Leptin Enhances, via AP-1, Expression of Aromatase in the MCF-7 Cell Line*

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Leptin, a product of adipocytes, is involved in the regulation of body weight and results strongly correlated to body fat content. An excess of fat mass represents a breast cancer risk factor particularly in postmenopausal women, where estrogen production by adipose tissue through its own aromatase activity stimulates tumor progression. Leptin stimulates estrogen production through the increase of aromatase expression and activity in human luteinized granulosa cells and adipose stromal cells. In the present study, we have examined the possible link that exists between leptin and breast cancer, focusing our attention on the direct effect of leptin on aromatase activity, which may enhance estrogen production and induce tumor cell growth stimulation. We have shown that leptin enhances aromatase mRNA expression, aromatase content, and its enzymatic activity in MCF-7. Aromatase expression appears to be regulated by tissue-specific promoter. It has been demonstrated that promoters II and 1.3 are the major promoters that drive aromatase expression in MCF-7. Transient transfection experiments using vector containing human aromatase promoters II and 1.3 sequence fused with luciferase reporter gene demonstrated that leptin is able to activate this promoter. In the presence of either mitogen-activated protein kinase inhibitor PD 98059 or ERK2 dominant negative as well as in the presence of STAT3 dominant negative, the stimulatory effects of leptin on aromatase promoter, enzymatic activity, and aromatase protein content were inhibited. Functional studies of mutagenesis and electrophoretic mobility shift assay revealed that the AP-1 motif is important in determining the up-regulatory effects induced by leptin on aromatase expression in MCF-7.

Leptin, the product of the ob gene, secreted by adipocytes, is involved in body weight control and results strongly correlated to body fat content (1–3). It is well known that an excess of fat mass represents a risk factor for breast cancer particularly in postmenopausal women, where estrogens produced by adipose tissue, through its own aromatase activity, stimulate breast tumor cells growth and progression (4, 5). Aromatase is not only highly expressed in adipose stromal cells surrounding the tumor but also in epithelial cancer cells, suggesting that local estrogen biosynthesis may promote breast cancer growth through an autocrine and paracrine mechanism (6, 7).

Although leptin is mainly synthesized by breast adipose tissue, its expression has also been detected in normal and tumors human mammary epithelial cells (8, 9). Some authors have reported that plasma leptin level and mRNA expression in adipose tissue in breast cancer patients were significantly higher than those in a healthy control group (10). However, the possibility that plasma leptin levels in breast cancer patients could be used as a prognostic index (10) was confuted by the evidence that no difference was demonstrated between leptin levels in premenopausal patients with in situ breast cancer and the control group (11).

In addition, it has been recently shown that leptin receptors (short and long isoforms) are expressed in normal mammary epithelial cells (12) and in human breast cancer cell lines (13, 14). The long form of leptin receptor is able, via Janus kinase 2, to activate STAT1, STAT3, STAT5, and STAT6 transcription factors (15, 16) and the MAPK proteins (16). In contrast, the short receptor isoform mainly activates the MAPKs (16, 17).

Previous studies have demonstrated that leptin stimulates estrogen production by increasing aromatase expression and activity in human luteinized granulosa (18) and adipose stromal cells (19). In our recent work we also demonstrated P450arom immunocytochemical localization together with mRNA and protein expression in MCF-7 cells (20). Therefore, the aim of the present study is to investigate whether leptin per se was able to stimulate aromatase expression and activity in breast cancer epithelial cells, addressing its potential role in enhancing local estrogen production and thereby inducing tumor cell growth stimulation.

In this study leptin has been shown to enhance aromatase mRNA expression, aromatase content, and its enzymatic activity in MCF-7. Aromatase expression appears to be regulated by tissue-specific promoter (21). Lately it has been demonstrated that promoters II and 1.3 are the major promoters driving aromatase expression in the MCF-7 cell line (22). Transient transfection experiments, using vector containing aromatase promoter II/1.3 sequence fused with luciferase reporter gene, demonstrated that leptin is able to directly activate this promoter.

In the presence of either MAPK inhibitor PD 98059 or ERK2

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1 The abbreviations used are: STAT, signal transducers and activators of transcription; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; DMEM, Dulbecco’s modified Eagle’s medium; PMSF, phenylmethylsulfonyl fluoride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ER, estrogen receptor; CREB, cAMP-responsive element-binding protein; P450arom, cytochrome P450 aromatase.

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dominant negative as well as in the presence of STAT3 dominant negative, the stimulatory effect of leptin on aromatase promoter, aromatase protein content, and enzymatic activity was inhibited. Functional studies of mutagenesis and electrophoretic mobility shift assay revealed that AP-1 motif is crucial in determining the up-regulatory effect induced by leptin on aromatase P11/P1.3 promoters activity in the MCF-7 cell line.

EXPERIMENTAL PROCEDURES

Materials—DMEM/Ham's F-12 medium, t-glutamine, Eagle's nes- sential amino acids, penicillin, streptomycin, calf serum, bovine serum albumin, and phosphate-buffered saline were purchased from Eurobio (Les Ulis, France). [1-3H]Androst-4-ene-3,17-dione and [14]H]thyymidine were provided by PerkinElmer Life Sciences, Triazol reagent was from Invitrogen, the RETROscript kit was from Ambion (Austin, Texas), and FuGENE 6 and poly[dI-dC] were from Roche Applied Science. Taq DNA polymerase, T4 poly(ADP-ribose) polymerase kinase, 50-bp DNA ladder, dual luciferase kit, and TK Renilla luciferase plasmid were provided by Promega (Madison, WI). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, and recombinant human leptin were purchased from Sigma. MAPK inhibitor PD 98059 was provided by Calbiochem (San Diego, CA). The ECL system, [γ-32P]ATP, and Sephadex G50 spin columns were from Amersham Biosciences. The PG5 vector containing human aromatase promoters II and I was ligated to a luciferase reporter gene (PG5 PI1/13) was a gift from Dr. E. R. Simpson and Dr. C. D. Clyne (Prince Henry's Institute of Medical Research, Clayton, Australia). PG5 vector containing the cDNA encoding dominant negative STAT3, which is a variant of the transcription factor STAT3 lacking an internal domain of 50 base pairs located near the C terminus (STAT−) was kindly given by Dr. J. Turkson (University of South Florida College of Medicine, Tampa, FL). pCMV5myc vector containing the cDNA encoding dominant negative ERK2 K52R (ERK2Δ) was generously provided by Dr. M. Cobb (Department of Pharmacology, Southwestem Medical Center, Dallas, TX). XETL is a plasmid containing an estrogen-responsive element conjugated with a luciferase reporter gene and has been described previously (23). The origins of the different antibodies used are described in the following paragraphs.

Cell Cultures—Wild-type human breast cancer MCF-7 cells were gifts from E. Surmacz (Philadelphia, PA). This cell line was cultured in DMEM/Ham's F-12 medium containing 5% calf serum, 1% t-glutamine, 1% Eagle's nonessential amino acids, and 1 mg/ml penicillin-streptomycin. The cells were cultured in phenol red-free DMEM/Ham's containing 5% charcoal-stripped fetal calf serum for 1, 3, 6, and 24 days in the presence of different concentrations of leptin (10 ng/ml) or control. To remove the last 6 h, [3H]thyymidine was added to the culture medium. After rinsing with phosphate-buffered saline, the cells were washed once with 10% and three times with 5% trichlo roactic acid. The cells were lysed by adding 0.1 ml NaOH and then incubated 30 min at 37 °C. Thymidine incorporation was determined by scintillation counting.

Aromatase Activity Assay—The aromatase activity in subconfluent MCF-7 cells medium was measured by the titrated water release assay using 0.5 μM [1-3H]androst-4-ene-3,17-dione as substrate (24). MCF-7 cells were cultured for 48 and 96 h in DMEM/Ham's F-12 medium in the absence or in presence of 10 and 100 ng/ml of leptin. The incubations were performed at 37 °C for 5 h under an air/CO2 (5%) atmosphere. The results obtained were expressed as fmol/h and normalized to mg of protein (fmol/h/mg protein). A set of experiments was carried out 24 h before each treatment, adding in the medium MAPK inhibitor PD 98059 (50 μM) overnight in the culture medium. At the next day, the cells were harvested and the cDNA encoding dominant negative ERK2 K52R (ERK2Δ) (0.5 μg/well) was used to normalize the efficiency of the transfection. The firefly and Renilla luciferase plasmids were normalized on the basis of transfection efficiency measured by pGL3 control vector.

Reverse Transcription-PCR Assay—Aromatase mRNA was analyzed by the reverse transcription-PCR method. cDNA was synthesized by oligo(dT) using a RETROscript kit as suggested by the manufacturer. The primers were further amplified by a PCR method using the following primers: 5′-CAAGGTTATTGATGGCTAGG-3′ (forward, nucleo tides 776–796) and 5′-TCTTACAGGTTTGCCGACGA-3′ (reverse, nucleotides 1261–1241) for human P450arom and 5′-CAACCCTATGCGACAATTCCATGCGCA-3′ and 5′-CTCAGACGCGGACTCCGTACC-3′ for the human housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH). The PCR was performed for 40 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min) for P450arom and 24 cycles (94 °C for 1 min, 64 °C for 1 min, and 72 °C for 2 min) to amplify the human G3PDH in the presence of 5 μl of first strand cDNA, 1 μl each of the primers mentioned above, 0.5 mM dNTp, Taq DNA polymerase (2 units/ tube), and 2 mM magnesium chloride in a final volume of 25 μl. To detect the presence of DNA contamination, a negative control PCR was performed on 1 μg of total RNA without Moloney murine leukemia virus reverse transcriptase (the negative control). The PCR products were analyzed on 2% agarose gel and stained with ethidium bromide. DNA quantity in each lane was analyzed by scanning densitometry. Standard DNA (50-bp DNA ladder) was run to provide the proper size marker. CYP19 encoding human P450arom (25) provided the positive control.

Western Blot Analysis—MCF-7 cells were grown in 10-cm dishes to 70–80% confluence and lysed in 500 μl of a 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 10% glycerol, 1% Triton X-100, and a mixture of protease inhibitors (aprotinin, PMSF, and soybean orthovanadate). Equal amounts of total protein were resolved on an 11% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane, probed with rabbit polyclonal antiserum directed against the human placental P450arom (1:4000) (Hauptman-Woodward, Medical Research Institute, Inc, Buffalo, NY). The antigen-antibody complex was detected by incubation of the membranes for 1 h at room temperature with peroxidase-coupled goat anti-rabbit IgG and revealed using the ECL System. The blots were then exposed to film, and the bands of interest were quantified by densitometer (model 620; Bio-Rad). The results obtained as optical density arbitrary values were transformed to percentages of the control (percent control) taking the samples from cells not treated as 100%. R2C cells were used as a positive control.

Transfection Assay—Transient transfection experiments were performed using human aromatase promoter II and 1.3 sequence ligated to a luciferase reporter gene into the pGL3 vector. The deletions of AP-1 sequences in the aromatase promoter were generated by PCR. The resulting plasmids encoding the aromatase promoter II and 1.3 containing the desired deletions were designated AP-1Δ (containing the deletions of AP-1 motifs at positions −498 and −935, respectively); the AP-1Δ−498/−935 plasmids contains deletions at both AP-1 sites. The desired deletions of sequences were confirmed by nucleotide sequence analysis. FuGENCE was used as directed by the manufacturer to cotransfect cells plated in 3.5-cm wells with the pGL3 vector control 0.5 μg/well). A set of experiments was performed cotransfecting pGL3 promoters II/I.3 (0.5 μg/well) and pSG5 vector containing the cDNA encoding dominant negative STAT3, which is a variant of the transcription factor STAT3 lacking an internal domain of 50 base pairs located near the C terminus (STAT−) (0.5 μg/well). Empty vectors were used to ensure that DNA concentrations were constant in each transfection. TK Renilla luciferase plasmid (25 ng/well) was used to normalize the efficiency of the transfection. Empty PGL3 Basic vector was used as a control vector to measure basal activity.

24 h after transfection, the medium was changed, and the cells were treated in DMEM/Ham's F-12 medium in the presence of 10 and 100 ng/ml of leptin for 48 h and 96 h. In another set of experiments, 24 h after transfection, we added MAPK inhibitor PD 98059 (50 μM) overnight in the medium before starting the treatment with leptin. Transient transfection experiments were also performed using XETL plasmid, a construct responsive to peroxide-coupled coupled anti-rabbit IgG and revealed using the ECL System. The blots were then exposed to film, and the bands of interest were quantified by densitometer (model 620; Bio-Rad). The results obtained as optical density arbitrary values were transformed to percentages of the control (percent control) taking the samples from cells not treated as 100%. R2C cells were used as a positive control.

Gel Mobility Shift Assay—The nuclear extracts were prepared from MCF-7 as previously described (26). Briefly, MCF-7 cells plated into 60-mm dishes were scraped into 1.5 ml of cold phosphate-buffered saline. The cells were pelleted for 10 s and resuspended in 400 μl of cold buffer A (10 mM HEPES-KOH, pH 7.9 at 4 °C, 1.5 mM MgCl2, 10 mM...
after incubation with 0.5 mM PMSF, 1 mM leupeptin) by flicking the tube. The cells were allowed to swell on ice for 10 min and then vortexed for 10 s. The samples were then centrifuged for 10 s, and the supernatant fraction was discarded. The cellular debris was removed by centrifugation for 2 min at 4 °C, and the supernatant fraction (containing DNA binding proteins) was stored at −70 °C. The yield was determined by the Bradford method (27). The probe was generated by annealing single-stranded oligonucleotides, labeled with [γ-32P]ATP and T4 polynucleotide kinase, and then purified using Sephadex G50 spin columns. The DNA sequences used as probe or as cold competitors are the following (the nucleotide motifs of interest are underlined, and mutations are shown as lowercase letters): AP-1, ATGGCCTGAGTGAGTCACTTTGAATTC; mutated AP-1, ATGGCCTGAGTtcaTCACTTTGAATTC. The oligonucleotides were synthesized by Sigma Genosys (Cambridge, UK). The protein binding reactions were carried out in 20 μl of buffer (20 mM HEPES, pH 8, 1 mM EDTA, 50 mM KCl, 10 mM DTT, 10% glycerol, 1 mg/ml bovine serum albumin, 50 μg/ml poly(dI-dC)) with 50,000 cpm of labeled probe, 20 μg of MCF-7 nuclear protein, and 5 μg of poly(dI-dC). The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotides. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25× Tris-borate-EDTA for 3 h at 150 V. The gel was dried and subjected to autoradiography at −70 °C.

Statistical Analysis—Each data point represents the mean ± S.E. of three experiments. The data were analyzed by analysis of variance using the STATPAC computer program.

RESULTS

Leptin Effect on Cell Proliferation in MCF-7 Cell Line—We evaluated the effects of leptin on MCF-7 cell line proliferation by measuring changes in the rate of DNA synthesis ([3H]thymidine incorporation). We confirmed, as previously demonstrated (14), that leptin induces proliferation of the human breast cancer cell line MCF-7. The [3H]thymidine incorporation was significantly increased upon treatment with 10 and 100 ng/ml of leptin (335 and 238% compared with control, which was set at 100%) (data not shown).

Stimulation of Aromatase Activity by Leptin in MCF-7 Cells—Aromatase activity was measured by tritiated water assay in MCF-7 cells incubated for 48 and 96 h in the presence of different concentrations of leptin (10 and 100 ng/ml). A significant increase of the enzymatic activity was observed upon 10 and 100 ng/ml of leptin (335 and 238% compared with control, which was set at 100%) (Fig. 1). It is noteworthy that this phenomenon was completely reversed by the aromatase inhibitor letrozole (p < 0.01) (Fig. 1). It is noteworthy that this phenomenon was completely reversed by the aromatase inhibitor letrozole (p < 0.01) (Fig. 1).

Leptin Enhances P450arom mRNA and Protein Expression in MCF-7 Cells—In the MCF-7 cell line we investigated the effects of leptin on P450arom mRNA levels by reverse transcription-PCR. As shown in Fig. 2A, the expected transcript of 465 bp was clearly detected using primers designated to amplify the highly conserved sequence of P450arom, which includes the helical and aromatic regions. Incubation with 100 ng/ml of leptin (335 and 238% compared with control, which was set at 100%) (data not shown).

Leptin Enhances Aromatase Expression in MCF-7 Cells. MCF-7 cells were cultured for 48 h (A) and 96 h (B) in the absence or presence of 10 and 100 ng/ml of leptin (Lep). 1 μM of aromatase inhibitor letrozole was used. Aromatase activity was evaluated by measuring the tritiated water released by MCF-7 cell cultures after incubation with 0.5 μM [1β-3H]androst-4-ene-3,17-dione at 37 °C for 5 h. The results obtained were expressed as fmol [3H]H2O/h released and were normalized for mg of protein (fmol/h/mg protein). The values represent the means ± S.E. of three different experiments, each performed with triplicate samples. *, p < 0.01 compared with control; **, p < 0.01 compared with leptin (100 ng/ml)-treated samples.

Leptin Enhances P450arom mRNA and Protein Expression in MCF-7 Cells. Total RNA was isolated from MCF-7 cell lines and reverse transcribed. cDNA was subjected to PCR using primers specific for P450arom (40 cycles) or G3PDH (25 cycles). Lane 1, control; lane 2, leptin (Lep, 100 ng/ml); lane 3, RNA sample without the addition of reverse transcriptase (negative control); lane 4, amplification of human CYP19 used as positive control. The histograms represent the means ± S.E. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *, p < 0.01 compared with control.

Fig. 1. Effects of leptin on aromatase activity in cultured MCF-7 cells. MCF-7 cells were cultured for 48 h (A) and 96 h (B) in the absence or presence of 10 and 100 ng/ml of leptin (Lep). 1 μM of aromatase inhibitor letrozole was used. Aromatase activity was evaluated by measuring the tritiated water released by MCF-7 cell cultures after incubation with 0.5 μM [1β-3H]androst-4-ene-3,17-dione at 37 °C for 5 h. The results obtained were expressed as fmol [3H]H2O/h released and were normalized for mg of protein (fmol/h/mg protein). The values represent the means ± S.E. of three different experiments, each performed with triplicate samples. *, p < 0.01 compared with control; **, p < 0.01 compared with leptin (100 ng/ml)-treated samples.

Fig. 2. Leptin enhances P450arom mRNA. Total RNA was isolated from MCF-7 cell lines and reverse transcribed. cDNA was subjected to PCR using primers specific for P450arom (40 cycles) or G3PDH (25 cycles). Lane 1, control; lane 2, leptin (Lep, 100 ng/ml); lane 3, RNA sample without the addition of reverse transcriptase (negative control); lane 4, amplification of human CYP19 used as positive control. The histograms represent the means ± S.E. of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control, which was assumed to be 100%. *, p < 0.01 compared with control.

three experiments. The data were analyzed by analysis of variance using the STATPAC computer program.
ng/ml of leptin for 48 h resulted in a significant increase of P450arom mRNA (p < 0.01) (Fig. 2). mRNA expression of the aromatase gene was normalized using the human housekeeping gene G3PDH (Fig. 2).

Next, we performed Western blot analysis using an antibody against human placental P450arom. A 55-kDa specific protein in MCF-7 cells comigrated with R2C cells used as positive control. Treatment with 100 ng/ml of leptin for 48 h enhanced aromatase protein content (p < 0.01) (Fig. 3).

Effect of Leptin on Expression of Human P450arom PII and P1.3/Luciferase Reporter Gene in MCF-7 Cells—Transient transfection experiments using vector containing human aromatase promoter II and 1.3 sequence fused to the luciferase reporter gene have been used to evaluate whether leptin was able to activate directly this promoter identified in epithelial breast cancer cells.

A significant enhancement of promoter activity was observed in the transfected cells exposed to both leptin concentrations tested at 48 and 96 h (p < 0.01) (Fig. 4). These findings led us to analyze the functional sequences present in the promoter and responsible of up-regulation induced by leptin. Because leptin signaling is able via Janus kinase 2 to activate STAT and MAPKs proteins, we focused our attention on the AP-1 motif as a possible effector of both signals.

Evidence That MAPK and STAT Signals Are Involved in the Leptin-induced Up-regulation of Aromatase Expression—Clear evidence of the crucial role of the MAPK in mediating leptin stimulation effects on aromatase has been pointed out by the fact that either in the presence of MAPK inhibitor PD 98059 or in the cells transiently transfected with ERK2 dominant negative, the up-regulatory effects on aromatase enzymatic activity (Fig. 5, A and B), aromatase protein content (Fig. 5C), and aromatase promoter activity (Fig. 5, D and E) were completely abrogated.

The role of the STAT proteins, as another signal transductional pathway activated by leptin, was investigated in MCF-7 cells transiently transfected with a dominant negative of STAT proteins. It is noteworthy that in these circumstances the enhancing effects induced by leptin on enzymatic activity (Fig. 6A), aromatase protein content (Fig. 6B), and aromatase promoter (Fig. 6C) were also inhibited.

AP-1 Motif Is Involved in the Regulation of Aromatase Promoter—Functional assays were performed also with PI/P1.3 aromatase promoter constructs with AP-1-deleted sites at positions −498 (AP-1 − 498), −935 (AP-1 − 935), and both (AP-1 − 498/−935). In these circumstances the leptin-induced activation on aromatase promoter was completely abrogated in all of the constructs tested (Fig. 7). These results address the AP-1 response element as a sequence crucial in mediating aromatase promoter activation upon leptin exposure.

Effect of in Vitro Leptin Treatment on AP-1 DNA Binding Activity in Nuclear Extracts from MCF-7 Evaluated by Electrophoretic Mobility Shift Assay—To further characterize the role of AP-1 in modulating promoter aromatase activity, we performed electrophoretic mobility shift assay using a [32P]AP-1 consensus sequence in the presence of nuclear extracts from MCF-7 cells. Leptin treatment induced a strong increase in AP-1 DNA binding activity (Fig. 8, lane 4) compared with basal levels (Fig. 8, lane 1).

The specificity of DNA binding activity for AP-1 was confirmed by the observation that a 100-fold excess of unlabeled
MAPK signal is involved in enhancing leptin aromatase activity, aromatase content, and aromatase promoters PII/1.3 activity. MCF-7 cells were serum-starved overnight with or without PD 98059 (PD) (A), then were untransfected or transiently transfected with ERK2 dominant negative (B), and then were treated for 48 h in the presence or absence of leptin (Lep). Aromatase activity was evaluated by measuring the tritiated water released by MCF-7 cell cultures after incubation with 0.5 μM [1H]androst-4-ene-3,17-dione at 37 °C for 5 h. The results obtained were expressed as fmol [3H]H2O/h released and were normalized for mg of protein (fmol/h/mg protein). The values represent the means ± S.E. of three different experiments, each performed with triplicate samples. *, p < 0.01 compared with control.

C, MCF-7 cells were treated in the absence (lane 2) or presence of leptin (100 ng/ml) for 48 h (lane 3). The cells were serum-starved overnight with MAPK inhibitor PD 98059 (50 μM) (lane 4) or transiently transfected with a dominant negative of ERK2 (lane 5) and then treated with leptin (100 ng/ml) for 48 h. R2C (lane 1) were used as positive control. Upper panel, representative autoradiograph; lower panel, the histograms represent the means ± S.E. of three separate experiments in which band intensity was evaluated in terms of optical density arbitrary units and expressed as percentages of the control assumed as 100%. D, MCF-7 cells were serum-starved overnight with or without MAPK inhibitor PD 98059 (50 μM) and then were transfected with human aromatase promoters II/1.3 luciferase reporter construct. E, MCF-7 were transfected with PGL3, promoter II/1.3 luciferase reporter gene alone or cotransfected with a dominant negative of ERK2. The cells were treated in the presence or absence of leptin (100 ng/ml) for 48 h and then were lysed and assayed for luciferase activity. These results represent the means ± S.E. of three different experiments, each performed with triplicate samples. *, p < 0.01 compared with control.
AP-1 probe inhibited the binding of the labeled probe to the nuclear proteins tested (Fig. 8, lane 2), whereas the use of mutated AP-1 homologous sequence as cold competitor revealed no change in this complex (Fig. 8, lane 3). In the presence of either MAPK inhibitor PD 98059 or in the MCF-7 transiently transfected with ERK2 dominant negative the AP-1 DNA binding activity induced by leptin treatment was drastically reduced (Fig. 8, lanes 5 and 6), whereas expression of dominant negative STAT3 determined a slight reduction of this binding complex (Fig. 8, lane 7).

The Enhanced Aromatization of Androst-4-ene-3,17-dione Induced by Leptin Potentiates Estradiol/ERα Signal in MCF-7 Cells—Transient transfection experiments using XETL plasmid was performed to investigate whether leptin was able to enhance the potency of an aromatizable androgen like androst-4-ene-3,17-dione (A) on estradiol/ERα signal in MCF-7 cells. As shown in Fig. 9, we observed that the addition of leptin (1000 ng/ml) was able to enhance XETL activation induced by androst-4-ene-3,17-dione alone (p < 0.001), which was inhibited by the aromatase inhibitor letrozole (data not shown).

DISCUSSION

Mammary adipose tissue is an important source of paracrine mitogens and anti-mitogens including insulin-like growth factor, transforming growth factor α, cytokines (tumor necrosis factor α and interleukin γ) and leptin (28). Recently leptin was reported to stimulate the proliferation of various cell types (29, 30) as a new growth factor. Indeed several studies have shown how leptin was able to activate the proliferation of pancreatic β cells (29), vascular endothelium (31), lung (32), gastric mucosa (33), keratinocytes cells (34), and, lately, breast cancer cells (14). Moreover, hyperleptinemia is a common feature of obese women, who are exposed to a higher risk in developing breast cancer than in normal weight women (35, 36).

This assumption is sustained by recent findings that show how normal mammary gland morphogenesis is impaired in both nontransgenic genetically obese leptin-deficient and ge-
Netically obese leptin receptor-deficient mice (37). Similar results were obtained for transgenic transforming growth factor α/lep<sup>ab</sup> lep<sup>ab</sup> mice that did not develop mammary tumors in contrast to the transgenic transforming growth factor α mice that were either homozygous lep<sup>+</sup> lep<sup>+</sup> or heterozygous lep<sup>+</sup> lep<sup>ab</sup> lean for the leptin gene that exhibited mammary tumor incidence rates of 50 and 67%, respectively, by 24 months of age (37). All of these findings seem to underscore an important role for leptin on mammary gland development and mammary gland tumorigenesis, giving more emphasis to the epidemiologic studies suggesting a relationship between obesity and breast carcinogenesis.

Obesity is an important health concern, because it is associated with a variety of metabolic disorders and an increased risk of developing cancer (38). It is now well established that postmenopausal women with upper body fat predominance (android obesity) experience a higher risk of breast cancer (4, 5).

The association between obesity and breast carcinoma is usually ascribed to estrogen excess, which derives from androgen aromatization in peripheral fat deposits (6, 7). Although estrogen receptor-positive breast tumors are usually more responsive to therapy than estrogen receptor-negative tumors, there is a report demonstrating that estrogen receptor-positive breast tumor status in obese women is actually associated with a poorer prognosis than is estrogen receptor-negative status (39). Also, the T-47D cell line, an estrogen-positive cell line, showed a dramatic increase in anchorage-independent growth after treatment with leptin (37).

Estrogens also appear to modulate leptin gene expression in adipose tissue (40, 41). It is interesting to note how two recent studies have reported increased serum leptin levels in women receiving tamoxifen as a treatment for breast cancer (42, 43). On the other hand, leptin was recently reported to increase aromatase activity in woman luteinized granulosa cells (18).
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**Fig. 9.** The estradiol/ERα signal activation induced by androst-4-ene-3,17-dione aromatization is potentiated by leptin in MCF-7 cells. MCF-7 cells were transiently transfected for 6 h with XETL plasmid and treated in the absence (control) or presence of leptin (100 ng/ml) for 72 h. For the last 24 h androst-4-ene-3,17-dione (colunm A) (100 nM) was added in the cells untreated or treated with leptin. These results represent the means ± S.E. of three different experiments. In each experiment the activities of the transfected plasmids were assayed in triplicate transfections. *, p < 0.01 compared with control; **, p < 0.01 compared with androst-4-ene-3,17-dione-treated samples.

and adipose stromal cells (19). Thus, it emerges from these findings that there is a potential relationship between leptin and estrogen.

Previously, we demonstrated P450arom immunocytochemical localization together with mRNA and protein expression in MCF-7 cells (20), and because both types of leptin receptors are present in this breast cancer cell line (13), it was reasonable to investigate whether leptin per se was able to stimulate aromatase gene expression.

Even though many aspects of potential relationship between leptin and estrogen can be addressed, in the present study, for the first time, we have provided evidence that leptin per se up-regulates aromatase gene expression in epithelial breast cancer cells and enhances both aromatase content and aromatase activity. In different tissues, leptin begins its actions by binding to a specific receptor (Ob-R) (44) localized in the cellular membrane activating RAS-dependent MAPK pathway and STAT1, STAT3, STAT5, and STAT6 transcription factors (15, 16).

Promoters II and 1.3 are the major aromatase promoters in MCF-7 cells (22), bearing consensus sequences for AP-1 at the sites −498 and −935. We focused our attention on this sequence as a potential target of leptin signal, because it has been reported that leptin induces mRNA for immediate early genes such as c-fos, c-jun, and junB, members of the AP-1 complex (45).

The crucial role of AP-1-responsive element in mediating the up-regulatory effects of leptin on aromatase promoter activity is demonstrated by the fact that in the presence of AP-1 deleted sites, these up-regulatory effects were completely abrogated. In addition, it is presumable that a strong increase in AP-1 DNA binding as evidenced in electrophoretic mobility shift assay following 48 h of leptin exposure is mainly attributable to the increase of mRNA transcription coding this dimeric protein. Leptin-induced increase of AP-1 DNA binding was drastically inhibited either in the presence of MAPK inhibitor or in the cells transiently transfected with ERK2 dominant negative.

The latter datum reveals previous findings showing how the same ERPε2 dominant negative (K52R) utilized in the present study was able to inhibit AP-1 transactivation and neoplastic transformation in other cell types (46). The transactivation and DNA binding of AP-1 are modulated by protein kinase cascades that terminate in phosphorylation of Jun and Fos family proteins. Members of the MAPK superfamily, the ERK, and the c-Jun N-terminal kinase, are thought to be responsible for phosphorylation of AP-1 proteins (47).

For instance, the RAS-MAPK pathway induces activation of CREB kinase, a member of the p90S6 kinase family that corresponds to RSK2, and thereby phosphorylating CREB serine 133 (48–50). This phosphorylated protein plays a multivalent role in inducing the DNA binding of AP-1: 1) as a component of this dimeric protein through its ethrodimerization with c-Jun (47); 2) as an inducer of c-Jun expression because the complex c-Jun/CREB ATPF2 recognizes the 12-O-tetradecanoylphorbol-13-acetate-responsive element consensus sequence located in the proximal region of the c-Jun promoter (51); and 3) as an inducer of c-Fos expression via the cAMP response element present in the c-Fos promoter region (52).

Also, it should be remembered that STAT proteins can also positively regulate the activity of AP-1 complex and thereby enhance aromatase activity because the c-Fos promoter is endowed with a sis-inducible enhancer element (53) that is recognized by the STAT group of transcription factors.

All of these observations fit with our present findings that demonstrate how MAPK inhibitor, ERK2 dominant negative, and STAT3 dominant negative inhibit the up-regulation of leptin on aromatase expression. Therefore all of our data taken together led us to postulate that both MAPK and STAT signals converge into stimulating aromatase expression upon leptin exposure; however, MAPK signal seems to be more involved in up-regulating aromatase expression in breast cancer cells.

The major outcome of the present study is the evidence that the aromatase gene is one of the genes whose transcription appears to be potentiated by leptin through an enhanced binding of AP-1 to specific DNA sites in the promoter region, and it addresses how leptin may be involved in the pathophysiology of breast modulating in situ estrogen production also in epithelial cells. The biological relevance of the enhanced aromatization induced by leptin is also demonstrated by the fact that the estradiol/ERα signal in MCF-7 cells transfected with XETL plasmid and exposed to aromatizable androgen (androst-4-ene-3,17-dione) was dramatically potentiated by leptin but inhibited by luteolone. On the basis of these findings we argue that an important role for leptin in promoting breast cancer in obese women increases its potential use of readily aromatizable androgens (androst-4-ene-3,17-dione, dehydroepiandrosterone) in breast tissue, enhancing in situ estradiol production not only by adipose tissue but also by epithelial breast cells, particularly when a drop in the levels of circulating estrogens occurs.

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Leptin Enhances, via AP-1, Expression of Aromatase in the MCF-7 Cell Line
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