The Angiogenic Factor Secretoneurin Induces Coronary Angiogenesis in a Model of Myocardial Infarction by Stimulation of Vascular Endothelial Growth Factor Signaling in Endothelial Cells

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Background—Secretoneurin is a neuropeptide located in nerve fibers along blood vessels, is upregulated by hypoxia, and induces angiogenesis. We tested the hypothesis that secretoneurin gene therapy exerts beneficial effects in a rat model of myocardial infarction and evaluated the mechanism of action on coronary endothelial cells.

Methods and Results—In vivo secretoneurin improved left ventricular function, inhibited remodeling, and reduced scar formation. In the infarct border zone, secretoneurin induced coronary angiogenesis, as shown by increased density of capillaries and arteries. In vitro secretoneurin induced capillary tubes, stimulated proliferation, inhibited apoptosis, and activated Akt and extracellular signal-regulated kinase in coronary endothelial cells. Effects were abrogated by a vascular endothelial growth factor (VEGF) antibody, and secretoneurin stimulated VEGF receptors in these cells. Secretoneurin furthermore increased binding of VEGF to endothelial cells, and binding was blocked by heparinase, indicating that secretoneurin stimulates binding of VEGF to heparan sulfate proteoglycan binding sites. Additionally, secretoneurin increased binding of VEGF to its coreceptor neuropilin-1. In endothelial cells, secretoneurin also stimulated fibroblast growth factor receptor-3 and insulin-like growth factor-1 receptor, and in coronary vascular smooth muscle cells, we observed stimulation of VEGF receptor-1 and fibroblast growth factor receptor-3. Exposure of cardiac myocytes to hypoxia and ischemic heart after myocardial infarction revealed increased secretoneurin messenger RNA and protein.

Conclusions—Our data show that secretoneurin acts as an endogenous stimulator of VEGF signaling in coronary endothelial cells by enhancing binding of VEGF to low-affinity binding sites and neuropilin-1 and stimulates further growth factor receptors like fibroblast growth factor receptor-3. Our in vivo findings indicate that secretoneurin may be a promising therapeutic tool in ischemic heart disease. *(Circulation. 2012;126:2491-2501.)*

Key Words: angiogenesis ■ gene therapy ■ myocardial infarction

Congestive heart failure represents an increasing global health problem that occurs predominantly as a result of coronary artery disease.1 Cardiac dysfunction after myocardial infarction (MI) results from 2 major mechanisms: loss of cardiac myocytes in the area of infarction and remodeling of the spared myocardium in the left ventricle (LV). Inadequate structural adaptation of the vascular bed in the area of cardiac myocyte hypertrophy accounts for the progression of LV dysfunction of the heart. Although ischemia induces endogenous myocardial angiogenesis, vascular growth is insufficient to maintain normal capillary density in the hypertrophied myocardium. Induction of neovascularization is recognized to be a valid approach to modify the pathological changes of ventricular remodeling. Gene transfer of growth...
factors known to induce angiogenesis such as vascular endothelial growth factor (VEGF), hepatocyte growth factor, sonic hedgehog, or basic fibroblast growth factor (bFGF) attenuates cardiac dysfunction after MI in animal models.\(^2\)\(^-\)\(^5\) In patients with coronary heart disease, randomized phase II and phase III gene therapy studies did not reach the primary end point, although in most studies signs of angiogenic bioactivity were observed.\(^6\)\(^-\)\(^8\) As in peripheral arterial disease, patient and study end point selection as well as determination of transgene expression will be important for planning future clinical studies evaluating treatment of ischemic cardiomyopathy. Additionally, a combination of growth factors or growth factors combined with cell therapy may be more promising than therapy with a single factor.\(^9\)

**Clinical Perspective on p 2501**

In this study, we present a plasmid gene therapy approach based on secretoneurin, a neuropeptide of 33 amino acids in length, derived from secretogranin-II (SG2), a member of the chromogranin/secretogranin family, which induces angiogenesis.\(^10\)\(^-\)\(^12\) In a recent study, we showed that secretoneurin gene therapy restored tissue integrity, function, and perfusion in the mouse hindlimb ischemia model by induction of angiogenesis, arteriogenesis, and vasculogenesis.\(^13\) We therefore hypothesized that secretoneurin may also exert beneficial effects in a rat model of MI. We provide evidence for improvement of cardiac function after MI by secretoneurin gene therapy in vivo and show a VEGF-dependent mechanism of secretoneurin action on human coronary artery endothelial cells (HCAECs) in vitro.

**Methods**

### Construction of the Human Secretoneurin Expression Plasmid

Synthetic oligonucleotides encoding secretoneurin and a signal peptide were cloned into an expression plasmid vector, as has been described previously.\(^13\)

### Rat Model of MI

Protocols described in this study were approved by the Austrian Committee for the Care and Use of Laboratory Animals. MI was induced by ligation of the left anterior descending coronary artery, as described previously.\(^14\) Animals received 100 μg of secretoneurin plasmid or control plasmid intramyocardially.

### Echocardiography

Echocardiograms were recorded under light anesthesia. Two-dimensional directed M-mode and Doppler transthoracic echocardiographic studies were performed 3 days, 2 weeks, and 4 weeks after ligation with the use of an Acuson Sequoia echocardiography system (Acuson Corporation, Mountain View, CA) with a commercially available 15-MHz linear-array transducer system (AcuNav, Acuson Corporation).\(^13\) Data were analyzed with the use of software resident on the ultrasonograph by an experienced researcher who was blinded to the treatment.

### Hemodynamic Analysis

Cardiac hemodynamics were measured with a 1.4F catheter (Millar Instruments), as described previously.\(^16\) Rats were anesthetized and placed in the supine position. After intubation, rats were mechanically ventilated, and the anterior chest wall was opened. During direct insertion of the catheter into the LV, ventilation was discontinued. Recordings of dP/dt and \(\tau\) were performed with the rats under ECG monitoring in a stable state during different cardiac cycles.

### Cell Culture and In Vitro Assays

The following cell lines were used and purchased from Promo Cell: HCAECs, human coronary artery smooth muscle cells (HCASMCs), and human cardiac myocytes. Migration and tube formation assays, Western blotting, and immunoprecipitation were performed as described previously\(^13\) or in detail in Methods in the online-only Data Supplement.

### Receptor Tyrosine Kinase Profiler and Immunoprecipitation

Human phospho-receptor tyrosine kinase (RTK) array kits (R&D, ARY001) were used for investigation of receptors involved in HCAEC and HCASMC signaling with secretoneurin as suggested by the manufacturer.

### 125I-VEGF Binding Assays on HCAECs

For binding assays, HCAECs were cultured in 24-well plates and incubated with 250 000 cpm 125I-VEGF (PerkinElmer), as detailed in the online-only Data Supplement.

### Statistical Analysis

All results are expressed as mean±SEM. Statistical comparisons between 2 groups were performed by Student’s t test. Multiple groups were analyzed by a 1-way ANOVA test followed by appropriate post hoc tests to determine statistical significance. \(P\) values <0.05 were considered statistically significant. All experiments were repeated at least in triplicate. For further details regarding materials and methods, see the online-only Data Supplement.

### Results

#### Secretoneurin Improves Cardiac Function After MI

To test whether secretoneurin gene therapy of ischemic myocardium improves cardiac function, echocardiographic investigations were performed 4 weeks after left anterior descending coronary artery ligation and treatment (\(n=12\) per group). We observed significant improvement of LV ejection fraction (LV ejection fraction: secretoneurin plasmid, 64.4±3.1% versus control plasmid, 40.3±2.7%, \(P=0.00001\); sham operation, 61.9±3.5%) and LV fractional shortening (secretoneurin plasmid, 39.8±4.6% versus control plasmid, 25.1±4.2%, \(P=0.04\); sham operation, 41.2±5.1%) in the secretoneurin-treated group compared with the control group (Figure 1B). Additionally, LV end-diastolic diameter (secretoneurin plasmid, 9.1±0.6 mm versus control plasmid, 10.5±0.3 mm; \(P=0.04\)) and LV end-systolic diameter (secretoneurin plasmid, 6.5±0.6 mm versus control plasmid, 9.1±0.5 mm; \(P=0.002\)) were significantly reduced in rats receiving secretoneurin plasmid compared with control treat-
Secretoneurin Induces Myocardial Angiogenesis and Arteriogenesis In Vivo

Vascular structures in ischemic myocardium (n=15 per group) were visualized by immunofluorescent staining for rat endothelial cell antigen (Figure 2A) and α-smooth muscle actin (Figure 2B). Rat endothelial cell antigen–positive capillaries showed significantly higher blood vessel density (number of capillaries per high-power field) in peri-infarct regions in secretoneurin-treated animals compared with control animals (secretoneurin plasmid, 381.7±100.1 versus control plasmid, 277.9±54.9; P=0.0005), and staining with anti-α-smooth muscle actin showed higher density for arteries and arterioles in the secretoneurin treatment group (secretoneurin plasmid, 9.9±3.0 versus control plasmid, 5.2±1.2; P=0.00004) (Figure 2C). These observations indicate induction of angiogenesis and arteriogenesis in the infarct border zone by secretoneurin gene therapy.

Secretoneurin Reduces Fibrosis After MI

Figure 3A shows Masson trichrome–stained myocardial sections from control plasmid– and secretoneurin plasmid–treated rats 4 weeks after left anterior descending coronary artery ligation. Treatment with secretoneurin plasmid resulted in a remarkable reduction of fibrosis in the LV after MI compared with controls. Figure 3B shows the percentage of fibrosis in hearts after permanent left anterior descending coronary artery occlusion. Hearts receiving secretoneurin gene therapy exhibited a significant reduction in fibrosis (secretoneurin plasmid, 21.7±1.6%) compared with controls (control plasmid, 35.4±2.8%; P=0.0007; n=13).
In Vitro Effects of Secretoneurin on Migration, Angiogenesis, and Apoptosis of HCAECs

Secretoneurin caused a dose-dependent induction of chemotaxis in HCAECs with a maximum effect at 100 ng/mL (relative chemotactic index, 2.4±0.2; \( P=0.0009 \) versus control; Figure 4A). Blockade of secretoneurin with a specific neutralizing antibody (secretoneurin antibody) completely inhibited secretoneurin-mediated HCAEC migration, indicating specificity of the observed effect (relative chemotactic index for secretoneurin 100 ng/mL vs secretoneurin antibody, 1.4±0.1; \( P=0.009 \) versus secretoneurin 100 ng/mL). Interestingly, the chemotactic effect of secretoneurin was also inhibited by a VEGF antibody (relative chemotactic index for secretoneurin 100 ng/mL + VEGF antibody, 1.4±0.1; \( P=0.006 \) versus secretoneurin 100 ng/mL) and the mitogen-activated protein kinase (MAPK) inhibitor PD98.059 (PD) (relative chemotactic index for secretoneurin 100 ng/mL + PD, 1.3±0.01; \( P=0.005 \) versus secretoneurin 100 ng/mL).

Secretoneurin-induced angiogenesis in vitro was demonstrated by increase in capillary-like tube formation in a Matrigel assay with HCAECs with a maximum effect at 10 ng/mL (relative tube formation, 2.2±0.1; \( P=0.00002 \) versus control; Figure 4B). Addition of secretoneurin antibody abolished secretoneurin-induced tube formation (1.1±0.1; \( P=0.001 \) versus secretoneurin 100 ng/mL), indicating specificity of observed effects. In accord with the observation in the migration assay, secretoneurin-induced tube formation was also impaired by VEGF antibody (relative tube formation, 1.3±0.1; \( P=0.005 \) versus secretoneurin 100 ng/mL). PD also inhibited secretoneurin-induced effects (relative tube formation, 1.5±0.1; \( P=0.007 \) versus secretoneurin 100 ng/mL), indicating a MAPK-dependent signaling of secretoneurin.

To investigate the effect of secretoneurin on HCAEC apoptosis, cells were starved and stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and 4',6-diamidino-2-phenylindole (DAPI). Secretoneurin significantly inhibited HCAEC apoptosis (% TUNEL-positive cells of DAPI-positive cells: control, 26.4±1.2 versus secretoneurin 100 ng/mL, 17.4±1.3; \( P=0.0008 \); Figure 4C). Secretoneurin antibody and VEGF antibody abrogated the antiapoptotic effect of secretoneurin (% TUNEL-positive cells of DAPI-positive cells: secretoneurin + secretoneurin antibody, 24.1±1.6; \( P=0.004 \) versus secretoneurin; secretoneurin + VEGF antibody, 26.5±1.4; \( P=0.005 \) versus secretoneurin).

Secretoneurin Signaling in HCAECs

Secretoneurin signaling was evaluated by Western blotting, which revealed activation of MAPK and phosphatidylinositol 3-kinase/Akt as judged by phosphorylation of extracellular...
signal-regulated kinase (ERK) and Akt. In HCAECs, secretoneurin stimulated MAPK phosphorylation at a concentration of 100 ng/mL, starting after 20 minutes with a continuing, long-lasting stimulation until 4 hours. Akt was activated by secretoneurin 100 ng/mL after 40 minutes with a 4-hour duration (Figure 5A). As observed in tube formation and migration assays, secretoneurin-induced effects on ERK activation were again blocked by VEGF antibody (Figure 5B).

Secretoneurin Stimulates VEGF Receptors
Because secretoneurin-mediated in vitro effects were blocked by a VEGF antibody, secretoneurin-induced signaling in HCAECs was further investigated with the use of RTK profiler assays (R&D). Results show an activation of VEGF receptor-1 and -2 (VEGFR1, VEGFR2, respectively) after a 40-minute treatment with secretoneurin 100 ng/mL (Figure 6A). This effect again could be blocked by VEGF antibody (1:500 dilution; Figure 6B), indicating that secretoneurin-induced VEGF receptor activation depends on endogenous VEGF.

Immunoprecipitation of HCAEC lysates with VEGFR2 antibody and immunoblotting for phosphotyrosine confirmed findings of the RTK profiler and revealed secretoneurin-induced VEGFR2 phosphorylation after 120 minutes (Figure 6C).

Western blot analysis of phosphorylated VEGFR2 additionally showed an increase of VEGF-induced (50 ng/mL) VEGFR2 activation by secretoneurin, comparable to the aforementioned effect of heparin (1 μg/mL), as shown in Figure 6D. These data indicate that secretoneurin stimulates VEGF receptor activation via a VEGF-dependent mechanism.

Immunohistochemical staining for phospho-VEGFR2 could be found only in ischemic rat hearts treated with secretoneurin plasmid 3 days after MI (Figure 6E) but not in control animals (data not shown).

In HCASMCs, we found activation of VEGFR1 and fibroblast growth factor receptor-3 (FGFR3) by secretoneurin 100 ng/mL after 40 minutes (Figure 6F). In contrast to HCAECs, however, we were not able to block secretoneurin-mediated effects by a neutralizing VEGF antibody (as demonstrated with a chemotaxis assay in HCASMCs; Figure IIA in the online-only Data Supplement).

Without starvation of HCAECs before stimulation with secretoneurin, a different picture was observed in RTK profiler assays: VEGFR1 and VEGFR2 were still stimulated by secretoneurin, but activation of FGFR3 and insulin-like
growth factor-1 receptor was also detectable (Figure 6G). To evaluate whether FGF also plays a role in secretoneurin-mediated effects on HCAECs, we inhibited bFGF with a neutralizing antibody but were not able to block secretoneurin-induced in vitro angiogenesis, in contrast to inhibition of VEGF (Figure IIB in the online-only Data Supplement). Therefore, secretoneurin-induced VEGF signaling in HCAECs was further evaluated.

To test the hypothesis that VEGFR2, which has been reported to be mainly responsible for angiogenic effects within the VEGF receptor family, is involved in secretoneurin signaling in HCAECs, a specific VEGFR2 inhibitor, SU1498, was tested. SU1498 (40 μmol/L) showed inhibition of secretoneurin-induced MAPK activation after 20, 40, and 60 minutes, indicating that secretoneurin-induced MAPK activation is mediated by VEGFR2 (Figure 7A).

Inhibition of secretoneurin-mediated in vitro angiogenesis by SU1498 was demonstrated by Matrigel assays: After addition of SU1498 (10 or 40 μmol/L; Figure 7B), secretoneurin-induced capillary tube formation was completely blocked (secretoneurin+SU1498, 10 μmol/L 1.1±0.03 versus secretoneurin, 2.1±0.2 μmol/L; *P*=0.0001), indicating that secretoneurin-induced MAPK activation is also mediated by VEGFR2. 5-Bromo-2′-deoxyuridine assays performed with HCAECs identified VEGFR2 and the MAPK pathway as essential modulators for secretoneurin-induced HCAEC proliferation. Both PD and SU1498 abolished secretoneurin-induced relative proliferation compared with secretoneurin 100 ng/mL (secretoneurin, 1.42±0.04 versus secretoneurin+PD, 0.58±0.04; *P*=0.00007; secretoneurin versus secretoneurin+SU1498, 0.69±0.07, *P*=0.00001; Figure 7C).

Secretoneurin Increases VEGF Binding to HCAECs, Heparin, and Neuropilin-1

To determine the mechanism of secretoneurin-induced VEGF receptor stimulation, we hypothesized that secretoneurin might interact with VEGFR2 and its coreceptor neuropilin-1 or with heparan sulfate proteoglycans, shown to be necessary for VEGF2 activation.17 However, we could not find direct binding of secretoneurin either to VEGF2 or to neuropilin-1 (data not shown) or to heparin with the use of heparin-coated Sepharose columns (data not shown). To test the hypothesis that secretoneurin increases the binding of VEGF to HCAECs, we performed binding studies with VEGF labeled by radioactive iodine (125I-VEGF) on HCAECs. Incubation with secretoneurin resulted in increased binding of 125I-VEGF to HCAECs with a maximum effect at 100 ng/mL secretoneurin (125I-VEGF, 1.7±0.1 fmol/10^5 cells versus secretoneurin 100 ng/mL+125I-VEGF, 2.1±0.1 fmol/10^5 cells; *P*=0.031; Figure 8A). On the basis of the effect of secretoneurin at a concentration of 100 ng/mL, we performed saturation studies in the presence or absence of secretoneurin, with increasing amounts of 125I-VEGF starting from 2.6 fmol/mL to a maximum concentration of 523 fmol/mL. B_{max} and K_{d} for VEGF binding to HCAECs were 4.7 fmol/10^5 cells and 290.3 fmol, respectively, whereas binding of VEGF in the presence of secretoneurin 100 ng/mL to HCAECs reached values of B_{max} of 5.2 fmol/10^5 cells and K_{d} of 228.2 fmol. These findings indicate that secretoneurin induces increased binding and increased affinity of HCAECs to VEGF.

Because secretoneurin did not affect binding of VEGF to VEGFR2 (Figure IIIA in the online-only Data Supplement), we further hypothesized that secretoneurin might stimulate binding of VEGF to heparan sulfate proteoglycans of the extracellular matrix. Therefore, a heparin–bovine serum albumin complex, as described previously,18 was used for analyzing the binding of VEGF to heparin in the presence of secretoneurin. Figure 8B illustrates a significant increase in 125I-VEGF binding to heparin, when secretoneurin 10 or 100 ng/mL was added to the binding buffer (secretoneurin 10 ng/mL, 1.09±0.01 pmol and secretoneurin 100 ng/mL, 1.12±0.01 pmol versus 125I-VEGF, 0.99±0.01 pmol; *P*=0.0003 and *P*=0.00007, respectively). A similar effect was observed when heparin-coated Sepharose beads were used (Figure IIB in the online-only Data Supplement).
To ascertain whether binding to heparan sulfate proteoglycans is responsible for increased secretoneurin-induced VEGF binding to HCAECs and secretoneurin-induced effects on HCAECs, we used heparinase I and heparinase III, which in combination digest heparin as well as heparan sulfates. Preincubation of cells with the mixture of heparinases resulted in complete loss of secretoneurin-mediated increase in VEGF binding (125I-VEGF/H1001 secretoneurin, 2.09 ± 0.08; P < 0.027; Figure 8C). The finding that secretoneurin-induced effects depend on heparan sulfate proteoglycans was confirmed by the observations that pretreatment of HCAECs by guest on April 21, 2017 http://circ.ahajournals.org/ Downloaded from

Figure 6. Secretoneurin (SN) stimulates vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) receptors. A, Treatment of human coronary artery endothelial cells (HCAECs) with SN induces phosphorylation of VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2). Profiler assays were used to investigate the role of receptor tyrosine kinases in SN-stimulated cells. HCAECs were incubated with SN 100 ng/mL for 40 minutes and further treated as recommended by the manufacturer. In comparison to untreated cells, SN induced phosphorylation of VEGFR1 and VEGFR2. B, SN-induced VEGF receptor activation is mediated by endogenous VEGF. For receptor tyrosine kinase profiler assays, HCAECs were treated with control medium (CTR) or SN 100 ng/mL with or without VEGF antibody (VEGF-Ab). SN-mediated VEGF receptor activation was blocked by the VEGF-Ab. C, SN induces phosphorylation of VEGFR2 (immunoprecipitation [IP]). HCAECs were treated with SN 100 ng/mL for different time periods, and cell lysates were collected and immunoprecipitated for VEGFR2. After blotting, membranes were probed with anti-phosphotyrosine antibody. SN-induced activation of VEGFR2 after 120 minutes. D, SN effects on VEGF-mediated VEGFR2 activation are comparable to those of heparin (Hep). HCAECs were treated with control medium, SN 100 ng/mL, and VEGF 50 ng/mL in combination with either heparin 1 μg/mL or SN 100 ng/mL. Levels of VEGFR2 phosphorylation were determined by Western blot analysis with the use of a specific phospho-VEGFR2 antibody. SN, like heparin, stimulated VEGF-mediated VEGFR2 phosphorylation. E, VEGFR2 phosphorylation in vivo. Frozen sections of rat heart 3 days after myocardial infarction and SN gene therapy showed positive staining for phospho-VEGFR2. F, Treatment of human coronary artery smooth muscle cells with SN induced phosphorylation of VEGFR1 and fibroblast growth factor receptor-3 (FGFR3). Human coronary artery smooth muscle cells were stimulated with SN 100 ng/mL for 40 minutes, and cell lysates were used for profiler assays. In comparison to untreated cells, SN induced phosphorylation of VEGFR1 and FGFR3. G, SN also activates FGFR3 and insulin-like growth factor-1 receptor (IGF-1R) in HCAECs. In unstarved HCAECs, profiler assays revealed stimulation of FGFR3 and IGF-1R with SN 100 ng/mL after 40 minutes, in addition to the already observed phosphorylation of VEGFR1 and VEGFR2.

Albrecht-Schgoer et al Secretoneurin in Myocardial Infarction 2497

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with heparinas I and III inhibited secretoneurin-mediated MAPK activation (Figure 8D) and antiapoptotic effects (secretoneurin relative to control, 0.57±0.02 versus secretoneurin+heparinase, 0.93±0.05, P=0.0002; secretoneurin versus secretoneurin+VEGF antibody, 0.97±0.02, P=0.0003; secretoneurin versus secretoneurin+PD, 0.85±0.05; P=0.0008; Figure 8E).

Although we found no effect of secretoneurin on binding of VEGF to its receptor VEGFR2 (Figure IIIA in the online-only Data Supplement), significantly increased VEGF binding to its coreceptor neuropilin-1 was observed with secretoneurin (125I-VEGF, 413.4±4.5 fmol/μg versus secretoneurin+125I-VEGF, 504±16.7 fmol/μg; P=0.012; Figure 8F).

**Discussion**

The main finding of our work is that gene therapy with the angiogenic factor secretoneurin improves outcome in a rat MI model in terms of LV function (evaluated by echocardiography and invasively by cardiac catheterization), LV remodeling, and scar formation. These effects were accompanied by an increase of capillary and arterial density in the infarct border zone, and we therefore hypothesized that this beneficial effect was largely due to induction of angiogenesis by secretoneurin, as shown before in other in vivo models, especially in the hindlimb ischemia model. As in this previous work, gene therapy was performed after ligation of the artery by intramuscular plasmid injection, and plasmid-derived secretoneurin was detectable for 2 weeks. Although we cannot exclude direct effects of secretoneurin on cardiac myocytes, we did not observe regulation of classic angiogenic factors such as VEGF by secretoneurin on these cells under either normoxic or hypoxic conditions (data not shown). First experiments also showed no effect of secretoneurin on RTKs in cardiac myocytes, as observed in this study for HCAECs and HCASMCs. Additionally, an intermediate time point after MI (14 days) showed a trend toward an increase, although it was not significant, of myocardial function and capillary density by secretoneurin, indicating that full induction of angiogenesis is necessary for the effects of secretoneurin on myocardial function (Figure IVA through IVC in the online-only Data Supplement). As in skeletal muscle cells, however, we also found in cardiac myocytes (but not in coronary endothelial cells) that prolonged hypoxia increases secretoneurin at messenger RNA and protein levels (Figure VA through VC in the online-only Data Supplement), which may indicate that secretoneurin, like other angiogenic factors, is increased as a physiological response to hypoxia in these cells to counteract the decrease of oxygen supply by the growth of new blood vessels. We also observed increased secretoneurin messenger RNA and protein in vivo after MI by polymerase chain reaction and immunofluorescence (Figure VIA and VIB in the online-only Data Supplement). In regard to the reduction in scar formation/fibrosis, we did not find effects of secretoneurin on human cardiac fibroblasts in vitro (regulation of secretoneurin by hypoxia, influence on cell proliferation or apoptosis by secretoneurin; Figure VIIA through VIIC in the online-only Data Supplement), and therefore we hypothesize that reduction of fibrosis is due to...
less damage of cardiac myocytes in the secretoneurin group rather than an inhibitory effect of secretoneurin on this process.

An interesting finding of our in vitro studies was that secretoneurin-induced effects on HCAEC migration, tube formation, proliferation, antiapoptosis, and ERK activation were blocked by a neutralizing VEGF antibody, whereas a bFGF antibody had no effect, at least on in vitro tube formation (Figure IIB in the online-only Data Supplement). This VEGF-dependent effect is in contrast to our data in human umbilical vein endothelial cells [10] (Figure VIII in the online-only Data Supplement), in which we did not observe stimulation of VEGF receptors by secretoneurin, indicating specificity of this observed effect for coronary arterial endothelial cells. The reason for this difference must be evaluated in future studies, and because we found that secretoneurin stimulates binding of VEGF to heparan sulfate proteoglycans, perhaps the composition of the extracellular matrix and/or differences in growth factors stored in the matrix are responsible for these observations. Nevertheless, to investigate whether secretoneurin stimulates RTKs, we used a commer-

Figure 8. Secretoneurin (SN) stimulates binding of vascular endothelial growth factor (VEGF) to heparan sulfate proteoglycans and neuropilin-1 (NP1). A, SN increases \( ^{125}\text{I}-\text{VEGF} \) binding to human coronary artery endothelial cells (HCAECs). For binding assays, 10 ng/mL \( ^{125}\text{I}-\text{VEGF} \) with or without increasing amounts of SN (1, 10, and 100 ng/mL) was added to HCAECs for 2 hours at 4°C. Binding of \( ^{125}\text{I}-\text{VEGF} \) was significantly increased in the presence of SN 100 ng/mL (\( p<0.05 \), \( ^{125}\text{I}-\text{VEGF} \) vs SN 100 ng/mL + \( ^{125}\text{I}-\text{VEGF} \); \( n=4 \)). B, SN enhances binding of \( ^{125}\text{I}-\text{VEGF} \) to heparin. Maxisorb tubes were coated with heparin–bovine serum albumin complex and incubated with 0.5 ng/mL \( ^{125}\text{I}-\text{VEGF} \) at 10 or 100 ng/mL SN for 2 hours at room temperature. In the presence of SN, binding of \( ^{125}\text{I}-\text{VEGF} \) to heparin was significantly increased compared with \( ^{125}\text{I}-\text{VEGF} \) alone (\( p<0.001 \), \( ^{125}\text{I}-\text{VEGF} \) vs SN + \( ^{125}\text{I}-\text{VEGF} \); \( n=5 \)). C, Heparinase (Hep.ase) pretreatment blocks SN-mediated \( ^{125}\text{I}-\text{VEGF} \) binding to HCAECs. To investigate the influence of heparan sulfate proteoglycans on \( ^{125}\text{I}-\text{VEGF} \) binding, HCAECs were preincubated with heparinase for 4 hours at 37°C. Thereafter, binding assays were performed with \( ^{125}\text{I}-\text{VEGF} \) + SN 100 ng/mL. Preincubation with heparinase diminished SN-induced increase of \( ^{125}\text{I}-\text{VEGF} \) binding to HCAECs (\( p<0.05 \), \( ^{125}\text{I}-\text{VEGF} \) vs SN + \( ^{125}\text{I}-\text{VEGF} \) and \( ^{125}\text{I}-\text{VEGF} + \) SN vs \( ^{125}\text{I}-\text{VEGF} + \) SN + heparinase; \( n=3 \)). D, Heparinase inhibits SN-mediated mitogen-activated protein kinase (MAPK) activation. After preincubation of HCAECs with heparinase, cells were stimulated with SN 100 ng/mL for different time periods, and lysates were processed for Western blotting. E, VEGF antibody (VEGF-Ab), PD98.059 (PD), and heparinases abrogate SN-induced antiapoptotic effects. HCAECs were starved overnight in medium without supplements + SN 100 ng/mL, PD (10 \( \mu \text{mol/L} \)), VEGF-Ab, and heparinase. VEGF 50 ng/mL served as positive control (\( p<0.001 \), SN vs control; **\( p<0.001 \), SN + heparinase, SN + VEGF-Ab, and SN + PD vs SN 100 ng/mL; \( n=4 \)). F, SN increases binding of \( ^{125}\text{I}-\text{VEGF} \) to its coreceptor NP1 (\( p<0.05 \), \( ^{125}\text{I}-\text{VEGF} \) vs \( ^{125}\text{I}-\text{VEGF} + \) SN; \( n=3 \)).
cially available profiler and found that secretoneurin stimulates phosphorylation of VEGFR1, VEGFR2, FGFR3, and insulin-like growth factor-1 receptor in coronary endothelial cells as well as VEGFR1 and FGFR3 in coronary smooth muscle cells. Additionally, stimulation of VEGFR2 was observed after MI and secretoneurin gene therapy in vivo by immunohistochemistry. These findings indicate that the angiogenic neuropeptide secretoneurin stimulates several RTKs in coronary vascular cells, which may explain the robust effects observed in our study. In particular, the fact that different RTKs shown to be important for angiogenesis such as VEGF and FGF receptors were activated by secretoneurin may be important because a recently published work was able to show that a combination of angiogenic growth factors (ie, FGF and hepatocyte growth factor) produced a more potent and sustained effect in the corneal angiogenesis and MI models compared with the single factors.9,20 Similar effects were reported previously with the combination of bFGF and platelet-derived growth factor.21 Additionally, a close interaction between VEGF and FGF pathways (FGF-dependent regulation of VEGF2) was shown to be necessary for posts ischemic neovascularization.22 Therefore, it is conceivable that combinations of growth factors or stimulation of several different RTKs exerts additive, long-lasting effects on therapeutic angiogenesis. This is also of particular interest for clinical studies of therapeutic angiogenesis, in which application of a single growth factor did not show positive results in phase III studies.23,24

Because VEGFR2 is considered to be the pivotal receptor for endothelial cell survival and proliferation and for angiogenesis25 and we found that the effects of secretoneurin on endothelial cells were blocked by a neutralizing VEGF antibody and by a VEGFR2 blocker (SU1498), we further characterized the mechanism of secretoneurin-induced action in regard to VEGFR2 signaling. Activation of VEGFR2 by VEGF is dependent on heparan sulfate proteoglycans,26 and matrix-bound VEGF was reported to activate more sustained VEGFR2 stimulation.26 VEGF signaling via VEGFR2 is also enhanced by a coreceptor (ie, neuropilin-1).27 We therefore hypothesized that secretoneurin may bind to one of these VEGF receptors, but we were not successful in demonstrating direct binding of secretoneurin to VEGF itself, to VEGFR2, to neuropilin, or to heparin (data not shown). We therefore analyzed whether secretoneurin increases binding of VEGF to its receptors, as was shown for heparin, which stimulates VEGF binding to VEGFR2.28 Indeed, we found that secretoneurin stimulated binding of VEGF to coronary endothelial cells, and this effect was abolished when cells were preincubated with heparinase, indicating that secretoneurin stimulates binding of VEGF to heparan sulfate proteoglycans. This finding was corroborated by the fact that secretoneurin-induced ERK activation and antiapoptotic effects were also blocked by heparinase. Secretoneurin did not increase binding of VEGF to VEGFR2 (Figure IIIA in the online-only Data Supplement) but stimulated binding of VEGF to its coreceptors heparin and neuropilin-1, indicating that secretoneurin-induced effects are mediated by increased VEGF binding to neuropilin-1 and heparan sulfate proteoglycans. It is known that C-terminal basic amino acids of VEGF are responsible for binding of VEGF-165 to heparin. Because secretoneurin contains several acidic amino acids, we plan to synthesize different secretoneurin derivatives to characterize the responsible sequence of secretoneurin necessary for increased binding of VEGF to heparin in future studies.

In contrast to endothelial cells, we were not able to block secretoneurin-induced effects in HCASMCs by a neutralizing VEGF antibody (Figure II A in the online-only Data Supplement), allowing the assumption that secretoneurin acts via a different mechanism on these cells. Because FGFR3 was also activated by secretoneurin in HCASMCs, we are planning to analyze the role of FGF in secretoneurin-mediated effects in smooth muscle cells as well as in endothelial cells.

Summarizing our data, we found that secretoneurin acts as an endogenous enhancer of VEGF binding to its coreceptor neuropilin-1 and to heparan sulfate proteoglycan binding sites. We would like to propose secretoneurin as a novel agent in the treatment of ischemic cardiomyopathy for several reasons. Secretoneurin acts as stimulator of several growth factor RTKs known to play essential roles in angiogenesis (ie, VEGFR2 and FGFR3) in the coronary vasculature. Furthermore, secretoneurin also affects coronary artery smooth muscle cells, as was shown in this and previous studies,10,13 to induce growth of smooth muscle cell–covered blood vessels that are known to be more stable than capillaries. Finally, secretoneurin improved LV function and LV remodeling in an in vivo model of MI. Future studies also should be able to determine appropriate vectors for efficient delivery of this peptide in large-animal models and in human trials.

Acknowledgments
We thank Erin Lambers and Veronica Ramirez (Feinberg Cardiovascular Research Institute, Northwestern University, Chicago, IL) for technical support.

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Disclosures
None.

References
Our work shows that the angiogenic factor secretoneurin improves left ventricular function in an animal model of myocardial infarction. Secretoneurin stimulates angiogenesis and arteriogenesis in the infarct border zone and reduces scar size after the infarct. Mechanistically, secretoneurin exerts beneficial effects on coronary endothelial cells via stimulation of growth factor receptors for angiogenic cytokines such as vascular endothelial growth factor or fibroblast growth factor. Secretoneurin thereby stimulates binding of vascular endothelial growth factor to its coreceptors neuropilin-1 and heparan sulfate proteoglycans. Despite promising preclinical and early clinical data for the application of angiogenic cytokines in the treatment of ischemic heart or limb disease (a therapeutic strategy termed therapeutic angiogenesis), randomized phase III clinical trials (Angiogenic GENe Therapy [AGENT] trial for coronary heart disease, Therapeutic Angiogenesis for Management of Arteriopathy in a Randomized International Study [TAMARIS] for critical limb ischemia) did not show the expected results. In addition to patient and study end point selection and pharmacokinetic considerations, it is also conceivable that administration of a single angiogenic factor is not sufficient to orchestrate a complex biological event like the growth of a new blood vessel. Indeed, a combination of angiogenic growth factors (such as basic fibroblast growth factor and platelet-derived growth factor or basic fibroblast growth factor and hepatocyte growth factor) exerted more potent and long-lasting angiogenic responses and beneficial effects in ischemic diseases compared with administration of single factors. In this context, it is of particular interest that secretoneurin, as a single compound, stimulated receptors for several of these cytokines like vascular endothelial growth factor or fibroblast growth factor. We therefore think that secretoneurin may be a promising candidate for therapeutic angiogenesis.
The Angiogenic Factor Secretoneurin Induces Coronary Angiogenesis in a Model of Myocardial Infarction by Stimulation of Vascular Endothelial Growth Factor Signaling in Endothelial Cells


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Supplemental Methods

Quantification of recombinant SN m-RNA expression levels in ischemic hearts

Quantitative real-time PCR was used to determine m-RNA of recombinant, plasmid derived SN content in ischemic myocardium (n=4) harvested 7, 14 and 28 days after plasmid delivery to the ischemic myocardium. Tissues were homogenized with an Ultra-Turrax® T25. Total cellular RNA was extracted using RNA-bee (amsbio). 1 μg of total RNA was transcribed using Superscript TM First-Strand Synthesis System (Invitrogen). cDNA was finally used as template for real time PCR using a BioRad C 1000 cycler with CFX96 optical reaction module and MESA GREEN qPCR Mastermix Plus for SYBR Assay (Eurogentec). For amplification, primer pairs specific for recombinant, plasmid derived SN not detecting endogenous SN m-RNA were used. Therefore, the forward primer was designed upstream of the SN sequence (forward primer: CCC AGC CGG CCA CAA; reversed primer: CCT GTC AGT TTC CCC AGC TC). The reaction sequence included denaturation for 10 min at 95°C before 40 cycles of denaturation for 15 sec at 95°C, annealing and extension for 30 sec at 61°C. Relative gene expression was calculated using the Ct method with normalization to GAPDH (forward primer: TCC TGG GCT ACA CTG AGG AC; reversed primer: GAG GGC CTC TCT CTT GCT CT).

Rat model of myocardial infarction

Briefly, Sprague-Dawley rats (Charles River Laboratories, Germany) weighing 250 to 280 g were anesthetized by intraperitoneal administration of a mixture of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (5 mg/kg). Rats were endotracheally intubated and mechanically ventilated. The heart was exposed through thoracotomy at the fourth intercostal space. Myocardial infarction was induced by LAD ligation with a 7-0 polypropylene suture at the level of the pulmonary artery. Animals were randomized in a blinded fashion to one of 2 experimental groups: control group (receiving the empty plasmid...
vector p-CTR) and SN-plasmid (p-SN) group. 10 min after LAD ligation, 5 depots of a total of 100 µl p-CTR (1 µg/µl) or 100 µl p-SN (1 µg/µl) were injected intramyocardially into the left ventricle. For sham operation the same procedure except LAD ligation was performed and 100 µl saline was injected.

**Morphological analysis**

Body-, heart- and lung-weight were determined after hemodynamic measurements.

**Ischemia-reperfusion model**

Ischemia-reperfusion model was generated as described previously,\(^1\) with some modification. Rats were anesthetized as for LAD ligation and ventilated. Left-side thoracotomy was performed to reach the heart. The LAD was occluded with 8-0 silk by help of a sterile PE plastic tubing. Ischemia was evident by discoloration of the left ventricle. After 30 minutes occlusion, the ligature was loosened, and reperfusion was confirmed visually by the rapid restoration of blood flow accompanied by a change in the appearance of the ischemic myocardium from pale to red. After 3 days of reperfusion, hearts were harvested and fixed with 10% (v/v) buffered formaldehyde as described next (histological analysis). After deparaffinitation and rehydration, sections were treated with proteinase K (10 µg/ml) for 15 minutes at room temperature and washed with PBS. Apoptotic cells were visualized with TUNEL kits (Roche) using alkaline phosphatase as suggested by the manufacturer.

**Histological analysis**

3, 14 or 28 days after LAD ligation, rats (for each treatment n=11) were sacrificed and hearts were quickly removed. Specimens were fixed in 10% (v/v) buffered formaldehyde, dehydrated with graded ethanol series and embedded in paraffin. Alternatively, fresh tissue was embedded in OCT compound (TISSUE-TEK®, Sakura Finetek) and snap-frozen in liquid nitrogen. Serial transverse sections of 5 µm were cut across the entire long axis of the heart and subsequently mounted on slides.
Capillary- and arteriole-density

For immunofluorescence staining, frozen sections were treated with RECA-1 (abcam 9774, dilution 1:30) and alexa fluor 594 goat anti mouse (Invitrogen, A11032) in a dilution of 1:200, for assessment of capillary density. Arterioles were visualized with alpha smooth muscle actin (abcam 5694, dilution 1:20) and alexa fluor 488 goat anti mouse (Invitrogen, A11029, dilution 1:200). Capillary- and arteriole-density was evaluated by counting the number of capillaries and arterioles in 5 random and nonrepeated fields of the muscle tissue section (n=10 for each treatment).

SN and VEGFR2 in vivo staining

For SN in vivo staining, frozen sections were incubated with rabbit polyclonal SN antibody (kindly donated by Prof. Fischer-Colbrie) in a dilution of 1:100 and alexa fluor 488 goat anti rabbit (Invitrogen, A11008, dilution 1:200). Stained sections were covered with fluorescent mounting medium (Dako) containing H33258 for nuclei visualisation (1:1000).

For phospho-VEGFR2 staining, frozen sections were treated with rabbit polyclonal p-Flk-1 (Santa cruz 101821) in a dilution of 1:20 and vectastain kit (Vector, PK-6101) as suggested by the manufacturer.

Evaluation of myocardial fibrosis and quantification of myocardial infarction

For analyzing collagen accumulation, Masson´s trichrome staining was performed to delineate collagen content as a percentage of the whole heart area. After staining, slides were scanned and computerized for digital image analysis. By using Image J software, fibrotic area was calculated as the sum of fibrotic area divided by the whole heart area.

Cell culture and in vitro assays

Human coronary artery endothelial cells (HCAECs), human coronary artery smooth muscle cells (HCASMC), human cardiac fibroblasts (HCF) and human cardiac myocytes (HCM) were purchased from Promo Cell. HCAECs were cultured in EGM-2 Medium (Lonza)
containing 4.76% (v/v) Fetal calf serum and EGM-2 Single Quots (Clonetics, Lonza). HCASMC, HCF and HCM were cultured as suggested by the manufacturer in the corresponding medium. For all in vitro assays, cells were incubated with medium without supplements containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich).

Cell migration assays were performed in a modified Boyden chemotaxis chamber in which an 8 µm-pore sized cellulose nitrate filter (Sartorius) separates the upper and the lower chamber. Cells were placed into the upper compartment of the chemotaxis chamber and were allowed to migrate toward SN 1, 10 and 100 ng/ml (with or without SN-Ab 1:500, VEGF-Ab (Sigma-Aldrich) 1:500, or PD98.059 10 µM (Sigma Aldrich)) placed in the lower chamber. Migration into the filter was quantified by measuring microscopically the distance from the surface of the filter to the leading front of cells. Data are expressed as chemotaxic index, which is the ratio between the distance of migration towards test attractants and that toward control medium into the nitrocellulose filter.

For tube-formation assays, an in-vitro angiogenesis kit from Chemicon was used. Cells were incubated on matrigel with SN (100 pg/ml; 1, 10 and 100 ng/ml) with or without SN-Ab, VEGF-Ab (1:500, respectively), PD98.059 or SU1498 10 µM and 40 µM (Calbiochem) and other test substances as indicated in the corresponding figures for 6 hours. VEGF (100 ng/ml) served as positive control. Capillary tubes were counted as described previously.

For apoptosis assay, HCAECs were incubated with EGM-2 medium without supplements containing SN 100 ng/ml or VEGF 50 ng/ml with or without SN-Ab or VEGF-Ab (1:500, respectively) for 24 hours (HCAEC). TUNEL assay was performed according to the manufacturer’s instructions (Roche) and cells positive for TUNEL staining and for DAPI staining were counted. Results are expressed as % TUNEL positive cells of all DAPI stained cells.

Caspase 3/7 assays were performed with cells seeded in 96 well plates, as described by the manufacturer (Promega), after 16 hours treatment with the corresponding substrates.
Proliferation assays were performed using a BrdU cell proliferation ELISA kit from Roche and cells were treated as recommended by the manufacturer and analyzed after 16 hours treatment with the corresponding substrates.

**Western Blotting**

HCAECs were maintained as described above, plated on 60 mm tissue culture dishes and starved with EGM-2 medium without supplements containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich) for 16 hours. The cells were stimulated with SN 100 ng/ml (with or without VEGF-Ab 1:500 or SU1498 40µM for different time periods. For investigation of the role of heparan sulfate proteoglycans, cells were preincubated with a mixture of heparinase I and III (both Sigma-Aldrich), 50 mU each, for 4 hours prior to stimulation with SN. Cells were lysed with TAT buffer containing 1% Triton X-100, protease inhibitor cocktail (complete, Mini, EDTA-free, Roche) and phosphatase inhibitors (Halt™ phosphatase inhibitor cocktail, Roche). Respectively 12 µg protein (determined with bicinchoninic acid, BCA from Thermo Scientific), was separated by gel-electrophoresis (Bio-Rad, 4–15% Tris–HCl Ready Gels), transferred to Protran Nitrocellulose Transfer membranes (Whatman) and blocked by 5% non-fat milk in PBST (Phosphate-buffered saline with 0,1% Tween 20). Blots were incubated with primary antibodies against phospho-p44/42 MAPK, phospho-Akt (Ser473) and tubulin (all from Cell signaling) diluted 1: 1000 in 5% BSA in PBST for 16 hours. For receptor blotting, p-Flk-1 (Tyr 951) and Flk-1 (C-1158) antibodies (both from Santa Cruz) were allowed to incubate on the membranes over night in 5% BSA in PBST. After washing, blots were incubated for 80 minutes with the respective secondary HRP-conjugated antibodies (goat anti-rabbit and goat anti-mouse from Jackson ImmunoResearch diluted 1:10.000) and washed again. Signals were visualized by ECL staining (GE Healthcare).

**RTK Profiler and Immunoprecipitation**

Human phospho-RTK array Kits (R&D, ARY001) were used for investigation of receptors involved in HCAECs and HCASMCs signaling with SN. After stimulation with SN 100 ng/ml ±
VEGF-Ab for 40 minutes, cells (with or without starvation for 16 hours in empty medium) were treated as recommended by the manufacturer and a total of 300 µg protein was used. For Immunoprecipitation (IP), cells were stimulated with SN 100 ng/ml for different time periods and lysed as described above. IP was performed by incubation of the lysates with VEGFR2 (55B11, Cell Signaling) coupled on G-sepharose (GE Healthcare) for 16 hours. After gel-electrophoresis (Bio-Rad, 5% Ready Gels), proteins were blotted to PVDF membranes (GE Healthcare) and incubated with phospho-tyrosine antibody (4G10, Upstate) for 16 hours. Total receptor was detected with Flk-1 rabbit polyclonal antibody diluted 1: 200 (C-1158, Santa Cruz). Signals were visualized by ECL staining (GE Healthcare).

I125VEGF-binding assays on HCAECs

For binding assays HCAECs were cultured in 24-well plates to near confluence and washed twice with ice cold PBS. Cells were then allowed to incubate with 250.000 cpm I125VEGF (10 ng/ml I125VEGF) ± SN (1 to 100 ng/ml) at 4°C for 2 hours in EBM-2 without supplements, containing 0.1% gelatin (G1393, Sigma-Aldrich). Thereafter, cells were washed 3 times with ice cold PBS, containing 1% BSA (Cohn V fraction, Sigma-Aldrich). Cells were lysed with 500 µl cold PBS with 1% Triton X-100 and collected with cell scrapers (Greiner). 300 µl of each sample were transferred to radio-immuno tubes and measured with a γ-counter (Perkin Elmer). For saturation curves, HCAECs were incubated with increasing amounts of I125VEGF (0.1 to 20 ng I125VEGF) with or without addition of SN 100 ng/ml. To determine specific binding a 500-fold excess of cold VEGF was added. Data from saturation were analyzed using GraphPad Prism 5.0. Investigation of influence of heparinase was performed by preincubation of the cells with a mixture of heparinase I and heparinase III, 50 mU each, for 4 hours at 37°C, prior to the experiment.

I125VEGF-binding assays to heparin, neuropilin 1 and VEGF-receptor 2

Maxisorb tubes (Nunc) were coated with 100 µl heparin-BSA complex (BSA: 0.2 µg/ml Tris-EDTA buffer, pH 7.4), or BSA-control complex over night at 4°C. Heparin-BSA and BSA-
control complex were synthesized as described previously. After 3 times washing with 350 µl PBST (phosphate-buffered saline with 0.1% Tween 20), tubes were blocked with 1% BSA (150 µl) for 1 hour at room temperature and washed again 3 times with PBST. Thereafter, I125VEGF 50,000 cpm (2.5 ng/ml I125VEGF) in 50 µl phosphate buffer (10 mM, pH 7.0) containing 1% BSA and 10 to 100 ng/ml SN, was added and allowed to incubate for 2 hours at room temperature. After 3 times washing with PBST, tubes were counted in a γ-counter.

For receptor binding assays, tubes were incubated with 1 µg/ml carrier free, recombinant human neuropilin 1, VEGF-receptor 2 (both R&D) or BSA as control over night at 4°C and then treated as specified above. To determine specific binding, a 500-fold excess of cold VEGF was added.

Real time PCR of SG2

Quantitative real-time PCR was used to determine m-RNA of SG2 levels in HCM, HCAEC and HCF (Promo Cell) after 72 hours in the corresponding culture medium (Promo Cell) under normoxic or hypoxic conditions. Normal humidified tissue culture incubators with 5% CO₂ were used for the normoxic cultures. For decreased oxygen cultures, plates were inserted into gas-tight modular incubator chambers (Billups-Rothenberg, Del Mar, CA, USA), which were flushed with a custom gas mixture containing 5% CO₂ and 95% N₂ for 15 minutes daily. RNA was isolated with RNeasy Mini Kits (Qiagen) as suggested by the manufacturer. 1 µg of total RNA was transcribed as described above and cDNA was finally used as template for real time PCR using a BioRad C 1000 cycler with CFX96 optical reaction module and SsoFast™ Eva Green® Supermix (BioRad). For amplification, primer pairs specific for human SG2 were used (forward primer: AGA ACG GGG AGG AAT ATG CT; reversed primer: GGT CTT TGC TTC AGC CAT GT). The reaction sequence included 35 sec at 95°C and 10 sec at 60°C for 40 cycles. Relative gene expression was calculated using the Ct method with normalization to tubulin (forward primer: CAG GCT GGT GTC CAG ATT GGC AA; reversed primer: CGT CTC ACT GAA GAA GGT GTT GAA GGA).
Radioimmunoassay (RIA) for SN

RIA was performed as described previously.⁶
Supplemental Results

SN gene therapy reduces organ congestion 4 weeks after MI

After SN-plasmid injection, heart-weight/body-weight ratio (HW/BW) and lung-weight/body-weight (LW/BW) were significantly lower than in the control plasmid group 4 weeks after MI (suppl. Table. 1).

SN gene therapy reduces apoptosis in a rat model of myocardial ischemia reperfusion

TUNEL staining (with alkaline phosphatase) for apoptotic cells 3 days after 30 minutes of ischemia showed significantly lower apoptotic nuclei in the SN-treated animal group compared to control group (p-SN: 34.9±1.4 vs. p-CTR: 39.5±1.2, #p=0.02; n=3; suppl. Fig. 1).

SN induces migration of HCASMCs

SN caused induction of chemotaxis in HCASMCs in concentrations of 10 and 100 ng/ml, with similar effects (SN 10 ng/ml relative CI: 1.75±0.13, SN 100 ng/ml 1.74±0.22, p=0.0002 vs. Control). SN mediated migration was comparable to the effects of VEGF 50 ng/ml (rel. CI: 1.89±0.19) and PDGF-BB 10 ng/ml (rel. CI: 1.91±0.13). SN induced migration could not be blocked with VEGF-Ab (rel. CI: 1.63±0.41; suppl. Fig. 2A).

SN mediated tube formation is not blocked by bFGF-Ab in HCAECs

SN 100 ng/ml induced angiogenesis in a matrigel assay with HCAECs (relative capillary tube formation 2.35±0.19, p=0.00005 vs. Control; suppl. Fig. 2B). Addition of VEGF-Ab abolished SN-induced tube formation (SN + VEGF-Ab 1:500, 1.24±0.13, p=0.0004 vs. SN and SN + VEGF-Ab 1:1000, 1.38±0.31, p=0.02 vs. SN). Interestingly, a bFGF-Ab did not influence SN-induced tube formation (SN + bFGF-Ab 1:500, 2.15±0.22, p=0.002 vs. Control). VEGF 50 ng/ml served as positive control.
SN does not increase binding of I125VEGF to VEGFR2

In order to test the possibility that SN increases the binding of VEGF to VEGFR2, we performed binding assays with I125VEGF. Maxisorb tubes were coated with recombinant, human VEGFR2 and binding assays in the presence or absence of SN 10 and 100 ng/ml were performed. SN 100 ng/ml did not increase I125VEGF binding to VEGFR2 (I125VEGF bound to VEGFR2: 745.6±15.2 fmol/µg; I125VEGF + SN 100 ng/ml bound to VEGFR2: 780.3±5.6 fmol/µg; suppl. Fig. 3A).

SN increases binding of I125VEGF to heparin

For heparin binding studies, heparin-coated beads were incubated with I125VEGF in presence or absence of SN 100 ng/ml. SN significantly increased the binding of I125VEGF to heparin (I125VEGF 2090.5±90.2 fmol vs. I125VEGF + SN 3177.6±52.7 fmol, p=0.009; suppl. Fig. 3B).

SN shows a tendency to improve cardiac function and blood vessel density 2 weeks after MI

Echocardiographic assessment of myocardial function 2 weeks after LAD ligation shows a tendency to improve left ventricular ejection fraction (LVEF: p-SN 54.3±7.3 % vs. p-CTR 38.8±3.9 %; sham operation 59.7±4.2 %) and fractional shortening (LVFS: p-SN 34.2±3.7 % vs. p-CTR 24.4±3.7 %; sham operation 42.9±2.6 %) in animals treated with SN-plasmid (p-SN) compared to control plasmid (p-CTR). Results did not reach statistical significance (suppl. Fig. 4A). Left ventricular end diastolic diameter (LVEDD: p-SN 7.8±0.4 mm vs. p-CTR 8.5±0.5 mm;) and systolic diameter (LVESD: p-SN 5.2±0.5 mm vs. p-CTR 6.4±0.5 mm) also revealed a trend for inhibition of left ventricular dilatation 2 weeks after MI by SN gene therapy compared to treatment with control vector. Results did not reach statistical significance (suppl. Fig. 4B). Quantification of RECA-positive capillaries (p-SN: 292.8±56.9 vs. p-CTR: 266.3±62.5, n=5) and α-SMA-positive arteries/arterioles (p-SN: 5.4±1.4 vs. p-
CTR: 4.6±1.2, n=5) in the rat myocardium border zone of the MI, 2 weeks after LAD ligation and treatment with SN-plasmid or control plasmid again showed a trend to improve with p-SN but did not reach statistical significance between groups (suppl. Fig. 4C).

**Hypoxia increases SN in human cardiac myocytes (HCM) but not in HCAECs in vitro**

SG 2 m-RNA is up-regulated under hypoxia in HCMs after 72 hours as measured by real time PCR (Hypoxia: 1.48±0.05, p=0.002 vs. Normoxia, suppl. Fig. 5A). SN is also increased at the protein level by hypoxia, as detected by radioimmunoassay (Hypoxia 57.2±1.6 fmol/10^5 cells vs. Normoxia: 39.3±1.1 fmol/10^5 cells, p=0.0009, suppl. Fig. 5B). In contrast to HCM, SG2 mRNA levels are not influenced by hypoxia in HCAECs (Hypoxia: 0.96±0.3, suppl. Fig. 5C).

**Hypoxia increases SN in rat cardiac myocytes in vivo**

Secretogranin 2 (SG2, pro-hormone of SN) m-RNA is up-regulated in ischemic rat heart in vivo 3 days after MI as measured by real time PCR (ischemic ventricle: 2.2±0.3 vs. CTR, p=0.009; n=3). Healthy heart tissue served as control (suppl. Fig. 6A). Immunofluorescent staining for SN (green) in the border zone of MI in rat hearts 3 days after LAD ligation confirms findings of real time PCR. H33258 was used for nuclei visualization (suppl. Fig. 6B).

**Hypoxia does not increase SG2 mRNA in HCF**

m-RNA of human cardiac fibroblasts (HCF) after 72 hours hypoxia was screened for Secretogranin 2 (pro-hormone of SN) by real time PCR (Hypoxia: 0.90±0.07; n=5), but did not reveal elevated SG2 m-RNA levels under hypoxic conditions (suppl. Fig. 7A).
**SN does not increase proliferation in HCF**

BrdU assays performed with HCF showed no SN-induced HCF proliferation (SN 10 ng/ml: 1.13±0.04; SN 100 ng/ml: 1.18±0.05). bFGF served as positive control (3.01±0.07; *p=0.00003 bFGF vs. Control, n=4). Results are shown as mean ± SEM (suppl. Fig. 7B).

**SN does not inhibit apoptosis in HCF**

SN showed no anti-apoptotic effects on HCF as measured by caspase 3/7 assays after 16 hours of starvation (SN 10 ng/ml: 0.96±0.02; SN 100 ng/ml: 0.97±0.06). bFGF was used as positive control (0.81±0.03; #p=0.047 bFGF vs. Control, n=4). Results are shown as mean ± SEM (suppl. Fig. 7C).

**SN induced tube formation in HUVECs is not influenced by bFGF-Ab or VEGF-Ab.**

Tube formation assays with SN 100 ng/ml in HUVECs revealed no influence of bFGF-Ab or VEGF-Ab on in vitro angiogenesis in these cells (*p<0.001 vs. Control; suppl. Fig. 8).
Supplemental Table

Table 1

<table>
<thead>
<tr>
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<th>HW/BW [mg/g]</th>
<th>LW/BW [mg/g]</th>
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p<0.005  p<0.05

Supplemental Figures

Suppl. Figure 1
Suppl. Figure 2

A

Suppl. Figure 3

A

B
Suppl. Figure 4

A

![Graph of LV EF and FS](#)

B

![Graph of LVEDD and LVESD](#)

C

![Graph of capillaries and arterioles](#)
Suppl. Figure 5

A

![Graph showing SG2 mRNA levels in 72h Normoxia and 72h Hypoxia](image)

B

![Graph showing SN [fmol/10^6 cells] in 72h Normoxia and 72h Hypoxia](image)

C

![Graph showing SG2 mRNA levels in 72h Normoxia and 72h Hypoxia](image)

Suppl. Figure 6

A

![Bar chart showing SG2 mRNA levels in CTR and 3d post OP](image)

B

![Images of fluorescence microscopy showing different conditions](image)
Suppl. Figure 7

A

B

relative proliferation

Normoxia Hypoxia

72h

72h

C

rel. apoptosis [caspase 3/7]

Control SN 10 ng/ml SN 100 ng/ml bFGF 50 ng/ml

relative tube formation

SN + tFGF-Ab 1:500 SN + VEGF-Ab 1:500

SN + bFGF-Ab 1:1000 VEGF-50 ng/ml

#
Supplemental Legends

**Suppl. Table 1 Morphological data at 4 weeks after MI** showed a significant difference in heart-weight/body-weight ratio (HW/BW) and lung-weight/body-weight ratio (LW/BW) in the SN treated group. Results are shown as mean ± SEM (HW/BW (n=16): p=0.0042 p-SN vs. p-CTR; LW/BW (n=11): p=0.029 p-SN vs. p-CTR).

**Suppl. Figure 1. SN gene therapy reduces apoptosis in a rat model of ischemia reperfusion.** TUNEL staining for apoptotic nuclei 3 days after 30 min ischemia (arrows indicate apoptotic (violet) nuclei). Quantification showed significant inhibition of cell apoptosis in the SN treated animal group vs. p-CTR group (#p<0.05; n=3 per group). Results are shown as mean ± SEM.

**Suppl. Figure 2. A SN induces migration of HCASMCs.** Migration assays with HCASMCs revealed chemotaxis towards SN (10 and 100 ng/ml), VEGF 50 ng/ml and PDGF-BB 10 ng/ml. A VEGF-Ab (1:500) did not significantly influence SN-activated migration of SMCs (*p<0.001 vs. Control; n=3). **B SN mediated tube formation is not blocked by bFGF-Ab in HCAECs.** HCAECs were allowed to form tubes in a matrigel assay in presence or absence of SN 100 ng/ml ± b-FGF or VEGF-Ab. VEGF served as positive control. SN induced tube formation is blocked by VEGF-Ab but not by bFGF-Ab (*p<0.001 SN and SN + bFGF-Ab vs. Control; SN+VEGF-Ab 1:500 vs. SN, ** p<0.001; SN+VEGF-Ab 1:1000 vs. SN, # p<0.05; n=3).

**Suppl. Figure 3. A SN does not increase binding of I125VEGF to VEGFR2.** Maxisorb tubes were coated with recombinant, human VEGFR2 and binding assays in the presence or absence of SN 10 and 100 ng/ml were performed and analyzed in a γ-counter. SN 100 ng/ml did not increase I125VEGF binding to VEGFR2 (n=4). **B SN increases binding of I125VEGF to heparin-sepharose.** Binding assays with heparin-coated sepharose beads
showed that SN 100 ng/ml significantly increased I125 VEGF binding to heparin (+p<0.01 VEGF vs.SN + VEGF; n=4). Results are shown as mean ± SEM.

**Suppl. Figure 4. A Effect of SN gene therapy on cardiac function 2 weeks after MI.**
Echocardiographic assessment of myocardial function showed a tendency to improvement of left ventricular ejection fraction and fractional shortening (LVEF and LVFS) 2 weeks after MI and treatment with SN-plasmid (p-SN) compared to control plasmid (p-CTR). Results are shown as mean ± SEM (n=9 per group) but did not reach statistical significance. **B Effects of SN gene therapy on left ventricular remodeling.** Echocardiographic analysis of left ventricular end diastolic and systolic diameter (LVEDD and LVESD) revealed a trend for inhibition of left ventricular dilatation 2 weeks after MI by SN gene therapy compared to treatment with control vector. Results are shown as mean ± SEM but did not reach statistical significance. **C Quantification of CD-31 positive capillaries and α–SMA positive arteries/arterioles** in the rat myocardium border zone of the MI, 2 weeks after LAD ligation and treatment with SN plasmid or control plasmid. Results are shown as mean ± SEM but did not reach statistical significance.

**Suppl. Figure 5. A Hypoxia increases SN in cardiac myocytes but not in HCAECs.** Secretogranin 2 (pro-hormone of SN) m-RNA is up-regulated under hypoxia in human cardiac myocytes after 72 hours as measured by real time PCR ($p<0.005$ vs. Normoxia; n=4). Results are shown as mean ± SD. **B SN** is also increased by hypoxia at the protein level as detected by radioimmunoassay (*p<0.001 vs. Normoxia; n=4). Results are shown as mean ± SEM. **C SG2 mRNA** is not increased in human arterial coronary ECs (HCAECs) after 72 hours of hypoxia. Results are shown as mean ± SD.

**Suppl. Figure 6. A Hypoxia increases SN in cardiac myocytes in vivo.** Secretogranin 2 (pro-hormone of SN) m-RNA is up-regulated in ischemic rat cardiac myocytes 3 days after MI as measured by real time PCR (+p<0.01 vs. CTR; n=3). Non-ischemic heart tissue served as control. Results are shown as mean ± SD. **B Immunofluorescent staining for SN** in
the border zone of MI in rat hearts 3 days after LAD ligation confirm findings of real time PCR. H33258 was used for nuclei visualization.

Suppl. Figure 7. A Hypoxia does not increase SG2 mRNA in HCF. Secretogranin 2 (pro-hormone of SN) m-RNA is not influenced by hypoxia in human cardiac fibroblasts after 72 hours as measured by real time PCR (n=5). Results are shown as mean ± SD. B SN does not increase proliferation in HCF. bFGF served as positive control (*p<0.01 bFGF vs. Control, n=4). Results are shown as mean ± SEM. C SN does not inhibit apoptosis in HCF. As positive control bFGF was used (#p<0.05 bFGF vs. Control, n=4). Results are shown as mean ± SEM.

Suppl. Figure 8. SN induced tube formation in HUVECs is not influenced by bFGF-Ab or VEGF-Ab. Tube formation assays with SN 100 ng/ml ± bFGF-Ab or VEGF-Ab in HUVECs (*p<0.001 vs. Control; n=3). Results are shown as mean ± SEM.
Supplemental References


