ORIGINAL PAPER

Eur. J. Histochem.
44: 325-334, 2000

Characterization of Leptin Intracellular Trafficking

M. Wilcke, and E. Walum

Molecular Biology & Genomics, Department of Biology, Pharmacia Corporation, S-112 87, Stockholm, Sweden

Accepted: 06/09/00

Key words: OB, obesity, leptin, endocytosis, intracellular transport

SUMMARY

Leptin is produced by adipose tissue, and its concentration in plasma is related to the amount of fat in the body. The leptin receptor (OBR) is a member of the class I cytokine receptor family and several different isoforms, produced by alternative mRNA splicing are found in many tissues, including the hypothalamus. The two predominant isoforms includes a long form (OBR_l) with an intracellular domain of 303 amino acids and a shorter form (OBR_s) with an intracellular domain of 34 amino acids. Since OBR_l is mainly expressed in the hypotalamus, it has been suggested to be the main signalling form. The peripheral production of leptin by adipocyte tissue and its effects as a signal of satiety in the central nervous system imply that leptin gains access to regions of the brain regulating in energy balance by crossing the blood-brain barrier. In an attempt to characterize the intracellular transport of leptin, we have followed binding internalization and degradation of leptin in HEK293 cells. We have also monitored the intracellular transport pathway of fluorescent conjugated leptin in HEK293 cells. Phenylarsine oxide, a general inhibitor of endocytosis, as well as incubation at mild hypertonic conditions, prevented the uptake of leptin, confirming a receptor-mediated internalization process. When internalized, ^{125}I-leptin was rapidly accumulated inside the cells and reached a maximum after 10 min. After 70 minutes about 40-50% of total counts in each time point were found in the medium as TCA-soluble material. Leptin sorting, at the level of early endosomes, did not seem to involve recycling endosomes, since FITC-leptin was sorted from Cy3-transferrin containing compartments at 37°C. At 45 minutes of continuos internalization, FITC-leptin appeared mainly accumulated in late endocytic structures colocalizing with internalized rhodamine coupled epidermial growth factor (EGF) and the lysosomal marker protein lamp-1. The transport of leptin was also shown to engage a monensin and bafilomycin sensitive degradation process in lysosomes. Together, our results provide novel data concerning the uptake, intracellular localization and transport of leptin.

The abbreviations used are: OB, leptin; OBR_s, the short form of the human OB-receptor; OBR_l the long form of the human OB-receptor; HEK293, human embryonal kidney cells; FITC, Fluorescein isothiocyanate; Cy3, cyanine dye 3; FCS, fetal calf serum; Tf. transferrin; TfR, transferrin receptor.

Correspondence to: M. Wilcke
E-mail: mona.wilcke@eu.pnu.com

INTRODUCTION

Leptin is a 16 kDa protein transcribed from the *obesity (ob) gene*, and it acts through binding to the leptin receptor (OBR). In strains of obese mice with normal leptin genes, plasma leptin levels are correlated with body fat mass but injections of recombinant leptin has little effect on the body weight. These observations suggests that other mutations, perhaps in leptin receptors or other mediators, may impair the animals ability to regulate energy intake and expenditure. Leptin down regulates the expression of mRNA for neuropeptide Y, which stimulates food intake and decreases thermogenesis [1]. The OBR, a member of the cytokine receptor family, is a product of the *diabetes* (*db*) gene, which encodes several different spliced forms of various lengths [2, 3]. The longest form of the receptor, OBR_l, has an extended cytoplasmic domain of 303 amino acids. OBR_l is mainly expressed in the hypotalamus where it is thought to mediate reduction in food intake and increased energy consumption followed by weight loss [4-8]. One of the shorter forms, OBR_s, is truncated at 34 amino acids in its cytoplasmic tail and is the most broadly expressed form [3, 9, 10]. The detailed mechanism of leptin transport into the CNS is unknown as well as the function of the OBR_s isoforms. However, it has been suggested that OBR_s may play a primary role in cellular leptin transport and in clearance of leptin from the circulation since a high expression of OBR_s has been seen at the blood-brain barrier [11, 12]. In the present study, we have studied the intracellular transport of the leptin in HEK293 cells. We have compared the trafficking of this protein with those of two marker proteins, human transferrin (Tf) and epidermal growth factor (EGF). Leptin was found sorted out from transferrin at an early stage, i.e. at the level of sorting endosomes. Upon continuous internalization, leptin appeared mainly accumulated in late endocytic structures, partially colocalizing with internalized Rhodamine coupled EGF and the lysosomal marker protein lamp-1.

MATERIALS AND METHODS

Reagents and antibodies

Rhodamine coupled EGF was obtained from Molecular Probes (Inc. Oregon, USA). RPMI 1640, fetal calf serum (FCS), penicillin, streptomycin, sodium pyruvate were purchased from Gibco (Paisley, Scotland). Protein-A Sepharose was from Pharmacia (Uppsala, Sweden). The following antibodies were used: the anti-human transferrin receptor was obtained from Boringer Mannheim. OBR polyclonal antibodies (anti-OBR_s) were produced by Neosystems (Strasbourg) and were raised against the amino acids 481-502 present in both the short and the long form of the human leptin receptor. Lamp-1 antibodies were from PharMingen. FITC- and Texas Red labelled donkey secondary antibodies were from Amersham Corp. Rhodamine conjugated EGF and FITC, marine blue and Cy3 labelling kits were all obtained from Molecular Probes (Inc. Oregon, USA). Iron-saturated human transferrin was from Sigma Immunochemicals (St.Louis, MO). Iodine was obtained from Amersham Corp.

Cell culture and internalization of endocytic markers

HEK293 were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Scotland) supplemented with 10% FCS and 5 mM glutamine (Gibco, Scotland) in a 5% humidified CO_2 incubator. Leptin and iron-saturated human transferrin (Sigma) was labelled with Cy3 (Amersham) or FITC (Molecular probes). For internalization of fluorophore-coupled proteins as Cy3-transferrin (Cy3-Tf), FITC-leptin and rhodamine-EGF, HEK293 cells grown on 12 mm round coverslips and were incubated in DMEM medium supplemented with 10% FCS, 4 mM glutamine in a 5% humidified CO_2 incubator for various times at 19°C or at 37°C. Cells were washed twice with ice cold PBS before fixation with 4% paraformaldehyde.

Immunofluorescence

For immunofluorescense experiments, cells were grown on 12-mm round glass coverslips, prior to fixation and quenching in 50 mM glycine. Cells were the permeabilized in 0.2% saponin containing PBS and 0.1% BSA. Cells were then double labelled with antibodies against various proteins. As secondary antibodies, we used donkey anti-IgG antibodies coupled to FITC or Texas Red (Amersham). When indicated cells were also treated with 80 μM Phenylarsine oxide (Sigma), 0.35 M sucrose, 50μM of monensin (Sigma) or 0.25μM

bafilomycin (Sigma) for 30 minutes before fixation. Coverslips were mounted in Mowiol (Sigma). Double immunofluorescence analysis were examined with a Leica DMR-RXA microscope (Leica Microsystems, Switzerland) equipped with a three-chip charged-coupled device (CCD) camera (C5810, Hamamatsu Photonics, Hamamatsu City, Japan). The images were captured using the Lida software and processed in a Quantimet 550 Pro Image Workstation (Leica Imaging Systems Ltd, Cambridge, UK) and Adobe Photoshop (Adobe Systems Inc, Mountain View CA).

Measurement of ^{125}I-leptin internalization and degradation

Cells were grown in DMEM-medium supplemented with 10% FCS in 24 wells plates to 90% confluence. To study ^{125}I-leptin internalization, HEK293 cells were incubated with 10 nM ^{125}I-leptin for 10 min as a pulse, to allow internalization but not degradation. Cells were then placed on ice and washed three times with ice cold DMEM-medium without FCS and further incubated at 37°C for different times with either DMEM-medium or DMEM-medium containing 10 nM unlabeled leptin or 50 μM monensin a well established blocker of receptor/ligand recycling and degradation [13-15]. Cells chased in 50 μM, monensin was first pre-incubated with the same amount of the drug for 20 minutes before internalization with ^{125}I-leptin. After different times of chase, cells were again placed on ice and the extracellular medium was collected and tricloroacetic acid (TCA) was added to final concentration of 10%. Intracellular ^{125}I-leptin was determined by measuring the cell-associated radioactivity after solubilization in 1 ml of 1 M NaOH and was calculated as percentage of total ^{125}I-leptin activity in each time point. The amount of TCA-soluble material released in the medium and cell associated radioactivity were determined. The amount of degraded ^{125}I-leptin was pelleted by centrifugation at 4°C and pellet and supernatant were counted in a gamma counter. The degradation of leptin (TCA soluble counts) was monitored as percentage of extracellular non precipitable ^{125}I-leptin added to extracellular and intracellular TCA-precipitable ^{125}I-leptin.

RESULTS

Immunofluorecsence localization of fluorophore labelled Leptin and OBR

HEK293 cells were incubated with medium containing 1-5 μg/ml of anti-OBR for 45 minutes at 37°C, followed by an additional incubation in the same medium together with 1-5 μg/ml of FITC-leptin for another 45 min (Fig. 1A-C). These results verify a receptor-mediated internalization process (Fig. 2). Arrows point on vesicles loaded with both FITC-leptin and anti-OBR$_s$ (Fig. 1C).

To study subcellular localization of leptin, cells were continuously internalized with FITC-leptin for 1 hour, FITC-leptin appeared in vesicles throughout

Fig. 1 - Immunofluorescence localization of fluorophore-labelled leptin and co-localization with anti-OBR$_s$. HEK293 cells were allowed to internalize 5 μg/ml FITC-coupled leptin (A, green fluorescence) together with antibodies against OBR$_s$ (B, red fluorescence) for two hours at 37°C before fixation. The partial localization of OBR immunoreactivity inside the cell indicates internalization of the receptor. This staining was partially colocalized with the vesicles labelled with FITC-leptin (C, arrow points on superimposition in yellow) indicating a receptor associated internalization of leptin with OBR.

Fig. 2 - HEK293 cells were internalized with 5 μg/ml of FITC-coupled leptin for 60 minutes at 37°C prior to fixation. FITC-leptin revealed a bright punctuate staining in intracellular vesicles indicating an intracellular localization (A). In cells pre-treated with 10 μM Phenylarsenic oxide, a potent inhibitor of protein internalization, FITC-leptin was emerged primarily in the plasma (B). In cells preincubated with 0.35 M sucrose for 30 minutes prior addition of FITC-leptin for 60 min, visualization of FITC-leptin in the plasma membrane confirmed that hypertonic medium blocks clathrin-mediated internalization of leptin (C).

the cell (Fig. 2A). On the contrary, in cells treated with phenylarsine oxide, a trivalent arsenical and a general inhibitor of receptor internalization [16], FITC-leptin emerged strongly in a bright plasma membrane staining (Fig. 2B). To establish a hypertonic environment and to inhibit clathrin-mediated internalization, cells were pre-treated with 0.35 M sucrose prior FITC-leptin internalization. This treatment strongly inhibited internalization of leptin (Fig. 2C), revealing a mechanism of leptin endocytosis dependent on clathrin-coated vesicles comparable to that mediating rapid internalization of e.g. transferrin and epidermial growth factor [17].

Leptin internalization and degradation is affected by Monensin and Bafilomycin

HEK293 cells were allowed to internalize ^{125}I-leptin for 10 minutes as a pulse, before washed and chased in medium for various lengths of time. As shown in Fig. 3A, ^{125}I-leptin was rapidly accumulated inside the cells and reached a maximum after 10 min. After 70 minutes about 40-50% of total counts in each time point were found in the medium as TCA-soluble material, indicating a leptin degradation process (Fig. 3B). Lysosomotropic reagents, such as the monovalent cation ionophore monensin, are known to increase the pH in intracellular organelles and thus to prevent receptor recycling and lysosomal degradation [18]. In cells pulsed and chased in the presence of 50μM monensin, the rate of internalization remained unchanged. However, more than 30% of the radioactivity resided cell associated after 70 minutes and less than 10% was found degraded in the medium. In HEK293 cells pre-treated with 0.25μM of bafilomycin, FITC-leptin and Cy3-Tf were allowed to contagiously internalize for 10 minutes as a pulse followed by two washes in ice-cold medium. The cells were then chased for another 60 minutes before fixation. At 10 minutes, FITC-leptin and Cy3-Tf was partially accumulated in early endosomes (Fig. 4A and B). This colocalization was however lost after 60 minutes of chase (Fig. 4C and D).

Incubation at 19°C induces FITC-leptin accumulation in early sorting endosomes

The subcellular distribution of leptin was compared to that of human transferrin and EGF. Tem-

Fig. 3 - *Leptin internalization and degradation in HEK293 cells expressing OBR.* Binding of ^{125}I-leptin to confluent monolayers was carried out as described in Materials and Methods. Briefly, 10 nM ^{125}I-leptin was internalized in HEK293 cells for 10 minutes as a pulse, cells were then washed and incubated in medium containing either only medium, medium supplemented with 1 nM cold leptin or with 50 μM monensin as a chase for the indicated time points. (A) Internalization of ^{125}I-leptin appeared more rapid in the presence of 1 nM leptin in the chase medium whereas 50 μM monensin drastically concentrated ^{125}I-leptin inside the cell. (B) Leptin degradation was evaluated as trichloroacetic acid-soluble radioactivity in the extracellular medium. The concentration of ^{125}I-leptin inside the cell upon treatment with monensin appears to be due to transport to a late endocytic/prelysosomal degradation compartment that is monensin sensitive. The results correspond to one representative experiment out of three.

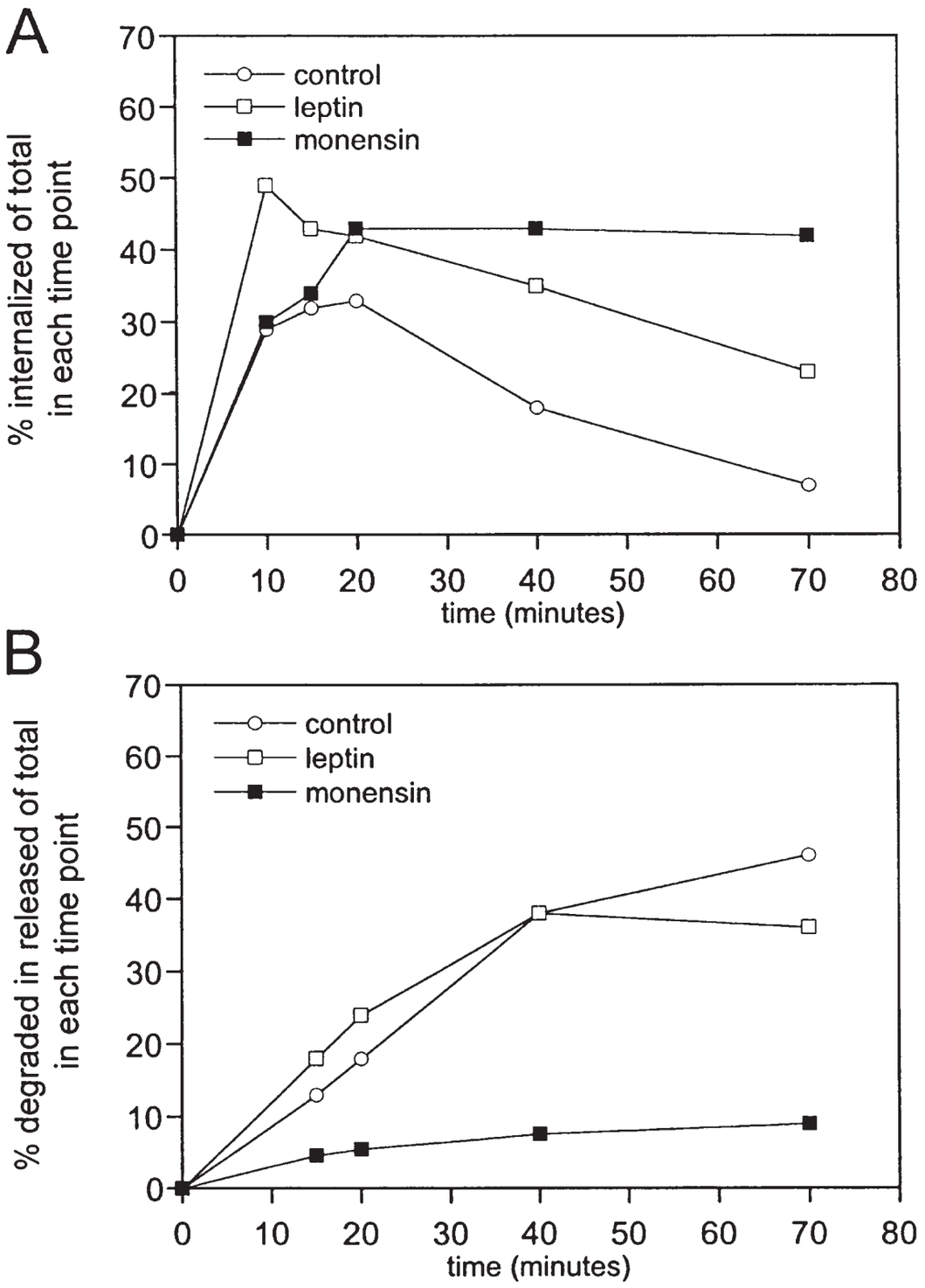

Fig. 4 - HEK293 cells were treated with 0.25μM of bafilomycin followed by continuous internalization of Cy3-Tf (A and C) and FITC-leptin (B and D) for 10 minutes as a pulse. Cells were then washes twice in ice-cold medium before chased in warm medium for another 60 minutes before fixation (60minutes of pulse). At 10 minutes, Cy3-Tf and FITC-leptin were partially accumulated in early endosomes (A and B), whereas no colocalization between Cy3-Tf and FITC-leptin was observerd after 60 minutes of chase (C and D).

peratures lower than 19°C have been shown to prevent recycling of proteins from early endosomes back to the plasma membrane [19, 20], as well as to block progression of endocytosed material along the endocytic pathway [21, 22]. FITC-leptin was continuously internalized together with Cy3-Tf into cells at 19°C. After 45 minutes of incubation both proteins were found in the same intracellular vesicular structures most probably representing part of the early sorting endocytic compartment (Fig. 5A). However, the matching of FITC-leptin and Cy3-Tf in the same structures was basically lost when cells were shifted from 19°C to 37°C for another 45 min as FITC-leptin remained disparaged in peripheral endosomes and did not reach the perinuclear recycling compartment comprising Cy3-Tf, suggesting that both proteins were sorted away from each other at the level of early sorting endosomes (Fig. 5B). In parallel, cells were incubated at 37°C with FITC-leptin together with rhodamine-EGF for 45 min. During this condition, FITC-leptin and rhodamine-EGF displayed a juxtapositional staining (Fig. 5C). In double labelling experiments, the lysosomal marker protein lamp-1 and internalized marine blue-leptin (for 60 min) was found in close proximity to each other (Fig. 5D).

DISCUSSION

We report here on the dynamics of the leptin intracellular trafficking. Inhibition of leptin internalization by phenylarsine reveal that endocytosis of leptin occurs in an energy dependent mechanism. Moreover, a hypertonic environment (0.35 sucrose) point to a clathrin-dependent receptor mediated mechanism for leptin to endocytose. A number of different ligand-receptor complexes are dissociated within the early endocytic compart-

Fig. 5 - *Study of leptin colocalization with established markers of the endocytic pathway during incubation at 19°C.* HEK293 cells were incubated with 5 μg/ml FITC-leptin (A, green fluorescence) at 19°C for 45 minutes together with 5 μg/ml of Tf-Cy3 (A, red fluorescence). Double immunofluorescence revealed leptin and transferrin mainly colocalizing together in the same vesicular pattern. In cells incubated with 5 μg/ml FITC-leptin together with 5 μg/ml rhodamine-EGF at 19°C for 45 min, the same intracellular matching between the two proteins was observed (B). The cells were then shifted to 37°C for another 45 minutes to allow further intracellular transport before fixation (C). Note that and FITC-leptin and Cy3-Tf labelling did basically not overlap any more, signifying leptin and transferrin to be sorted out from each other, while FITC-leptin and rhodamine-EGF remained partially juxtaposed in late endocytic/pre lysosomal vesicles (D).

ment at a pH of 6.2-6.5 [23], allowing free receptors to recycle back to the plasma membrane and the ligand protein to shuttle to lysosomes for degradation. Two hypotheses have been proposed for the organization of the endocytic compartment. The “maturation model” suggests a gradually maturation of clathrin-coated vesicles during their intracellular transport [24]. The “vesicle shuttle model” suggests transport between pre-existing endocytic organelles to be mediated by transport [25, 26]. Independent of the organization of the endocytic compartment, it is evident that it contains sorting functions. Monensin is a cation ionophore with characteristics of increasing the pH of endocytic compartments, affecting receptor/ligand dissociation and degradation [18, 27]. We observed both an increase in the internalization and concentration of ^{125}I-leptin inside the cell upon treatment with monensin. The intracellular accumulation is thus most likely due to an inhibition of the transport of leptin into a degrading lysosomal compartment as reported for other proteins before [15], since as much as 40% of the internalized ^{125}I-leptin were found released and degraded at 40 minutes in nontreated cells. Additionally, in recent publications, OBR$_s$ have been reported to mediate degradation of leptin via a lysosomal pathway, further supporting our conclusions [28, 29]. Moreover, in immunofluorescence experiments with FITC-leptin and OBR, leptin positive vesicles only partially colocalized with OBR These leptin-positive but OBR negative vesicles may well represent a part of leptin sorted out from OBR containing vesicles on their way for degradation in lysosomes, since a significant part of leptin was seen in lamp-1 positive compartments.

However, in an attempt to further test this hypothesis, we also performed experiments in bafilomycin