Leptin induces, via ERK1/ERK2 signal, functional activation of estrogen receptor alpha (ERα) in MCF-7 cells.

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Running title: Leptin induces ERα transactivation in MCF-7 cells.
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Summary

Leptin is a hormone with multiple biological actions, produced predominantly by adipose tissue. In humans, plasma levels correlate with total body fat, and high concentrations occur in obese women. Among its functions, leptin is able to stimulate normal and tumor cell growth. We demonstrated that leptin induces aromatase activity in MCF-7 cells evidencing its important role in enhancing in situ estradiol production and promoting estrogen-dependent breast cancer progression. Estrogen receptor α (ERα), which plays an essential role in breast cancer development, can be transcriptionally activated in ligand-independent manner. Taking into account that unliganded ERα is an effector of mitogen-activated protein kinase (MAPK) signal and that leptin is able, via janus-kinase (JAK 2), to activate the ras dependent MAPK pathway, in the present study we investigate the ability of leptin to transactivate ERα. We provided evidence that leptin is able to reproduce the classical features of ERα transactivation in breast cancer cell line: a nuclear localization, a down-regulation of its mRNA and protein levels, and the up-regulation of a classic estrogen dependent gene like pS2.

Transactivation experiments with a transfected reporter gene for nuclear ER showed an activation of ERα either in MCF-7 or in HeLa cells. Using an ERK2 dominant negative or the MAPK inhibitor PD 98059, we showed that leptin activates the ERα through the MAPK pathway. The N-terminal transcriptional activation function 1 (AF-1) appears essential for the leptin response. Finally, it is worth to note that leptin exposure potentiates also the estradiol induced activation of ERα. Thus, we are able to demonstrate that the amplification of estrogen signal induced by leptin occurs through an enhancing in situ E₂ production as well as a direct functional activation of ERα.
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Introduction

Leptin, the product of the ob gene, mainly secreted by adipocytes, is involved in the control of body weight and results strongly correlated to the body fat mass (1-4). Recently, leptin was reported to stimulate the proliferation of various cell types (5-11) leading to consider leptin as a novel growth factor. Indeed several studies have shown how leptin was able to activate the proliferation of pancreatic β cells (5), vascular endothelium (7), lung (8), gastric mucosa (9), keratinocytes cells (10) and, recently, breast cancer cells (11).

Although leptin is mainly synthesized by breast adipose tissue, its expression has also been detected in normal and tumoral human mammary epithelial cells (12, 13). In addition, it has been shown that leptin receptors (short and long isoforms) are expressed in normal mammary epithelial cells (14) as well as in human breast cancer cell lines (11, 15). These data suggest an important role of leptin on mammary gland development and tumorogenesis, giving more emphasis to the epidemiological studies that evidence 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 (16). It is now well established that postmenopausal women with upper body fat predominance experience a higher risk of breast cancer (17, 18). The association between obesity and breast carcinoma is usually ascribed to estrogen excess, derived from androgen aromatization in peripheral fat deposits (19, 20). In our recent work we have demonstrated that leptin is able to stimulate, through mitogen-activated protein kinase (MAPK) and signal transducers and activators of transcription (STAT) signals, aromatase expression in MCF-7 cell line evidencing its important role in enhancing in situ estradiol production and...
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promoting cell proliferation (21). Besides, a potential relationship between leptin and estrogens stems also from the evidences that estrogens appear to modulate leptin gene expression in adipose tissue (22, 23). 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 estrogen receptor-negative status (24). Also, the T-47D cells, an estrogen receptor positive cell line, evidenced a dramatic increase in anchorage-independent growth after treatment with leptin (25).

Estrogen receptors (ERα and ERβ) are members of the superfamily of nuclear steroid hormone receptors, which are able to regulate the transcriptional activity of target genes by interacting with different DNA response elements (26). The estrogen receptor alpha (ERα) signaling plays an essential role in promotion and progression of steroid hormone dependent breast cancer (27). In addition to mediating the classical transcriptional effect of estrogen, ERα can be transcriptionally activated in the absence of estrogen, a process referred to as ligand-independent activation (28). Ligand-independent activation of ERα has been reported in response to a variety of stimuli [e. g. serum (29), dopamine (30), cAMP (31), caveolin 1 (32), Akt kinase (33), epidermal growth factor (EGF) (34) and specific cyclins (35, 36)]. The most completely studied pathway for ligand-independent ERα activation involves MAPK-mediated activation of ERα in tumor-derived cell lines (34, 37). In Cos1 cells, for example, growth factor-induced activation of ERα results from MAPKinase-mediated phosphorylation of serine 118 in the A/B domain of the ER (34).

Taking into account that unliganded ERα is an effector of MAPKinase signal and that leptin is able, via janus-kinase (JAK 2), to activate the ras dependent MAPKinase
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pathway (6) the aim of the present study was to investigate whether leptin was able to induce the functional transactivation of ERα using as model systems the estrogen dependent MCF-7 breast cancer cells and steroid receptor negative HeLa cells. Our results have demonstrated, for the first time, the ability of leptin to induce ERα nuclear localization together with the typical features of ERα functional transactivation in breast cancer cells.
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**Materials and methods**

**Materials**

Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12 Ham (DMEM/F12), L-Glutamine, Eagle’s non essential amino acids, penicillin, streptomycin, calf serum (CS), bovine serum albumine (BSA), phosphate-buffered saline (PBS) were purchased from Eurobio (Les Ullis Cedex, France). Triazol Reagent by Invitrogen (Carlsbad, California), FuGENE 6 by Roche (Indianapolis, Indiana). Taq DNA polimerase, 50 bpDNA ladder, Dual luciferase kit and TK renilla luciferase plasmid were provided by Promega (Madison, Wisconsin). Aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and recombinant human leptin were purchased by Sigma (Milan, Italy). MAPK inhibitor PD98059 was provided by Calbiochem (San Diego, CA). Antibodies against ERα and α-actin were provided by Santa Cruz Biotechnology (Santa Cruz, California). Biotinylated horse-anti-mouse IgG and ABC complex/HRP were provided by Vector Laboratories (Burlingame, CA). Chromogen 3-di-aminobenzidine tetrachloride dihidrate was purchased by Bio-Optica, MI. ECL System, [γ³²P]ATP, and Sephadex G50 spin columns were purchased by Amersham Pharmacia (Buckinghamshire, UK).

**Plasmids**

Firefly luciferase reporter plasmid is XETL a construct containing an estrogen-responsive element (37). The wild type human ERα expression vector (HEGO) consists of the full-length ERα cDNA fused with the SV40 early promoter and expressed in the pSG5 vector (38). pSG5/HE15 and pSG5/HE19 plasmids codify for amino-terminal ERα (AF-1, amino acid 1-281) and for carboxyl-terminal ERα (AF-2, amino acids 179-595)
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respectively (a gift from Dr. D. Picard, University of Geneve, Switzerland). S104/106/118A-ER plasmid is mutated in serine residues 104, 106, 118 to Ala (a gift from Dr. D.A. Lannigan, University of Virginia, Charlottesville); HE241G ERα plasmid mutant that lacks a nuclear translocation signal (NLS) (Ä250-303) (kindly provided by Dr. P. Chambon, I.G.B.M.C., CNRS-INSERM, ULP, Strasbourg, France). pCMV5myc vector containing the c-DNA encoding dominant negative ERK2 K52R (ERK2-) (gift from Dr M. Cobb, Department of Pharmacology, Southwestern Medical Center, Dallas; Texas).

Cell cultures

Wild-type human breast cancer (MCF-7) cells were a gift from E. Surmacz (Philadelphia, USA). Human uterin cervix adenocarcinoma (HeLa) cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). MCF-7 and HeLa cells were maintained in DMEM/F12 containing 5% CS, 1% L-Glutamine, 1% Eagle’s non essential amino acids and 1 mg/ml penicillin-streptomycin. Cells were cultured in Phenol red-free DMEM containing 5% charcoal-stripped FCS (CS-FCS), 0.5% BSA and 2 mM L-Glutamine (PRF-SFM), for 24 hrs before each experiment.

Immunocytochemical staining

Paraformaldehyde fixed MCF-7 and HeLa cells (2% PFA for 30 min) were used for immunocytochemical staining. Endogenous peroxidase activity was inhibited by hydrogen peroxide (3% in absolute methanol for 30 min) and non specific sites were blocked by normal horse serum (10% for 30 min). ERα immunostaining was then performed using as primary antibody a mouse monoclonal antiserum (1:40, overnight at
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4°C), while a biotinylated horse-anti-mouse IgG (1-600, for 1 hr at RT) was utilized as secondary antibody. Avidin-biotin-horseradish peroxidase complex (ABC complex/HRP) was applied (30 min) and the chromogen 3-3’-di-aminobenzidine tetrachloride dihydrate was used as detection system (5 min). TBS-T (0.05 M Tris-HCl plus 0.15 M NaCl, pH 7.6 containing 0.05%-Triton X-100) served as washing buffer. The primary antibody was replaced by normal mouse serum at the same concentration in control experiments on MCF-7 cultured cells.

RNA isolation

Total cellular RNA was extracted from MCF-7 cells using ‘TRIAZOL Reagent’ as suggested by the manufacturer. The purity and integrity of the RNA were checked spectroscopically and by gel electrophoresis before carrying out the analytical procedures.

RT-PCR assay

The evaluation of ERα and pS2 mRNA expression was performed by semi-quantitative RT-PCR (39).

For ERα, pS2 and the internal control gene 36B4 the primers were: 5’-GTGTACAACTACCGAGG-3’ (ERα forward) and 5’-CAGATTCATCATGCAGGAACCGAATG-3’ (ERα reverse), 5’-TTCTATCCTAATACCAGTGACG-3’ (pS2 forward) and 5’-TTTGAGTAGTCAAAGTCAGACG-3’ (pS2 reverse), and 5’-CTCAACATCTCCCCCTTCTC-3’ (36B4 forward) and 5’-CAAATCCCATATCCGTGTCGT-3’ (36B4 reverse) to yield products of 1172 bp, 210 bp and 408 bp, with 20, 15 and 15 PCR cycles respectively.
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Western blot analysis

MCF-7 cells were grown in 100 mm dishes up to 70-80% confluence and then lysed. Protein lysates were obtained with a buffer containing 50mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl2, 1mM EGTA, 10% glycerol, 1% Triton X-100, a mixture of protease inhibitors (Aprotinin, PMSF and Na-orthovanadate). Equal amounts of total protein were resolved on a 11% SDS-polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane, probed with the antibodies F-10 against ERα or β-actin. The antigen-antibody complex was detected by incubation of the membrane at room temperature with a peroxidase-coupled goat anti-mouse IgG and revealed using the ECL System.

Transfection assay

MCF-7 cells were transferred into 24-well plates with 500 µl of regular growth medium/well the day before transfection. The medium was replaced with DMEM lacking phenol red as well as serum on the day of transfection, which was performed using the FuGENE 6 Reagent as recommended by the manufacturer with the mixture containing 0.5 µg of reporter plasmid XETL.

A set of experiments was performed cotransfecting XETL and pCMV5myc vector containing the cDNA encoding dominant negative ERK2 K52R (ERK2-) (0.5 µg/well).

HeLa cells were cotransfected with XETL, HEGO and ERK2- (0.5 µg/well).

Another set of experiments was carried out by using 0.5 µg/well of pSG5/HE15, pSG5/HE19, S104/106/118A-ER and HE241G plasmids.
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Six hours after transfection, the medium was changed and the cells were treated in DMEM-F12 in the presence of leptin 100 ng/ml and 1000 ng/ml or estradiol (E₂) 100nM for 48 hours. 10 µM of the pure antiestrogen ICI 182,780 was used.

In another set of experiments, after transfection, we added MAPK inhibitor PD 98059 (50 µM) overnight in the medium before starting the treatment with leptin.

TK renilla luciferase plasmid (25 ng/well) was used to normalize the efficiency of the transfection. Firefly and renilla luciferase activities were measured using Dual Luciferase Kit. The firefly luciferase data for each sample were normalized on the basis of transfection efficiency measured by renilla luciferase activity.

Gel mobility shift assay

Nuclear extracts were prepared from MCF-7 as previously described (40). Briefly, MCF-7 cells plated into 60 mm dishes were scraped into 1.5 ml of cold phosphate-buffered saline (PBS). Cells were pelleted for 10 seconds and resuspended in 400 µl cold buffer A (10 mM HEPES-KOH pH 7.9 at 4°C, 1.5mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) by flicking the tube. The cells were allowed to swell on ice for 10 minutes and then vortexed for 10 seconds. Samples were then centrifuged for 10 seconds and the supernatant fraction discarded. The pellet was resuspended in 50 µl of cold Buffer B (20 mM HEPES-KOH pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM PMSF, 1 mM leupeptin) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 minutes at 4°C and the supernatant fraction (containing DNA binding proteins) was stored at −70°C. The yield was determined by Bradford method (41). The probe was generated by annealing single stranded oligonucleotides and
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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 competitor is the following (the nucleotide motif of interest is underlined) 5’-TCCCCCTGCAAGGTCAC GGTGGCCACCCCGTG-3’. Oligonucleotides were synthesized by Sigma Genosys. 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% glicerol, 1 mg/ml BSA, 50 µg/ml poli dI/dC) with 50000 cpm of labeled probe, 20 µg of MCF-7 nuclear protein and 5 µg of poly (dI-dC). The above-mentioned mixture was incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotide. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25 X Tris borate-EDTA for 3 h at 150 V. Gel was dried and subjected to autoradiography at –70° C.

Statistical Analysis

Each datum point represents the mean ± S.E. of three different experiments. Data were analyzed by ANOVA test using the STATPAC computer program.
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Results

Leptin modulates ERα nuclear immunoreactivity in MCF-7 cells.

It is well documented that ERα is predominantly localized in the nucleus (42, 43, 44) and upon ligand activation, undergoes conformational changes leading to homodimerization, and target gene regulation (45).

To provide evidence that leptin is able to modulate ERα nuclear localization in MCF-7 cells we did perform two sets of immunostaining experiments using different culture conditions.

Figure 1 shows that in MCF-7 cells, maintained in medium without serum for 24 hrs, ERα immunoreactivity was well detectable in the nuclear compartment (Fig 1A) and downregulated in cells treated for 24 hrs with 100 nM of E2 (Fig 1B) and 1000 ng/ml of leptin (Fig 1C).

In the other set of experiments, MCF-7 cells were cultured in serum deprivation conditions for 96 hrs (Fig 2). We observed that ERα immunoreactivity was not longer detectable in the control (Fig 2A), while, in the same experimental conditions, the treatment with either E2 (Fig 2B) or leptin (Fig 2C) for 24 hrs induced a strong ERα immunoreactivity in the nuclear compartment.

No immunoreactivity was observed either by replacing the anti-ERα antibody by irrelevant mouse IgG (small squares in Fig. 1 and 2) or by using the primary antibody preabsorbed with an excess of receptor protein (data not shown).

Leptin downregulates ERα expression.
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E2 is known to downregulate the levels of ERα in breast cancer cell line through an increased turnover of the E2-activated ERα protein and a reduced transcription rate of its own gene (46). This downregulation represents an additional hallmark of ERα activation by an agonist. To evaluate if leptin may exhibit a like-estrogen action, we investigated the downregulatory effects of ERα mRNA and total protein levels in MCF-7 cells. A treatment of 24 hrs with either 1000 ng/ml of leptin or E2 100 nM displayed in both circumstances a similar pattern of response consistent with a down-regulation of both ERα mRNA (Fig 3A and B) and protein content (Fig 3C and D). ERα mRNA levels were compared by semiquantitative RT-PCR and standardized on the mRNA levels of the house-keeping gene 36B4 (Fig 3A and B).

Leptin upregulates pS2 mRNA.

To provide further evidence about the ability of leptin to activate per se ERα we investigated upon leptin exposure the expression of a classic estrogen-dependent gene, like pS2. We observed, by RT-PCR, in MCF-7 cells treated with 1000 ng/ml of leptin for 24 hrs, a strong increase of pS2 mRNA which was inhibited by the addition of the pure antiestrogen ICI 182,780 (Fig 4A and B).

Leptin induces functional activation of ERα in MCF-7 and HeLa cells.

In order to corroborate the specificity of leptin to transactivate the endogenous ERα, we transiently transfected MCF-7 cells with the gene reporter XETL, which carries firefly luciferase sequences under the control of an estrogen response element upstream of the thymidine kinase promoter. A significant enhancement of XETL expression was observed in the transfected cells exposed to 1000 ng/ml of leptin for 48 hrs (p<0.01) (Fig
Leptin induces ERα transactivation in MCF-7 cells. Similar results were obtained in estrogen receptor negative HeLa cells cotransfected with HEGO and XETL plasmids tested in the same experimental conditions (Fig 5). Remarkably the antiestrogen ICI 182,780 was shown to efficiently antagonize the stimulatory effect of leptin on ERα regulated transactivation in MCF-7 and HeLa cells (Fig 5).

Leptin is able, via JAK2, to activate the ras dependent MAPkinase pathway. Thus, a potential role of ERK1/ERK2 pathway in mediating the stimulatory effects of leptin on ERα has been reasonably investigated since MAPK signal is generally involved in enhancing ERα functional activation in a ligand-independent manner. In the presence of MAPK inhibitor PD 98059 or in the cells transiently transfected with ERK2 dominant negative plasmid in MCF-7 and HeLa cells, the upregulatory effects induced by leptin on XETL luciferase activity through ERα activation were completely abrogated (Fig 6).

Leptin increases ERα transcriptional activation through AF-1 domain.

In order to specify which functional domain of ERα was mainly involved in ERα transactivation, HeLa cells were cotransfected with XETL reporter gene and PSG5/HE15 or PSG5/HE19 plasmids codifying for AF-1 and AF-2 domains, respectively. The treatment with 1000 ng/ml of leptin for 48 hrs induced an increased transcriptional activation only in transfected cells bearing the plasmid codifying for AF-1 domain (p<0.01) (Fig 7 B).

Our results clearly demonstrate that the N-terminal AF-1, but not the C-terminal AF-2, is necessary for the leptin response.
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Leptin is unable to induce a transcriptional activation of ERα mutated in Ser 104/106/118 or lacking of nuclear localization sequence.

ERα is predominately phosphorylated on Ser-118 and to a lesser extent on Ser-104 and Ser-106. These serine residues, which are effectors of ERK1/ERK2 signal, are all located within the AF-1 region of the N-terminal domain of ERα (47). To confirm the involvement of AF-1 domain in the activation of ERα by leptin and to demonstrate that the activation of ERα occurs at the genomic level, HeLa cells were cotransfected with XETL and either HEGO or S104/106/118A-ER or HE241G. As shown in Fig 7C, we observed how in transfectants with mutants 1000 ng/ml of leptin for 48 hrs was no longer able to elicit any substantial activation on ERE luciferase signal as compared to the cells bearing wild-type ERα.

Leptin amplifies ERα activation by estradiol.

It is worth to note how in HeLa cells expressing ectopically ERα the combined treatment of E₂ and leptin synergized in upregulating transactivation of ERα (Fig 8). In the presence of ERα mutated in serine residues 104/106/118, the upregulatory effects on ERα activation by E₂ still persisted, while the potentiating effect induced by the combined presence of E₂ and leptin was no longer noticeable (Fig 8).

Effect of in vitro leptin treatment on ERE DNA binding activity in MCF-7 cells.

The results obtained with the functional studies were corroborated by electrophoresis mobility shift (EMSA). Nuclear extracts from MCF-7 cells were analyzed by EMSA using a 32 base pair DNA probe containing an estrogen-responsive element
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(ERE) from the human pS2 gene. In the EMSA shown in Fig 9 we obtained a specific protein-DNA complex using nuclear extracts prepared from MCF-7 cells (lane 1). The formation of this complex was abolished by the addition of a 200-fold molar excess of non-radiolabeled probe (lane 2). A treatment of 48 hrs with 1000 ng/ml of leptin or E$_2$ 100 nM induced a strong increase in ERE DNA binding activity (lane 3 and 4) which was reversed in the presence of the pure antiestrogen ICI 182,780 when compared with basal level (lane 5).

Using nuclear extracts from MCF-7 cells either treated with MAPK inhibitor PD 98059 or transiently transfected with ERK2 dominant negative, the ERE DNA binding activity induced by leptin treatment was drastically reduced (lanes 6 and 7).
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Discussion

An association between breast cancer and obesity has been recognized for at least 40 years, even though the mechanism underlying such relationship remains to be fully elucidated (16). Although in situ estrogen production by adipocytes has been considered an important risk factor for breast cancer progression (16-18), an additional candidate which may play a major role in the same scenario is leptin. Leptin is a hormone with multiple biological actions which is produced predominantly by adipose tissue and is present at high concentrations in obese women who are exposed to a higher risk in developing breast cancer (1-3). In the same vein several actions of leptin including the stimulation of normal and tumor cell growth, migration and invasion, and enhancement of angiogenesis, suggest that this hormone is involved in breast cancer progression (5-11, 48). This assumption is sustained by recent findings that show how normal mammary gland morphogenesis is impaired in both nontransgenic genetically obese leptin-deficient and genetically obese leptin receptor deficient mice (25). Similar results were obtained for transgenic TGF-α/lepob lepob mice which did not develop mammary tumors in contrast to the transgenic TGF-α mice that were either homozygous lep+ lep+ or heterozygous lep+ lepob lean for the leptin gene that exhibited mammary tumor incidence rates of 50% and 60% respectively by 24 months of age (25). All these findings seem to underscore an important role for leptin on mammary gland development and mammary gland tumorogenesis giving new interpretative clues to understand the epidemiological relationship between obesity and breast carcinogenesis.

The two isoforms of leptin receptor are both present in different breast cancer cell lines (11, 14, 15). The short and long isoforms induce the activation of one or more members of the Janus (or JAK) family of tyrosine kinases which form a complex with the
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cytokine receptor subunits, thereby inducing autophosphorylation as well as phosphorylation of the receptor. These phosphorylated tyrosines form binding sites for various signaling molecules, which are themselves thought to be phosphorylated by JAK kinases like STAT proteins. The same phosphorylated tyrosine sites bind SHP2 protein containing phosphatase. SHP2 is proposed as a positive regulator of leptin signaling through MAPK activation by the recruitment of the adapter protein growth receptor bound 2 (Grb-2) and the activation of the Ras/Raf pathway. A secondary pathway for leptin induced MAPK signaling is mediated directly via JAK2 (49).

Stemming from the evidence that MAPK signal induces the functional activation of unliganded ERα (34, 37) it was reasonable to investigate the potential role of leptin in stimulating ERα. In the present study we have demonstrated for the first time that leptin was able to induce functional transactivation of ERα that was abrogated in the presence of either MAPK inhibitor or ERK2 dominant negative. This addresses a crucial role of MAPK signal to stimulate ERα upon leptin exposure. In different breast cancer cell lines, it has been demonstrated how the interaction between insulin/IGF-1 and estradiol signaling occurs also through the direct transcription activation of ERα via MAPK (34).

In this concern, it is well documented that the human ERα is phosphorylated by ERK on Ser^{118} (34, 37, 50). The phosphorylation of this serine is required for full activity of the ERα AF-1 domain. Overexpression of active ERK kinase (MEK) or the active p21^{ras}, resulting in the ERK1/ERK2 activation, enhances estrogen-induced transcriptional activity of the wild type ERα, but not of a mutant ERα with an alanine in place of Ser^{118} (34).
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All these observations fit with our data demonstrating that: 1) only the construct bearing N-terminal domain (AF-1) was able to activate ERE reporter gene; 2) ERα mutated in serine residues 104/106/118 is no longer stimulated by leptin and it is still transactivated by E2 even though in a lesser extent with respect to wild type. This finding recalls a recent report (51) evidencing how the full activation of liganded receptor requires the integrity of phosphorylated serine residues 104/106/118. Besides, it has been demonstrated, by the same authors, how the mutation of serine 104/106/118 affects the physical and functional interaction of full length ERα with p160/SRC and CBP in the absence of ligand. Thus, it is reasonable to assume how the ERα mutated in Ser104/106/118 being unable to recruit cofactor fails to activate cell transcription machinery upon leptin stimulation.

It emerges from recent findings that in human vascular smooth muscle cells MAPKinase activation per se results in a nuclear translocation of ERα which supports how the MAPK-mediated phosphorylation of ERα is a prerequisite for its nuclear localization (50). Thus the sustained MAPKinase activation induced by leptin may explain why even after prolonged incubation of MCF-7 cells in serum free medium, leptin treatment for 24 hrs is able to induce ERα nuclear localization as revealed by our immunostaining data, while ERα immunoreactivity was just scantily detectable in untreated cells.

This finding appears to be not related to the cell type specificity since were reproduced in HeLa cells where leptin was able to induce ERα wild type nuclear localization but failed to do that in the presence of ERα lacking nuclear localization signal (NLS) (data not show).
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Therefore, in MCF-7 cells, concomitantly with leptin treatment, the classic biological features of ER\(\alpha\) wild type functional transactivation were observed: 1) a nuclear compartmentalization of ER\(\alpha\); 2) the downregulation of its mRNA and total protein content; 3) the upregulation of a classic estrogen dependent gene like pS2 which was inhibited by the pure antiestrogen ICI 182, 780.

These results well fit with EMSA findings. In this assay using \(^{32}\)P ERE sequence of pS2 promoter in the presence of nuclear extracts from MCF-7 cells, we observed that upon leptin treatment a strong increase in DNA binding occurred in the same extent of that induced by estradiol, and was markedly reduced in the presence of either MAPK inhibitor or ERK2 dominant negative.

These data broaden furthermore the potential relationship existing between leptin and estrogens. Indeed it has been reported that estrogens appear to modulate leptin gene expression in adipose tissue (22, 23). On the other hand, we recently reported for the first time, how leptin is able to induce the aromatase gene expression in MCF-7 cells via AP-1 (21) addressing unconfutable how leptin may be involved in the pathophysiology of breast modulating in situ estrogen production also in epithelial cells.

Now we are able to demonstrate that the potential amplification of estrogens signal induced by leptin has two active components: 1) the enhanced aromatase activity, and 2) a direct activation of ER\(\alpha\) in the absence of the natural ligand. Besides, the potentiating effects of leptin on E\(_2\)-induced activation of ER\(\alpha\) addresses how different functional domains as effectors of two distinct signals may cooperate in a synergistic way. This gives a great emphasis to the role of leptin in promoting breast cancer in obese women through the potential use of readily aromatizable androgens in breast tissue.
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enhancing in situ estradiol production together with its ability to activate directly ERα in epithelial breast cancer.

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References


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Legend of figures

Fig. 1 Leptin downregulates ERα expression in MCF-7 cells.
MCF-7 cells were incubated in serum-free medium for 24 hrs (A-C) and then treated with vehicle (A) or 100 nM of E2 (B) or 1000 ng/ml of leptin (C) for 24 hrs. No immunodetection was observed replacing the anti-ERα antibody with an irrelevant mouse IgG (small squares). Each experiment is representative of at least 10 tests. Scale bars: 5 μm.

Fig 2 ERα nuclear signaling induced by leptin in long term deprived MCF-7 cell line.
MCF-7 cell line was incubated in serum-free medium for 96 hrs (A-C) and then treated with vehicle (A) or 100 nM of E2 (B) or 1000 ng/ml of leptin (C) for 24 hrs. No immunodetection was observed replacing the anti-ERα antibody with an irrelevant mouse IgG (small squares). Each experiment is representative of at least 10 tests. Scale bars: 5 μm.

Fig. 3 Effect of leptin on ERα mRNA and protein levels.
A, semiquantitative RT-PCR of ERα mRNA. MCF-7 cells were stimulated for 24 hrs with E2 (100 nM) or leptin (1000 ng/ml); 36B4 mRNA levels were determined as a control. C, immunoblot of ERα from MCF-7 cells treated with E2 (100 nM) or leptin (1000 ng/ml) for 24 hrs; β-Actin serves as loading control. B and D, the histograms represent the mean ± S.E. of three separate experiments in which bands intensity were
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evaluated in term of optical density arbitrary units and expressed as percentage of the control assumed as 100%. * = p<0.05 compared with control; ** = p<0.01 compared with control.

Fig 4 Leptin up-regulates pS2 mRNA.
A, semiquantitative RT-PCR of pS2 mRNA. MCF-7 cells were treated in the absence (Control) or in the presence of E2 (100 nM) or leptin (1000 ng/ml) for 24 hrs. The pure antiestrogen ICI 182,780 (10 μM) was used. 36B4 mRNA levels were determined as a control. B, the histograms represent the mean ± S.E. of three separate experiments in which bands intensity were evaluated in term of optical density arbitrary units and expressed as percentage of the control assumed as 100%. * = p<0.01 compared with control;  = p<0.01 compared with E2-treated samples;  = p<0.01 compared with leptin-treated samples.

Fig. 5 Leptin activates ERα in MCF-7 and HeLa cells.
MCF-7 cells were transfected with the luciferase reporter plasmid XETL. HeLa cells were cotransfected with XETL and HEGO plasmids. The cells were treated in the absence (Control) or in the presence of 100 and 1000 ng/ml of leptin (Lep) for 48 hrs. 10 μM of the pure antiestrogen ICI 182,780 was used. The values represent the means ± SE of 3 different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. * = p<0.01 compared with control.

Fig. 6 MAPKinase signal is involed in the activation of ERα.
Leptin induces ER\(\alpha\) transactivation in MCF-7 cells.

MCF-7(A) and HeLa (B) cells were transiently transfected with XETL or cotransfected with XETL and HEGO respectively. The cells were serum-starved overnight with or without PD 98059 (PD) and were untransfected or transiently transfected with ERK2 dominant negative plasmid, and then were treated for 48 hrs in the presence or absence of leptin (1000 ng/ml). The values represent the means ± SE of 3 different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. * = p<0.01 compared with control.

Fig. 7 Effects of le

A, schematic illustration of ER\(\alpha\) constructs. HEGO is a wild type ER\(\alpha\)-expressing vector that encodes a 595 amino acid protein. HE15 (1-282) contains AF-1 and the DNA binding domain (DBD). HE19 (179-595) contains DBD and AF-2 domains. S104/106/118A-ER plasmid is mutated in serine residues 104, 106, 118 to Ala. HE241G encodes a mutated ER\(\alpha\) which has the nuclear translocation signal (NLS) deleted (Δ 250-303).

B, HeLa cells were transiently cotransfected with XETL and either HEGO or PSG5/HE15 or PSG5/HE19 plasmids.

C, HeLa cells were transiently cotransfected with XETL and either HEGO or S104/106/118A-ER or HE241G.

The cells were treated for 48 hrs in the absence (Control) or in the presence of leptin (1000 ng/ml). The values represent the means ± SE of 3 different experiments. In each experiment, the activities of the transfected plasmids were assayed in triplicate transfections. * = p<0.01 compared with control.
Leptin induces ERα transactivation in MCF-7 cells.

**Fig 8 Leptin amplifies ERα activation by estradiol.**

HeLa cells were transiently cotransfected with XETL and either HEGO or S104/106/118A-ER plasmids.

The cells were treated for 48 hrs in the absence (Control) or in the presence of leptin (1000ng/ml) or E₂ (100 nM) or leptin and E₂. The values represent the means ± SE of 3 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 E₂-treated samples.

**Fig. 9 Effects of in vitro leptin treatment on ERE DNA binding activity in MCF-7 cells.**

Nuclear extracts from MCF-7 cells were incubated with a double-stranded ERE-specific consensus sequence probe labeled with [γ-32P] ATP and subject to electrophoresis in a 6% polyacrilamide gel (lane 1). Competition experiments were performed by adding as competitor a 200-fold molar excess of unlabeled probe (lane 2). MCF-7 nuclear extracts treated with 100 nM of E₂ or 1000 ng/ml of leptin (lep) for 48 hrs incubated with probe is shown in lane 3 and 4. The pure antiestrogen ICI 182,780 (10 iM) (lane 5) was added in leptin-treated MCF-7 nuclear extracts. MCF-7 cells were serum-starved overnight with PD 98059 (lane 6) or transiently transfected with ERK2 dominant negative (lane 7) and then treated for 48 hrs with leptin.
Leptin induces ERα transactivation in MCF-7 cells.
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Leptin induces ERα transactivation in MCF-7 cells.

**Fig. 6**

![Graphs showing the effect of Leptin on ERα transactivation in MCF-7 and HeLa cells.](image-url)
Leptin induces ERα transactivation in MCF-7 cells.
Leptin induces ERα transactivation in MCF-7 cells.

**Fig. 8**
Leptin induces ERα transactivation in MCF-7 cells.

Fig. 9

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Leptin induces ERα transactivation in MCF-7 cells.
Leptin induces, via ERK1/ERK2 signal, functional activation of estrogen receptor alpha (ERα) in MCF-7 cells
Stefania Catalano, Loredana Mauro, Stefania Marsico, Cinzia Giordano, Pietro Rizza, Vittoria Rago, Daniela Montanaro, Marcello Maggiolini, Maria Luisa Panno and Sebastiano Ando

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