Peptides 32 (2011) 232–240

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

# *In vitro* leptin treatment of rainbow trout hypothalamus and hindbrain affects glucosensing and gene expression of neuropeptides involved in food intake regulation

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ARTICLE INFO

*Article history:*
Received 15 October 2010
Received in revised form 8 November 2010
Accepted 8 November 2010
Available online 18 November 2010

*Keywords:*
Trout
Glucosensing
Leptin
Hypothalamus
Hindbrain
CART
POMC
CRF
NPY

ABSTRACT

The aim of the present study was to evaluate in hypothalamus and hindbrain of rainbow trout *in vitro* the effect of leptin treatment on glucosensing capacity and the expression of orexigenic and anorexigenic peptides involved in the control of food intake. In a first experiment, the response of parameters involved in glucosensing (GK, PK and GSase activities; GK expression and glucose; glycogen and DHAP levels) and the expression of orexigenic (NPY) and anorexigenic (POMC, CART, CRF) peptides was assessed in hypothalami and hindbrain incubated for 1 h with 2, 4 or 8 mM D-glucose alone (controls) or with 10 nM leptin, or with 10 nM leptin plus inhibitors of leptin signaling pathways (50 nM wortmannin and 500 nM AG490). Leptin treatment increased levels in parameters involved in glucosensing. Leptin treatment decreased NPY mRNA levels in hypothalamus without affecting the expression of the other peptides assessed. Leptin effects were reverted in the presence of inhibitors for all parameters assessed suggesting the involvement of JAK/STAT and IRS-PI(3)K pathways. In a second experiment, we observed time-dependent (1–3 h) and dose (10, 20 and 50 nM)- effects of leptin treatment in decreasing NPY mRNA levels without affecting expression of the other peptides assessed. Considering the orexigenic action of NPY in fish, it seems that the anorexic effect of leptin can be mediated by reduced expression of NPY occurring in hypothalamus, and that change can be related to the activation of the glucosensing system occurring simultaneously.


## 1. Introduction

In mammals, brain regions including hypothalamus, medulla oblongata and mesencephalon integrate endocrine and metabolic information to elaborate a coordinated response using neural effectors pathways that produce factors that either stimulate (orexigenic) or inhibit (anorexigenic) food intake [21]. These areas contain specialized neurons that through glucokinase (GK) activity utilize glucose as a signaling molecule [17]. Thus, glucose-excited (GE) neurons increase, whereas glucose-inhibited (GI) neurons decrease their firing rate as glucose levels rise [17,21]. In previous studies, we provided evidence for the existence of glucosensor systems in hypothalamus and hindbrain of a carnivorous teleost species like the rainbow trout [29] being activated when glucose levels increase in parallel with decreased food intake; conversely, when glucose levels decrease the glucosensors are inactivated and food intake increases [31,32]. The mechanisms through which these glucosensor systems operate are similar to those described in mammals in brain regions though quite different in pancreatic cells [30]. We also provided information for the molecular characterization of these systems [32,33], their specific location in hypothalamus [34], their responses under stress conditions [5], and their possible presence in gut [35].

Mammalian glucosensing neurons are considered as metabolic sensors since besides glucose they also respond to a host of other metabolic, hormonal and neural signals, including fatty acids, lactate, monoamines, GABA, vasopressin and oxytocin, leptin, insulin, catecholamines, serotonin, opioids and glutamate [17]. Leptin has been shown to interact with glucosensing neurons in mammalian brain, modulating their functions, and acting as an indicator of the energy status of the organism [21].

In fish, leptin gene has been identified in several species [11–13,19,36], and there is evidence for the expression of leptin receptors in different tissues including brain [15,20,36,46]. The effect of leptin treatment on food intake is somewhat controversial but appears to be inhibitory in most species [1,7,24]. We demonstrated in a previous study that ICV leptin treatment in rainbow trout induced in hypothalamus and hindbrain dose-dependent changes in parameters related to glucosensing (GK activity and expression, glycogen and glucose levels, etc.) comparable with those occurring under hyperglycemic conditions, a situation that is known to produce anorexia, the same effect than that produced by ICV leptin treatment in the same species [1]. However, we cannot eliminate that those effects of leptin could be attributed to an

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doi:10.1016/j.peptides.2010.11.007

indirect effect mediated by changes in other hormone systems. Moreover, we cannot also discard that changes were dependent on glucose concentration since in mammals leptin effects may be dependent upon ambient glucose concentration [18].

It is well known in mammals that neurons in glucosensing areas produce peptides involved in the control of food intake [39]. Thus, for instance neurons from the arcuate nucleus in the hypothalamus producing NPY/AgRP are GI whereas neurons producing POMC/CART are GE [9] resulting in increased expression of POMC and CART and decreased expression of NPY and AgRP when glucose levels rise [4,22]. We recently obtained evidence in rainbow trout for changes in the expression of several neuropeptides involved in the regulation of food intake in central glucosensing areas (hypothalamus and hindbrain) when rainbow trout was subjected to changes in glycemia [6] including decreased mRNA levels of NPY and increased levels of CART and POMC mRNA in hypothalamus of hyperglycaemic fish, whereas in hindbrain increased mRNA levels of CART and CRF were also noted. In mammals, leptin influences the production of neuropeptides in glucosensing neurons of hypothalamus and hindbrain [39] inhibiting activity of orexigenic (NPY, AgRP) and stimulating activity of anorexigenic (POMC, CART) neurons [8,23]. In fish, the available studies were carried out under *in vivo* conditions demonstrating that ICV injection of leptin reduces NPY mRNA levels in the hypothalamus and telencephalon of goldfish [44] or in the whole brain of grass carp [19] whereas in rainbow trout IP injection of leptin induced in hypothalamus a transient reduction and elevation of NPY and POMC mRNA levels, respectively [24]. Since all those studies were carried out *in vivo* the possibility that the effects of leptin in peptide expression can be attributed to an indirect action through other hormone systems cannot be discarded.

Therefore, we aimed in the rainbow trout (1) to assess *in vitro* (under different glucose conditions) the responses of parameters involved in glucosensing in hypothalamus and hindbrain to leptin treatment to corroborate previous results obtained *in vivo* [1], and (2) to evaluate under those conditions the effect of leptin treatment in the expression of those orexigenic (NPY) and anorexigenic (POMC, CART, CRF) peptides involved in the control of food intake in fish [43] whose expression is known to change in hypothalamus and hindbrain of rainbow trout when subjected to different glycemic conditions [6].

## 2. Materials and methods

### 2.1. Fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) were obtained from a local fish farm (Soutorredondo, Spain). Fish were maintained for 1 month in 100 l tanks under laboratory conditions and a natural photoperiod in dechlorinated tap water at 15 °C. Fish mass was 112 ± 3 g. Fish were fed once daily (10:00 AM) to satiety with commercial dry fish pellets (Dibaq-Diprotg SA, Segovia, Spain; proximate food analysis was 48% crude protein, 6% carbohydrates, 25% crude fat, and 11.5% ash; 20.2 MJ/kg of feed). The experiments described comply with the Guidelines of the European Union Council (2010/63/EU), and of the Spanish Government (RD 1201/2005) for the use of research animals.

### 2.2. Experimental protocol

#### 2.2.1. Experiment 1: effects of leptin treatment alone and leptin plus inhibitors of leptin signaling on glucosensing function and neuropeptide expression

Every morning of experiment, fish were dipnetted, anaesthesized with MS-222 (50 mg $l^{-1}$) buffered to pH 7.4 with sodium bicarbonate, weighed and sacrificed by decapitation. Hypothalamus and hindbrain were dissected as described previously [30,40]. Tissues were washed with modified Hank's medium (92.56 mM NaCl; 3.63 mM KCl, 2.81 mM $NaHCO_3$, 0.85 mM $CaCl_2$, 0.55 mM $MgSO_4$, 0.4 mM $KH_2PO_4$, 0.23 mM $Na_2HPO_4$, 7.5 mM HEPES, 50 U $ml^{-1}$ penicillin, and 50 mg $ml^{-1}$ streptomycin sulfate, pH 7.0; referred to a basal medium), sliced in chilled Petri dishes, placed in a chilled Petri dish containing 100 ml of modified Hank's medium $g^{-1}$ tissue, and gassed with a 0.5% $CO_2$/99.5% $O_2$ mixture. In order to have enough mass, tissues were pooled from different fish resulting in pools of 3–4 hypothalami and 3–4 hindbrains. On each pool, tissue was finely sliced and mixed and then placed in 48-well culture plates (25 mg of tissue in 250 μl of modified Hank's medium per well).

On each experiment, freshly obtained tissues were incubated as previously described [30] in 48-well culture plates at 15 °C for 1 h with 250 μl of modified Hank's medium per well containing 25 mg of tissue that was gassed with a 0.5% $CO_2$/99.5% $O_2$ mixture. In control wells, medium contained three different concentrations of D-glucose: 2, 4 and 8 mM (indicative of hypo-, normo- and, hyperglycaemic conditions in rainbow trout). In treated wells medium contained the same glucose concentration and 10 nM human leptin (L, leptin from Sigma Chemical Co.) alone, or 10 nM leptin plus a phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor (50 nM wortmannin; L+W), or 10 nM leptin plus a Janus kinase (JAK) type 2 inhibitor (500 nM AG490; L+A). The inhibitors were added 15 min prior to the addition of leptin. The concentration of leptin was selected according to previous *in vitro* studies carried out in fish [26,27] whereas those of the different inhibitors used were selected on the basis of *in vitro* studies carried out in mammals [23,37,45]. No effects on the parameters assessed were observed due to the vehicle alone (data not shown) or to the action of the different inhibitors (wortmannin and AG-490) alone (data not shown). After 1 h incubation, tissues were quickly removed, freeze-clamped in liquid nitrogen, and stored at −80 °C until assayed.

On each experiment, one set of 12 tissue pools per tissue were assessed (4 treatments × 3 glucose concentrations) for enzyme activities (GK, GSase and PK), whereas another set of 12 tissue pools were used for the assay of tissue metabolites (glucose, glycogen and DHAP levels). Another set of 12 tissue pools were used for the assay of mRNA levels (GK, NPY, POMC, CART, and CRF). The number of independent experiments (one set of 12 tissue pools each) carried out was four ($N = 4$).

#### 2.2.2. Experiment 2: dose-dependent effects of leptin on neuropeptide expression

Hypothalami and hindbrains were obtained and incubated with different D-glucose concentrations (2, 4, and 8 mM) as described in experiment 1, but incubated for 1 h or 3 h with increased leptin concentrations: 10, 20 and 50 nM. After incubation, tissues were sampled and processed as described above. On each time (1 or 3 h), one set of 12 tissue pools were assessed (4 treatments × 3 glucose concentrations) for mRNA levels (GK, NPY, POMC, CART, and CRF). The number of independent experiments carried out was four ($N = 4$).

### 2.3. Assessment of metabolite levels and enzyme activities

The pieces of tissue used for the assessment of metabolite levels were homogenized immediately by ultrasonic disruption with 7.5 vol. of ice-cooled 6% perchloric acid, and neutralized (using 1 mol $l^{-1}$ potassium bicarbonate). The homogenate was centrifuged, and the supernatant used for assays. Tissue glycogen levels were assessed using the method of Keppler and Decker [14]. Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined with a commercial kit