Original Research

The activation of leptin-mediated survivin is limited by the inducible suppressor SOCS-3 in MCF-7 cells

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Abstract

Although leptin has been found to be implicated in obesity-related breast carcinogenesis in postmenopausal women, the molecular mechanisms involved are yet to be defined. Recently, the antiapoptotic gene *survivin* has been recognized as a target gene for leptin in breast cancer. The aim of this study was to investigate the effect of leptin on the expression of survivin and on the transcriptional activity of its promoter in MCF-7 breast cancer cells. We also studied the potential involvement of SOCS-3 (a negative regulator of leptin's main signaling pathway JAK2/STAT3) in the expression of leptin-mediated survivin. Our results showed a significant increase in the mRNA (dose-dependent increase of 40–70%) and protein expression levels of survivin 24 h post-leptin treatment, which was followed by a significant decrease at 48 and 72 h (of 60–70%). In accordance, a chromatin immunoprecipitation assay revealed an initial strong binding of STAT3 to the *survivin* promoter, which was no longer detected after 24 h. Myc/mad/max network proteins and histone H3 acetylation status were not found to contribute to the expression of leptin-mediated survivin. Furthermore, a protein immunoprecipitation assay detected an enhanced SOCS-3 binding to the long isoform of leptin's receptor (Ob-Rb) 48 and 72 h after leptin administration, thus conferring inhibition to leptin signaling. In conclusion, our findings suggest, for the first time to our knowledge, that the effect of leptin on the antiapoptotic gene *survivin* is limited by the inhibitory role of SOCS-3 in the leptin-activated JAK2/STAT3 signaling pathway in MCF-7 breast cancer cells.

Keywords: leptin, survivin, STAT3, SOCS-3, MCF-7 cells

Experimental Biology and Medicine 2011; **236**: 70–76. DOI: 10.1258/ebm.2010.010224

Introduction

Accumulating epidemiological data indicate that obesity is associated with increased risk for breast cancer in postmenopausal women; however, the molecular links are yet to be clarified.[1] Adipose tissue-derived hormones, called adipokines, are the main body weight regulators and have been found to promote breast cancer development and progression.[2] Among them, leptin, the 16-kDa non-glycosylated protein product of the Ob gene, is a multifunctional hormone with a prominent role in breast carcinogenesis.[3,4] Leptin exerts its actions in human tissues through its at least four isoforms of receptors, Ob-Ra, Ob-Rb, Ob-Rc and Ob-Re, with OB-Rb being the main signaling form and reported to act as a mitogen on many cell types.[5–10] In several breast cancer cell models, leptin has been shown to induce survival and cancer cell proliferation, while in breast cancer, leptin and its receptor have been found to be overexpressed and have been correlated with poor prognosis.[11–17]

Leptin signaling is transmitted by multiple signaling pathways, including the Janus-activated kinase 2/signal transducers and activators of transcription 3 (JAK2/STAT3) pathway.[18] JAK2/STAT3 pathway activation is considered to be the primary signal transduction pathway mediated by leptin, leading to STAT3 phosphorylation and its translocation to the nucleus and thus consequent transactivation of specific target genes.[19] STAT3 has been shown to contribute to oncogenesis by promoting cell cycle progression and inhibiting apoptosis.[20] Survivin, a member of the inhibitor of apoptosis protein (IAP) family, has been shown to be an immediate target gene of STAT3 in human breast cancer cells.[21] Survivin is invariably upregulated in human malignancies and has been linked to poor clinical outcome.[22]

The suppressors of cytokine signaling (SOCS) proteins are negative regulators of the JAK/STAT pathway and inhibit cytokine signaling.[23] It is considered that SOCS proteins can attenuate signaling by inhibiting JAK activity or by promoting protein degradation.[24–26] SOCS-3 has been identified as an inducible suppressor of leptin signaling and it has been proposed that prolonged Ob-Rb stimulation can attenuate leptin signaling via SOCS-3 in human embryonic kidney cells and in the mouse hypothalamus.[27–30] In breast cancer cells, SOCS-3 over-expression has been shown to decrease proliferation and anchorage-independent growth.[31]

In this study, we investigated the effect of leptin on the expression profile and the transcriptional regulation of the antiapoptotic gene *survivin* in MCF-7 breast cancer cells.

Materials and methods

Cell culture

The human breast cancer cell line MCF-7, previously reported to respond to leptin treatment, was selected for this study.[18,32] MCF-7 cells were maintained in RPMI 1640 medium (Gibco, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin (Gibco) at 37°C under a humidified 5% CO_2 atmosphere.

Leptin treatments

Cells were cultured in 25-cm^2 flasks and were allowed to attach overnight in serum-containing medium. Cells were then deprived of serum and treated with 25, 50, 100 or 200 ng/mL of human recombinant leptin (R&D Systems, Minneapolis, MN, USA) for 24, 48 or 72 h. Controls included serum-free, leptin-untreated cells and serum-supplemented (10% of fetal bovine serum in the medium) leptin-untreated cells.

Cell viability

Cells were plated at 1×10^4 cells/well in a 96-well plate and allowed to attach overnight. The following day, cells were shifted to serum-free medium and then treated with 25, 50, 100 or 200 ng/mL of human recombinant leptin (R&D Systems) for 24, 48 or 72 h. Controls included serum-free leptin-untreated cells. Cell viability was estimated using the TACS MTT assay kit (R&D Systems) according to the manufacturer's instructions as previously described.[33]

Study of apoptosis

The percentages of apoptotic cells after leptin treatment and at different time points were determined by flow cytometry. The study of apoptosis was performed using the TACS AnnexinV-FITC Apoptosis Detection Kit (R&D Systems). Stained cells were analyzed using the FACSCalibur (Beckton Dickinson, Franklin Lakes, NJ, USA). At least 10,000 events were collected for each sample.

Quantitative real-time reverse transcriptase-polymerase chain reaction

Total cellular RNA was extracted from MCF-7 cells using Trizol reagent (Gibco) according to the manufacturer's instructions. Preservation of 28S and 18S rRNA species was used to assess RNA integrity. Only samples with prominent 28S and 18S rRNA components were included in the study. Of the total RNA, 1 μg from each sample was reverse transcribed to complementary DNA (cDNA) using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen, Life Technologies, Paisley, UK) and random primers (Invitrogen). Survivin mRNA expression was evaluated by real-time polymerase chain reaction using FastStart Universal SYBR Green Master (ROX) (Roche, Mannheim, Germany) in a iCycler Optical Module (Bio-Rad, Hercules, CA, USA). Reactions were performed in triplicate using 2 μL of cDNA per reaction and primers specific for survivin (forward: 5′ CGA GGC TGG CTT CAT CCA 3′; reverse: 5′ GCA ACC GGA CGA ATG CTT T 3′′), while human porphobilinogen deaminase was used as a housekeeping gene (forward: 5′ AGA GTG ATT CGC GTG GGT ACC 3′; reverse: 5′ GGC TCC GAT GGT GAA GCC 3′).

Western blot analysis

MCF-7 cells were trypsinized, collected and centrifuged for three minutes at 2000 rpm. Cell pellets were lysed using Nonidet P-40 lysis buffer containing 30 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40 and a cocktail of protease inhibitors for 30 min on ice, followed by centrifugation for 20 min at 13,000 rpm. Supernatants were transferred in new tubes and stored at −80°C. The protein concentration was quantified using the Bio-Rad Bradford protein assay (Bio-Rad), with bovine serum albumin as standard. Equal amounts of protein were heated at 95°C for five minutes and then electrophoresed and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad) and transferred to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, USA). The membrane was probed with antibodies against survivin (1:100 dilution) (sc-10811, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and SOCS-3 (1:400 dilution) (sc-9023, Santa Cruz Biotechnology) and signals were detected using anti-rabbit immunoglobulin IgG conjugated with horseradish peroxidase (1: 1000 dilution). Nitrocellulose membranes were then exposed to photographic film and the chemiluminescence was resolved by an enhanced chemiluminescence ECL kit (Amersham, Milan, Italy). Survivin and SOCS-3 protein levels were normalized to β-actin protein levels.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed using a ChIP assay kit (Upstate USA, Inc, Charlottesville, VA, USA) on leptin-treated and -untreated cells. Briefly, cells were cross-linked by incubating them in 1% (vol/vol) formaldehyde-containing medium for 10 min at 37°C and then sonicated to make soluble chromatin with DNA

fragments between 200 and 1000 bp. Samples of total chromatin were taken at this point to use as a positive control in the PCRs (input chromatin). Cell lysates were precleared by incubation with G-Sepharose beads and then incubated with anti-STAT3 (sc-483), anti-max (sc-197), anti-c-myc (sc-764) and anti-mad1 (sc-222) (Santa Cruz Biotechnology) polyclonal antibodies overnight at 4°C. For acetylation status analysis, antibodies against acetylated histone H3 (06–599) (Upstate Biotechnology, Lake Placid, NY, USA) were used for immunoprecipitations. DNA–protein complexes were collected with G-Sepharose beads followed by several rounds of washing, eluted and reverse cross-linked. Following the treatment with Protease K (Sigma-Aldrich, Munich, Germany), samples were extracted with phenol–chloroform and precipitated with ethanol. The recovered DNA was resuspended in TE buffer and used for PCR amplification. The following primer sets were used for the amplification of human *survivin* promoter region for STAT3 binding: forward primer, 5′ CAG TGA GCT GAG ATC ATG CC 3′ and reverse primer, 5′ TAT TAG CCC TCC AGC CCC AC 3′ and for myc/mad/max binding and histone H3 acetylation: forward primer, 5′ CTG CAC GCG TTC TTT GA 3′ and reverse primer, 5′ GCG GTG GTC CTT GAG A 3′. PCR generated a 232- and 327-bp fragment of the *survivin* promoter, respectively. The PCR products were fractionated on 3% agarose gels and were stained with ethidium bromide.

Protein immunoprecipitation assay

Protein was extracted from untreated and leptin-treated MCF-7 cells as described above. Equal amounts of protein were incubated with antibody against Ob-R (sc-1834) or SOCS-3 (sc-9023) (Santa Cruz Biotechnology) for two hours at 4°C with rotation. Protein–protein complexes were collected with A/G agarose beads. After several rounds of washing and elution, the proteins were detached from the agarose beads upon incubation at 95–100°C. Samples were then centrifuged at 8000 rpm for 10 min and supernatants were transferred in new tubes and stored at −80°C. Western blot analysis was performed with antibodies against SOCS-3 (sc-9023) or Ob-R (sc-1834) (Santa Cruz Biotechnology).

Statistical analysis

All calculations were performed using the SPSS software (version 11.0). Data were analyzed by the unpaired *t*-test, as well as analysis of variance and the Tukey's honestly significant difference (HSD) test as the *post hoc* test was applicable. A two-sided P value <0.05 was considered statistically significant.

Results

Cell viability after leptin treatment

We first studied the effect of leptin on MCF-7 cell viability. In order to do so, MCF-7 cells were treated with increasing doses (25–200 ng/mL) of leptin and cell viability was measured at 24, 48 and 72 h post-treatment. As illustrated in Figure 1, treated cells demonstrated dose-dependent increase of their proliferation compared with serum-starved cells, as evidenced by the MTT assay. More specifically, a dose-dependent increase of 20–30% was observed at 24 h and a dose-dependent increase of 40–50% occurred at 48 and 72 h post-treatment.

Alteration in the survivin expression profile in response to leptin treatment

Because survivin is an immediate target of STAT3, which exerts its action downstream of leptin, we investigated the effect of leptin on survivin mRNA and protein expression. As shown in Figure 2, treatment of MCF-7 cells with increasing doses (25–200 ng/mL) of leptin led to a significant (of 40–60%) dose-dependent up-regulation of survivin mRNA expression 24 h post-treatment compared with untreated cells ($P < 0.05$), whereas a significant down-regulation (of 60–70%) of survivin mRNA expression was observed at 48 and 72 h post-treatment ($P < 0.05$) (Figure 2a). This effect of leptin was preserved at the protein level as well, as demonstrated in Figure 2b.

Study of apoptosis after leptin treatment

The fluctuation of survivin expression after leptin administration in MCF-7 cells prompted us to investigate the apoptotic index of treated and untreated cells. Apoptosis was measured in serum-starved, serum-supplemented and leptin-treated (100 and 200 ng/mL) cells at 24, 48 and 72 h post-treatment. As demonstrated in Figure 3, a significant dose-dependent decrease of apoptosis was observed at 24 h post-leptin administration, in agreement with the observed increase of survivin mRNA and protein expression. Percentages of apoptosis at 48 and 72 h did not differ significantly between leptin-treated and serum-starved cells.

Survivin's promoter activity after leptin treatment

The alteration in survivin mRNA and protein expression profiles upon leptin treatment prompted us to investigate the differential binding of transcription factors c-myc, mad1, max and STAT3 to the *survivin* promoter, all found to participate in *survivin* gene's expression regulation, as well as the acetylation status of histone H3, in leptin-treated and -untreated cells at 24, 48 and 72 h. The ChIP assay did not reveal any changes between leptin-treated and -untreated cells in the acetylation status of H3 histone, or in the binding of transcription factors c-myc, mad1 and max to the *survivin* promoter at any time point. On the contrary, the ChIP assay revealed a strong binding of the transcription factor STAT3 to the gene's promoter 24 h after leptin treatment, which was interestingly no longer detected 48 or 72 h post-treatment, in accordance with survivin expression profiles (Figure 4).

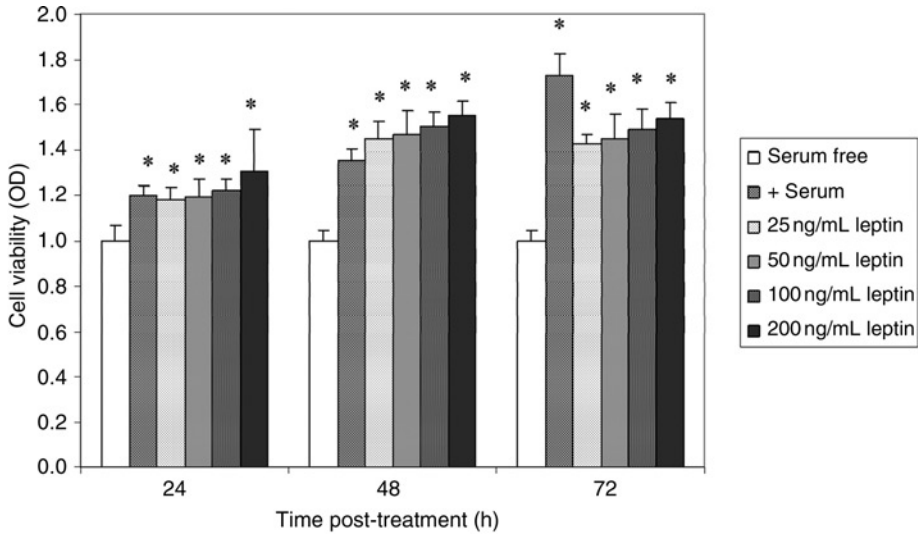

Figure 1 MCF-7 cells were incubated with increasing doses of leptin (25–200 ng/mL) for 24, 48 and 72 h. Cell viability was measured by the MTT assay. Serum-free cells were used as controls and their cell viability was set to 1. The cell viability of cells cultured in serum supplemented (10% of fetal bovine serum) medium was also measured. Histograms represent mean values of six different measurements, bars represent standard errors and asterisks designate statistically significant results ($P < 0.05$)

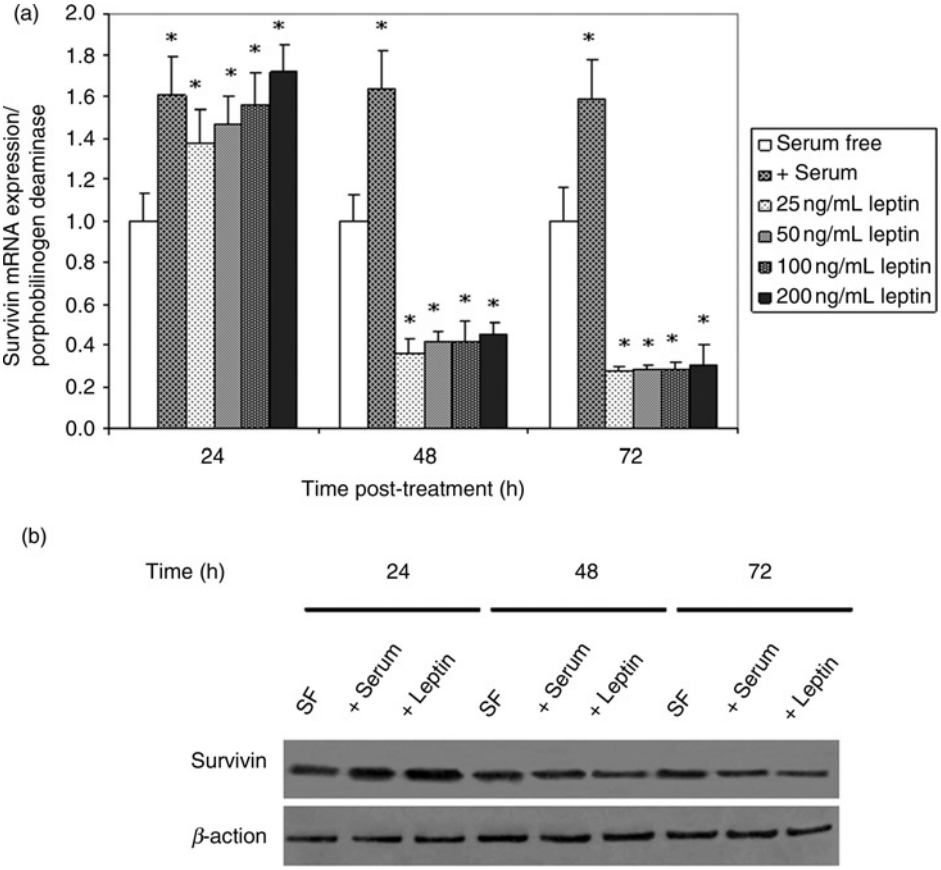

Figure 2 (a) Histograms represent the increase or decrease of survivin expression in MCF-7 cells incubated with increasing doses of leptin (25–200 ng/mL) for 24, 48 and 72 h. Measurements were performed in triplicate. Bars represent standard errors and asterisks designate statistically significant results ($P < 0.05$). (b) Western blot analysis of survivin protein expression in serum-free, serum-treated (10% fetal bovine serum) and leptin-treated (200 ng/mL) MCF-7 cells. Analysis for β-actin was performed to show equal loading

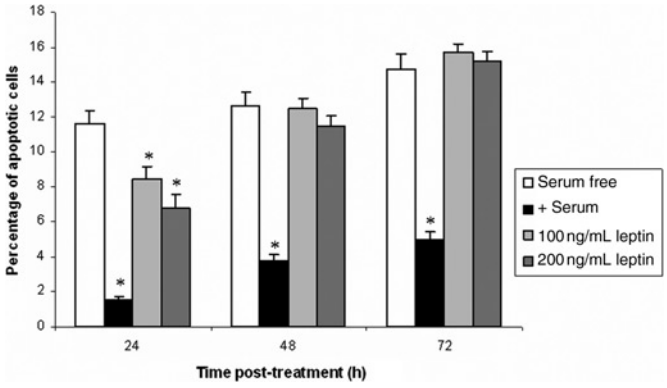

Figure 3 Measurement of apoptosis in serum-starved, serum-supplemented (+ serum) and leptin-treated (100 ng/mL and 200 ng/mL) MCF-7 cells at 24, 48 and 72 h post-treatment using the Annexin V assay. Histograms represent mean values of three different measurements, bars represent standard errors and asterisks designate statistically significant results ($P < 0.05$)

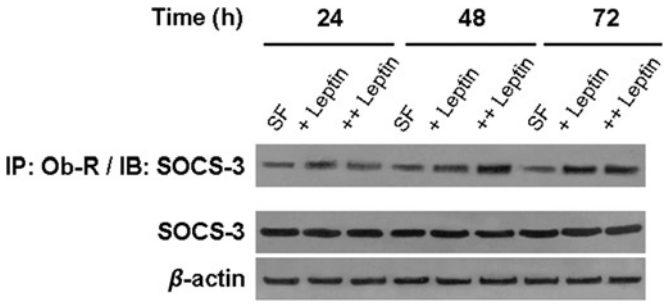

Figure 5 Upper panel: the immunoprecipitation (IP) assay was performed to examine the recruitment of SOCS-3 on the intracellular domain of Ob-R. Ob-R receptor from leptin-treated and -untreated cells was immunoprecipitated with an antibody against Ob-R, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% [w/v]) acrylamide and immunoblotted using an antibody against SOCS-3. Lower panel: Western blot analysis of SOCS-3 protein expression in serum-free and leptin-treated (100 [+] and 200 ng/mL [++]) MCF-7 cells. Analysis for β-actin was performed to show equal loading

SOCS-3 binding to Ob-Rb after leptin treatment

The observed decrease in survivin expression levels and the simultaneous low binding of STAT3 to the *survivin* promoter 48 and 72 h after leptin treatment led us to examine the possible implication of SOCS-3 in the negative regulation of leptin signaling in MCF-7 breast cancer cells. We thus studied the interaction between SOCS-3 and the Ob-Rb intracellular domain. As shown in Figure 5, SOCS-3 binding to Ob-Rb was found to be enhanced in leptin-treated MCF-7 cells at 48 and 72 h compared with leptin-untreated cells, whereas no changes were detected 24 h after leptin administration. The study of SOCS-3 protein levels did not reveal significant changes between leptin-treated and -untreated cells (Figure 5).

Discussion

Increasing evidence suggests that leptin may act as a mitogenic, transforming, angiogenic, migration factor, and that it might be involved in the pathogenesis of different malignancies including breast cancer.[6,11] However, the molecular mechanisms underlying the impact of leptin on breast cancer development and progression are not fully understood. In this study, we investigated the mitogenic effect of leptin on MCF-7 breast cancer cells and examined survivin expression and transcriptional regulation in leptin-treated breast cancer cells.

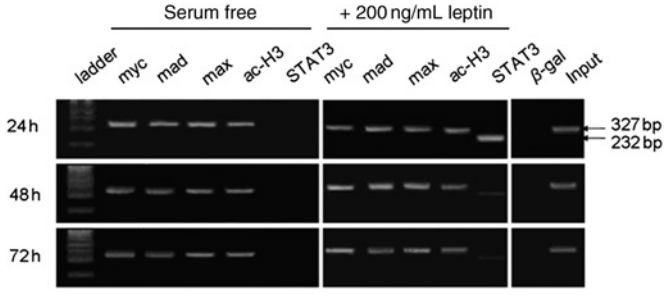

Figure 4 Chromatin immunoprecipitation analysis showing binding of transcription factors c-myc, mad1, max and STAT3, as well as acetylation status of histone H3 to the *survivin* promoter in leptin-treated or serum-starved cells

We found that increasing doses of leptin induced cell proliferation in serum-starved MCF-7 cells in a dose-dependent manner. This finding is in accordance with previous relative observations in the normal breast epithelial cell line HBL100 and in breast cancer cell lines MCF-7, T-47D and ZR75–1.[7,18,32] Moreover, leptin has been found to induce survival and anchorage-independent growth of MCF-7 and T-47D human breast cancer cell lines.[6,32]

It is considered that leptin exerts its proliferative activity through a combination of mechanisms leading to proliferation stimulation and apoptosis inhibition.[18,32] The recent finding that survivin, a member of the IAPs, has been recognized as a target gene for leptin in breast cancer,[34] prompted us to investigate survivin mRNA and protein expression in MCF-7 cells after leptin treatment. We found that leptin increased survivin expression, 24 h post-treatment, but a decrease in both survivin mRNA and protein levels was observed when exposure to leptin was extended to 48 or 72 h. In a previous study, Jiang *et al.*[35] treated MCF-7 cells with leptin up to two hours and observed an increase in survivin expression. In this study, we showed that this initial increase in survivin expression lasted for at least 24 h post-leptin treatment and was followed by a subsequent decrease in survivin mRNA and protein expression levels. The measurement of the apoptotic index of MCF-7 cells revealed a reduction in apoptosis at 24 h post-leptin treatment (100 and 200 ng/mL), in agreement with previous observations,[34] compared with 48 and 72 h, in accordance with the changes in survivin expression at the same time points.

The differential survivin expression levels after leptin treatment at 24 h, and 48 and 72 h prompted us to investigate the possible differential binding of transcription factors, as well as the acetylation status of histone H3 to the *survivin* promoter. c-myc over-expression has been found to increase survivin expression, while survivin has been recognized as an immediate downstream target of STAT3 in breast cancer cells.[21,35,36] It is known that the *survivin* promoter contains a response element for proteins of the myc/mad/max complex,[36] as well as two STAT3

binding sites.[21] We observed that leptin administration initially induced STAT3 binding to the *survivin* promoter, followed by a reduction at 48 h and afterwards, whereas no profile modification was detected concerning H3 acetylation status or myc/mad/max binding, suggesting that leptin treatment has no effect on these molecular pathways. Previous studies have reported a strong STAT3 binding to the *survivin* promoter 30 min post-leptin treatment.[35] In this study, we provide, for the first time to our knowledge, information about *survivin* promoter activity up to 72 h after leptin administration and we demonstrated that STAT3 activated survivin expression at least up to 24 h post-treatment. STAT3 was not found to bind to the *survivin* promoter 48 or 72 h post-leptin treatment, in accordance with survivin expression levels.

The above finding led us to examine the potential inhibitory role of SOCS-3 protein, being a negative regulator of leptin signaling by inhibition of JAK2/STAT3 signaling pathway through recruitment to the intracellular domain of the activated Ob-Rb.[30] We found that stimulation of Ob-Rb by leptin did augment the binding of SOCS-3 to Ob-Rb at 48 and 72 h post-leptin treatment in concordance with our findings on *survivin* gene expression and regulation by STAT3. The absence of changes in SOCS-3 protein levels suggests an activation of SOCS-3 protein upon extended leptin presence. Previous studies in cultured cells and in the mouse hypothalamus had suggested that chronic high levels of leptin can attenuate leptin signaling through the initiation of SOCS-3 transcription.[29,37–39] However, this was not the case in our study, because leptin treatment up to 72 h did not modify SOCS-3 protein levels, but only seemed to enhance SOCS-3 binding to the leptin receptor. Moreover, over-expression of SOCS proteins has been found to reduce STAT activity in cancer cells, inhibit proliferation and induce apoptosis of these cells.[23] More specifically, in breast cancer cells, it has been found that long-term over-expression of SOCS-3 suppresses STAT3 expression and activation.[31] However, it seems that the inhibitory effect of leptin-mediated SOCS-3 on STAT3 activation and survivin expression does not have a significant impact on the proliferation and survival of breast cancer cells probably owing to the multiple signaling pathways activated by leptin.

In conclusion, in this study we provide novel evidence for the limited contribution of the antiapoptotic gene *survivin* to leptin-mediated MCF-7 breast cancer cell proliferation and progression due to the inducible suppressor SOCS-3, suggesting that other STAT3-independent pathways might be responsible for leptin's proliferative effect on breast cancer cells.

Authors contributions: All authors participated in the design of the study. MP conducted most of the experiments, interpreted the results, analyzed data and drafted the manuscript; VP and NS conducted part of the experiments; and AT participated in data analysis and finalized the manuscript.

Funding: The present work was financed by the Institute of Biomedical Research and Technology, Larissa, Greece.

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(Received July 26, 2010, Accepted September 30, 2010)