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Leptin does not act on proliferation but increases the expression of cell immunity- related genes MX-1, MX-2, and IFI-27 in the glioblastoma cell line T98G. Does leptin protect the cell from a viral intruder?

Buse TÜREGÜN ATASOY[1], Mehmet KIYAN[1], Cengiz TOPTAŞ[2], Ahmet ERGÜN[3], Eyüphan ŞEKEROĞLU[2], Egemen SARIMADEN[2], Shameem AKHTER[1], Fikret ŞAHİN[1]

Departments of [1]Medical Microbiology, [3]Physiology, Ankara University, School of Medicine, Turkey

[2]Private Arı Anatolian High School

Keywords

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ABSTRACT • Background and Aims: Leptin is an adipocyte-derived hormone with roles in the central control of energy metabolism and many pleiotropic effects in different physiological systems. Studies indicate that leptin is associated with an increased risk of several cancers. In addition, increasing evidences show that leptin has role in innate and adaptive immunity to prevent viral infections. Nonetheless, no study has investigated the role of IFN-stimulated genes (ISGs) in single-cell immunity. In this study we first investigated how leptin acts on the proliferation of T98G cells and the expression of some of the proliferation and apoptosis-related genes. Secondly, we searched the effect of leptin on the expression of some of the major ISGs, which protect the cell from viral intruders in T98G cells. **Materials and Methods:** The effect of leptin on the proliferation of T98G cells was investigated using the MTT assay. The expression of proliferation-related genes and ISGs genes were investigated by reverse-transcriptase polymerase chain reaction. **Results:** Results showed that while there is no effect on the proliferation of T98G cells, leptin dramatically increased the expression of antiapoptotic protein IXAP in a dose-dependent manner. Importantly, leptin increased the expression of MX1, MX2, and IFI27, which have a broad range of antiviral activity. **Conclusion:** Leptin may protect the individual cell from viral intruders by expressing antiviral IGS molecules. However, further studies including animal models are necessary to precisely define the effect of leptin on the individual cell for protection against viruses.

Correspondence: Fikret ŞAHİN, Department of Microbiology, Ankara University School of Medicine, Basic Science Building, 3rd Floor, Sihhiye-Ankara,
Phone: +90 312 595 82 77 • Fax: +90 312 310 63 70 • E-mail: fsahin29@hotmail.com

INTRODUCTION

Leptin is a hormone with a similar structure to that of the cytokine family, including interleukin (IL)-6, IL-11, IL-12, leukemia inhibitory factor, granulocyte colony stimulating factor (G-CSF), and ciliary neurotropic factor (CNTF) (1,2). Leptin is synthesized mainly in adipose cells. It regulates weight control in a central manner (1,3). Circulating leptin levels (normal range 1–15 ng/mL) are related to body fat mass in both mice and humans. Obesity or overweight conditions are associated with a significantly increased risk of development of various diseases, particularly cardiovascular disease, type 2 diabetes, hypertension, dyslipidemia, liver disease, and cancer (2). Increasing evidences indicate that obesity is associated with over 13 different cancers, including breast, cervical, colon or rectal, esophageal, gall bladder, kidney, liver, ovarian, pancreatic, stomach, and uterine cancer, as well as multiple myeloma (4-8).

In addition to functioning in homeostasis, metabolism, and tumorigenesis, studies indicate that leptin involves the activation of the immune system. Leptin affects both innate and adaptive immunity (9,10). In innate immunity, leptin modulates the activity and function of mast cells by enhancing their migratory capacity and survival rate (11). Leptin stimulates the release of inflammatory cytokines (including IL-1b, IL-6, and IL-8) and chemokines (monocyte chemotactic protein-1). Leptin also modulates the activity and function of neutrophils by increasing chemotaxis and the secretion of oxygen radicals (such as hydrogen peroxide, H_2O_2, and superoxide, O_2) (12-14). In adaptive immunity, leptin affects the generation, maturation, and survival of T cells by reducing their rate of apoptosis. On memory T cells, leptin promotes the switch toward T helper 1 (Th1) cell immune responses by increasing interferon-γ (IFN-γ) secretion (2,9).

In this study we searched the effect of leptin on proliferation and the expression of proliferation-related genes of the glioblastoma cell line, T98G. We also investigated the expression of genes mainly related to single-cell innate immunity. We found that leptin has no effect on T98G proliferation but results in a dramatic increase in the expression of Mx proteins, which are known as protectors of the single cell against viruses.

MATERIALS and METHODS

Cells and cell growth conditions

Human glioblastoma multiform cells (T98G, European Collection of Cell Cultures) were used in these experiments. The cells were grown in Dulbecco's modified Eagle medium (Biochrom-Germany) supplemented with 10% (vol/vol) fetal bovine serum (Biochrom-Germany) and penicillin-streptomycin 1% (Biochrom-Germany) in a 5% CO_2 atmosphere at 37 °C. Growing T98G cells were collected after trypsinization and counted and splitted 4×10^4 to each of well of 24-well plates for cell viability. Also, the T98G cells were plated into 10 mm plates for the RNA extraction. Leptin was added after 24 h.

Drug and doses

Three different doses of metreleptin (Amylin Pharmaceutical, Inc, USA) were applied. The first dose (D1) was determined to be at the normal plasma level of non-obese people (10 ng/L). The second dose (D2) was 5–10 times higher than the normal level (200 ng/L). The third dose (D3) was 50 times higher than the normal level (1000 ng/L).

Cell survival assay

The cell survival assay (MTT) test was used for this purpose. MTT stock solution was prepared by diluting 25 mg MTT (3- (4,5-dimethyl diazole-2-yl) -2,5 diphenyl tetrazolium bromide) in 5 ml phosphate-buffered saline. At 48 h after the leptin treatment, 500 µl medium containing 10% w/w MTT was added to the cells. Three hours later, the medium was removed, and a lysis solution of 1% sodium dodecyl sulfate and 6% acetic acid dimethyl sulfoxide (DMSO) was added. After 10 min of moderate shak-

ing, the absorbance was measured by spectrophotometry at a wavelength of 570/630 nm.

Total RNA isolation

Total cell RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA quantification was carried out by spectrophotometry, and each sample was aliquoted and frozen at –80°C until required.

cDNA synthesis

The RNA samples were used as a template for reverse transcription with the RevertAid First Strand cDNA Synthesis Kit according to the protocol supplied by the manufacturer (MBI, Fermentas, Germany).

Primers used in the multiplex RT-PCR

All primers for cell viability, immunity-related genes, and the internal control were designed with the Primer3 primer tool program (http://primer3.ut.ee). The primer sequences are shown in Table 1.

Polymerase chain reaction

Polymerase chain reaction (PCR) was used first to determine the linear range of target genes. Second, the linear range of the internal standard was determined using actin primers. Conditions for PCR were optimized so that the target genes and actin internal control would be in the linear range. Reactions contained Taq DNA polymerase, reaction buffer, dNTP mix, target gene specific upstream and downstream primers or actin rRNA primers, MgCl2, and cDNA template. The thermocycler was programmed as follows: 94 °C for 2 minutes to denature template; 94 °C for 45 seconds for denaturation; at 60 °C for 45 seconds for annealing; and 68 °C for 1 minute for extension. The PCR products were electrophoresed in a 1.5% agarose gel, and the gel was stained with EtBr. EtBr signals were analyzed by using the ImageJ computer-assisted program, and the ratio of the target genes and internal control were used for the graphs.

Table 1 Used primers and sequences

Primer name	Sequences
BID F	GCTGTATAGCTGCTTCCAGTGTA
BID R	GCTATCTTCCAGCCTGTCTTCTC
BAD F	GCACAGCAACGCAGATGC
BAD R	AAGTTCCGATCCCACCAGG
XIAP F	TCAGCATCAACACTGGCACGAG
XIAP R	TCTCTTGGGGTTAGGTGAGCATAG
CIAPI F	GCACATTCATTATCTCCCACCTTG
CIAPI R	CATCATCCTTTGGTTCCCAGTTAC
P21 F	GGGGACAGCAGAGGAAGAC
P21 R	CGGCGTTTGGAGTGGTAGA
IFI6 F	AGCTGGTCTGCGATCCTGAATG
IFI6 R	ATCCTCCTCACTATCGAGATAC
IFI27 F	ACCTCATCAGCAGTGACCAGTG
IFI27 R	AATGACAGCCGCAATGGCAGAC
IFIT3 F	TCAAGGAAGACAGTGTCTCAAG
IFIT3 R	ATCTGAGCATCTGAGAGTCTGC
MX1 F	TGCATCGACCTCATTGACTC
MX1 R	CCGAAATCTCAATCTCGTAG
MX2 F	AATGAATTCCTTCCAGCAACA
MX2 R	ACTGGCTGTACAGGTTGTTC
IL28B F	TGACTGGAGCAGTTCCTGTC
IL28B R	GCAGAAGCGACTCTTCTAAG

RESULTS

Leptin does not affect T98G cell viability

Three different doses of leptin were used as described in Materials and Methods. There was no effect of leptin on cell proliferation, even in high-dose leptin-treated cells (Figure 1).

Leptin increases the expression of the antiapoptotic gene XIAP but has no effect on the expression of CIAP1, CIAP2, p21, BAD, and BID

After T98G cells were treated with the different doses of leptin, cells were extracted for total RNA. Following RNA extraction, cDNAs were synthe-

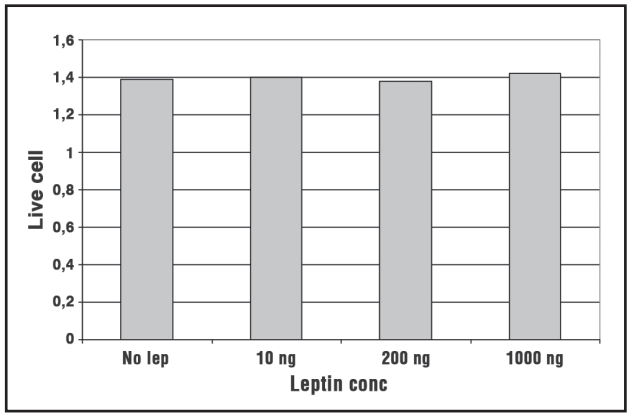

Figure 1 MTT result of cells 48 h after leptin treatment. (Lep: Leptin)

sized and used as a template for the individual RNA expression of apoptosis- and proliferation-related genes including XIAP, CIAP1, CIAP2, p21, BAD, and BID. Analysis of the expression of the CIAP1, CIAP2, p21, BAD, and BID showed that leptin has no effect on the expression of these genes. However, the expression of the anti apoptotic protein XIAP was increased in a dose-dependent manner (Figure 2).

Effects of leptin on the expression of cell immunity-related genes including IFI-6, IFI-27, IFIT-3, IL-28, MX-1, and MX-2

The expression of genes including IFI-6, IFI-27, IFIT-3, IL28, MX-1, and MX-2 related to single-cell immunity toward invading viruses were investigated. While leptin has no effect on the expression of IFI-6 and IFIT-3, it changed the expression of MX1, MX-2, and IFI-27 dose dependently. Leptin increased the expression of IL-28 at a low dose but decreased its expression at higher doses.

DISCUSSION

Leptin is an activator of cell proliferation, an anti-apoptotic molecule, and an inducer of cancer cells in many cell types. Its critical roles in tumorigenesis are based on its oncogenic, mitogenic, pro-inflammatory, and pro-angiogenic actions (5,7,15). Different human cancer cells exhibit differential responses to treatment with leptin. Leptin has a proliferative effect in some cancer cell types, such as esophageal and breast cells, hepatocytes, and Kupffer cells (15,16). Moreover, leptin controls apoptosis and the cell cycle of hepatic cancer cells. However, treatment with different concentrations of leptin did not result in cell proliferation in some cancers, including prostate cancer (17). In agreement with the results obtained for prostate cancer, our results showed that leptin has no effect on the proliferation of the T98G cell line, even with higher doses of leptin. However, leptin increased the expression of XIAP, which is an antiapoptotic protein, in a dose-dependent manner. Our results indicate that there is no effect of XIAP expression on T98G proliferation after 48 h.

In addition to systemic effects related to energy homeostasis and functioning in cancer formation, studies indicate that leptin also has roles in innate and adaptive immunity (2,9). The first evidence of a possible involvement of leptin in regulation of the immune system was obtained from the study of its structure and receptor, which belongs to the class I cytokine superfamily (9,16-18, 36). Leptin receptors have the signaling capabilities of IL-6-type cytokine receptors, activating the JAK-STAT, PI3K, and MAPK signaling pathways (18,19). In fact, leptin receptors have been found in monocytes, granulocytes, and natural killer (NK) cells. Further studies indicate that leptin up-regulates the phagocytic function of monocytes and maturation of dendritic cells and activates granulocytes and NK cells (10,12,14).

Leptin also plays a role in adaptive immunity by affecting T cells and B cells (9,10,20,21). One important systemic response to infection is the ability to fight it by activating the immune system. The endocrine and immune systems are linked by a network of cytokines and neuropeptides, which modulate the response to infection (2).

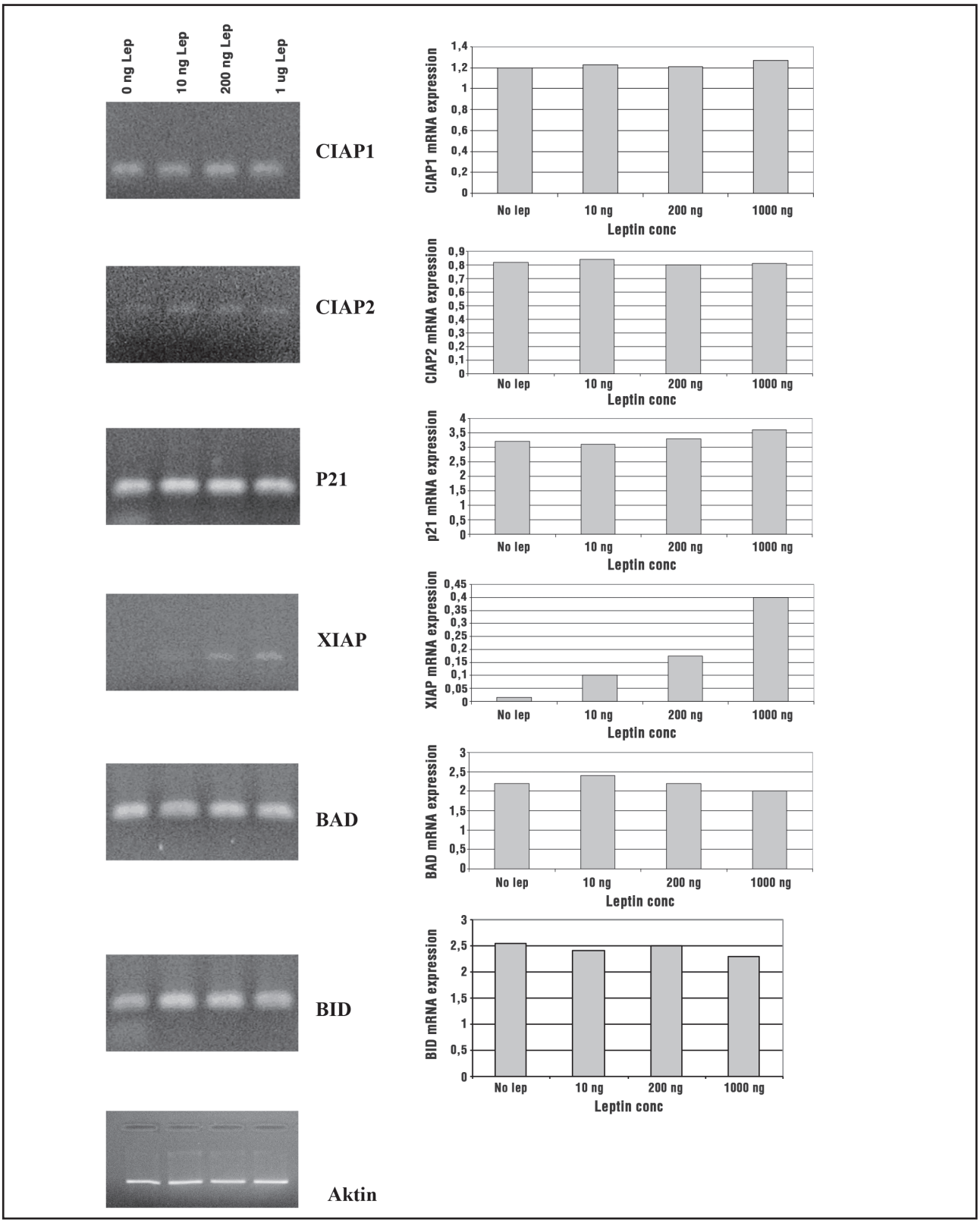

Figure 2 Expression analysis of proliferation-related genes. (Lep: Leptin)

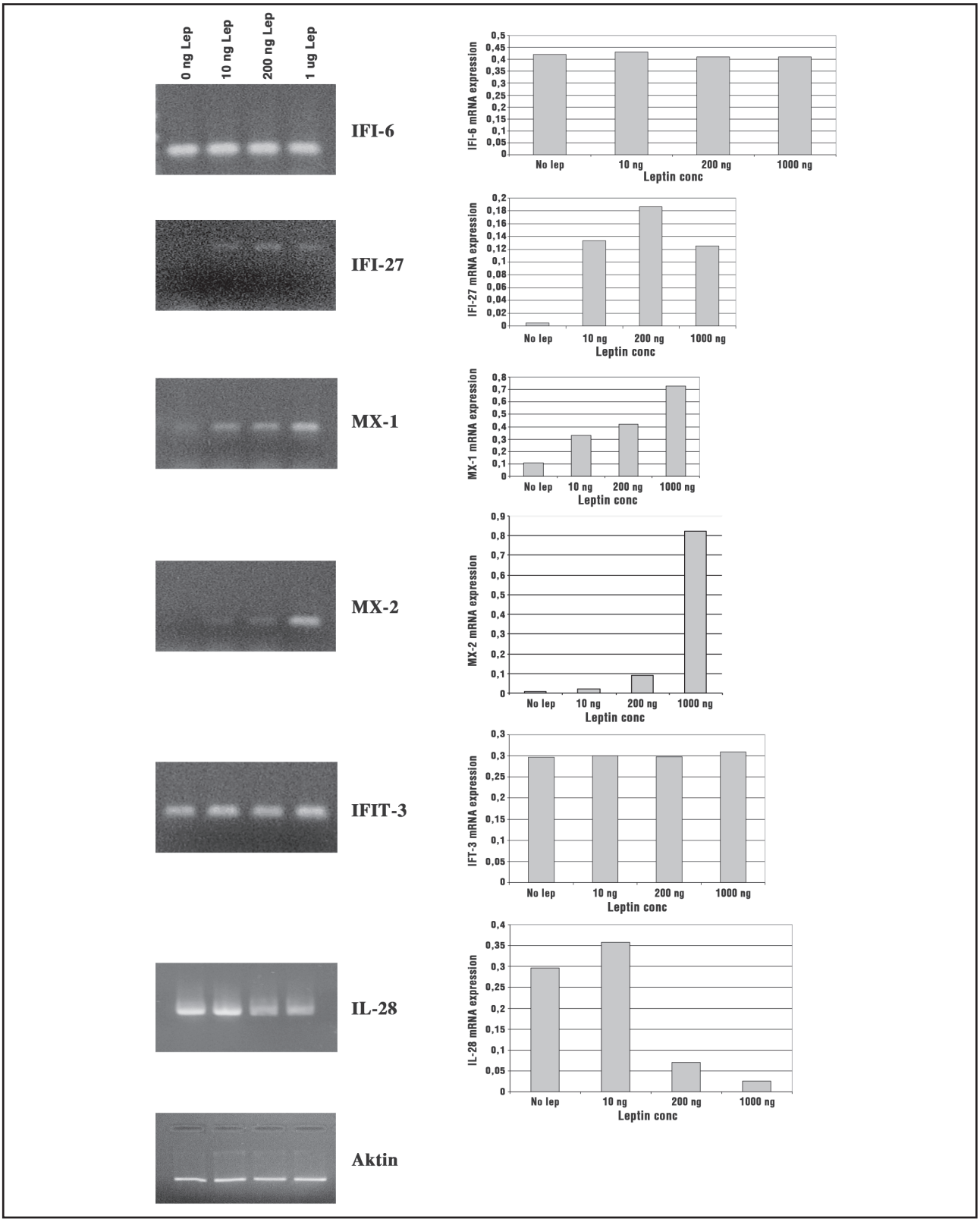

Figure 3 Expression analysis of ISG genes. (Lep: Leptin)

In addition to adaptive and innate immunity provided by T cells and B cells, monocytes, granulocytes, and NK cells, each cell has its own innate immunity against viral infection. Upon virus infection, viral pathogen-associated molecular patterns are sensed by host pattern recognition receptors (PRRs) (22). Activation of these PRRs leads to signaling through adaptor proteins that induce the expression of type I and III interferons (IFNs). In response to viral infection, secretion of type I interferon (IFN-I) is a key step in the host cells' innate immune responses. The antiviral actions of IFN-I are followed by the products of the IFN-stimulated genes (ISGs). To date, it is estimated that 2,000 human and mouse ISGs have already been identified, including IFI-6, IFI-27, Il-28, IRF-1, IRF-9, ISG-20, MX-1, OAS-1, PKR, and IFITM. However, most of these genes which have antiviral functions remain uncharacterized (23).

Although leptin has been investigated for a long time and increased evidence has been gathered in support of the effect of leptin on innate and adaptive immunity, no study has investigated the effect of leptin on the role of ISGs' in single-cell immunity. In this study we investigated some of the well-known ISGs, including IFI-6, IFI-27, MX-1, MX-2, IFIT-3, and IL-28. Our results showed that the expression of MX proteins 1 and 2 were increased in the presence of leptin with the drug in a dose-dependent manner (Figure 3). After the discovery of mouse and human MX proteins, it was found that MX proteins are present in almost all vertebrates (24). The studies described that Mx proteins have a broad range of antiviral activity, such as preventing the nucleocapsid protein form, viral RNA polymerase complex, inhibiting the accumulation of viral transcripts, and particularly the longer ones, suggesting an effect on elongation (24). MX proteins from different species possess distinct antiviral activities, and the subcellular localization of MX protein contributes to its antiviral specificity. In general, nuclear MX proteins (e.g., mouse MX-1) protect against viruses that replicate in the nucleus, such as influenza virus (24,25) whereas cytoplasmic forms (e.g., mouse MX-2) inhibit replication of vesicular stomatitis virus (VSV) and other viruses that replicate in the cytoplasm (26-28).

We found that the expression of IFI-27 was also increased in the presence of leptin. IFI-27 (interferon-stimulated gene 12a, interferon alpha inducible protein 27) is another ISG-related molecule which is induced by type I IFN in many cell types (29). Experimentally, it has been shown that IFI-27 mediates the cell response to Newcastle disease viral infection (29). The other ISG-related protein, IL-28 is associated with response to chronic hepatitis C. Hepatitis virus (HCV) infection induces a unique hepatic innate immune response associated with robust production of IL-28 (30). We found that IL-28 mRNA expression was increased by leptin at a normal serum concentration. IL-28 expression was suppressed by higher doses of leptin.

Among these ISGs, the family of IFN-induced proteins with tetratricopeptide repeats (IFITs) plays an important role in antiviral processes and restricts viral replication through altering protein synthesis, binding to viral RNAs, or interacting with structural or nonstructural viral proteins (31). IFIT-3 was demonstrated to inhibit the replication of many DNA and RNA viruses, such as hepatitis B virus, human papillomavirus, hepatitis C virus, West Nile virus, porcine reproductive and respiratory syndrome virus, Dengue virus, VSV, and others (32,33). The other ISG is interferon-α inducible protein 6 (IFI-6), which impairs EGFR activation by CD81 and inhibits hepatitis C virus infection (34,35). According to our result, lepton has no effect on IFIT-3 and IFI-6 expression in the T98G cell line.

In conclusion, our results indicate that leptin may protect the individual cell against viral intruders by expressing antiviral ISG molecules. However, further studies including animal models are necessary to define precisely the effect of leptin on the individual cell in terms of protection against viruses.

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