Endothelium

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713617829

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Online Publication Date: 01 March 2006

To cite this Article Lazarovici, Philip, Gazit, Aviv, Staniszewska, Izabela, Marcinkiewicz, Cezary and Lelkes, Peter I.(2006)'Nerve Growth Factor (NGF) Promotes Angiogenesis in the Quail Chorioallantoic Membrane',Endothelium,13:1,51 — 59

To link to this Article: DOI: 10.1080/10623320600669053

URL: http://dx.doi.org/10.1080/10623320600669053

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Nerve Growth Factor (NGF) Promotes Angiogenesis in the Quail Chorioallantoic Membrane

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Angiogenesis, the formation of new blood vessels, is tightly regulated by growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). The authors hypothesize that nerve growth factor (NGF), a well known neurotrophin, may play a direct angiogenic role. To test this hypothesis, the authors measured the effects of NGF on the natural vascularization of the quail chorioallantoic membrane (CAM). The angiogenic effect of NGF was compared to that of human recombinant VEGF165 (rhVEGF) and basic FGF (rhbFGF). In comparison to phosphate-buffered saline–treated controls, NGFs from different biological sources (mouse, viper, and cobra) increased the rate of angiogenesis in a dose-dependent fashion from 0.5 to 5 µg. For quantitative morphometry, grayscale images of the blood vessels end points of the CAM arteries were binarized for visualization and skeletonized for quantization by fractal analysis. In mouse NGF-treated embryos the fractal dimension (Df), indicative of arterial vessel length and density, increased to 1.266 ± 0.021 compared to 1.131 ± 0.018 (p < .001) for control embryos. This effect was similar to that of 0.5 µg rhVEGF (1.290 ± 0.021, p < .001) and 1.5 µg rhbFGF (1.264 ± 0.028, p < .001). The mouse NGF–induced angiogenic effect was blocked by 1 µM K252a (1.149 ± 0.018, p < .001), an antagonist of the NGF/trkA receptor, but not by 1 µM SU-5416 (1.263 ± 0.029, p < .001), the VEGF/Fk1 receptor antagonist, indicating a direct, selective angiogenic effect of NGF via quail embryo trkA receptor activation. These results confirm previous observations that NGF has angiogenic activity and suggest that this neurotrophin may also play an important role in the cardiovascular system, besides its well-known effects in the nervous system. The angiogenic properties of NGF may be beneficial in engineering new blood vessels and for developing novel antiangiogenesis therapies for cancer.

Keywords  bFGF, CAM Assay, K252a, NGF, SU-5416, VEGF

Received 25 February 2006; accepted 15 March 2006.

This study was supported by grants from the Space Administration (NASA NNN04HC81G-01 to PIL), the Nanotechnology Institute of Southeastern Pennsylvania (NTI to PIL), the Stein Family Foundation (PIL and PL), and the Calhoun Endowment (PIL). PL is affiliated with and supported in part by the David R. Bloom Center for Pharmacy at the Hebrew University of Jerusalem.

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Angiogenesis, the generation of capillaries from existing blood vessels, is a tightly controlled biological process driven largely by endothelial cell proliferation. Current in vivo angiogenesis models, such as the quail chorioallantoic membrane (CAM), allow for the assessment of both stimulation of angiogenesis by angiogenic growth factors and inhibition by antiangiogenic molecules (Gonzalez-Iriarte et al. 2003; Parsons-Wingerter et al. 1998). The concept that such chemicals could be used to induce therapeutic vessel growth in ischemic cardiovascular diseases (Freedman and Isner 2001), or inhibit vessel growth in pathological tumor angiogenesis (Folkman 2004), has generated intensive experimental and clinical studies. Amongst the best characterized proangiogenic growth factors are members of the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) families. In the quail, four VEGF isoforms (Finkelstein and Poole 2003a) and the VEGF receptor 2 (flk-1) are expressed during both vasculogenesis and
angiogenesis (Flamme et al. 1995). Exogenous delivery of human recombinant VEGF165 resulted in enhanced angiogenesis and, with that, in significant changes in the vascular pattern of the embryo (Finkelstein and Poole 2003b). Similarly, basic FGF (bFGF) and its receptors have been detected in quail embryos (Cox and Poole 2000; Sarkar et al. 2001). Exogenous application of bFGF in the quail CAM assay selectively stimulated arteriogenesis (Parsons-Wingerter et al. 1998, 2000). In view of the large number of stimulatory and inhibitory growth factors involved in regulation of angiogenesis processes (Carmeliet 2000), characterization of potential novel angiogenic factors constitutes a priority area of active investigation.

Nerve growth factor (NGF) is a member of the neurotrophin family of growth factors that induce survival, proliferation, differentiation, and neuroprotective effects on sensory, sympathetic, and certain cholinergic neurons in the nervous system (Sofroniew et al. 2001). A large number of NGFs have been discovered from different species and tissues, characterized by an evolutionary conserved consensus sequence (Jaaro et al. 2001), which, depending on the species, may differ in 40 out of 118 amino acids of their β-chain, the chain that is solely responsible for the neurotrophic activity of NGF. The majority of research on NGF has been performed using NGF isolated from the male mouse submaxillary gland (NCBI, NP38637). Mouse NGF is very similar in its biological activities to human NGF (NCBI, AAA59931) (Ulrich et al. 1983). Recent studies indicate that in addition to its neurotrophic property, NGF also promotes endothelial cell proliferation (Jiang et al. 1997) and migration (Dolle et al. 2005) in vitro, and angiogenesis in the chicken CAM assay (Cantarella et al. 2002), suggesting that this neurotrophin may have complementary functions as a dual and angiogenic growth factor (Lazarovici et al. 2006).

The biological activities of NGF are mediated by two receptors, a low-affinity p75 NTR and the high-affinity tyrosine kinase, trkA (Kaplan and Miller 2000). An important tool for investigating the biological actions of NGF is K252a, a relative selective trkA antagonist, which has been widely used to distinguish specific effects of NGF in a variety of physiological systems (Koizumi et al. 1988; Lazarovici et al. 1997).

In the present study we provide the first quantitative analysis of the angiogenic properties of NGF using the quail CAM model of angiogenesis (Henry et al. 1998; Richardson and Singh 2003) and comparing the angiogenic effects of the various NGFs to those of VEGF165 and bFGF. Using different NGF agonists and selective antagonists of flk-1 and trkA receptors, we provide evidence that NGF directly and selectively stimulates angiogenesis of small capillaries in the CAM arterial tree.

**MATERIALS AND METHODS**

**Growth Factors**

Recombinant human vascular endothelial growth factor (rhVEGF165) was purchased from Sigma (St. Louis, MO). Recombinant human basic fibroblast growth factor (rhbFGF) was kindly provided by Cytolab (Rehovot, Israel). Recombinant human nerve growth factor (rhNGF) and mouse nerve growth factor (2.5S-mNGF) were kindly supplied by Alomone Labs (Jerusalem, Israel). Viper nerve growth factor (vNGF–β) and cobra nerve growth factor (cNGF) were purified in the laboratory from the venom of *Vipera russelli russelli* and *Naja naja kaouthia*, respectively, as previously described (Hayashi et al. 1996; Katzir et al. 2003). During the present study, the neurotrophic activities of the different NGFs were routinely validated using the PC12 bioassay (Katzir et al. 2003). The growth factors were stored, lyophilized, and prepared in phosphate-buffered saline (PBS) before the start of experiment.

**Drugs**

The high-affinity NGF-receptor (trkA) antagonist K-252a, (8R*, 9S*, 11S*)-(-)-(1-)-9-hydroxy-9-methoxy carbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo(a,g)cycloocta (c,d,e)trindene-1-one, an alkaloid isolated from the culture broth of *Nocardiosis sp.*, was a gift from either Kyowa Hakko Kogyo (Tokyo, Japan) or Dr. Yosef Behrend (Fermentek, Jerusalem, Israel) (both batches were 99% pure as analyzed by thin-layer chromatography [TLC], high-performance liquid chromatography [HPLC] and mass spectroscopy methods). The trkA inhibitory activity of K252a was estimated in the PC12 bioassay (Katzir et al. 2003). The selective VEGF receptor (flk-1/KDR) antagonist SU-5416, 3-[(2,4-dimethylpyrrol-5-yl)methylidenyl]-indolin-2-one, was synthesized according to a published procedure from commercially available 3,5-dimethylpyrrol-2-carbaldehyde by aldocondensation with indolin-2-one in ethanol in the presence of piperidine (Sun et al. 1998). Analytical and functional characterizations were performed by TLC, ultraviolet-visible (UV-VIS) spectroscopy, nuclear magnetic resonance, and flk kinase assay (Strawn et al. 1996). Drugs were dissolved in DMSO at stock concentrations of 1 mM (K252a) and 10 mM (SU-5416), aliquoted, and stored in the dark at −20°C. Working solutions of the drugs in experimental media were prepared fresh, before the start of the experiment. Control experiments were treated with vehicle only.

**Quail Embryo Cultures**

For the experiments described in this article, several batches of fertilized eggs of Japanese quail (*Coturnix coturnix japonica*) were purchased from Boyd Bird (Pullman, Washington). All eggs were cleaned, sterilized with 70% ethanol, and maintained in an incubator at 37°C and 100% relative humidity for 3 days in a horizontal position, which allows the embryos to reorient near the middle of the egg, along the horizontal axis. All subsequent manipulations were performed in a heated, sterile laminar flow hood. At embryonic day 3 (E3), the eggs were wiped lightly with diluted iodine solution and allowed to dry. An incision was made in the middle ventral part of the shell using sterile scissors. The eggs were then opened, the embryos transferred to...
six-well tissue culture dishes, and then returned to the humidified incubator for 4 additional days, until the start of the experiments (Parsons-Wingerter et al. 1998, 2000).

CAM Assay
At day E7, growth factors and drugs were prepared to the desired concentration in prewarmed PBS; 0.5 mL of solutions containing either the drugs/factors or the vehicle control was immediately applied in drops, in several places on the surface of each CAM. The embryos were then incubated at 37°C for another 24 to 28 h, at which time they were fixed in prewarmed fixative (4% paraformaldehyde/2% glutaraldehyde/PBS) for 2 days at room temperature before dissection. The CAM of a fixed embryo was carefully dissected and mounted without folds onto a glass slide and coverslipped with a solution of polyvinyl alcohol/glycerol (Parsons-Wingerter et al. 1998). The mortality of the embryos upon treatment was between 16% and 25% and independent of growth factor/drug/control treatment. CAMs of dead embryos were not included in the sample cohorts.

CAM Morphometry—Imaging and Fractal Dimension Analysis
Aldehyde fixation of the embryos preferentially delineates the arterial tree due to the retention of erythrocytes within the arterial vessels, in contrast to the venous tree in which the erythrocytes leak from the venous vessels during dissection (Parsons-Wingerter et al. 1998). Images of distal arterial branches located in the central region of the CAM were acquired with a computer-controlled scanner in grayscale mode at a resolution of 520 × 250 pixels. The images were transferred to Adobe Photoshop and adjusted for brightness and size. The CAM picture, magnified 10× (sufficient to image virtually the entire arterial vascular tree; Parsons-Wingerter et al. 1998) was printed and a square region of 4.5 × 5 cm, which included the middle region of the terminal arterial vessels, was selected in each CAM. Thereafter, the vascular tree was traced on the printed picture using a thin marker pen, which helped to more readily distinguish the smaller vessels. The CAM window was rescanned, transferred to Adobe Photoshop, and rescaled to 514 × 514 pixels to fit the format required for the fractal analysis by NIH Image J (http://rsb.info.nih.gov/ij/). Images of the arterial tree were cleaned from venous contributions using Adobe Photoshop, adjusted for threshold (to increase contrast and sharpness) and saved as jpeg files. All subsequent image processing was performed with NIH Image J software. The images of the vessels in the CAMs were binarized to facilitate visual inspection of the vascular morphology and skeletonized to calculate the fractal coefficient D₁, following the procedures described by Parsons-Wingerter et al. (1998, 2000). D₁, a statistical descriptor of space-vessel filling area and length (Kirchner et al. 1996), ranging in our experiments from 1 to 1.5, was determined using an NIH Image J subroutine, implementing the method of box counting. In this method, the CAM image is overlaid with a series of square boxes of decreasing size (denoted in pixel, p) and the number of boxes (N_p) containing at least one pixel is counted. The negative value of the least-squares regression slope of the plot of log N_p versus log p yields D₁ (Kirchner et al. 1996). The structure was considered fractal if the r² value of regression was >.95 (Kirchner et al. 1996; Parsons-Wingerter et al. 1998). Because in our hands fractal analysis was a more sensitive representation of changes in vasculature pattern in the skeletonized images (a direct representation of total vessel length) than analysis of binary images, only the D₁ values of skeletonized images are presented.

Statistics
A total of 340 CAMs were analyzed. The quantitative data, expressed as mean ±SD (n = 6–11 CAMs), represent results from two to five independent experiments. Comparisons between groups were analyzed by one-way analysis of variance (ANOVA) and/or Student’s t test, with p < .05 considered as statistically significant.

RESULTS
The morphology of small blood vessels in the arterial vasculature of the quail CAM was assessed by visual inspection and evaluated by quantitative image analysis of the digitized images, as described by Parsons-Wingerter et al. (1998, 2000). The angiogenic potential of NGF, bFGF, and VEGF can be inferred from the typical appearance of the arterial vessels under the various experimental conditions (Figure 1A). Visual inspection of the binary images suggests that the potent angiogenic effect of the growth factors is mainly manifested as a significant increase in the pattern and extent of vessel branching and density, as previously reported for rhbFGF (Parsons-Wingerter et al. 1998, 2000). At embryonic day 7 (E7), CAMs exposed to mNGF showed a significant increase in the vascular density and exhibited a more elaborate network of branching vessel (Figure 1A), as compared to PBS (Figure 1B). Quantitative analysis (Figure 1B) indicates that at the dose used (5 µg mNGF, 1.5 µg rhbFGF, or 0.5 µg rhVEGF), NGF-induced angiogenesis (D₁ = 1.266 ± 0.05) was similar to that due to either rhbFGF (D₁ = 1.264 ± 0.03), or rhVEGF (D₁ = 1.290 ± 0.02), two of the established “standard” angiogenic growth factors. When applied at E8, mNGF also stimulated CAM arterial growth, with a D₁ of 1.252 ± 0.017 compared to a D₁ of 1.153 ± 0.020 (p < .05) in the PBS control group. However, at this stage in development, NGF-enhanced angiogenesis was less effective than at E7, probably due to the enhanced spontaneous angiogenesis in the quail CAM at E8 (Henry et al. 1998; Parsons-Wingerter et al. 1998; and data not shown).

Shown in Figure 2 is the dose response of angiogenesis induced by NGFs from different biological sources and compared to the effects of rhVEGF. For all NGF species, the angiogenic effect was dose dependent in the range of 0.5 to 5.0 µg/CAM.

NGF-INDUCED ANGIOGENESIS IN THE QUAIL CAM 53
rhVEGF also induced sizeable increases in vascular density at doses of 0.5 to 1.0 µg/CAM. When comparing the different agonists, we noted their angiogenic potential as rhVEGF > cNGF > mNGF ≫ vNGF.

A major question is whether the observed NGF-enhanced angiogenesis involved a direct activation of the trkA neurotrophin receptor, or rather was caused indirectly by NGF stimulation of VEGF production. To investigate these issues, the embryos were incubated with mNGF in the presence of two specific inhibitors of NGF and VEGF, K252a and SU-5416, respectively. Prior studies indicated that trkA receptors for NGF are expressed in early embryonic tissues of the quail (Yao et al. 1994; Zhang et al. 1996). Because both K252a and SU-5416 are solubilized in DMSO before dilution in PBS, the effect of 0.05% DMSO (vehicle) on CAM angiogenesis was also evaluated and found to be insignificant (Figure 3).

As presented in Figure 3 and Tables 1 and 2, the effect of K252a on CAM vasculature is complex. In the absence of exogenous NGF, K252a induced a strong, NGF-like, angiogenic stimulation. On the other hand, concomitant application of 5 µg mNGF and 1 µM K252a for 24 h resulted in a complete inhibition of mNGF-induced angiogenic stimulation. In contrast, application of the VEGF receptor 2 (flk-1) antagonist SU-5416 (1 µM) alone neither significantly affected arterial growth relative to PBS/DMSO-treated controls nor impaired mNGF stimulation of angiogenesis when applied together with 5 µg mNGF (Figure 3). This observation is significant, because when 1 µM SU-5416 was applied to the CAM concomitant with rhVEGF, rhVEGF-induced angiogenesis was reduced by 60% (Table 1).

At a higher dose of 5 µM (Table 2), but not at 1 µM (Table 1), K252a blocked both mNGF- and rhVEGF-induced angiogenesis, indicating a nonselective effect of K252a on flk-1 receptor at this higher dose. In contrast, SU-5416, at both 1 µM (Table 1) and 5 µM (Table 2) selectively inhibited rhVEGF- but not NGF-induced angiogenesis. Also,
NGF-INDUCED ANGIOGENESIS IN THE QUAIL CAM

Fig. 2. Dose-response of different NGF agonist-induced angiogenesis in the quail CAM, compared to rhVEGF. Quantitative image analyses expressed as change in fractal dimension (Df) in response to growth factors. The embryos were treated at E7 for 26 hr with PBS, 0.5 µg, 2.0 µg, and 5.0 µg mouse NGF (mNGF), 0.5 µg and 2.0 µg viper NGF (vNGF), 0.5 µg and 2.0 µg cobra NGF (cNGF) and 0.5 µg and 1.0 µg rhVEGF. Df values are presented as mean ± SD (n = 6 in each group); statistical significance (*p < .05) by ANOVA and Student’s t tests compared to PBS.

**DISCUSSION**

In this study, we present a quantitative evaluation of the selective, proangiogenic effect of NGF in vivo, as assessed by the significant increase in number and length of small capillaries of the quail CAM arterial tree. The proangiogenic effect of NGF was quantified as the change in fractal dimension in response to different doses of NGF agonists isolated from different species. Importantly, the angiogenic effect of NGF was efficiently blocked by 1 µM K252a, the sole trk receptor–specific and –selective antagonist available today. This inhibition suggests that NGF stimulation of angiogenesis is mediated by activation of trkA receptor signaling, because at this dose neither rhVEGF nor rhbFGF angiogenic-stimulatory effects were modulated by K252a. Our findings strongly support the notion of a direct angiogenic effect of exogenous NGF on the quail CAM arterial tree, independent of endogenous quail bFGF and VEGF. However, these data do not exclude the possibility that the proangiogenic effects of NGF are mediated indirectly by influencing the balance of other endogenous pro- and antiangiogenic factors in the quail CAM. Interestingly, K252a alone mimicked the proangiogenic effects of NGF. This property is reminiscent of the well-documented, NGF-like agonistic effects of K252a in neurons, characterizing this reagent as a mixed agonist-antagonist compound (Lazarovici et al. 1997).

The pleiotropic effects of NGF, which acts on both neurons and capillaries, are reminiscent of those of VEGF and bFGF which besides their eminent role in cardiovascular system, also possess neurotropic, neurotrophic, and neuroprotective effects in the nervous system (Lazarovici et al. 2006). During embryonic and fetal development there is a cross-talk between the vascular and the neuronal systems (Weinstein 2005), in which blood vessel development guides the development of the peripheral nervous system. For example, the migration of sympathetic (and sensory) neurons will occur along preformed vascular beds, such as the aorta (Reissmann et al. 1996). Similarly, in vivo experiments demonstrated that neovascularization along an FGF-1 (acidic FGF)–treated, collagen-coated
the effect of trkA (K252a) and flk-1 (SU-5416) antagonists on the morphometry of mouse NGF-stimulated angiogenesis in the quail CAM. (A) Representative pictures of the arterial vasculature in quail CAMs. The embryos were treated at E7 for 24 h with PBS, 0.05% DMSO in PBS, 5 µg mNGF without or with 1 µM K252a, or K252a alone and with 1 µM SU-5416 or SU-5416 alone. (B) Quantitative image analyses expressed as change in fractal dimension (Df) in response to growth factor and drug treatments described in A. Df values are presented as mean ± SD of two (SU-5416) and four (K252a) experiments (n = 6–10 in each group); statistical significances (*p < .05 compared to PBS; **p < .01, compared to respective growth factor alone) were analyzed by ANOVAs and Student’s t tests.

polytetrafluoroethylene fiber is accompanied/followed by de novo innervation of this synthetic conduit (Thompson et al. 1989).

More recent studies suggest that the cross-talk between the vascular and the nervous systems is truly bidirectional: the nervous system plays an important role in the development of the embryonic cardiovascular system (Scholz et al. 2001). For example, sensory nerves determine the pattern of arterial differentiation and blood vessel branching (Miller 2002; Mukouyama et al. 2002).
NGF-INDUCED ANGIOGENESIS IN THE QUAIL CAM

TABLE 2
Morphometry of growth factors stimulation of arterial angiogenesis in the quail CAMs treated with a high dose of K252a or SU-5416

<table>
<thead>
<tr>
<th>Drug</th>
<th>PBS (n = 7)</th>
<th>mNGF (n = 5)*</th>
<th>rhVEGF (n = 8)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>1.117 ± 0.023</td>
<td>1.237 ± 0.024</td>
<td>1.230 ± 0.038</td>
</tr>
<tr>
<td>K252a</td>
<td>1.225 ± 0.031</td>
<td>1.163 ± 0.029</td>
<td>1.172 ± 0.035</td>
</tr>
<tr>
<td>SU-5416</td>
<td>1.134 ± 0.034</td>
<td>1.242 ± 0.049</td>
<td>1.174 ± 0.056</td>
</tr>
</tbody>
</table>

*The embryos were treated at E7 with 5 µg mNGF or 0.5 µg rhVEGF in the absence or presence of 5 µM of either K252a or SU-5416 and fixed after 26 h. CAMs were dissected, mounted, and analyzed as described in Materials and Methods. Df values are presented as mean ± SD: Student’s t test statistical significance: *p < .001 compared to DMSO–PBS; **p < .005 compared to rhVEGF alone.

Taken together, all these findings suggest that the nervous system might contribute to the control of embryonic vascularization, most probably by a paracrine route involving neurotrophins such as NGF. Reparative neovascularization in the adult occurs mainly through capillary endothelial cells sprouting, enlargement of existing arterioles by proliferation of endothelial cells and smooth muscle cells (arteriogenesis), and de novo vascularization from circulating endothelial cell precursors (Carmeliet 2000). Recent studies, mostly in vitro but also in vivo, provide mounting evidence for a direct involvement of NGF in angiogenic processes, in addition to the well-established role of “classical” angiogenic growth factors such as VEGF and basic FGF. For example, NGF induces in vitro proliferation of umbilical cord (Cantarella et al. 2002), brain capillary (Moser et al. 2004), choroidal (Steinle and Granger 2003), arterial (Tanaka et al. 2004), and dermal microvasculature endothelial cells (Raychaudhuri et al. 2001). Furthermore, NGF increases the migration of aortic endothelial cells (Dolle et al. 2005; Rahbek et al. 2005) and up-regulates expression of adhesion proteins such as intercellular adhesion molecule (ICAM) (Raychaudhuri et al. 2001). The few studies in animal models to-date indicate that NGF up-regulates VEGF production (Calza et al. 2001; Manni et al. 2005) and induces reparative neangiogenesis, arteriogenesis, and wound healing (Emanuelli et al. 2002; Graiani et al. 2004; Lambiase et al. 2000; Seo et al. 2001; Yoo et al. 2004).

Whether these processes occur cumulatively, directly via NGF-induced angio/arteriogenesis or indirectly via the induction of classical “angiogenic factors” such as VEGF, remains to be clarified. Our data (Figure 3) suggest a direct role of NGF-trkA signaling in these angiogenesis-dependent events. Recent studies examining the angiogenic actions of the related neurotrophin, brain-derived neurotrophic factor (BDNF), demonstrated the requirement of this neurotrophin for maintenance of cardiac wall stability during embryonic development (Donovan et al. 2000) and its important role in revascularization of ischemic skeletal muscle (Kermani et al. 2005).

Together with prior studies characterizing NGF effects on endothelial cells, our findings using the quail CAM assay provide new evidence for a role of NGF, and neurotrophins in general (Kraemer and Hempstead 2003), as pleiotropic proangiogenic growth factors in the embryonic cardiovascular and nervous systems, as well as during inflammation and tumor growth in adult organisms. In addition to yielding some insight into the role of NGF as a novel proangiogenic growth factor crossing the boundary between the nervous and the vascular systems, our findings may also be relevant for pharmaceutical purposes.

REFERENCES


