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Disturbed secretion of mutant adiponectin associated with the metabolic syndrome☆

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Abstract

Adiponectin, an adipocyte-derived protein, consists of collagen-like fibrous and complement C1q-like globular domains, and circulates in human plasma in a multimeric form. The protein exhibits anti-diabetic and anti-atherogenic activities. However, adiponectin plasma concentrations are low in obese subjects, and hypoadiponectinemia is associated with the metabolic syndrome, which is a cluster of insulin resistance, type 2 diabetes mellitus, hypertension, and dyslipidemia. We have recently reported a missense mutation in the adiponectin gene, in which isoleucine at position 164 in the globular domain is substituted with threonine (I164T). Subjects with this mutation showed markedly low level of plasma adiponectin and clinical features of the metabolic syndrome. Here, we examined the molecular characteristics of the mutant protein associated with a genetic cause of hypoadiponectinemia. The current study revealed (1) the mutant protein showed an oligomerization state similar to the wild-type as determined by gel filtration chromatography and, (2) the mutant protein exhibited normal insulin-sensitizing activity, but (3) pulse-chase study showed abnormal secretion of the mutant protein from adipose tissues. Our results suggest that I164T mutation is associated with hypoadiponectinemia through disturbed secretion into plasma, which may contribute to the development of the metabolic syndrome.
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Adipose tissue was originally considered as a storage site of excess energy in the form of triglycerides, but recent studies indicate that the tissue is a hormonally active system involved in the control of metabolism [1,2]. The term 'adipocytokines' refers to a series of adipocyte-derived biologically active molecules, which influence the function as well as the structural integrity of other tissues [2]. In humans under over-nutrient environment, however, exhibit enlarged functional impairment and dysregulated production of adipocytokines. For example, overproduction of tumor necrosis factor α (TNFα) interferes with insulin signaling in muscle, resulting in insulin resistance [3,4] and overproduction of plasminogen activator inhibitor type 1 (PAI-1) inhibits the fibrinolytic system, resulting in thrombosis [5]. The metabolic syndrome, a cluster of insulin resistance, hypertension, and dyslipidemia, is a common basis of type 2 diabetes and atherosclerotic vascular diseases in industrial countries [1,2,5]. Identification of a key adipocytokine will profound our understanding on the metabolic syndrome.

☆ *Abbreviations:* TNFα, tumor necrosis factor α; PAI-1, plasminogen activator inhibitor type 1; PCR, polymerase chain reaction; ELISA, the enzyme-linked immunosorbent assay system; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FCS, fetal calf serum; 2-DG, 2-deoxy-[^{3}H]glucose; PLSD, protected least significant difference.

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On the other hand, adiponectin, also known as gelatin-binding protein-28 [6], AdipoQ [7], and ACRP30 [8], is a plasma protein specifically produced by adipose tissues [9]. The protein belongs to the soluble defense collagen superfamily, composed of a collagen-like fibrous domain and complement C1q-like globular domain [9–12]. Adiponectin forms trimers, hexamers, and high-molecular weight structures [13,14], and its level in peripheral blood in normal human ranges from 5 to 30 μg/ml [15]. Adiponectin suppresses the expression of adhesion molecules in various vascular endothelial cells [16], lipid accumulation and secretion of TNFα by human monocyte-derived macrophages [17], growth factor-induced proliferation of vascular smooth muscle cells in vitro [18], and reduces atherosclerotic vascular lesions in vivo [19]. Adiponectin also promotes glucose uptake by C2C12 myocytes [20] and improves insulin resistance in vivo [20]. In humans, low adiponectin plasma concentrations have been reported in obese subjects [15]. There is evidence to suggest that hypoadiponectinemia is associated with the metabolic syndrome [21,22]. In contrast, high level of plasma adiponectin protects against the development of type 2 diabetes [23–25] and the risk of cardiac death [26]. These studies suggest that adiponectin could play a key role in the metabolic syndrome.

In general, plasma adiponectin concentrations are decreased under conditions of overnutrition [15]. In this regard, identification of a genetic cause for hypoadiponectinemia should provide important information regarding our understanding of the functions of this molecule. Mice genetically lacking adiponectin are prone to develop diet-induced insulin resistance [20] and severe neointimal thickening in response to vascular injury [27]. In other words, these animals exhibit several features of the insulin resistance syndrome [20].

During the screening of mutations in the adiponectin gene, we recently identified several missense mutations in the globular domain [21]. Of these, we focused on a missense mutation in which isoleucine at position 164 is substituted with threonine, because the subjects carrying this mutation showed markedly low levels of plasma adiponectin. Interestingly, these subjects frequently exhibited the clinical features of the metabolic syndrome [21]. The present study is an extension to the above work and reports the molecular characteristics and mechanism of low plasma level of the mutant adiponectin.

Materials and methods

Cell culture, transfection, and preparation of recombinant protein. Quantum's 293A (QBI-293A) cells (Qbiogene, Funakoshi Life Science, Tokyo, Japan) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). DNAs containing the human full-length adiponectin with wild-type and I164T mutation were amplified by polymerase chain reaction (PCR) engineered for subcloning into the pTARGET mammalian expression vector (Promega, Tokyo). An expression vector containing a DNA with opposite direction of the wild-type adiponectin was used as control. Plasmids for transfection were purified using the EndoFree plasmid kit (QIAGEN, Tokyo). The integrities of all plasmids were verified by DNA sequencing. For each 6-well culture plate, each 1 μg of expression vector was dissolved with Lipofectamine plus (Life Technologies, Tokyo) according to the protocol provided by the manufacturer and used for transfection. One milliliter of fatty acids-free DMEM containing 0.5% bovine serum albumin was replaced 3 h later. The lysates of transfected cells were harvested 24 h later and subjected to Western blotting assay to investigate the oligomeric state of adiponectin.

For glucose uptake experiments, wild-type and mutant recombinant proteins were synthesized. Glutathione *S*-transferase (GST) fusion vector containing globular domain of wild-type (gWT) or I164 mutant (gI164T) adiponectin was constructed with pGEX-6P-1 (Amersham Pharmacia Biotech, Tokyo), as described previously [20]. GST-gWT or -gI164T protein was produced in strain BL21 of *Escherichia coli* and purifying using glutathione sepharose 4B (Amersham Pharmacia Biotech). GST was cleaved from GST-gWT or -gI164T protein by PreScission Protease (Amersham Pharmacia Biotech).

C2C12 myoblasts were purchased from the Riken Cell Bank (Tsukuba city, Ibaraki, Japan). Differentiation of C2C12 myocytes was performed as previously described [28]. Briefly, C2C12 myoblasts were cultured in 100-mm dishes in an atmosphere of 5% CO_2 at 37 °C in DMEM supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin (100 U/ml) till 100% confluence. Myoblast differentiation was induced with DMEM supplemented with 5% horse serum and L-glutamine for 72 h. Differentiated myotubes were then starved for 5 h in serum-free DMEM before treatment.

Plasma adiponectin concentrations. Written informed consent was obtained from all subjects before enrollment in the study [21]. The study was approved by the Osaka University Ethics Review Board. Blood samples were obtained from each subject and genomic DNA was isolated from peripheral blood leukocytes. We screened for mutants in the adiponectin gene by PCR using adiponectin gene specific primers, as reported previously [21]. DNA sequences were analyzed by ABI377 automatic sequencer.

Enzyme-linked immunosorbent assay. The concentration of plasma adiponectin was measured by ELISA, as described previously [15]. In brief, a 96-well plate was coated with 5 μg/ml mouse monoclonal antibody, ANOC9108, at 4 °C and blocked with 0.1% bovine serum albumin (BSA) and 0.05% sodium azide. Human plasma was diluted with five volumes of the sample buffer (31.25 mmol/l Tris–HCl, pH 6.8, and 2.3% SDS) and boiled for 5 min. Each sample was then diluted with the sample buffer, and 50 μl of the sample, at a final 1:5000 dilution, was applied to each well of the anti-adiponectin monoclonal antibody (ANOC9108)-coated plate and incubated overnight at room temperature. A recombinant adiponectin protein without a leader peptide, NH_2-terminal 11 amino acids, was used as the standards. The wells were washed three times with 5 mmol/l Tris–HCl (pH 8.0) containing 15 mmol/l NaCl and 0.05% Tween 20 and then 100 μl of a final 1:10,000 dilution of rabbit polyclonal antibody, the optimal cutting temperature (OCT)9104 was determined by the *O*-phenylenediamine dihydrochloride–horseradish peroxidase method.

Western blotting analysis. The molecular forms of plasma adiponectin in humans with wild-type (W) or heterozygous form of the missense mutation with a substitution from isoleucine to threonine at position 164 (I164T: I) were analyzed by Western blotting analysis. Briefly, an equal aliquot of plasma (a final 1:50 dilution with Dulbecco's PBS free of calcium and magnesium chloride (PBS (−))) was lysed in Laemmli sample buffer (Bio-Rad Technologies, Richmond, CA) in the absence or presence of β-mercaptoethanol and subjected to 10% SDS–PAGE.

The molecular form of adiponectin protein in cell lysates of QBI-293A cells transfected with pTARGET(Promega)-WT or -I164T were

also analyzed by Western blotting analysis. The cells were washed twice with PBS (−), and then resuspended in 300 μl of disruption buffer (20 mM Tris–HCl, pH 7.5, 145 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 200 μM sodium orthovanadate, 200 μM PMSF, 1 μg/ml aprotinin, and 1 μg/ml leupeptin), and lysed with three repetitive freeze–thaw cycles. The proteins (5 μg) were lysed in Laemmli Sample Buffer (Bio-Rad Technologies) in the absence or presence of β-mercaptoethanol and separated on a 10% SDS–PAGE and transferred onto nitrocellulose transfer membrane (Schleicher and Schuell, Keene, NH). Western blot analysis was performed using anti-human adiponectin monoclonal antibody (ANOC9108) at a dilution of 1:1000. As a secondary antibody, a 1:2000 dilution of affinity-purified anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) was used. The signal was detected by chemiluminescence using the ECL system (Amersham Pharmacia Biotech). The gel was exposed to X-ray film, X-OMAT AR (Kodak, Tokyo).

Pulse-chase study. Pulse-chase studies were performed to analyze the secretion steps of the newly synthesized mutant and wild-type of adiponectin proteins, according to the procedure described previously [29,30]. Briefly, Quantum's 293A (QBI-293A) cells were subcultured at a density of 1.0×10^5 cells/well in a 6-well plate on the day before transfection. The cells were transiently transfected with 1 μg of each pTARGET construct using Lipofectamine plus. One day after transfection, the medium was replaced with fresh complete medium (DMEM supplemented with 10% FCS) and incubated for further 24 h. Cells were washed with PBS (−) and then were incubated with 1 ml of FCS, methionine, and cysteine-free DMEM supplemented with 100 μCi/ml of L-[^{35}S]methionine and L-[^{35}S]cysteine (Pro-mix L-[^{35}S], Amersham Pharmacia Biotech) in vitro cell labeling mixture and incubated for 30 min. The cells were chased with fresh FCS-medium containing cold methionine and cysteine for the indicated period. At each time point, the medium was collected, and the cells were washed with PBS (−) and suspended in 300 μl of disruption buffer (10 mmol/l Tris–HCl [pH 7.5], 150 mmol/l NaCl, 5 mmol/l EDTA, 10 mmol/l benzamidine, 1 mmol/l PMSF, and 1% Nonidet P-40), and lysed with three repetitive freeze–thaw cycles. The cell lysates were adjusted to equal protein concentration with disruption buffer and subjected to immunoprecipitation. A total of 500 μl of cell lysates (100 μg of total protein) was mixed with equal volume of disruption buffer lacking EDTA and Nonidet P-40, and immunoprecipitated with 5 μl of rabbit antibody against human adiponectin, ANOC9108, overnight at 4 °C, followed by incubation with 40 μl of protein G beads (Amersham Biosciences) for 2 h at 4 °C. For medium, aliquots (450 μl) of metabolically-labeled culture medium were added by 1/10 volume of 10× immunoprecipitation buffer (IPB; 1 × IPB = 10 mmol/l Tris–HCl [pH 7.5], 150 mmol/l NaCl, 1 mmol/l EDTA, 1%, Nonidet P-40, 10 mmol/l benzamidine, and 1 mmol/l PMSF) and immunoprecipitated as described above. Then, the immunoprecipitates were washed three times with 1× IPB, followed by solubilization with a sample buffer, and subjected to SDS–PAGE. After electrophoresis, the gel was dried, and radiolabeled proteins were analyzed by autoradiography. The band intensities were quantified by densitometry.

Gel filtration chromatography. Plasma samples (100 μl each) were drawn from subjects with wild-type or heterozygous form of I164T adiponectin and adjusted with PBS (−) up to 2 ml. Following dialysis against PBS (−), purified protein samples were fractionated in a Hi-Load 16/60 Superdex 200 column (Amersham Biosciences) and eluted with PBS (−). The concentration of adiponectin in each 1-ml fraction was determined by ELISA [15].

Determination of glucose uptake by C2C12 skeletal muscle cells. 2-Deoxy-[^{3}H]glucose (2-DG) was purchased from NEN Life Science Products (Boston, MA). 2-DG uptake assay was performed using C2C12 myocytes, as described previously [28]. Briefly, after 5 h of serum starvation at day 5 after differentiation induction, 10 μg/ml of recombinant empty, gWT or gI164T was added and incubated for 24 h and then stimulated with or without insulin (100 nM) for 30 min, before cell collection. After insulin treatment, cells were washed twice with wash buffer (20 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, and 1 mM $CaCl_2$). Cells were then incubated in buffer transport solution (wash buffer containing 0.5 mCi 2-[^{3}H]DG/ml and 10 mM 2-DG) for 10 min. Uptake was terminated by aspiration of the solution. Cells were then washed three times, and radioactivity associated with the cells was determined by cell lysis in 0.05 M NaOH, followed by scintillation counting. Aliquots of cell lysates were used for protein content determination. 2-DG uptake was expressed as picomoles per minute per milligram of protein.

Statistical analysis. Statistical analysis was performed using the StatView software (Statview, Abacus Concept, Berkeley, CA). Group differences were determined using analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) test. All variables are expressed as means ± SEM. An α level of 0.05 was used to determine statistical significance.

Results

Isoleucine at position 164 of human adiponectin is conserved in all of the species investigated

Isoleucine residue substituted in I164T mutation was located in the globular domain of adiponectin. Sequence alignment of adiponectin in five mammals (human, mouse, bovine, monkey, and dog) revealed that Isoleucine 164 was the third amino acid in the highly conserved sequence, YHITVY motif (Fig. 1).

Antibody reactivity and oligomeric state of mutant adiponectin in plasma

In our previous study, plasma adiponectin concentrations in subjects with I164T mutation measured by ELISA [15] were markedly low compared to those in subjects without such mutation (1.9 ± 1.1 vs. 6.3 ± 0.3 μg/ml). We used a monoclonal antibody ANOC9108 for the first antibody to immobilize adiponectin to the ELISA plate. First, we examined whether the low plasma concentration of adiponectin in subjects with I164T mutation was due to a low reactivity of ANOC9108 to

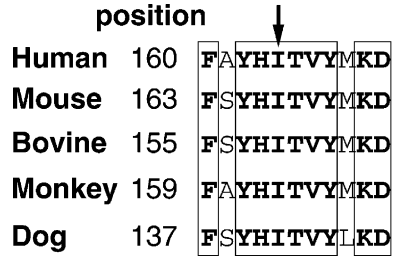

Fig. 1. Amino acid sequence alignment of mammals surrounding Isoleucine at position 164. Human, mouse, bovine, monkey, and dog adiponectin sequences relate to GenBank/EBI Accession Nos. NP_004788, BAB22597, AAK58902, AAK92202, and AAL09702, respectively. The conserved amino acids are shown in bold letters and surrounded by boxes. Arrow indicates the isoleucine at position of human adiponectin.

the mutant protein. We tried to detect mutant adiponectin by Western blotting analysis using two monoclonal antibodies, each of which recognizes a different epitope, ANOC9108 and ANOC9131, and a polyclonal antibody, OCT9104. Fig. 2A shows the positions of epitopes recognized by each antibody. Adiponectin in plasma has been identified as a monomer and several oligomeric states in SDS–PAGE under reducing condition [13–15]. Equal amounts of plasma from subjects with the mutation and subjects with low plasma adiponectin but without the mutation were subjected to SDS–PAGE. Plasma adiponectin concentrations determined by ELISA using ANOC9108 were 3.66 and 3.99 μg/ml in subjects without the mutation, and 1.18 and 2.90 μg/ml in subjects with the mutation, respectively. Although the reactivity to a different oligomeric form was different, each of the three antibodies recognized adiponectin in plasma in the control subjects and subjects with the mutation in a similar manner comparable to the concentration (Fig. 2B). Thus, the low plasma concentration of adiponectin in subjects with the mutation was not due to the low reactivity of the monoclonal antibody used in ELISA.

High-ordered structure is necessary for the maximal function of adiponectin. Therefore, we next analyzed the oligomeric state of adiponectin in subjects with the mutation by Western blotting analysis under reducing and non-reducing conditions. Adiponectin migrated to a monomer position under reducing condition. Under non-reducing condition, the bands shifted to positions of the high molecular weight form (HMW) and a hexamer (Fig. 2C). Similar results were obtained in the plasma of subjects with the mutation (Fig. 2C, number 4).

For further characterization, we analyzed the oligomeric state of wild-type and mutant adiponectin in plasma by gel filtration chromatography. In the wild-type, adiponectin was eluted with three peaks (HMW > hexamer > trimer). Plasma from subjects with I164T mutation showed a similar elution profile (Fig. 2D). Thus, the oligomeric state of adiponectin in the plasma of subjects with the mutation did not differ from those with wild-type.

Oligomeric state and secretion of mutant adiponectin in cultured cells

Next, we investigated the oligomeric state and secretion of the mutant adiponectin expressed in cultured QBI-293A cells. The oligomeric state of the I164T mutant protein was observed by Western blotting analysis. The majority of mutant adiponectin expressed in QBI-293A cells was transformed to the monomer form under reducing condition, and shifted to dimer, trimer, hexamer, and HMW forms under non-reducing conditions, similar to the wild-type protein (Fig. 3A). These results indicate that the I164T mutation does not interfere with the oligomeric processing of adiponectin.

Next, we investigated the secretion process of the newly synthesized I164T mutant protein using the pulse-chase study [30]. After a 2-h metabolic labeling, the media were replaced with complete DMEM containing non-radiolabeled methionine and cysteine, and chased for the indicated time. The L-[^{35}S]methionine and L-[^{35}S]cysteine-labeled adiponectin in the media (Fig. 3B, left) and in the cell lysates (Fig. 3B, right) were immunoprecipitated and quantified by densitometry. The wild-type adiponectin was secreted efficiently. Approximately, a half of the protein was secreted after 4-h chase. In contrast, disruption of the secretion of I164T adiponectin was noted; the mutant protein was retained in the cells compared with the wild-type, and gradually disappeared from the cells.

Finally, we investigated the function of I164T mutant adiponectin by 2-deoxy-[^{3}H]glucose (2-DG) uptake experiments in C2C12 myocytes. The globular adiponectin

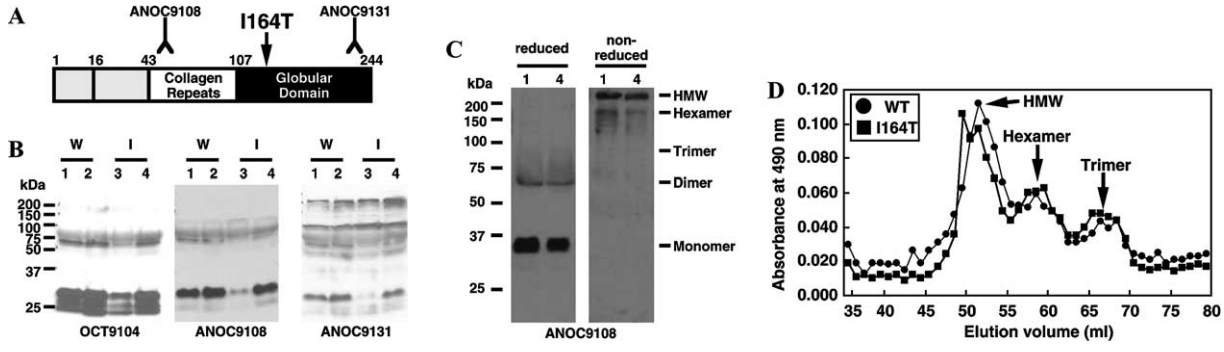

Fig. 2. Antibody cross-reactivity and oligomeric state of adiponectin in plasma. (A) Epitope positions of monoclonal antibodies against human adiponectin (ANOC9108 and ANOC9131). (B) Western blotting analysis of adiponectin in plasma from wild-type (W) and I164T mutation (I) using different antibodies. Numbers 1 and 2, W; 3 and 4, I. Plasma adiponectin levels in subjects 1–4 were 3.66, 3.99, 1.18, and 2.90 μg/ml, respectively. (C) Western blotting analysis of adiponectin in plasma from wild-type (W; number 1) and I164T mutation (I; number 4) under reducing and non-reducing conditions. (D) Elution profiles of plasma from wild-type (WT) and I164T mutation by gel filtration column. The concentration of adiponectin in each fraction was determined by ELISA.

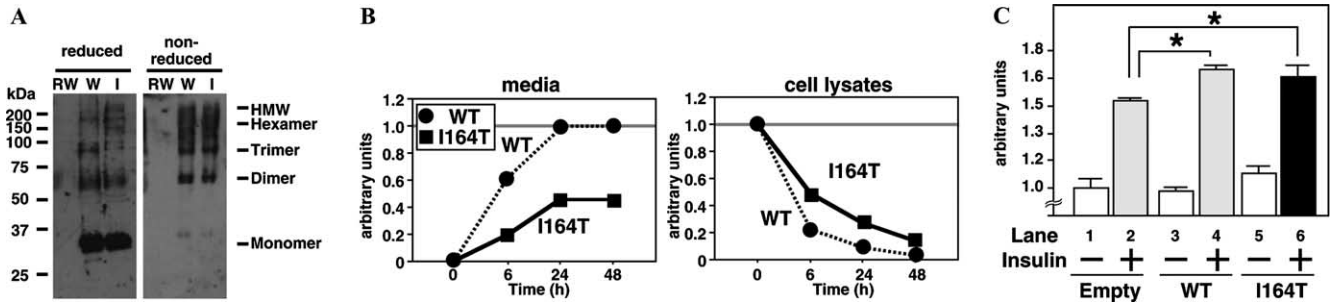

Fig. 3. Oligomeric state, secretion, and function of mutant adiponectin. (A) Western blot analysis of adiponectin in QBI-293A cells transfected with reversed type (RW, control), wild-type (W), or I164T mutant (I) adiponectin under non-reducing and reducing conditions. (B) Pulse-chase labeling of adiponectin in QBI-293A cells. ^{35}S-labeled adiponectin was immunoprecipitated, subjected to SDS–PAGE, and visualized by autoradiography. Band intensities quantified by densitometry in the media (left) and cell lysates (right) at the indicated time. Results were obtained from triplicate. Similar results were obtained in two other independent experiments. (C) Effects of wild or I164T mutant adiponectin on glucose uptake in C2C12 myocytes in the presence or absence of insulin. Data are means ± SEM values of four experiments; $*p < 0.05$, by ANOVA with Fisher's PLSD test. This experiment was performed three times with similar results.

is known to upregulate glucose uptake in muscle [20,31,32]. Insulin stimulated 2-DG uptake by approximately 1.5-fold (Fig. 3C, lanes 1 and 2), as reported previously [20,28]. WT adiponectin significantly enhanced insulin-stimulated glucose uptake (Fig. 3C, lanes 2 and 4), as reported previously [20]. I164T mutant adiponectin also enhanced insulin stimulated glucose uptake at a level similar to that seen with WT (Fig. 3C, lanes 2 and 6).

Discussion

Adipocyte orchestrates various adipocytokines in the self-defense system [2]. Substantial reports demonstrated that the decreased production of adiponectin by overnutrition might play a fundamental role in the metabolic syndrome in humans [15,22,23] and rodents [33]. In a previous study, we reported that the I164T missense mutation is associated with low plasma adiponectin levels and clinical features of metabolic syndrome [21]. Although the precise mechanisms remain unclear, it was speculated that the normal assembly or secretion of the protein might be disturbed in this mutation. In the present study, we demonstrated that the assembly of the protein was normal but the secretion was abnormal in I164T mutation. The disturbed secretion of the mutant protein may explain, at least in part, the associated hypoadiponectinemia and frequent association with the metabolic syndrome in subjects with this mutation.

Adiponectin forms oligomer intracellularly through disulfide bond formation mediated by Cys39 [34]. Oligomerization state of adiponectin is important to activate nuclear factor-κB (NF-κB) in C2C12 myocytes [35]. In the plasma of subjects with I164T mutation, adiponectin showed a similar distribution to the oligomerization state (trimer < hexamer < HMW form). In subjects carrying the I164T mutation in a heterozygous form, only the wild-type adiponectin might be secreted into the plasma. However, this is unlikely because the mutant adiponectin produced by cultured cells also formed multimeric structure similar to the wild-type adiponectin.

Our results also showed impaired secretion of mutant adiponectin and a slow secretion of the mutant protein from the cells. The mechanism underlying the disrupted secretion of the protein in I164T mutation is not clear at present. The disturbed secretion of mutant protein could contribute, at least in part, to the low plasma adiponectin concentration in subjects with I164T mutation. Plasma adiponectin concentrations in subjects with the mutation were approximately 30% of normal, even in heterozygotes [21]. It is possible that degradation or catabolism of the mutant protein is enhanced under such conditions. At this stage, it is difficult to estimate the half-life of the protein because iodination of the protein affects the normal high-ordered structure of the protein. Finally, the mutant protein may be less active for lipid and glucose metabolism. However, the activities of the recombinant adiponectin with I164T mutation in C2C12 cells were not different from those of the normal adiponectin.

In conclusion, we have demonstrated in the present study abnormal secretion of mutant adiponectin in subjects with the metabolic syndrome.

Acknowledgments

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