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Leptin enhances STAT-3 phosphorylation in HC11 cell line: Effect on cell differentiation and cell viability

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Abstract

Leptin is produced in the mammary gland by the fat tissue or by the mammary epithelium. The aim of this study was to investigate the role of leptin on mammary epithelial cell differentiation and cell viability. This study was conducted using the mouse mammary epithelial cell line HC11. We show that leptin, synergizes with prolactin to increase β-casein gene expression during mammary epithelial cell differentiation. This was correlated with increased phosphorylation of the signal transducer and activator of transcription 3 (STAT-3). Inactivating the function of STAT-3 by expression of a short hairpin RNA demonstrated that the effect of leptin on β-casein expression is mediated by STAT-3. Secondarly, cells in which STAT-3 had been inactivated showed increased cell viability compared to controls and were resistant to the negative effect mediated by leptin. Further, leptin triggers apoptosis in mammary epithelial cells cultivated in non-differentiating conditions. Taken together, these results suggest that leptin, by activating STAT-3, may act as a paracrine factor modulating mammary epithelial cell function.

Keywords: Leptin; STAT-3; Mammary epithelial cell; β-Casein; Differentiation; Cell viability

1. Introduction

Leptin is a polypeptide hormone initially discovered as a regulator of food intake and energy expenditure that emerged subsequently as a pleiotropic molecule with a variety of physiologic and pathologic roles. Alterations in leptin levels or responsiveness were reported during starvation and obesity (Zhang et al., 1994; Chen et al., 1996; Considine et al., 1996; Wang et al., 1999).

Growing evidence support a role for leptin in the periphery (for reviews, see Harris, 2000; Baratta, 2002) and an important role in mammary gland development has newly been uncovered. Mice homozygous for a loss of function mutation of the leptin gene (*ob/ob* mice) display obesity, hyperglycaemia, insulin resistance and infertility that can be corrected by injection of recombinant leptin. Withdrawal of leptin therapy from pregnant *ob/ob* females that had been leptin-treated to allow reproduction results in death of the newborn pups (Malik et al., 2001) probably due to a lack of development of the mammary gland. In a recent study, Bates et al. (2003a,b) observed that the offspring of fertile female mice defective in leptin-induced activation of STAT-3, due to a single point mutation in the leptin receptor, died within 24 h of birth without milk in their stomachs unless fostered to wild-type mothers. These results suggest that signaling through the leptin receptor is important for development of the mammary gland. However, the molecular mechanism of this action is still unclear. During pregnancy intense proliferation of mammary epithelial cells results in the formation of lobulo-alveoli composed of differentiated cells capable to respond to lactogenic hormones and to synthesize milk components. Leptin has been detected in mammary adipocytes during early pregnancy and in mammary epithelial cells during lactation, while expression of its receptor is restricted to the epithelial compartment (reviewed in Baratta et al., 2003). This suggests that leptin may act as a paracrine factor on mammary epithelial cell proliferation,

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differentiation and/or apoptosis via adipocyte/epithelial cell interactions.

Multiple signaling pathways have been identified which contribute to the cellular phenotypes of mammary epithelial cells. Cytokines action, particularly the function of prolactin, which activates the prolactin receptor and its downstream effectors Jak2 and Stat5 (Wakao et al., 1994), has been studied in great detail. It is evident from the plethora of signaling molecules that are important for mammary gland differentiation (Hennighausen and Robinson, 2005) that Jak2/Stat5 pathway has to be augmented by other components. Leptin activates a transmembrane receptor belonging to the gp130 family of cytokine receptors. Like prolactin, binding of leptin to its receptor stimulates gene transcription via activation of STAT proteins. Injection of leptin activates STAT-3 in the hypothalamus in wild type and in leptin mutant mice (*ob/ob*) but not in mice lacking leptin receptor (Vaisse et al., 1996). While STAT-3 signaling is essential for the regulation of food intake and energy expenditure by leptin, STAT-3-independent signals mediate leptin regulation of reproduction, growth and glucose homeostasis (Bates et al., 2003a,b). These signals might also be mediated by other STAT proteins, like STAT-5 and STAT-6 (Ghilardi et al., 1996) or by the mitogen-activated protein kinase (MAPK) cascade (Yamashita et al., 1998; Bjorbaek et al., 2001). In this paper we used mouse mammary epithelial cell line HC11 as a model to investigate if the STAT-3 pathway is involved in the biological effect of leptin during differentiation and viability of mammary cells.

2. Materials and methods

2.1. Materials

Recombinant human leptin was kindly provided by Ely Lilly (Indianapolis, IN, USA); RPMI 1640, insulin, ovine recombinant prolactin, epidermal growth factor (EGF) and dexamethasone were purchased from Sigma–Aldrich Inc. (St Louis, MO, USA). Master mix for real-time PCR was purchased from BioRad Laboratories Inc. (Hercules, CA, USA); fetal calf serum, glutamine and penicillin were purchased from EuroClone LtD. (West York, UK).

2.2. Cell culture

HC11 cell line was derived from midpregnant BALB/c mouse mammary tissue and is considered to retain important characteristics of normal mammary epithelial cells such as the ability to produce milk protein in response to lactogenic hormones without cultivation on exogenous extracellular matrix or cocultivation with adipocytes or fibroblasts (Marte et al., 1995). These cells were maintained in RPMI 1640 medium containing 10% fetal calf serum, 5 μg/ml insulin, 10 ng/ml EGF, 2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin (growth medium). To induce differentiation, cells were grown to confluence and cultured for an additional day in growth medium. Subsequently, the cells were incubated for up to 4 days in differentiation medium RPMI 1640 medium containing 3% FCS and the lactogenic hormones dexamethasone (1 μM), insulin (5 μg/ml) and prolactin (5 μg/ml). Differentiation was monitored by measuring expression of the milk protein β-casein (20). To study effect of leptin on differentiation, leptin (1 or 100 nM) was included in differentiation medium in the presence or absence of prolactin.

Lentiviral vectors for expression of small hairpin RNAs Vectors for stable shRNA-mediated gene inactivation were created by cloning duplex small hairpin RNA (shRNA) oligos against STAT-3 (shSTAT-3) (Chatterjee et al., 2004) or control oligos (TM2) (Taulli et al., 2005) into pSUPER vector (OligoEngine). Lentiviral constructs were generated by subcloning the H1-promoter-shSTAT-3/TM2 cassette from the derived vectors into the EcoRV-XhoI sites of the pCCL.sin.PPT.hPGK.GFPWpre lentiviral vector (Follenzi et al., 2000).

2.3. Production of lentiviruses

High titer lentiviruses were obtained by calcium phosphate-mediated co-transfection of 293T cells with the modified pCCL.sin.PPT.hPGK.GFPWpre transfer vectors and the packaging vectors pMDLg/pRRE, pRSV-Rev and pMD2.VSVG. Supernatants were harvested after 30 h, filtered through 0.22 μm pore size filters (Millipore) and concentrated by ultracentrifugation (50,000 × *g* for 2 h).

2.4. Transduction of HC11 cells

HC11 cells (1×10^5 in 35 mm diameter culture dishes) were incubated with viruses in the presence of 8 μg/ml polybrene for 24 h, after. Infectivity was determined after 72 h by FACS analysis of EGFP positive cells. Sorting of GFP positive cells was performed on MoFlo Cell Sorter using Summit™ software (MoFlo; DakoCytomation Inc., Fort Collins, CO). To maximize purification efficiency, the flow speed used during cell sorting corresponded to ($\sim 2 \times 10^4$) cells/s. The purity of cells in the sorted samples was routinely greater than 98%.

2.5. β-Casein gene expression measurement by real-time PCR

Cells were lysed and total RNA was extracted with Macherey–Nagel NucleoSpin® RNA II (Düren, Germany) as described by the manufacturer. One microgram of total RNA was reverse transcribed with Ready-To-Go First Strand Beads (Amersham Pharmacia Biotech Uppsala, Sweden) as described by the manufacturer. Diluted cDNAs (1:5 and 1:50) were used for real-time PCR amplification. Primers for mouse β-casein were: forward 5′-TCACTCCAGCATCCAGTCACA-3′, reverse 5′-GGCCCAAGAGATGGCACCA-3′; primers for 18S (reference gene) were: forward 5′-CGTTTGTGTGGGGAGTGAATGGTG-3′, reverse 5′-GCGTGGGGGTTGGCGGAAAGAGAA-3′. Real-time PCR parameters were: cycle 1, 95 °C for 90 s; cycle 2, 95 °C 60 s, 59 °C 10 s for 40 cycles. The 2-ΔΔCT method was used to analyze the data as described by Kenneth et al. (2001).

2.6. Cell viability

Cell viability was evaluated using WST-1 assay (Roche Diagnostic, Penzberg, Germany). The intensity of the coloured compound formed (formazan dye) was quantified with an ELISA microplate reader (Biorad 680). Briefly, 300 cells/well were seeded in a 96-wells microplate. After 12 h of culture, control cells ($n = 6$) were quantified by WST-1 reagent (day 0) to normalize the successive measurements of treated and not-treated groups. When WST-1 reagent was added the wells were subjected to further incubation for a period of 60 min to facilitate the reaction between mitochondrial dehydrogenase released from viable cells and tetrazolium salt of WST-1 reagent. The absorbance was measured at 450 nm, with the reference at 620 nm. Cell viability was routinely evaluated after 24, 48 and 72 h of treatments.

2.7. Western blotting

Cells were lysed with EB buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with Protease Inhibitor Cocktail (Sigma), 1 mM PMSF and 1 mM sodium orthovanadate. The protein concentration was determined using the BioRad Dc Protein Assay. Twenty micrograms of total protein were run on 10% SDS-PAGE and transferred to Hybond-C extra membranes (Amersham Pharmacia Biotech). Bound antibodies were detected with horseradish peroxidase-conjugated secondary antibody and Supersignal ECL (Pierce) and recorded by X-ray film. Antibodies were obtained as follows: phospho STAT-3, STAT-3 (9131, 9132 Cell Signaling); phospho STAT-5 (05-495 UpState); STAT-5 (SC-835 Santa Cruz); cleaved caspase-3 (Cell Signaling, 9661); α-tubulin (T5168 Sigma).