EXPERIMENTAL STUDY

Catecholamines suppress leptin release from *in vitro* differentiated subcutaneous human adipocytes in primary culture via β_1 - and β_2 -adrenergic receptors

Dorothee Scriba, Irene Aprath-Husmann, Werner F Blum¹ and Hans HaunerGerman Diabetes Research Institute at the Heinrich-Heine-University Düsseldorf, Clinical Department, D-40225 Düsseldorf, Germany and ¹Department of Pediatrics, University of Gießen, and Lilly Deutschland GmbH, Bad Homburg, Germany

(Correspondence should be addressed to H Hauner, German Diabetes Research Institute at the Heinrich-Heine-University Düsseldorf, Auf'm Hennekamp 65, D-40225 Düsseldorf, Germany; Email: hauner@dfi.uni-duesseldorf.de)

Abstract

Objective: Circulating leptin, the product of the *ob* gene, is known to be closely correlated with adipose tissue mass, but it is also subject to short-term regulation by a variety of hormones including catecholamines. The aim of this study was to investigate the contribution of the three β -adrenergic receptors to leptin secretion from cultured human adipocytes.

Design and methods: The model of *in vitro* differentiated human subcutaneous adipocytes was used in this study. The presence of the β -adrenoceptor subtypes was studied by RT-PCR. The functional role of the receptor subtypes was determined by stimulation of lipolysis by selective β -adrenergic agonists and by measuring glycerol release. Leptin secretion into the medium of cultured human adipocytes from young normal-weight females was measured by radioimmunoassay.

Results and conclusion: In a first set of experiments, the expression of the three β -adrenergic receptor subtypes in cultured human adipocytes was demonstrated. To test their functional activity, the effect of the β -adrenoceptor agonists isoproterenol (non-selective agonist), dobutamine (β_1 -selective), fenoterol (β_2 -selective) and the β_3 -selective agonists BRL 37344 and CGP 12177 was studied. All agonists exhibited a dose- and time-dependent stimulation of glycerol release into the medium in a rather uniform manner. Isoproterenol rapidly reduced leptin secretion from cultured subcutaneous adipocytes in a dose-dependent fashion. Incubation with 10^{-6} mol/l isoproterenol for 24 h resulted in a reduction of the leptin concentration by 48% ($P < 0.01$). A similar, but less pronounced suppressing effect was seen for dobutamine and fenoterol, whereas both BRL 37344 and CGP 12177 were not effective. These data provide evidence that catecholamines are able to suppress leptin release from differentiated human adipocytes, supporting the concept that leptin secretion is acutely regulated by surrounding hormones. This inhibition is obviously mediated via β_1 - and β_2 -adrenergic receptors.

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Introduction

Leptin is the product of the recently discovered *ob* gene, which is mainly expressed by white and brown adipocytes (1). This protein is secreted into the circulation and after passing the blood–brain barrier is involved in the central regulation of food intake and energy expenditure (2–4). Although circulating serum leptin levels are closely correlated with adipose tissue mass (5), it is now well known that leptin is also subject to short-term regulation by metabolic and hormonal factors (6–8).

It was originally reported by Trayhurn and coworkers that exposure of mice to cold is followed by a rapid decrease of *ob* mRNA levels (9). The results of their study also suggested that this inhibition is mediated by an

increase in catecholamine secretion. This observation was confirmed in a number of subsequent studies in various animal models of obesity and under a variety of experimental conditions (10–16). In rodents, the suppression of leptin was mainly due to an activation of β_3 -adrenergic receptors (13–16).

There is now the first evidence from *in vitro* and clinical studies for a suppressive effect of catecholamines on leptin secretion in humans (17–23), although some discrepancies remain not fully resolved (24, 25). However, to date, it is still undefined which specific adrenoceptor subtypes are responsible for the inhibition of leptin release from human adipocytes.

It was the aim of this study to investigate the contribution of the three β -adrenergic receptor subtypes to the regulation of leptin release from human

adipocytes using the model of *in vitro* differentiated adipocytes. For this purpose, it was necessary to demonstrate the presence of the three β -adrenergic receptor subtypes as well as their functional role in terms of stimulation of lipolysis in cultured human adipocytes.

Materials and methods

Materials

Collagenase CLS type 1 was obtained from Worthington (Freehold, NJ, USA); human insulin and cortisol were kindly donated by Hoechst Marion Roussel (Frankfurt, Germany); culture media were obtained from Gibco (Berlin, Germany); isoproterenol, dobutamine, fenoterol, propranolol, and gentamycin were purchased from Sigma (Munich, Germany). BRL 37344 was obtained from RBI (Natick, MA, USA) and CGP 12177 was a gift from Ciba-Geigy (Basel, Switzerland). Superscript RT reverse transcriptase, random hexamers, Taq polymerase, PCR buffer, dCTP, dGTP, dTTP, dATP were obtained from Gibco/BRL (Berlin, Germany); for sequencing of the transcripts we used the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit from Perkin Elmer (Weiterstadt, Germany). Troglitazone was kindly provided by Sankyo (Munich, Germany). All other chemicals were from Boehringer (Mannheim, Germany) or Merck (Darmstadt, Germany). Sterile plasticware for tissue culture was purchased from Flow Laboratories (Irvine, Strathclyde, UK).

Subjects

Subcutaneous adipose tissue samples (50 to 100 g wet weight) were obtained from 9 normal-weight young females (body mass index (BMI) < 27 kg/m²; age 31 $\pm$ 8 years) undergoing elective mammary reduction. All subjects were of Caucasian origin. They were otherwise healthy according to clinical examination and laboratory tests and did not take any medication. Informed consent was obtained from the patients and the use of the adipose tissue for scientific studies was approved by the Ethical Committee of the Heinrich-Heine-University.

Isolation and *in vitro* differentiation of the stromal cell fraction from human adipose tissue

After removal, the adipose tissue samples were immediately transported to the laboratory in DMEM/F12 medium. The stromal cell fraction was isolated by collagenase digestion according to the method described recently with some minor modifications (26). Briefly, the fat specimens were cut into small pieces (1–2 mm³) under sterile conditions and incubated in a collagenase solution (0.5 mg collagenase per ml phosphate-buffered

saline (PBS) containing 20 mg/ml bovine serum albumin) for approximately 90 min in a shaking waterbath at 37°C. After removal of the floating adipocyte fraction and a short centrifugation at 200 *g*, the sedimented stromal cells were resuspended in an erythrocyte lysing buffer containing 154 mmol/l NH₄Cl, 5.7 mmol/l K₂HPO₄ and 0.1 mmol/l EDTA for 10 min to remove contaminating red blood cells. Finally, the dispersed cell material was filtered through a nylon mesh of 150 μ m pore size. After repeated washing steps, the stromal cells were inoculated in a medium consisting of DMEM/Ham's F12 (v/v, 1:1) supplemented with 10% fetal calf serum. After 16 to 20 h for cell attachment, cultures were washed two times with PBS to remove non-adhering cells, cell detritus and serum. Cells were then refed with a serum-free medium consisting of DMEM/Ham's F12 (v/v, 1:1) supplemented with 15 mmol/l NaHCO₃, 15 mmol/l Hepes, 33 μ mol/l biotin, 17 μ mol/l pantothenate, 10 mg/ml human transferrin and 50 μ g/ml gentamycin. To induce adipose differentiation, the medium was also supplemented with adipogenic factors including 66 nmol/l insulin, 100 nmol/l cortisol, 0.2 nmol/l tri-iodothyronine and, for the first three days, with 1 μ g/ml troglitazone. The culture medium was changed 3 times per week. Full adipose differentiation was obtained after 16 days in culture with 50–70% of cells developing the adipocyte phenotype. These *in vitro* differentiated fat cells were used to study the effects of exposure to β -adrenoceptor agonists on leptin secretion into the culture medium.

RNA preparation and RT-PCR

Total RNA from cultured cells was prepared according to the method described by Chomczynski & Sacchi (27). After incubation with the respective β -adrenoceptor agonist for 24 h, cells were harvested in TRIzol (Life Technologies, Eggenstein, Germany) and 200 μ l chloroform were added. After centrifugation, the aqueous phase was mixed with an equal volume of isopropyl alcohol. After 12 h at –20°C, the RNA was pelleted for 15 min at 10 000 *g*, redissolved in LiCl₄, washed twice with 70% ethanol, dried and redissolved in H₂O. To exclude contamination by genomic DNA, a DNase digestion (Promega, Madison, WI, USA) was performed according to the instructions of the manufacturer. The total RNA was diluted to 0.2 μ g/ μ l in H₂O, and first strand cDNA prepared using 1 μ g total RNA, Superscript RT reverse transcriptase, and random hexamers, according to the manufacturer's instructions.

Qualitative RT-PCR

cDNA was diluted 1:8 with H₂O, and PCR was carried out using 8.75 μ l diluted cDNA and a PCR reaction mix containing 2.0 U Taq polymerase in its 1 $\times$ buffer, 200 μ mol/l dCTP, dGTP, dTTP, and dATP, and 500 pmol/l of each primer in a 25 μ l volume with 50 μ l mineral oil.

PCR conditions were a denaturation step at 95 °C for 1 min followed by 33 cycles of 94 °C, 45 s; 61 °C, 45 s; 72 °C, 45 s for the detection of β_1 - and β_3 -adrenoceptor; and by 33 cycles of 94 °C, 45 s; 66 °C, 45 s; 72 °C, 45 s for the detection of β_2 -adrenoceptor. PCR products were transferred on a 2% TBE-agarose gel, stained with ethidium bromide, and were analyzed by the Lumi-Analyst system obtained from Boehringer. In this qualitative RT-PCR the following specific primers were used: β_1 -adrenoceptor specific primers (161 bp): 5'-GTG GCC CTA CGC GAG CAG AAG-3' sense; 5'-GCG TAG CCC AGC CAG TTG AAG A-3' antisense; β_2 -adrenoceptor specific primers (295 bp): 5'-TCT GGC CGC GTT TCT GTG TTG G-3' sense; 5'-GCT CTT CTG TGG CCG CTA CCT G-3' antisense; β_3 -adrenoceptor specific primers (178 bp): 5'-CCC AAT ACC GCC AAC ACC AGT-3' sense; 5'-CGG CCA GCG AAG TCA CGA A-3' antisense. The sequence of all transcripts was confirmed using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions.

Measurement of glycerol and leptin concentration in the culture medium

Glycerol release and accumulation in the culture medium was used as an index of lipolysis. The measurement was carried out using a bioluminescent assay developed by Hellmer *et al.* (28) using commercially available solutions (BioThema AB, Dalarö, Sweden). Leptin was measured in the medium using a specific highly sensitive radioimmunoassay (RIA) as described recently (29). The intra- and interassay coefficients of variation of the leptin RIA were 0.8% and 8.5% respectively.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Differences between groups were tested using Student's *t*-test for paired data. Differences with a *P* value <0.05 were regarded as significant.

Results

Expression of β -adrenergic receptors and lipolytic response to agonists in *in vitro* differentiated human adipocytes

First, to address the question of whether *in vitro* differentiated human adipocytes express the full lipolytic system, we examined the presence of the three β -adrenoceptor subtypes in differentiated fat cells. By using a qualitative RT-PCR technique we were able to demonstrate specific transcripts for the three subtypes according to transcript length and sequence (Fig. 1).

In order to assess the functional role of the three receptors, we studied the effect of specific β -adrenoceptor agonists on lipolysis. As depicted in Fig. 2, the

Figure 1 Specific transcripts of the mRNA of the three β -adrenoceptor subtypes in *in vitro* differentiated subcutaneous human adipocytes on day 18 as assessed by qualitative RT-PCR. One representative example out of four agarose gels is shown as an inverted image. A 100 bp ladder (600 bp band is 2–3 $\times$ pronounced) was used as standard (Std.).

non-selective agonist, isoproterenol, was found to induce a dose-dependent release of glycerol into the culture medium after a 12-h exposure. Similar effects were observed for the selective β_1 -agonist, dobutamine, the β_2 -agonist, fenoterol, the β_3 -adrenoceptor agonist CGP 12177 and BRL 37344 (Fig. 2). At a concentration of 10^{-6} mol/l for 12 h, isoproterenol caused a 6.3-fold increase in glycerol concentration above controls. The respective increase for dobutamine was 5.5-fold, for fenoterol 4.4-fold, for BRL 37344 6.3-fold and for CGP 12177 5.2-fold (Fig. 2). Similar data were obtained when cells were exposed to the β -adrenoceptor agonists for 4 and 24 h respectively (data not shown).

Effect of β -adrenoceptor agonists on leptin secretion from human adipocytes

Figure 3 shows the time-course of leptin secretion into the culture medium in the absence and presence of 10^{-7} mol/l isoproterenol. After 4 and 24 h respectively there was a statistically significant reduction of leptin accumulation in the medium after exposure to isoproterenol. After the 4-h incubation, leptin concentration was reduced by 35% ($P < 0.05$) and after a 24-h exposure by 34% ($P < 0.01$). The inhibitory effect of isoproterenol was independent of the presence of troglitazone during the initial three days of culture for induction of adipose differentiation. However, in the presence of troglitazone the differentiation rate was clearly higher than in those without the thiazolidinedione (data not shown).

Figure 4 demonstrates the dose-response relationship between isoproterenol and leptin release. A significant reduction of leptin release was already detectable at a concentration of 10^{-8} mol/l isoproterenol after a

Figure 2 Dose-dependent effect of selected β -adrenoceptor agonists on glycerol release into the culture medium from *in vitro* differentiated human subcutaneous adipocytes on day 18. Cells were exposed to the agonists for 12 h. Results are means $\pm$ S.E.M. of three separate experiments evaluated by Student's *t*-test for paired data. * $P < 0.05$, ** $P < 0.01$, compared with control (C).

24-h incubation. In the presence of 10^{-6} mol/l dobutamine for 24 h, leptin concentration was reduced by 25% ($P < 0.05$) (Fig. 5). A smaller but still significant reduction by 16% was seen after a 24-h exposure to 10^{-6} mol/l fenoterol ($P < 0.05$) (Fig. 5). In contrast, the two β_3 -adrenoceptor agonists, BRL 37344 and CGP 12177, were without an effect on leptin release (Fig. 5) indicating that the β_3 -adrenoceptors did not contribute to the suppression of leptin in human adipose tissue. This conclusion is also substantiated by another

experiment ($n = 4$) where cells were incubated with 10^{-7} mol/l propranolol and simultaneously with 10^{-7} mol/l isoproterenol. The presence of propranolol was found to completely prevent the inhibitory effect of isoproterenol on leptin secretion (10^{-7} mol/l isoproterenol for 24 h: $63.3 \pm 6.4\%$ of control, $P < 0.001$; 10^{-7} mol/l propranolol and 10^{-7} mol/l isoproterenol: $95.0 \pm 4.5\%$ of control), also arguing against a role of β_3 -adrenoceptors in the regulation of leptin secretion from human adipocytes.

Figure 3 Time-course of the effect of isoproterenol on leptin release into the culture medium of *in vitro* differentiated human adipocytes. Cells were exposed to 10^{-7} mol/l isoproterenol for the time intervals indicated. Results are means $\pm$ S.E.M. of four separate experiments in triplicate evaluated by Student's *t*-test for paired data. C, control (no isoproterenol).

Figure 4 Dose–response relationship of the effect of isoproterenol on leptin release into the culture medium of *in vitro* differentiated human adipocytes. Cells were exposed to isoproterenol for 24 h. Results are means $\pm$ S.E.M. of three separate experiments in triplicate evaluated by Student's *t*-test for paired data. **P* < 0.05, compared with control (C).

Discussion

The results of these experiments clearly show that the non-selective β -adrenoceptor agonist, isoproterenol, exerts an inhibitory action on leptin protein release from cultured human adipocytes from young normal-weight females. This effect was observed in the presence of insulin in the culture medium. The inhibition of leptin release from human adipocytes by isoproterenol was time- and dose-dependent and it is important to note that this effect was rapid and had already reached statistical significance after 4 h.

These data are in good agreement with the results from a recent study by Ricci & Fried (23) who also found a decrease of leptin secretion and steady-state mRNA levels in adipose tissue from obese humans after exposure to isoproterenol. Furthermore, in this and other studies a decrease of leptin protein by catecholamines was also shown for omental adipose tissue (21, 23) which is another, but less important source of circulating leptin in humans (30).

In view of pronounced differences in the BMI of the donors and the culture conditions between the study of Ricci & Fried (23) and our study, these results indicate that the inhibitory effect of isoproterenol is independent

of the relative weight of the donor and of the experimental conditions.

In additional experiments, we also addressed the question of which β -adrenergic receptors contribute to this suppression. Our results suggest that the isoproterenol-induced suppression of leptin production is mediated via β_1 - and β_2 -adrenoceptors, although the β_3 -adrenoceptor subtype was clearly detectable at the mRNA level using a qualitative RT-PCR technique. However, the two selective β_3 -adrenoceptor agonists, BRL 37344 and CGP 12177, despite their possible interaction with other adrenoceptor subtypes (31) were unable to suppress leptin secretion and mRNA expression.

This finding is in clear contrast to the results of earlier reports in various animal models of obesity, and in clonal cell lines from rodent origin. In these studies, it was shown that the inhibitory effect of catecholamines was mainly mediated via the β_3 -adrenergic receptor which is the predominant β -adrenoceptor subtype in rodents (31). For instance, Moinat *et al.* (10) showed that administration of a β_3 -adrenoceptor agonist inhibits leptin expression in white and brown adipose tissue of lean Zucker rats. Similar findings were also reported by other groups (13–16).

Figure 5 Effect of selective β -adrenoceptor agonists on leptin release into the culture medium of *in vitro* differentiated human adipocytes. Cells were exposed to the agonists for 24 h. Each agonist was used at a concentration of 10^{-6} mol/l. Results are means $\pm$ S.E.M. of six separate experiments in triplicate evaluated by Student's *t*-test for paired data. **P* < 0.05, compared with control (C).

In cultured isolated rat adipocytes, isoproterenol and, less efficiently, a β_3 -adrenoceptor agonist decreased leptin mRNA expression and secretion into the medium (11). This study also established that addition of dibutyl cAMP mimicked this effect suggesting that the effect of β_3 -adrenoceptor agonists is exerted by its classical receptor pathway (11, 14, 16). Similar findings were reported for the 3T3-L1 adipocyte cell-line, where isoproterenol also potently reduced leptin mRNA levels, with a maximum effect at 10^{-7} mol/l. In addition, propranolol was found to partially block this inhibition (12).

According to our results incubation of human adipocytes with β_3 -adrenoceptor agonists did not affect leptin release despite a potent stimulation of lipolysis. This finding may indicate that regulation of leptin release by catecholamines is not linked to the lipolytic pathway and may involve other, not yet identified, mechanisms. The physiological role of the β_3 -adrenoceptor in human adipose tissue is still a matter of debate, as controversial findings have been reported for both the expression and function of this subtype in humans (32–35).

In conclusion, our experiments suggest an acute inhibitory effect of catecholamines on leptin secretion from cultured human adipocytes via β_1 - and β_2 -adrenoceptor agonists. In contrast to animal studies, the β_3 -adrenergic receptor subtype, although detectable and functionally active in cultured human adipocytes, does not appear to be involved in the inhibition of leptin expression.

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