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Am J Physiol Endocrinol Metab 279:116-123, 2000.

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Biological activities of recombinant chicken leptin C4S analog compared with unmodified leptins

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Dridi, S., N. Raver, E. E. Gussakovsky, M. Derouet, M. Picard, A. Gertler, and M. Taouis. Biological activities of recombinant chicken leptin C4S analog compared with unmodified leptins. *Am J Physiol Endocrinol Metab* 279: E116–E123, 2000.—The chicken leptin sequence, in contrast to mammalian leptins, contains an unpaired Cys at position 3 of the original cDNA (AF012727). The presence of an extra Cys may confer a different structure and affect the leptin's biological activity. To address this, we studied the effects of wild-type and mutated (C4S) chicken leptins *in vitro* and *in vivo* and compared them with mammalian leptin prepared from ovine leptin cDNA. The prokaryotic expression vector pMON, encoding full-size A(-1) chicken leptin (AF012727), was mutated using a mutagenesis kit, yielding the C4S analog. *Escherichia coli* cells transformed with this vector overexpressed large amounts of chicken leptin C4S upon induction with nalidixic acid. The expressed protein, found in the inclusion bodies, was refolded and purified to homogeneity on a Q-Sepharose column, yielding three electrophoretically pure fractions, eluted from the column by 100, 125, and 150 mM NaCl, respectively. All three fractions showed a single band of the expected molecular mass (16 kDa) and were composed of >95% monomeric protein. Proper refolding was evidenced by comparing the circular dichroism spectrum of the analog with spectra of nonmutated chicken and ovine leptins. The biological activity of the C4S analog was evidenced by its ability to stimulate proliferation of leptin-sensitive BAF/3 cells transfected with a long form of human leptin receptor construct similar to its nonmutated counterpart, indicating that Cys4 plays no role in leptin activity. The *in vitro* activity of both wild-type and mutated chicken leptins was ~10-fold lower than that of ovine leptin. After intravenous or intraperitoneal injections, C4S analog and the nonmutated chicken and ovine leptins all lowered the food intake of starved 9-day-old broiler or 5-wk-old layer male chickens by 11–34%. Monitoring food behavior revealed that the attenuated food intake resulted not from a decreased number of approaches to the feeders but from a decrease in the average time spent eating during each approach.

food intake; unpaired cysteine; mutagenesis; hormone

OBESE PROTEIN (OB), also known as leptin, is a 146-amino acid hormone secreted by adipose tissue that serves as a signal acting on the central nervous system to regulate food intake and the body's energy balance (3, 8–10, 13, 21) through specific receptors. This protein, which belongs to the cytokine family (6, 17), exhibits high homology in mammalian species. We recently cloned and sequenced the first nonmammalian leptin, chicken leptin (AF012727; see Ref. 16), and its sequence was confirmed by Ashwell et al. (1). Chicken leptin cDNA exhibits >83% homology to human leptin but has only 145 amino acids (16). More interestingly, in avian species, leptin is expressed in liver and adipose tissue, as evidenced by the presence of leptin mRNA (1, 16). The cDNA encoding chicken leptin was subcloned into a prokaryotic expression vector, expressed as Ala-leptin, and was purified to homogeneity, yielding up to 400 mg protein/10 l of bacterial culture (14). The biological activity of the recombinant chicken leptin was demonstrated by its ability to stimulate the proliferation of leptin-sensitive BAF/3 cells transfected with a long form of human leptin receptor construct and by its effect on lowering the food intake of starved chickens after intravenous or intraperitoneal injection (14). One unique structural feature of chicken leptin is that, in contrast to mammalian leptins, it contains an unpaired Cys at position 3 of the original cDNA (or position 4 in the Ala-leptin). To test whether this residue is important for biological activity, we mutated it to serine, prepared the C4S analog, and tested its *in vitro* and *in vivo* activities. An additional aim of the present work was to compare the *in vivo* activities of chicken and ovine leptins, as the latter lacks the unpaired Cys at position 3, and to elucidate whether their inhibitory effect on food intake results from a decreased number of approaches to the feeders or from a decrease in the average time spent eating during each approach.

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MATERIALS AND METHODS

Materials

Ovine (fraction SP) and chicken leptins were prepared in our laboratory as described previously (5, 14). Molecular weight markers for gel electrophoresis, RPMI 1640 medium, and nalidixic acid were obtained from Sigma Chemical (St. Louis, MO). SDS-PAGE reagents were purchased from Bio-Rad Laboratories (Richmond, CA). FCS was purchased from Labotal (Jerusalem, Israel), and a Superdex 75 HR 10/30 column and Q-Sepharose (fast flow) were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other chemicals were of analytical grade.

Construction of Chicken Leptin Analog C4S Expression Vector

The chicken leptin expression vector (14) was modified with the Stratagene Quickchange mutagenesis kit (La Jolla, CA) according to the manufacturer's directions using two complementary primers: the sense primer 5'-GGCAGTGC-CGTCCCAAATCTTCCAGGATGAC-3' (the mutated base is underlined) and the antisense primer 5'-GTCATCCTGGAA-GAATTGGGACGGCACTGCC-3'. The procedure included 12 PCR cycles as specified by the manufacturer's manual and the use of *Pfu* polymerase enzyme for the reaction. The template used for mutant construction was wild-type chicken leptin in the prokaryotic expression vector pMON (14). The mutated construct was then digested with *Dpn* I restriction enzyme, which is specific to methylated and hemimethylated DNA (target sequence: 5'-G^{m6}ATC-3'), to digest the template and to select for mutation-containing synthesized DNA. The vector was then transfected into XL1 competent cells. Two colonies were sequenced and confirmed to contain the mutation without any undesired misincorporation of nucleotides.

Expression, Refolding, and Purification of Chicken Leptin Analog C4S

A preliminary experiment was performed with four clones in a 250-ml flask containing 30 ml terrific broth medium (18). Four hours after nalidixic acid induction, *Escherichia coli* MON105 cells transformed with the expression plasmids containing C4S chicken leptin analog cDNA expressed it (~16 kDa) as a major cell protein. One of the colonies expressing the analog was selected for large-scale preparation. Expression and refolding were carried out as described previously for the unmodified chicken leptin (14).

Determination of Purity and Monomer Content of the Isolated Protein

SDS-PAGE was carried out according to Laemmli (11) in a 15% polyacrylamide gel under reducing conditions. Gels were stained with Coomassie brilliant blue R. HPLC gel-filtration chromatography was performed on a Superdex 75 HR 10/30 column with 0.2-ml aliquots of Q-Sepharose column-eluted fractions using 25 mM Tris · HCl buffer, pH 8, containing 150 mM NaCl. Freeze-dried samples were dissolved in H₂O.

Circular Dichroism Spectra

The circular dichroism (CD) spectra in degrees were measured with an AVIV model 62A DS CD spectrometer (Aviv Associates, Lakewood, NJ) with a 0.020-cm rectangular QS Hellma cuvette. The spectrometer was calibrated with camphorsulfonic acid (19). Lyophilized protein samples were dissolved in 55–65 mM sodium carbonate buffer, pH 7.5, at concentrations of 0.55–0.75 mg/ml and were cleared by cen-

trifugation at 18,000 *g* for 10 min. The CD measurements were performed at 25.0°C, as controlled by thermoelectric Peltier elements with an accuracy of 0.1°C. The CD spectra were measured in five repetitions, resulting in an averaged spectrum for each protein. SD of the averaged CD signal at 222 nm was in the 0.5–2.0% range. For the secondary structure determination, the CD data were expressed in degrees per square centimeter per decimole per mean residue of the molecular weight, which was calculated to be 109.7 for ovine Ala-leptin and 109.5 for chicken Ala-leptin and its C4S analog, resulting in respective molecular masses of 16,124, 15,984 and 15,968 kDa. The protein concentration of the cleared solutions was determined using a buret method in five repetitions at different dilutions for each protein and with bovine pancreatic RNase A as a reference (protein concentration E = 0.715 ml/mg at 277 nm). The SD of the concentration determination was in the 1.6–7.1% range.

BAF/3 Cell Proliferation Bioassay

The proliferation rate of leptin-sensitive BAF/3 1442-C1 4 cells transfected with the long form of human leptin receptor was used as an *in vitro* leptin bioassay (5, 14). In stationary cultures, these cells were grown in RPMI 1640 medium supplemented with 5% FCS and 0.1 ng human interleukin-3 (IL-3)/ml at 37°C in 95% air-5% CO₂. Before the experiment, the cells were collected by centrifugation, washed two times, and resuspended in the same medium without IL-3. The cells were then diluted to 50,000 cells/ml and divided into 24-well plates at 1 ml/well. No or various concentrations of ovine, chicken, or chicken leptin C4S analog were added, and the cells were grown for an additional 72 h. Cell growth was determined by counting the cells with a Coulter counter (Coulter Electronics, Hialeah, FL), and the number of doublings was calculated according to the following equation: log (no. of cells in wells treated with leptin/no. of cells in wells without leptin)/log 2. Ovine leptin (leptin-SP), which is as active as human or mouse leptin in this bioassay (5), was used as a control.

Animal Experiments

To avoid any misleading conclusions concerning a general effect of leptin on food intake in chickens, we used two different strains characterized by slow (layer) or fast (broiler) growth. Both chicken strains were used in three different protocols. In *protocol A*, 9-day-old broiler (Shaver) male chickens (body weight ~100 g) were used. They were housed in individual cages in a temperature-, humidity-, and light-controlled room with free access to H₂O and a regular diet (3,100 kcal, 19% protein). To synchronize feeding behavior and to create a high-appetite state, chickens were accustomed to daily 2-h fasting periods (0900–1100) for 3 days before each assay. On the day of the experiment, chickens that had been fasted for 90 min received a single intravenous (0.1 ml in wing vein) injection of 1 mg chicken leptin or its C4S analog per kilogram body weight. They were refed 30 min later, and food intake was measured manually every 30 min for 5 h. In *protocol B*, 5-wk-old semiheavy layer genotype, ISA Brown male chickens (body weight ~600 g) were housed under the same conditions except that each individual cage was placed on an electronic balance linked to a computer for continuous recording of food intake, namely by recording throughout the experiment in real time (at 1-s intervals), as described previously (21). Chickens were divided into two groups of nine chickens each, and to synchronize feeding behavior and to create a high-appetite state, chickens were accustomed to daily 2-h fasting periods (0900–

1100) for 3 days before each assay, as described in *protocol A*. On *day 1*, the two groups received an intraperitoneal injection of isotonic saline solution (placebo), and food intake was monitored continuously for 24 h using a computerized system. On *day 2*, each group received 1 mg of chicken or ovine leptin per kilogram. On *day 3*, the groups that had received ovine leptin received chicken leptin and vice versa, resulting in 18 chickens used for each treatment. In an additional experiment carried out in the same way, food intake was also continuously monitored for 24 h, and we report values obtained for the first 2.5–10 h of treatment. Feeding activity and time spent eating were measured using constantly connected electronic balances. The number of approaches was estimated by counting the number of uninterrupted eating periods lasting >5 s (21). In *protocol C*, two groups of 5-wk-old layer chickens ($n = 3$) were accustomed to fasting and refeeding as described above. On *day 1* of the experiment, both groups received a placebo, and food intake was continuously measured after the treatment. On *day 2*, each group received 1 mg of either wild-type or mutant chicken leptin per kilogram, respectively, and cumulative food intake was measured continuously using the computerized system.

Statistical Analysis

Statistical analyses were performed with Statistical Analysis System (SAS) software (SAS Institute, Cary, NC) using the Student-Newman-Keuls test for variables. The results obtained according to *protocol C* were also analyzed by paired *t*-test.

RESULTS

Purification of Chicken Leptin Analog C4S

The protein contained in the inclusion bodies prepared from 2.5 liters of induced cells was refolded in 4.5 M urea according to the protocol described for wild-type chicken leptin (14). The refolded protein was purified on a Q-Sepharose column by stepwise elution with increasing concentrations of NaCl in Tris · HCl buffer, pH 9 (Fig. 1), as described previously for the

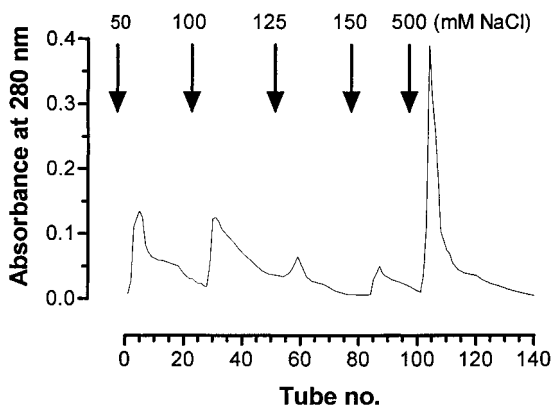


Fig. 1. Purification of inclusion body extracted and refolded chicken leptin C4S analog on a Q-Sepharose column. The fraction containing inclusion-body proteins was solubilized in 45 M urea in 40 mM Tris · HCl at pH 11.3, was dialyzed against 10 mM Tris · HCl, pH 9, buffer (600 ml), and then was applied to the Q-Sepharose column pre-equilibrated with the same buffer. The flow rate was 120 ml/h, and 5-ml fractions were collected. The nonabsorbed fraction was not collected, and after absorption the column was washed with 50, 100, 125, 150, and 500 mM NaCl in 10 mM Tris · HCl, pH 9, buffer.

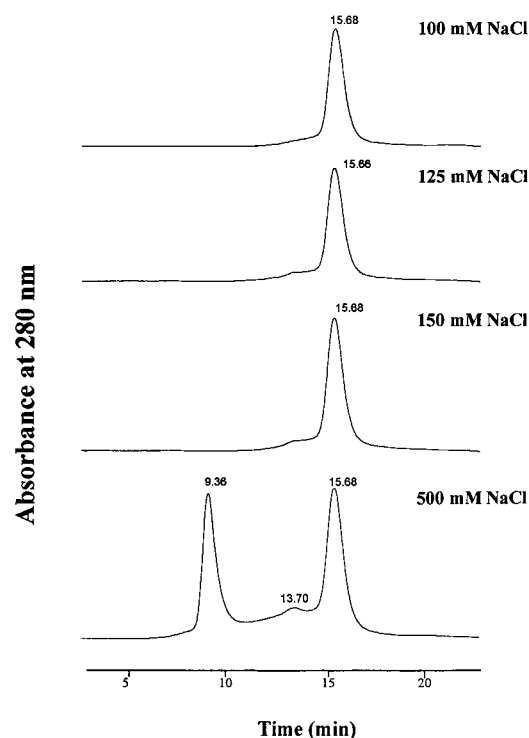


Fig. 2. Gel filtration of purified chicken leptin analog C4S fractions eluted from the Q-Sepharose column by 100, 125, 150, and 500 mM NaCl. Aliquots (200 μ l) of lyophilized fractions (0.2 mg/ml) were applied to Superdex 75 HR 10/30 column, and the eluate was monitored by absorbance at 280 nm. The column was developed with 25 mM Tris · HCl buffer, pH 8, containing 150 mM NaCl at 0.8 ml/min and was calibrated with BSA [66 kDa, retention time (RT) = 1,113 min], egg albumin (45 kDa, RT = 1,258 min), extracellular domain of human growth hormone receptor (28 kDa, RT = 1,352 min), and ovine placental lactogen (23 kDa, RT = 1,392 min).

wild-type hormone (14). After washing the column with 50 mM NaCl, four fractions were eluted with, respectively, 100, 125, 150, and 500 mM NaCl in the same buffer. The fractions were pooled, dialyzed against 0.2% NaHCO_3 (5 \times 5 liters), and lyophilized. Fractions eluted with 100, 125, and 150 mM NaCl contained >95% monomeric leptin, and the fraction eluted with 500 mM NaCl consisted of a mixture of oligomers, dimers, and monomers (Fig. 2). All four fractions yielded only one band of ~15–16 kDa after 15% SDS-PAGE performed in the presence of a reducing agent (Fig. 3). Inclusion bodies prepared from 2.5 liters of fermentation culture yielded 40 mg leptin in the 100 mM NaCl eluate, 18 mg in the 125 mM NaCl eluate, 14 mg in the 150 mM NaCl eluate, and 45 mg in the 500 mM NaCl eluate. Because the mutation did not change the expected molar extinction at 280 nm, the value calculated for unmodified chicken leptin (2,394 M/cm^{-1}) was used.

Figure 4 shows the CD spectra of chicken, C4S analog, and ovine leptins at neutral pH. All of the spectra resembled the spectrum of α -helix with its characteristic two minima at 208 and 222 nm and positive band at ~191 nm (20). The measured spectra had minima at ~209 and 220 nm. The CD spectra of the leptins enabled a calculation of their secondary structure ac-

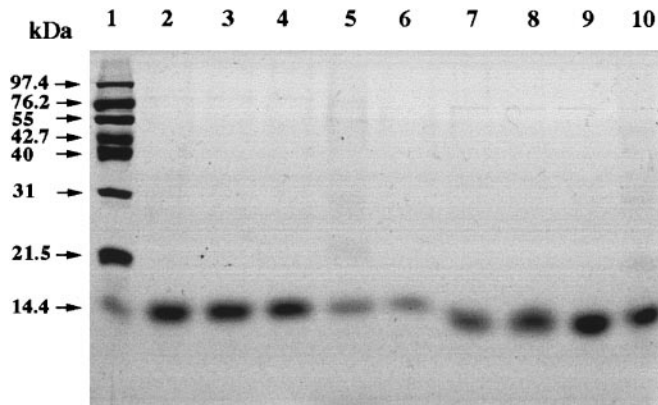


Fig. 3. SDS-PAGE analysis of recombinant chicken leptin C4S analog on a 15% gel run in the presence (lanes 6–10) or absence (lane 2–5) of β -mercaptoethanol. Lane 1, molecular mass markers in kDa; lanes 2 and 7, pool 100 mM; lanes 3 and 8, pool 125 mM; lanes 4 and 9, pool 150 mM; lanes 5 and 10, pool 500 mM; lane 6, chicken leptin.

according to the procedure and computer program CONTIN (20). The program determines α -helix, β -strands, and β -turns as percentage of amino acid residues involved in these ordered forms. The constraint that the sum of all elements of the secondary structure in a protein must be equal to unity resulted in a remainder that might be interpreted as random coil content (20). In the present study, for calculations by the CONTIN program, a set of standard CD spectra of 17 proteins was employed. The results are shown in Table 1. A high α -helix content, no β -strands, and $\sim 10\%$ content of β -turns were clearly characteristic for all the proteins, as expected from the structural analysis of human leptin (22).

Biological Activity In Vitro

The biological activity of the 100 mM NaCl-eluted fraction of chicken leptin analog C4S was compared with that of unmodified leptin and ovine leptin in a BAF/3 proliferation bioassay (Fig. 5). The results were analyzed by PRISM software (7), according to a nonlinear regression sigmoidal dose-response curve. In all

analyses, the degree of match for the nonlinear correlation was very high ($R^2 > 0.97$). EC_{50} for ovine leptin, chicken leptin, and chicken leptin analog C4S (100 mM NaCl-eluted fraction) were, respectively, 2.80×10^{-11} , 2.86×10^{-10} , and 2.45×10^{-10} M. The difference between the unmodified chicken leptin and the chicken leptin analog C4S was not statistically significant. The activity of the chicken analog C4S fractions eluted with 125 and 150 mM NaCl was identical to that eluted with 100 mM NaCl, whereas the activity of the fraction eluted with 500 mM NaCl was 35% lower (not shown).

Biological Activity In Vivo

Experiment 1: Effect of chicken and ovine leptin on food intake in 5-wk-old layer chickens. Five-week-old layer chickens were divided into two groups ($n = 9$) and were treated with saline (placebo) on day 1, according to protocol B. After 90 min of fasting, saline was injected intraperitoneally, and animals were refed 30 min later. Food intake was continuously measured 60 min after the injection, as described in MATERIALS AND METHODS. On day 2, one group was treated with chicken leptin and the other with ovine leptin (both at 1 mg/kg), and, on day 3, the group that had received chicken leptin was treated with ovine leptin and vice versa. Because there was no significant effect for giving ovine before chicken leptin or vice versa (data not shown), the results for each treatment (saline, chicken, or ovine leptin) were pooled, providing 18 animals for each treatment. The data presented in Fig. 6 (mean \pm SE) show food intake every 60 min, chosen from the computerized data. No statistically significant difference was observed within the first 60 min. In contrast, both hormones significantly reduced ($P < 0.05$ to $P < 0.001$) cumulative food intake during the next 2–8 h of feeding (Fig. 6). It should be noted that chicken and ovine leptin had comparable effects, with a mean reduction of ~ 20 – 31% compared with the placebo. The maximum effect was observed after 4 h of refeeding, with a 31.5 and 24.6% reduction in cumulative food intake after chicken and ovine leptin administration, respectively.

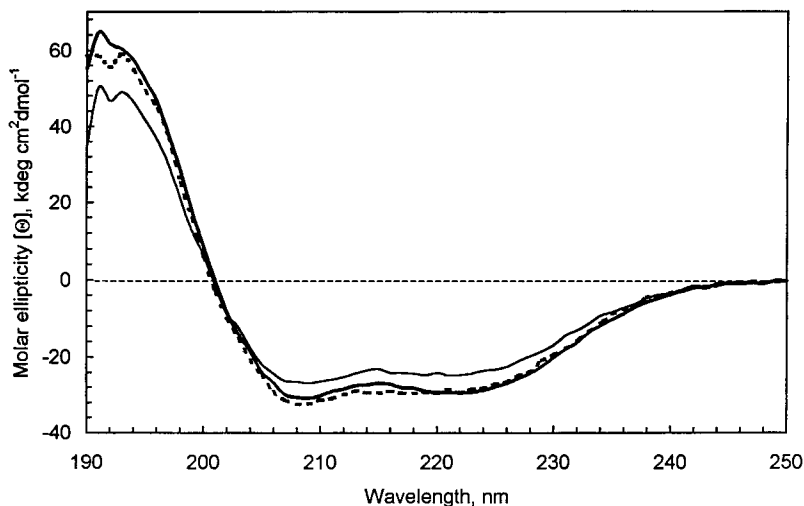


Fig. 4. Circular dichroism spectra of leptins in 60 mM sodium carbonate buffer at 250°C. Solid line, chicken leptin at pH 7.8; bold line, chicken leptin C4S at pH 7.5; broken line, ovine leptin at pH 7.8.

Table 1. *The secondary structure of leptins at neutral pH*

Secondary Structure	Chicken (pH 7.8)	Chicken C4S (pH 7.5)	Ovine (pH 7.8)
α -Helix	71 \pm 0.7	81 \pm 0.6	81 \pm 0.5
β -Strands	2 \pm 0.8	0	0
β -Turns	14 \pm 0.3	10 \pm 0.5	10 \pm 0.5
Remainder	13 \pm 0.5	9 \pm 0.5	9 \pm 0.5

Data are means \pm SD. Errors arose only from an uncertainty of the fitting of the experimental circular dichroism (CD) spectrum by the set of standard protein CD spectra in the CONTIN program. Errors of both the CD measurement and the protein concentration determination were not included.

The cumulative food intake 4 h after the treatment was as follows: controls 14.6 \pm 0.67 g; chicken leptin 10.0 \pm 0.88 g; and ovine leptin 11.0 \pm 0.66 g (mean \pm SE, $P < 0.001$).

Continuous cumulative food intake and accumulated time spent eating were also measured according to protocol B. Figure 7A, which shows the average cumulative food intake (mean of 18 animals obtained from computerized data), indicates that the inhibitory effects of chicken and ovine leptin on food intake began \sim 1,000 s after refeeding and dramatically increased for the first 5,000 s. The significant reduction in cumulative food intake was maintained for 5.5 h after the refeeding. With regard to time spent eating, a similar pattern was recorded (Fig. 7B), with the same maximum effect zone localized within the first 90 min after treatment. Evidently, the time spent eating was consistently reduced by treatment with both chicken and ovine leptins. For instance, the cumulative time spent eating after 2.5 h in groups treated with chicken leptin, ovine leptin, and controls (mean \pm SE) was, respectively, 2,184 \pm 317, 2,385 \pm 318, and 3,119 \pm 392 s. The corresponding values after 4.5 h were 3,592 \pm 525, 4,099 \pm 604, and 5,141 \pm 681 s. These differences at those specific points were significant at $P < 0.05$ (Fig.

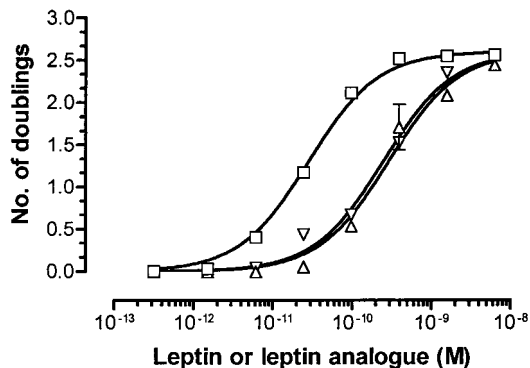


Fig. 5. Biological activity of chicken leptin (Δ), chicken leptin analog C4S (∇), and ovine leptin (\square) in BAF/3 cells transfected with the long form of human leptin receptor. Synchronized cells were grown for 72 h in the presence of various concentrations of the various leptins, and cell counting using a Coulter counter monitored the proliferation. The results represent means \pm SE (3 replicates). In most cases, the SE was too small to be seen. The number of doublings was equal to \log (no of cells in wells treated with leptin/no of cells in wells not treated with leptin)/ $\log 2$.

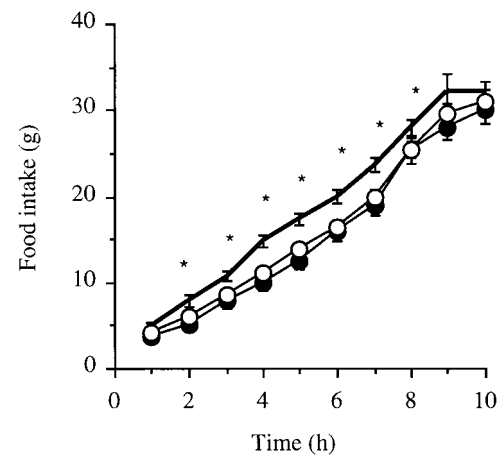


Fig. 6. Effect of intraperitoneally injected chicken (\bullet) or ovine (\circ) leptin or saline (solid line) on cumulative food intake of 5-wk-old layer chickens. Chickens (2 groups of 9 chickens each) were previously accustomed to a 2-h daily fast (0900–1100) for 3 days. On the first day of the experiment, chickens starved for 90 min were injected with saline and refed 30 min later, and food intake was recorded continuously. On the second day, one group was injected with chicken, the other was injected with ovine leptin, and the food intake was recorded similarly. On the third day, the groups that had received ovine leptin received chicken leptin and vice versa, resulting in 18 chickens used for each treatment. Data are presented as means \pm SE and show food intake every 60 min chosen from the computerized data.

7B). For both measurements (food intake and time spent eating), chicken leptin was slightly more potent than ovine leptin, but the difference between the two hormones was not statistically significant (Fig. 7, A and B). The number of approaches to the feeders, defined as uninterrupted period of active pecking at the feed particles lasting >5 s, was not significantly different for the three experimental treatments (Table 2). It should be noted that food intake before leptin injection was almost identical in the three groups used in this experiment.

Experiment 2: Effect of chicken leptin C4S on broiler (9-day-old) and layer (5-wk-old) chickens. Two different chicken strains [broilers (9 days old) and layer chickens (5 wk old)] were treated with either wild-type chicken leptin or its mutated (C4S) analog. In broiler chickens (protocol A), immediately after food was restored, 120-min control-fasted animals began to eat regularly at varying rates throughout the 5-h observation period (Fig. 8A). After intravenous injection of chicken leptin or its C4S analog, cumulative food intake decreased after 30 min, but the difference was not statistically significant. However, during the next 4.5 h, wild-type and mutant chicken leptin significantly ($P < 0.05$ to $P < 0.001$) reduced cumulative food intake (Fig. 8A). Inhibition varied between 11 and 24% for wild-type leptin and between 19 and 34% for mutant leptin. C4S leptin was slightly more efficient than the wild type, but the effect was not statistically significant. After 2 h of treatment, wild-type and C4S leptins inhibited food intake by 23 and 34%, respectively, compared with the controls. This result indicates that Cys4 in the wild-type leptin is not required for biological activity.

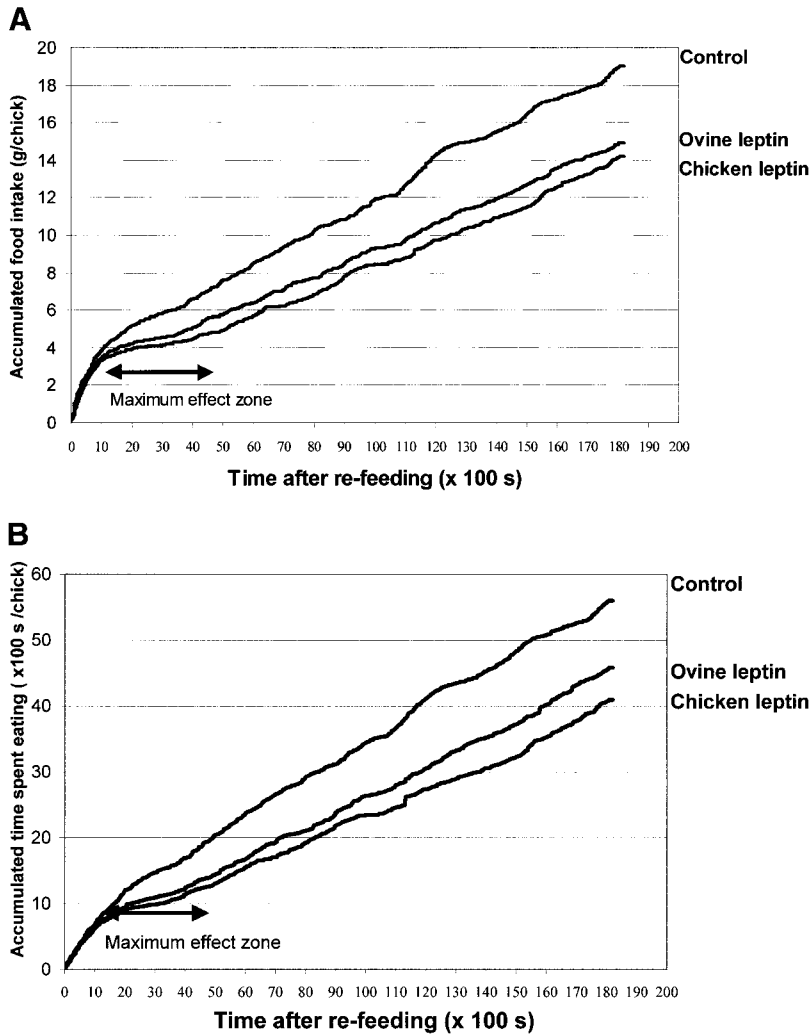


Fig. 7. Effect of intraperitoneally injected chicken leptin, ovine leptin, or saline (control) on cumulative food intake (A) and accumulated time spent eating (B) of 5-wk-old layer chickens. Chickens were accustomed to a 2-h daily fast (0900–1100) for 3 days. On *day 1*, the two groups received an ip injection of isotonic saline solution (placebo), and food intake was monitored continuously for 24 h by a computerized system. On *day 2*, each group received 1 mg of chicken or ovine leptin/kg. On *day 3*, the groups that had received ovine leptin received chicken leptin and vice versa, resulting in 18 chickens used for each treatment. Constantly connected electronic balances monitored feeding activity, and the results show the average continuous food intake or accumulated time spent eating (mean of 18 animals, calculated from the computer-collected data).

To verify whether the anorexogenic effect of chicken leptin and the C4S analog is dependent upon genotype and age, two groups of 5-wk-old male chickens of a layer genotype received either 1 mg chicken leptin or 1 mg C4S/kg body wt by single intraperitoneal injection (*protocol C*). The same groups had been treated 24 h earlier with a placebo (saline), and food intake was measured. Figure 8B shows that, immediately after food was restored after a 120-min fast, both groups began to eat regularly, and the cumulative food intake

of groups treated with chicken leptin or its C4S analog was, respectively, 10.52 ± 2.27 and 9.22 ± 4.04 g (mean \pm SE) within 150 min. The corresponding cumulative food intake determined the previous day was 17.32 ± 2.25 and 15.74 ± 3.32 g, indicating respective decreases of 39 and 41%. The results of that experiment were also analyzed by paired *t*-test, yielding the respective *t* values of 4.24 and 4.29 for the effects of chicken and chicken C4S leptin. Despite the small number of animals, these *t* values are very close to the *t* value of 4.30 that is indicative of statistical significance of the $P = 0.05$ level (degrees of freedom = 2). Because wild-type and mutant leptin gave almost identical results, we have also analyzed the results by pooling both treatments. The corresponding *t* value for paired *t*-test analysis was 5.73 (degrees of freedom = 5), which is indicative of a highly ($P < 0.001$) significant difference.

Table 2. Cumulative number of approaches to feeders after injection of chicken or ovine leptins

Time After Injection, h	Chicken Leptin	Ovine Leptin	Controls
1.5	45.2 \pm 8.0	50.2 \pm 9.5	54.7 \pm 7.7
2.5	70.9 \pm 13.9	80.2 \pm 15.9	84.6 \pm 13.3
3.5	102.1 \pm 19.5	116.2 \pm 22.0	124.6 \pm 18.6
4.5	130.8 \pm 25.6	147.8 \pm 22.0	157.8 \pm 23.9
5.5	164.2 \pm 31.9	185.0 \pm 33.3	192.2 \pm 29.6
6.5	203.1 \pm 37.3	227.3 \pm 40.4	225.8 \pm 34.2

Data are means \pm SE. No statistically significant difference between various treatments was found.

DISCUSSION

Chicken leptin is characterized by the presence of an extra unpaired Cys at position 3 of the mature protein or position 4 in the Ala-leptin produced by us (14). To dem-

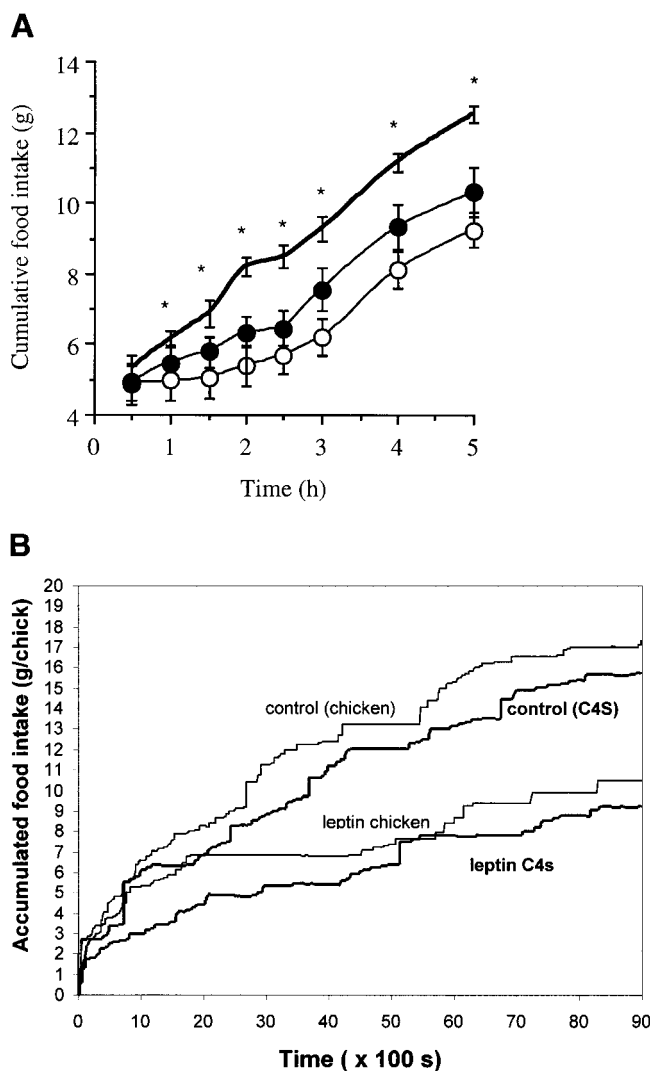


Fig. 8. Effect of chicken leptin and C4S analog on cumulative food intake of 9-day-old broiler chickens (A) and 5-wk-old layer chickens (B). A: chickens were accustomed to a 2-h daily fast (0900–1100) for 3 days. On the day of the experiment, chickens starved for 90 min were injected iv with chicken leptin (●), C4S analog (○), or saline (solid line; time 0) and refed 30 min later, and food intake was recorded every 30 min. Data are presented as means \pm SE; $n = 9$ chickens/treatment. * $P < 0.05$. B: 2 groups of layer chicken were accustomed as described above. On day 1, the two groups ($n = 3$) were treated with saline, and food intake was monitored continuously as described in MATERIALS AND METHODS. On day 2, the groups were treated ip with 1 mg chicken leptin or C4S analog. Results are presented as average cumulative food intake calculated from the computer-collected data.

onstrate the putative role of this Cys, wild-type chicken leptin cDNA was mutated (Cys4 to Ser), and the mutant leptin mass was produced in the same way as the wild-type hormone with a yield of ~ 120 mg/2.5 l fermentation culture. The yield of mutated chicken leptin was similar to that of the unmodified leptin. CD spectra (Fig. 2 and Table 1) indicated proper refolding. The studied leptins did not show β -strand content but had a notable amount of β -turns. According to the Protein Data Bank, human leptin (PDB code: 1ax8) contains 61.0% α -helix, 11.0% β -turns, and no β -strands, as deduced from X-ray data

with 2.40 Å resolution (22). Our data on the secondary structure of leptins correlated with the PDB data for human leptin, albeit with a higher α -helix content. The difference in α -helix content, up to 20% between human and ovine or C4S leptins and 10% between human and chicken leptins, could result from both low resolution of the X-ray data and errors in the CD spectra determinations. Therefore, at present, there is no significant evidence that the secondary structures of the leptins studied in the present work and human leptin differ. In any case, even if such differences do exist, their extent may not exceed 10% of α -helix and $\sim 3\%$ of β -turns. Evidently, chicken leptin, its C4S analog, and ovine leptin do not contain β -strands, and the 2% in chicken leptin is likely to have resulted from a determination error.

The effect of mutated leptin was compared with that of wild-type chicken leptin both in vitro and in vivo. In addition, ovine and chicken leptins were compared because ovine leptin lacks a Cys at position 3. In BAF/3 cells stably transfected with the long form of human leptin receptor, wild-type and mutant chicken leptins exhibited equivalent or similar effects on cell proliferation, whereas ovine leptin was ~ 10 -fold more efficient, in agreement with our former result (14). This indicates that, at least for the purpose of cell proliferation, the Cys3 (or Cys4 in Ala-leptin) in chicken leptin plays no role. Furthermore, the differences between ovine and chicken leptin cannot be attributed to improper refolding of the recombinant proteins but rather to differences in their primary structure, which likely affect their affinity toward human leptin receptor. Because the receptor in BAF/3 cells is a human leptin receptor, this hypothesis can only be confirmed once the chicken leptin receptor has been identified and characterized.

The role of Cys4 in chicken Ala-leptin was also indirectly investigated in vivo by comparing the effects of ovine leptin, chicken leptin, and C4S analog of the latter on reducing in food ingestion capacity. In 5-wk-old layer-genotype chickens, a single intraperitoneal injection of ovine or chicken leptin exerted a significant reduction in food intake in animals being refed after 2 h of fasting (Figs. 6 and 7A). The effect lasted >5 h but disappeared after 9–10 h (Fig. 6). Moreover, both ovine and chicken leptins exerted similar effects on feeding behavior, with a similar impact on time spent eating but with no effect on the number of approaches to the feeders compared with placebo-administered controls (Table 2). Because the total cumulative food intake (Fig. 7A) and the accumulated time spent eating were reduced by both chicken and ovine leptin treatments (Fig. 7B), whereas the number of approaches was not affected by the hormonal treatment (Table 2), we conclude that the reduced feed intake resulted mainly from the decreased average meal duration during each approach and not from the change in meal frequency. This is somewhat different from rodents, in which leptin injection inhibited food intake by reducing the meal size without affecting meal duration or meal frequency (4). Hence, chicken sensitivity toward leptin is clearly demonstrated. In wild-type mice, the effect of

leptin lasted longer (>12 h) but was maintained by daily injections (8–10), with a significant reduction of 35% in food intake after 4 days of treatment (10 mg leptin · kg⁻¹ · day⁻¹). These data show that ovine and chicken leptin are equipotent in reducing food intake and hint at similar affinities toward chicken leptin receptor, although this has yet to be demonstrated. As already shown, the ovine and chicken leptins exhibited different biological activities toward human leptin receptor in a bioassay based on cell proliferation. In addition, this result indicates, at least indirectly, that the presence of Cys4 has no effect. Treatment of two chicken strains differing in their food ingestion capacity further supported this hypothesis, despite the fact that at the same age broiler chickens ingest at least four times more food than layer chickens.

However, it should be noted that, when mouse leptin was injected directly in the chicken central nervous system, no alteration in food intake was observed (2). This observation raises the question if the difference between the present and other (2) results originates from a different way of application or is related to a different source of leptin. The difference between the central and peripheral effects of leptin on food intake may be partially explained by the absence of leptin receptor in chicken brain or by the notion that mouse leptin does not bind to chicken leptin receptor. Another possibility is that the infusion period was too short. For instance, in ewes given intracerebroventricular infusion of ovine leptin, a considerable decrease of activity could be seen only after 72 h (D. H. Keisler and A. Gertler, unpublished data). Moreover, we also suggest that the peripheral leptin injection may allow leptin to bind to binding protein and then act centrally. In addition, a specific peripheral effect of leptin on liver, stomach, and β-cells of the pancreas and others may also play a role in regulating food intake. Indirect evidence also suggests that food intake control in the central nervous system of chickens is slightly different from that in mammals. For instance, electrocoagulation of the ventromedian hypothalamus in chicken or goose (12, 15) induces moderate hyperphagia but not as strongly as in mammals.

In conclusion, the present results indicate that Cys3 in chicken leptin (Cys4 in Ala-leptin) is not crucial for its biological effect in vitro or in vivo. Moreover, the discrepancy between the efficiencies of ovine and chicken leptin in vitro and in vivo suggests that the chicken leptin receptor recognizes both molecules but human receptors discriminate between them. Confirmation of this hypothesis awaits the identification of the chicken leptin receptor.

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