

# Leptin stimulates pituitary prolactin release through an extracellular signal-regulated kinase-dependent pathway

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## Abstract

Leptin was initially identified as a regulator of appetite and weight control centers in the hypothalamus, but appears to be involved in a number of physiological processes. This study was carried out to examine the possible role of leptin in regulating prolactin (PRL) release using the teleost pituitary model system. This advantageous system allows isolation of a nearly pure population of lactotrope cells in their natural, *in situ* aggregated state. The rostral pars distalis were dissected from tilapia pituitaries and exposed to varying concentrations of leptin (0, 1, 10, 100 nM) for 1 h. Release of PRL was stimulated by leptin in a potent and concentration-dependent manner. A time-course experiment showed that the strongest response in PRL release with leptin occurs within the first hour (approximately sixfold), and stimulation was sustained after 16 h (approximately twofold). Many of the actions of

leptin are mediated by the activation of extracellular signal-regulated kinase (ERK1/2) but nothing is known about the cellular mechanisms by which leptin might regulate PRL secretion in vertebrates. We therefore tested whether ERK1/2 might be involved in the leptin PRL response and found that the ERK inhibitor, PD98059, hindered leptin-induced PRL release. We further analyzed leptin response by quantifying tyrosine and threonine phosphorylation of ERK1/2 using western blots. One hour incubation with leptin induced a concentration-dependent increase in phosphorylated, and thus active, ERK1/2. Our data show that leptin is a powerful stimulator of *in vitro* PRL release and that its actions occur in part through stimulation of ERK1/2.

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## Introduction

Among the pituitary hormones, prolactin (PRL) is the most versatile in the spectrum and number of functions it regulates. PRL modulates virtually every aspect of vertebrate physiology, including osmoregulation, growth, metabolism, development, reproduction, parental behavior, and immune function (Freeman *et al.* 2000). The central biological importance of PRL highlights the need for defining those factors and intracellular mechanisms that govern its secretion from the pituitary gland. Among its numerous functions, PRL plays a critical role in freshwater osmoregulation (Manzon 2002) as well as in the modulation of energy metabolism in teleost fishes (Sheridan 1986, Leena *et al.* 2001, Sangio-Alvarellos *et al.* 2006). PRL is regulated by a complex array of hormonal factors and neurotransmitters, including the osmoregulatory and metabolic hormones, insulin-like growth factor-I (IGF-I), and cortisol (Nishioka *et al.* 1988, Fruchtman *et al.* 2000, Hyde *et al.* 2004). Gut stomach-derived ghrelin also modulates teleost PRL release (Riley *et al.* 2002, Kaiya *et al.* 2003a) and in mammals this hormone may act in cooperation with leptin to transfer information to the brain about the caloric state of the animal (Popovic & Duntas 2005).

Leptin is the 16 kDa product of the *ob* gene, and the hormone is primarily expressed and secreted by white adipose tissue in mammals (Zhang *et al.* 1994). In teleosts, putative leptin has been detected with mammalian antibodies and is particularly abundant in the bloodstream and liver (Johnson *et al.* 2000). Recently, a teleost ortholog of mammalian leptin was found in three fish species (Kurokawa *et al.* 2005) and shown to be expressed in various tissues including liver and adipose (Kurokawa *et al.* 2005, Huising *et al.* 2006a). Leptin receptors are also present in the genome of teleosts examined thus far (Huising *et al.* 2006b).

Since its discovery, the biology of leptin has been extensively studied and in mammals it seems important as a humoral ‘lipostat’, by increasing energy expenditure and decreasing appetite (Sahu 2004). Accordingly, food intake is suppressed in response to treatment with exogenous leptin in mammals (Campfield *et al.* 1995, Sahu 2004) and birds (Denbow *et al.* 2000, Lohmus *et al.* 2003). In fish some ambiguity exist as to leptin’s role, since treatment with mammalian hormone was reported to have either anorexigenic actions (sunfish, Johnson *et al.* 2000; goldfish, Volkoff *et al.* 2003), or no effect at all (catfish, Silverstein & Plisetskaya 2000; coho salmon, Baker *et al.* 2000; sunfish, Londraville & Duvall 2002). Food deprivation in

mammals induces a drop in leptin expression (Bertile *et al.* 2003) but a similar treatment elicits little effect in carp (Huisling *et al.* 2006a), suggesting possible evolutionary differences in leptin regulation among endotherms and ectotherms.

In mammals it appears that leptin works on hypothalamic neurons to induce luteinizing hormone release, and when energy stores are sufficient, promotes the initiation of sexual maturation (Smith *et al.* 2002, Harvey & Ashford 2003). Leptin treatment also stimulates circulating PRL levels in rats (Gonzalez *et al.* 1999, Watanobe 2002). Melanocortin-4 receptor antagonists decrease the magnitude of leptin-induced PRL surges *in vivo* indicating interaction with this receptor and that hypothalamic control of the rat pituitary plays a significant role in the response (Watanobe *et al.* 1999). In addition to leptin's effect via the hypothalamus, leptin appears to act directly at the level of the rat pituitary to stimulate gonadotropin and PRL release from anterior pituitary cells (Yu *et al.* 1997). Leptin also stimulates PRL secretion from bovine pituitary explants (Accorsi *et al.* 2007), but has no effect on PRL release from primary cultures of porcine anterior pituitary cells (Nonaka *et al.* 2006). Whether leptin stimulates PRL release in nonmammalian vertebrates is unknown. In isolated pituitary cells from teleosts, recombinant mammalian leptin induces release of gonadotropins (European sea bass, Peyon *et al.* 2001; rainbow trout, Weil *et al.* 2003) and somatolactin (Peyon *et al.* 2003). We examined whether leptin might modulate PRL secretion from the teleost pituitary, which contains a naturally, highly enriched population of lactotopes within the rostral pars distalis (RPD, Nishioka *et al.* 1988, see Fruchtmann *et al.* 2000, Kasper *et al.* 2006). The pituitary RPD, containing 95–99% PRL cells, can be easily isolated and allows the study of a nearly pure population of PRL cells in their naturally aggregated *in situ* state using completely defined, hormone-free culture medium. We characterized the concentration-dependent response and time-course of leptin's possible effect on PRL release from the RPD. Extracellular signal-regulated protein kinases (ERK1/2) have been reported to be essential signal transducers of leptin responses in several tissues (Harvey & Ashford 2003) and it is involved in IGF-I induced PRL release from the teleost lactotrope (Fruchtmann *et al.* 2000, 2001). Whether this signaling pathway might be involved in mediating potential actions of leptin on PRL release in vertebrates is unknown. To this end, we examined if the selective blockage of ERK1/2 activation might alter PRL release evoked by leptin and whether leptin might regulate the phosphorylation, and thus activation of ERK1/2 within the teleost RPD.

## Materials and Methods

### Static incubations

Adult male tilapia (*Oreochromis mossambicus*; 100–200 g) were maintained in freshwater at a constant photoperiod (12 h light:12 h darkness) for a minimum of 3 weeks prior to all experiments. Fish were killed by decapitation, the RPD was dissected from the pituitary and placed in separate wells of a

Falcon 96-well plate (Becton Dickinson, Oxnard, CA, USA) containing 100 µl Krebs bicarbonate Ringers. Ringers solution contained glucose, glutamine and Eagle's minimal essential medium (355–360 mOsm, pH 7.2; Hyde *et al.* 2004). Tissues were incubated at 27 °C in a humidified chamber (95% O<sub>2</sub>/5% CO<sub>2</sub>). The chamber was continuously agitated on a gyratory platform at 60 r.p.m. To allow PRL release to stabilize to baseline levels, the tissues were pre-incubated for 2 h, after which, medium was removed and replaced with treatment medium. Samples were taken after 1, 4, and 16 h, and medium and tissue were collected. Tissues were sonicated in RIA buffer (0.01 M sodium phosphate, 1% BSA, 0.01% NaN<sub>3</sub>, and 0.1% Triton X-100; pH 7.3) and stored at –20 °C. For measures of ERK1/2, tissues were directly placed into reducing sample buffer for subsequent western blot analyses.

### PRL measurements

The tilapia pituitary releases two PRLs (PRL<sub>177</sub> and PRL<sub>188</sub>) and both are regulated in a similar fashion by all secretagogue examined to date (see Hyde *et al.* 2004). Thus, in the present study only the release of the more abundant PRL<sub>188</sub> was measured in a concentration–response and time–course experiment. Tilapia PRL<sub>188</sub> was quantified using a homologous RIA as previously described (Ayson *et al.* 1993, Tipsmark *et al.* 2005) and hormone release is expressed as a percentage of the total amount of hormone in the incubations (tissue + media). In a separate experiment, the effect of a specific ERK1/2 inhibitor, PD98059, on leptin-induced PRL release was examined. In this experiment, PRL was quantified by colorimetric gel detection according to our previously described and validated procedures where both tilapia PRLs, due to their differences in sizes, could be measured simultaneously (Borski *et al.* 1991, Hyde *et al.* 2004). In short, tissue and media samples were run on SDS-PAGE and the gels stained with Coomassie brilliant blue. After destaining, both PRL<sub>177</sub> and PRL<sub>188</sub> bands were quantified using an Odyssey scanner (LI-COR, Lincoln, NE, USA). Data were calculated as percentage of total hormone released or the amount of hormone released in media divided by total hormone (media + tissue) in the incubation.

### ERK1/2 analysis

Tissues were sonicated in reducing sample buffer (NuPAGE, Invitrogen; final concentration in the loaded samples in mmol/l: 141 Tris base, 106 Tris HCl, 73 LDS, 0.5 EDTA, 50 1,4-dithiothreitol and 8% glycerol (v/v), 0.019% Serva blue G250 (w/v), 0.006% phenol red (w/v)), and frozen at –20 °C before western analysis. After heating at 80 °C for 10 min, proteins were resolved by SDS-PAGE. Proteins were separated by gel electrophoresis using 4–12% Bis–Tris gels (NuPAGE), and MES/SDS–buffer (in mmol/l: 50 2-(*N*-morpholino)-ethanesulfonic acid, 50 Tris, 3.5 SDS, 1 Na<sub>2</sub>-EDTA; with addition of NuPAGE antioxidant) at 200 V (Xcell II SureLock; Invitrogen). Molecular size was estimated by including

a pre-stained marker (Bio-Rad). Following electrophoresis, the gel was soaked for 30 min in transfer buffer (in mmol/l: 25 Tris, 192 glycine and 20% methanol) and immunoblotted onto nitrocellulose membranes (0.45  $\mu$ m; Invitrogen) by submerged blotting for 1 h at 30 V (XCell II; Invitrogen). Membranes were blocked in TBS-T with LI-COR blocking buffer (1:1) and washed in TBS-T.

For detection of dual phosphorylated (active) ERK1/2, monoclonal phospho-p44/42 MAPK (Thr202/Tyr204) from Cell Signaling was used (Beverly, MA, USA; dilution 1:2000). For detection of total ERK1/2, membranes were probed with polyclonal anti-MAPK ( $\alpha$ -94; Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:1000). Following washing, membranes were incubated 1 h with goat anti-mouse and anti-rabbit secondary antibodies conjugated to Alexa IRDye 680 or IRDye 800CW (LI-COR). Phosphorylated and total (nonphosphorylated and phosphorylated) ERK1/2 was detected on the same blot at 680 and 800 nm respectively. Active (dual phosphorylated) ERK1 and ERK2 were normalized to total ERK1 and ERK2 content. Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR).

### Statistical analysis

Statistical differences were analyzed using Statistica 7.0 (Tulsa, OK, USA). When appropriate, one-way ANOVA in conjunction with Dunnett's test or two tailed *t*-test were used to analyze differences among treatment groups. In all cases, a significance level of  $P=0.05$  was used.

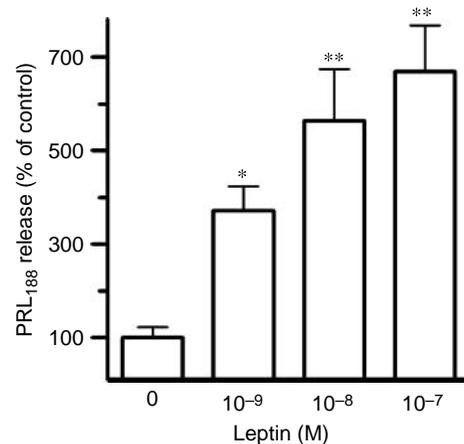
## Results

### Effect of leptin on PRL release

We tested the hypothesis that leptin may modulate PRL release directly at the level of the pituitary by first testing the possible effect of a range of leptin concentrations ( $10^{-9}$ – $10^{-7}$  M) on *in vitro* PRL<sub>188</sub> release from isolated RPDs. As shown in Fig. 1, leptin stimulates PRL<sub>188</sub> release many fold over basal levels (basal release:  $0.57 \pm 0.12\%$  of total content) during 1-h incubation. The effect was concentration dependent with  $10^{-9}$ – $10^{-7}$  M dosages stimulating PRL<sub>188</sub> release by four- to sevenfold. We also evaluated the time-course over which leptin stimulates PRL<sub>188</sub> release, using a  $10^{-8}$  M leptin concentration. As shown in Fig. 2, leptin stimulates PRL<sub>188</sub> release by 600% over the first hour (basal release:  $1.17 \pm 0.23\%$ ) of incubation, with a more modest increase in PRL<sub>188</sub> release during longer-term incubations (1–4 h, 220%,  $P=0.08$ , basal release:  $3.09 \pm 0.90\%$ ; 4–16 h, 160%,  $P<0.05$ , basal release:  $21.53 \pm 2.77\%$ ).

### Effect of an ERK1/2 blocker on leptin-induced PRL release

The role of ERK in transducing leptin's effect in the RPD was examined using PD98059, which blocks the activation of

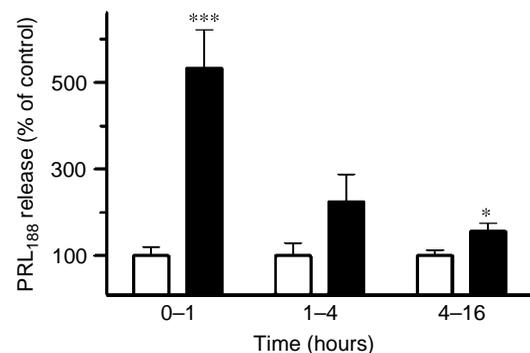


**Figure 1** Effect of different concentrations of leptin on PRL<sub>188</sub> release from lactotropes in isolated RPDs during 1-h incubation. Values are means  $\pm$  S.E.M. ( $n=10$ ). Asterisks indicate significant difference from control (\*\* $P<0.01$ ; \* $P<0.05$ ).

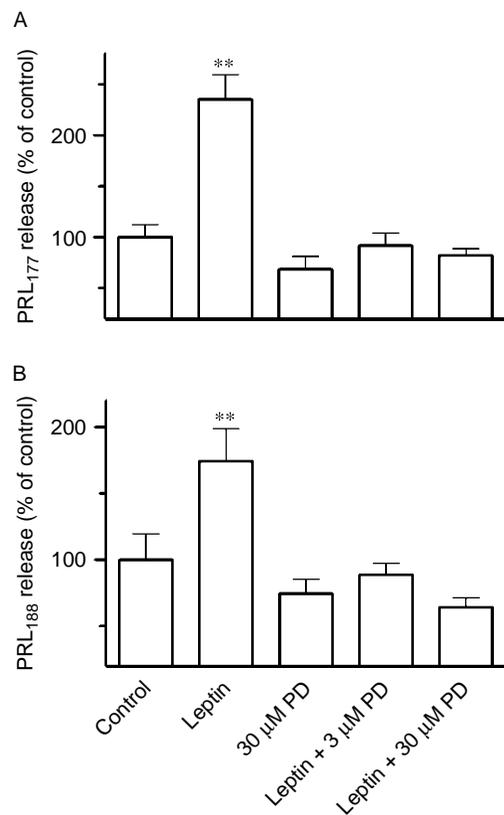
ERK1 and ERK2 by inhibiting ERK kinase (MEK). As shown in Fig. 3, the two concentrations of PD98059 (3 and 30  $\mu$ M) examined were both effective in suppressing the stimulatory actions of leptin on the release of both PRLs during a 4-h incubation (PRL<sub>177</sub> basal release:  $3.92 \pm 0.48\%$ ; PRL<sub>188</sub> basal release:  $4.70 \pm 0.93\%$ ).

### Effect of leptin on ERK1/2 phosphorylation

Whether the leptin-induced increase in PRL secretion might be linked to an increase in ERK signaling was further assessed by quantifying changes in dual phosphorylation, and thus activity of ERK1/2. Tilapia RPDs were treated with different concentrations of leptin for 1 h and the samples were analyzed using double fluorescent western blotting techniques. In Fig. 4, western blotting using the ERK1/2 specific antibody and an antibody specific to the dual phosphorylated kinase revealed two protein bands of 47 kDa (ERK1) and 43 kDa (ERK2). In Fig. 4A the dual phosphorylated epitope



**Figure 2** Time-course effects of leptin (10 nM) on PRL<sub>188</sub> release from lactotropes in isolated RPDs (control media, open bars; leptin, closed bars). Values are means  $\pm$  S.E.M. ( $n=10$ ). Asterisks indicate significant difference from control (\*\*\*) $P<0.001$ ; \*) $P<0.05$ .

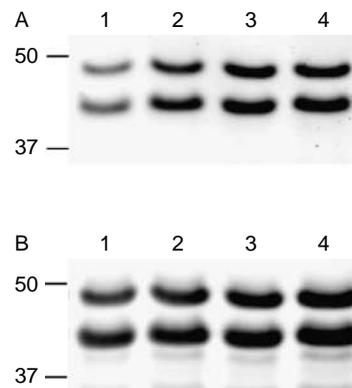


**Figure 3** Effects of the specific MEK inhibitor, PD98059, on leptin induced PRL<sub>177</sub> (A) and PRL<sub>188</sub> (B) release from lactotropes in isolated RPDs. Values are means  $\pm$  S.E.M. ( $n=7$ ). Asterisks indicate significant difference from control (\*\* $P<0.01$ ).

of ERK1 and ERK2 was detected at 700 nm. In Fig. 4B, the total ERK1 and ERK2 was detected on the same blot at 800 nm. The relative phosphorylated values normalized to the total ERK abundance are depicted as relative ERK1 and ERK2 activity in Fig. 5A and B. One-hour incubation with leptin induced more than twofold higher phosphorylation of ERK1/2 than that observed in the absence of hormone. The dose-dependent increase in ERK1/2 phosphorylation with leptin paralleled that observed with the PRL release response (PRL release and ERK1 activity:  $r^2=0.9981$ ,  $P=0.0010$ . PRL release and ERK2 activity:  $r^2=0.9997$ ,  $P=0.0001$ ). There were no significant changes in total (phosphorylated + nonphosphorylated) protein expression of ERK1 and ERK2 during the 1-h incubation (Fig. 5C and D).

## Discussion

The present data demonstrate that leptin is a potent stimulator of PRL release in teleosts and that its actions are mediated, at least in part, through stimulation of ERK1/2. Since its discovery (Zhang *et al.* 1994), research has mainly focused on leptin's actions on the brain in regulating appetite suppression

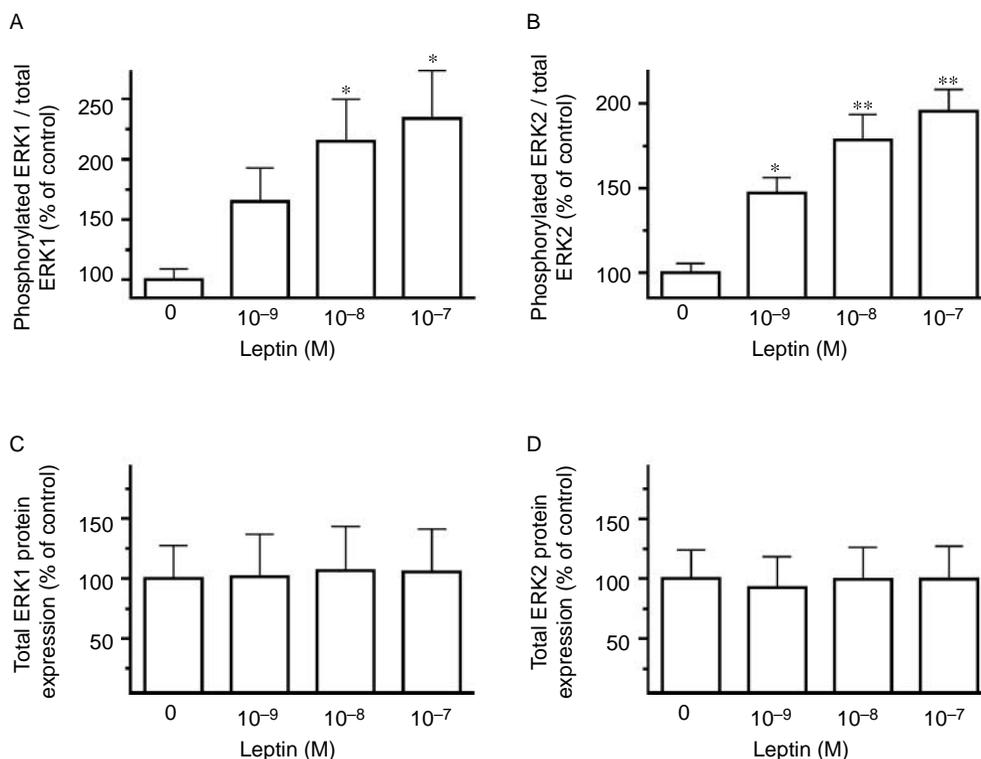


**Figure 4** Western blot showing detection of double phosphorylated ERK1/2 (A) and total ERK1/2 (B) in RPDs treated for 1 h with control Ringer's (lane 1), 1 nM leptin (lane 2), 10 nM leptin (lane 3), and 100 nM leptin (lane 4). Bars designate molecular weight markers (kDa).

and thermogenesis (Sahu 2004). It is increasingly clear that leptin also plays important roles in control of neuroendocrine function (Smith *et al.* 2002) by stimulating pituitary hormone release in mammals either at the level of the pituitary or indirectly through regulation of the hypothalamus (Inui 1999, Watanabe 2002). Likewise, leptin receptors are highly expressed in both the mammalian hypothalamus and pituitary (Jin *et al.* 2000, Czaja *et al.* 2002). In isolated anterior pituitaries from rats, nanomolar concentrations of leptin stimulate gonadotropin release (Yu *et al.* 1997). In teleosts, mammalian leptin in the micromolar range has been shown to induce gonadotropin release from dispersed pituitary cells (Peyon *et al.* 2001, Weil *et al.* 2003). The authors suggested that the apparent insensitivity of the cells to nanomolar concentration of leptin could be explained by the use of heterologous human and mouse hormone respectively. In addition to its effect on gonadotropes, leptin has also been shown to regulate release of GH and PRL from isolated adenohypophysis preparations in rodents and cows (Yu *et al.* 1997, Zieba *et al.* 2003). In other vertebrates, data are largely lacking, although one study has shown that micromolar concentrations of leptin stimulate somatotactin release by sixfold during the initial hours of treatment in European sea bass (Peyon *et al.* 2003).

In the current study, we found leptin to be a very potent PRL secretagogue, inducing a sixfold initial increase in PRL release during the first hour followed by a lower sustained twofold elevation during 1–4 and 4–16 h of treatment. This temporal dynamics in PRL stimulation with leptin likely represents a classic biphasic response observed by many endocrine cells during stimulus–secretion coupling and is similar to that previously shown to occur with exposure to IGF-I, ghrelin, and angiotensin II in tilapia (Kajimura *et al.* 2002, Eckert *et al.* 2003, Kaiya *et al.* 2003b).

In contrast to the effects of mammalian leptin on teleost gonadotrope and somatotactotropes as mentioned above, we found that the tilapia lactotrope is highly sensitive to



**Figure 5** Effect of different concentrations of leptin on phosphorylation/activation of ERK1 (A) and ERK2 (B) during 1-h incubation. Dual phosphorylated (active) ERK1 (A) and ERK2 (B) normalized to total ERK1/2 in RPDs. Abundance of total ERK1 (C) and ERK2 (D) per RPD in the same experiment. Values are means  $\pm$  S.E.M. ( $n=4$ ). Asterisks indicate significant difference from control (\*\* $P<0.01$ ; \* $P<0.05$ ).

recombinant human leptin, responding to the hormone at low nanomolar concentrations. Our studies are in agreement with previous investigations on rat anterior pituitary cells and bovine pituitary explants where leptin also stimulates PRL secretion, albeit with less potency than that observed here (Yu *et al.* 1997, Accorsi *et al.* 2007). By contrast there was no effect of leptin on PRL release from porcine pituitary cells (Nonaka *et al.* 2006), suggesting potential species differences in responsiveness to leptin. The use of serum-supplemented medium prior to or during exposure to test compounds might also contribute to potential differences in responsiveness of lactotropes among studies. However, this is largely eliminated in the present investigations, where a nearly pure population of lactotropes was incubated in a completely defined medium. This reduces any confounding variables associated with exposure to undefined serum factors and the potential effects it may have on leptin regulation of PRL secretion. In the present study, the effect of leptin is most likely receptor mediated, since there is a clear concentration-dependent action of leptin on PRL release and ERK1/2 activation. Whether the actions of leptin on PRL release reflects a direct regulation of lactotropes or is mediated by paracrine factors secreted from other pituitary cell types (adrenocorticotropin secreting or stellate cells in the RPD) is uncertain. Future studies co-localizing leptin receptors to lactotropes are required to address this question.

The effects of leptin observed in the present study appear physiologically relevant since the effective concentrations fall within circulating values measured in mammals (Accorsi *et al.* 2005) and that of the putative hormone measured in teleosts based on heterologous RIA (Mustonen *et al.* 2002, Nagasaka *et al.* 2006).

Leptin receptors belong to the cytokine receptor superfamily (Huisling *et al.* 2006b) and the activation of JAK tyrosine kinase via the receptors intracellular signaling domain has been reported to stimulate several signal transduction pathways. Leptin receptor-driven signaling includes the JAK-STAT pathway, and both the PI 3-kinase and Ras-ERK pathways, both of which are downstream of JAK (Harvey & Ashford 2003, Frühbeck 2006). In addition, we have previously found that the specific inhibitor of ERK activation, PD98059, blocks IGF-I evoked PRL release from striped bass RPDs (Fruchtman *et al.* 2001) and therefore PRL secretion can in some instances be controlled by this signaling kinase. In the current study, evidence suggests that that leptin-induced PRL release is dependent on ERK activation. The potent inhibitor of ERK, PD98059 was able to block leptin-induced PRL release. Additionally, we directly analyzed ERK1/2 activity by quantifying double tyrosine and threonine phosphorylation of ERK1/2, using western blotting and dual infrared imaging. One-hour

incubation with leptin induced an increase in double phosphorylation, and thus activity, of ERK1/2. The concentration–response curve of leptin and RPD ERK1/2 activity corresponded well with that observed for leptin and PRL release, further suggesting leptin's actions are mediated by ERK activation.

In summary, findings demonstrate that leptin is a very potent *in vitro* secretagogue of PRL release in ectotherms and are the first to suggest in vertebrates that leptin stimulates PRL release through ERK1/2 activation. However, the physiological significance of leptin's actions in stimulating PRL release including any similar effects it may have on PRL secretion *in vivo* remains uncertain. Considering the multitude of actions of PRL and as yet the poorly defined role of leptin in teleosts, it is possible that leptin may interact with PRL to regulate metabolism, growth, osmotic homeostasis, or maturation. Since PRL has been shown to modulate lipid metabolism in teleosts (Sheridan 1986, Leena *et al.* 2001, Sangiao-Alvarellos *et al.* 2006), it is possible that leptin may alter PRL secretion to change fat deposition or other metabolic processes in fish. Because of the significant expression of the ghrelin gene in gill and kidney, it has been suggested that this appetite regulatory hormone could be involved in modulating the function of these osmoregulatory organs (Kaiya *et al.* 2003b). PRL is an essential hormone for freshwater osmoregulation in teleosts (Manzon 2002) and it is possible that leptin could interact with PRL in regulating osmotic homeostasis.

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