Unique Profile of Chicken Adiponectin, a Predominantly Heavy Molecular Weight Multimer, and Relationship to Visceral Adiposity

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Adiponectin, a 30-kDa adipokine hormone, circulates as heavy, medium, and light molecular weight isoforms in mammals. Plasma heavy molecular weight (HMW) adiponectin isoform levels are inversely correlated with the incidence of type 2 diabetes in humans. The objectives of the present study were to characterize adiponectin protein and quantify plasma adiponectin levels in chickens, which are naturally hyperglycemic relative to mammals. Using gel filtration column chromatography and Western blot analysis under nonreducing and non-heat-denaturing native conditions, adiponectin in chicken plasma, and adipose tissue is predominantly a multimeric HMW isoform that is larger than 669 kDa mass. Under reducing conditions and heating to 70–100 C, however, a majority of the multimeric adiponectin in chicken plasma and adipose tissue was reduced to oligomeric and/or monomeric forms. Immunoprecipitation and elution under neutral pH preserved the HMW adiponectin multimer, whereas brief exposure to acidic pH led to dissociation of HMW multimer into multiple oligomers. Mass spectrometric analysis of chicken adiponectin revealed the presence of hydroxyproline and differential glycosylation of hydroxylysine residues in the collagenous domain. An enzyme immunoassay was developed and validated for quantifying plasma adiponectin in chickens. Plasma adiponectin levels were found to be significantly lower in 8- compared with 4-wk-old male chickens and inversely related to abdominal fat pad mass. Collectively, our results provide novel evidence that adiponectin in chicken plasma and tissues is predominantly a HMW multimer, suggesting the presence of unique multimerization and stabilization mechanisms in the chicken that favors preponderance of HMW adiponectin over other oligomers. **(*Endocrinology* 150: 3092–3100, 2009)**

Adiponectin is an adipokine hormone secreted primarily from adipose tissue in several mammalian species (1–4). It is composed of four distinct domains that include a signal peptide at the N terminus followed by a short variable region, a collagenous domain, and a C-terminal globular domain (5). During biosynthesis in mammalian adipocytes, the 30-kDa adiponectin monomers are assembled into oligomers and multimers and secreted as three distinct isoforms denoted as low (LMW), medium (MMW), and heavy molecular weight (HMW) isoforms (6–8). The LMW adiponectin isoform represents a homotrimer of three adiponectin monomers, whereas the MMW isoform is a hexamer composed of two homotrimers and the HMW adiponectin isoform is likely to contain 18 or more adiponectin monomers (7).

Oligomerization status appears to dictate the function of adiponectin in humans and rodents (6). A number of studies have elucidated the role of adiponectin, in particular that of the HMW isoform, in metabolic diseases of humans and rodent models. The circulating levels of the HMW isoform, rather than total adiponectin, are inversely correlated with the incidence of type 2 diabetes and insulin resistance (8). A selective down-regulation of the HMW form of adiponectin has been reported in human subjects with hyperinsulinemia and in type 2 diabetes (9). In contrast, treatment of diabetic human subjects with insulin-sensitizing thiazolidinedione drugs increased the blood levels of the HMW adiponectin isoform that correlated with increased insulin sensitivity (10, 11). In addition, weight reduction by calorie restriction or gastric bypass sur-

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Abbreviations: EIA, Enzyme immunoassay; HMW, heavy molecular weight; LMW, low molecular weight; MMW, medium molecular weight; MS/MS, tandem mass spectrometry; PVDF, polyvinylidene difluoride; TBST, Tris, sodium chloride, and Tween 20; UPLC, ultra-performance liquid chromatography; Vo, void volume.

gery results in a selective elevation of the HMW adiponectin but not the LMW or MMW isoforms (12–14).

The chicken is a useful model to study adiponectin as it is naturally hyperglycemic compared with humans and rodents, with blood glucose levels averaging three times those of humans, and is considered to be insulin resistant (15). Both insulin and glucose affect plasma adiponectin levels because hyperglycemia was found to prevent the suppressive effect of hyperinsulinemia on plasma adiponectin levels in healthy human subjects (16). Moreover, commercial broiler chickens are genetically selected to achieve rapid body growth and muscle accretion, which also leads to an unintended increase in visceral adiposity (9). Because visceral adiposity and diabetes are associated with hypoadiponectinemia, studies on chicken adiponectin may provide critical information on the biosynthesis, stability, and secretion of adiponectin multimers.

Whereas the biology of mammalian adiponectin isoforms is beginning to be elucidated, there are no reports on the properties of adiponectin protein in any nonmammalian species. We previously cloned and characterized the chicken genes that encode adiponectin and two of its receptors, adiponectin receptor-1 and -2 (17, 18). The deduced protein sequence of chicken adiponectin cDNA contains both collagenous and globular domains, similar to mammalian adiponectin (17). Unlike humans and rodents, however, the chicken adiponectin gene is widely expressed in a variety of tissues (17, 19). Our efforts to characterize adiponectin protein or to quantify circulating adiponectin levels in the chicken using a variety of antibodies against human or mouse adiponectin failed due to poor conservation of chicken and mammalian adiponectin protein sequences (17). Therefore, one of our objectives was to develop appropriate reagents and characterize chicken adiponectin protein under native conditions using nonreducing and non-heat-denaturing PAGE and mass spectrometry to determine posttranslational modifications. Additionally, we sought to develop an enzyme immunoassay (EIA) to quantify plasma adiponectin levels in the chicken and determine whether plasma adiponectin levels would be influenced by an increase in visceral adiposity in chickens between 4 and 8 wk of age or by fasting. Based on human and rodent studies, we hypothesized that the chicken adiponectin protein would occur as three different multimeric forms in the circulation and in adipose tissue protein extracts. Refuting our hypothesis, however, our results indicate that adiponectin found in chicken plasma, adipose tissue, skeletal muscle, and liver is predominantly a multimeric HMW isoform with little to none of the other isoforms. Similar to human adiponectin, however, the chicken adiponectin collagenous domain contained several hydroxyproline and glucosylgalactosyl hydroxylysine residues that are critical for biosynthesis of the HMW adiponectin multimer. We also provide evidence that plasma adiponectin levels in chickens are influenced by visceral adiposity or age in the chicken.

Materials and Methods

Animals

Commercial strain male broiler (Cobb) or leghorn (Hy-Line W36) chickens (1–8 wk old) were maintained at the Poultry Education and Research Center (The Pennsylvania State University, University Park, PA). The chickens were provided with 16-h light, 8-h dark photoperiod and were provided with water and feed *ad libitum* unless indicated otherwise. All animal procedures were carried out in accordance with the Institutional Animal Care and Use Committee approved protocol.

Western blot analysis of adiponectin under native conditions

A polyclonal antiserum was developed in rabbits against a keyhole limpet hemocyanin conjugated synthetic peptide (EMADQADQSDPK-MSC) in the N-terminal hypervariable domain of chicken adiponectin (GenBank accession no. AAX40986). Adiponectin antiserum was affinity purified using chicken adiponectin peptide and used for characterization of the chicken adiponectin protein. Protein extracts of chicken abdominal fat pad, liver, and pectoral muscle were prepared as described previously (20). Blood samples were collected from 4- and 8-wk-old male leghorn or broiler chickens into EDTA-coated syringes. Aliquots of adipose, liver and skeletal muscle protein extracts, and plasma were stored at −80 C. One-dimensional PAGE was performed with the NativePAGE Novex minigel system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Samples were prepared by adding NativePAGE buffer and NativePAGE G-250 additive to approximately 0.5 μl chicken plasma, 20 μg each of adipose tissue and skeletal muscle, or 40 μg liver protein extracts. NativeMark protein standard (Invitrogen) was used to provide molecular mass determination between 20 and 1236 kDa. Proteins were separated on a NativePAGE 4–16% Bis-Tris polyacrylamide gel (Invitrogen) under nonreducing and non-heat-denaturing conditions and electrotransferred onto Immun-Blot polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). Membranes were incubated in SuperBlock T20 blocking buffer (Pierce, Rockford, IL) for 1 h at room temperature and then with rabbit antichicken adiponectin antibody (1:40,000) or affinity purified antichicken adiponectin IgG (0.1 μg/ml) at 4 C for 18 h. After several washes, the membranes were incubated with horseradish peroxidase conjugated to goat antirabbit IgG (Pierce; 0.08 μg/ml) for 1 h at room temperature. The membranes were then incubated with ECLPlus chemiluminescence detection reagent (GE Healthcare, Piscataway, NJ) and scanned using the Storm 860 optical scanner (Amersham Biosciences, Piscataway, NJ). To determine the specificity of the antichicken adiponectin antibody, the primary antibody was preadsorbed with chicken adiponectin peptide and centrifuged at 50,000 × *g*. The supernatant was used in place of antichicken adiponectin antibody for Western blot analysis.

Gel filtration column chromatography

To confirm the molecular weight of the HMW adiponectin isoform, chicken plasma (150 μl) or adipose tissue protein extract (150 μl; 10 μg/μl) was applied to a Superose 6 column (GE Healthcare) attached to an HPLC apparatus (Shimadzu Scientific Instruments Inc., Columbia, MD) connected to a diode array UV detector (HP 1040M; PerkinElmer Life and Analytical Sciences, Waltham, MA) set at 280 nm. Fractionated proteins were eluted with HEPES-calcium buffer [25 mM HEPES, 150 mM NaCl, 1 mM $CaCl_2$ (pH 7.4)] at a flow rate of 0.3 ml/min for 70 min. Each 0.3-ml fraction was subjected to dot-blot analysis, and fractions 21–58 were analyzed for the presence of adiponectin by nonreducing and nonheat denaturing PAGE and Western blot analysis as described above. Before separating plasma or adipose protein extract, molecular mass standards ranging from 669,000 to 12,327 Da (GE Healthcare, Sigma-Aldrich, St. Louis, MO) were separated on the Superose 6 column (GE Healthcare) under identical conditions. Elution volumes were recorded for each protein standard. The void volume (Vo) of the column was determined by using blue dextran 2000 (GE Healthcare). A standard curve (log molecular weight *vs.* elution volume/Vo) was generated to calculate the molecular weights of adiponectin isoforms.

Western blot analysis of chicken adiponectin under reducing conditions

To determine the behavior of chicken adiponectin in the presence of reducing agent and exposure to heat, a Western blot analysis was done using

the NuPAGE Novex minigel system (Invitrogen) as previously described (20). Chicken plasma (0.5 μl) and adipose tissue protein extract (20 μg) were subjected to heat (70 or 100 C) or no heat (ambient temperature) in the presence or absence of NuPAGE reducing agent. Proteins were separated on a 10% Bis-Tris polyacrylamide gel (Invitrogen) using 3-[*N*-morholino] propanesulfonic acid buffer and electrotransferred onto PVDF membranes. Adiponectin immunoreactive bands were identified using the antichicken adiponectin antibody as described above.

Chicken adiponectin immunoprecipitation and mass spectrometry

Adiponectin was immunoprecipitated from chicken adipose tissue protein extract using ProFound coimmunoprecipitation kit (Pierce) following the manufacturer's protocol. Briefly, 100 μg of affinity purified antichicken adiponectin IgG was coupled to 50 μl of settled gel for 18 h at 4 C. Adipose tissue protein extract (13 mg) was incubated with the antibody-coupled gel for 18 h at 4 C. After several washes, the immunoprecipitate complex was eluted five times each with 50 μl glycine buffer (pH 2.8) supplied with the kit. Eluted fractions were neutralized by 2.5 μl 1 M Tris (pH 9.5) placed at the bottom of each collection tube. To avoid harsh acidic conditions while eluting, a separate immunoprecipitation was conducted as above but adiponectin was eluted with 3 M sodium chloride (pH 7.2). Eluted fractions were dialyzed against 1× PBS (pH 7.4) and stored at 4 C until analyzed by Western blot as described above.

Mass spectrometric analysis of chicken adiponectin immunoprecipitated from adipose tissue protein extracts was conducted at the Protein Sciences Facility, Carver Biotechnology Center, University of Illinois (Urbana, IL) using a Waters Q-ToF API-US mass spectrometer integrated with a Waters nanoACQUITY ultraperformance liquid chromatography (UPLC) system (Waters Corp., Milford, MA). Chicken adiponectin was digested with trypsin, quenched with 50% acetonitrile/5% formic acid, and dried using a Speedvac (ThermoFisher Scientific, Waltham, MA). The trypsin-digested peptides (2 μg) were subjected to UPLC using an Atlantis dC18 nanoACUITY column (3 μm beads, 75 μm inner diameter × 150 mm length; Waters). A gradient of 0–60% acetonitrile in 0.1% formic acid was applied over 60 min at a flow rate of 0.25 μl/min. Peptide mixtures separated by UPLC were infused directly into the mass spectrometer via a nanoelectrospray ion source (Waters). The resultant peptide mass data were filtered, deisotoped, and further analyzed by MASCOT software (Matrix Science, Boston, MA). Posttranslational modifications in peptide fragments were detected using tandem mass spectrometry (MS/MS) ion analysis for the addition of hydroxyl, galactosyl, and glucosyl-galactosyl hydroxyl moieties in lysine or proline residues with mass difference of 15.99, 178.05, and 340.28 Da, respectively.

Development of a chicken adiponectin competitive EIA

A competitive EIA was developed, validated, and used to quantify adiponectin levels in chicken plasma. Plastic 96-well plates coated with goat antirabbit IgG (Pierce) were first washed three times with 25 mM Tris, 150 mM sodium chloride, and 10% Tween 20 (TBST; pH 7.2). Adiponectin peptide standards (0–20,000 ng/ml; > 95% pure; Sigma-Genosys, Woodlands, TX) were made in StartingBlock dilution buffer (Pierce) and plated in triplicate. After plating unknown plasma samples, affinity purified antichicken adiponectin IgG (0.1 μg/ml) was added to each well. Biotinylated adiponectin peptide (>95% pure; Sigma-Genosys; 200 ng/ml) was added to each well except the blank. The plate was incubated for 2 h at room temperature while shaking at 300–400 rpm. The wells were washed six times with TBST, and streptavidin-conjugated horseradish peroxidase (GE Healthcare; 1 μg/ml) was then added to each well. The plate was incubated for 1 h at room temperature while shaking. Each well was then washed six times with TBST, and 3,3′,5,5′-tetramethylbenzidine substrate solution (Pierce) was added and incubated for 30 min while shaking at room temperature. Absorbance was read at 450 nm using a Victor^3 1420 multilabel plate reader (PerkinElmer). Preliminary studies were conducted to determine the appropriate conditions for the EIA such as levels of primary antibody, biotinylated peptide, secondary antibody, and chromogen as well as length of incubation. To determine parallelism within the assay, plasma samples from 4- and 8-wk-old broiler chickens were pooled (n = 4/age group), and serial dilutions were made to represent 10, 20, 40, 60, 80, and 100% pooled plasma samples. To determine whether chicken plasma interfered with the sensitivity of the assay, pooled plasma samples were spiked with a known amount of chicken adiponectin peptide to yield a final expected adiponectin concentration within the linear portion of the standard curve. All samples were assayed in triplicate to establish parallelism and any possible binding interference. To determine specificity of immunodetection of chicken adiponectin, 5 μg of eukaryotically expressed recombinant human adiponectin (R&D Systems, Minneapolis, MN) or prokaryotically expressed human adiponectin (GenWay Biotech, San Diego, CA) were included in the chicken adiponectin EIA.

Blood samples were collected from 9-wk-old male broiler chickens that were either fed *ad libitum* or fasted for 48 h (n = 6 chickens/treatment) with water being provided throughout treatment or from *ad libitum*-fed 4- or 8-wk-old broiler chickens. Samples were taken from the wing vein into EDTA-coated syringes and plasma was separated by centrifugation at 1500 × *g* for 15 min at 4 C and stored in aliquots at −80 C. Blood glucose levels were determined at the time of blood collection as described previously (20). The entire abdominal fat pad, including fat under the gizzard, was excised, weighed, and expressed as a proportion to the body weight.

Statistical analysis

Data were analyzed using Student's *t* test using Statistical Analysis Software (SAS Institute, Cary, NC). A probability level of $P \leq 0.05$ was considered statistically significant. All data are represented as mean ± SE.

Results

Detection of adiponectin in chicken plasma and tissues under native conditions

To detect chicken adiponectin oligomeric and multimeric isoforms in chicken plasma and primary metabolic tissues (adipose, liver, and skeletal muscle), a Western blot analysis under nonreducing and non-heat-denaturing conditions was performed. A strong immunoreactive band with a molecular mass of approximately 720 kDa was detected in broiler chicken plasma, adipose tissue, and skeletal muscle, representing the HMW adiponectin isoform (Fig. 1A). The same HMW adiponectin isoform appeared as a less intense band in the liver protein extract despite loading twice as much protein (40 μg) as skeletal muscle or adipose tissue. Adiponectin detected in skeletal muscle and liver is most likely a result of local production as we and others have reported expression of adiponectin mRNA or protein in a variety of chicken tissues (17, 19, 21, 22). Nevertheless, residual blood may have contributed to the detection of adiponectin in liver and skeletal muscle. Preadsorption of the antichicken adiponectin antibody with the chicken adiponectin peptide completely abolished immunostaining (Fig. 1A). Nonreducing and nonheat denaturing Western blot analysis of plasma collected from two 4- and two 8-wk-old male leghorn and broiler chickens revealed the presence of a major HMW adiponectin isoform of approximately 720 kDa size (Fig. 1B). Additionally, traces of an oligomeric adiponectin isoform at approximately 242 kDa was noticed in leghorn chicken or 4-wk-old broiler chicken (Fig. 1B).

Gel filtration chromatography

Separation of chicken plasma and adipose tissue protein extract using gel filtration and Western blot analysis of the fractions

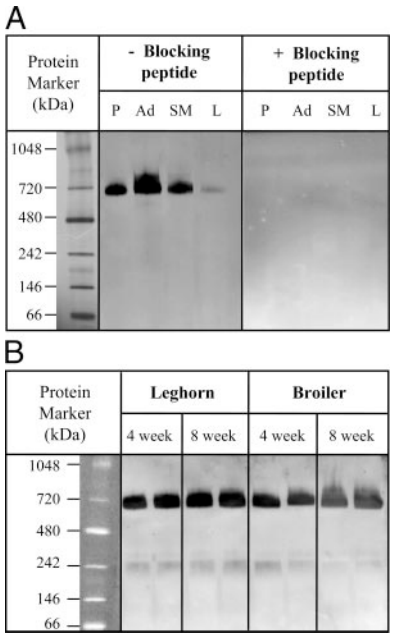


FIG. 1. A and B, Nonreducing and non-heat-denaturing Western blot analysis of chicken adiponectin under native conditions. A, Male broiler chicken plasma (0.5 μl) and protein extracts of adipose tissue (Ad; 20 μg), skeletal muscle (SM; 20 μg), or liver (L; 40 μg) were separated by electrophoresis under native conditions (*see Materials and Methods*). Adiponectin was detected using antichicken adiponectin antibody that was either preadsorbed with chicken adiponectin peptide (+ blocking peptide) or not preadsorbed (− blocking peptide). B, Plasma (0.5 μl) from two chickens belonging to two genetic lines (leghorns, broiler) at two different ages (4 and 8 wk old) were separated under nonreducing and non-heat-denaturing conditions and adiponectin was detected as described in A. A protein molecular weight standard was included in the electrophoresis to identify the molecular weight of adiponectin.

revealed that adiponectin in the chicken is predominantly a HMW isoform of masses of 669 kDa or greater (Fig. 2A). In addition to the HMW isoform, chicken plasma contained traces of an additional adiponectin multimer of approximately 330 kDa mass (Fig. 2A) as described in Fig. 1B, based on the molecular weight calibration generated using protein standards (Fig. 2B). Adipose tissue protein extract exhibited aggregates of adiponectin that eluted in the void volume of the column (Fig. 2A), possibly due to the high protein concentration of adipose tissue protein extract applied to the gel filtration column.

Effect of reducing agent and/or heat on plasma and adipose tissue adiponectin

To determine the behavior of the HMW adiponectin isoform under reducing conditions, broiler chicken plasma and adipose tissue extracts were subjected to electrophoresis with or without reducing agent and/or heating. Omission of heat and reducing agent resulted in a major immunoreactive band that is greater than 191 kDa and is representative of the HMW isoform as well as a less intense 64-kDa oligomeric adiponectin isoform in both plasma and adipose tissue extracts (Fig. 3A). Addition of reducing agent, however, led to a complete disappearance of the HMW isoform that is reduced primarily to a 64-kDa oligomer in plasma and as a 64-kDa oligomer as well as a 30-kDa monomer in the adipose tissue extract (Fig. 3A). Dissociation of the HMW adiponectin isoform in the presence of reducing agent suggests that the HMW isoform is held by disulfide bonds, whereas the 64-kDa oligomeric complex is held by noncovalent interactions. Such noncovalent interactions among adiponectin monomers are stronger in chicken plasma compared with adipose tissue protein extract. Heating of chicken plasma to 100 C in the presence of reducing agent led to a complete dissociation of adiponectin 64-kDa oligomeric isoforms into monomeric adiponectin of approximately 30 kDa molecular mass, while heating to 70 C did not completely dissociate the noncovalent interactions, resulting in both 64-kDa oligomeric and 30-kDa monomeric isoforms. There were a small number of less intense bands that may represent partially dissociated adiponectin complexes. Figure 3B confirms the specificity of the immunoreactions as the use of preadsorbed antibody completely abolished all the immunoreactive bands.

Immunoprecipitation and mass spectrometric analysis

Using affinity purified antichicken adiponectin IgG, adiponectin was immunoprecipitated from chicken adipose tissue protein extracts. Elution of adiponectin using a glycine buffer under acidic conditions (pH 2.8) and subsequent neutralization with 1 M Tris hydrochloride (pH 9.5) on elution resulted in dissociation of the HMW adiponectin isoform into several multimers ranging in size from 720 to 146 kDa (Fig. 4). This suggests that brief exposure to acidic conditions results in irreversible dissociation of the HMW adiponectin isoform, which does not reaggregate after restoring neutral pH conditions. Elution of adiponectin using 3 M sodium chloride solution (pH 7.2) preserves the HMW adiponectin isoform, although such elution was not efficient (Fig. 4).

To characterize posttranslational modifications, tryptic digests of chicken adiponectin immunoprecipitated from adipose tissue were subjected to UPLC/MS/MS analysis. An adiponectin peptide mass fingerprint was produced that spanned 90% of the collagenous domain of chicken adiponectin (Fig. 5, A and B). The remaining 10% of the collagenous domain (DGKDGKDGQK; Fig. 5A, *boxed*) could not be detected in our analysis, possibly due to cleavage at the C-terminal end of lysine residues by trypsin, resulting in short oligopeptides of two to four amino acids. An MS/MS ion analysis as well as comparison of the theoretical mass of the peptides that spanned the collagenous domain with that of observed mass revealed several modified peptides with mass changes of +47 to +874 Da, corresponding to the addition of hydroxyl groups and/or differential glycosylation (Fig. 5B). Peptide A in Fig. 5B reveals the presence of hydroxylation of Pro42, Pro45, and Pro51 residues that corresponds to an aggregate mass difference of +47.261 Da. Four different masses of Peptide B were detectable representing differential hydroxylation of Pro69 and hydroxylation or glycosylation of Lys66, Lys75, and Lys81. Hydroxyl, galactosyl hydroxyl or glucosylgalactosyl hydroxyl groups were added to Lys66 and Lys81, whereas Lys75 was found to be variably modified with the addition of either galactosyl hydroxyl or glucosylgalactosyl hydroxyl moieties. Similarly, Peptide C revealed lysine residues (Lys99 and Lys102) that are variably glycosylated or hydroxylated, in addition to hydroxylation of Pro93. Trypsin cleaves the C-terminal peptide bond of lysine but not that of glucosylgalactosyl hydroxylysine (23). The lysine residues in Peptides B and C were not cleaved by trypsin digestion, which confirms the pres-

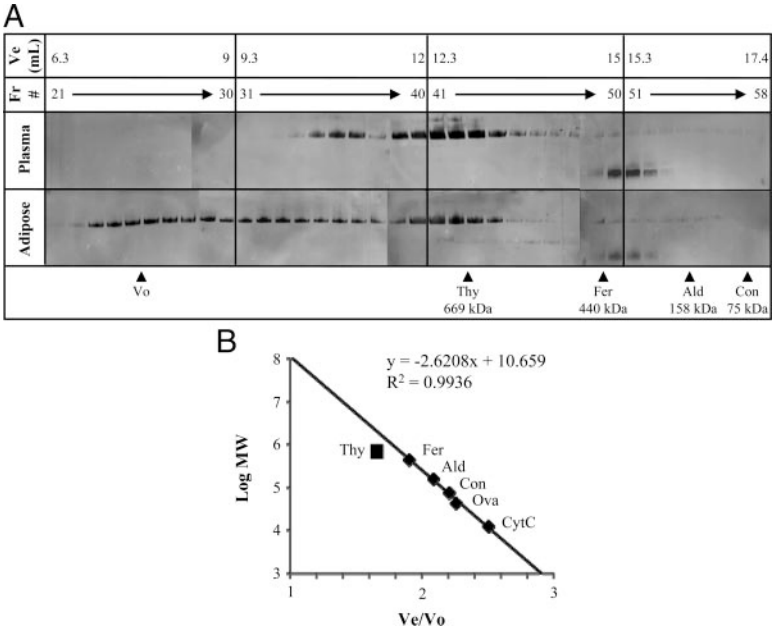


FIG. 2. A and B, Gel filtration analysis of adiponectin in chicken plasma and adipose tissue protein extract. Chicken plasma or adipose tissue protein extracts (A) and molecular weight standards (B) were separated on gel filtration column and the resultant fractions (Fr#) of plasma and adipose tissue protein were subjected to nonreducing and non-heat-denaturing PAGE and Western blot analysis to detect adiponectin. *Arrows* in A indicate the fractions that contained Vo or elution of protein molecular weight standards. Thy, thyroglobulin; Fer, ferritin; Ald, aldolase; Con, conalbumin; Ova, ovalbumin; CytC, cytochrome C; Ve, elution volume; MW, molecular weight.

ence of glycosylation of hydroxylysine residues. The lysine and proline residues in Peptide E or F (Fig. 5B) derived from the globular domain did not appear to be modified. Alignment and comparison of the adiponectin protein sequence revealed all of the five conserved hydroxyproline residues are in the Yaa position of the Gly-Xaa-Yaa tripeptide repeats, whereas two additional nonconserved hydroxyproline found only in human adiponectin are in the Xaa position (Fig. 6). Similarly, lysine residues in the collagenous domain of chicken and human adiponectin showed considerable variation. A greater number of lysine residues were found in the chicken adiponectin collagenous domain than in the collagenous domain of human adiponectin (eight *vs.* four). In particular, Thr83 and Pro104 in human adiponectin are replaced by Lys81 and Lys102 and are modified as glucosyl galactosyl hydroxylysine (Lys81 and Lys102) or hydroxylysine (Lys102) in chicken adiponectin.

Development and validation of chicken-specific adiponectin enzyme immunoassay

To quantify adiponectin in chicken plasma, we developed and validated an EIA using affinity-purified antichicken adiponectin IgG. Figure 7A depicts the standard absorbance curve generated using a range of adiponectin peptide concentrations (50–20,000 ng/ml). To measure parallelism, serial dilutions of pooled broiler plasma were assayed and yielded a linear relationship with an R^2 value of 0.9733 ($P < 0.01$; Fig. 7B). In addition, supplemental adiponectin peptide was recovered (90.9–96.6%) from chicken plasma that was spiked with a known amount of chicken adiponectin peptide (Table 1), thereby indicating that chicken plasma did not interfere with the sensitivity of the assay. Interassay coefficient of variation, as determined by quantifying adiponectin in broiler chicken plasma (pooled from eight animals) in every plate, was found to be 4.33%, whereas intraassay coefficient of variation was determined to be 4.48%. All standards and samples were run in duplicates or triplicates that consistently yielded an interreplicate coefficient of variation of less than 1%. In our chicken adiponectin EIA, human adiponectin levels remained below the detection range and such levels were not different from the blank wells.

Effect of fasting, age, and/or abdominal fat pad size on plasma adiponectin levels

Plasma adiponectin levels were not significantly different in response to fasting for 48 h relative to the *ad libitum*-fed chickens

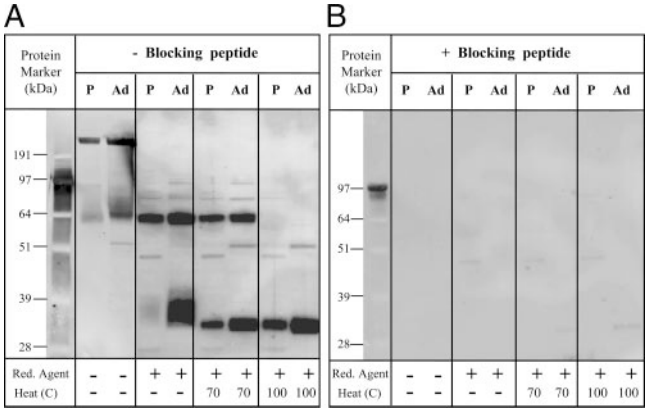


FIG. 3. A and B, Western blot analysis of adiponectin under reducing and denaturing conditions. Chicken plasma (P; 0.5 μl) and adipose tissue protein extracts (Ad; 20 μg) were treated with (+) or without (−) reducing agent, heat denatured (70 or 100 C) or not heat denatured (−), separated by electrophoresis, and blotted onto PVDF membrane. Adiponectin was detected by immunostaining using a rabbit antichicken adiponectin antibody (A; − blocking peptide). Specificity of the adiponectin immunostaining was determined by preadsorption of the antichicken adiponectin antibody with chicken adiponectin peptide (B; + Blocking peptide).

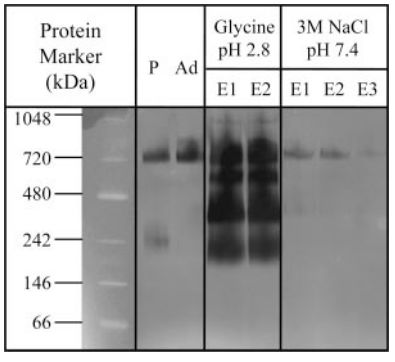
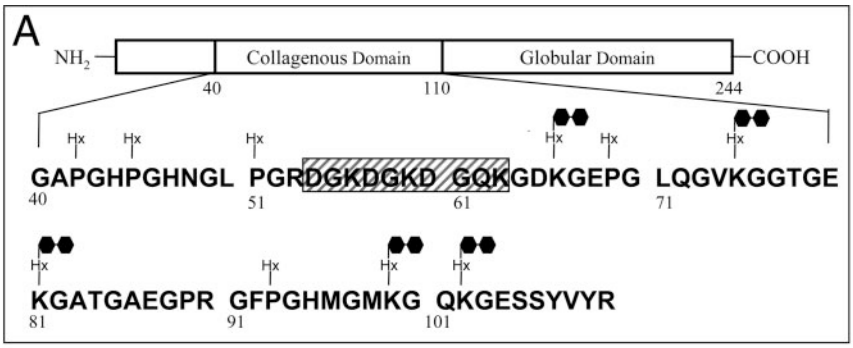


FIG. 4. Effect of acidic or neutral pH on the chicken adiponectin heavy molecular weight isoform. Adiponectin was immunoprecipitated from the chicken adipose tissue protein extracts and subjected to elution with a glycine buffer (pH 2.8) or 3 M sodium chloride solution (pH 7.2) (NaCl). The eluted adiponectin fractions (E1, E2, and E3) were separated by electrophoresis under nonreducing and non-heat-denaturing native conditions as described in Fig. 1 legend. Leghorn chicken plasma (P; 0.5 μl) and protein extracts of adipose tissue (Ad; 20 μg) were also included for comparison. Adiponectin was detected using antichicken adiponectin antibody.



B

Peptide	Position	Fragment Sequence	Observed Mass (Da)	Theoretical Mass (Da)	Mass Difference (ΔDa)
A	32-53	MSCANWMGGAPGHP GHNGLPGR	2250.2233	2202.9622	**+47.2611** +3 OH (P42; P45; P51)
B	64-90	GDKGEPGLQGVKGGT GEKGATGAEGPR	3043.3953	2509.2520	**+532.3953** + 1 OH (K66 or K81) +1 Glu-Gal-O (K75) +1 Gal-O (K66 or K81)
	64-90	GDKGEPGLQGVKGGT GEKGATGAEGPR	3059.1196	2509.2520	**+549.8676** + 1 OH (P69) +3 Gal-O (K66; K75; K81)
	64-90	GDKGEPGLQGVKGGT GEKGATGAEGPR	3367.2051	2509.2520	**+857.9531** +2 Glu-Gal-O (K66; K81) +1 Gal-O (K75)
	64-90	GDKGEPGLQGVKGGT GEKGATGAEGPR	3383.1961	2509.2520	**+873.9441** + 1 OH (P69) +2 Glu-Gal-O (K66 or K81; K75) +1 Gal-O (K66 or K81)
C	91-110	GFPGHMGMKGQKGE SSYVYR	2586.8673	2215.0302	**+371.8371** +1 Glu-Gal-O (K99) +2 OH (P93; K102)
	91-110	GFPGHMGMKGQKGE SSYVYR	2910.9454	2215.0302	**+695.9152** +2 Glu-Gal-O (K99; K102) +1 OH (P93)
D	111-120	SAFSVGLTER	1065.4266	1065.5454	**-0.1188**
E	121-132	APHPNVPIRFTK	1375.6426	1375.7724	**-0.1298**
F	133-147	IFYNEQNHYDSSTGK	1801.6168	1801.7907	**-0.1739**

FIG. 5. A and B, Mass spectrometric analysis of chicken adiponectin. A, Schematic of the collagenous domain of chicken adiponectin and the amino acid sequence of collagenous domain showing hydroxylation (Hx) of proline residues (42, 45, 51, 69, and 93) and glycosylation of hydroxylysine residues (66, 75, 81, 99, and 102) derived from MS/MS analysis. A short peptide sequence DGKDGKDGQK (*boxed*) deduced from the chicken adiponectin cDNA sequence [GenBank accession no. NM_206991; (17)] within the collagenous domain could not be detected in the liquid chromatography/MS/MS analysis. B, Identification of peptide fragments and posttranslational modifications of amino acids in the chicken adiponectin protein by peptide mass fingerprinting and MS/MS ion analysis. Peptide A demonstrates hydroxylation of three proline residues (42, 45, and 51) that account for a difference of 47.2611Da representing three hydroxyl groups. Peptide B was found in four different masses each representing various combinations of hydroxylation of proline residue (69) as well as hydroxylation and/or glycosylation of lysine residues (66,75, and 81). Peptide C was found in two different masses with various combinations of hydroxylation (P93 and K102) and/or glycosylation (K99 and K102). Peptides D–F, Fragments from the N-terminal end of the globular domain of chicken adiponectin wherein the proline and lysine residues were not hydroxylated or glycosylated. Amino acids that are *underlined* in Peptides A-C denote modified residues.

($P > 0.05$; Fig. 8A). Plasma collected from 8-wk-old chickens contained significantly lower levels of adiponectin when compared with that of 4-wk-old chicken plasma ($P < 0.05$, Fig. 8B), at a time when abdominal fat pad mass relative to body weight was significantly increased (0.35 *vs.* 0.55%; $P < 0.05$). The blood glucose levels were not significantly different ($P > 0.05$) between these two age groups.

Discussion

This is the first report on the characterization of adiponectin protein in any species other than mammals. Using gel filtration chromatography and Western blot analysis, we show that chicken adiponectin expressed in adipose tissue or in plasma, unlike mammalian adiponectin, is predominantly a multimeric HMW isoform that is larger than 669 kDa mass. In contrast, human and mouse adiponectin from serum or adipocytes exists as three different molecular mass species (67, 136, and >300 kDa) termed LMW, MMW, and HMW isoforms, respectively (8). Using velocity sedimentation and gel filtration, the human HMW adiponectin multimeric isoform is found to be much larger than 669 kDa (24). A preponderance of HMW adiponectin isoforms in chicken plasma and tissues, without significant amounts of LMW and MMW oligomeric forms is intriguing. The mechanism(s) that aids in the assembly of this single, major HMW multimeric isoforms in the chicken is currently unknown. However, the amino acid sequence as well as posttranslational modifications of chicken adiponectin combined with physiological factor(s) found in the chicken may favor such unique multimerization.

Chicken adiponectin, similar to mammalian adiponectin, contains an N-terminal collagenous and a C-terminal globular domain (17). The collagenous domain is considered to be essential for the assembly and stability of multimeric forms of adiponectin larger than trimers (7). Based on our mass spectrometric analysis of chicken adiponectin, the unique amino acid sequence observed in the collagenous domain of chicken adiponectin is likely to aid in multimerization. For instance, the chicken adiponectin collagenous domain contains twice as many lysine residues (eight *vs.* four) compared with human or mouse adiponectin. Mutations in some or all of the lysine residues in the collagenous domain of mouse adiponectin leads to a progressive loss of multimerization (25, 26), suggesting that a greater number of lysine residues are likely to favor multimerization. Our mass spectrometric analysis of chicken adiponectin revealed the presence of five lysine residues (three conserved and two nonconserved; Fig. 5) that carried glucosylgalactosyl hydroxyl moiety, a posttranslational modification that is likely to aid in multimerization of chicken adiponectin. In support of this hypothesis, all four lysine residues in the collagenous domain of mammalian adiponectin are modified as glu-

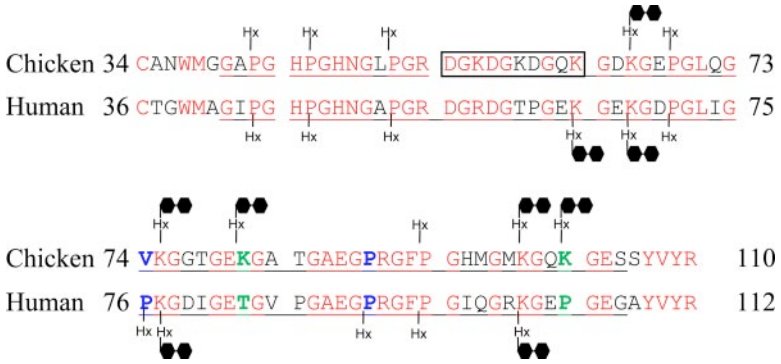


FIG. 6. A comparison of chicken and human adiponectin protein sequences emphasizing posttranslational modification to proline and lysine residues. A part of the hypervariable region and entire collagenous domain of the adiponectin protein sequence is shown. Gly-Xaa-Yaa repeats in the collagenous domain are *underlined*. Conserved amino acids are highlighted in *red letters*. Chicken adiponectin has two additional glycosylated hydroxylysines (K81 and K102; *green*) but lacks the hydroxyproline residues (V74 and P89; *blue*) compared with human adiponectin. Human adiponectin sequence and posttranslational modifications are obtained from another report (25). A short peptide sequence DGKDGKDGQK (*boxed*) deduced from the chicken adiponectin cDNA sequence [GenBank accession no. NM_206991 (17)] could not be detected in the liquid chromatography/MS/MS analysis. Hx, Addition of hydroxyl group.

cosylgalactosyl hydroxylysine, and such posttranslational modifications were found to be essential for improving stability of the HMW adiponectin isoform and for conferring certain biological activities (25–29). Variable glycosylation of lysine residues in the collagenous domain found in chicken adiponectin is consistent with differential glycosylation reported for human adiponectin (25).

The collagenous domain of chicken adiponectin consists of Gly-Xaa-Yaa tripeptide repeats as found in collagen. Within this tripeptide, presence of hydroxyproline in Yaa position enhances the stability of the triple helix aggregates of collagen, whereas occurrence of hydroxyproline in Xaa position has the opposite effect (30, 31). In the human adiponectin collagenous domain, two of the seven hydroxyproline residues are in Xaa position, whereas all hydroxyproline residues are in Yaa position in chicken adiponectin, a feature that is likely to favor greater stability of adiponectin multimers and partly explain the predominance of the HMW isoform in chickens.

Our results indicate that addition of reducing agent to chicken plasma without heating led to the formation of an adiponectin oligomer of approximately 64 kDa size, suggesting that disulfide bonds are necessary to hold chicken adiponectin in its HMW isoform. This observation is consistent with human adiponectin, as the presence of a reducing agent without heat dissociates the human HMW adiponectin isoform into a 67-kDa oligomer in SDS-PAGE (8). The 67-kDa human adiponectin oligomer was deduced to represent a trimer using a variety of experimental techniques to determine electromobility and migration of human serum adiponectin (8). The two cysteine residues in the collagenous and globular domains of chicken (Cys34 and Cys150), human (Cys36 and Cys152), and mouse (Cys39 and Cys155) adiponectin are highly conserved. Mutation of Cys155 in mouse adiponectin did not impair multimerization or formation of hexameric and HMW multimeric isoforms (7), whereas mutation of Cys39 results in a HMW isoform that is unstable under acidic conditions (24). Whereas it is not clearly understood how the oligomers of chicken adiponectin are assembled into multimers and held stably in the circulation as a predominant HMW isoform, noncovalent interactions and a heat-labile plasma-borne substance are likely to be involved in the stability of oligomeric forms of chicken adiponectin. This hypothesis is based on our results that chicken adiponectin in plasma remained largely as a 64-kDa oligomer without further dissociation under reducing conditions, whereas adipose tissue yielded both a 64-kDa oligomer and a 30-kDa monomer.

In addition to the Cys36 residue in human adiponectin protein, substitution of other amino acids, as occurring in natural mutations of the adiponectin gene (32–35), can result in impaired multimerization and/or secretion of adiponectin in humans, leading to hypoadiponectinemia and diabetes (8). Site-directed mutagenesis of Arg112 into Cys, Tyr159 into Asp, and Ile164 into Thr in mouse adiponectin leads to a complete lack of multimerization, and furthermore, the monomeric adiponectin was not secreted into the culture medium (8). Whereas Arg112 and Tyr159 are conserved in chicken adiponectin, the hydro-

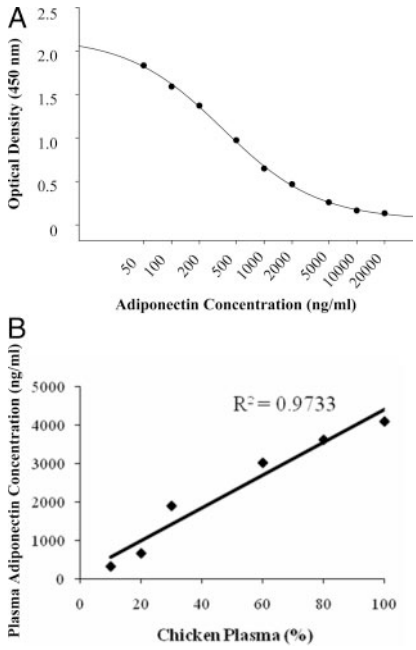


FIG. 7. A and B, Validation of a competitive EIA for quantifying plasma adiponectin levels in chickens. A, Representative absorbance curve at 450 nm obtained from triplicate wells containing a range of chicken adiponectin peptide between 50 and 20,000 ng/ml. B, Parallelism was determined by serial dilutions of pooled chicken plasma in EIA assay buffer to obtain 100, 80, 60, 40, 20, and 10% plasma. Each sample was assayed in triplicate for determining adiponectin levels and a linear regression applied. *Data points* represent mean values obtained.

TABLE 1. Determination of binding interference in chicken adiponectin EIA

Adiponectin added (ng)	Expected recovery (ng/ml)	Actual recovery (ng/ml)	Recovery (%)
200	7083.78	6840.72	96.6
400	11083.78	10079.34	90.9
600	15083.78	13963.78	92.6

Chicken plasma was spiked with known amount of adiponectin and total adiponectin levels (basal + spiked) were determined by immunoassay.

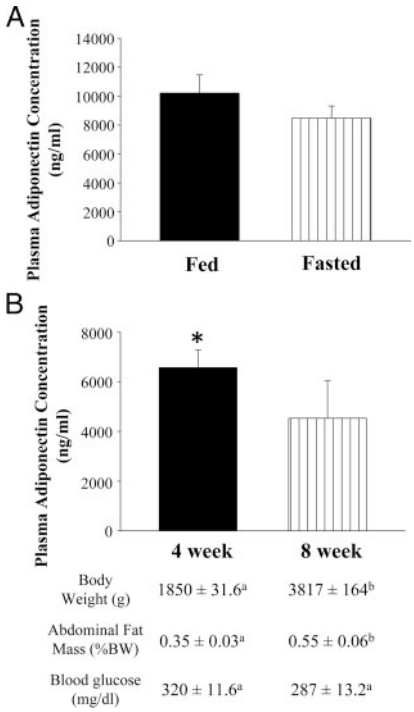


FIG. 8. A, Plasma adiponectin levels in chickens that were fed or fasted for 48 h. B, Relationship among plasma adiponectin levels, age, abdominal fat pad mass, and blood glucose levels in the broiler chicken. Plasma adiponectin levels in 4- and 8-wk-old chickens were determined by EIA. Data are represented as mean ± SEM (n = 6). *, $P < 0.05$; a, b, $P < 0.05$.

phobic Ile164 is replaced by another hydrophobic amino acid, leucine. All of these amino acids may together play a major role in the formation of trimeric forms of adiponectin that multimerize into a HMW isoform, perhaps through disulfide bonding and involvement of the Gly-Xaa-Yaa tripeptide repeats found in the collagenous domain. This seems plausible as mutations in Gly84 and Gly90 within the collagenous domain results in a complete lack of the multimeric HMW adiponectin isoform synthesis (8). Our current mass spectrometric data as well as our previous report (17) suggest that the Gly-Xaa-Yaa tripeptide repeats in the adiponectin collagenous domain are conserved.

The oligomeric LMW and MMW isoforms are suggested to act as chaperones to increase the solubility of the HMW complex because mouse HMW adiponectin isoform becomes insoluble once it is eluted from gel filtration columns (24). Absence of significant amounts of MMW or LMW adiponectin isoforms in chickens offers an unique model to study the physiochemical properties of the HMW adiponectin isoform. Our results indicate that chicken HMW adiponectin isoform is dissociated into relatively smaller multimeric forms when exposed to acidic conditions. This is consistent with previous reports that the HMW isoform of recombinant mouse adiponectin collapses to hexameric or lower molecular weight (≤220 kDa) isoforms under acidic conditions (36). Similar observations have been reported for the dissociation of HMW adiponectin isoform purified from human plasma (37) or for mouse HMW adiponectin isoform (24).

In addition to the unique amino acid sequence within the adiponectin protein, other environmental factors such as high levels of blood glucose in chickens could likely affect multimerization. Chickens are hyperglycemic relative to humans and their high blood glucose levels, often exceeding 300 mg/dl (16.7 mM), could favor the formation of HMW adiponectin isoform. In support of this hypothesis, a recent report suggests that glucose is likely to favor multimerization of adiponectin, at least in glucose-responsive tissues (25). Subcutaneous human adipose tissue explants cultured in 15 mM glucose (compared with 5 mM) from three of 11 human subjects resulted in an increase in total adiponectin secreted as well as a greater proportion of HMW adiponectin in the media (25). Furthermore, a different migration pattern of adiponectin isoforms was reported in two-dimensional gel electrophoresis in response to 15 mM glucose, indicating that environmental factors such as glucose concentration could result in differential posttranslational modification of adiponectin (25). Insulin treatment of male mice results in a selective decrease in HMW adiponectin isoform, an effect that was counteracted by glucose replacement (36). In addition to high blood glucose levels, chickens are insulin resistant (15), which may synergistically favor the formation of the HMW adiponectin isoform as well.

This is the first report on quantifying circulating levels of adiponectin in any nonmammalian species. The adiponectin EIA that we developed revealed that adiponectin levels are in the range of 4–10 μg/ml in chicken plasma. Such high levels of adiponectin (1.9–17 μg/ml) have similarly been reported in human serum (38). In the present study, fasting did not affect plasma adiponectin levels in chickens, as reported in humans (39), suggesting that circulating adiponectin levels are resistant to metabolic changes occurring in response to fasting. Our results, however, indicate that 8-wk-old chickens had significantly lower plasma adiponectin levels at a time when their body weight and abdominal fat pad mass increased by 2- and 1.5-fold, respectively, compared with 4-wk-old chickens. In support of our data, plasma adiponectin levels are significantly lower in obese human subjects (38). In addition to increased abdominal fat pad mass, changes associated with age or rapid growth may have also led to the decline of circulating adiponectin levels.

Collectively, our results indicate that adiponectin in chicken plasma and tissues is predominantly a HMW isoform, suggesting the presence of unique multimerization and stabilization mechanisms in the chicken that favors preponderance of HMW adiponectin isoform over other oligomers. Further studies are required to determine the relative contribution of the amino acid sequence of chicken adiponectin, posttranslational modification of adiponectin, and environmental factors that lead to the secretion of HMW adiponectin isoform.

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