The Neuropeptide Catestatin Acts As a Novel Angiogenic Cytokine via a Basic Fibroblast Growth Factor–Dependent Mechanism


Rationale: The neuropeptide catestatin is an endogenous nicotinic cholinergic antagonist that acts as a pleiotropic hormone.

Objective: Catestatin shares several functions with angiogenic factors. We therefore reasoned that catestatin induces growth of new blood vessels.

Methods and Results: Catestatin induced migration, proliferation, and antiapoptosis in endothelial cells and exerted capillary tube formation in vitro in a Matrigel assay, and such effects were mediated via G protein, mitogen-activated protein kinase, and Akt. Catestatin-induced endothelial cell functions are further mediated by basic fibroblast growth factor, as shown by blockade of effects by a neutralizing fibroblast growth factor antibody. Furthermore, catestatin released basic fibroblast growth factor from endothelial cells and stimulated fibroblast growth factor signaling. In addition to its function on endothelial cells, catestatin also exerted effects on endothelial progenitor cells and vascular smooth muscle cells. In vivo, catestatin induced angiogenesis in the mouse cornea neovascularization assay and increased blood perfusion and number of capillaries in the hindlimb ischemia model. In addition to angiogenesis, catestatin increased density of arterioles/arteries and incorporation of endothelial progenitor cells in the hindlimb ischemia model, indicating induction of arteriogenesis and postnatal vasculogenesis.

Conclusion: We conclude that catestatin acts as a novel angiogenic cytokine via a basic fibroblast growth factor–dependent mechanism. (Circ Res. 2010;107:1326-1335.)

Key Words: angiogenesis ■ blood vessels ■ endothelium ■ endothelial progenitor cells
tion of angiogenesis often result in pathological conditions such as inflammatory diseases, ischemic heart, peripheral vascular diseases, proliferative retinopathy, and solid tumors. The most potent angiogenic factors are basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Besides bFGF and VEGF, a variety of neuropeptides like substance P (SP), secretoneurin (SN), and neuropeptide (NP)Y exert effects on endothelial cells (ECs) and induce angiogenesis.

CST shares several features with SP such as vasodilation by CST11a and SP12 and having comparable pI (CST, 12.16; versus SP, 11.14). In addition, CgA was shown to be upregulated by hypoxia in neuronal cells after transient hypoxia, a typical characteristic of angiogenic factors like VEGF. Based on the above similarities of CST with angiogenic peptides, we reasoned that CST would exert effects on ECs in vitro and induce angiogenesis in vivo. The present report establishes CST as a novel angiogenic cytokine with the potential of inducing therapeutic effects in animal models of ischemia.

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

CST Peptide and Antiserum
Human CST and antiserum have been described previously.

Endothelial Progenitor Cell Isolation
Endothelial progenitor cells (EPCs) were isolated and cultured, and EPC chemotaxis assay was performed as described.

Tube Formation Assay
Tube formation assay was performed as described. Some Matrigel assays were performed with a mixture of 5000 HUVECs and 3000 Dil LDL-labeled EPCs. The association of EPC to capillary structures was quantified using Image J.

Cornea Neovascularization Assay
Pellets containing 500 ng of CST, scrambled CST, or VEGF were implanted in C57BL/6J mice as described. On postoperative day 7, mice received a IV injection of 500 μg of BS1 lectin conjugated to FITC (Vector Laboratories). After euthanasia, enucleated eyes were fixed in 1% paraformaldehyde and corneas dissected and examined by fluorescence microscopy.

Mouse Hindlimb Ischemia Model
C57BL/6 wild-type mice were subjected to unilateral hindlimb surgery. Mice were injected with saline or 20 μg of CST into thigh and calf muscles after operation and every other day for weeks 1 and 2 and 2 times per week for weeks 3 and 4. Limb necrosis status (see also the Online Data Supplement) was determined on days 7, 14, and 28.

Bone Marrow Transplantation Model
Bone marrow transplantation (BMT) was performed as described. Labeling of functional vessels and of EPCs in BMT mice was performed 3 weeks after induction of ischemia as described. EPCs are expressed as number of double positive cells per high-power field (magnification, ×100).

For confocal microscopy, a Broadband Confocal Leica TCS SP5 microscope (lenses: HCX PL APO CS 20×0.7 [dry] and HCX PL APO CS 63×1.2 [water]) was used.

Blood Flow Measurement
Blood flow measurements were performed using a laser Doppler perfusion imaging (LDPI) analyzer (Moor Instruments). Blood perfusion is expressed as the LDPI index representing the ratio of left (operated, ischemic leg) versus right (not-operated, not-ischemic leg) limb blood flow.

Immunohistochemistry
CD31 and α-smooth muscle actin (SMA) staining were performed as described. For fluorescent microscopy, appropriate secondary antibodies (Alexa 488 for SMA and Alexa 594 for CD31; both from Invitrogen 1:200) were used.

Results

CST Effects on Migration, Proliferation, and Apoptosis in ECs In Vitro
CST caused dose-dependent induction of chemotaxis in human umbilical vein endothelial cells (HUVECs) with a maximum effect at 1 mmol/L (relative chemotaxis index [CI], 1.67±0.06, P<0.01 versus control; Figure 1a) comparable to other angiogenic factors like VEGF or SN. Blockade of CST by a specific neutralizing antibody completely inhibited CST-mediated EC migration, indicating specificity of the observed effect (relative CI: CST 1.74±0.06;
CST+antibody, 1.12±0.06; P<0.01, CST versus CST+Ab; Figure 1b). CST-induced chemotaxis was completely abolished by inhibition of G-protein coupled receptors by pertussis toxin (PTX) and mitogen-activated protein kinase (MAPK) pathway by PD 98059 (PD), indicating CST signaling via G protein–coupled receptors and MAPK pathway (relative CI: CST 1.74±0.06; CST+PTX 1.0±0.04; CST+PD 1.08±0.05; P<0.01, CST versus CST+PTX and CST+PD; Figure 1b). Tyrosine kinase inhibitor genistein (1 µmol/L) caused only partial inhibition of CST-induced EC migration (relative CI: CST 1.73±0.05; CST+genistein 1.41±0.04; P<0.05, CST versus CST+genistein).

CST also induced proliferation of HUVECs (as determined by cell number) with a maximum effect at 1 nmol/L (relative cell number 1.85±0.11; P<0.01 versus control), and such effect was blocked by pretreatment with CST antibody (relative cell number 0.97±0.06; P<0.05 versus CST) and blockade of MAPK by PD (relative cell number, 1.19±0.09; P<0.05 versus CST), indicating specificity of CST effect and its signaling through MAPK pathway (Figure 1c). CST-induced EC proliferation was also determined by bromodeoxyuridine (BrdUrd) staining (Figure 1d). CST, like VEGF, significantly increased BrdUrd incorporation. ECs were stimulated with CST at 1 nmol/L and 300 nmol/L, with VEGF (50 ng/mL) (all 10 minutes) with the combination of VEGF and CST 300 nmol/L or with 20% FBS, and extracts were analyzed for ERK or Akt activation by Western blotting. CST induced ERK and Akt phosphorylation at low and even more at high concentrations and did not block VEGF-induced stimulation of these pathways. Actin and tubulin stains were used as loading controls.

To test the potential effects of CST on EC apoptosis, HUVECs were serum-starved and stained for TUNEL and DAPI. CST caused significant inhibition of apoptosis (percentage of TUNEL-positive apoptotic cells of DAPI-positive cells) at 1 nmol/L, whereas coincubation with WM (10 nmol/L) inhibited CST-induced protective effect. *P<0.05 vs control; §P<0.05 vs CST; n=3. Representative images of TUNEL and DAPI stains are shown on the right; arrowheads are examples of double positive cells; arrow in control DAPI, cell positive for DAPI and negative for TUNEL. f, CST- and VEGF-induced ERK and Akt activation. ECs were stimulated with CST at 1 nmol/L and 300 nmol/L, with VEGF (50 ng/mL) (all 10 minutes) with the combination of VEGF and CST 300 nmol/L or with 20% FBS, and extracts were analyzed for ERK or Akt activation by Western blotting. CST induced ERK and Akt phosphorylation at low and even more at high concentrations and did not block VEGF-induced stimulation of these pathways. Actin and tubulin stains were used as loading controls.
revealed activation of extracellular signal-regulated kinase (ERK) and Akt, as judged by phosphorylation of ERK and Akt (Figure 1f). CST was able to stimulate ERK and Akt phosphorylation at 1 and 300 nmol/L; VEGF was used as positive control. In contrast to the N-terminal CgA peptide vasostatin, CST did not inhibit VEGF-induced ERK activation in ECs.

CST Induction of Angiogenesis In Vitro
CST induced angiogenesis in vitro, as demonstrated by increments in capillary tube formation in a Matrigel assay (Figure 2), with a maximum effect at 1 nmol/L (relative capillary tube formation, 1.77±0.08; *P<0.01 versus control) comparable to VEGF or SN. Conversely, a scrambled peptide exerted no effect. Pretreatment with CST antibody completely abolished CST-induced tube formation (tube formation, 0.87±0.1; *P<0.01 versus CST), indicating specificity of observed effects. In addition, CST-induced tube formation was totally inhibited by coinubcation of CST with PD (tube formation 0.89±0.11, *P<0.01 versus CST), indicating CST signaling through MAPK pathway in in vitro angiogenesis.

In Vivo Results

CST Effect on Cornea Neovascularization Assay
To further establish the role of CST in angiogenesis, we took advantage of the in vivo cornea neovascularization assay. We found that scrambled CST (Figure 3, left) did not induce growth of new vessels toward the pellet, whereas CST induced growth of arteries out of the limbus artery leading to a capillary network around the pellet. Of note, vessels reaching the limbus vein and presumably representing veins were also observed (Figure 3, middle). VEGF was used as positive control and showed a distinct pattern of neovascularization with higher density of capillaries at the leading front of vessels (Figure 3, right).

CST Effects on Hindlimb Ischemia Model
Intramuscular injection of CST into the ischemic limb significantly inhibited tissue necrosis and amputation as shown by reduced necrosis score 14 and 28 days after induction of ischemia and CST treatment (necrosis score day 28: control, 1.8±0.12; CST, 1.3±0.1; *P<0.05; Figure 4a). Furthermore, CST improved blood perfusion, as judged by LDPI (Figure 4b). Three and 4 weeks after induction of ischemia, LDPI ratio increased significantly in CST-treated group as compared with saline-treated group (4 weeks: saline, 0.76±0.04 versus CST, 0.94±0.03, *P<0.01; Figure 4b). Blood vessel density in ischemic muscle immunohistochemistry was determined by CD31 staining (detecting ECs) and by α-SMA (detecting pericytes/smooth muscle cells). Significantly higher

Figure 2. CST induces capillary tube formation in vitro. ECs were seeded onto Matrigel, and capillary tubes were counted. VEGF (50 ng/mL) and SN (1 nmol/L) were used as positive control, and CST induced capillary tube formation with a maximum effect at 1 nmol/L (M). Scrambled CST (CST scramb.) was not effective. PD or a neutralizing antibody blocked CST-induced tube formation. *P<0.05; **P<0.01 capillary tubes vs control; §P<0.01 vs CST; n=3 to 5.

Figure 3. CST induces angiogenesis in vivo in the mouse cornea neovascularization assay. Pellets (P) containing 500 ng of scrambled CST, CST, or VEGF were implanted into the cornea of mice. After 7 days, mice received IV FITC-BS1-lectin and were euthanized, and corneas were dissected and subjected to fluorescent microscopy. CST and VEGF induced growth of arteries (red arrows) originating from the limbus artery (red arrowhead), forming a capillary plexus (white arrow) at the bottom of the pellet and draining the blood via veins (blue arrows) to the limbus vein (blue arrowhead).
densities of capillaries (CD31-positive vessels) were noticeable in CST-treated mice 4 weeks after surgery (capillaries/mm²: CST, 845.5±47.8; saline, 530.4±41.8; \( P < 0.01 \)). Representative images are shown. CST improved perfusion compared with saline 3 and 4 weeks after operation \( (n=10) \). Representative pictures are shown. CST increases capillary density. Sections from ischemic muscles (after 4 weeks) were stained for capillary density by CD31. Values are expressed as CD31-positive capillaries/mm². CST significantly increased capillary density \( (n=10) \). CST increases density of arteries/arterioles. Sections were also stained for \( \alpha \)-SMA. Values are expressed as SMA-positive arteries or arterioles/mm² \( (n=10) \). \( *P<0.05; \ **P<0.01, \) saline vs CST \( \) for a through d. e, Immunofluorescence staining. Adjacent sections of CST-treated ischemic limb stained for CD31 and \( \alpha \)-SMA. Merging of images shows CD31-positive endothelial cells lining the inner surface of the vessels, which are surrounded by \( \alpha \)-SMA-positive smooth muscle cells.

CST-Induced Effects Are Mediated by bFGF
To assess effects of VEGF or bFGF on CST-mediated in vitro angiogenesis, CST was incubated with neutralizing VEGF and bFGF antibodies. Inhibition of VEGF did not influence CST-induced tube formation, but bFGF antibody completely inhibited CST-mediated effects (tube formation CST, 2.05±0.08; CST+VEGF-Ab, 1.98±0.12; CST+bFGF-Ab, 1.3±0.06; \( P < 0.05 \), CST versus CST+bFGF-Ab; Figure 6a). Additionally, bFGF antibody inhibited CST-induced MAPK activation in ECs. When we performed a time course of CST-induced MAPK activation in HUVECs, a biphasic activation of MAPK was observed with a maximum at 10 and 50 minutes (Figure 6b). Whereas early activation of MAPK was not influenced by bFGF antibody, late activation of MAPK was blocked by bFGF inhibition, indicating that CST-mediated late activation of MAPK is mediated by bFGF.
Because bFGF mRNA was not upregulated by CST over a broad range of concentrations and time points (data not shown and Online Data Supplement), we evaluated whether CST releases bFGF protein from ECs. We found that ECs stimulated by 1 nmol/L CST released bFGF into cell supernatant from 30 minutes to 6 hours, with a maximum at 1 hour (pg/mL: control, 28.6±2.2; CST, 53.8±3.4; P<0.01; Figure 6c). Concentration of bFGF was not changed in heparin wash solutions or cell lysates after CST treatment (data not shown).

To evaluate whether CST stimulates bFGF signaling, we performed studies on FGF receptor (FGFR)1 activation. We observed that CST (after 60 minutes), like bFGF (after 30 minutes), stimulated phosphorylation of FGFR1 (Figure 6d).

To investigate the mechanism of bFGF release, cells were
incubated with substances inhibiting different signaling transduction pathway. We observed that inhibition of MAPK by PD blocked CST-induced bFGF release, whereas inhibition of PI3-kinase (by WM), of protein kinase C (by GFX), or of Rho kinase (by Y26732) had no effect (Figure 6e). We also found that PD and FGF antibody inhibited CST-induced FGFR1 activation indicating that CST transactivates FGF receptor by release of bFGF (Figure 6f).

In vivo, inhibition of bFGF by a neutralizing antibody inhibited CST-induced effects on angiogenesis, as shown by increased necrosis score (Figure 6g) and impaired blood perfusion in LDPI measurements (day 28: CST+IgG, 0.75±0.3; CST+bFGF-Ab, 0.46±0.04; n=7; P<0.01; Figure 6h; control day 28: 0.55±0.08; P<0.05 versus CST+IgG, n=3, data not shown). Additionally, CST-induced vasculogenesis in the GFP BMT mouse was inhibited by blockade of bFGF, as demonstrated by reduced GFP+lectin+ EPCs in ischemic limbs (EPCs per high-powered field: control, 8.8±0.8; CST+IgG, 15.9±1.2; CST+bFGFAb, 10.8±0.8; P<0.05, CST+IgG versus CST+bFGF-Ab; Figure 6i) and reduced circulating flk-1+/GFP+ cells (percentage of GFP+ cells; control, 1.2±0.25; CST+IgG, 3.1±0.4; CST+bFGFAb, 1.5±0.3; P<0.05, CST+IgG versus CST+bFGF-Ab; Figure 6j).

Discussion

Overview

CST was initially described as a potent endogenous nicotinic cholinergic antagonist inhibiting nicotine-evoked catecholamine secretion from PC12 cells and primary cultures of bovine chromaffin cells. Subsequently, CST was established as a pleiotropic peptide. CST acts as a negative regulator of hypertension, inotropy, and lusitropy. CST was initially described as a potent endogenous nico-
induces histamine release in vivo in rat and in vitro from mast cells. Furthermore, CST induces directed migration of human blood monocytes, which qualifies CST to be an inflammatory cytokine. Recently, CST has been shown to induce vasodilation in human dorsal hand vein. The present findings establish CST as an angiogenic peptide.

**CST Induces Effects in ECs**

Inflammation and hypoxia are usually accompanied or followed by increased generation of blood vessels. The precursor of CST, has been shown to be upregulated in brain in response to hypoxia, as has been reported previously for angiogenic factors like VEGF. Here, we found that CST exerts several effects on ECs including EC migration and proliferation, and consistent with previous findings in well-established angiogenic peptides VEGF and bFGF, CST also signals through MAPK pathway to induce these effects. Furthermore, inhibition of CST-induced chemotaxis of ECs by PTX indicates CST signaling through G proteins. PI3-kinase/Akt pathway plays a pivotal role in EC survival and we show that CST stimulates Akt phosphorylation, that CST inhibits EC apoptosis, and that inhibition of PI3-kinase by WM abrogates CST-mediated effect. These data suggest that CST signals through the PI3-kinase/Akt pathway to inhibit HUVEC apoptosis.

**CST Effects on Other Vascular Cells (EPCs, SMCs), Vasculogenesis, and Arteriogenesis**

Besides its effects on ECs, CST also induced chemotaxis on other vascular cells like EPCs or SMCs (see the Online Data Supplement). CST also exerted incorporation of EPCs into vascular structures in vitro, and inhibition of bFGF blocked this effect, indicating that bFGF, a factor reported to attract EPCs, mediates CST-induced effects (see below). These findings indicate that this peptide may also induce postnatal vasculogenesis. Consistent with this, we found that CST increases incorporation of EPCs in ischemic hindlimbs in GFP BMT mice, which is a well-characterized model to study these cells. Additionally, we found increased numbers of arteries/arterioles in CST treated ischemic hindlimbs, consistent with induction of arteriogenesis. Upregulation of PDGF-B in ECs might indicate that CST induces maturation of capillaries. In contrast, MCP-1, an important regulator of maturation of preexisting collaterals, was not increased by CST.

**CST Effects on Angiogenesis In Vivo**

Consistent with angiogenic peptides, CST induces angiogenesis in vivo in the mouse cornea neovascularization assay. This in vivo finding further strengthened the ability of CST to induce angiogenesis in the hindlimb ischemia model. Serial measurements of blood perfusion point out that CST increases perfusion to levels before ligation of the femoral artery, yielding a significant better value compared with saline-injected animals. These observations establish CST as a novel angiogenic cytokine. In this context, it is also interesting that the CST precursor CgA was found in motor nerve endplates of skeletal muscles. Future studies will have to reveal whether CST plays a role in physiological processes like nerve–muscle signal transmission or in the pathophysiology of injured skeletal muscle cells, in addition to its ability to increase blood perfusion to these cells.

**CST Effects Are Mediated by bFGF**

Indirect angiogenic factors like sonic hedgehog induce their effects by upregulation of other, direct angiogenic cytokines like VEGF. Furthermore, effects of factors like PDGF-BB or prostaglandin E on vascular cells or angiogenesis have been shown to be mediated by bFGF. We therefore tested whether CST-mediated effects on ECs depend on VEGF or bFGF and found that inhibition of bFGF by a neutralizing antibody indeed blocked CST-mediated in vitro angiogenesis and late MAPK activation. Additionally, we found that CST releases bFGF from ECs with a maximum as early as 1 hour and stimulates FGFR1 activation. Release of bFGF was blocked by inhibition of MAPK, indicating that this signaling pathway is necessary for CST-induced bFGF release. Furthermore, inhibition of bFGF and MAPK blocked CST-induced FGFR1 activation. This finding, as well as the observation that CST-induced FGFR1 activation was delayed compared with bFGF, indicates that CST transactivates FGFR1 by release of bFGF. Therefore, we propose a model for CST action in which bFGF is released from ECs by CST via a MAPK-dependent mechanism, stimulates FGFR1, leading to a second, late activation of MAPK after 50 minutes, in addition to a first, direct CST-mediated MAPK activation independent of bFGF after 10 minutes. This prolonged MAPK stimulation seems to be necessary for CST-mediated EC function, as shown by blockade of CST-induced capillary tube formation by inhibition of bFGF. A similar effect of bFGF on PDGF-BB–induced vascular SMC function has been reported previously. Additionally, we also were able to demonstrate that CST-induced effects in vivo depend on bFGF, as shown by increased necrosis, decreased blood perfusion, reduced EPCs in ischemic muscles, and reduced flk-1/GFP+ cells in the circulation in mice treated with a neutralizing bFGF–Ab. These findings might indicate that bFGF mediates CST-induced mobilization and homing to ischemic muscle tissue.

It should be pointed out that the N-terminal CgA peptide vasostatin inhibits VEGF-induced angiogenesis. In fact, vasostatin blocked VEGF-induced MAPK activation in ECs and inhibited angiogenesis exerted by this factor in vivo in the Matrigel assay. These findings indicate selective inhibition of EC function and angiogenesis by the CgA peptides. Future studies will determine why 2 peptides (vasostatin and CST) derived from CgA exert opposite effects on angiogenesis. It is yet to be determined whether CgA exhibits differential processing to vasostatin and CST in response to different physiological demands. In this context, it is also interesting that TNF-α exhibits dose-dependent, opposing actions on angiogenesis.
Of note, SN, a peptide derived from another member of the chromogranin/secretogranin family, secretogranin II, also induces therapeutic angiogenesis. Several angiogenic cytokines have been used to treat patients experiencing peripheral arterial or coronary heart disease and CST, as well as SN, is emerging as a promising novel candidate in the therapy of these diseases.

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Disclosures

None.

References


**Novelty and Significance**

**What Is Known?**

- Neuropeptides such as substance P, neuropeptide Y, or secretoneurin induce angiogenesis.
- Catestatin (human chromogranin A(362–372); bovine chromogranin A(344–364)) was initially identified as a catecholamine release-inhibitory peptide inhibiting catecholamine secretion in an autocrine/paracrine manner by acting as a nicotinic cholinergic antagonist.
- Subsequent studies established catestatin as a pleiotropic peptide that lowers blood pressure. It induces vasodilation, is a negative inotrope, and has antimicrobial activity. It also stimulates monocyte chemotaxis.

**What New Information Does This Article Contribute?**

- Catestatin induces migration and proliferation of endothelial cells (ECs) and stimulates chemotaxis in vascular smooth muscle cells or endothelial progenitor cells in vitro. These effects are mediated by the activation of Akt and mitogen-activated protein kinase (MAPK) in ECs.
- Catestatin acts as an angiogenic factor in vivo, specifically in the corneal neovascularization model and the hindlimb ischemia model. It stimulates the incorporation of endothelial progenitor cells into ischemia hindlimbs.
- Both the in vitro and in vivo effects of catestatin were blocked by a neutralizing basic fibroblast growth factor (bFGF) antibody, implicating the induction of bFGF-mediated signaling by catestatin via stimulation of bFGF release from ECs and activation of FGF receptor-1.

We hypothesized that the neuropeptide catestatin induces the formation of new blood vessels because of its reported effects on vasodilation coupled with hypoxia-induced upregulation of the catestatin precursor chromogranin A. We found that catestatin induces chemotactic, proliferative, and antiapoptotic effects on ECs in vitro by activating Akt and MAPK. Catestatin induced angiogenesis in the mouse cornea neovascularization assay. In the hindlimb ischemia model, catestatin therapy improved blood flow and reduced necrosis. Immunofluorescent studies revealed increased density of capillaries and arteries and incorporation of endothelial progenitor cells by catestatin, implying that the peptide induces angiogenesis, arteriogenesis, and postnatal vasculogenesis. The in vitro and in vivo effects of catestatin were inhibited by a neutralizing bFGF antibody, indicating that bFGF mediates the action of catestatin. Of note, catestatin releases bFGF from ECs and stimulates FGF receptor-1 in these cells. These findings indicate that catestatin induces therapeutic angiogenesis in the hindlimb ischemia model. Additional studies are required to evaluate the therapeutic potential of this peptide in other ischemic conditions such as ischemic heart disease.
The Neuropeptide Catestatin Acts As a Novel Angiogenic Cytokine via a Basic Fibroblast Growth Factor–Dependent Mechanism

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The Neuropeptide Catestatin Acts As a Novel Angiogenic Cytokine via a Basic Fibroblast Growth Factor–Dependent Mechanism: Correction

In the article that appears on page 1326 of the November 26, 2010 issue, the following sentence should have been included in the Sources of Funding section:

M. Theurl was supported by the intramural funding program of the Medical University Innsbruck for young scientists MUI-START, project 2010012022.

The authors apologize for this error, and the error has been noted and corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/107/11/1326.full.

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Materials and Methods

Materials

Neutralizing basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) antibodies, PD 98059, pertussis toxin, genistein and DAPI were purchased from Sigma (Germany). Anti-phospho tyrosine antibody was from Millipore, antibodies against FGF Receptor 1 and 3 were from Santa Cruz. Scrambled CST peptide was from PiChem, Austria. SDF-1 was from Bachem.

Catestatin peptide and antiserum

Human catestatin CGA_{352-372} (SSMKLSFRARGYFGRPGPQL) was synthesized by the solid-phase method, using 9-fluorenylmethoxycarbonyl protection chemistry, as described previously. Peptides were purified to 95% homogeneity by preparative reversed-phase high-performance liquid chromatography on C-18 silica columns. Authenticity and purity of peptides were further verified by analytical chromatography (reversed phase high-performance liquid chromatography), and electrospray-ionization or matrix-assisted laser desorption ionization mass spectrometry. Polyclonal rabbit antisera recognizing human catestatin were developed by a modification of protocols previously described for other chromogranin peptides.

Endothelial cell (EC) and smooth muscle cell (SMC) chemotaxis assay:

The migratory response of human umbilical vein endothelial cells (HUVEC) and human umbilical artery smooth muscle cells (HUASMC) (both from Promo Cell, Germany)
was measured using a modified 48-well Boyden chemotaxis chamber in which a 8 µm pore-sized cellulose nitrate filter (Sartorius, Göttingen, Germany) separated the upper and the lower chamber. Cells were detached from the tissue flask with 0.05% trypsin and EDTA (Gibco) and re-suspended at a density of 3x10⁵ cells/well in chemotaxis medium [EBM-2 (Clonetics®) for HUVEC or smooth muscle cell medium with 0.75% bovine serum albumin; PAA] before being placed in the upper wells of the chemotaxis chamber (Neuroprobe, Bethesda, Maryland).

Cells were allowed to migrate toward different soluble concentrations of catestatin in a humidified atmosphere (37°C, 5% CO₂) for 8 hours (HUASMC) and 16 hours (for HUVEC). After the incubation time the nitrocellulose filters were dehydrated, fixed and stained with haematoxilin-eosin. Migration into the filter was quantified by measuring microscopically the distance from the surface of the filter to the leading front of the cells. Data are expressed as relative chemotaxis index, which is the ratio between the distance of migration towards test attractants and that toward control medium into the filters. Basal migration of cells typically was 20-30 µm.

To investigate the involvement of mitogen activated protein kinases, G-protein coupled receptors and tyrosine kinases-receptors HUVECs were pre-treated for 90 minutes with PD 98059 (Sigma) 10µmol/L, pertussis toxin (Sigma) 1µg/ml or genistein (Sigma),10⁻⁶M before the chemotaxis assay. For some experiments cells were incubated with CST antibody (1:1000).

**EC proliferation assays:**

To investigate a possible effect of catestatin on endothelial cell proliferation, cells (30,000/well) were seeded in multiwell plates (24 well) and incubated for 36 hours in EBM-2
(Clonetics®) containing 3% FBS (PAA) and solvent, different concentrations of catestatin or 10 ng/ml secretoneurin (Sigma). Subsequently, cells were stained with DAPI (Sigma) and cell numbers were counted using ImageJ 1.4g. Three randomly selected fields were counted per well and each condition was performed in quadruplicates. Three independent experiments were performed. Additionally, EC proliferation was determined by BrdU Assay (Cell Proliferation ELISA BrdU Kit from Roche) as determined by the manufacturer. Briefly, cells were incubated with dilution medium (EBM2+1.5% FBS), VEGF (50 ng/ml) or CST (1nmol/L) for 20 hours. Afterwards cells were labeled with BrdU for 4 hours and processed as suggested by the manufacturer. Signals were analyzed using an Anthos Lucy 1 luminometer.

**Apoptosis Assay:**

For apoptosis assay cells were incubated with control medium or CST 1 nmol/L in serum free EBM-2 for 18 hours. To investigate the effect of inhibition of phosphatidylinositol-3 (PI-3) kinase/Akt pathway on CST mediated effects on EC apoptosis ECs were treated with wortmannin (WM, from Sigma) at a concentration of 10 nM alone or in combination with CST. TUNEL assay was performed according to the manufacturer’s instructions (Roche) and cells positive for TUNEL staining and for DAPI staining were counted and results are expressed as % of TUNEL positive cells of all DAPI stained cells.

**Western Blotting:**

All primary antibodies were purchased from Cell Signaling. HUVECs were plated on 60 mm tissue culture dishes coated with gelatine 0,2 % (Sigma), starved for 12 hours in serum free EBM-2 (Clonetics®), treated with catestatin or VEGF (50 ng/ml), and processed for western blotting, as suggested by the manufacturer. Serum free medium served as negative control and 20% FBS (PAA) as positive control. For some experiments cells were co-incubated with CST and bFGF Antibody 1:1000.
bFGF-ELISA:

ELISA was purchased from R&D. HUVEC were starved for 6 hours and treated with 1nmol/L CST for 30 minutes to 6 hours. Supernatants were removed from cells, cells washed with PBS and a heparin solution and finally lysed as described. bFGF ELISA was performed from supernatants, heparin washes and cell lysates.

FGF-Receptor-1 and -3 activation

HUVEC were stimulated with bFGF (20 ng/mL) for 30, 60 and 90 minutes or CST (1 nmol/L) for 30, 60, 90 and 180 minutes. For some experiments CST was co-incubated with PD 98059 (10 µM) or a bFGF antibody (1:1000). Afterwards cells were processed for FGF-Receptor-1 phosphorylation by immunoprecipitation for FGF-Receptor-1 and subsequent phosphor-tyrosine immunoblotting and immunoblotting for FGF-Receptor-1 (antibodies from Santa Cruz, USA) as described. For FGF-Receptor-3 activation similar experiments using FGF-Receptor-3 antibody instead of FGF-Receptor-1 antibody were performed for control and 60 minutes stimulation with bFGF and CST as described above.

PCR:

PCR was performed as described to detect mRNA expression of respective proteins and RNA of cells was extracted (Qiagen, RNasy Mini Kit, Vienna, Austria). To eliminate genomic DNA, samples were digested with DNase I (desoxyribonuclease I, Amplification Grade, Invitrogen, Vienna, Austria), then subjected to reverse transcription (Superscript TM First-Strand Synthesis System for RT-PCR, Invitrogen). cDNA was finally used as template for PCR reactions using Biorad C 1000 cycler with CFX96 optical reaction module. As mastermix MESA GREEN qPCR Mastermix Plus for SYBR Assay (Eurogentec) was used. Omitting reverse transcriptase enzyme or cDNA template was used as negative control.
Following primers were used:

human MCP-1 (256 bp)

    fwd CACCAATAGGAAGATCTCAGTGC
    rev AACAGGGTGTCCTGGGAAAG

Following primers were used according to published literature

human SDF-1 (77 bp):

    fwd GAAGCGAAAAAATCAGTGAATAAACC
    rev TGGAACCTGAAACCCTGCTG

human PDGF-B (111 bp):

    fwd CATTCCCCGAGGAGCTTTATGAG
    rev TCCA ACTCGGC CCCCATCT

human VEGF-A (72 bp):

    fwd GTGCCCACTGAGGAGTCCA
    rev TCCTATGTGCTGGCCTTG

human FGF2 (85 bp):

    fwd AGCGACCCTCACATCAAGCTA
    rev CCAGGTAACGGTTAGCACA CACT

**Endothelial progenitor cell (EPC) isolation**

    EPCs were isolated and cultured and EPC chemotaxis assay was performed as we described before. Cultured cell were analyzed by FACS for P1H12 and CD31 and by PCR for KDR and CD31.
**FACS analysis**

FACS analysis of blood samples was performed in GFP bone marrow transplanted mice 3 weeks after induction of hindlimb ischemia and treatment with CST or saline i.m. For some experiments mice also received bFGF-neutralizing antibody or IgG (see above). 200 µl of blood was withdrawn by puncture of the retro-orbital plexus. Cells positive for GFP were analyzed also for VEGF-receptor 2 (flk-1) by incubating samples with PE-conjugated antibody against flk-1 (BD Pharmingen) for 15 min in the dark. Appropriate fluorochrome-conjugated isotype control was used (BD Pharmingen). After incubation, erythrocytes were lysed using FACS lysing solution (BD Pharmingen) and washed with PBS before analysis. Cells positive for flk-1 and GFP are expressed as percentage of all GFP+ cells. Cells were analyzed using a FacsCalibur (Beckton-Dickinson) and CellQuest software (Beckton-Dickinson).

**Tube formation assay:**

For tube formation assays a growth factor reduced matrigel was used (Chemicon). 96-well plates were coated with matrigel and HUVECs (5000/well) were seeded in serum free EBM-2 with or without test substances (SN, CST, CST+PD 98059; CST+Cat Antibody 1:1000; CST+bFGF Antibody 1:1000; CST+VEGF Antibody 1:1000) and incubated for 8 to 12 hours at 37°C and 5% CO₂. Images of tube formation were obtained using an inverted light microscope (Olympus CK30) and the degree of tube formation was quantified by measuring the intersection points in a grid (Plugin „Analyze-Grid Image J 1.4g). Some matrigel assays were performed with a mixture of 5000 HUVECs and 3000 DiI LDL-labelled EPCs as described⁹,¹⁰. All EPCs per high power field were counted using Image J 1.4g (Plugin-Analyze-Cell counter) and cells associated with tubes (in the same field) were expressed as percentage of total EPCs. For more detailed studies of EPC –EC interaction ECs
were labelled with PKH67 green fluorescent cell linker kit (Sigma) as described by the manufacturer.

**Animal models**

All protocols were approved by the Animal Care and Use Committee of the Medical University Innsbruck and Hannover. Mice were purchased from Charles River Laboratories, Kisslegg, Germany. Anesthesia was performed with 2,2,2-tribromoethanol (880 mmol/kg body wt i.p.; Sigma-Aldrich).

**Cornea Neovascularisation Assay:**

Pellets containing 500 ng scrambled CST, CST or VEGF were implanted in C57/BL/6J mice (age of 8 weeks) as described. On postoperative day 7 mice received an intravenous injection of 500 µg BS1 lectin conjugated to FITC (Vector Laboratories). After euthanasia, enucleated eyes were fixed in 1% paraformaldehyde, corneas dissected and examined by fluorescence microscopy.

**Mouse hindlimb ischemia model**

Male C57BL/6 wild-type mice at the age between 12 and 15 months were subjected to unilateral hind-limb surgery as described. Briefly, the left femoral artery was exposed, ligated with 5-0 silk ligatures, and excised. Mice were injected with saline or 20 µg cestatin (n=10 mice/group) into thigh and calf muscles after operation and every other day for weeks 1 and 2 and 2 times per week for weeks 3 and 4 according to previously published work. All further measurements and analyses were performed by investigators blinded to the treatment of the animals. For some experiments mice were treated with a mouse monoclonal neutralizing bFGF antibody or respective IgG control (both from Abcam) via subcutaneously
implanted osmotic minipumps (Alzet) to allow continuous release of a total of 10 µg antibody over 4 weeks. In some mice hindlimb muscles were homogenized 3 days after ischemia and CST content of ischemic and control hindlimbs was determined by CST ELISA (Bachem). In another group of mice CST was injected into hindlimbs (20 µg) and CST content in muscles and serum was analyzed by ELISA after different time-points.

Limb necrosis score was determined as described previously in detail \(^{14}\). Briefly, mice were investigated at day 7, 14 and 28 and scored with 1 point if no necrosis or defect was observed, with 2 points if skin necrosis or below ankle amputation was present and with 3 points if above ankle amputation was present. Scores were averaged for catestatin or saline group.

**Bone marrow transplantation model**

Bone marrow transplantation (BMT) was performed as described \(^{8,15,10}\). Briefly, 8 weeks old wild-type C56BL/6J recipient mice were lethally irradiated. ß-actin/GFP mice (C56BL/6J background) that ubiquitously express GFP were used as bone marrow donors. After irradiation, the recipient mice received bone marrow cells (5 x 10\(^6\)) from the ß-actin/GFP mice by tail vein injection. After 3 weeks reconstitution of transplanted marrow was confirmed by FACS analysis of peripheral leukocytes and >98% of cells were GFP positive (data not shown). 6 weeks after transplantation, hind-limb ischemia surgery and injection of catestatin or saline was performed. Mice were injected with saline or 20 µg catestatin into thigh and calf muscles after operation and every other day for weeks 1 and 2 and 2 times per week for week 3. For some experiments mice were treated with a mouse monoclonal neutralizing bFGF antibody or respective IgG control (both from Abcam) via osmotic minipumps (Alzet) to allow continuous release of a total of 10 µg antibody over 3 weeks. Labelling of functional vessels and of EPCs in BMT-mice was performed 3 weeks after induction of ischemia as described \(^{15}\). Briefly, host-derived vessels were labelled by
intra-venous (i.v.) injection of 100 µL of rhodamine-conjugated BS-1 lectin 20 minutes before euthanasia. Hind-limb muscles were embedded in OCT compound (TISSUE-TEK®, Sakura Finetek) and snap-frozen in liquid nitrogen. 5 µm-thick frozen sections were analysed for red (rhodamine) labelled host derived vessels and for green (GFP) labelled graft bone marrow derived cells. EPCs are considered as double positive stained cells (red for incorporation into the host vasculature and green for bone-marrow origin). EPCs are expressed as number of double positive cells per high-power field (magnification x100). Each muscle was divided into 2 parts and EPCs were counted and averaged from 5 sections of each part. A group of mice was also treated with bFGF neutralizing monoclonal antibody or IgG as described above.

For confocal microscopy a Broadband Confocal Leica TCS SP5 microscope (lenses: HCX PL APO CS 20x0.7 (dry) and HCX PL APO CS 63x1.2 (Water)) was used.

**Blood flow measurement**

Blood flow measurements were performed using a laser Doppler perfusion image (LDPI) analyzer (Moor Instruments, USA) as described \(^\text{12}\). To minimize data variables attributable to ambient light and temperature animals were kept on a heating plate at 37°C before measurement and blood perfusion is expressed as the LDPI index representing the ratio of left (=operated, ischemic leg) versus right (=not-operated, not-ischemic leg) limb blood flow. A ratio of 1 before operation indicates equal blood perfusion of both legs, whereas after femoral artery excision this ratio drops to values between 0.15 and 0.26, indicating severe attenuation of leg blood supply in the operated leg.

**Immunofluorescence**
For tissue staining, mice were sacrificed and ischemic limb tissues were retrieved after 4 weeks. 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. Tissues were sliced into 5-µm sections. Vascular endothelial cells were identified by CD-31 (Pharmingen) or FITC-isolectin-B4 (Vector, dilution 1:100) staining and for assessment of artery/arteriole density sections were stained with a mouse monoclonal alpha-smooth muscle actin (SMA) antibody (Pharmingen) as described. For fluorescent microscopy appropriate secondary antibodies (Alexa 488 or 594 for SMA and Alexa 594 for CD31; both from Invitrogen 1:200) were used. Adductor muscle samples of each leg were divided into 2 parts and capillaries (CD31 positive) and arteries (alpha-smooth muscle actin positive) were counted in five sections of each part and are expressed as capillary and arteriole density per mm².

For staining of myelo-monocytic cells a CD11b antibody was used (clone M1/70 from Bioscience) and detected by a goat-anti-rat Alexa 647 secondary antibody (Invitrogen).

For cell apoptosis assays sections of muscles subjected to hind-limb ischemia and treated by CST or saline after 7 days were stained using the TUNEL assay from Roche (In-Situ Cell Death Detection Kit, Fluorescein) as described by the manufacturer. Sections were additionally stained for DAPI and CD-31.

**Statistical Analysis**

All results are expressed as mean ± SEM. Statistical comparisons between 2 groups were performed by Student-\( t \) test. Multiple groups were analysed by 1-way ANOVA test followed by appropriate post hoc tests to determine statistical significance. Probability values <0.05 were considered statistically significant. All experiments were repeated at least in triplicate.
Supplemental Results

1. Effects of CST on EPCs in-vitro.

To determine potential effects of CST on EPCs we performed cell migration experiments. CST induced migration of EPCs with the maximum effect at 1 nmol/L (relative CI CST 1.64±0.04; P<0.05; Online Figure Ia). SDF-1 and SN were used as positive controls. CST also caused significant increase in percentage of EPCs associated with capillary tubes in the co-culture matrigel assay of HUVEC with Dil-labelled EPCs and this effect was blocked by a bFGF antibody (Ctr 57.6±2.7% of EPCs associated with capillary tubes; CST 1 nmol/L 78.8±2.7%; CST+bFGF Ab 53.4±2.6%; P<0.01 CST vs. Ctr; and CST+bFGF Ab; Online Figure Ib). These findings indicate that CST-mediated EPC-EC interaction in-vitro is mediated by bFGF like other effects of CST on ECs in vitro and on angiogenesis and vasculogenesis in-vivo (Fig. 6).

For more detailed studies of EPC –EC interaction in co-culture EPCs were labelled with DiI-acetylated LDL and ECs with PKH67. Online Figure Ic shows close interaction of EPC (red) and EC (green) (arrowheads), some EPCs are also without contact to tubes (arrow).

2. CST effects on smooth muscle cells (SMCs)

Since formation of arteries or arteriogenesis, requires migration of SMCs, we tested whether cestatin could induce chemotaxis of SMCs. Like the well-established SMC chemotactic agent platelet-derived growth factor (PDGF)-BB, CST also induced chemotaxis of SMCs with a maximum effect at 10^{-9} mol/L (CI 1.64±0.05, P<0.05 vs. Ctr; Online Figure IIa). We additionally evaluated if CST induces release of bFGF from SMCs and found significant release after 1 hour but not at later time-points (pg bFGF/ml: Ctr. 21.9±2.4, CST 1 hour: 35.1±1.7; P<0.05, n=3). Addition of PD but not of bFGF antibody inhibited CST-
induced SMC chemotaxis (Online Figure IIa) indicating that SMC chemotaxis by CST is independent of bFGF like also shown for PDGF-BB\textsuperscript{2}. We also investigated if CST stimulates MAPK in SMCs and found activation of ERK with a maximum after 10 minutes (Online Figure IIb).

3. Regulation of angiogenic factors in ECs by CST

To evaluate if CST up-regulates angiogenic factors in ECs cells were incubated with CST in different concentrations (1 nmol/L, 10 nmol/L) and time-points (3, 6 and 12 hours) and respective m-RNAs were measured by real-time PCR. We found up-regulation of PDGF-B after 6 hours of stimulation with 1 nmol/L CST (rel. m-RNA/18S: 1.62±0.14; P<0.05 vs. Ctr). We did not detect regulation of other factors including VEGF, bFGF, MCP-1 or SDF-1 (Online Figure III).

4. Isolectin staining of capillaries in ischemic muscles

Beside CD31 we also used isolectin to identify ECs in ischemic muscles treated with saline or catestatin. Co-staining with α-SMA was used to identify arterioles/arteries. We found that catestatin increased density of capillaries and arteries using isolectin staining (Online Figure IV).

5. Characterisation of GFP-positive cells after BMT

To evaluate CST-induced post-natal vasculogenesis we used the GFP-bone marrow transplanted mouse. As described above in the Methods section (bone marrow transplantation model) mice were subjected to hindlimb ischemia operation and received i.v. injection of rhodamine-labelled BS-1 lectin before euthanasia. Sections of ischemic muscles were analyzed for GFP+ cells (bone marrow origin) and rhodamine-lectin positive cells. Double
positive cells were considered as EPCs. To additionally characterize cells for monocyte origin we stained cells for CD11b. As shown in Online Figure V we found cells positive for CD11b and GFP (arrows) and, however distinct cells, positive for GFP and BS-1 lectin (arrowheads). This finding indicates that EPCs and CD11b positive monocytes are different cells.

6. CST concentration in ischemic limbs, CST tissue half life

After 3 days of ischemia hindlimb muscles of ischemic and control side were homogenized and CST content analyzed by ELISA. In the non-ischemic limb CST level was below the detection limit of the assay. In the ischemic limb CST concentration was 1.04±0.34 ng/1g wet weight (n=5). This corresponds to an approximately 0.5 nmol/L concentration.

To evaluate half life of GST in the limb tissue 20 µg CST was injected i.m. and CST content was measured in tissue homogenates 1, 2, 4 and 6 hours after injection as well as 48 hours after injection (the time-point when the next injection was performed). From the slope of the curve a half-life of CST of approximately 12 hours can be calculated (Online Figure VI).

7. FGF Receptor 3 activation

FGF Receptor 3 (FGFR3) activation was studied by immunoprecipitation as described above. We found that bFGF and CST activate FGFR3 (Online Figure VII).

8. Catestatin effects on p38 and JNK activation.

Possible activation of p38 and JNK by CST was evaluated in HUVEC by western blotting. Only a slight activation of p38 after 10 and 20 minutes was observed whereas JNK was not activated by CST (Online Figure VIII).

9. Akt-activation of catestatin is not blocked by bFGF antibody.
HUVEC were treated with CST in the presence or absence of a neutralizing bFGF antibody for different time-points and evaluated for Akt activation by western blotting. Akt activation by CST was not blocked by the bFGF-Ab (Online Figure IX).

10. Catestatin inhibits apoptosis in ischemic hindlimbs

Sections of ischemic hindlimbs 7 days after induction of ischemia were stained with DAPI and TUNEL. Calculations revealed significantly decreased apoptotic cells by CST treatment (%TUNEL-positive cells of DAPI-positive cells: Ctr. 2.0±0.1, CST 1.46±0.1; P<0.05; n=4, Online Figure X).
References


Online Figure I

a.

![Graph showing chemotaxis index](image)

b.

![Images showing control (Ctr), CST, CST+FGF-Ab](image)

![Bar graph showing %EPCs associated with tubes](image)
Online Figure I. Catestatin exerts effects on EPCs.

I.a. Catestatin induces EPC migration. Results are expressed as chemotactic index relative to untreated cells. Catestatin induces chemotaxis of EPCs with a maximum effect at 1 nmol/L. *P<0.05, **P<0.01 vs. Ctr.; n=3

I.b. Catestatin increases EPC incorporation into capillary tubes. ECs were seeded onto matrigel together with acylated LDL-DiI labeled EPCs. EPCs incorporated and associated with capillary tubes per high power field were counted and compared to total EPCs in the same field. Catestatin (10^{-7} mol/L, 10^{-9} mol/L increased numbers of EPCs associated with capillary tubes and effect was blocked by bFGF antibody. * P<0.05; ** P<0.01 vs. Ctr.; § P<0.05 CST vs. CST+bFGF-Ab; n=3.

EC and EPC co-culture: double fluorescent labelling (right lower picture). EPCs were labelled with DiI-acetylated LDL and ECs with PKH67. After co-culture picture reveals close interaction of both cell types (arrowheads), some EPCs are located between tubes (arrow).
Online Figure II. Catestatin induces effects on vascular smooth muscle cells.

II.a. Catestatin induces SMC migration. Migration was evaluated for PDGF-BB (10 ng/ml) and different concentrations of catestatin. Catestatin induced migration of these cells with a
maximum effect at a concentration of $10^{-9}$ mol/L. Effect was blocked by PD but not by bFGF-Ab.*$P<0.05$ vs. Ctr.; § $P<0.05$ CST vs. CST+PD; n=3

II.b. Catestatin induces MAPK activation in SMC. CST at a concentration of 1 nmol/L stimulated ERK activation in SMCs with a maximum effect at 10 and 20 minutes.
Online Figure III: Regulation of angiogenic factors in ECs by CST.

ECs were serum starved and treated with 1 nmol/L CST for 6 hours. Real time PCR was used to detect respective m-RNAs. * P<0.05 PDGF-B vs. Ctr, n=3.
Online Figure IV: Catestatin increases lectin-positive capillaries and arteries.

Sections of ischemic hindlimb muscles treated by saline or catestatin were stained by isolectin (FITC labelled, left panels) or SMA antibody (labelled by a red fluorescent secondary ALEXA antibody, middle panels); merged pictures: right panels. Catestatin increases lectin-positive vessels.
Online Figure V: Characterisation of GFP positive cells in the GFP-bone marrow transplanted mouse.

After BMT mice were subjected to hindlimb ischemia operation and treated with CST. Before sacrifice host blood vessels were stained with i.v. injection of rhodamine labelled BS1-lectin. Sections of ischemic hindlimbs were stained with DAPI and CD11b antibody and analysed for GFP+ cells and rhodamine BS1 lectin positive vessels. Merging of pictures (right lower picture) revealed GFP+/CD11b+ cells (arrows) and, distinct from them, GFP+/lectin+ cells (arrowheads).
Online Figure VI: CST tissue half life after i.m. injection

CST levels in hindlimbs were determined by ELISA in tissue homogenates at different time-points after injection of 20 μg CST. From the slope of the curve a half-life of approximately 12 hours can be calculated.
Online Figure VII: Activation of FGF-receptor 3 by CST

HUVEC were stimulated with bFGF (20 ng/ml) and CST (1nmol/L) for 60 minutes and immunoprecipitation using a FGF-receptor 3 antibody was performed followed by immunoblotting for phospho-tyrosine and FGF-receptor 3. CST and bFGF stimulate FGF receptor 3 activation.
Online Figure VIII: Catestatin and p38 and JNK

HUVEC were stimulated with 1 nmol/L CST for different time-points and evaluated for activation of p38 and JNK by western blotting. A slight activation of p38 but no activation of JNK was observed.
Online Figure IX: Akt activation by CST is not blocked by inhibition of bFGF.

HUVEC were stimulated with 1 nmol/L CST for different time points with or without a neutralizing bFGF antibody. CST-induced Akt activation was not blocked by bFGF-Ab.
Online Figure X: Catestatin inhibits apoptosis in-vivo.

7 days after induction of ischemia and treatment with CST or saline sections of ischemic muscles were analyzed for apoptotic cells by TUNEL staining. Fewer cells were TUNEL-positive after CST treatment.
Correction

The Neuropeptide Catestatin Acts As a Novel Angiogenic Cytokine via a Basic Fibroblast Growth Factor–Dependent Mechanism: Correction

In the article that appears on page 1326 of the November 26, 2010 issue, the following sentence should have been included in the Sources of Funding section:

M. Theurl was supported by the intramural funding program of the Medical University Innsbruck for young scientists MUI-START, project 2010012022.

The authors apologize for this error, and the error has been noted and corrected in the online version of the article, which is available at http://circres.ahajournals.org/content/107/11/1326.full.

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