Peptide-Mediated Disruption of Calmodulin–Cyclin E Interactions Inhibits Proliferation of Vascular Smooth Muscle Cells and Neointima Formation

Sonya Hui,* Jaehyun Choi,* Syed Zaidi, Abdul Momen, Sarah K. Steinbach, Al-Muktafi Sadi, Kiwon Ban, Mansoor Husain

Rationale: Cell cycle progression in vascular smooth muscle cells (VSMCs) is a therapeutic target for restenosis. Objective: Having discovered that calmodulin (CaM)-dependent cyclin E/CDK2 activity underlies Ca2+-sensitive G1-to-S phase transitions in VSMCs, we sought to explore the physiological importance of the CaM–cyclin E interaction.

Methods and Results: A peptide based on the CaM binding sequence (CBS) of cyclin E was designed to interfere with CaM–cyclin E binding. Compared with control peptides, CBS blocked activating Thr160 phosphorylation of CDK2, decreased basal cyclin E/CDK2 activity, and eliminated Ca2+-sensitive cyclin E/CDK2 activity in nuclear extracts from mouse VSMCs. Nucleofection with CBS, or treatment with CBS conjugated to the HIV-1 TAT protein transduction domain to improve bioavailability, inhibited G1-to-S cell cycle progression in a dose-dependent manner. These effects were not observed with control peptides. TAT-CBS inhibited 3H-thymidine incorporation in primary human aortic SMCs (HA-SMCs) in vitro, manifested greater transduction into HA-SMCs compared with endothelial cells in vitro, and limited decreased SM22α expression, neointima formation, and medial thickening without affecting collagen deposition or reendothelialization in a mouse model of carotid artery injury in vivo. The antiproliferative effects of CBS remained evident in mouse embryonic fibroblasts derived from wild-type mice but not cyclin E1/E2 double knockout mice.

Conclusions: A synthetic peptide designed to disrupt CaM–cyclin E binding inhibits Ca2+/CaM-dependent CDK2 activity, cell cycle progression, and proliferation in VSMCs and limits arterial remodeling following injury. Importantly, this effect appears to be cyclin E–dependent and may form the basis of a potentially novel therapeutic approach for restenosis. (Circ Res. 2011;108:1053-1062.)

Key Words: vascular smooth muscle cell | cell cycle | restenosis | calmodulin | cyclin E/CDK2

Vascular smooth muscle cells (VSMCs) normally proliferate at very low rates in the media of adult arteries, remaining in the growth arrested (G0) phase of the cell cycle. A shift in the balance between growth stimulatory and inhibitory factors can lead to cell cycle reentry and transformation from contractile and quiescent to proliferative and synthetic phenotypes. Thus activated, VSMCs can remodel the artery by altering the extracellular matrix, replicating in the media, and migrating to the intima to undergo further cycles of proliferation. Indeed, unregulated proliferation of VSMCs is a principal mechanism underlying the pathogenesis of common vascular diseases, such as atherosclerosis and restenosis.1,2

Decades of work have implicated ionic calcium (Ca2+) as a regulator of eukaryotic cell cycle progression.3 In VSMCs, we previously made 3 related discoveries regarding Ca2+-mediated cell cycle regulation: (1) a coordinated increase in the free intracellular Ca2+ concentration is required for G1-to-S phase cell cycle transition;4,5 (2) this occurs through cell cycle–associated expression and activation of specific Ca2+ pumps and channels;5–7; and (3) is at least partly mediated by Ca2+/calmodulin (CaM)-dependent cyclin E/CDK2 activity.8

Our findings suggested that Ca2+ sensitivity of the G1-to-S phase cell cycle transition requires the direct binding of the major Ca2+ signal transducer CaM to cyclin E, through a specific and conserved CaM-binding motif in cyclin E. The functional importance of this motif was accentuated by the observation that a cyclin E mutant lacking this motif was unable to produce Ca2+/CaM-stimulated activity of CDK2.8

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1053
Non-standard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
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<tr>
<td>Ca²⁺</td>
<td>ionic calcium</td>
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<tr>
<td>CaM</td>
<td>calmodulin</td>
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<tr>
<td>CBS</td>
<td>calmodulin binding sequence</td>
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<tr>
<td>CMZ</td>
<td>calmidazolium</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>G₀</td>
<td>growth-arrested phase of cell cycle</td>
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<tr>
<td>IP</td>
<td>immunoprecipitation</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
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<tr>
<td>NC</td>
<td>negative control</td>
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<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<tr>
<td>SM22-α</td>
<td>smooth muscle 22-α</td>
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<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<tr>
<td>sm-MHC</td>
<td>smooth muscle myosin heavy chain</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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These data shed light on a mechanistic basis for Ca²⁺-sensitive cell cycle progression and predicted other possible Ca²⁺/CaM-sensitive cell cycle targets.⁹

Based on the discovery of a functional CaM–cyclin E interaction, we hypothesized that blocking CaM–cyclin E binding through the use of a synthetic peptide will inhibit Ca²⁺-sensitive G₁-to-S phase transitions and slow the proliferation of VSMCs by competing with cyclin E for binding to CaM. The present report details our characterization of such a peptide and provides data supporting the physiological importance of CaM–cyclin E interactions in VSMCs. Together, the results suggest a novel therapeutic approach to inhibiting proliferation in VSMCs.

**Methods**

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

**Cell Culture**

Isolation and culture of primary mouse aortic smooth muscle cells (SMCs) have been described.⁶ Cells and reagents for primary human aortic SMC and primary human aortic endothelial cell (EC) culture were purchased from Invitrogen. Wild-type and cyclin E1/E2 double knockout mouse embryonic fibroblasts (MEFs) were kindly provided by Dr P. Sicinski (Harvard Medical School, Boston, MA).

**Cyclin E/CDK2 Assay**

Histone H1 kinase activity of complexes immunoprecipitated with anti–cyclin E antibody were performed as described previously.⁸ Nucleofection and TAT fusion were used to deliver CBS peptide into cells.

**Western Blot**

Primary antibodies, including anti-CDK2 (07-631; Upstate Biotech, Billerica, MA), anti–cyclin E (ab7959; Abcam, Cambridge, MA), anti–phosphorylated CDK2 on Thr160 (Upstate Biotech, Danvers, MA), anti-CaM (sc-1989; Santa Cruz Biotechnology, Santa Cruz, CA), anti–phosphorylated CDK2 on Thr14/Tyr15 (sc-28435-R; Santa Cruz Biotechnology), and anti-actin (sc-7210; Santa Cruz Biotechnology) were used to detect levels of CaM-binding and CDK2 phosphorylation and to confirm peptide delivery.

**Immunostaining**

Confocal microscopy was used to track fluorescein isothiocyanate (FITC)-labeled peptides in cells following nucleofection and to characterize the phenotype of TAT-CBS–treated SMCs in human aortic SMCs and mouse carotid artery by immunostaining for SMC marker proteins. TAT domain–mediated delivery of His-tagged peptides into human aortic SMCs and mouse carotid artery was also confirmed by immunostaining. To assess reendothelialization, carotid artery sections harvested 7 days after wire-denudation injury were immunostained with anti-CD31 antibody.

**Proliferation Assays**

Cell counting and the MTT and the ³H-thymidine incorporation assays were used to measure cell number and proliferation of CBS peptide–treated cells.

**LDH, Caspase-3, and TUNEL Assays**

The LDH-based in vitro toxicology assay kit (Sigma) was used to assess cytotoxicity of nucleofected peptides and TAT-conjugated peptides.¹⁰ The caspase-3 Colorimetric Assay Kit (Sigma) and In Situ Cell Death Detection Kit (Roche) were used to assess apoptosis of TAT-peptide–treated cells.

**Flow Cytometry**

To track cell cycle stages, flow cytometric analysis was performed as described previously.⁵

**Mouse Carotid Artery Injury**

All animal experimentation conformed to protocols approved by the institutional animal care committee. The model used has been described previously.¹¹

**Tissue Processing and Analysis**

At 14 days after injury, lethally anesthetized mice were perfusion fixed. Previously injured and sham common carotid arteries were harvested, postfixed, and embedded in paraffin or OCT. Sections were then stained, imaged, and quantified for analyses.

**Results**

**Design of the CBS Peptide**

We hypothesized that a 22-aa synthetic peptide based on the Calmodulin Binding Sequence (CBS) of human cyclin E1 would compete with cyclin E for binding to CaM and thus inhibit the CaM–cyclin E interaction (Figure 1A). A “5A” peptide, in which each hydrophobic residue of CBS was substituted with alanine, and a negative control peptide (NC) composed of a random sequence of 22 aa were also synthesized. First, we tested the effects of these peptides on cyclin E/CDK2 activity as measured by histone H1 kinase activity of nuclear extracts immunoprecipitated with anti–cyclin E antibody. In these experiments, all peptides were added during the immunoprecipitation (IP) step. CBS reduced cyclin E/CDK2 activity by 40%, whereas the NC peptide had no effect as compared with untreated controls. 5A had less inhibitory effect on cyclin E/CDK2 activity than CBS, suggesting functional importance of the hydrophobic residues in CBS (Figure 1B). Co-IP analysis revealed that cyclin E/CDK2 complex formation was not affected by addition of the CBS peptide (Figure 1C). By measuring histone H1 kinase activity over a range of Ca²⁺ concentrations, we found that Ca²⁺-sensitive enhancement of cyclin E/CDK2 activity was abolished by CBS but not NC (Figure 1D).
Delivery of the CBS Peptide to VSMCs

To investigate the effects of the CBS peptide on cell cycle progression and proliferation of VSMCs, CBS and NC were delivered to primary mouse aortic SMCs via nucleofection. For tracking purposes, both peptides were tagged with FITC. Confocal microscopy for FITC confirmed delivery of CBS to VSMC nuclei with approximately 40% efficiency (data not shown). The MTT cell viability assay was used to determine the effect of peptide nucleofection on VSMC number, as an indirect indicator of proliferation (Online Figure I).

Because the absence of NC effect on cyclin E/CDK2 kinase activity (Figure 1B) may have resulted from differences in secondary structure and biochemical properties (pH, hydrophobicity, polarity) of the NC peptide, MTT assays were also performed with a scrambled CBS, harboring all of the original residues at different locations. CBS treatment significantly decreased the number of VSMCs compared with untreated and NC–treated cells as measured by MTT assay (Figure 2A). Importantly, the scrambled peptide was unable to decrease VSMC number, confirming that the amino acid sequence of CBS is critical to its antiproliferative function (Figure 2A).

The CBS peptide also delayed and inhibited the number of primary mouse aortic SMCs in a dose-dependent manner (Figure 2B). To define an IC50, different concentrations of CBS were delivered to asynchronous primary mouse aortic SMCs tracked with the MTT assay. A sigmoidal dose response curve (Boltzmann model) yielded an IC50 of 186 μmol/L for the parent CBS peptide (Online Figure II).

Mechanisms Underlying the Observed Effects of CBS

To explore mechanisms underlying CBS-induced inhibition of VSMC number, LDH levels were examined as a measure
of cytotoxicity. LDH release into the culture media of each treatment group did not differ (untreated-, CBS-, and NC-treated VSMCs), indicating that nucleofection of CBS peptide did not increase cell death (Online Figure III).

Densitometric quantification of co-IP and Western blot data revealed that CBS inhibited both (1) CaM–cyclin E interactions (2.25 versus 1.11) without affecting levels of CaM (1.84 versus 1.68) or cyclin E (2.36 versus 2.48); and (2) phosphorylation of CDK2 on Thr160, a critical event in the activation of CDK2 (2.63 versus 0.94). It was also observed that the amount of inhibitory CDK2 phosphorylation at Thr14/Tyr15 sites (0.41 versus 0.38), and the total amount of CDK2 (1.04 versus 0.98) were not altered by treatment with CBS. Furthermore, binding of CaM to calcineurin, another CaM target protein, was unaltered by CBS (1.44 versus 1.40), suggesting selectivity of CBS for its target interaction (Figure 3A).

FACS-driven analysis of cell cycle progression confirmed that the effect of CBS on cell number was attributable to inhibition of proliferation by G1 cell cycle arrest. Starvation of primary mouse aortic SMCs resulted in 80% cell synchronization in G0/G1. CBS versus NC peptides were delivered to starved cells via nucleofection followed by serum stimulation with 10% FBS/50 ng/mL PDGF for 24 hours. Without peptide treatment, 22.4% of serum-stimulated cells entered S phase, whereas only 11.7% of CBS-treated cells entered S phase (Figure 3B).

Animal models were obtained. Absence of cyclin E1 protein in double knockout cells was confirmed by Western blot (Online Figure IV). MTT assays revealed that wild-type MEF cell number was reduced by CBS at a level similar to that observed in VSMCs. However, the number of MEFs from cyclin E1/E2 double knockout mice was unaffected by CBS, supporting a cyclin E–dependent mechanism of action (Figure 4A).

We next tested whether the effects of CBS on cell cycle progression of MEFs were also cyclin E–dependent. CBS or NC peptides were delivered to starved MEFs via nucleofection, followed by serum stimulation with 10% FBS for 24 hours. FACS analysis indicated that cell cycle progression into S phase was inhibited by CBS in wild-type, but not in cyclin E1/E2 double knockout, MEFs. By contrast, calmidazolium, a nonselective CaM-inhibitor, was able to inhibit cell cycle progression of double knockout MEFs, possibly through other CaM-dependent pathways (Figure 4B). These results further supported cyclin E–target specificity of the CBS peptide.

Modifications to Increase Bioavailability

Because nucleofection is not possible in vivo, we fused the TAT protein transduction domain from HIV-1 to CBS to enable potential in vivo therapy (Online Figure V, A). This approach was based on reports of the ability of the TAT domain to successfully deliver a size-independent variety of molecules into cell nuclei. Although the exact mechanism of TAT-mediated protein transduction is unknown, the large cationic charge of arginine residues is believed to allow TAT-conjugates to effectively cross cell membranes by receptor-mediated endocytosis.14,15 Cells were exposed to...
TAT-CBS-His peptide in serum-free cell culture media for 1 hour, as serum decreases the transfection efficiency of the TAT domain. Delivery of TAT-CBS-His or TAT-NC-His was confirmed by both Western blot and immunostaining analyses using an anti–His tag antibody (Online Figure V, B and C).

To investigate putative antiproliferative effects of TAT-CBS-His, primary mouse aortic SMCs were treated with peptide, washed twice with PBS, supplied with fresh media containing 10% FBS, and analyzed by MTT assay 2 days later. As expected, the TAT-CBS-His peptide inhibited the number of VSMCs, whereas TAT-NC-His peptide showed no effect (Figure 5A). Treatment with various concentrations of peptide revealed a dose-dependent relationship between TAT-CBS-His and decrease in cell number. Nonlinear regression analysis (Boltzmann sigmoid curve model) of 3 separate experiments revealed an IC$_{50}$ of 8.89 $\pm$ 1.24 µmol/L (Online Figure VI, A), demonstrating that the antiproliferative effect of CBS was significantly enhanced by TAT domain fusion compared with CBS delivered by nucleofection (IC$_{50}$: 8.9 versus 186.2 µmol/L). The maximum level of inhibition was also increased by TAT-CBS fusion as compared with CBS delivered by nucleofection (0.70 versus 0.50).

Effects of CBS Peptide Treatment on Human Vascular Cells

To validate results in mouse and explore physiological relevance of TAT-CBS in human, we demonstrated an antiproliferative effect of the peptide in human aortic SMCs by cell counting (Online Figure VI, B). Confirming that the antiproliferative effect of TAT-CBS was attributable to cell cycle arrest and not cell death, neither extracellular LDH release, caspase-3 activation, nor TUNEL staining were detected in human aortic SMCs at 24 hours (data not shown) or 72 hours following treatment (Online Figure VII). Moreover, immunostaining for contractile SMC marker proteins smooth muscle 22-α (SM22-α), smooth muscle myosin heavy chain (sm-MHC), and α-smooth muscle actin (α-SMA) revealed that TAT-CBS treatment did not significantly alter the phenotype of human aortic SMCs in vitro (Online Figure VIII).

To further establish that CBS works via an inhibitory effect on S-phase entry and to ascertain whether this was evident in
human VSMCs, we next examined DNA synthesis as measured by 3H-thymidine incorporation in human aortic SMCs treated with TAT-CBS. Compared with untreated cells, TAT-CBS, but not TAT-NC, produced a dose-dependent inhibition of S-phase entry in human aortic SMCs (Figure 5B).

To explore the clinical potential of TAT-CBS as a novel therapeutic agent, we also tested the effect of TAT-CBS on 3H-thymidine incorporation in human aortic ECs. Although TAT-CBS did appear to dose-dependently inhibit human aortic EC proliferation, the effect only differed from TAT-NC–treated cells at the highest concentration administered (1 mmol/L), which may be cytotoxic (Figure 5B). To compare transduction efficiency of TAT-CBS in HA-SMCs versus HA-ECs, immunostaining for the His-tagged version of the peptide was performed at several time points following treatment in vitro. Results showed that HA-SMCs manifest a similar extent but greater degree of His-staining than HA-ECs (Online Figure IX). Consistent with the results from nucleofection experiments with CBS (MTT assays and flow cytometry; Figure 3C and 3D), the ability of TAT-CBS to block S-phase entry as measured by 3H-thymidine incorporation was only evident in wild-type MEFs (Online Figure X, A) and not cyclin E double knockout MEFs (Online Figure X, B). Together, these data strongly support a cyclin E–dependent effect of the CBS peptide sequence on S-phase progression in both mouse and human cell types.

**CBS Inhibits Neointima Formation In Vivo**

The TAT-CBS-His peptide was also tested in a mouse common carotid artery injury model via pluronic gel administration. In aqueous solution, the surfactant pluronic F-127 transforms from liquid to nonfluid hydrogel above room temperature. This property enables in vivo peptide delivery, because the semisolid solution of peptide and F-127 allows the peptide to remain concentrated and protected by the surfactant matrix. Carotid artery injury was performed by wire denudation. Injuries were performed on the left common carotid artery, with the right common carotid artery serving as an uninjured control. After injury, common carotid arteries were immersed in 100 μL of 25% (wt/vol) pluronic F-127 gel, either containing peptide (TAT-CBS-His or TAT-NC-His), 250 μmol/L or no peptide (6 mice per treatment group) before wound closure. Delivery of TAT-CBS-His into VSMCs of the mouse carotid artery was confirmed by immunostaining (Online Figure XI).

Because migration of VSMCs from media to intima following initial proliferation in media is an essential step in the development of restenosis or atherosclerosis, our quantitative analysis of arterial sections included: total area of arterial (1) media, (2) intima, and (3) intima to media (I/M) ratio. H&E staining of arteries harvested 14 days after injury showed that treatment with TAT-CBS-His significantly decreased neointima formation and arterial media thickening compared with treatment with F-127 alone or TAT-NC-His control peptide groups (Figure 6A). Morphometry confirmed that injured arteries treated with TAT-CBS-His manifest reduced medial and intimal areas and I/M ratio, indices consistent with reduced VSMC proliferation and migration (Figure 6B).

Proliferating cell nuclear antigen (PCNA) staining was performed as an additional measure of the level of proliferation in injured arteries. Arteries treated with TAT-CBS-His had a decreased percentage of PCNA-positive nuclei, further confirming the ability of TAT-CBS-His peptide to inhibit cell proliferation in injured arteries (Figure 7A). Separate analyses of intima versus media revealed significantly more PCNA-positive nuclei in the intima than media of injured arteries. Indeed, TAT-CBS-His treatment specifically decreased PCNA-positive nuclei in the intima (I/M) ratio, indices consistent with reduced VSMC proliferation and migration (Figure 6B).
may prevent injury-driven decreases in the expression of VSMC-specific contractile markers (ie, “dedifferentiation”) in vivo, a strategy recently shown to have promise in a similar model in rat.16

CBS Does Not Affect Extracellular Matrix Formation or Reendothelialization In Vivo

Because extracellular matrix deposition also contributes to the formation of restenosis, we quantified collagen in injured carotid arteries (Online Figure XII). Compared with controls, TAT-CBS treatment did not significantly alter type I, type III, or total collagen deposition. Given our data with ECs in vitro, we also investigated the effect of CBS on the reendothelialization of injured carotid arteries in vivo. CD31 staining of sectioned arteries revealed that TAT-CBS–treated arteries did not affect reendothelialization at 7 days after injury, as compared with gel-only and TAT-NC–treated arteries (Figure 8).

Discussion

In this study, we have shown that CBS, a specific 22-aa peptide inhibits (1) the binding of CaM to cyclin E; (2) Ca\(^{2+}\)-sensitive cyclin E/CDK2 activity; (3) G\(_1\)-to-S cell cycle progression of VSMCs; and (4) VSMC and EC proliferation in vitro, with (5) greater peptide transduction in VSMCs versus ECs, and without increasing (6) cytotoxicity, (7) apoptosis, or (8) dedifferentiation of VSMCs. It was also shown that CBS inhibits (9) the activating phosphorylation of CDK2 at Thr160 without altering the inhibitory phosphorylation on Thr14/Tyr15 by (10) selectively interfering with CaM–cyclin E interactions. Importantly, (11) the binding of
dysfunction manifesting itself in the form of residual vasodilatory deficits and/or long-term thrombosis risks remain a concern. Indeed, alternative strategies for more cost-effective and less toxic treatments of conditions such as restenosis remain of interest to interventional cardiology.

A distinguishing characteristic of the CBS peptide versus antiproliferative agents used in existing drug eluting stents is its focused mechanism of action. Targeting cyclin E–dependent Ca$^{2+}$-sensitive CDK2 activity, the actions of CBS peptide are restricted to proliferating cells, whereas the actions of agents such as sirolimus and paclitaxel (on mTOR and microtubules respectively) are not. As such, the CBS peptide may be a less toxic alternative to these agents. Although we cannot state with certainty that CBS does not alter the binding of CaM to any of the $>300$ known target proteins of CaM, our experiments in cyclin E knockout cells strongly support the target specificity of CBS. In these studies, CBS only reduced proliferation of MEFs with intact cyclin E, suggesting that even if CBS does alter CaM-binding to proteins other than cyclin E, these off-target effects do not affect proliferation.

Our dose–response studies of TAT-CBS–treated human aortic ECs and SMCs in vitro reveal IC$_{50}$ values that suggest similar antiproliferative potency in these cell types. However, adventitial delivery of TAT-CBS in vivo did not retard reendothelialization following injury. This discrepancy may be attributable to 2 factors. First, the ability of adventitial TAT-CBS to gain access to the luminal EC compartment may be less than its ability to penetrate VSMCs. Second, circulating EC progenitors have been shown to contribute to reendothelialization, and their attachment and spread to denuded areas may be insensitive to TAT-CBS.

Although TAT-CBS treatment was effective at inhibiting VSMC proliferation and neointima formation in mice, our peptide did not confer therapeutic benefits on other aspects of vascular remodeling such as collagen deposition and endothelial repair. Therefore, as a potential next generation agent in drug-eluting stents, treatment with CBS, by itself, may not be sufficient to prevent adverse remodeling in more complex large animal models or clinical cases of atherosclerosis. Combining the CBS peptide with therapies promoting endothelial repair and/or inhibiting extracellular matrix deposition may be required for synergistic benefits.

Based on our mouse carotid injury model data, we anticipate that the CBS peptide could be delivered to patients at the time of coronary intervention and continuously administered afterward as a novel agent in drug eluting stents. VSMC-targeted delivery of CBS peptide (or its next generation surrogates) may have the potential to selectively block rapidly proliferating VSMCs, while not interfering with reformation of the antithrombotic endothelial cell lining at the site of injury. For instance, a gene therapy method could be used in which a plasmid containing the CBS sequence is under the transcriptional control of a SMC-specific promoter. This approach has proven effective with the SM22-α promoter for SMC-specific transfection in a rat model of carotid injury. Additionally, virus retargeting techniques that modify surface proteins and moieties for binding to a cell surface
MLCK.34 The success of these related drugs supports further NFAT-mediated proliferation and inflammation of VSMCs.31 Small peptides that block the interaction of cyclin A/CDK2 with substrates such as E2F1 have also been investigated in a number of tumor cell lines. These inhibitory peptides induced S-phase arrest and abrupt apoptosis. Cell death was selective to transformed cells; although a normal human fibroblast cell line did not undergo apoptosis, a T antigen–transformed subclone derived from it was killed.32 Similar to the CBS peptide, other molecules have been developed to inhibit the binding of CaM to its target proteins, such as ATPase33 and MLCK.34 The success of these related drugs supports further drug development based on CBS.

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Disclosures
J.C. and M.H. are coinventors of the CBS and have filed a provisional patent for its use as an antiproliferative agent. The authors have no other conflicts of interest to disclose.

References


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**Novelty and Significance**

**What Is Known?**

- Tightly coordinated increases in intracellular calcium are required for cell cycle progression in vascular smooth muscle cells (VSMCs).
- Calcium dependence of cell cycle in VSMCs is regulated in part by protein–protein interaction between the calcium signal-transducer calmodulin (CaM) and the cell cycle regulator cyclin E.

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

- A synthetic peptide corresponding to the calmodulin binding sequence (CBS) of cyclin E disrupts CaM–cyclin E interactions and inhibits calcium-dependent activation of the cyclin E–cell cycle kinase CDK2 complex in VSMCs.
- The CBS peptide exerts a cyclin E–dependent inhibitory effect on cell cycle progression and cell proliferation.
- Adventitial application of the CBS peptide following carotid artery denudation injury in the mouse prevented loss of contractile marker gene expression and inhibited VSMC proliferation and neointima formation in vivo without affecting collagen deposition or endothelial repair.

VSMC proliferation contributes to the adverse remodelling of atherosclerosis and restenosis. Elucidating mechanisms regulating cell cycle progression in VSMCs can form the basis of new antiproliferative therapies for these conditions. Here, we demonstrate the physiological significance of the CaM–cyclin E interaction and the relevance of calcium-sensitive cell cycle progression in VSMCs. A synthetic peptide based on the CaM-binding sequence (CBS) of cyclin E disrupted CaM–cyclin E interactions and inhibited cell cycle progression and proliferation of mouse and human VSMCs in vitro. The CBS peptide significantly inhibited proliferation of VSMCs and neointima formation following carotid wire injury in vivo without affecting matrix deposition or endothelial repair. What distinguishes the CBS peptide from agents used in existing drug-eluting stents for restenosis is its focused mechanism of action. CBS inhibits calcium-sensitive but not baseline cyclin E/CDK2 activity and is unable to inhibit proliferation of cells lacking cyclin E. Because existing drugs for restenosis broadly affect mTOR signaling (eg, sirolimus) or microtubule stability (paclitaxel), their toxicity includes impaired endothelial function and repair, which pose long-term risks. The present study identifies a novel antiproliferative agent that prevents adverse vascular remodeling without inducing cell death or loss of contractile marker gene expression.
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SUPPLEMENTAL MATERIAL

Materials. Purified CaM, calmidazolium, DTT, aprotinin, leupeptin, thiazolyl blue tetrazolium bromide, and other chemicals were purchased from Sigma. Pluronic F-127 Gel was obtained from BASF Corp (Mount Olive, NJ).

Peptide synthesis. All synthetic peptides (TAT-CBS-His, TAT-NC-His, TAT-CBS, TAT-NC and TAT-Scramble, see Supplemental Table 1 for sequences) were purchased from GenScript Corp, had greater than 95% purity, were prepared by dissolution in milli-Q water to a concentration of 2 mmol/L and stored at -20°C. Peptides are stable for one year following synthesis and were used within this time period.

Generation of CBS sequences. Amino acid sequences of mouse and human cyclin E1 were obtained from UniProt Knowledgebase (ID# Q61457 and P24864) and analyzed with the CaM-target database (http://calcium.uhnres.utoronto.ca/ctdb). A putative CaM-binding motif was listed when the motif was at least 16 amino acids in length and displayed a probability score >3.

Primary mouse aortic SMC. Briefly, VSMC were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT), 50 ng/mL PDGF (Sigma) and 1% penicillin-streptomycin (Invitrogen). For serum starvation, cells were grown to 70% confluence, washed twice with PBS, and cultured in starvation medium (DMEM with 0.25% FBS) for 72 h to achieve G0 arrest. Progression to S phase was induced with media containing 10% FBS and 50 ng/mL PDGF for 24 h.

Cell culture. Cells and reagents for primary human aortic SMC culture were purchased from Invitrogen. Human VSMC (C-007-5C) were grown in Medium 231 with Smooth Muscle Growth Supplement (S-007-25) and 1% penicillin-streptomycin. Cells were serum-starved by culturing in Media 231 with 2% SGMS. Cells and reagents for primary human aortic endothelial cell (EC) culture were purchased from Invitrogen. HAEC (C-006-5C) were grown in Medium 200 with Low Serum Growth Supplement (S-003-10) and 1% penicillin-streptomycin (Invitrogen). Wild-type and cyclin E1/E2 double knockout mouse embryonic fibroblasts (MEF) were kindly provided by Dr. P. Sicinski (Harvard Medical School), and maintained in DMEM with 10% FBS (Hyclone) and 1% penicillin-streptomycin. G0 arrest of MEF was achieved by starvation for 48 h in medium lacking FBS.

Cyclin E/CDK2 assay. SMC (5×10^6) were harvested and resuspended with 2 mL of lysis buffer (50 mmol/L Tris [pH 7.4], 250 mmol/L NaCl, 5 mmol/L EDTA, 0.1% NP-40, 0.5 mmol/L DTT, 0.1 mmol/L Na3VO4, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 2 mmol/L PMSF, and 10% glycerol). After homogenization, cell lysates were clarified by centrifugation at 12x10^3 rpm, 4°C for 30 minutes, and the supernatant collected. An aliquot was taken for protein concentration determination using the BCA protein assay kit (Sigma). Cell extracts (200 µg) were then incubated for 2 hours at 4°C with saturating concentrations of polyclonal antibodies. Immune complexes were collected by incubation with GammaBind G Sepharose resin (Amersham Pharmacia, Piscataway, NJ) for 1 hour at 4°C. The beads were then washed three times with washing buffer (50 mmol/L Tris [pH 7.4] and 1 mmol/L DTT).

For in vitro kinase assays, 20 µL of kinase reaction mixture (20 mmol/L Tris [pH 7.4], 5 mmol/L MgCl2, 2.5 mmol/L MnCl2, 1 mmol/L DTT, 10 µg of C terminal of human Rb protein or 4 µg of histone-H1 [both Upstate Biotechnology] as substrates, 20 µmol/L ATP, and 2.4 µCi [γ-32P]ATP [Amersham Pharmacia]) was added to CDK2-, cyclin E–, or cyclin A–immunoprecipitated (IPd) complexes after removing the washing buffer from GammaBind G Sepharose resin. Based on the total calcium content of kinase reaction mixtures (as determined by inductively coupled plasma atomic emission spectrometry), the amount of EGTA required to yield final concentrations of free Ca2+ ion (0, 100 or 500 nmol/L) was calculated by Winmol/LaxC (http://www.stanford.edu/~cpatton/winmaxc2.html). After 30 min incubation at 37°C, reactions were stopped by adding 20 µL of 2X sodium dodecyl sulfate (SDS) loading buffer and heating at 100°C for 5 min. Labelled proteins were resolved by 16% SDS-PAGE. Phosphorylated Rb or histone–H1 bands were visualized by autoradiography and quantified in a Scintillation Counter LS6500 (Beckman Coulter, Fullerton, CA) after being cut from the gel.
Western blot. 20-30 µg of cell extracts were resolved by 12 % SDS-PAGE and transferred to nitrocellulose membranes (Sigma, 0.2 µm pore size). Blots were blocked with 5% non-fat dry milk in TBS-T (10 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, and 0.05 % Tween 20) overnight at 4°C and incubated for 3 h at 25°C in TBS-T plus 3% non-fat dry milk containing primary antibodies: anti-CDK2 (07-631, Upstate Biotech, Billerica, MA), -cyclin E (ab7959, Abcam, Cambridge, MA), -phosphorylated CDK2 on Thr160 (2561, Cell Signaling Technology, Danvers, MA), -CaM (sc-1989, Santa Cruz Biotechnology, Santa Cruz, CA), -phosphorylated CDK2 on Thr14/Tyr15 (sc-28435-R, Santa Cruz Biotechnology), and -actin (sc-7210, Santa Cruz Biotechnology). Protein bands were detected with ECL Reagents (GE Healthcare, Piscataway, NJ) and horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (sc-2054, Santa Cruz Biotechnology). Quantification of band intensity was performed using Quantity One (Bio-Rad, Hercules, CA).

Nucleofection. To deliver unconjugated synthetic peptides to cells in vitro, we employed Nucleofector technology (Amaxa GmBH, Germany). 1×10⁶ cells were harvested, resuspended in 100 µl of Nucleofector solutions (VSMC: VPI-1004; MEF: VPD-1005) and mixed with 100 nmol (in 10 µl) of each peptide. Cell solutions were transferred to customized cuvettes for the Nucleofector device, in which a programmed single pulse was applied (A-33 for VSMC; T-20 for MEF) as per the manufacturer’s protocol. Cells were transferred to 96-well plates (2,000-5,000 per well for MTT assays) or T-25 culture flasks (for FACS analysis) containing pre-warmed medium and cultured at 37°C in 5% CO₂.

TAT-conjugated peptide delivery. For peptide treatment, cells were seeded in 24-well plates, grown to 60% confluence, washed twice with PBS and administered solutions containing varying doses of peptide and their appropriate serum-free medium (Medium 231 without SMGS for human aortic SMC, Medium 200 without LSGS for human aortic EC, DMEM without FBS for MEF) for 1 h at 37°C. Cells were then washed twice with PBS, and immediately given complete medium (Medium 231 containing SMGS, Medium 200 containing LSGS or DMEM supplemented with FBS).

Immunostaining. Confocal microscopy was used to track FITC-labeled peptides following nucleofection. VSMCs harboring FITC-labeled peptides were fixed with 2% paraformaldehyde for 30 min and washed with PBS 3 times for 5 min each. Confocal microscopy was also used to examine phenotype of TAT-CBS-treated SMC in human aortic SMC or injured carotid arteries by SMC marker immunostaining using rabbit anti-SM22-α (ProteinTech, 10493-1-AP), mouse anti-sm-MHC (Abcam, ab683) and mouse anti-αSMA (Sigma, A2547), donkey anti-rabbit-Cy3 (Jackson ImmunoResearch, 711-165-152) or goat anti-mouse-Cy2 (Jackson ImmunoResearch, 115-225-146) antibodies. Cells were seeded on sterile coverslips in 6-well plates and treated with 100 µmol/L TAT-CBS or TAT-NC. Untreated controls were cultured in complete media or serum-starved. Cells were immunostained 72 h post-treatment. Cells and frozen sections were fixed with Cytofix (BD, 554655) for 15 min at RT, and washed 5 times with PBS. Mounted slides were examined with an Olympus Fluoview 1000 confocal microscope and images were obtained with FV10-ASW software. For TAT domain mediated delivery of His-tagged peptides into human aortic SMC and mouse carotid artery was confirmed by immunostaining using rabbit anti-His antibody (sc-804, Santa Cruz). To assess re-endothelialization, carotid artery sections harvested 7 days post wire-denudation injury were immunostained with goat anti-CD31 antibody (sc-1506, Santa Cruz). Antigen-antibody complexes were visualized by using rabbit or goat Vectastain kits (PK6101, PK6105, Vector Laboratories).

Proliferation assays. Active mitochondria from living cells are able to cleave 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to produce formazan, in amounts directly proportional to the number of living cells. Cells in a 96-well plate were washed twice with PBS and incubated for 4 h at 37°C with 1 ng/ml of MTT (in phenol red-free DMEM, L-glutamine 2 mmol/L, sodium pyruvate 1 mmol/L) in a reaction volume of 100 µl. MTT solution was removed and DMSO added to dissolve
formazan crystals. The plate was shaken for 5 min at 55°C for complete dissolution. Dye absorbance in viable cells was measured at 595 nm with reference wavelength 630 nm. All experiments were repeated at least 3 times, and each condition was repeated in at least quadruplicate. Validity of MTT assay was confirmed by cell counting (Supplementary Fig. 1). Incorporation of $^3$H-thymidine during DNA synthesis was used as another marker of cell proliferation. After treatment with peptides, 0.5µCi of $^3$H-thymidine (NET027Z, Perkin-Elmer, Waltham, MA) was administered to each well of a 24-well plate. Cells were grown at 37°C and analyzed at 24, 48 and 72 h after $^3$H-thymidine exposure. At the time of harvest, cells were washed twice with ice-cold PBS, and incubated with 10% trichloroacetic acid (TCA) on ice for 10 min to precipitate macromolecules. Two more incubations with fresh 10% TCA on ice were carried out for 5 min each. TCA was removed, and precipitates were dissolved by vigorous shaking at room temperature for 5 min with a solution of 0.2mol/L NaOH and 1% SDS. Solubilized contents of wells were removed and added to 2.5 ml of scintillation fluid (ReadySafe™ Cocktails, 141349, Beckman Coulter, Fullerton, CA). Radioactivity was measured in a scintillation counter (LS 6500, Beckman Coulter). Measured counts were taken as relative indices of proliferation by normalizing to counts from untreated cell controls.

Cell counting. For validation of MTT assay, cells (either primary mouse aortic SMC or MEF) were seeded in a 96-well plate. Each experiment was done in triplicate. After 24 h incubation, cells were trypsinized, and counted with a haemocytometer. For TAT-CBS treatment, human aortic SMC were treated with 100 µmol/L TAT-CBS, TAT-NC or TAT-Scramble. Cells were counted 72 h post-treatment.

LDH assay. The LDH-based in vitro toxicology assay kit (Sigma) was used to assess cytotoxicity of nucleofected peptides and TAT-peptide-treated cells. After peptide nucleofection, 10⁶ cells were grown on a 24-well plate with phenol red-free DMEM. After 24 and 72 h, 500 µl of cell culture media was collected from each well, followed by centrifugation (12,000 rpm, 30 min, 4°C) to completely remove debris. Subsequent enzymatic assay was performed as per the manufacturer’s protocol.

Caspase-3 assay. The caspase-3 Colorimetric Assay Kit (Sigma) was used to assess apoptotic activity of TAT-peptide-treated cells. Human aortic SMC were plated 2 x 10⁴ cells per well on a 24-well plate. Cell lysates were collected 24 and 72 h post-treatment for caspase-3 activity. Subsequent enzymatic assay was performed as per the manufacturer’s protocol.

TUNEL assay. The In Situ Cell Death Detection Kit (Roche) was used to assess in situ apoptotic activity of TAT-peptide treated cells. Human aortic SMC were seeded onto sterile coverslips in a 6-well plate. Cells were fixed 72 h post-treatment and TUNEL staining performed as per the manufacturer’s protocol.

Flow cytometry. Cells were plated at medium density (~5x10⁵ cells per 25-cm² tissue culture flask) in multiple parallel flasks and allowed to attach overnight in 10% FBS-DMEM. The next day, cells were washed twice in PBS and synchronized via growth arrest by serum starving them in 0.25% FBS-DMEM for 48 to 72 hours. Parallel cultures of growth-arrested cells were serum-stimulated with 10% FBS-DMEM and then serially harvested and ethanol-fixed at 4- to 8-hour intervals (G₀, G₀+8, G₀+12, G₀+16, G₀+24, and G₀+32). This process involved two washes in PBS, trypsinization, centrifugation for 10 minutes at 500g, and resuspension of the cell pellet at a density of 4x10⁶ cells/mL in serum-free DMEM containing 0.1% sodium azide. With gentle vortexing, an equal volume of 100% ethanol was slowly added to the suspension. Cells were stored at 4°C until all specimens were similarly fixed. The entire batch was pelleted as outlined above and resuspended at 2x10⁶ cells/mL in PBS with 0.1% Triton X-100. Cells were then incubated for 1 hour at 37°C with 100 µg/mL of RNase A. After another centrifugation, cells were resuspended in PBS containing 50 µg/mL propidium iodide and incubated overnight at 4°C in the dark. Cells were counted in a flow cytometer (FACScan; BD Biosciences, Franklin Lakes, NJ), and G₀/G₁, S, and G₂/M cell percentages were calculated with Cell Quest software (BD Biosciences).

Mouse carotid artery injury. All animal experimentation was conducted in accordance with operating protocols approved by the Toronto General Hospital Animal Care Committee. 8-12 weeks age (20-25 g body weight) C57bl6 male mice were purchased from the Charles River Co. (Wilmington, MA) and
housed for 1-2 wks before experimentation. Animals were anesthetized using intraperitoneal ketamine-HCl (100 mg/kg IP) xylazine-HCl (10 mg/kg, IP), and placed on a warming pad to regulate temperature. The primary bifurcation of the right common carotid artery was isolated after midline neck incision, and two ligatures were placed around the external branch. Next, the distal ligature was tied, and flow through the common carotid artery was temporarily occluded with a vascular clamp. An incision was made in the external carotid artery between the two ligatures, and a curved polished copper wire (0.3mm diameter) introduced into the lumen. The wire was advanced past the primary bifurcation into the common carotid artery, and vessel systematically injured by simultaneously rotating the curved copper wire while passing along the vessel four times. The wire was removed, and the external carotid artery tied off proximal to the incision with the second ligature. The vascular clamp was next removed restoring flow through the common carotid artery. Post-procedure, the injured left common carotid artery was surrounded by 100 µl of 25 % (wt/vol) pluronic F-127 gel with or without peptides prior to wound closure. The skin was closed with a single suture, and animals were allowed to recover on a warming pad.

**Tissue processing and analysis.** At 14 days post-injury, animals were lethally anesthetized with pentobarbital (100 mg/kg) and perfusion-fixed at physiological pressure with 4% buffered paraformaldehyde via LV puncture. Injured and sham common carotid arteries were harvested and fixed in 10% formalin in PBS and embedded in paraffin. Starting 50 µm from the bifurcation of the common carotid, three 4 µm thick sections, each 50 µm apart (i.e. covering a 150 µm span) were taken to represent the ‘distal’ common carotid. Moving 1 mm below this point (i.e. at the 20th 50 µm interval below), another three 4 µm thick sections, each 50 µm apart were collected as the ‘mid’ portions of the common carotid. Finally, leaving aside another 1 mm, we analyzed a final three 4 µm sections, each 50 µm apart, to represent the ‘proximal’ portion of the artery. Sections were stained with H&E for planimetry and with immunohistochemistry to detect expression of the proliferating cell nuclear antigen (PCNA; sc-56, Santa Cruz; 1:50 dilution). Images were analyzed by planimetry using Image J software. The mean of 3 different measurements was obtained by averaging the triplicate values obtained at the proximal, middle, and distal portions of each collected arterial sample: lumen area (A), the area surrounded by the inner elastic lamina (i.e. lumen + intima, B), and the area surrounded by the most external elastic lamina (i.e. lumen + intima + media, C). Intimal area was calculated by subtracting A from B, whereas the medial area was calculated by subtracting B from C. The I/M ratio was calculated as (B-A) / (C-B).

Paraffin-embedded sections from the same 3 points of maximal injury were also stained for Picro-Sirius Red, and polarized light microscopy used to quantify collagen. Images were analyzed using Adobe Photoshop CS4 Software.

EC staining was performed on a 4µm thick section obtained from 1 mm below the bifurcation of common carotid artery.

Harvested carotid arteries were also embedded in OCT and cut into 5 µm sections 200 µm below the bifurcation of the common carotid for SM22-α immunostaining.

**Statistical analyses.** One-way ANOVA was followed by Student’s t-test or Bonferroni’s test for multiple comparisons. Data shown are mean ± SE. Analyses were performed on SPSS v13.0 (SPSS Inc, Chicago, IL) and Graphpad Prism v4.0 (GraphPad Software Inc, La Jolla, CA). Curve-fitting and IC50 calculations were performed using Origin 6.0 (Microcal Software Inc, Northampton, MA).
Online Figure I. Validation of MTT assay by cell counting. Linear relationship between cell number as determined by counting and MTT assay results was shown in both (A) Primary mouse aortic SMC and (B) MEF.
Online Figure II. Dose-dependent effect of CBS on SMC number. Representative plot of dose-dependent effects of CBS on the number of primary mouse aortic SMC at 2 days post-serum-stimulation (N=3, IC$_{50}$ = 186.21 μmol/L).
Online Figure III. Effect of CBS peptide on LDH release. Lactate dehydrogenase (LDH) assay performed following peptide delivery. Peptides (1 mmol/L) were delivered into asynchronized primary mouse aortic SMC by nucleofection. Cell culture media were collected after 72 h for LDH assay. One-way ANOVA was applied followed by Student’s t-test for multiple comparisons. (P=NS)
Online Figure IV. Validation of cyclin E1/E2 double knock-out MEFs by Western bloting. A representative Western blot from wild type- (WT) and cyclin E1/E2 double knock out- (KO) MEFs is shown (N=2).
Online Figure V. TAT-CBS-His peptide delivery in vitro. (A) Sequence of TAT-CBS-His peptide. The 10 amino acid sequence of the TAT domain of HIV-1 was fused to the N-terminus of CBS, while a 6X His tag was fused to its C-terminus. (B) Western blot on primary mouse aortic SMC extracts following peptide treatment (both 100 µmol/L) reveal uptake of the peptides. A representative blot is shown (N=2). (C) TAT domain mediated delivery of CBS peptide into human aortic SMC. Human aortic SMC were treated with TAT-CBS-His (left panel) or TAT-CBS (right panel) peptides, cells were fixed with 4% paraformaldehyde, and immunostained using rabbit anti-His antibody.
Online Figure VI. Anti-proliferative effect of TAT-CBS as measured by cell counting. (A) Representative dose-response curve of TAT-CBS-His on primary mouse aortic SMC number measured by MTT (IC_{50} = 8.89 ± 1.24 µmol/L, N=3). (B) Primary human aortic SMC were treated with 100 µmol/L TAT-CBS, TAT-NC or TAT-Scramble. Proliferation was assayed by cell counting at 72 h. One-way ANOVA was applied followed by Bonferroni’s test for multiple comparisons. This experiment was repeated once (each condition was performed with five replicates, *P<0.05, **P<0.01).
A
LDH Release Assay

B
Caspase-3 Activity Assay

Online Figure VII
Online Figure VII

C

HA-SMC (p7)

- DAPI+
- TUNEL+

- PBS (100 µM volume)
- TAT-CBS (100 µM)
- TAT-NC (100 µM)
- PBS (10 µM volume)
- TAT-CBS (10 µM)
- TAT-NC (10 µM)
- (-) Control
- (+) Control

(xii)
Online Figure VII. Analysis of TAT-CBS-mediated effects on cell death in HA-SMC. (A) LDH release and (B) caspase-3 activity assays were performed 72 h following peptide treatments. TAT-CBS treatment did not increase LDH release or caspase-3 activity compared to TAT-NC or untreated controls. Similar results were obtained at 24 h (not shown). One-way ANOVA was applied (n=4 for both studies). P=NS. (C) Effect of TAT-CBS on in situ apoptosis determined by TUNEL staining. HA-SMC were treated with either 10 or 100 µmol/L of TAT-CBS, TAT-NC or an equal volume of PBS. In situ cell death was assessed 72 h post-treatment. TAT-CBS treatment causes a dose-dependent reduction in cell number, but does not increase the amount of apoptosis compared to controls. Representative fluorescent images are shown.
Online Figure VIII. TAT-CBS did not appreciably alter expression of smooth muscle cell markers in human aortic SMC. Human aortic SMC were treated with 100 μmol/L TAT-CBS or TAT-NC. Untreated (PBS) controls were serum-starved or exposed to serum-supplemented media. Cells were immunostained for SMC markers SM22-α, sm-MHC and α-SMA 72 h post-treatment. Representative confocal microscopy images are shown.
Online Figure IX. In vitro delivery of TAT-CBS in human aortic SMC and EC. Cells were treated with either TAT-CBS-His or TAT-CBS (100 µmol/L). Cells were immunostained 1, 4, 8, 24 and 72 h post-treatment with rabbit anti-His antibody. Representative images are shown.
Online Figure X. Cyclin E–dependent effect of TAT-CBS as measured by $^3$H-thymidine incorporation. (A) MEF treated with TAT-CBS or TAT-NC peptides. Proliferation was assayed by $^3$H-thymidine incorporation at 48 h (N=6, *P<0.05, **P<0.01). Wild-type MEF demonstrate an anti-proliferative effect of TAT-CBS vs. TAT-NC. This experiment was repeated twice. (B) In Cyclin E1/E2 double knockout (DKO) MEF, TAT-CBS did not inhibit proliferation (each condition was done in triplicate; P=NS). This experiment was repeated once. One-way ANOVA was applied followed by Bonferroni’s test for multiple comparisons.
Online Figure XI. TAT-CBS-His peptide delivery to SMC of an injured carotid artery. Arteries were harvested 7 days post-injury. Sections were immunostained using rabbit anti-His antibody (right panel) or rabbit IgG (left panel). Top (20X) and bottom (4X) low magnification images show eccentric retention of TAT-CBS-His by mural VSMC.
Online Figure XII. TAT-CBS-His does not affect collagen deposition in vivo. Injured carotid arteries were harvested 14 days post-injury. Picro-Sirius Red staining, polarized light microscopy and collagen quantification was performed. Representative images are shown. One-way ANOVA was applied for TAT-CBS (n=6), TAT-NC (n=6), uninjured TAT-CBS (n=6) and uninjured TAT-NC (n=7), (P=NS).
Supplemental Table 1. Amino acid sequences of synthetic peptides used.

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Amino Acid Sequence</th>
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<tr>
<td>TAT-CBS-His</td>
<td>RRRQRRKKRGGAEEFSARSRKANVTFLQDDHHHHHH</td>
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<tr>
<td>TAT-CBS</td>
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<tr>
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<td>TAT-NC</td>
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<tr>
<td>TAT-Scramble</td>
<td>RRRQRRKKRGFAFGRQVNKARSEKALGVSDRT</td>
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