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Leptin directly regulates exocrine pancreas lipase and two related proteins in the rat

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Leptin, a metabolic regulator of energy expenditure, exerts its peripheral effects primarily by altering lipid metabolism. The exocrine pancreas has a key role in the digestion of dietary lipids, but the role of leptin in regulating pancreatic lipases remains unknown. Using the exocrine pancreas *in vitro* AR42J cell model, we studied the direct effects of leptin on pancreatic lipase (PL) secretion and on the mRNA levels of PL and PL-related proteins 1 and 2 (PLRP1, PLRP2). Leptin directly, rapidly (within 30 min) and significantly inhibited both the secretion and intracellular activity of PL. Leptin downregulated mRNA levels of PL and PLRP1, and upregulated transcripts of PLRP2. This study provides the first evidence that leptin directly regulates exocrine lipases at the levels of synthesis, activity and secretion. This rapid regulation may be associated with a short-term control of energy balance.

Leptin: Pancreatic lipase: Pancreatic lipase-related protein 1: Pancreatic lipase-related protein 2

Leptin is a 167-amino-acid protein that is synthesised and secreted mainly by adipose tissue, as well as by the reproductive tract and stomach (Bado *et al.* 1998; Margetic *et al.* 2002). Leptin exerts its metabolic regulation both centrally (via the central nervous system) on appetite regulation and energy expenditure, and peripherally (on the blood system, reproduction, etc.) (Margetic *et al.* 2002). The discovery of leptin production by the stomach pinpointed the possible role of leptin in digestive physiology, in particular in the short-term control of energy balance (Picó *et al.* 2003; Sanchez *et al.* 2005). In contrast to adipogenic leptin, leptin from gastric cells reacts quickly (within 15 min) to short-term nutritional changes (meals) and to gastrointestinal hormones. Short-term and rapid changes, both nutritional and hormonal, are also key regulators of the synthesis and secretion of digestive enzymes by the exocrine pancreas (Rothman, 1976; Jakob *et al.* 2000; Rothman *et al.* 2002).

Leptin receptors are expressed abundantly in the brain (Margetic *et al.* 2002), but they are also widely distributed in the peripheral tissues, including the gastrointestinal tract: in the islets of the Langerhans in the endocrine pancreas (Kieffer & Habener 2000), in the mucosa of the stomach (Bado *et al.* 1998; Sobhani *et al.* 2000; Goiot *et al.* 2001), in the small intestine (Morton *et al.* 1998; Buyse *et al.* 2001) and on neuronal structures in the rat pancreas (Sha & Szurszewski 1999). Two functional forms of the leptin receptor (long and short) have recently been found in *in vitro* (AR42J cells) and *in vivo* models (Harris *et al.* 1999; Guilmeau *et al.* 2002; Konturek *et al.* 2003).

Extensive investigations of the endocrine pancreas β-cells indicate that leptin inhibits insulin secretion both directly (via leptin receptors) and indirectly, via the autonomous nervous system (Kieffer & Habener, 2000). Several recent *in vivo* studies in dog and rat models (Sha & Szurszewski, 1999; Guilmeau *et al.* 2002; Konturek *et al.* 2003) suggest that leptin plays a role in the physiological regulation of pancreas amylase secretion by the exocrine pancreas; it is, however, not known whether leptin affects other pancreatic enzymes, especially as different pancreatic enzymes are regulated independently of and differently from each other.

The digestion of dietary nutrients is achieved mainly through the activity of enzymes from the exocrine pancreas. The exocrine pancreas synthesises, stores and secretes a panel of digestive enzymes. The synthesis and secretion of pancreatic enzymes are regulated by both nutrients and hormones, mainly gastrointestinal, in order to maximise the utilisation of dietary nutrients. Pancreatic lipases are known to be regulated differently and independently by diet, hormones and development even when they share high homology, as do pancreatic lipase (PL) and the two PL-related proteins 1 and 2 (PLRP1, PLRP2; Dagorn & Mongeau 1977; Dagorn *et al.* 1977; Dagorn & Estival 1979; Schick *et al.* 1984*a*,*b*; Iovanna *et al.* 1986; Saab *et al.* 1986; Pradel *et al.* 1993; Lowe, 1997; Birk & Brannon 2004; Birk *et al.* 2004). In the present study, we used the most abundant and best-studied exocrine pancreas model cells – the AR42J cell line – to investigate whether leptin directly regulated the secretion and mRNA levels of the pancreatic lipases PL, PLRP1 and PLRP2.

Abbreviations: GAPDH, glyceraldehyde-3 phosphate dehydrogenase; PL, pancreatic lipase; PLRP, pancreatic lipase-related protein.
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Materials and methods

Cell culture

Rat pancreatoma AR42J cells (American Type Culture Collection, Rockville, MD, USA) were maintained as a subconfluent monolayer culture in Dulbecco's Eagle's medium (Rhenium, Jerusalem, Israel) containing 10 % (v/v) fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 1 % (v/v) penicillin–streptomycin. Cells were grown in 5 % CO_2 at 37°C. Dexamethasone (100 nM; Sigma-Aldrich, Rehovot, Israel) was added to the cells for 48 h before the experiments to induce cell differentiation. AR42J cells were incubated with different concentrations of rat recombinant leptin (R&D Systems, Minneapolis, MN, USA) for the times indicated.

For the secretion studies, leptin (0–400 ng/ml) was added to the AR42J cells for 0·5, 1, 3 and 5 h. At the indicated time points, both medium (for secreted PL activity) and cells (for intracellular PL activity) were collected and frozen immediately in the presence of soya trypsin inhibitor (0·01 % v/v; Sigma) until enzyme analysis was performed. Prior to freezing, AR42J cells were sonicated (15s × 4, 4°C; Fisher Sonic Dismembretor 150; Artek System Corporation, NY, USA) in the presence of soya trypsin inhibitor (0·01 % v/v). The secretion studies were carried out in four experiments.

For the transcript studies, leptin (100 or 1000 ng/ml) was added to the AR42J cells for 0·5 and 1 h. At the end of the indicated time period, cells were frozen for later RNA isolation. The transcript studies were carried out in three independent experiments in duplicate.

Enzyme analysis

Lipase activity was determined using an autotitrimetric method (Saab *et al.* 1986). Briefly, using a gum-stabilised emulsion of neutralised triolein as a substrate and with excess crude co-lipase, hydrolysed free fatty acids were titrated automatically (Radiometer America, Cleveland, OH, USA) with 20 mmol/l NaOH to a constant pH. Enzyme activity was expressed as U/mg protein (1 U lipase is μmol fatty acid released per min per mg protein). Total protein was determined according to the method of Bradford (1976).

RNA isolation and cDNA synthesis

Total RNA was isolated from cultures of AR42J cells using slight modifications of the protocol of Chomczynski & Sacchi (1987). This method is based on a single extraction with an acid guanidinium thiocyanate–phenol–chloroform mixture. In short, AR42J cells were homogenised in 4 M-guanidinium thiocyanate, 26 mM-sodium citrate, pH 7, 0·5 % (v/v) sarcosyl and 0·7 % (v/v) 2-mercaptoethanol. The RNA was then sequentially extracted by adding 0·2 M-sodium acetate (pH 4) and phenol and chloroform–isoamyl alcohol mixture (49:1). RNA was precipitated with isopropanol, reprecipitated with 75 % (v/v) ethanol and dissolved in sterile diethylpyrocarbonate (Sigma) treated water. RNA integrity was tested by agarose gel electrophoresis (0·8 % w/v) with ethidium bromide staining. RNA was quantitated by UV absorption at 260 nm using a spectrophotometer (NanoDrop ND-1000 UV-Vis; NanoDrop Technologies, Wilmington, DE, USA). The mRNA (2 μg/μl) were subject to first-strand cDNA synthesis by random hexamers using a cDNA mix containing the following: 0·05 mg/ml random primers (Bioline, London, UK); 0·5 mM each of dNTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA); 5 mM-DTT (Bioline), 500 U reverse transcriptase (Bioline); 5X first-strand buffer (Bioline). Quantitative PCR was subsequently performed.

Quantitative PCR for pancreatic lipase and pancreatic lipase-related proteins 1 and 2

Transcript levels of PL, PLRP1 and PLRP2 mRNA were determined using quantitative PCR (ABI Prism 7000; Applied Biosystems, Foster City, CA, USA). Primers were designed using Primer express software (Applied Biosystems). The following primers were used: PL, forward 5′-TCCAT AGAACCCGACGGATC-3′, reverse 5′-TCCCAGCAGCACTG CAAA-3′; PLRP1, forward 5′-AACTGCTTTTCAGAC TCTCCAGCT -3′, reverse 5′- TGAAAATTTGAGGCCCCAAT-3′; PLRP2, forward 5′-TTGTTCACA TTCCGCAGTGC -3′, reverse 5′-GGCCAGACTCAAAGAAGCAGTT-3′; housekeeping gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH), forward 5′-GGAAGGGCTCATGACGACCACAGT-3′, reverse 5′-CACAGT CTTCTGAGTGGCAGTGAT-3′.

Amplification reactions were carried with preincubation at 50°C for 2 min followed by denaturation at 95°C for 10 min and 40 cycles of 95°C for 15 s, with annealing and elongation at 60°C for 1 min. Quantitative PCR reactions were performed using a mix kit (Applied Biosystems) in microtubes (Applied Biosystems) in a total reaction volume of 20 μl. The final concentration of primers was 300 nM for PLRP1, PLRP2 and GAPDH, and 100 nM for PL. Primer and cDNA concentrations were optimised following the supplier's guidelines. All quantitative PCR reactions were performed in duplicate. The internal reference was ROX. The reaction was carried out using the ABI Prism 7000 sequence detection system (Applied Biosystems).

Statistics

All results were expressed as means with their standard errors. For the secretion studies, results were analysed using two-way ANOVA. For the expression studies, results were analysed using one-way ANOVA. Results were considered significantly different if $P < 0·05$.

Results

Modulation of pancreatic lipase secretion in in vitro *AR42J cells by leptin*

AR42J cells exposed to various concentrations of leptin (10–400 ng/ml) for different periods (0·5–5 h) showed a significant decrease in secreted PL activity compared with control untreated cells (Fig. 1(A)). There was a significant ($P < 0·05$) independent effect of leptin concentration (10–400 ng/ml significantly smaller than control) on the activity of secreted PL. There was no significant independent effect of leptin exposure time or interactive effect of concentration and time on secreted PL activity. There was a significant ($P < 0·05$) independent effect of leptin concentration (10–400 ng/ml significantly

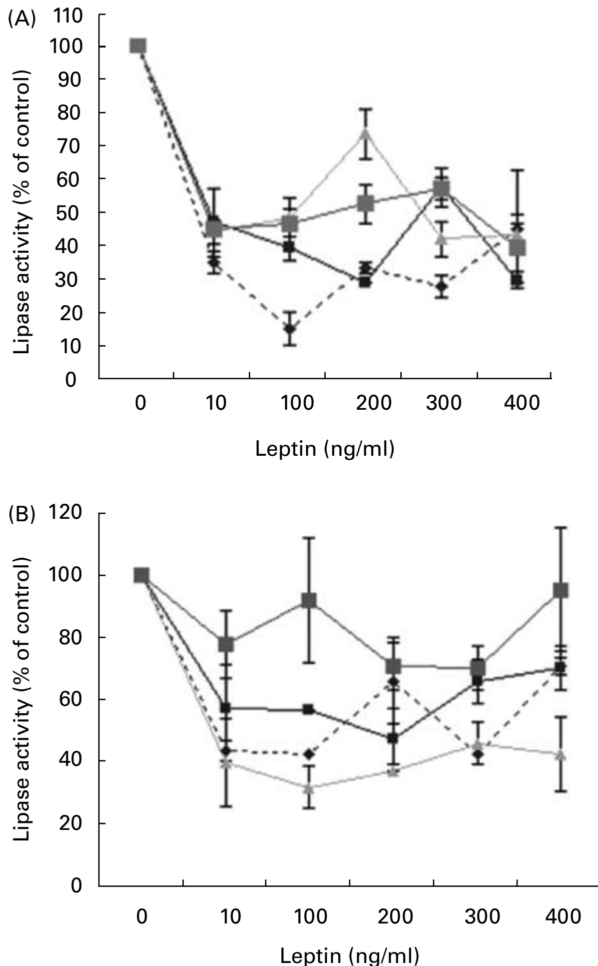

Fig. 1. Effect of leptin on pancreatic lipase secretion (A) and intracellular activity (B) in AR42J cells. Cells were treated with rat leptin for different time periods (···◆···, 0·5 h; –■–, 1 h; –▲–, 3 h; –■–, 5 h), after which incubation media and cells were collected and assayed for lipase activity. Results are expressed as mean values with their standard errors of duplicates from two independent experiments represented by vertical bars. All treatments with leptin were significantly different from the control ($P < 0·05$).

smaller than control) on intracellular PL activity. There was a significant ($P < 0·001$) independent effect of time of exposure to leptin ($0·5\,h = 1\,h = 3\,h < 5\,h =$ control) on intracellular PL activity. There was not a significant interaction effect of concentration × time on intracellular PL activity. (Fig. 1(B)).

Modulation of transcripts levels of pancreatic lipases in AR42J cells by leptin

The mRNA levels of PL in the AR42J cells were measured in response to treatment with leptin (100 and 1000 ng/ml) for 0·5 and 1 h. Leptin (1000 ng/ml) significantly decreased PL mRNA levels at 30 min (by approximately 50 %; Fig. 2) compared with control levels. Lower leptin levels (100 ng/ml) did not alter PL mRNA levels compared with the control. Exposure to leptin for 1 h did not affect PL mRNA levels compared with the control (data not shown).

AR42J cells showed a significant decrease in PLRP1 mRNA levels in response to treatment with leptin (by approximately 50 %; 100 ng/ml) for 30 min compared with the

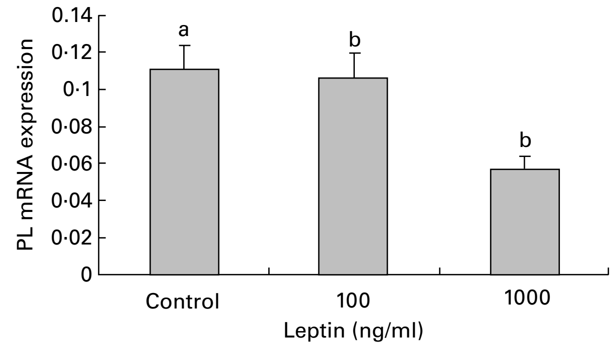

Fig. 2. The effect of leptin (100, 1000 ng/ml) on mRNA levels of pancreatic lipase (PL) in AR42J cells after 30 min exposure. mRNA expression levels were determined by real-time quantitative PCR. For each sample, results were normalised to the levels of the internal housekeeping control gene (glyceraldehyde-3 phosphate dehydrogenase). Results are expressed as means with their standard errors of duplicates from three independent experiments. Mean values with unlike superscript letters were significantly different ($P < 0·05$).

control. Exposure to higher levels of leptin (1000 ng/ml) did decrease PLRP1 mRNA levels, but the results were not significantly different from the control or from treatment with 100 ng/ml leptin (Fig. 3). Incubation for 1 h did not affect PLRP1 mRNA levels (data not shown).

AR42J cell PLRP2 mRNA levels were significantly elevated in response to treatment with leptin (200 % and 300 % for 100 and 1000 ng/ml, respectively) for 30 min and 60 min (400 %; 1000 ng/ml) compared with the control (Fig. 4).

Discussion

The regulation of the exocrine pancreas is achieved at several levels: neural; nutritional; hormonal. The central role of leptin as a satiety factor is well documented, but its peripheral regulative role in the gastrointestinal tract is not fully understood. Leptin is known as a regulator of enzymes involved in lipid metabolism in other peripheral tissues (Liang & Tall, 2001; Houseknecht & Spurlock, 2003). Pancreatic lipases are key

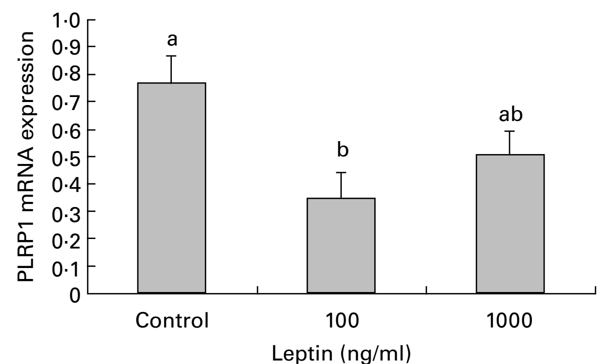

Fig. 3. The effect of leptin (100, 1000 ng/ml) on the mRNA levels of pancreatic lipase-related protein 1 (PLRP1) in AR42J cells after 30 min exposure. mRNA expression levels were determined by real-time quantitative PCR. For each sample, results were normalised to the levels of the internal housekeeping control gene (glyceraldehyde-3 phosphate dehydrogenase). Results are expressed as means with their standard errors of duplicates from three independent experiments. Mean values with unlike superscript letters were significantly different ($P < 0·05$).

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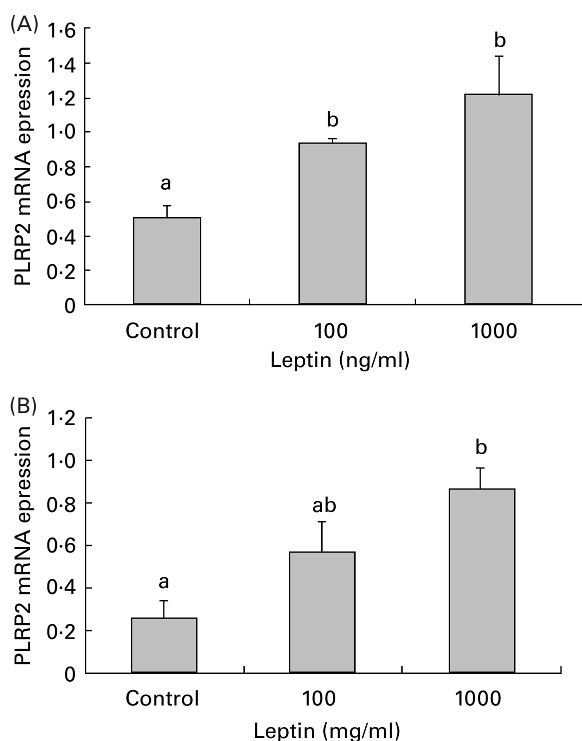

Fig. 4. The effect of leptin (100, 1000 ng/ml) on the mRNA levels of pancreatic lipase-related protein 2 (PLRP2) in AR42J cells after 30 min (A) and 60 min (B) exposure. mRNA expression levels were determined by real-time quantitative PCR. For each sample, results were normalised to the levels of the internal housekeeping control gene (glyceraldehyde-3 phosphate dehydrogenase). Results are expressed as means with their standard errors of duplicates from three independent experiments. Mean values with unlike superscript letters were significantly different ($P < 0.05$).

enzymes in the digestion of dietary lipids. The direct regulatory effect of leptin on the mRNA levels and secretion of these enzymes is unknown. In the present study, experiments using the most abundant and best-studied exocrine *in vitro* pancreas model, AR42J cells, showed that leptin directly regulated pancreatic lipases at the different levels of mRNA and secretion. We showed for the first time that leptin regulates PL activity and mRNA levels, as well as PLRP1 and PLRP2 at the mRNA level. This regulation was found to be rapid, occurring within 30 min, in line with the known rapid (over minutes) regulation of the *de novo* synthesis and secretion of exocrine pancreas enzymes following stimulation (rapid nutritional and hormonal changes; Rothman, 1976; Bado *et al.* 1998; Margetic *et al.* 2002; Rothman *et al.* 2002).

PL is the main enzyme responsible for the digestion of dietary triacylglycerols and digests up to 56 % of dietary triacylglycerols consumed in a test meal, allowing the full absorption of dietary triacylglycerols. PL and its related proteins are members of the lipase family, which are very similar in both their amino acid sequence and gene organisation, and include among others lipoprotein lipase, hepatic lipase and endothelial lipase (Wong & Schotz, 2002).

Members of the lipase family are, however, regulated by nutritional status in a tissue-specific manner. For instance, in the fed state, lipoprotein lipase is activated in white adipose tissue and downregulated in skeletal muscle and heart; fatty acids will therefore be directed to adipose tissue for esterification and storage in a time of energy surplus (Cryer *et al.* 1976). On fasting, however, the situation is reversed, with upregulation of lipoprotein lipase in muscle and suppression in adipose tissue, so that fatty acids are directed to the tissue in which they are needed as an oxidative fuel (Cryer *et al.* 1976). Moreover, in the exocrine pancreas, PL, PLRP1 and PLRP2 are regulated differently in various nutritional, hormonal and developmental states (Lowe, 1997; Birk *et al.* 2004; Birk & Brannon, 2004).

Leptin is known to regulate lipid metabolism, reducing lipid storage by altering the activity of enzymes involved in lipogenesis and fatty acid oxidation in adipocytes as well as in skeletal muscle, liver and endocrine pancreas (Houseknecht & Spurlock, 2003). It is not yet clear, however, whether such effects of leptin might be mediated proximally by changes in the activity of lipoprotein lipase and other components of the fatty acid storage pathway; as direct effects of leptin on peripheral tissues are increasingly being recognised, this becomes more likely (Fielding & Frayn, 1998). In the present study, leptin was shown to significantly downregulate the activity of secreted PL as well as intracellular PL activity, starting at 10 ng/ml (similar to normal physiological concentrations) and continuing up to 400 ng/ml. The reduction in PL secretion by leptin diminished as the time increased; although not significant, the largest inhibition was found at 0·5 h and the smallest at 5 h. Intracellular PL activity levels were also reduced at 0·5–3 h, and by 5 h there was no longer any effect.

As both intracellular and extracellular (secreted) PL activity was reduced following leptin administration, the mRNA levels of PL were studied to test whether the inhibition of PL activity was due to a reduction in its synthesis. We demonstrated that leptin at high concentrations (1000 ng/ml) significantly diminished PL mRNA levels. PL mRNA levels were not significantly affected by leptin at lower concentration (100 ng/ml). The non-parallel effect of leptin at 100 ng/ml on secreted PL levels and transcript levels might indicate that leptin regulates PL at the post-transcriptional level.

The metabolic effects of leptin were assessed in a concentration range from 10 to 1000 ng/ml (the mammalian physiological plasma leptin range ranging from 0 to over 100 ng/ml depending on metabolic state). Experiments evaluating the effects of leptin on metabolic activity, both *in vivo* and *in vitro*, have used a wide range of leptin concentration (Muller *et al.* 1997). For example, in rat adipocytes, high leptin levels (480 ng/ml) were required for maximal inhibition of the effects of insulin (Muller *et al.* 1997). Aiston & Agius (1999) reported that glycogen synthesis in rat hepatocytes was maximally stimulated by 800 ng/ml leptin. In skeletal muscle, Ceddia *et al.* (1999) demonstrated that 1600 ng/ml leptin stimulated glycogen synthesis twofold, whereas glucose oxidation increased by 75 % with a concentration of only16 ng/ml. It should be noted that higher doses of leptin might be physiologically relevant because leptin levels could vary between tissues, and leptin levels in fat tissue and/or stomach might be greater than those found in serum. As was elegantly shown by Weigle *et al.* (1998), adipocyte leptin levels of 392 ng/ml were found in rodent models of obesity.

In addition, recombinant leptin is known to have a lower potency than native leptin, which may be due to a different

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pattern of glycosylation (Cohen *et al.* 1996). AR42J cells express both the long and the short leptin receptor isoforms (Harris *et al.* 1999). It was recently demonstrated that the short forms of the leptin receptor can also activate the leptin signalling pathway, raising the possibility that the dose–response of these receptors is different from that of the long form of the receptor (Yamashita *et al.* 1998). The absence of a relative standard for the biological activity of recombinant leptin makes it difficult to determine whether this wide range of leptin levels is the result of apparent differences in sensitivity between tissues or species, differences in experimental methodology or varying quality between recombinant leptin preparations.

To date, studies on the effect of leptin on secretion from the exocrine pancreas focused on amylase secretion, and the information was limited and somewhat contradictory. Using the same *in vitro* model (AR42J cells), Harris *et al.* (1999) found that leptin alone (0–200 ng/ml) did not affect amylase release, but it directly inhibited cholecystokinin-induced amylase secretion and enhanced (100–1000 ng/ml) Ca mobilisation by cholecystokinin. In accordance with the present findings, Konturek *et al.* (2003) found that, in dogs, pancreatic amylase secretion stimulated by basal and sham feeding was significantly inhibited by exogenous leptin (10–50 μg/kg daily by subcutaneous injection). In contrast, a study by Guilmeau *et al.* (2002) showed a stimulatory effect of leptin (100 nmol/kg intravenous injection) on pancreatic amylase secretion in fistulated Wistar rats.

Although PLRP share high homology with PL, previous studies have shown different patterns of regulation (both developmental and nutritional) of the different lipases (Payne *et al.* 1994). For instance, the expression of PL, PLRP1 and PLRP2 is developmentally discoordinate. Whereas PL is mainly expressed during adulthood, PLRP1 and PLRP2 are mainly expressed during the suckling phase, indicating a physiologically different, not fully understood, role in fat digestion (Lowe, 1997). PLRP1 is highly homologous to PL (65 %) (Payne *et al.* 1994; Roussel *et al.* 1998), but although it is secreted into the duodenum, no known lipolytic activity of PLRP1 has yet been reported. The site-directed mutagenesis of two amino acids in PLRP1 to those seen in PL restored the co-lipase-dependent lipolytic activity of PLRP1 (Giller *et al.* 1992; Crenon *et al.* 1998), suggesting that PLRP1 might be a non-functional homologue of PL. In this study, leptin decreased PLRP1 mRNA levels. The physiological significance of this effect is not clear and has yet to be proven.

The second related protein, PLRP2, exhibits lipolytic properties different from those of PL. PLRP2 has significant lipolytic activity in the absence of co-lipase (PL's essential co-factor) and has both phospholipase and galactolipase activities (Roussel *et al.* 1998). It has been proposed that PLRP2 may play a role in the digestion of milk fat (rich in phospholipids) during the early postnatal suckling phase, as its expression is high at this developmental age, declining sharply to minimal levels after weaning (Payne *et al.* 1994). This suggestion is supported by Lowe *et al.* (1998), who found decreased neonatal dietary fat absorption and digestion in PLRP2 knockout mice, and by Birk *et al.* (2004), who showed a regulation of PLRP2 by high levels of long-chain PUFA in the rat early postnatal period. The leptin-induced, upregulated PLRP2 mRNA levels that were found in the present study may result from a suggested role of PLRP2 in the digestion of phospholipid-coated fat globules in breast milk (Lowe *et al.* 1998). This upregulation of PLRP2 by leptin might point toward an additional intriguing role of leptin as a modulator of pancreatic enzymes at an early age. This possibility is of special interest as leptin is known to be functional immediately after birth (Trottier *et al.* 1998; Birk *et al.* 2003).

Leptin was found to regulate the mRNA levels of PL, PLRP1 and PLRP2 in an anti-coordinated manner: whereas leptin down-regulated the mRNA levels of PL and PLRP1, it upregulated transcript levels of PLRP2. The different patterns of regulation of mRNA level for the various enzymes were not expected but were not surprising. The nutritional, hormonal and developmental anti-coordinated regulation of the activity, synthesis and secretion of pancreatic enzymes is a well-documented phenomenon. Although the influence of chronic overnutrition, typical of high leptin levels, on the exocrine pancreas is not known, the intake of a diet high in fat is a risk factor for acute pancreatitis and pancreatic cancer. Moreover, obese Zucker rats exhibit reduced function of the exocrine pancreas, as expressed in terms of a reduction in enzyme secretion (Habara *et al.* 1991; Chowdhury *et al.* 2000).

In conclusion, this is the first report indicating that leptin directly and rapidly modulates PL activity and the mRNA levels of the pancreatic digestive enzymes PL, PLRP1 and PLRP2. The regulation of synthesis of the pancreatic lipases PL. PLRP1 and PLRP2 by leptin occurs in an anti-coordinated manner. This direct modulation is likely to be of physiological significance in nutrient digestion.

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