The Neuropeptide Secretoneurin Acts as a Direct Angiogenic Cytokine In Vitro and In Vivo

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Background—Secretoneurin is an abundant neuropeptide of the central, peripheral, and autonomic nervous systems, located in nerve fibers characterized by a close interaction with blood vessels and known to stimulate endothelial cell migration.

Methods and Results—We hypothesized that secretoneurin might act as an angiogenic cytokine and tested for these effects in vivo using a mouse cornea neovascularization model and in vitro by assessing capillary tube formation in a matrigel assay. In vivo, secretoneurin-induced neovascularization is characterized by a distinct pattern of arterial and venous vessels of large diameter and length. Immunohistochemical staining for CD-31 revealed endothelial lining of the inner surface of these vessels, and recruitment of α-smooth muscle actin–positive perivascular cells suggests vessel maturation. In vitro, secretoneurin-induced capillary tube formation was dose dependent and specific, confirming that effects of secretoneurin occur directly on endothelial cells. Secretoneurin also stimulated proliferation and exerted antiapoptotic effects on endothelial cells and activated intracellular phosphatidylinositol 3’ kinase/Akt and mitogen-activated protein kinase pathways, as demonstrated by increased phosphorylation of Akt and extracellular signal–regulated kinase.

Conclusions—These data show that secretoneurin represents a novel direct angiogenic cytokine and reiterate the coordinated relationship between nervous and vascular systems. (Circulation. 2004;109:777-783.)

Key Words: angiogenesis • endothelium • nervous system

Angiogenesis, the growth of new blood vessels from the preexisting vasculature, plays an important role in wound healing, the menstrual cycle, and pathological conditions such as tumor growth, diabetic retinopathy, tissue ischemia, and inflammatory diseases.1–3 Cytokines such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) mediate this process in vivo, where they stimulate formation of capillaries and exert mitogenic and antiapoptotic effects on endothelial cells (ECs) in vitro.4–6

Recently, application of EC growth factors as proteins or genes has gained scientific and clinical interest to treat limb or myocardial ischemia to induce augmented collateral growth or improve endothelial function, a procedure that has been termed therapeutic angiogenesis.7–9

Secretoneurin (SN) is a neuropeptide derived from secre togranin II, an abundant protein in neuroendocrine storage vesicles and a member of the chromogranin/secretogranin family.10,11 SN acts as an inflammatory peptide released from primary afferent C-fibers of the peripheral nervous system and has been shown to induce directed migration of ECs.12,13 It has been demonstrated additionally that SN-containing nerve fibers are closely associated with blood vessels, in particular in the female reproductive system and in skeletal muscles.14,15 Furthermore, transient hypoxia of the brain induced a strong upregulation of SN production in neuronal cells.16

Because of the chemotactic properties of SN on ECs in vitro, the close interaction of SN-containing nerve fibers with blood vessels, and the increased SN production in the setting of tissue ischemia, we hypothesized that SN might act as an angiogenic cytokine.

In this study we demonstrate that SN exhibits potent angiogenic properties in vivo in a mouse cornea neovascularization assay and in vitro using a matrigel tube formation assay. In vitro, SN showed antiapoptotic and proliferative effects on ECs and activated the Akt/protein kinase B pathway and the mitogen-activated protein kinase (MAPK) system.
Methods

Secretoneurin Peptide and Antibody

The peptide secretoneurin contains 33 amino acids and is synthesized by standard t-butoxycarbonyl chemistry. It is purified by HPLC to approximately 97%, and purity is verified by mass spectrometry and amino acid analysis. The peptide does not contain endotoxin, as measured in a previous study (tested by Limulus amebocyte lysate test for endotoxins from Sigma) and for this study (same method). The peptide was dissolved in PBS containing 0.1% BSA. This solvent was used as control. SN was purchased from Neosystems (Strasbourg, France). The SN antibody was raised in rabbits against the murine SN peptide (synthesized as reported above) coupled to keyhole limpet hemocyanin and described in detail previously.

Figure 1. SN induces angiogenesis in vivo in a cornea neovascularization assay. a and b, Representative images of slit-lamp biomicroscopy after implantation of control (a) or SN (b) pellets (P). c through f, Whole-mount images of in vivo FITC-BS1 lectin stained corneas (c and d, ×40; e and f, ×100) with control (c and e) and SN (d and f) pellets. White arrowhead indicates leading edge of neovascularization; red arrowhead, limbus artery; yellow arrowhead, limbus vein; and blue arrowhead, venous structure.

Figure 2. Immunohistochemical characterization of SN-induced vessels. Immunohistochemistry of corneal cross sections was performed at the front of neovascularization using an antibody against CD-31 (a) and of adjacent sections with an irrelevant antibody (negative control, b). Staining of cross sections of the area between the limbus artery and the leading edge of SN-induced neovascularization for CD-31 (c) and (in an adjacent section) for α-SM actin (d).
Animal Models
All protocols were approved by the St Elizabeth’s Medical Center Institutional Animal Care and Use Committee. Anesthesia was performed with 2,2,2-tribromoethanol (880 mmol/kg body weight IP; Sigma-Aldrich).

Mouse Cornea Neovascularization Model
Pellets containing 600 ng SN or solvent (0.1% BSA in PBS) were implanted in C57/BL/6J mice as described. Corneas were examined by slit-lamp biomicroscopy on postoperative day 7. Total area of corneal neovascularization was calculated as described. Afterward, mice received an intravenous injection of 500 μg BS1 lectin conjugated to FITC (Vector Laboratories). After euthanasia, enucleated eyes were fixed in 1% paraformaldehyde and examined by fluorescence microscopy. Some corneas were fixed in 100% methanol, and cross sections of corneal hemispheres (between the limbus artery and the pellet) were immunohistochemically stained for CD31 and α-smooth muscle (SM) actin as described.

Tube Formation Assays
Human umbilical vein ECs (HUVECs) were isolated and cultured as described. The formation of vascular-like structures by HUVECs was assessed on a basement membrane matrix preparation (matrigel) using the ECMatrix in vitro angiogenesis kit (Chemicon); 96-well plates were coated with matrigel, and HUVECs (5000 cells/well) were seeded in serum-free medium 199 (M199) with or without test substances (SN, IgG-purified anti-SN antibody; VEGF, neutralizing anti-bFGF and neutralizing anti-VEGF antibodies, both from Sigma, Vienna, Austria) and incubated at 37°C for 8 to 12 hours.

Cell Culture
HUVECs in 24-well plates were starved in M199 without serum and treated with different concentrations of SN, 100 ng/mL VEGF, 10 mmol/L Wortmannin (WM, inhibitor of phosphatidylinositol 3 [PI3] kinase), 10 μmol/L PD98059 (PD, inhibitor of MAPK) (all from Sigma), or different combinations for 16 hours. Cells were fixed by 4% paraformaldehyde, stained with DAPI (4′,6-diamidino-2-phenylindole, Sigma), and examined by fluorescence microscopy, and total cell numbers as well as numbers of apoptotic cells, characterized by condensation and fragmentation of the nucleus, were counted.

Statistics
All results are expressed as mean±SEM. Statistical comparisons between groups were performed by ANOVA. P<0.05 was considered to denote statistical significance.
Figure 4.

(a) Images showing cell number and percent pyknotic nuclei under different conditions:

- **Ctr**: Control
- **SN 10 ng/ml**: Test condition 1
- **VEGF 100 ng/ml**: Test condition 2

(b) Images of control and SN (10 ng/ml) groups showing cell morphology.

(c) Bar graph showing cpm (% control) for different conditions:

- **Ctr**: Control
- **SN (10 ng/ml)**: Test condition 1
- **VEGF (100 ng/ml)**: Test condition 2

(d) Bar graph showing relative cell number for different SN concentrations.

Significance levels:

- *p < 0.01
- **p < 0.001
Kirchmair et al  
Secretoneurin and Angiogenesis  781

Results

In Vivo Cornea Neovascularization Assay
Slit-lamp biomicroscopy revealed prominent neovascularization in corneas of the SN group but not in those of the control group (Figures 1a and 1b). After injection of FITC-labeled BS1 lectin as an EC marker, robust corneal neovascularization from the limbus artery toward the pellet was observed for SN but not for control pellets (Figures 1c and 1d). The neovascularature induced by SN (Figure 1f) was characterized by large vessels, often reaching the cytokine pellet and forming a network of capillaries at the leading edge of neovascularization (Figure 1f, white arrowhead). Several SN-induced vessels did not originate from the limbus artery (Figure 1f, red arrowhead) but were connected with the limbus vein (Figure 1f, yellow arrowhead) and appear to be part of venous structures (Figure 1f, blue arrowhead). The area of neovascularization was 1.92±0.31 mm² for SN and 0.75±0.1 mm² for control (P<0.01; n=4 per group).

Immunohistochemistry of corneal cross sections at the front of neovascularization for the endothelial marker CD 31 confirmed the results from BS1 lectin perfusion staining, showing that SN induced vessels of large diameter (Figure 2a, see insets for higher magnifications). Additionally, when cross sections of the area between the limbus artery and the leading edge of neovascularization were stained for CD-31 (Figure 2c) and adjacent sections for α-SM actin (Figure 2d), we found that SN-induced vessels clearly stained positive for both markers (arrows in Figures 2c and 2d), indicating recruitment of perivascular cells.

In Vitro Matrigel Tube Formation Assay
To examine the effects of SN on EC differentiation into vascular structures in vitro, a matrigel tube formation assay in the absence or presence of various concentrations of SN (0.1, 1, 10, and 100 ng/mL; not all data shown) was performed. SN at a concentration of 10 ng/mL was most effective in promoting HUVEC tube formation, and the extent of this effect was comparable to that of VEGF at 100 ng/mL, which was used as a positive control (Figure 3a). Specificity of this SN-induced effect was tested by using a SN antiserum, which abrogated the SN-induced effect, whereas the SN antiserum did not affect VEGF-induced tube formation (data not shown). When the incubation media contained SN (10 ng/mL) in combination with either anti-VEGF or anti-bFGF antibodies, the SN-induced tube formation was not affected (Figure 3b).

SN Inhibits HUVEC Apoptosis and Stimulates HUVEC Proliferation In Vitro
For assessing effects of SN on apoptosis in serum-starved HUVECs, 3 independent methods were used. First, when HUVECs were treated with SN, VEGF, or solvent before DAPI staining, SN induced a dose-dependent increase in cell number compared with control (140.3±13.9 cells/mm²) ranging from 207.7±14.2 cells/mm² (SN 0.1 ng/mL) to 248±6 cells/mm² (SN 10 ng/mL), which was similar to the effect of VEGF (257.3±8.7 cells/mm²) (Figure 4a). Increasing the dose of SN (100 ng/mL) did not additionally improve the SN-mediated effect.

Second, cells with pyknotic, condensed nuclei per total number of cells were reduced by SN (6.3±1.2% for 10 ng/mL) and VEGF (7.5±1.9%) compared with control media (18.1±3.4%) (Figure 4a, arrows). Again, increase of SN to 100 ng/mL showed no additional decrease in apoptotic nuclei. Therefore, for subsequent studies, SN at a dose of 10 ng/mL was used.

Third, to investigate effects of SN on an earlier stage of apoptosis, we stained HUVECs with annexin V after serum starvation (Figure 4b). SN at a dose of 10 ng/mL reduced annexin V–positive cells, consistent with decreased EC apoptosis.

To differentiate antiapoptotic from additional proliferative properties of SN, we investigated the effect of SN on DNA synthesis in HUVECs (Figure 4c). SN (10 ng/mL) significantly increased [3H]thymidine incorporation in HUVECs (117±2.9%) versus control media (100±3.5%) and exerted an effect similar to that of 100 ng/mL VEGF (123±2.5%) (P<0.001 for control versus SN or VEGF, n=5). Additionally, HUVEC proliferation by various concentrations of SN was studied under identical conditions (addition of 10% FBS) by direct cell counting. Again, SN dose-dependently increased cell numbers, with a maximum effect at 10 ng/mL (control, 1±0.04; SN, 1.5±0.1; P<0.001). At very high doses (1 and 10 μg/mL), proliferative effects of SN were not observed (P versus control, NS) (Figure 4d). Results from the above experiments suggest that the SN-induced increase in cell number (Figure 4a) is not only a result of an antiapoptotic mechanism but also attributable to proliferative actions of SN on HUVECs.

SN Activates Intracellular Signal Transduction Pathways
To assess effects of SN on Akt and ERK activation, HUVECs were incubated with 10 ng/mL SN before protein extracts were used for Western blotting with specific antibodies against the phosphorylated (activated) form of Akt or ERK. SN stimulated phosphorylation of both ERK (maximum after 10 minutes) and Akt (maximum after 20 minutes) (Figure 5a); however, ERK phosphorylation seemed to be much more prominent.

Figure 4. SN inhibits EC apoptosis and stimulates EC proliferation. a, Representative photographs of HUVECs incubated for 16 hours with control buffer (Ctr), 10 ng/mL SN, or 100 ng/mL VEGF and subsequent DAPI staining (top, white arrows indicate pyknotic, condensed nuclei). Quantification of total cell numbers per square millimeter and percentage of cells with pyknotic nuclei (bottom) of HUVECs treated with control buffer, different concentrations of SN, or 100 ng/mL VEGF (P<0.01 vs control, *P<0.001 vs control; n=4). b, Representative pictures of serum-starved (10 hours) HUVECs after staining with annexin V and propidium iodide (PI); solvent (control), top panel; SN (10 ng/mL), bottom panel; bright field, left; fluorescent images for annexin V (FITC) and PI, right. c, Incubation of HUVECs with SN (10 ng/mL) or VEGF (100 ng/mL) for 48 hours and incorporation of [3H]thymidine into cells compared with controls (P<0.001 control vs SN or VEGF, n=5). d, HUVECs were cultured in 10% FBS for 48 hours and incubated with control buffer, different concentrations of SN, or 100 ng/mL VEGF, and cells were counted after DAPI staining (P<0.001 vs Ctr, n=4).
To functionally confirm these findings, HUVECs were incubated with different concentrations of PI3-kinase inhibitor WM and the MAPK inhibitor PD. Whereas WM at 100 nmol/L inhibited basal cell growth (data not shown), WM at a low concentration of 10 nmol/L and PD at 10 μmol/L did not (relative cell number: control, 1 ± 0.02; WM, 1.03 ± 0.04; PD, 1.03 ± 0.05) (Figure 5b). However, the SN-induced increase in cell number (1.55 ± 0.09; P < 0.001 versus control) was completely inhibited by coincubation with 10 nmol/L WM or 10 μmol/L PD (SN+WM, 0.97 ± 0.08; SN+PD, 1.05 ± 0.07; P < 0.001 SN+WM and SN+PD versus SN; n = 4). These data confirm a role of MAPK pathway and possibly also PI3-kinase/Akt system in the SN-mediated mechanism (Figure 5b).

**Discussion**

Recent reports reveal a close interaction between regulators of angiogenesis and cytokines responsible for trophic actions in the nervous system. This interaction seems to be the consequence of the requirement for oxygen supply of nervous tissues and the lack of regenerative capacity of these cells after hypoxic damage; it was demonstrated that nerve growth factor, beside its effect on neurons, has angiogenic activity in a chorioallantoic membrane assay and in the hindlimb ischemia model.\(^{20,21}\) Furthermore, other members of the family of neuropeptides, such as substance-P and neuropeptide-Y, have been reported to induce angiogenesis.\(^{22,23}\) VEGF, on the other hand, a classical endothelial mitogen, has been shown to exert trophic functions on neuronal cells and to improve nerve function in ischemic and diabetic neuropathy by acting directly on Schwann cells, in addition to angiogenic effects on nerve vasculature.\(^{24-26}\) These reports provide abundant evidence to support the interaction of nerve and vessel formation in neuronal regeneration.

In this study, we report that the abundantly expressed neuropeptide SN has potent angiogenic activity in vitro and in vivo, comprising features of classical direct angiogenic cytokines. SN exerts antiapoptotic and mitogenic effects on ECs, activates Akt and MAPK pathways, induces capillary tube formation in vitro, and stimulates angiogenesis in vivo.

Angiogenic cytokines such as VEGF or bFGF are mitogens and antiapoptotic agents for ECs in vitro and stimulate Akt and MAPK pathways in these cells.\(^{4,5,27,28}\) This study demonstrates in HUVECs that SN acts as an antiapoptotic agent and induces cell proliferation (as shown by \[^{3}H\]thymidine incorporation and by direct cell counts) at a low nanomolar concentration. At higher concentrations (1 and 10 μg SN/mL) this proliferative effect was not observed, but basal cell counts were not reduced at these concentrations in low-passage HUVECs used in our experiments. This finding is in contrast to a previous study where we reported inhibitory effects of SN on rat and bovine EC proliferation at high concentrations (10\(^{-7}\) and 10\(^{-6}\) mol/L, corresponding to approximately 500 and 5000 ng/mL).\(^{13}\) These differences might be attributable to different cell types, different passages of cells, and different methods used.

Stimulation of HUVECs with SN led to the activation of ERK and, to a lesser extent, of Akt, and inhibition of these pathways abrogated the effect of SN, suggesting involvement of especially the MAPK pathway in SN-mediated angiogenesis. Possible involvement of Akt seems not to be mediated by VEGF, because angiogenic effects by SN were not inhibited by neutralizing VEGF antibodies.

Although the SN receptor has not been cloned yet, functionally active binding sites for SN have been identified on leukocytes recently.\(^{29}\) Functional and biochemical responses of ECs to SN, as observed in a previous\(^{13}\) and the present study, implicate the presence of a putative SN receptor on these cells.

In vitro angiogenesis assays demonstrated that SN induces capillary tube formation in a dose-dependent and specific manner, because an anti-SN antibody inhibited this effect. Interestingly, antibodies against VEGF or bFGF did not inhibit the angiogenic response, suggesting a direct angiogenic mechanism of SN.

In vivo SN-induced neovascularization was characterized by long vessels of a large diameter. Interestingly, SN-induced neovascularization apart from the leading front of angiogenesis was characterized by positive staining for \(\alpha\)-SM actin, suggesting recruitment of pericytes or SM cells as markers of vessel maturation.\(^{3}\) The clinical significance of this induction of large and long capillaries remains to be determined.
might speculate that these vessels might be stable because of the recruitment of perivascular cells.

It has been shown that SN-containing nerve fibers are closely associated with blood vessels in the rat uterus,\textsuperscript{14} an organ in which angiogenesis occurs on a physiological basis in the adult organism. Angiogenesis is also involved in inflammatory processes, and neuropeptides with angiogenic properties such as SN or substance-P are released from secretory granules of afferent sensory nerve fibers contributing to diseases such as rheumatoid arthritis.\textsuperscript{1,30} We also observed increased SN-immunoreactivity in skeletal muscle cells in the mouse ischemic hindlimb model (Rudolf Kirchmair, unpublished data, 2003), additionally supporting the hypothesis that this peptide plays a role in physiological/pathophysiological angiogenesis.

Finally, a recent study reported the effect of SN on neurite outgrowth (differentiation) without affecting neuronal survival or proliferation.\textsuperscript{31} This is in contrast to our findings on ECs and to the observations made with VEGF on neuronal cells.\textsuperscript{24} Presently, we can only speculate what mechanisms cause these differences. One possible explanation would be the use of different neuronal cells in these studies (superior cervical ganglia and dorsal root ganglion cells\textsuperscript{24} versus granular cells of the cerebellum\textsuperscript{31}).

In summary, our data indicate that the neuropeptide SN represents a novel, potent angiogenic growth factor of neuronal origin.

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References

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