BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **244,** 30–34 (1998)
ARTICLE NO. RC988199

Autocrine Inhibition of Leptin Production by Tumor Necrosis Factor-α (TNF-α) through TNF-α Type-I Receptor *in Vitro*

Masaaki Yamaguchi,[1] Takashi Murakami,* Takuji Tomimatsu, Yukihiro Nishio, Nobuaki Mitsuda, Tohru Kanzaki, Hirohisa Kurachi, Kenji Shima,* Toshihiro Aono,† and Yuji Murata

*Department of Obstetrics and Gynecology, Osaka University Medical School, Suita 565, Japan; and *Department of Laboratory Medicine and †Department of Obstetrics and Gynecology, School of Medicine, University of Tokushima, Kuramotocho 3-chome, Tokushima 770, Japan*

Received January 27, 1998

The aim of this study was to find factors which regulate m-leptin secretion during pregnancy. Mouse parametrial adipocytes from day 13 of pregnancy were cultured with or without mouse placental lactogen (mPL)-I, mPL-II, or mouse tumor necrosis factor-α (mTNF-α) and mouse-leptin (m-leptin) concentration in the medium was assessed by RIA. Up to four days of mPL-I or mPL-II treatment did not affect m-leptin secretion. However, mTNF-α, which is produced by adipocytes, significantly inhibited m-leptin secretion in a dose- and time-dependent manner. Antibody to mTNF-α completely blocked the inhibitory effect of mTNF-α on m-leptin secretion. mTNF-α significantly inhibited the expression of m-leptin messenger RNA. Agonistic polyclonal antibody directed against the mTNF-type-I receptor (mTNF-RI) significantly inhibited m-leptin secretion, but the anti-mTNF-RII antibody did not change m-leptin secretion. Moreover, human TNF-α (h-TNF-α) also inhibited human-leptin (h-leptin) secretion by cultured human adipocytes collected from the subcutaneous fat of pregnant women. These results suggest that TNF-α, which is secreted by adipocytes, inhibits m-leptin secretion through mTNF-RI and suggest the presence of an autocrine or paracrine regulation of leptin secretion in human and mouse adipose tissue *in vivo*.

Leptin, mainly produced by adipocytes, is known to be involved in the regulation of body weight and food intake in humans and rodents (1,2). Recently, it was found that leptin also affects ovarian function and the development of sexual organs (3,4). Human placenta, but not mouse or rat placenta, also expresses leptin mRNA and produces the leptin protein (5,6). Serum human-leptin (h-leptin) and rat-leptin (r-leptin) concentrations increase about 2-3 fold (6-8), whereas serum mouse -leptin (m-leptin) concentration increases more than 20 fold during pregnancy (9,10). These findings suggest that the physiological importance of leptin during pregnancy may be different among these three species

In the rat, several factors, such as food intake, insulin, and glucocorticoids, are known to regulate serum r-leptin concentration (11–14). In the mouse, serum m-leptin concentration dramatically increases after mid-pregnancy (9,10), but factors that regulate m-leptin secretion during pregnancy have not been determined. We demonstrate here that although mouse placental hormones do not affect m-leptin secretion, tumor necrosis factor-α (TNF-α), which is produced by adipocytes, strongly inhibits leptin secretion from adipocytes through the TNF-type-I receptor (TNF-RI), suggesting the presence of an autocrine or paracrine regulation of m-leptin secretion by TNF-α.

MATERIALS AND METHODS

Reagents. RIA kits for mouse and human leptin were purchased from Linco Research (St. Louis, MO). β-actin and leptin cDNAs were described previously (9,15). Anti-mTNF-α antibody and recombinant mTNF-α were purchased from R&D Systems (Minneapolis, MN). Recombinant hTNF-α was a gift from Dainippon Pharmaceutical Co. Ltd (Osaka Japan). Anti-mTNF-RI and mTNF-RII antibodies were gifts from Genentech (South San Francisco, CA). mPL-II and recombinant mPL-I were kind gifts from Dr. F. Talamantes (University of California, Santa Cruz, CA).

Adipocyte cell cultures. Time-pregnant ICR mice were purchased from Japan SLC Inc. (Hamamatsu City, Shizuoka, Japan). The para-

[1] To whom correspondence should be addressed. Fax: 81-6-879-3359.

Abbreviations: h-leptin, human leptin; m-leptin, mouse leptin; r-leptin, rat leptin; hTNF-α, human tumor necrosis factor-α; mTNF-α, mouse TNF-α; mPL, mouse placental lactogen; mTNF-RI, mTNF type-I receptor, mTNF-RII, mTNF type-II receptor.

0006-291X/98 $25.00

metrial adipose tissue was collected from day 13 pregnant mice. The presence of a vaginal plug indicated day 0 of pregnancy. Human subcutaneous adipose tissue was collected from pregnant women when they underwent repeated Cesarean section at 37-39 weeks of gestation. All patients gave informed consent to take their adipose tissue. Adipocyte cultures were performed as described previously with several modifications (12). The adipose tissues were minced finely and incubated in dissociation medium (medium 199, 20 mM HEPES, 10 mM $NaHCO_3$, 50 μg gentamicin sulfate/ml, pH 7.4) containing 4.4 mg/ml collagenase and 0.002% DNAse at 37 C for 15 min. The cells were then filtered through mesh (400 μM) and centrifuged for 1 min at 100 rpm. The floating adipocytes were collected and were cultured in a culture medium (NCTC-109 with 20 mM Hepes, 50 μg gentamicin sulfate/ml) containing 10% fetal calf serum (FCS) at a density of 2×10^5 cells/cm^2. The cells were incubated at 37 C under an atmosphere of 95% air/5% CO_2. The medium was changed daily and stored at -20 C until assayed.

Leptin RIAs. Leptin concentration was determined by RIAs using the procedure described previously (9). The intra- and inter-assay coefficients of variation for the mouse leptin RIA were 6.8 and 9.2%, respectively. The intra- and inter-assay coefficients of variation for the human leptin RIA were 6.3 and 8.5%, respectively.

Northern blot analysis. Total RNAs were isolated from cultured adipocyte and Northern blot analysis was performed as described previously (16). The RNAs were hybridized with ^{32}P-labeled rat leptin cDNA and then re-hybridized with ^{32}P-labeled β-actin cDNA. Autoradiographs were scanned and band intensities were determined by a densitometer (Imaging Research Inc. St. Catharines, Ontario, Canada) as previously described (17). The leptin mRNA band intensity of each lane was normalized to that of the β-actin.

Statistical analysis. Data were analyzed for homogeneity of variance with Bartlett's test. Then the data were analyzed by analysis of variance for completely randomized design. Subsequent analysis was carried out with Scheffe's multiple range test, Dunnett's test, and unpaired *t*-test as required. A *P* value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

As previously reported, serum m-leptin concentration increases more than 20 fold after midpregnancy (9,10). Since the gestational profile of serum m-leptin concentration is very similar to that of serum mPL-II concentration (18), we first examined whether mouse placental hormones directly regulate m-leptin secretion *in vitro*. Cultured mouse parametrial adipocytes from day 13 of pregnancy were treated with or without mPL-I or mPL-II for 5 days and m-leptin concentrations in the medium were assessed by the RIA. Neither mPL-I nor mPL-II at a concentration of 5 μg/ml, which is close to the peak serum concentration during pregnancy (19,20), affected m-leptin concentration up to the fourth day of culture [mean $\pm$ SE (ng/ml), m-leptin concentration in the medium on the fourth day of culture: control, 11.9 $\pm$ 0.7; mPL-I, 11.1 $\pm$ 0.4; mPL-II, 10.5 $\pm$ 0.4; $n = 4$, $P > 0.05$]. These results suggest that placental hormones may not be the factor regulating m-leptin secretion, although other mouse placental hormones, such as proliferin (PLF), PLF-related protein, and GHRF, were not tested. We next examined whether TNF-α regulates m-leptin secretion, since it is known that adipocytes produce TNF-α in humans and mice (21,22). The m-leptin concentration in the medium was 16.0 $\pm$ 0.69 ng/ml ($n = 4$) on the first day of the adipocyte culture. Then the m-leptin concentration somewhat decreased and was 11.0 $\pm$ 0.83 ng/ml ($n = 4$) on the fourth day of the culture (Fig. 1). mTNF-α at a concentration of 100 ng/ml significantly inhibited m-leptin secretion by the second day of culture (Fig. 1). The effect was dose-dependent and the lowest concentration of mTNF-α which caused significant inhibition of m-leptin secretion was 1 ng/ml with greater inhibition at 10 and 100 ng/ml on the third day of culture (Fig. 2). Similar results were obtained when parametrial adipocytes collected from virgin mice were treated with mTNF-α (data not shown). To confirm that the effect of m-TNF-α on m-leptin secretion was specific, we added 5 ng/ml mTNF-α to cells simultaneously with 10 μg/ml anti-mTNF-α antibody. The non-specific effect of IgG on m-leptin secretion was taken into account by adding 10 μg/ml nonimmune IgG to the control and mTNF-α only incubations. The anti-mTNF-α antibody almost completely blocked the ability of mTNF-α to inhibit m-leptin secretion on the third day of culture (Fig. 3).

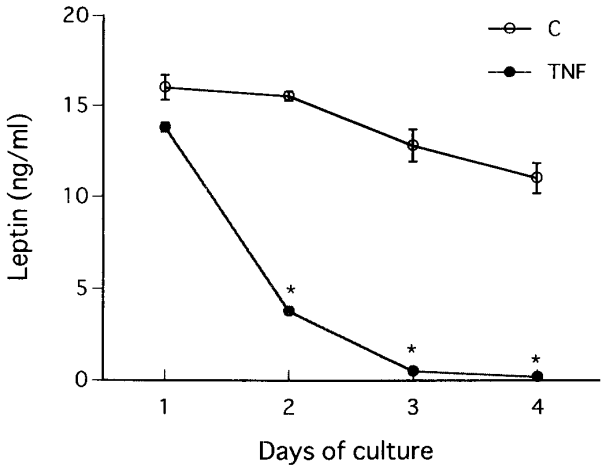

FIG. 1. Effect of mTNF-α on m-leptin secretion. 4×10^5 parametrial adipocytes from day 13 of pregnancy were incubated in the absence or presence of 100 ng/ml mTNF-α for 4 days. The medium was changed daily and assayed for the m-leptin concentration. Values represent the mean $\pm$ SE of 4 wells. *, Significantly different from control on the given day of culture ($P < 0.05$). The data shown are from representative experiments. The experiment was repeated three times with similar results.

To determine whether mTNF-α affects m-leptin mRNA, adipocytes were incubated with or without 10 ng/ml mTNF-α for 2 days and Northern blot analysis for m-leptin was performed as described in Materials and Methods. As shown in Fig. 4, mTNF-α significantly reduced steady-state levels of m-leptin mRNA [mean $\pm$ SE (arbitrary units), normalized band intensity: control, 1.08 $\pm$ 0.02; mTNF-α, 0.0731 $\pm$ 0.0136; $n = 3$, $P < 0.05$].

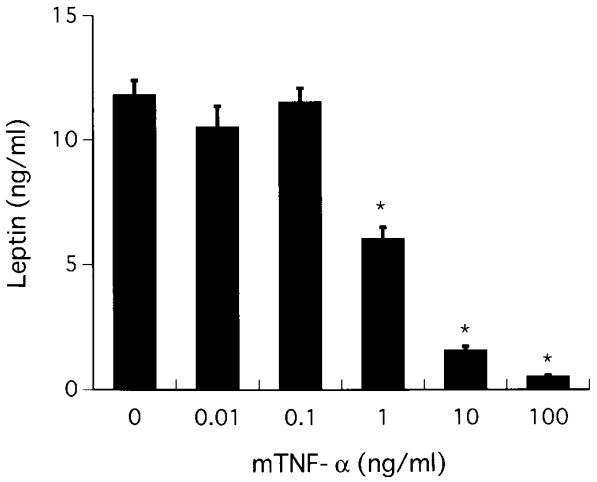

FIG. 2. Dose-dependent effect of mTNF-α concentration on m-leptin secretion. 4×10^5 parametrial adipocytes from day 13 of pregnancy were incubated for 3 days. The medium was changed daily and the m-leptin concentration was determined in the medium collected on the third day of culture. Each value represents the mean ± SE of 4 wells. *, Significantly different from control ($P < 0.05$). The data shown are from representative experiments. The experiment was repeated three times and similar results were obtained.

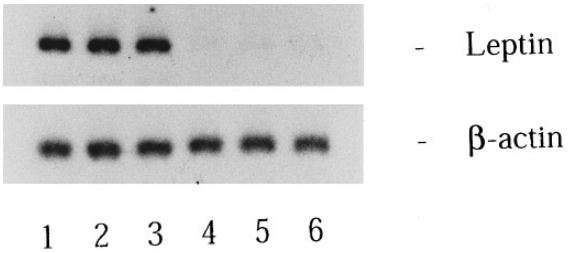

FIG. 4. Effect of mTNF-α on the m-leptin mRNA level. Cells from day 12 of pregnancy were plated at 2×10^6 cells per well in 6-well plates and incubated without (lanes 1-3) or with 10 ng/ml mTNF-α (lanes 4-6) for 2 days. Ten μg of total RNA from each well was subjected to Northern blot analysis. The upper panels show the blots hybridized with the ^{32}P-labeled r-leptin probe, and the lower panels show the same blots rehybridized with the ^{32}P-labeled β-actin probe. The data shown are from representative experiments. The experiment was repeated three times and similar results were obtained.

Two distinct receptors for TNF-α, TNF-RI and TNF-RII, have been identified (23–26) and they have no apparent similarity in their cytoplasmic domains (27). Moreover, interaction between the two types of receptor is not required for TNF-α signal transduction (23,24,28). These findings suggest that these two receptors employ different signal transduction pathways (27) and that each receptor mediates distinct physiological responses (29,30). To determine which receptor is employed by mTNF-α to inhibit m-leptin secretion, mouse adipocytes were incubated with agonistic polyclonal antibodies (31) for mTNF-RI or mTNF-RII for up to 3 days. Addition of 10 mg/ml of anti-TNF-RI antibody, but not anti-TNF-RII antibody, significantly inhibited m-leptin secretion (Fig. 5), suggesting that the

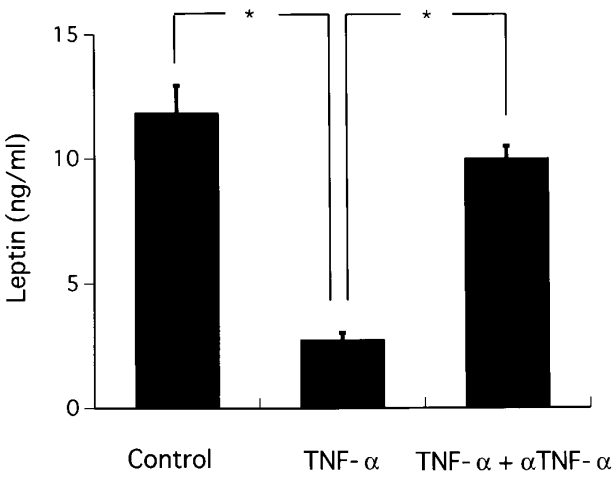

FIG. 3. Effect of anti-mTNF-α antibody on the inhibition of m-leptin secretion by mTNF-α. 4×10^5 parametrial adipocytes from day 13 of pregnancy were incubated for 3 days in a medium containing nonimmune IgG (10 μg/ml) (control), nonimmune IgG (10 μg/ml) with 5 ng/ml mTNF-α (TNF-α), and anti-mTNF-α antibody (10 μg/ml) with 5 ng/ml mTNF-α (TNF-α + αTNF-α). The medium was changed daily and m-leptin concentration was determined in the medium collected on the third day of culture. Each value represents the mean ± SE of 4 wells. *, Significantly different from control ($P < 0.05$). The data shown are from representative experiments. The experiment was repeated three times and similar results were obtained.

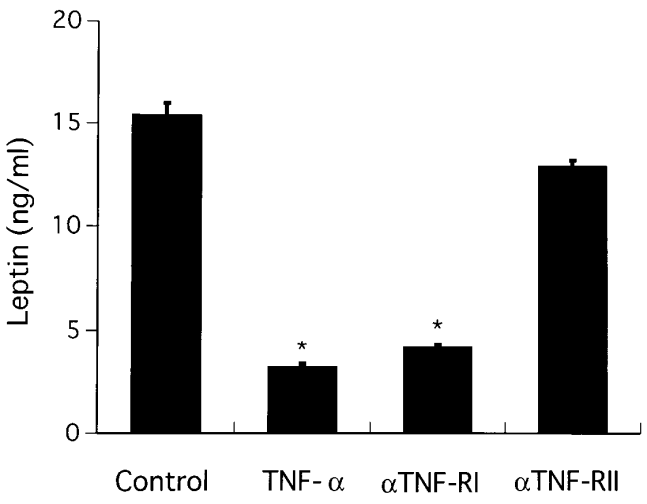

FIG. 5. Effect of mTNF-α and agonistic antibodies for mTNF-RI and mTNF-RII on m-leptin secretion. 4×10^5 parametrial adipocytes from day 13 of pregnancy were incubated in a medium containing nonimmune IgG (10 μg/ml) (control), nonimmune IgG (10 μg/ml) with 10 ng/ml mTNF-α (TNF-α), 10 μg/ml anti-mTNF-RI antibody (αTNF-RI), or 10 μg/ml anti-mTNF-RII antibody (αTNF-RII) for 3 days. The medium was changed daily and m-leptin concentration was determined in the medium collected on the third day of culture. Each value represents the mean ± SE of 4 wells. *, Significantly different from control ($P < 0.05$). The data shown are from the representative experiments. The experiment was repeated three times and similar results were obtained.

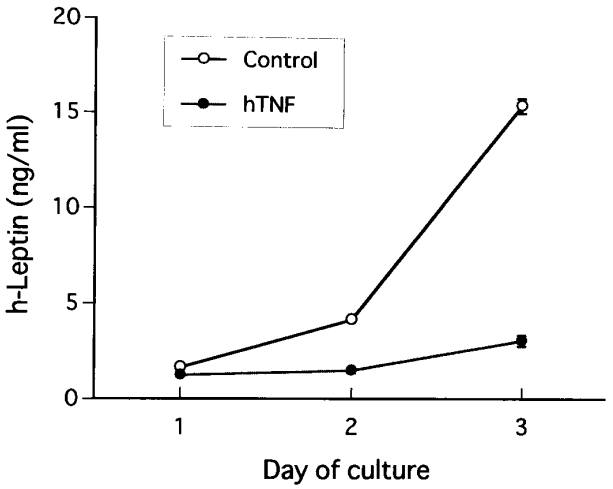

FIG. 6. Effect of hTNF-α on h-leptin secretion. 4×10^5 subcutaneous adipocytes from pregnant women at 37-39 weeks of gestation were incubated in the absence or presence of 100 ng/ml hTNF-α for 3 days. The medium was changed daily and assayed for the h-leptin concentration. Values represent the mean ± SE of 4 wells. Significant differences were observed from the second day of culture ($P < 0.05$). The data shown are from representative experiments. The experiment was repeated three times with similar results.

inhibitory effect of mTNF-α on m-leptin secretion acts through stimulation of mTNF-RI signal transduction.

To determine whether the effect of TNF-α on leptin secretion is specific for the mouse, human subcutaneous adipocytes collected from pregnant women were cultured with or without 100 ng/ml hTNF-α for three days and secreted h-leptin in the medium was determined by the RIA. hTNF-α significantly inhibited h-leptin secretion by the second day of culture (Fig. 6), suggesting that the effect of TNF-α on leptin secretion is not species specific.

In the human and rat, serum concentration of leptin increases only 2-3 fold during pregnancy (6-8), but that of m-leptin increases more than 20 fold (9,10). Mouse adipocytes *in vitro* increase m-leptin production only 2-3 fold and other tissues, including placenta, do not produce m-leptin. These findings suggest an increase in m-leptin half-life time or a reduction in clearance. In fact, Gavrilova et al. recently reported that dramatic increase of serum m-leptin concentration during pregnancy may result from production of soluble leptin receptor by placenta (10). In general, complexes of a protein and its binding protein increase the protein half-life and reduce the clearance (32).

In conclusion, we have shown that TNF-α, but not placental hormones, strongly inhibits leptin secretion by adipocytes through binding to TNF-RI in an autocrine or paracrine mechanism. However, factors which increase m-leptin secretion and the physiological roles of soluble leptin receptor during gestation remain to be identified.

ACKNOWLEDGMENTS

The authors thank Ms. Kayo Yamamoto for her technical assistance. We thank Dr. F. Talamantes for providing the mPL-II and recombinant mPL-I.

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