Leptin reduces Atlantic salmon growth through the central pro-opiomelanocortin pathway

Koji Murashita a,b,⁎, Ann-Elise Olderbakk Jordal a, Tom Ole Nilsen a, Sigurd Olav Stefansson a, Tadahide Kurokawa b, Björn Thrandur Björnsson c, Anne-Grethe Gamst Moen a, Ivar Rønnestad a

a Department of Biology, University of Bergen, Box 7803, NO-5020 Bergen, Norway
b Tohoku National Fisheries Research Institute, Fisheries Research Agency, 3-27-5, Shinhama, Shiogama, Miyagi 985-0001, Japan
c Fish Endocrinology Laboratory, Department of Zoology/Zoophysiology, University of Gothenburg, Box 463, S-405 30 Gothenburg, Sweden

A R T I C L E   I N F O
Article history:
Received 19 June 2010
Received in revised form 6 September 2010
Accepted 7 September 2010
Available online 16 September 2010

Keywords:
Growth rate
Recombinant protein
Appetite
Pro-opiomelanocortin

A B S T R A C T
Leptin (Lep) is a key factor for the energy homeostasis in mammals, but the available data of its role in teleosts are not conclusive. There are large sequence differences among mammalian and teleost Lep, both at the gene and protein level. Therefore, in order to characterize Lep function in fish, the use of species-specific Lep is crucial. In this study, the cDNA sequence of salmon leptin A1 (lepa1) was used to establish a production protocol for recombinant salmon LepA1 (rsLepA1) in Escherichia coli, that enabled a final yield of 1.7 mg pure protein L−1 culture. The effects of 20-day administration of rsLepA1 on growth and brain neuroendocrine peptide gene expression [npy, cart, agrp (-1 and -2), pomic (-a1, -a2, -a2s, and -b)] were studied in juvenile, immature Atlantic salmon (96.5±2.1 g). In the highest dosage group (10 ng g−1 h−1), the growth rate was significantly reduced, and pomic-a1 gene expression was higher than in controls. The results support the lipostatic hypothesis and suggest that lepA1 reduces growth in Atlantic salmon by affecting food intake through the central pro-opiomelanocortin pathway.

© 2010 Elsevier Inc. All rights reserved.

1. Introduction

The non-mammalian leptin (Lep) homolog gene was first identified in the teleost torafugu, Takifugu rubripes, and the major lep expression site was the liver (Kurokawa et al., 2005), whereas it is well known that mammalian Lep is principally produced in adipose tissue (Zhang et al., 1994). In teleosts, there seem to be two main types of Lep genes; a and b type. These have low amino acid (aa) identity and are derived from the whole-genome duplication event in the teleost lineage (Gorissen et al., 2009; Kurokawa and Murashita, 2009). So far lep b has only been identified in medaka, Oryzias latipes (Kurokawa and Murashita, 2009) and zebrafish, Danio rerio (Gorissen et al., 2009). Both these species also have a gene for lepa. In several species a single gene for lepa sequence has been identified, including torafugu, green pufferfish, Tetraodon nigroviridis (Kurokawa et al., 2005), rainbow trout, Oncorhynchus mykiss (Murashita et al., 2008), Arctic char, Salvelinus alpinus (Froiland et al., 2010), silver carp, Hypophthalmichthys molitrix, and grass carp, Ctenopharyngodon idellus (Li et al., 2010). In common carp, Cyprinus carpio (Huisings et al., 2006) and Atlantic salmon, Salmo salar (Rønnestad et al., 2010) two parologue lepa genes with higher identity (82 and 72%, respectively) have been identified.

Pro-opiomelanocortin (POMC) is a prohormone that undergoes extensive post-translational modification to release several smaller, bioactive peptides. These include adrenocorticotropic hormone (ACTH), lipotropin (LPH) and melanophore-stimulating hormones (α-, β-, γ- and δ-MSH) that are characterized by the core amino acid (aa) sequence HFRW and are derived from the ACTH or LPH segments. Corticotropin-like intermediate lobe peptide (CLIP) is also derived from the ACTH, and β-endorphin (β-END) with the core aa sequence YYGF from LPH (Takahashi and Kawauchi 2006). The peptides derived from POMC have a wide range of physiological functions such as steroid synthesis (Lamers et al., 1992), stress response (Slominski et al., 2000), and lipolysis (Yada et al., 2000). Furthermore, POMC plays an anorexigenic role in hypothalamus, through its role as a precursor for the MSH peptide (Nahon 2006). In mammals, Lep acts on anorexigenic pathways in the central nervous system (CNS), and the brain is the major target organ (Klok et al., 2007). In mammals, Lep inhibits food intake through hypothalamic neuropeptides such as agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART), neuropeptide Y (NPY) and POMC (Schwartz et al., 2000). POMC neurons in the hypothalamus contain the long form of leptin receptor (Cheung et al., 1997), and leptin raises mRNA levels of POMC (Schwartz et al., 1997; Thornton et al., 1997; Elias et al., 1998).
Although the tertiary structure of Lep appears to be conserved among mammals and fish, the amino acid conservation is low, with only 22.4 and 24.1% sequence identity between slepa1 and slepa2 and human Lep, respectively (Rønnestad et al., 2010). The large differences among mammalian and fish leptins raise the question whether the physiological functions of leptin are conserved, or if it reflects differential roles in regulation of energy metabolism and, especially, if there are fundamental differences between ectothermic and endothermic vertebrates (Crespi and Denver, 2006). Further, questions have been raised whether there are differences between different fish species due to differences in the physiology of energy balance and life history (Fröiland et al., 2010). Due to these differences, elucidation of Lep function in fish can only be achieved using homologous research tools, and previous studies using mammalian Lep orthologs to examine the relationship between Lep and energy regulation in fish (Londraville and Duvall, 2002; Volkoff et al., 2003; de Pedro et al., 2006) need to be interpreted with caution. Research on rainbow trout and grass carp using homologous Lep proteins shows short-term anorexic Lep effects in both species (Murashita et al., 2008; Li et al., 2010). However, Lep plasma levels increase in food-deprived rainbow trout (Kling et al., 2009), and there is no clear correlation between splea plasma levels and growth rate in Atlantic salmon (Rønnestad et al., 2010). Thus, the role of Lep in the regulation of energy homeostasis in fish is still obscure.

Atlantic salmon is an important aquaculture species, as well as an important model for studies on teleost energy homeostasis. Recently, the genes for a series of appetite-related peptide hormones in Atlantic salmon have been characterized, including agrp (-1 and -2), cart, and npy (Murashita et al., 2008). The aim of the present study was to further elucidate the regulatory role of Lep in Atlantic salmon growth and energy balance, with focus on brain neuropeptides likely to be involved in control of appetite, metabolism and growth. In order to achieve this objective, the present study initially established a protocol for production of recombinant salmon LepA1 (rsLepA1), as this paralogue is highly expressed in tissues involved in energy allocation (e.g. white muscle, liver, visceral fat) while splea2 has a more pronounced expression in the gastrointestinal (GI) tract (Rønnestad et al., 2010). The long-term (20-day) effects of rsLepA1 on growth in Atlantic salmon were examined using intraperitoneal osmotic pumps. In order to get a better understanding the interactions of Lep with brain peptides, three novel pomc transcripts were isolated from Atlantic salmon and analyzed with qPCR gene expression assays together with agrp-1 and -2, cart, and npy.

2. Materials and methods

2.1. Expression and purification of rsLepA1

The rsLepA1 was expressed and purified according to a modified method of Murashita et al. (2008) using slepa1 sequence (GenBank accession no. FJ830677) (Rønnestad et al., 2010). The fragment encoding the putative mature region of sLepA1 was cloned to the N-terminal glutathione S-transferase (GST) fusion protein expression vector (Murashita et al., 2008). The vector was transformed into the BL21-AI Escherichia coli strain (Invitrogen, Carlsbad, CA, USA) for protein expression. Transformed cells were grown in 1 L of Luria-Broth (LB) medium containing 20 μg mL−1 ampicillin at 37 °C. When absorbance at 600 nm reached between 0.5 and 0.8, arabinose was added to the flask to a final concentration of 0.2% to induce the expression of the GST-rsLepA1 protein. Cells were grown for an additional 6 h at 26 °C and collected by centrifugation at 3700 × g for 20 min at 4 °C. The cell pellet was resuspended in 8 mL of Celllytic B (Sigma-Aldrich, St. Louis, MO, USA) and broken up by sonication. Phenylmethylsulfonyl fluoride (PMSF) was then added to a final concentration of 0.2 mM. The soluble (supernatant) fraction was collected after centrifugation at 15,000 × g for 15 min at 4 °C. The GST-rsLepA1 fusion protein was purified from the soluble fraction using Glutathione Sepharose 4B (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and the rsLepA1 was cut off from the fusion protein using PreScission Protease (GE Healthcare Bio-Sciences) by a batch method according to the manufacturer’s instructions. The purified rsLepA1 was dialyzed against 10 mM ammonium bicarbonate, freeze-dried and stored at −20 °C until use. Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) using BSA as a standard. SDS-PAGE was carried out to confirm the expression and purity of rsLepA1.

2.2. Fish and sampling

Juvenile Atlantic salmon postsmolts (mean body mass 96.5 ± 2.1 g) were used to study the effects of long-term rsLepA1 administration on growth and appetite. The fish were reared at the Bergen High-Technology Centre (Bergen, Norway) in a indoor tank supplied with a continuous flow of fresh water at 13.5 °C with continuous light. The fish were fed a commercial dry pellet diet to satiation (EWOS, Bergen, Norway) by an automatic feeder. The fish were weighted and killed by an overdose of MS-222, and the whole brain was collected and stored in RNA Later at −20 °C until RNA isolations were performed.

2.3. Cloning of salmon pomc

cDNA for salmon pomc was cloned from total RNA isolated from salmon pituitary using TRI reagent (Sigma, St. Louis, MO, USA) according to the manufacturer’s instructions. The isolated total RNA was treated with DNase using Turbo DNase (Ambion). The integrity of the RNA was verified by its optical density (OD) absorption ratio (OD260nm/OD280nm) using NanoDrop 1000 (Thermo Fisher Scientific, DE, USA), and samples with a ratio >1.8 were used for the cDNA synthesis. First-strand pituitary cDNA was synthesized from the total RNA using oligo (dT) primer with a reverse transcription kit (Invitrogen). As the Atlantic salmon pomc b sequence was already available in GenBank (accession no: DQ508935), the pomc-a type was targeted. In order to obtain the reading frame of salmon pomc a sequences, one forward primer (SSPOMCA Fw1, Table 1) and two reverse primers (SSPOMCA Rv1 and SSPOMCA Rv2, Table 1) were designed and RT-PCR was performed. The SSPOMCA Fw1 primer was designed based on the rainbow trout pomc-a sequence (GenBank accession no: X69808). The SSPOMCA Rv1 and SSPOMCA Rv2 primers were based on the sequences of Atlantic salmon EST contigs of TC61095 and TC72756 (Atlantic salmon Gene Index, http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=salmon), which have a high degree of similarity to the rainbow trout pomc-a gene. The PCR product was purified from agarose gel using QAguck Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the pCR 4-TOPO vector (Invitrogen). The inserts were sequenced at the University of Bergen Sequencing Facility (Bergen, Norway). One

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSPOMCA Fw1</td>
<td>AAGAAAGACAACTCTCCGAAGAAGAAAAGT</td>
<td>Cloning</td>
</tr>
<tr>
<td>SSPOMCA Fw2</td>
<td>TGAGGCGGAGAGCGGAGAGAG</td>
<td>qPCR for pomc-a</td>
</tr>
<tr>
<td>SSPOMCA Fw3</td>
<td>CTGGAGGCTGAGGACTCCGGA</td>
<td>qPCR for pomc-a2</td>
</tr>
<tr>
<td>SSPOMCA Fw4</td>
<td>AACAGCAAGCTGGGGGCA</td>
<td>qPCR for pomc-a2s</td>
</tr>
<tr>
<td>SSPOMCA Rv1</td>
<td>ATAGCAAGACTAACTCAATTCGCCGG</td>
<td>Cloning</td>
</tr>
<tr>
<td>SSPOMCA Rv2</td>
<td>ACTTATTAAACTAAATCAATGCCTGCA</td>
<td>Cloning</td>
</tr>
<tr>
<td>SSPOMCA Rv3</td>
<td>CCCTCCGGCTCTCTGAGAC</td>
<td>qPCR for pomc-a1/a2/a2s</td>
</tr>
<tr>
<td>SSPOMCA Rv4</td>
<td>GAGTACCAAATGCTCCAGAAGAGAA</td>
<td>qPCR for pomc-b</td>
</tr>
<tr>
<td>SSPOMCA Rv5</td>
<td>GACACGCTGCTGCTGGGGGGA</td>
<td>qPCR for pomc-b</td>
</tr>
<tr>
<td>SSPOMCA Rv6</td>
<td>AGAAGATGCTGCTGCGGGA</td>
<td>qPCR for pomc-b</td>
</tr>
<tr>
<td>SSPOMCA Rv7</td>
<td>AGAAGATGCTGCTGCGGGA</td>
<td>qPCR for pomc-b</td>
</tr>
<tr>
<td>SSPOMCA Rv8</td>
<td>AGAAGATGCTGCTGCGGGA</td>
<td>qPCR for pomc-b</td>
</tr>
<tr>
<td>SSPOMCA Rv9</td>
<td>AGAAGATGCTGCTGCGGGA</td>
<td>qPCR for pomc-b</td>
</tr>
<tr>
<td>SSPOMCA Rv10</td>
<td>AGAAGATGCTGCTGCGGGA</td>
<td>qPCR for pomc-b</td>
</tr>
<tr>
<td>SSPOMCA Rv11</td>
<td>AGAAGATGCTGCTGCGGGA</td>
<td>qPCR for pomc-b</td>
</tr>
</tbody>
</table>
The pomc sequence was obtained from the PCR primer set of SsPOMCA Fw1 and SsPOMCA Rv1, and a further two different pomc sequences were obtained from the PCR primer set of POMCA Fw1 and SsPOMCA Rv2; these were named pomc-a1, pomc-a2 and pomc-a2s on the basis of their sequential similarity to rainbow trout pomc-a1, pomc-a2 and pomc-a2s, respectively (Leder and Silverstein, 2006).

**Fig. 1.** Nucleotide sequence and deduced amino acid sequence of salmon pomc-a1, -a2 and -a2s. A broken line indicates signal peptide, which was estimated using SignalP Ver. 3.0 program (http://www.cbs.dtu.dk/services/SignalP/). Solid lines indicate hormonal segments in POMC. Double underlining indicates the deduced processing amino acid sites, which was estimated according to Takahashi et al. (2006). The core amino acid sequences for MSH (HFRW) and β-END (YGGFM) segments are boxed. The pomc-a2-specific sequence is shaded; which sequence is lacked in pomc-a2s.
2.4. Effect of rsLepA1 on growth in Atlantic salmon

On the initiation of the experiment, 10 fish were euthanized and sampled for analysis (see below) and another 40 fish were anesthetized, weighted and implanted with a micro-osmotic pump (Model 1007D, Alzet, Palo Alto, CA, USA), containing 75 ng μL⁻¹, 750 ng μL⁻¹, 7.5 μg μL⁻¹ of rsLepA1 dissolved in teleost saline (200 mg Na₂CO₃ L⁻¹ of 0.6% NaCl) or teleost saline alone (sham), total volume was 100 μL per pump (10 fish per group). Based on manufacturer’s data, the calculated pumping rates were 0 (sham), 0.1, 1.0 and 10 ng g⁻¹ h⁻¹, respectively. To implant the osmotic pump, an incision (approximately 7 mm) was made in the dorsoventral side of the abdominal musculature surrounding the peritoneal cavity, and the pump was carefully inserted together with a PIT tag (passive integrated transponder, for individual identification,Destron-Fearing, MN, USA) and massaged into the peritoneal cavity of each fish. Fish were allowed to recover and returned to the tank. To compare the groups under identical conditions, all fish were reared in the same tank and fed ad libitum using an automatic feeder. On day 20, fish were euthanized and sampled. The experimental period (20 days) was determined based on estimated osmotic pump duration. No mortality occurred during the experiment. The specific growth rate (SGRw) was calculated as: 100 × [ln final body weight (g) − ln initial body weight (g)]/interval days. Condition factor (CF) was calculated as: [body mass (g)/body length (cm)³] × 100. Hepatosomatic index (HSI) was calculated as: [liver mass (g)/body mass (g)] × 100. Viscera-somatic index (VSI) was calculated as: [internal organ mass (g)/body mass (g)] × 100. The internal organs included heart, gallbladder, GI tract, spleen and fat surrounding the GI tract.

2.5. Effect of rsLepA1 on brain neuroendocrine peptide gene expression

First-strand cDNA of the whole brain was prepared from the rsLepA1 treated fish (see Section 2.4) as described above (Section 2.3). The mRNA levels for salmon npy, cart and agrp (-1 and -2) in brain were analyzed by real-time quantitative RT-PCR (qPCR) according to Murashita et al. (2009). The mRNA for salmon pomc in brain were analyzed by qPCR using SYBR Green assays (Chromo 4 System, Bio-Rad Laboratories Inc., CA, USA) according to the manufacturer’s instructions. Primer sets for the analysis of pomc-a1, -a2 and -a2s were designed in the nucleotide sequence obtained (primer set for pomc-a1, SsPOMCA Fw2 and SsPOMCA Rv3; primer set for pomc-a2, SsPOMCA Fw3 and SsPOMCA Rv3, primer set for pomc-a2s, SsPOMCA Fw4 and SsPOMCA Rv3, Table 1). In addition to the pomc-a isolated, the mRNA level of Atlantic salmon pomc-b was also analyzed. A primer set for the qPCR for pomc-b was designed in the salmon pomc-b nucleotide sequence available in GenBank accession no. DQ508935 (SsPOMCB Fw1 and SsPOMCB Rv1, Table 1). The PCR parameters were 40 cycles at 94 °C for 30 s, 62 °C for 30 s and 72 °C for 30 s for 30 s. Atlantic salmon elongation factor 1α (ef1α; GenBank accession no. AF321836) was also amplified as an internal standard (primer set for ef1α, SsEF1α Fw1 and SsEF1α Rv1, Table 1). The ef1α gene has been shown to be the most stable reference gene in Atlantic salmon (Olsvik et al., 2005), and Ct values for ef1α in this study was confirmed to be stable among

Fig. 2. Alignment of the amino acid sequences of Atlantic salmon POMCs. The core amino acid sequences for MSH (HFRW) and β-END (YGGFM) segments are boxed. The Atlantic salmon POMC-B was referred from GenBank accession no. DQ508935.

Fig. 3. Expression and purification of recombinant salmon leptin. (A) SDS-PAGE stained with Coomassie Brilliant Blue. M, molecular maker; lane 1, un-induced bacterial cells transformed with expression vector; lane 2, induced bacterial cells transformed with expression vector; lane 3, soluble fraction from induced bacterial cells; lane 4, insoluble fraction from induced bacterial cells; lane 5, affinity purified GST-rsLepA1; and lane 6, purified rsLepA1 after digestion with PreScission protease. (B) Reduced and non-reduced purified rsLepA1. A 13.5% gel was used in SDS-PAGE.
the treatment groups (Ct values of ef1a/amount of RNA used, one-way analysis of variance). Non-amplification of negative control (non-reverse transcribed RNA sample) from each qPCR primer set was also confirmed.

2.6. Statistical analysis

The results were analyzed by one-way analysis of variance (ANOVA) followed by Turkey’s multiple comparison test using InStat 3.0 (GraphPad Software, CA, USA). The results were considered statistically significant at \( p < 0.05 \). Correlation coefficient between the delivered leptin concentration and the SGRw, CF, HSI or VSI was analyzed using Microsoft Office Excel (Microsoft, WA, USA).

3. Results

3.1. cDNA identification of salmon POMCs

From the PCR, full-length cDNA sequences of pomc-a1, pomc-a2 and pomc-a2s were obtained. The Atlantic salmon pomc-a1 nucleotide sequence (GenBank accession no. AB462418) was 1153 bp in length, and contained a 90 bp of 5′-untranslated region (5′-UTR), a 364 bp of 3′-untranslated region (3′-UTR) and a reading frame (RF) of 699 bp encoding a prepro-POMC-A1 with 232 aa (Fig. 1). The Atlantic salmon pomc-a2 nucleotide sequences (GenBank accession no. AB462419) was 991 bp in length, and contained a 103 bp of 5′-UTR, a 189 bp of 3′-UTR and a 699 bp of RF encoding a prepro-pomc-a2 with 232 aa (Fig. 1). The salmon prepro-pomc-a2s (922 bp, GenBank accession no. AB462420) nucleotide encoded a protein with 209 aa residues, which was identical to the prepro-pomc-a2 sequence except for the deletion of 69 nucleotides, which encode 23 amino acid residues (Fig. 1). The salmon POMC-A1, POMC-A2 and POMC-A2s contained the 21-residue putative signal peptide. The core sequences for MSH (HFRW) and \( \beta \)-END (YGGFM) segments were well conserved in the salmon POMC (Figs. 1 and 2).

3.2. Expression and purification of the rsLepA1

The rsLepA1 was expressed in BL21-AI E. coli cells following arabinose induction and purified. A unique band of GST-rsLepA1 fusion protein was observed in induced bacterial cells in comparison of uninduced bacterial cells (Fig. 3A, lanes 1 and 2). Most of the expressed GST-rsLepA1 fusion protein was found in the soluble fraction compared with the insoluble fraction (Fig. 3A, lanes 3 and 4). The GST-rsLepA1 fusion protein was purified by affinity purification (Fig. 3A, lane 5). The subsequent purification with PreScission protease produced pure rsLepA1, and the band was observed around the estimated rsLepA1 molecular weight (17.5 kDa) from the deduced amino acid sequence (Fig. 3A, lane 6). Non-reduced (without 2-mercaptoethanol) purified rsLepA1 band was found in to have a smaller size compared with reduced (with 2-mercaptoethanol) rsLepA1, which indicates that the purified rsLepA1 was successfully folded (Fig. 3B). The production of rsLepA1 yielded 1.7 mg pure protein L\(^{-1}\) culture of E. coli.

3.3. Effect of rsLepA1 on growth

The SGRw of the highest rsLepA1 dosage (10 ng g\(^{-1}\) h\(^{-1}\)) group was significantly suppressed compared with the sham-treated group (Fig. 4A), while HSI of the 10 ng g\(^{-1}\) h\(^{-1}\) dosage group was higher than that of the controls (Fig. 4C). There were no significant differences among the groups in CF and VSI (Fig. 4C and D). There was a significant negative correlation \( (R = -0.804; P < 0.01) \) between rsLepA1 dose and SGRw. A positive correlation \( (R = 0.461; P < 0.01) \) was found between rsLepA1 dose and HSI, and a negative correlation

Fig. 4. Effect of rsLepA1 on growth. (A) SGRw, (B) CF, (C) HSI, and (D) VSI. Bars with different letters are significantly different \( (p < 0.05) \). Data presented as mean ± SEM \( (n = 7) \).
with CF (R = -0.382; P = 0.015). There was no significant correlation between leptin dose and VSI.

3.4. Effect of rsLepA1 on brain-gut neuroendocrine peptide gene expression

The brain pomc-a1 mRNA levels were significantly increased in the highest rsLepA1 dosage (10 ng g\(^{-1}\) h\(^{-1}\)) group (Fig. 5), but none of the rsLepA1 treatments significantly affected gene expression of pomc-a2, pomc-a2s, pomc-b, agrp-1, agrp-2, npy, cart in the brain (Fig 5).

4. Discussion

To further explore the role of Lep in Atlantic salmon, recombinant sLepa1 was produced and used to examine its long-term effects on growth. The highest (10 ng g\(^{-1}\) h\(^{-1}\)) sLepA1 dosage for 20 days significantly decreased growth rate. The dosage compares well with mammalian studies (rodents), where effective Lep delivery using osmotic pump peripheral infusion is usually 15-20 ng g\(^{-1}\) h\(^{-1}\) (Rousseau et al., 2005; Harris et al., 2007; Kusakabe et al., 2009).

Lep treatment reduces food intake and thereby body weight in many mammalian species such as rats, mice, pigs and monkeys.

Fig. 5. Effect of rsLepA1 on brain appetite-related genes. The salmon brain agrp (-1 and -2), npy, cart, pomc (-a1, -a2, -a2s and -b) gene expression levels were measured. The mRNA levels were normalized as the average of saline dosed fish mRNA levels = 1. Bars with different letters are significantly different (*p < 0.05, **p < 0.01). Data presented as mean ± SEM (n = 7).
(Seeley et al., 1996; Sahu 1998; Sahu 2004; Wetzler et al., 2004), and the Lep-induced decrease in growth rate in the present study suggests that the role of LepA1 is at least to some extent comparable to that in mammals. This is in contrast with a study on coho salmon (Oncorhynchus kisutch), where human Lep did not cause changes in body weight or any of the assessed physiological parameters (Baker et al., 2000). A likely reason for this discrepancy was the use of human Lep in the coho salmon study with its low sequence identity with salmon Lep.

The present study identified cDNA sequences for several novel pmc genes (pmc-a1, -a2 and -a2s) in Atlantic salmon. The salmon pmcs identified in the present study were well conserved with POMC features such as (α- and β-) MSH, CLIP, and β-END (Figs 1 and 2). In mammals, Lep decreases food intake through hypothalamus orexigenic NPY/AgRP and anorexigenic POMC/CART neurons (Schwartz et al., 2000). Lep decreases hypothalamic NPY/AgRP mRNA and increases POMC/CART mRNA (Stephens et al., 1995; Schwartz et al., 1997; Korner et al., 2001; Kristensen et al., 1998). In a previous study, intraperitoneal injection of recombinant rainbow trout Lep reduced short-term food intake with increasing hypothalamic pmc expression (Murashita et al., 2008). Increased pmc-a1 levels were also found by 20-day treatment in the present study, which suggests that Lep decreases food intake in salmonids through the pmc pathway as indicated in the short-term treatment Lep study of rainbow trout (Murashita et al., 2008). However, the 20-days rsLepA1 treatment did not affect npy, agpr and cart expression in the current study. This use of the whole brain in the current study might have masked regional differences within the brain, since many of these peptides have appetite-related functions localized to the hypothalamus. It is also possible that there are responses of genes that only are apparent on shorter timescales of hours–days, and are not observed in longer-term, such as the current 20-day experiment. Long-term fasting increases plasma leptin levels in rainbow trout (Kling et al., 2009). In carp, lep mRNA levels do not respond to long-term fasting, but show an increase after feeding (Huisings et al., 2006). Furthermore, long-term treatment of grass carp with species-specific Lep does not affect food intake and body weight, whereas a short-term treatment decreases food intake (Li et al., 2010). This suggests that species differences may exist among fish groups in regard to Lep function. It should be noted that food intake was not quantified in the present study. However, quantification of the food intake is an important experimental parameter in order to reveal the underlying mechanisms of energy regulation and the involvement of Lep and should be included in new studies.

In the context of energy balance, fat distribution and related regulatory mechanisms in teleost fish, it should certainly be noted that there are at least two major strategies. “Fatty” or “oily” fish, which include salmon, tuna, herring, mackerel and sardines, store large amount of fat in the muscle tissue, with fillet fat content up to 30%, whereas “lean” fish or “whitefish” such as gadoids and carp have much less total as well as muscle fat, and store most of the body fat in the liver (Oehlenschläger and Rehbein, 2009). The relative liver size (HSI) in salmon is in general lower than in carp (Nordgarden et al., 2002; Young et al., 2006; Hossaina et al., 2001a, b) whereas muscle fat content in salmon (12–17%) is much higher than in carp (0.6–6.9%) (Young et al., 2005; Wang et al., 2005; Kocour et al., 2007; Bauer and Schlott, 2009). Moreover, lep is highly expressed in liver and muscle in salmon (Rønnestad et al., 2010) while the lep expression levels in muscle in carp are much lower than in liver (Huisings et al., 2006). Muscle may be a more important part for energy storage in salmon than carp, and this is reflected in the lep expression profiles.

In this study, rsLepA1 led to an increased relative liver size. In mammals, leptin activates enzymes involved in catabolism of carbohydrates and lipids, and increases the metabolic rate through uncoupling protein (UCP) (Shimabukuro et al., 1997; Zhou et al., 1997; Cusin et al., 1998; Unger et al., 1999). In grass carp, acute injection of recombinant grass carp Lep increases mRNA levels of ucp-2 (Li et al., 2010). In Atlantic salmon, although the liver has a high expression of slepa1, the long-form lep receptor (slep) has only very low expression levels in the liver (Rønnestad et al., 2010). It is therefore possible that as a long-term effect, LepA1 in salmon might work on energy catabolism in non-liver organs and tissues, and may explain the apparent HSI increase in this study since liver has fewer slep. In fact, VSI did not change among the groups though SGWR decreased by leptin implanting; this means weight gain of the internal organs also was reduced together with whole fish BW, while the liver weight was not, so the HSI increased in the Lep treated fish. A study in frog showed that in the early larval–premetamorphic stages the expected anorectic effect of Lep was not apparent and this was explained by the underdeveloped state of fat stores and the neuroendocrine system (Crespi and Denver, 2006). The fish used in the current study were juveniles with a fully developed neuroendocrine system and these data are therefore not directly comparable. However different life stages and strategies may strongly affect the effects of Lep. Many teleosts, including Atlantic salmon, may fast for extended periods in specific life stages and the different demands for allocation of energy to growth and energy stores may have unexplored effect on Lep function.

In conclusion, the rsLepA1 which was successfully produced in E. coli, reduces SGWR of Atlantic salmon when infused intraperitoneally over 20 days at a rate of 10 ng g⁻¹ h⁻¹. Moreover, this Lep treatment increases gene expression of the newly identified salmon pmc-a1. The rsLepA1 will be a useful tool for the continued elucidation of leptin function in Atlantic salmon. However, as a closely-related paraloge (slepa2) is also found in salmon (Rønnestad et al., 2010), additional work is required to explore the possible regulatory divergence of the two forms. Taken together, the present study indicates that salmon LepA1 may have an anorexigenic role in the regulation of body weight in Atlantic salmon that compares with that in mammals.

Acknowledgements

We thank Frank Midtøe and Tharmi Kalanathan (BIO, UiB) for the assistance during the experiment. This work has been supported by a research fellowship of the JSPS for Young Scientists to KM, Research Council of Norway grant #172548/40 (IR and SOS), UiB and HelgøeVest grant to IR (NettMettBac), in part by “The promotion of basic research activities for innovative biosciences” of Bio-oriented Technology Advancement Institution (BRAIN), Japan to TK, and in part by the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas) grant #2008-1258 (BThB). The research leading to these results has also received funding from the European Community’s Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 222719 – LIFECYCLE (IR, SOS and BThB).

References


