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CORRECTION

Pegylated leptin antagonist with strong orexigenic activity in mice is not effective in chickens

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There was an error published in *J. Exp. Biol.* **217**, 180-184.

The last author's name was spelled incorrectly as M. Freidman-Einat. The correct spelling is above. This has been corrected online, but not in the print version of the article.

We apologise to the readers for any inconvenience this may have caused.

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SHORT COMMUNICATION

Pegylated leptin antagonist with strong orexigenic activity in mice is not effective in chickens

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ABSTRACT

A chicken gene orthologous to human leptin receptor (LEPR) has been characterized and found to be active in leptin signaling *in vitro* in response to a variety of recombinant leptins and leptin-containing blood samples. However, the endogenous ligand of chicken LEPR (cLEPR) – the putative chicken leptin – has been reported by us and others to be undetectable at the DNA, mRNA, protein and activity levels. These reports have raised questions as to cLEPR's role. Here we analyzed the effects of a pegylated superactive mouse leptin antagonist (PEG-SMLA) in chicken. We showed that the leptin antagonist efficiently and specifically blocks leptin signaling through the cLEPR *in vitro*. The effect of the leptin antagonist was then studied *in vivo* by daily administration of 10 mg kg^{-1} for 10 consecutive days to white leghorn female chickens (*Gallus gallus*) at the age of 2 weeks. Despites the efficient attenuation of the cLEPR *in vitro*, no effect was observed on body mass, feed intake, feed efficiency or fat accumulation in the treated birds. Because similar treatment in rodents leads to a highly pronounced increase in appetite and body mass that are observed from the first day of treatment, it is concluded that the cLEPR is not implicated in the control of appetite or adipose homeostasis in chickens.

KEY WORDS: Chicken leptin, Chicken leptin receptor, Leptin antagonist, Energy balance

INTRODUCTION

The mammalian leptin receptor (LEPR) (Tartaglia et al., 1995), a class I cytokine receptor, was cloned a year after discovering its ligand, the satiety hormone leptin (Zhang et al., 1994). LEPR and leptin play a key role in controlling the body's energy balance (Friedman, 2011), and a mutation in either the LEPR or leptin leads to extreme obesity and diabetes in rodents and humans (Farooqi et al., 2007; Montague et al., 1997; Tartaglia et al., 1995; Zhang et al., 1994). Leptin is a 16-kDa peptide hormone secreted by adipose tissue in proportion to its mass. It was cloned by positional cloning as the mutated gene in obese (*ob*) mice (Zhang et al., 1994). LEPR is a single transmembrane receptor with 20 exons and at least six isoforms (LEPRa–f), primarily resulting from alternate splicing (Huising et al., 2006; Tartaglia, 1997). The main (and longest) signaling isoform, LEPRb, is expressed predominantly in the hypothalamus and immune cells, and signals via the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. Although the shorter forms of the receptor are more widely expressed than LEPRb, their precise functions are unknown. A soluble form that lacks all of the intracellular and transmembrane domains has also been characterized and is thought to have a role in the stability and activity of circulating leptin (Schaab et al., 2012; Sinha et al., 1996).

We have cloned, characterized and mapped the chicken LEPR (cLEPR) (Dunn et al., 2000; Horev et al., 2000), which showed only 60% average sequence similarity to the mammalian LEPRs at both the nucleotide and amino acid levels. Nevertheless, similarities in the pattern of mRNA expression, as well as in the number of exons, and the sequences and positions of all of the well-characterized functional motifs and consensus sequences were found also in the cLEPR (Horev et al., 2000). Our mapping of cLEPR to chicken chromosome 8, at a position syntenic to the human LEPR (Dunn et al., 2000), indicated their common origin. The cloning and characterization of cLEPR was also reported by others (Ohkubo et al., 2000), and a highly similar LEPR was also found in turkeys (Richards and Poch, 2003). Furthermore, we and others showed that, when introduced into cell cultures together with a reporter gene, cLEPR specifically activates the reporter gene through the JAK/STAT pathway in response to a variety of leptins (Adachi et al., 2008; Hen et al., 2008), and that a recombinant peptide encompassing the predicted leptin-binding domain of cLEPR specifically binds leptins of several origins *in vitro* (Niv-Spector et al., 2005). However, despite these characteristics of the cLEPR, its ligand seems to be missing from the chicken genome (Friedman-Einat et al., 1999; Hen et al., 2008; Pitel et al., 2010; Sharp et al., 2008). Substantial genomic, transcriptomic and proteomic analyses (Burt, 2006; Carre et al., 2006; Cogburn et al., 2003; Friedman-Einat et al., 1999; Hen et al., 2008; Pitel et al., 2010; Sharp et al., 2008), as well as the aforementioned functional assays in cultured cells (Adachi et al., 2008; Hen et al., 2008; Yosefi et al., 2010), have failed to reveal the leptin gene, mRNA, protein or activity in chicken samples.

In other non-mammalian vertebrates such as fish, lizards and frogs, highly divergent leptin and LEPR genes have been characterized with ~30% sequence similarity, or even less (Boswell et al., 2006; Crespi and Denver, 2006; Johnson et al., 2000; Kurokawa and Murashita, 2009; Kurokawa et al., 2008; Spanovich et al., 2006). Despite this low sequence similarity, cross-binding between the known leptins and LEPR from various sources has been reported in our and others' laboratories (Crespi and Denver, 2006; Hen et al., 2008; Niv-Spector et al., 2005). These reports are compatible with the predicted structures of the known leptins and leptin-binding domains in the LEPRs (Crespi and Denver, 2006; Prokop et al., 2012), showing high structural similarities, although with increased variations in binding properties in fish with multiple leptin proteins (Prokop et al., 2012).

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In the present study, a further step in the effort to reveal the role of cLEPR was taken by using a long-acting leptin antagonist recently developed in our laboratory (Elinav et al., 2009; Mela et al., 2012; Niv-Spector et al., 2012). Because cross-reactivity of the cLEPR with divergent types of leptin, such as human and frog, has been well established (Crespi and Denver, 2006; Hen et al., 2008; Niv-Spector et al., 2005), we expected that injection of leptin antagonist would block, and thereby reveal, the activity of cLEPR in chickens.

RESULTS AND DISCUSSION

To determine whether our well-established leptin antagonist (Benoit et al., 2013; Chapnik et al., 2013; Niv-Spector et al., 2012; Shpilman et al., 2011), which blocks mammalian LEPRs, also blocks the cLEPR, we used our previously described leptin bioassay in HEK-293 cells (Hen et al., 2008; Marikovsky et al., 2002; Yosefi et al., 2010) (Fig. 1). HEK-293 cells expressing exogenous cLEPR and luciferase reporter genes were stimulated with human leptin and with interferon αA (IFNA2), both activating the reporter gene through the cLEPR or an endogenous IFNR, respectively (Hen et al., 2008; Rosenblum et al., 1998). The dose–response curves of luciferase activation by leptin and IFNA2 were used to select a concentration within the linear range (Fig. 1A,B) for stimulation of the reporter gene in the presence of increasing amounts of leptin antagonist (Fig. 1C,D). Luciferase activation by 625 pmol l^{-1} human leptin was completely blocked by 10^4 ng ml^{-1} of the antagonist and significant partial inhibition could be observed at 10^3 and 2×10^3 ng ml^{-1} (Fig. 1C). Under the same assay conditions, the leptin antagonist did not block or inhibit activation of luciferase by IFNA2 (Fig. 1D). This efficient and specific inhibition of cLEPR activation is compatible with the reported resemblance of the tertiary structures of known leptins and binding domains in the known LEPRs (Denver et al., 2011; Prokop et al., 2012), and with our and others' previous results of cross-binding of various leptins to a variety of LEPRs (Crespi and Denver, 2006; Hen et al., 2008; Niv-Spector et al., 2005).

Administration of leptin antagonist to chickens

To explore the leptin antagonist's potential to induce appetite and enhance body growth rate in chickens, as we previously showed in mice (Benoit et al., 2013; Chapnik et al., 2013; Elinav et al., 2009; Niv-Spector et al., 2012; Niv-Spector et al., 2010; Shpilman et al., 2011), lean male chickens at 2 weeks of age were subjected to daily administration of the leptin antagonist. As demonstrated in Fig. 2, during the 10 days of treatment, the pegylated leptin antagonist had no observable effect on body mass (BM), feed intake, feed efficiency (as indicated by feed conversion rate) or fat accumulation.

Injection of 10 mg antagonist per kilogram BM is expected to give a circulating concentration of approximately 80 μg ml^{-1} (based on an estimated blood volume of 8% of total BM). This concentration is 8 times higher than that leading to complete blockage of the cLEPR signaling *in vitro* at the presence of 50 ng ml^{-1} leptin, which corresponds to the circulating level of leptin in obese subjects (Silha et al., 2003). Moreover, the same preparation of the antagonist (Chapnik et al., 2013; Solomon et al., 2013), as well as other preparations (Elinav et al., 2009; Niv-Spector et al., 2012; Shpilman et al., 2011), and a very similar administration protocol led to an approximately 30 to 50% increase in BM and feed intake in rodents at day 10 of treatment, as well as an increase in food intake and fat deposition. The effect on BM and feed intake were significant from the first measurement (the first or second day of treatment). Therefore, the results in Fig. 2 can be taken as an indication that in sharp contrast to the mammalian LEPR, the cLEPR is not implicated in the control of food intake, body growth or fat deposition.

We have previously shown that blocking of leptin activity in rodents by the pegylated leptin antagonist is mediated both by blocking its transport to the brain and by direct blocking of LEPR signaling in the brain (Elinav et al., 2009). In addition, we have demonstrated relatively high levels of the short form of the cLEPR in the choroid plexus [the main site of leptin transport to the brain

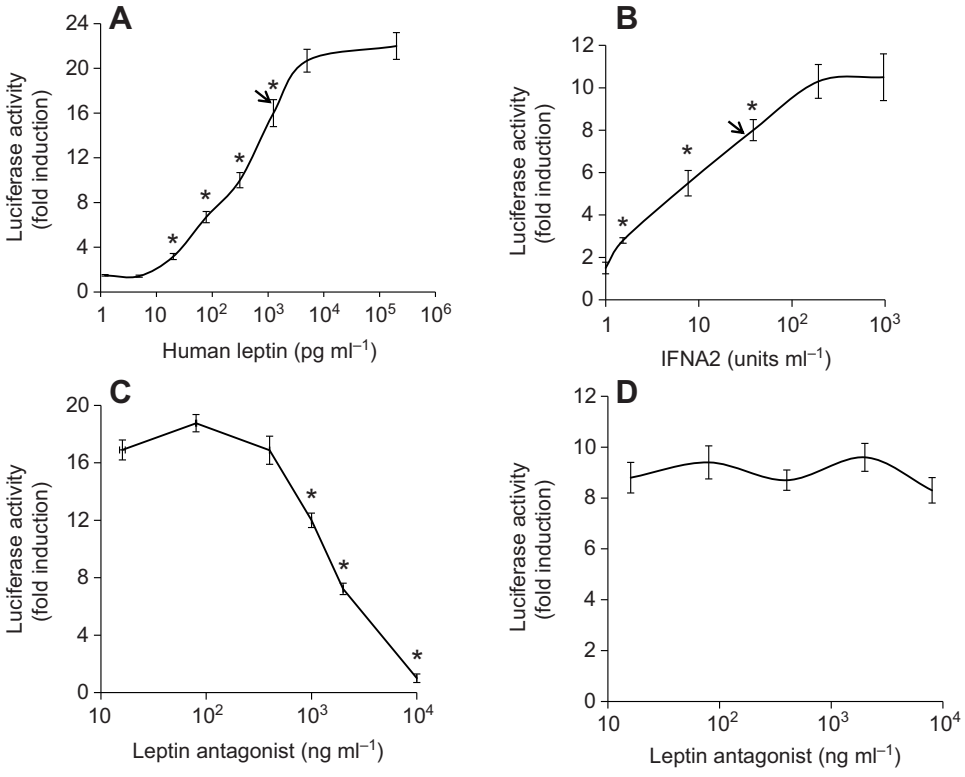

Fig. 1. Inhibition of leptin signaling though the chicken leptin receptor (cLEPR) *in vitro*. HEK-293 cell line stably expressing the cLEPR and STAT3-derived luciferase (described in Materials and methods) was used to establish dose–response curves for leptin (A) and IFNA2 (B), and for analysis of inhibition by the leptin antagonist for human leptin (C) and IFNA2 (D). Human leptin at 50 ng ml^{-1} (625 pmol l^{-1}) and IFNA2 at 100 units ml^{-1} (0.35 ng ml^{-1}; 18.5 pmol l^{-1}), which were at the linear range of the dose–response curves (indicated by arrows), were chosen for the inhibition assays with the indicated amounts of leptin antagonist. Error bars indicate ± s.e., *N*=3. Asterisks indicate significant differences from all other treatments (**P*<0.05). All experiments were repeated twice with similar results, and one representative experiment is shown.

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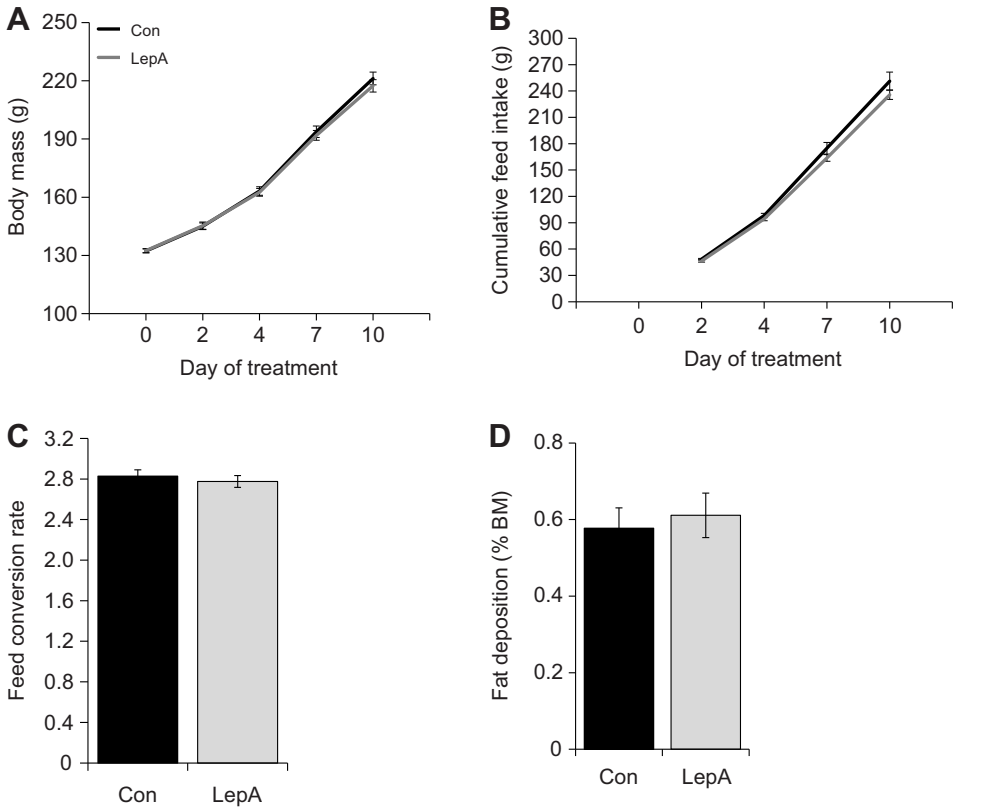

Fig. 2. Leptin antagonist has no effect on feed intake or body growth rate in chickens. Leghorn-type chicks were administered leptin antagonist (LepA) or vehicle control (Con) at 10 mg kg^{-1} body mass (BM) daily, by subcutaneous injection, for 10 days. Body mass (A) and feed intake (B), measured every 2 days, were used to calculate feed conversion rate for the 10 days of treatment (C). Visceral fat deposition (D) was measured on day 10 of the experiment as described in Materials and methods. No significant difference was observed (P<0.05), in contrast to experiments in mice using the same leptin antagonist preparation and a similar experimental protocol (Chapnik et al., 2013; Solomon et al., 2013). The results are presented as means ± s.e.m.; N=8 for each treatment.

(Zlokovic et al., 2000)], and of the long form in the hypothalamus (Horev et al., 2000). This pattern of cLEPR expression, which is highly similar to the LEPR expression in mice (Tartaglia et al., 1995), indicates its accessibility to blocking by the leptin antagonist at the same sites as in mice.

Does the cLEPR have a ligand?

The unsuccessful efforts to identify the ligand of the cLEPR are detailed in the Introduction. While the erroneous identification of the chicken leptin has been removed from GenBank, leptin has recently been annotated in *Taeniopygia guttata* (Gene ID: 101233729), suggesting that leptin is expressed in these birds. However, this gene sequence did not show significant similarity to the published chicken genome sequence or to our own full chicken genome sequencing (at ~20-fold coverage, obtained with an Illumina HiSeq2000 sequencing platform; data not shown). It should be noted that the template DNA for our genome sequencing was of the exact chicken strain used for injection in the present study. Hence, the lack of detectable leptin-like sequences in the genome of these chickens is compatible with the finding of no effect of the leptin antagonist on the indicated energy balance aspects. Nevertheless, it is likely that this receptor, which has retained its functional properties through evolution, may have another role (yet to be determined) that is not equivalent to the crucial function of the mammalian ortholog in the control of appetite and adipose homeostasis.

Conclusions

Our experiments indicate that inhibition of the cLEPR *in vivo* using a highly potent antagonist does not affect feed intake, feed efficiency or fat accumulation in leghorn chickens. The observation is in line with our previous reports demonstrating no circulating leptin activity in fat or lean birds (Hen et al., 2008; Yosefi et al., 2010), as well as with the indication from our and others' laboratories that leptin gene or gene products cannot be detected in chickens (Burt 2006; Carre et al., 2006; Cogburn et al., 2003; Friedman-Einat et al., 1999; Hen et al., 2008; Pitel et al., 2010; Sharp et al., 2008). Therefore, questions about the evolutionary preservation of active cLEPR remain open.

MATERIALS AND METHODS

Animals

Male Lohmann Selected Leghorn chicks [Gallus gallus (Linnaeus 1758)] were purchased from a local breeder (Mekorit, Hasolelim, Israel) at 1 day of age and maintained at the ARO Volcani Center in Bet Dagan, Israel. Lighting and temperature conditions were set to the recommendations of the Lohmann breeding company (Lohmann GB, Worcester, UK). Food (Brown and Sons, Hod Hasharon, Israel) was formulated according to National Research Council (1994) recommendations. Food and water were provided ad libitum. After brooding, the chickens were moved to individual cages. All animal procedures were carried out in accordance with the National Institutes of Health Guidelines on the Care and Use of Animals and approved by the Animal Experimentation Ethics Committee of the ARO, Volcani Center (protocol no. 356-0616).

Leptin antagonist

The leptin antagonist (PEG-SMLA) described by us previously (Elinav et al., 2009; Mela et al., 2012; Niv-Spector et al., 2012; Shpilman et al., 2011) is a pegylated superactive mouse leptin mutant (L23D/L39A/D40A/F41A) that exhibits high affinity to mammalian LEPRs because of the L23D mutation (Shpilman et al., 2011), and an efficient leptin blocking activity because of the three other replacements (L39A, D40A and F41A).

Leptin bioassay

Leptin activity was measured using a bioassay consisting of HEK-293 cells expressing exogenous full-length cLEPR cDNA (Hen et al., 2008) and the firefly luciferase gene under the control of a trimer of the STAT3-binding element. This STAT3 responsive promoter, derived from interferon regulatory factor 1, precedes the herpes simplex virus thymidine kinase

minimal promoter in the reporter construct (Rosenblum et al., 1998). The assay was performed as described previously (Hen et al., 2008; Marikovsky et al., 2002; Yosefi et al., 2010). Briefly, cells were plated in 48-well tissue-culture plates (Nunc, Danyel Biotech, Rehovot, Israel) at a concentration of 2×10^5 cells per well and a final volume of 300 ml of DMEM (Gibco/BRL, Rhenium, Modi'in, Israel). The following day, medium was replaced with fresh DMEM or with medium containing either human leptin or IFNA2, either alone or in combination with the indicated doses of the leptin antagonist. After 4 h incubation at 37°C and 5% CO_2, the medium was aspirated, and the cells were lysed by adding 100 µl Promega cell lysis reagent (Biological Industries, Beit-Haemek, Israel). Luciferase activity was measured using the TD20e luminometer (Turner Designs, Mountain View, CA, USA).

Injection of chickens with leptin antagonist

At 15 days of age, 16 leghorn males were assigned to control and treated groups with similar mean and distribution of BM (132.3±1.02 and 132.4±1.07 g, respectively). Chicks were placed in individual cages to allow for individual measuring of feed intake. Each morning at 09:00 h the birds were injected subcutaneously with either vehicle (control) or leptin antagonist (10 mg kg^{-1} BM; treated). On days 2, 4, 7 and 10, each bird was weighed and its individual feed intake was recorded. At the end of the 10 days treatment (25 days of age), hens were killed and their abdominal fat pads were weighed. Percent of abdominal fat weight was calculated from total BM. Feed conversion ratio was calculated by dividing total net food intake by cumulative net mass gain during the 10 days of treatment.

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Competing interests

The authors declare no competing financial interests.

Author contributions

A.G., M.F.-E. and E.S. were involved in the conception and design of the study. S.Y., D.S., M.S., M.R. and C.I.R. were involved in collecting and interpreting the experimental data. E.S. collected and interpreted the bioinformatic data. M.F.-E. and E.S. wrote the manuscript with editorial input from A.G and C.I.R.

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