponectin. Molecular weight markers (kDa) are shown at left.

quantities, it failed to alter the electrophoretic distribution of adiponectin. Evidence supporting a change in conformation due to the destruction of glycosylated residues came with the demonstration that incubation with 10 mM metaperiodate affected the pattern of cleavage generated by proteinase K. This enzyme cleaves at the carboxyl side of hydrophobic, aliphatic, and aromatic amino acids, and changes to the cleavage pattern suggest alterations in the accessibility of some of these residues to the external environment.

The hexamer and HMW isoforms are mostly composed of disulfide-linked dimers, while the native trimer contains one monomer and one dimer, or three non-covalently associated monomers when reduced by DTT (Tsao *et al.* 2003). Our results show that once reduced, trimers derived from HMW isoforms tended to reassociate on removal of DTT, while the LMW

trimer remained unchanged. This process was unaffected by oxidation of sugar residues and presumably depends upon non-covalent interactions between different subunits. The reassembly of trimers to HMW isoforms was incomplete, suggesting that glycosylation of the conserved lysine residues alone is insufficient for complete reconstitution of the HMW isoforms. Appropriate formation of disulfide bonds would appear crucial, since the HMW isoform contains nine disulfide-linked dimers (Tsao *et al.* 2003, Suzuki *et al.* 2007), implying that it could not be readily assembled from the trimer, which contains a dimer and a monomer with a single available sulfhydryl group. The process appears to require additional intracellular events, and evidence suggests that the HMW isoform is secreted via a pathway separate from that of other isoforms (Xu *et al.* 2005).

Figure 5 (A) A western blot following non-reducing SDS gel electrophoresis showing adiponectin antigenically detected by a monoclonal antibody. Recombinant adiponectin is shown in lane 1 (arrowhead), and affinity-purified HMW and LMW fractions are shown from two different plasmas in lanes 2 and 3, and lanes 4 and 5 respectively. (B) A non-reducing SDS gel showing total sugars from HMW and LMW fractions of adiponectin detected by ProQ Emerald 300 after treatment with 44 mM metaperiodate. Recombinant adiponectin is shown in lane 1 (arrowhead), and analyses of affinity-purified HMW and LMW fractions from the two plasmas in (A) are shown in lanes 2 and 3, and lanes 4 and 5 respectively. (C) A non-reducing SDS gel treated showing sialic acid residues from HMW and LMW adiponectin detected by ProQ Emerald 300 after treatment with 1 mM metaperiodate. Recombinant adiponectin is shown in lane 1 (arrowhead), and analyses of affinity-purified HMW and LMW fractions from the two plasmas in Fig. 5A are shown in lanes 2 and 3, and lanes 4 and 5 respectively.

We found that LMW adiponectin readily dissociated to a dimer in the presence of SDS, irrespective of whether or not its carbohydrates had been oxidized, whereas the HMW and MMW isoforms were more stable. However, these became susceptible to disruption subsequent to complete oxidation of their carbohydrate moieties, but not when sialic acid was preferentially destroyed. Sugars are thought to increase the stability of the collagen helix (Bann & Bachinger 2000), and the glucosylgalactosyl-modified lysine residues may act cooperatively in the formation of HMW isoforms (Wang *et al.* 2006b). Since electron microscopy of the hexamer and HMW isoforms indicates that they contain a single stalk of collagen domains (Tsao *et al.* 2003, Suzuki *et al.* 2007), our data suggest that glucosylgalactosyl-modified lysines stabilize interactions between the collagenous regions of dimers within the HMW and MMW isoforms, but not between the dimer and monomer within the LMW trimer. Further investigation is needed to define the full range of variants in hydroxylation and glycosylation of plasma adiponectin, and the effect such modifications may have on receptor binding, and interactions with other proteins (Wang *et al.* 2006a).

In summary, we showed that sialic acid was present in human plasma adiponectin and identified differences between the HMW and LMW isoforms in their degree of glycosylation, and in the influence of glycosylation on their structure and stability. In particular, it was the glucosylgalactosyl moiety, rather than sialic acid, which was crucial in these roles. The HMW and LMW isoforms had the same amount of sialic acid, but the former had a greater degree of glycosylation, and thus of the glucosylgalactosyl moiety. In addition, destruction of

Figure 6 (A) Levels of glycosylation of affinity-purified HMW and LMW fractions of plasma adiponectin. Gels were incubated with 44 or 1 mM metaperiodate, to detect total glycosylation, or sialic acid respectively. Results are expressed relative to the glycosylation of recombinant adiponectin as the mean $\pm$ S.E.M. (B) Levels of total sugars and sialic acid in affinity-purified HMW and LMW fractions of adiponectin from nine individual normal plasmas. Data are expressed as the ratio of the amount of sugars in HMW to LMW fractions in each plasma for both total sugars and sialic acid.

sialic acid had no effect on the stability or conformation of adiponectin, while modification of the glucosylgalactosyl moiety led to significant changes in the conformation of adiponectin and in the stability of the HMW and MMW complexes.

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