Increased Expression of Leptin and the Leptin Receptor as a Marker of Breast Cancer Progression: Possible Role of Obesity-Related Stimuli

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Abstract

Purpose: Recent *in vitro* studies suggested that the autocrine leptin loop might contribute to breast cancer development by enhancing cell growth and survival. To evaluate whether the leptin system could become a target in breast cancer therapy, we examined the expression of leptin and its receptor (ObR) in primary and metastatic breast cancer and noncancer mammary epithelium. We also studied whether the expression of leptin/ObR in breast cancer can be induced by obesity-related stimuli, such as elevated levels of insulin, insulin-like growth factor-I (IGF-I), estradiol, or hypoxic conditions.

Experimental Design: The expression of leptin and ObR was examined by immunohistochemistry in 148 primary breast cancers and 66 breast cancer metastases as well as in 90 benign mammary lesions. The effects of insulin, IGF-I, estradiol, and hypoxia on leptin and ObR mRNA expression were assessed by reverse transcription-PCR in MCF-7 and MDA-MB-231 breast cancer cell lines.

Results: Leptin and ObR were significantly overexpressed in primary and metastatic breast cancer relative to noncancer tissues. In primary tumors, leptin positively correlated with ObR, and both biomarkers were most abundant in G3 tumors. The expression of leptin mRNA was enhanced by insulin and hypoxia in MCF-7 and MDA-MB-231 cells, whereas IGF-I and estradiol stimulated leptin mRNA only in MCF-7 cells. ObR mRNA was induced by insulin, IGF-I, and estradiol in MCF-7 cells and by insulin and hypoxia in MDA-MB-231 cells.

Conclusions: Leptin and ObR are overexpressed in breast cancer, possibly due to hypoxia and/or overexposure of cells to insulin, IGF-I, and/or estradiol.

Obesity increases postmenopausal breast cancer risk by 30% to 50% (1). The exact mechanism of this phenomenon is not known, but it is assumed that different biologically active factors that are secreted by adipose tissue, such as estrogens, insulin, insulin-like growth factor-I (IGF-I), and leptin, might be implicated (1–5). Although the role of estrogens, insulin, and IGF-I in breast tumorigenesis has been extensively studied, the potential role of leptin is just being recognized (2).

The adipokine leptin (obesity protein) acts as a neurohormone-regulating energy balance and food intake in the hypothalamus. Additionally, leptin has been shown to influence various processes in peripheral organs (6, 7). In the breast, leptin is required for normal mammary gland development and lactation (8), but it might also contribute to mammary tumorigenesis (2). In support of the latter, there is evidence that different breast cancer cell lines can express various isoforms of the leptin receptor (ObR), including the long signaling form ObRl (9–13). Furthermore, in breast cancer cells, leptin has been shown to stimulate DNA synthesis and cell growth acting through multiple signaling cascades, such as the Janus-activated kinase 2/signal transducers and activators of transcription 3, extracellular signal-regulated kinase 1/2, protein kinase Cα, and Akt/GSK3 pathways (9–16). Leptin-induced cell cycle progression was accompanied by up-regulation of cyclin-dependent kinase2 and cyclin D1 levels (15) and hyperphosphorylation/inactivation of the cell cycle inhibitor pRb (13). Noteworthy, in T47D breast cancer cells, but not in normal mammary epithelial cells, leptin stimulated not only cell growth but also cellular transformation (10).

The involvement of leptin in breast carcinogenesis could be additionally supported by the fact that the hormone can potentiate estrogen signaling. Specifically, in MCF-7 cells, leptin induced aromatase gene expression, elevating aromatase activity and increasing estrogen synthesis (16). Leptin was also

able to enhance estrogen receptor α (ERα)–dependent transcription by decreasing ERα ubiquitination and degradation, especially in the presence of the antiestrogen ICI 182,780 (13). Furthermore, leptin has been shown to transactivate ERα via the extracellular signal-regulated kinase 1/2 pathway (17).

Limited studies on cancer and noncancer breast biopsies indicated that both leptin and ObR are present in the human breast tissue, suggesting that mammary gland can be influenced by leptin not only through paracrine or endocrine mechanisms but also by via autocrine pathways (18–21). Importantly, one previous report (18) and our present study suggest that leptin and ObR are overexpressed in primary and metastatic invasive ductal breast carcinoma compared with noncancer mammary tissue.

The mechanism regulating leptin/ObR overexpression in mammary epithelium is not known. The synthesis of leptin in other cellular systems is influenced by different humoral factors, among them insulin (22, 23), IGF-I, and estrogens (24). In addition, leptin expression can be up-regulated by hypoxia via hypoxia-inducible factor 1–mediated transcription (25, 26). The regulation of ObR is much less understood, but preliminary evidence suggested that ObR expression can be stimulated by estradiol and hypoxia in rodents (27). Because estrogens, insulin, and IGF-I are overabundant in obese subjects (5), and obesity is associated with tissue hypoxia (28), we explored whether the expression of the leptin/ObR loop in breast cancer cells can be affected by these stimuli.

Materials and Methods

Tissue samples

The expression of leptin, ObR, and other breast cancer markers was assessed in breast cancer and noncancer mammary epithelium. Tissue samples were obtained from 148 women who underwent partial or total mastectomy and lymph node dissection for primary breast cancer as well as from 48 women treated surgically for intraductal proliferative lesions. Immediately after excision, tissue samples were fixed in 10% buffered formaldehyde solution, embedded in paraffin blocks at 56°C, and stained with H&E. Histopathologic examination of sections was based on the WHO and pT_N classification of breast tumors (29). The protocol of the present study was reviewed and approved by the local ethical committee.

Breast cancer samples. Breast cancer samples included invasive ductal carcinomas in grades G_2 (57.4%) and G_3 (42.6%); in stages pT_1 (54.7%) and pT_2 (45.3%); 52.7% (78 of 148) of patients had involved lymph nodes at the time of diagnosis; 66 cases of lymph node metastases were analyzed in parallel with primary tumors. The age of patients with breast cancer ranged from 30 to 80 years (mean, 54.5 years); 55.4% of women were premenopausal, and 44.6% were postmenopausal.

Noncancer samples. Ninety cases of intraductal proliferative lesions were analyzed: 48 cases without accompanying breast cancer (37 usual ductal hyperplasias and 11 atypical ductal hyperplasias) and 42 cases of noncancer tissue adjacent to breast cancer. The latter group included 20 cases of usual ductal hyperplasia and 22 cases of atypical ductal hyperplasia. The age of patients with intraductal proliferative lesions ranged from 24 to 68 years (mean, 46.8 years); 76.2% of the subjects were premenopausal, 23.8% postmenopausal.

Immunohistochemistry

The immunohistochemical analysis of leptin, ObR, ERα, ERβ, and Ki-67 expression was carried out using 5-μm consecutive tissue sections obtained from tissue samples, as described by us previously in detail (30). The sections were dewaxed in xylene and rehydrated in graded alcohols. After antigen unmasking and endogenous peroxidase removal, nonspecific binding was blocked by incubating the slides for 1 hour with 1.5% normal serum in PBS. Next, the sections were incubated with the primary antibodies. The following antibodies were used for immunohistochemistry: for leptin, rabbit polyclonal antibody A-20 (Santa Cruz Biotechnology, Santa Cruz, CA), dilution 1:100; for ObR, rabbit polyclonal antibody H-300 (Santa Cruz Biotechnology), dilution 1:75; for ERα, mouse monoclonal antibody F-10 (Santa Cruz Biotechnology), dilution 1:200; for ERβ, rabbit polyclonal antibody H-150 recognizing primarily the cytoplasmic form of ERβ (Santa Cruz Biotechnology), dilution 1:200; and for Ki-67, mouse monoclonal antibody MIB-1 (DAKO, Copenhagen, Denmark), dilution 1:100. The studies for leptin, ObR, ERα, and ERβ were done with avidin-biotin-peroxidase complex (ABC Staining System, Santa Cruz Biotechnology), and for Ki-67 with streptavidin-biotin-peroxidase complex (LSAB kit, DAKO) to reveal antibody-antigen reactions. All slides were counterstained with hematoxylin. Breast specimens previously classified as positive for the expression of the studied markers were used for control and protocol standardization. In negative controls, primary antibodies were omitted. The expression of leptin, ObR, ERα, ERβ, and Ki-67 was analyzed by light microscopy in 10 different section fields, and the mean percentage of tumor cells displaying positive staining was scored. The expression of leptin and ObR in cancer samples was classified using a four-point scale: 0, <10% positive cells; 1+, 10 to 50% positive cells with weak staining; 2+, >50% positive cells with weak staining; 3+, >50% positive cells with strong staining. The expression of leptin and ObR in noncancer tissues was classified as negative (<5% of positive cells) or positive (≥5% positive cells). ERα and ERβ were classified as follows: 0, <10% cells with positive staining; 1+, 10% to 50% cells with positive staining; 2+, 50% to 80% cells with positive staining; 3+ >80% cells with positive staining. Ki-67 expression was classified as follows: 0, <10% cells with positive staining; 1+, 10% to 40% cells with positive staining; 2+ >40% cells with positive staining.

Cell lines

MCF-7 ERα-positive and MDA-MB-231 ERα-negative breast cancer cell lines were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM:F12 plus 5% calf serum, as described by us before (31).

Cell treatments

Eighty percent confluent cell cultures were placed in phenol red–free serum-free DMEM/F12 medium for 24 hours and then treated for 4 hours with 10 nmol/L 17-β-estradiol (E2), 50 ng/mL IGF-I, 100 ng/mL insulin, or 100 nmol/L $CoCl_2$ (to induce hypoxia). The time and dose response for all treatments was tested in advance and the conditions eliciting the maximal leptin and ObR induction were applied.

Reverse transcription-PCR

RNA was isolated from untreated and treated cells using Trizol (Invitrogen, San Diego, CA). Total RNA (2 μg) was reverse transcribed with Superscript2 (Invitrogen). RT product (2 μL) was amplified by PCR using the following conditions: for leptin, 95°C for 5 minutes, and then 40 cycles of 95°C for 50 seconds, 60°C for 60 seconds, 72°C for 80 seconds, extension 72°C for 10 minutes. Leptin primers: forward, 5′-CTGTGCCCATCCAAAAAGTCC-3′; reverse, 5′-CCCCCAGGCTGTC-CAAGGTC-3′ (product size 336 bp). Primers for ObR (common domain ObR and ObRl): 95°C for 5 minutes, and then 30 cycles of 95°C for 40 seconds, 60°C for 50 seconds, 72°C for 50 seconds, 72°C for 10 minutes. Primers for ObR common domain: forward, 5′-CATTTTAT-CCCCATTGAGAAGTA-3′; reverse, 5′-CTGAAAATTAAGTCCTTGTGC-CCA-3′ (product size 270 bp). Primers for ObRl: forward, 5-CAGAAG-CCAGAAACGTTTCAG-3′; reverse, 5-AGCCCTTGTTCTTCACCAGT-3′ (product size 344 bp). The expression of a constitutive 36B4 mRNA was assessed as control of RNA input using primers described before (32). The PCR products were run on a 2% agarose gel, and the intensity of bands was quantified by Scion Image laser densitometry program, as described before (31).

Q2

Q3

Q4

Statistical analysis

Spearman test was used to analyze correlations among studied biomarkers in primary breast cancer and in lymph node metastases. Analyses of correlations were not corrected for multiple comparisons. The associations of leptin and ObR with clinicopathologic features were evaluated using χ^2 and Spearman tests. The significance of reverse transcription-PCR results was assessed by Student's t test. Ps < 0.05 were taken as statistically significant.

Results

Low expression of leptin in benign mammary lesions. The characteristics of leptin immunostaining in usual and atypical ductal hyperplasias were similar; therefore, all intraductal proliferative lesions were treated as one group. Within this group, positive cytoplasmic leptin immunoreactivity was found in 15 of 48 (31.3%) of intraductal proliferative lesions without accompanying breast cancer and in 24 of 42 (57.1%) of benign mammary lesions adjacent to breast cancer (Table 1; Fig. 1).

Enhanced expression of leptin in breast cancer. In primary breast cancers, leptin was detected in 128 of 148 (86.4%) cases. Most frequently (64 of 128, 50.0%), leptin immunostaining was classified as 2+, whereas lower expression (1+) was observed in 45 of 128 (35.2%) of samples, and high (3+) expression was found in 19 of 128 (14.8%) of positive tissues (Table 2; Fig. 1).

In lymph node metastases, the presence of leptin was noted in 62 of 66 (93.9%) of cases. Like in primary breast cancer, the expression of leptin in metastatic cancer was most frequent at the 2+ level (33 of 62, 53.2% of leptin-positive lymph node metastases) and less frequent at 3+ (16 of 62, 25.8%) and 1+ (13 of 62, 20.9%) levels (Table 2; Fig. 1). The expression of leptin was undetectable in primary and metastatic cancer samples when immunostaining was done with the omission of the primary antibody.

Expression of ObR is elevated in breast cancer. The expression of ObR was examined with the antibody recognizing a common domain of ObRl and ObRs, allowing for detection of all ObR isoforms. ObR immunostaining was negative in almost all studied noncancer tissues (Table 1; Fig. 1). Only in five specimens of intraductal proliferative lesions, focally positive cytoplasmic immunostaining for ObR was observed.

In contrast, ObR was often expressed in primary breast cancers, where cytoplasmic immunoreactivity for ObR was noted in 61 of 148 (41.2%) of cases. Most frequently (41 of 61, 67.2%), the expression of ObR was weak; however, ObR staining at 2+ and 3+ levels was also noted in some tissues (15 of 61 and 5 of 61 of positive samples, respectively; Table 2; Fig. 1).

In lymph node metastases, ObR was found in 34 of 66 (51.5%) of specimens. In the majority of positive samples, the expression of ObR was weak (14 of 34, 41.2%) or medium (14 of 34, 41.2%). Some metastatic cancers (6 of 34, 17.6% of positive cases) expressed high levels of ObR (Table 2; Fig. 1). ObR immunoreactivity was undetectable in the control samples where the primary antibody was omitted.

Leptin and ObR are coexpressed in primary breast cancer. The expression of leptin in the group of all primary tumors as well as in the subgroups of ERα-positive and ERα-negative primary tumors positively correlated with the expression of ObR (P = 0.002, r = 0.275; P = 0.005, r = 0.393; P = 0.003, r = 0.411, respectively). In all lymph node metastases as well as in subgroups derived from ERα-positive or ERα-negative tumors, the expression of leptin was not significantly associated with ObR expression (Table 3).

Expression of leptin and ObR is maintained during metastasis to lymph nodes in ERα-positive tumors. In the group of all cancer cases, the presence of leptin in primary breast cancer positively correlated with its expression in matched cases of lymph node metastases (P = 0.046, r = 0.270; Table 3). After division of samples into ERα-positive and ERα-negative subgroups (according to the initial diagnosis of primary tumor), a strong link between leptin expression in primary tumor and its metastasis was found only in the subgroup of ERα-positive tumors (P = 0.008, r = 0.507; Table 3). Similarly, the expression of ObR in primary tumors positively correlated with its expression in lymph node metastases only in the subgroup of ERα-positive tumors (Table 3).

Relationships between the leptin/ObR system and ERα, ERβ, and Ki-67 in primary breast cancers. Because leptin is a mitogen for breast cancer cells, we assessed the relationship between the leptin/ObR system and cell proliferation (Ki-67 expression). Furthermore, because leptin is a modulator of ERα function, we explored the association between leptin/ObR and ER.

ERα, ERβ, and Ki-67 were found in 60.8%, 80.4%, and 64.2% of primary tumors, respectively. In primary tumors, leptin positively correlated with ERβ (P = 0.001, r = 0.327) but not with ERα or Ki-67 (Table 4). A positive correlation (P = 0.006, r = 0.378) between leptin and ERβ was also found in the subgroup of ERα-positive but not ERα-negative primary tumors (Table 4). The expression of ObR in primary tumors was not significantly associated with the expression of ERα, ERβ, or Ki-67 (Table 4).

Relationships between the leptin/ObR system and ERα, ERβ, and Ki-67 in lymph node metastases. ERα, ERβ, and Ki-67 expression were detected in 60.6%, 83.3%, and 68.2% of

Table 1. Leptin and ObR expression levels in non-cancer mammary epithelium

Tissue type	Leptin expression		ObR expression	
	Negative	Positive	Negative	Positive
Noncancer tissue without accompanying breast cancer (n = 48)	33	15	47	1
Noncancer tissue adjacent to breast cancer (n = 42)	18	24	38	4

NOTE: The expression of leptin and ObR was determined in noncancerous mammary tissue, as described in Materials and Methods. The number of cases (n) in each staining category is shown.
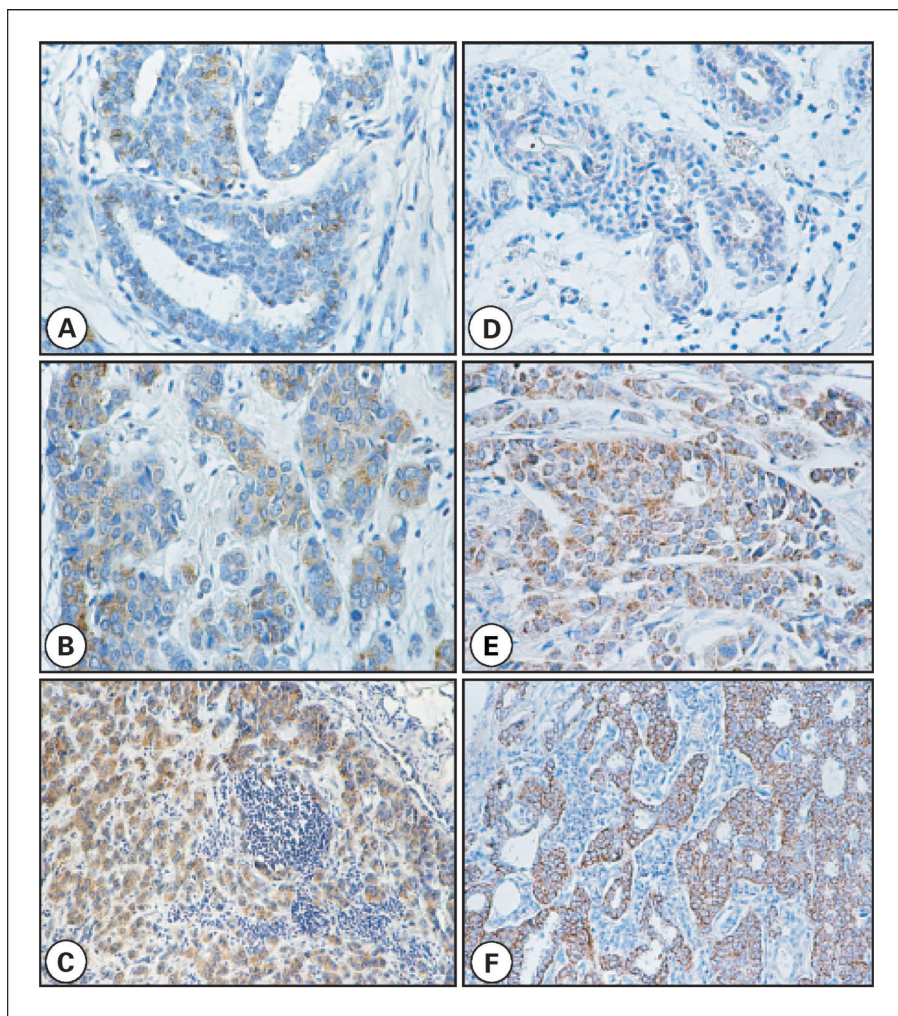
Fig. 1. Immunohistochemical detection of leptin and ObR expression. The expression of leptin (*A-C*) and ObR (*D-F*) in noncancer and breast cancer tissues was studied by immunohistochemistry, as described in Materials and Methods. In this representative image, cytoplasmic leptin immunostaining is seen in intraductal proliferative lesion (*A*). In primary breast cancer (*B*), weak leptin immunostaining is observed in >50% of cancer cells (assessed as 2+); strong staining is found in lymph node metastasis (*C*) in >50% of cancer cells (assessed as 3+). A weak ObR immunoreactivity is present in a few epithelial cells in noncancer mammary gland (*D*), whereas strong ObR immunostaining can be seen in primary tumor (*E*) and lymph node metastasis (*F*). Original magnification, ×200 (*A, B, D,* and *E*) and ×100 (*C* and *F*).

lymph node metastases, respectively. Like in primary tumors, leptin expression in lymph node metastases was associated with T5 ERβ (*P* = 0.014, *r* = 0.338; Table 5) but not with ERα. This relationship was also noted in lymph node metastases derived from ERα-positive (*P* = 0.029, *r* = 0.400) but not ERα-negative primary tumors (Table 5). Interestingly, a negative association between leptin expression and Ki-67 was found in the subgroup of metastases derived from ERα-positive but not ERα-negative primary tumors (Table 5).

The expression of ObR in lymph node metastases positively correlated with ERα (*P* < 0.0001, *r* = 0.442; Table 5) but not ERβ. In addition, ObR negatively correlated with Ki-67 (*P* = 0.021, *r* = −0.310; Table 5). These relationships were lost when we separately analyzed subgroups of lymph node metastases derived from ERα-positive or ERα-negative primary tumors (Table 5).

Associations of leptin/ObR with clinicopathologic features. We studied associations between the leptin/ObR system and lymph node involvement (pN), tumor size (pT), histologic differentiation (G), menopausal status, and patient age. Notably, elevated leptin expression was characteristic for less differentiated tumors, specifically high (3+) leptin content positively correlated with G3 grade (*P* = 0.031), whereas in tumors with medium (2+) leptin expression, there was a trend toward a positive correlation with G3 grade (*P* = 0.069). On the other hand, weak (1+) leptin expression was not significantly associated with tumor differentiation. Similarly, high ObR expression in primary cancers was more frequent in G3 tumors, but the association did not reach statistical significance (*P* = 0.074). No statistically significant correlations were found between leptin or ObR and lymph node involvement, tumor size, menopausal status, and age of patients.

Table 2. Leptin and ObR expression levels in primary and metastatic breast cancer

Tissue type	Leptin expression				ObR expression			
	0	1+	2+	3+	0	1+	2+	3+
PT (*n* = 148)	20	45	64	19	87	41	15	5
			128				61	
LNM (*n* = 66)	4	13	33	16	32	14	14	6
			62				34	

NOTE: The expression of leptin and ObR was determined in primary breast cancers and in lymph node metastases, as described in Materials and Methods. The number of cases (*n*) in each staining category is shown.
Abbreviations: PT, primary tumors; LNM, lymph node metastases.

Table 3. Associations between leptin and ObR in primary tumors and lymph node metastases

Compared biomarkers	Leptin (PT), ObR (PT), $n = 148$, P (r)	Leptin (LNM), ObR (LNM), $n = 66$, P (r)	Leptin (PT), leptin (LNM), $n = 66$, P (r)	ObR (PT), ObR (LNM), $n = 66$, P (r)
All tumors ($n = 148$)	**0.002 (0.275)**	0.154 (0.186)	**0.046 (0.270)**	0.144 (0.191)
ERα^+ tumors ($n = 90$)	**0.005 (0.393)**	0.120 (0.290)	**0.008 (0.507)**	**0.046 (0.355)**
ERα^- tumors ($n = 58$)	**0.003 (0.411)**	0.419 (0.308)	0.449 (0.271)	0.818 (0.055)

NOTE: The associations between the expression of leptin and ObR in primary tumors and lymph node metastases in the group of all tumors and in the subgroups of ERα-positive and ERα-negative tumors (according to the initial diagnosis of primary tumors) were evaluated by Spearman correlation; P, statistical significance; r, correlation coefficient; n, number of cases. Statistically significant values are in bold.
Abbreviations: PT, primary tumors; LNM, lymph node metastases.

Leptin and ObR expression can be induced by different stimuli in ERα-positive and ERα-negative breast cancer cells. We studied the possible mechanism of leptin/ObR overexpression in breast cancer using ERα-positive MCF-7 and ERα-negative MDA-MB-231 breast cancer cell lines. We focused on factors and conditions that are known to induce leptin or ObR expression in other cell systems, especially insulin, IGF-I, E2, and hypoxia. Insulin, IGF-I and E2 are mitogens for breast cancer cells, and their levels are often elevated in obese women.

The induction of leptin, ObR (common domain), and ObRl mRNAs were assessed by reverse transcription-PCR in cells stimulated with E2, IGF-I, insulin, or CoCl$_2$. In MCF-7 cells, all stimuli significantly induced leptin mRNA expression, whereas ObRl and ObR mRNAs were increased by E2, IGF-I and insulin but not by hypoxia (Fig. 2).

In MDA-MB-231 cells, leptin and ObR mRNAs, but not ObRl mRNA, were induced by hypoxia. In addition, insulin stimulated the expression of leptin, ObR, and ObRl mRNAs. E2 and IGF-I did not produce significant effects on the leptin/ObR system (Fig. 2). In both cells lines, the expression of the control gene 36B4 was not affected by the treatments (Fig. 2).

Discussion

Recent reports suggested that leptin, a hormone whose expression is elevated in overweight and obese individuals, might be involved in the development and/or progression of different cancers. This concept is supported by experimental evidence that leptin can stimulate cell growth, counteract apoptosis, and induce migration and expression of matrix degrading enzymes and angiogenic factors in different cellular cancer models (2). For instance, in different breast cancer cell lines, leptin has been shown to stimulate cell proliferation, survival, and transformation, acting through ObRl, the signaling form of the leptin receptor (2, 10, 11, 13).

The involvement of leptin in mammary carcinogenesis awaits further validation in animal models and human clinical material. In this context, new data suggested that leptin is necessary for mammary tumor development in transforming growth factor-α transgenic Lep(ob)Lep(ob) mice (33). In addition, preliminary immunhistochemistry studies described the expression of ObR and/or leptin in human breast tumors and normal mammary gland (19). One recent report suggested that leptin and ObRl are overexpressed in primary breast tumors relative to normal mammary epithelium (18). No prior studies were done using clinical samples obtained from matched pairs of primary breast tumors and lymph node metastases. Similarly, the regulation of leptin/ObR expression in breast cancer cells has never been characterized.

Consequently, our goals were (*a*) to examine the relative expression of leptin and ObR in primary and metastatic breast cancer versus noncancer tissue; (*b*) to evaluate whether the expression of leptin/ObR system is maintained during metastasis to lymph nodes; (*c*) to assess the association between leptin/ObR and other clinicopathologic features, especially tumor differentiation, expression of ER, and cell proliferation; (*d*) to examine whether the expression of the leptin system can be influenced by obesity-related stimuli, such as high levels of insulin, IGF-I, estradiol, and hypoxic conditions in ERα-positive and ERα-negative cells.

We found that leptin and ObR were expressed at low levels in noncancer tissues, and both markers were overexpressed in primary breast tumors as well as in lymph node metastases. The notion that leptin is overexpressed in primary breast tumors is

Table 4. Relationships between the leptin system and ERα, ERβ, and Ki-67 in primary breast cancers

Compared biomarkers	Leptin ERα, P (r)	Leptin ERβ, P (r)	Leptin Ki-67, P (r)	ObR ERα, P (r)	ObR ERβ, P (r)	ObR Ki-67, P (r)
All PT ($n = 148$)	0.523 (−0.056)	**0.001 (0.327)**	0.611 (−0.056)	0.705 (0.032)	0.353 (0.091)	0.291 (−0.103)
ERα^+ PT ($n = 90$)	0.836 (−0.024)	**0.006 (0.378)**	0.289 (−0.150)	0.346 (−0.102)	0.175 (0.173)	0.263 (−0.143)
ERα^- PT ($n = 58$)	—	0.683 (0.092)	0.456 (−0.166)	—	0.451 (−0.164)	0.246 (−0.252)

NOTE: The associations were evaluated in ERα-positive and ERα-negative primary breast tumors by Spearman correlation; P, statistical significance; r, correlation coefficient; (−), no cases in this category. Statistically significant values are in bold.
Abbreviations: PT, primary tumors; LNM, lymph node metastases.

Table 5. Relationships between the leptin system and ERα, ERβ, and Ki-67 in lymph node metastases

Compared markers	Leptin ERα, P (r)	Leptin ERβ, P (r)	Leptin Ki-67, P (r)	ObR ERα, P (r)	ObR ERβ, P (r)	ObR Ki-67, P (r)
All LNM (n = 66)	0.334 (0.124)	**0.014 (0.338)**	**0.016 (−0.331)**	**<0.0001 (0.442)**	0.092 (0.230)	**0.021 (−0.310)**
LNM derived from ERα⁺ PT (n = 40)	0.282 (0.172)	**0.029 (0.400)**	**0.031 (−0.394)**	**0.001 (0.507)**	0.099 (0.292)	0.388 (−0.155)
LNM derived from ERα⁻ PT (n = 26)	0.356 (0.207)*	0.433 (0.280)	0.512 (−0.236)	0.965 (0.010)*	0.176 (0.494)	0.296 (−0.393)

NOTE: The associations were evaluated in lymph node metastases derived from ERα-positive and ERα-negative primary tumors using Spearman correlation; P, statistical significance; r, correlation coefficient. Statistically significant values are in bold.
Abbreviations: PT, primary tumors; LNM, lymph node metastases.
*In several cases, ERα-positive metastases originated from ERα-negative primary tumors.

consistent with the results of Ishikawa et al. (18), whereas the present finding of increased expression of leptin and ObR in lymph node metastasis versus noncancer breast epithelium is original. We also report for the first time that in intraductal proliferative lesions bordering on breast cancer, leptin expression is higher relative to proliferative lesions without accompanying breast cancer, which might imply that leptin abundance is related to disease progression.

The above results further indicate that breast cancer cells can be influenced not only by endocrine and/or paracrine leptin but also via a potent autocrine leptin loop. The function of the leptin autocrine system might be especially important in primary tumors where the expression of leptin correlated with the presence of ObR in both ERα-positive and ERα-negative tumors. This observation is in agreement with the results of Ishikawa et al. who found coexpression of leptin and ObRl in primary ductal breast cancer (18). Here, we additionally identified a correlation between a less differentiated phenotype (G3 grade) and the expression of the leptin system in primary

tumors. This notion is consistent with the fact that breast cancer dedifferentiation can be promoted by hypoxia (34, 35), which also can induce leptin/ObR expression (see also below).

Notably, the expression of both leptin and ObR in lymph node metastases was more frequent than their levels in primary tumors. Whether leptin is truly involved in breast cancer metastasis is still not known, but a limited analysis of Ishikawa et al. (18) suggested that the expression of leptin and ObRl is associated with cancer recurrence in distant organs and a shorter 5-year disease-free survival. Interestingly, in metastases, but not in primary tumors, both leptin and ObR negatively correlated with Ki-67, which could suggest that in metastases the leptin system is not involved in proliferation.

The mechanisms responsible for leptin/ObR overexpression in primary and metastatic breast cancer are not clear. Our results suggest that different stimuli associated with obesity can induce leptin and ObR mRNA. Most notably, high concentrations of insulin and hypoxia stimulated leptin mRNA in both ERα-positive MCF-7 and ERα-negative MDA-MB 231 cell lines.
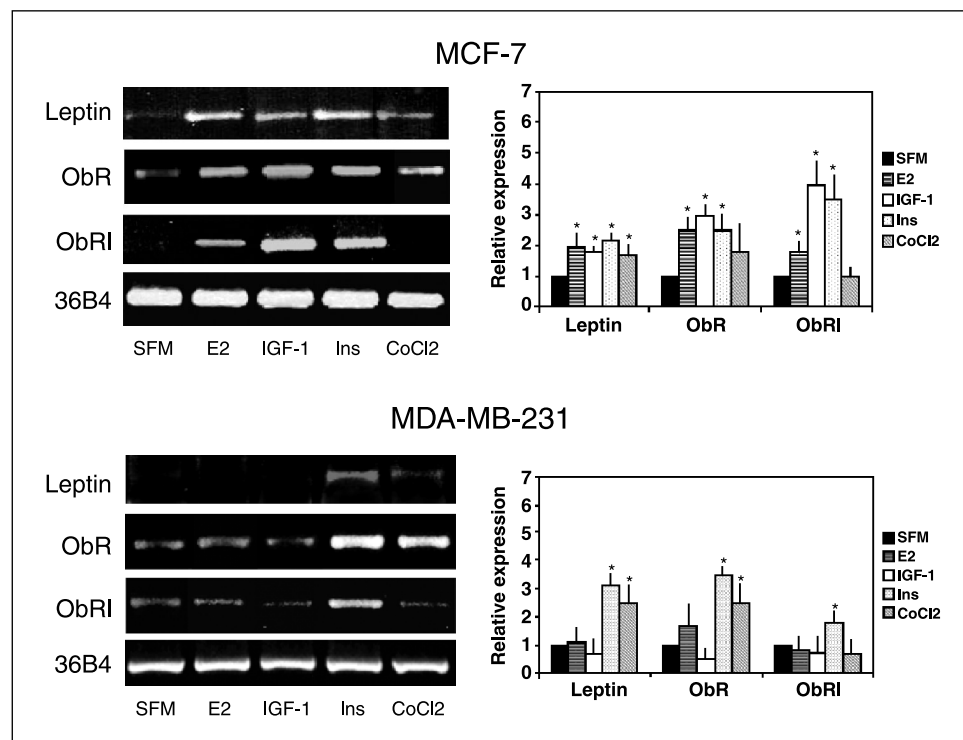
Fig. 2. Effects of E2, IGF-I, insulin, and hypoxia on leptin and ObR mRNA expression in breast cancer cells. MCF-7 and MDA-MB-231 cells were placed in serum-free medium (SFM) for 24 hours and then stimulated with E2, IGF-I, insulin (Ins), or CoCl₂, as described in Materials and Methods. The expression of leptin, ObR (all isoforms), and ObRl (the long form of the receptor only) mRNAs was probed by reverse transcription-PCR using conditions and primers listed in Materials and Methods. Obtained from at least three independent experiments. The abundance of leptin, ObR, and ObRl mRNAs is shown relative to the levels of 36B4 control mRNA. In all cases, the relative expression in serum-free medium is taken as 1. Bars, SD. *, statistically significant differences between treated and untreated cells.

ObRl mRNA was induced by hypoxia only in MDA-MB-231 cells. On the other hand, IGF-I and E2 stimulated leptin and ObR mRNAs in MCF-7 cells. The differential response of MCF-7 and MDA-MB-231 cells to E2 and IGF-I is in agreement with our previous results (36, 37).

Previous reports suggested a link between leptin and ER. Leptin has been found to enhance ERα activity and stimulate the synthesis of estradiol (13, 16, 17). Reciprocally, estradiol can induce leptin and ObR expression, as shown by this study and earlier reports in other models (24, 27, 38). It is possible that ERα effects on leptin/ObR is mediated in part by IGF-I and insulin systems, as E2 is known to up-regulate both pathways in breast cancer cells (39–41). Interestingly, in our study, the expression of leptin and ObR in primary tumors positively correlated with their presence in matched lymph node metastases but only in ERα-positive cases, which might

suggests greater stability of the leptin system in this cell context.

Our study also suggested a relationship between leptin/ObR and ERβ (in particular the cytoplasmatic pool of ERβ recognized by our antibody). The significance of this link is not clear, especially in light of the controversial role of ERβ in breast cancer (42). However, some reports suggested the association of ERβ with poor prognostic features in breast cancer (42, 43), which would agree with our and other findings that the leptin system might be involved in metastasis (2).

In summary, we show that leptin and ObR are overexpressed in primary breast cancer and lymph node metastasis. This overexpression could be related to exposure of cells to high levels of insulin, IGF-I, and estradiol as well as due to hypoxic conditions. Thus, targeting leptin signaling could be beneficial for breast cancer therapy and prevention.

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