Nerve Growth Factor Promotes Angiogenesis and Arteriogenesis in Ischemic Hindlimbs

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Background—The neurotrophin nerve growth factor (NGF) regulates neuron survival and differentiation. Implication in neovascularization is supported by statement of NGF and its high-affinity receptor at vascular level and by NGF property of stimulating vascular endothelial cell proliferation. The present study investigated the involvement of endogenous NGF in spontaneous reparative response to ischemia. Mechanisms and therapeutic potential of NGF-induced neovascularization were examined.

Methods and Results—Unilateral limb ischemia was produced in CD1 mice by femoral artery resection. By ELISA and immunohistochemistry, we documented that statement of NGF and its high-affinity receptor is upregulated in ischemic muscles. The functional relevance of this phenomenon was assessed by means of NGF-neutralizing antibody. Chronic NGF blockade abrogated the spontaneous capillarization response to ischemia and augmented myocyte apoptosis. Then we tested whether NGF administration may exert curative effects. Repeated NGF injection into ischemic adductors increased capillary and arte riole density, reduced endothelial cell and myofiber apoptosis, and accelerated perfusion recovery, without altering systemic hemodynamics. In normoperfused muscles, NGF-induced capillarization was blocked by vascular endothelial growth factor–neutralizing antibodies, dominant-negative Akt, or NO synthase inhibition.

Conclusions—These results indicate that NGF plays a functional role in reparative neovascularization. Furthermore, supplementation of the growth factor promotes angiogenesis through a vascular endothelial growth factor–Akt–NO–mediated mechanism. In the setting of ischemia, potentiation of NGF pathway stimulates angiogenesis and arteriogenesis, thereby accelerating hemodynamic recovery. NGF might be envisaged as a utilitarian target for the treatment of ischemic vascular disease. (Circulation. 2002;106:2257-2262.)

Key Words: angiogenesis ▪ ischemia ▪ muscles

Postnatal neovascularization occurs through capillary sprouting, a process named angiogenesis, proliferation of preexisting arteriolar connections into collateral arterioles and arteries, a process called arteriogenesis, and de novo capillary formation from endothelial cell (EC) precursors, ie, vasculogenesis.1,2 Recently, the concept has been introduced that in the setting of ischemia, neural and vascular factors may cooperate to promote tissue repair. For instance, vascular endothelial growth factor (VEGF), a prototypical angiogenic molecule, is involved in the maintenance and restoration of peripheral nerve integrity.3,4 Reciprocally, a neural drive for vascular growth may occur during postnatal development. Nerve growth factor (NGF), the first isolated member of a growing family of neurotrophins, is indeed regarded for its potential involvement in physiological and pathologic angiogenesis. Binding sites for NGF have been detected on cultured ECs5 and vascular smooth muscle cells (VSMCs),6 and activation of NGF receptors influences vascular cell biology and fate in vitro.6–9 Furthermore, NGF is present in circulating monocytes,10 regarded as important players in muscularization of nascent capillaries.11 Moreover, NGF may act as an indirect activator of EC growth by stimulating the release of other vascular growth factors (GFs). For instance, in superior cervical ganglia of newborn rats, capillary sprouting is promoted by NGF via the release of VEGF.12 Finally, a subsidiary clue for NGF acting as a proangiogenic agent emerges from preclinical and clinical studies documenting the wound-healing properties of the GF.13–15

In consideration of all of the above, we investigated the hypothesis that endogenous NGF may be implicated in reparative angiogenesis in extraneural tissue. Furthermore, we challenged the healing potential of NGF supplementation in the setting of limb ischemia.

Methods

Procedures and Operative Model
All procedures complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory

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Animal Resources, National Academy of Sciences, Bethesda, Md, 1996). Unilateral hindlimb ischemia was induced in male 8-week-old CD1 mice (Charles River, Comerio, Italy) by surgically excising the left femoral artery under 2,2,2-trichloroethanol anesthesia (880 mmol/kg body weight IP, Sigma-Aldrich).16,17

Effects of Ischemia on NGF and NGF Receptor Expression
NGF content was determined in homogenates of adductor muscles harvested at 0, 1, 3, 7, and 14 days from ischemia induction (n = 4 for each time point) using a highly sensitive ELISA.16 NGF tyrosine kinase A (trkA) receptor expression was evaluated in adductors collected at 0 or 7 days (n = 5 each group). To this aim, anesthetized mice were perfused with PBS (1 minute) and then with buffered 4% formalin (10 minutes) at 100 mmHg via the abdominal aorta. Immunohistochemistry was performed in 3-μm transverse muscular sections. Rabbit anti-rat trkA antiserum (Santa Cruz, Calif) was used as primary antibody. The specific binding was detected using a biotinylated anti-rabbit IgG and the avidin-biotin peroxidase technique (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, Calif). In negative controls, the primary antibody was substituted with rabbit IgG (Vector Laboratories).

Histologic Assessment of Microvascular Effects Induced by NGF or NGF Antibody
Mice were randomly attributed to the following protocols. First, the functional relevance of endogenous NGF in spontaneous postischemic neovascularization was evaluated. Neutering anti-NGF antibody (100 μg in 100 μL PBS, n = 8 mice) or control goat IgG (n = 6) was injected intraperitoneally every 5 days starting the day preceding femoral artery excision. Mice were killed at 21 days after ischemia induction. The antibody was raised against ultra-pure murine 2.5S NGF as previously described.19

Second, the proangiogenic properties of NGF supplementation were tested in the setting of limb ischemia. Mouse 2.5S NGF (20 μg in 20 μL PBS) or vehicle was daily injected into ischemic adductors for 5 (n = 7 and 10, respectively) or 14 (n = 8 and 10, respectively) days starting on the same day of surgery. Mice were euthanized at 21 days from ischemia induction. NGF was extracted from mouse submaxillary glands, following the method of Bocchini et al.20

Third, the mechanisms implicated in the proangiogenic action of NGF were investigated. Mice were given the following treatments for 7 days: (1) daily injection of NGF (20 μg in 20 μL PBS) or PBS into normoperfused adductors (n = 5 each group); (2) intramuscular NGF plus VEGF neutralizing antibody (2.5 μg IP twice a week, R&D Systems, Minneapolis, Minn) or control IgG (n = 5 each group); (3) intramuscular NGF plus the NO synthase inhibitor L-nitroarginine methyl ester (L-NAME) or the inactive isomer (D-NAME) (both at 1.4 mmol/kg per day in drinking water, Sigma-Aldrich, n = 5 each group); and (4) intramuscular NGF plus adenovirus carrying dominant-negative Akt (Ad.Akt shakt, prepared at the Kimmel Cancer Center, Philadelphia, Pa) or reporter gene Luciferase (Ad.Luc) (each at 5 × 10^11 pfu in a single intramuscular injection, n = 5 each group).

On the day of euthanasia, the limbs of anesthetized mice were perfusion-fixed and both adductors were harvested and processed for histology.16,17 Capillary and myofiber density was determined in transverse muscular sections stained with H&E. Consecutive sections were stained with a mouse monoclonal anti-α-smooth muscle actin antibody (Sigma) for assessment of arteriole density. Histological analyses were performed in a blinded fashion. Capillaries and myofibers were counted using an ocular reticle (9604-μm² area) at ×1000 magnification. Twenty-five fields were randomly examined and averaged. The number of capillary (n_cap) and myofiber (n_fiber) per field was used to compute capillary and myofiber density per mm² of section. Capillary density was normalized to myofiber density (n_cap/n_fiber). The number of arterioles (n_art) in the entire section was counted at ×1000 magnification. Sections were acquired at 400× with a videocamera (Pixera), and section areas were measured by using an image analysis program (Image ProPlus, Media Cybernetics, Silver Spring, Mass). Arteriolar density per mm length of section area (n_art/mm) was then calculated.

Ischemia-induced apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay in muscle sections harvested from limbs of mice given systemic NGF antibody or control IgG (n = 7 and 5, respectively) or intramuscular NGF or vehicle (n = 4 each group). Sections 3 μm thick were stained with streptavidin-conjugated peroxidase (using DAB as a chromogen) and counterstained with methyl green. Positive controls were pretreated with 1 μg/mL DNaase I, and negative controls were incubated in the absence of the terminal deoxynucleotidyl-transferase. Sections were examined at ×1000 magnification by a scientist (G.G.) unaware of the randomization protocol. TUNEL-positive ECs and myofibers were separately counted. Apoptotic density was expressed as the number of apoptotic ECs per 1000 capillaries and of apoptotic myofibers per 1000 fibers, respectively.

Effects of NGF Supplementation or NGF Blockade on Postischemic Hindlimb Blood Flow Recovery
Studies were performed on the same mice used for evaluation of microvascular effects of NGF or NGF antibody. Hindlimb blood flow (BF) was measured in anesthetized animals by laser Doppler flowmetry (Lisca Inc) before surgery and weekly thereafter. The ischemic to nonischemic foot BF ratio was calculated.16,17 To avoid any confounding effect related to drug delivery, measurements were performed 24 hours apart from the last injection of each agent. A supplementary group of 6 mice, submitted 7 days in advance to limb ischemia, underwent consecutive measurements of limb BF under basal conditions and up to 30 minutes after injection of 20 μg NGF into ischemic adductors.

Effects of NGF on Systemic Hemodynamics
Tail-cuff systolic blood pressure (SBP) and heart rate (HR) were measured in conscious mice under basal conditions and at weekly intervals from surgery. The effect of IV NGF (10 ng to 20 μg) on mean blood pressure was evaluated in anesthetized mice via a catheter passed to the thoracic aorta through the left carotid artery (n = 3).21

Akt Phosphorylation by NGF in Normoperfused Muscles
Anesthetized mice were given a single intramuscular injection of NGF (20 μg in 20 μL PBS) or vehicle. Ten or 30 minutes later, adductors were harvested, pulverized in N2, and solubilized in lysis buffer. Akt phosphorylation was detected by the use of primary antibody specific for serine 473 phosphorylated Akt (phospho-Akt-Ser473). Total Akt was recognized by an antibody raised against phosphorylation-state independent Akt (Cell Signaling Technology, Milan, Italy). To verify the amount of protein loaded, blots were reprobed with anti-tubulin antibody (Promega, Milan, Italy). Western immunoblot density was analyzed using a dedicated image program (Scion Corporation).

Statistical Analysis
All results are expressed as mean ± SEM. Multivariate repeated-measures ANOVA was performed to test for interaction between time and grouping factor. In multiple comparisons among independent groups in which ANOVA and F test indicated significant differences, the statistical value was determined according to the Bonferroni’s method. Differences within and between groups were determined using paired or unpaired Student’s t test, respectively. P < 0.05 was interpreted to denote statistical significance.

Results
Impact of Muscular Ischemia on NGF and NGF Receptor Expression
As shown in Figure 1, NGF content increased strikingly on ischemia induction, peaking at 1 day and then remaining
elevated up to 14 days from surgery. As shown in Figure 2, ischemia enhanced the expression of trkA receptor in skeletal muscles (right panel) compared with normoperfused conditions (left panel).

Effect of NGF Blockade on Reparative Angiogenesis and Hemodynamic Recovery

In otherwise healthy animals, limb ischemia is generally followed by an increase in muscular capillarity. Native angiogenic response was profoundly impaired under NGF blockade but not disturbed by control IgG (Figure 3). The results were confirmed after normalization of capillarity by myofiber density (data not shown). However, the growth of collateral arterioles and the hindlimb perfusion recovery were not affected by NGF blockade (data not shown).

Ischemia-induced apoptosis was augmented by NGF antibody at the level of myofibers (2.47 ± 0.48 versus 0.99 ± 0.24 TUNEL-positive cells every 1000 myofibers in controls, P < 0.05), whereas the number of apoptotic ECs remained unaltered (data not shown).

Effect of Local NGF Supplementation on Reparative Angiogenesis and Hemodynamic Recovery

As shown in Figure 4, daily injection of NGF for 14 days enhanced the native microvascular response to ischemia both at capillary (panel A) and arteriole (panel B) level, without disturbing the vascularity of contralateral muscles. A shorter protocol (daily injection for 5 days) was also capable of increasing arteriole density of ischemic muscles, but less than the 14-day treatment. The results were confirmed after normalization by fiber density (data not shown). Stimulation of vascular growth by NGF was associated with inhibition of ischemia-induced apoptosis at the EC and myofiber level (Figure 5).

Potentiation of reparative neovascularization accelerated the rate of perfusion recovery. As shown in Figure 6, 14-day treatment with NGF restored perfusion ratio (0.89 ± 0.11) to the levels recorded before ischemia (0.98 ± 0.03, P = NS), whereas at the same time point the recovery of vehicle-treated group was still incomplete (0.58 ± 0.06 versus 1.00 ± 0.03 before ischemia, P < 0.05). At 21 days from ischemia, NGF-treated mice showed a higher perfusion ratio (1.00 ± 0.15) compared with controls (0.79 ± 0.11, P < 0.05). Administration of NGF for 5 days was not enough to ameliorate recovery (data not shown).

Effect of NGF on Local and Systemic Hemodynamics

Intravenous NGF injection did not alter mean blood pressure or HR (data not shown). Injection of NGF into ischemic limbs produced an acute increase in BF that was extinguished within 30 minutes (data not shown). In chronic protocols using NGF or NGF antibody, no effect of treatment was detected on SBP or HR (data not shown).

Mechanisms Implicated in the Angiogenic Action of NGF

Local injection of NGF was able to increase muscular capillarity even in the absence of an ischemic environment (1078 ± 94 versus 629 ± 25 in noninjected and 617 ± 25 cap/
NGF is regarded as a pleiotropic molecule, involved in a wide variety of functions, such as neuropeptide modulation, wound healing, and tissue cicatrization. Part of the healing effects might be attributable to stimulation of neoangiogenesis, even if the theory has not been firmly validated. A proliferative response to NGF was shown in cultured dermal ECs or HUVECs (Emanueli, unpublished results, 2002), suggesting that vascular endothelium constitutes a biologic

Figure 4. Bar graph shows the effect of exogenous NGF administration on reparative neovascularization. Local daily injections of NGF (full columns) or vehicle (V, dotted columns) into ischemic muscles were repeated over 5 (n=7 and 10, respectively) or 14 (n=8 and 10, respectively) days, starting from the day of ischemia induction. Capillary (A) and arteriole (B) density was evaluated 14 days thereafter. Microvascular density of untouched adductors (open column) is shown as a reference. Neovascularization was potentiated at arteriole and capillary level by 14-day NGF treatment. Five days of treatment exerted an effect on arteriole growth only. Values are mean±SEM. *P<0.05 vs controls; §P<0.05 vs V; +P<0.05 vs 5-day treatment.

Figure 5. Bar graph shows the effect of NGF (full columns, n=4) or vehicle (V, open columns, n=4) on apoptosis in ischemic adductors. NGF or vehicle was intramuscularly injected every day starting on the day of femoral artery excision. Muscles were harvested 5 days later. Apoptosis found at the level of capillary endothelial cells (ECs) and myocytes was expressed as the number of TUNEL-positive cells per 1000 capillaries (A) or myofiber (B), respectively. Values are mean±SEM. §P<0.05 vs V.

Figure 6. Line graph shows the effect of NGF supplementation for 14 days on foot postischemic BF recovery, expressed as ischemic to contralateral BF ratio. Perfusion recovery was more rapid in mice given NGF (full symbols, n=8) compared with vehicle (open symbols, n=10). Values are mean±SEM. *P<0.05 vs time zero; §P<0.05 vs vehicle.
apoptosis by 123%, suggesting that the GF may exert prosurvival effects on skeletal myocytes exposed to ischemia. Recent studies point to trkA as the receptor mediating the antiapoptotic action of NGF in neural tissue. It cannot be ruled out that potentiation of capillary sprouting by NGF contributes to preventing excessive myocyte death.

In the setting of peripheral ischemia, the success of a supply-side strategy based on administration of exogenous NGF was documented by anatomic and functional evidence. Continuous treatment for 14 days enhanced the spontaneous neovascularization of ischemic adductors, encompassing increased capillary sprouting and arteriole growth. Furthermore, hemodynamic outcome was significantly improved, as documented by BF measurements. Interestingly enough, we found that NGF itself behaves as a local vasodilator when injected into the ischemic muscle, although being devoid of effects on systemic blood pressure. It cannot be excluded that NGF-mediated vasodilatation may contribute in triggering arteriogenesis, a growth process strongly influenced by local hemodynamic and mechanical effects.

One major limitation of protein or polypeptide GF-based therapeutic angiogenesis consists in the necessity of repeated injections of the curative substance for all the duration of the healing process. Thus, we decided to evaluate whether short-duration treatment, limited to the mounting phase of reparative neovascularization, would provide equal therapeutic benefit compared with more prolonged administration. We found that 5-day NGF treatment does not improve capillarity but is enough to produce a mild increase in arteriole density of ischemic muscles. Apart from therapeutic implications, our findings reinforce the opinion that arteriogenesis is rapidly initiated after arterial occlusion11 and also shed light on the plasticity of this neovascularization process in response to short-term supplementation with exogenous GF.

Various mechanisms may be implicated in the vascular effects of NGF. The GF influences EC growth and, as shown here, exerts a prosurvival effect on ECs. It may also act as an indirect activator of EC proliferation by stimulating the release of other endothelial mitogens, including VEGF and substance P, or modulators of angiogenesis, such as NO. We found that in normoperfused muscles, NGF enhances the phosphorylation of Akt, a protein kinase acting downstream of VEGF and angiopoietin to confer EC survival and ensure proper blood vessel development. In addition, Akt functions as an activator of NO production in response to VEGF through its ability to phosphorylate endothelial NOS on serine 1179 or 1177. Using inhibitors of this putative pathway, we documented that NGF can indeed act to improve neovascularization through a VEGF-Akt-NO-dependent mechanism.

In summary, we have found that spontaneous surge of NGF after hindlimb vascular occlusion is instrumental to reparative angiogenesis. Potentiation of NGF signaling by exogenous supplementation has a profound impact on both angiogenesis and arteriogenesis, thereby accelerating the rate of tissue recovery. Thus, the present studies provide new insights into the vascular action of NGF and give evidence of curative properties in the setting of peripheral ischemia.
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References
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