Nerve growth factor is critical requirement for in vitro angiogenesis in gastric endothelial cells

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Ahluwalia A, Jones MK, Brzozowski T, Tarnawski AS. Nerve growth factor is critical requirement for in vitro angiogenesis in gastric endothelial cells. Am J Physiol Gastrointest Liver Physiol 311: G981–G987, 2016. First published October 13, 2016; doi:10.1152/ajpgi.00334.2016.—Angiogenesis is critical for the healing of gastric mucosal injury and is considered to be primarily regulated by vascular endothelial growth factor (VEGF), the fundamental proangiogenic factor. The role of nerve growth factor (NGF) in gastric angiogenesis is unknown. We examined the expression of NGF and its TrkA receptor in endothelial cells (ECs) isolated from gastric mucosa of rats (GMECs), the effect of NGF treatment on in vitro angiogenesis in GMECs, and, the mechanisms underlying NGF’s proangiogenic actions. Isolated GMECs from Fisher rats were treated with vehicle, NGF (10–1,000 ng/ml), VEGF (20 ng/ml), or NGF+VEGF. To determine whether and to what extent NGF is critical for angiogenesis in GMECs, we silenced NGF expression using specific siRNA and examined in vitro angiogenesis with and without treatment with exogenous NGF and/or VEGF. Treatment of GMECs with NGF significantly increased in vitro angiogenesis similar to that seen in GMECs treated with VEGF. Silencing of NGF in GMECs abolished angiogenesis, and this effect was reversed only by exogenous NGF but not VEGF, which indicates a direct proangiogenic action of NGF on GMECs that is, at least in part, distinct and independent of VEGF. NGF’s proangiogenic action on GMECs was mediated via PI3-K/Akt signaling. This study showed for the first time that gastric mucosal ECs express NGF and its receptor TrkA and that NGF is critical for angiogenesis in these cells.

endothelial cells; nerve growth factor; angiogenesis; PI3-K/Akt

NEW & NOTEWORTHY

Angiogenesis—new blood vessel formation—is critical for gastric mucosal injury healing. The endothelial cells lining blood vessels are the targets and effectors of angiogenesis, and vascular endothelial growth factor is recognized as the main regulator. The role of nerve growth factor (NGF) in gastric angiogenesis is unknown. Our study uncovered a critical role of NGF in gastric angiogenesis and identified some of the underlying mechanisms.

ANGIOGENESIS—NEW BLOOD VESSEL FORMATION—is sine qua non for tissue growth and healing (7, 15–17, 21, 22) in general and is critical for maintaining integrity and healing of gastric mucosa. The main targets and effectors of angiogenesis are the endothelial cells (ECs) that line blood vessels, are integral components of all tissues, and are essential for the delivery of oxygen and nutrients. ECs carry genetic information to proliferate and form tubes, branches, anastomoses, and a capillary network (7, 17, 21). Under normal physiological conditions, this genetic information is suppressed in most tissues, and ECs within microvessels remain quiescent (resting phenotype), and their turnover rate is very low (7, 17, 21). In certain situations (e.g., gastric injury healing), the resting phenotype of ECs is changed to the angiogenic phenotype (2, 3).

Previous studies have demonstrated the important role of vascular endothelial growth factor (VEGF, recognized as a fundamental regulator of angiogenesis) in all essential steps of angiogenesis: EC migration (24), tube and lumen formation (2, 37), and proliferation (28). Our previous studies identified the cellular, molecular, and ultrastructural features of angiogenesis in gastric mucosa, its dependence on VEGF, and its impairment in aging (2, 3, 22, 23).

NGF is an evolutionarily conserved peptide, which is crucial for the growth, survival, and maintenance of sensory and sympathetic neurons, septal cholinergic neurons in the brain, and neurite outgrowth (18, 29–31). NGF binding to its high-affinity tropomyosin-related kinase (TrkA) receptor signals neuroprotective and repair functions in physiological and pathological conditions of the nervous system (9, 29, 30). Although the actions of NGF were originally defined with respect to the central and peripheral nervous systems, recent data indicate that NGF also exerts actions on nonneuronal cells and that in addition to neural cells, other cell types, including fibroblasts, epithelial cells, and ECs produce NGF (5, 26).

Limited studies have indicated a possible role for NGF in angiogenesis of regenerating adult thymus (33), cultured brain capillary ECs (32), and human umbilical vein endothelial cells (HUVECs) (5). However, NGF’s proangiogenic action on ECs is not universal to all vascular systems. For example, NGF stimulates migration and proliferation of human choroidal endothelial cells (ECs), but not retinal ECs (36). Because the role of NGF in gastric angiogenesis is unknown, we examined the expression of NGF and its TrkA receptor in ECs isolated from gastric mucosa of rats (GMECs), the effect of NGF treatment on angiogenesis and proliferation of GMECs, and the molecular mechanisms underlying NGF’s proangiogenic actions. To determine whether and to what extent NGF is critical for angiogenesis in GMECs, we silenced NGF expression using specific siRNA and examined in vitro angiogenesis with and without treatment with exogenous NGF and/or VEGF.

This study showed for the first time that GMECs express NGF and its receptor TrkA and that NGF is critical for these cells’ angiogenesis. Treatment of GMECs with NGF significantly increased in vitro angiogenesis, and this NGF action was mediated via PI3-K/Akt signaling. The essential role of NGF in
gastric angiogenesis was clearly demonstrated by the fact that silencing NGF in GMECs using specific siRNAs abolished angiogenesis, and this effect was reversed only by exogenous NGF but not VEGF.

**MATERIALS AND METHODS**

All experimental studies in rats were approved by the institutional animal review committee: Subcommittee for Animal Studies (Institutional Animal Care and Use Committee) of the VA Long Beach Healthcare System, Long Beach, California.

**Isolation of GMECs.** GMECs were isolated from Fisher F-344 rats (purchased from the National Institute on Aging, Bethesda, MD) at 3 mo of age using platelet endothelial cell adhesion molecule 1 (CD31) selection and magnetic bead separation, as described previously (3). ECs were identified by positive staining for CD31, Factor VIII-related antigen, and VEGF-receptor 2, as well as by the absence of staining for the myofibroblast marker, smooth muscle α-actin. Endothelial cells were grown on collagen-coated dishes in endothelial cell growth media containing 20% FBS, heparin, and endothelial cell growth supplements. Culture dishes were coated with rat tail collagen type 1 diluted to 50 μg/ml in 0.02 M acetic acid (6 μg/cm² surface area) at 37°C for 30 min, and the remaining collagen solution was removed. Collagen-coated dishes were washed with PBS and used for culture of GMECs. For some studies, ECs were treated with specific PI-3 kinase inhibitor LY294002 (50 μM for 30 min; Cell Signaling Technology, Danvers, MA), recombinant rat NGF (10-1,000 ng/ml; R&D Systems, Minneapolis, MN), or recombinant rat VEGF (20 ng/ml; R&D Systems). LY294002 was dissolved in DMSO, which also served as a vehicle control where appropriate.

**In vitro angiogenesis assay.** Endothelial tube formation on growth factor-reduced Matrigel was determined using in vitro angiogenesis assay similar to our previous studies (3, 23). ECs were grown in complete growth media in 60-mm tissue culture dishes, until they were about 80% confluent. The growth medium was replaced with basal medium supplemented with 1% FBS and antibiotics, and the cells were incubated for 18 more hours. The cells were then trypsinized, counted, resuspended in basal medium supplemented with 1% FBS and antibiotics, and the number of BrdU-positive cells were determined in five randomly selected fields for each well under ×200 magnification.

**GMEC cell proliferation.** Cell proliferation was determined by bromodeoxyuridine (BrdU) assay using a commercially available kit (EMD Millipore, Billerica, MA), according to the manufacturer’s instructions. The total cell count and number of BrdU-positive cells were determined in five randomly selected fields. Cell proliferation was expressed as percent of BrdU-positive cells.

**Reverse transcription real-time quantitative PCR.** Total cellular RNA was isolated from GMECs using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA (1 μg) was treated with deoxyribonuclease I and reverse transcribed using the GeneAmp RNA-PCR kit (Applied Biosystems, Foster City, CA), as described in our previous studies (3, 37). Real-time quantitative PCR was performed using prevalidated QuantiTect assays for NGF, TrkA, and β-actin (Qiagen, Valencia, CA) and the iCycler real-time PCR detection system (Bio-Rad, Hercules, CA). For negative controls, we used no template control and no reverse transcriptase controls, which did not show any detectable mRNA expression in the RT-PCR assays. Relative mRNA levels were calculated using the 2-ΔΔCt method and normalized to β-actin.

**Western blot analysis.** Proteins were isolated from GMECs using standard RIPA buffer supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL). Total cellular proteins (50 μg) were resolved on premade 10% Tris glycine gels (Bio-Rad, Hercules, CA) and electrophroblotted onto Immobilon polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA), as described in our previous studies (1, 3, 37). Western blot analysis was performed to examine the expression of NGF, TrkA, pTrkA, Akt, and pAkt proteins. Immunoblots were blocked with Odyssey blocking solution (LI-COR Bioscience, Lincoln, NE) followed by overnight incubation with primary antibodies. β-actin was used as a reference. Antibodies used for these studies were NGF (1:250, sc 548; Santa Cruz Biotechnology, Santa Cruz, CA), TrkA (1:500, sc 2056; Cell Signaling Technology), pTrkA (1:250, no. 9141, Cell Signaling Technology), phosphorylated Akt (pAkt, 1:500, no. 9271, Cell Signaling Technology), and Akt (1:1,000, no. 9272; Cell Signaling Technology) or β-actin (1:1,000; Sigma, St. Louis, MO). Immunoreactive bands were detected by using goat anti-rabbit IR Dye 800 secondary antibodies for NGF, TrkA, pTrkA, Akt, and pAkt (1:20,000, LI-COR Bioscience, Lincoln, NE), and goat anti-mouse IRDye-680 secondary antibodies for β-actin (1:20,000; LI-COR Bioscience). Signals were detected using the Odyssey infrared imaging system (LI-COR Bioscience). Immunostaining. Protein expression and localization in GMECs were analyzed by immunostaining, as previously described (3, 4).
Briefly, GMECs were cultured on collagen-coated coverslips, fixed in paraformaldehyde, and then permeabilized with methanol. The expression of NGF and TrkA in GMECs was examined by immunofluorescence staining with specific antibodies for NGF (sc 548; Santa Cruz Biotechnology) and (sc 118; Santa Cruz Biotechnology). GMECs cells stained in the absence of primary antibody were used as negative controls. The staining signal intensity was quantified using MetaMorph 7.0 (Molecular Devices, Downingtown, PA).

NGF silencing. GMECs were treated with either nonsilencing scrambled control or NGF-specific siRNA (100 nM; Qiagen, CA) for 72 h using methods similar to those described in our previous study (3). Transfected GMECs were used to study effects of NGF silencing on in vitro angiogenesis and cell proliferation. The siRNA transfection efficiency in GMECs was determined using incorporation of Alexa Fluor 488-labeled siRNA and was 85%.

Statistical analysis. Data are presented as means ± SD. Statistical significance was analyzed by either Student’s t-test to compare data between two groups, one-way ANOVA with Tukey’s multiple-comparison post-hoc testing for evaluating differences between multiple groups, or Pearson’s correlation using Prism (GraphPad Software, La Jolla, CA). A P value of < 0.05 was considered statistically significant.

RESULTS

Gastric mucosal ECs express NGF and TrkA. Gastric mucosal endothelial cells (GMECs) isolated from rat stomach expressed NGF and its high-affinity TrkA receptor mRNAs and proteins, as determined by real-time RT-PCR (Fig. 1A) and Western blot analysis (Fig. 1B). Immunostaining using specific NGF and TrkA antibodies showed strong expression of these proteins’ signals and their cellular localization in GMECs (Fig. 1C).

NGF stimulates in vitro angiogenesis and cell proliferation in gastric mucosal endothelial cells. NGF dose dependently stimulated in vitro angiogenesis in gastric mucosal endothelial cells (GMECs), and the maximal threshold dose of NGF was 100 ng/ml (Fig. 2). We also compared the ability of exogenous NGF and VEGF to stimulate angiogenesis in GMECs. Treatment of GMECs with NGF 100 ng/ml or VEGF 20 ng/ml significantly increased angiogenesis by ~1.4-fold (all P < 0.001) vs. PBS control (Fig. 3A). Concomitant treatment with NGF plus VEGF increased angiogenesis significantly more than either NGF or VEGF alone (Fig. 3A), demonstrating the additive effects of NGF and VEGF on in vitro angiogenesis in GMECs. NGF treatment significantly increased proliferation of GMECs (P < 0.001) (Fig. 3B).

NGF treatment of GMECs increases phosphorylation of TrkA, stimulates phosphorylation of Akt, and promotes in vitro angiogenesis by a mechanism involving PI3 kinase. Treatment of GMECs with exogenous NGF increased phosphorylation of TrkA protein (Fig. 4). NGF treatment also induced formation of lamellipodia and filopodia (Fig. 5), which are characteristic features of angiogenic phenotype of ECs (11, 19, 20).

We next examined whether NGF mediates its angiogenic action on GMECs through a PI3-K/Akt signaling. To determine the extent to which PI3-K signaling is critical for NGF-induced angiogenesis, we treated GMECs with PBS (solvent; control) or recombinant rat NGF (100 ng/ml) in the presence of DMSO (solvent; control) or the specific PI3 kinase inhibitor, LY294002 (50 µM). Treatment of GMECs with exogenous NGF resulted in a significant increase in Akt phosphorylation (Fig. 6A). Treatment with LY294002 significantly reduced phosphorylation of Akt in GMECs (Fig. 6A). Treatment with exogenous NGF significantly increased in vitro angiogenesis in...
NGF treatment of GMECs increases TrkA phosphorylation. A: representative Western blots for pTrkA, TrkA, and β-actin expression in GMECs treated with exogenous NGF (100 ng/ml) for 4 h. Values are expressed as means ± SD of three independent experiments performed in duplicate (n = 6). Treatment of GMECs with exogenous NGF increased phosphorylation of TrkA receptor. *P < 0.01.

Fig. 4. NGF treatment of GMECs increases TrkA phosphorylation. A: representative Western blots for pTrkA, TrkA, and β-actin expression in GMECs treated with exogenous NGF (100 ng/ml) for 4 h. Values are expressed as means ± SD of three independent experiments performed in duplicate (n = 6). Treatment of GMECs with exogenous NGF increased phosphorylation of TrkA receptor. *P < 0.01.

NGF treatment of GMECs increases lamellipodia and filopodia formation. Immunostaining is shown for NGF in GMECs cultured on collagen-coated coverslips in response to NGF (100 ng/ml) treatment for 4 h. NGF treatment induced formation of lamellipodia (arrowheads) and filopodia (arrows) in GMECs.

Fig. 5. NGF treatment of GMECs increases lamellipodia and filopodia formation. Immunostaining is shown for NGF in GMECs cultured on collagen-coated coverslips in response to NGF (100 ng/ml) treatment for 4 h. NGF treatment induced formation of lamellipodia (arrowheads) and filopodia (arrows) in GMECs.

GMECs, and this action of NGF was completely abolished by pretreatment of these cells with LY294002 (Fig. 6B).

Silencing of NGF expression inhibits in vitro angiogenesis and cell proliferation in GMECs. To determine whether and to what extent NGF is critical for angiogenesis in GMECs, we silenced NGF expression in these cells using specific NGF siRNA. Silencing NGF expression with specific NGF siRNA (but not a nonsilencing scrambled control RNA) in GMECs reduced NGF protein expression by 2.3-fold (Fig. 7A). NGF silencing reduced in vitro angiogenesis by 2.6-fold in GMECs, and this reduction was reversed by exogenous NGF, but not by VEGF (Fig. 7B). Silencing NGF expression also significantly reduced proliferation of GMECs by 76% (P < 0.001), and this reduction was reversed by exogenous NGF but not by exogenous VEGF (Fig. 7C).

DISCUSSION

Our present study showed for the first time that GMECs isolated from rat stomachs strongly express NGF and its TrkA receptor mRNAs and proteins. It also showed that treatment with NGF significantly increases in vitro angiogenesis in these cells and that concomitant treatment of GMECs with exogenous NGF and VEGF increased in vitro angiogenesis significantly more than either NGF or VEGF alone. NGF also stimulated proliferation of GMECs. Furthermore, this study demonstrated that NGF treatment increases phosphorylation of TrkA receptor. Serum NGF concentration in healthy humans is 19.68 pg/ml with an interquartile range of 11.06–41.74 pg/ml (25). While the concentration of NGF used in our study exceeds that reported in serum, it should be pointed out that NGF is also generated locally by ECs, neuronal cells, and epithelial cells, and that NGF is also released during tissue injury that triggers angiogenesis.

The critical role of NGF in in vitro angiogenesis in GMECs was clearly demonstrated in this study by the fact that NGF silencing in GMECs with specific siRNA abolished in vitro angiogenesis. This abolishment was reversed by exogenous NGF and not by exogenous VEGF treatment, which indicates a direct proangiogenic action of NGF on GMECs that is at least, in part, distinct and independent of VEGF.

Our previous studies demonstrated the sequential steps of angiogenesis in vivo in gastric mucosa in response to injury and in vitro in cultured gastric mucosal ECs (2, 3, 22, 23). We demonstrated EC migration, endothelial tube and lumen formation, and the regulation of these processes by VEGF (2, 3, 22, 23). Endothelial tube and lumen formation in angiogenesis studies in vitro have direct relevance to in vivo angiogenesis in gastric mucosa, where endothelial lumen formation is an important feature of angiogenesis, as shown in our recent paper.
Limited studies indicate that NGF can stimulate angiogenesis in some ECs, e.g., HUVECs and brain capillary ECs (5, 27). However, NGF’s proangiogenic action on ECs is not universal to all vascular systems, e.g., NGF stimulates migration and proliferation of human choroidal ECs, but not retinal ECs (36).

Our present study showed that the NGF-induced angiogenesis was dependent on PI3-K/Akt signaling pathway. Treatment with exogenous NGF significantly increased in vitro angiogenesis in GMECs, and this action of NGF was completely abolished by pretreatment of these cells with LY294002, a specific PI3 kinase inhibitor. We focused in this paper on PI3-K/Akt signaling since the activation of this pathway appears to be important for angiogenesis (12, 34). We previously demonstrated the critical role of MAPK/Erk1/2.
signaling in angiogenesis (23) and are planning to explore that and other signaling pathways in future studies.

The formation of filopodia is an important step in endothelial tube formation and branching in angiogenesis (8, 11, 20, 24, 38). Previous studies demonstrated that VEGF-induced filopodia formation in branching vessels during angiogenesis (19, 35). This study showing that NGF stimulates lamellipodia and filopodia formation in GMECs is the first demonstration that NGF induces an angiogenic phenotype in ECs.

The findings of our current study may have important mechanistic implications for our understanding of EC biology and angiogenesis and the specific role of NGF. Dysregulation of angiogenesis underlies various pathological conditions. Reduced angiogenesis results in inadequate revascularization and delayed healing of a variety of injured or diseased tissues, including stomach (2, 7, 13, 14). Conversely, increased, abnormal angiogenesis is encountered in pathological conditions, such as inflammatory bowel disease (10) and cancer (6). This study has potential therapeutic implications. NGF therapy can be used for stimulating angiogenesis, while NGF siRNA or specific inhibitors of NGF signaling will likely inhibit pathologic angiogenesis.

REFERENCES


34. Park MJ, Kwak HJ, Lee HC, Yoo DH, Park IC, Kim MS, Lee SH, Rhee CH, Hong SI. Nerve growth factor induces endothelial cell invasion and cord formation by promoting matrix metalloproteinase-2 expression.


