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Adipokine resistin promotes in vitro angiogenesis of human endothelial cells

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Abstract

Objective: Resistin may be associated with obesity and cardiovascular diseases. However, it is unknown whether resistin directly contributes to angiogenesis. In the present study, we evaluated the effects of resistin on angiogenic potential, including endothelial cell proliferation, migration, and capillary-like tube formation.

Methods: Human coronary artery endothelial cells (HCAECs) were treated with resistin. Cell proliferation was evaluated by [^{3}H]thymidine incorporation and MTS assays. Cell migration was assessed by a modified Boyden chamber assay. Capillary-like tube formation was studied with a Matrigel model. Several gene expression levels were determined by real-time PCR. Activation of mitogen-activated protein kinases (MAPKs) was determined by Bio-Plex luminex analyzer. Basic fibroblast growth factor (bFGF) was used as a control. Human umbilical vein endothelial cells (HUVECs) and human lung microvascular endothelial cells (HMVEC-L) were also included.

Results: Resistin induced both endothelial proliferation and migration in a dose- and time-dependent manner with the maximal effect at 40 ng/ml. Both resistin-induced cell proliferation and migration could be effectively blocked by a resistin-neutralization antibody. In addition, resistin promoted capillary-like tube formation of HCAECs on Matrigel. Resistin also significantly upregulated the mRNA expression of vascular endothelial growth factor receptors (VEGFR-1 and VEGFR-2) and matrix metalloproteinases (MMP-1 and MMP-2) at both mRNA and protein levels. Furthermore, transient phosphorylation of ERK1/2 and p38 was observed after the addition of resistin to HCAECs. The resistin-induced cell proliferation and migration were both completely blocked by specific ERK1/2 and p38 inhibitors.

Conclusions: Resistin induces human endothelial cell proliferation and migration, promotes capillary-like tube formation, upregulates the expression of VEGFRs and MMPs, and activates ERK1/2 and p38 pathways. Thus, resistin may play an important role in angiogenesis-associated vascular disorders.

Keywords: Angiogenesis; Cell proliferation; Endothelial cell; ERK1/2; Migration; p38; Resistin; Tube formation

1. Introduction

Cardiovascular disease (CVD) remains the leading cause of death in industrialized nations [1]. The prevalence of obesity, as a cardiovascular disease risk factor, contributes to the morbidity of CVD [2]. Adipose tissues release a number of bioactive molecules (adipokines) such as leptin, adiponectin, tumor necrosis factor-α (TNF-α), interleukin-6, plasminogen activator inhibitor type 1, and resistin [3,4]. These adipokines can influence not only body weight homeostasis but also inflammation, coagulation, fibrinolysis, insulin resistance, diabetes, atherosclerosis and some forms of cancer [5,6].

Resistin belongs to a family of proteins named FIZZ (found in inflammatory zone) or RELM (resistin-like molecules) and is known to link insulin resistance and

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obesity [4]. A number of studies have demonstrated that serum resistin levels are elevated in patients with obesity-associated diabetes [7,8] or CVD [9] and that plasma resistin levels correlate with markers of inflammation. For example, resistin plasma levels in healthy individuals are around 15 ng/ml, while resistin plasma levels in diabetic patients are around 40 ng/ml [10,11]. Thus, resistin may be considered a predictive factor for coronary atherosclerosis. Several studies have shown that resistin could upregulate chemokines and adhesion molecules on endothelial cells [12] and promote smooth muscle cell proliferation [13], while the underlying cellular mechanisms have not been fully elucidated.

Dysregulated angiogenesis is involved in pathological conditions such as ischemic heart diseases, cancer, diabetes, or chronic inflammation including atherosclerosis [14]. Angiogenesis may promote the formation of plaque and thrombosis by facilitating inflammation in the lesion [15]. Several clinical studies demonstrated that high levels of tissue factor, vascular endothelial growth factor (VEGF) and von Willebrand factor (vWF) were observed in patients with risk factors of atherosclerosis or coronary artery diseases, indicating the simultaneous presence of abnormal angiogenesis and endothelial cell dysfunction [16].

In the present study, we investigated the direct effect of resistin on endothelial cells. We found that human recombined resistin induced human endothelial cell proliferation, migration, and capillary-like tube formation, thereby suggesting a potential for increased angiogenesis. We further examined the involvement of some angiogenic molecules and mitogen-activated protein kinases (MAPKs) in the role of resistin. The data from this study provides experimental evidence that resistin may induce endothelial dysfunction and contribute to cardiovascular disease and angiogenic disorders.

2. Methods

2.1. Chemicals and reagents

Human recombinant resistin and anti-resistin antibody were obtained from Phoenix Pharmaceuticals, Inc. (Belmont, CA, USA). Anti-resistin antibody was the rabbit polyclonal antibody against 51–108 aa of resistin. In our study, we tested 1:250 and 1:1000 concentrations of this antibody and showed its neutralization activity in human endothelial cells. Inhibitors of ERK (PD98059) and p38 (SB203580) were obtained from Calbiochem Inc. (San Diego, CA, USA). For human resistin (MW 11.42 kDa), the concentrations of 10, 20, 40, 60, and 80 ng/ml equate 0.88, 1.75, 3.50, 5.25, and 7.01 nM, respectively. Human basic fibroblast growth factor (bFGF) was a kind gift from Prizm Pharmaceuticals (San Diego, CA, USA). Since the molecular weight of bFGF is 18 kDa, the concentrations of bFGF (2.5, 5, 10, and 20 ng/ml) equate 0.14, 0.28, 0.56, and 1.11 nM, respectively. Anti-MMP-1 and MMP-2 antibodies, PE-conjugated mouse anti-human vascular endothelial growth factor-1 and -2 (VEGFR-1 and VEGFR-2) were purchased from R and D Systems, Inc. (Minneapolis, MN, USA). Anti-integrins β3, αV, and α3 antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). PE-conjugated mouse IgG1, PE-conjugated anti-mouse IgG1, mouse IgG1, cytoperm/cytofix and perm wash buffer were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

2.2. Cell culture

Human coronary artery endothelial cells (HCAECs), human umbilical vein endothelial cells (HUVECs), and human lung microvascular endothelial cells (HMVEC-L) were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA). The cells were routinely cultured in Endothelial Basal Medium-2 (EBM-2) with growth factors and antibiotic (EGM-2-MV BulletKit, Cambrex Bio Science Walkersville, Inc.) supplemented with 10% fetal bovine serum (Gibco, Invitrogen Corporation, Grand Island, NY, USA) at 37 °C in humidified air and CO_2. Prior to each experiment, cells were placed in the EBM-2 medium with 1% fetal bovine serum (FBS), without addition of growth factors, for 16 h (serum starvation).

2.3. Cell proliferation

The effect of resistin on endothelial cell proliferation was first measured by [^{3}H] thymidine incorporation assay. Briefly, cells were seeded onto a 96-well plate (4×10^4 cells/well). After 24 h, cells were serum-starved for 16 h, and then treated with various doses of resistin for an additional 24 h. At 4 h, 1 ìCi/ml [^{3}H] thymidine was added to each well and the cells were further incubated for 20 h. [^{3}H] thymidine incorporation was measured in a scintillation solution using a microplate scintillation and luminescence counter (Topcount; Packard Biosciences, Shelton, CT, USA).

Cell proliferation was also determined with CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega, Madison WI, USA) according to the manufacturer's instructions. Briefly, cells were then treated with various doses of resistin for 24, 48 and 72 h. After completion of the treatment, 20 μl MTS reagent was pipetted into each well of the 96-well assay plate containing 100 μl of fresh culture medium. The absorbance at 490 nm was recorded using an ELISA plate reader (EL800, Bio-Tek Instruments, Inc., Winooski, VT, USA). The percentage of the absorbance was calculated against untreated cells.

2.4. Cell migration assay

Cell migration was measured using a modified Boyden chamber assay, following a protocol provided by BD BioCoat Angiogenesis System. Briefly, cell suspension (250 μl,

Table 1
Sequence details of individual pairs of primers

Gene	Gene bank No.	Forward primer	Reverse primer
VEGFR-1	NM_002019	TCTCACACATCGACAAACCAATACA	GGTAGCAGTACAATTGAGGACAAGA
VEGFR-2	AF063658	GCAGGGGACAGAGGGACTTG	GAGGCCATCGCTGCACTCA
MMP-1	NM_002421	TCACAGCTTCCCAGCGACTC	TCAACTTGCCTCCCATCATTCTTC
MMP-2	AY738117	CAACTACAACTTCTTCCCTCGCA	GGTCACATCGCTCCAGACTTG
Integrin β3	NM_000212	ATGCTTCATCCATCATCGGTGTC	GGTCACCTGGTCAGTTAGCGT
Integrin αV	NM_002210	GATTTCTTCGTGCCCAGCG	GCGGGTAGAAGACCAGTCAC
Integrin α3	NM_005501	CTAGAGTGCCCCATCCCTGAT	TGTAATCCTCGATGAAGGTGCTG
β-actin	BC013835	CTGGAACGGTGAAGGTGACA	GGAAGCACTTCCTGGACTTGAT

5×10^5 cells/well) was added to the transwell insert (3-μm pore size, Costar, Cambridge, MA). Then, 750 μl of starvation medium was added to the lower chamber and incubated for a certain period of time. After 4 to 24 h incubation at 37 °C in a 5% CO_2 atmosphere, the chambers were then incubated in Hank's Balanced Salt Solution (HBSS) with 50 nM Calcein-AM, a fluorescence dye, for an additional 90 min to label living cells. The cells were fixed in 2% paraformaldehyde for 5 min and washed in PBS. The non-migrating cells in the upper chamber were scraped off using blunt-ended forceps and swabs, and washed with PBS. The fluorescence from the cells migrated to the lower chamber was measured using a fluorescence microplate reader (FLx800, Bio-Tek Instruments, Inc.) from the bottom at 485/535 nm wavelength. The migrated cells were represented by the ratio of fluorescence as compared to the control.

2.5. Capillary-like tube formation assay

The formation of capillary-like structures by HCAECs on Matrigel (BD Biosciences, San Jose, CA, USA) was studied as previously described [17]. Twenty-four-well culture plates were coated with Matrigel according to the manufacturer's instructions. HCAECs were pretreated with or without resistin (40 ng/ml) or bFGF (20 ng/ml) in the starvation medium for 24 h. Trypsin-harvested HCAECs were then seeded onto the coated plates at 5×10^3/well in the fresh assay medium, and incubated at 37 °C for 3, 6 or 18 h. Tube formation images were captured at a magnification of 100× with a digital microscope camera system (Olympus, Tokyo, Japan). The level of the tube formation was quantified by measuring the length of tubes in three randomly chosen fields from each well using an Image-Pro Plus software and was calculated against untreated groups.

2.6. RNA isolation and real-time RT-PCR

Serum-starved HCAECs were treated with resistin (40 ng/ml) for 24 h, and total RNA was isolated using Trizol reagent (Invitrogen). One microgram of total RNA was reverse-transcribed in 20 μl reaction solution using the iScript™ cDNA Synthesis Kit. Human VEGF receptor-1 (VEGFR-1), VEGFR-2, matrix metalloproteinase-1 (MMP-1), MMP-2, and integrins β3, αV and α3 as well as β-actin primers were designed using Beacon Designer software (Table 1). The real-time PCR reactions were performed with the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories) in 96-well reaction plates. Reaction volumes were 25 μl, containing 2 μl cDNA and 100 μM of each pair of primers and iQ™ SYBR Green Supermix (Bio-Rad). Thermal cycling conditions included pre-incubation 95 °C for 3 min followed by 40 PCR cycles at 95 °C for 20 s and 60 °C for 1 min. All reactions were run in triplicate. iCycler software was used to analyze the calibration curve by plotting the threshold cycle (Ct) vs. the logarithm of the number of copies for each calibrator. The relative amount of mRNA for each gene was normalized based on that of the housekeeping gene β-actin [2^(Ct β-actin-Ct gene of interest)].

2.7. Flow cytometry analysis

The cells were detached with trypsin/EDTA solution and then suspended in cold staining buffer (PBS containing 2% FBS and 0.09% sodium azide). Before incubating with primary antibody, the cell suspension was immnunoblocked in 10% human serum in 4 °C for 20 min. Before immunolabeling with antibodies, the cells were permeablized with cytoperm/cytofix buffer in 4 °C for 20 min. Similar numbers of cells (1×10^6) were immunostained with manufacturer-recommended concentrations of antibodies: PE-conjugated anti-human VEGFR-1 and VEGFR-2 antibodies, anti-human MMP-1 and MMP-2 antibodies, and anti-integrin β3, αV and α3 antibodies in 4 °C for 30 min. PE-conjugated mouse IgG1 and mouse IgG1 were used as isotype controls, respectively. PE-conjugated anti-mouse IgG1 was used as secondary antibodies for MMPs and integrins. The cells were analyzed by FACScalibur flow cytometer (Becton Dickinson, San Jose, CA) with the CellQuest software. Each analysis included at least 5×10^3 cell events. Data are presented as the percentage of positive cells corresponding to the mean fluorescence intensity in each experiment.

2.8. MAPK activation

The MAPK phosphorylation state was analyzed by Bio-Plex phosphoprotein and total target protein assays (Bio-Rad) according to manufacturer's instructions. Luminex is a groundbreaking technology which has been recently used to

a wide range of bioassays including MAPKs. Briefly, serum-starved HCAECs were treated with resistin (40 ng/ml) and the cell lysates were collected at different time points of 0, 5, 10, 20, 30, 45, 60, 90, and 120 min with Bio-Plex Cell Lysis Kit. The protein concentration was adjusted to 600 μg/ml. Fifty microliters of coupled beads, which recognize phosphorylated and total ERK1/2, p38, and JNK, respectively, were added to the 96-well filter plate, followed by washing twice. Same volume of the cell lysates were added and incubated with the beads for 15–18 h. Next, 25 μl of detection antibodies (1×) were added after washing and incubated for 30 min. Fifty microliters of streptavidin-PE (1×) was added followed washing and incubated in the dark for 10 min. After rinsing, 125 μl of resuspension buffer was added, and the phosphoprotein and total proteins of MAPKs were analyzed by a Luminex 100TM analyzer and Bio-Plex Manager software (BioRad).

2.9. Statistical analysis

Data are presented as mean±SEM as compared to the negative control. Statistical significance was determined by a Student's *t*-test (two tailed). A value of $P<0.05$ was considered significant.

3. Results

3.1. Resistin induces endothelial cell growth

Resistin has been reported to promote endothelial cell activation [12,18], but no report has shown that resistin affects endothelial cell proliferation and migration. To evaluate the direct effects of resistin on endothelial cell proliferation, we first performed a [^{3}H]thymidine incorporation assay. Serum-starved cells were treated for 24 h with increasing concentrations (10 to 80 ng/mL) of resistin, and the [^{3}H]thymidine incorporation was then analyzed by a topcount machine. As shown in Fig. 1A, resistin significantly induced HCAEC proliferation in a dose-dependent manner, with the maximum effect at 40 ng/ml ($P<0.01$; increased up to 150%, as compared to the untreated control; $n=6$). Higher doses (60 or 80 ng/ml of resistin) also significantly induced HCAEC proliferation over the untreated control ($P<0.05$; $n=6$), respectively. As a positive control, bFGF significantly increased ^{3}H-thymidine incorporation in a concentration dependent manner (Fig. 1B). The effect of bFGF at 5 ng/ml (0.28 nM) is comparable to that of resistin at 40 ng/ml (3.50 nM) for ^{3}H-thymidine incorporation in HCAECs.

In another set of experiments, the effect of resistin on HCAEC proliferation was studied by the nonradioactive colorimetric assay (MTS). After 24 h of the treatment with increasing concentrations (10 to 80 ng/ml) of resistin or 20 ng/ml bFGF as a positive control, a dose-dependent proliferative effect was also observed in the treated

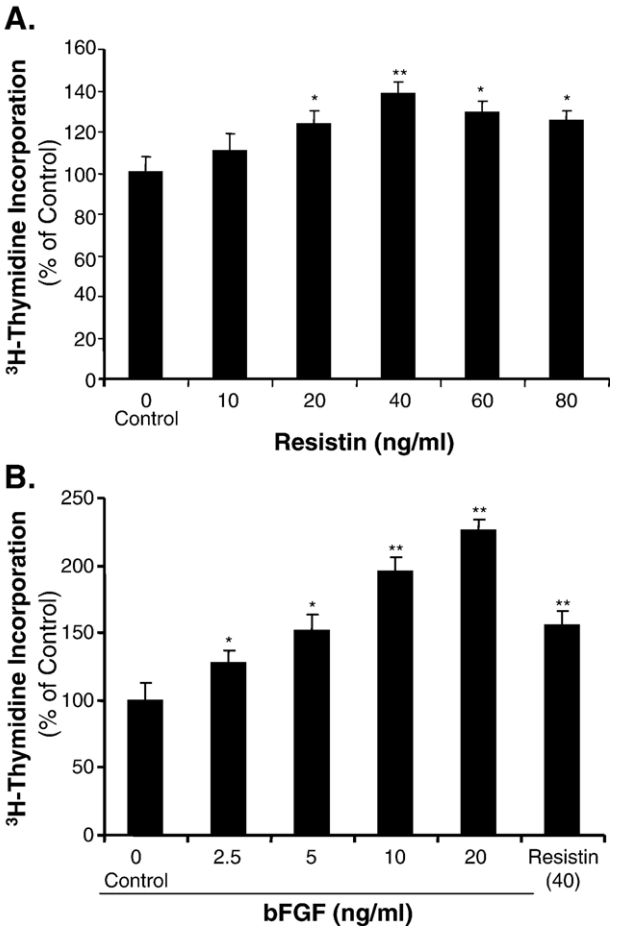

Fig. 1. Effect of resistin and bFGF on [^{3}H]thymidine incorporation in HCAECs. Serum-starved HCAECs were treated with different doses of resistin (0–80 ng/ml) (A) or bFGF (0–20 ng/ml) (B) for 24 h. [^{3}H]Thymidine incorporation by HCAECs was measured in scintillation solution. Results are expressed as a percentage of the [^{3}H] incorporation of the treated HCAECs vs. the untreated cells. Each bar represents the mean±SEM. *$P<0.05$ and **$P<0.01$ as compared to untreated cells, $n=6$ per group.

samples, respectively (Fig. 2A; the maximum effect up to 120% at the dose of 40 ng/ml and 140% by bFGF as compared to the negative control; $P<0.01$; $n=6$). Thus, by performing 2 different methods of [^{3}H]thymidine incorporation and MTS, we confirmed that resistin could induce HCAEC proliferation in a dose-dependent manner with its maximum effect at the concentration of 40 ng/ml.

The time course study revealed that resistin had the maximal effect at 24 h and was slightly declined with time (Fig. 2B, $P<0.01$, from 119% down to 114% from 24 to 72 h, respectively; $n=6$), whereas the effect of bFGF increased with time (up to 134% to 193% from 24 to 72 h, respectively; Fig. 2B; $n=6$).

To confirm the specificity of the effect of resistin on HCAECs, we blocked resistin actions with a resistin-neutralizing antibody for 60 min and then tested cell proliferation with MTS assay. As shown in Fig. 2C, resistin-neutralizing antibody (1:1000 and 1:250) alone

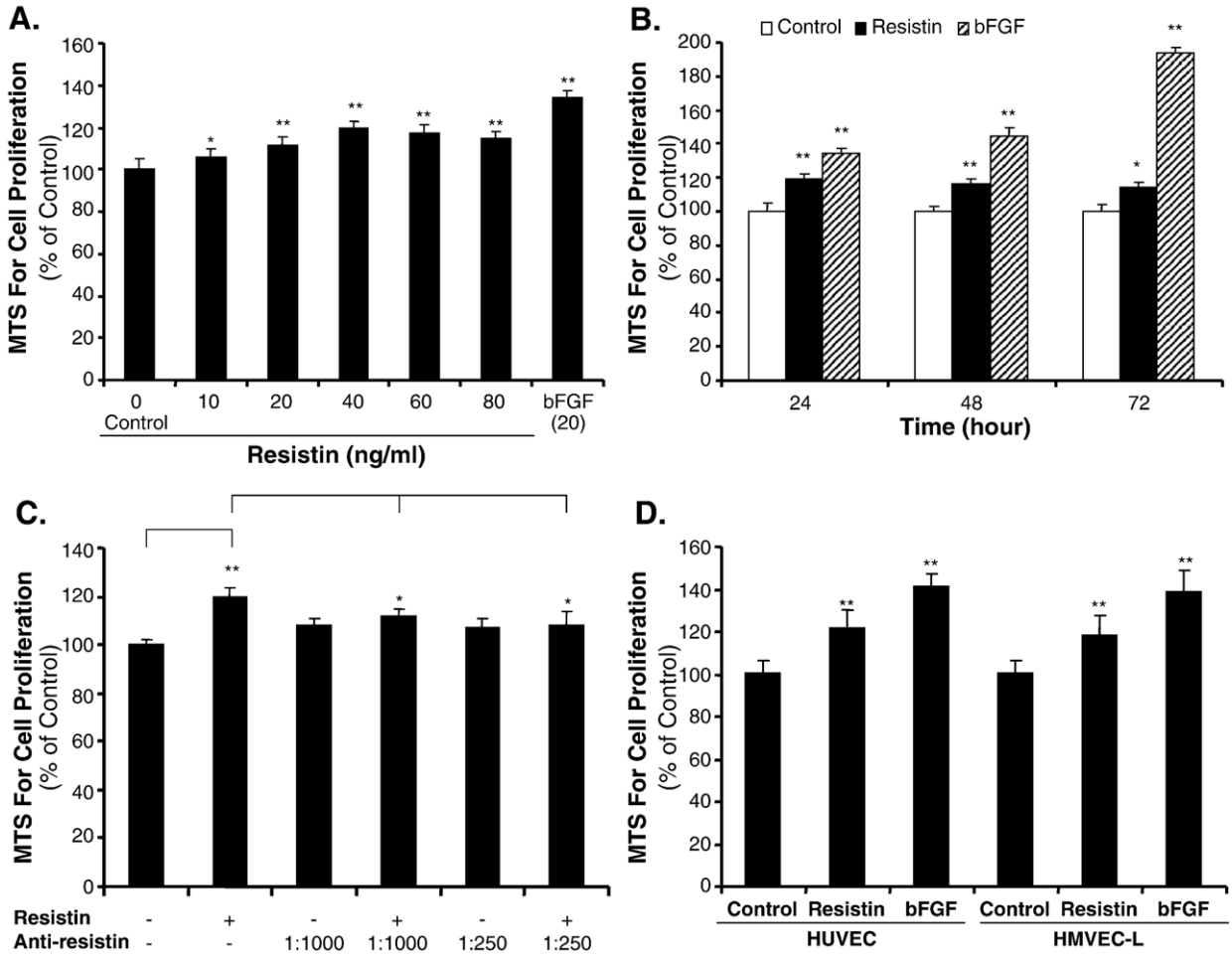

Fig. 2. Effect of resistin and bFGF on human endothelial cells by MTS assay. A. Serum-starved HCAECs were treated with measured doses of resistin (0–80 ng/ml) and human bFGF (20 ng/ml) for 24 h and the cell growth was assessed by MTS assay. B. Time course study of HCAEC proliferation. Serum-starved HCAECs were treated with or without 40 ng/ml of resistin, and 20 ng/ml of bFGF for 24, 48 and 72 h. The cell proliferation activity was assessed by MTS assay. C. Effect of resistin-neutralization antibody on resistin-induced HCAEC proliferation. Serum-starved HCAECs were treated with measured doses of resistin-neutralization antibody (1 : 250 and 1 : 1000) with the presence or absence of resistin (40 ng/ml) for 24 h. HCAEC proliferation activity was assessed by MTS assay. D. Serum-starved HUVECs or HMVEC-L were treated with or without resistin (40 ng/ml) or bFGF (20 ng/ml) for 24 h and the cell growth were assessed by MTS assay. *$P<0.05$ and **$P<0.01$ as compared to untreated cells, $n=6$ per group.

did not show any significant effect on HCAECs growth, but it significantly blocked the effect of resistin on HCAEC proliferation ($P<0.05$ as compared to the resistin treatment; $P>0.05$ as compared to the control; $n=6$). This suggests that resistin specifically increased HCAEC proliferation.

In addition to HCAECs, the effect of resistin on endothelilal cell proliferation was also tested in the other cell lines including HUVECs and HMVECs-L by MTS assay. As shown in Fig. 2D, resistin (40 ng/ml) significantly increased HUVEC and HMVEC-L proliferation up to 121.9% and 118.7% as compared to the untreated control ($P<0.01$, $n=6$). These data confirm the effect of resistin on proliferation in several types of human endothelial cells.

3.2. Resistin induces endothelial cell migration

Since endothelial proliferation and migration are critical steps of angiogenesis, we were interested in determining whether resistin could affect endothelial cell migration. Cells were treated with increasing concentrations of resistin (10–80 ng/ml) in the upper chambers in a modified Boyden chamber assay. The cells migrated through a polystyrene-membrane with 3 μm-size pores were stained with calcein-AM, a fluorescence dye, and measured with a fluorescence reader after incubation with calcein-AM. As shown in Fig. 3A, resistin (10–80 ng/ml) significantly increased HCAEC migration in a dose dependent manner with the maximum effect at 40 ng/ml (up to 149% as compared to the medium alone control, $P<0.01$). As a positive control, bFGF also significantly increased HCAEC migration in a dose dependent manner (Fig. 3B). The effect of bFGF at 5 ng/ml (0.28 nM) is comparable to that of resistin at 40 ng/ml (3.50 nM) for HCAEC migration.

To confirm the above finding, we performed a time course study at 4, 8, 12 and 24 h. We observed a detectable

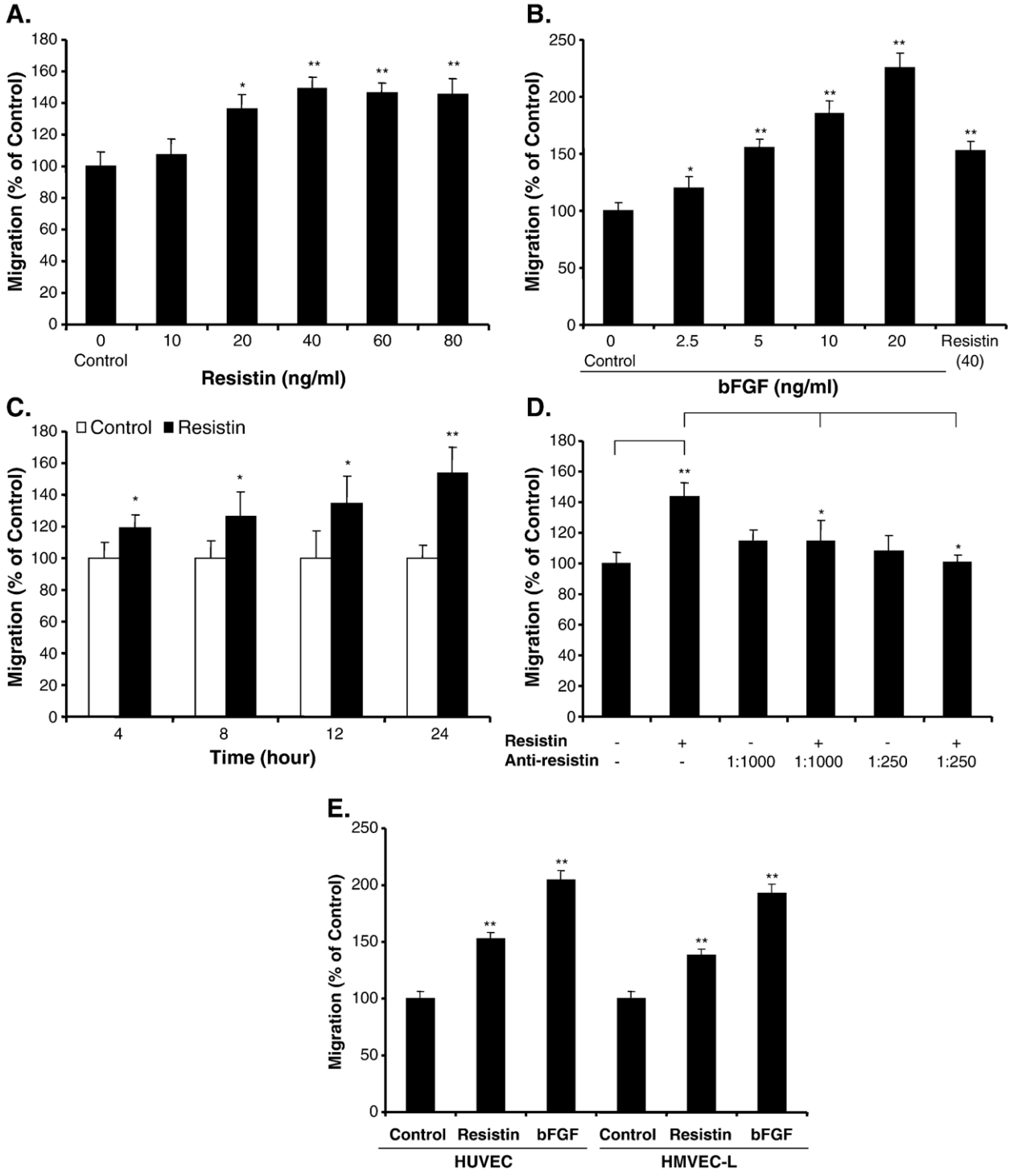

Fig. 3. Effect of resistin and bFGF on human endothelial cell migration. A. Resistin dose-dependent study on HCAECs. Serum-starved HCAECs were seeded onto a transwell plate with resistion (0–80 ng/ml). After a 24 h incubation, HCAECs that migrated to the lower chamber were measured by fluorescence staining and quantitation. Results are expressed as a percentage of the fluorescence on resistin-treated vs untreated groups. B. bFGF dose-dependent study on HCAECs. C. Time course study. Serum-starved HCAECs were seeded onto a transwell plate with or without 40 ng/ml resistin. HCAEC migration was studied at 4, 8, 12, and 24 h. D. Effect of resistin-neutralization antibody on resistin-induced HCAEC migration. Serum-starved HCAECs were seeded onto a transwell plate with measured doses of resistin-neutralization antibody (1:250 and 1:1000) with the presence or absence of resistin (40 ng/ml). HCAEC migration was studied at 24 h. E. Effect of resistin on HUVECs and HMVEC-L migration. Serum-starved HUVECs or HMVEC-L were seeded onto the transwell plate with or without resistin (40 ng/ml) or bFGF (20 ng/ml). After 24 h incubation, cells migrated to the lower chamber were measured by a fluorescence staining and quantitation. Each bar represents the mean ± SEM. *$P<0.05$ and **$P<0.01$ as compared to untreated cells, $n=6$ per group.

effect of resistin on cell migration as early as 4 h after cell seeding, and the most significant effect was observed at 24 h (Fig. 3C). The promoting effect of resistin on HCAEC migration was effectively blocked by the resistin-neutralization antibody (Fig. 3D, $P<0.05$ as compared to the resistin-treated group, and $P>0.05$ as compared to the control; $n=6$), indicating this effect is resistin specific.

The effect of resistin on endothelial cell migration was also tested in the other cell lines including HUVECs and HMVEC-L by migration assay. As shown in Fig. 3E, resistin (40 ng/ml) also increased HUVEC and HMVEC-L migration up to 152.9% and 138.3% as compared to the untreated control ($P<0.01$, $n=6$). These data confirm the effect of resistin on migration in several types of human endothelial cells.

3.3. Resistin accelerates capillary-like structure formation in vitro

To investigate whether resistin could affect the angiogenic property of endothelial cells, we performed a capillary-like tube formation assay on Matrigel, a most commonly used method for in vitro angiogenesis. HCAECs were pretreated with or without resistin (40 ng/ml) or bFGF (20 ng/ml) for 24 h and then seeded 5×10^4 cells/well on Matrigel in the 24-well plate with fresh medium. The capillary-like tube was developed and photos were taken at 3, 6 and 18 h incubation. Treatment with resistin (40 ng/ml) promoted the capillary-like tube formation as compared to the controls at each time point (Fig. 4A). Quantitative analyses revealed the total length of capillary-like tubes

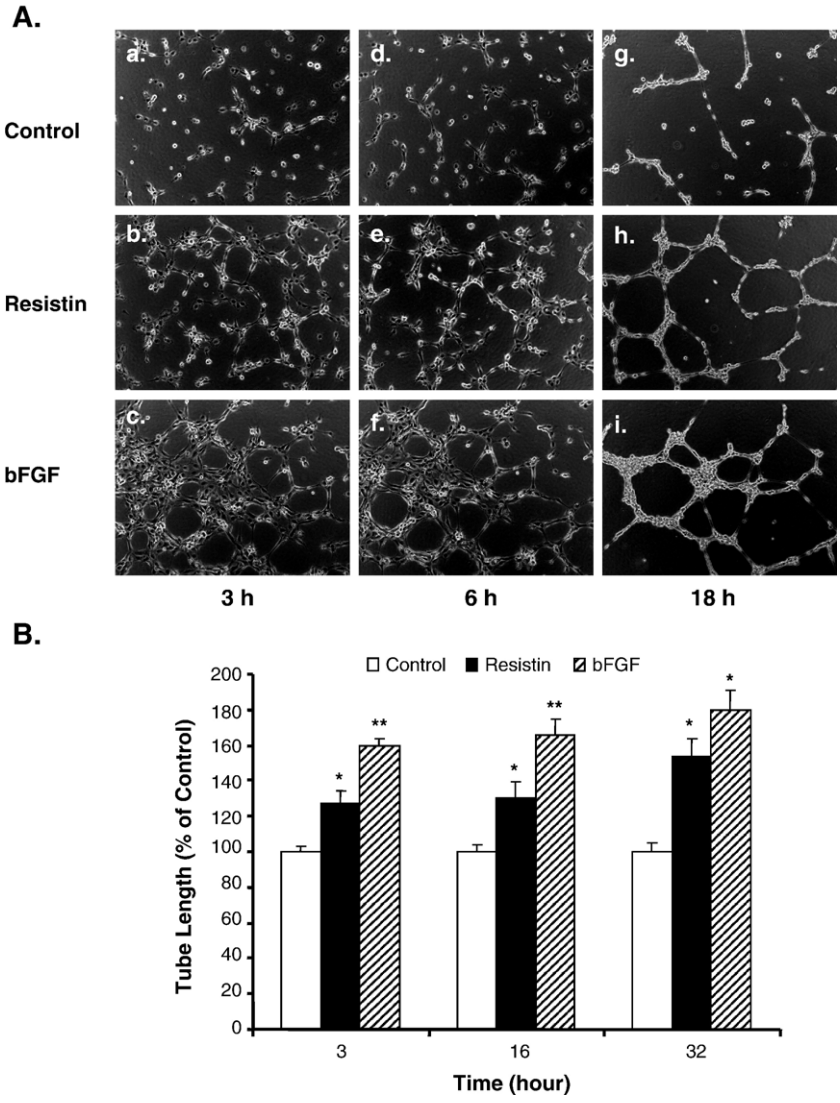

Fig. 4. Effect of resistin on capillary-like tube formation of HCAECs. Serum-starved HCAECs were treated with or without resistin (40 ng/ml) or bFGF for 24 h before they were seeded onto Matrigel with fresh medium. Capillary-like tube formation was observed and photos were taken at 3, 6, and 18 h after seeding. The total length of capillary-like tubes was measured and normalized with controls. A. Typical photos of HCAECs capillary-like tube formation, on Matrigel, after 3 h of treatment (a–c), 6 h (d–f) and 18 h (g–i). Untreated control: a, d, g; resistin-treated: b, e, h; bFGF -treated: c, f, i. B. Tube length measurement. $^{**}P<0.01$ as compared to the control, $^{*}P<0.05$ vs. control, $n=3$ per group.

induced by resistin (40 ng/ml) was significantly higher than that of the untreated control (Fig. 4B; increased up to 127%, 130% and 154% at 3, 6 and 18 h, respectively, $P<0.05$; $n=3$), but shorter than that of bFGF treatment. These results suggest that resistin promotes in vitro angiogenesis of HCAECs.

3.4. Resistin increases the expression of VEGFRs,MMPs, and integrins in HCAECs

VEGF, mediated through two major receptors as VEGFR1 (Flt-1) and VEGFR2 (Flk-1), is a major regulator of endothelial cell proliferation, migration and angiogenesis [19]. MMPs and integrins play an essential permissive role in cell migration and angiogenesis. Expression of VEGFR-1 and VEGFR-2, MMPs and integrins in HCAECs before and after resistin or bFGF treatment were evaluated by real-time PCR. As shown in Fig. 5, the mRNA levels of MMP-1, MMP-2, VEGFR-1, and VEGFR-2 in resistin-treated HCAECs were significantly increased up to 157%, 255%, 246% and 238%, respectively, as compared to untreated controls (100%; $P<0.05$; $n=3$). MMP-9 mRNA level was very low and did not show any changes with resistin treatment. In addition, the mRNA levels of integrins β3, αV and α3 in resistin-treated cells were also significantly increased by 168%, 146%, and 146%, respectively, as compared to untreated controls (100%; $P<0.05$; $n=3$). Resistin-induced upregulation of MMP-1, MMP-2, VEGFR-1, and VEGFR-2 were also confirmed at protein levels by flow cytometry analysis (Fig. 5B, $P<0.05$, $n=3$). Thus, we demonstrated that resistin upregulates angiogenic factors such as VEGFRs and MMPs at both mRNA and protein levels in HCAECs.

3.5. Resistin activates ERK1/2 and p38, but not JNK

To better understand the signal pathways involved in resistin-induced HCAEC proliferation, migration and in vitro angiogenesis, we investigated the possible involvement of MAPKs including, extracellular signal-regulated kinase (ERK1/2), p38, and the c-Jun N-terminal protein kinase (JNK). To address this issue, serum-starved HCAECs were incubated with resistin (40 ng/ml) and cell lysates were collected at different time points. A bio-plex assay (Bio-Rad bioscience) was used to detect phospho- and total proteins of p38, ERK1/2 and JNK. The ratio of phosphoproteins to total proteins at each time point was used to evaluate the phosphorylation of ERK1/2, p38, and JNK. As shown in Fig. 6A, ERK1/2 increased at 20 min and reached its peak of up to 10-fold at 30 min, and declined after 90 min treatment. In addition, p38 was also found to increase up to 1.47-fold at 3 min and declined after 10 min. But no significant change was observed for JNK. The results suggest that resistin activated ERK1/2 and p38 signaling pathways on HCAECs.

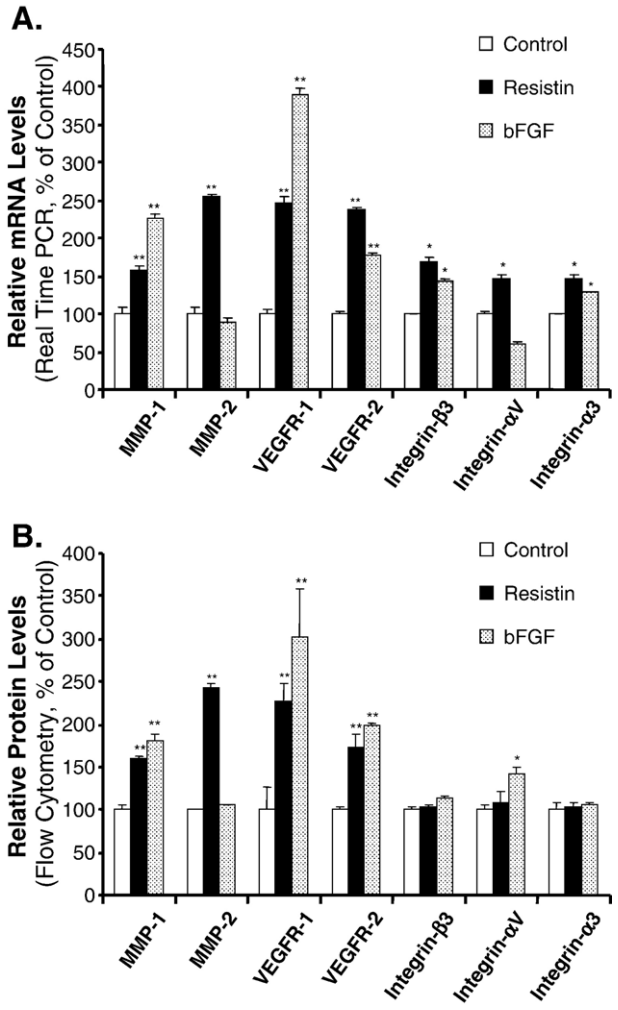

Fig. 5. Effect of resistin on the expression of several angiogenic factors on HCAECs. Serum-starved HCAECs were treated with or without resistin (40 ng/ml) or bFGF (20 ng/ml) for 24 h. A. At mRNA level. After treatment, the cDNA was synthesized from total mRNA of the cells by reverse transcription. The mRNA levels of MMP-2, MMP-1, VEGFR-1, VEGFR-2, and integrins β3, αV and α3 were analyzed by real-time PCR. The relative amount of mRNA for each gene was normalized based on that of the housekeeping gene β-actin [2^(Ct β-actin-Ct gene of interest)]. B. At protein level. The cells (1×10^6) were immunostained with antibodies: PE-conjugated anti-human VEGFR-1 and VEGFR-2 antibodies, anti-human MMP-1 and MMP-2 antibodies, anti-integrin β3, αV and α3 antibodies in 4 °C for 30 min. PE-conjugated mouse IgG1 and mouse IgG1 were used as isotype controls, respectively. PE-conjugated anti-mouse IgG1 was used as secondary antibodies for MMPs and integrins. The cells were analyzed by FACScalibur flow cytometer with the CellQuest software. $^{**}P<0.01$ as compared to the control, $^{*}P<0.05$ vs. control, $n=3$ per group.

To test the impact of ERK1/2 and p38 activation on the effect of resistin, we used specific inhibitors of ERK1/2 and p38 in resistin-induced HCAEC proliferation and migration. HCAECs were incubated with ERK1/2 inhibitor PD98059 and p38 inhibitor SB203580 for 1 h before and during resistin treatment. Cell growth and migration were evaluated by [^{3}H]thymidine incorporation and a modified Boyden chamber method, respectively. Although PD98059 (25 μM)

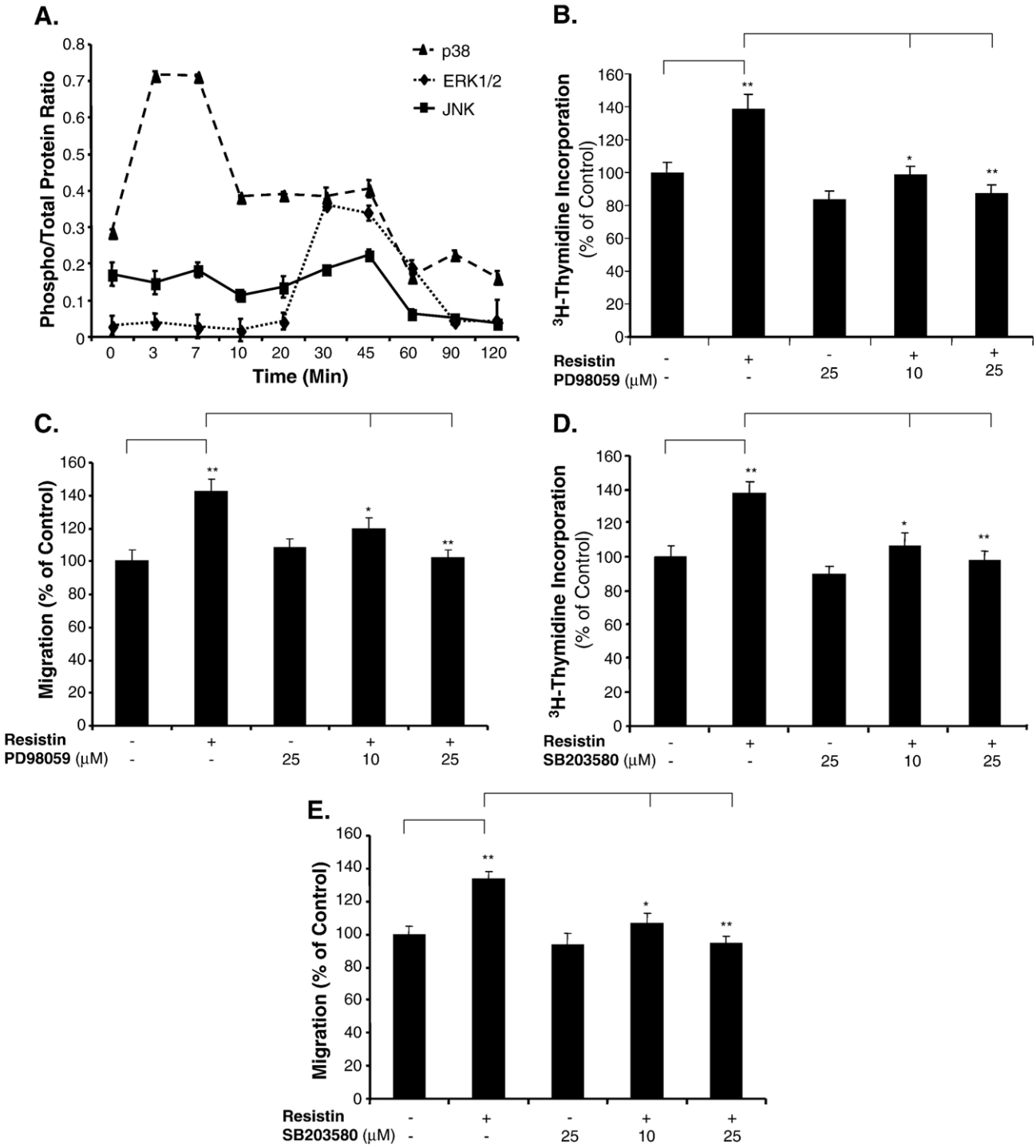

Fig. 6. Effect of resistin on MAPK activation. A. Bio-plex assay. Serum-starved HCAECs were treated with resistin (40 ng/ml), and cell lysates were harvested at different time points as shown, $n=3$ per group. B. Effect of ERK1/2 inhibitor on resistin-induced HCAEC proliferation, $n=6$ per group. C. Effect of ERK1/2 inhibitor on resistin-induced HCAEC migration, $n=6$ per group. D. Effect of p38 inhibitor on resistin-induced HCAEC proliferation, $n=6$ per group. E. Effect of p38 inhibitor on resistin-induced HCAEC migration, $n=6$ per group. Serum-starved HCAECs were treated with ERK1/2 inhibitor PD98059 (10 and 25 μmol/L) or p38 inhibitor SB203580 (10 and 25 μmol/L) for 60 min before and during resistin treatment for 24 h. HCAEC proliferation and migration were assessed using the [^{3}H]thymidine incorporation and the modified Boyden chamber, respectively. *$P<0.05$ and **$P<0.01$ as compared to untreated cells.

alone did not show any effect on HCAECs, it suppressed resistin-induced HCAEC proliferation completely at 25 μM ($P<0.01$ as compared to the resistin treatment; $n=6$) but partly at 10 μM ($P<0.05$ as compared to the resistin treatment, $n=6$) (Fig. 6B and C; $P>0.05$ as compared to the control; $n=6$). In addition, SB203580 (25 μM) alone did not affect the cell proliferation and migration. However, it did block the promoting effect of resistin on both HCAEC

proliferation and migration (Fig. 6D and E; $P<0.05$ as compared to the resistin treatment and $P>0.05$ as compared to the control; $n=6$). These findings suggest that resistin induces HCAEC proliferation and migration through ERK1/2 and p38 signaling pathways.

4. Discussion

In the present study, we attempted to determine whether human resistin could have direct effects on human endothelial cells. Indeed, we demonstrated that resistin was able to induce human endothelial cell proliferation, migration and in vitro angiogenesis through activation of ERK1/2 and p38. In addition, resistin could upregulate several angiogenesis-related factors such as VEGFR-2, VEGFR-1, MMP-1 and MMP-2. These findings provide a direct link between resistin and angiogenesis, which may contribute to vascular lesion formation.

Angiogenesis is involved in many diseases such as ischemic heart diseases, cancer, diabetes, and chronic inflammation including atherosclerosis [19,20]. The microvessels in the intima and plaque of atherosclerosis facilitate activation and infiltration of macrophages, T-lymphocytes, and plasma proteins to the lesion, with promotion of plaque thrombosis [21]. Moreover, in clinical studies, the number of foam cells was correlated with the numbers of neovessels, suggesting an important role for plaque angiogenesis in plaque thrombosis and clinically relevant diseases. Several chemokines and molecular pathways were reported to be the link between angiogenesis and atherosclerosis or atherothrombosis. Monocyte chemoattractant protein-1 (MCP-1) was reported to induce angiogenesis in vivo [22]. Thrombin is another molecule that exhibits angiogenic activity in vitro and in vivo [23]. Platelet microparticles, which are directly correlated with atherosclerotic disease, promote angiogenesis of endothelial cells [24]. Several clinical studies demonstrate high levels of tissue factor, VEGF and vWF in patients with abnormal thrombogenesis, angiogenesis, and endothelial cell dysfunction [16]. Our data showed that resistin increased cell proliferation and migration, as well as in vitro angiogenesis in HCAECs, in a dose-dependent manner. This effect was also observed in other types of human endothelial cells such as HUVECs and HMVEC-L. The maximal effect of resistin was observed at the concentration of 40 ng/ml, which is relevant to the clinical plasma levels of resistin in obesity and diabetic patients [10,11]. Two independent studies showed that resistin treatment increased ET-1 promoter activity and upregulated VCAM-1 and MCP-1 expression, with concomitant reductions in TRAF-3 expression on endothelial cells [12]. Our recent study using porcine coronary arteries showed that resistin reduces the vasorelaxation, which is associated with increased superoxide radical production and decreased eNOS expression. Taken together, human resistin-induced angiogenesis and inflammation responses may contribute to cardiovascular diseases such as atherosclerosis and other angiogenic disorders.

VEGF, through two major receptors of VEGFR1 (Flt1) and VEGFR2 (Flk-1) in endothelial cells, plays a major role in angiogenesis due to its unique biologic capacity to induce migration and proliferation of endothelial cells, to enhance vascular permeability, and to modulate thrombogenity [20]. MMPs are capable of degrading both the collagenous and noncollagenous components of the extracellular matrix [25]. Emerging evidence suggests that MMPs play an important role in angiogenesis [25,26], particularly in endothelial cell migration, capillary basement membrane breakdown, and pericellular fibrinolysis [27]. Integrins are MMPs receptors and also play important roles in the process of angiogenesis [28]. We showed that resistin-treated HCAECs significantly increased expression of VEGFR-1, VEGFR-2, MMP-2, and MMP-1 in both mRNA and protein levels. The mRNA levels of several integrins in HCAECs were also increased after resistin treatment. These data could help us to understand the molecular mechanisms of resistin-induced angiogenesis. More detailed investigations are warranted.

Phosphorylation of MAPKs was determined by Bio-Plex assays with Lumixen technology, which contain dyed beads conjugated with monoclonal antibodies specific for a target protein or peptide such as a cytokine or a phosphoprotein. The antibodies used in these assays undergo rigorous optimization to ensure the highest degree of sensitivity, specificity, and reproducibility. The Bio-Plex assay can detect as less as 10 μg/ml in 25 μl of total protein of cell culture lysate with a minimal intra-assay or inter-assay variability. MAPKs including ERK1/2 p38, and JNK are involved in many signal transduction pathways of several cellular responses to various cytokines, growth factors, and environmental factors. ERK1/2 pathway plays a central role in angiogenesis by the finding that specific inhibitors of MEK1/2, the kinase responsible for ERK activation, reduce endothelial tubulogenesis in vitro [29]. Additionally, a role for VEGF-induced p38 activation is suggested in regulating angiogenesis because the p38 inhibitor SB203580, inhibited actin reorganization and cell migration [30]. In our current study, we found that ERK1/2 and p38 pathways were activated and the inhibition of either signaling pathway by its specific inhibitor completely blocked resistin-induced HCAEC proliferation and migration. This suggested that resistin promotes in vitro angiogenesis in HCAECs via ERK1/2 and p38 signaling pathways. Another recent study also showed that p38 is involved in VEGF-induced cell angiogenesis, which is consistent with our observation [31]. There is some evidence for cross-talk between the ERK and JNK pathways in VEGF mitogenic signaling. VEGF activates JNK and the expression of a dominant-negative JNK-1 mutant inhibited VEGF-induced [^{3}H]thymidine incorporation [32]. In our study, however, the JNK was not activated in resistin-treated HCAECs.

In current study, resistin induced a 1.47-fold increase of p38 phosphorylation at 3 min of resistin treatment, while resistin increased a 10-fold increase of ERK1/2 phosphorylation at 30 min of resistin treatment. The time points of the phosphorelation of MAPKs varies according to different stimuli. Several reports showed that p38 was activated at early time point as 3–5 min after stimulation, while ERK1/2 was activated around 30 min [33,34], and these data are consistent with our findings. However, detailed downstream and upstream signaling molecules of p38 and ERK1/2 activated by resistin are not known and warranted further investigations.

In summary, we have demonstrated for the first time that a newly characterized adipokine, resistin, directly induces endothelial human endothelial cell proliferation and migration, while promoting in vitro angiogenesis through activation of ERK1/2 and p38. These effects are correlated with resistin-induced upregulation of VEGFR-1 and VEGFR-2 as well as MMP-1 and MMP-2. Our findings provide direct evidence that resistin causes endothelial dysfunction, which may be linked to obesity and cardiovascular disease.

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