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

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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 experiencing 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 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–stimulated proliferation of human aortic SMCs via binding to somatostatin receptors (sst2 and sst5) and ghrelin receptor, induction of cAMP and p38-mitogen–activated protein kinase, and inhibition of Akt activity. Moreover, cortistatin impaired lamellipodia formation and migration of human aortic SMCs toward platelet-derived growth factor by inhibiting, in a ghrelin-receptor–dependent manner, Rac1 activation and cytosolic calcium increases. These effects on SMC proliferation and migration correlated with an inhibitory action of cortistatin on the neointimal formation in 2 models of carotid arterial ligation. Endogenous cortistatin seems to play a critical role in regulating SMC function because cortistatin-deficient mice developed higher neointimal hyperplasic 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. (*Cir Res*. 2013;112:1444-1455.)**

Key Words: atherosclerosis ■ cortistatin ■ migration ■ neointimal formation ■ proliferation ■ smooth muscle cell

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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 occur 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.[2] PDGF is the most potent mitogen for vascular SMCs and critically drives their migration.[3] After its production by multiple cells at the site of the injury, including SMCs, activated endothelial cells, platelets, and monocytes,[2] PDGF binds to its receptor in SMCs and activates multiple signal transduction pathways inducing the entry in cell cycle and reorganization of cytoskeleton.[2,4–6] Thus, compounds able to reestablish any or several of the pathologically signaling pathways upregulated by PDGF are desirable to combat the abnormal proliferation and migration of SMCs that occur in many vasculopathies.

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Nonstandard Abbreviations and Acronyms	
CDK	cyclin-dependent kinase
ERK	extracellular signaling–activated kinase
GHSR	ghrelin receptor
hAoSMC	human aortic smooth muscle cell
MAPK	mitogen-activated protein kinase
PDGF	platelet-derived growth factor
SMA	smooth muscle actin
SMC	smooth muscle cell
Sst	somatostatin receptors

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.[7,8] Somatostatin acts through a family of 5 members of G-protein–coupled receptors named somatostatin receptors (ssts) 1 to 5.[9] Ssts are widespread distributed in many cell types, including SMCs, and regulate multiple signal transduction pathways that lead to inhibition of cell proliferation.[9–11] Although various evidences indicate that somatostatin inhibits proliferation of SMCs in vitro,[7,11] there is still controversy regarding the failure of somatostatin or its synthetic analogues to regulate SMC proliferation in vivo.[12–14] 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,[15] with which shares many functions, especially concerning regulation of hormonal secretion.[16,17] This apparent functional redundancy relies in the ability of both peptides to indistinctly bind/activate all the ssts.[17] 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.[15,18–20] Similarly, in the cardiovascular system, cortistatin, but not somatostatin, attenuates vascular calcification.[21] 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.[19–23] This is partially relevant because arterial SMCs express GHSR and ghrelin regulates angiotensin-stimulated SMCs proliferation.[24,25] Moreover, patients experiencing coronary heart disease show elevated serum cortistatin levels.[26] 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 using cortistatin-deficient mice and the therapeutic potential of cortistatin to regulate the formation of neointimal lesions in 2 different models of carotid artery ligation.

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Methods

Complete and Partial Ligations of Carotid Artery

To induce SMC hyperplasia in vivo, we subjected FVB/NJ, C57BL/6, and cortistatin-deficient mice to permanent complete ligation of the left common carotid artery near its bifurcation or alternatively to partial ligation of left carotid artery (Online Figure I).[27,28] Animals received intraperitoneally saline (control) or mouse cortistatin-14 (100 ng) every 2 days starting 1 day after artery ligation. At different times, we isolated the ligated and contralateral unligated arteries for morphometry, neuropeptide content determination, and Western blot analysis (Online detailed Methods). For morphometric analysis, we fixed the isolated carotid arteries in 4% formaldehyde/0.1 mol/L phosphate buffer at 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 optimal cutting temperature compound and freezing. We obtained 5 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.[27] 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 [^{3}H]-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, LY294002, PD98059, or SB203580 (all at 1 µmol/L) before the neuropeptides. We added Alamar-Blue (10% vol/vol) during the last 3 hours of the 24-hour culture and measured its reduction by fluorescence (ex 560-em 590) in a BioTek fluorescence plate reader. In parallel cultures, we added 1 µCi [^{3}H]-thymidine/well for the last 12 hours of the 24-hour culture and determined the incorporation of [^{3}H]-thymidine on a MicroBeta Trilux counter. For cell-cycle analysis, cells were detached at the end of the 24-hour 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 Online 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% CO_2), removed the nonmigrated cells with a cell scraper, fixed the filter bottom face with cold methanol, stained the cells with 4',6-diamidino-2-phenylindole, 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 antihuman α-smooth muscle actin (SMA) monoclonal antibody (1:100; 10 minutes; 20°C) and then with antihuman cortistatin-29 polyclonal antibody (1:500; 30 minutes; 20°C) using a Plus Double 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 antihuman cortistatin-29 (1:500) and antihuman αSMA (1:2000) antibodies for 8 hours at 4°C, followed by detection with Alexa Fluor-594–conjugated antirabbit and Alexa Fluor-488–conjugated antimouse secondary antibodies (1:500 each; 1 hour; 20°C). Sections were 4',6-diamidino-2-phenylindole-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 antimouse cortistatin-29 (1:200), Cy3-conjugated antimouse αSMA (1:1000), and anti-Ki-67 (1:500) antibodies (8 hours; 4°C) and detected with goat Alexa Fluor-546–conjugated antirabbit (1:1000) or fluorescein isothiocyanate–conjugated antimouse-IgG1 (1:400) secondary antibodies (1 hour; 20°C). Nuclei were Hoechst-counterstained and slices examined in a fluorescence microscope.

Rac1 Activation and Western Blot

We evaluated Rac1 activation in hAoSMCs by pulling-down the cdc42/Rac interactive binding domain that precipitates activated Rac1 (Rac1-GTP) and detecting Rac1 by Western blot and determined phosphorylation/activation of extracellular signaling–activated kinase (ERK)1/2, Akt, and p38-mitogen–activated protein kinase (MAPK) by Western blot using specific antibodies that recognize the phosphorylated and total forms of the kinases (Online detailed Methods).

Statistical Analysis

All data are mean±SEM. We analyzed data for statistical differences using Student *t* test or 1-way ANOVA followed by a test for multiple comparisons, considering $P<0.05$ 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). 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, because of 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 and in the endothelial layer (Figure 1B; Online Figure IIA).

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 >50% of the plaque-forming cells. Figure 1C shows that many cells of the atherosclerotic plaque expressed cortistatin. Although cortistatin mostly colocalized 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 reverse transcriptase-polymerase chain reaction 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 on activation with PDGF (Figure 1E).

We next analyzed the expression of the receptors through which cortistatin exerts its actions in most cells, ssts and GHSRs. Among the ssts, sst2 and the truncated receptor sst5transmembrane domain (TMD)4 were the most expressed receptors in hAoSMCs. They also expressed sst1, sst5, the truncated receptor sst5TMD5, and 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 3 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 (CDK) 2 and CDK4, and increased the levels of the CDK-inhibitor $p27^{kip1}$ (Figure 2D).

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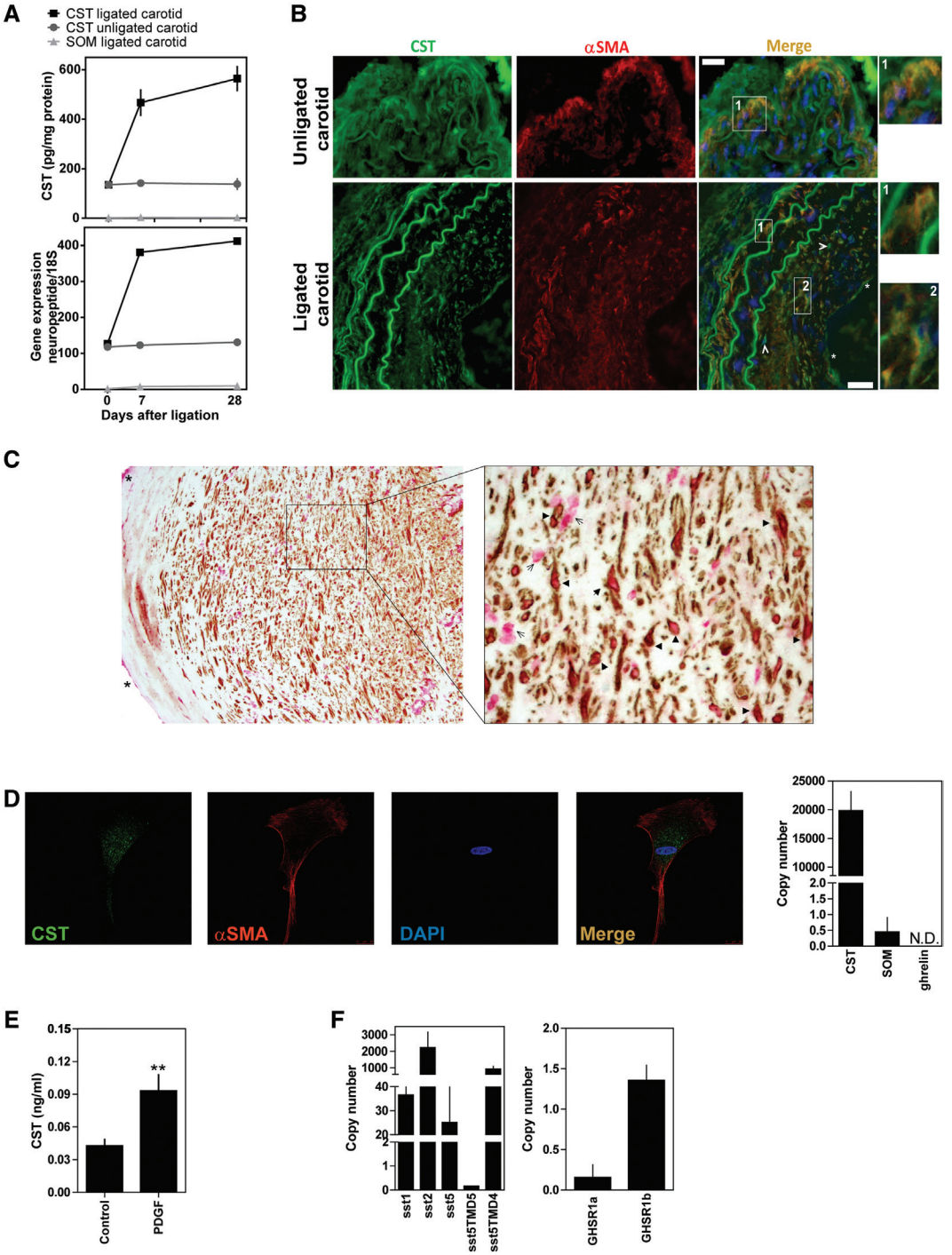

Figure 1. Expression of cortistatin in arterial smooth muscle cells (SMCs). A and **B**, Neointimal hyperplasia was induced by complete ligation of left carotid artery of FVB/NJ mice. mRNA and protein levels of cortistatin (CST) and somatostatin (SOM) were determined by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and ELISA on unligated and ligated carotids at different times after ligation (**A**; n=5). Immunofluorescence analysis (28 days after ligation) shows colocalization of CST with αSMA⁺ cells (**B**; n=7). Note some CST immunostaining in infiltrating SMA-negative cells in intima layer (arrows) and in endothelial cell line (asterisks). 4',6-diamidino-2-phenylindole (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 CST-deficient mice and ligated carotids incubated only with fluorescein isothiocyanate–labeled and phycoerythrin-labeled secondary antibodies. **C**, Immunohistochemical analysis for CST (pink-staining) and αSMA (brown-staining) in human femoral arteries bearing atherosclerotic plaques (n=3). Note numerous cells coexpressing CST and αSMA (dark pink staining pointed by solid arrowheads), some SMA-negative infiltrating cells expressing CST (open arrowheads), and endothelium expressing CST (asterisks). See Online Figure III for additional images and negative control of immunostaining. **D**, Detection of CST protein and mRNA in primary human aortic SMCs (hAoSMCs) by immunofluorescence and qRT-PCR (n=3). **E**, Secretion of CST quantified by ELISA after 2-hour incubation of hAoSMSs in medium (control) or with platelet-derived growth factor (PDGF, 10 ng/mL). ***P*<0.01 (n=4). **F**, Expression of SOM-receptors (sst) 1, sst2, sst5, sst5TMD5, sst5TMD4, ghrelin-receptor (GHSR) 1A, and GHSR1B mRNAs quantified by qRT-PCR in hAoSMCs (n=3). ND indicates nondetected; and SMA, smooth muscle actin.

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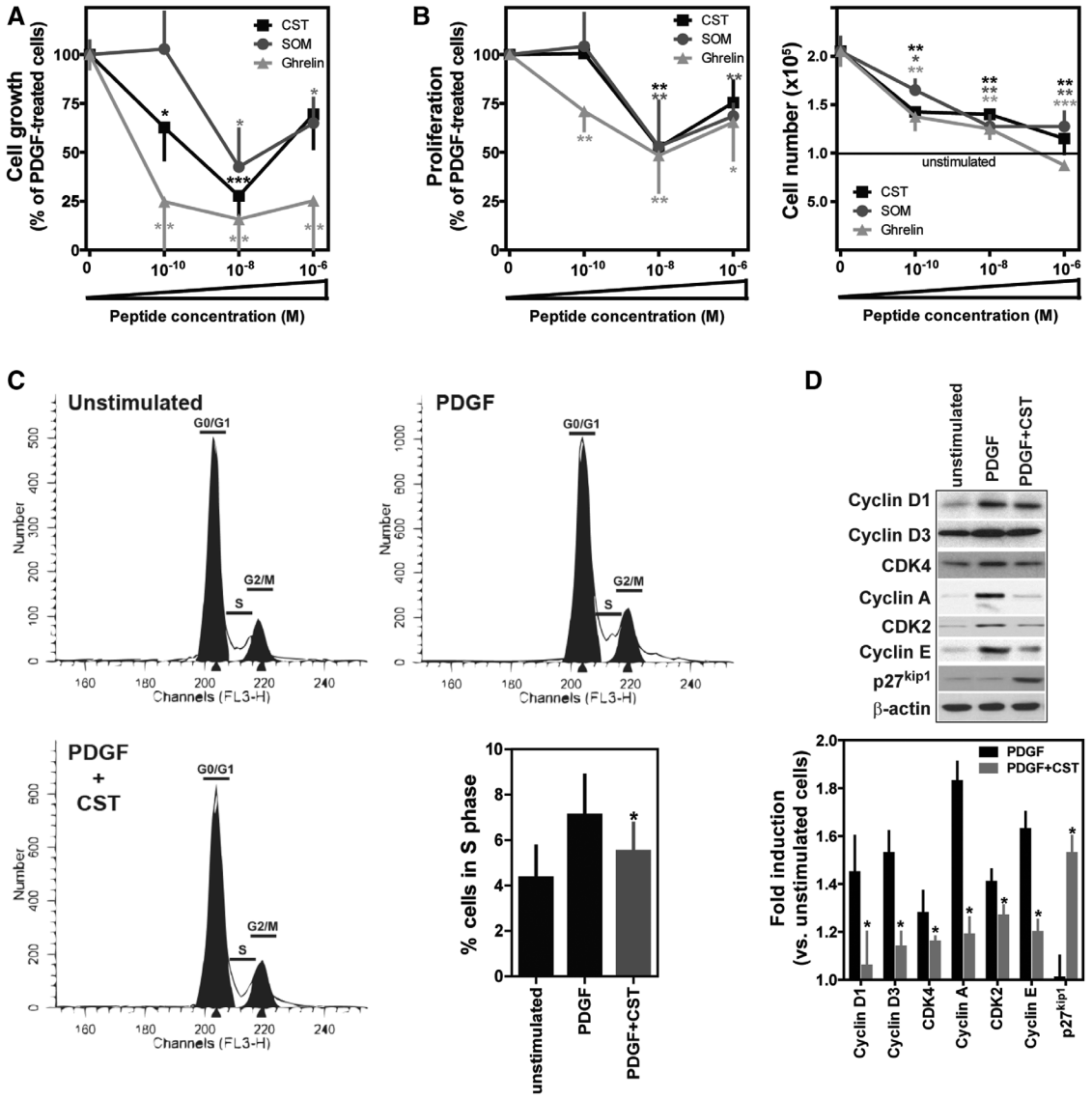

Figure 2. Effects of cortistatin (CST) in cell growth and proliferation of human aortic smooth muscle cells. Cells were incubated with platelet-derived growth factor (PDGF) and different doses of CST, somatostatin (SOM), or ghrelin (10 nmol/L CST in **D**). **A**, Cell growth determined by measuring Alamar-Blue reduction and expressed as percentage of PDGF-stimulated cells (n=5, in triplicate). **B**, Cell proliferation evaluated by quantifying [^{3}H]-thymidine-incorporation into DNA and expressed as percentage of PDGF-stimulated cells (**left**) or by counting cell numbers in Neubauer chambers (**right;** 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, cyclin-dependent kinase (CDK) 2/4, and p27^{kip1} assayed by Western blot and expressed as fold-induction vs unstimulated cells (n= 4–5). **P*<0.05; ***P*<0.01; and ****P*<0.001 vs PDGF-treated cells.

However, 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 GHSRs in hAoSMCs

We next investigated the potential involvement of ssts and GHSRs on the effects of cortistatin using specific peptide antagonists for GHSR, sst2, and sst5 (BIM-28163, BIM-23627, and BIM-23867, respectively). The 3 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 antagonist 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

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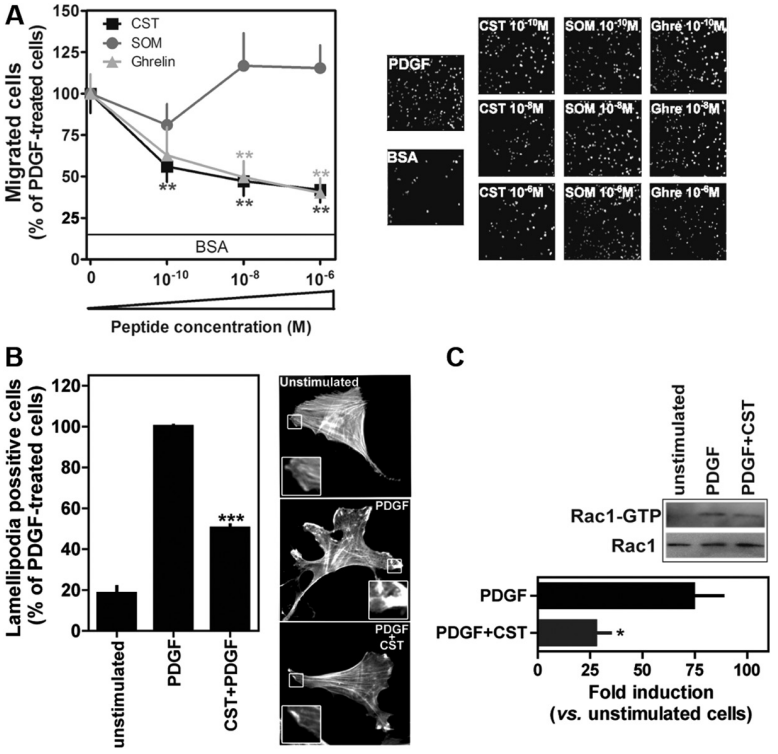

Figure 3. Effects of cortistatin (CST) in migration of human aortic smooth muscle cells (hAoSMCs). A, Effects of CST, ghrelin, and somatostatin (SOM) on the migration of hAoSMCs placed onto the upper side of collagen/vitronectin-coated membranes toward platelet-derived growth factor (PDGF, 10 ng/mL, located at lower chamber). Results show percentage of migrating cells of control samples without neuropeptide treatment. We used migration toward BSA as basal control (n=3, in triplicate). **B**, Inhibitory effect of CST in the formation of lamellipodia (insets) induced by PDGF in hAoSMCs assayed by immunofluorescence for α-smooth muscle actin. 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; and ****P*<0.001 vs PDGF-treated cells.

sst antagonist, 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/protein kinase A pathway is a major signal that inhibits SMC proliferation,[24] and that free cytosolic calcium concentration ($[Ca]_i^{2+}$) 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 3 neuropeptides significantly increased the levels of intracellular cAMP (Figure 5A), only cortistatin and ghrelin, but not somatostatin, blocked the rapid and transient increase in $[Ca]_i^{2+}$ 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 adenylate cyclase with MDL-12330A or of protein kinase A with H89 fully reversed the effects of cortistatin, somatostatin, and ghrelin on hAoSMC proliferation (Figure 5C), confirming the involvement of cAMP/protein kinase A 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 $[Ca]_i^{2+}$ 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). In 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/protein kinase B and ERK1/2 are critically involved in PDGF-stimulated proliferation and migration of SMCs.[4–6] 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 3 neuropeptides because 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.[27] 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

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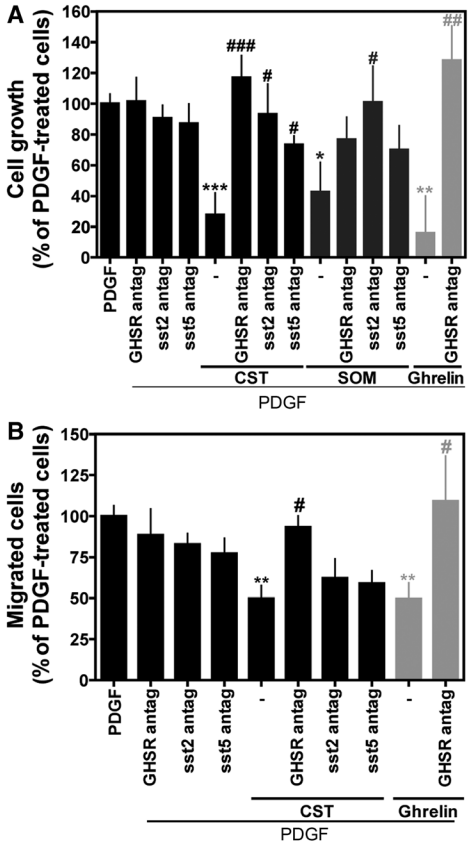

Figure 4. Involvement of somatostatin (SOM) receptors (ssts) and ghrelin receptors (GHSRs) in the inhibitory effects of cortistatin (CST). Effects of the antagonists for GHSR (BIM-28163), sst2 (BIM-23627), or sst5 (BIM-23867) in the inhibitory actions of CST, SOM, or ghrelin (10 nmol/L each) on platelet-derived growth factor (PDGF)–stimulated human aortic smooth muscle cell (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; and ###P<0.001 vs neuropeptide-treated cells without antagonists. *P<0.05; **P<0.005; and ***P<0.001 vs PDGF-treated cells.

remodeling that has relevance to human carotid intima-media thickening and atherosclerosis, in which 3 of the 4 caudal branches of the left common carotid artery are ligated.[28] This partial ligation causes a reduced and oscillatory blood-flow in carotid, resulting in outward remodeling that involves inflammatory infiltration, SMC proliferation, and extracellular matrix reorganization.[28] 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). Terminal deoxynucleotidyl transferase dUTP nick end-labeling 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 using mice deficient for cortistatin subjected to ligation of carotid artery. We observed that cortistatin-deficient 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 the proliferation of these cells and their migration to the intima layer in an autocrine/paracrine manner. In fact, treatment with cortistatin partially reversed the exacerbated neointimal lesion observed in the ligated arteries of cortistatin-deficient 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.[1] 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. On the basis of its high homology with somatostatin, since its discovery, cortistatin was believed a mere somatostatin analogue, in particular, regarding the inhibition of neuroendocrine secretions.[16,17] Nowadays, recent evidence indicates that cortistatin has its own functions, not shared with somatostatin,[15,18–20] 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.[21] As in the immune system,[18–20] the capacity of cortistatin to bind to GHSRs seems to play a major role in this differential effect.[22] Similarly, using new selective receptor antagonists,

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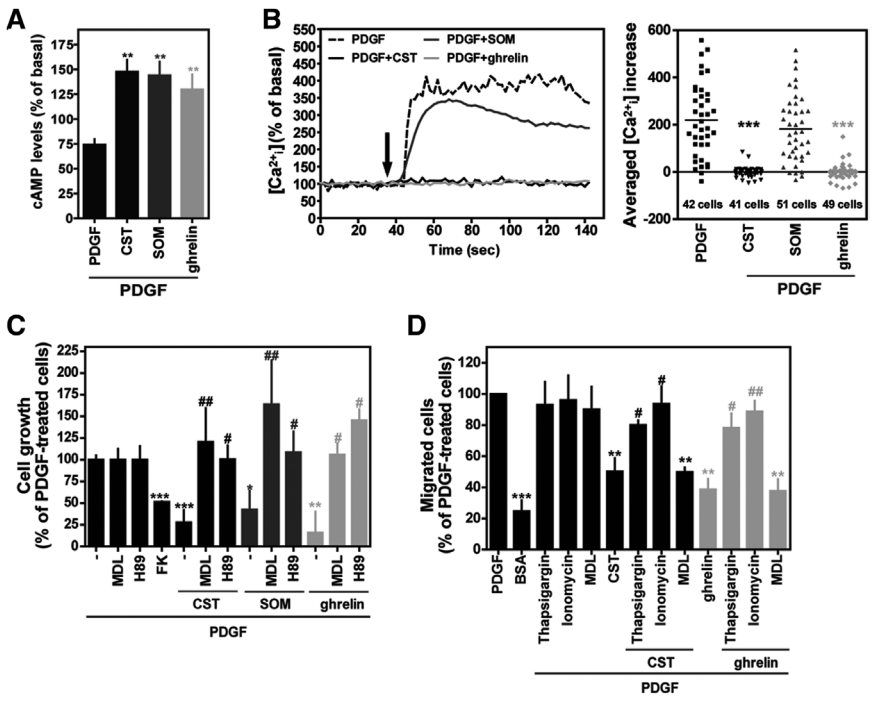

Figure 5. Effects of cortistatin (CST), somatostatin (SOM), and ghrelin in cAMP- and calcium-mediated signaling. Human aortic smooth muscle cells (hAoSMCs) were incubated with medium (unstimulated) or stimulated with platelet-derived growth factor (PDGF, 10 ng/mL) in the absence or presence of CST, SOM, 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 4 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 CST, SOM, ghrelin, forskolin (FK, 10 μmol/L), MDL-12330A (1 μmol/L), and 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 CST 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; and ****P*<0.001 vs PDGF-treated cells. #*P*<0.05; and ##*P*<0.005 vs neuropeptide-treated cells. $[Ca]_i^{2+}$ indicates free cytosolic calcium concentration.

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.[7,9,12,24,25,31,32] Moreover, we found high expression of sst5TMD4, a recently cloned truncated sst5 isoform 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 $p27^{kip1}$, a cell-cycle inhibitor that acts during late G1-phase by impairing CDK2-cyclin A and CDK2-cyclin E complexes. These findings suggest that the cortistatin-treated SMCs

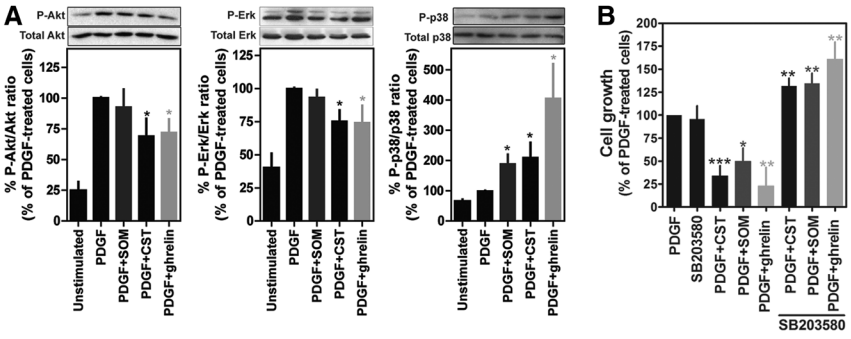

Figure 6. Effect of cortistatin (CST), somatostatin (SOM), and ghrelin in the signaling mediated by Akt, Erk, and p38. A, Effects of CST, SOM, or ghrelin (10 nmol/L each) on Akt, extracellular signaling–activated kinase (ERK) and p38 phosphorylation (assayed by Western blot) in human aortic smooth muscle cells (hAoSMCs) cultured in medium alone (unstimulated) or stimulated with 10 ng/mL platelet-derived growth factor (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 CST, SOM, or ghrelin on PDGF-stimulated hAoSMC proliferation by the p38-mitogen–activated protein kinase (MAPK) inhibitor SB203580. Results show cell growth relative to PDGF-treated cells (n=3, in triplicate). **P*<0.05; ***P*<0.005; and ****P*<0.001 vs PDGF-treated cells.

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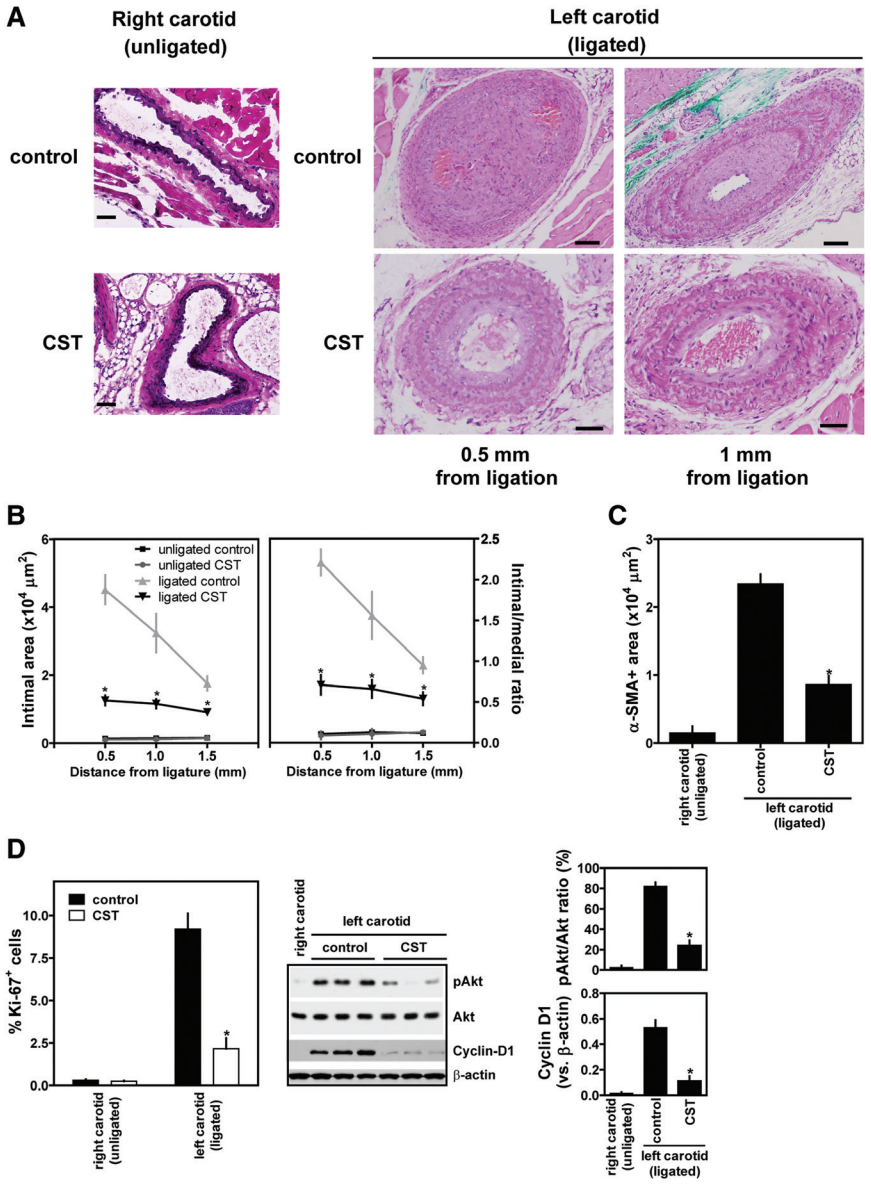

Figure 7. In vivo inhibitory effect of cortistatin (CST) on smooth muscle cells (SMC) proliferation in a model of neointimal hyperplasia caused by ligation of carotid artery. FVB/NJ mice (6–9/group, 3 independent experiments) received intraperitoneally saline (control) or CST (100 ng) every 2 days starting the day after ligation of left carotid artery. **A** and **B**, Treatment with CST 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**, CST decreased the number of SMCs (stained with α smooth muscle actin [SMA]) in the intima layer of ligated carotids. Results are from arteries isolated 4 weeks after ligation analyzed in cross-sections located at 0.8 to 1 mm from ligature. **D**, Treatment with CST 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.

fail to progress into the late G1-phase and enter the S-phase of the cycle. Activation of the cAMP/protein kinase A 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 $p27^{kip1}$.[4–6,33] Moreover, p38-MAPK inhibits cyclin D1 expression and degrades cyclin D3.[34] Beside its effect in proliferation, p38-MAPK activation causes growth arrest by inducing apoptosis in various cell types, including AoSMCs.[35] 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 $[Ca]_i^{2+}$ induced by PDGF in hAoSMCs, which is mandatory for their migratory activity.[5] 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.[29] 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 3 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,[4–6] the effects of cortistatin and ghrelin versus 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

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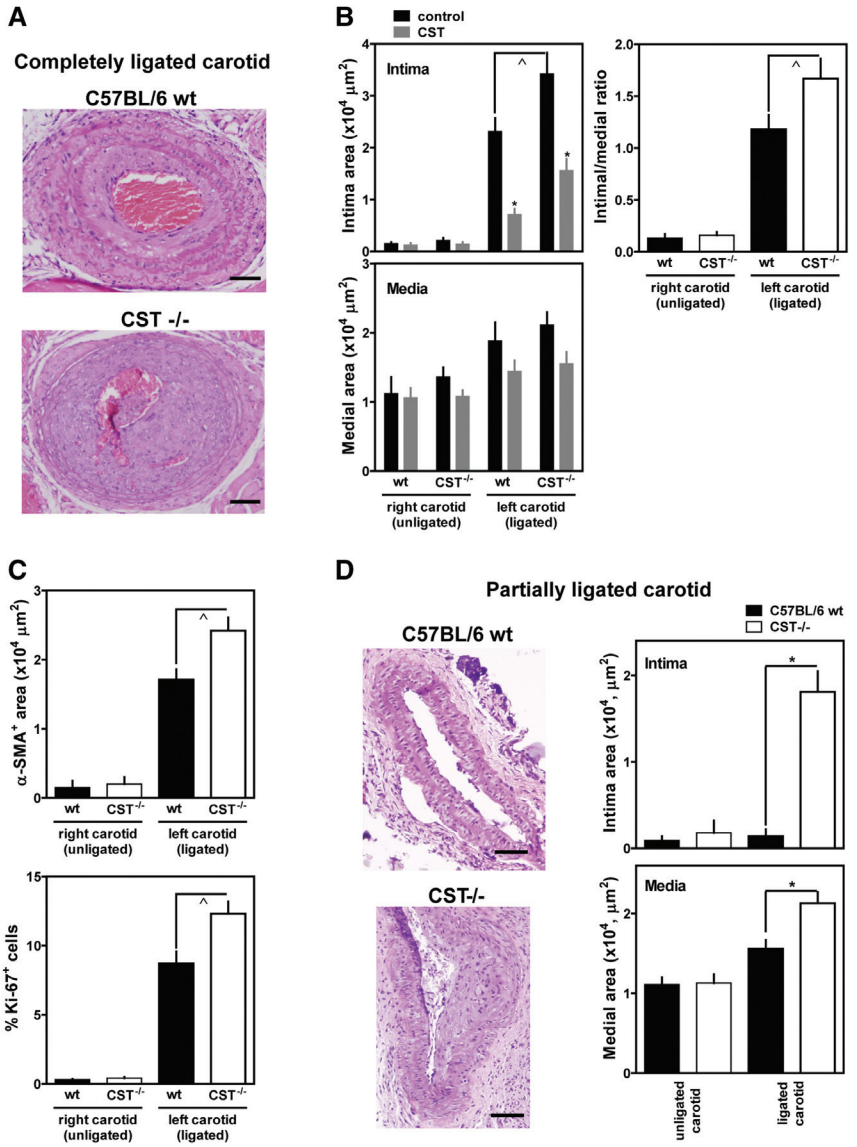

Figure 8. Lack of cortistatin (CST) exacerbates neointima lesion formation. A, Representative photomicrographs showing cross-sectional areas (at 0.8–1 mm from ligature) of the completely ligated carotid artery of wild-type (WT, C57Bl/6) and CST-deficient ($CST^{-/-}$) mice isolated 21 days after ligation. Scale bars, 50 µm. **B**, Lack of CST significantly increased intimal area and intimal/medial ratio vs WT controls. Treatment with CST (200 ng, every 2 days, gray bars) partially reversed this phenotype. **P*<0.05 vs saline; and ^*P*<0.05 vs WT mice. **C**, Mice deficient for CST 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 CST showed marked intima-media thickening after partial carotid ligation compared with WT mice. Scale bars, 50 µm (n=6–8/group; 2 independent experiments). **P*<0.05 vs WT mice.

where somatostatin is absent.[17,36] 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 seems 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 experiencing coronary heart disease.[23] 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),[17] and of cortistatin concentration found in human plasma (0.8 nmol/L)[23] 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 antiatherosclerotic agent. Importantly, the safety and efficacy of the infusion of cortistatin (in the same dose range of 4 µg/kg that is used in our study) demonstrated in humans with Cushing disease will help the translation of our findings

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to the clinic.[37] 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 2 structurally and functionally related neuropeptides, especially in pathological conditions, supports its role as an endogenous factor involved in autocrine/paracrine regulation of vascular homeostasis.

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Disclosures

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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.

SMC 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 high levels of cortistatin, but not other structurally/functionally related peptides, especially in pathological 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.

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