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Leptin Upregulates β3-Integrin Expression and Interleukin-1β Upregulates Leptin and Leptin Receptor Expression in Human Endometrial Epithelial Cell Cultures

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Human endometrium and endometrial epithelial cells (EECs) either cultured alone or cocultured with human embryos express leptin and leptin receptor. This study compares the effect of leptin with that of interleukin-1β (IL-1β) on the expression of β3-EEC integrin, a marker of endometrial receptivity. Both cytokines increased the expression of β3-EEC at concentrations in the range of 0.06–3 n*M*; however, leptin exhibited a significantly greater effect than IL-1β. We also determined the regulatory effects of IL-1β on leptin secretion and on the expression of leptin and leptin receptor at the protein level in both EEC and endometrial stromal cell (ESC) cultures. In EEC cultures, IL-1β upregulated secretion of leptin and expression of both leptin and leptin receptors. No effect of IL-1β was found in the ESC cultures. However, leptin exhibited marginal upregulation of leptin receptor. The upregulation of β3-integrin and leptin/leptin receptor expression by IL-1β in EEC cultures indicates that both cytokines may be implicated in embryonic-maternal cross-talk during the early phase of human implantation. Our present data also raise the possibility that leptin is an endometrial molecular effector of IL-1β action on β3-integrin upregulation. Thus, a new role for leptin in human reproduction as an autocrine/paracrine regulator of endometrial receptivity is proposed.

Key Words: Leptin; leptin receptor; interleukin-1β; β3 integrin; endometrial receptivity; implantation.

Introduction

Coordinated effects of an array of cytokines and growth factors allow the acquisition of uterine receptivity by endometrial cells previously primed by the actions of steroid hormones. Molecular communication between the preimplantation embryo and maternal endometrium is also thought to be related to cytokine actions *(1)*.

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The interleukin-1 (IL-1) system, composed of IL-1α and IL-β agonists, receptor types I and II (IL-R tI and tII), and receptor antagonist (IL-1ra), has been shown to be an important factor in this process. The IL1-system is expressed by preimplantation embryos *(2,3)*. IL-1β can modulate the growth of bovine embryos in vitro at early stages of development *(4)*. Successful implantation in humans has been positively correlated with the potential for IL-1 embryo secretion *(5)*. IL-1β is secreted at higher levels by preimplantation embryos cocultured with endometrial epithelial cells (EEC) or EEC-conditioned media *(2)*, suggesting a role of the endometrium in the regulation of the embryonic IL-1 system. Synthesis of the IL-1 system is shared by embryo and mother. The IL-1β receptor is mainly expressed by EECs during the luteal phase while IL-1β is expressed by endothelial and stromal cells throughout the menstrual cycle *(6)*. In addition, immunoreactive IL-1β is present in the human maternotrophoblast unit and maternal stromal decidual cells after implantation occurs *(7)*. Interestingly, the blockade of IL-1β action by injection of IL-1ra in mice significantly decreases the implantation rate *(8)*. Moreover, in vitro IL-1β upregulates the expression of β3-EEC integrin, a marker of endometrial receptivity. Impairment of embryonic adhesion with IL-1ra is mediated through a direct effect on transformation of the epithelial plasma membrane at the time of implantation as a result of downregulation of α4, αv, and β3 integrins *(9)*. Recently, it was reported that the blockade in mice of β3 integrin by antibodies or peptide antagonists leads to a decrease in the number of implantation sites, impairing reproductive outcome *(10)*. The expression of integrin molecules α1β1, α4β1, and αvβ3 within endometrial tissue has been proposed as a marker of uterine receptivity during the implantation window *(11,12)*. Temporal and spatial differences in expression of the cycle-dependent integrins have been found between fertile and infertile endometria *(13,14)* and in diverse conditions that adversely affect endometrial receptivity *(15–17)*.

Leptin, the product of the *ob* gene, originally described as a regulator of food intake and energy balance, was further described to have a role in human reproduction (for review see ref. *18*). Leptin is also linked to the inflammatory response, proliferation, apoptosis (for review see ref. *19*), and angiogenesis *(20)*. Leptin is a small adipocyte-derived cytokine (16 kDa) also expressed by many reproductive

tissues together with its receptor (Ob-R) (i.e., follicular cells *[21,22]*, oocytes and preimplantation embryos *[23]*, endometrium *[24–26]*, placenta *[27,28]*). Recent evidence suggests a role of leptin in testicular function *(29)*.

The leptin receptor, the product of the *db* gene, is related to class I cytokine receptors and has a similar helical structure and signaling capabilities as IL-6 receptor *(30)*. Several splice variants of the leptin receptor have been identified in humans, the long isoform (Ob-RL) with full intracellular signaling capabilities and shorter isoforms with less biologic activity (Ob- RS) *(31,32)*.

Leptin rescue of fertility in both genders of sterile leptin-defective mice (*ob*/*ob*) suggests that it is required for normal reproductive function *(33,34)*. A role for leptin at the early stages of human implantation has been proposed since the human blastocyst cocultured with EECs differentially regulates leptin secretion *(26)*. Much evidence shows that the IL-1 and leptin system are intrinsically related in several cellular functions *(35–38)*. Leptin production by placental cells is modulated by IL-1α *(39)* and IL-6 *(40)*. In addition, both leptin *(41,42)* and IL-1 *(42)* are regulators of the invasive phenotype of cytotrophoblast cells. These data taken together strongly suggest that leptin and IL-1β could also be involved in the achievement of endometrial receptivity and preimplantation embryomaternal molecular crosstalk. Therefore, we investigated whether leptin regulates the endometrial expression of β3 integrin, and the regulation of leptin and leptin receptor by IL-1β. Our findings demonstrate that leptin, like IL-1β, upregulates β3 integrin expression by EECs. IL-1β upregulates the secretion and expression of leptin and its receptor.

Results

Effects of Leptin and IL-1β on Expression of β3 Integrin by EEC Cultures

Flow cytometry (FC) was performed to quantify the regulatory effect of leptin and IL-1β on β3-integrin expression by EEC cultures. Basal expression of β3-integrin was assessed in the control cultures and compared with EEC cultures in media containing the cytokines. Figure 1 shows a representative FC analysis of β3-integrin expression in control EECs (Fig. 1A) compared with EEC cultures containing leptin (0.06 n*M*; Fig. 1B) and IL-1β (0.06 n*M*; Fig. 1C). Quantitative results of β3-integrin expression by EECs from five different experiments are shown in Fig. 1D. Interestingly, leptin (0.06–3 n*M*) significantly increased the expression of β3 integrin by EECs. However, maximal β3-integrin expression (2.7-fold increment) was achieved with 0.06 n*M* leptin concentration.

IL-1β also increased β3-integrin expression. Maximal upregulation of β3-integrin expression was observed at doses of IL-1β between 0.3 and 0.6 n*M* (Fig. 1D). By comparison, leptin, at lower doses than IL-1β, upregulated to a greater degree the β3-integrin expression.

Effects of Leptin and IL-1β on Expression of Leptin Receptor by Stromal Endometrial Cell and EEC Cultures

The expression of leptin receptor in endometrial stromal cells (ESC) and EEC cultures was quantified by FC. The addition of leptin to EEC cultures did not significantly change the expression of its own receptor (Fig. 2A). Only a marginal upregulatory effect of leptin on the expression of leptin receptor by ESCs was observed (Fig. 2A). By contrast, IL-1β at a dose of 3 n*M* upregulated the expression of leptin receptor by EECs (Fig. 2B). However, no significant effect of IL-1β on leptin receptor expression was observed in ESC cultures (Fig. 2B).

Effects of IL-1β on Expression and Secretion of Leptin by ESC and EEC Cultures

The effects of IL-1β on the expression and secretion of leptin by endometrial cell cultures were assessed by FC (Fig. 3) and enzyme-linked immunosorbent assay (ELISA) (Fig. 4), respectively. Basal expression of leptin was higher in EEC than in ESC cultures (Fig. 3). However, basal leptin secretion was similar in both cultures (ESC = 307 pg/mL and EEC = 353 pg/mL; see Fig. 4). IL-1β did not affect leptin expression (Fig. 3) or secretion by ESCs (Fig. 4). Interestingly, leptin expression by EEC cultures was upregulated by IL-1β at doses between 0.6 and 3 n*M* (Fig. 3). Similarly, IL-1β upregulated the secretion of leptin into the conditioned medium by EECs at doses between 0.03 and 3 n*M* (Fig. 4).

Cell Viability After Cytokine Treatment

Quantitative FC measurement of propidium iodide (PI) staining demonstrated a negative effect of IL-1β treatment on endometrial cell viability. Interestingly, after a 24-h cytokine incubation at all doses, viability was significantly higher in EECs treated with leptin (96%) than in IL-1β (82%) compared with the control cells. ESCs treated with IL-1β also exhibited lower viability (72%) than those treated with leptin (92%).

Immunocytochemical Studies

Expression of leptin and its receptor was also assessed qualitatively by immunocytochemistry using optical and fluorescence microscopy. Basal expression of β3 integrin from control wells was increased by adding leptin (0.06 n*M*; Fig. 5A) and IL-1β (0.06 n*M*; Fig. 5B). Leptin treatment showed a higher degree of β3-integrin upregulation by EECs. A low expression of leptin and leptin receptor was detected in ESCs and EECs under basal conditions and after cytokine cultures, but from immunocytochemical results, no change was noticed in leptin expression on adding IL-1β. Figure 5C,D show leptin and leptin receptor staining after IL-1β treatment, at 0.6 and 3 n*M*, respectively. A higher degree of leptin receptor staining was observed after culture of EECs with IL-1β at 3 n*M* (Fig. 5D).

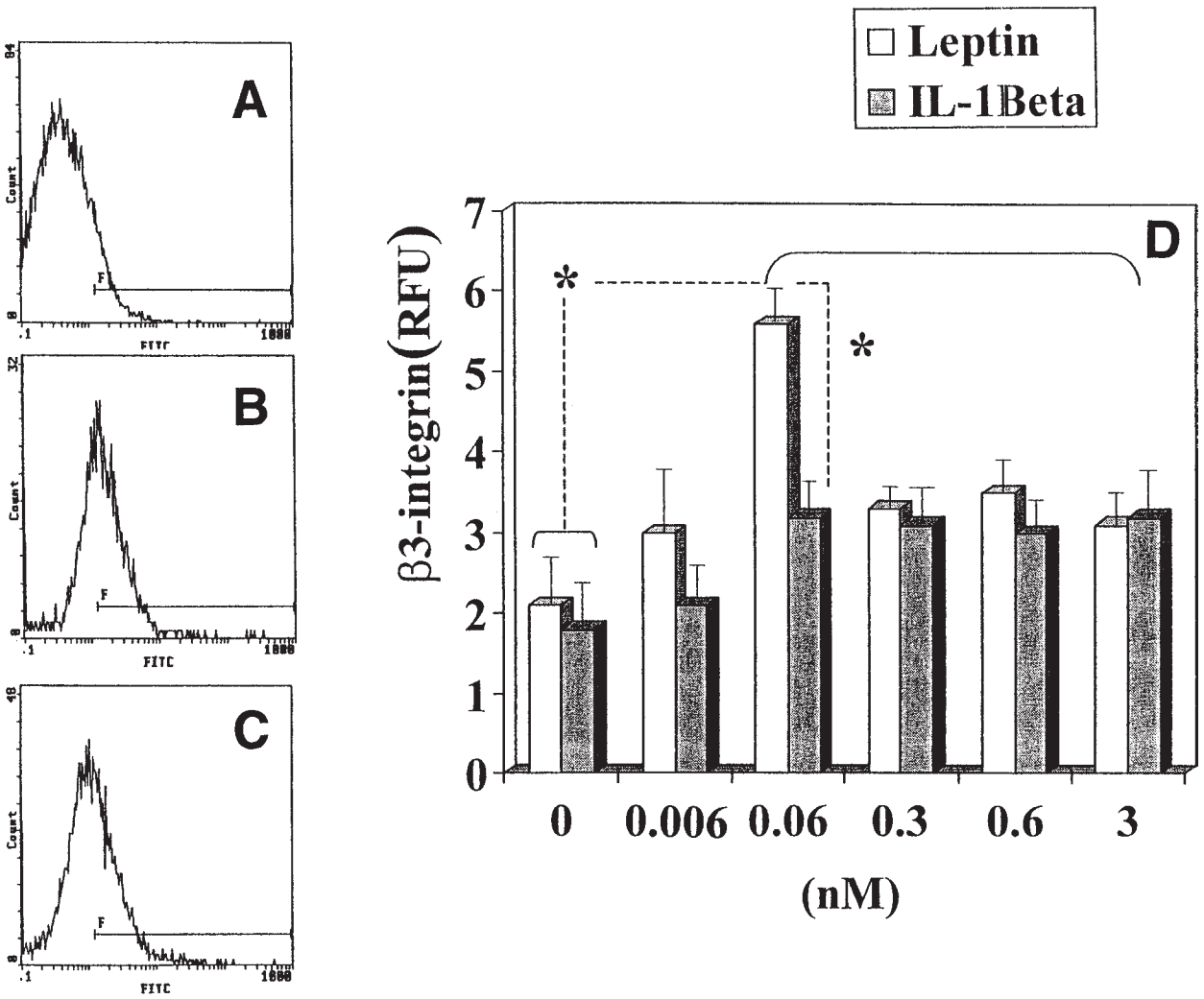

Fig. 1. Leptin and IL-1β upregulation of β3-integrin expression by EECs. Representative scatters from FC measurements for β3-integrin expression in (**A**) control EECs, (**B**) leptin (0.06 n*M*)-treated EECs, and (**C**) IL-β (0.06 nM)-treated EECs. The horizontal axis shows fluorescence intensity of β3 integrin (log scale), and the vertical axis shows the number of stained cells. EECs were stained with monoclonal mouse antibody anti–β3 integrin (clone SS A6; 1:100) and antimouse-fluorescein isothiocyanate (FITC) conjugate. A negative control with nonspecific IgG isotype-matched monoclonal antibody (MAb) (Dako, Carpinteria, CA) and a positive control with antihuman leukocyte antigen-I MAb (anti-HLA-I, clone w6/32; 1:100; Dako) were introduced in each determination. (**D**) Effects of increasing doses (0–3 n*M*) of leptin and IL-1β on EEC β3-integrin expression ($n = 5$). β3-Integrin concentrations were expressed in relative fluorescence units (RFU) (see Materials and Methods). Data are expressed as mean ± SEM. *$p < 0.05$ was considered statistically significant.

Discussion

To the best of our knowledge, we describe here for the first time the leptin upregulation of β3 endometrial epithelial integrin, one of the most reliable markers of endometrial receptivity, and the IL-1β upregulation of endometrial leptin synthesis and leptin receptor expression. These findings reinforce our hypothesis for a role of leptin in the early phase of human implantation.

Despite a substantial number of investigations, the functions of leptin in human reproduction are still not very well understood *(18)*. Decisive evidence of a leptin requirement for normal reproduction was demonstrated by Chehab and colleagues *(33)* using the *ob*/*ob* sterile mice model. However, these investigators further reported that withdrawal of exogenous leptin treatment by 0.5 d of postcopulatory plug detection did not impair implantation in *ob*/*ob* mice *(43)*. The full relevance of these observations in the human model cannot be completely established since they did not evaluate the presence of leptin in oocytes and embryos. In human oocytes and embryos, a particular cell-surface polarization of immunoreactive leptin has been detected. Leptin accumulation in oocytes and embryos may be derived from the oocyte or embryo itself or from a maternal source *(23)*. Recently, we *(26)* and others *(24,25)* demonstrated that leptin receptor is expressed by the human endometrium. However, in contrast to our findings, these authors *(24,25)* were

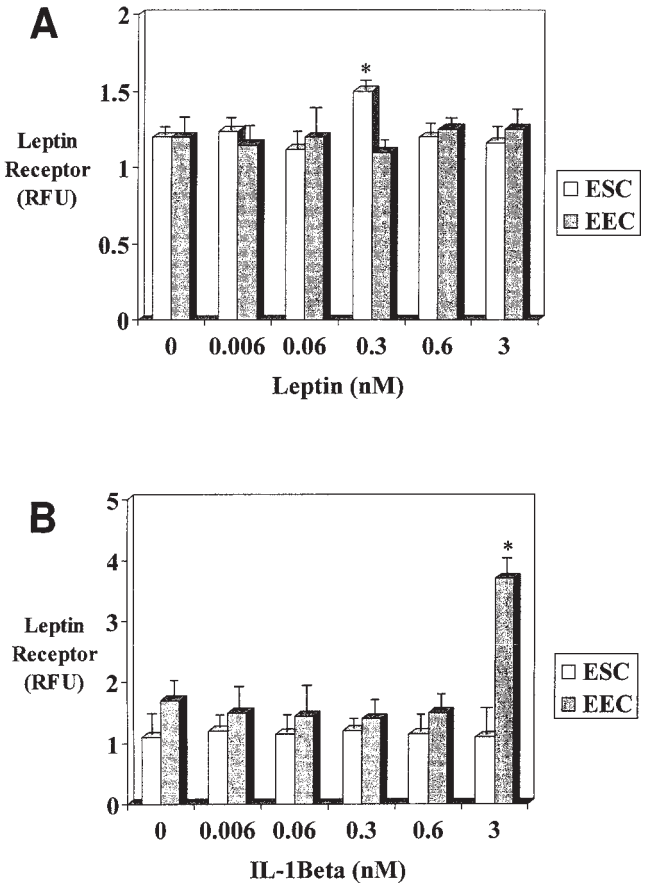

Fig. 2. Flow cytometric determination of (**A**) effects of leptin and (**B**) IL-1β on the expression of leptin receptor (Ob R) by EECs and ESCs. Cells were cultured in a fetal bovine serum (FBS)-enriched medium, washed twice with buffer and cultured an additional 2 d in FBS-free medium, and washed again and cultured in the same FBS-free medium containing IL-1β (0–3 n*M*) for 24 h. Cell suspensions were incubated with goat anti-Ob R antibody (Ob R Sc-20; 1.5 μg/mL; Santa Cruz Biotechnology) followed by incubation with antigoat IgG-FITC-conjugated antibody. Irrelevant goat IgG was used as negative control and antihuman leukocyte antigen-I MAb (anti-HLA-I, clone w6/32; 1:100; Dako) as positive control. Ob R concentrations were expressed in relative fluorescence units (RFU) (see Materials and Methods). Data are expressed as mean ± SEM. **p* < 0.05 was considered statistically significant.

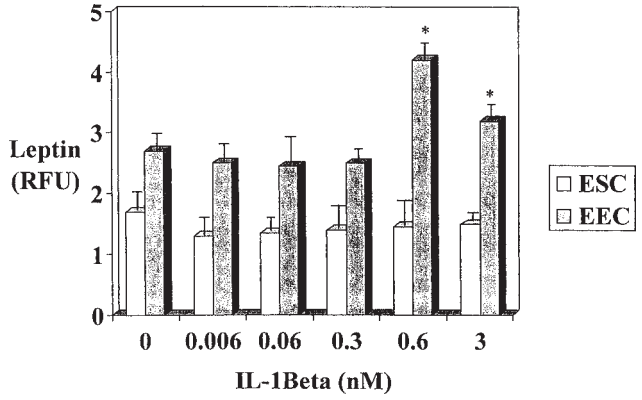

Fig. 3. IL-1β regulation of leptin expression by endometrial cells. Effects of IL-1β on leptin (Ob) expression by EECs and ESCs were determined by FC. Cells were cultured in an FBS-enriched medium, washed twice with buffer and cultured an additional 2 d in FBS-free medium, and washed again and cultured in the same FBS-free medium containing IL-1β (0–3 n*M*) for 24 h. Cell suspensions were permeabilized with 70% ethanol and incubated with rabbit antileptin antibody (ObY-20; 1 μg/mL; Santa Cruz Biotechnology) followed by incubation with antirabbit IgG-FITC-conjugated antibody. Irrelevant rabbit IgG was used as negative control. Ob concentrations were expressed in relative fluorescence units (RFU) (see Materials and Methods). Data are expressed as mean ± SEM. **p* < 0.05 was considered statistically significant.

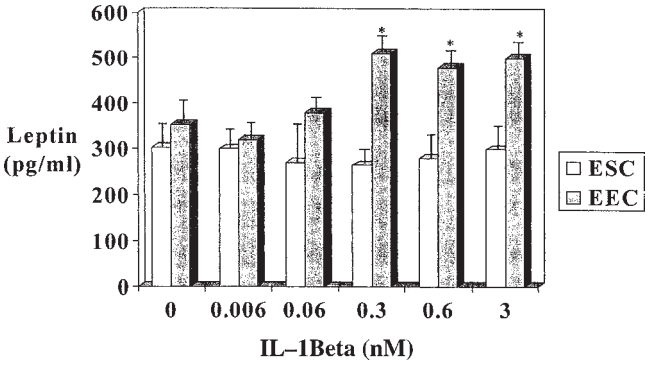

Fig. 4. IL-1β regulation of leptin secretion by endometrial cells. Effects of IL-1β on leptin (Ob) secretion by EECs and ESCs were determined by ELISA (DRG Diagnostic). Cells were cultured in an FBS-enriched medium, washed twice with buffer and cultured an additional 2 d in FBS-free medium, and washed again and cultured in the same FBS-free medium containing IL-1β (0–3 n*M*) for 24 h. Conditioned medium was lyophilized ($n = 6$ per treatment) and resuspended in 120 μL of deionized water at basic pH. Leptin concentrations in conditioned media were divided by the lyophilization concentration factor and expressed in picograms/milliliter. Standards, controls, and samples were assayed in duplicate. The intra- and interassay coefficients of variation (CVs) were between 0.54–6.10 and 7.30–7.52%, respectively. Data are expressed as mean ± SEM. **p* < 0.05 was considered statistically significant.

unable to demonstrate leptin or leptin mRNA expression in endometrium. Positive immunohistochemical staining for immunoreactive leptin found in endometrial biopsies was thought to be owing to leptin from an adipose source bound to short isoforms of the leptin receptor or to nonspecific staining *(25)*. The present data confirm our previous findings that leptin is expressed by endometrial cells. Data from current experiments showing leptin secretion and immunoreactive leptin staining in endometrial cell cultures are not likely to be from any serum contamination since after the confluent layer was obtained by culturing in FBS-enriched medium, these cells were thoroughly washed and cultured for an additional period of 3 d in FBS-free medium.

Previously we also demonstrated that in vitro the human blastocyst and EECs secrete leptin. Leptin secretion could be related to implantation potential and be a marker of cell viability. Hatched blastocysts cultured alone secreted significantly higher concentrations of leptin than arrested embryos.

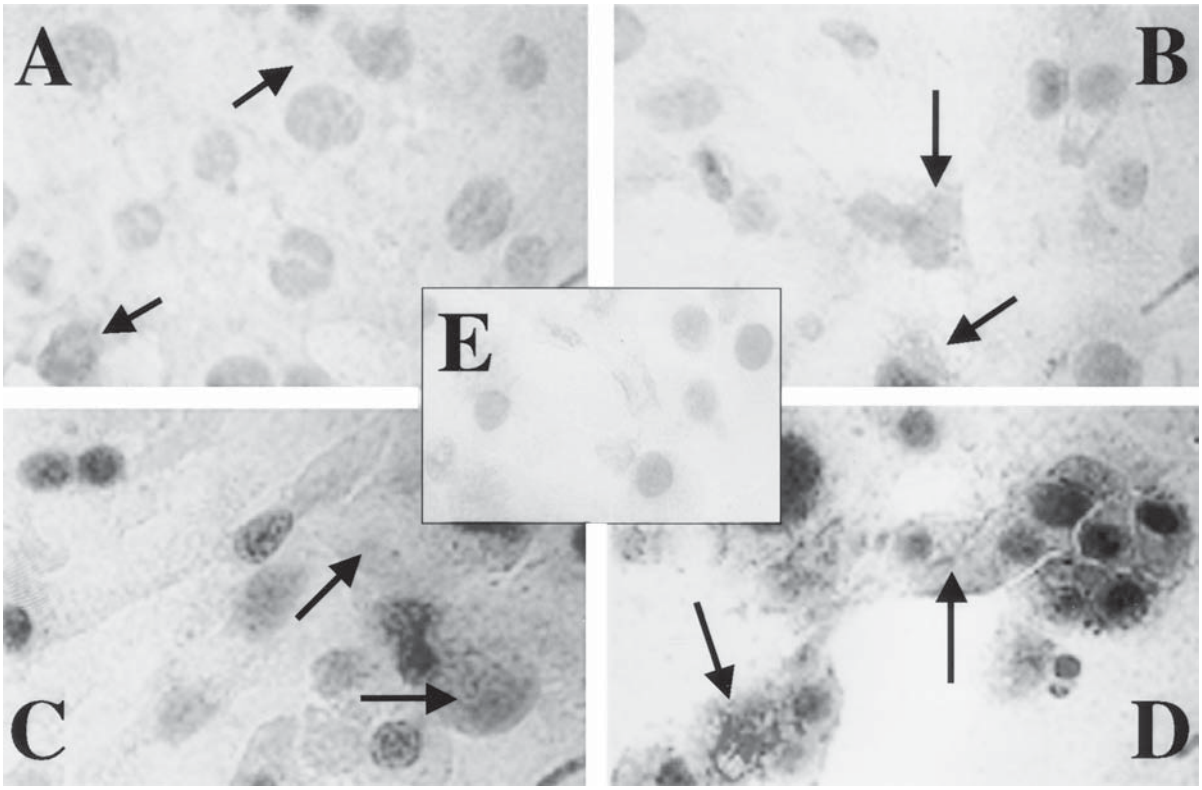

Fig. 5. Immunocytochemical detection of β3-integrin, leptin, and leptin receptor in EECs cultured with leptin and IL-1β. β3-Integrin staining in EECs cultured with (**A**) leptin and (**B**) IL-1β, both at 0.06 nM. (**C**) Leptin and (**D**) leptin receptor detection in EECs cultured with IL-1β at 0.6 and 3 n*M*, respectively. EECs were cultured for 24 h in an FBS-free medium containing leptin or IL-1β. Arrows show specific staining for antigens. Note that there is a higher degree of staining for β3-integrin in (A) EEC-leptin-treated compared with (B) IL-1β-treated cells. Leptin staining (C) was weak to moderate at all IL-β concentrations assayed. Leptin receptor staining (D) increased in IL-1β-treated EECs. Cells were acetone fixed and assayed to detect β3-integrin, leptin, and leptin receptor expression using specific antibodies and a streptavidin–biotin–horseradish peroxidase system (LSAB kit system; Dako). EECs were stained by amine-ethyl-carbazol (AEC) enzymatic product and counterstained with hematoxylin. (**E**) Negative controls were incubated with irrelevant antibodies species matched to primary antibodies. Immunostained cells were evaluated on a Kodak microscope. Magnification: ×400.

Leptin secretion is differentially secreted when competent or arrested embryos are cocultured with EECs, suggesting a regulation in the secretion of leptin or binding to leptin receptor in the cocultures of human embryos and EECs. These data provided evidence for a possible role of leptin in human implantation *(26)*.

On the other hand, substantial evidence of a role of an IL-1 system in human implantation has been published by Simon et al. *(44)* and others. The human embryo expresses the complete IL-1 system (agonist, receptor, and antagonist). However, IL-1ra mRNA synthesis is likely to be arrested in early embryo development because IL-1ra-positive embryos fail to reach the blastocyst stage *(3)*. Using the same co-culture model employed in the present investigation, De los Santos et al. *(2)* demonstrated that competent human embryos secrete higher IL-1β in presence of EECs. IL-1β has been shown to play a role in maternal-embryonic cross talk. Endometrial luminal cells express IL-1R tI *(6)*. IL-1 in these cells upregulates the expression of β3 integrin *(45)*, an adhesion molecule, which, in turn, could increase the possibility of successful implantation. In addition, human endometrial epithelium expresses IL-1ra *(46–48)*, which regulates the effects of IL-1 on decidualization and cell proliferation of human ESCs *(49)*. Thus, IL-1 may play a crucial role in embryomaternal interaction by regulating stromal cell expression of IL-1β and IL-1ra, resulting in an appropriate ratio during the process of embryonic implantation *(50)*.

The present data demonstrate that the regulatory effects of IL-1β on Ob-R expression in endometrial cell cultures are in contrast to those reported for expression of its own receptor. IL-1β upregulates the Ob-R in EECs but does not affect its expression in ESCs. IL-1β did not upregulate IL-1R tI expression by EECs but increased IL-1R tI mRNA in ESCs *(51)*.

In addition, IL-1β upregulates leptin expression and secretion by EECs. However, IL-1β does not affect leptin expression in ESCs. The upregulatory effect of IL-1β on β3-integrin expression by EECs *(45)* was confirmed by us. Interestingly, leptin upregulates β3-EECs to a greater degree than does IL-1β. Therefore, it might be possible that a primary effect

of IL-1β in the endometrium is to upregulate leptin and its receptor, which, in turn, effectively upregulate the β3 expression, conferring a higher receptive status to the endometrial epithelium. Our present data raise the possibility that leptin is a molecular effector of IL-1β for the achievement of endometrial receptivity. Further investigations on blocking endometrial leptin receptor are needed to establish the relative importance of leptin on the upregulatory effect of IL-1 on β3-integrin expression and its role in the embryo-maternal cross talk.

Leptin is also linked to IL-1 actions during trophoblast invasion. In fact, IL-1 upregulates leptin secretion by trophoblast cells *(39)*. Previously, we showed that both cytokines positively affect the invasive phenotype of cytotrophoblast cells by increasing the expression of α5 and α6 integrins and the activity of metalloprotease-9 *(42)*.

Nevertheless, it remains to be assessed whether leptin could also regulate the IL-1 system in embryos and endometrial tissue. Leptin has been reported as an inductor of IL-1ra in human monocytes *(52)*, IL-1β mRNA in mouse glial cells *(53)*, and inflammatory cytokines in rodents *(54)*.

Our data suggest that leptin and the IL-1 system are closely related at the early stage of human implantation. Because the human embryo and endometrium could communicate through the IL-1 system *(44)*, leptin might also participate in this cross talk *(26)*, IL-1 upregulates leptin and leptin receptor expression, and both cytokines increase endometrial epithelial β3 expression, a new role for leptin in human reproduction is proposed: leptin could be an endometrial effector of IL-1β and a new autocrine/paracrine regulator of endometrial receptivity.

Materials and Methods

Isolation of Endometrial Cells and Culture Conditions

All chemicals and cultured media were obtained from Sigma (St. Louis, MO) and Worthington. Human recombinant leptin and IL-1β were provided by R&D Systems. Endometrial tissues were obtained from hysterectomies of nonmalignant etiologies. Tissues were treated with collagenase I (0.1%)–deoxyribonuclease I (DNase, 0.005%) dissolved in Dulbecco's modified Eagle's medium (DMEM) for 90 min at 37°C *(55)*. Following sedimentation of glandular structures and separation of ESCs, the glands were purified of ESCs and macrophage contaminants by incubating at 37°C in a Falcon flask. Stromal and epithelial cell dispersions were counted in a hemocytometer, and cell viability was assessed by optical microscopy using the Trypan Blue exclusion method. Means of viability for ESCs and EECs were approx 90%. To examine the homogeneity of cell preparations, specific MAbs to vimentin (Vm; ESC+), cytokeratin (Ck; EEC+), and CD45 (leukocyte +) were used in cell smears *(14)*, followed by incubating with rabbit antimouse IgG-FITC-conjugated antibody (all antibodies were from Dako). Maximum cross-contamination found between the endometrial cells (ESC-EEC) was <1%. Cells were cultured in 12-well plates for 5–7 d with medium containing DMEM-MCDB105 plus 10% FBS, 1% amphopthericin B, 100 μg/mL streptomycin, and 100 U/mL of penicillin until confluent layers were obtained. To eliminate any cytokine effect from the FBS supplement, the cells were washed twice with phosphate-buffered saline (PBS)-2% bovine serum albumin (BSA) and cultured an additional 2 d in DMEM-MCDB105-2% BSA. Next, the cells were washed out three times with the culture medium and cultured in the same medium containing leptin or IL-1β at doses of 0.006, 0.06, 0.3, 0.6, and 3 n*M*. Conditioned media were collected after 24 h and the culture stopped. Endometrial cells were detached from the plates by incubating with trypsin (0.025%)-EDTA (1 m*M*) for 5 min at room temperature. After washing with PBS-2% BSA, the cells were incubated with 1 μg/mL of PI solution in PBS-2% BSA for 15 min at room temperature, and the viability was evaluated by FC.

Conditioned media from endometrial cell cultures were lyophilized and kept at 4ºC until assayed for leptin concentrations. Duplicate wells were run for each treatment with leptin and IL-1β at the concentrations described, and the experiments were repeated at least three times with different endometrial preparations. Controls were the same cellular preparations cultured in the absence of leptin or IL-1β.

Flow Cytometric Measurements

All of the following incubation steps were performed at 4ºC in an ice bath using 500,000 cells dispersed in 100 μL of PBS-2% BSA.

Expression of β3 Integrin by EECs

EEC suspensions were incubated for 1 h with a β3-integrin specific mouse MAb (anti CD61, clone SS A6; 1:100; for references see ref. *11*). After washing with PBS-2% BSA, the cells were incubated for 1 h with a 1:20 diluted rabbit antimouse IgG-FITC-conjugated antibody. A negative control with nonspecific IgG isotype-matched MAb (Dako) and a positive control with antihuman leukocyte antigen-I MAb (anti HLA-I, clone w6/32; 1:100; Dako) were introduced in each determination *(14)*.

Expression of Leptin Receptor and Leptin

ESC and EEC suspensions were incubated for 2 h with a goat antileptin receptor antibody (ObR Sc-20; 1.5 μg/mL; Santa Cruz Biotechnology) and further incubated for 45 min with an antigoat-FITC-conjugated antibody (Dako). Cell suspensions were also permeabilized with 70% ethanol for 15 min. Then the cells were incubated for 1.5 h with a rabbit antileptin antibody (ObY-20; 1 μg/mL; Santa Cruz Biotechnology) following by incubation for 1 h with an anti-rabbit IgG-FITC-conjugated antibody (Dako). Irrelevant rabbit and goat IgGs (Santa Cruz Biotechnology) were used as controls.

FC measurements were taken in an FASCcan Flow Cytometer (Becton Dickinson) using the CellQuest software for data acquisition and analysis. Concentration of antigens was expressed in relative fluorescence units. One relative fluorescence unit was defined as the ratio (*F*/*Fo*) of the mean of fluorescence (*F*) obtained from the labeled antigen and the background of the mean of fluorescence of the negative control (*Fo*) *(14)*.

Immunocytochemistry

Cell suspensions from cultures were acetone fixed for 20 min at −20ºC. Fixed-cell smears were blocked for nonspecific binding with PBS-2% BSA for 30 min at room temperature and assayed to detect β3 integrin *(14)*, leptin, and leptin receptor expression using specific antibodies diluted in PBS-2% BSA. EECs were incubated for 1 h at room temperature with the anti–β3 integrin MAb (diluted 1:80 in PBS-2% BSA) and further with a streptavidin–biotin–horseradish peroxidase system (LSAB kit system; Dako). EECs were stained by AEC enzymatic product and counterstained with hematoxylin. ESC- and EEC-fixed smears were also assayed for leptin and leptin receptor detection using an immunofluorescence method. Cells were incubated for 45 min at room temperature with specific antibodies to leptin (2 μg/mL) and leptin receptor (2.5 μg/mL) already described and detected with secondary FITC-labeled antibodies. Negative controls for β3-integrin, leptin, and leptin receptor were incubated with irrelevant mouse MAb, and normal rabbit and goat serum, respectively, instead of primary antibodies. Immunostained and immunofluorescence-labeled cells were evaluated on a Kodak microscope.

Leptin Secretion by Endometrial Cell Cultures

Lyophilized conditioned media (n = 6 per treatment) from EECs and EECs cultured in the presence of different concentrations of IL-1β were resuspended in 120 μL of deionized water at basic pH, and leptin concentrations were measured by ELISA (DRG Diagnostic; Instruments GmbH, Germany). Recovery experiments were conducted by adding human recombinant leptin to fresh culture medium, following lyophilization and resuspension as described for conditioned media. Total recovery of leptin ranged between 90 and 93%. Leptin concentrations as determined by ELISA were within the dynamic range of the standard curve. Leptin concentrations in conditioned media were divided by the lyophilization concentration factor and expressed in picograms/milliliter. Standards, controls, and samples were assayed in duplicate. The intra- and interassay CVs were between 0.54–6.10 and 7.30–7.52%, respectively. According to the manufacturer, the performance characteristics of ELISA are as follows: 100% specificity for human leptin; sensitivity of 200 pg/mL; <0.2% crossreactivity with rat and mouse leptin; and no detectable crossreactivity with human insulin, human proinsulin, rat insulin, human C-peptide, glucagons, or insulin-like growth factor 1.

Statistical Analyses

Differences in β3-integrin, leptin, and leptin receptor expression and leptin protein concentrations were evaluated by analysis of variance using Analyse-it software (Microsoft Excel, Leeds, UK; htpp://www.analyse-it.com). All data are presented as mean ± SEM, $p < 0.05$ was considered statistically significant.

References

1. Tabibzadeh, S. S. (1991). *Endocr. Rev.* **12,** 272–290.
2. De los Santos, M. J., Mercader, A., Frances, A., Portoles, E., Remohi, J., Pellicer, A., and Simón, C. (1996). *Biol. Reprod.* **54,** 563–574.
3. Russel, J. S., Simón, C., Rubio, M. C., Pape, A. R., Wen, Y., Huang, H.-Y., Bielfeld, P., and Polan, M. L. (1998). *Hum. Reprod.* **3,** 2206–2211.
4. Paula-Lopes, F. F., de Moraes, A. A., Edwards, J. L., Justice, J. E., and Hansen, P. J. (1998). *Biol. Reprod.* **59,** 1406–1412.
5. Sheth, K. V., Rovca, G. L., Al-Sedairy, S. T., Parher, R. S., Hamilton, C. J. C. M., and Al-Abdul Jabbar, F. (1991). *Fertil. Steril.* **55,** 952–957.
6. Simón, C., Piquette, G. N., Frances, A., and Polan, M. L. (1993). *J. Clin. Endocrinol. Metab.* **77,** 549–555.
7. Simón, C., Frances, A., Piquette, G., Hendriclson, M., Milki, A., and Polan, M. L. (1994). *J. Clin. Endocrinol. Metab.* **78,** 847–854.
8. Simón, C., Frances, A., Piquette, G. N., el Danasouri, I., Zurawski, G., Dang, W., and Polan, M. L. (1994). *Endocrinology* **134,** 521–528.
9. Simón, C., Valbuena, D., Krussel, J., Bernal, A., Murphy, C. R., Shaw, T., Pellicer, A., and Polan, M. L. (1998). *Fertil. Steril.* **70,** 896–906.
10. Illera, M. J., Cullinan, E., Gui, Y., Yuan, L., Beyler, S. A., and Lessey, B. A. (2000). *Biol. Reprod.* **62,** 1285–1290.
11. Lessey, B. A., Damjanovich, L., Coutifaris, C., Castelbaum, A., Albelda, S. M., and Buck, C. A. (1992). *J. Clin. Invest.* **90,** 188–195.
12. Lessey, B. A. (1994). *Fertil. Steril.* **61,** 812–814.
13. Lessey, B. A., Castelbaum, A. J., Sawin, S. J., and Sun, J. (1995). *Fertil. Steril.* **63,** 535–542.
14. Gonzalez, R. R., Palomino, A., Boric, A., Vega, M., and Devoto, L. (1999). *Hum. Reprod.* **4,** 2485–2492.
15. Lessey, B. A. and Castelbaum, A. J. (1995). *Br. J. Obstet. Gynaecol.* **102,** 347–348.
16. Meyer, W. R., Castelbaum, A. J., Somkuti, S., Sagoskin, A. W., Doyle, M., Harris, J. E., and Lessey, B. A. (1997). *Hum. Reprod.* **12,** 1393–1398.
17. Gonzalez, R. R., Palomino, A., Vantman, D., Gabler, F., and Devoto, L. (2001). *Early Preg. Biol. Med.* **5,** 132–143.
18. Gonzalez, R. R., Simón, C., Caballero-Campo, P., Norman, R., Chardonnens, D., Devoto, L., and Bischof, P. (2000). *Hum. Reprod. Update* **6,** 290–300.
19. Fantuzzi, G. and Faggioni, R. (2000). *J. Leuk. Biol.* **68,** 437–446.
20. Sierra-Honigmann, M. R., Nath, A. K., Murakami, C., Garcia-Cardena, G., Papetropoulos, A., Sessa, W. C., Madge, L. A., Schechner, J. S., Schwabb, M. B., Polverini, P. J., and Flores-Riveros, J. R. (1998). *Science* **282,** 1683–1686.
21. Karlsson, C., Lindell, K., Svensson, E., Bergh, C., Lind, P., Billig, H., Carlsson, L. M., and Carlsson, B. (1997). *J. Clin. Endocrinol. Metab.* **82,** 4144–4148.
22. Cioffi, J. A., Van Blerkom, J., Antczak, M., Shafer, A., Wittmer, S., and Snodgrass, H. R. (1997). *Mol. Hum. Reprod.* **3,** 467–472.
23. Antczak, M. and Van Blerkom, J. (1997). *Mol. Hum. Reprod.* **3,** 1067–1086.

24. Kitawaki, J., Koshiba, H., Ishihara, H., Kusuki, I., Tsukamoto, K., and Honjo, H. (2000). *J. Clin. Endocrinol. Metab.* **85,** 1946–1950.
25. Alfer, J., Muller-Schottle, F., Classen-Linke, I., von Rango, U., Happel, L., Beier-Hellwig, K., Rath, W., and Beier, H. M. (2000). *Mol. Hum. Reprod.* **6,** 595–601.
26. Gonzalez, R. R., Caballero-Campo, P., Jasper, M., Mercader, A., Devoto, L., Pellicer, A., and Simón, C. (2000). *J. Clin. Endocrinol. Metab.* **85,** 4883–4888.
27. Masuzaki, H., Ogawa, Y., Sagawa, N., Hosoda, K., Matsumoto, T., Hiroko, M., Nishimura, M., Yoshimasa, Y., Tanaka, I., Mori, T., and Nakao, K. (1997). *Nat. Med.* **3,** 1029–1033.
28. Senaris, R., Garcia-Caballero, T., Casabiell, X., Gallego, R., Castro, R., Considine, R. V., and Casanueva, F. F. (1997). *Endocrinology* **138,** 4501–4504.
29. Tena-Sempere, M., Pinilla, L., Zhang, F. P., Gonzalez, L. C., Huhtaniemi, I., Casanueva, F. F., Dieguez, C., and Aguilar, E. (2001). *Biol. Reprod.* **64,** 634–643.
30. Baumann, H., Morella, K. K., White, D. W., Dembski, M., Bailon, P. S., Kim, H., Lai, C. F., and Tartaglia, L. A. (1996). *Proc. Natl. Acad. Sci. USA* **93,** 8374–8378.
31. Campfield, L. A., Smith, F. J., and Burn, P. (1996). *Horm. Metab. Res.* **28,** 619–632.
32. Wang, Y., Kuropatwinski, K. K., White, D. W., Hawley, T. S., Hawley, R. G., Tartaglia, L. A., and Baumann, H. (1997). *J. Biol. Chem.* **272,** 16,216–16,223.
33. Chehab, F., Lom, M., and Lu, R. (1996). *Nat. Genet.* **12,** 318–320.
34. Mounzih, K., Lu, R., and Chehab, F. F. (1997). *Endocrinology* **138,** 1190–1193.
35. Grunfeld, C., Zhao, C., Fuller, J., Pollack, A., Moser, A., Friedman, J., and Feinggold, K. R. (1966). *J. Clin. Invest.* **97,** 2152–2157.
36. Sarraf, P., Frederich, R. C., Turner, E. M., Ma, G., Jaskowiak, N. T., Rivet, D. J. III, Flier, J. S., Lowell, B. B., Fraker, D. L., and Alexander, H. R. (1997). *J. Exp. Med.* **185,** 171–175.
37. Janik, J. E., Curti, B. D., Considine, R. V., Rager, H. C., Powers, G. C., Alvord, W. G., Smith, J. W. II, Gause, B. L., and Kopp, W. C. (1997). *J. Clin. Endocrinol. Metab.* **82,** 3084–3086.
38. Faggioni, R., Fantuzzi, G., Fuller, J., Dinarello, C. A., Feingold, K. R., and Grunfeld, C. (1998). *Am. J. Physiol.* **274(1)(Pt. 2),** R204–R208.
39. Chardonnens, D., Cameo, P., Aubert, M. L., Pralong, F. P., Islami, D., Campana, A., Gaillard, R. C., and Bischof, P. (1999). *Mol. Hum. Reprod.* **5,** 1077–1082.
40. Meisser, A., Cameo, P., Islami, D., Campana, A., and Bischof, P. (1999). *Mol. Hum. Reprod.* **5,** 1055–1058.
41. Castelluci, M., De Matteis, R., Meisser, A., Canullo, R., Monsurro, V., Islami, D., Sarzani, R., Marzioni, D., Cinti, S., and Bischof, P. (2000). *Mol. Hum. Reprod.* **6,** 951–958.
42. Gonzalez, R. R., Devoto, L., Campana, B., and Bischof, P. (2001). *Endocrine*, in press.
43. Mounzih, K., Qiu, J., Wwart-Toland, A., and Chehab, F. (1998). *Endocrinology* **139,** 5259–5262.
44. Simon, C., Moreno, C., Remohi, J., and Pellicer, A. J. (1998). *J. Reprod. Immunol.* **39,** 117–131.
45. Simon, C., Gimeno, M. J., Mercader, A., O'Connor, J. E., Remohi, J., Polan, M. L., and Pellicer, A. (1997). *J. Clin. Endocrinol. Metab.* **82,** 2607–2616.
46. Tabibzadeh, S. and Sun, X. Z. (1992). *Hum. Reprod.* **7,** 1214–1221.
47. Sahakian, V., Anners, J., Haskill, S., and Halme, J. (1993). *Fertil. Steril.* **60,** 276–279.
48. Simon, C., Frances, A., Lee, B. Y., Mercader, A., Huynh, T., Remohi, J., Polan, M. L., and Pellicer, A. (1995). *Hum. Reprod.* **10,** 2472–2477.
49. Tanaka, T., Sakamoto, T., Mizuno, K., Umesaki, N., and Ogita, S. (2000). *Horm. Res.* **53,** 300–304.
50. Huang, H. Y., Wen, Y., Kruessel, J. S., Raga, F., Soong, Y. K., and Polan, M. L. (2001). *J. Clin. Endocrinol. Metab.* **86,** 1387–1393.
51. Simon, C., Piquette, G. N., Frances, A., el-Danasouri, I., Irwin, J. C., and Polan, M. L. (1994). *J. Clin. Endocrinol. Metab.* **78,** 675–682.
52. Gabay, C., Dreyer, M. G., Pellegrinelli, N., Chicheportiche, R., and Meier, C. A. (2001). *J. Clin. Endocrinol. Metab.* **86,** 783–791.
53. Hosoi, T., Okuma, Y., and Nomura, Y. (2000). *Biochem. Biophys. Res. Commun.* **273,** 312–315.
54. Loffreda, S., Yang, S. Q., Lin, H. Z., Karp, C. L., Brengman, M. L., Wang, D. J., Klein, A. S., Bulkley, G. B., Bao, C., Noble, P. W., Lane, M. D., and Diehl, A. M. (1998). *FASEB J.* **12,** 57–65.
55. Satyaswaroop, P. G., Bressler, R. S., de la Pena, M. M., and Gurpide, E. (1979). *J. Clin. Endocrinol. Metab.* **48,** 639–641.