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Tissue-Specific Regulation of Leptin Expression and Secretion by All-*Trans* Retinoic Acid

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Abstract In addition to hormones and the sympathetic nervous system, specific nutrients may regulate leptin mRNA expression and adipose tissue metabolism. However, little is known regarding the effect of nutrients on leptin mRNA expression. Retinoic acid (RA) is a ligand of some nuclear receptors and previous reports have demonstrated contradictive effects on plasma leptin levels. Thus, we examined the effect of RA on expression of leptin in adipocytes of murine and human origin. After 48 h incubation of murine 3T3-L1 adipocytes with 1 and 10 μM all-*trans* RA, the expression of leptin mRNA was reduced by 56% and 65%, respectively, whereas the secretion of leptin was reduced by 38% and 77%, respectively. In human adipose tissue explants, 1 μM all-*trans* RA reduced leptin mRNA expression levels by 55% and leptin secretion by 25% after 24 h incubation. We observed an increased mRNA expression level of the transcription factors peroxisomal proliferator activated receptor γ (PPARγ), retinoid X receptor α (RXRα), and RA receptor α (RARα) in 3T3-L1 cells, whereas the mRNA level of these transcription factors was unchanged in human adipose tissue explants after incubation with RA. In two other leptin-expressing cell systems, the human placental throphoblast cell line BeWo and normal human primary osteoblasts, there was no effect of RA on leptin mRNA expression, but leptin secretion was reduced by 64% after 24 h incubation with 10 μM all-*trans* RA in BeWo cells. In conclusion, all-*trans* RA reduced both expression and secretion of leptin in human and rodent adipose tissue. In human BeWo cells or primary osteoblasts, leptin mRNA expression levels was not changed by all-*trans* RA, indicating a tissue-specific regulation of leptin mRNA expression by all-*trans* RA. J. Cell. Biochem. 92: 307–315, 2004.

Key words: all-*trans* retinoic acid; leptin; adipose tissue; placenta cells; bone

Leptin is a peptide hormone mainly secreted from adipose tissue with an important function in regulation of the amount of body fat [Zhang et al., 1994]. Plasma leptin concentration is proportional to body fat mass [Considine et al., 1996], and synthesis and secretion of leptin by adipocytes is under complex regulation. In addition to adipocytes, other tissues and cells, such as placenta [Masuzaki et al., 1997], osteoblasts [Reseland et al., 2001c], muscle [Wang et al., 1998], breast epithelium [Smith-Kirwin et al., 1998], and stomach [Mix et al., 2000] produce leptin. Except for inhibiting food intake and activating thermogenesis via the central nervous system [Havel, 2000; Schwartz et al., 2000], an important function of circulating leptin is probably to protect tissues from overload of triacylglycerol by stimulation of fatty acid oxidation [Wang et al., 1999]. Furthermore, leptin may be a growth hormone stimulating proliferation of several cell types, such as hematopoietic cells [Gainsford et al., 1996], endothelial cells [Sierra-Honigmann et al., 1998], and osteoblasts [Reseland et al., 2001c; Gordeladze et al., 2002]. In addition, leptin secreted by the placenta may function as a fetal growth factor during pregnancy [Hassink et al., 1997].

Fasting and refeeding, as well as insulin and glucocorticoids, markedly influence leptin production [Saladin et al., 1995; Kolaczynski et al., 1996]. In a previous study, it was shown that polyunsaturated fatty acids may regulate the production and secretion of leptin in vivo as well as in vitro [Reseland et al., 2001a,b]. All-*trans*

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retinoic acid (RA) is an active metabolite of vitamin A, and may increase uncoupling-protein 1 (UCP1) gene expression in brown adipose tissue and decrease leptin expression in white adipose tissue in rats [Kumar and Scarpace, 1998]. Reduction of leptin secretion by all-*trans* RA has also been demonstrated in human adipose tissue in vitro [Menendez et al., 2001] by binding to the retinoid acid receptor (RAR) [Giguere et al., 1987; Petkovich et al., 1987] or the retinoid X receptor (RXR) [Heyman et al., 1992; Levin et al., 1992]. Both RAR and RXR can heterodimerize with peroxisomal proliferator activated receptor γ (PPARγ) [Kliewer et al., 1992] that may repress leptin expression [De Vos et al., 1996; Kallen and Lazar, 1996].

In the present study, we investigated the effect of all-*trans* RA on leptin expression and secretion in adipose cells of human and murine origin. We also examined whether other leptin-expressing cells were subject to regulation of leptin production by all-*trans* RA. Furthermore, we monitored expression of PPARγ, RXRα, and RARα in adipocytes incubated with all-*trans* RA to reveal mechanisms involved in the regulation of leptin mRNA expression.

MATERIALS AND METHODS

Cells

BeWo and 3T3-L1 cell lines were purchased from the American Type Culture Collection. The cell lines were cultured in Ham's F12 or Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co, St. Louis, MO), respectively, and supplemented with 10% heat-inactivated FCS (Integrob, Zaandam, The Netherlands), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (BioWhittaker, Walkersville, MD). BeWo cells were grown to 80% confluency prior experiments. 3T3-L1 cells were grown to confluency and differentiated essentially as described [Benito et al., 1991]. Briefly, the cells were incubated in DMEM added 20% FCS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthin, and 2 μM insulin for 4 days. The following days, cells were incubated in DMEM added 10% FCS and 200 nM insulin. All-*trans* RA (Sigma Chemical Co.) was added at day 13 after differentiation start. Commercially available human osteoblasts from NHOst cell system (Clonetics, San Diego, CA) were grown in osteoblast growth media (OGM) (Clonetics) and exposed to β-glycerophosphate and ascorbic acid in the ambient medium according to the manufacturer's instructions to induce differentiation. Cells were tested for the expression of osteoblast markers and experiments were conducted after a differentiation period of 2 weeks. All-*trans* RA dissolved in ethanol was added to the cells at the indicated concentrations, with $<1\%$ ethanol final concentration in the culture media. Control cells were added ethanol only. Medium was changed daily and saved for measurements of leptin secretion.

Cell viability was confirmed both by cell staining with propidium iodide (PI) and Hoechst 33342, as well as by measurement of release of lactate dehydrogenase (LDH) to the medium. For microscopic analysis, cell cultures were first incubated with PI (0.5 mg/ml) in the dark for 20 min. Thereafter, Hoechst 33342 (1 mg/ml) was added to the same cultures and incubated for another 20 min in the dark. After staining, the cells were evaluated in a Leitz Ortholux II fluorescence microscope (Leica, Wetzlar, Germany). Apoptosis and necrosis were not increased due to incubation with all-*trans* RA. LDH was measured using the Cytotoxicity Detection Kit (LDH) (Boehringer, Mannheim, Germany). LDH activity was not more than 5% of positive control with detergent in any of the cell systems, indicating that the cell viability was satisfactory after incubation with all-*trans* RA.

Isolation and Culture of Human Adipose Tissue Explants

Subcutaneous adipose tissue was collected from healthy women undergoing mammoplastic surgery at the "Volvat Medical Center" in Oslo. Written informed consent was obtained from the subjects. The Regional Ethics committee approved the study. The subjects were 28 ± 9 years of age, with a body mass index (BMI) of 24 ± 3 kg/m^2 with no diagnosed metabolic diseases. Pieces of adipose tissue were prepared under sterile conditions and used for incubation in plastic tubes essentially as described elsewhere [Ottosson et al., 1994]. Briefly, 60–80 mg adipose tissue was cut into pieces of 5–20 mg and preincubated for 3 days in a control medium (Parker199 medium, Sigma M-9163) supplemented with 10 mM HEPES, 10 g/L bovine serum albumin (BSA) (Sigma A-4503), 12.5 mM $NaHCO_3$, 7.175 nM human insulin (Sigma I-0259), 100 U/ml penicillin, and 100 μg/ml streptomycin prior to addition of all-*trans* RA. pH in the medium was adjusted to 7.4 daily.

mRNA Isolation, Northern Blotting, and Semi-Quantitative RT-PCR

Adipose tissue was lysed and mRNA was extracted with magnetic oligo dT particles (Genovision, Oslo, Norway) as described by the manufacturer. Two micrograms mRNA was analyzed by Northern blotting and hybridization [Reseland et al., 2001b]. A human probe for leptin and PPARγ was made by cloning the RT-PCR products described [Reseland et al., 2001b], using the TOPO TA Cloning kit (Invitrogen Corp., Carlsbad, CA). The identity of the probes was confirmed by sequencing. The probe for RXRα was as described by Mangelsdorf et al. [1992]. Hybridization signals were monitored in a Phospho Imager (Molecular Dynamics, Inc., Sunnyvale, CA) and normalized to the signals for L27 (ATCC-107385). mRNA from cell cultures was purified using magnetic oligo dT particles (Dynabeads, Dynal AS, Oslo, Norway or Genovision, Oslo, Norway) as described by the manufacturer. A sample of the mRNA was used in a semi-quantitative RT-PCR using the GeneAmp EZ rTth RNA PCR kit (Perkin Elmer, Applied Biosystems, Foster City, CA). Each reaction contained 2.0 μCi α^{32}P-CTP, 22.5 pmol of each primer and 2.5 mM $Mn(OAc)_2$. Oligonucleotide sequences of sense and antisense primers are presented in Table I. Linearity of the PCR reaction was confirmed for each primer set and cell type prior to the experiments.

Temperature cycles were as follows: 60°C for 30 min, 94°C for 1 min followed by 32 cycles of 94°C for 30 s, and 60°C for 1.5 min. Finally the samples were incubated at 60°C for 7 min. PCR products were separated on 2% agarose gels. The cDNA bands were excised from the gel and allowed to elute for 2 h in scintillation liquid before counting in a WinSpectral 1414 Liquid scintillation Counter (Wallac, Turku, Finland). Relative mRNA abundance was calculated as the ratio between the specific mRNA and β-actin or α-tubulin, for murine or human samples respectively, and is presented as % of control incubations.

Leptin Secretion

Medium from cell cultures was concentrated 10× using a MICROSEP microconcentrator with exclusion limit of 3 kDa. Medium from human adipose tissue explants was used without concentration. Leptin was measured in 100 μl medium using a competitive radioimmunoassay (Linco Research, St. Charles, MO). The data are presented as % of control incubations.

Statistics

Results are presented as means ± standard deviation. Student's *t*-test analysis, or when normality test failed, Mann–Whitney Rank Sum test, were used to determine the significance level of differences among sample groups, with a significance criterion of $P \leq 0.05$.

RESULTS

RA Reduced Leptin mRNA Expression Levels and Secretion in Adipose Tissue

Leptin mRNA expression levels in mature murine adipocytes (3T3-L1) was reduced by 56% ($P = 0.028$) after 48 h incubation with 1 μM all-*trans* RA (Fig. 1A). A tenfold increase in all-*trans* RA concentration (10 μM) gave similar effects reducing the leptin mRNA expression levels, and maintaining the reduced leptin mRNA expression levels after 72 h incubation. One and 10 μM all-*trans* RA significantly reduced leptin secretion to the media after 24 h

TABLE I. Primer Pairs Used for Semi-Quantitative RT-PCR

Product designation	Estimated size (bp)	Sense and antisense primer sequences
Murine β-actin	540	5′-GTGGGCCGCTCTAGGCACCAA-3′ 5′-CTCTTTGATGTCACGCACGATTTC-3′
Murine leptin	250	5′-AGCAGTGCCTATCCAGAAAGT-3′ 5′-ATTCTCCAGGTCATTGGCTAT-3′
Murine PPARγ	674	5′-TTGAGTGCCGAGTCTGTGGGGATAA-3′ 5′-CAGGGAGGCCAGCATCGTGTAGA-3′
Murine RXRα	165	5′-ATGAAGCGGGAAGCTGTG-3′ 5′-CATGTTTGCCTCCACGTATG-3′
Murine RARα	166	5′-CAGTTCCGAAGAGATAGTACC-3′ 5′-TACACCATGTTCTTCTGGATGC-3′
Human leptin	197	5′-GGCTTTGGCCCTATCTTTTC-3′ 5′-GGATAAGGTCAGGATGGGGT-3′
Human α-tubulin	527	5′-CACCCGTCTTCAGGGCTTCTTGGTTT-3′ 5′-CATTTCACCATCTGGTTGGCTGGCTC-3′

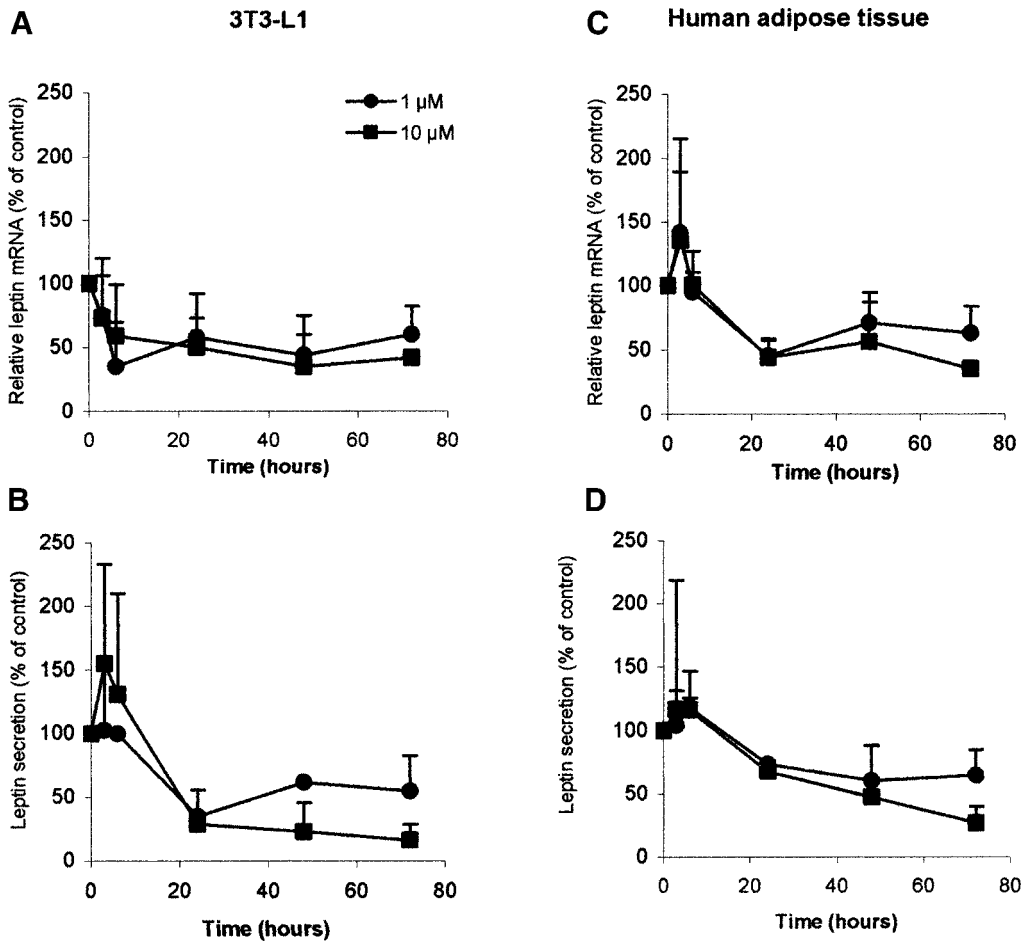

Fig. 1. The effect of all-*trans* retinoic acid on leptin mRNA expression levels and secretion in 3T3-L1 adipocytes (**A**, **B**) and human adipose tissue explants (**C**, **D**). Leptin mRNA expression levels (A, C) and secretion (B, D) are presented as means ± SD (% of control), based on three separate experiments performed in triplicates. Leptin mRNA expression levels are investigated by RT-PCR (3T3-L1) or Northern hybridization (human adipose tissue).

(Fig. 1B). The effect was significant, but more pronounced with 10 μM all-*trans* RA on leptin secretion after 48 and 72 h, as compared to 1 μM all-*trans* RA. Viability of the cells were confirmed by measuring LDH release to the medium, and DNA staining followed by microscopic analysis (data not shown).

In human adipose tissue explants leptin mRNA expression levels (Fig. 1C) as well as leptin secretion to the medium (Fig. 1D) was significantly reduced after 24 h incubation with 1 and 10 μM all-*trans* RA. This suggests that the regulation of leptin mRNA expression and secretion by all-*trans* RA in adipose tissue is similar in humans and mice.

After 3 h incubation with 1 or 10 μM all-*trans* RA, the mRNA expression levels of PPARγ in 3T3-L1 cells was significantly increased (Fig. 2A). Furthermore, RXRα mRNA expression levels were significantly increased after 6 h incubation with all-*trans* RA (Fig. 2B). Ten μM of all-*trans* RA increased RARα mRNA expression by 59% ($P = 0.029$) after 24 h, whereas 1 μM all-*trans* RA had no significant effect (Fig. 2C). In contrast, the mRNA expression levels of PPARγ (Fig. 2D) and RXRα (Fig. 2E) were unchanged in human adipose tissue explants after incubation with 1 or 10 μM all-*trans* RA at all time points.

Leptin mRNA Expression Levels Were Not Regulated by All-*Trans* RA in Human Placental Cells and Osteoblasts

Leptin mRNA expression levels were not altered by all-*trans* RA in BeWo cells (Fig. 3A), but we observed a significant reduction in leptin

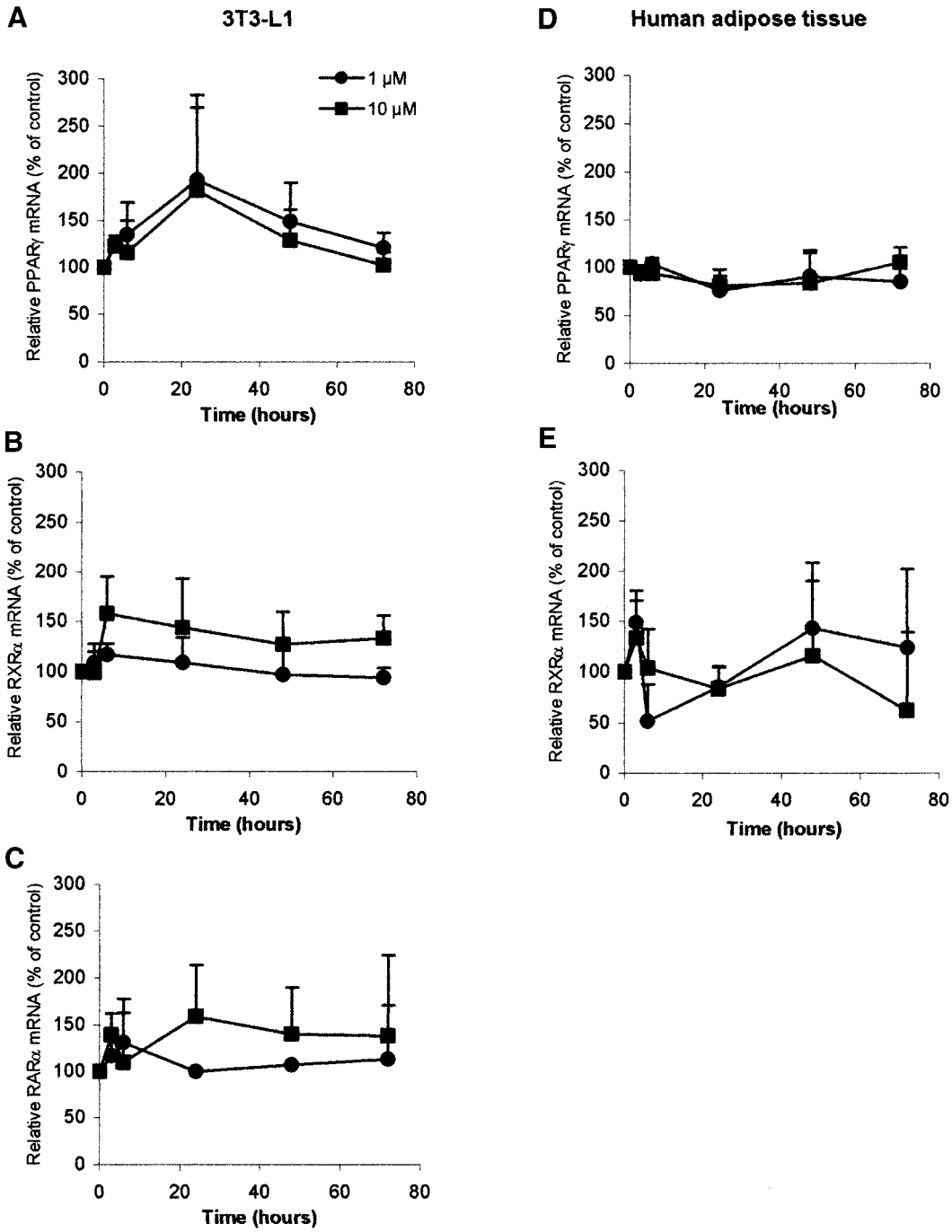

Fig. 2. The effect of all-*trans* retinoic acid on the expression of PPARγ, RXRα, and RARα mRNA in 3T3-L1 adipocytes (**A–C**) or human adipose tissue explants (**D**, **E**). PPARγ (A, D), RXRα (B, E), and RARα (C) mRNA expression levels are presented as means ± SD (% of control), based on three separate experiments performed in triplicates. mRNA expression was investigated by RT-PCR (3T3-L1) or Northern hybridization (human adipose tissue).

secretion after 24 h of incubation with 1 and 10 μM all-*trans* RA. After 48 h, only the cells incubated with 10 μM all-*trans* RA maintained a significant reduction of leptin secretion (Fig. 3B). Incubation of all-*trans* RA had no significant effect on leptin mRNA expression levels in primary cultures of human osteoblasts during 72 h periods (Fig. 3C).

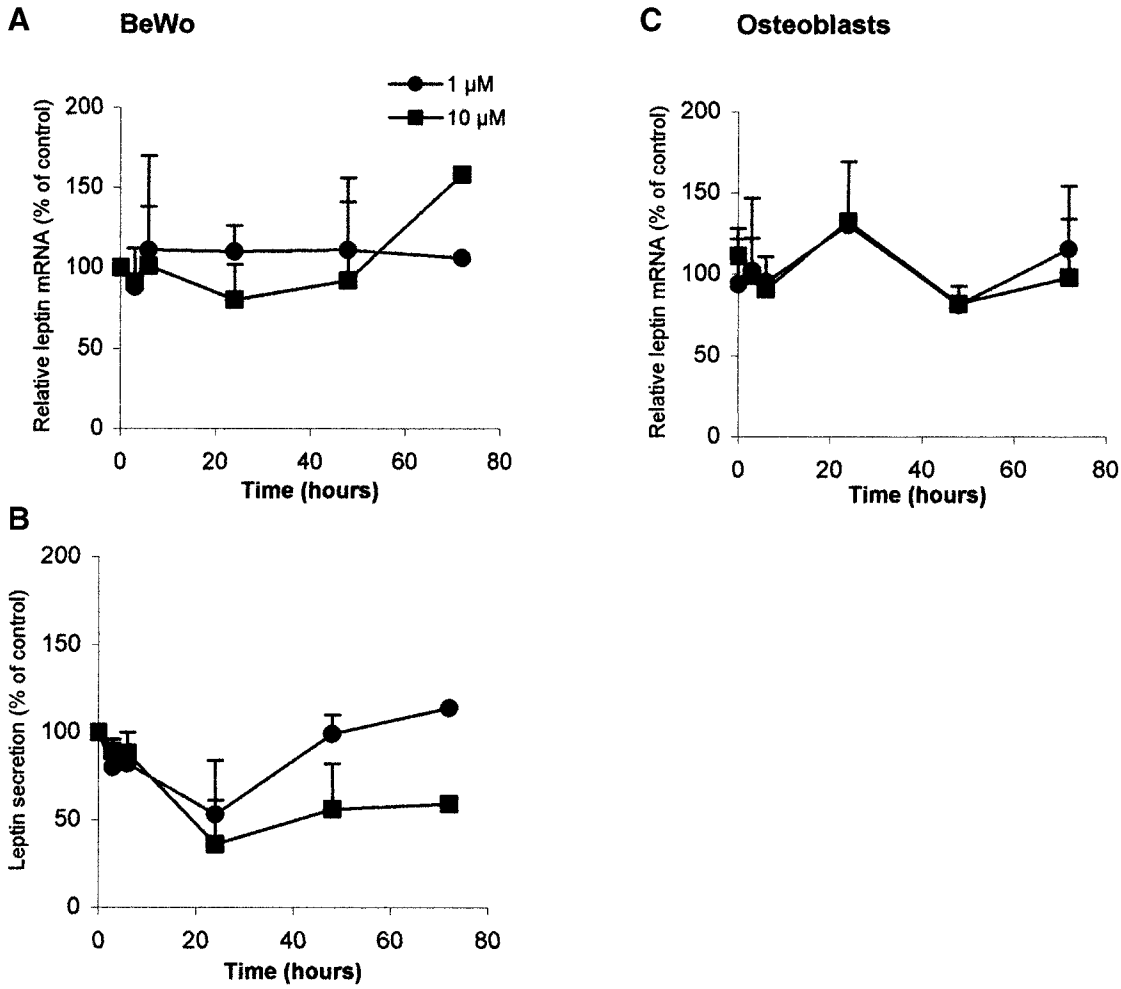

Fig. 3. The effect of all-*trans* retinoic acid on leptin mRNA expression and secretion in BeWo cells (**A**, **B**) and osteoblasts (NHO) (**C**). Leptin mRNA expression levels (A, C) were presented as means ± SD (% of control), based on three separate experiments performed in triplicate. The NHO experiments (C) were based on two separate experiments performed in duplicates. Leptin mRNA expression was investigated by RT-PCR.

DISCUSSION

In this study, we show that all-*trans* RA reduced leptin mRNA expression levels as well as leptin secretion in adipose tissue. The reduction is observed in human adipose tissue explants as well as in mouse 3T3-L1 cells, suggesting a similar effect by all-*trans* RA on leptin production in the two species. Menendez et al. [2001] also observed reduced leptin secretion from cultured human adipose tissue incubated with all-*trans* RA. Administration of retinol and RA may also reduce circulating leptin levels and leptin expression in rats [Kumar and Scarpace, 1998; Kumar et al., 1999] and mice [Bonet et al., 2000].

All-*trans* RA is a ligand for the RAR transcription factor [Giguere et al., 1987; Petkovich et al., 1987], which can heterodimerize with nuclear receptors such as RXR or PPAR [Kliewer et al., 1992]. All-*trans* RA may induce elevated RXR and RAR mRNA levels in F9 teratocarcinoma cells [Wan et al., 1994] or in retinoblastoma cell lines [Li et al., 2002]. Activation of PPARγ has previously been shown to inhibit leptin expression in rats as well as in cultured adipocytes [De Vos et al., 1996; Kallen and Lazar, 1996]. In 3T3-L1 cells, all-*trans* RA pro-

moted increased mRNA expression levels of PPARγ, RXRα, and RARα, indicating that leptin mRNA expression in 3T3-L1 cells may be regulated directly or indirectly via these nuclear receptors. In contrast, the transcript levels of PPARγ and RXRα were not significantly changed at any time point in human adipose tissue explants after all-*trans* RA incubation in our experiments. In brown adipose tissue, it was observed that RA isomers reduced PPARγ mRNA [Valmaseda et al., 1999], and expression of PPARγ as well as leptin was reduced in murine adipose tissue by subcutaneous injection of all-*trans* RA [Ribot et al., 2001]. The findings reported so far indicate that the effect of RA on PPARγ may be time-, dose-, tissue-, and species-dependent.

We did not observe any effects of RA on leptin mRNA expression in neither osteoblasts nor placenta cells. This implies that leptin mRNA expression probably is regulated by other mechanisms in these cells than adipose tissue. Our results in BeWo cells are supported by Guibourdenche et al. [2000] demonstrating that leptin expression was unaltered in primary cultures of human syncytiotrophoblasts after incubation of 0.1 μM all-*trans* RA for 48–96 h. In contrast, we found that all-*trans* RA caused a significant reduction in leptin secretion to the medium after 24 h from BeWo cells. The discrepancy between mRNA expression and secretion of leptin in BeWo cells could be due to stability of the product or inhibition of release of leptin from intracellular compartments, without affecting expression of the leptin gene. Placenta-produced leptin probably acts as a fetal growth hormone during pregnancy [Hassink et al., 1997], and production and secretion from placental cells might be regulated differently than in other tissues, to protect the fetus against rapid shifts in leptin exposure.

The concentrations of *all-trans* RA used in our experiments are high, but neither apoptosis nor necrosis were increased due to this exposure in neither of the cell systems tested. LDH activity was <5% of positive control with detergent in all cell systems, indicating that the cell viability was satisfactory after all-*trans* RA incubation.

Rather high doses of all-*trans* RA are used routinely for treatment of newly diagnosed acute promyelocytic leukemia [Douer, 2000]. Moreover, RA is negatively associated with bone density [Melhus et al., 1998; Promislow et al., 2002] and bone resorption may be induced by vitamin A in vitro [Scheven and Hamilton, 1990; Promislow et al., 2002]. We have previously shown that leptin is produced and secreted by human osteoblasts [Reseland et al., 2001c], that leptin stimulates osteoblast differentiation and proliferation, and that leptin may inhibit all-*trans* RA-induced apoptosis [Gordeladze et al., 2002]. All-*trans* RA might inhibit bone growth by affecting osteoblast proliferation, differentiation, and production of leptin or other secreted factors such as interleukines targeting osteoclasts. We observed no effect of all-*trans* RA on leptin mRNA expression in primary osteoblasts, and we were unable to detect apoptosis in our cells, although this could be due to the relatively short incubation times.

In conclusion, all-*trans* RA reduced expression of leptin mRNA as well as secretion of leptin in human and murine adipose tissue. In human BeWo cells or primary osteoblasts, leptin mRNA expression was not changed by all-*trans* RA, suggesting a tissue-specific regulation of leptin by all-*trans* RA.

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