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RhoA/ROCK pathway mediates leptin-induced uPA expression to promote cell invasion in ovarian cancer cells

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ABSTRACT

Previous studies have shown that leptin, an adipocyte-secreted hormone, stimulates ovarian cancer invasion. Here, we investigated the contribution of uPA in leptin-induced ovarian cancer cell invasion. The cell invasion and migration experiments were carried out using matrigel invasion and wound healing assays in ovarian cancer cell lines (OVCAR3, SKOV3and CaoV-3). The mechanism underlying the invasive effect of leptin was examined using cell transfection with Ob-Rb siRNA, pre-treatment with a specific inhibitor of RhoA and ROCK, RhoA activation assay, OB-Rb, Rock and upA protein expression. Our results show that leptin induced ovarian cancer cell invasion via up-regulating upA in a time and dose-dependent manner, which was attenuated using knockdown of OB-Rb by siRNA. Moreover, pre-incubation with C3 (inhibitor of RhoA) and Y-27632 (inhibitor of ROCK) effectively attenuated leptin-induced upA expression and inhibited invasive ability of ovarian cancer cells. We also found that pretreatment with inhibitors of PI3K/AKT (LY294002), JAK/STAT (AG490) and NF-kB (BAY 11-7082) significantly reduced leptin-induced upA expression. Collectively, our findings demonstrate that OB-Rb, RhoA/ROCK, PI3K/AKT, JAK/STAT pathways and NF-kB activation are involved in leptin-induced upA expression. These results may provide a new mechanism that facilitates leptin-induced ovarian cancer invasion.

1. Introduction

Ovarian cancer is the second most common female reproductive tract cancers with >200.,000 new cases reported each year and about 125,000 deaths per year worldwide [1]. High mortality rate of ovarian cancer is strongly due to the fact that approximately 75% of patients have advanced stage (III or IV) accompanied by widely metastatic disease [2–5]. Metastasis begins with detaching cancer cells from the primary tumor and migrates to neighboring organs, a process called cancer cell invasion [6–8]. Despite extensive efforts to identify modifiable factors contributing to metastasis of cell cancer, little is known about the molecular aspects of this process.

A number of studies have demonstrated that obesity is a risk factor for the development of women cancers such as ovarian, breast and endometrium cancers [9]. Obesity is associated with adipokines deregulation, which was secreted by adipocytes, and regulates various biologic processes include energy expenditure and control of appetite [10]. Leptin, a member of adipokines family, is encoded by the obese (ob) gene. Apart from the primary function in the hypothalamic modulator of food intake, body weight, and fat stores, leptin seems to play an important role in the pathogenesis of different types of cancer, including ovarian cancer [10–12]. It has been demonstrated that the adipocyte expression and serum concentration of leptin elevate in both gynecological and breast cancer patients [13]. Leptin exerts its function through leptin receptor (ObR) which belongs to the cytokine receptor superfamily [10]. The long isoform (OB-Rb) has full signaling capability responsible for biological effects of leptin [12,14]. The binding of leptin to OB-Rb activates several signaling pathways such as JAK/STAT and PI3K/AKT [15]. Previous studies have shown that overexpression of leptin receptor correlates with an unfavorable outcome in ovarian cancer [12,15]. Urokinase plasminogen activator (uPA), a serine protease, contributes to many pathophysiological behaviors, including cell metastasis, tissue repair and vasculature [8,16,17]. Previous studies have shown that high uPA protein levels correlate with poor prognosis and associated with advanced stage and distant metastasis in ovarian cancer [8,17–21]. Numerous signaling pathways such as Rho (the small G protein Ras homolog gene family) and its immediate downstream effector Rho-associated coiled-coil-forming protein kinase (ROCK) can induce expression of uPA [22]. Past studies have shown that Rho/ROCK

Abbreviations: uPA, urokinase plasminogen activator; ObR, leptin receptor; OB-Rb, The long form of leptin receptor; RhoA, Ras homolog gene family, member A; ROCK, Rho-associated coiled-coil-forming protein kinase; siRNA, small interfering RNA; C3, C3 transferase; JAK, Janus kinase; STAT, signal transducers and activators of transcription; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; NF-kB, nuclear factor-κB.

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pathway may be regulated by leptin [23,24]. Recent studies also have reported that the expression of uPA may be regulated by NF-kB, a downstream of Rho/ROCK pathway, in ovarian cancer [22].

Based on these previous findings, we hypothesize that leptin/OB-Rb pathway may be contribute to ovarian cancer cell invasion by upregulating uPA via the involvement of Rho/ROCK, JAK/STAT3, PI3/AKT pathway and NF-kB activation.

2. Materials and methods

2.1. Materials

Culture media and growth supplements were obtained from Gibco (Germany). Recombinant human leptin (rhleptin) was purchased from R&D Systems (Minneapolis, MN, USA). C3 transferase, a clostridial toxin that selectively inhibits Rho and Y-27632 dihydrochloride, a cell permeable inhibitor of ROCK-1 and ROCK-2, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). BAY 11-7082, an inhibitor of NF-kB was purchased from Merck (Biosciences, Darmstadt, Germany). LY294002, PI3-kinase inhibitor, and AG490, JAK inhibitor were obtained from Abcam Company (Cambridge, MA, USA). Goat *anti*-mouse IgG-HRP: sc-2005, monoclonal Antibodies against uPA (PGM2001): sc-59728, GAPDH (G-9): sc-365062 and NF-κB p65 Antibody (F-6): sc-8008 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ROCK antibody and Phospho-ROCK antibody were obtained from Abcam (Cambridge, MA, USA). Anti-Human OB-R Antibody: MAB867 was purchased from R&D Systems. siRNA (sc-36115) and siRNA transfection reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rho activation assay kit (17-294) was obtained from EMD Millipore (Lake Placid, NY, USA).

2.2. Ovarian cell lines and cell culture

The ovarian cancer cell lines, OVCAR3, SKOV3and CaoV-3 were obtained from National Cell Bank of Iran (NCBI, Pasteur Institute of Iran). The cells were cultured in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin, incubated in 95% air and 5% CO_2 at 37 °C.

2.3. MTT assay

The growth effect of leptin in OVCAR3, SKOV3 and CaoV-3 was measured using the MTT assay. The cells were cultured in 96-well plates at 5×10^3 cells/well in medium containing 10% FBS and incubated overnight. To determine the stimulatory effect of leptin on cell growth, fresh medium containing 1.5% FBS was added and the cells were treated with leptin (0, 10, 50, 100 and 200 ng/ml) for 48 h. After treatment with leptin, MTT tetrazolium salt (50 μl, of 2 mg/ml) was added to each well and the cells were incubated at 37 °C for 4 h. The MTT containing medium was removed and the produced formazan crystals in the viable cells were dissolved in DMSO and the optical density was read at 570 nm. The cell viability percentage was calculated using the following formula: (mean OD of treated group/mean OD of non-treated group) × 100.

2.4. BrdU cell proliferation assay

Cell proliferation was also analyzed using colorimetric immunoassay based on bromodeoxyuridine (BrdU) according to the manufacturer's instructions. Briefly, the cells were seeded at a density of 5000 cells/well in 96-well plates for 24 h and then treated with various concentrations of leptin for 48 h. Subsequently, the medium was supplemented with 20 ml of BrdU-labeling solution and the cells were incubated for 4 h. After removing the growth medium, cells were incubated with Fixodent solution for 30 min. The samples were then incubated with peroxidase-conjugated *anti*-BrdU antibody for 90 min. Following removing of unbound anti-BrdU-POD, color reaction was initiated by the addition of substrate solution and stopped with sulfuric acid addition after 3–5 min. Absorbance values of the samples were assessed using a spectrophotometric plate reader at 450 nm.

2.5. Wound healing assay

Cell migration was examined using the scratch wound assay as described previously [25]. Briefly, ovarian cancer cells were seeded in six-well plates and incubated for 24 h. Cells were allowed to grow in complete medium containing %10 FBS to reach %100 confluency, washed with serum-free medium and serum starved for 16 h. A 10 μl pipette tip was used to scratch the cells to make a wound. The cells were rinsed two times with PBS and then treated with leptin (100 ng/ml) for various time intervals of time. The spread of the wound closure was photographed after 6, 12 and 24 h under a microscope. Results were quantified with image J software.

2.6. Matrigel invasion assay

Cell invasion was determined using an invasion assay kit with Matrigel-coated inserts (8 μm, BD Biosciences, San Jose, CA, USA). Briefly, 2×10^5 cells per insert were cultured in the upper chamber containing a serum-free medium. Cells were treated with different concentrations of leptin (0, 10, 50, 100 and 200 ng/mL) for 24 h. In another set of experiments, cells were pre-incubated with Y-27632 (10 μm) or C3 (0.25 μg/ml) for 60 min before leptin treatment. Medium containing 10% FBS was applied to the lower chamber as a chemoattractant. After 24 h incubation at 37 °C and 5% CO_2, cells remaining above the insert were gently scraped off by cotton swab. The invaded cells that were adherent to the lower surface of the filter were fixed in methanol for 10 min, washed in PBS, stained with crystal violet and counted in ten different fields under a light microscope at ×400 magnification. The experiment was repeated three times.

2.7. Small interfering RNA (siRNA)-mediated knockdown of OB-Rb

SKOV3 and OVCAR3 cells were cultured in 6-well plates and transfected with an OB-Rb siRNA (40 nM) using a siRNA transfection reagent (Santa Cruz Biotechnology). A scrambled siRNA containing a random sequence of nucleotides with no known specificity was used as a negative control according to the manufacturer's instructions. After the initial incubation (7 h), medium containing 2× FBS (e.g. 20% FBS) was added to each well without removing the transfection mixture and samples were incubated for 24 h. The transfection mixtures were then replaced with fresh medium with 10% FBS and incubated for an additional 24 h before experimental treatment with leptin (100 ng/ml). Cells were harvested 24 h after leptin treatment and applied for cell invasion assays, Real-time PCR, and Western blotting analysis.

2.8. Real-time RT-PCR experiments

mRNA expression levels were assessed using Quantitative Real-Time RT-PCR. RNA was extracted from cultured cells with Trizol reagent (Invitrogen, USA). Total RNA (2 μg) was reverse transcribed using the first-strand cDNA synthesis kit (Takara Shuzo, Otsu, Japan) according to the manufacturer's protocol. Quantitative RT-PCR of the first-strand cDNA was performed using the SYBR Green kit (Amplicon) in an ABI 7500 Sequence Detection System (Applied Biosystems) in accordance with the manufacturer's recommendations. PCRs were carried out for 40 cycles under the following Conditions: denaturation at 95 °C for 15 min, annealing at 57 °C for 5 s, and elongation at 72 °C for 5 s. A melting point curve was generated at the end of each PCR reaction to confirm amplification specificity. The relative gene expression level was determined using the $2^{-\Delta\Delta Ct}$ analysis for target gens and endogenous housekeeping gene GAPDH. The primers used for Real-time RT-PCR were as follows: for OB-Rb, Forward primer, TGAGGTATCATAGGAGCAGCC, and Reverse

primer, TGTTGGTGGAGAGTCAAGTGA; for GAPDH, Forward primer, CTCCCGCTTCGCTCTCTG, and Reverse primer, TCCGTTGACTCCGACCTTC.

2.9. Western blotting

OB-Rb, uPA, ROCK, Phospho-Rock and NF-kB protein content were detected by Western blot analysis. The cultured cells were suspended in ice-cold RIPA buffer (20 mM Tris–HCl pH 7.5, 0.5% Nonidet P-40, 0.5 mM PMSF, 100 mM β-glycerol 3-phosphate, and 0.5% protease inhibitor cocktail). The suspensions were vortexed every 15 min for 2 h, the extracts were clarified by centrifugation at 10,000 *g* for 10 min at 4 °C and the supernatant was served as the cytosolic extract. The plates were re-suspended in RIPA buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 5% glycerol and 0.5% protease inhibitor cocktail). The samples were incubated on ice for 15 min, centrifuged in 15,000 *g* for 20 min and supernatant was served as nuclear extract. The total amount of proteins in supernatants were determined using Bradford reagent assay and the protein concentration was determined based on the standard curve using bovine serum albumin. The same amounts of protein from each samples were subjected to 10% SDS–PAGE gels and transferred onto a PVDF membrane (Amersham Pharmacia Biotech.). The membranes were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween 20 (PBST) for 2 h. Membranes were incubated with specific primary antibody against OB-Rb, uPA, ROCK, and Phospho-ROCK and NF-kB p56 overnight at 4 °C. After three times washing with PBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. The immunoreactivity of the target proteins was detected with ECL detection reagent (Amersham Corp., Arlington Heights, IL, USA).

2.10. RhoA activity assay

Determination of the amount active or GTP-RhoA content compared with total RhoA content was performed using Rho activation assay kit (Upstate, Lake Placid, NY) according to the manufacturer's instructions. Following treatment with leptin in a time-dependent (100 ng/ml for 5–120 min), the cells were harvested and lysed with cell lysis buffer. An equal amount of protein from each sample was incubated with RBD–agarose for 45 min at 4 °C with gentle agitation and was pelleted the agarose beads by brief centrifugation (10 s, 14,000 ×*g*, 4 °C). The beads were washed, resuspended in 25 μL of 2× Laemmli reducing sample buffer and boiled for 5 min. Lysates were processed Western blotting with *anti*-RhoA.

2.11. Statistical analysis

All values are shown as the mean ± SD of three individual experiments performed in triplicate and presented as the mean. Statistical analysis was performed by nonparametric one-way analysis of variance (ANOVA) followed by Dunnett's test using the statistical program SPSS 18.0. $P < 0.05$ was considered statistically significant.

3. Results

3.1. OVCAR3 and SKOV3 cells express the active form of the leptin receptor

Previous studies have shown that leptin exerts its actions through binding to OB-Rb. Therefore, we first examined protein level of OB-Rb expression in human ovarian cancer cell lines including OVCAR3, SKOV3 and CaoV-3 using Western blotting analysis. As shown in Fig. 1A, we found that OVCAR3 and SKOV3 cells express significantly high level of OB-Rb, whereas Caov-3 cell line dose not express substantially the OB-Rb protein.

To determine whether leptin could influence the OB-Rb expression, OVCAR3 and SKOV3 cell lines were incubated with leptin for 24 h and OB-Rb mRNA and protein expression were examined using real-time PCR and Western blotting respectively. Leptin treatment resulted in an induction of OB-Rb mRNA and protein levels in both cell lines. 100 ng/mL of leptin significantly induced mRNA expression of OB-Rb by 3 fold in OVCAR3 and 3.2 fold in SKOV3 compared to untreated cells (Fig. 1B). Moreover the same concentration of leptin exposure increased the protein levels of OB-Rb 1.5 fold in OVCAR3 ($P < 0.05$) and

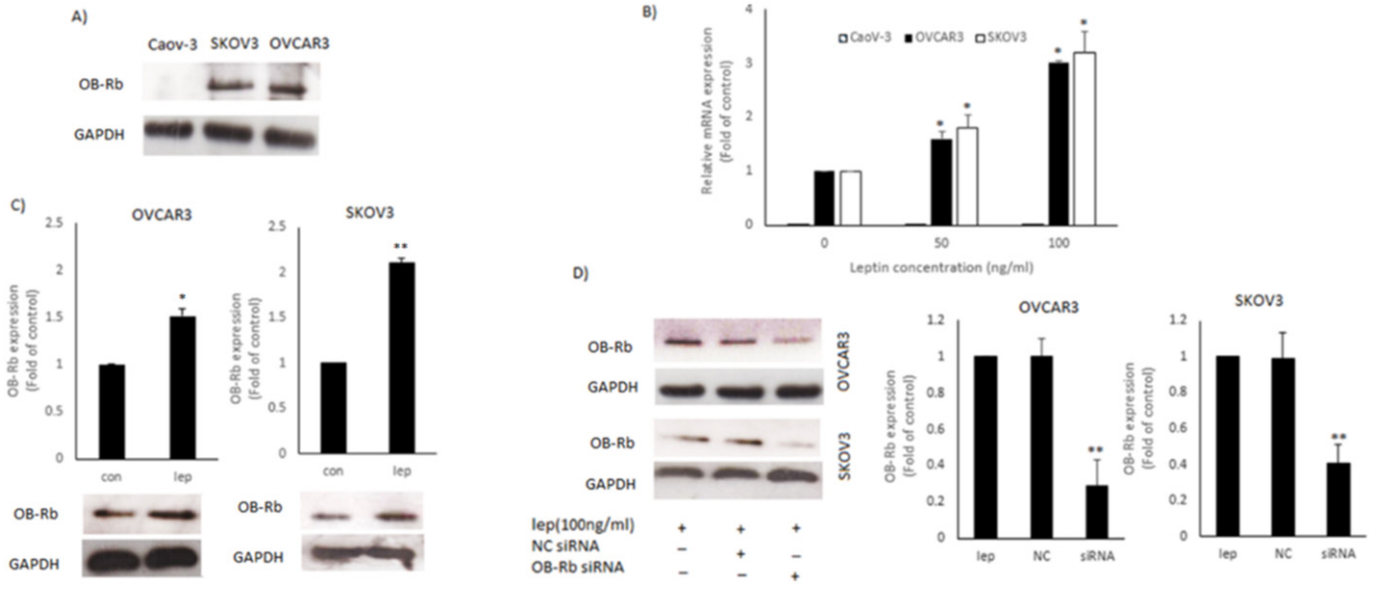

Fig. 1. Leptin induces the OB-Rb expression in ovarian cancer cell lines and OB-Rb siRNA transfection attenuated OB-Rb receptor expression: (A) Total protein was isolated from cells, and equal amounts of proteins were subjected to Western blotting using specific antibodies for OB-Rb. (B) cells were treated with leptin (0, 50, 100 ng/ml) for 24 h, total RNA was extracted and mRNA gene expression was analyzed by real-time PCR. Values of each experiment was normalized using GAPDH as an endogenous control. Quantitative analysis of data was performed using the$2^{-\Delta\Delta Ct}$ method. (C) Cells were treated with leptin (100 ng/ml), total protein was extracted and subjected to Western blotting. (D) Cells were transfected with nonspecific control (NC) and OB-Rb siRNA (40 nM) for 72 h. Cell lysates were collected and subjected to Western blotting to detect expression of OB-Rb. The histogram represents the quantitative analysis of relative level of OB-Rb expression, which was normalized for GAPDH expression. The data represent the mean ± SD of at least three different experiments. (ANOVA, * $P < 0.05$, ** $P < 0.01$ vs control).

2.1 fold in SKOV3 ($P < 0.01$) (Fig. 1C). These results indicate that OB-Rb mRNA expression are reflected at the protein level.

We transfected OVCAR3 and SKOV3 cells with different concentrations of OB-Rb siRNA and determined the optimal concentration of siRNA to maximum OB-Rb blocking. In both cell lines, 40 nM was the optimal concentration of siRNA for efficient reduction of OB-Rb mRNA ($P < 0.01$). Using a higher concentrations of OB-Rb siRNA induced unwanted cell detachment and death (data not shown). In addition, the transfection of OB-Rb siRNA (40 nM) in OVCAR3 and SKOV3 showed an efficient reduction of the target protein level ($P < 0.05$). However, transfection of nonspecific control (NC) showed no inhibitory effect (Fig. 1D).

3.2. Leptin induces cell proliferation of OVCAR3 and SKOV3 cells

We next measured the effect of leptin on the viability of ovarian cancer cell lines. The serum leptin level may be increase to 10 ng/ml in non-obese subject, whereas in obese individuals leptin concentration are elevated to 100 ng/ml [15,26–29]. So, to cover all physiological/pathophysiological concentration range, we used 0, 10, 50,100 and 200 ng/ml of leptin for proliferation experiments. OVCAR3, SKOV3 and CaoV-3 ovarian cancer cells were seeded in complete medium containing 1.5% FBS, incubated with different concentrations of leptin (0, 1, 10, 50, 100 and 200 ng/ml) for 48 h and cell growth was analyzed using the MTT assay. As illustrated in Fig. 2A, B the response of these cell lines following exposure to leptin for 48 h. The growth effects of leptin on OVCAR3 cells at 50, 100 and 200 ng/ml were significantly higher than untreated cells (141.1 ± 7% for 50 ng/ml, 170.4 ± 7% for 100 ng/ml and 147.6 ± 11% vs. control 100%, respectively; $P < 0.05$,). Leptin also induced a significant increase in the SKOV3 cells viability (136.9 ± 1.7% for 50 ng/ml, 165.4 ± 16% for 100 ng/ml and 148.6 ± 11% vs. control 100%, respectively; $P < 0.05$). No significant effect of leptin was seen on cell growth in CaoV-3 cells.

The Brdu assay results (Fig. 2C, D) shown that cell proliferation was significantly increased in response to leptin in a dose-dependent manner in OVCAR3 ($P < 0.05$) and SKOV3 ($P < 0.05$). BrdU results are in agreement with the results of MTT assay.

3.3. Leptin induces migration in OVCAR3 and SKOV3 ovarian cancer cell lines

An important step in the metastatic process involves the ability of cancer cells to invade and migrate to points far from a given primary tumor mass. In order to investigate the effect of leptin on ovarian cancer cell migration the cells were treated with leptin for 6, 12 and 24 h and migration was measured by wound healing assay. As shown in Fig. 3 both OVCAR3 and SKOV3 cells, treated with leptin, were migrated rapidly and covered the wound during 24 h. These results demonstrated that treatment of ovarian cancer cells with leptin resulted in increased migration potential in a time-dependent manner.

3.4. OB-Rb gene silencing suppress leptin-induced cell invasion in OVCAR3 and SKOV3

Matrigel invasion assay showed a dose-dependent increase in cell invasion in the both cell lines following exposure to leptin. As depicted in Fig. 4A, stimulative effects of leptin on cell invasion in OVCAR3 cells started at 10 ng/ml and reached to maximum at100 ng/ml (from 1.75 ± 0.45 fold for 10 ng/ml; $P < 0.05$, to 4.7 ± 0.2 fold for 100 ng/ml; $P < 0.01$, vs. control 1 fold, respectively). As depicted in

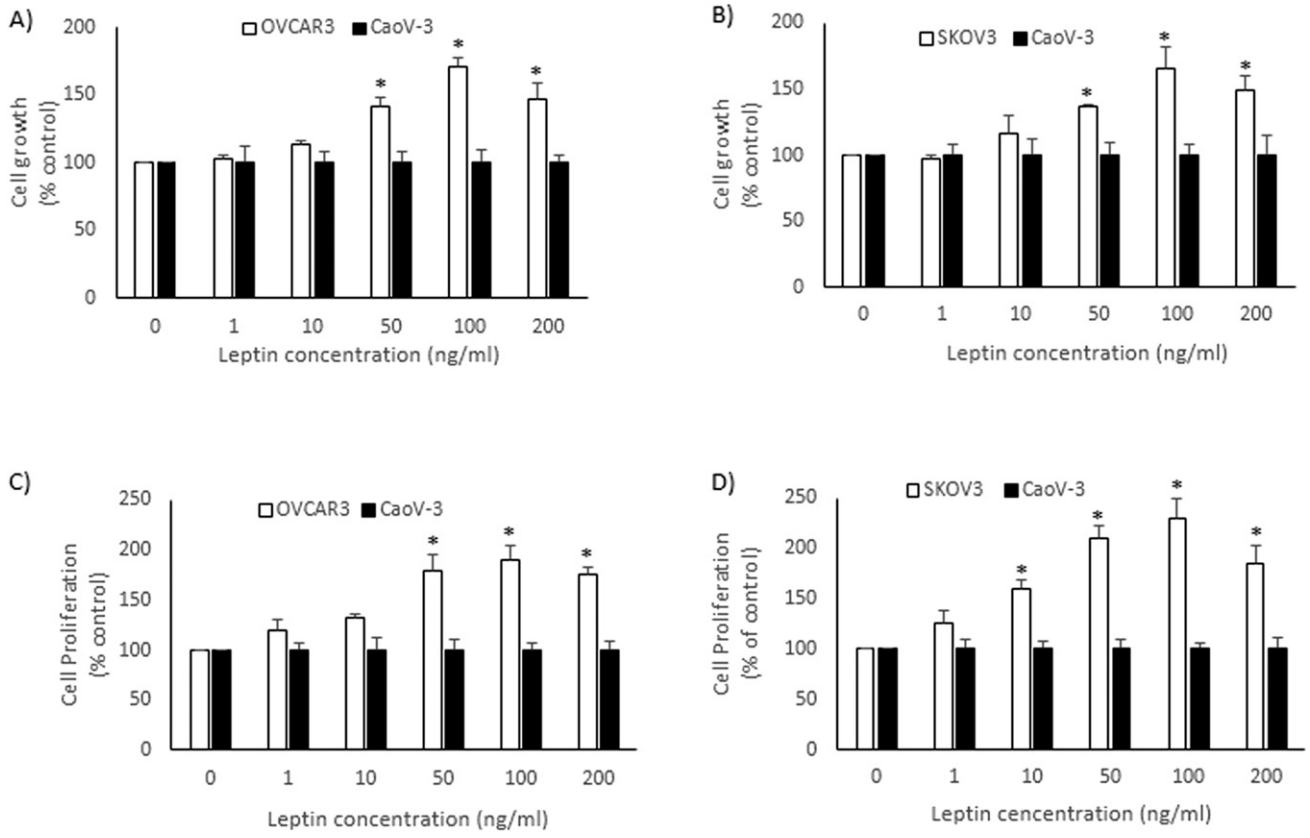

Fig. 2. Leptin enhances the cell proliferation in the ovarian cancer cell lines: Cells were treated with different concentrations (0, 1, 10, 50, 100 and 200 ng/ml) of leptin for 48 h, and proliferation was assessed by MTT assay (A, B) And Brdu assay (C, D). Leptin induce cell proliferation in a dose-dependent manner. Results (mean ± SD) were calculated as percent of corresponding control values. Each point represents four repeats, each triplicate. (ANOVA,* $P < 0.05$ vs control).

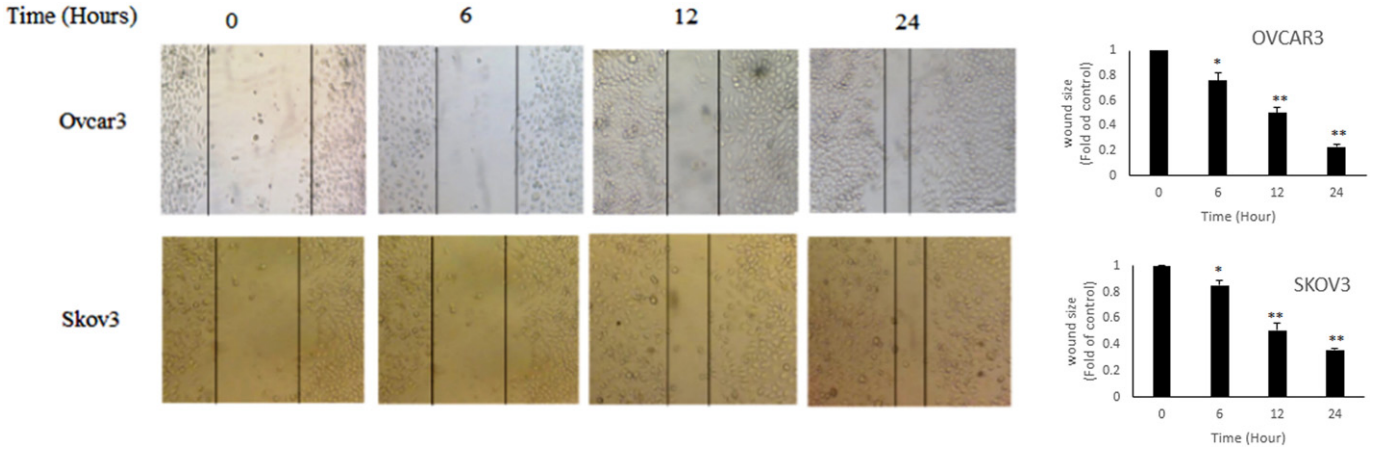

Fig. 3. Leptin induces cell migration in OVCAR3 and SKOV3: cells were grown to reach 100% confluency, serum-starved for 12 h, were scratched with a 10-μl pipette tip and were photographed immediately following scratching (0 h). Culture media was replaced with media containing leptin (100 ng/ml). The plates were photographed at the identical location of the initial image at 6, 12 and 24. The histogram represents the quantitative analysis of the relative migration which calculated by measuring the area of the scratch at the indicated time and fold of reduction of initial wound area was calculated relative to time 0.

Fig. 4B, leptin also induced a significant increase in the SKOV3 cells invasion (from 2.09 ± 0.3 fold for 10 ng/ml; $P < 0.05$, to 4.3 ± 0.35 fold for 100 ng/ml; $P < 0.01$, vs. control 1 fold, respectively). No additional increasing was seen at higher concentration of leptin in both cell lines. These results indicated that leptin significantly increased the invasive potential of both OVCAR3 and SKOV3 cell lines. To explore whether OB-Rb is directly involved in leptin-induced cell invasion of OVCAR3 and SKOV3 cell lines, cells were transfected with OB-Rb specific siRNA or scrambled non-specific siRNA and cell invasion was investigated with matrigel invasion assay. As shown in Fig. 4C, D the silencing of OB-Rb significantly decreased the cell invasion of ovarian cancer cell line in comparison with control cells (OVCAR3: 2.1 ± 0.3 fold; $P < 0.01$, SKOV3: 1.8 ± 0.07 fold; $P < 0.01$,). The transfection with NC siRNA had no inhibitory effect. Treatment with leptin (100 ng/ml) showed no effect of cell invasion in CaoV-3 cells (Fig. 4E).

3.5. Leptin induces uPA expression in OVCAR3 and SKOV3 cells through OB-Rb receptor

Previous studies have shown that uPA has an essential role in ovarian cancer cell invasion [17]. To determine whether uPA is regulated by leptin, we evaluated uPA expression after treatment with various concentrations of leptin for 24 h. The results of Western blot assay demonstrated that leptin treatment (10–100 ng/mL) significantly increased

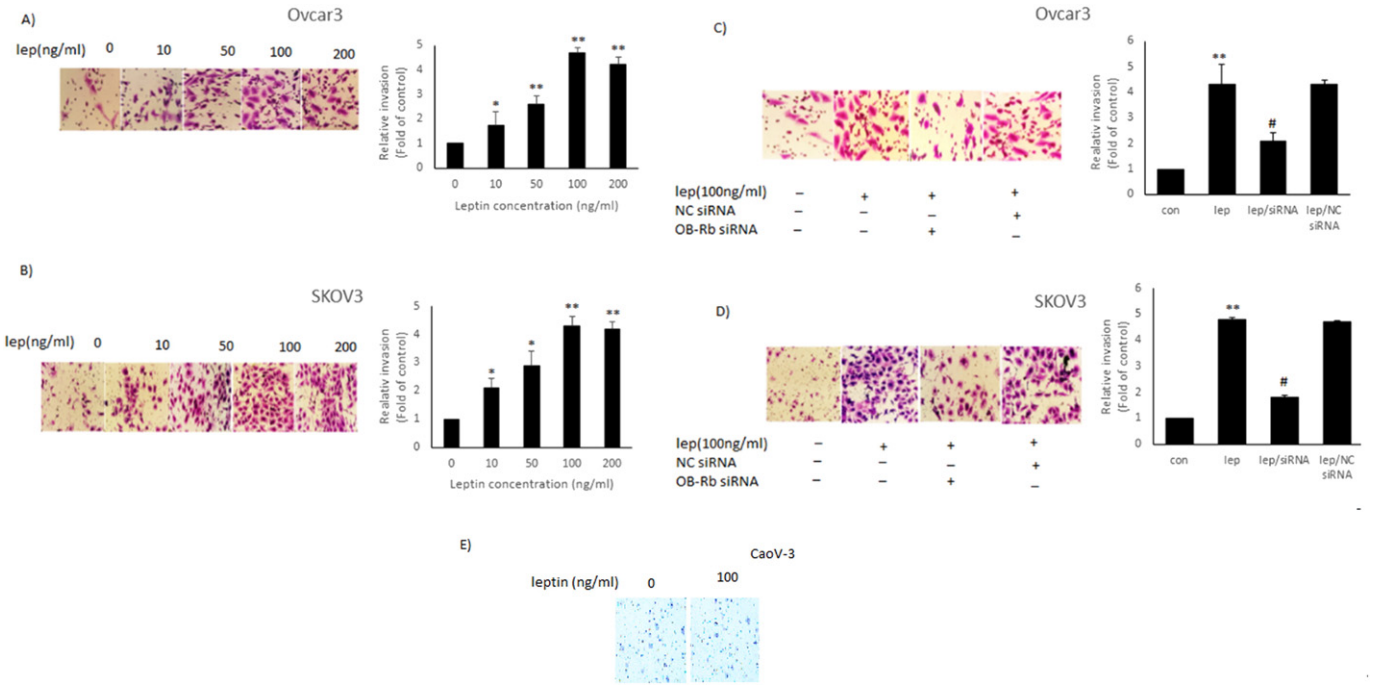

Fig. 4. Leptin enhances cell invasion and transfection of Ob-RB siRNA inhibited leptin-induced cell invasion in OVCAR3 and SKOV3 cells: (A, B) 1×10^5 cells were seeded in Matrigel-coated chambers, cells were treated with leptin in indicated concentration for 24 h. The histogram represents the quantitative analysis of the matrigel invasion assay which was calculated by counting the invaded cells in six randomly selected fields within each membrane, and the values were averaged. (C, D) In another experiment, cells were transfected with Ob-RB siRNA (40 nM) or scrambled control (NC) siRNA (40 nM), transfected cells were seeded in Matrigel-coated chambers, were treated with leptin (100 ng/ml), and the cell invasion was detected at 24 h. (E) Leptin has no effect on cell invasion in CaoV-3 cell line as negative control. All data shown were expressed as mean ± SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs control. # $P < 0.01$ vs leptin (100 ng/ml). Statistical analysis was performed by ANOVA.

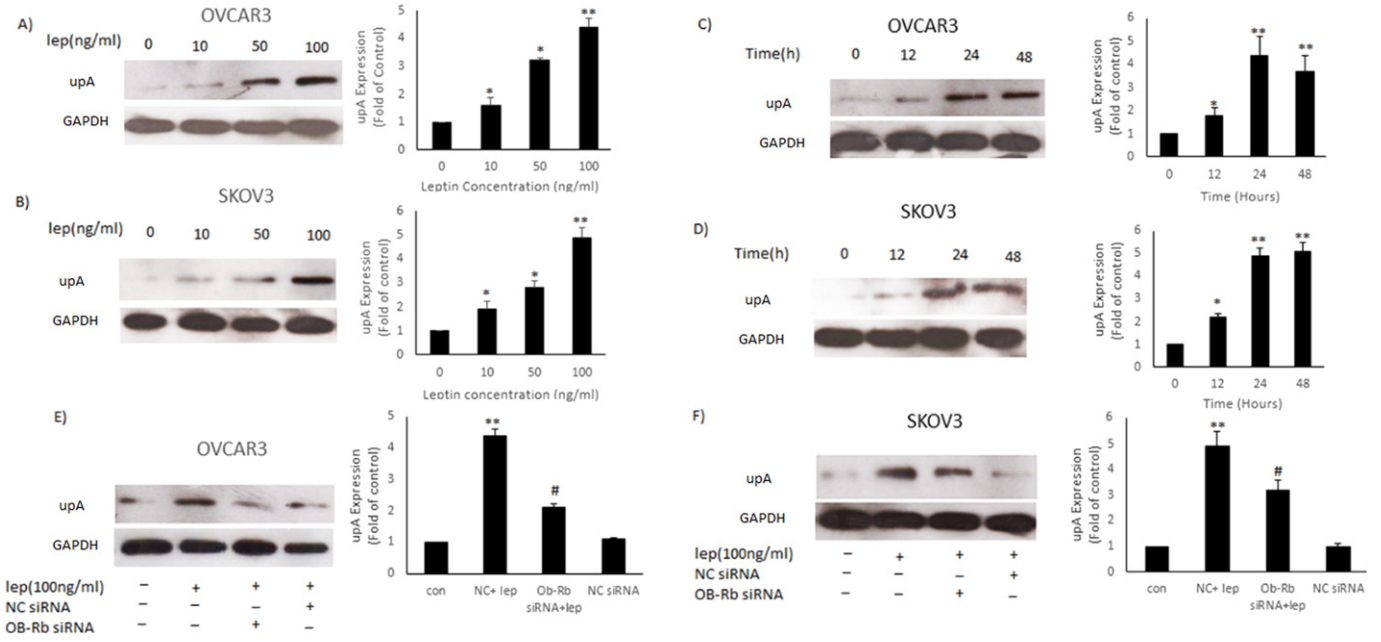

Fig. 5. Leptin stimulates upA expression and transfection of Ob-RB siRNA attenuated leptin induced-upA expression in ovarian cancer cell lines: (A, B) Cells were treated with various concentrations of leptin (0, 10, 50, 100, and 200 ng/ml) for 24 h, total protein was isolated and equal amounts of proteins were subjected to Western blotting using specific antibodies for upA. (C, D) In a separate experiment, cells were treated with leptin (100 ng/ml) for 0, 12, 24, and 48 h and the upA expression was detected by Western blotting. (E, F) Cells were transfected with Ob-RB siRNA (40 nM) or scrambled control (NC) siRNA (40 nM), transfected cell were treated with leptin (100 ng/ml) for 24 h, total protein was isolated and then analyzed the upA expression by Western blotting. All data shown were expressed as mean ± SD. of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs control. # $P < 0.05$ vs leptin (100 ng/ml). Statistical analysis was performed by ANOVA.

uPA expression in a dose- dependent manner (Fig. 5A, B). Maximum response was seen at 100 ng/ml in comparison to unstimulated cells (4.4 ± 0.3; $P < 0.01$ and 4.9 ± 0.34; $P < 0.01$ fold in OVCAR3 and SKOV3 cell lines respectively). In a separate experiment, the cells were treated with leptin (100 ng/ml) for different time intervals (0, 12, 24, and 48 h) and expression of uPA was evaluated using Western blotting. As depicted in Fig. 5C, D, a maximal response to leptin (100 ng/ml) was observed at 24 h. No additional increase was seen after treatment with leptin at 48 h.

To address whether OB-Rb was involved in leptin-induced uPA protein up-regulation, we tested the level of uPA expression of OVCAR3 and SKOV3 cells after OB-Rb gene silencing. The results showed that OB-Rb

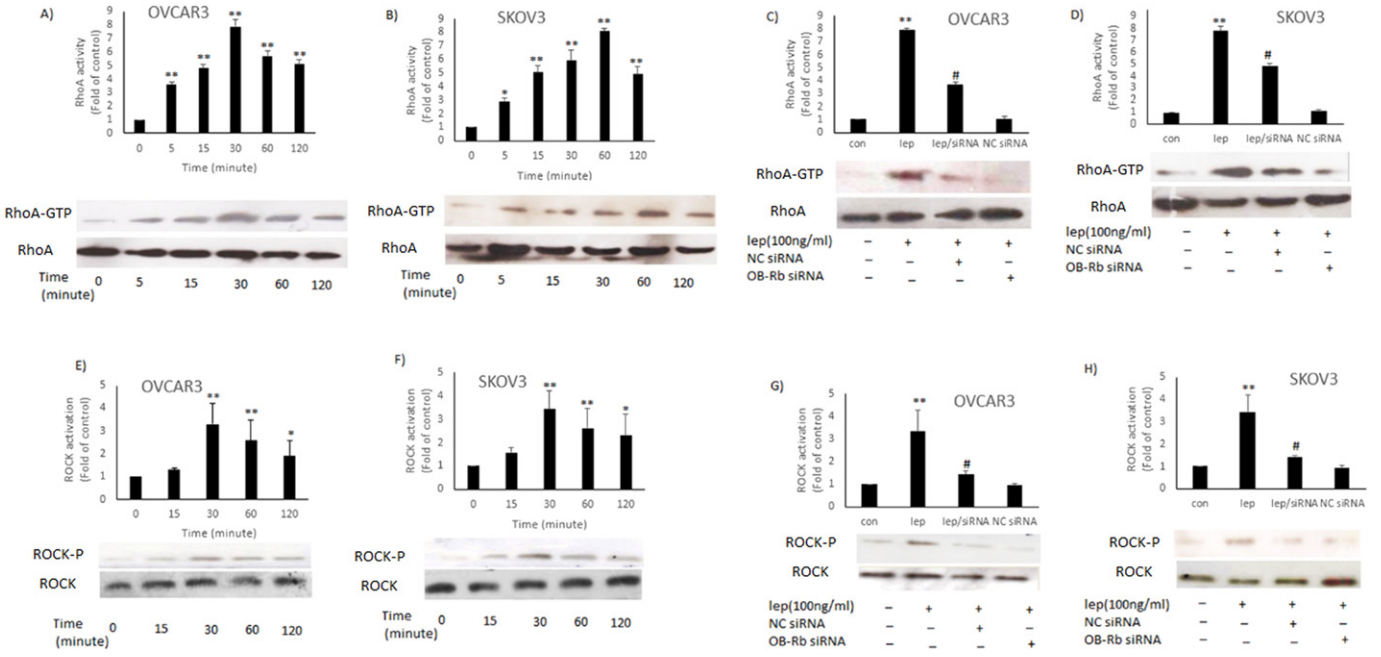

Fig. 6. Leptin induces RhoA activation and Rock phosphorylation through OB-Rb in ovarian cancer cells: (A, B) Cells were treated with leptin (100 ng/ml) for various periods as indicated. RhoA activation was determined by Western blot with RhoA-GTP antibody following the pull-down assay. The blots were analyzed by densitometry, and the intensity of the RhoA-GTP bands was normalized to the intensity of the corresponding total RhoA band. (C, D) In a another experiment, cells were transfected with OB-Rb siRNA (40 nM) or scrambled control (NC) siRNA (40 nM), transfected cell were treated with leptin (100 ng/ml) for 30 min (in OVCAR3) or 60 min (in SKOV3) and then analyzed the RhoA activation by pull-down assay. (E, F) The phosphorylation levels of ROCK in cell lysates were determined by Western blotting. (G, H) In a separate experiment, transfected cells were treated with leptin (100 ng/ml) for 30 min in both cell lines and ROCK phosphorylation levels were determined in cell lysates by Western blotting. All data shown were expressed as mean ± SD. of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs control. # $P < 0.01$ vs leptin (100 ng/ml). Statistical analysis was performed by ANOVA.

siRNA transfection significantly reduced uPA protein level in both cell lines (Fig. 5E, F; OVCAR3: 2.1 ± 0.11 fold; $P < 0.05$, SKOV3: 3.2 ± 0.33 fold; $P < 0.05$).

3.6. Leptin activates the RhoA/ROCK pathway through OB-Rb receptor in OVCAR3 and SKOV3

RhoA cycle acts as the binary switch between active GTP-bound and inactive GDP-bound forms. To evaluate whether leptin modulated RhoA activation in OVCAR3 and SKOV3 cells, we measured Rho-GTP binding at various time points in the presence of leptin (100 ng/ml). As shown in Fig. 6A, B, leptin significantly increased the active RhoA, peaked at 30 min after incubation in OVCAR3 (7.9 ± 0.5 fold; $P < 0.01$) and 60 min in SKOV3 (8.1 ± 0.22 fold; $P < 0.01$). OB-Rb siRNA transfection significantly attenuated leptin-induced RhoA activation in OVCAR3 (3.7 ± 0.18 fold; $P < 0.01$) and SKOV3 cells (5.12 ± 0.19 fold; $P < 0.015$), indicating OB-Rb plays a key role in leptin-induced RhoA activation (Fig. 6C, D).

Furthermore, we determined the phosphorylation of ROCK, downstream molecule in the RhoA pathway, at various time intervals after leptin incubation using Western blotting. As depicted in Fig. 6E, F, phosphorylation of ROCK was also induced by leptin in ovarian cancer cell lines which peaked 30 min after incubation (3.3 ± 0.9 fold; $P < 0.05$ in OVCAR3, 3.4 ± 0.8 fold; $P < 0.05$ in SKOV3). As shown in Fig. 6G, H, the phosphorylation of ROCK was significantly blocked by OB-Rb siRNA (1.43 ± 0.17 fold in OVCAR3and 1.42 ± 0.05 fold in SKOV3; $P < 0.01$).

3.7. Leptin increased the cell invasion and uPA up-regulation through Rho/ROCK pathway in OVCAR3 and SKOV3 cells

To examine whether leptin regulates the ovarian cancer cell invasion through the Rho/ROCK pathway, OVCAR3 and SKOV3 cells were pretreated with specific Rho inhibitor (C3) and ROCK inhibitor (Y27623) for 60 min. As shown in Fig. 7A, B, pretreatment with C3 (10 μm) and Y27632 (10 μm) significantly reduced the leptin-mediated cell invasion in OVCAR3 (C3: 2 ± 0.37 fold; $P < 0.01$, Y27623: 2.1 ± 0.37 fold; $P < 0.01$) and SKOV3 (C3: 1.7 ± 0.16 fold; $P < 0.01$, Y27623: 1.5 ± 0.3 fold; $P < 0.01$) cell lines. In another experiment, to assess whether leptin regulates uPA expression through the Rho/ROCK pathway, cells were pretreated with RhoA and ROCK inhibitors for 60 min. Although a decrease in leptin-induced uPA expression was detected after pretreatment with Y-27632 in OVCAR3 cell line, ($P < 0.05$), C3 did not affect the expression levels of uPA (Fig. 7C). As shown in Fig. 7D, pretreatment with either Rho (C3) or ROCK (Y-27632) inhibitor prevented the leptin-induced expression of uPA in SKOV3 cells ($P < 0.05$).

3.7.1. JAK/STAT and PI3K/AKT are involved in leptin-induced upA expression in ovarian cancer cells

Recently Kato et al. showed that JAK/STAT and PI3K/AKT are contributed in leptin-dependent ovarian cancer cell invasion [15]. We examined possible roles of JAK/STAT and PI3K/AKT in leptin-induced upA expression in ovarian cancer cells. SKOV3 and OVCAR3 cells pretreated with AG490 (JAK inhibitor) and LY294002 (PI3K inhibitor) for 1 h. As shown in Fig. 8, leptin-induced upA expression was decreased after pretreatment with AG490 (2.8 ± 0.29 for OVCAR3; 2.7 ± 0.3 for SKOV3) and LY294002 (2.2 ± 0.14 for OVCAR3; 3.1 ± 0.32 for SKOV3). These results indicated that JAK/STAT and PI3K/AKT have important roles in leptin-dependent upA expression.

3.7.2. Modulation of NF-kB alters leptin-induced upA expression in ovarian cancer cells

Previously, Jeong et al. demonstrated that lysophosphatidic acid-induced upA production was shown to involve NF-kB, downstream of RhoA/ROCK pathway, in ovarian cancer cell lines [22]. Thus, we investigated whether leptin regulates NF-kB activity in OVCAR3 and SKOV3 cells. As shown in Fig. 9A, B the NF-κB P56, active form of NF-κB, expression was significantly stimulated after leptin incubation for 60 min (2.9 ± 0.35-fold for OVCAR3; 3.3 ± 0.8-fold for SKOV3). Also, Pretreatment by BAY 11-7082 (NF-kB inhibitor), followed by leptin treatment significantly decreased upA expression in OVCAR3 ($P < 0.05$) and SKOV3 ($P < 0.01$), suggesting NF-κB is involved in leptin-induced upA expression.

4. Discussion

The prevalence of obesity has risen over the past 30 years and reaching epidemic proportions [30]. Obesity is primarily characterized by excess fat storage, adipocyte mass, and is a risk factor for initiation and progression of cancer. Several mechanisms have been proposed to explain the association of obesity with cancer risk including elevated lipids and lipid signaling, inflammation, insulin signaling and adipokines [31]. Leptin has been found as a proliferation-stimulating factor in ovarian cancer and has critical roles in tumor metastasis [12, 15,32,33]. However, the exact effect of leptin on cell invasion and the mechanism of the response to leptin in ovarian cancer is still unclear. In the present study, we demonstrate the effect of various concentrations of leptin on proliferation and invasion of ovarian cancer cell lines (OVCAR3, SKOV3, and CaoV-3). Our data indicated that leptin induces the proliferation and migration of OVCAR3 and SKOV3 ovarian cells in a dose and time-dependent manner. Our data showed that OB-Rb is expressed in OVCAR3 and SKOV3 cell lines, whereas we did not detect significant OB-Rb protein in CaoV-3 cell line.

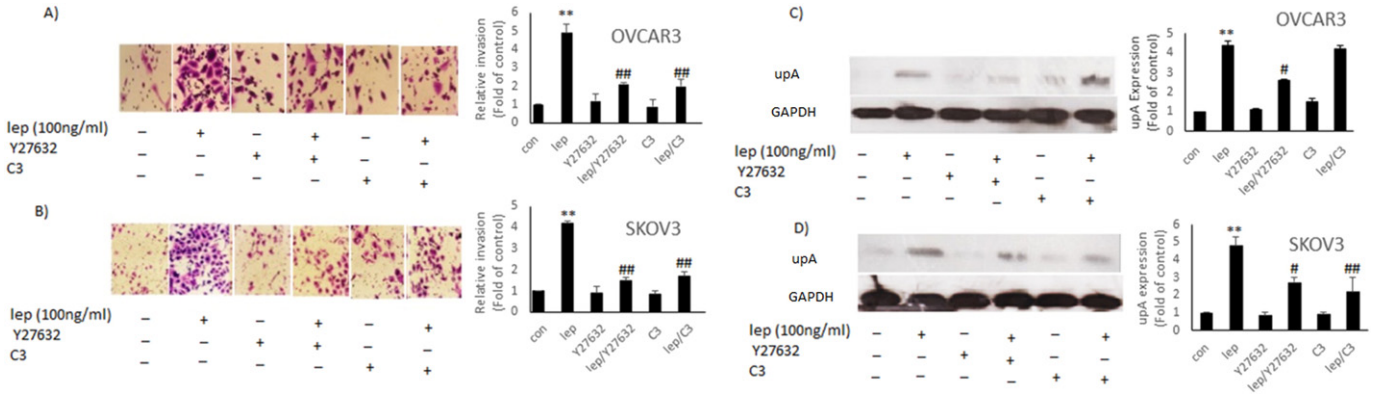

Fig. 7. The RhoA/ROCK signaling pathway is involved in leptin-mediated increase of ovarian cell invasion and upA expression: (A, B) OVCAR3 and SKOV3 cells (1×10^5) were seeded in Transwell inserts, pretreatment with C3 transferase (0.25 mg/ml) or Y-27632 (10 μM) for1 h and cell invasion was analyzed in the absence (control) or presence of leptin (100 ng/ml) by matrigel invasion assay as indicated. (C, D) ovarian cancer cells pretreatments with C3 transferase (0.25 mg/ml) or Y-27632 (10 μM) for1 h, the cells were treated with leptin (100 ng/ml) for 24 h, total protein was isolated and equal amounts of proteins were subjected to Western blot. All data shown were expressed as mean ± SD. of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs control. # $P < 0.05$, ## $P < 0.01$ vs leptin (100 ng/ml). Statistical analysis was performed by ANOVA.

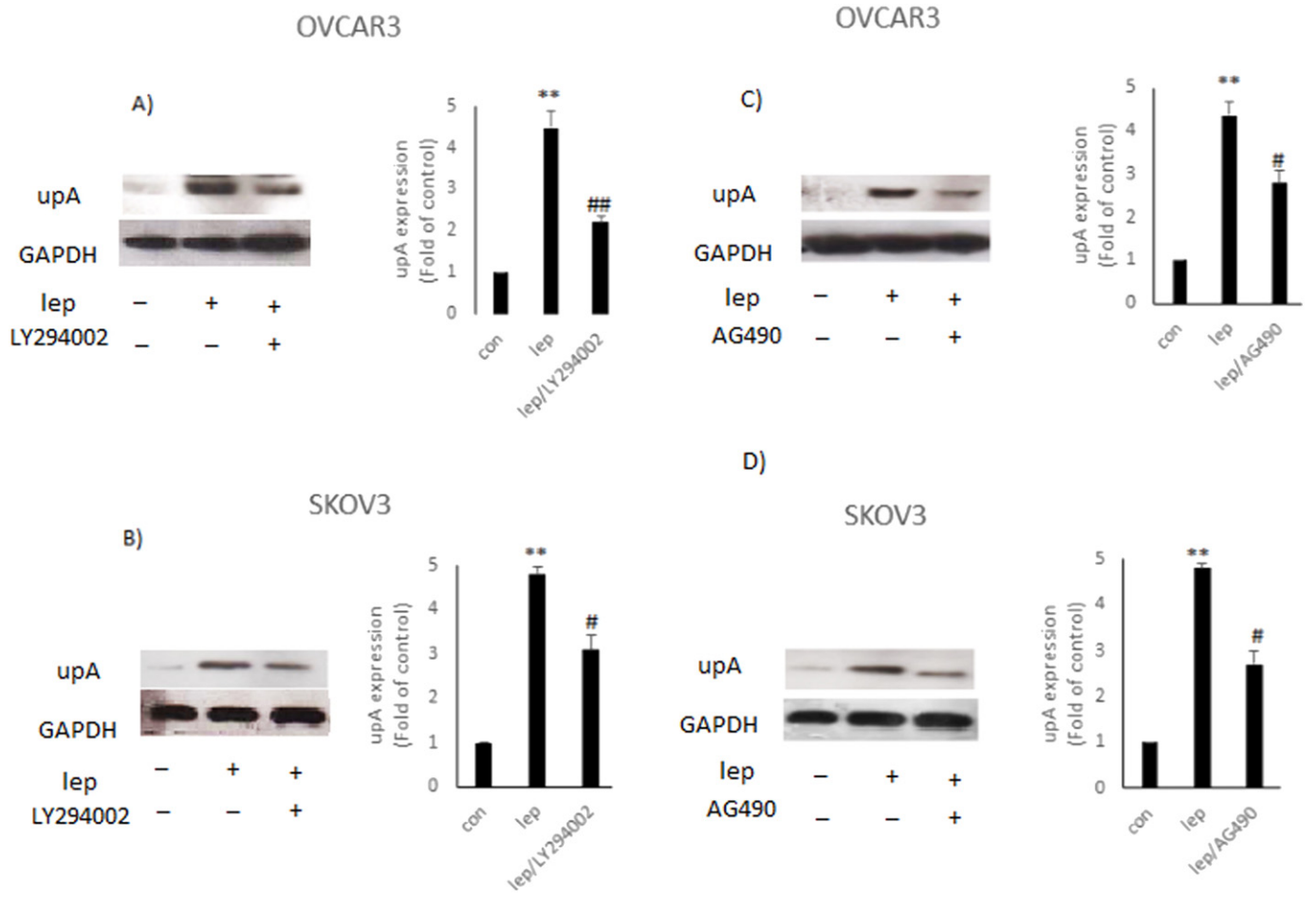

Fig. 8. JAK/STAT and PI3K/AKT signaling pathways are involved in leptin-induced upA expression: Cells were pretreated with AG490 (10 μm) or LY294002 (100 μm) for 1 h and upA expression was measured in absence or presence of leptin (100 ng/ml) using Western blotting in OVCAR3(A) and SKOV3(B) cells. All data shown were expressed as mean ± SD. of three independent experiments. ** $P < 0.01$ vs control. # $P < 0.05$, ## $P < 0.01$ vs leptin (100 ng/ml). Statistical analysis was performed by ANOVA.

Previous studies have shown a positive correlation between overexpression of uPA and aggressive biological behaviors of ovarian cancer cells [19,21]. We hypothesize that leptin-induced ovarian cancer invasion may be mediated by uPA up-regulation. For this reason, we also evaluated the effect of various concentrations of leptin on the uPA expression in ovarian cancer cell lines. Our results demonstrate that leptin increases uPA expression of ovarian cancer cells in a dose and time-dependent manner. In addition, our data show that the Rho/ROCK pathway is involved in leptin-induced uPA expression and OB-Rb is required for Rho/ROCK activation. We also demonstrated that JAK/STAT, PI3K/AKT, and NF-kb pathways are involved in upA up-regulation. Given the great impact of leptin (100 ng/ml) on cancer cell growth and invasion, our results are in the line with some of the previous reports. Leptin induces cancer cell invasion in breast cancer [34], colon cancer [27] and pancreatic cancer [33]. Choi et al. reported that treatment with leptin (100 ng/ml) resulted in a significant increase in the growth of BG-1 ovarian cancer cell line via the activation of ERK, STAT-3 and PI3K signaling pathways [29,35]. Neeraj et al. demonstrated that leptin (100 ng/ml) induced the growth and cell invasion of HepG2 and Huh7 human hepatoma cell lines [25]. Sharma et al. also reported that 100 ng/ml of leptin significantly enhanced cell growth and the invaded cell number in endometrium cancer cell lines, whereas higher doses (150 and 200 ng/ml) showed no additional stimulation [36]. In another study, Dong et al. indicated that leptin treatment (10–500 ng/ml) significantly increased cell migration in gastric cancer cell lines [24]. Consistent with our study, Kato et al. recently postulated that leptin treatment (100 ng/ml) stimulates cell migration and invasion in SKOV3 and HEY cells [15].

The principal mediator of leptin's actions is OB-Rb, which is the long isoform of leptin receptors and distributed in the whole body [14]. We also found that leptin treatment of the cells significantly increased the mRNA and protein levels of OB-Rb. Previous studies demonstrated that overexpression of OB-Rb is associated with worse outcome in some cancers including renal [37], breast [38], prostate [39] and brain tumor [40]. Uddin et al. also reported that high OB-Rb expression in the ovarian cancer correlate with poor outcomes, suggesting a role for OB-Rb in tumor progression [12]. In agreement with our data, Choi et al. detected OB-Rb in various ovarian cell lines including BG1, OVCAR3 and SKOV3 [35]. In another study, Kato et al. demonstrated that the ovarian cancer cell lines (SKOV3, HEY, UCI101, A2780, and OVCAR3) expressed the OB-Rb isoform. Moreover, it was shown that leptin treatment significantly decreased the membrane OB-Rb due to receptor down regulation [15]. Choi et al. indicated that Leptin at 0.1–1.0 mg/mL induced increase in ObR mRNA and protein expression in ovarian endometrioses [41]. In another study, Tang et al., reported that leptin enhanced the mRNA and protein level of OB-Rb in microglia, whereas OB-Rb siRNA transfection had an inhibitory effect [42]. Moreover, Dong et al. reported that leptin treatment stimulated OB-Rb mRNA and protein expression in gastric cancer [43]. Our results shown that using OB-Rb gene silencing markedly impaired leptin-induced invasion. These findings suggest an important role for OB-Rb and leptin in ovarian cancer invasion and metastasis.

Here, we shown, for the first time, that leptin induces uPA expression in a dose and time-dependent manner and was inhibited by OB-Rb gene silencing. Past investigations indicated that overexpression of uPA correlates with the aggressiveness of ovarian cancer [16–20,44,

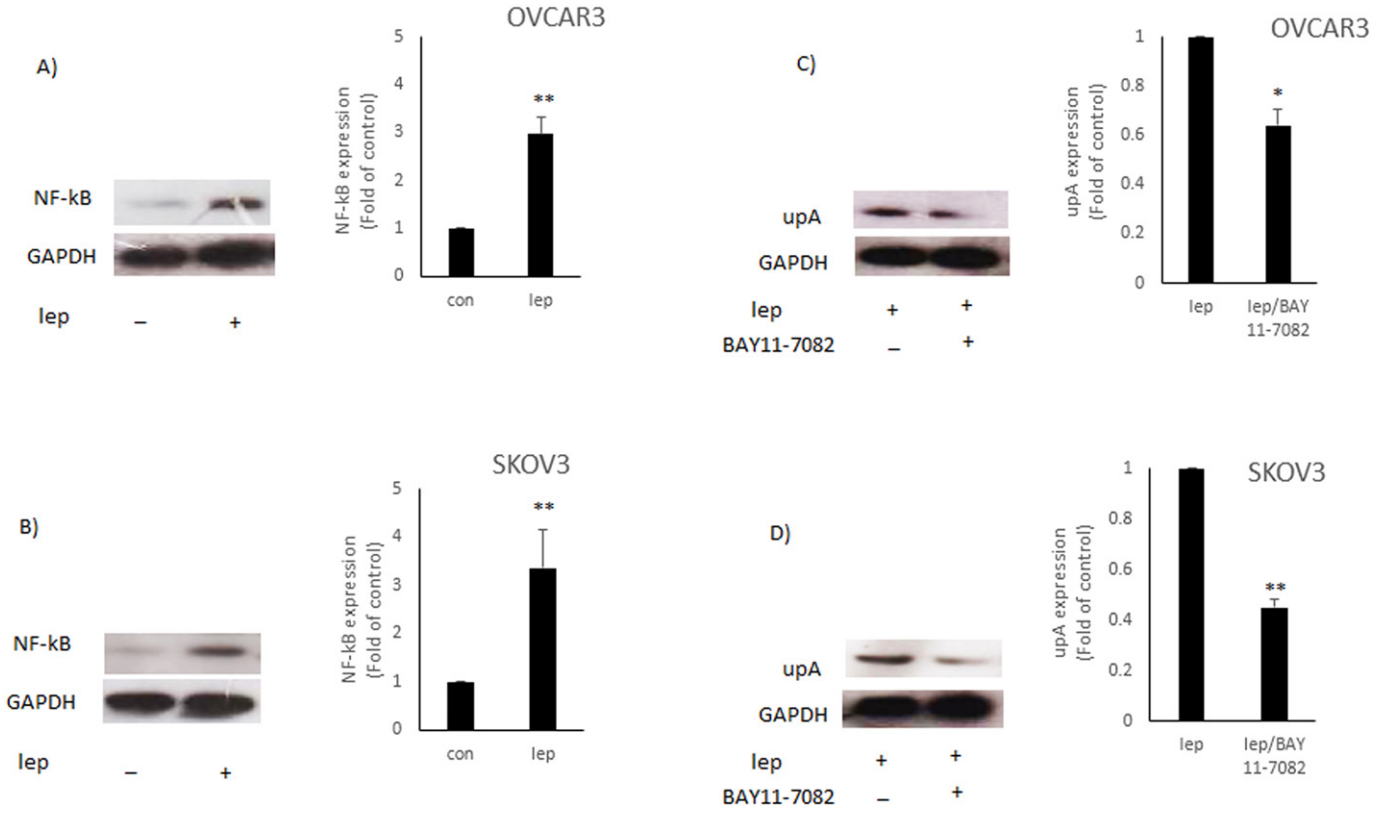

Fig. 9. Leptin stimulate NF-kB expression and NF-kB contributed in leptin-induced upA expression: (A) Cells were incubated with leptin (100 ng/ml) for 1 h, nuclear extracts were isolated, equal amounts of proteins were subjected to Western blotting and NF-kB activation was detected using specific antibodies for NF-kB p56. (B) Cell were pre-incubated with BAY 11-7082 (5 μm) for 1 h and then were stimulated by leptin (100 ng/ml) for 24 h, cell lysates were extracted and upA expression was analyzed using Western blotting. All data shown were expressed as mean ± SD of three independent experiments. ** $P < 0.01$ vs control. # $P < 0.05$, ## $P < 0.01$ vs leptin (100 ng/ml). Statistical analysis was performed by ANOVA.

45]. Zhang et al. demonstrated that uPA overexpression increased the abilities of cell invasion, migration and adhesion in SKOV3 cells, suggesting an important role in ovarian cancer metastasis [21]. Jeong et al. indicated that lysophosphatidic acid (LPA), a lipid mediator, stimulated cell invasion of SKOV3 cell line by upA activation via RhoA/Rock pathway. They also demonstrated that uPA gene silencing significantly attenuated LPA-induced ovarian cancer cell invasion [22]. The binding of uPA to its plasma membrane receptor, uPAR, activates conversion of plasminogen to plasmin. Then plasmin participates to the extracellular matrix proteolysis, and finally, facilitates cell metastasis [21]. It is, therefore, conceivable that leptin-induced uPA overexpansion may promote the remodeling of the ECM and subsequently cell invasion and metastasis in ovarian cancer.

Accumulating evidences suggest a key role of the Rho/ROCK signaling pathway in cancer invasion and metastasis. The Rho family GTPase proteins have been known to reorganize cytoskeletons and to regulate the cell migration via activation of effector proteins such as ROCK [46–48]. RhoA and ROCK are overexpressed and promote the invasive phenotype in cancer cells. In this regards, LPA stimulates ovarian cancer progression through coordinated activation of a Rho/ROCK signaling pathway [22]. In another study, Leve et al. also demonstrated LPA-induced cell migration in colon cancer depends on Src and Rho–Rock signaling [49]. Moreover, Liu et al. demonstrated that Wnt5a promoted gastric cancer cell migration via RhoA activation [47]. Our findings also demonstrated that leptin treatment increases RhoA activation as well as ROCK phosphorylation, starting between 5 and 60 min after treatment in, OVCAR3 and SKOV3 cell lines. In consistent with these results OB-Rb gene silencing significantly antagonized the leptin-induced RhoA activation and phosphorylation of Rock. Although, pretreatment with Y27632 (ROCK inhibitor) significantly decreased upA expression as well as cell invasion in both cell lines, but C3 (RhoA inhibitor) pretreatment had no effect on upA expression in OVCAR3 cells. This difference is probably due to differences in cell type. In the line with our results, Kato describes that 30 min leptin incubation enhanced protein levels of both the total RhoA as well as active GTP-bound form, in HEY cells [15]. Also, Dong et al. reported that RhoA activity and phosphorylation of ROCK was significantly increased after leptin stimulation at 15–60 min in gastric cancer cell lines, whereas total levels of RhoA and ROCK remained unaltered [24]. Inconsistent these reports, Jaffe et al. indicated that leptin remarkably increased GTP-bound RhoA levels only at a high dose (2000 ng/ml) in colon cancer, whereas different doses of leptin had no effect on total RhoA levels [50]. Zeidan et al. reported that 50 ng/ml of leptin incubation stimulated RhoA activation at 2–60 min in Rat neonatal ventricular myocytes and 5 min in vascular smooth muscle. In addition, pretreatment with an *anti*-OB-Rb antibody significantly inhibited leptin-induced RhoA activation, suggesting a possible role for OB-Rb receptor in RhoA activation [23,51]. RhoA/ROCK pathway may contribute to leptin-induced cell invasion via mechanism that are independent of upA expression. In this regards it was postulated that LPA stimulates Caov-3 cell invasion via direct MMP9 activation [22].

Our results, also confirmed that pretreatment with AG490 or LY294002 significantly decreased upA expression, demonstrating the role of these pathways in leptin-induced upA production. However, it remains to be clarified if leptin-induced RhoA/Rock activation has any relationship with JAK/STAT and PI3K/AKT signaling pathway. In the present study, we also showed that leptin activates NF-kB, which is in agreement with previous findings [52–54]. Further analysis indicates that BAY 11-7082 attenuated the effect of leptin in upA expression.

5. Conclusion

In summary, our results provide the in vitro evidences, for the first time, uPA contributes to the leptin-mediated invasion of ovarian cancer.

It also suggests that leptin, increases cell invasion by binding to the OB-Rb and activation of RhoA-ROCK, JAK/STAT, PI3K/AKT and NF-kB which are involved in uPA overexpression in ovarian cancer. This study suggests that leptin probably plays an important role in the association between obesity and advanced ovarian cancer. Present study also may provide the basis for future development of useful targets to reduce the metastasis in obese patients with ovarian cancer.

Conflict of interest

The authors declare no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found, in the online version.

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