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Leptin mediates a proliferative response in human MCF7 breast cancer cells☆

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Abstract

Obesity is a risk factor of breast cancers. As leptin, a hormone mainly secreted by white adipocytes, elicits proliferative effects in some cell types, we tested the hypothesis that leptin could influence human breast cancer MCF-7 cell growth. Here we show that MCF-7 cells express leptin receptors and respond to human recombinant leptin by STAT3 and p42/p44 MAPkinase activations and by increased proliferation. These findings suggest that leptin could act in vivo as a paracrine/endocrine growth factor towards mammary epithelial cells thus contributing to explain why obesity is a risk factor of developing breast cancers. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Leptin; Leptin receptors; MCF-7 proliferation; Signal transduction

Obesity is an important health concern, as it is associated with a variety of metabolic disorders and with an increased risk of developing cancer. It is now well established that post-menopausal women with upper body fat predominance (android obesity) experience a higher risk of breast cancer [1,2]. The association between obesity and breast carcinoma is usually ascribed to oestrogen excess, derived from androgen aromatization in peripheral fat deposits. However, adipose tissue secretes other products like complement factors, components of the coagulation/fibrinolytic cascade, and various cytokines such as TNFα, interleukins, and leptin (for review see [3]).

Leptin, a hormone mainly produced in adipose tissue, acts centrally in the hypothalamus to regulate body weight and peripheral energy expenditure [4,5]. Circulating leptin levels are strongly correlated to the body fat content and are higher in obese than in normal weight individuals [6–8].

Recently, leptin was reported to stimulate the proliferation of various cell types [9,14] leading to consider leptin as a novel growth factor. Cellular actions of leptin are initiated by leptin binding to specific receptors (Ob-R) localized in the cell membrane of various tissues [15,16] followed by the activation of the ras-dependent MAP-kinase pathway and STAT3 phosphorylation.

The aim of the present study was to investigate the hypothesis that leptin may play a role in the development and progression of breast cancer. We have thus tested the influence of human recombinant leptin, in vitro, on the proliferation capacities of human breast cancer cells (MCF-7). At first, we have attempted to identify the presence of Ob-R in MCF-7 cells. These receptors being expressed, we have then studied the MAPK and STAT pathways in these cells. These pathways being reactive to leptin, we have finally compared the influence of leptin and of 17-β-estradiol chosen as a reference on MCF-7 proliferation. The results presented herein clearly show that leptin stimulates the proliferation of MCF-7 cells. The latter finding strongly suggests that leptin secreted by mammary adipose cells and more

☆ *Abbrevations*: MAPK, mitogen-activated protein kinase; STAT3, signal transducers and activators of transcription; RT-PCR, reverse transcription-polymerase chain reaction; FCS, fetal calf serum.

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generally by adipose tissue could play the role of a paracrine/endocrine growth factor towards mammary epithelial cells and could thus contribute to explain the increased risk of breast cancer with obesity.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, hepes, leupeptin, aprotinin, phenylsulfonylfluoride (PMSF), and bovine serum albumine (BSA) were purchased from Sigma Chemical (St. Louis, MO, USA). Collagenase was from Roche Molecular Biochemicals (Mannheim, Germany). Recombinant human leptin was provided by R&D Systems Europe (Abingdon, UK), Superscript II Rnase H-RT by Gibco BRL (Grand Island, NY, USA), Taq polymerase and RNA guard by Pharmacia Biotechnology (Uppsala, Sweden), and the MEK inhibitor, U0126 was by Promega (Charbonnières, France). Origins of the different antibodies used are described in the following paragraphs.

Cell culture. The human breast cancer cells, MCF-7, were obtained from European Collection of Cell Cultures (Salisbury, UK). These cells were maintained routinely in phenol-red free DMEM with hepes (20 mM), 10% fetal calf serum (FCS), streptomycin (0.1 mg/ml), and penicillin (100 U/ml) at 37 °C under 5% CO_2 atmosphere. Forty-eight hours later, medium was removed and replaced by a phenol-red free DMEM supplemented with 8% charcoal-stripped FCS until starting assays.

[^{3}H]thymidine incorporation. MCF-7 cells were propagated in 12-well plates in DMEM supplemented with 8% charcoal-stripped FCS. During the exponential phase of growing, cells were exposed to various concentrations of human recombinant leptin for 24 h. For the six last hours, [^{3}H]thymidine (1 mCi/ml) was added to the culture medium. After washing three times with saline, cells were lysed for 5 min with 1% SDS and treated with 10% trichloroacetic acid for 45 min at 4 °C. After filtration on GF/C filters (Whatman, Clifton, NY, USA), radioactivity was counted.

Immunocytochemistry. MCF-7 cells were grown to semi-confluency on glass coverslips. At this stage, cells were washed with phosphate buffered saline (PBS) and fixed in acetone for 5 min at room temperature. After two washes with PBS, cells were treated with 1% H_2O_2 in PBS for 10 min and then blocked with PBS containing 3% BSA for 20 min. Cells were treated with a 1:50 dilution of goat polyclonal anti-human Ob-R antibody C-20 (sc-1832, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in PBS overnight at 4 °C. Cells were washed three times in PBS for 5 min and the immunocytochemical visualization was obtained using the streptavidin–biotin peroxydase complex (Vector Laboratories, Burlingame, UK). Finally, cells were stained with hematoxylin. Control studies were performed using the above-described methods with deletion of primary antiserum.

RT-PCR. To detect Ob-R mRNA, total RNA was isolated using Trizol reagent [17] from MCF-7 and preadipocytes cultures and 2.5 μg total RNA were reverse transcribed using standard reagents (Life Technologies). In this semi-quantitative RT-PCR method, the following primer sets were designed for the amplification of the human Ob-Rt (short and long isoforms) or Ob-Rb (long isoform): Ob-Rt sense 5′-CAT TTT ATC CCC ATT GAG AAG TA-3′, Ob-Rt antisense 5′-CTG AAA ATT AAG TCC TTG TGC CCA G-3′, Ob-Rb sense 5′-TCA CCC AGT GAT TAC AAG CT-3′, and Ob-Rb antisense 5′-CTG GAG AAC TCT GAT GTC CG-3′. PCR generated a 273-bp and a 1071-bp fragments of the Ob-Rt and Ob-Rb genes, respectively. To ensure that amplification of these genes was within the exponential range, different numbers of cycles (25–40 cycles) were run. Finally, 30 and 35 cycles of PCR amplification were chosen to study Ob-Rt and Ob-Rb mRNA expressions, respectively. PCR was performed with a thermocycler Gene Amp PCR 2400 (Perkin–Elmer, USA).

PCR products were analysed on a 2% agarose gel in 90 mM Tris–borate, 2 mM EDTA buffer (TBE), pH 8, and visualized by staining with ethidium bromide and ultraviolet transillumination. Quantification was realized with the Bio-1D software (Vilber Lourmat, Marne la Vallée, France). Controls without reverse transcriptase were systematically performed in order to detect eventual genomic DNA contaminations.

For comparison, Ob-R mRNA expression was also investigated in T47D and MDA-231 human breast cancer cell total RNA (provided by Dr. R. Lidereau) using the RT-PCR protocol described above.

MAPkinase activation. To study MAPkinase pathway, cells were serum deprived overnight and then exposed during 2, 5, 15, and 30 min to human recombinant leptin (20 nM) or 2 min to 10% FCS or 100 nM 17-β-estradiol. Then, cells were scrapped and sonicated on ice in buffer containing 50 mM Tris, 120 mM NaCl, 1 mM EDTA, 1% Nonidet-P40, 0.5 mM desoxycholate, 0.1% sodium dodecyl sulfate, 1 mM sodium vanadate, 0.57 mM PMSF, 30 mM β-glycerophosphate, 5 μg/ml aprotinin, and 12.5 μg/ml leupeptin. After centrifugation at 100,000*g* for 10 min at 4 °C, supernatants were diluted in Laemmli's buffer (vol/vol). Equal amounts (10 μg) of cellular extracts were subjected to SDS–PAGE (12.5%). Proteins were transferred to PVDF membrane and blocked in buffer A with 2.5% gelatin during 2 h. Then membranes were incubated overnight at room temperature with rabbit polyclonal p42/p44 MAPkinase phosphorylated antibody (pTEpY, V8031) (1:7000 dilution, Promega, Charbonnières, France) or with mouse monoclonal anti-total ERK (pan-ERK) antibody (1:500 dilution, Transduction Laboratories, Lexington, KY, USA) in buffer A with 2.5% gelatin. The antigen–antibody complexes were detected as described above.

STAT3 activation. To study the STAT pathway, cells were maintained overnight in a serum-free culture medium. After exposure to human recombinant leptin (20 nM) during 5, 15, and 30 min, cells were washed one time with PBS, scrapped in cold immunoprecipitation buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8, 0.2 mM sodium ortho-vanadate, 0.2 mM PMSF, and 0.5% Nonidet-P40), and sonicated on ice before to be maintained under shaking at 4 °C during 30 min. After centrifugation at 16,000*g* for 15 min at 4 °C, 100 μl supernatant (400 μg total protein) was incubated overnight at 4 °C with 4 μg polyclonal rabbit anti-STAT3 C20 (sc-482, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1 ml final volume of immunoprecipitation buffer. The immunocomplexes were then adsorbed on protein A:G agarose beads for 3 h at 4 °C, pelleted by centrifugation at 16,000*g* for 5 min and, washed three times with immunoprecipitation buffer. The recovered pellets were suspended in electrophoresis buffer, boiled for 5 min, and centrifuged at 16,000*g* for 5 min at 4 °C. Then, supernatants were transferred to new tubes and finally loaded onto SDS–PAGE (7.5%). Proteins were transferred to PVDF membranes and blocked in buffer A with 2.5% gelatin during 2 h. Then membranes were incubated overnight at 4 °C with A 1/300 dilution of mouse monoclonal p-Tyr antibody (PY99, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit polyclonal STAT3 C-20 antibody. The antigen–antibody complexes were detected as described above.

Protein concentration was measured according to Bradford [18] with BSA as standard.

Statistical analysis. All values were expressed as means ± SEM of 3–4 different experiments, and statistical analysis was performed using unpaired Student's *t* test.

Results

Leptin receptor expression in MCF-7 cells

Using two different primer sets in RT-PCR analysis, we found that Ob-R mRNA are expressed in MCF-7

cells as they are in primary cultured human preadipocytes shown as controls (Fig. 1). Moreover, the full length form (Ob-Rb) subtype, is readily detected in MCF-7 cells as in human preadipose cells (Fig. 1). These data were confirmed by immunocytochemical analysis. In the presence of Ob-R antibody, Fig. 2 shows indeed a positive brown membranous and cytoplasmic staining in MCF-7 cells (photomicrograph 2) which is comparable to that observed in human preadipocytes (control cells) (photomicrograph 4). In the absence of the specific primary antiserum, no staining could be observed in both cell types (photomicrographs 1 and 3).

Interestingly, in two other human breast cancer cells, T47D and MDA-231 cells, RT-PCR experiments revealed Ob-Rt but not Ob-Rb mRNA expression in T47D and no expression of these isoforms in MDA-231 cells (data not shown).

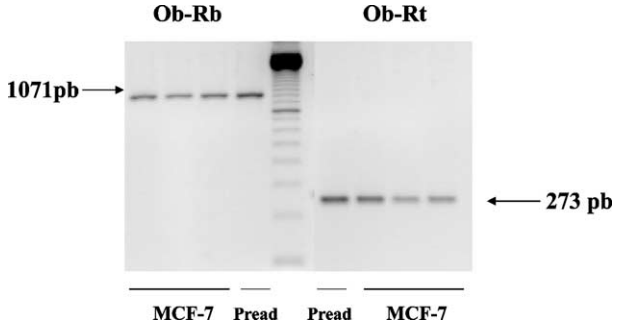

Fig. 1. Ob-Rb and Ob-Rt mRNA expressions in MCF-7 cells. Total RNA was extracted from MCF-7 cells and human primary cultured preadipocytes and analyzed by RT-PCR with the primers as described under Materials and methods. This figure shows one RT-PCR representative of three separate experiments on MCF-7 cells and one experiment on human preadipocytes as control (Pread).

Activation of STAT3 and MAPkinase pathways by leptin

As the phosphorylation-dependent activation of STAT3 is a major transduction pathway for leptin signaling [10,16,19], expression of phosphorylated STAT3 was investigated in cellular extracts from MCF-7 cells immediately after their in vitro exposure to leptin. Lysates were immunoprecipitated with STAT3 antibody and immunoblotted with phosphotyrosin antibody. This protocol has previously been used to demonstrate leptin signaling in rat hypothalamus [20]. Data in Fig. 3 show the kinetics of STAT3 activation due to 20 nM leptin exposure. This dosage was chosen because dose–response experiments (5–50 nM) revealed that 20 nM was the lowest leptin concentration giving the maximal

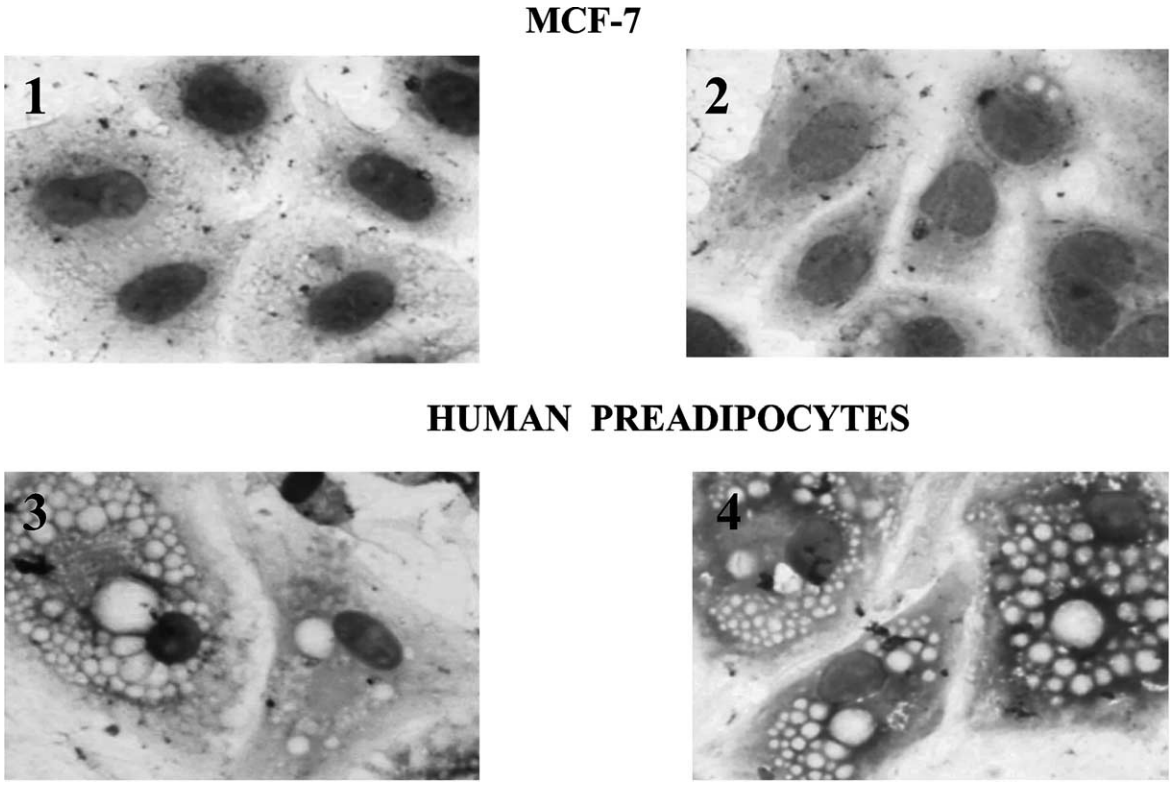

Fig. 2. Ob-R protein expression in MCF-7 cells. The presence of Ob-R immunoreactivity on MCF-7 cells (photomicrographs 1 and 2) and primary cultured human preadipocytes (photomicrographs 3 and 4) was analysed as described under Materials and methods. 1 and 3: cells were incubated without primary antiserum overnight at +4 °C (negative control). 2 and 4: cells were incubated with the specific anti-Ob-R antiserum under the same conditions.

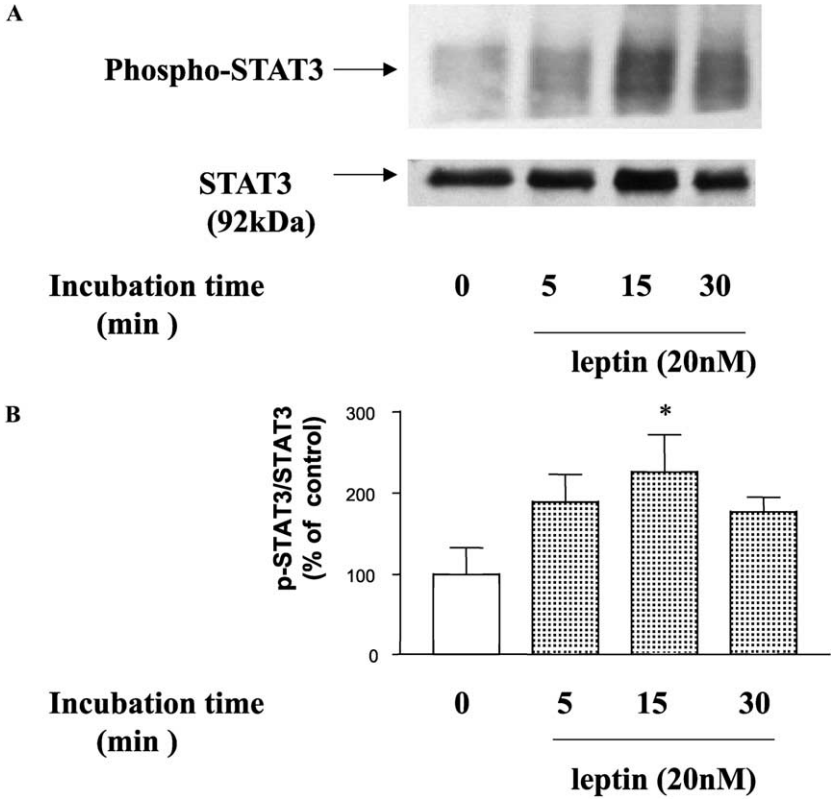

Fig. 3. Tyrosine phosphorylation of STAT3 by leptin in MCF-7 cells. Cells were maintained in a serum-free culture medium overnight prior to be exposed to human recombinant leptin (20 nM) as described under Materials and methods. At the indicated times, immunoprecipitates in presence of anti-STAT3 antibody were prepared and immunoblotted with anti p-Tyr or anti STAT3 antibodies. (A) Western-blot analysis from one representative experiment of three. (B) Densitometric analysis of the phosphorylated STAT3/STAT3 protein immunoblots. Values are the means ± SEM obtained from three separate experiments and are expressed as percentages of control value without leptin (0). $* = p < 0.05$, ns = non-significant.

STAT3 and MAPkinase activation (data not shown). As can be seen, exposure to leptin induced a rapid increase in the phosphorylated STAT3 form in MCF-7 cells. Activation was maximal after 15 min and then decreased after 30 min exposure to leptin. Stripping of the immunoblots and reprobing with STAT3 antibody confirmed the presence of equal amounts of STAT3 in each lane (Fig. 3).

Previous reports have shown that leptin binding to Ob-Rb also leads to the phosphorylation-dependent activation of the MAPkinase pathway [21]. Therefore, the phosphorylated form of p42/p44 MAPkinase was investigated in cytosolic extracts of MCF-7 cells following their in vitro exposure to leptin. As shown in Fig. 4, exposure to leptin (20 nM) induced a rapid and clear increase in the phosphorylated p42/p44 MAPkinase isoforms starting after 2 min and reaching its maximum after 15 min. It is important to notice that the maximal effect of leptin was of the same magnitude as those found after MCF-7 cell exposure to either 10% FCS or 17-β-estradiol (100 nM). Moreover, exposure of MCF-7 cells for 5 min to both leptin (20 nM) and the specific MAPkinase inhibitor UO126 (10 μg/ml) [22], resulted in a complete abolition of the positive effect of leptin on the p42/p44 MAPkinase isoform phosphorylation (data not shown).

Cell proliferation

Cell proliferation was studied by measuring changes in the rate of DNA synthesis ([^{3}H]thymidine incorporation). As shown in Fig. 5, leptin, in vitro, increased [^{3}H]thymidine incorporation in a dose-dependent manner. Maximal stimulation ($\times 1.62 \pm 0.028$) was observed within a 20–50 nM leptin concentration range. For comparison, the magnitude of the increase in [^{3}H]thymidine incorporation caused by 100 nM 17-β-estradiol was more than two times higher ($\times 4.04 \pm 0.97$). However, MCF-7 cell exposure to both 20 nM leptin and 100 nM 17-β-estradiol failed to produce any further stimulation of [^{3}H]thymidine incorporation when compared to the effect of 17-β-estradiol alone.

Discussion

Accumulating evidence suggests that the local cellular environment and particularly the mammary adipose

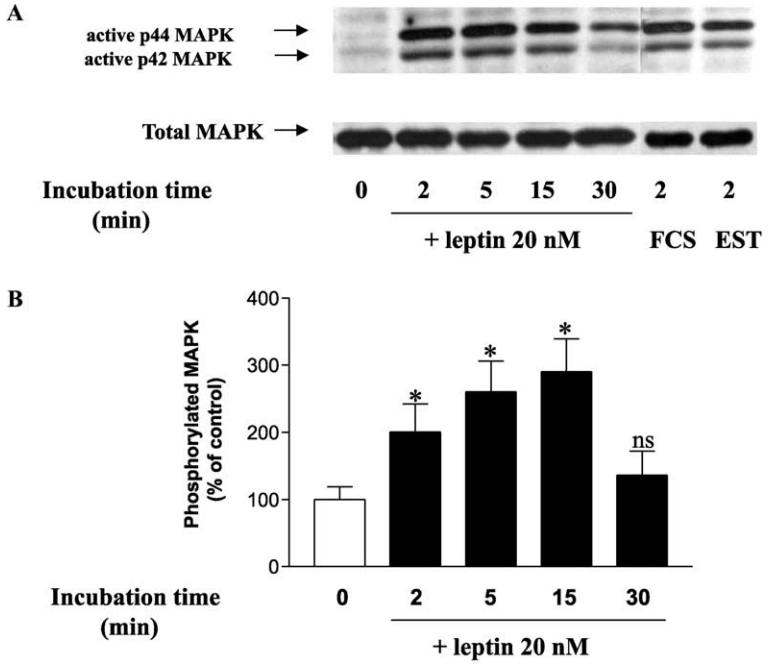

Fig. 4. Activation of p42/p44 MAPkinase by leptin in MCF-7 cells. Cells were maintained in a serum-free culture medium overnight prior to be exposed to human recombinant leptin (20 nM) or 10% FCS or 17-β-estradiol (100 nM). At the indicated times, cellular extracts were prepared and immunoblotted with either anti-p42/p44 MAPkinase active or anti ERK (pan-ERK) antibodies. (A) Western-blot analysis from one representative experiment of three. (B) Densitometric analysis of MAPK immunoblots. Values are the means ± SEM obtained from three separate experiments and are expressed as percentages of control value without leptin (0). $* = p < 0.05$, ns = non-significant.

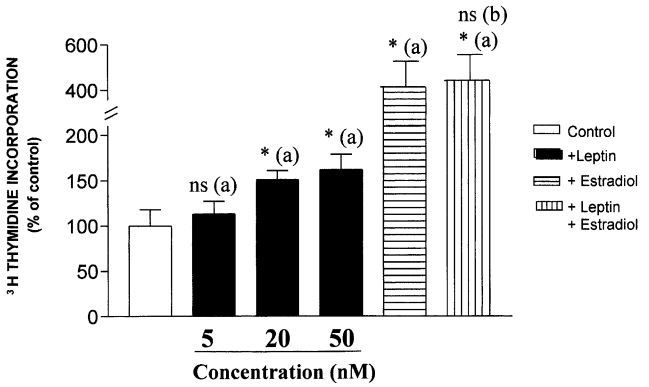

Fig. 5. Effects of leptin on DNA synthesis Cells were exposed to 5, 20, or 50 nM human recombinant leptin or to 100 nM 17-β-estradiol with or without 20 nM leptin in the presence of [^{3}H]thymidine as described under Materials and methods. Results are means ± SEM of three experiments and are normalized as percentages of the control value (without leptin) (a) vs control, (b) leptin +17-β-estradiol vs 17-β-estradiol. $* = p < 0.05$, ns = non-significant.

cells play an integral role in controlling the proliferation of both normal and neoplastic mammary epithelial cells in vivo. Mammary adipose tissue is an important source of paracrine mitogens or anti-mitogens including, insulin-like growth factor 1 (IGF1), transforming growth factor α (TGFα), estrogens, and cytokines (TNFα, IL6). Leptin is another cytokine, mainly produced by adipose tissue. From the recent literature, leptin appears to be a potential candidate to promote cell growth. Indeed, leptin was reported to activate the proliferation of pancreatic-β [9], vascular endothelial [10], lung [11], gastric mucosa [12], keratinocytes [13], and preadipose cells [14]. Moreover, hyperleptinemia is a common feature of obese women who are exposed to a higher risk of breast cancer than normal weight women. Thus besides other signals, leptin could be an additional factor contributing to mammary epithelium hyperplasia. To test this hypothesis, we have examined the influence of human recombinant leptin in vitro on the proliferation of MCF-7 cells.

As a prerequisite for this type of study, it was essential to demonstrate the presence of leptin receptors in these cells. Characterization of these receptors has been performed by using RT-PCR and immunocytochemistry. These experiments provided clear evidence that both Ob-R mRNA and protein are expressed in MCF-7 cells. These results are consistent with those recently reported by Laud et al. [23] showing both the presence and specific regulation of Ob-R in ovine mammary epithelial cells during pregnancy and lactation. The functionality of the Ob-R was next examined by testing the activation of STAT3 and MAPK signaling pathways by human recombinant leptin. These experiments clearly showed that leptin in vitro rapidly and directly stimulates the STAT3 and MAPK pathways in MCF-7 cells. Because the maximally efficient leptin concentration (20 nM) was

higher than the normal human blood leptin level, the physiological relevance of the present data could be questionable. However, the leptin concentration used here is in the lower part of the range of those tested in other in vitro studies (10–200 nM) [12,24], and due to the lack of soluble leptin receptor in our culture medium, the stability of recombinant leptin is certainly lower than in the plasma in vivo. Moreover, a recent in vivo study reported much higher leptin levels in human fat interstitial fluid than in blood [25] suggesting that the leptin concentrations tested in the present study range within the physiological concentrations found in the interstitial fluid surrounding adipose tissue.

In various cell types, MAPK pathway activation by leptin is associated with a stimulation of cell proliferation [11,12,21]. The present study extends these findings to the MCF-7-cell line by showing a dose-dependent increase in their number following an exposure to human recombinant leptin. Although the maximal increase in proliferation due to leptin was weaker than that due to 17-β-estradiol, it was at least comparable in magnitude with those caused by leptin in other cell types [9,11–14,21]. The potency of 17-β-estradiol to increase MCF-7 DNA synthesis was unchanged by leptin suggesting a common mechanism of action for leptin and 17-β-estradiol. As a matter of fact, recent studies on various cell types have demonstrated rapid and non-genomic cellular actions of estrogens involving activation of various transduction pathways including the MAPkinase cascade [27,28]. Our present experiments (see Fig. 4) confirmed that 17-β-estradiol like leptin induces a rapid stimulation of the phosphorylation-dependent MAPK activation in MCF-7 cells.

It is important to notice that despite our efforts, we were unable to detect any leptin mRNA signal using RT-PCR in our MCF-7 cells (data not shown). This negative finding which confirms a previous report [29], suggests that leptin can act as a paracrine/endocrine growth factor on mammary epithelial cells. Such a role for leptin might be particularly important in situations like the post-menopausal state where leptin could relay the deficient estrogen production by exerting its mitogenic actions in breast tissue.

The P-450 aromatase catalyses the aromatization of androgens to estrogens. Various cytokines (IL6, LIF, TNFα, etc.) regulate the aromatase gene expression in breast cancer cells [30] and in adipose stromal cells [31] through STAT3 pathway activation and via an interferon-γ activation site (GAS) [32]. Recently, leptin was reported to increase aromatase activity in human luteinized granulosa cells [33] and adipose stromal cells [34]. From these and our present studies, it can be postulated that leptin, through STAT3 activation, may also stimulate the aromatase gene expression in MCF-7 cells in which a recent study clearly demonstrated activity and expression of P-450 aromatase mRNA and protein [35]. If so, leptin in vivo could control estrogen biosynthesis and, by the way, promote further mammary cell proliferation.

In conclusion, this study leads to consider leptin as a functional signal linking adipocytes to epithelial cells in mammary gland. By acting as an paracrine factor on epithelial cell growth, leptin could play directly and/or indirectly an important role in normal and neoplastic mammary gland growth.

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