Leptin Induces Migration and Invasion of Glioma cells through MMP-13 Production

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Running title: leptin induces MMP-13 production

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Number of words in text: 3838

Number of figures: 7

Key words: glioma cells; invasion; leptin; matrix metalloproteinase

Abstract

Leptin, the product of the *obses* gene, plays an important role in the regulation of body weight by coordinating metabolism, feeding behavior, energy balance and neuroendocrine responses. However, central regulation of leptin gene expression is different from that in the adipocytes. In addition, leptin has been found in many tumor cell lines and has been shown to have mitogenic and angiogenic activity in a number of cell types. Glioma is the most common primary adult brain tumor with poor prognosis due to the spreading of tumor cell to the other regions of brain easily. Here we found that malignant C6 glioma cells expressed more leptin and leptin receptors than non-malignant astrocytes. Furthermore, it was found that exogenous application of leptin enhanced the migration and invasion of C6 glioma cells. In addition, we found that the expression of matrix metalloproteinase-13 (MMP-13) but not of MMP-2 and MMP-9 was increased in response to leptin stimulation. The leptin-induced increase of cell migration and invasion was antagonized by MMP-13 neutralizing antibody or silencing MMP-13. The up-regulation of MMP-13 induced by leptin was mainly through p38 MAP kinase and NF-κB pathway. In addition, migration-prone sublines demonstrate that cells with increasing migration ability had more expression of MMP-13 and leptin. Taken together, these results indicate that leptin enhanced migration and invasion of C6 glioma cells through the increase of

MMP-13 production.

Introduction

The hormone leptin, the product of the *obese* (Ob) gene, is a 16 kDa polypeptide which plays an important role in the regulation of body weight homeostasis by coordinating metabolism, feeding behavior, energy balance and neuroendocrine responses (Friedman and Halaas 1998). Leptin is secreted predominantly from adipocytes, and the blood-cerebrospinal fluid barrier at the choroids plexus and the blood-brain barrier at the cerebral endothelium are two major controlling sites for the entry of circulating leptin into the brain (Zhang et al. 1994; Zlokovic et al. 2000). Nevertheless, recent works indicate that leptin gene is also expressed in placenta (Senaris et al. 1997), stomach (Bado et al. 1998), heart (Matsui et al. 2007), skeletal muscle (Wang et al. 1998), and mammary gland (Smith-Kirwin et al. 1998). Morash *et al*. (1999) also report that gene and protein of leptin are expressed in rat cortex, cerebellum, hypothalamus, and pituitary gland in the CNS(Morash et al. 1999). Either overexpression or lack of leptin can result in phenotypic abnormalities, such as obesity (Hsing et al. 2007) or hypertension (Tumer et al. 2007). Leptin elicits its effects by interacting with specific receptors (ObR), which are found in many tissues with several spliced forms (Fei et al. 1997). The long isoform of leptin receptor (ObRl) has signaling characteristics while other shorter spliced variants (ObRt) are thought to serve mainly as leptin transporters or to

mediate leptin degradation (Uotani et al. 1999). However, central regulation of leptin gene expression is different from that in the adipocytes. The co-localization of leptin and leptin receptors in multiple brain regions is consistent with reports that leptin regulates brain development, neuronal projections, and the expression of neuronal and glial proteins (Ahima et al. 1999), and possesses both anti-apoptotic (Russo et al. 2004) and neuroprotective properties (Dicou et al. 2001). Leptin has also been shown to have mitogenic (Wolf et al. 1999) and angiogenic activity (Sierra-Honigmann et al. 1998) in a number of cell types.

Glioblastoma multiform is the most frequent and malignant tumor of the central nervous system (CNS) with aggressive histological features and an exceptionally poor clinical outcome. At early stages, while the tumor mass is small and seems well limited macroscopically, tumor cells have already invaded the surrounding brain (Kleihues et al. 1995). This pathological characteristic of their insidious infiltration of the brain leads to the poor prognosis after surgery and/or radiation therapy. Invasion of glioma cells into adjacent brain structures occurs through the activation of multigenic programs, including matrix metalloproteinases (MMPs). MMPs play a central role in tumor invasion due to their ability to degrade many extracellular matrix (ECM) components and other substrates (Ala-aho and Kahari 2005), and tumor cells consequently invade into the surrounding stroma consisting primarily of fibrillar

collagens. The MMPs can be divided into subgroups of collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs according to their substrate specificity and function (Yong et al. 2001). MMP-13 is a collagenolytic MMP characterized by a wide substrate specificity, and it is expressed by many types of invasive tumors, such as breast carcinomas (Freije et al. 1994), squamous cell carcinomas of the head and neck (Leivonen et al. 2006). The correlation between MMP-13 expression level and invasive capacity suggests that MMP-13 is an important molecule for cancer progression.

The expression of both leptin and leptin receptors detected in C6 glioma cells suggests a possible autocrine role for leptin homeostasis (Morash et al. 2000). In this study, we hypothesized that leptin plays an important role in the functional regulation of tumor. We demonstrate that exogenous application of leptin enhanced the production of MMP-13 but not of MMP-2 or MMP-9 in C6 glioma cells, and the leptin-induced MMP-13 expression increased cell migration and invasion of C6 glioma. This is the first evidence to demonstrate the relationship between leptin and MMP-13 on tumor progression.

Materials and Methods

Materials

Human recombinant leptin, LY294002 (PI3 kinase inhibitor), Wortmannin (PI3 kinase inhibitor), PD98059 (ERK inhibitor), SB203580 (p38 MAPK inhibitor), SP610025 (JNK inhibitor), PDTC (NF-κB inhibitor), TPCK (IκB protease inihibitor), and Bay 11-7082 (IκBα-kinase inhibitor) were purchased from Sigma (St. Louis, MO). Antibodies against MMP-13 and normal IgG were purchased from Santa Cruz Biotechnology (CA, USA).

Cell Cultures

C6 cells, originating from a rat brain glioma, were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in 75 cm^2 flasks with F12 (Gibco, Grand Island, NY, USA) supplemented with 15% heat-inactivated horse serum (HS; Hyclone, Logan, UT, USA) and 5% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator under 5% CO_2 and 95% air.

RBA1, adult rat brain astrocytes, was also purchased from American Type Culture Collection (ATCC, Manassas, VA). NRA (Neonatal rat astrocytes) was kindly provided by the Institute for Biological Sciences, National Research Council of Canada. Both cells were maintained in 75 cm^2 flasks with DMEM containing 10%

FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator under 5% CO_2 and 95% air.

Reverse Transcriptase-PCR

RNA extraction and reverse transcriptase-PCR were performed as described previously (Yeh et al. 2007) with mild modification. In brief, PCR was performed using an initial step of denaturation (5 min at 95°C), 35 cycles of amplification (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min), and an extension (72°C for 5 min). PCR products were analyzed on 2% agarose gels. The oligonucleotide primers were:

leptin: forward: 5'-TCACCCCATTCTGAGTTTGT-3' and

reverse: 5'-CGCCATCCAGGCTCTCT-3'

leptin receptor short form: forward: 5'-ACACTGTTAATTTCACACCAGAG-3' and

reverse: 5'-AGTCATTCAAACCATAGTTTAGG-3'

MMP-2: forward: 5'-GACCTTGACCAGAACACCATCG-3' and

reverse: 5'-GCTGTATTCCCGACCGTTGAAC-3'

MMP-9: forward: 5'-CCCCACTTACTTTGGAAACGC-3' and

reverse: 5'- AGCCACGACCATACAGATGCTG-3'

MMP-13: forward: 5'-GCCCTGAATGGGTATGACAT-3' and

reverse: 5'-GCATGACTCTCACAATGCGA-3'

GAPDH: forward: 5'-GCCATCAACGCCCCTTCATT-3' and

reverse: 5'-ACGGAAGGCCATGCCAGTGAGCTT-3'

Western Blotting

Protein extraction was performed as described previously (Yeh et al. 2007). After electrophoresis, protein was transferred from sodium dodecyl sulphate-polyacrylamide gels to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were incubated for 1 h with 4% dry skim milk in PBS buffer to block nonspecific binding and then immunoblotted overnight at 4℃ with the following primary antibodies: rabbit anti-Ob, mouse anti-ObR, rabbit anti-MMP-13, rabbit anti-p-Akt, mouse anti-p-ERK, mouse anti-p-p38, mouse anti-α-tubulin, rabbit anti-IKKα/β (1:1000; Santa Cruz Biotechnology, CA), rabbit anti-p-IKKα/β, and rabbit anti-p-JNK (1:1000; Cell Signaling Technology, MA). After PBST washing, the membranes were then incubated with goat anti-rabbit or anti-mouse peroxidase-conjugated secondary antibody (1:1000; Santa Cruz Biotechnology, CA) for 1 h. The blots were visualized by enhanced chemiluminescence (ECL; Santa Cruz Biotechnology, CA) using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY).

Enzyme-linked immunosorbent assay (ELISA)

C6, RBA1, and NRA were seeded onto 6-well plates, and 100 μl of culture medium was collected and cell lysate were extracted after 48 hr's culture. Leptin produced by different cell lines was measured by Rat leptin Immunoassay kit (BioSource,

Camarillo, CA) according to the procedure in protocol booklet. The absorbance was measured at 450 nm by an ELISA reader (Bio-Tek, Winooski, VT).

Gelatin Zymography

Samples of supernatant medium conditioned by cell culture under different experimental condition were centrifuged. Samples in equal volume were separated on a 10% sodium dodecyl sulphate (SDS) -polyacrylamide gel containing 0.1% gelatin. After electrophoresis, gels were washed with 2.5% Triton X-100 (in 50 mM Tris-HCl) for 30 min to remove SDS. Substrate digestion was formed by incubating the gel in a developing buffer (50 mM Tris-Hcl containing 5 mM $CaCl_2$, 1 mM Zn Cl_2, 0.02% NaN_3, and 1% Triton X-100) at 37°C for 24 hr. Gels were subsequently stained with Coomassie brilliant blue and destained in buffer containing 50% methanol and 10% acetic acid (v/v), and the location of gelatinolytic activity was detected as clear bands.

Immunocytofluorescent Staining

C6 glioma cells were grown on glass cverslips. After exposure to drugs for 1 hr, cells were washed with PBS and fixed with PBS containing 4% paraformaldehyde for 15 min, and then permeabilized with 1% Triton X-100 for 20 min. After blocking with 4% dry skim milk in PBS buffer, cells were incubated with rabbit antibodies against NF-κB p65 and p50 (1:100, Santa Cruz Biotechnology, CA) overnight at 4°C. Following a brief wash, cells were then incubated with goat anti-rabbit

FITC-conjugated secondary antibody (1:200; Leinco Tec. Inc., St. Louis, MO) for 1 h. Finally, cells were washed again, mounted, and visualized with Zeiss fluorescence microscope.

Migration and Invasion Assay

C6 glioma cells suspended in F12 serum free medium ($1x10^4$ cells for migration, $1x10^5$ cells for invasion) were seeded onto the cell culture inserts with 8 μm pore size polycarbonate filters (Coring, NY, USA). For invasion assay, filters were precoated with 25 μl Matrigel^{TM} basement membrane matrix (BD Biosciences, Bedford, MA) for 30 min. The following procedures were the same for both migration and invasion assays. F12 medium containing 50% FBS was used as a chemoattractant in the lower chambers. Cells were incubated 1 hr for adherence, leptin or neutralizing antibodies was then added and incubated for another 24 hr at 37℃ in a humidified incubator. Non-migratory cells on the upper surface of the filters were removed by wiping with a cotton swab. Invasive cells that penetrated through pores and migrated to the underside of the filters were stained with 0.05% crystal violet solution containing 20% methanol. The cell number of three fields per well was counted under microscopic at 100x magnification, and the average cell number was determined and was expressed as the "fold of control".

Establishment of Migration-Prone Sublines

Subpopulations from C6 glioma cells were selected according to their differential migration ability, using cell culture insert system as described above. After 24 hrs' migration, cells that penetrated through pores and migrated to the underside of the filters were trypsinized and harvested for second-round selection. The original cells which did not pass through membrane pores were designated as S0. After ten rounds of selection, the migration-prone subline was designated as S10.

Transfection of C6 Glioma Cells with MMP-13-shRNA

The MMP-13-shRNA conjugated on the vector of pLKO.1 with puromycin resistant region was kindly provided by National RNAi Core Facility located at the Institute of Molecular Biology / Genomic Research Center. MMP-13-shRNA and Lipofectamine 2000 (LF2000; 10 μg/ml; Invitrogen) were premixed with Opti-MEM I (Gibco, Grand Island, NY) separately for 5 min and then mixed with each other for 25 min and then applied to C6 glioma cells. The control shRNA was used as negative control. For transient transfection, cells were transfected with MMP-13-shRNA at different doses for 24 hr and exposed to 250 ng/ml leptin for another 6 hr. For stable transfection, medium was changed to F12 growth medium after 24 hrs' transfection and C6 glioma cells were recovered for 6 hr, and puromycin (10 μg/ml; MDBio, Inc., Taipei, Taiwan) was then added to the cultures for selection. Within 15 days, puromycin-resistant colonies appeared. We selected 2 colonies (sh1 and sh2) to confirm the silencing

effects by different assay and used the better one in migration and invasion assay. The sequence of MMP-13-shRNA and control shRNA is described as follow:

MMP-13-shRNA:

5’-CCGGCCGTGACCTTATGTTTATCTTCTCGAGAAGATAAACATAAGGTCACGGTTTTTG-3

Control shRNA:

5’-CCGGTCACAGAATCGTCGTATGCAGCTCGAGCTGCATACGACGATTCTGTGATTTTTG-3’

Statistics

Values are expressed as mean ± SEM of at least three experiments. Results were analyzed with one-way analysis of variance (ANOVA), followed by Neuman-Keuls. Significance was defined as $p < 0.05$.

Results

Leptin increases the migration and invasion of C6 glioma cells

It has been reported that leptin maintains neuronal stem cells and progenitor cells and is related to neuronal and glial development in the mouse embryonic brain (Udagawa et al. 2006a; Udagawa et al. 2006b), suggesting that leptin plays an important role in embryonic cerebrocortical development. It is also reported that factors expressed during the developmental stage of the CNS exhibits an overexpression pattern by neoplastic cells in mature brain (Merrill and Oldfield 2005; Plata-Salaman 1991; Trojan et al. 2007). Since Morash *et al.* (2000) demonstrate that C6 glioma cells do not express long form of leptin receptor, we thus compared the expression of leptin and short form of leptin receptor between cancer and normal cell line of astrocytes. It was found that as compared with non-malignant astrocytes RBA1 and NRA, malignant C6 glioma cells expressed more leptin and leptin receptor in both mRNA and protein levels (Fig. 1A). The leptin secreted into culture medium after 24 hr's culture was 16.8 ± 1.0 pg/ml / 1 x 10^6 control C6 glioma cells. However, the production of leptin by RBA1 and NRA was too low to be detected. We also measured the leptin content using cell lysates and it was found that the leptin content was 1030.1 ± 42.4 pg/ml, 192.6 ± 39.7 and 605.1 ± 81.8 pg/ml per 1 x 10^6 cells for C6 glioma, RBA1, and NRA, respectively (n=3 for each) (Fig. 1B). As malignant C6

glioma cells expressed more leptin than non-malignant cells, we further examined whether leptin induced cell mobility. Application of human recombinant leptin to C6 glioma cells, we found that leptin enhanced cell migration in a dose-dependent manner, and reached a 2.1 ± 0.1-fold (n=3) when stimulated by 250 ng/ml leptin (Figs. 1C, E). It was also found that the invasive ability of C6 glioma cells through Matrigel^{TM} basement membrane matrix was increased up to 2.3 ± 0.1-fold (n=3) at 250 ng/ml leptin (Figs. 1D, F).

Increase of MMP-13 expression is involved in the potentiation of migration and invasion by leptin in C6 glioma cells

Matrix metalloproteinases (MMPs) are strongly related to tumor invasion and metastasis due to their ability to degrade extracellular matrix (ECM) components (Ala-aho and Kahari 2005). As shown in Figs. 2 (A and B) human recombinant leptin dose-dependently increased MMP-13 expression at both mRNA and protein levels in C6 glioma cells, and reached the maximal effect at the dose of 250 ng/ml (3.9 ± 0.5-fold and 2.4 ± 0.2-fold, respectively; n=3). Furthermore, gelatin zymography also demonstrated the similar potentiating action by leptin (Fig. 2C; 2.9 ± 0.1-fold as compared with control at the dose of 250 ng/ml; n=3). However, the mRNA expression and the zymograph showed that both MMP-2 and MMP-9 were not affected by leptin stimulation (Figs. 2D, E).

We further used the antibody against MMP-13 to neutralize its function to examine the relationship between leptin-induced increase of migration and invasion of C6 glioma cells and MMP-13 expression. As shown in Fig. 3, leptin-induced increase of migration and invasion of C6 glioma cells was antagonized by MMP-13 neutralizing antibody (49.1 ± 3.0% and 69.0 ± 7.0% (n=3), respectively) but not by control IgG. Moreover, we confirmed the relationship between leptin-induced increase of cell mobility and MMP-13 expression using MMP-13-shRNA. First of all, transient transfection of MMP-13-shRNA dose-dependently inhibited leptin-induced increase of MMP-13 mRNA expression (Fig. 4A). We then selected two puromycin-resistant stable clones containing MMP-13-shRNA and designated as sh1 and sh2. As shown in Fig. 4B, both sh1 and sh2 exhibited lower basal level of MMP-13 expression (0.19 ± 0.03-fold and 0.14 ± 0.02-fold, as compared with non-transfected control C6, respectively; n=3). Both migration and invasion ability of sh1 and sh2 subclone were also decreased, as compared with non-transfected control C6 glioma cells (data not shown). Furthermore, the enhancement of mRNA and protein expression of MMP-13 in response to leptin stimulation was also antagonized in sh1 and sh2 subclones (mRNA was reduced by 94.1 ± 4.4% and 98.4 ± 9.1% in sh1 and sh2 subclones, respectively; and protein was reduced by 93.0 ± 1.1% and 99.2 ± 3.4%, respectively; n=3) (Figs. 4C, D). In addition, the leptin-induced

increase of MMP-13 enzyme activity was reduced by 96.4 ± 3.5% and 99.1 ± 1.9% (n=3), respectively in sh1 and sh2 (Fig. 4E). We further used sh2 to examine the activity of migration and invasion. The leptin-induced increase of cell migration and invasion was decreased by 76.4 ± 13.4% and 87.3 ± 12.2% (n=3), respectively (Figs. 4F, G). All experiments as mentioned above, control shRNA was transfected as negative control (NC).

Involvement of p38 MAPK and NF-κB signaling pathways in leptin-induced increase of MMP-13 expression in C6 glioma cells

Leptin time-dependently phosphorylated phosphoinositide-3 kinase (PI3K/Akt) and mitogen-activated protein kinases (MAPKs) pathway (Fig. 5A). The phosphorylation reached peak level 30 min after leptin stimulation in C6 glioma cells. Pretreatment of LY294002 (10 μM) and wortmannin (100 nM) for 30 min effectively inhibited the phosphorylation of PI3K/Akt. PD98059 (30 μM), SB203580 (10 μM), and SP610025 (10 μM) [all presented as (inh) in Fig. 5A] inhibited leptin-induced phosphorylation of extracellular signal-regulated kinases (ERK), p38, and Jun-amino-terminal kinase (JNK), respectively. As shown in Figs. 5 (B to D), leptin-induced increase of mRNA, protein expression and enzyme activity of MMP-13 was markedly inhibited by SB603580 (94.1 ± 10.1%, 98.8 ± 6.4%, and 71.5 ± 3.2%, respectively; n=3). However, inhibitors of PI3 kinase, ERK and JNK did not affect the expression of

MMP-13. These results indicate that p38 MAPK is involved in leptin-induced increase of MMP-13 production.

Nuclear factor κB (NF-κB) is one of the transcription factors which can drive MMP-13 expression (Liacini et al. 2003; Ohno et al. 2006). Here we further examined whether NF-κB is involved in the action of leptin. As shown in Fig. 6A, leptin induced nuclear translocation of NF-κB subunits of p65 and p50 1 hr after drug application. In addition, IκB kinase α/β (IKKα/β) phosphorylation increased in a time-dependent manner in response to leptin application (Fig. 6B). Leptin-induced increase of mRNA, protein expression and enzyme activity of MMP-13 was significantly antagonized by PDTC (25 μM), TPCK (10 μM), and Bay 11-7082 (2 μM) (Figs. 6, C to E). These results indicate that NF-κB transcription factor is involved in leptin-induced increase of MMP-13 production.

Increase of MMP-13 and leptin expression in migration-prone cells

We selected C6 glioma sublines with higher cell mobility according to the procedures as described in "*Methods*". Migration-prone subline S10 had higher cell mobility and migrated more easily through the membrane of cell culture inserts or Matrigel^{TM} basement membrane matrix as compared with original C6 glioma cells designated as S0 (2.0 ± 0.1-fold and 2.4 ± 0.3-fold, respectively; n=3) (Figs. 7A, B). Moreover, it was found that S10 markedly increased the expression of MMP-13 and leptin at both

mRNA and protein levels (3.0 ± 0.2-fold and 1.4 ± 0.1-fold in mRNA expression, respectively; 1.9 ± 0.3-fold and 2.6 ± 0.4-fold in protein expression, respectively; n=3). There was no significant change of leptin receptor (Figs. 7C, D). Therefore, cells with higher ability to migrate and invade expressed more MMP-13 and leptin.

Discussion

The elucidation of the molecular biology of cancer cells in recent years has identified various molecular pathways altered in different cancers. This information is currently being exploited to develop potential therapeutic targets. In this study, we found that malignant C6 glioma cells indeed expressed more leptin and leptin receptor than non-malignant astrocytes RBA1 and NRA, indicating that leptin and/or leptin receptor play a significant role in neoplastic cells. It has been reported that leptin increases the migration and invasion of hepatocellular carcinoma cells and prostate cancer cells (Frankenberry et al. 2004; Saxena et al. 2007). Here we found that exogenous application of human recombinant leptin enhanced the migration and invasion ability of C6 glioma cells.

Although the endogenous leptin level in control C6 glioma cell is much lower than that of exogenous application, the leptin concentration was chosen according to some references. It has been demonstrated that leptin production is elevated under some pathological states. For example, eutopic stromal cells do not express leptin under normoxia, whereas, it produces 37.5 ± 5.3 pg/ml under 48 hr's hypoxia. On the other hand, stromal cells derived from endometriotic lesions express significantly high amounts of leptin under both normoxic and hypoxic conditions (44.3 ± 11.8 pg/ml and 191.5 ± 31.4 pg/ml, respectively) (Wu et al. 2007). To take obesity as

another example, plasma leptin is an excellent index of obesity and correlated with some obesity-related diseases, such as diabetes, coronary heart disease, and obesity-linked carcinoma (Sader et al. 2003; Saxena et al. 2007). The extracellular space is quite small so that the local extracellular concentration of leptin may reach a much higher concentration in the brain.

Different families of matrix metalloproteinases (MMPs) have been found to be involved in the migration and invasion. Expression of MMP-2 and MMP-9 is elevated by leptin in cytotrophoblastic cells and adipocytes (Castellucci et al. 2000; Moon et al. 2007). However, we found that production and activity of MMP-13, but not of MMP-2 and MMP-9, were markedly increased in response to leptin application in C6 glioma cells. The leptin-induced cell mobility was further correlated to the elevated MMP-13 production by using MMP-13 neutralizing antibody and MMP-13 silencing. MMP-13-silenced C6 glioma cells exhibited less cell mobility compared with original C6 glioma cells. Upon leptin stimulation, MMP-13-silenced cells showed the inhibition of migration and invasion. On the other hand, migration-prone sublines were selected and showed higher migration ability. Our results suggest that cells expressing more MMP-13 had higher migration and invasion activity. The more prominent expression of leptin in migration-prone cells further indicates that leptin may play an autocrine or paracrine function to enhance migration and invasion.

To investigate the downstream signaling pathways involved in the action of leptin, different pharmacological inhibitors were used. Although leptin is able to activate different signaling pathways including PI3K/Akt and MAPK pathways (Cui et al. 2006; El Homsi et al. 2007; Tang et al. 2007), MMP-13 elevation was only antagonized by p38 MAPK inhibitor in C6 glioma cells. Furthermore, inhibition of NF-κB p65 pathways by PDTC, TPCK and Bay 11-7082 also reversed the leptin-induced increase of MMP-13 production. MMP-13 expression is also reported to be induced via NF-κB activation in chondrocytes (Liacini et al. 2003; Ohno et al. 2006). Since Morash *et al*. (2000) reported that C6 glioma cells do not express long form of leptin receptor (ObRl), all the signal transduction observed in this study is due to the activation of short form of leptin receptor (ObRt). These results indicate that other than the role in leptin transporter, short form of leptin receptor also plays a notable role in mediating signal transduction (Bjorbaek et al. 1997; Murakami et al. 1997).

A variety of evidence indicates that leptin is highly correlated with obesity-related carcinoma, such as breast cancer (Rose et al. 2002) and hepatocellular carcinoma (Saxena et al. 2007). High leptin levels are associated with advanced tumor stage and higher tumor grade in breast cancer (Goodwin et al. 2005). Moreover, Frankenberry *et al*. (2004) demonstrate that leptin contributes to the progression of

prostate cancer by the induction of vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF-β1), and basic fibroblast growth factor (bFGF) expression, which are important mitogenic and angiogenic factors. As glioma is not one of the well documented obesity-related carcinoma, our findings suggest that leptin can also play a tumor-encouraging role regardless of obesity. Furthermore, increase of MMP-13 production is related to leptin-induced tumor progression.

Acknowledgments

This work was supported by research grants from the National Science Council of Taiwan. We thank the National Research Council of Canada for providing us the cell line NRA. We also thank National RNAi Core Facility located at the Institute of Molecular Biology / Genomic Research Center, Academia Sinica for providing us shRNA targeting MMP-13 and control shRNA.

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Figure Legends

Fig. 1. Increase of migration and invasion by leptin in C6 glioma cells.

(A) Malignant C6 glioma cells expressed more leptin and leptin receptors than non-malignant astrocytes RBA1 and NRA, as demonstrated by RT-PCR (upper panels) and Western blotting (lower panels). The summarized data were shown in the right two panels. (B) Endogenous levels of leptin in cell lysates measured by ELISA in C6, RBA1, and NRA cells. *, $p<0.05$, as compared with C6. (C) Exogenous application of human recombinant leptin (125 ng/ml and 250 ng/ml) enhanced migration of C6 glioma cells. (D) Exogenous application of human recombinant leptin (250 ng/ml) enhanced invasion of C6 glioma cells. The quantitative results were shown in (E) and (F), respectively. *, $p<0.05$, as compared with control. All data are presented as mean ± SEM from three independent experiments. Abbreviations: con, control; leptin R, leptin receptor.

Fig. 2. Increase of MMP-13 expression by leptin in C6 glioma cells.

Leptin dose-dependently increased the expression of MMP-13 at mRNA (A) and protein (B) levels. (C) MMP-13 enzyme activity was also increased by leptin administration. MMP-13 expression reached the maximal levels at the dose of 250 ng/ml. The mRNA expression (D) and enzyme activity (E) of both MMP-2 and MMP-9 was not affected by leptin in C6 glioma cells. Data are presented as mean ±

SEM from three independent experiments. *, $p<0.05$, as compared with control. Abbreviations: con, control; MMP, matrix metalloproteinase.

Fig. 3. Leptin-induced migration and invasion of C6 glioma cells was antagonized by MMP-13 neutralizing antibody.

Application of MMP-13 neutralizing antibody (10 μg/ml) but not normal IgG significantly decreased leptin-induced cell migration (A) and invasion (B) in C6 glioma cells. Data are presented as mean ± SEM from three independent experiments. *, $p<0.05$, as compared with control group. #, $p<0.05$, as compared with leptin-treated group. Abbreviations: Ab, MMP-13 neutralizing antibody; con, control; IgG, normal IgG.

Fig. 4. Leptin-induced increase of migration and invasion of C6 glioma cells was inhibited by the transfection of MMP-13-shRNA.

(A) MMP-13-shRNA was first confirmed to have adequate efficacy in silencing MMP-13 mRNA expression in C6 glioma cells in a dose-dependent manner in response to 250 ng/ml leptin application. (B) Two puromycin-resistant stable clones (sh1 and sh2) were selected and exhibited lower basal level of MMP-13 mRNA expression. (C-E) Leptin-induced increase of mRNA and protein expression as well as enzyme activity of MMP-13 was significantly reduced in both sh1 and sh2 clones. (F, G) Cell migration or invasion assay was performed using sh2 clone and it is shown

that both migration and invasion were markedly inhibited in response to the administration of leptin (250 ng/ml). Data are presented as mean ± SEM from three independent experiments. *, $p<0.05$, as compared with control group. #, $p<0.05$, as compared with leptin-treated non-transfected group. Abbreviations: con, non-transfected control; NC, negative control group of cells transfected with control shRNA.

Fig. 5. Involvement of p38 MAPK in leptin-induced increase of MMP-13 expression in C6 glioma cells.

(A) Application of leptin (250 ng/ml) to C6 glioma cells gradually increased the phosphorylation of PI3K/Akt (upper panel) and MAPK (lower panel) in a time-dependent manner. Pretreatment of LY294002 (10 μM) and wortmannin (100 nM) for 30 min effectively inhibited the phosphorylation of PI3K/Akt (upper panel). Treatment of PD98059 (30 μM), SB203580 (10 μM), and SP610025 (10 μM) (presented as "*inh*") inhibited leptin-induced phosphorylation of extracellular signal-regulated kinases (ERK), p38, and Jun-amino-terminal kinase (JNK), respectively (lower panel). Leptin-induced increase of MMP-13 mRNA (B), protein expression (C), and also enzyme activity (D) was markedly antagonized by SB203580. Data are presented as mean ± SEM from three independent experiments. *, $p<0.05$, as compared with control group. #, $p<0.05$, as compared with leptin-treated alone.

Abbreviations: con, control; inh, inhibitors (according to different experimental conditions); LY, LY294002; PD, PD98059; SB, SB203580; SP, SP610025; Wor, Wortmannin.

Fig. 6. Involvement of NF-κB signaling in leptin-induced increase of MMP-13 expression in C6 glioma cells.

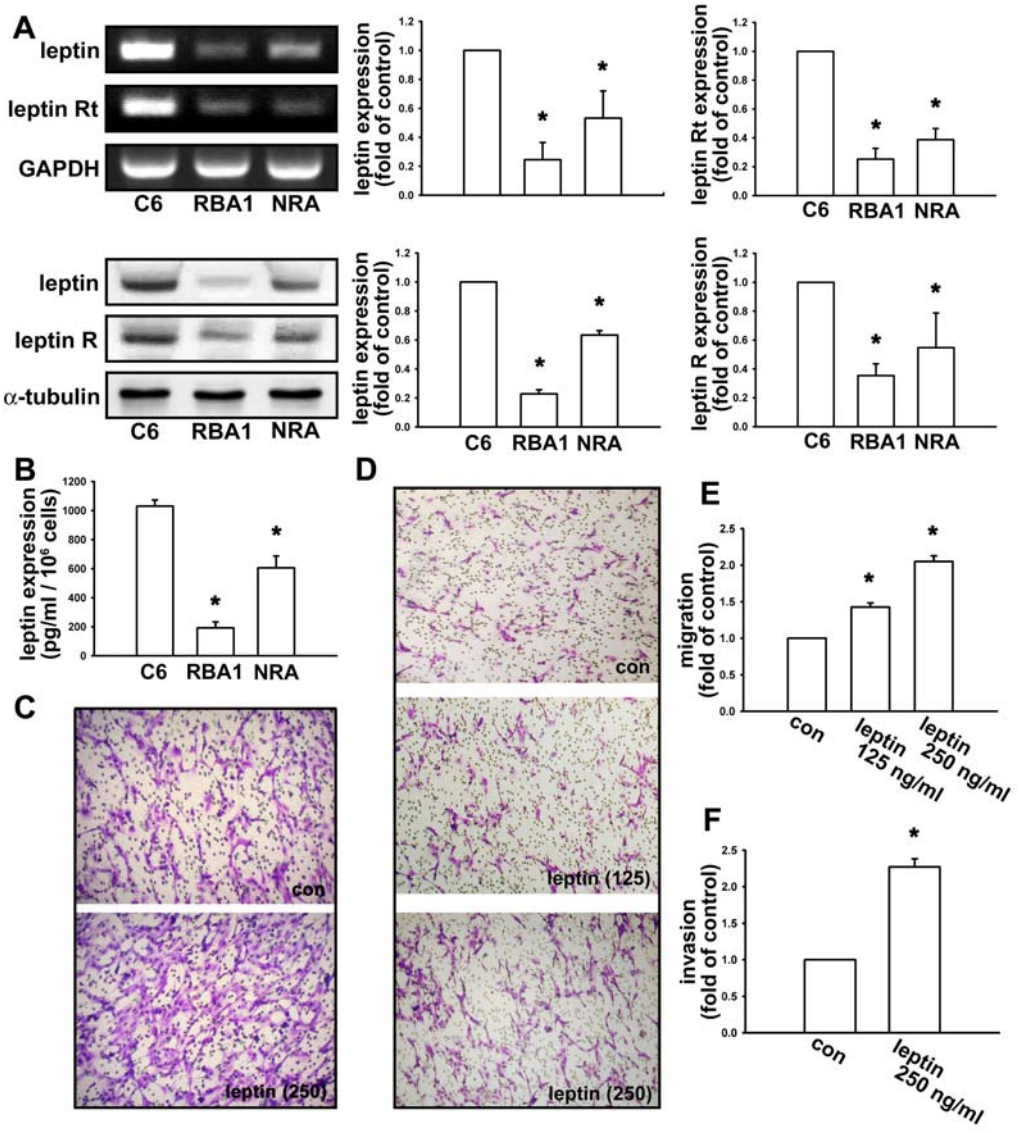
(A) Leptin (250 ng/ml) induced translocation of p65 and p50 subunits of NF-κB from cytoplasm to nucleus as shown by immunofluorescent staining. (B) Leptin time-dependently increased IKKα/β phosphorylation as demonstrated by Western blotting. Leptin-induced increase of MMP-13 expression at mRNA (C), protein (D), and enzyme activity (E) was significantly antagonized by PDTC (25 μM), TPCK (10 μM), and Bay 11-7082 (2 μM). Data are presented as mean ± SEM from three independent experiments. *, $p<0.05$, as compared with control group. #, $p<0.05$, as compared with leptin-treated alone. Abbreviations: con, control; Bay, Bay 11-7082.

Fig. 7. Upregulation of MMP-13 and leptin expression in migration-prone cells.

After ten rounds of selection of C6 glioma cells by cell culture insert system, migration-prone subline (S10) exhibited more migration (A) and invasion ability (B) than original C6 glioma cells (S0). S10 expressed more MMP-13 and leptin but not leptin receptor at both mRNA (C) and protein (D) levels. The summarized data were shown in the lower panels. Data are presented as mean ± SEM from three

independent experiments. *, $p<0.05$, as compared with S0. Abbreviations: S0, original C6 glioma cells; S10, migration-related subline after ten rounds of selection.

Figure.1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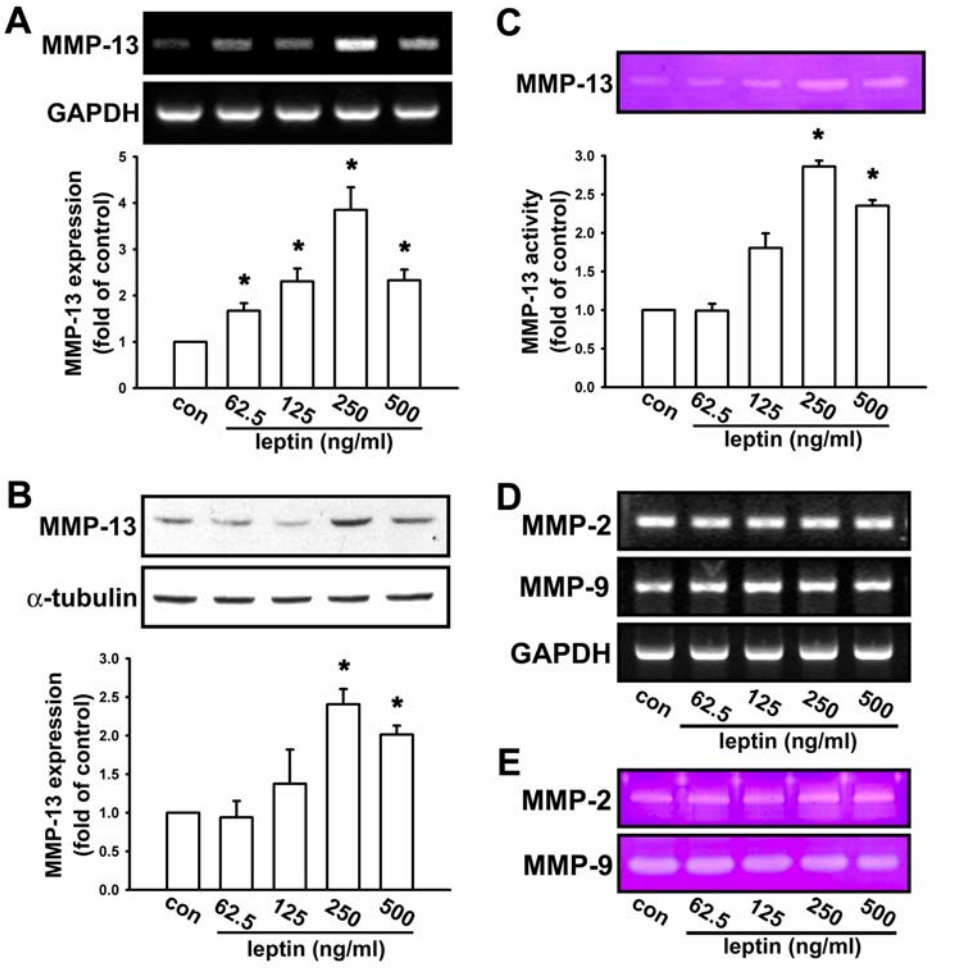
leptin
leptin Rt
GAPDH
C6 RBA1 NRA
leptin expression (fold of control)
leptin Rt expression (fold of control)
leptin
leptin R
α-tubulin
leptin R expression (fold of control)
B
leptin expression (pg/ml / 10^6 cells)
C
con
leptin (250)
D
con
leptin (125)
leptin (250)
E
migration (fold of control)
con
leptin 125 ng/ml
leptin 250 ng/ml
F
invasion (fold of control)
con
leptin 250 ng/ml

Figure.2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MMP-13
GAPDH
MMP-13 expression (fold of control)
con
62.5
125
250
500
leptin (ng/ml)
B
MMP-13
α-tubulin
MMP-13 expression (fold of control)
con
62.5
125
250
500
leptin (ng/ml)
C
MMP-13
MMP-13 activity (fold of control)
con
62.5
125
250
500
leptin (ng/ml)
D
MMP-2
MMP-9
GAPDH
con
62.5
125
250
500
leptin (ng/ml)
E
MMP-2
MMP-9
con
62.5
125
250
500
leptin (ng/ml)

Figure.3

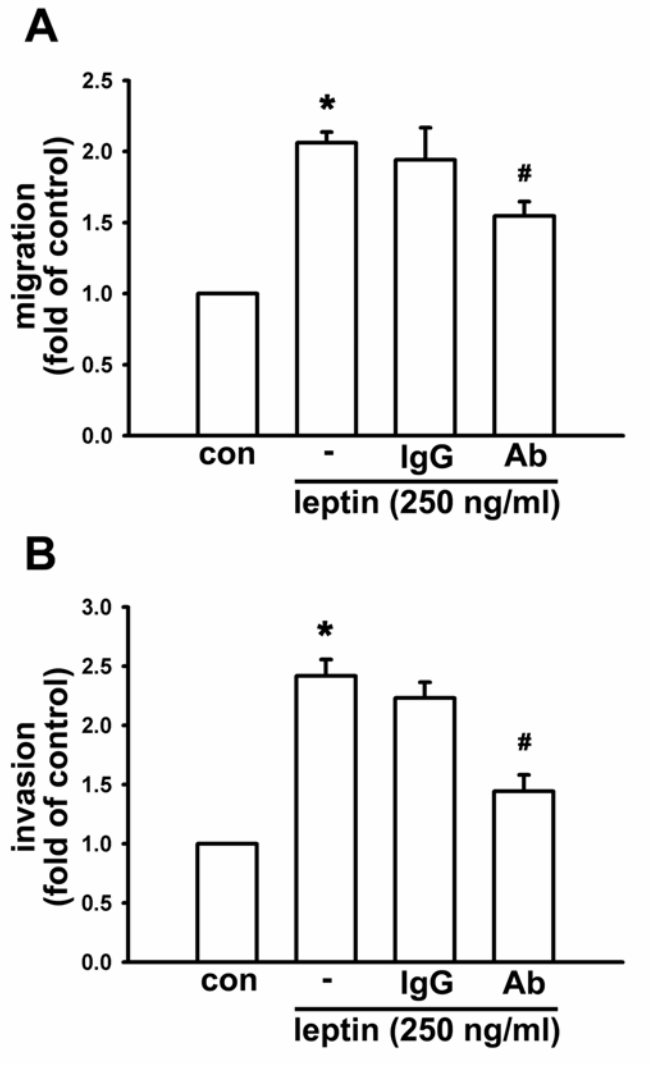

A
migration
(fold of control)
2.5
2.0
1.5
1.0
0.5
0.0
*
#
con
-
IgG
Ab
leptin (250 ng/ml)
B
invasion
(fold of control)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
#
con
-
IgG
Ab
leptin (250 ng/ml)

Figure.4

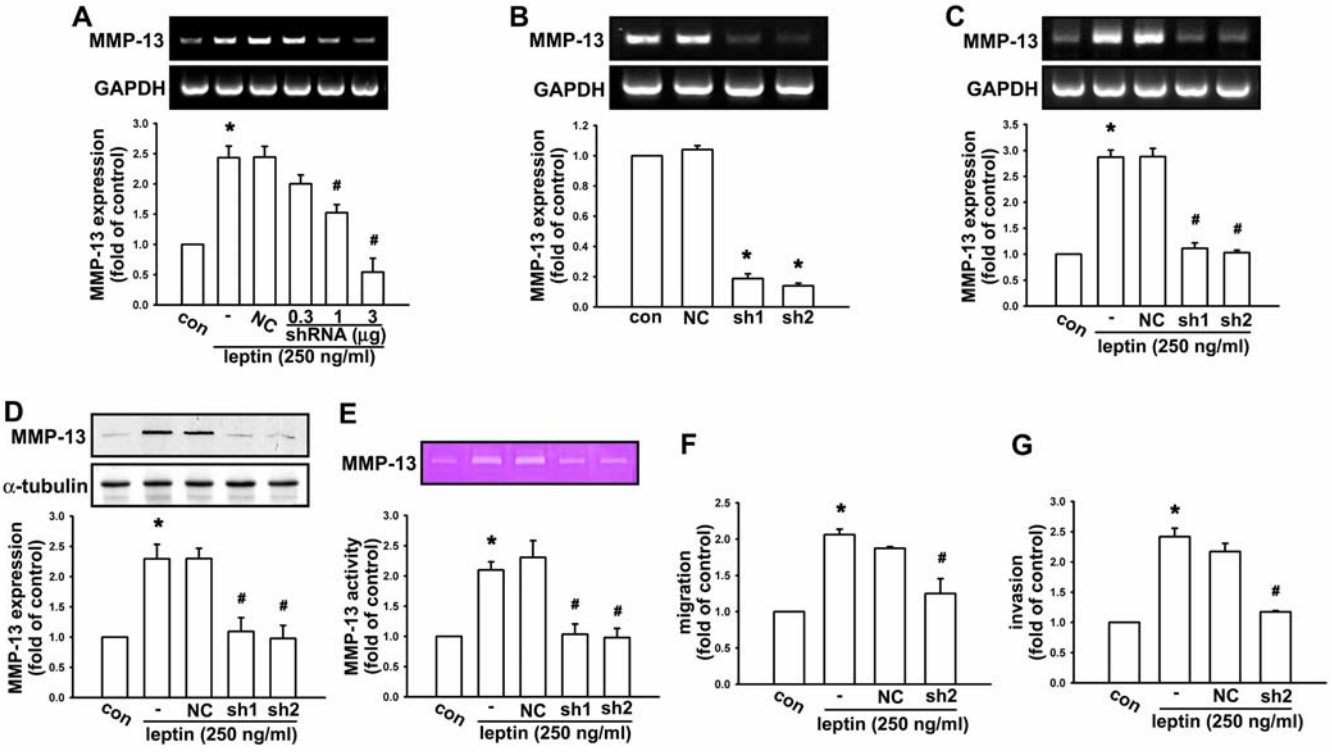

A
MMP-13
GAPDH
MMP-13 expression (fold of control)
con
-
NC
0.3
1
3
shRNA (μg)
leptin (250 ng/ml)
B
MMP-13
GAPDH
MMP-13 expression (fold of control)
con
NC
sh1
sh2
C
MMP-13
GAPDH
MMP-13 expression (fold of control)
con
-
NC
sh1
sh2
leptin (250 ng/ml)
D
MMP-13
α-tubulin
MMP-13 expression (fold of control)
con
-
NC
sh1
sh2
leptin (250 ng/ml)
E
MMP-13
MMP-13 activity (fold of control)
con
-
NC
sh1
sh2
leptin (250 ng/ml)
F
migration (fold of control)
con
-
NC
sh2
leptin (250 ng/ml)
G
invasion (fold of control)
con
-
NC
sh2
leptin (250 ng/ml)

Figure.5

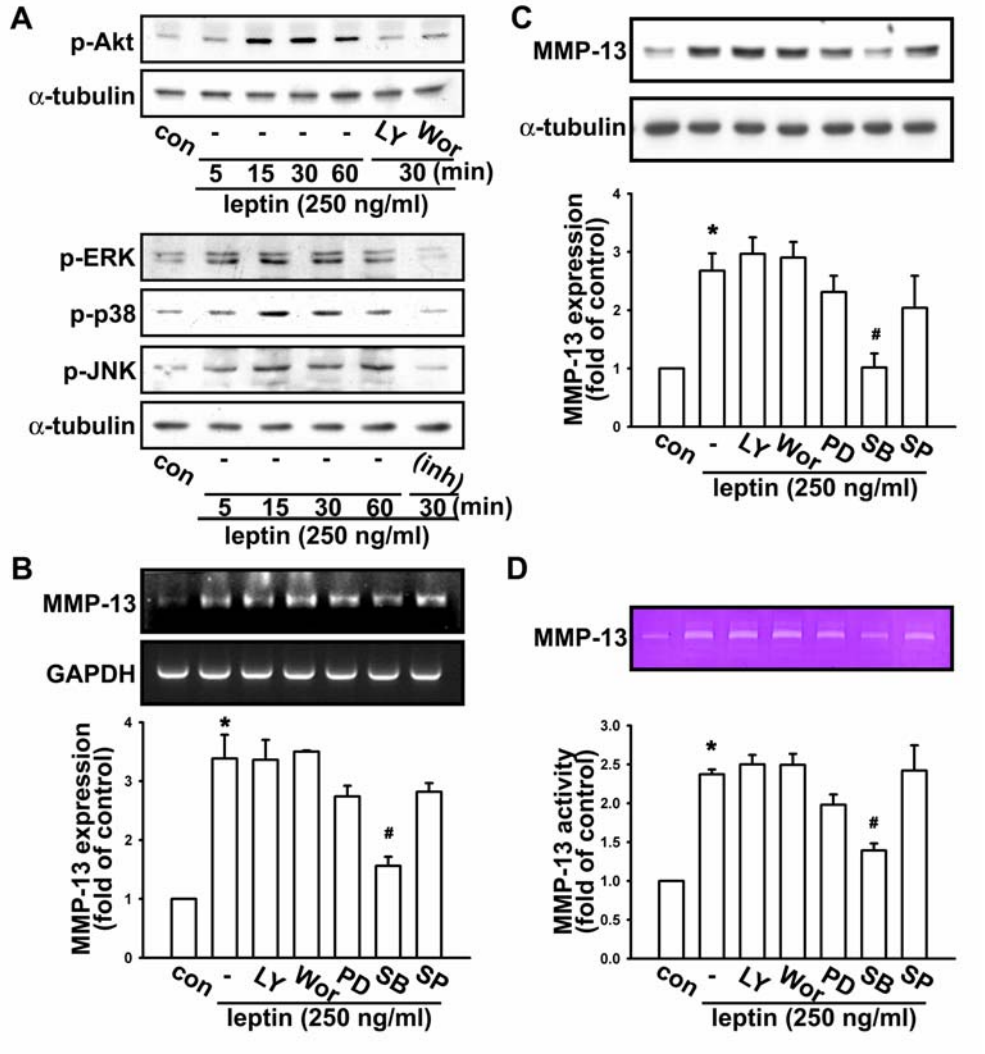

A
p-Akt
α-tubulin
con - - - - LY Wor
5 15 30 60 30 (min)
leptin (250 ng/ml)
p-ERK
p-p38
p-JNK
α-tubulin
con - - - - (inh)
5 15 30 60 30 (min)
leptin (250 ng/ml)
C
MMP-13
α-tubulin
MMP-13 expression (fold of control)
con - LY Wor PD SB SP
leptin (250 ng/ml)
B
MMP-13
GAPDH
MMP-13 expression (fold of control)
con - LY Wor PD SB SP
leptin (250 ng/ml)
D
MMP-13
MMP-13 activity (fold of control)
con - LY Wor PD SB SP
leptin (250 ng/ml)

Figure.6

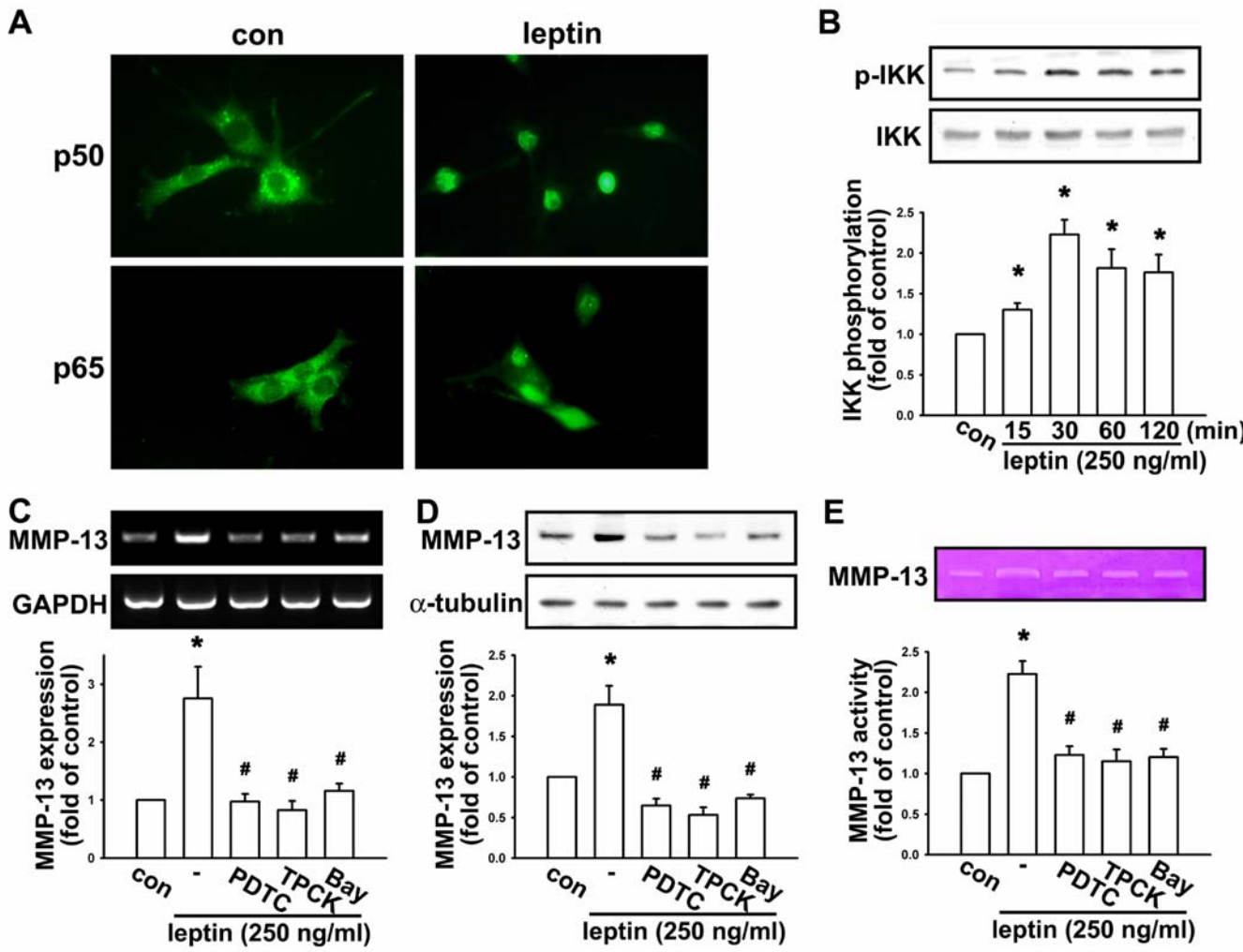
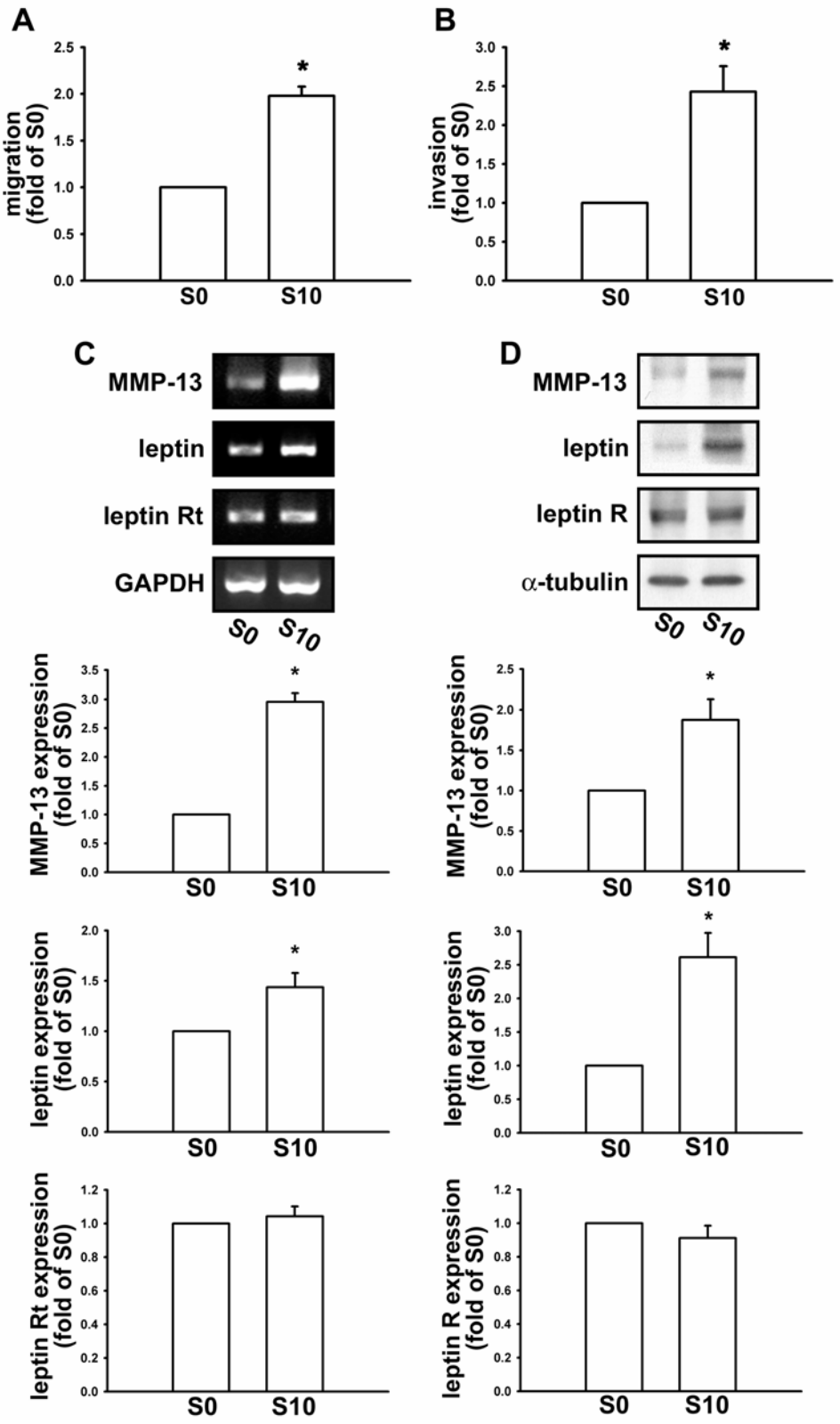

A
con
leptin
p50
p65
B
p-IKK
IKK
IKK phosphorylation (fold of control)
con
15
30
60
120
(min)
leptin (250 ng/ml)
C
MMP-13
GAPDH
MMP-13 expression (fold of control)
con
-
PDTC
TPCK
Bay
leptin (250 ng/ml)
D
MMP-13
α-tubulin
MMP-13 expression (fold of control)
con
-
PDTC
TPCK
Bay
leptin (250 ng/ml)
E
MMP-13
MMP-13 activity (fold of control)
con
-
PDTC
TPCK
Bay
leptin (250 ng/ml)

Figure.7


A
migration (fold of S0)
S0
S10
B
invasion (fold of S0)
C
MMP-13
leptin
leptin Rt
GAPDH
D
MMP-13
leptin
leptin R
α-tubulin
MMP-13 expression (fold of S0)
leptin expression (fold of S0)
leptin Rt expression (fold of S0)
leptin R expression (fold of S0)