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Title: Molecular and cellular endocrinology.
Title Abbrev: Mol Cell Endocrinol
Citation: 2001 May 15;176(1-2):67-75
Article: Effects of estrogen on leptin gene promoter activa
Author: ONeil JS; Burow ME; Green AE; McLachlan JA; Henson MC
NLM Unique ID: 7500844 Verify: PubMed
PubMed UI: 11369444
ISSN: 0303-7207 (Print)
Publisher: North Holland Publishing, Limerick :
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Molecular and Cellular Endocrinology 176 (2001) 67–75

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Effects of estrogen on leptin gene promoter activation in MCF-7 breast cancer and JEG-3 choriocarcinoma cells: selective regulation via estrogen receptors α and β☆

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Received 13 December 2000; accepted 20 February 2001

Abstract

Leptin is a potential regulator of conceptus development. We have previously suggested that in primate pregnancy, leptin biosynthesis is regulated by estrogen in a tissue-specific manner. Therefore, the objective of the current study was to determine the mechanism of estrogen action on *LEP* promoter activation in divergent cell types. The effects of estrogen were investigated in estrogen receptor (ER)-positive MCF-7 breast cancer cells and in ER-negative JEG-3 choriocarcinoma cells. Cells were transfected with a leptin-luciferase or an estrogen responsive element (ERE)-luciferase reporter construct, in conjunction with ERα, ERβ, or empty vector expression plasmids. Cells were treated with estradiol and/or the specific estrogen antagonists, ICI-182,780 or 4-hydroxytamoxifen. In MCF-7 cells, estradiol stimulated ($P < 0.05$) ERE-luciferase activity and was inhibited by ICI-182,780, but did not stimulate leptin-luciferase activity. However, leptin-luciferase was stimulated by estradiol ($P < 0.05$) and inhibited by antiestrogens in JEG-3 cells that were co-transfected with ERα. Both antiestrogens stimulated leptin-luciferase activity ($P < 0.05$) in JEG-3 cells co-transfected with ERβ. Results suggested that *LEP* promoter activation may depend upon co-activators present in leptin-producing cells and may be inhibited by repressors present in non-leptin producing cells. Divergent effects of estrogen may be owed to differences in the type of ER (α or β) expressed in target tissues.

Keywords: Leptin; Estrogen; Antiestrogens; MCF-7 (breast cancer cells); JEG-3 (choriocarcinoma cells)

1. Introduction

Leptin is a hormone product of the *LEP* (formerly *obese*) gene, that is of potential importance to body weight regulation in many species, including humans (Mantzoros, 1999) and non-human primates (Tang-Christensen et al., 1999). Predominantly produced by adipose tissue, leptin is secreted into the circulation in amounts correlated with adipose mass and communicates the status of somatic energy reserves to the brain via specific hypothalamic receptors (Tartaglia, 1997). In addition to its known role in energy homeostasis, the production of both leptin (Senaris et al., 1997; Henson et al., 1998, 1999) and its receptor (Henson et al., 1998; Green et al., 2000) by the primate placental syncytiotrophoblast and the correlation between umbilical cord leptin and conceptus size imply that it may also regulate conceptus growth or placental function (Henson and Castracane, 2000). Enhanced maternal leptin con-

☆ This work was presented in part at the 48th annual meeting of the Society for Gynecologic Investigation; Toronto, Ontario, Canada

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0303-7207/01/$ - see front matter
PII: S0303-7207(01)00473-7

centrations early in gestation, prior to significant increases in adiposity, indicate that factors other than simple changes in adipose mass influence leptin levels during human gestation (Highman et al., 1998). To this end, estrogen has been reported to stimulate leptin production (Slieker et al., 1996; Brann et al., 1999) through transcriptional activation of the *LEP* gene (Machinal et al., 1999). Pregnancy-associated, maternal hyperleptinemia has been linked to a potential increase in leptin production by maternal adipose tissue under the influence of elevated estrogen levels typical of primate pregnancy (Sivan et al., 1998). Clearly, a better understanding of the specific mechanisms regulating leptin biosynthesis is requisite to elucidating its physiological relevance during pregnancy.

Recently, we investigated the effects of estrogen on leptin production during primate pregnancy in the baboon (O'Neil et al., 2001), an established model for the study of human pregnancy (Henson, 1998). Leptin production was enhanced in maternal adipose tissue during pregnancy, and increased further with advancing gestation, commensurate with the rise in maternal serum estrogen levels typical of primate pregnancy. To further examine estrogen's role in leptin biosynthesis, maternal serum estrogen levels were reduced via fetectomy, the surgical removal of the fetus at midgestation. Because the primate placenta relies upon fetal androgen precursors for optimal estrogen biosynthesis, removal of the fetus dramatically reduces serum estrogen concentrations (Albrecht et al., 1991; Henson, 1998). In fetectomized baboons, *LEP* mRNA transcript abundance and leptin protein levels were reduced in maternal adipose tissue and enhanced in placental villous tissue, suggesting that estrogen affects leptin production in a tissue-specific manner. Certainly, a precedent exists in this regard since regulation of *LEP* transcription varies by cell type (Bi et al., 1997; Yura et al., 1998), suggesting that cell-specific regulatory mechanisms are involved in leptin biosynthesis.

Although fetectomy has been extensively used to examine the specific effects of estrogen during pregnancy, decreases in other gestational hormones and the potential effects of undetermined fetal endocrine contributions cannot be dismissed. Therefore, to elucidate the specific effects of estrogen and determine its molecular mechanism of action, we employed the luciferase reporter gene system to directly investigate *LEP* promoter activation. Because estrogen responsive element (ERE) consensus sequences have been identified in the *LEP* promoter region (Machinal et al., 1999), we hypothesized that estrogen could activate the *LEP* promoter and that this activation would be inhibited by an estrogen receptor antagonist. Thus, we utilized estrogen receptor (ER) positive MCF-7 breast cancer cells (Levenson and Jordan, 1997) and JEG-3 choriocarcinoma cells, which normally produce leptin, to examine activation of the *LEP* promoter in divergent cell types.

2. Materials and methods

2.1. Cells and plasmid constructs

MCF-7 (N variant, passage 52) breast cancer cells were maintained as previously described (Burow et al., 1999) in 90% Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Grand Island, NY) plus 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) in a 37°C incubator with 5% CO_2. Media was changed every 2–3 days and cells were subcultured into charcoal stripped FBS (HyClone Laboratories Inc., Logan, UT) 2–3 days prior to experiments. JEG-3 cells (American Tissue Culture Collection, Manassas, VA) were initiated from frozen stock (# 984725) by rapid thaw in a 37°C water bath. Cells were maintained in 90% DMEM plus 10% FBS (Sigma) in a 37°C incubator with 10% CO_2. Media was changed every 2–3 days and cells were subcultured into charcoal stripped FBS (HyClone) 2–3 days prior to experiments.

The ERE-luciferase plasmid, pERE-2-luciferase (Burow et al., 1999), the human ERα plasmid construct, pSG5-ERα (Ignar-Trowbridge et al., 1993), and the human ERβ plasmid construct, pSG5-ERβ (Collins-Burow et al., 2000), have been described. The pSG5-ERβ construct was generously provided by Jan-Ake Gustafsson, M.D., Ph.D. (Karolinska Institute, Stockholm, Sweden). The leptin-luciferase plasmid, p1774, was generously provided by Marc Reitman, Ph.D. (Diabetes Branch, NIDDK, National Institutes of Health, Bethesda, MD). The luciferase reporter construct was based on the pGL3-basic (Promega, Madison, WI) construct and contains the promoter region of the *LEP* gene. The construction of p1774 has been reported (Gong et al., 1996). The ERE and AP1 consensus sequences were identified within the *LEP* promoter utilizing the MatInspector V2.2 computer program (Quandt et al., 1995).

2.2. Transient cell transfection

MCF-7 cells were split into 12-well culture plates and transfected with 3 μl of LipofectAMINE (Life Technologies) and 1 μg p1774 reporter plasmid, or 1 μg ERE-luciferase reporter plasmid, per well. Briefly, DNA and lipid were coincubated in 50-ml conical polystyrene tubes with DMEM (no supplements) for 30 min at room temperature. Culture media was aspirated from cells and 1 ml/well of transfection media was applied. JEG-3 choriocarcinoma cells were split into 12-well culture plates and transfected with 5 μl of LipofectAMINE (Life Technologies) and 1 μg p1774

reporter plasmid or 1 μg ERE-luciferase reporter plasmid, 500 ng human PGS5-ERα or 500 ng human PSG5-ERβ, per well. DNA and lipid were coincubated in 50-ml conical polystyrene tubes with DMEM (no supplements) for 30 min at room temperature. Culture media was aspirated from cells and 1 ml/well of transfection media was applied. The following control experiments were also performed: (1) transfection with 1 μg ERE-luciferase and 500 ng pCDNA3.1 vector demonstrated that JEG-3 cells are not estrogen responsive without co-transfection of an estrogen receptor; and (2) transfection with pCMV-β-galactosidase served as a transfection efficiency control.

2.3. Treatments

Approximately 4 h post-transfection, transfection media was removed by aspiration and 1 ml of charcoal stripped media was added per well. MCF-7 and JEG-3 cells were treated with 20 ng/ml phorbal myristate acetate (PMA), 1 nM or 1 μM 17β-estradiol (E_2), 100 nm ICI 182,780 (Zeneca; Cheshire, UK) and 100 nM ICI-182,780 + 1 nM E_2. JEG-3 choriocarcinoma cells were further treated with 100 nM 4-hydroxytamoxifen and 100 nM 4-hydroxytamoxifen + 1 nM E_2. Treatments were applied in triplicate wells, and experiments performed in three separate cultures.

2.4. Luciferase assay

Culture media was removed by aspiration and cells were treated with 200 μl Reporter Lysis Buffer (Promega), diluted 1:4 with water and incubated with shaking for 30 min at room temperature. Following lysis, cells and lysis buffer were transferred to 1.5-ml microfuge tubes and cell debris pelleted by centrifugation at $12\,000 \times g$ for 2 min. Thirty microliters of cleared supernatant was pipetted into luminometer cuvettes (PharMingen, Becton Dickinson, San Diego, CA) and luciferase activity was measured with Luciferase Assay Substrate (Promega) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Relative luciferase units were normalized to control.

2.5. RNA extraction and RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Life Technologies), according to Chomczynski and Sacchi (1997) and Chirgwin et al. (1979) as adapted in our laboratory (Henson et al., 1998). All samples were treated with DNase (Life Technologies) to eliminate DNA contamination and reprecipitated with sodium acetate and 100% ethanol. Oligonucleotide primers were synthesized (Midland Reagent Company, Midland TX) for *LEP*, ERα, ERβ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Sequence analysis of PCR products (Biotech Core, Palo Alto, CA) confirmed that primers specifically amplified regions of *LEP* complimentary DNAs (cDNA). cDNAs were synthesized from 2 μg total RNA using SuperScript Kit (Life Technologies) and PCR was performed in a Temp-Tronic Thermocycler (Barnstead/Thermolyne, Dubuque, IA), as we have previously described (Henson et al., 1999).

Conditions for PCR were as follows: *LEP* (30 cycles for amplification, denaturation at 94°C for 60 s, annealing at 55°C for 30 s, extension at 72°C for 90 s) (Henson et al., 1998), ERα (35 cycles for amplification, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s) (Speirs et al., 1999), ERβ (40 cycles for amplification, denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 70°C for 60 s) (Pedeutour et al., 1998), GAPDH (24 cycles for amplification, denaturation at 94°C for 30 s, annealing at 58°C for 60 s, extension at 72°C for 60 s) (Henson et al., 1998). PCR products were visualized under UV light on 2% agarose gels with ethidium bromide. PCR reactions were accompanied by the following controls: (1) constitutively expressed GAPDH affirmed consistent cDNA synthesis; (2) sterile water blanks served as a reagent control; and (3) RNA that had not been transcribed into cDNA was used as a control for DNA contamination.

2.6. Statistical analyses

One-sample, one way *t*-tests were performed to establish statistical significance between groups. Significant differences were understood to exist when $P < 0.05$.

3. Results

The expression of *LEP*, ERα and ERβ mRNA transcripts in cultured MCF-7 breast cancer cells and JEG-3 choriocarcinoma cells was determined by RT-PCR.

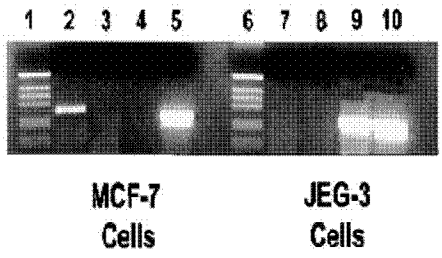

Fig. 1. Expression of ERα (lanes 2 and 7), ERβ (lanes 3 and 8), *LEP* (lanes 4 and 9) and GADPH (lanes 5 and 10) mRNA in MCF-7 breast cancer cells (lanes 2–5) and JEG-3 choriocarcinoma cells (lanes 7–10). Lanes 1 and 6 are 1-kb DNA ladders.

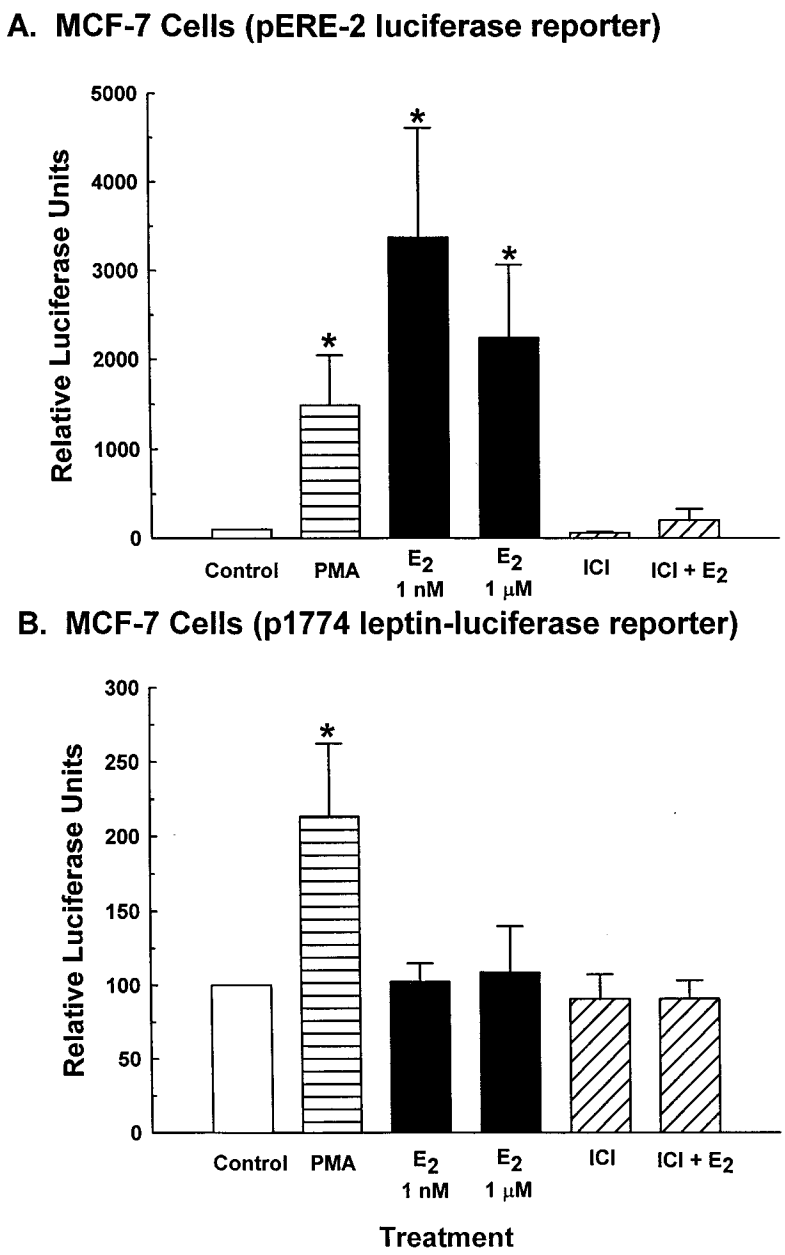

Fig. 2. Relative luciferase activity in MCF-7 cells transfected with the ERE-luciferase (panel A) or leptin-luciferase (panel B) reporter and treated with 20 ng/ml phorbal myristate acetate (PMA), 1 nM or 1 μM 17β-estradiol (E_2), 100 nM ICI-182,780 (ICI) or 100 nM ICI + 1 nM E_2. Values represent the means ± S.E.M. of three separate cultures. *Values are significantly different from control ($P < 0.05$).

As illustrated in Fig. 1, ERα transcripts were expressed in MCF-7 breast cancer cells, although ERβ and *LEP* transcripts were not. Conversely, JEG-3 cells expressed *LEP* mRNA, but were ERα and ERβ negative.

To determine the ability of estrogen to activate the *LEP* promoter, MCF-7 cells were transfected with either pERE-2-luciferase reporter construct or p1774, the leptin-luciferase reporter construct, and the effects of PMA, E_2 and ICI-182,780 were determined. Fig. 2A depicts the effects of PMA, E_2 and the estrogen receptor antagonist, ICI-182,780, on pERE-2-luciferase activity. Each bar represents the relative luciferase units (mean ± S.E.M.) from triplicate determinations from separate cultures ($n = 3$), normalized to control. As expected, E_2 stimulated pERE-2-luciferase activity ($P < 0.05$) and was inhibited by co-incubation with the estrogen receptor antagonist, ICI-182,780. Fig. 2B depicts the effects of PMA, E_2 and ICI-182,780, on p1774 activity. Although the *LEP* promoter was stimulated ($P < 0.05$) by PMA, E_2 and ICI-182,780 had no effect. No differences ($P > 0.05$) existed between estrogen dosages, in respect to elicited effects.

To determine the ability of estrogen to activate the *LEP* promoter within cells capable of *LEP* transcription, JEG-3 choriocarcinoma cells were transfected with pERE-2-luciferase or p1774 and co-transfected with ERα or ERβ to elicit estrogen responsiveness. JEG-3 cells, that were not co-transfected with ER, functioned as negative controls. In contrast to the studies using MCF-7 cells, PMA did not stimulate p1774 activity in JEG-3 cells, suggesting that specific cellular context is a critical component in determining leptin expression within tissue or cell types. Fig. 3A portrays the effects of PMA, E_2, and the estrogen receptor antagonists, ICI-182,780 and 4-hydroxytamoxifen, on luciferase activity in cells co-transfected with pERE-2-luciferase and ERα. E_2 stimulated luciferase activity ($P < 0.05$) and was inhibited by both ICI-182,780 and 4-hydroxyta-

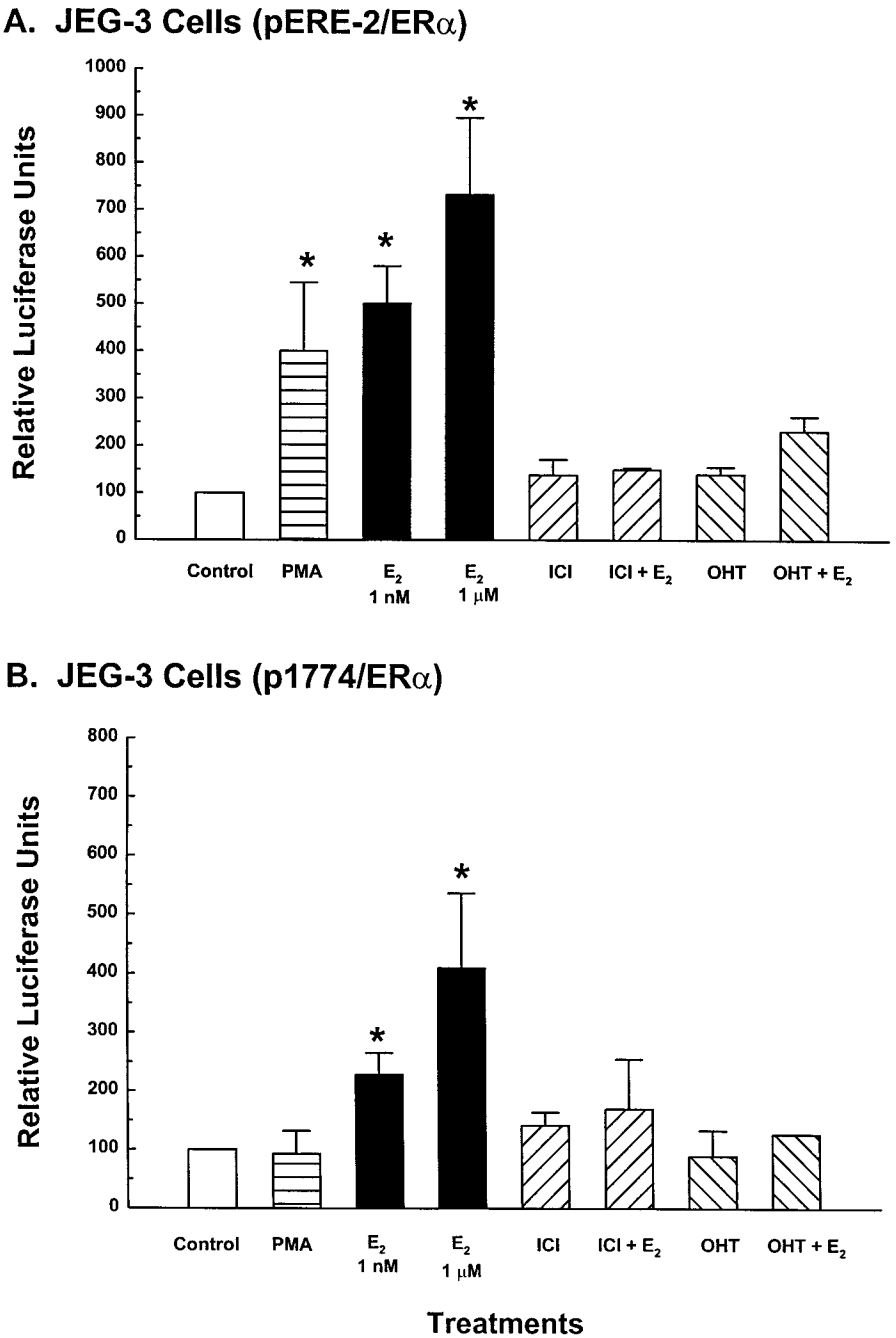

Fig. 3. Relative luciferase activity in JEG-3 cells co-transfected with the ERE-luciferase reporter and ERα (panel A) or leptin-luciferase and ERβ (panel B) and treated with 20 ng/ml PMA, 1 nM or 1 μM E_2, 100 nM ICI-182,780 (ICI), 100 nM ICI + 1 nM E_2, 100 nM 4-hydroxytamoxifen (OHT) or 100 nM OHT + 1 nM E_2. Values represent the means ± S.E.M. of three separate cultures. *Values are significantly different from control ($P < 0.05$).

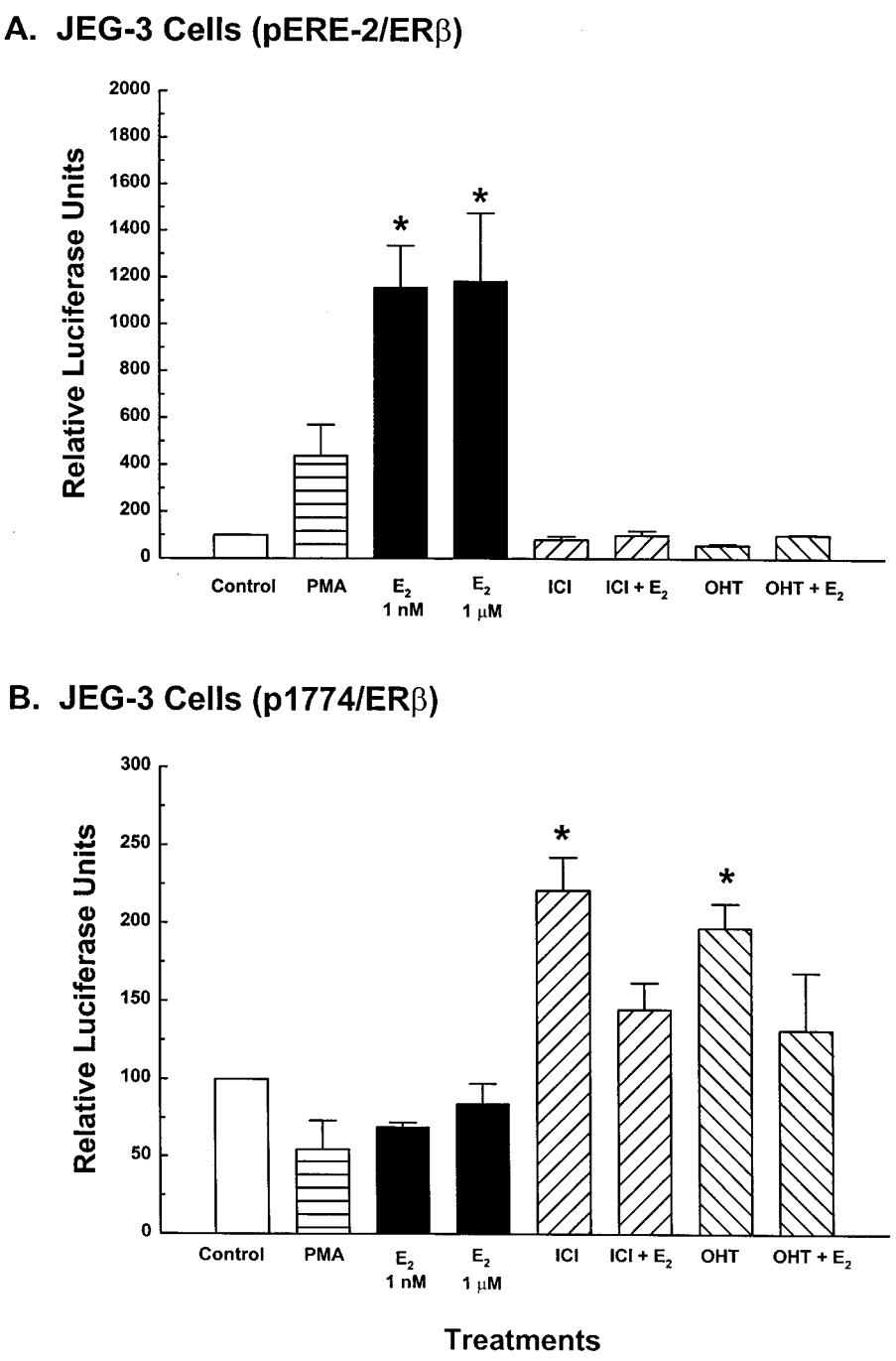

Fig. 4. Relative luciferase activity in JEG-3 cells co-transfected with the ERE-luciferase reporter and ERα (panel A) or leptin-luciferase and ERβ (panel B) and treated with 20 ng/ml PMA, 1 nM or 1 μM E_2, 100 nM ICI-182,780 (ICI), 100 nM ICI + 1 nM E_2, 100 nM 4-hydroxytamoxifen (OHT) or 100 nM OHT + 1 nM E_2. Values represent the means ± S.E.M. of three separate cultures. *Values are significantly different from control ($P < 0.05$).

moxifen. Fig. 3B portrays the effects of PMA, E_2, and the estrogen receptor antagonists, ICI-182,780 and 4-hydroxytamoxifen on cells co-transfected with p1774 and ERβ. E_2 stimulated luciferase activity ($P < 0.05$) and was inhibited by the estrogen receptor antagonists. Fig. 4A depicts the effects of PMA, E_2, and the estrogen receptor antagonists, ICI-182,780 and 4-hydroxytamoxifen on luciferase activity in cells co-transfected with pERE-2-luciferase and ERβ. E_2 stimulated luciferase activity ($P < 0.05$) and was inhibited by both ICI-182,780 and 4-hydroxytamoxifen. Fig. 4B depicts the effects of PMA, E_2, and the estrogen receptor antagonists, ICI-182,780 and 4-hydroxytamoxifen on cells co-transfected with p1774 and ERβ. E_2 did not stimulate luciferase activity. However, both estrogen receptor antagonists activated the *LEP* promoter ($P < 0.05$).

4. Discussion

The current study sought to identify the specific effects of estrogen on leptin biosynthesis. Therefore, the luciferase reporter gene system was utilized to demonstrate estrogen activation of the *LEP* promoter in vitro. Because ERE consensus sequences have been identified in the *LEP* promoter (Machinal et al., 1999), we hypothesized that estrogen may activate the *LEP* promoter and be inhibited by an estrogen receptor antagonist. Thus, we transfected ER positive MCF-7 breast cancer cells, a commonly used estrogen-responsive cell line (Levenson and Jordan, 1997), with either pERE2-luciferase or p1774, a leptin luciferase reporter construct. Transfected cells were treated with E_2 and a specific estrogen receptor antagonist. As postulated, E_2 stimulated luciferase activity in cells transfected with ERE-luciferase and that activity was inhibited by an estrogen receptor antagonist. Conversely, E_2 elicited no response in leptin-luciferase transfected cells, suggesting that consistent with a lack of endogenous *LEP* expression, estrogen does not activate the *LEP* promoter in MCF-7 cells.

As previously discussed, the *LEP* gene is present in all cell types, yet gene expression is highly regulated and occurs only in specific cells. Therefore, we suggest that successful promoter activation and gene transcription may rely on co-activators within leptin producing cells and that the presence of repressors within cell types that do not endogenously produce leptin may inhibit activation of the *LEP* promoter. In the present study, the entire *LEP* promoter was included in the luciferase construct; therefore, all potential activation and/or repressor sites were available. Thus, based on the potential importance of co-activators and repressors in promoter activation, we examined the effects of E_2 on the activation of leptin-luciferase in leptin-producing JEG-3 choriocarcinoma cells. Performing these experiments in leptin-producing cells ensured that co-activators, as well as potentially unknown factors imperative to promoter activation, were present. Because JEG-3 cells do not express ER mRNA (Gehm et al., 2000) it was necessary to co-transfect cells with an ER construct in addition to leptin or ERE-luciferase.

The ER is a member of the nuclear receptor superfamily. Until recently, only one receptor was thought to mediate the effects of estrogens. However, the identification of a second ER (ERα), with a tissue distribution different from that of classical ER (ERβ), may provide an explanation for the selective actions of estrogens within diverse tissues (Kuiper et al., 1996). Therefore, in the current study, we transfected JEG-3 choriocarcinoma cells with ERα or ERβ. As a negative control, we also co-transfected cells with leptin-luciferase or ERE-luciferase and a vector plasmid to ensure that the cells were not estrogen responsive without the addition of ER. In both cases, no luciferase activity was detected. As expected, E_2 treatment stimulated luciferase activity, which was inhibited by the ER antagonists, ICI-182,780 and 4-hydroxytamoxifen, in JEG-3 cells co-transfected with ERE-luciferase and either ERα or ERβ. These results confirmed the ability of JEG-3 cells to become estrogen responsive by transfection with ERα or ERβ and demonstrated the capacity of those receptors to respond appropriately to both E_2 and ER antagonists.

In JEG-3 cells co-transfected with leptin-luciferase and ERα, E_2 stimulated luciferase activity that was inhibited by both ICI-182,780 and 4-hydroxytamoxifen, suggesting that E_2 regulates *LEP* gene expression via promoter activation. Unexpectedly, however, we found that in JEG-3 cells co-transfected with leptin-luciferase and ERβ, E_2 had no effect; although both estrogen receptor antagonists stimulated luciferase activity. Furthermore, this stimulation was reduced by co-treatment with E_2. Although the differential activation of the luciferase construct was unanticipated, other investigators have also reported differential activation of ERα and ERβ at AP1 sites (Paech et al., 1997).

The traditional activation of an ERE involves ligand-bound receptor binding at a specific palindromic sequence of DNA. However, ligand-bound ER also mediates gene transcription from AP1 enhancer elements, when complexed with the AP1 transcription factors Fos and Jun (Umayahara et al., 1994). It is well known that certain estrogen receptor antagonists behave as estrogen-like ligands in specific tissues (Jones et al., 1984). However, it has been demonstrated that although these antagonists inhibit transcription of genes that are regulated by the classical ERE, they activate the transcription of genes under the control of AP1 (Umayahara et al., 1994; Gaub et al., 1990). It has been reported that ERα and ERβ signal in opposite ways when complexed with E_2 at an AP1 site (Paech et al., 1997). Specifically, E_2 stimulated reporter gene activity in HeLa cells transfected with ERα, but antiestrogens stimulated reporter gene activity in cells transfected with ERβ. These findings are consistent with our results in JEG-3 cells, where E_2 stimulated leptin-luciferase through ERα, while 4-hydroxytamoxifen and ICI-182,780 stimulated activity through ERβ. Similarly, Saville et al. (2000) recently demonstrated that the regulation of transcription by Erβ-Sp1 is inhibited by E_2 and stimulated by antiestrogens. These results, combined with those of Zou et al. (1999), using the RARα1 promoter, further confirm our findings of functional differences in transcriptional regulation between estrogen-bound and antiestrogen-bound ERα/β. The *LEP* promoter region contains ERE half sites, as well as AP1 sites. Therefore, it is conceivable that in JEG-3 cells, luciferase activation occurred through the classical ERE pathway via ERα and that AP1 activation occurred through ERβ.

In conclusion, we have demonstrated that estrogen can activate the *LEP* promoter in JEG- 3 cells through ERα and have suggested that regulation of leptin biosynthesis may depend upon a functional ER. In contrast, ERβ activation of the *LEP* promoter, although slightly repressed by E_2, was stimulated by the antiestrogens, 4-hydroxytamoxifen and ICI-182,780. This suggests that the tissue-specific regulation of leptin biosynthesis by estrogen may depend upon the type and amount of ER present in target tissues. The divergent roles of estrogen (through ERα) and antiestrogens (through ERβ) in regulating the *LEP* promoter and potentially, leptin biosynthesis, may have serious implications for patients receiving estrogen-replacement or selective estrogen receptor modulator (SERM) therapy. Collectively, results of the current study demonstrate the molecular mechanism of estrogen activation on the *LEP* promoter and further attest to the potentially tissue-specific nature of mechanisms regulating leptin biosynthesis.

Acknowledgements

The authors wish to thank Dr Jan-Ake Gustafsson (Karolinska Institute, Stockholm, Sweden), for providing us with the pSG5-ERβ plasmid construct and Dr Marc Reitman (Diabetes Branch, NIDDK, National Institutes of Health, Bethesda, MD) for providing us with the p1774 leptin-luciferase plasmid for these studies. We also thank Mrs Nathlynn Dellande for her contributions to manuscript preparation.

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