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Angiogenic role of orexin-A via the activation of extracellular signal-regulated kinase in endothelial cells

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ABSTRACT

Orexin-A, a neuropeptide originally discovered in the hypothalamus, is found in peripheral organs, as well as in the central nervous system, and is involved in the regulation of food intake, energy homeostasis, and cardiovascular functions. In this study, we report that orexin-A induces *in vivo* neovascularization in a mouse Matrigel plug and *ex vivo* sprouting of endothelial cells in rat aortic rings. We also show that orexin-A increases migration and tube formation in human umbilical vein endothelial cells (HUVECs), and this effect is mediated by orexin receptors on endothelial cells. Moreover, orexin-A activates the extracellular signal-regulated kinase 1/2 (ERK1/2) in HUVECs, which is closely linked to angiogenic responses. The inhibition of ERK activation significantly suppresses orexin-A-stimulated endothelial angiogenesis. Taken together, our results indicate that orexin-A functions as a new proangiogenic peptide and requires MEK/ERK-dependent pathway for its angiogenic actions. These results suggest orexin-A and its receptor may act as important modulators of angiogenesis under pathophysiological conditions.

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1. Introduction

Angiogenesis, the formation of new blood vessels from a preexisting vasculature, plays a critical role in embryonic development, wound healing, and adult tissue regeneration [1,2]. Aberrant angiogenesis contributes to pathological processes, including chronic inflammation and tumor growth [3]. Under pathophysiological conditions, the angiogenic process is regulated by a variety of positive and negative regulators, including "classic" pro- and anti-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and thrombospondin-1 [4]. There is increasing evidence that many endogenous peptides such as neuropeptides play regulatory roles in endothelial angiogenesis [5].

Orexin-A and orexin-B, also known as hypocretins, are neuropeptides that are cleaved from a common precursor, prepro-orexin, and were first identified in the rat hypothalamus [6,7]. Orexins are known to be involved in the central regulation of sleep/wakefulness, feeding, and energy homeostasis [8–10]. Orexins mediate their effect by binding to G-protein coupled receptors, orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R). OX1R has a high affinity for orexin-A, whereas OX2R has similar affinities for both orexin-A and orexin-B [6]. Orexins and their receptors are found in not only the central nervous system but also the peripheral tissues such as the gastrointestinal tract, kidneys, reproductive tract, adipose tissue, and cardiovascular system [11]. Particularly, orexin-A has been detected in the plasma and its level varies depending on metabolic states [12–14]. More recently, central and peripheral effects of orexin-A on cardiovascular function have been extensively investigated [14–16]. The endothelium lining the vasculature is in direct contact with the plasma and plays key roles in angiogenesis and the short- and long-term regulation of the cardiovascular system [17]. However, the exact role of orexin-A in endothelial cells remains unknown.

In this study, we investigated the effects of orexin-A on angiogenesis *in vivo* and *in vitro* and determined its underlying molecular mechanism(s). Our study showed for the first time that orexin-A exerts angiogenic properties both *in vivo* and *ex vivo* and directly stimulates migration and tube formation in endothelial cells via the orexin receptors. We further found that orexin-A-induced angiogenic action is mediated by activating the MEK/ERK signaling pathway in endothelial cells.

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2. Materials and methods

2.1. Reagents

Orexin-A was purchased from Tocris. Antibodies for phospho-ERK and ERK were obtained from Cell Signaling. Human α-tubulin antibody was acquired from Biogenex. Rat CD31 (PECAM-1) antibody was supplied by BD Pharmingen. Antibodies to orexin-1 receptor and orexin-2 receptor were purchased from Alpha Diagnostic International. SB334867 and TCS-OX2-29 were purchased from Tocris. U0126 was purchased from A.G. Scientific Inc.

2.2. In vivo mouse Matrigel plug assay

C57/BL6 mice were subcutaneously injected with 0.5 mL of Matrigel (BD Bioscience) containing the indicated amounts of orexin-A with heparin (10 units/μL). After 7 days, the mice were killed, and the Matrigel plugs were recovered, fixed with 4% paraformaldehyde/phosphate-buffered saline, and embedded in paraffin. Each plug was fixed, sectioned, and stained with hematoxylin–eosin or Trichrome-Masson. The hemoglobin level was measured using the Drabkin method for quantifying blood vessel formation.

2.3. Intravital fluorescence microscopic analysis

Neovascularization was determined by intravital fluorescence microscopy as described previously [18,19]. 100 μL of Matrigel with or with orexin-A was injected into the inner space of window, which was surgically implanted between the skin and abdominal wall of male C57/BL6 (8 weeks old). After 6 days, microvasculature was recorded by a fluorescence intravital microscope (Carl Zeiss, Inc., Thornwood, NY) after intravenous injection dextran (molecular weight, 250,000) labeled with fluorescein isothiocyanate (FITC) via the tail vein.

2.4. Rat aortic ring sprouting assay

The aorta was excised from 6-week-old male Sprague–Dawley rats, and the fibroadipose tissue was removed. The aorta was sectioned into 1-mm-thick slices; the aorta rings were placed on Matrigel-coated wells, covered with an additional 50 μL Matrigel, and allowed to gel for 30 min at 37 °C. Orexin-A was added to the wells to a final volume of 200 μL of human endothelial cell media. Microvessel outgrowth was photographed under a phase contrast microscope. Quantitative analysis was used to count the number of microvessel outgrowth. The data reported are the mean ± SD from three different experiments carried out in triplicate.

2.5. Immunostaining of sprouted endothelial cells from rat aortic rings

Sprouted endothelial cells cultured on a coverglass were fixed with 10% formalin for 20 min, washed with phosphate-buffered saline (PBS) three times, treated with 0.2% Triton X-100 in PBS for 5 min at 4 °C, washed with PBS-T three times, and blocked with 5% normal goat serum. The cells were incubated with anti-PECAM-1 antibody, the primary antibody, at 4 °C overnight with gentle shaking. After overnight incubation, the cells were washed with PBS three times and incubated with Alexa 594-conjugated secondary antibodies for 1 h at room temperature. Coverglasses were mounted in Vectastain containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Confocal microscopy was performed using an Olympus FV-1000 laser-scanning microscope.

2.6. Cell culture

Primary human umbilical vein endothelial cells (HUVECs) (passage 5–8) were purchased from CLONETICS. HUVECs were plated onto a 0.2% gelatin-coated dish and grown in sterile endothelial growth medium (EGM-2; CLONTECTICS). EGM-2 consisted of endothelial basal media (EBM-2; CLONTECTICS), trace elements, growth factors, and antibiotics. The cells were grown at 37 °C in a humidified 95% air/5% CO_2 atmosphere. A human microvascular endothelial cell line (HMEC-1) was obtained from the Centers for Disease Control and Prevention (CDC) (Atlanta, GA).

2.7. Reverse transcription-polymerase chain reaction

Total RNA was isolated from the HUVECs using a TRIzol reagent kit (Invitrogen). cDNA synthesis was carried out using 2 μg of the total RNA by using a reverse transcription kit (Promega). The following oligonucleotide primers were used for polymerase chain reaction (PCR): β-actin: 5′-GACTACCTCATGAAGATC-3′ and 5′-GATCCACATCTGCTGGAA-3′; OX1R: 5′-TCATGGAATGCAGCAGTGTG-3′ and 5′-GACATTGAGGACGCTGATGG-3′; OX2R: 5′-TCCTGGTTTGTGT GGCAGTGT-3′ and 5′-AGGTGATGGTCACGAGCACAT-3′.

2.8. Chemotactic migration assay

Transwells with polycarbonate membrane inserts of pore size of 8 μm were coated with 10 μg gelatin. The HUVECs were suspended in EGM-2 media at a concentration of 1×10^5 cells/100 μL and added to the upper chamber. Orexin-A (100 nM) in EGM-2 media was added to the lower chamber. HUVECs migrating through the filter to the lower side were fixed by careful immersion of the filter into methanol for 1 min, followed by staining with hematoxylin/eosin solution and counting in three random fields per well. Each experiment was performed in duplicate, and three separate experiments were carried out for each group.

2.9. Tube formation assay

Growth factor-reduced Matrigel (300 μL) was pipetted into 24-well culture plates and polymerized for 30 min at 37 °C. HUVECs were seeded onto the surface of the Matrigel; orexin-A was then added, and the plates were incubated for 6 h at 37 °C. Morphological changes of the cells were observed under a microscope and photographed.

3. Results

3.1. Orexin-A stimulates angiogenesis in vivo and ex vivo

To investigate the *in vivo* angiogenic activity of orexin-A, we performed a mouse Matrigel plug assay. Matrigel with or without orexin-A was subcutaneously injected into male C57BL/6 mice and then excised from the mice at 7 days after implantation. As shown in Fig. 1A, orexin-A induced newly formed blood vessels inside the Matrigel. The number of functional vasculatures was quantified by measuring the hemoglobin content in the Matrigel plug. Hemoglobin levels of the Matrigel containing orexin-A were four times higher than those of the negative control gel. Histological analysis using hematoxylin–eosin or Trichrome-Masson staining showed that Matrigel in the plug containing orexin-A produced more neovessels than in the control gel (Fig. 1B). We also confirmed the *in vivo* angiogenic activity of orexin-A using fluorescence-based intravital microscopic analysis (Fig. 1C). However, orexin-B was unable to elicit an *in vivo* angiogenic response when used at equimolar concentrations of orexin-A which showed angiogenic

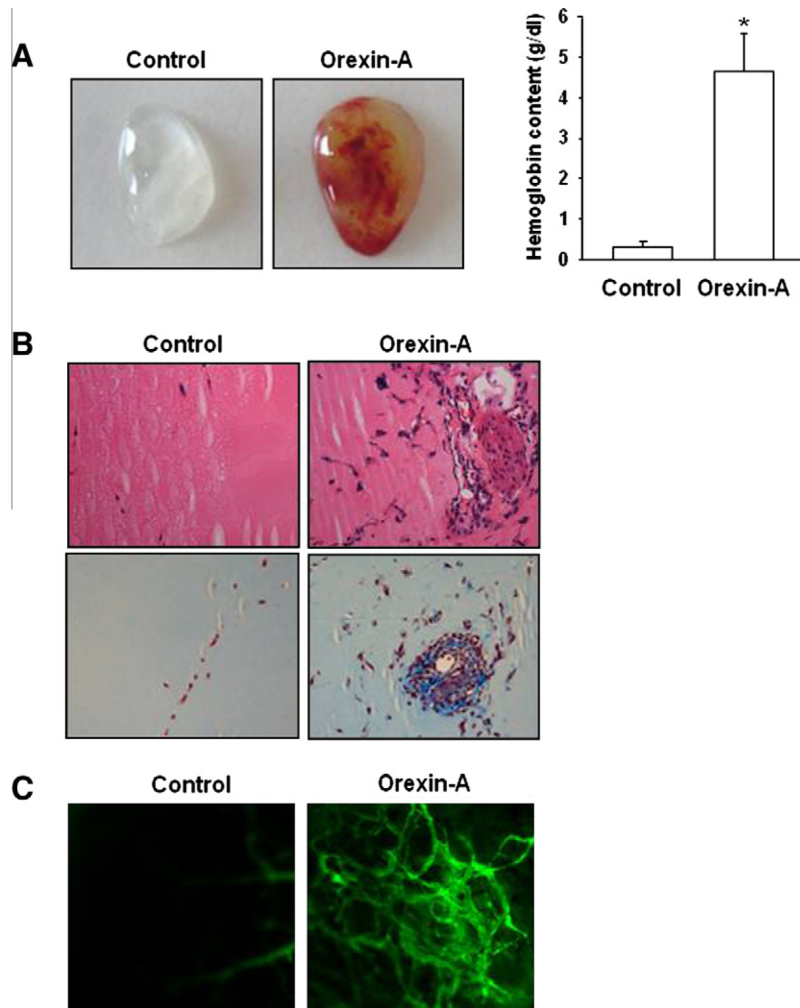

Fig. 1. Angiogenic activity of orexin-A in mouse Matrigel plug assay. (A) Matrigel plugs mixed with PBS or orexin-A (1 μg) were injected into mice. After 7 days, Matrigel plugs were removed and photographed (left). Hemoglobin concentration of the plugs was determined using a parallel assay with a known amount of hemoglobin (right). Each value represents the mean of at least five animals, and similar results were obtained in four different experiments. * $P < 0.05$, compared to the control. (B) Plugs were cross-sectioned and stained using hematoxylin–eosin (upper) or Trichrome-Masson (lower). A stained plug was photographed under a phase contrast microscope at 20× magnification. (C) Matrigel containing orexin-A was injected into the abdominal window of mice, which was surgically prepared. Neovessels within the Matrigel with or without orexin-A were visualized using an *in vivo* intravital microscope.

activity (Supplementary Fig. 1). We further investigated the angiogenic activity of orexin-A in an *ex vivo* angiogenesis model, a rat aortic ring sprouting assay. As shown in Fig. 2A, treatment with orexin-A increased the amount of microvessel outgrowth from the adventitia of the aortic rings in a dose-dependent manner. This was quantified by counting the number of sprouting microvessels (Fig. 2B). Immunostaining of PECAM-1 on outgrowths from the aortic explants treated with orexin-A supported the presence of endothelial cells (Fig. 2C).

3.2. Orexin-A increases chemotactic motility and tube formation in endothelial cells: the role of orexin receptors

To assess the angiogenic activity of orexin-A *in vitro*, *in vitro* angiogenesis assays were performed using HUVECs. Orexin-A mediates diverse cellular responses in many cell types by binding to two types of orexin-A receptors, OX1R and OX2R [6]. The presence of OX1R and OX2R on vascular endothelial cells has not yet been reported. In this report, we demonstrated that mRNAs and proteins of orexin-A receptors are present on both HUVECs and HMEC-1 (Fig. 3A). Amplification of OX1R and OX2R cDNA products was confirmed by DNA sequencing analysis. Next, we investigated the effect of orexin-A on chemotactic motility of HUVECs using a modified Boyden chamber assay. As shown in Fig. 3B, orexin-A enhanced the chemotactic motility of HUVECs by ∼2.5-fold relative to the untreated control cells. We then determined whether orexin-A stimulates the formation of capillary-like network of HUVECs on the Matrigel. Tube-like structures of HUVECs were quantified by measuring the number of tube branches and their area using an image analysis program. As shown in Fig. 3C, orexin-A stimulated tube formation in HUVECs by two-fold over the control. To elucidate whether orexin-A promotes angiogenesis by activating orexin receptors, we investigated the effect of orexin receptor antagonists. We used the OX1R-specific antagonist SB334867 and the OX2R-specific antagonist TCS-OX2-29 to examine orexin-A-induced endothelial angiogenesis. As shown in Fig. 3D, both SB334867 and TCS-OX2-29 effectively attenuated orexin-A-induced migration and tube formation in HUVECs. These results suggest that orexin-A induces essential angiogenic events, including endothelial migration and capillary tube formation, which are mediated by the activation of orexin receptors on endothelial cells.

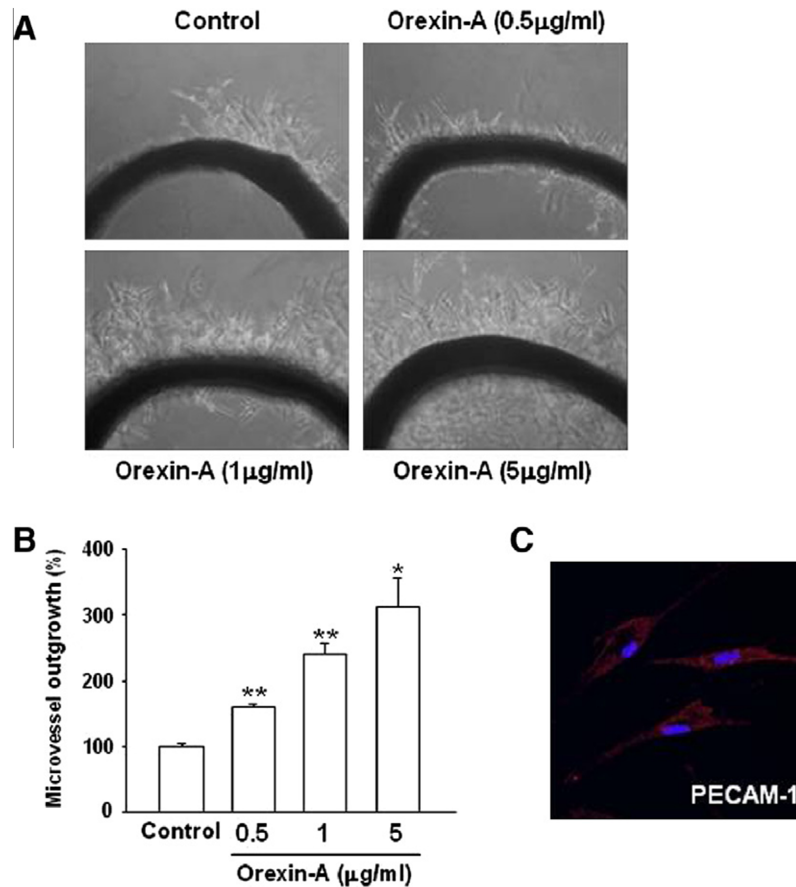

Fig. 2. Orexin-A induces microvessel sprouting *ex vivo*. (A) Rat aortic rings embedded in Matrigel were treated with the indicated concentrations of orexin-A. On day 4, representative microvessel outgrowths were photographed under a phase contrast microscope at 10× magnification. (B) The number of microvessels formed by the newly formed endothelial cell sprouting from the margin of aortic rings was counted. Each value represents the mean of at least three independent experiments. $^{**}P < 0.01$; $^{*}P < 0.05$ as compared to the control. (C) Orexin-A-treated aortic segments were removed from the Matrigel and immunostained with PECAM-1 (red) and DAPI (blue) and observed by confocal microscopy at 80× magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

3.3. Orexin-A-induced angiogenesis requires MEK/ERK-dependent signaling pathway

Several studies have shown that the activation of ERK is closely involved in the major steps of the endothelial angiogenic process, such as endothelial migration and tube formation [18–20]. Therefore, we determined whether orexin-A induces phosphorylation of ERK in HUVECs. Treatment of HUVECs with orexin-A increased the level of phosphorylated ERK1/2 (p44 ERK1 and p42 ERK2), with maximal activation after 5 min of orexin-A stimulation (Fig. 4A). As shown in Fig. 4B, orexin-A stimulated phosphorylation of ERK1/2 in endothelial cells in a dose-dependent manner. The MEK-specific inhibitor U0126 completely suppressed orexin-A-induced ERK1/2 phosphorylation (Fig. 4C), indicating that the phosphorylation of ERK1/2 was mediated by the activation of orexin-A-dependent MEK. Moreover, specific antagonists for OX1R and OX2R, SB334867 and TCS-OX2-29, respectively, efficiently blocked orexin-A-stimulated activation of ERK1/2 (Supplementary Fig. 2). Next, we investigated the functional involvement of ERK in orexin-A-induced angiogenesis. As shown in Fig. 4D, capillary-like tube formation induced by orexin-A was significantly blocked when orexin-A was applied to HUVECs with U0126. In addition, pretreatment of cells with U0126 efficiently suppressed orexin-A-induced microvessel sprouting in an *ex vivo* aortic ring model (Fig. 4E). Thus, these results suggested that ERK mediates the angiogenic response to orexin-A.

4. Discussion

Orexins were first isolated from the lateral hypothalamus and considered as central regulators of energy homeostasis and food intake. Recently, growing evidence suggests the presence of orexins and their receptors in peripheral tissues, but their precise roles in the periphery remain relatively unexplored [11,21]. Orexin-A is detectable in human and rat plasma, although the main source of plasma orexin-A remains to be identified [12,13,22]. Moreover, orexin-A, and not orexin-B, crosses the blood–brain barrier by simple diffusion [23]. Our RT-PCR, western blotting, and sequencing analysis data confirmed the presence of orexin receptors, OX1R and OX2R, in the vascular endothelial cells. We also found that exogenous orexin-A stimulates orexin receptor-mediated endothelial cell migration and tube formation *in vitro* and elicits *in vivo* and *ex vivo* angiogenic responses.

Several studies have shown that the activation of ERK1/2 in endothelial cells is considered as a proangiogenic event induced by many direct angiogenic factors [24]. We found that orexin-A induced ERK1/2 activation in HUVECs. In different cell types, binding of orexins to their G-protein-coupled receptors triggers the phosphorylation of ERK1/2 via the activation of multiple intracellular signaling pathways, including Ras, phosphatidylinositol-3 kinase, protein kinase C, Src, and calcium [25–27]. Engagement of orexin-A to the orexin-1 receptor, the heterodimeric Gq protein, triggers the exchange of GDP for GTP on the Gα subunit, followed by

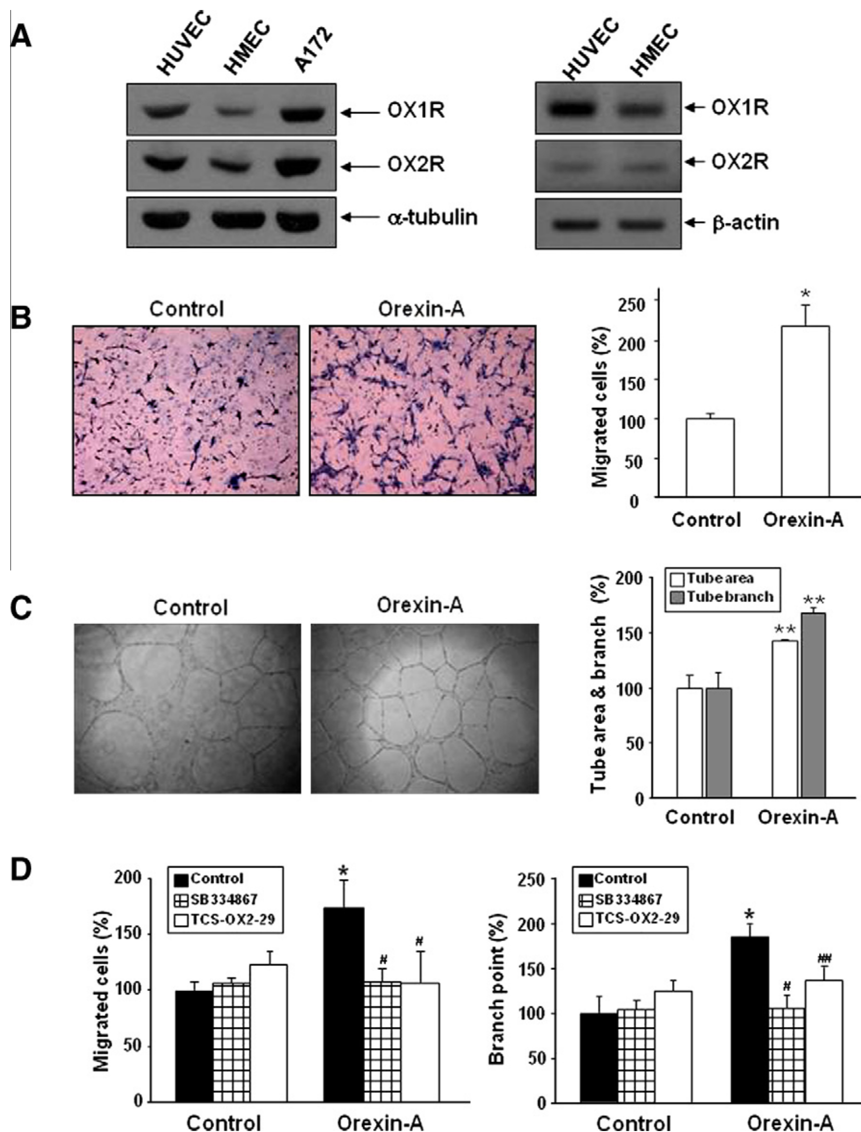

Fig. 3. Orexin-A induces migration and tube formation in HUVECs. (A) Protein expression levels of orexin receptors were confirmed by western blotting using anti-orexin-1 receptor (OX1R) and anti-orexin-2 receptor (OX2R) antibodies. α-tubulin served as the loading control. Human glioblastoma A172 cells have been reported to express high levels of OX1R and OX2R proteins (left). Total RNA was isolated, and then analyzed by RT-PCR using primers specific to human OX1R and OX2R. β-actin served as an internal control (right). (B) HUVECs were seeded into the upper chamber and incubated on Transwell chambers for 4 h in the absence or presence of orexin-A (100 nM). Migrated cells were stained with hematoxylin/eosin and photographed (left). The number of migrated cells was counted (right). $^{*}P < 0.05$ compared to the control. (C) HUVECs were incubated at 37 °C on growth factor reduced (GFR)-Matrigel without (control) or with orexin-A (100 nM) for 3 h. The tube area and tube branch number were counted. $^{**}P < 0.01$ compared to the control. (D) HUVECs were treated with orexin-A in the absence and presence of SB334867 (1 μM) or TCS-OX2-29 (1 μM) on Transwell. After 3 h, optical microscopy was used to count the cells that migrated to the lower side of the filter (left). HUVECs were seeded on Matrigel and pretreated for 30 min with SB334867 (1 μM) or TCS-OX2-29 (1 μM) before treatment with orexin-A. Capillary-like tube formation was observed, and photographs were taken after 3 h. The branch point number was counted (right). $^{*}P < 0.05$ vs. control; #$P < 0.05$ vs. orexin-A alone; ##$P < 0.2$ vs. orexin-A alone.

the dissociation of this subunit from the Gβγ dimer, which stimulates the Src-like enzyme to mediate the cellular response in cancer cell lines [28]. Activation of Src family kinases contributes to the stimulation of endothelial cell proliferation, migration, and vascular permeability [29]. In our preliminary study, we found that inhibition of Src with a specific inhibitor downregulates orexin-A-stimulated ERK activity in endothelial cells. Therefore, the activation of Src is probably required for the orexin-A-induced endothelial angiogenesis.

Many typical angiogenic molecules, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), are known to have gastroprotective and neuroprotective functions mediated by stimulating neovascularization [30,31]. Angiogenesis after cerebral ischemia facilitates the delivery of oxygen and nutrients to the ischemic zone and provides neurotrophic support for neurogenesis, leading to effective neurorestorative treatments [32]. The formation of a microvascular network is essential for gastrointestinal wound healing [33]. Recent studies indicate that orexin-A exerts gastroprotective actions against stress-induced gastric injury and provides neuroprotective effects in cerebral ischemia [34,35]. Further investigations are necessary to elucidate the role of orexin-A in therapeutic angiogenesis for regenerative treatments of brain ischemia and gastric damage.

In general, the effect of proangiogenic peptides on endothelial angiogenesis is mediated by either direct action on endothelial cells or indirectly via the induction of other angiogenic promoters [5]. We showed that orexin-A directly stimulates chemotactic

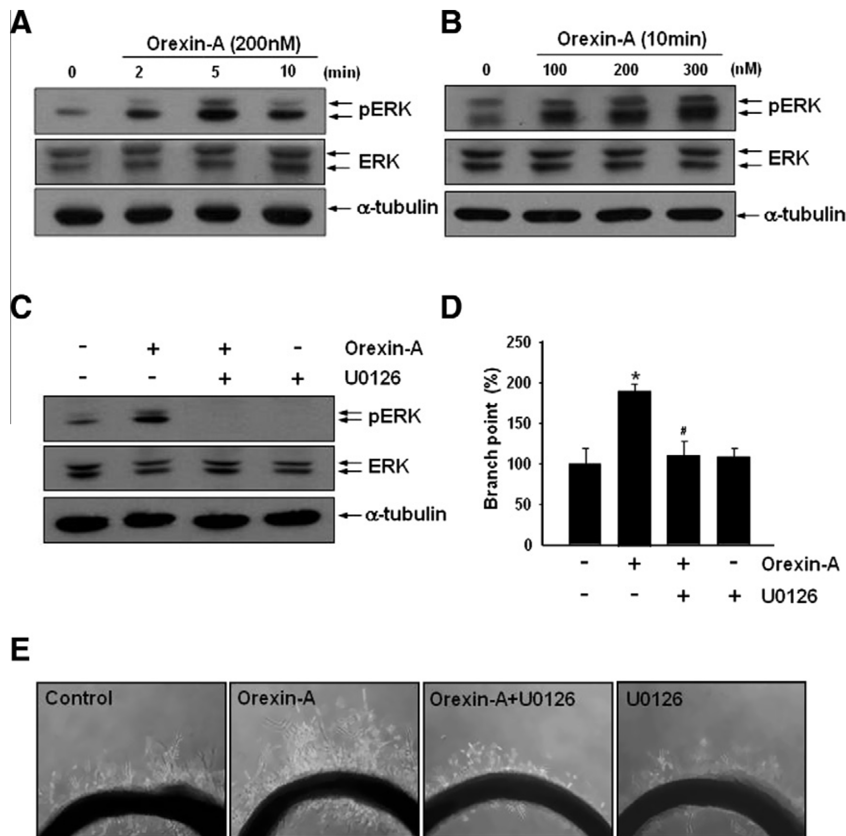

Fig. 4. Involvement of MEK/ERK pathway in orexin-A-induced angiogenesis. (A) HUVECs were incubated with orexin-A (200 nM) for the indicated times. (B) HUVECs were treated with various concentrations of orexin-A (100, 200, and 300 nM) for 10 min. (C) HUVECs were preincubated with U0126 (10 μM) for 30 min, and then incubated with orexin-A (200 nM). Protein levels were measured by Western blot analysis using anti-phosphorylated ERK, anti-ERK, and anti-β-tubulin antibodies. (D) HUVECs were plated onto GFR-Matrigel. HUVECs were pretreated with U0126 (10 μM), and then incubated with orexin-A for 3 h. Capillary-like tube formation was assessed using a phase contrast microscope. The branch point number was counted. *$P < 0.05$ vs. control; #$P < 0.05$ vs. orexin-A alone. (E) Rat aortic rings were embedded in Matrigel. Rat aortic rings in the presence or absence of U0126 (10 μM) were cultured with orexin-A (1 μg/mL). After 4 days, the outgrowth of microvessels was photographed under the microscope. Results shown are representative of at least three independent experiments.

migration and tubular morphogenesis of endothelial cells. Orexin-A was previously shown to induce the activation of hypoxia-inducible factor-1 (HIF-1), a major transcriptional factor that activates many genes involved in angiogenesis, in OX1R-overexpressing HEK293 cells and hypothalamic neurons [36]. Therefore, we investigated whether orexin-A influences the expression of VEGF, a potent HIF-1 target angiogenic factor in endothelial cells, but it had no effect on VEGF expression level in HUVECs (data not shown). Future studies are necessary to determine whether orexin-A has an indirect angiogenic effect on vascular endothelial cells mediated in part by other angiogenic factors produced by nonendothelial cells.

In summary, our findings provide the first evidence that orexin-A exerts *in vitro* and *in vivo* angiogenic activity via the orexin receptor-mediated MEK/ERK1/2 signaling pathway. These results not only increase the understanding of the novel orexin-A function in the regulation of physiological and pathological angiogenesis but also provide an important therapeutic strategy for angiogenesis-related diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.10.115.

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