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A Peptide Derived from the Human Leptin Molecule Is a Potent Inhibitor of the Leptin Receptor Function in Rabbit Endometrial Cells

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In this article we show that rabbit endometrial cells express leptin receptor and that human leptin triggers phosphorylation of signal transducer and activator of transcription 3 and up-regulates the expression of interleukin-1 receptor type I as was previously found in human endometrial cells. Interestingly, leptin also up-regulates the secretion of leukemia inhibitory factor and expression of its receptor by rabbit endometrial cells. Analysis of a structural model of the leptin–leptin receptor complex suggested that helices I and III of the human leptin structure were likely sites of interaction with the cytokine binding domain of leptin receptor. Accordingly, we synthesized a peptide (LPA-2) comprising helix III (residues 70–95) and investigated its ability to inhibit leptin receptor function. The effects of LPA-2 were assayed in rabbit endometrial cells, and an antileptin receptor antibody and a scrambled version of LPA-2 were used as positive and negative controls, respectively. LPA-2 binds specifically and with high affinity ($K_i \sim 0.6 \times 10^{-10}$ *M*) to leptin receptor and is a potent inhibitor of its functions in rabbit endometrial cells. Because leukemia inhibitory factor and interleukin-1 have been implicated in embryo implantation, our results raise the possibility that the LPA-2-induced inhibition of leptin receptor may be exploited to study the actions of leptin in endometrium and in other tissues under conditions characterized by abnormal leptin production.

Key Words: Leptin peptide antagonist; leptin receptor; interleukin 1; leukemia inhibitory factor; endometrial receptivity; implantation.

Introduction

Leptin plays a critical role in reproductive function, inflammation, and angiogenesis *(1–4)*. To date leptin, a pleiotropic and ubiquitous molecule, has been implicated in the pathology of a variety of conditions including but not limited to diabetes mellitus *(5),* acquired immune disease *(6)*, cancer *(7)*, endocrine disorders of the adrenal cortex and pituitary glands *(8),* preeclampsia *(9)* endometriosis *(10,11),* polycystic ovarian syndrome (PCOS) *(12)*, and infertility *(13)*—all of these in addition to its most recognized role in regulation of body weight and food intake *(14)*.

Leptin binding to its receptor (OB-R, the product of the *db* gene) promotes JAK-2 activation and triggers the phosphorylation of signal transducer and activator of transcription 3 (Stat3), that, in turn, activates a number of downstream signaling pathways *(15)*.

Mice expressing a non-functional leptin molecule (*ob/ob*) or those which are unable to express OB-R (*db/db*) are sterile. However, fertility in *ob/ob* mice can be restored by exogenous leptin treatment *(16)*. Studies from *ob/ob* female mice have shown that leptin is essential for normal preimplantation and/or implantation processes *(17)*. In addition, leptin promotes preimplantation embryo development *(18)*.

Human blastocyst and endometrial epithelial cells (hEEC) secrete leptin in vitro and, when co-cultured, these cells regulate leptin secretion *(19)*. Leptin and OB-R are up-regulated by interleukin-1 (IL-1) *(20)*, but leptin can also up-regulate the IL-1 system (ligand, receptor and antagonist) in human endometrial cell cultures *(21)*. Interestingly, both leptin and IL-1 cytokines up-regulate β3 integrin expression (a marker for endometrial receptivity) by hEEC *(20–22)* and the expression of integrins and proteases in human trophoblasts *(23)*. The blockade of OB-R function by antibodies impairs both leptin and IL-1 functions *(21)* suggesting that leptin could substitute for some of the IL-1 functions in the endometrium and trophoblast.

Both IL-1 *(24,25)* and leukemia inhibitory factor (LIF) *(26,27)* have been suggested to play important roles in embryo implantation. However, studies on the ability of interleukin-1 receptor antagonist (IL-1Ra) to disrupt embryo implantation in mice capabilities have yielded conflicting results *(28–30)*.

In contrast, LIF is essential for mouse reproduction *(27)* and has been suggested to play an important role in embryo implantation in the rabbit *(31–33)*. Human endometrial cells (hEC) differentially secrete immunoreactive LIF depending

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on the stage of the menstrual cycle *(34,35)* and maximal LIF secretion by these cells coincides with the time corresponding to the implantation window *(34)*. Furthermore, infertility in humans has been related to abnormal expression of LIF by the endometrium *(36)*.

Structural analyses of the leptin binding sites *(37)* and of a homology-based model of the leptin/OB-R complex *(38)* suggest that specific interactions between certain leptin loops and OB-R lead to the homodimerization of the leptin/OB-R complex, which is required for its biological activity. Our previous studies indicated that leptin function at the endometrial level could be regulated using anti OB-R antibodies. However, relatively high doses of antibodies were required to block OB-R which could potentially generate undesired immunological side effects in vivo *(21)*.

In this study, we have investigated OB-R expression and leptin regulation of IL-1R tI and LIF (ligand and receptor) expression by rEC. We also investigated whether a peptide comprising a segment of the sequence of native human leptin can inhibit the function of OB-R in these cells. Therefore, we synthesized a peptide, LPA-2, corresponding to amino acid residues 70–95 from one of the putative sites of interaction of human leptin with OB-R. We compared the effects of blocking OB-R with specific antibodies vs LPA-2 or its scrambled version (LPA-2Sc). Our results show that rEC express OB-R, human leptin up-regulates protein expression of IL-1R tI and LIF (ligand and receptor), and that LPA-2 binds specifically to OB-R and is a potent inhibitor of OB-R function in these cell cultures.

Results

Design, Synthesis, Purification, and Characterization of LPA-2

LPA-2 and LPA-2Sc were synthesized using solid-phase peptide synthesis. After HPLC purification, the peptides were ≥98% pure. Determination of molecular weight (MW) of LPA-2 peptides by mass spectral analysis demonstrated a unique peak corresponding to the calculated MW of 2982 for both LPA-2 and LPA-2Sc.

Results from circular dichroism spectroscopy measurements used to predict LPA-2 structure showed that this peptide conserves the alpha-helical structure found in the native human leptin molecule *(39)*. Analysis of the secondary structures and hydrophobicity/hydrophilicity profiles of LPA-2 and its scrambled version using the program Protean (DNA STAR) indicated that the two peptides have a significantly different secondary structure. Therefore, LPA-2Sc is a reliable negative control for testing LPA-2 binding to OB-R and its specific effects on rEC cultures.

Expression of Functional OB-R by Rabbit Endometrial Cells and Binding of LPA-2 to OB-R

After the purification steps, homogeneous preparations of rabbit endometrial stromal (rESC) and epithelial cells (rEEC) were successfully obtained. Immunocytochemical studies showed that rESC expressed vimentin (Fig. 1Aa), but not cytokeratin. In contrast, rEEC did not express vimentin but were positive for the epithelial cell marker, cytokeratin (Fig. 1Ac).

Interestingly, both cellular types constitutively expressed OB-R (Figs. 1Ab and 1Ad). Addition of leptin, LPA-2, or anti-OB-R antibody to cultures of rESC or rEEC did not affect the expression of OB-R. Negative controls using cells incubated with non-specific species-matched IgGs showed no staining for any of the antigens tested (Fig. 1e).

Western blot analysis of lysates from cell cultures corroborated the immunocytochemical findings. A main band of approx 190 kDa corresponding to the full length of OB-R was found in all protein lysates from rESC and rEEC cultured in basal medium or medium containing the tested compounds (Fig. 1B).

Results from competitive binding studies between human leptin and LPA-2 show that ^{125}I-leptin bound to OB-R from cellular lysates was displaced by addition of LPA-2 (Fig. 1C). LPA-2 ($K_i \sim 0.6 \times 10^{-10}$ *M*) but not LPA-2Sc specifically binds to OB-R expressed by rabbit endometrial cells (rEEC and rESC).

Up-Regulation of p-Stat3 Expression by Leptin in Rabbit Endometrial Cells and Inhibition by Anti-OB-R Antibody and LPA-2

To test the functionality of OB-R expressed by the rabbit endometrial cells, p-Stat3 phosphorylation was investigated following leptin treatment. Figure 2 shows the immunocytochemical results obtained with rESC. The basal expression of p-Stat3 (Fig. 2A) was up-regulated by leptin (Fig. 2B). Similar results were obtained for rEEC (not shown).

LPA-2 significantly inhibits the leptin up-regulation of Stat3 phosphorylation (Fig. 2D) to a degree similar to that found with anti-OB-R antibody treatments (data not shown). The LPA-2 blockade of p-Stat3 expression appears to be specific, because this effect was found at very low concentrations of LPA-2 (doses ranged from 3 to 300 n*M*) and was not observed with LPA-2Sc (Fig. 2C) at the same concentrations. Negative controls using cells incubated with non-specific species matched IgGs showed no staining for any of the antigens tested (Fig. 2E).

Results from Western blot determinations of p-Stat3 expression by rESC and rEEC confirmed the immunocytochemical findings. An 81 kDa band corresponding to p-Stat3 was found in samples from rESC (Fig. 2F and 2G) and rEEC (Figs. 2H and 2I). Both cell types exhibited increased p-Stat3 expression after incubation with leptin (3 n*M*).

The antibody specific for the NH_2 terminal end of human OB-R (at all concentrations assayed) effectively blocks the receptor function in the cell cultures by decreasing the leptin up-regulation of Stat3 phosphorylation in rESC (Fig. 2F) and rEEC (Fig. 2H). This effect appears to be specific since

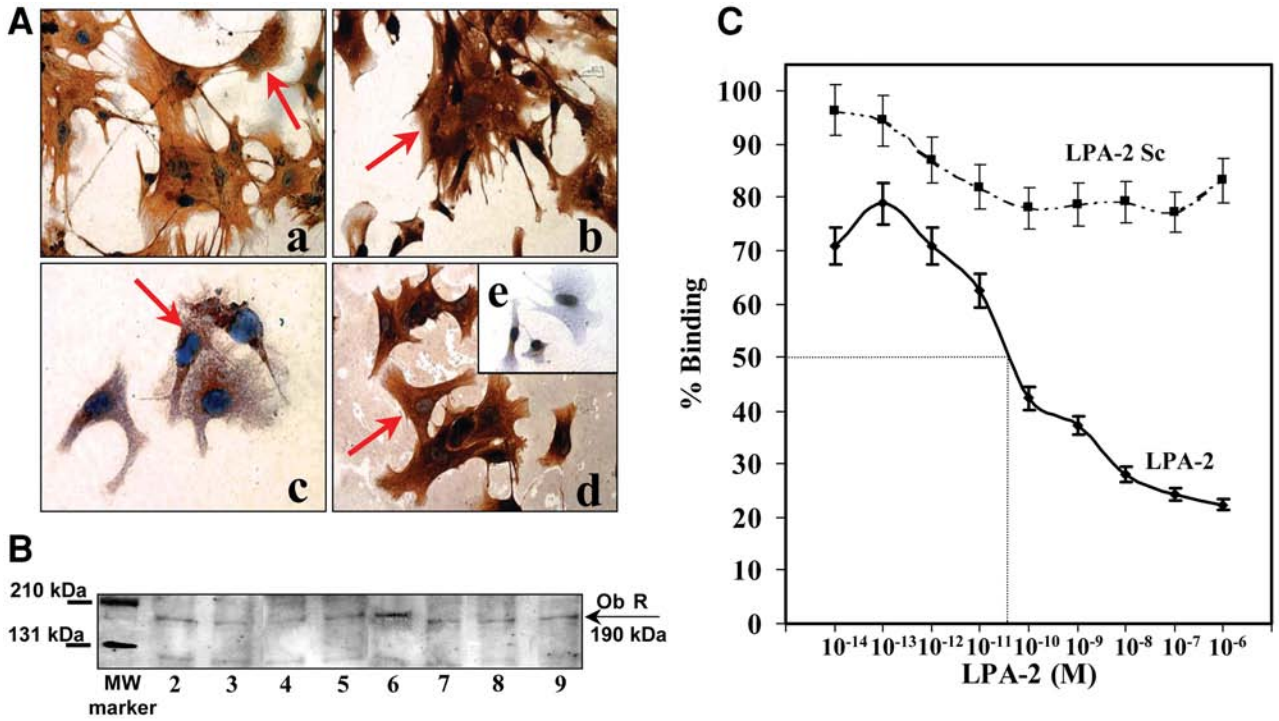

Fig. 1. Differential expression of vimentin, cytokeratin, and OB-R by rabbit endometrial stromal (rESC) and epithelial cells (rEEC). LPA-2 binds to OB-R in rabbit endometrial cells. (**A**) Immunocytochemical results for the expression of various antigens in rabbit endometrial cells. (**Aa**) Expression of vimentin was found only in rESC cultures. (**Ab**) rESC express OB-R. (**Ac**) Expression of cytokeratin was found only in rEEC cultures. (**Ad**) rEEC also express OB-R. (**Ae**) The cells were incubated with nonspecific-species-matched IgGs to primary antibodies (negative control). Expression of OB-R was not changed in any cell type by effects of leptin or LPA-2 (data not shown). Arrows indicate positive staining for the different antigens tested. (**B**) Western blot determination of OB-R expression by rabbit endometrial cell cultured in medium containing leptin or LPA-2. rESC: (1) MW marker, (2) basal medium, (3) leptin (3 n*M*), (4) LPA-2 (300 n*M*), (5) leptin (3 n*M*) plus LPA-2 (300 n*M*). rEEC: (6) basal medium, (7) leptin (3 n*M*), (8) LPA-2 (300 n*M*), (9) leptin (3 n*M*) plus LPA-2 (300 n*M*). A 190 kDa band corresponding to the OB-R monomer (long form of functional OB-R) was detected in all samples. In addition, several bands corresponding to OB-R isoforms with lower MW were detected. However, neither the treatment with leptin nor LPA-2 changes the expression of the OB-R isoforms. (**C**) LPA-2 but not its scrambled version (LPA-2Sc) competes with ^{125}I-leptin for binding to OB-R in protein extracts from endometrial rabbit cells. LPA-2 binds with high affinity to OB-R ($K_i \sim 0.6 \times 10^{-10}$ *M*) expressed by rabbit endometrial cells (rEEC and rESC).

the cells incubated with non-specific goat IgGs did not down-regulate p-Stat3 after leptin treatment (See last lane in Figs. 2F and 2H).

As it was found in the immunocytochemical studies, LPA-2 (3–300 n*M*) effectively inhibited the basal expression of p-Stat3 in rESC (Fig. 2G) and rEEC (Fig. 2I). No inhibitory effect on p-Stat 3 expression was seen when the cells where incubated with LPA-2Sc (See last lane in Figs. 2G and 2I).

Leptin Regulation of IL-1R tI Expression by Endometrial Rabbit Cells

Results from immunocytochemical and Western blot studies on leptin regulation of IL-1R tI expression and the antibody and LPA-2 effects were similar in rESC and rEEC. Figure 3 shows results from rESC. Rabbit endometrial cells cultured in basal medium weakly express IL-1R tI (Fig. 3Aa). Leptin (3 n*M*) substantially up-regulates IL-1R tI expression (Fig. 3Ab). Moreover, as was found for p-Stat3 expression, the leptin effect on IL-R tI expression was completely abolished by the addition of LPA-2 (Fig. 3Ac). However, this LPA-2 inhibitory effect was more evident in rESC than in rEEC cultures (data not shown). Cells incubated with non-specific mouse IgGs did not show any positive staining for IL-1R tI (Fig. 3Ad).

The Western blot analysis of IL-1R tI expression from the cellular lysates of rESC cultures (Figs. 3B and 3C) confirmed the immunocytochemical results. The incubation of rESC cultures with anti-OB-R antibody was used as a positive control for the OB-R inhibition (Fig. 3B). The increase in intensity of the 80 kDa band corresponding to IL-1R tI in the blot from protein extracts of rESC following incubation of the cells with leptin (3 n*M*, number 2 in Fig. 3B) compared to cells cultured in basal medium (number 1 in Fig. 3B) demonstrates the leptin up-regulation of IL-1R tI expression. The addition of anti-OB-R antibody neutralized the leptin up-regulation of IL-1R tI (number 3 in Fig. 3B), further demonstrating the specificity of the leptin effect.

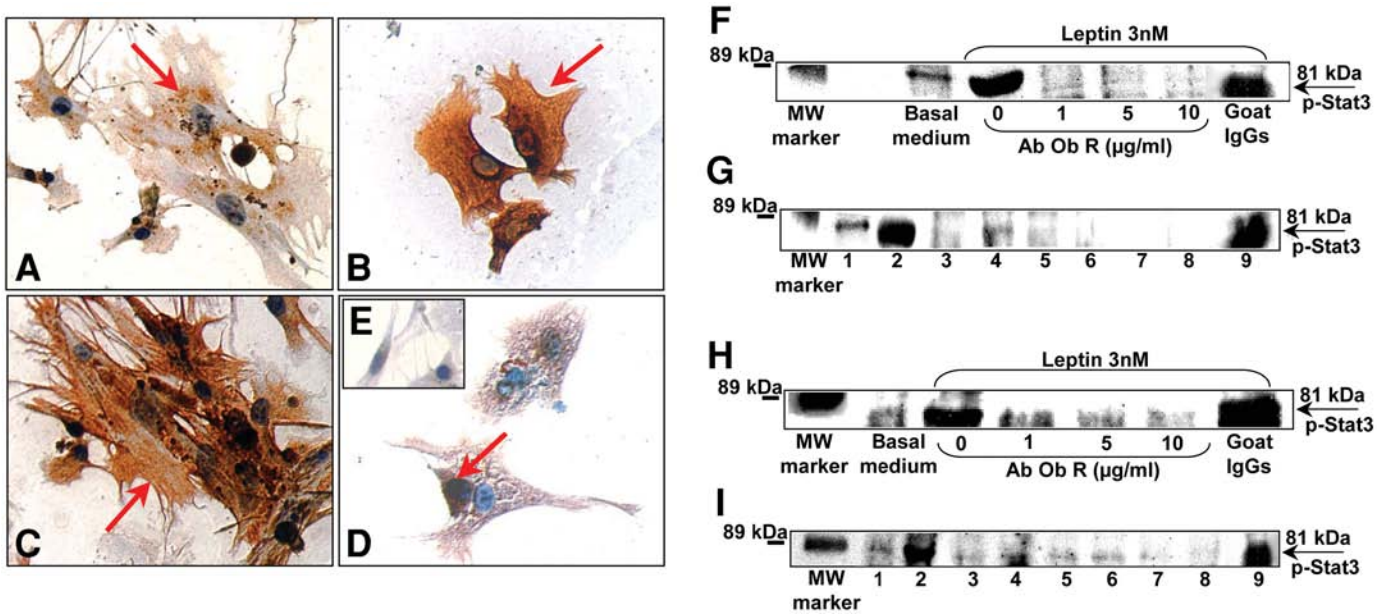

Fig. 2. Inhibition of leptin up-regulation of p-Stat3 expression in rabbit endometrial cells by an anti-OB-R antibody and LPA-2. (**A–E**) Immunocytochemical results from the expression of p-Stat3 by rabbit endometrial stromal cells (rESC) cultured in medium containing leptin, LPA-2 or LPA-2Sc (**A–E**). (**A**) Basal expression of p-Stat3. (**B**) pStat3 up-regulation by leptin (3 n*M*). (**C**) LPA-2Sc did not affect the leptin up-regulation of p-Stat3. (**D**) In contrast, LPA-2 significantly inhibits the leptin up-regulation of p-Stat3. (**E**) Negative control using nonspecific-species-matched IgGs to primary antibodies. Arrows indicate positive staining for the different antigen tested. (**F–I**) Western blot analysis of the leptin up-regulation of p-Stat3 expression and inhibition of this leptin effect by an anti-OB-R antibody (1–10 µg/mL) and LPA-2 (3–300 n*M*) in rESC and rabbit endometrial epithelial cells (rEEC). (**F**) rESC: Inhibition of the leptin up regulation of p-Stat3 in rESC by anti-OB-R antibodies. (**G**) rESC: Inhibition of the leptin up regulation of p-Stat3 in rESC by LPA-2. (1) Basal medium; (2) leptin (3 n*M*); (3) LPA-2 (3 n*M*); (4) leptin (3 n*M*) plus LPA-2 (3 n*M*); (5) LPA-2 (30 n*M*); (6) leptin (3 n*M*) plus LPA-2 (30 n*M*); (7) LPA-2 (300 n*M*); (8) leptin (3 n*M*) plus LPA-2 (300 n*M*); and (9) leptin (3 n*M*) plus LPA-2Sc (300 n*M*). (**H**) rEEC: Inhibition of the leptin up regulation of p-Stat3 in rESC by anti OB-R antibodies. Leptin up-regulated p-Stat3 expression. (**I**) rEEC: Inhibition of the leptin up-regulation of p-Stat3 in rESC by LPA-2. (1) Basal medium; (2) leptin (3 n*M*); (3) LPA-2 (3 n*M*); (4) leptin (3 n*M*) plus LPA-2 (3 n*M*); (5) LPA-2 (30 n*M*); (6) leptin (3 n*M*) plus LPA-2 (30 n*M*); (7) LPA-2 (300 n*M*); (8) leptin (3 n*M*) plus LPA-2 (300 n*M*); and (9) leptin (3 n*M*) plus LPA-2Sc (300 n*M*). Endometrial cells were cultured for 24 h in basal medium alone or containing leptin, anti-OB-R antibody or LPA-2. Negative control includes the cells cultured in medium containing leptin (3 n*M*) plus 10 µg/mL of nonspecific goat IgGs or leptin (3 n*M*) plus LPA-2Sc (300 n*M*). P-Stat3 (81kDa band) was detected by Western blot.

Furthermore, incubation of rESC with non-specific goat IgGs and leptin did not prevent the leptin up-regulation of IL-1R tI expression (number 4 in Fig. 3B).

Interestingly, LPA-2 exhibited inhibitory effects on leptin up-regulation of IL-R tI expression in rESC cultures similar to those found with anti-OB-R antibody (Fig. 3C). LPA-2 (number 3 in Fig. 3C) inhibited the basal expression of IL-1R tI by rESC (number 2 in Fig. 3C). Moreover, leptin up-regulation of IL-1R tI (number 1 in Fig. 3C) was significantly decreased by LPA-2 in a dose-response manner (number 4 and 5 in Fig. 3C).

In contrast, LPA-2Sc did not alter the leptin effect on IL-1R tI expression (number 6 in Fig. 3C). Results from Western blot of rEEC lysates were similar (data not shown).

Leptin Regulation of LIF Secretion and LIF-R Expression by Rabbit Endometrial Cells

The effects of leptin on LIF-R expression in rESC and rEEC were also investigated. We found that leptin up-regulates the expression of LIF-R in both cell types. The immunocytochemical results from LIF-R staining in rESC are shown in Figs. 4A–D. The basal expression of LIF-R (Fig. 4A) was increased by leptin (Fig. 4B). Once again, the incubation of rESC cultures with leptin plus LPA-2 showed that this peptide also inhibited the leptin effect on LIF-R expression (Fig. 4C). Negative controls for LIF-R staining with rESC incubated with nonspecific goat IgGs exhibited no staining (Fig. 4D).

Western blot analysis of rESC and rEEC lysates for LIF-R were also performed. Figure 4E shows representative results from rEEC. Leptin at 3 n*M* significantly increases the expression of LIF-R (90 kDa band; number 2 in Fig. 4E) relative to the basal level (number 1 in Fig. 4E). The addition of anti-OB-R antibody to rEEC cultures containing leptin (3 n*M*) completely inhibited LIF-R expression (number 3 in Fig. 4E) in contrast to the negative control, consisting of cells incubated with nonspecific goat IgGs (number 4 in Fig. 4E). LPA-2 totally inhibited the leptin effect on LIF-R expression at 30 and 120 n*M* doses (number 5 and 6 in Fig. 4E, respectively). Incubation of rEEC with LPA-2Sc did not prevent the leptin up-regulation of LIF-R (number 7 in Fig. 4E).

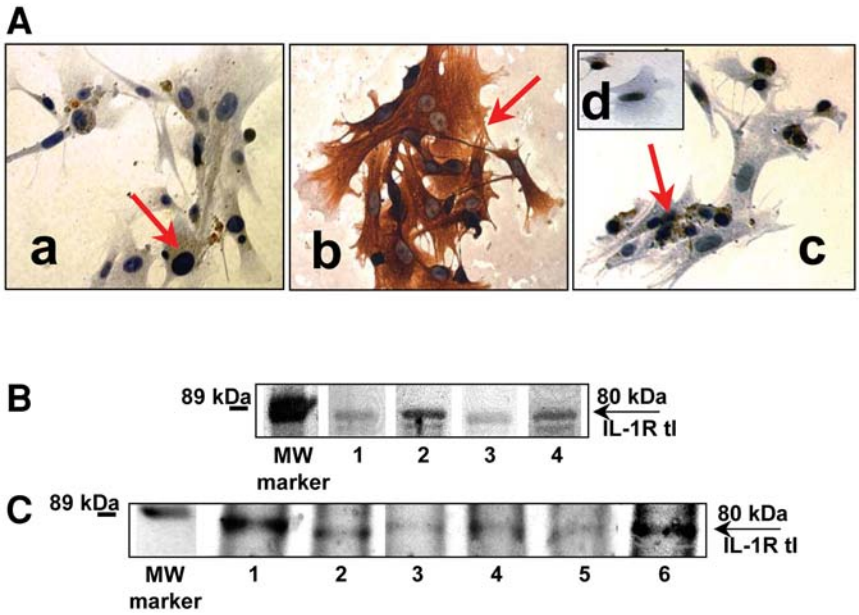

Fig. 3. Leptin up regulation of IL-1R tI by rabbit endometrial stromal cells (rESC). Inhibition of leptin effects by an anti-OB-R antibody and LPA-2. (**A**) Immunocytochemical results from the expression of IL-1R tI. (**Aa**) Basal expression of IL-1R tI. (**Ab**) IL-1R tI up-regulation by leptin (3 n*M*). (**Ac**) LPA-2 significantly inhibits the leptin up regulation of IL-1R tI. (**Ad**) Negative control using nonspecific mouse IgGs. Arrows indicate positive staining for IL-1R tI. (**B**) Western blot analysis of the effects of leptin and anti-OB-R antibody on IL-1R tI- expression by rESC. (1) Basal medium; (2) leptin (3 n*M*); (3) leptin (3 n*M*) plus anti-OB-R antibody (20 µg/mL); (4) leptin (3 n*M*) plus nonspecific mouse IgGs (20 µg/mL). (**C**) Western blot analysis of the effects of leptin and LPA-2 on the IL-1R tI expression by rESC. (1) leptin (3 n*M*); (2) basal medium; (3) LPA-2 (120 n*M*); (4) leptin (3 n*M*) plus LPA-2 (30 n*M*); (5) leptin (3 n*M*) plus LPA-2 (120 n*M*); and (6) leptin (3 n*M*) plus LPA-2Sc (120 n*M*). Endometrial cells were cultured for 24 h in basal medium alone or containing leptin, anti-OB-R antibody, or LPA-2. Negative control includes the cells cultured in medium containing leptin (3 n*M*) plus 20 µg/mL of nonspecific goat IgGs or leptin (3 n*M*) plus LPA-2Sc (300 n*M*). IL-1R tI (80 kDa band) was detected by Western blot using a specific monoclonal antibody (R&D System). Incubation of rESC with nonspecific IgGs or LPA-2Sc did not prevent the leptin up-regulation of IL-1R tI. Similar results were obtained with rabbit endometrial epithelial cells (rEEC).

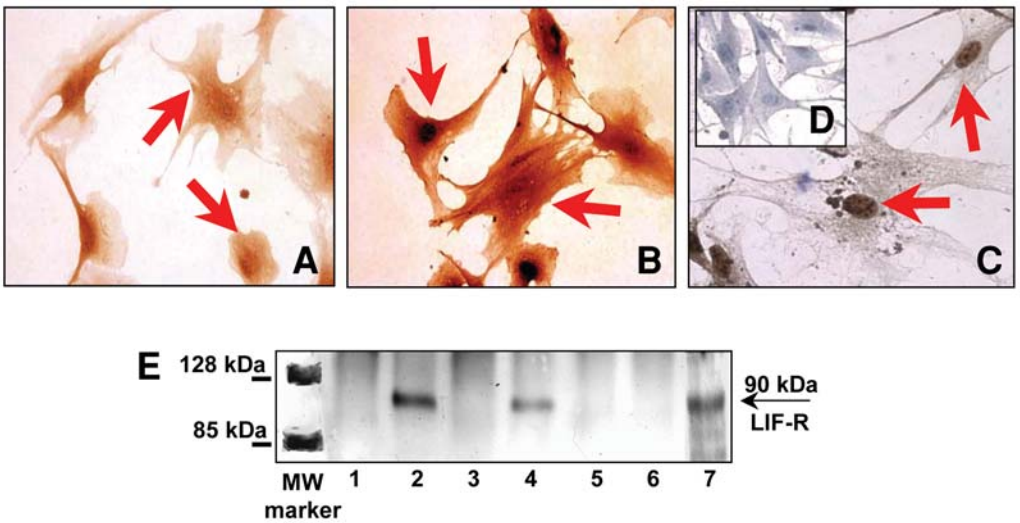

Fig. 4. Leptin up-regulation of LIF-R by rabbit endometrial cells. Inhibition of leptin effects by an anti OB-R antibody and LPA-2. Immunocytochemical results from the expression of LIF-R. (**A**) Basal expression of LIF-R in rabbit endometrial stromal cells (rESC). (**B**) LIF-R up-regulation by leptin (3 n*M*). (**C**) LPA-2 significantly inhibits the leptin up-regulation of LIF-R. (**D**) Negative control using nonspecific goat IgGs. Arrows indicate positive staining for LIF-R. Similar results were found in rabbit endometrial epithelial cells (rEEC). (**E**) Western blot analysis of the effects of leptin, anti OB-R antibody and LPA-2 on LIF-R expression by rEEC. (1) Basal medium; (2) leptin (3 n*M*); (3) leptin (3 n*M*) plus anti-OB-R antibody (20 µg/mL); (4) leptin (3 n*M*) plus nonspecific goat IgGs (20 µg/mL); (5) leptin (3 n*M*) plus LPA-2 (3 n*M*); (6) leptin (3 n*M*) plus LPA-2 (30 n*M*); and (7) leptin (3 n*M*) plus LPA-2Sc (300 n*M*). Endometrial cells were cultured for 1 h in basal medium alone or containing leptin, anti- OB-R antibody or LPA-2. Negative control includes the cells cultured in medium containing leptin (3 n*M*) plus 20 µg/mL of nonspecific goat IgGs or leptin (3 n*M*) plus LPA-2Sc (300 n*M*). LIF-R (90 kDa band) was detected by Western blot using a specific monoclonal antibody (R&D System). Incubation of rabbit endometrial cells with nonspecific IgGs or LPA-2Sc did not prevent the leptin up-regulation of LIF-R.

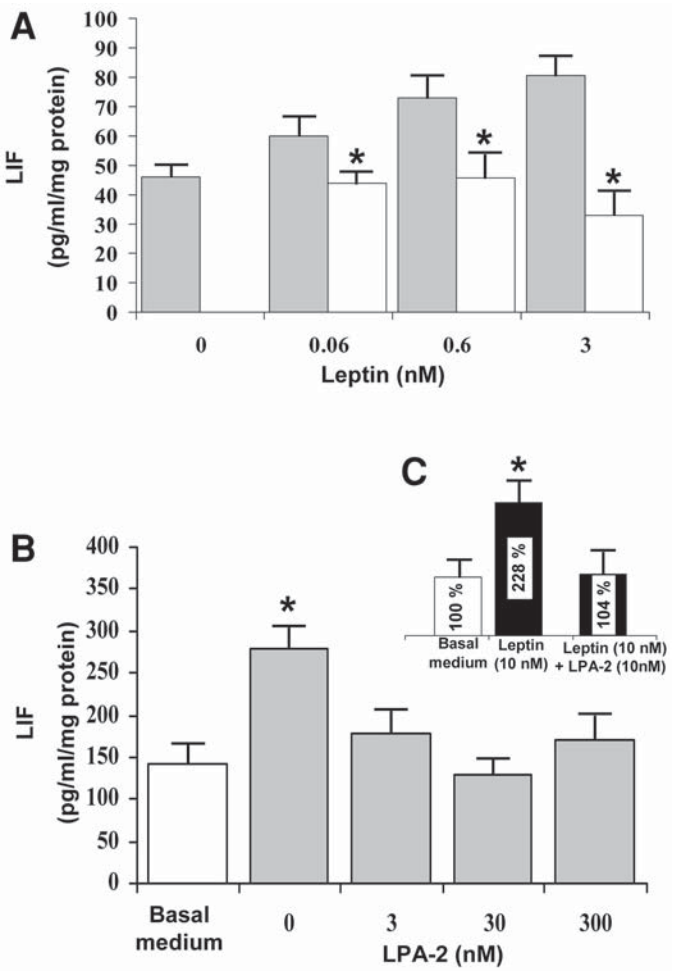

Fig. 5. Leptin regulation of LIF secretion by rabbit endometrial epithelial cells (rEEC). Inhibition of leptin effects by an anti-OB-R antibody and LPA-2. (**A**) Basal secretion of LIF by rEEC is up-regulated in a dose-response manner by leptin (filled bars) and the addition of the OB-R antibody (20 μg/mL) blocks this leptin effect (empty bars). (**B**) The up-regulation of LIF secretion by leptin (3 n*M*) is effectively inhibited by LPA-2 (3–300 n*M*). (**C**) Higher dose of leptin (10 n*M*) provoked a significant increase of LIF secretion (more than twofold) by rEEC cultures. However, the addition of LPA-2 (10 n*M*) completely inhibited the leptin up-regulatory effects on leptin induced LIF secretion by rEEC. $^*p < 0.05$.

LIF secretion by rabbit endometrial cell cultures was low but detectable by the ELISA kit. LIF concentrations were divided by the milligrams of total protein present in the cell lysates and expressed as pg/mL/mg protein.

Secretion of LIF by rEEC was higher than in the case of rESC (not shown) under all-experimental conditions. Moreover, leptin increased LIF secretion in a dose-dependent manner, the effect being more evident in rEEC. Moreover, the blockade of OB-R with the OB-R antibody abolished the leptin effect (Fig. 5A).

Interestingly, the addition of LPA-2 at doses from 3 to 300 n*M* significantly inhibited the leptin (3 n*M*) induction of LIF secretion by rEEC (Fig. 5B). A higher leptin dose (10 n*M*) resulted in a twofold increase in LIF secretion by rEEC, which was effectively inhibited by the addition of equimolar LPA-2 (Fig. 5C).

Discussion

The human endometrium expresses both leptin *(11,19)* and functional OB-R *(13,19,40).* OB-R expression in human endometrium fluctuates during the menstrual cycle *(40).* Low expression of OB-R in the human endometrium has been related to subfertility *(13)*.

To further study leptin functions in the endometrium, we investigated whether rEC express functional OB-R and whether leptin regulates IL-1R tI and LIF (ligand and receptor) protein expression in these cells.

Here we show for the first time that rEC constitutively express OB-R and that human leptin can up-regulate IL-1R tI, LIF and LIF-R protein expression in these rEC cultures. Furthermore, in order to study leptin actions in the endometrium, we produced and tested a potential leptin peptide antagonist (LPA-2). This peptide appears to be an efficient inhibitor of OB-R function in rEC.

A number of options exist that might be used to block leptin actions including (i) blockade of either leptin or functional OB-R using specific antibodies, (ii) modulation of free leptin levels by addition of exogenous inactive isoforms of leptin receptor (OB-Re), or (iii) production of leptin antagonists that can bind to OB-R without transmitting appropriate cellular signals.

In spite of the fact that in vivo leptin actions can be modulated by a soluble form of leptin receptor (OB-Re) *(5,41, 42)*, there are no available data on in vitro leptin inhibition by soluble OB-Re.

Only a few studies on the blockade of leptin or OB-R with specific antibodies have been published that provide information on the regulatory effects of leptin. The blockade of OB-R with a polyclonal antibody against the extracellular domain inhibits the rates of formation of expanded mouse blastocyst and hatched blastocyst in an in vitro model of embryo development *(18)*. We have also found that the blockade of OB-R with a specific antibody inhibits the up-regulatory effects of leptin on the expression of β3-integrin and the IL-1 system in endometrial cell cultures *(21).*

The large extracellular domain of OB-R (816 amino acids) is common to all OB-R forms, and the variable length cytoplasmatic tail (300 amino acid residues) distinguishes the several isoforms *(43)*. The extracellular region of cloned OB-R differs from that of many other cytokine receptors in that it contains several homologous segments representing potential ligand binding sites. This region takes on a mosaic structure, which consists of an immunoglobulin C2-like (C2) domain, four fibronectin type 3-like (F3) domains, and two-cytokine receptor (CK) domains. Deletion and substitution mutants of OB-R demonstrate that first binding motif is not required for leptin binding and receptor activation, whereas

modification of the second potential binding motif can lead to inactive receptor mutants. The leptin binding site has been suggested to involve residues 323–640, which encompasses the second segment of CK and F3 domains *(37)*.

The main goal of this study was to design and generate an LPA capable of inhibiting OB-R functions in the endometrium. Comparison of leptin amino acid sequences available from the Swiss Protein Sequence Data Base (Protein Data Bank) reported to date using the CLUSTAL W program indicated that the leptin sequence is conserved in a majority of mammalian species. Although the sequence of rabbit leptin has not yet been published, our studies assume it to be homologous to these other mammalian leptins. Therefore, in this investigation we used recombinant human leptin to assess OB-R function in rabbit endometrial cell cultures.

A structural model of leptin binding to OB-R was constructed by superimposing the four-helix bundle structure of leptin *(40)* on that of a homologous protein whose structure is known, viz., granulocyte-colony stimulator factor (G-CSF) complexed to its receptor G-CSF R *(38)* using program "O" *(44)*. Analysis of this model suggested that leptin interacts with the CK binding domain *(37)* of its receptor, and regions of its sequence corresponding to helices I and III formed the interacting surfaces. These helices correspond to amino acids 3–34 and 70–95, respectively, of the leptin sequence. All these amino acids have been previously suggested to participate in leptin/OB-R interactions *(38)*. Interestingly, all amino acids found in helices I and III of leptin belong to the highly conserved regions of the protein and are located in the N-terminal region which exhibits the highest biological activity *(45)*.

Consequently, we hypothesized that a peptide derived from these putative leptin binding sequences will compete with leptin for binding to OB-R and may block the homodimerization of the receptor, which is thought to be required for biological activity *(46)*. An ideal LPA, therefore, should have two principal properties; (i) high binding affinity for OB-R and (ii) effective blockade of leptin signaling.

New efforts in drug development have focused on leptin-related synthetic peptide agonists as potential anti-obesity drugs *(47)*. The fact that a single base mutation of the *ob* gene in codon 105 (*ob/ob* mice) results in the replacement of arginine by a premature stop codon and hence the production of a truncated inactive form of leptin suggests that leptin activity may be localized, at least in part, to domains distal to amino acid residue 104. Leptin-derived peptides containing amino acid sequences between residues 106 and 140 of the leptin molecule have been shown to significantly reduce weight gain by *ob/ob* mice *(48)*.

It has also been reported that the R128Q leptin mutant binds to OB-R but is unable to trigger intracellular signaling *(40,49)*. However, further investigations on R128Q effects in vitro and in vivo have so far been inconclusive *(50)*. Moreover, analysis of the 2:2 (leptin:OB-R) model proposed by Hiroike et al. *(38)* shows that Arg-128 does not participate in leptin binding to its receptor. This suggests that an effective leptin peptide antagonist should comprise sequences of leptin binding sites.

Therefore, we synthesized, purified, and tested a peptide, LPA-2 that contains amino acids 70–95 from helix III of human leptin. To assess the effects of LPA-2 on leptin function, we used rEC. Indeed, the addition of leptin to rEC cultures triggers the phosphorylation of Stat3 and up-regulates the expression of components of two cytokine systems (LIF and IL-1) that have been suggested to be important for mammalian reproduction.

Unfortunately, the amino acid sequence of rabbit LIF has not been determined nor do commercial kits exist for measuring LIF secretion by rabbit cells. However, recombinant human LIF (hrLIF) has been used to study reproductive function in other mammals *(51)*. Therefore, for measuring LIF secretion by rEC cultures, we used an ELISA kit with hrLIF as the standard. We found significant increases in secretion of LIF by rEEC and rESC after leptin treatments. The increases of LIF were blocked by anti-OB-R antibody and LPA-2 treatments. Levels of LIF were low in the conditioned media possibly as a consequence of the lack of proper reactivity between the antibodies in the ELISA kit with rabbit LIF. On the other hand, rEC may secrete low concentrations of LIF.

The anti-human OB-R antibody, used as positive control, effectively blocks the up-regulatory effects of leptin found in rEC cultures. Interestingly, LPA-2 binds to OB-R with high affinity suggesting that it is able to interact with the CK binding site of OB-R as was postulated from the leptin-binding model. The affinity of LPA-2 for OB-R binding was comparable to those reported for human leptin binding to human OB-R *(52,53)*. Moreover, LPA-2 effectively abolishes the leptin up-regulation of immunoreactive p-Stat3 in rEC.

These data indicate that LPA-2 binds to OB-R without initiating intracellular signaling in rEC. Consequently, LPA-2 also inhibits the leptin induced up-regulation of IL-1R tI and LIF-R expression as well as LIF secretion by rEC cultures. We have recently observed similar results with human endometrial cells (unpublished results).

Leptin up-regulates the expression of IL-1R tI by human ESC and EEC. The addition of OB-R antibody to these cell cultures blocks the leptin effects. However, higher concentrations of the OB-R antibody are needed to completely inhibit the leptin up-regulatory effects on IL-1R tI expression by EEC than ESC *(21)*. Similarly, in this investigation we found that the LPA-2 inhibitory effects on leptin up-regulation of IL-1R tI expression by rabbit endometrial cells were more evident in ESC than in EEC cultures. This fact could suggest that ESC cultures are more sensitive to leptin effects, therefore the blockage of OB-R function is found at lower concentrations of the OB-R specific inhibitors.

Leptin is well known as a regulator of food intake and energy balance. Much research has been directed toward

understanding and exploiting the effects of leptin on the control of body weight. Our research over the past several years, however, has focused on the role of leptin in human reproduction, specifically in the regulation of implantation and placentation. We have previously proposed that the leptin system, ligand and receptor, could have autocrine/paracrine roles in preimplantation embryo development and implantation that could also affect placentation *(1,19,20,23).*

The actions of IL-1β and leptin can be related at the endometrial level. Both cytokines have been reported to up-regulate β3-integrin expression *(20–22)* in the endometrium which suggests they could improve endometrial receptivity *(54,55)*.

In addition to adipose tissue *(56)* and endometrium *(11, 13,19,40),* leptin, together with its receptor, is expressed in many reproductive tissues, i.e., follicular cells *(57,58)*, oocytes and preimplantation embryos *(18,59)*, and placenta *(60, 61)*. Leptin produced by placental or fetal tissues could play a role in the regulation of fetal growth and development. We have previously shown that leptin also promotes the expression of markers of the invasive trophoblast phenotype *(23)*.

Leptin could be also involved in the development of endometriotic lesions where up-regulation of leptin synthesis *(11)* together with IL-1, IL-6, and TNF-α secretion *(62)* have been found. In addition, leptin levels have been found positively associated with endometrial cancer *(7).* However, it is unknown whether leptin plays a specific role in endometrial carcinogenesis or whether it is a simple correlate of obesity *(7).*

The specific mechanisms whereby leptin modulates reproductive function are unknown *(1,4)*. Moreover, the extent to which leptin per se mediates fetal growth and developmental abnormalities associated with disease states such as diabetes, hypoxia, or preeclampsia remains to be fully clarified by future studies in humans *(63)*.

Paradoxically, human obesity is not characterized by leptin deficiency. On the contrary, leptin levels are very high in obese individuals. An attractive idea has been suggested, that obesity could be a state of leptin resistance, but evidence for this view is limited *(64–66)*. We found that a peptide, LPA-2, which comprises a sequence of human leptin (from the leptin binding region to OB-R) can inhibit OB-R function. It is tempting to speculate that one or more similar peptides resulting from normal leptin catabolism in humans could be a natural inhibitor of OB-R in vivo. However, further research is needed to validate this hypothesis.

In conclusion, we found that rEC express OB-R and that in these cells leptin up-regulates IL-1R tI protein expression as was previously found in hEC *(21)*. Leptin also up-regulates the secretion of immunoreactive LIF and the expression of LIF-R by rEC. Interestingly, LPA-2 inhibits these leptin effects that could regulate critical steps in the development of endometrial receptivity and/or implantation. LPA-2 might therefore be useful as a novel tool to study leptin functions in the endometrium and an agent for fertility control. Moreover, LPA-2 may be useful to study leptin regulation in disease states such as preeclampsia, endometriosis, diabetes mellitus, and obesity, all conditions associated with abnormalities in leptin function.

Materials and Methods

Hormones, Antibodies, and Reagents

Human recombinant leptin, monoclonal antibodies anti-IL-1R tI, anti-LIF-R, and goat polyclonal antibody to the NH_2 terminal end of human OB-R were provided by R & D System Inc., MN. Monoclonal antibody (B-7) for phosphorylated Stat3 (p-Stat3) and nonspecific mouse and goat sera were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Anti-vimentin, anti-cytokeratin (pan-cytokeratin), and anti-CD45 antibodies were from Dako Corporation, Carpinteria, CA. ^{125}I-human leptin was obtained from New England Nuclear (NEN, MA), and human recombinant insulin and MCDB-105 culture medium were obtained from Sigma Chemical Co., St Louis, MO. Fetal bovine serum (FBS) was obtained from Gemini Bioproducts, Woodland, CA, and Dulbecco's modified Eagle's medium (DMEM) and antibiotic–antimycotic mixture were provided by GIBCO BRL Products, Gaithersburg, MD. Other chemicals were from Sigma.

Design of LPA-2

To design a potential leptin peptide antagonist (LPA), we analyzed data from the following sources: (a) Comparison of leptin sequences from different species using CLUSTAL W 1.81 (a multiple sequence alignment program). (b) Reported studies on the binding and biological activities of leptin analogs (deletion and mutants) (*45,49,50,52)*. (c) Building of a model for the leptin–leptin receptor complex using program "O" *(44)* based on a model previously proposed for leptin binding to OB-R *(38)*. For this purpose we superimposed the four-helix bundle structure of leptin *(39)* onto G-CSF in the complex with its receptor G-CSF R. From the analysis of these data we designed LPA-2 to contain residues 70–95 of helix III of the human leptin molecule.

Synthesis and Purification of LPAs

LPA-2 (26 amino acids residues; SRNVIQISNDLENL RDLLHVLAFSKS) and a scrambled version of this peptide (LPA-2Sc; VAEVLNRSDLIQRISFSLDLNNSKLH) were synthesized by solid-phase peptide synthesis (Applied Biosystems, Model 431A Peptide Synthesizer) using 9-fluorenylmethoxycarbonyl (fmoc) chemistry. The peptides were purified using a C18 preparative high performance liquid chromatography (HPLC) column. The program Protean (DNA STAR) was used to compare the LPA-2 and LPA-2Sc peptides to ensure that the two peptides had dissimilar secondary structures.

Because LPA-2 peptides proved to be sparingly soluble in water, dimethyl sulfoxide (DMSO) was used as solvent

for the preparation of concentrated solutions of these peptides. After solubilization with DMSO, it was then possible to dilute the peptides either in 50 m*M* Tris and 100 m*M* NaCl, pH 8.5, to promote proper re-folding or directly in culture medium to obtain the desired concentrations for the binding and cell culture experiments.

Protein Analytical Procedures

The purities of LPA-2 and LPA-2Sc were evaluated by reversed-phase HPLC. Circular dichroism spectroscopy was used to assess LPA-2 and LPA-2Sc secondary structures. The molecular masses of the peptides were determined by mass spectral analysis on a Voyager-RP Biospectrometer MALDI-TOF Workstation (Perseptive Biosystems, Cambridge, MA). Spectra were averages of approximately 200 scans.

Endometrial Tissues

Uteri from non-mated female New Zealand white rabbits were kindly provided by Dr. M. Ortega (Schepens & Eyes Institute, Harvard University, Boston, MA). The rabbit uteri used for preparation of endometrial cells were obtained under an approved IACUC protocol. Endometrial tissues were scraped from the uteri and then digested with proteases for isolation of endometrial cells as described elsewhere *(55)*. Briefly, endometrial tissues were minced and treated with collagenase I (0.1%)–DNAse I, 0.005% for 1 h at 37°C. rEEC were purified of rESC and macrophage contaminants by repeated incubation for 1–2 h at 37°C in a Falcon flask. rEEC and rESC dispersions were counted in a hemocytometer and cell viability was assessed by optical microscopy using the Trypan Blue exclusion method. The mean of cell viability was higher than 95%. The homogeneity of cell preparations was assessed by the expression of cytokeratin (EEC +), vimentin (ESC +), and CD45 (leukocytes +) using specific antibodies *(55)*. Homogeneity of cell preparations was approximately 98%.

Cell Cultures

Rabbit endometrial cells (5×10^5 cells/well) were cultured for 5–9 d in DMEM-MCDB105 (3:1) medium containing 10% fetal bovine serum (FBS), 5 µg/mL insulin, 1% amphopthericin B, 100 µg/mL streptomycin, and 100 U/mL penicillin until confluent layers were obtained. The cells were washed twice with 100 m*M* phosphate saline buffer (PBS)–2% BSA (w/v), pH 7.2 and cultured for an additional 2 d in the same medium but without FBS (basal medium). This procedure was performed to reduce any effects of cytokines from FBS on the phosphorylation rate of Stat3 as well as the expression of other cytokines and their receptors. Cells were washed as described before and cultured in basal medium containing leptin (0–10 n*M*), OB-R antibody (1–20 µg/mL) and/or LPA-2 peptides (0–300 n*M*). Cultures were stopped at 24 h and the cells were used for immunocytochemistry, binding studies, and Western blot analysis. The conditioned media were collected, lyophilized, and stored at –80°C for ELISA determinations. Duplicate wells were run for each treatment, and the experiments were repeated at least three times with different cell preparations from different rabbits. Controls were the same cellular preparations cultured in basal medium containing nonspecific goat IgGs and LPA-2Sc.

Determination of LIF Secretion by Rabbit Endometrial Cell Cultures

IL-1β from rabbits and humans have sequence differences, but no data are available concerning the sequence of rabbit LIF. In addition, there are no commercial kits for measuring rabbit IL-1β or LIF. However, because LIF has been suggested to be important for mammalian reproduction, we attempted to measure LIF in rabbit culture supernatants using a quantitative method designed for determining human LIF. Conditioned media ($n = 3$ per treatment) from rESC and rEEC cultured in the experimental conditions described above were used to quantify the secretion of LIF by ELISA (LIF-Quantikine®, R&D Systems). Standards, controls, and samples were assayed in duplicate. The intra- and interassay coefficients of variations were between 0.7–12% and 3–7%, respectively. According to the manufacturer, the performance characteristics of the ELISA were as follows: 100% specificity and sensitivity less than 8 pg/mL for both natural and recombinant human LIF; no significant crossreactivity or interference was observed with a great diversity of human and mouse cytokines and growth factors.

Immunocytochemistry

The rabbit endometrial cells were cultured as described above in duplicate on 8-well glass-bottom culture plates (Nalgene Nunc International, Naperville, IL) and fixed with methanol at –20°C for 20 min for immunocytochemical studies. All the antibodies tested were diluted in PBS-2% BSA (w/v; buffer A). The expression of OB-R was assessed by incubation of rEC for 1 h at room temperature with goat antibodies directed toward the amino terminal ends of human OB-R (R&D system) diluted 1:80 in buffer A. Anti-IL-1R tI (1 µg/mL), anti-LIF-R (2 µg/mL), anti-vimentin (1:50), anti-cytokeratin (1:50), and anti-p-Stat3 (1 µg/mL) antibodies were used to assess the expression of the respective antigens by rEEC and rESC cultures. After incubation with primary antibodies the cells were incubated with a streptavidin-biotin-alkaline phosphatase system according to the manufacturer's directions (Vectastain, ABC-AP kit, Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin (Dako). Negative controls included cell preparations in which the primary antibodies were substituted by irrelevant species matched IgGs.

Preparation of Cell Lysates

After culture in the presence of leptin, LPAs or antibodies, the endometrial cells were washed with ice-cold PBS and lysed by homogenization on ice with lysis buffer (20 n*M* Tris, pH 7.4, containing 137 *nM* NaCl, 2 m*M* EDTA, 10%

glycerol, 50 m*M* b-glycerophosphate, 50 n*M* NaF, 1% Nonidet P-40, 2 m*M* phenyl-methylsulfonyl fluoride, and 2 m*M* sodium orthovanadate plus protease inhibitors *[21]*). Cellular lysates were centrifuged at 24,000*g* at 4°C for 10 min. Protein concentrations were determined using the Bradford protein assay (BioRad Laboratories Inc., Hercules, CA).

Western Blot Analysis

Protein extracts from cell lysates were combined (1:1) with Laemmli buffer (2X concentrated) and 10 µg of proteins were loaded per lane on 7.5% (for p-Stat3, IL-1R tI, and LIF-R) and 10% (for OB-R) SDS-PAGE gels. Electrophoresis was performed at 65 V for 1–1.5 h (BioRad, electrophoresis apparatus). Electroblotting onto 0.2 µm nitrocellulose membranes was performed at 22 V overnight at 4°C in 48 n*M* Tris–39 n*M* glycine buffer containing 0.037% SDS and 20% methanol. Membranes were blocked for 1 h at room temperature in 20 m*M* Tris, 137 m*M* NaCl pH 7.4 buffer containing 0.5% Tween 20 (v/v) (wash buffer) supplemented with 5% Amersham blocking reagent (blocking buffer) and rinsed three times with wash buffer. The membranes were subsequently incubated at room temperature for 1 h with 2 µg/mL of anti-OB-R, IL-1R tI, LIF-R, and p-Stat3 antibodies in blocking buffer. Detection was performed by incubation with biotinylated anti-mouse or anti-goat antibodies followed by incubation with streptavidin-horseradish peroxidase-conjugate (Amersham Pharmacia Biotech) for 30 min at room temperature. Positive specific antigen–antibody reactions in the blots were visualized using an ECL-chemiluminescent assay (Amersham) followed by exposure on KODAX X-Omat AR film (IBI-Kodak Ltd, Cambridge, U.K.). Nonspecific mouse and goat IgGs (Santa Cruz Biotechnology) were used instead of primary antibodies to produce negative control blots.

Binding Assays

^{125}I-human leptin served as ligand and LPA-2 and LPA-2Sc as competitors for the binding to OB-R in cellular extracts. All compounds were diluted in assay buffer (50 m*M* Tris and 100 m*M* NaCl, pH 8.5) to promote proper re-folding. Each assay tube contained 50 µL of cellular lysate (100 µg protein from 10^6 cells cultured for 2 d in basal medium), 50 µL of ^{125}I-human leptin (100,000 cpm), and 50 µL of LPAs (providing 10^{-14}–10^{-5} *M*/tube). The tubes were incubated for 24 h at room temperature. Then, 250 µL of 1% BSA (w/v) and 500 µL of 20% polyethylene glycol (w/v) in PBS were added and the tubes were incubated for an additional 12-h period at 4°C. The tubes were centrifuged at 12,000*g* for 15 min at 4°C. After careful aspiration of supernatant and complete drying of the tube walls with filter paper, ^{125}I-human leptin binding in the precipitates was measured (Beckman 5000, counter). The binding of ^{125}I-leptin to OB-R in absence of LPAs was set at 100%, and LPA competitive binding values were expressed as percentages of total cpm. Affinity binding constants for LPAs were calculated from the competition curves.

Statistical Analysis

A one-way ANOVA test with Dunnett error protection and a confidence interval of 95% was used from the Analyse-it for Microsoft Excel (Leeds, UK, htpp://www.analyse-it.com) for data analysis. Data are expressed as mean ± SEM. Values for $p < 0.05$ were considered statistically significant.

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