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ORIGINAL PAPER

Leptin regulates chondrogenic differentiation in ATDC5 cell-line through JAK/STAT and MAPK pathways

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Abstract Leptin, the satiety hormone, has been found to affect growth-plate cartilage development. In the present study, some of the signal transduction pathways that mediate leptin signaling in the ATDC5 chondrogenic cell-line, a model for endochondral ossification, were analyzed. For this purpose, real-time PCR, Western blots and immunofluorescence techniques were used. It was found that leptin increased phosphorylation of ERK1/2, p38, and STAT3 in a time- and dose-dependent manner. Specific inhibition of STAT3 or ERK1/2, but not of P38, blocked the stimulatory effect of leptin on type X collagen mRNA levels. Moreover, leptin induced the translocation of ERK1/2 into the nucleus, as well as c-fos expression, indicating full activation of this cascade. Leptin-induced JNK phosphorylation was not observed, although leptin significantly and rapidly increased JNK protein levels and c-jun mRNA levels. In addition, ERK5 was identified in these cells, but there was no apparent effect of leptin on either its phosphorylation or protein level. The study indicates that the effects of leptin on growth-plate chondrocytes are specifically mediated through ERK1/2 and STAT3, while P38 is not essential for leptin-induced type X collagen expression. This is the first demonstration that these pathways are involved in leptin-induced growth.

Keywords Leptin · JAK/STAT · MAPK · Chondrocyte · ATDC5

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Introduction

Leptin is a 16 kDa protein which regulates food intake and energy balance through its central signaling in the hypothalamus. It was first identified in obese ob/ob mice as the product of the ob gene [1]. Leptin is produced mainly by adipocytes, although the placenta, ovaries, skeletal muscles, abdomen, and pituitary secrete small amounts as well [2]. The wide distribution of the Ob receptor (Ob-R) in different tissues indicates that leptin has numerous peripheral effects [3]. The contribution of leptin to growth-plate cartilage development has been reported by our group [4, 5], as well as by others [6–8]. Our previous study showed that chondrocytes of murine mandibular condyle, an established organ culture model for endochondral ossification, express leptin receptors, and that leptin stimulates chondrocyte proliferation and differentiation as well as insulin-like growth factor I receptor expression. Accordingly, we suggested that leptin may play a role in the process of endochondral ossification [4]. This was supported by in vivo findings of similar effects of leptin in growth plates of ICR mice [5].

The objective of the present study was to identify the signal transduction pathways that mediate leptin signaling in growth-plate chondrocytes. ATDC5 cells, which are capable of differentiating into chondrocytes in vitro in a multi-step process, from mesenchymal condensation to calcification [9, 10], were used as a model of endochondral

cartilage. The study focused on two main signal transduction pathways: janus kinases (JAK)/signal transducers and activators of transcription (STAT) and mitogen-activating protein kinase (MAPK).

STAT transcription factors are known to play a role in the signal transduction mechanism of leptin in different tissues [11], but their involvement in leptin-induced chondrogenesis in growth-plate cartilage has not been studied.

The MAPK group of signal transduction pathways transmits extracellular signals which stimulate mitogenic and stress responses. Each MAPK cascade is composed of several protein kinases that specifically phosphorylate and activate each other in a hierarchical manner, thereby forming a complex signaling network. Four distinct MAPK cascades have been recognized: extracellular signal-regulated kinase 1/2 (ERK1/2), c-jun N-terminal kinase (JNK), p38MAPK, and big mitogen-activated kinase 1 (BMK1), known also as ERK5 [12]. Previous studies using ATDC5 cells showed that ERK1/2 and p38MAPK are activated during chondrogenesis induced by growth/differentiation factor 5 (GDF–5) [13], whereas JNK apparently functions as a chondrogenesis suppressor [14]. However, their involvement in leptin-induced chondrogenesis has not been investigated.

Results

Effects of leptin on ATDC5 culture

Non-stimulated ATDC5 culture showed a clear proliferation phase, followed by an accelerated differentiation phase. On differentiation, the cells initially expressed increased levels of type II collagen mRNA, which eventually decreased and were replaced by type X collagen mRNA, a unique marker of differentiated growth-plate chondrocytes. These observations were in agreement with previously shown results [15, 16].

In 2 weeks old differentiated cultures treated with leptin (50 ng/ml) for the last several hours of incubation (0, 2, 4, 6, or 8 h), a significant increase in type X collagen mRNA levels was observed after 8 h (Fig. 1).

As type X collagen is expressed exclusively by mature growth-plate chondrocytes, and its expression is characteristic of the endochondral ossification process, we used it as a differentiation marker of the ATDC5 cell model in the following experiments.

Leptin signaling through the JAK/STAT pathway

We confirmed that ATDC5 cells expressed the long isoform of leptin receptor (Ob-Rb), starting from the first day of culture and throughout its duration, as was observed by others [6, 17]. On the basis of previous findings that the Ob-Rb transmits signals through the JAK/STAT pathway [18], and that complete STAT3 transcription activity depends on the phosphorylation of two amino acid residues, tyrosin 705 and serine 727 [19], we assessed the phosphorylation of STAT3. As depicted in Fig. 2 (panels a and b), Western blot analysis clearly showed that leptin stimulated the phosphorylation of STAT3 on both residues. Densitometry analysis of the Western blots, using total STAT3 as a reference showed that maximal intensity was achieved after 10 min and declined thereafter. The phosphorylation level was higher for tyrosine than serine at all time points.

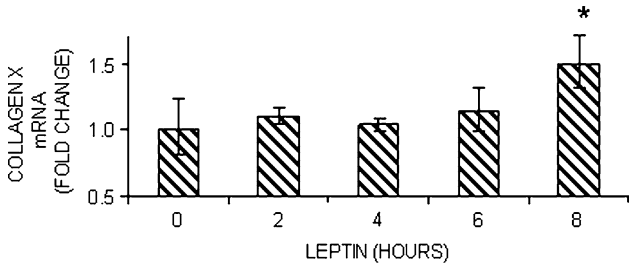


Fig. 1 Effect of leptin on type X collagen mRNA levels. ATDC5 cells were cultured for 14 days, and then treated with leptin (50 ng/ml) for several hours, as indicated. All the cultures were harvested together at the end of the whole experiment—to neutralize any effects of cell age on the results. Leptin significantly increased the level of type X collagen mRNA after 8 h of incubation. Values are the means derived from a representative real-time PCR experiment carried out in triplicate and repeated independently thrice. Asterisks indicate the statistically significant change compared to the untreated control group (0)

Leptin also affected native STAT3 protein levels, which increased by 51% after 4 h of treatment and twofold after 6–8 h ($P < 0.05$) (Fig. 2, panels c and d).

To evaluate whether STAT3 activation is crucial for leptin signaling, we used a cell-permeable analog of the STAT3-SH2 domain-binding phosphopeptide, which interferes with STAT3 binding to DNA, thereby suppressing transcription in a noncompetitive manner [20]. In the presence of the STAT3-specific inhibitor, the effect of leptin on type X collagen mRNA levels was suppressed (Fig. 2e). Interestingly, the STAT3 inhibitor itself appeared to have a stimulatory effect on the level of type X collagen mRNA in the control cultures ($P < 0.05$).

Using several types of transcription-factor site-prediction software programs, including TFsearch [21] and Consite (Karolinska Institute, Center for Genomics and Bioinformatics, Stockholm, Sweden), we identified a conserved-sequence STAT3 recognition site, AAGTGA, 1,003 bp upstream to the first exon of type X collagen gene, in the predicted promoter region. This finding suggests that leptin's effects on type X collagen may be mediated through the STAT3 transcription factor.

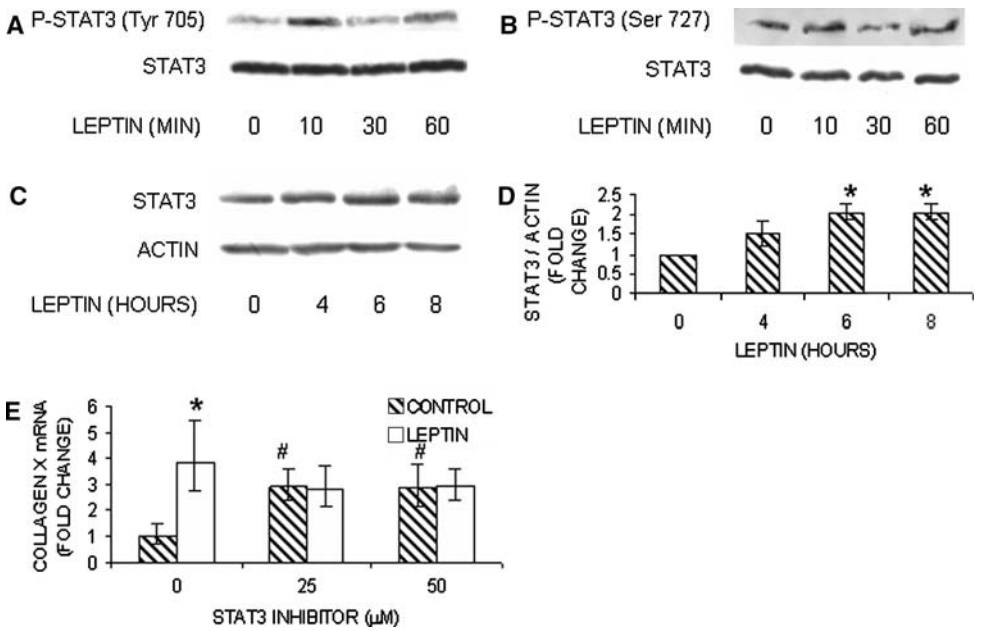


Fig. 2 Effect of leptin on STAT3 phosphorylation and level in ATDC5 cells (**a–d**) and the effect of STAT3 inhibitor on leptin-induced type X collagen mRNA (**e**). Two weeks old cultures were treated with leptin (50 ng/ml) for the indicated times. Western blot analysis performed with (**a**) anti-phospho-tyrosine 705 STAT3 or (**b**) anti-phospho-serine 727 STAT3 showed phosphorylation of both residues. Leptin also increased native STAT3 levels (compared to actin internal control) (**c**). Findings were confirmed by densitometry measurements (**d**). Real-time PCR analysis of the cultures indicated that STAT3 inhibitor (25 or 50 μM) blocked the leptin-induced increase in type X collagen mRNA levels (**e**). *Statistically significant difference between the leptin-treated and control cultures. #Statistically significant difference between the inhibited and non-inhibited control

Effect of leptin on ERK1, ERK2, and c-fos activation

Using antibodies specific for phospho-ERK1 and 2, we found that treatment with 50 ng/ml leptin doubled the levels of phosphorylated ERK1 and ERK2 in ATDC5 cells after 10 min (Fig. 3a). Total ERK1/2 levels, as well as actin levels, remained unchanged. Phospho-ERK returned to basal levels after 30 min of incubation (data not shown). ERK1 (p44mapk) was more phosphorylated than ERK2, although their increase in phosphorylation in response to leptin was proportionate. Moreover, leptin treatment activated the immediate-early gene encoding c-fos (Fig. 3b), a transcription factor commonly induced by the ERK cascade.

To analyze whether the ERK1/2 cascade mediates leptin-induced type X collagen mRNA expression, we used U0126, a specific inhibitor of MEK1, the upstream regulator of ERK1/ERK2. Western blot analysis with native and phosphorylated anti-ERK1/2 antibodies revealed a considerable inhibitory effect of U0126 on leptin-induced phosphorylation (Fig. 3c). Even low concentrations (5 μM) decreased phosphorylation levels by 80% compared to the leptin-induced, non-inhibited culture. Moreover, inhibition of MEK1 completely blocked leptin-induced type X collagen mRNA expression. Figure 3d shows that when ATDC5 cells were treated with U0126 before exposure to leptin, the effect of leptin on type X collagen was abolished. Total inhibition was observed already with the lowest concentration of U0126 used (5 μM), in agreement with the effect on phosphorylation. Interestingly, like for the STAT3 inhibitor, addition of the inhibitor itself stimulated type X collagen mRNA levels in the control cultures ($P < 0.05$).

To determine complete activation of the ERK1/2 pathway, we used immunofluoroscence staining with a specific anti-ERK2 antibody that recognizes the c-terminus of the kinase, and hence, does not interfere with its activity. Three hours after leptin stimulation, most of the ERK (67%) migrated to the nucleus (Fig. 4).

Effect of leptin on p38 MAPK in ATDC5 cells

The effect of leptin on p38 phosphorylation was investigated with anti-phospho-p38 and anti-p38 antibodies. As shown is Fig. 5a, leptin-induced p38 phosphorylation in the ATDC5 cells in a time-dependent manner. Phosphorylation was maximal after 1 min and decreased thereafter.

To analyze p38 mediation of leptin-induced type X collagen mRNA expression, we used SB203580, a specific competitive inhibitor, which functions via blockage of

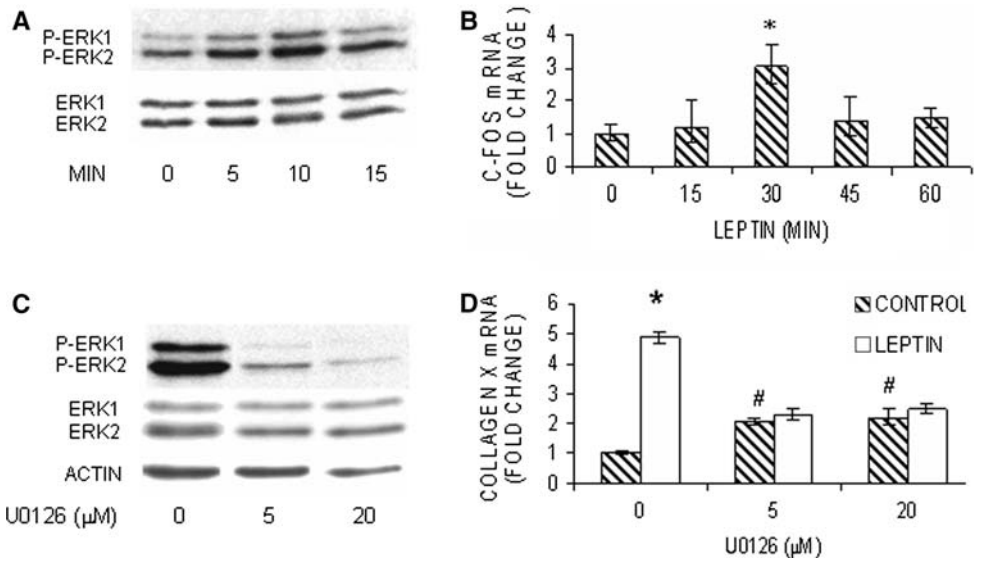


Fig. 3 The significance of leptin to the ERK1/2 pathway in ATDC5 cells. (**a** and **b**) Effect of leptin on ERK1/2 phosphorylation (**a**) and on c-fos expression (**b**). (**c** and **d**) Effect of ERK1/2 inhibition on leptin-induced ERK 1/2 phosphorylation (**c**) and type X collagen mRNA (**d**). Cells were cultured for 2 weeks and treated with leptin (50 ng/ml) for the indicated times. (**a**) Western blot analysis performed with a specific anti-phosph-ERK1/2 antibody showed increased ERK1/2 phosphorylation, while total levels remained unchanged. (**b**) Real-time PCR showed increased levels of c-fos mRNA. (**c**) Western blot analysis performed with MEK inhibitor U0126 (5 or 20 μM) demonstrated a reduction in leptin-induced ERK1/2 phosphorylation, even bellow the baseline level. (**d**) Real-time PCR analysis of cultures treated with the MEK inhibitor showed complete inhibition of the leptin-induced increase in type X collagen mRNA levels. Data for Western blot experiments are representative of 4–6 independent experiments which yielded essentially the same results. The real-time PCR data are means of three different experiments carried out in triplicate. *Statistically significant difference between the leptin-treated and control cultures. #Statistically significant differences between the inhibited and the non-inhibited control cultures

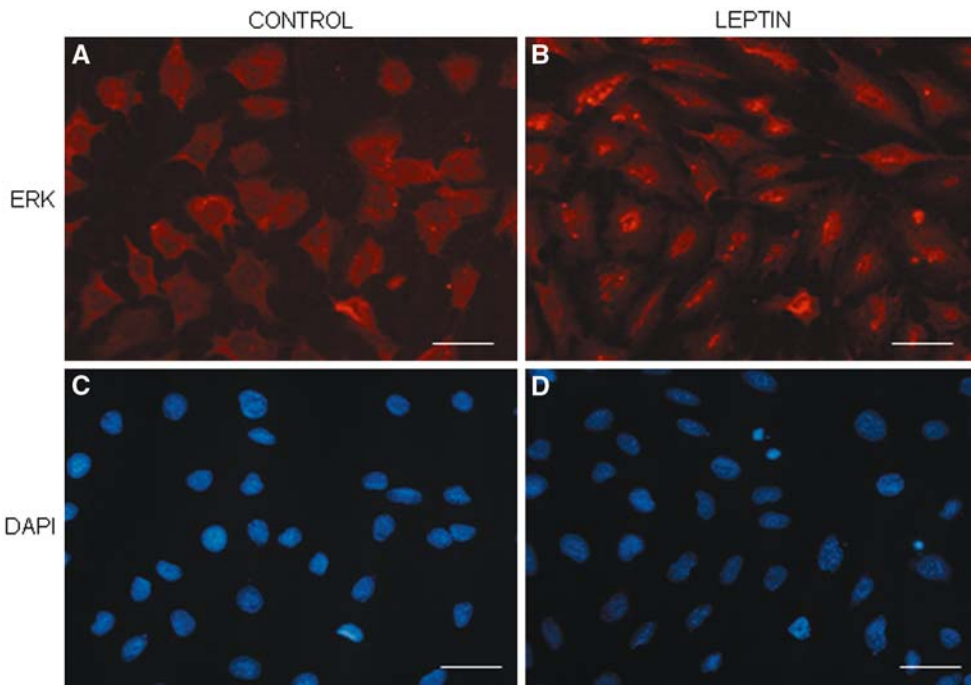


Fig. 4 Leptin-induced translocation of ERK. ATDC5 cells were cultured in serum-free medium for 3 days, after which leptin (50 ng/ml) was added for additional 3 h. (**a** and **b**) Immunofluorescence assay performed with a specific anti-ERK2 antibody localized cellular-ERK before and after treatment (**a** and **b**, respectively). (**c** and **d**) DAPI staining confirmed the nuclear location within the cells before and after treatment (**c** and **d**, respectively). The experiment was repeated thrice in duplicate wells ($n = 6$) (magnification 400×, bar = 20 μm)

ATP binding to p38, thereby preventing its ability to phosphorylate downstream effectors [22]. In the presence of this inhibitor, type X collagen mRNA levels were not attenuated (Fig. 5b). Interestingly, once again we observed a slight increase in type X collagen level in response to the inhibitor itself, at concentration of 10 μM ($P < 0.05$).

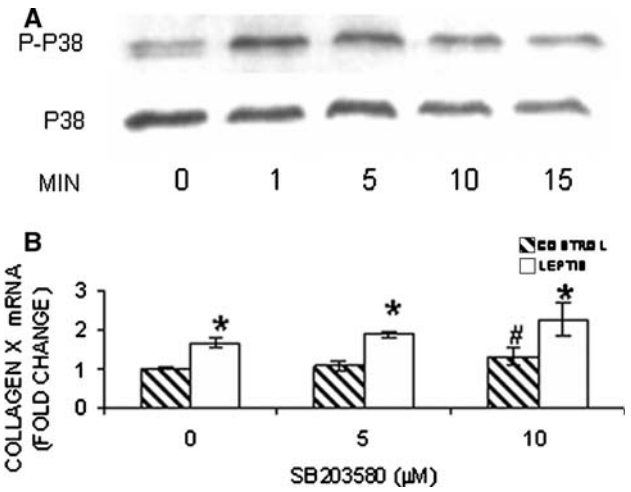


Fig. 5 Significance of leptin to P38 pathway in ATDC5 cells. (**a**) Effect of leptin on p38 phosphorylation. (**b**) Effect of p38 inhibition on leptin-induced type X collagen mRNA. ATDC5 cells were cultured for 2 weeks and then treated with leptin (50 ng/ml) for the indicated times. (**a**) Western blot performed with a specific anti-phospho-p38 antibody showed an increase in p38 phosphorylation compared to total p38 protein level. Data are representative of three independent experiments which yielded essentially the same results. (**b**) p38 inhibitor SB203580 (5 or 10 μM) had no effect on the leptin-induced increase in type X collagen mRNA levels. Data are means of 3 different experiments carried out in triplicate. *Statistically significant difference between the leptin-treated and the corresponding control cultures. #Statistically significant difference between the inhibited and the non-inhibited control cultures

Effect of leptin on JNK levels in differentiating chondrocytes

Leptin increased JNK protein levels (Fig. 6a). Although JNK was significantly phosphorylated after exposure to peroxyvanadatae solution (VOOH), a tyrosine phosphatase inhibitor, we did not detect leptin-induced phosphorylation of JNK (data not shown). In addition, leptin increased c-jun mRNA levels, which is the down-stream nuclear transcription-factor activated directly by JNK. This effect was time-dependent, with a peak at 15 min after stimulation (Fig. 6b).

Effect on ERK5 in differentiating chondrocytes

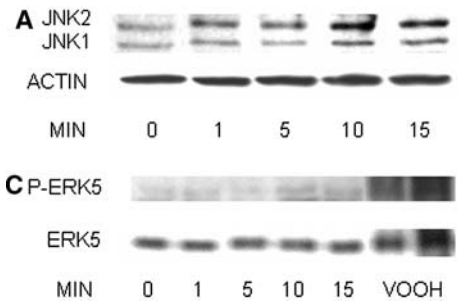
Although ERK5 in the ATDC5 cell line was significantly phosphorylated after exposure to VOOH, there was no change in either its level or its phosphorylation in response to leptin (Fig. 6c).

Discussion

In the present study, we utilized the ATDC5 chondrogenic cell-line to study leptin signaling in endochondral ossification. We found that leptin increased the phosphorylation of ERK1/2, p38, and STAT3 in a time- and dose-dependent manner, and that the specific inhibition of either ERK1/2 or STAT3 abolished leptin's effect on type X collagen expression, whereas inhibition of p38 did not.

Leptin signal transduction through the JAK/STAT pathway depends on the presence of the long leptin receptor OB-Rb, since this is the only isoform that contains a STAT3 binding site [11]. Our observation of continuously expressed Ob-Rb receptors in ATDC5 cells is in agreement with Kishida et al. [6], and with previous findings of the leptin receptor isoforms Ob-Ra, Ob-Re, and Ob-Rb in these cells [17].

To specifically examine the effect of leptin on differentiation at the molecular level, we studied mRNA expression of type X collagen, a unique marker of hypertrophic chondrocytes. Our results may suggest a direct positive control of leptin on this gene. The significance of



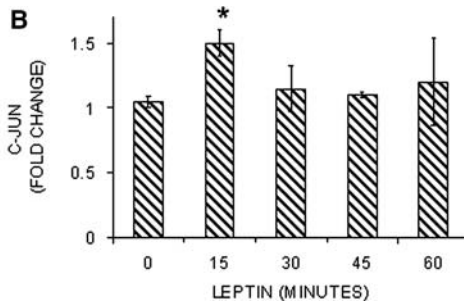


Fig. 6 Effect of leptin on JNK, c-jun and ERK5. ATDC5 cells were cultured for 2 weeks and then treated with leptin (50 ng/ml) for the indicated times. (**a**) Western blot analysis performed with anti-JNK antibody showed that leptin increased the levels of JNK 1 and 2, as compared to the level of actin. (**b**) Real-time PCR detected increased mRNA levels of c-jun. (**c**) Western blot analysis performed with anti-ERK5 and anti-phospho-ERK5 antibodies showed no effect of leptin on either total ERK5 level or ERK5 phosphorylation, compared to the positive effect of VOOH exposure after 10 and 30 min. The Western blot experiments were repeated thrice with the same results. The real-time PCR data are means of three different experiments carried out in triplicate. Asterisk indicate statistically significant difference between the leptin-treated and control cultures

leptin to type X collagen expression was previously described by Kishida et al. [6], who observed reduced type X collagen expression in growth plates from ob/ob mice, as well as a less organized pattern of the collagen fibrils, compared to wt mice.

Signal transduction by leptin has been studied extensively in numerous experimental systems, in vitro and in vivo (see reviews [11, 23]), but only one study examined leptin-activated signal transduction pathways in chondrocytes [24]. The authors found that JAK2, PI3K, p38, and ERK were activated in the ATDC5 cell model during synergistic stimulation of nitric oxide type II by leptin and interleukin-1 (IL-1). Here, we studied leptin-induced activation of two major pathways: JAK/STAT and MAPK.

It was previously shown that phosphorylation of STAT3 on tyrosine 705 induces dimerization, nuclear translocation, and DNA binding [25], whereas transcriptional activation seems to be regulated by phosphorylation at serine 727 (via the MAPK or mTOR pathway) [19]. Accordingly, we found that leptin stimulated STAT3 phosphorylation on both residues in differentiated ATDC5 cultures (14 days), as well as in mature hypertrophic ATDC5 cultures (30 days). Published results concerning STAT3 phosphorylation of ser727 residue in response to leptin are scarce and ambiguous, and may be cell-specific [26, 27]. Besides the increase in STAT3 phosphorylation, in the present study, leptin treatment also increased STAT3 levels—either directly or as a result of positive auto-regulation of STAT3 itself [28]. Similarly, increased STAT3 levels have been observed in hepatocytes of ob/ob mice after leptin administration [29], as well as in ES cells expressing the BCR-ABL gene [30]. These findings suggest that this effect is not restricted to chondrocytes, but is a part of a general mechanism of action of leptin and a common mechanism for STAT3 activation. These findings are of special importance considering the scarce data on the JAK/STAT pathway in chondrocytes [31–33].

The MAPK pathway is involved in growth and differentiation and is known to mediate leptin signaling in other tissues [23]. In the present study, leptin stimulated the MAPK cascades ERK1/2, p38, and JNK. The activation of the ERK1/2 pathway was demonstrated by ERK1/2 phosphorylation and translocation to the nucleus and the initiation of transcription from the c-fos immediate-early gene. Translocation to the nucleus is an essential step for the appropriate transcription factors' activation. The presence of ERK in the nucleus as long as 3 h after stimulation is in accordance with the theory of Pouyssegur et al. [34] of a rapid entrance of ERK into the nucleus followed by its slow accumulation. Indeed, ERK nuclear accumulation has been reported even 6 h after stimulation [35].

The p38 MAPK cascade is activated during chondrogenesis as well as in more advanced stages of endochondral ossification [36]. Ohlsson et al. [37] found that p38 phosphorylation increased during chondrogenesis of chick mesenchymal cells, and that inhibition of p38 activity blocked chondrogenesis. Zhen et al. [38] reported that the inhibition of p38 signaling suppressed expression of type X collagen in chicken growth-plate chondrocytes, and Stanton et al. [39], in a study of a micromass of mesenchymal limb bud cells, concluded that p38 is required for hypertrophic chondrocyte differentiation. In this study we have shown that leptin-induced phosphorylation of p38 MAPK, although the inhibition of this kinase did not block leptin's effect on type X collagen. Therefore, the involvement of this pathway in leptin-induced type X collagen expression is not essential.

The available data on the activation of the JNK cascade during chondrogenesis are incomplete and controversial. Nakamura et al. [13] reported that JNK was not phosphorylated during GDF5-induced chondrogenesis in ATDC5 cell, whereas Tuli et al. [40] detected JNK phosphorylation during TGF-β-induced chondrogenic differentiation in human mesenchymal progenitor cells. Nakajima et al. [14] concluded that the JNK cascade is apparently involved in negative regulation of chondrogenesis. We found, in the ATDC5-cell model, that leptin increased the levels of JNK immediately, without affecting its phosphorylation. Thus, although JNK phosphorylation is not always detected during chondrocyte differentiation, the JNK cascade probably does play a regulatory role in chondrogenesis. Further support for this notion is provided by a previous finding that the inhibition of JNK activity enhanced the synthesis of glycosaminoglycan and expression of chondrogenic differentiation markers (type II collagen, type X collagen, and aggrecan) in the ATDC5 cell-line [14]. In addition, Chu et al. [41] found that JNK inhibition facilitated the recovery of human articular cartilage metabolism following thermal stress. Enhanced JNK protein levels have been reported in condylar chondrocytes following 48 h of mechanical loading stress, which triggers the differentiation process [42], as well as in osteoarthritic chondrocytes subjected to IL-1beta (after 30 min) [43]. However, the ATDC5 cell-line in our study showed a particularly rapid change in the level of JNK, probably suggesting a change in protein stability rather than in synthesis. This may have been due to cell-culture differences or it may suggest that the JNK cascade is more active in the differentiation process of these transformed cells.

ERK5/BMK is the least studied of the MAPK cascades. Findings in different tissues indicated that ERK5 is activated in response to growth factors (vascular endothelial growth factor, endothelial growth factor, brain-derived neurotrophic factor, fibroblast growth factor, and nerve growth factor) and stress (hyperosmotic shock, oxygenic stress, laminar flow shear stress, and ultraviolet radiation)

[44]. Despite the great structural similarity between ERK5 and ERK1/2, these kinases do not respond to the same stimuli. Furthermore, ERK5 is not phosphorylated by MEK1/2, and it has different cellular compartmentalization, being nuclear and not cytoplasmic [34]. As growth-plate cartilage is known to be hypoxic, at least in some parts [45], we speculated that ERK5 may be a reasonable target for leptin stimulation. To our knowledge, this is the first study that shows ERK5 expression in chondrocytes. However, leptin had no effect on ERK5 phosphorylation or level.

To identify the relative roles and interactions of the different pathways involved in leptin signaling in chondrocytes, we used specific inhibitors. We found that the STAT3-specific inhibitor completely blocked the stimulatory effect of leptin on type X collagen mRNA levels. This result is reinforced by our finding in silico of a putative ISRE/GAS binding site for STAT3 in the type X collagen promoter. Considering that the functionality of type X collagen distal promoter is not fully known [46] the latter finding, although needs further validation by experimental data, is important, because it may suggest a direct effect of leptin-activated STAT3 on type X collagen expression. At the same time, our finding that the STAT3 inhibitor stimulated type X collagen mRNA level in untreated cultures may indicate opposing actions of this pathway. This effect has not been reported before, and more research is needed to elucidate its mechanism and importance.

Similarly, the addition of a specific ERK1/2 inhibitor (U0126) abolished the effect of leptin on type X collagen mRNA. It also increased type X collagen mRNA levels in control cultures, suggesting a negative regulation of ERK1/2 cascade on chondrogenic differentiation. Accordingly, Murakami et al. in a study of mice that constitutively express activated MEK1, the upstream regulator of ERK1/2, demonstrated delayed endochondral ossification, leading to a dwarf phenotype similar to human achondroplasia [47]. The authors concluded that the MAPK pathway inhibits hypertrophic chondrocyte differentiation and longitudinal bone growth, without affecting chondrocyte proliferation. Our results are in line with this observation and show that ERK1/2 may be also involved in stimulation of chondrogenic differentiation.

Inhibition of ERK by either a specific MEK inhibitor or a dominant negative form of MEK1 was previously shown to abrogate STAT3 Ser272 phosphorylation and DNA binding in J744.2 macrophages, suggesting hierarchical relationship between these two factors [26]. Accordingly, we have shown that inhibition of either ERK or STAT3 activity completely blocked leptin-induced type X collagen stimulation.

Harada et al. [48] found a potential binding site for MEF2, a target transcription factor for p38, in the promoter of type X collagen. Nevertheless, p38 inhibition in our study failed to block leptin's effects on type X collagen expression in the ATDC5 cells.

Finally, although the activation of the signaling pathways described here agrees with the study of Takahashi et al. [49] on leptin's effects in C3H10T1/2 cells, it was low compared to some other cell models. The difference might be attributable to the constant activation of the signaling pathways that mediate chondrogenesis in ATDC5 cells, owing to their transformed nature. The relatively high basal phosphorylation levels of ERK1/2, p38, and STAT3, even after 24–48 h of serum-starvation, indicate that these results were not due to the presence of insulin in the culture medium. Moreover, FCS activation of the studied pathways also yielded low responses (data not shown). The finding that leptin affects signaling pathways that are already active during ATDC5-cell chondrogenic differentiation suggests that leptin may play a regulatory role in the normal endochondral ossification process.

In conclusion, this study further supports the direct effect of leptin on growth-plate chondrocytes, and identifies the intracellular events that occur in response to leptin. We found that leptin induced the activation of the MAPK cascades ERK1/2, p38, and JNK and that inhibition of the STAT3 and ERK1/2 pathways abolished the effect of leptin on type X collagen, a marker of chondrocyte differentiation. The inhibition of p38 yielded no such effect, and its role in this setting warrants further investigation.

Endochondral ossification is a complex process, which requires the coordinated function of numerous factors. Our results suggest that leptin may be an important link between nutrition and growth, and may be one of the mediators of the "growth without growth hormone" phenomenon which is often seen in obese children [50].

Materials and methods

Reagents

Recombinant ovine leptin was produced by Prof. A. Gertler (The Hebrew University, Jerusalem, Israel). ATDC5 cells were obtained from the European Collection of Cell Culture (ECACC) (Wiltshire, UK); insulin/transferrin/sodium-selenite mixture from Sigma (St. Louis, MO, USA); MEK inhibitor (U0126) from Cell Signaling Technology (Danvers, MA, USA); p38 inhibitor (SB203580) from Alexis Biochemicals (Lausen, Switzerland); cell-permeable STAT3 inhibitor from Calbiochem (San Diego, CA, USA) (Cat. No. 573095); anti-phosphorylated and anti-non-phosphorylated ERK1/2, ERK5, P38, JNK and STAT3 antibodies from Cell Signaling Technology; anti-ERK2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA);

anti-β-actin from ABcam (Cambridge, UK); reverse transcriptase polymerase chain reaction (RT-PCR) reagents and equipment from Applied Biosystems (Foster, CA, USA).

Cell culture

ATDC5 cells were plated at a density of 0.7×10^4 cells/cm^2 at 37°C and 5% CO_2. For routine expansion, cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM)/F12, containing 5% fetal calf serum (FCS), 2 mM glutamine and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The medium was changed every second day. For the experiments, the medium was supplemented with 10 μg/ml insulin, 10 μg/ml transferrin and 3×10^{-8} mol/l sodium selenite; leptin (10–100 ng/ml) was added for the indicated periods. To test inhibition, inhibitors at the indicated concentrations were added 2 h before treatment with leptin (50 ng/ml), which was then added for another 8 h. As a positive control for the phosphorylation experiments, cells were stimulated for 10–30 min with peroxyvanadatae solution (VOOH) [containing 100 μM sodium orthovanadate (Na_3VO_4) and 200 μM H_2O_2 in Phosphate Buffered Saline (PBS)].

RNA isolation and cDNA preparation

Total RNA was isolated by the acid–guanidinium–thiocianate–phenol–chloroform method [51]. In brief, cells were lysed with a denaturing solution (containing 4 M guanidinium–thiocyanate, 25 mM sodium-citrate pH 7.0 and 0.1 M β-mercaptoethanol), and treated with 2 M sodium-acetate (pH 4.0) and then phenol and isoamyl-alcohol/chloroform (1:49). After centrifugation, the pellet was precipitated with isopropanol, and RNA was dissolved in RNase-free water (Qiagen, Hilden, Germany). The RNA yield was spectrophotometrically determined, and its quality was assessed on denaturing agarose gel. A digestion step with DNase I (Promega, Madison, WI, USA) was introduced to prevent any genomic DNA from interfering with the PCR reaction. For reverse transcription, the Superscript III First-Strand Synthesis System and random hexamer primers were used, according to the manufacturer's instructions (Invitrogen, Gaithersburg, MD, USA).

Real-time PCR

PCR analyses were done with an ABI prism 7000 sequence detection system. The amount of cDNA used for the reaction was determined for each gene individually, prior to the experiment, to avoid great differences between the endogenous control and the examined gene. In practice, cDNA (10–100 ng) was used as a template to determine the relative amounts of mRNA. For each experiment, cDNA of 3–4 independent cultures was used in triplicate. The real-time PCR reaction was carried out using the TaqMan assay, with specific pre-made primers. The signals from the leptin and control groups were normalized to ribosomal protein S3, which showed no significant difference between the two groups. TaqMan primers were used for the following genes: S3 (Assay ID: Mm00656272_m1), type II collagen (Mm00491889_m1), type X collagen (Mm00487041_m1), c-jun (Mm00495062_s1), and c-fos (Mm00487425_m1).

Western blot

Protein extracts were assayed for protein content using the DC protein assay (Bio-Rad, Hercules, CA, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were performed with 50 μg of total protein extracts. Specific antibodies were used for phosphorylated and non-phosphorylated ERK1/2, ERK5, p38, JNK and STAT3. The secondary anti-rabbit horseradish peroxidase-conjugated antibodies were diluted 1:10,000 in Tris-buffered saline containing 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween-20. Antigenic expression was visualized by Super Signal West Pico chemiluminescence substrate (Pierce Biotechnology, Rockford, IL, USA) after exposure to X-ray film (Kodak, Rochester, NY, USA). Protein expression was quantified by densitometry analysis using the VersaDoc Imaging System (Bio-Rad Laboratories). Blots were assayed for β-actin content as standardization of sample loading. Quantitative densitometric values of each protein of interest were normalized to β-actin or to the non-phosphorylated form of the protein.

Immunofluorescence cell staining

ATDC5 cells were seeded on glass cover-slips in 24-well plates, maintained in low-serum-containing medium (0.5% FCS) for 24 h, and then placed in serum-free medium for an additional 48 h and treated with 50 ng/ml leptin for 3 h or left untreated. Translocation was shown in 3-day-old cultures because of technical considerations: In order to show isolated cells, the culture needs to be very young; otherwise, the cells are too condensed and tend to grow on top of each other and form nodules. The cells were fixed with 4% (w/v) paraformaldehyde, permeabilized with ice-cold methanol and blocked with 10%

non-immunized goat serum (Zymed by Invitrogen). Subsequently, the cells were incubated overnight, first with anti-ERK2 antibody (1:50), and then with goat anti-rabbit Cy3 (1:100) (Jackson Laboratory, West Grove, PA, USA). Nonimmunized rabbit serum was used as a negative control. To locate the nuclei, the cells were incubated with 3 μg/ml of 4′-6-diamino-2-phenylindole (DAPI) (Sigma) for 5 min. The cells were visualized with a fluorescence microscope and captured by an Olympus DP50 camera. Image-Pro plus 4.5.1 software (Media Cybernetics, Silver Spring, MD, USA) was used for quantification.

Statistical analysis

All data represent three independent experiments. Real-time PCR was also performed in triplicate samples. Values are presented as mean ±SD. Statistical differences were determined with Student's *t*-test. $P < 0.05$ was considered significant.

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