Hormonal regulation of leptin expression in broiler chickens

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Ashwell, Christopher M., Susan M. Czerwinski, Donna M. Brocht, and John P. McMurtry. Hormonal regulation of leptin expression in broiler chickens. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R226–R232, 1999.—Leptin, the polypeptide hormone encoded by the obese gene, is secreted by adipose tissue and has been shown to induce satiety and increase energy expenditure in mammals. In this study, we confirmed the presence of a leptin homolog in liver and adipose tissues of broiler chickens. Leptin expression was also detected in chicken embryonic liver and yolk sac. The effects of hormone treatment on leptin expression in chickens were also investigated. Leptin expression in the liver is increased by insulin and dexamethasone and decreased by glucagon and estrogen. There was no induction of leptin expression in adipose tissue by any treatment, whereas only estrogen decreased adipose expression. The differential effect on liver and adipose tissue suggests that adipocytes in chickens may be expressing leptin at a maximal rate or that its mechanism of expression regulation differs from liver. The localization of leptin expression and tissue-specific effects of hormone treatments on leptin expression observed in chickens may indicate a relationship between leptin and avian lipid metabolism.

avian embryo; yolk sac; insulin; dexamethasone; estrogen; glucagon; insulin-like growth factor I

The polyepitope hormone leptin has been shown to play an important role in food intake regulation, energy metabolism, and reproduction in mammals. Leptin is secreted by mammalian adipocytes and functions as a hormonal sensing mechanism for fat deposition (3, 8). Adipocytes produce and secrete more leptin as fat storage increases, signaling the brain to reduce food intake and increase energy expenditure. The homeostatic nature of this mechanism has been the subject of many studies over the last few years.

These previous studies have investigated the role of insulin in regulating leptin expression. Insulin has been shown to increase leptin mRNA and circulating leptin levels in rodents and humans (5). Additionally, it has been reported that glucocorticoids increase leptin mRNA and circulating leptin levels in rodents and humans (19). Estrogen has been reported to increase leptin production in rats and humans (22). Other hormonal regulators that have been investigated include cAMP, insulin-like growth factor (IGF) I, growth hormone, and testosterone (6, 15). These studies have demonstrated the direct relationship between leptin mRNA expression levels and circulating leptin concentrations. In addition, these studies have shown definitively that leptin expression is responsive to energy intake and the hormonal environment.

The reported sequence of leptin from human, cow, pig, sheep, mouse, rat, dog, and, most recently, chicken, shows a high degree of sequence conservation. This sequence similarity suggests a common function or mechanism of the peptide hormone across species. The role leptin plays in metabolism and food intake regulation in domestic animals, selected for increased growth and energy conversion, is largely unknown.

Our interest lies in the function and regulation of leptin in broiler chickens. A recent report of the cloning of chicken leptin by Taouis et al. (26) demonstrated the presence of a leptin homolog in birds as well as its unique expression in both liver and adipose tissues. This observation is thought to be due to the role of the avian liver as the primary source of avian lipogenesis (16). Leptin expression in both liver and adipose tissue suggests that birds may have a different mechanism for leptin regulation of feed intake and energy expenditure than that of mammals. To investigate the regulation of avian leptin expression, we investigated the effect of several hormone treatments to modulate leptin expression in broiler chickens.

METHODS

Animals. All animal studies were conducted with research protocols approved by the Beltsville Animal Care and Use Committee. One-day-old male broiler chicks were purchased from Shaver Poultry Breeding Farms (Cambridge, Ontario, Canada) and grown in brooder batteries until 3 wk of age, at which time the birds were transferred to individual cages. Standard commercial starter and grower diets and water were available ad libitum. Tissues from 8-wk-old males were harvested and snap frozen in liquid nitrogen for isolation of tissue-specific RNAs and genomic DNA.

Cloning of chicken leptin. Oligonucleotide primers for amplification of a chicken homolog of mammalian leptin were selected on the basis of regions of high sequence identity between known leptin sequences (human, mouse, rat, dog, monkey, gorilla, cow, sheep, and pig). The regions selected for primer design correspond to sense LepUp, 5′-CACCCAGGAT-CAATGACATTTCAC, and antisense LepDn, 5′-CACCTCCTGTTGAGTAGAGTGGAGC. Total RNA was isolated using the Tri-Reagent procedure (Life Technologies, Rockville, MD). Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI) was used to reverse transcribe 10 µg of total RNA isolated from both liver and adipose tissue of 8-wk-old male broiler chickens using an oligo dT18 primer. A subsequent PCR amplification was performed using an MJ Research PTC-100 programmable thermal controller (Water-
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town, MA) with Taq polymerase (Promega). PCR reactions contained 10 µl of cDNA produced from the RT reaction (50 µl), and the LepUp/LepDn primers. The thermal cycling parameters used were: 30 cycles; 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min. The resulting 312-bp product was cloned using the TOPO-cloning procedure (Invitrogen, San Diego, CA) and sequenced by fluorescent BigDye-terminator chemistry using an ABI 377 automated DNA sequencer (Perkin-Elmer, Foster City, CA).

The primary chicken leptin amplification product was used as the driver in a 5'-3'-extension procedure. A second set of oligonucleotide primers was generated from the cloned chicken leptin sequence described previously: sense LepUp2, 5'-CGTCGTTATCGCCAACGAGGG, antisense LepDn2 5'-CCAGGACGCCATCCAGGCTCTGGC that produces a 261-bp amplification product. RT-PCR was performed as described above with a higher annealing temperature of 60°C. This amplification product was gel purified and isolated by the QiAquick procedure (Qiagen, La Jolla, CA). The isolated double-stranded product was used to amplify cDNA generated by RT of liver RNA (10 µg) with oligo dT18. This product was extended by 60 cycles of 94°C for 30 s and 68°C for 30 s using Taq polymerase under standard PCR conditions in 100 µl. The reaction mixture was ethanol precipitated, and the entire reaction was subjected to TOPO cloning. Escherichia coli HB101, ultracompetent cells (Promega) were transformed with the entire TOPO reaction, and, of the clones isolated, three were able to have their 3'-ends repaired and contained the entire gene coding sequence.

RT-PCR assay for leptin expression. Using RT-PCR, we have assayed for the presence of leptin mRNA in various tissues, including adipose (abdominal fat pad), brain, bursa, heart, gizzard, kidney, liver, muscle, and spleen of 8-wk-old male broilers, day 5 embryo, day 17 embryonic liver, and day 17 embryonic yolk sac. Embryonic tissue samples were harvested from Leghorn eggs (n = 3) incubated for the times stated and pooled. Total RNA was isolated using the Trizol procedure as before. RT-PCR amplification products were produced from reverse transcriptase reactions including 10 µg of total RNA, MMLV reverse transcriptase, and oligo dT18 primer in a total reaction volume of 50 µl. PCR amplification was performed using 10 µl of the RT reaction and Taq polymerase (30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min), similar to that previously described and separated by agarose gel electrophoresis (1%). The product size of 261 bp was as expected. Leptin mRNA expression was detected in both adipose and hepatic tissues with liver being apparently the major source.

Northern blot analysis. Total RNA (40 µg) isolated from various tissues of 8-wk-old male broilers by the method previously described was separated by agarose gel electrophoresis and transferred to a nylon (Hybond N+) membrane. Hybridization was accomplished using a chicken leptin DNA probe (cDNA) labeled with [α-32P]dATP random prime labeled; Amersham-Pharmacia Biotech using the RapidHyb procedure (Amersham-Pharmacia Biotech). Radioactive labeling was detected by emission storage on a phosphor screen using a Storm 860 imager from Molecular Dynamics (Carlsbad, CA).

Genomic Southern analysis. To determine the number of leptin isoforms present in the chicken genome, we generated a genomic Southern blot. Genomic DNA was isolated from both liver and whole blood (24). DNA was digested (10 µg) with the restriction enzymes BamHI, EcoRI, HindIII, and XhoI (Promega) at 37°C for 16 h. The digestion products were then separated by agarose gel electrophoresis (1%) and transferred to a nylon (Hybond N+) membrane, as previously described. After UV cross linking, the blots were probed with random prime labeled chicken leptin and the hybridization pattern was analyzed as previously described.

Animal treatments. In the first experiment, 12-wk-old males were injected intramuscularly with either dexamethasone (Sigma) at a dose of 100 µg·kg body wt·day⁻¹·day⁻¹ (n = 4) or porcine insulin (Sigma) at 200 µg·kg body wt·day⁻¹·day⁻¹ (n = 4) for a period of 4 days. Both hormones were dissolved in saline. Sham-injected controls were treated with an equal volume of saline.

In the second experiment, 4-wk-old males were injected intramuscularly with 20 mg of estradiol 17β (Sigma) dissolved in ethaneglycol (n = 4) or ethaneglycol alone (controls, n = 4) on days 1 and 3. An additional group of birds (n = 4) was injected daily with porcine glucagon (Sigma) at a dose of 50 µg·kg body wt·day⁻¹·day⁻¹ for 4 days, whereas control birds were treated with an equal volume of 10 mM HCl. To test the effect of IGF-I on tissue leptin expression, 4-wk-old males (n = 4) were implanted with osmotic minipumps (model 2001; Alza, Palo Alto, CA) scheduled to deliver 400 µg·kg body wt·day⁻¹·day⁻¹. All birds were killed 24 h after the final injection (day 5), and the tissues were snap frozen in liquid nitrogen, except for the estrogen-treated birds that were autopsied 48 h (day 5) after the final injection. IGF-I-treated chickens were killed 5 days after implantation. All tissues were stored at −80°C until processed.

Quantification of differential leptin expression levels. Total RNA was isolated using the Tri-Reagent protocol and quantitated spectrophotometrically. Reverse transcriptase reactions were carried out using an oligo dT18 primer, MMLV reverse transcriptase, and 5 µg of total RNA in 50 µl. After the RT reaction, 2.5- and 5-µl aliquots were used as a template for the PCR amplification with the leptin-specific primers, as described previously, in duplicate. As an internal control for the integrity of the mRNA in each sample, additional PCR amplifications were performed using oligonucleotide primers specific for chicken β-actin that produce a product of 612 bp. The reaction products were separated by agarose gel electrophoresis (2%), stained with ethidium bromide, and quantified by UV transillumination and video capture by an Alpha Innotech gel documentation workstation (San Leandro, CA). Samples were normalized to the quantity of β-actin signal produced by RT-PCR and quantified for leptin expression by arbitrary units per microgram of starting total RNA (n = 4). C57BL/6J mice were weighed over the linear range of UV detection of ethidium bromide staining.

Hormone analysis. Double antibody radioimmunoassays were used to determine plasma concentrations of the following hormones: insulin, intra-assay coefficient of variation of 3.2% (14); IGF-I, intra-assay coefficient of variation of 2.9% (21); chicken IGF-II, intra-assay coefficient of variation of 3.4% (13); and triiodothyronine (T3) and thyroxine (T4), intra-assay coefficients of variation of 2.5 and 3.1%, respectively (12). Radiolabeled T3 and T4 were purchased from ICN Pharmaceuticals (Costa Mesa, CA). Plasma glucagon was determined using a kit purchased from Linco Research (St. Charles, MO). The intra-assay coefficient of variation for this assay was calculated to be 1.6%.

Statistical analysis. Statistical analyses were performed using Student’s t-test to determine the significance of hormone treatment on leptin expression. Correlation between
RESULTS

Cloning and expression of chicken leptin. Using a RT-PCR amplification strategy, we demonstrated the presence of a leptin homolog in chickens. This homolog is >90% identical to murine leptin and >80% identical to most of the other known leptin sequences. The sequence for chicken leptin that was reported by Taouis et al. (26) is nearly identical to that which we have cloned and sequenced (GenBank no. AF082500). A single nucleotide difference occurring at position 342 (C for T) appears consistently in all analyses (n = 6) (Fig. 1). This difference does not alter the predicted amino acid sequence of the leptin protein.

In addition to finding that leptin is expressed in hepatic and adipose tissue, we observed leptin expression in the developing chick embryo. RT-PCR of total RNA isolated from various tissues in adult birds as well as embryonic chick tissues were used to look for the presence of leptin mRNA (Fig. 2A). In adult birds, leptin mRNA was detected only in adipose and liver tissue. Leptin expression was detected in whole day 5 embryos and day 17 embryonic liver and embryonic yolk sac membrane. To support these observations, we performed Northern analysis on the same tissues.

Radiolabeled leptin sequence hybridized to total RNA isolated from the various tissues detected leptin expression in identical tissues to that of RT-PCR. Quantification of the levels of leptin mRNA by RT-PCR and Northern analysis yielded similar results. The hybridization pattern observed in the Northern analysis indicated the possibility of two different mRNA transcript sizes present in the RNA isolated from different tissues. Adult hepatic and adipose tissue appear to contain a predominantly longer leptin transcript (1.5 kb; Fig. 2B), whereas the day 5 embryo and, specifically, the day 17 yolk sac appear to have a higher percentage of the shorter leptin transcript (0.9 kb). This observation, in addition to the presence of leptin expression in both liver and adipose tissues, indicates the potential for multiple isoforms of leptin being present in chickens. To partially address this question, we performed a genomic Southern analysis. Genomic DNA was fragmented by restriction digestion and probed with radiolabeled leptin sequence. The hybridization pattern observed when the digested genomic DNAs were probed with chicken leptin suggests a single copy gene within the chicken genome (Fig. 3).

Hormone effects on leptin expression. Leptin expression was measured by an RT-PCR-based assay in which the internal standard  β-actin was used to estimate equivalent amounts of mRNA pools. The RT-PCR amplification products (Fig. 4) were quantified by densitometry and expressed as arbitrary units per microgram of total RNA starting material. The RT-PCR assay was validated by determining the linear range of leptin amplification from 12–40 thermocycles with varying amounts of leptin cDNA. Tissues with maximal observed leptin expression levels were used to determine the upper limits of the linear range of mRNA detection.
The use of 30 amplification cycles allows for substantial signal detection of low-level tissue expression and is at the upper limit for measurement of high expression levels, possibly underestimating actual values (Fig. 5A). The use of two amounts of cDNA in the PCR reactions as template (2.5 and 5 µl) also allowed for measurement of leptin expression over the linear range of amplification, even in the highest-expressing tissues (Fig. 5B).

The leptin expression level in adipose tissue of control chickens was significantly different \((P < 0.001)\) and consistently about one-half of that found in an equivalent amount of total RNA isolated from liver (Table 1). After treatment with insulin and dexamethasone, leptin expression in hepatic tissue was significantly increased \([3.1- and 2.3\text{-fold}, \text{respectively} (P < 0.001); \text{Fig.} \ 6]\). The level of leptin expression in adipose tissue was unchanged with either dexamethasone or insulin treatment. Conversely, treatment with glucagon and estrogen decreased the level of hepatic leptin expression \(25\% (P < 0.01)\) and \(40\% (P < 0.02)\), respectively. Leptin expression in adipose tissue was significantly decreased only by estrogen treatment \((P < 0.002)\). Treatment with IGF-I had no effect on leptin expression in either tissue (Fig. 6 and Table 1).

Analysis of circulating hormone levels revealed several correlations to leptin expression levels. Leptin expression in liver was correlated to IGF-II levels \((P < 0.0003, \ r^2 = 0.355\); Fig. 7A). On comparison of the individual hormone treatment groups, their leptin expression levels, and circulating hormones, additional correlations were observed. Expression levels of leptin in the liver of birds treated with insulin showed a correlation with corticosterone levels \((P < 0.037, \ r^2 = 0.934\); Fig. 7B). Glucagon, insulin, \(T_3\), \(T_4\), and IGF-I were not significantly correlated to leptin expression in either liver or adipose tissue.

**DISCUSSION**

This study addressed the presence, localization, and hormonal regulation of leptin in broiler chickens. We confirmed the presence of a leptin homolog in chickens and verified its sequence. The observed difference between our leptin clone and the sequence reported by Taouis et al. (26), although it does not change the predicted amino acid sequence of the protein, may have some significance. The single base change may be due to differences between strains of chickens. If this difference is real on the evaluation of several strains of bird, it may be an important naturally occurring polymorphism, which could be useful in genetic mapping experiments.

We also confirmed previous findings that leptin is expressed in both chicken hepatic and adipose tissue (26). The presence of leptin mRNA in chicken adipose tissue is readily explained by its similarity to mammalian leptin expression (6). Leptin expression in chicken liver can possibly be related to the function of the avian
liver in lipogenesis (16). The avian liver is responsible for as much as 95% of de novo fatty acid synthesis in birds and is also the site of considerable lipid storage in obese chickens (17). Lipogenesis in humans, unlike

Table 1. Tissue-specific leptin expression values for various hormonal treatments of male broiler chickens

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leptin, U/µg RNA</th>
<th>Expression, Significance Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Adipose</td>
</tr>
<tr>
<td>Dex</td>
<td>4,635 ± 392</td>
<td>813 ± 13</td>
</tr>
<tr>
<td>Ins</td>
<td>6,295 ± 370</td>
<td>832 ± 14</td>
</tr>
<tr>
<td>Ins/Dex control</td>
<td>1,995 ± 143</td>
<td>797 ± 24</td>
</tr>
<tr>
<td>Estrogen</td>
<td>1,244 ± 180</td>
<td>608 ± 17</td>
</tr>
<tr>
<td>Estrogen control</td>
<td>2,018 ± 209</td>
<td>762 ± 33</td>
</tr>
<tr>
<td>Glucagon</td>
<td>1,581 ± 80</td>
<td>776 ± 45</td>
</tr>
<tr>
<td>Glucagon control</td>
<td>2,077 ± 192</td>
<td>804 ± 29</td>
</tr>
<tr>
<td>IGF-I</td>
<td>1,746 ± 108</td>
<td>819 ± 67</td>
</tr>
<tr>
<td>IGF-I control</td>
<td>2,127 ± 272</td>
<td>799 ± 34</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 for all experimental groups. Dex, dexamethasone; IGF, insulin (Ins)-like growth factor. *Significantly different from control treatment.
that in rodents, is also predominantly a hepatic process, but leptin expression has not been previously described in the human liver. The reason for expression of leptin in the liver of birds is still unknown and warrants further study.

An additional finding in this study was the demonstration by both RT-PCR and Northern analysis that leptin mRNA is present in the developing chick embryo. This finding is similar to that previously reported in rodent embryos, suggesting that leptin has paracrine and endocrine effects on embryogenesis (9). Moreover, the presence of leptin mRNA in the embryonic yolk sac membrane suggests it may have a similar role in the chick embryo to that hypothesized for the human placenta (21). The role of leptin in the development of the chick embryo is unknown but may be similar to that in mammals. Further studies are required to determine the leptin expression pattern in the embryo throughout development.

The Northern analysis of leptin mRNA indicated that there were two different transcript lengths. These different transcript lengths appear to segregate by tissue distribution and stage of development. The long form is predominantly present in mature birds and in the liver of late-stage (day 17) embryos. The short form appears to only be present in the day 5 embryo and the yolk sac, suggesting its function in embryogenesis. Leptin expression in the embryonic yolk sac may be explained by its regulation of energy expenditure by nutrient transfer to the developing embryo (4). Multiple transcript lengths of leptin have not been previously described. The possibility of a second distinct leptin gene being responsible for these different transcript lengths is unlikely because of the results of the genomic Southern analysis performed in this study indicating a single gene in the chicken genome.

In general, the regulation of leptin expression appears to be similar to that of mammals, with the exception that the response to certain hormones is tissue specific. It is conceivable that the significance of avian leptin expression in the liver is directly related to its role in lipogenesis. Leptin has often been referred to as being regulated by the lipid storage status of an organism. The extent of the changes in leptin expression to the different hormone treatments used in this study suggests different mechanisms of leptin expression regulation in hepatic versus adipose tissue. This is supported by the ability to induce leptin expression in liver by insulin and dexamethasone but not in adipose tissue.

Treatment with estrogen and glucagon decreases leptin expression levels in both tissues, which supports previous data involving the effects of estrogen on the liver and feed intake in chickens (20, 25). It is possible that adipose tissue expression of leptin under normal conditions is at some maximal rate that is unable to be significantly induced by hormone treatment. It is plausible that leptin mRNA turnover in adipose tissue is increased in response to dexamethasone and insulin treatment, negating the inductive effect of the hormone. The effects of these hormone treatments on leptin expression agree well with their previously shown effects on feed intake in chickens (7). The engagement of leptin in regulating feed intake in birds is therefore a likely event and has been observed in one study to date (18).

The hormone levels present in the birds studied compared with leptin expression levels in adipose and liver tissue showed several relationships. Previous studies have suggested that IGF-II is involved in some aspect of lipid metabolism in chickens (10). The results of the current study suggest a possible relationship between IGF-II and hepatic leptin expression levels. Because it is apparent that IGF-II is involved in lipid metabolism, our data suggest that this may involve leptin function. This relationship is most likely confined to some component of energy metabolism, because feed intake is unchanged after IGF-II treatment (23). A definitive answer to this hypothesis awaits further study.

In those chickens treated with insulin, there was a distinct relationship between leptin expression in adipocytes and circulating corticosterone levels. This relationship has also been shown to exist in rodents and humans (1) and may also be related to energy metabolism in birds.

The present study is the first to report the effect of various hormones on leptin gene expression in birds, and, in general, the leptin mRNA response is similar to what has been reported to occur in mammals. In addition, this is the initial report demonstrating the presence of leptin expression in the developing avian embryo and an extraembryonic membrane (yolk sac), suggesting that leptin has a functional role in avian embryogenesis. With the development of a reproducible assay, it has become possible to look at the regulation of chicken leptin expression in relation to changes in metabolism and growth in embryonic and posthatch maturation.

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