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Leptin Secretion and Protein Kinase A Activity

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Summary

Leptin is an adipocyte-derived hormone participating in the regulation of food intake and energy balance. Its secretion from fat cells is potentiated by insulin and by substrates providing ATP, whereas factors increasing cAMP level attenuate hormone release stimulated by insulin and glucose. The present experiments were aimed to determine the effect of cAMP on leptin secretion stimulated by glucose, alanine or leucine in the presence of insulin. Moreover, the effect of protein kinase A inhibition on leptin secretion was tested. To stimulate leptin secretion, isolated rat adipocytes were incubated for 2 h in the buffer containing 5 mmol/l glucose, 10 mmol/l alanine or 10 mmol/l leucine, all in the presence of 10 nmol/l insulin. Inhibition of protein kinase A (PKA) by H-89 (50 μmol/l) slightly enhanced leptin release stimulated by glucose and leucine but not by alanine. Activation of this enzyme by dibutyryl-cAMP (1 mmol/l) substantially restricted leptin secretion stimulated by glucose, alanine and leucine. The inhibitory influence of dibutyryl-cAMP on leptin secretion was totally (in the case of stimulation induced by glucose) or partially (in the case of stimulation by alanine and leucine) suppressed by H-89. These results demonstrate that leptin secretion induced by glucose, alanine and leucine is profoundly attenuated by cAMP in PKA-dependent manner. Therefore, the action of different stimulators of leptin secretion may be restricted by agents increasing the cAMP content in adipocytes. Moreover, it has also been shown that inhibition of PKA evokes the opposite effect and enhances leptin release.

Key words
Adipocytes • Leptin • Secretion • PKA • cAMP

Introduction

Leptin, the product of the *ob* gene, is secreted predominantly by adipose tissue and participates in the regulation of food intake, body weight, fat stores, and energy expenditure (Trayhurn and Rayner 1996, Flier 1998, for review see Janečková 2001). The adipocyte *ob* gene expression and plasma leptin level are both positively correlated with fat stores in the organism (Friedman and Halaas 1998). Leptin released by adipocytes into the circulation reaches the brain and provides information about the adiposity. In healthy subjects, the proper regulation of leptin secretion, which follows the energetic state of the organism, seems to be an important element in the so-called lipostat theory (Holst 1996, Friedman and Halaas 1998).

Blood leptin level is influenced by several factors. The stimulation of β-adrenergic receptors by isoproterenol diminishes its concentration (Donahoo *et al.* 1997, Stumvoll *et al.* 2000). The nutritional status of

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the organism also has an important effect on blood leptin. Fasting suppresses leptin gene expression, attenuates leptin secretion from isolated fat cells and lowers its blood level (Becker *et al.* 1995, Boden *et al.* 1996, Gettys *et al.* 1996, Li *et al.* 1998), whereas refeeding exerts the opposite effect (Becker *et al.* 1995, Saladin *et al.* 1995, Thompson 1996). Leptin release from adipocytes was found to be potentiated by glucose and insulin (Barr *et al.* 1997). Levy *et al.* (2000) proved that glucose and other substrates providing energy and increasing ATP production enhanced leptin secretion from isolated fat cells, whereas substrates depleting energy restricted this process. The stimulatory effect of insulin on leptin secretion was postulated to result predominantly from the insulin-induced rise in glucose transport and metabolism in adipocytes (Mueller *et al.* 1998). However, insulin itself is also able to enhance leptin secretion (Gettys *et al.* 1996, Levy *et al.* 2000, Wang-Fisher *et al.* 2002).

Experiments with isolated rat cells revealed that leptin secretion stimulated by glucose and insulin was inhibited when the cAMP content was enhanced (Gettys *et al.* 1996, Cammisotto and Bukowiecki 2002, Moreno-Aliaga *et al.* 2002). However, it is not proven whether this inhibitory action of cAMP results from the activation of protein kinase A (PKA) or is due to PKA-independent events induced by this nucleotide. The latter possibility cannot be excluded since several experiments have provided evidence that cAMP affects some cellular processes independently on PKA (Renstrom *et al.* 1997, Cass *et al.* 1999, Staples *et al.* 2001).

It has recently been demonstrated that, apart from glucose and insulin, alanine (Levy *et al.* 2000) and leucine (Roh *et al.* 2003) are also able to stimulate leptin secretion. However, there are no literature data concerning the influence of cAMP on leptin secretion stimulated by alanine and leucine. This problem seems to be significant, especially in the case of leucine, because this amino acid is recognized as an important dietary factor stimulating protein synthesis in adipose tissue (Lynch *et al.* 2002) and probably participates in postprandial increase of blood leptin levels (Roh *et al.* 2003).

The purpose of the studies was to determine whether the rise in cAMP content in isolated rat adipocytes activating PKA affects leptin secretion stimulated by glucose, alanine or leucine in the presence of insulin. Moreover, the effect of PKA inhibition under basal conditions and conditions of increased cAMP level on leptin secretion was tested.

Methods

Preparation of adipocytes

Adipocytes were isolated from male Wistar rats weighing 200-220 g and kept under standard laboratory conditions. Animals were killed by decapitation, their epididymal fat tissue was pooled and cells isolated according to the method of Rodbell (1964) with minor modifications (Szkudelska *et al.* 2000). The fat tissue was rinsed with 0.9 % NaCl, cut into small pieces with scissors and transferred into a plastic flask with Krebs-Ringer buffer (in mmol/l: 118 NaCl, 4.8 KCl, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 24.8 $NaHCO_3$) containing 3 mmol/l glucose, 3 % bovine serum albumin (BSA, fraction V), 10 mmol/l HEPES and 2 mg/ml collagenase (from Clostridium histolyticum, type II). Before use the buffer was aerated with carbogen (95 % O_2/5 % CO_2) and its pH was adjusted to 7.4. Incubation of fat tissue with this buffer was performed for 90 min by gentle shaking at 37 °C. Thereafter, adipocytes were filtered through a nylon mesh and rinsed four times with a collagenase-free Krebs-Ringer buffer.

Leptin and glycerol release

Adipocytes (10^6 cells/ml) were incubated in plastic tubes with Krebs-Ringer buffer (3 % BSA, 10 mmol/l HEPES, pH 7.4, 37 °C) containing 5 mmol/l glucose, 10 mmol/l alanine or 10 mmol/l leucine and 10 nmol/l insulin. Furthermore, fat cells were incubated with glucose, alanine or leucine and insulin in the presence of dibutyryl-cAMP (DB-cAMP, non-hydrolysable cAMP analogue, 1 mmol/l), H-89 (selective inhibitor of PKA, 50 μmol/l) or both. Each treatment was performed in five repetitions, i.e. in 5 tubes incubated simultaneously and repeated in two separate experiments. All incubations were performed for 2 h at 37 °C with gentle shaking. Immediately after that, adipocytes were removed and samples of the incubation medium were stored (–80 °C) until leptin and glycerol determination. Leptin was determined using a radioimmunoassay kit specific for rat hormone derived from Linco Research, Inc., USA and glycerol was measured according to Foster and Dunn (1973). H-89 was from ICN Pharmaceuticals, Inc., USA. D-glucose, L-alanine, L-leucine and all other reagents were purchased from Sigma. The experiments were performed according to rules accepted by the Local Ethical Commission for Investigation on Animals. Results were statistically evaluated using one-way analysis of variance and

Duncan's multiple range test ($p \leq 0.01$).

Results

Incubation of adipocytes with H-89, an inhibitor of PKA, restricted basal lipolysis by 16 % and caused an increase by 17 % in leptin secretion stimulated by glucose and insulin. Activation of PKA by DB-cAMP evoked the opposite but more profound effect. Lipolysis was potentiated by more than 5-times and leptin secretion was restricted by 78 %. Lipolysis induced by DB-cAMP was markedly suppressed (by 66 %) due to inhibition of PKA activity by H-89. Inhibition of this enzyme restored the release of leptin to the level observed in adipocytes incubated without DB-cAMP (Fig. 1).

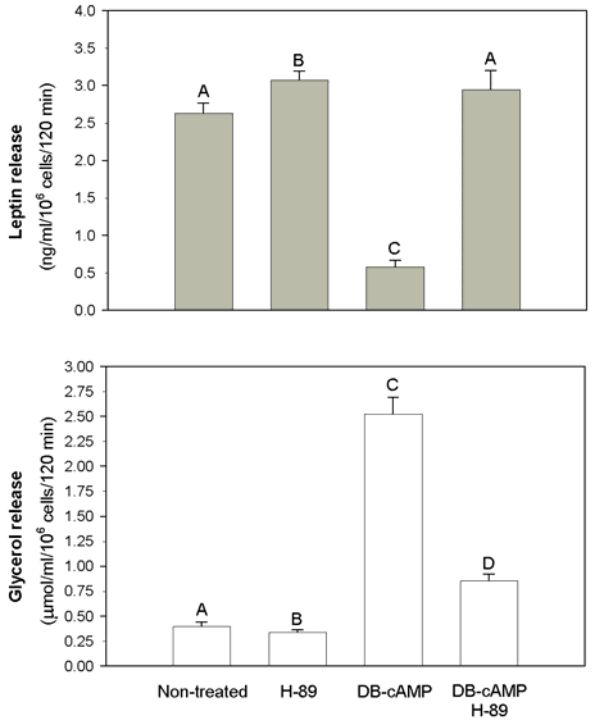

Fig. 1. The effect of H-89 (50 µmol/l), dibutyryl-cAMP (DB-cAMP, 1 mmol/l) and their combination on leptin secretion induced by glucose (5 mmol/l) and insulin (10 nmol/l) from isolated rat adipocytes (upper plot) and on lipolysis (lower plot). Values are means ±S.E.M. from two separate experiments (n=5). Means marked by different letters differ significantly ($p \leq 0.01$).

Leptin secretion stimulated by alanine and insulin was not significantly altered by H-89 in spite of restriction of lipolysis by 35 %. DB-cAMP increased glycerol release more than 8-times with simultaneous diminution of leptin secretion by half. Restriction of PKA activity by H-89 in the presence of DB-cAMP diminished lipolysis by 70 % and potentiated leptin release by 33 %. The inhibitory effect of DB-cAMP on hormone secretion was not fully suppressed (Fig. 2).

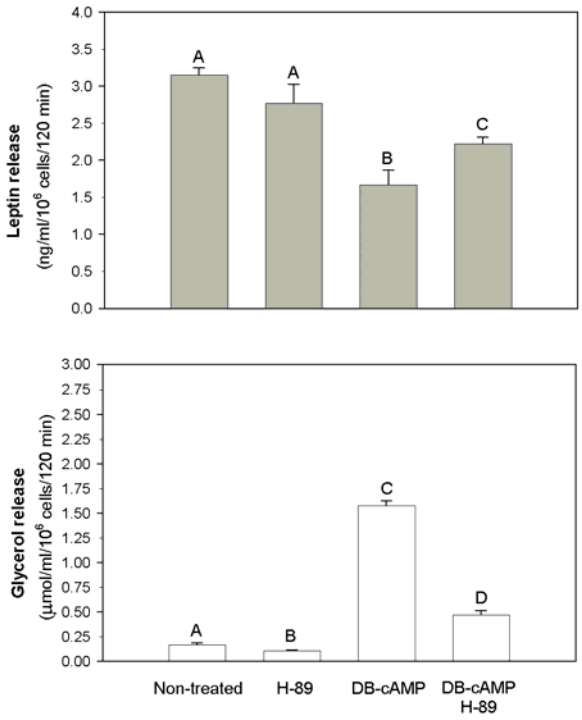

Fig. 2. The effect of H-89 (50 µmol/l), dibutyryl-cAMP (DB-cAMP, 1 mmol/l) and their combination on leptin secretion induced by alanine (10 mmol/l) and insulin (10 nmol/l) from isolated rat adipocytes (upper plot) and on lipolysis (lower plot). Values are means ±S.E.M. from two separate experiments (n=5). Means marked by different letters differ significantly ($p \leq 0.01$).

Secretion of leptin stimulated by leucine and insulin was, similarly as in the case of glucose and insulin, slightly augmented (by 14 %) when fat cells were incubated with H-89 and concomitant restriction of lipolysis by 46 % was found. DB-cAMP-induced activation of PKA evoked a drop in leptin release by half and a rise in lipolysis by more than four times. Lipolysis caused by DB-cAMP was reduced by 26 % as a result of H-89 action. Simultaneously, hormone secretion was augmented by 90 % in comparison to the level observed in adipocytes incubated with DB-cAMP alone (Fig. 3).

Discussion

Studies on isolated adipocytes revealed the inhibitory action of cAMP on leptin release from these cells. This effect was evoked by agents stimulating cAMP production, inhibiting its degradation or mimicking its action in fat cells (Gettys *et al.* 1996, Wang-Fisher *et al.* 2002, Cammisotto and Bukowiecki 2002). The

physiological significance of this phenomenon was confirmed *in vivo* in experiments demonstrating that agents augmenting intracellular cAMP content decrease plasma leptin level (Donahoo *et al.* 1997, Stumvoll *et al.* 2000).

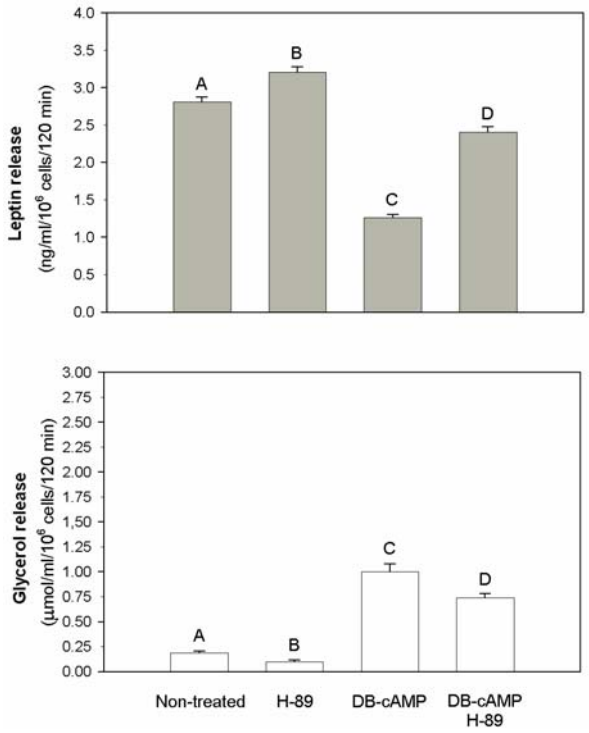

Fig. 3. The effect of H-89 (50 µmol/l), dibutyryl-cAMP (DB-cAMP, 1 mmol/l) and their combination on leptin secretion induced by leucine (10 mmol/l) and insulin (10 nmol/l) from isolated rat adipocytes (upper plot) and on lipolysis (lower plot). Values are means ±S.E.M. from two separate experiments (n=5). Means marked by different letters differ significantly ($p \leq 0.01$).

In the experiments performed previously the inhibitory effect of cAMP was observed when leptin secretion was stimulated by insulin, insulin and glucose or nicotinic acid. In the present studies we demonstrated a clear-cut inhibitory action of DB-cAMP, a non-hydrolysable cAMP analogue, on leptin secretion potentiated not only by glucose but also by alanine and leucine in the presence of insulin. This effect was accompanied by enhanced lipolysis reflecting the increase in PKA activity (Figs 1-3). The mechanism of the inhibitory action of cAMP on leptin secretion is not clear. One can suppose that the observed restriction of leptin secretion is not the result of the deterioration in the action of insulin since leptin release stimulated by nicotinic acid or its analogue Acipimox was also strongly depressed by DB-cAMP (Wang-Fisher *et al.* 2002). Kelada *et al.* (1992) demonstrated that DB-cAMP at 1 mmol/l substantially restricted glucose transport in adipocytes by inhibiting the ability of GLUT4 to transport this sugar. This effect is certainly responsible, at least partially, for the observed restriction of glucose-stimulated leptin secretion. However, the diminution of alanine-induced leptin release caused by DB-cAMP proves the existence of other effects of cAMP that strongly attenuate hormone release. These results indicate that the restriction of leptin secretion by cAMP is not characteristic solely for glucose and insulin, but also persists in the presence of other stimuli. It is likely that cAMP may restrict different steps of leptin secretion that are crucial for this process.

The stimulatory influence of glucose, and probably alanine, on leptin secretion is thought to be the result of their metabolism providing ATP (Levy *et al.* 2000). This assumption was confirmed by findings demonstrating that the inhibition of glucose transport and glycolysis resulted in restriction of leptin secretion (Mueller *et al.* 1998). Leucine potentiates leptin release acting at the level of translation *via* activation of protein kinase called target of rapamycin (mTOR) (Lynch 2001, Roh *et al.* 2003). The stimulatory influence of this amino acid on leptin secretion from isolated rat adipocytes was found to be without any rise in leptin mRNA and was not suppressed by antinomycin D, the inhibitor of transcription. Leucine-induced hormone secretion was, however, sensitive to rapamycin. These results confirm that mTOR is activated by this amino acid resulting in leptin secretion (Roh *et al.* 2003). Glucose and insulin may also be able to activate mTOR in adipocytes (Lin *et al.* 1994, Pause *et al.* 1994). However, in the case of leucine and insulin the activation of this process is not achieved by the identical way (Lynch 2001). Scott and Lawrence (1998) demonstrated that the activation of mTOR by insulin was inhibited by increased cAMP content in adipocytes. This finding may explain, at least partially, the suppressive effect of DB-cAMP on leptin secretion observed in our experiment. However, it is not clear whether PKA is involved in this action of cAMP. To test this, adipocytes were incubated with both specific PKA inhibitor and DB-cAMP. The restriction of PKA activity strongly attenuated lipolysis induced by DB-cAMP and simultaneously fully suppressed its inhibitory action on leptin secretion stimulated by glucose and insulin (Fig. 1). In the case of alanine and leucine the inhibitory action of DB-cAMP on leptin secretion was also significantly attenuated as a result of restriction of

PKA activity, however, total recovery of hormone secretion was not reached (Figs 2 and 3). Since the inhibition of PKA activity resulted in a significant rise of leptin secretion despite the presence of DB-cAMP in adipocytes, one can conclude that the action of cAMP on leptin release is PKA-dependent. This is in agreement with the results obtained by Cammisotto *et al.* (2003) indicating that the inhibition of leptin secretion is achieved by fatty acids released upon stimulation of lipolysis.

Apart from the influence of the restriction of PKA activity stimulated by DB-cAMP on leptin secretion, we attempted to determine whether the inhibition of basal activity of this enzyme affects hormone release. It was previously demonstrated that insulin is unable to suppress lipolysis and PKA activity when cAMP content is not increased by any agent (Morimoto *et al.* 1998). However, basal lipolysis may be substantially attenuated by H-89 (Szkudelski and Szkudelska 2000). The incubation of fat cells with this compound resulted in a partial restriction of non-stimulated lipolysis. Simultaneously, leptin secretion was slightly enhanced when potentiated by glucose and leucine in the presence of insulin (Fig. 1 and 3). These results indicate that the basal activity of PKA may be attenuated, at least by pharmacological agents, leading to a rise in leptin secretion. However, secretion of this hormone stimulated by alanine and insulin was unaffected by PKA inhibition in spite of reduced lipolysis (Fig. 2). The difference between glucose and alanine may suggest that the mechanism of their action as leptin secretagogues is not identical.

The results obtained in this experiment clearly demonstrated that leptin secretion stimulated by glucose, alanine and leucine in the presence of insulin is profoundly attenuated by DB-cAMP. It was also shown that the inhibitory effect of cAMP on leptin release is PKA-dependent. Therefore, the inhibition of PKA activity is of major importance for potentiation of leptin secretion than cAMP degradation *per se*. Under physiological conditions, several factors can enhance the cAMP content in adipocytes and thereby restrict leptin secretion. On the other hand, the rise in cAMP content is counteracted by insulin *via* activation of cAMP phosphodiesterase resulting in cAMP decomposition (Eriksson *et al.* 1995). Since leptin secretion strongly depends on PKA activity it is likely that the inhibition of this enzyme by insulin substantially contributes to the insulin-induced rise of leptin secretion from adipocytes.

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