Cortistatin Inhibits Migration and Proliferation of Human Vascular Smooth Muscle Cells and Decreases Neointimal Formation upon Carotid Artery Ligation

Mario Duran-Prado, Maria Morell, Virginia Delgado-Maroto, Justo P. Castaño, Jose Aneiros-Fernandez, Luis de Lecea, Michael D. Culler, Pedro Hernandez-Cortes, Francisco O’Valle, Mario Delgado

1Institute of Parasitology and Biomedicine Lopez-Neyra, CSIC, Granada, Spain; 2Department of Cell Biology, Physiology, and Immunology, University of Cordoba, Reina Sofia University Hospital, Institute Maimonides of Biomedical Research of Cordoba, and CIBER Physiopathology, Obesity and Nutrition, Spain; 3Pathology, Hospital Complex and Northwest and South Areas of Granada, Spain; 4Stanford University School of Medicine, Stanford, California; 5Ipsen Group, Milford, Massachusetts; 6Department of Traumatology and Orthopedic Surgery, San Cecilio University Hospital, Granada, Spain, and; 7Department of Pathology, School of Medicine, University of Granada, Granada, Spain.

Running title: Functional Characterization of Cortistatin on SMCs

Subject codes:
[93] Receptor pharmacology
[96] Mechanism of atherosclerosis/growth factors
[162] Smooth muscle proliferation and differentiation

Address correspondence to:
Dr. Mario Delgado
Institute of Parasitology and Biomedicine Lopez-Neyra
CSIC, 18100
Granada, Spain
mdelgado@ipb.csic.es

In March 2013, the average time from submission to first decision for all original research papers submitted to Circulation Research was 14.5 days.
ABSTRACT

Rationale: Proliferation and migration of smooth muscle cells (SMCs) are key steps for the progression of atherosclerosis and restenosis. Cortistatin is a multifunctional neuropeptide belonging to the somatostatin family that exerts unique functions in the nervous and immune systems. Cortistatin is elevated in plasma of patients suffering coronary heart disease and attenuates vascular calcification.

Objective: To investigate the occurrence of vascular cortistatin and its effects on the proliferation and migration of SMCs in vitro and in vivo, and to delimitate the receptors and signal transduction pathways governing its actions.

Methods and Results: SMCs from mouse carotid and human aortic arteries (hAoSMCs) and from human atherosclerotic plaques highly expressed cortistatin. Cortistatin expression positively correlated with the progression of arterial intima hyperplasia. Cortistatin inhibited platelet-derived growth factor (PDGF)-stimulated proliferation of hAoSMCs via binding to somatostatin-receptors (sst2 and sst5) and ghrelin-receptor (GHSR), induction of cAMP and p38-MAPK and inhibition of Akt activity. Moreover, cortistatin impaired lamellipodia formation and migration of hAoSMCs towards PDGF by inhibiting, in a GHSR-dependent manner, Rac-1 activation and cytosolic calcium increases. These effects on SMC proliferation and migration correlated with an inhibitory action of cortistatin on the neointimal formation in two models of carotid arterial ligation. Endogenous cortistatin seems to play a critical role in regulating SMC function since cortistatin-deficient mice developed higher neointimal hyperplastic lesions than wild-type mice.

Conclusions: Cortistatin emerges as a natural endogenous regulator of SMCs under pathological conditions and an attractive candidate for the pharmacological management of vascular diseases that course with neointimal lesion formation.

Keywords: Cortistatin, smooth muscle cell, atherosclerosis, proliferation, migration, neointimal formation

Nonstandard Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>[Ca(^{2+})](_i)</td>
<td>free cytosolic calcium concentration</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CST(^{-/-})</td>
<td>cortistatin-deficient mice</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular-signaling activated kinase</td>
</tr>
<tr>
<td>GHSR</td>
<td>ghrelin receptor</td>
</tr>
<tr>
<td>hAoSMC</td>
<td>human aortic smooth muscle cell</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>Sst</td>
<td>somatostatin receptors</td>
</tr>
</tbody>
</table>

INTRODUCTION

The process of neointima formation is common to various forms of vascular diseases such as atherosclerosis, restenosis and transplant vasculopathy.\(^1,2\) In response to vascular injury, the medial smooth muscle cells (SMCs) proliferate and migrate into the intima, where they proliferate and secrete abundant extracellular matrix to form the neointima.\(^1\) This exacerbated proliferation and migration of vascular SMCs occurs in response to inflammatory cytokines and growth factors produced in excess after injury.\(^1\) Among them, platelet derived growth factor (PDGF) plays an imperative role in this
coordinated response. PDGF is the most potent mitogen for vascular SMCs and critically drives their migration. Following its production by multiple cells at the site of the injury, including SMCs, activated endothelial cells, platelets and monocytes, PDGF binds to its receptor in SMCs and activates multiple signal transduction pathways inducing the entry in cell cycle and reorganization of cytoskeleton. Thus, compounds able to re-establish any or several of the pathologically signaling pathways up-regulated by PDGF are desirable to combat the abnormal proliferation and migration of SMCs that occurs in many vasculopathies.

Two decades ago, the hypothalamic peptide somatostatin emerged as an interesting putative molecule for the management of cardiovascular diseases, in particular, for the treatment of atherosclerosis and restenosis. Somatostatin acts through a family of five members of G-protein coupled receptors named ssts1-5. Ssts are widespread distributed in many cell types, including SMCs, and regulate multiple signal transduction pathways that lead to inhibition of cell proliferation. Although various evidences indicate that somatostatin inhibits proliferation of SMCs in vitro, there is still controversy regarding the failure of somatostatin or its synthetic analogues to regulate SMC proliferation in vivo. Moreover, there is no actual evidence for the ability of somatostatin to regulate SMC migration.

Cortistatin is a recently discovered peptide that shows a remarkable sequential and structural resemblance with somatostatin, with which shares many functions, especially concerning regulation of hormonal secretion. This apparent functional redundancy relies in the ability of both peptides to indistinctly bind/activate all the ssts. However, cortistatin exerts unique functions in the brain and immune system. Thus, cortistatin, but not somatostatin, decreases locomotor activity, promotes sleep and deactivates inflammatory and autoimmune responses. Similarly, in the cardiovascular system, cortistatin, but not somatostatin, attenuates vascular calcification. These unique functions of cortistatin relate to its ability to bind to receptors other than ssts, such as ghrelin-receptor (GHSR) or the human Mas-related gene X-2. This is partially relevant because arterial SMCs express GHSR and ghrelin regulates angiotensin-stimulated SMCs proliferation. Moreover, patients suffering coronary heart disease show elevated serum cortistatin levels. These findings suggest a potential role for cortistatin in the cardiovascular system. In the present work, we examined the occurrence of cortistatin in the arterial system in normal and pathological conditions and its effect on the proliferation and migration of human aortic SMCs (hAoSMCs) activated with PDGF and we identified the receptors and signal transduction pathways involved in such response. We also evaluated the capacity of endogenous cortistatin to regulate SMC function in vivo by using cortistatin-deficient mice and the therapeutic potential of cortistatin to regulate the formation of neointimal lesions in two different models of carotid artery ligation.

METHODS

Complete and partial ligations of carotid artery.
To induce SMC hyperplasia in vivo, we subjected FVB/NJ, C57BL/6 and cortistatin-deficient (CST-/-) mice to permanent complete ligation of the left common carotid artery near its bifurcation, or alternatively to partial ligation of left carotid artery (see Online Figure I). Animals received intraperitoneally saline (control) or mouse cortistatin-14 (100 ng) every two days starting one day after artery ligation. At different times, we isolated the ligated and contralateral unligated arteries for morphometry, neuropeptide content determination and western blot analysis (see Detailed methods). For morphometric analysis, we fixed the isolated carotid arteries in 4% formaldehyde/0.1 mol/L phosphate buffer pH 7.4 for 6 hours, and then subjected them to cryopreservation in 30% sucrose/0.1 mol/L phosphate buffer at 4°C, embedding in OCT-compound and freezing. We obtained five cryosections (6-µm thick) at 0.5 to 1.5 mm proximal to the ligation site from each animal. We measured areas of intima and media in sections stained with hematoxylin/eosin in a blinded manner using the ImageJ software. Briefly, we calculated the intimal area by subtracting the luminal area from the internal elastic lamina area, and the medial area by subtracting the internal elastic lamina area...
from the external elastic lamina area. We also used cryosections for immunofluorescence analysis (see below).

**Cell growth and proliferation assays.**

We evaluated cell growth of hAoSMCs by measuring reduction of Alamar-Blue reagent and proliferation by measuring incorporation of [3H]-thymidine. hAoSMCs (10^4/well) were cultured for 12 hours in SmGM-2 medium in 96-well plates, and serum-starved for 24 hours in SmBM medium before stimulation with PDGF (10 ng/mL) in the absence or presence of different concentrations of cortistatin, somatostatin or ghrelin (10 nmol/L, except when indicated). We used hAoSMCs incubated with medium alone as unstimulated controls. When indicated, hAoSMCs were simultaneously treated with the neuropeptides and the receptor antagonists BIM-28163, BIM-23627 and BIM-23867, or pretreated for 30 minutes with H89, MDL-12330A, thapsigargin, ionomycin, LY294,002, PD98,059 or SB203580 (all at 1 µmol/L) before the neuropeptides. We added Alamar-Blue (10% v/v) during the last 3 hours of the 24 hours-culture and measured its reduction by fluorescence (ex 560-em 590) in a BioTek fluorescence plate reader. In parallel cultures, we added 1 µCi [3H]-thymidine/well for the last 12 hours of the 24 hours-culture, and determined the incorporation of [3H]-thymidine on a MicroBeta Trilux counter. For cell cycle analysis, cells were detached at the end of the 24 hours-culture with 1 mmol/L EDTA-PBS, fixed in 70% ethanol, treated with RNase (100 µg/mL, 30 minutes, 37°C), stained with propidium iodide (50 µg/mL) and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). We also determined viability of cultured AoSMCs using an annexin V-based apoptosis detection kit (R&D Systems). The effect of the neuropeptides in the levels of intracellular cAMP and free cytosolic calcium is described in **Detailed methods.**

**Migration assays.**

hAoSMCs expanded in SmGM-2 medium were trypsin-detached and cultured in SmBM medium containing 0.1% BSA at 10^4 cells/well in the upper chamber of a 48-well migration system with 8-µm pore size polycarbonate filters (NeuroProbe, Gaithersburg, MD) coated with 100 µg/mL collagen type-I and 10 µg/mL vitronectin. We added PDGF (10 ng/mL) as chemoattractant in the lower chamber, the neuropeptides to the cells immediately before placing them in the upper chamber and the inhibitors/antagonists 30 minutes before neuropeptides. We allowed cell migration for 6 hours (37°C, 5% CO₂), removed the non-migrated cells with a cell scraper, fixed the filter bottom face with cold-methanol, stained the cells with DAPI and counted migrated cells with a Nikon fluorescence microscope.

**Immunohistochemistry and immunofluorescence.**

Three human femoral arteries bearing atherosclerotic plaques were fixed in 10% buffered-formalin for 24 hours, embedded in paraffin and sectioned. Deparaffined sections (4-µm thick) were heat-treated (95°C, 20 minutes, in 1 mmol/L EDTA pH 8.0) for antigenic unmasking and incubated with anti-human α-smooth muscle actin monoclonal antibody (αSMA, 1:100, 10 minutes, 20°C) and then with anti-human cortistatin-29 polyclonal antibody (1:500, 30 minutes, 20°C) using a Plus Dobule Stain Polymer Kit. We then performed the immunohistochemical study on an Autostainer 480 using the polymer-peroxidase-based method and development with diaminobenzidine to detect SMA expression and permanent alkaline phosphatase Red kit to detect cortistatin-positive cells.

hAoSMCs grown onto glass coverslips for 24 hours were fixed for 5 minutes in cold-methanol, immunolabeled with anti-human cortistatin-29 (1:500) and anti-human αSMA (1:2000) antibodies for 8 hours at 4°C, followed by detection with Alexa Fluor-594-conjugated anti-rabbit and Alexa Fluor-488-conjugated anti-mouse secondary antibodies (1:500 each, 1 hour, 20°C). Sections were DAPI-counterstained and images acquired using a Leica-SP5 confocal microscope.

To study the lamellipodia formation, hAoSMCs were grown to subconfluence onto collagen/vitronectin-coated glass coverslips, starved for 24 hours in SmBM, and then treated for 15 minutes with 10 ng/mL PDGF in the absence or presence of neuropeptides (10 nmol/L). Cells were fixed for 5 minutes with cold-methanol and immunostained for αSMA as described above. We
quantified lamellipodia using a fluorescence microscope giving 0 and 1 scores to negative and positive cells, respectively.

Sections of carotid arteries were blocked with 10% goat serum (30 minutes, 20°C), incubated with rabbit anti-mouse cortistatin-29 (1:200), Cy3-conjugated anti-mouse αSMA (1:1000) and/or anti-Ki-67 (1:500) antibodies (8 hours, 4°C) and detected with goat Alexa Fluor-546-conjugated anti-rabbit (1:1000) or FITC-conjugated anti-mouse-IgG1 (1:400) secondary antibodies (1 hour, 20°C). Nuclei were Hoechst-counterstained and slices examined in a fluorescence microscope.

**Rac-1 activation and Western blot.**

We evaluated Rac1 activation in hAoSMCs using the GST-CRIB binding domain that precipitates activated Rac1 (Rac1-GTP) and detecting Rac1 by western blot and determined phosphorylation/activation of ERK1/2, Akt and p38-MAPK by western blot using specific antibodies that recognize the phosphorylated and total forms of the kinases (see *Detailed methods*).

**Statistical analysis.**

All data are mean±SEM. We analyzed data for statistical differences using Student’s *t*-test or one-way ANOVA followed by a test for multiple comparisons, considering *P*-values <0.05 as significant.

**RESULTS**

**Vascular SMCs express cortistatin.**

Cortistatin is a neuropeptide widely distributed in many tissues; however, its expression in the vascular system remains largely unknown. Therefore, we first investigated the presence of cortistatin in mouse and human arteries. Mouse carotid arteries highly expressed cortistatin (mRNA and protein), but not somatostatin (Figure 1A). Immunofluorescence analysis mainly localized cortistatin in αSMA+ SMCs of the muscular layer of the carotid artery (Figure 1B, Online Figure IIA). In order to investigate whether the content of cortistatin varies in pathological conditions of vascular remodeling, we used a model of neointimal hyperplasia. Complete ligation of the left carotid artery of FVB and C57BL/6 mice resulted in increased cortistatin expression (Figure 1A, Online Figure IIC). Interestingly, cortistatin levels were higher in FVB mice, which yield greater neointimal response than C57BL/6 mice.29 Probably, due to the proliferation and migration of SMCs occurring after artery ligation, we observed an increase in the number of cortistatin-positive SMCs in the intima layer of ligated arteries (Figure 1B, Online Figure IIC). Moreover, we found cortistatin-staining in some non-SMC infiltrating cells in the intimal lesion as well as in the endothelial layer (Figure 1B, Online Figure IIA).

In order to confirm the relevance of these findings to human disease, we analyzed the expression of cortistatin in human arteries with atherosclerotic plaques, where SMCs represent more than 50% of the plaque-forming cells. Figure 1C shows that many cells of the atherosclerotic plaque expressed cortistatin. Although cortistatin mostly co-localized with SMA+ SMC cells, some non-SMC infiltrating inflammatory cells also expressed cortistatin (Figure 1C, Online Figure III), as occurred in mouse carotid arteries.

We next confirmed the expression of cortistatin in isolated hAoSMCs. Quantitative rtRT-PCR analysis indicated that hAoSMCs expressed high levels of cortistatin mRNA (Figure 1D). However, the other cortistatin-related peptides, somatostatin and ghrelin, were weakly expressed and undetectable in hAoSMCs, respectively (Figure 1D). Confocal microscopy analysis revealed a vesicular and perinuclear distribution of cortistatin in hAoSMCs (Figure 1D) and ELISA showed that hAoSMCs constitutively release cortistatin and increase its secretion upon activation with PDGF (Figure 1E).
We next analyzed the expression of the receptors through which cortistatin exerts its actions in most cells, ssts and ghrelin-receptors. Among the ssts, sst2 and the truncated receptor sst5TMD4 were the most expressed receptors in hAoSMCs. They also expressed sst1, sst5, the truncated receptor sst5TMD5 and ghrelin receptors (GHSR1a and GHSR1b) at low levels (Figure 1F); however, we did not detect sst3 nor sst4 expression (not shown).

Cortistatin impairs the proliferation and migration of hAoSMCs.

These findings suggest that cortistatin is an endogenous factor that could exert autocrine/paracrine actions on SMCs. Because proliferation and migration of SMCs are key steps for the progression of atherosclerosis and restenosis, we investigated the effects of cortistatin and related peptides (somatostatin and ghrelin) on both processes in primary hAoSMCs stimulated with PDGF. The three neuropeptides significantly inhibited, in a dose-dependent manner, PDGF-induced cell growth of hAoSMCs (Figure 2A), by affecting cell proliferation (Figure 2B), but not apoptosis (Online Figure IV). Inhibition on cell proliferation correlated with the induction of a partial cell cycle arrest by cortistatin, characterized by a reduction on cells in S-phase (Figure 2C). Moreover, cortistatin decreased the expression of factors that drive cell cycle progression, including the cyclins D1, D3, E and A and the cyclin-dependent kinases CDK2 and CDK4, and increased the levels of the CDK-inhibitor p27kip1 (Figure 2D).

On the other hand, cortistatin significantly inhibited PDGF-induced migration of hAoSMCs (Figure 3A). Whereas ghrelin mimicked the cortistatin effects, somatostatin did not affect hAoSMC migration (Figure 3A). The inhibitory role of cortistatin in cell migration correlated with its effect in the reorganization of actin cytoskeleton of hAoSMCs. A short exposition to PDGF induced a rapid polarization of the actin cytoskeleton and the formation of lamellipodia at the leading edge of hAoSMCs, and treatment with cortistatin reduced in 50% the number of cells with lamellipodia (Figure 3B). In agreement, cortistatin significantly reduced PDGF-induced Rac1 activity (Figure 3C), which is the GTPase responsible for the formation of lamellipodia.30

Cortistatin acts through somatostatin and ghrelin receptors in hAoSMCs.

We next investigated the potential involvement of ssts and/or GHSRs on the effects of cortistatin by using specific peptide antagonists for GHSR, sst2, and sst5 (BIM-28163, BIM-23627 and BIM-23867, respectively). The three antagonists reversed the inhibitory effect of cortistatin in cell proliferation, being the GHSR-antagonist the most potent, followed by sst2- and sst5-antagonists (Figure 4A). Conversely, whereas the sst2-antagonist completely abrogated the antiproliferative effect of somatostatin, neither sst5- nor GHSR-antagonists affected it (Figure 4A). These results indicate that cortistatin exerts its antiproliferative effects in AoSMCs through both ssts and GHSRs. However, only the GHSR-antagonist, but not the sst-antagonists reversed the inhibitory effect of cortistatin in the migration of AoSMCs (Figure 4B). This agrees with the lack of effect of somatostatin in the chemotaxis of these cells (Figure 2D). As expected, the GHSR-antagonist fully reversed the inhibitory effect of ghrelin on cell growth and migration (Figure 4).

Intracellular signaling involved in the inhibition of hAoSMC proliferation and migration by cortistatin.

Considerable evidence suggests that the cAMP/PKA pathway is a major signal that inhibits SMC proliferation,24 and that [Ca2+]i is essential to reorganize actin cytoskeleton and promote migration of these cells in response to PDGF.5 We first confirmed the ability of cortistatin, somatostatin and ghrelin to regulate these second messenger pathways. Whereas the three neuropeptides significantly increased the levels of intracellular cAMP (Figure 5A), only cortistatin and ghrelin, but not somatostatin, blocked the rapid and transient increase in [Ca2+]i induced by PDGF in hAoSMCs (Figure 5B, Online Figure V). Regulation of both intracellular messengers by cortistatin seems to be involved in its inhibitory action on vascular SMCs. Thus, inhibition of adenylyl cyclase with MDL-12330A or of PKA with H89 fully reversed the effects of cortistatin, somatostatin and...
ghrelin on hAoSMC proliferation (Figure 5C), confirming the involvement of cAMP/PKA signaling in their effects on hAoSMC proliferation. In agreement, the adenylate cyclase activator forskolin mimicked the inhibitory effect of cortistatin in PDGF-stimulated cell proliferation (Figure 5C). Moreover, increase of [Ca^{2+}], with thapsigargin, which releases calcium from endoplasmic reticulum, or with ionomycin that facilitates the entry of extracellular calcium, significantly abrogated the inhibitory effect of cortistatin and ghrelin on PDGF-induced hAoSMC migration (Figure 5D). Contrary, adenylate cyclase inhibition did not affect the inhibitory action of both peptides on hAoSMC migration (Figure 5D).

It is widely reported that phosphorylation and subsequent activation of Akt/PKB and ERK1/2 are critically involved in PDGF-stimulated proliferation and migration of SMCs. Cortistatin and ghrelin, but not somatostatin, reduced PDGF-induced phosphorylation/activation of Akt and ERK in hAoSMCs (Figure 6A). Interestingly, cortistatin, ghrelin and somatostatin increased the active form of p38-MAPK (Figure 6A), an effect linked to the cell growth arrest induced by the three neuropeptides, since the p38-inhibitor SB203580 reversed their inhibitory effects on hAoSMC proliferation (Figure 6B).

**Cortistatin inhibits SMC proliferation in vivo.**

To investigate whether cortistatin affects SMC proliferation and migration in vivo, we assayed its effect on an established model of neointima hyperplasia induced by complete ligation of the carotid artery. In this model of blood-flow cessation, after an early phase of inflammatory cell recruitment, medial SMCs rapidly proliferate and migrate toward the lumen, leading to extensive neointima formation after 4 weeks (Figure 7A). Systemic injection of cortistatin strongly reduced neointima formation in the ligated artery (Figure 7A and 7B) and reduced vascular stenosis from 80.5±4.6% (in untreated mice) to 12.6±2.5%. The presence of thrombus, endothelial denudation and decreased vessel diameter lessen the physiological relevance of this model to investigate human cardiovascular disease. Therefore, we confirmed the effect of cortistatin in a complementary model of vascular remodeling that has relevance to human carotid intima-media thickening and atherosclerosis, in which three of the four caudal branches of the left common carotid artery are ligated. Similarly that in the blood-flow cessation model, systemic treatment with cortistatin avoided the intima-media thickening caused by partial carotid ligation (Online Figure VI).

Because SMC proliferation seems to drive vascular remodeling after carotid artery ligation, we evaluated the SMC content by immunostaining for αSMA and the cell proliferation marker Ki-67. Treatment with cortistatin significantly impaired the increase in the content of SMCs and the number of proliferating SMCs (double staining for Ki-67 and αSMA) in the intima layer observed after carotid ligation (Figure 7C and 7D). As observed in vitro, this reduction in SMC proliferation correlated with diminished Akt activity (measured by its phosphorylated form) and cyclin D1 expression in the ligated artery (Figure 7D). TUNEL staining revealed very few apoptotic cells in ligated arteries of both untreated (6.2±0.7%) and cortistatin-treated mice (6.4±0.8%). These data indicate that cortistatin protects from the formation of the neointima lesion mainly by inhibiting the proliferation of SMCs.

**Endogenous cortistatin regulates neointima lesion formation.**

Our findings indicate that SMCs respond to pathological vascular conditions by producing cortistatin (Figure 1). Therefore, we finally evaluated whether the endogenous cortistatin plays a role in the control of vascular remodeling in such conditions by using mice deficient for cortistatin (CST−/−) subjected to ligation of carotid artery. We observed that CST−/− mice developed higher neointimal lesions and had more proliferating SMCs in the ligated artery than wild-type C57BL/6 mice (Figure 8A-8C), with no significant changes in the contralateral nonligated artery. Moreover, whereas wild-type mice showed minimal intima hyperplasia and outward remodeling after partial carotid ligation, lack of cortistatin favored intima-media thickening in the ligated carotid (Figure 8D). This suggests
that cortistatin produced by SMCs regulates in an autocrine/paracrine manner the proliferation of these cells and their migration to the intima layer. In fact, treatment with cortistatin partially reversed the exacerbated neointimal lesion observed in the ligated arteries of CST−/− mice (Figure 8B).

DISCUSSION

The accumulation of SMCs in the intimal space of arteries as a result of their migratory and proliferative activities is a critical event in atherogenesis and restenosis. Indeed, SMCs can turn into more than one half of the cells present in atherosclerotic plaque. Although their role on the stabilization of the plaque is somehow controversial, the control of the proliferation and migration of SMCs is a proposed strategy to limit the progression of atherosclerosis. In this study, we show that the neuropeptide cortistatin emerges as an attractive candidate to keep under control this particular step on the pathology of atherosclerosis and other vasculopathies. We found that cortistatin is able to inhibit in vitro and in vivo the proliferation and migration of SMCs in response to PDGF and to reduction of blood flow.

Importantly, we found that cortistatin exerted unique functions not shared with its natural analogue, somatostatin, especially related to regulation of SMC migration. Based on its high homology with somatostatin, since its discovery, cortistatin was believed a mere somatostatin analogue, in particular regarding the inhibition of neuroendocrine secretions. Nowadays, recent evidence indicates that cortistatin has its own functions, not shared with somatostatin, and its action in the cardiovascular system represents another example. Beside our results, a recent study reported that cortistatin, but not somatostatin, attenuated vascular calcification. As in the immune system, the capacity of cortistatin to bind to GHSRs seems to play a major role in this differential effect. Similarly, by using new selective receptor antagonists, we demonstrate that cortistatin mediates its effects on SMC proliferation through both ssts (sst2 and sst5) and GHSR and on SMC migration through GHSR. Our study confirmed the expression of these receptors in hAoSMCs, as previously reported in rat and human arterial SMCs. Moreover, we found high expression of sst5TMD4, a recently cloned truncated sst5 isof orm that responds preferentially to cortistatin, although its implication in cortistatin effects in this system is unknown.

Cortistatin binding to both ssts and GHSR is mechanistically linked to the inhibition of the molecular players that are activated by PDGF in SMCs in pathological conditions. Our data indicate that cortistatin decreases the expression of cyclins D1, D3 and E, which rise in the early G1-phase, in the late G1-phase, and in the G1-checkpoint, respectively, and reduces the expression of CDK2 and CDK4 in PDGF-activated hAoSMCs. Both CDK4-cyclins D and CDK2-cyclin E complexes participate in the activation of E2F transcription factor, the expression of S-phase genes and cell cycle progression. Indeed, cortistatin decreased the S-phase cyclin A in hAoSMCs. Moreover, cortistatin upregulated the levels of p27kip1, a cell cycle inhibitor that acts during late G1-phase by impairing CDK2-cyclin E/A complexes. These findings suggest that the cortistatin-treated SMCs fail to progress into the late G1-phase and enter the S-phase of the cycle. Activation of the cAMP/PKA and p38-MAPK pathways and inhibition of Akt and ERK activities by cortistatin seem to be critically involved in this cell growth arrest. Indeed, evidence shows that Akt and ERK1/2 play a critical role in the induction of SMC proliferation by PDGF, by activating cyclin D-cdk4 complexes and deactivating p27kip1. Moreover, p38-MAPK inhibits cyclin D1 expression and degrades cyclin D3. Beside its effect in proliferation, p38-MAPK activation causes growth arrest by inducing apoptosis in various cell types, including AoSMCs. However, this is not the case on the effect of cortistatin in AoSMCs. Regarding the inhibitory action of cortistatin in SMC migration in response to PDGF, our data indicate that cortistatin and ghrelin, but not somatostatin, blocked increase in [Ca2+]i induced by PDGF in hAoSMCs, which is mandatory for their migratory activity. Importantly, cortistatin and ghrelin impaired the rapid activation of the GTPase Rac1 and reorganization of the actin cytoskeleton in lamellipodia, which initiates the directional migration toward the PDGF gradient. Interestingly, somatostatin, which affects only p38-MAPK but not Akt or ERK1/2 activities, was able to inhibit...
proliferation, but not migration of SMCs, and ghrelin that affects the activation of the three kinases and only acts through GHSRs was as efficient as cortistatin in inhibiting proliferation and migration. Because Akt and ERK1/2 pathways are critically involved in lamellipodia formation and chemotaxis in SMCs, the effects of cortistatin and ghrelin vs. somatostatin on both kinases could explain their differential actions on SMC migration. Therefore, the various kinase cascades analyzed herein may act in concert to regulate precisely/distinctly cell growth and migration in response to this family of related peptides.

Beside its potential therapeutic implications, our study is also relevant from a physiological point of view. It is noteworthy that cortistatin exhibits a more widespread expression pattern than somatostatin, being present in many tissues where somatostatin is absent. Therefore, cortistatin should have a distinct functional role rather than being a mere somatostatin sibling. The cardiovascular system is not an exception and we presented evidence that cortistatin is the endogenous regulator of this family of peptides in the arterial system. Thus, mouse arterial SMCs and hAoSMCs express and secrete high cortistatin levels. Moreover, cortistatin is upregulated by PDGF and its expression appears linked to the progression of the atherosclerotic plaque and neointimal lesion, where PDGF is abundant. In contrast, the expression of somatostatin and ghrelin is marginal or absent in this system. In line with our results is the fact that cortistatin, but not somatostatin, is elevated 2-fold in plasma of patients suffering coronary heart disease. This finding initially suggested a direct correlation between cortistatin levels and the disease and that the peptide could play a causal role on this pathology. However, the results presented herein, prompt us to propose that the increased cortistatin release by SMCs is not the origin of the disease, but a likely consequence, and it would represent an attempt of the cells to defend the tissue against a pathological state. In fact, we found that lack of cortistatin exacerbates the formation of neointimal lesion. This scenario would attribute cortistatin an important role in the homeostasis of SMCs and vascular function. Importantly, cortistatin regulated SMC proliferation and migration at doses between 0.1 and 10 nmol/L, which are in the range of Kd affinity of cortistatin for ssts and GHSR1 (0.1-1 nmol/L), and of cortistatin concentration found in human plasma (0.8 nmol/L) and in hAoSMC cultures (0.1 nmol/L, Figure 1).

In conclusion, our study demonstrates that the natural peptide cortistatin shows potent inhibitory effects on growth and migration of SMCs and emerges as an attractive candidate to treat vasculopathies that course with neointima formation and intima-media thickening. Moreover, the potent anti-inflammatory activity showed by cortistatin is a desirable characteristic for any anti-atherosclerotic agent. Importantly, the safety and efficacy of the infusion of cortistatin (in the same dose range of 4 μg/Kg that used in our study) demonstrated in humans with Cushing’s disease will help the translation of our findings to the clinic. The differential effects of cortistatin and somatostatin in hAoSMCs, as well as the different receptors and intracellular signals involved in such functions, also support the concept that cortistatin is not a somatostatin sibling at the cardiovascular level. Thus, elucidation of the receptors and molecular players involved in the cortistatin effects will help in the design of stable cortistatin analogues more suitable for cardiovascular therapy. Finally, the fact that cortistatin is highly expressed by hAoSMCs in comparison with the other two structurally-and functionally-related neuropeptides, especially in pathologic conditions, supports its role as an endogenous factor involved in autocrine/paracrine regulation of vascular homeostasis.

SOURCES OF FUNDING
Work supported by grants from Spanish Ministry of Economy and Competitiveness and Sara Borrell Program.

DISCLOSURES
JPC received funds from Ipsen and Novartis. MDC is employed by Ipsen.
REFERENCES


FIGURE LEGENDS

Figure 1. Expression of cortistatin in arterial SMCs. A-B, Neointimal hyperplasia was induced by complete ligation of left carotid artery of FVB/NJ mice. mRNA and protein levels of cortistatin (CST) and somatostatin (SOM) were determined by qrtPCR and ELISA on unligated and ligated carotids at different times after ligation (A, n=5). Immunofluorescence analysis (28 days after ligation) shows co-localization of cortistatin with αSMA+ cells (B, n=7). Note some cortistatin-immunostaining in infiltrating SMA-negative cells in intima layer (arrows) and in endothelial cell line (asterisks). DAPI (blue): nuclear counterstaining. Scale bars: 25-µm. See Online Figure II for similar results in C57BL/6 mice, and for negative controls consisting in carotids of cortistatin-deficient mice and ligated carotids incubated only with FITC- and PE-labeled secondary antibodies. C, Immunohistochemical analysis for cortistatin (pink-staining) and αSMA (brown-staining) in human femoral arteries bearing atherosclerotic plaques (n=3). Note numerous cells co-expressing cortistatin and αSMA (dark pink-staining pointed by solid arrow heads), some SMA-negative infiltrating cells expressing cortistatin (open arrow heads) and endothelium expressing cortistatin (asterisks). See Supplementary Figure 3 for additional images and negative control of immunostaining.

Figure 2. Effects of cortistatin in cell growth and proliferation of hAoSMCs. Cells were incubated with PDGF and different doses of cortistatin, somatostatin or ghrelin (10 nmol/L cortistatin in panel D). A, Cell growth determined by measuring AlamarBlue reduction and expressed as percentage of PDGF-stimulated cells. n=5, in triplicate. B, Cell proliferation evaluated by quantifying [3H]-thymidine-incorporation into DNA and expressed as percentage of PDGF-stimulated cells (left panel) or by counting cell numbers in Neubauer chambers (right panel). n=5, in triplicate. C, Cell cycle distribution analyzed by flow cytometry and expressed as percentage of cells in S-phase. n=4, in duplicate. D, Expression of cyclins D1/D3/E/A, CDK2/4 and p27kip1 assayed by Western blot and expressed as fold-induction vs. unstimulated cells. n=4-5. *P<0.05, **P<0.01, ***P<0.001 vs. PDGF-treated cells.

Figure 3. Effects of cortistatin in migration of hAoSMCs. A, Effects of cortistatin, ghrelin and somatostatin on the migration of hAoSMCs placed onto the upper side of collagen/vitronectin-coated membranes towards PDGF (10 ng/mL, located at lower chamber). Results show percentage of migrating cells of control samples without neuropeptide-treatment. We used migration towards BSA as basal control. n=3, in triplicate. B, Inhibitory effect of cortistatin in the formation of lamellipodia (insets) induced by PDGF in hAoSMCs assayed by immunofluorescence for α-SMA. Results show percentage of cells with lamellipodia relative to PDGF-treated cells. n=3, in triplicate. C, Rac1 activation assayed by Western blot of immunoprecipitated GTP-Rac1 (Rac1 active form) and expressed as fold-induction vs. unstimulated cells. n=3. *P<0.05, **P<0.01, ***P<0.001 vs. PDGF-treated cells.

Figure 4. Involvement of ssts and GHSRs in the inhibitory effects of cortistatin. Effects of the antagonists for GHSR (BIM-28163), sst2 (BIM-23627) or sst5 (BIM-23867) in the inhibitory actions of cortistatin, somatostatin or ghrelin (10 nmol/L each) on PDGF-stimulated hAoSMC cell growth (A, n=4, in triplicate) and migration (B, n=3, in triplicate). Results show cell growth and migration relative to PDGF-treated cells. #P<0.05, ##P<0.005, ###P<0.001 vs. neuropeptide-treated cells without antagonists. *P<0.05, **P<0.005, ***P<0.001 vs. PDGF-treated cells.

Figure 5. Effects of cortistatin, somatostatin and ghrelin in cAMP- and calcium-mediated signaling. hAoSMCs were incubated with medium (unstimulated) or stimulated with PDGF (10 ng/mL) in the absence or presence of cortistatin, somatostatin or ghrelin (10 nmol/L each). A, Intracellular cAMP levels after 30 minutes of culture. Results show percentage of cAMP concentration
relative to unstimulated cells. n=3, in duplicate. B, Intracellular calcium levels determined by confocal microscopy using the calcium indicator Fluo-4. Results are the averaged percentage of variation of fluorescence after treatment vs. baseline, in the indicated number of cells, assayed in four independent experiments. C, cAMP is involved on the antiproliferative response of the neuropeptides. Cell growth of hAoSMCs stimulated with PDGF in the absence or presence of cortistatin, somatostatin, ghrelin, forskolin (FK, 10 μmol/L), MDL-12330A (1 μmol/L), and/or H89 (1 μmol/L). Results show cell growth relative to PDGF-treated cells. n=3, in triplicate. D, Migration toward PDGF (10 ng/mL) of hAoSMCs placed onto the upper side of collagen/vitronectin-coated membranes in the absence or presence of cortistatin or ghrelin (10 nmol/L), plus thapsigargin, Ionomycin or MDL-12330A (1 μmol/L each). We used BSA in lower chamber as a negative control. Results show percentage of migrating cells relative to PDGF-stimulated samples. n=3, in triplicate. *p<0.05, **p<0.005, ***p<0.001 vs. PDGF-treated cells, #p<0.05, ##p<0.005 vs. neuropeptide-treated cells.

Figure 6. Effect of cortistatin, somatostatin and ghrelin in the signaling mediated by Akt, Erk, and p38. A, Effects of cortistatin, somatostatin or ghrelin (10 nmol/L each) on Akt, ERK and p38 phosphorylation (assayed by Western blot) in hAoSMCs cultured in medium alone (unstimulated) or stimulated with 10 ng/mL PDGF. Results show the ratio between the phosphorylated form and the total amount of each kinase, expressed relative to PDGF-treated samples. n=5-6. B, Reversal of the inhibitory effect of cortistatin, somatostatin or ghrelin on PDGF-stimulated hAoSMC proliferation by the p38-MAPK inhibitor SB203580. Results show cell growth relative to PDGF-treated cells. n=3, in triplicate. *P<0.05, **P<0.005, ***P<0.001 vs. PDGF-treated cells.

Figure 7. In vivo inhibitory effect of cortistatin on SMC proliferation in a model of neointimal hyperplasia caused by ligation of carotid artery. FVB/NJ mice (6-9/group, 3 independent experiments) received intraperitoneally saline (control) or cortistatin (100 ng) every two days starting the day after ligation of left carotid artery. A-B, Treatment with cortistatin reduced intimal area and intimal/medial ratio of the ligated carotid artery isolated 4 weeks after ligation at different distances from the ligature. Photomicrographs show representative cross-sectional areas of the contralateral (unligated) and ligated artery. Scale bars: 50-μm. C, Cortistatin decreased the number of SMCs (stained with αSMA) in the intima layer of ligated carotids. Results are from arteries isolated 4 weeks after ligation analyzed in cross-sections located at 0.8-1 mm from ligature. D, Treatment with cortistatin decreased the percentage of Ki67+-proliferating cells and the expression of cyclin D1 and pAkt in ligated arteries isolated 2 weeks after ligation. *P<0.05 vs. controls.

Figure 8. Lack of cortistatin exacerbates neointima lesion formation. A, Representative photomicrographs showing cross-sectional areas (at 0.8-1 mm from ligature) of the completely ligated carotid artery of wild-type (wt, C57Bl/6) and cortistatin-deficient (CST−/−) mice isolated 21 days after ligation. Scale bars: 50-μm. B, Lack of cortistatin significantly increased intimal area and intimal/medial ratio vs wt controls. Treatment with cortistatin (200 ng, every two days, grey bars) partially reversed this phenotype. *P<0.05 vs. saline. ^P<0.05 vs. wt mice. C, Mice deficient for cortistatin showed more αSMA+ and proliferating cells in the intima layer of completely ligated arteries than wt mice. ^P<0.05 vs. wt mice. D, Mice deficient for cortistatin showed marked intima-media thickening after partial carotid ligation compared to wt mice. Scale bars: 50-μm. n=6-8/group, two independent experiments. *P<0.05 vs. wt mice.
Novelty and Significance

What Is Known?

- In response to vascular injury, the medial smooth muscle cells (SMCs) migrate into the intima, where they proliferate and secrete extracellular matrix to form the neointima.
- Factors that regulate neointima formation are critical targets in the treatment of various forms of vascular diseases such as atherosclerosis, restenosis and transplant vasculopathy.
- Cortistatin, a recently discovered neuropeptide that exerts multiple effects on the neuroendocrine and immune systems, regulates vascular calcification and is elevated in patients with coronary disease.

What New Information Does This Article Contribute?

- Human and mouse arterial SMCs produce cortistatin, especially in response to activation and in vasculopathies associated with neointima hyperplasia.
- Cortistatin impairs proliferation and migration of human aortic SMCs induced by platelet-derived growth factor by regulating several transduction pathways at multiple levels.
- Treatment with cortistatin reduces intima-media thickening in carotid arteries subjected to blood-flow cessation or reduced-oscillatory blood-flow. Conversely, lack of cortistatin exacerbates the formation of neointimal lesions.

Smooth muscle cell accumulation in the intimal space of arteries contributes to the progression of various vasculopathies such as atherosclerosis and restenosis. In this study, we demonstrate that the neuropeptide cortistatin regulates intimal thickening. We found that cortistatin has potent inhibitory effects in vitro and in vivo on the growth and the migration of arterial SMCs in response to growth factors and to blood-flow reduction. We also identify the receptors and molecular mediators involved in the cortistatin action. SMCs express highly levels of cortistatin, but not other structurally/functionally-related peptides, especially in pathologic conditions. Indeed, lack of cortistatin results in exacerbated neointima lesions in carotid arteries exposed to blood-flow alterations. These findings support the role of cortistatin as an endogenous factor involved in autocrine/paracrine regulation of vascular homeostasis.
Figure 4

A

Cell growth (% of PDGF-treated cells)

B

Migrated cells (% of PDGF-treated cells)
Figure 5
Figure 6

A

- % P-Akt/Akt ratio (% of PDGF-treated cells)

- % P-Erk1/2 ratio (% of PDGF-treated cells)

- % P-ERK ratio (% of PDGF-treated cells)

- % P-p38 ratio (% of PDGF-treated cells)

B

- Cell growth (% of PDGF-treated cells)

- Treatment conditions include:
  - Unstimulated
  - PDGF
  - SB203580
  - PDGF + SB203580
  - PDGF + Somatostatin
  - PDGF + Somatostatin + SB203580

- Graphs show changes in protein expression and cell growth after treatment with different conditions.
Cortistatin Inhibits Migration and Proliferation of Human Vascular Smooth Muscle Cells and Decreases Neointimal Formation upon Carotid Artery Ligation

Mario Duran-Prado, Maria Morell, Virginia Delgado-Maroto, Justo P Castaño, Jose Aneiros-Fernandez, Luis de Lecea, Michael D. Culler, Pedro Hernandez-Cortes, Francisco O’Valle and Mario Delgado

Circ Res. published online April 17, 2013;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2013/04/17/CIRCRESAHA.112.300695

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2013/04/17/CIRCRESAHA.112.300695.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplemental Material

Detailed Methods

Animals, culture media and reagents.

Animals: We used male FVB/NJ and C57Bl/6 mice (22-25 g, 8-weeks-old, Charles River, France) throughout the study. Mice lacking the gene for cortistatin (CST−/−) were generated in a C57BL/6 background and backcrossed with C57BL/6 mice for ten generations as previously described. The experiments reported in this study followed the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental animals approved by the Animal Care Unit Committee IPBLN-CSIC (# protocol 202-10-2).

Cells and Media: hAoSMCs and the SmGM-2 and SmBM media to expand and culture them were purchased from Clonetics (Verviers, Belgium).

Reagents: Human somatostatin-14, human cortistatin-17 and human ghrelin were purchased from Phoenix Pharmaceuticals (Burlingame, CA), mouse cortistatin-14 from Bachem AG (Bubendorf, Switzerland) and human PDGF-BB from PreproTech (Rocky Hill, NJ). Selective peptide antagonists for GHSR, sst2 and sst5 (BIM-28163, BIM-23627 and BIM-23867, respectively) were provided by Ipsen Biomeasure (Boston, MA). H89, MDL-12330A, thapsigargin, ionomycin, SB203580, collagen I and vitronectin were obtained from Sigma (Barcelona, Spain). [3H]-thymidine and cAMP Biotrack EIA system were obtained from GE Healthcare (Barcelona, Spain) and Alamar-Blue reagent from Biosource International. Fura-2 AM and Fluo-4 AM dyes were obtained from Molecular Probes (Barcelona, Spain). Specific ELISA kits for human cortistatin-29 (EK-060-12), mouse cortistatin-29 (EK-060-05) and human/mouse somatostatin-28 (EK-060-14) were purchased to Phoenix Pharmaceuticals. Annexin V-based apoptosis detection kit was obtained from R&D Systems (Madrid, Spain). Plus Dobule Stain Polymer and Permanent alkaline phosphatase Red Kits were purchased to Monosan (Uden, Netherlands).

Antibodies: The antibodies used in this study were: anti-human cortistatin-29 (H-060-12, Phoenix Pharmaceuticals), anti-mouse cortistatin-29 (H-06-05, Phoenix Pharmaceuticals), unconjugated and Cy3-conjugated anti-α-smooth muscle actin (clone 1A4, Sigma), Alexa Fluor-594-conjugated anti-rabbit, Alexa Fluor-488-conjugated anti-mouse, goat Alexa Fluor-546-conjugated anti-rabbit and FITC-conjugated anti-mouse-IgG1 (Molecular Probes), anti-Ki67 (clone B56, BD Bioscience), anti-ERK1/2, anti-phospho-ERK1/2, anti-Akt, anti-phospho-Akt, anti-p38 and anti-phospho-p38 (Cell Signaling, Boston, MA), peroxidase-conjugated anti-rabbit and peroxidase-conjugated anti-mouse (Sigma), anti-Rac1 (BD Biosciences), anti-cyclin A, anti-cyclin E, anti-cyclin D1, anti-cyclin D3, anti-cdk2, anti-cdk4, anti-p27Kip1 and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Determination of neuropeptide contents.

hAoSMCs were seeded at subconfluence onto 12-well plates, starved for 24h in SmBM, and then treated for 24h with medium or PDGF (10 ng/mL). We determined the amount of cortistatin secreted to the culture supernatant using a specific competitive ELISA kit.

We isolated protein extracts from unligated and ligated carotid arteries by homogenization of 10 mg tissue in 200 μL of lysis buffer (50 mmol/L Tris-HCl, pH 7.4, with 0.5 mmol/L DTT, and 10 μg/mL of a cocktail of proteinase-inhibitors containing phenylmethylsulfonyl fluoride, pepstatin and leupeptin). We centrifuged the samples (20,000g, 15 min, 4°C) and assayed the supernatants for the contents of cortistatin and somatostatin with two specific commercial ELISA kits. Western blot analysis of artery extracts is described below.

We determined the content of cortistatin in cultured hAoSMCS, human aortas and carotid arteries by immunodetection as described in Methods, and we quantified gene expression of cortistatin, somatostatin, ghrelin and their receptors in total RNA extracted from hAoSMC cultures by qrtRT-PCR using specific primers, as described below.

Quantitative real time PCR (qrtRT-PCR).

RNA was isolated using the Trizol reagent (Life Technologies, Gaithersburg, MD). Total RNA (1 μg) was reverse transcribed (RT) in a 20 μL volume using the reagents supplied in the cDNA First-Strand Synthesis kit (Fermentas, Madrid, Spain), and the resulting cDNA (1 μL) amplified
by real-time PCR using primers described and validated previously for ssts, and the following primer pairs for ghrelin receptors, GHSR1A_S 5'-ACACCACCTACACGCGCATTT-3', GHSR1A_AS 5'-ACACCACTACAGCCGATTTA-3', human somatostatin, hSOM_S 5'-AACCCAACCAGCACGCCA-3', hSOM_AS 5'-TAGCGGGTTGGAGTTGAGA-3', human cortistatin, hCST_S 5'-ATGCACTTCAGCAACAGACC-3', hCST_AS 5'-TCTGCGCTAAAGCTACACATC-3', mouse somatostatin, mSOM_S 5'-TGCTGCGCTTTGGGCGGCTTC-3', mSOM_AS 5'-TGCACTCCAGCTCAGCTCTTCG-3', mouse cortistatin, mCST_S 5'-AAGACCCTCTGTCCCACCAA-3', mCST_AS 5'-ACCAAGGCAAGGAAATCGAAG-3' and the housekeeping gene 18S used as internal standard, human 18s_S 5'-CCCATTCGAACGTCTGCCCTATC-3' and 18s_AS 5'-TGCTGCGCTTTGGAGTTGAGA-3'; mouse 18s_S: 5'-CCCATTCGAACGTCTGCCCTATC-3'; mouse 18s_AS: 5'-TGCTGCGCTTTGGAGTTGAGA-3'. All PCR reactions were carried out in an iCycler IQ™ thermal cycler (BioRad, Barcelona, Spain). Thermal cycling profile consisted of a pre-incubation step at 95°C for 10 minutes, followed by 40 cycles of denaturation (95°C, 30 seconds), annealing (61–64°C, 1 minute), and extension (72°C, 30 seconds). At the end of the amplification, the final product was subjected to graded temperature-dependent dissociation to verify that only one product was amplified. To estimate the starting copy number of cDNA, sample signal was compared with that of the standard curve run on a separate plate.

**Determination of free cytosolic calcium ([Ca^{2+}]_i) and cAMP.** hAoSMCs were plated onto collagen-coated 25 mm-coverslips, grown for 24h and incubated for 30 minutes at 37°C with 2.5 µmol/L of the Ca^{2+} indicator dyes Fura-2 AM or Fluo-4 AM in SmBM medium. After washing, we mounted the coverslips treated with Fura-2 on the stage of a Nikon-Eclipse microscope (coupled to a thinned-CCD camera) and those treated with Fluo-4 on a Leica confocal microscope. We examined the cells under a 40X objective during exposure to alternating 340- and 380-nm light beams, and measured the intensity of light emission at 505 nm every 5 seconds. We recorded changes in [Ca^{2+}]_i after the PDGF/neuropeptide-treatments as background subtracted ratios of the corresponding excitation wavelengths using MetaFluor Software (Imaging Corporation).

For cAMP determination, hAoSMCs (10^4/well, 96-well plates) were preincubated for 30 min with 1 mmol/L 3-isobutyl-1-methylxanthine before the PDGF/neuropeptide-treatment, and 30 minutes later, cells were HCl-lysed and intracellular cAMP accumulation determined using a cAMP Biotrack EIA kit.

**Western blot and Rac-1 activation assays.** hAoSMCs were cultured to sub-confluence in SmGM-2 medium in 6-well plates, assayed for 10 minutes in SmBM with the indicated treatments and immediately lysed in pre-warmed SDS-DTT sample buffer (62.5 mmol/L Tris-HCI, 2% SDS, 20% glycerol, 100 mmol/L DTT and 0.005% bromophenol blue) followed by sonication for 10 seconds and boiling for 5 minutes. Protein extracts from unligated and ligated carotid arteries were prepared by lysing 10 mg tissue in 200 µL of lysis buffer (50 mmol/L Tris-HCI pH 8.0, 100 mmol/L NaCl, 5 mmol/L EDTA, 50 mmol/L glycerolphosphate, 1% Triton X-100, 5% glycerol, 1 mmol/L EDTA, 2 µg/mL leupeptin, 5 µg/mL apronin, 1 mmol/L PMSF, 5 mmol/L NaF, 10 mmol/L NaHPO_4, and 1 mmol/L Na_2VO_4) for 30 minutes on ice and mixing gently. Insoluble fragments were pelleted by centrifugation for 30 minutes at 10,000g. Proteins from cell and artery extracts (60–80 µg/lane) were separated by 12.5% SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (Millipore) using a semidry system. Membranes were blocked with TBS/Tween-20/3% nonfat dry milk for 1 hour at room temperature and subsequently probed overnight at 4°C with primary antibodies against cyclin A, cyclin D1, cyclin D3, cyclin E cdk2, cdk4, p27kip1, ERK1/2, P-ERK1/2, Akt, P-Akt, p38 and P-p38 (at 1:1000, 8 hours, 4°C). Immunodetection was performed by incubation with the corresponding peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (at 1:2000, 2 hours, 20°C) and developed with an enhanced chemiluminescence detection system (GE Healthcare). Bands were quantified by densitometry using Image J software. Equal protein loading was controlled by reprobing with anti-β-actin antibody. Relative phosphorylated ERK, Akt and p38 levels were obtained from normalization of P-ERK1/2, P-Akt and P-p38 values to the total ERK1/2, Akt and p38 values, respectively. Rac1 activation was evaluated using the GST-CRIB binding domain that precipitates the activated form of Rac1 (Rac1-GTP). To this end, hAoSMCs were grown to subconfluence in SmGM-2 onto culture Petri dishes, starved 24 hours in SmBM and assayed for 5 minutes with...
medium (unstimulated) or stimulated with PDGF (10 ng/mL) in the absence or presence of 10 nmol/L cortistatin. Cell lysates (obtained as described above) were incubated with 4 µg GST-CRIB beads for 30 minutes at 4° C, washed and then bound Rac1 was eluted by boiling the samples in Laemli buffer. Samples were separated on 15% SDS-polyacrylamide gels and immunoblotted with a monoclonal anti-Rac1 antibody (at 1:2000) and developed with peroxidase-conjugated anti-mouse antibody as described above. Rac1 activation was determined as ratio of the bands obtained from the immunoprecipitates normalized to total Rac1 protein present in the initial cell lysate.

References for Extended Methods
Online Figures

Online Figure I: Scheme depicting the surgical procedure of complete and partial ligations of left carotid artery. Complete ligation of left common carotid artery (LCA) was performed with a single 6-0 silk suture (A) near to the bifurcation in internal carotid artery (ICA) and external carotid artery (ECA). Partial ligation of LCA was performed by ligating with 6-0 silk suture three branches of the LCA (ECA and superior thyroid artery (STA) with suture B1, and ICA with suture B2), while leaving the occipital artery (OCA) open. In both models, the contralateral right common carotid artery (RCA) was left unligated and served as control.
Online Figure II: Expression of cortistatin in carotid artery of C57BL/6 mice. A-C, C57BL/6 and cortistatin-deficient (CST-/-) mice were subjected to complete ligation of the left common carotid artery. A, Immunofluorescence analysis shows co-localization (pointed by close arrow heads) of cortistatin with αSMA⁺ SMCs of the muscular layer of unligated arteries and of media and intima layers of ligated arteries in C57Bl/6 mice. Note cortistatin-immunostaining in the endothelial cell line (asterisks). B, Lack of cortistatin-positive cells in carotid arteries of CST-/-
mice was used as a negative control for cortistatin-immunostaining. Note the non-specific fluorescent elastic layer and SMA-positive cells in the same sample. Scale bars: 20-µm. n=6 mice, in two independent experiments. C, mRNA and protein levels of cortistatin were determined by qRT-PCR and ELISA on unligated and ligated carotids in C57BL/6 wild-type (WT) and CST-/- mice two weeks after ligation. ND, non-detected. n=3, in duplicates. D, Negative control of immunofluorescence assays performed in ligated carotid arteries of FVB/NJ mice incubated only with FITC- and PE-labeled secondary antibodies, and omitting incubation with primary antibodies against cortistatin and SMA (to compare with images depicted in Figure 1B). Scale bar: 50-µm. Nuclei were counterstained with DAPI (blue staining).
Online Figure III: Expression of cortistatin in human atherosclerotic plaques. A, Micrography of one of the femoral arteries bearing an atherosclerotic plaque used in the study. Staining with hematoxylin and eosin. Scale bar: 100-μm. B, Negative control of immunohistochemistry: atherosclerotic plaque incubated with rabbit antiserum instead rabbit anti-human cortistatin antibody and then detected with permanent alkaline phosphatase. Scale bar: 50-μm. C, Double immunohistochemistry analysis for cortistatin (pink-staining) and αSMA (brown-staining) in different areas of a human femoral artery bearing an atherosclerotic plaque. Solid arrow heads point to some examples of cortistatin-SMA co-localization. SMA-negative infiltrating cells expressing cortistatin are pointed by open arrow heads. Scale bar: 20-μm.
Online Figure IV: Neuropeptides did not affect apoptosis in hAoSMCs activated with PDGF. Cells were starved for 24 hours in SmBM, and then treated for 24 hours with medium (unstimulated), or stimulated with 10 ng/mL PDGF in the absence or presence of 10 nmol/L cortistatin (CST), somatostatin (SOM) or ghrelin. Apoptotic cells were quantified by immunostaining with FITC-labeled anti-Annexin V and flow cytometry analysis. Results are expressed as percentage of apoptotic cells vs. total cells (n=4).
Online Figure V: Cortistatin and ghrelin, but not somatostatin, impaired the increase of free cytosolic calcium in PDGF-activated hAoSMCs. A, Free cytosolic calcium levels were determined by confocal microscopy using the calcium indicator Fluo-4. Pictures illustrate cells in resting conditions before (T20) and after treatments administration (T60 and T120). B, Free cytosolic calcium levels were also determined by fluorescence microscopy using the calcium indicator Fura-2. Results are expressed as percentage of variation of Fura-2 fluorescence after treatment vs. time 0. **P<0.005, ***P<0.001 vs. PDGF-treated cells.
Online Figure VI: Cortistatin reduced intima-media thickening in a model of low and oscillatory flow caused by partial ligation of carotid artery. FVB/NJ mice (8/group, two independent experiments) were subjected to partial ligation of left common carotid as described in Online Figure I. Animals were treated intraperitoneally with saline (control) or with cortistatin (100 ng, three times per week) starting the following day to ligation. Unligated (-) and ligated (+) carotid arteries were collected 10 or 20 days after ligation and analyzed morphometrically as described in Methods. Scale bars: 50-µm. *P<0.001 vs. control.