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Is Visfatin an Adipokine or Myokine? Evidence for Greater Visfatin Expression in Skeletal Muscle than Visceral Fat in Chickens

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Visfatin, an adipokine hormone produced primarily by visceral adipose tissue in mammals, has been implicated in the immune system, cellular aging, and glucose metabolism. Increased visceral adiposity and hyperglycemia have been correlated with elevated plasma visfatin levels in humans. The present study investigated visfatin cDNA and protein expression as well as plasma visfatin levels in chickens that are selected for rapid growth and are naturally hyperglycemic relative to mammals. By RT-PCR, we detected visfatin cDNA in multiple tissues in the chicken. The deduced amino acid sequence of full-length chicken visfatin was 92–93% homologous to mammalian visfatin. Using real-time quantitative PCR and Western blotting, chicken skeletal muscle was found to contain 5- and 3-fold greater quantities of visfatin mRNA and protein than abdominal fat pad, respectively. Visfatin mRNA and protein quantities were not significantly different among sc and visceral adipose tissue depots. Skeletal muscle visfatin mRNA and protein quantities as well as plasma visfatin levels determined by enzyme immunoassay were significantly higher in 8-wk-old compared with 4-wk-old chickens, possibly due to rapid skeletal muscle growth and visceral fat accretion occurring in broiler chickens during this period. However, fasting and refeeding did not affect plasma visfatin levels in the chicken. Collectively, our results provide novel evidence that skeletal muscle, not the visceral adipose tissue, is the primary source of visfatin in chickens, thereby raising the possibility that visfatin may be acting as a myokine affecting skeletal muscle growth and metabolism. (*Endocrinology* 149: 1543–1550, 2008)

THE GROWING OBESITY epidemic has sparked numerous studies on the identification of hormones secreted from adipose tissue and their influence on energy metabolism. Recently, visfatin has been identified as an adipokine hormone that is associated with obesity (1, 2), type II diabetes (3), and rheumatoid arthritis (4). Previously known as pre-B cell colony-enhancing factor (PBEF) and nicotinamide phosphoribosyltransferase (Nampt), visfatin was found to be a cytokine-like growth factor for B lymphocytes (5) as well as a mediator in the salvage pathway of nicotinamide adenine dinucleotide (6, 7). Since its original discovery, visfatin has been identified in multiple tissues of humans (5), dogs (8), laboratory rodents (9), and pigs (10). Visfatin was found to induce tyrosine phosphorylation of insulin receptors and increase glucose uptake in cultured human osteoblasts (11). In addition, glucose has been found to stimulate visfatin release from cultured adipocytes and to increase circulating plasma visfatin levels in healthy adult men (12). Furthermore, human subjects with type II diabetes mellitus, a condition characterized by insulin resistance and chronic hyperglycemia, exhibit elevated plasma visfatin levels (3). Similarly, circulating visfatin levels were found to be elevated in patients with type I diabetes (13). However, the exact role of visfatin in glucose metabolism has yet to be elucidated.

Chickens are naturally hyperglycemic compared with mammals, with their blood glucose levels averaging three times that found in humans (300 *vs.* 100 mg/dl). Genetic selection and diet optimization driven by economic demands have resulted in broiler chickens that are approximately four times heavier than those raised five decades ago (14). To achieve greater body weight and muscle yield, broiler chickens are selected for rapid growth to occur from hatch until market weight in approximately 42 d. During this period, broiler chickens voraciously eat approximately 4.1 kg of feed to achieve a 40-fold increase in body weight arising from increases in skeletal muscle mass (15), primarily in the breast (pectoralis) muscle, as well as in visceral adipose tissue (16). In addition, chickens are considered to be insulin resistant (17), requiring insulin doses greater than four times that required in mammals to achieve hypoglycemia (18). Since visceral adiposity and higher blood glucose levels have been shown to influence visfatin expression in mammals, studies on chicken visfatin may provide critical information on the physiological role of visfatin in glucose and lipid metabolism. In addition, visfatin in chicken adipose tissue and liver would provide unique information on the role of visfatin in lipogenesis and lipid storage because the liver, not adipose tissue, is the primary site of lipid synthesis in chickens (19). However, there are no reports currently available on chicken visfatin.

The objectives of the present study were to clone visfatin

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Abbreviations: C_T, Cycle threshold; DNase, deoxyribonuclease; EIA, enzyme immunoassay; M-MuLV, Moloney murine leukemia virus; Nampt, nicotinamide phosphoribosyltransferase; PVDF, polyvinylidene difluoride.

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mRNA and characterize visfatin gene and protein expressions in the primary metabolic tissues (adipose, liver, and skeletal muscle) as well as to evaluate changes in visfatin expression with age in the broiler chicken. Furthermore, we sought to validate a human visfatin enzyme immunoassay (EIA) to quantify plasma visfatin levels in the chicken and determine whether plasma visfatin levels would be influenced by the dramatic increase in body weight in chickens that occurs between 4 and 8 wk of age. Based on human and rodent studies, we hypothesized that visceral fat would be the primary source of visfatin in the chicken and that plasma visfatin levels would become elevated with increased body weight. We provide novel evidence that visfatin expression in the chicken is severalfold greater in skeletal muscle than visceral adipose tissue, and such expression is further elevated with age. Furthermore, our data suggest that plasma visfatin levels are also increased with age in chickens, whereas fasting did not affect plasma visfatin levels.

Materials and Methods

Antibodies and reagents

Trizol and RNeasy kits used to isolate total RNA were obtained from Invitrogen (Carlsbad, CA) and QIAGEN (Valencia, CA), respectively. Moloney murine leukemia virus (M-MuLV) reverse transcriptase and random primers (RP12) used for the RT reactions were purchased from New England Biolabs (Beverly, MA). Additional PCR and RT materials (SYBR GreenER qPCR SuperMix and RNAseOut) were obtained from Invitrogen, whereas dNTP mixture was purchased from Roche Applied Sciences (Indianapolis, IN). Affinity-purified antihuman visfatin antibody and human visfatin blocking peptide were purchased from Bethyl Laboratories (Montgomery, TX). Monoclonal anti-chicken α-tubulin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Visfatin C-terminal EIA kit was purchased from Phoenix Pharmaceuticals (Burlingame, CA).

Animals

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

Cloning of chicken visfatin cDNA and RT-PCR

Broiler chickens were killed by decapitation to collect diencephalon, pituitary, myocardium, kidney, spleen, liver, skeletal muscle (pectoralis), and abdominal fat pad. Total RNA was extracted from tissues using Trizol (Invitrogen) and/or the RNeasy kit. After on-column deoxyribonuclease (DNase I) (QIAGEN) treatment, first-strand cDNA was synthesized by reverse transcribing 1 μg total RNA using random primers and 2 U M-MuLV reverse transcriptase in a 20-μl reaction. Approximately 50 ng of single-stranded cDNA were used as template to amplify a 140-bp product of visfatin with the following primer sequences: forward 5′-AGTCCAGAGGCACCACTCAT-3′ and reverse: 5′-CTGAGATATGGTGGCAGCAA-3′. A PCR was performed using SYBR GreenER qPCR SuperMix and 300 nM forward and reverse primers, with the following thermocycle parameters: 50 C for 2 min (to incubate with uracil DNA glycosylase and prevent amplification of deoxyuracil-containing PCR products), 95 C for 10 min, followed by 35 cycles of 95 C for 15 sec, 55 C for 30 sec, and 72 C for 30 sec. The PCR products were subjected to agarose gel electrophoresis and ethidium bromide staining for visualization. For negative controls, RT reactions using 1 μg total RNA from each tissue with no reverse transcriptase (−RT) were used as a template in place of RT reactions that contained reverse transcriptase (+RT). In addition, the full-length visfatin cDNA sequence was amplified using skeletal muscle cDNA as a template with the following primers (GenBank accession no. AY946242): forward 5′-ATGGAGTGCGCGGCGGCGGGCGCCGAGTTCAA-3′ and reverse 5′-TTAGTGAGACGCCGTTTCTAGTTCACTGTTCTTCA-3′. The resultant 1482-bp product was sequenced (Davis Sequencing, San Diego, CA) to confirm the authenticity of chicken visfatin cDNA and for determining its homology with mammalian visfatin cDNA sequence.

Effect of age on visfatin expression

To determine the effect of age on tissue visfatin mRNA and protein expression as well as plasma visfatin levels, male chickens at 4 and 8 wk of age (n = 6) were weighed, and blood samples from the wing vein were collected into syringes coated with 10% EDTA. Broiler chickens at 4 and 8 wk of age were selected to represent two age groups that are drastically different in body weight. Blood glucose levels were measured by the glucose oxidase method using OneTouch Ultra blood glucose meter (LifeScan, Milpitas, CA). Blood samples were centrifuged at 1500 × *g* for 15 min at 4 C, and plasma was collected and stored at −80 C until further analysis by EIA. Chickens were euthanized by decapitation. Skeletal muscle (pectoralis), liver, and abdominal fat pads were then excised, frozen in liquid nitrogen, and stored at −80 C until further analysis by real-time quantitative PCR and Western blotting.

Quantification of visfatin mRNA by real-time quantitative PCR

One microgram of total RNA from abdominal fat pad, liver, or skeletal muscle was reverse transcribed using random primer 12 and 2 U M-MuLV reverse transcriptase in a 20-μl reaction. Chicken visfatin mRNA and chicken 18S mRNA were quantified using 2.5 μl of the RT reaction (equivalent to 50 ng single-stranded cDNA) as template in the real-time quantitative PCR analysis with the following primers: visfatin forward 5′-AGTCCAGAGGCACCACTCAT-3′ and reverse 5′-CTGAGATATGGTGGCAGCAA-3′ and 18S forward 5′-GTATGGTTGCAAAGCTGAAACTTA-3′ and reverse 5′-AAGAGCTCTCAATCTGTCAATCCT-3′. The real time-quantitative PCR mixture consisted of 1× SYBR GreenER qPCR SuperMix (Invitrogen) and 300 nM forward and reverse primers. The reactions were carried out in the DNA Engine Opticon II (MJ Research, Waltham, MA) with the following thermocycle parameters: 50 C for 2 min (to incubate with uracil DNA glycosylase and prevent amplification of deoxyuracil-containing PCR products), 95 C for 10 min, followed by 35 cycles of 95 C for 15 sec, 55 C for 30 sec, and 72 C for 30 sec. At the end of amplification, a melting-curve analysis was done by heating the PCR products from 65 to 95 C, held for 15 sec at increments of 0.2 C, and the fluorescence detected to confirm the presence of a single amplification product. In addition, the resultant 140-bp visfatin product was sequenced (Davis Sequencing) to confirm the authenticity of this partial visfatin cDNA. Tissue samples from each animal were run in duplicate to obtain average cycle threshold (C_T) values for visfatin mRNA and 18S mRNA. The log-linear threshold values (C_T) during the exponential phase of the PCR for visfatin mRNA were subtracted from that of 18S mRNA. Visfatin mRNA quantity was expressed as a proportion of 18S mRNA quantity following $2^{-\Delta\Delta C_T}$ method for converting log-linear C_T values to linear term (20). The relative amounts of visfatin mRNA in the various tissues were then compared.

Quantification of visfatin protein by Western blot analysis

Approximately 0.2–0.3 g of abdominal fat pad, liver, or skeletal muscle were homogenized using the Tekmar Tissumizer (Tekmar Co., Cincinnati, OH) in 2 ml lysis buffer [10 mM Tris-HCl, 150 mM NaCl (pH 8.0), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] containing protease inhibitor cocktail and phosphatase inhibitor cocktail 2 (Sigma-Aldrich). The homogenate was then passed through a 22-gauge needle and shaken in a thermomixer (Eppendorf, Westbury, NY) at 1000 rpm for 30 min at 4 C. The lysate was centrifuged at 14,000 × *g* for 15 min at 4 C, and the supernatant was collected. Total protein concentration was estimated by a protein dye-binding assay (21) using a commercial kit (Bio-Rad, Hercules, CA) with chicken ovalbumin as the standard. Aliquots of skeletal muscle, liver, and adipose protein extracts were stored at −80 C until analyzed by Western blotting.

One-dimensional gel electrophoresis was performed with the Nu-

PAGE Novex minigel system (XCell *SureLock* Mini Cell; Invitrogen) according to manufacturer's recommendations. Samples were prepared by combining approximately 20 μg total protein extract with 4× NuPAGE LDS sample buffer and 10× NuPAGE reducing agent and heating for 10 min at 70 C before electrophoresis. Proteins were separated on a 10% Bis-Tris polyacrylamide gel (Invitrogen) using MOPS running buffer under denaturing conditions and electrotransferred onto Immun-Blot polyvinylidene difluoride (PVDF) membranes (0.20 μm; Bio-Rad). Membranes were incubated in blocking buffer (1× casein solution; Vector Laboratories, Burlingame, CA) for 2 h at room temperature before incubating overnight in rabbit antihuman visfatin antibody (0.1 μg/ml in blocking solution) at 4 C with gentle agitation. To determine the specificity of the visfatin antibody, the antibody was preabsorbed with 40 μg human visfatin blocking peptide. Immunoreactive proteins were detected by incubation in horseradish peroxidase conjugated to goat antirabbit IgG (Pierce, Rockford, IL; 0.08 μg/ml in blocking solution) for 1 h at room temperature. The membrane was treated with ECLPlus Chemiluminescence Detection Reagent (Amersham Biosciences, Piscataway, NJ). Chemiluminescent signals were detected using the Storm 860 optical scanner (Amersham Biosciences), and the signals were analyzed using Image Quant TL software (Amersham Biosciences). The chemiluminescence signal intensity of each band was calculated using a local average background correction. To normalize for protein load on immunoblots comparing samples of 4- and 8-wk-old broiler chickens, membranes were reprobed using mouse anti-α-tubulin antibody (0.7 μg/ml followed by incubation in horseradish peroxidase conjugated to goat antimouse IgG (Pierce; 0.08 μg/ml), and the chemiluminescence signal was determined as described previously. Visfatin protein quantity was expressed as a proportion of α-tubulin, and relative amounts of visfatin protein in respective tissues of 4- and 8-wk-old broiler chickens were then compared.

Quantification of visfatin mRNA and protein in visceral and sc adipose tissue

To determine whether visfatin expression would differ in visceral *vs.* sc adipose tissue, the abdominal fat pad, gizzard fat, and sc fat in the neck region were collected from 8-wk-old broiler chickens (n = 6). Total RNA and protein were extracted and subjected to visfatin mRNA and protein quantification, respectively, as described above.

Quantification of plasma visfatin concentrations

Plasma visfatin concentrations were determined using a human visfatin (COOH-terminal) EIA kit (Phoenix Pharmaceuticals). To quantify chicken visfatin, the kit was first validated by determining parallelism as well as any possible interference of chicken plasma with the assay sensitivity. To determine parallelism within the assay, plasma samples from 8-wk-old broilers (n = 8) were pooled, and serial dilutions were prepared in EIA buffer to obtain 100, 80, 60, 40, 20, and 10% plasma levels. To determine whether chicken plasma interfered with assay sensitivity, pooled plasma samples were spiked with a known amount of the human visfatin standard to yield a final expected visfatin concentration within the linear portion of the standard curve. All samples were assayed in triplicate to establish parallelism and any possible binding interference.

After validation, chicken blood samples collected at 4 and 8 wk of age were analyzed to quantify plasma visfatin concentrations. All samples were run in triplicate, with sample comparisons run in the same assay to eliminate between-assay variation. The intraassay coefficient of variation was found to be 6.46%.

Effect of fasting and refeeding on broiler chicken plasma visfatin concentrations

Male broiler chickens were fed *ad libitum*, fasted for 48 h, or fasted for 48 h followed by refeeding for 24 h (n = 6 chickens per treatment), with water being provided throughout treatments. Body weights were taken after treatments. Blood was collected as previously described to determine blood glucose levels, and plasma was separated by centrifugation at 1500 × *g* for 15 min at 4 C. Plasma was collected and stored at −80 C until further analysis by EIA.

Statistical analysis

All analyses were conducted by ANOVA using Minitab Statistical Software, version 13.1 (Minitab, Inc., State College, PA). Before performing the ANOVA, normality and homogeneity of variance within the data were confirmed. Differences between individual means were partitioned further by performing Tukey's multiple comparison analysis. A probability level of $P \leq 0.05$ was considered statistically significant. For analysis of real-time quantitative PCR data, relative visfatin mRNA quantity to 18S mRNA quantity was first converted from log-linear to linear terms. All data are represented as mean ± SE.

Results

Cloning of chicken visfatin mRNA and RT-PCR

A full-length 1482-bp chicken visfatin cDNA was cloned using skeletal muscle RNA, and its sequence was compared with that of other species. We found that the skeletal muscle visfatin mRNA sequence was identical to the previously reported chicken visfatin mRNA sequence (GenBank accession no. AY946242). The chicken visfatin cDNA is 82–84% homologous to mammalian visfatin cDNA, whereas the deduced amino acid sequence is 92–93% similar to mammalian visfatin (Table 1). A much lesser degree of homology exists between the sequences of chicken and zebrafish visfatin cDNA, with nucleotide and amino acid similarities of 60 and 57%, respectively (Table 1). Using RT-PCR, a 140-bp partial visfatin cDNA corresponding to nucleotides 910-1049 (GenBank accession no. AY946242) was detected in the total RNA extracted from male broiler chicken diencephalon, pituitary, myocardium, kidney, spleen, skeletal muscle, liver, and abdominal fat pad (Fig. 1). The use of either water in place of cDNA or RNA that was not reverse transcribed did not produce any PCR product (Fig. 1), confirming the absence of genomic DNA contamination.

Detection of visfatin protein

To detect visfatin protein in the primary metabolic tissues (adipose, liver, and skeletal muscle), a Western blot analysis was performed. A rabbit antihuman visfatin antibody raised against a 51-amino-acid synthetic human visfatin peptide that is 98.1% homologous to the corresponding chicken visfatin sequence was used for immunodetection. A 52-kDa immunoreactive visfatin was observed in protein extracts from chicken abdominal fat pad, liver, and skeletal muscle (Fig. 2). Additionally, a smaller immunoreactive band, with an approximate molecular mass of 28 kDa, was detected in abdominal fat pad and liver extracts. Preabsorption of the antivisfatin antibody with the visfatin peptide greatly reduced the 52-kDa band while completely eliminating the 28-kDa band (Fig. 2). The protein sample integrity was con-

TABLE 1. Similarity of chicken visfatin to mammalian or nonmammalian visfatin

Species	GenBank accession no.	Nucleotide % identity	Amino acid % identity
Human	BC106046	83.9	93.1
Mouse	NM_021524	82.4	92.7
Rat	NM_177928	82.6	93.1
Pig	DQ020218	84.1	93.7
Cow	XM_878444	84.1	93.1
Zebrafish	NM_212668	60.2	56.8

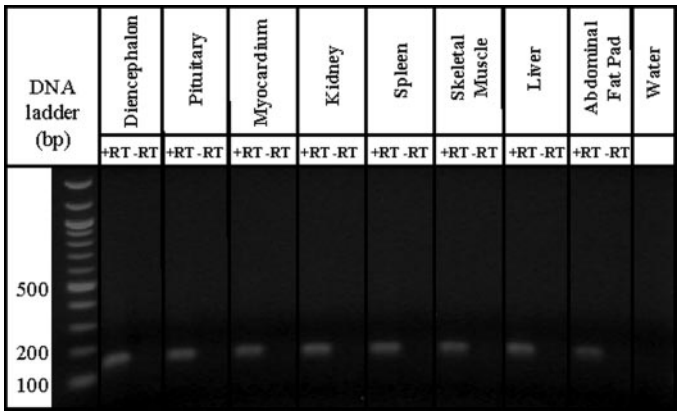

FIG. 1. RT-PCR analysis of visfatin gene expression in various tissues of the chicken. Total RNA extracted from each tissue was DNase I digested and reverse transcribed (+RT). Approximately 50 ng cDNA were used as template to amplify a 140-bp chicken visfatin cDNA. Contamination controls consisted of RNA from each tissue without reverse transcriptase addition (−RT) or substitution of water for the cDNA template.

firmed by the presence of a 50-kDa α-tubulin immunoreactive band (data not shown).

Relative quantity of visfatin mRNA and protein in primary metabolic tissues

Real-time quantitative PCR analysis of adipose, liver, and skeletal muscle revealed that skeletal muscle contains the highest amount of visfatin mRNA, followed by liver, whereas abdominal fat pad contained the least visfatin mRNA quantity (Fig. 3A). These findings were confirmed with a second, independent set of chickens (n = 6) that yielded identical results (data not shown). Melting-curve analyses showed the presence of a single PCR product for

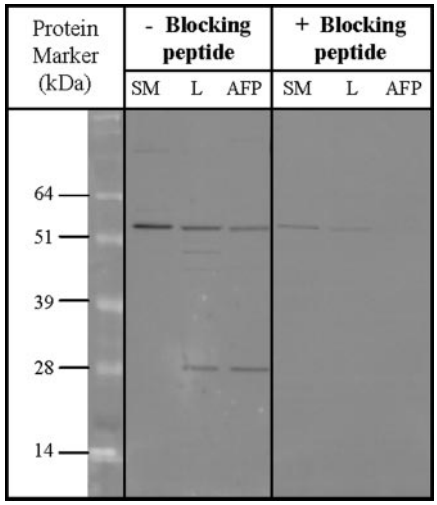

FIG. 2. Western blot analysis of chicken visfatin in skeletal muscle (SM), liver (L), and abdominal fat pad (AFP). Approximately 20 μg total protein extracted from each tissue were electrophoresed and blotted onto PVDF membrane. Visfatin was detected by immunostaining using a rabbit antihuman visfatin antibody. Specificity of the visfatin immunostaining was determined by preabsorption of the antihuman visfatin antibody with human visfatin peptide (blocking peptide).

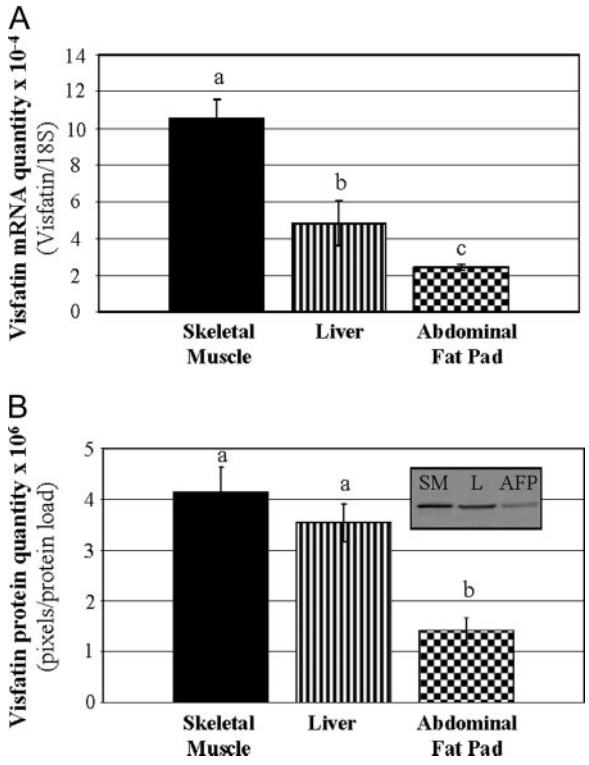

FIG. 3. Quantification of visfatin mRNA and protein in skeletal muscle, liver, and abdominal fat pad. A, Visfatin mRNA quantity relative to 18S mRNA in skeletal muscle, liver, and abdominal fat pad. Total RNA from each tissue was DNase I digested and reverse transcribed, and 50 ng cDNA was used in real-time quantitative PCR (n = 6). B, Visfatin protein abundance within skeletal muscle (SM), liver (L), and abdominal fat pad (AFP) as measured by Western blot analysis. Approximately 20 μg total protein extract from each tissue were electrophoresed and blotted onto PVDF membrane. Visfatin was detected by immunostaining using a rabbit antihuman visfatin antibody, and immunoreactivity was analyzed by densitometric quantification (n = 4). *Inset*, Representative blot of visfatin-immunoreactive bands in various tissues. Data in A and B are represented as mean ± SEM. *Different letters above each bar* indicate significant difference at $P < 0.05$.

visfatin mRNA or 18S mRNA, confirming the specificity of the reaction (data not shown). Furthermore, sequencing of the real-time quantitative PCR product for visfatin indicated 100% homology to chicken visfatin cDNA (GenBank accession no. AY946242). Skeletal muscle and liver were also found to contain significantly greater levels of visfatin protein than did adipose tissue ($P < 0.05$, Fig. 3B) as determined by Western blot analysis. Both visfatin mRNA and protein levels were not different among sc fat, gizzard fat, and abdominal fat pad collected from 8-wk-old broiler chickens ($P > 0.05$, Fig. 4).

Effect of age on tissue-specific visfatin expression

To determine the influence of age on tissue-specific visfatin expression, adipose, liver, and skeletal muscle were collected from chickens at 4 and 8 wk of age. The 8-wk-old chickens were more than twice as heavy as those at 4 wk of age (3828 ± 0.27 *vs.* 1618 ± 0.05 g; $P < 0.01$). Blood glucose levels between the 4- and 8-wk-old chickens were not significantly different (307 ± 12.3 and 297 ± 7.85 mg/dl; $P >$

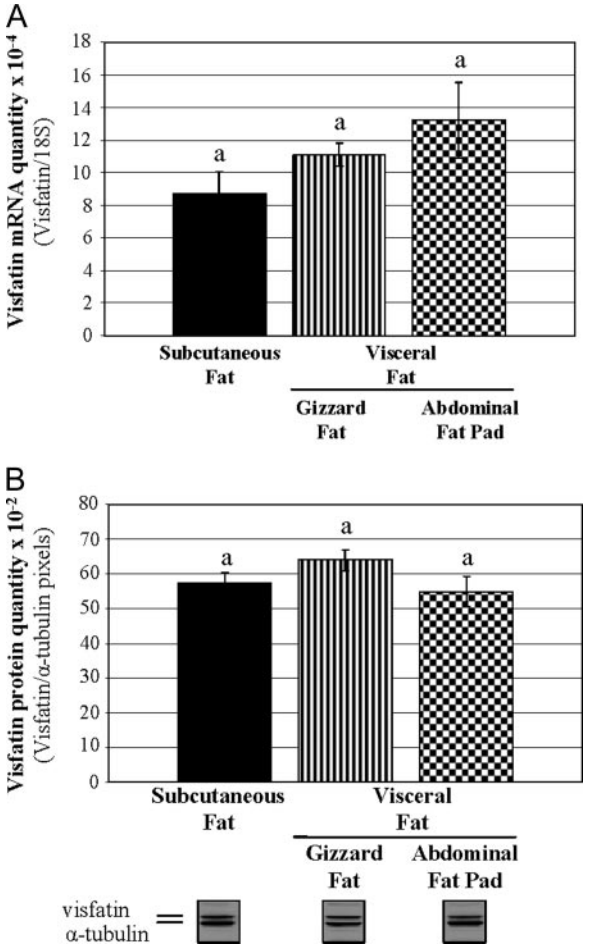

FIG. 4. Quantification of visfatin mRNA and protein in various fat depots. A, Visfatin mRNA abundance relative to 18S mRNA within sc fat, visceral gizzard fat, and abdominal fat pad. Total RNA from each tissue was DNase I digested and reverse transcribed, and 50 ng cDNA was used in real-time quantitative PCR. B, Visfatin protein abundance within various fat depots as measured by Western blot analysis. Approximately 20 μg total protein extract from each tissue were electrophoresed and blotted onto PVDF membrane. Visfatin was detected by sequential immunostaining using a rabbit antihuman visfatin antibody and mouse antichicken α-tubulin antibody, and immunoreactivity was analyzed by densitometric quantification. *Inset*, Representative blots of visfatin and α-tubulin immunostaining. Data in A and B are represented as mean ± SEM. *Different letters above each bar* indicate significant difference at $P < 0.05$ (n = 6).

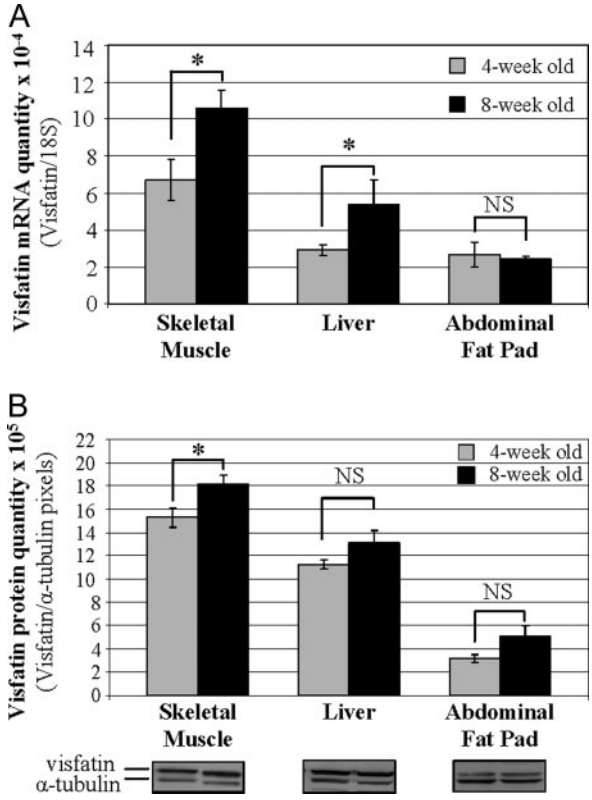

FIG. 5. Quantification of visfatin mRNA and protein in skeletal muscle, liver, and abdominal fat pad tissues of 4- and 8-wk-old chickens. A, Visfatin mRNA abundance relative to 18S mRNA within skeletal muscle, liver, and abdominal fat pad. Total RNA from each tissue was DNase I digested and reverse transcribed, and 50 ng cDNA was used in real-time quantitative PCR (n = 4–6). B, Visfatin protein abundance in 4- and 8-wk-old chicken skeletal muscle, liver, and abdominal fat pad as measured by Western blot analysis described in Fig. 4 (n = 4). Data in A and B are represented as mean ± SEM. *, Significant differences at $P < 0.05$. NS, Not significant.

0.05). However, visfatin mRNA levels were significantly greater in the skeletal muscle and liver of 8-wk-old chickens when compared with those of 4-wk-old chickens ($P < 0.05$, Fig. 5A). Abdominal fat pad visfatin mRNA levels remained unchanged with age. In addition, 8-wk-old chicken skeletal muscle also contained significantly greater amounts of visfatin protein compared with that of 4-wk-old chicken skeletal muscle ($P < 0.05$, Fig. 5B), whereas adipose and liver visfatin protein levels were not affected by age ($P > 0.05$, Fig. 5B).

Validation of visfatin EIA

A commercial human visfatin EIA kit was validated for detecting chicken plasma visfatin by determining parallelism as well as possible interference of chicken plasma with assay sensitivity. To measure parallelism, serial dilutions of pooled broiler plasma were assayed and yielded a linear relationship with an R^2 value of 0.9872 ($P < 0.01$; Fig. 6). In addition, supplemental human visfatin was completely recovered

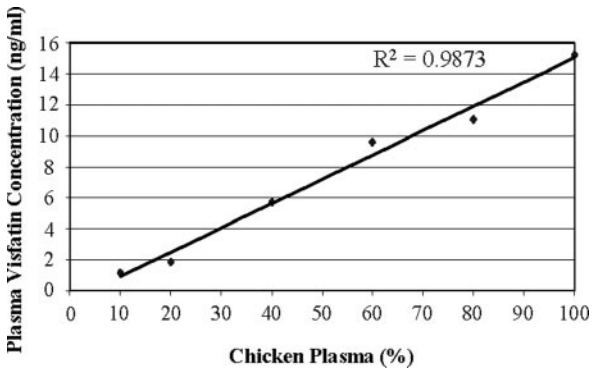

FIG. 6. Validation of human visfatin EIA for quantifying plasma visfatin levels in chickens. Parallelism was determined by serial dilutions of pooled chicken plasma in EIA buffer to obtain 100, 80, 60, 40, 20, and 10% plasma. Each sample was assayed in triplicate for visfatin concentrations, and a linear regression was applied. *Data points* represent mean values obtained.

(26.44 ± 0.486 ng/ml) from chicken plasma that was spiked with a known amount of human visfatin that would result in a final expected concentration of 20 ng/ml. These results thereby indicate that the chicken plasma did not interfere with the sensitivity of the assay.

Effect of age on plasma visfatin concentrations

To determine whether age affects plasma visfatin levels, we quantified plasma visfatin in chickens at 4 and 8 wk of age. Plasma collected from 8-wk-old chickens contained significantly greater levels of visfatin when compared with that of 4-wk-old chicken plasma ($P < 0.05$, Fig. 7A) despite having blood glucose levels that were not significantly different ($P > 0.05$).

Effect of fasting and refeeding on chicken plasma visfatin concentrations

Fasting for 48 h resulted in a significant decrease in body weight compared with broiler chickens in the control or re-fed groups (data not shown), whereas blood glucose levels remained unchanged regardless of treatment (Fig. 8). In addition, plasma visfatin levels were not significantly different in response to fasting or fasting and refeeding relative to the fed animals ($P < 0.05$, Fig. 8).

Discussion

The present study is the first to report visfatin gene and protein expression in the chicken. We provide novel evidence that chicken skeletal muscle expresses greater amounts of visfatin compared with visceral adipose tissue, thereby raising the possibility that visfatin may be acting as a myokine as well as an adipokine. We found that the visfatin cDNA and the deduced amino acid sequences are highly homologous among various species of animals, indicating that visfatin is evolutionarily conserved and may serve important biological functions. Using RT-PCR, we identified visfatin mRNA in multiple tissues such as the diencephalon, pituitary, myocardium, kidney, spleen, skeletal muscle, liver, and adipose tissues. Originally identified in human peripheral blood lymphocytes, visfatin has since been shown to be ubiquitously expressed in multiple tissues in the human (5, 22), pig (10), and dog (8). In the present study, a 52-kDa visfatin protein was identified in chicken skeletal muscle, liver, and adipose tissues, which is in accordance with previous reports on the human pre-B cell colony-enhancing factor (PBEF)/visfatin protein (5). In addition, we consistently found a smaller 28-kDa band in adipose and liver extracts. In support of our findings, a low molecular weight band was observed in the culture media from COS-7 cells transfected with human visfatin cDNA (5), where it was suggested to be the result of proteolytic degradation. Alternatively, this smaller band may indicate alternative splicing of visfatin within chicken liver and adipose tissues, because tissue-specific visfatin splice variants have been previously reported in the pig (10).

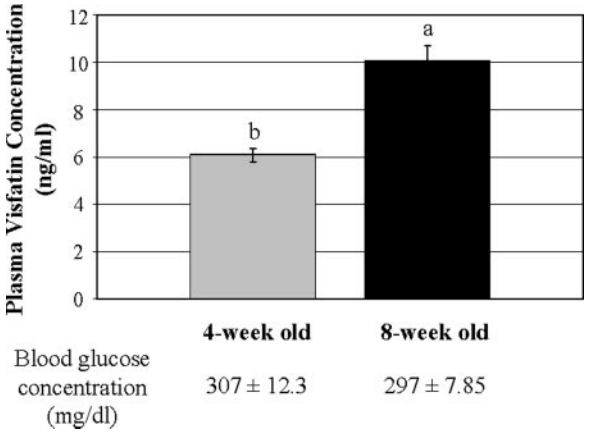

FIG. 7. Relationship between plasma visfatin and age in chickens. Plasma visfatin levels in 4- and 8-wk-old chickens as determined by EIA. *Different letters above each bar* indicate significant difference at $P < 0.05$. Data are represented as mean ± SEM (n = 6).

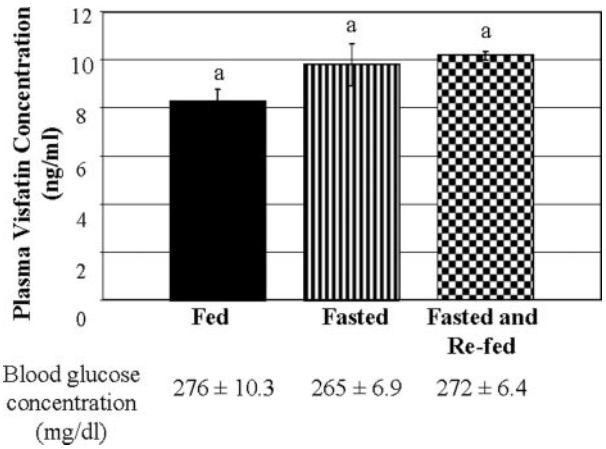

FIG. 8. Effect of fasting and refeeding on plasma visfatin levels in chickens. Chickens were fed *ad libitum*, fasted for 48 h, or fasted for 48 h followed by refeeding for 24 h (n = 6). Plasma samples were subjected to EIA. Data are represented as mean ± SEM. *Different letters above each bar* indicate significant difference at $P < 0.05$.

In the present study, skeletal muscle was found to contain greater quantities of visfatin mRNA compared with adipose and liver, whereas visfatin protein quantity was higher in skeletal muscle and liver compared with adipose tissue. These findings are in contrast to previous studies on mammalian visfatin, which identified visceral adipose tissue as the primary source for visfatin (23). To our knowledge, however, such studies did not include both skeletal muscle and adipose tissue for comparing visfatin mRNA levels (5, 8, 22). It is therefore difficult to assess whether similar results may exist in mammals, or whether the altered expression reflects species specificity. In addition to visfatin, skeletal muscle has previously been identified as a source for several other adipokines, including adiponectin (24), resistin (25), and TNF-α (26), where these adipokines have been implicated in regulating energy homeostasis and insulin sensitivity within the muscle. Similarly, visfatin may be acting within the chicken skeletal muscle in an autocrine/paracrine manner to regulate energy metabolism, because visfatin has been shown to increase glucose uptake in cultured human osteoblasts (11). The greater expression of visfatin in the chicken liver compared with adipose tissue may reflect the altered site of lipid synthesis in the chicken, wherein lipogenesis occurs primar-

ily in the liver rather than in the adipose tissue as in rodents (19). In the present study, visfatin mRNA and protein quantity were not different among fat depots in the sc, gizzard, and abdominal fat pad in the chicken. Although these results are in contrast to a previous reports indicating greater visfatin mRNA expression in visceral adipose tissue than sc (23), our results support those found by Berndt *et al.* (22) that visfatin expression is similar in various fat depots of humans.

To determine the effect of age on visfatin expression, skeletal muscle, liver, and adipose tissues of 4- and 8-wk-old chickens were analyzed to quantify visfatin mRNA and protein levels. Interestingly, both visfatin gene and protein expression were further elevated in skeletal muscle with age. Although visfatin mRNA levels in the liver were also significantly elevated, there was no significant difference at the protein level between the two age groups. The increase in skeletal muscle visfatin with age may be due in part to the rapid growth of skeletal muscle in chickens, which have been selectively bred for enlarged breast (pectoralis) muscle. Tang *et al.* (27) demonstrated that visfatin mRNA expression within rat skeletal muscle is up-regulated during development, with the greatest expression found in adulthood (27). Indeed, the skeletal muscle of chickens is undergoing rapid growth and development during the ages of 4 and 8 wk (15), which may account for the increased visfatin expression within the 8-wk-old chicken skeletal muscle. Taken together, we propose that visfatin may be linked to skeletal muscle myogenesis, possibly by either stimulating proliferation (11) or by inhibiting apoptosis much like the myokine IL-15 (28). Conversely, visfatin may be involved in glucose metabolism within the muscle. Visfatin has previously been shown to stimulate glucose uptake by human osteoblasts similar to insulin. It is also possible that increased muscle growth, as is occurring in the broiler chicken, may increase muscular visfatin expression to augment glucose transport into the myocytes.

In the present study, we validated a human visfatin EIA kit for quantifying circulating visfatin levels in chickens. We observed that the plasma visfatin level in chickens was significantly increased with age, such that 8-wk-old chickens had approximately 67% greater circulating visfatin levels than the 4-wk-old chickens. Glucose-clamp studies in humans revealed that increases in blood glucose levels resulted in increases in plasma visfatin levels (12). However, in the current study, blood glucose levels remained unchanged with age, whereas plasma visfatin levels were significantly elevated. Previous studies have suggested that elevated plasma visfatin levels are associated with greater content of visfatin mRNA and protein in the visceral adipose tissue of women with polycystic ovarian syndrome (23). The increased plasma visfatin levels observed in the 8-wk-old chickens may therefore be the result of increased abdominal adipose tissue with age. The current study found that the visfatin gene is expressed in the chicken visceral adipose tissue, and the size of this abdominal fat pad increases with age (16). Visfatin mRNA quantity was previously found to be elevated during differentiation of preadipocytes to mature adipocytes (29, 30). In the present study, however, visceral adipose tissue had the lowest level of visfatin gene and protein expression of the tissues tested, and neither was altered with age. Conversely, the breast muscle, which was used in this study, also undergoes a significant increase with age in chickens (15) and may therefore serve as the primary source for visfatin in chickens. Because skeletal muscle was found in this study to contain the greatest quantity of visfatin mRNA and protein when compared with adipose tissue, and the breast muscle undergoes rapid growth, it is reasonable to hypothesize that the increased breast muscle mass is one of the major sources for the elevated plasma visfatin levels. The ability of visfatin to be secreted from skeletal muscle, or any other tissue, is not unequivocal (6, 31). Although lacking a signal peptide, it has been suggested that visfatin may be secreted through a nonclassical pathway (32), whereas other studies argue that it is not at all secreted, but rather released as a result of cellular apoptosis (33). Our identification of visfatin in chicken plasma, as well as numerous studies identifying visfatin in the plasma of other species and in bovine milk (34), confirm that visfatin is indeed present in extracellular fluids, although a secretory pathway remains to be identified.

Our findings indicate that food deprivation did not alter plasma visfatin levels and blood glucose levels in chickens, despite causing significant body weight loss. This is the first report on the effect of fasting on plasma visfatin levels in any species. However, Nampt (visfatin) quantity was found to be dramatically increased in the liver mitochondrial protein extracts obtained from rats that were fasted for 48 h, suggesting that Nampt is possibly functioning as a stress- and nutrient-sensitive molecule (35).

In conclusion, we have identified skeletal muscle, not visceral adipose tissue, as the primary source for visfatin in chickens, thereby raising the possibility that visfatin may be functioning as a myokine. Furthermore, we show that the skeletal muscle visfatin gene and protein expression, as well as plasma visfatin concentrations, are significantly elevated in 8-wk-old *vs.* 4-wk-old broiler chickens, possibly reflecting greater breast muscle and/or visceral adipose tissue accretion in the older chickens. Additional studies are necessary to characterize the functional role of visfatin on skeletal muscle metabolism in the chicken.

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