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SCIENCE DIRECT

Metabolism Clinical and Experimental 55 (2006) 8–12

Metabolism
Clinical and Experimental

www.elsevier.com/locate/metabol

Leptin-mediated inhibition of the insulin-stimulated increase in fatty acid uptake in differentiated 3T3-L1 adipocytes

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Received 7 February 2005; accepted 16 June 2005

Abstract

The effects of insulin and leptin on fatty acid uptake in differentiated (adipocytes) and undifferentiated 3T3-L1 cells were investigated. It was demonstrated that in undifferentiated 3T3-L1 cells, insulin and leptin have no effect on fatty acid uptake. In differentiated 3T3-L1 adipocytes, insulin had a concentration-dependent stimulatory effect on fatty acid uptake, whereas leptin on its own had no effect. Leptin, when coincubated with 10 nmol/L insulin, resulted in a concentration-dependent inhibition of the insulin-stimulated fatty acid uptake in differentiated 3T3-L1 cells. These results indicate that leptin has a direct inhibitory effect on the stimulation of fatty acid uptake by insulin in differentiated murine adipocytes.

1. Introduction

Leptin plays an important role in the etiology and the maintenance of obesity, for the most part due to a resistance to leptin developed by obese subjects [1,2]. Understanding how leptin can influence and regulate biomarkers of metabolic activity, such as glucose and fatty acids, is therefore crucial for the development of therapies destined to tackle metabolic disorders. Fatty acids are used for diverse cellular processes, including mitochondria oxidation, membrane synthesis, and energy storage. Pathologically increased intracellular fatty acid concentrations can cause cellular apoptosis and have been linked to insulin desensitization, type 2 diabetes, obesity, and cardiovascular disease [3]. All of these intracellular processes are dependent upon fatty acids traversing the plasma membrane to get into the cell. Therefore, understanding how fatty acid transport proteins regulate this process and how agents can modulate them is of great importance in biomedical research and drug discovery.

Evidence shows that insulin can stimulate fatty acid uptake in dissociated normal rat adipocytes [4]. Likewise, the uptake of fatty acids is up-regulated in adipocytes of Zucker *fa/fa* rats, which have a nonsignaling mutated form of the leptin receptor, suggesting that leptin could be involved in the regulation of fatty acid transport [5]. Similarly, long-term leptin administration to rats has an inhibitory effect on fatty acid uptake and fatty acid transporters in skeletal muscle [6]. The combined results from these reports point toward a possible modulatory effect of leptin and insulin on fatty acid uptake.

In this study, a fluorescence-based method was used to investigate both the individual and combined effects of insulin and leptin on fatty acid uptake in undifferentiated and differentiated 3T3-L1 cells.

2. Methods

2.1. Cell culture

3T3-L1 cells (LGC Promochem, Teddington, UK; ATCC, Manassas, USA) were differentiated according to a 3-stage protocol that requires a fresh media change (composition: Dulbecco's modified Eagle's medium containing 5.5 mmol/L glucose, 10% fetal bovine serum, 2 mmol/L L-glutamine,

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doi:10.1016/j.metabol.2005.06.013

100 U/mL penicillin, 0.1 mg/mL streptomycin) every 2 to 3 days. Stage 1: Flasks of cells (80%-90% confluent) were incubated in media containing insulin (5 μg/mL), isobutylmethylxanthine (0.5 mmol/L), and dexamethasone (0.25 μmol/L) for 2 days. Stage 2: Media was then replaced by fresh media containing insulin (5 μg/mL) for 2 days. Stage 3: Cells should be more than 90% differentiated into adipocytes; otherwise, the flask was discarded (adipocyte phenotype verified by accumulation of lipid droplets). Cells were then placed in fresh media with no additives for 7 days. After this period, the adipocytes were ready for assay. Cells were then plated into a 96-well plate (black wall/clear bottom, Costar) at 50 000 cells per well in 100 μL media for 4 hours before the experiment. Before the experiment, undifferentiated 3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium containing 5.5 mmol/L glucose, 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin until 80% to 90% confluent.

2.2. Detection of ObRb in 3T3-L1 cells

2.2.1. RNA extraction and complementary DNA synthesis

Total RNA was extracted from differentiated and undifferentiated 3T3-L1 cells before assay for fatty acid uptake using the Promega SV total RNA isolation kit (Promega, Southampton, UK) according to the manufacturer's instructions. Ten microliters of the extracted RNA sample was used to synthesize first-strand complementary DNA (cDNA) after heating at 70°C for 5 minutes in the presence of oligo dT. Nine microliters of cDNA mix (composed of 1 μL of 10 mmol/L deoxy-NTP, 4 μL of 5× concentrate first-strand buffer [Promega], 2 μL of 0.1mol/L dithriothreitol, 1 μL of RNase/nuclease-free water, and 1 μL of Superscript II reverse transcriptase [Invitrogen, Paisley, UK]) was then added to the RNA–oligo dT mixture and incubated at 42°C for 60 minutes followed by 5 minutes at 70°C.

2.2.2. Polymerase chain reaction

One microliter of cDNA sample was added to the polymerase chain reaction (PCR) mix comprising 7.2 μL sucrose, 2 μL 10× reaction buffer, 1 μL (10 mmol/L) deoxy-NTPs, 1 μL primer, 7.67 RNase/nuclease-free water, and 0.125 μL (25 U) of Taq polymerase and then heated at 94 ° C for 2 minutes. It then underwent 35 thermal cycles (2 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C). Finally, the samples were heated for 10 minutes at 72°C and then rested at 4°C until removed. Primers used to amplify rat ObRb cDNA were specific forward primer for the long form (CACCCAGGGAACCTGTGAGG) combined with a specific reverse primer for the long form (AGTGAGCTGGGAATGGGCAC). As a control for messenger RNA (mRNA) quality, β-actin mRNA was amplified from RNA samples using primers ATAGGCCTGTCTGTCGGCCC and ACAAGGAGTGGACGGCACG. The amplified samples were then resolved in a 1.5% agarose gel with ethidium bromide. Images were obtained using a GelDoc UV system (BioRad, Hemel Hempstead, UK).

2.3. Fatty acid uptake assay

2.3.1. Assay principle

The homogeneous fatty acid uptake assay (QBT; Molecular Devices, Sunnyvale, CA) consists of a fluorescence-based assay for the detection of fatty acid uptake in cells containing fatty acid transporters [7]. The BODIPY label provides an ideal long-chain fatty acid analogue that behaves much like natural fatty acids: it becomes activated by acyl-CoA attachment, is incorporated into di- and triglycerides, and accumulates in intracellular lipid droplets. The QBT fatty acid uptake assay kit consists of a formulation of quenching agent Q-Red.1 (Molecular Devices) [8] and a pretitered amount of BODIPY-FA (4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid; Molecular Probes, Carlsbad, USA). QBT fatty acid uptake assay stock solutions were dissolved completely by adding 10 mL of 1× HBSS buffer (1× Hanks balanced salt solution with 20 mmol/L HEPES and 0.2% fatty acid–free BSA).

Uptake of the fluorophore occurs through mainly active transport, as the BODIPY analogue is a known substrate for fatty acid transporters because its uptake by adipocytes can be competed by nonlabeled fatty acids [7]. The quenching dye eliminates the need to repeatedly wash the fluorophore away from the cells, thus allowing computer data acquisition (referred to as relative fluorescence units [RFUs]) of real-time uptake as well as less perturbation to the cells.

2.3.2. Uptake assay

Cells were incubated in serum-free medium (90 μL per well) for 1 hour. Ten microliters of leptin (0.1 pmol/L to 100 nmol/L) or vehicle solution was then added. After an incubation period of 60 minutes, a further 10 μL of insulin (0.1 pmol/L to 100 nmol/L) or vehicle was added. After a period of 30 minutes, the reaction was initiated by the addition of 100 μL of QBT dye. Fatty acid uptake was monitored using a Flexstation (Molecular Devices; excitation, 485 nm; emission, 515 nm; cutoff, 495 nm) for 60 minutes. For experiments where the effect of leptin was tested on the insulin-stimulated uptake, a single concentration (submaximal) of 10 nmol/L insulin was used chosen from the concentration-response curve.

2.3.3. Drugs and reagents

Leptin (mouse) and insulin (bovine) were obtained from Sigma (Poole, UK). QBT fatty acid uptake assay kit was obtained from Molecular Devices. Cell culture reagents and all other reagents used were obtained from Sigma.

2.4. Data analysis

Visual determination of the level of ObRb (presence or absence) expression was used to evaluate the respective amounts of ObRb mRNA in the undifferentiated and differentiated cells. RFU data were analyzed as mean area

under the curve (AUC ± SE) using Softmax Pro 4.3DD software from Molecular Devices. Curve and statistical analysis (sigmoidal curve fitting option and Student *t* test) were performed with GraphPad Prism version 3.03 (San Diego, USA). Concentration-response curves were generated from experiments that were performed 4 to 5 times on different days. In the case of the curve showing the inhibitory effect of leptin on the insulin-stimulated fatty acid uptake (expressed as percentage of insulin effect), the vehicle data were subtracted from all values.

3. Results

3.1. Leptin receptor expression in differentiated and undifferentiated 3T3-L1 adipocytes

The presence of the ObRb receptor (signaling form of the leptin receptor) was verified in differentiated and undifferentiated 3T3-L1 cells. Reverse transcription–PCR was used to detect the presence of ObRb in RNA isolated from differentiated and undifferentiated 3T3-L1 cells. ObRb was only expressed in adipocyte-differentiated 3T3-L1 cells (Fig. 1). This detection of the signaling form of the leptin receptor in differentiated 3T3-L1 cells suggests that they can be used to study the effects of leptin on adipocyte metabolism.

3.2. Insulin, but not leptin, increases fatty acid uptake in differentiated 3T3-L1 adipocytes

When incubated for 30 minutes with different concentrations of insulin (0.1 pmol/L to 100 nmol/L) before reading, differentiated 3T3-L1 adipocytes displayed a concentration-dependent increase in the fatty acid uptake (EC_{50} = 0.17 nmol/L, 95% confidence interval [CI], 0.031-0.99; maximal stimulation, 112% at 100 nmol/L) (Fig. 2). Leptin tested under the same conditions (but using an incubation time of 90 minutes instead of 30 minutes used for insulin) had no effect on the fatty acid uptake (0.1 pmol/L to 100 nmol/L) (Fig. 2).

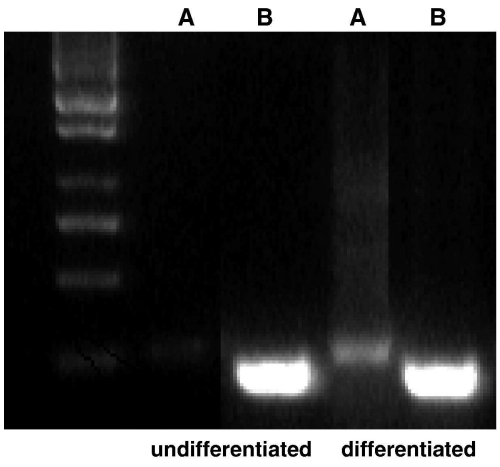

Fig. 1. Characterization of the leptin receptor ObRb (long form) expression in differentiated, and undifferentiated 3T3-L1 cells. The reverse transcription–PCR was performed on mRNA from both differentiated and undifferentiated 3T3-L1 cells. A indicates mRNA ObRb, B, mRNA actin.

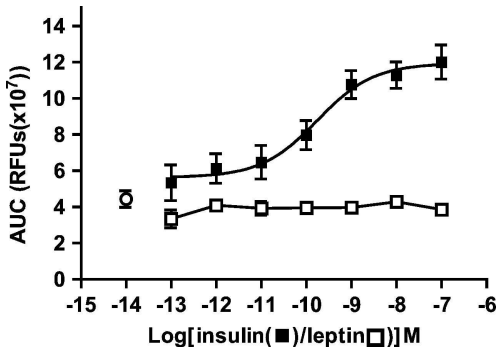

Fig. 2. Fatty acid uptake concentration-response curve showing an increase in AUC fluorescence in the presence of insulin in differentiated 3T3-L1 cells. The EC_{50} for the effect of insulin in differentiated cells (filled squares) is 0.175 nmol/L (95% CI, 31-994 pmol/L). Leptin (open squares) did not cause any changes in fatty acid uptake in this assay when compared with vehicle (open circle). The data were obtained from 4 different experiments performed on different days and expressed as RFUs.

3.3. Insulin and leptin have no effect on fatty acid uptake in undifferentiated 3T3-L1 adipocytes

Undifferentiated 3T3-L1 cells were incubated for 30 minutes in the presence of a range of concentrations of insulin (0.1 pmol/L to 100 nmol/L). Unlike the differentiated adipocytes, 3T3-L1 fibroblasts did not exhibit any response to insulin stimulation of fatty acid uptake (Fig. 3). In the same conditions (but using an incubation time of 90 minutes instead of 30 minutes), leptin (0.1 pmol/L to 100 nmol/L) had no effect on the uptake. Leptin tested in the presence of insulin again had no effect on the uptake of fatty acid in undifferentiated 3T3-L1 cells.

3.4. Leptin inhibits the insulin-stimulated increase in fatty acid uptake in differentiated 3T3-L1 adipocytes

A concentration of 10 nmol/L insulin caused a submaximal response and was chosen to characterize the effects of

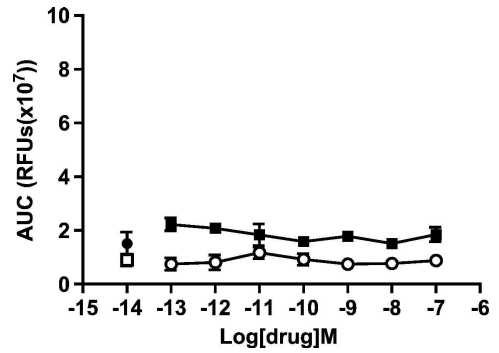

Fig. 3. Insulin (filled squares) and leptin (open circles) did not cause any change in fatty acid uptake in undifferentiated 3T3-L1 cells (0.1 pmol/L to 100 nmol/L). Filled circle represents vehicle. A concentration of 10 nmol/L leptin incubated with 10 nmol/L insulin (open square) did not cause any changes in fatty acid uptake compared with vehicle (filled circle) (Student *t* test, $P > .05$). The data were obtained from 4 different experiments performed on different days and expressed as RFUs.

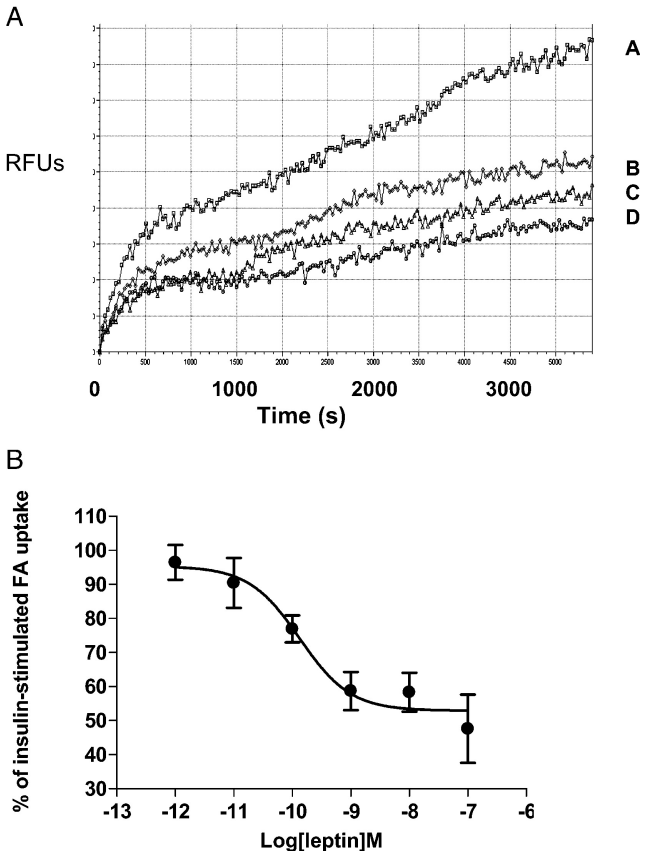

Fig. 4. A, Typical time course (1 hour) showing the increase in fluorescence (and expressed as RFUs) caused by 10 nmol/L insulin (A) in differentiated 3T3-L1 adipocytes. This translates to an increase in fatty acid uptake. Leptin (10 nmol/L), when administered with 10 nmol/L insulin (B), decreases the uptake of fatty acid by differentiated 3T3-L1 adipocytes. Leptin (10 nmol/L) on its own (C) does not elicit significant changes in fatty acid uptake vs vehicle (D). The mean AUC was obtained using similar graphs. B, Concentration-response curve of the effect of a range of concentrations of leptin (1 pmol/L to 100 nmol/L) on the increase in fluorescence elicited by 10 nmol/L insulin. The IC_{50} for this effect is 0.13 nmol/L (95% CI, 0.03-0.57 nmol/L). Background/vehicle is first subtracted from the data and then expressed as a percentage of the insulin response for each experiment. This graph was constructed from 5 different experiments performed on different days.

leptin on fatty acid uptake. A typical example of the effect of 100 nmol/L leptin on 10 nmol/L insulin (Fig. 4A) shows that the rate of fatty acid uptake is decreased in the presence of leptin (leptin on its own had no effect on the uptake). This inhibitory effect of leptin is concentration-dependent as seen in Fig. 4B. For this analysis, the vehicle control obtained for each separate experiment was subtracted from all values. Leptin caused a maximal decrease of 47% at 100 nmol/L of the insulin-stimulated fatty acid uptake (IC_{50} = 0.13 nmol/L; 95% CI, 0.03-0.57 nmol/L).

4. Discussion

A wealth of evidence is now available to indicate that leptin serves as a key mediator in the neuroendocrine cascade regulating energy expenditure and food intake. Injections of leptin in the hypothalamus (ventromedial nucleus) result in a decreased glucose uptake in the rat skeletal and heart muscle as well as brown adipose tissue, but not in the white adipose tissue [9]. Further studies show that fatty acid transport can be rapidly increased in the muscle after contraction and by the effect of insulin increasing the plasma translocation of the FAT/CD36 fatty acid transport–associated protein [10]. The hypothesis that a similar action can occur in myocytes was initially tested by Bonen et al [11], who reported that insulin facilitates the translocation of FAT/CD36 to the cell membrane, thus facilitating fatty acid transport. In this study, a new fluorescence-based assay relying on the use of a fatty acid analogue dye coupled to a specific quencher was used. The results reported show the stimulatory action of insulin on fatty acid transport in differentiated 3T3-L1 adipocytes expressing the long form of the leptin receptor with subnanomolar concentrations. The uptake of fatty acid by 3T3-L1 cells has been reported to be predominantly transporter-mediated [4]. This leads to the hypothesis that the observations made in this study may be occurring mainly via active transport and not diffusion. In undifferentiated 3T3-L1 fibroblasts, insulin had no effect on the fatty acid uptake, suggesting that the intracellular signaling necessary for fatty acid transport modulation by insulin is not yet in place or is different in these cells. Furthermore, despite the long form of the leptin receptor being expressed in the differentiated 3T3-L1 adipocytes, leptin was shown to be ineffective at modulating the uptake of fatty acid when tested. It is hypothesized that in this system, insulin stimulation of fatty acid uptake is a precondition for observing the inhibitory effects of leptin.

This contrasts with a previous study where insulin and leptin are shown to have opposite effects on the transport of long-chain fatty acid in dissociated adipocytes [12]. In this previous study, an effect of leptin was only observed after several days of treatment of the animals, whereas in the present study, the effect was measured after 30 minutes of incubation. Such temporal difference might explain the dissimilarity of the results. We suggest that the mechanisms described in this study may involve fatty acid transport modulation and probably not modifications in the fatty acid partitioning, which affect adipose tissue. In this study, we have demonstrated an inhibitory effect of leptin on insulin-stimulated fatty acid uptake in differentiated 3T3-L1 adipocytes, therefore reaffirming the tight links that exist between the 2 hormones.

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