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Leptin is synthesized in the liver and adipose tissue of the dunlin (*Calidris alpina*)

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Abstract

Fat is the main source of energy for birds during a long-distance flight. Migration routes are usually divided into several steps. In stopover sites migratory birds restore energy reserves needed for continuation of migration. During a long-distance flight and when foraging at a stopover site birds should be able to assess their actual reserves accumulated in the form of fat stores. The information about energy being stored in body reserves may be provided by circulating factors involved in body mass regulation, such as adipose-derived hormone leptin. To date, little is known about the expression and potential role of leptin in birds. The aim of the present study was to determine whether leptin is synthesized in the liver and adipose tissue of the dunlin (*Calidris alpina*), a long-distance migrant. Western blot analysis with leptin-specific antibody detected a protein with a molecular mass of approximately 15–16 kDa in dunlin liver and adipose tissue. To our knowledge, this is the first report demonstrating leptin expression in the liver and adipose tissue of a migratory bird. This finding raises the possibility that in birds leptin may signal the status of energy reserves during migratory flight.

Keywords: Leptin; Adipose tissue; Liver; Dunlin; *Calidris alpina*; Bird migration

1. Introduction

Leptin, a cytokine-like hormone, has been discovered in rodents by Zhang and co-workers as a 16-kDa protein synthesized primarily in adipose tissue and released into the bloodstream (Zhang et al., 1994). It has been established that in mammals leptin signals body energy status to the central nervous system (the hypothalamus) where it inhibits the synthesis and release of orexigenic peptides, thus reducing food intake (Halaas et al., 1995; Friedman and Halaas, 1998). The effects of leptin on food intake have been documented in rodents and primates (Tang-Christensen et al., 1999; Friedman, 2002). However, little is known about the expression and potential role of leptin in birds. A few years ago, Ashwell et al. (1999a) reported the induction of leptin gene expression in chicken liver and adipose tissue in response to meal feeding. Furthermore, Lohmus et al. (2003) recently demonstrated that leptin administered via intramuscular injection reduced food intake in great tits (*Parus major*). To date, this is the only study concerning leptin in wild birds.

The dunlin (*Calidris alpina*) is a migratory bird that winters thousands of kilometres from its breeding grounds. Such as most long-distance migrants, the dunlin is unable to cross the whole distance between breeding and wintering grounds in one long non-stop flight. Instead, dunlins divide their migration route into multiple steps between sites with abundant food resources. At these stopover sites, they restore energy reserves needed for continuation of migration, accumulating fat as the main source of energy (McLandress and Raveling, 1981; Lindstrom and Piersma, 1993; Jenni and Jenni-Eiermann, 1998). Leptin, regarding its role in mammals, might provide migratory birds with information on their energy reserves. The aim of the

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present study was to determine whether leptin is synthesized in the liver and adipose tissue of the dunlin, a long-distance migrant.

2. Materials and methods

2.1. Animals

Studies on autumn migration of waders were conducted in the mouth of the Reda river (54°21′N, 18°57′E) by the Waterbird Research Group KULING. A fragment of the right lobe of the liver (*lobus hepatis dexter*) and adipose tissue from an interclavicular region were taken from a juvenile male dunlin which died accidentally during ringing. The amount of body fat in the dunlin has been estimated from the regression described by Meissner (1998):

$$\text{fat mass} = 0.51\ \text{total body mass} - 0.15\ \text{wing length} - 0.20\ \text{total head length} + 7.13$$

This equation takes into consideration the well-known relation between the lean body mass of a bird and its linear measurements. The model involving total body mass, wing length, and total head length was chosen because it has the highest coefficient of determination ($R^2 = 0.957$).

The induction of leptin expression in chicken liver and adipose tissue in response to meal feeding has been reported previously (Ashwell et al., 1999a). Thus, in order to verify the specificity of leptin detection, we determined leptin levels in the liver, adipose tissue and muscle of broiler chickens. Since leptin gene expression does not occur in muscle (Ashwell et al., 1999a), we used this tissue as a negative control. White adipose tissue of male Wistar rat was used as an additional positive control.

In our study, six Hubbard broiler chickens were used. They were fed on a starter diet (Cargill Polska, Warszawa, Poland) for 3 weeks. Then chickens were randomly divided into two groups: deprived of food for 12 h (FD group, $n = 3$) and ad libitum fed controls (F group, $n = 3$). The animals were killed by cervical dislocation. All tissue samples were frozen in liquid nitrogen and stored at −80 °C. The experimental protocol was approved by the Local Animal Ethics Committee.

2.2. Tissue homogenisation

Tissue specimens were homogenised in 2 ml of ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 0.2% Triton X-100 and protease inhibitor mixture) per 200 mg of tissue using an Ultra-Turrax homogeniser (IKA-Werke, Staufen, Germany), as described by Kochan (2003). The homogenate was then centrifuged at 20000*g* for 20 min at 4 °C to remove all insoluble material. The supernatant was collected and the protein concentration of each sample was determined by the Bradford assay (Bio-Rad, Hercules, USA) with BSA as a standard.

2.3. Western blot analysis of leptin

Small aliquots of homogenates containing 20 μg of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in 15% gels (Bio-Rad) and transferred to Hybond membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). To check transfer of proteins and assess the uniformity of loading the membranes were stained with Ponceau S (Sigma, St. Louis, USA). After destaining, the membranes were blocked with 1% blocking reagent (Roche Diagnostics, Mannheim, Germany) and subsequently incubated in buffers containing primary polyclonal antibodies against leptin (PA1-051, Affinity Bioreagents, Golden, USA), diluted 1:5000, followed by AP-conjugated secondary antibodies (Sigma), as previously described (Karbowska et al., 2003). Primary PA1-051 antibodies were raised against a synthetic peptide corresponding to amino acids 25–44 of mouse leptin (QKVQDDTKTLIKTIVTRIND). This sequence is highly conserved in vertebrates, including human, mouse, rat, carp, duck, turkey, and chicken. The proteins of interest were detected by enhanced chemiluminescence with CDP-Star as a substrate (Roche Diagnostics). The membranes were exposed to Kodak film (Kodak BioMax Light) for 1–5 min at room temperature. The developed films were analyzed using Quantity One software (Bio-Rad).

3. Results

3.1. Estimation of body fat content in the dunlin

The dunlin had relatively small amount of adipose tissue. Body fat content, as estimated from the equation based on the total body mass, wing length and total head length, was 2.5 g (5.0% of body mass).

3.2. Detection of leptin in the liver and adipose tissue of the dunlin

Western blot analysis of dunlin liver and adipose tissue using leptin-specific antibody detected a protein with a molecular mass of approximately 15–16 kDa (Fig. 1). Notably, leptin levels were higher in the liver than adipose tissue.

3.3. The effect of food deprivation on leptin levels in chicken liver and adipose tissue

To verify changes in leptin concentration in response to food deprivation, we quantified leptin levels in the liver and adipose tissue of chickens deprived of food for 12 h relative to fed chickens (control). Leptin levels determined by Western blot analysis with leptin-specific antibody were approximately 30% lower in livers of food-deprived chickens than those detected in livers of fed (control) chickens. After 12 h of food deprivation leptin levels in the liver decreased from 1 ± 0.037 to 0.66 ± 0.096, $P < 0.05$ (Fig. 2). As shown in Fig. 3, in adipose tissue of chickens deprived of food for 12 h there was a trend for leptin to be lower (0.87 ± 0.09 vs. 1 ± 0.01 in the control group, $P = 0.2182$). The concentration of leptin was higher in the liver than adipose tissue in all chickens analyzed. In contrast, leptin was undetectable in muscle samples from both food-deprived and control chickens (Fig. 1).

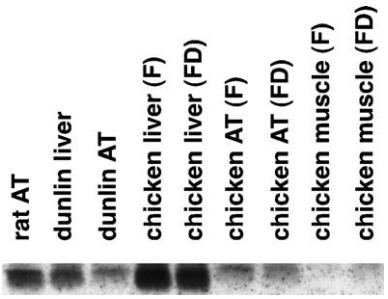

Fig. 1. Leptin detected by Western blot analysis with leptin-specific antibody in the liver and adipose tissue (AT) of the dunlin. To verify the specificity of leptin detection, leptin levels were also determined in the liver and adipose tissue of fed (F) and food-deprived (FD) chickens. Rat adipose tissue provided an additional positive control. Chicken muscle was used as a negative control.

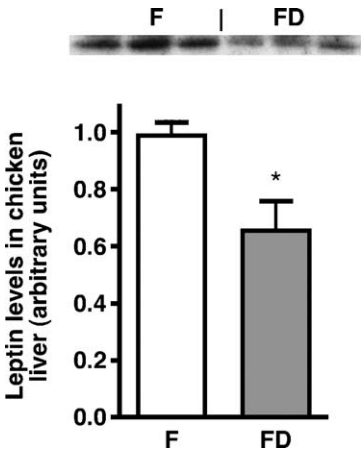

Fig. 2. Leptin levels in livers of food-deprived (FD) and fed (F) chickens. Leptin levels were determined by Western blot analysis with leptin-specific antibody. Loading uniformity was assessed by Ponceau S staining of the membranes after blotting. Data are reported as means ± SEM, $n = 3$; $^{*}P < 0.05$.

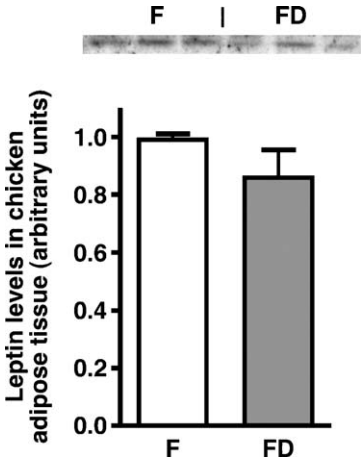

Fig. 3. Leptin levels in adipose tissue of food-deprived (FD) and fed (F) chickens. Leptin levels were determined by Western blot analysis with leptin-specific antibody. Loading uniformity was assessed by Ponceau S staining of the membranes after blotting. Data are reported as means ± SEM, $n = 3$; $P = 0.2182$.

4. Discussion

The body mass of migratory birds is the result of a trade-off between accumulating enough fat to complete their journeys and carrying excessive fat reserves that could reduce flight ability and increase predation risk. Thus, to choose the optimal migratory behaviour birds should be able to assess their body reserves (such as fat stores) during a long-distance flight and when foraging at a stopover site. The information about energy being stored in body reserves may be provided by circulating factors involved in body mass regulation, such as leptin.

Leptin has been characterized as a protein hormone which in mammals is synthesized and secreted primarily by adipocytes in response to increased energy storage in adipose tissue (Zhang et al., 1994; Friedman and Halaas, 1998). Circulating levels of this hormone are associated with body fat and reflect the amounts of fat tissue, i.e., they are reduced by the decrease in fat mass (Maffei et al., 1995; Kochan and Karbowska, 2004). Most of what we know about leptin comes from studies in rodents and humans, whereas the expression pattern and potential role of this hormone in birds remain to be elucidated.

Leptin expression in the liver and adipose tissue of broiler chickens was first reported by Ashwell et al. (1999a). However, leptin synthesis in wild birds has not been studied before. The present study was designed to determine if leptin is expressed in a migratory bird, the dunlin (*Calidris alpina*). Using Western blot analysis with specific antibodies against leptin we demonstrated that leptin is synthesized in the liver and adipose tissue of the dunlin, a long-distance migrant. This finding raises the possibility that leptin may signal the status of body energy reserves and/or may be implicated in regulating foraging behaviour of migratory birds. The effects of leptin on food intake in birds have been documented. In chickens, injection of leptin (both intracerebroventricular and intraperitoneal) resulted in a decrease in food intake (Denbow et al., 2000; Dridi et al., 2000). More recently, Lohmus et al. (2003) demonstrated that the intramuscular injection of leptin reduced food intake in caged great tits (*Parus major*).

Little is known about the hormonal regulation of leptin gene expression in birds. It has been demonstrated that in chickens leptin gene expression is regulated by insulin, which increases leptin synthesis, and glucagon, which reduces leptin gene expression (Ashwell et al., 1999a). Notably, both hormones modified leptin synthesis in chicken liver but not in adipose tissue (Ashwell et al., 1999a,b). In our studies 12-h food deprivation decreased leptin levels in chicken liver by approximately 30% (Fig. 2). In adipose tissue, alterations in leptin levels in response to food deprivation were less pronounced. There was a trend for leptin to be lower in adipose tissue of chickens deprived of food for 12 h (Fig. 3). Further studies are needed to establish whether leptin synthesis and secretion from the liver in birds is under hormonal control and whether leptin levels in adipose tissue depend on body fat reserves. A hormone-independent mechanism of leptin synthesis and secretion from adipose tissue would allow this adipokine to signal the status of energy reserves stored in fat tissue to the hypothalamus. An analogous mechanism has previously been proposed by Levy et al. (2000), who suggested that leptin secretion from mammalian adipocytes is regulated by the amount of triacylglycerols stored in adipose tissue.

To our knowledge, the results presented here are the first experimental evidence that leptin is synthesized in the liver and adipose tissue of a migratory bird. In previous experiments, leptin was found in chicken tissues (Ashwell et al., 1999a,b; Denbow et al., 2000; Dridi et al., 2000). Our results support the idea of leptin as a signalling molecule which in birds may provide information on fat reserves and overall energy status during migratory flight.

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