Sustained Inhibition of ε Protein Kinase C Inhibits Vascular Restenosis After Balloon Injury and Stenting

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Background—ε Protein kinase C (εPKC) is involved in vascular smooth muscle cell (VSMC) activation, but little is known about its function in vascular pathology. We aimed at assessing the role of εPKC in the development of restenosis.

Methods and Results—Rat models of aortic balloon injury with or without subsequent stenting were used. Rats were treated with the selective εPKC activator ψε receptor for activated protein kinase C (ψεRACK), the selective εPKC inhibitor εV1–2, or saline. Both down-stream cascades of the platelet-derived growth factor receptor via extracellular signal-regulated kinase and Akt, respectively, were evaluated in vivo and in VSMC cultures. Intimal hyperplasia with luminal obliteration developed in saline-treated balloon-injured rat aortas (20.3±8.0%), and ψεRACK significantly promoted neointima development (32.4±4.9%, P=0.033), whereas εV1–2 significantly inhibited luminal narrowing (9.2±4.3%, P=0.093). εPKC inhibition led to significantly reduced VSMC extracellular signal-regulated kinase phosphorylation in vivo, whereas Akt phosphorylation was not markedly affected. Neointimal proliferation in vivo and platelet-derived growth factor-induced VSMC proliferation/migration in vitro were significantly inhibited by εV1–2. The inhibition of the platelet-derived growth factor pathway was mediated by inhibiting down-stream extracellular signal-regulated kinase and Akt phosphorylation. In vitro, εV1–2 showed inhibitory properties on endothelial cell proliferation, but that did not prevent reendothelialization in vivo. εV1–2 showed proapoptotic effects on VSMC in vitro. After stent implantation, luminal restenosis (quantified by optical coherence tomography imaging) was significantly reduced with εV1–2 (8.0±2.0%) compared with saline (20.2±9.8%, P=0.028).

Conclusions—εPKC seems to be centrally involved in the development of neointimal hyperplasia. We suggest that εPKC inhibition may be mediated via inhibition of extracellular signal-regulated kinase and Akt activation. εPKC modulation may become a new therapeutic target against vascular restenosis. (Circulation. 2010;122[suppl 1]:S170–S178.)

Key Words: ε protein kinase C ■ vascular restenosis ■ platelet-derived growth factor pathway

Eleven related serine/threonine protein kinases form the protein kinase C (PKC) family. Recent developments of isozyme-specific activators and inhibitors provided us with tools to differentially study isozyme-specific functions. It appears that αPKC and εPKC, together with the atypical PKCs, mediate cell proliferation and survival, whereas δPKC and εPKC are important regulator of apoptosis. δPKC has been shown to play a role in promoting early medial infiltra-
tion in a carotid artery balloon injury model, thus aiding in controlling neointimal inflammation and growth. The δPKC and εPKC isozymes have been shown to play opposing roles in cardiac ischemia and reperfusion, with εPKC mediating cardioprotection against ischemic injury and δPKC mediating reperfusion-induced cell damage. Selective sustained εPKC inhibition has been shown to suppress chronic inflammation and the development of perivascular fibrosis in a murine cardiac transplantation model. In hypertensive rats, εPKC inhibition prolonged survival, reduced cardiac hypertrophy, excessive fibrosis, vascular remodeling, inflamma-
tion, and corrected cardiac dysfunction. However, the selec-
tive εPKC activator ψε receptor for activated protein kinase C (ψεRACK) conferred cardioprotection from ischemia-
reperfusion injury under cell culture conditions and in animal models of acute myocardial infarction, when delivered acutely before the ischemic event. εPKC activation may appear as a “double-edged sword”, acutely increasing mito-
chondrial function and preventing cell death, but chronically increasing inflammatory responses. The complex involvement of PKC isoforms in cardiovascular physiology needs further research before targets for therapeutic interventions can be identified. Especially the role of ePKC on vascular remodeling is not known. In this study, we sought to investigate the role of ePKC on the development of intimal hyperplasia after mechanical injury.

**Methods**

**Rat Restenosis Models**

Male Sprague–Dawley rats weighing 550 to 600 g were purchased from Harlan. All rats were housed under conventional conditions in the animal care facilities. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. Aortic endothelial denudation and stent placement (Yukon Plus, 2.5 × 12 mm; Translumina) was performed as previously reported and described in the supplemental data, available online at http://circ.ahajournals.org.

Animals were treated with the selective ePKC activator, ϕeRACK, the selective ePKC inhibitor, ϕV1–2, the cell-penetrating TAT carrier peptide (TAT47–57), or saline. Both PKC modifying peptides were conjugated to TAT47–57, which greatly enhances cell permeation of these intracellularly acting peptides in vivo and in vitro. The selectivity of the peptides to ePKC was previously reported in many independent studies in vitro and in vivo. All drugs were administered in a sustained fashion via implanted osmotic pumps (Alzet), which were replaced every second week. Peptides were each delivered at 3 mg/kg per day. Denuded aortas were harvested after 4 weeks and stented aortas after 6 weeks.

**Histology**

Cross-sections of denuded aortas were stained with Elastica van Giesson and Masson–Goldner trichrome (Merck). Histology revealed a high density of spindle-shaped cells and areas of neointimal fibrosis (35% of neointimal area; Figure 1E). Although the ϕeRACK group was significantly greater than with phosphate-buffered saline, measurements showed that luminal obliteration in saline-affected by the carrier peptide TAT47–57. Interestingly, sustained treatment with the ePKC activator, ϕeRACK, significantly promoted neointima development, whereas sustained ePKC inhibition with ϕV1–2 significantly inhibited luminal narrowing (Figure 1D). Accordingly, the maximal plaque thickness in the ϕeRACK group was significantly greater than with ϕV1–2 (Figure 1E).

**Results**

**In Vitro VSMC and Endothelial Cell (EC) Culture Assays**

Proliferation assays with mitogen-stimulated rat aortic VSMC (PDGF 25 ng/mL) and EC (VEGF 10 ng/mL) were performed in the presence of TAT47–57 only (control), ϕeRACK, or ϕV1–2 (1 μmol/L each). To assess VSMC migration, a standard scratch was made across a VSMC culture slide, and the number of cells that migrated into this “wound” within 4 days was counted. The TUNEL assay (Invitrogen) was used to detect apoptotic cells. For details, please see supplemental data.

**In Vitro PKC Translocation Assay and Western Blot Analysis**

Levels of active ePKC in VSMC were determined by cell fractionation and Western blot analysis. Cells were pretreated with ϕV1–2 or TAT47–57 and stimulated with PDGF as outlined in supplemental data. Cells were homogenized, spun, and supernatants were collected. All samples were separated by 10% SDS-PAGE, and the proteins were transferred to immobilon-P transfer membranes for immunoblotting. Akt, ERK, phosphorylated Akt, and ERK levels were measured as described above.

**Statistical Analysis**

Data are presented as mean±SD. Comparisons were done by ANOVA between groups with Bonferroni post hoc tests (SPSS). P<0.05 was considered significant.

**Optical Coherence Tomography (OCT) Imaging**

OCT images were obtained with the M2 OCT imaging system (LightLab Imaging). Motorized pullback was performed at a rate of 1.0 mm per second as described in the supplemental data. Neointimal cross-sectional areas were measured, and luminal obliteration was calculated.

**Western Blot Analysis**

Rat aortic tissue was homogenized in homogenization buffer (50 mmol/L Tris-HCl, pH 7.5/150 mmol/L NaCl/1% SDS/protease inhibitor cocktail [Sigma-Aldrich]). After removal of tissue debris by centrifugation, 20 or 50 μg of proteins were separated on an 8 or 10% SDS-PAGE and were transferred to immobilon-P transfer membrane (Millipore). Antibodies against platelet-derived growth factor (PDGF) receptor-β, phosphorylated Akt (S453) and extracellular signal-regulated kinase (ERK), and β-actin (Santa Cruz Biotechnology, Inc; Cell Signaling Technology) were used for immunoblotting followed by HRP-conjugated antimouse, rabbit or goat IgG antibodies (GE Healthcare; Santa Cruz Biotechnology, Inc). All samples were loaded equally for each gel based on protein concentration.

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Figure 1. Intimal hyperplasia in balloon-injured aortas. Aortic cross-sections in hematoxylin and eosin staining are shown depicting the degree of intimal hyperplasia 4 weeks after balloon injury (magnification, ×15; n=6 animals per group) (A). Neointimal fibrosis was visualized by trichrome staining (magnification, ×400) (B), and cell proliferation was detected by PCNA staining (magnification, ×600) (C). Mean percentage luminal obliteration (D) and maximal plaque thickness (E) of the aortas are presented. Treatment with the εPKC activator ψεRACK significantly promoted neointima development, whereas εPKC inhibition with εV1–2 significantly inhibited luminal narrowing. Sections were stained for α-actin (red), DAPI (blue), and elastin (green) (F). Reendothelialization (RECA-1, green; DAPI, blue) was complete in all groups (G).
tion in all slides without differences related to the treatment with εPKC modulators (Figure 1G).

In Vivo VSMC PDGF Pathway Activation
Both down-stream cascades of the PDGF pathway controlling VSMC cell cycle (Figure 2A) were explored. The levels of PDGF receptor expression were not affected by εPKC modulation (Figure 2B). A significant reduction of ERK phosphorylation (C), but not for Akt phosphorylation (D), could be shown after the administration of εV1–2. Western blot analysis images from the same gel are rearranged in the presented order (n=6 samples per group).

In Vitro VSMC and EC Culture Assays
We set out to determine whether εPKC plays a direct role in VSMC and EC proliferation and VSMC migration. First, PDGF-induced εPKC activation was determined. PKC translocation (or movement from the cell soluble to the cell particulate fraction) is an established method to assess PKC activation. We found that PDGF stimulation significantly activated εPKC (Figure 3A) and that this activation was blocked by pretreatment of the cells with εV1–2. We further show that VSMC proliferation induced by PDGF treatment was moderately stimulated by εRACK and significantly inhibited by εV1–2 treatment (Figure 3B). We also performed the assay with 100 ng/mL of PDGF stimulation and observed similar magnitudes of growth enhancement and significant inhibition by treatment with εV1–2 (data not shown). VSMC migration was assessed in culture slide scratch assays (Figure 3C) and revealed that VSMC migration was similarly influenced by εPKC modulation. We found significant stimulation of VSMC migration by εRACK in vitro and significant inhibition by εV1–2 (Figure 3D). Cell proliferation of VEGF-stimulated EC in vitro is quantified in Figure 3E and depicted in Figure 3F. EC proliferation was significantly stimulated by εRACK and significantly inhibited with εV1–2. A possible implication of εPKC modulators on VSMC apoptosis was further investigated using the TUNEL assay (Figure 3G). There was mild reduction of apoptosis by εRACK, but we found significantly increased numbers of apoptotic VSMCs with εV1–2 treatment (Figure 3H).

Effect of εV1–2 Treatment on Down-Stream Signaling of PDGF
To investigate the molecular mechanism of VSMC growth inhibition by εV1–2 treatment, we next determined ERK and Akt activation, which are postulated to play a role in VSMC proliferation. As demonstrated by Western blot analysis, 5 minutes of PDGF stimulation significantly increased the levels of phosphorylated ERK and Akt, but εV1–2 treatment did not have a significant effect at this time point (Figure 4A). However, after 6 hours of εV1–2 treatment, there was a significant decrease in phosphorylated ERK levels in cells treated with or without PDGF as compared with cells treated with control peptide (Figure 4B). Similar effects of εV1–2 on

Figure 2. In vivo VSMC PDGF pathway activation. PDGF pathway-mediated VSMC cell cycle control involves the ERK/MEK and phosphatidylinositol 3-kinase/Akt down-stream cascades (A). PDGF receptor expression was not affected by εPKC modulation (B). A significant reduction of ERK phosphorylation (C), but not for Akt phosphorylation (D), could be shown after the administration of εV1–2. Western blot analysis images from the same gel are rearranged in the presented order (n=6 samples per group).
Akt and phosphorylated Akt were also found. Although there was no effect of eV1–2 treatment after 5 minutes with PDGF, there was a significant decrease in the levels of phosphorylation ratio of Akt after 6 hours. Because PDGF signaling is long lasting in injured vessels, such changes may be physiologically relevant. Together, our data suggest that ePKC inhibition of PDGF-induced VSMC proliferation is likely mediated, at least in part, through inhibition of ERK and Akt activation.

**Intimal Hyperplasia in Stented Aortas**

Figure 5A shows histological cross-sections of the stented aortas. The stent struts were eliminated during the processing, leaving behind open spaces at their former locations. The systemic release of eV1–2 significantly reduced the development of the neointimal plaque area from 1.0±0.4 to 0.5±0.8 mm² (P=0.047) (Figure 5B). Representative OCT frames are shown in Figure 5C. OCT-derived luminal obliteration (Figure 5D) was 12.2±9.8% in saline-treated rats and
was significantly reduced by a 6-week treatment with εV1–2 to 8.0±2.0% (P=0.028).

Discussion
Clinical results of percutaneous coronary interventions with or without stenting have been limited by restenosis and patients frequently require repeat revascularization procedures. VSMC proliferation and migration with subsequent synthesis of extracellular matrix are central cellular events resulting in neointimal formation. Rat models of aortic balloon injury with and without subsequent stent placement are established models for the development of arterial restenosis and have been used in this study.

Role of εPKC in the Development of Intimal Hyperplasia
Although the importance of PKC signaling for VSMC growth and restenosis has been shown previously, the specific roles

Figure 4. Effect of εV1–2 treatment on downstream signaling of PDGF. Five minutes of PDGF stimulation (25 ng/mL) significantly increased ERK and Akt phosphorylation (n=3 per group). εV1–2 treatment did not show a significant early effect on these cascades (*P<0.05 vs TAT; †P<0.05 vs εV1–2) (A). However, after 6 hours, there was a significant decrease in phosphorylated ERK and Akt levels (P<0.05 vs TAT; †P<0.05 vs εV1–2; §P<0.05 vs TAT+PDGF) (B), suggesting that εPKC inhibition of PDGF-induced VSMC proliferation may be mediated via inhibition of ERK and Akt activation.
of its isoforms remained unknown. We used a selective εPKC activator and inhibitor, respectively, to elucidate the role of εPKC in vascular restenosis. In a rat model of balloon injury, we here show that sustained εPKC activation significantly increases neointimal hyperplasia, whereas it is reduced with sustained εPKC inhibition. The suppression of neointimal development by εPKC inhibition could be shown to result from an antiproliferative capacity of εV1–2 in vivo. The cellularity of the neointima and the neointimal fractional area of fibrosis were found to be similar among groups. Therefore, both cellular proliferation and the increase of extracellular fibrosis seem to be affected to similar extents by εPKC modulation. Reendothelialization is crucial for physiological healing of mechanically injured arteries. εV1–2 treatment, although shown to inhibit in vitro EC proliferation, did not prevent reendothelialization until 4 weeks after injury. Our data, however, cannot determine whether endothelial healing was slowed down by εPKC inhibition, because only 1 time point was assessed.

**Molecular Basis of εPKC Inhibition on PDGF-Stimulated VSMC Proliferation**

PDGF induces VSMC proliferation by triggering the activation of both the ERK/mitogen-activated protein kinase and phosphatidylinositol 3-kinase-Akt pathways. To further examine the molecular basis for the inhibition of VSMC proliferation in rat aortas treated with εV1–2, we used VSMC in primary cultures. εV1–2 treatment decreased PDGF-induced εPKC activation and cell proliferation in VSMC cultures in vitro. ERK is a key transducer of extracellular signals and promotes cell growth and migration. ERK1 and ERK2 play pivotal roles in proliferation of VSMC in vitro and in vivo. PDGF-induced proliferation is inhibited by dominant negative mutants or antisense oligonucleotides of ERKs. In addition, dominant negative mutation of Akt causes delays in G1 exit in the cell cycle and controls proliferation of primary rat aortic VSMC. Independent but synergistic effects of the phosphatidylinositol 3-kinase-Akt pathway and the MEK1/2-ERK pathway in PDGF-induced responses were observed using inhibitors of these kinases, LY294002 and PD98059, respectively. In addition to the relationship between ERK activity and VSMC proliferation, a correlation between ERK and neointimal hyperplasia in rat aorta has been previously observed following balloon angioplasty-induced injury. Mechanical injury-induced VSMC proliferation involves ERK phosphorylation, and ERK activation is among the earliest of biochemical changes detected after arterial injury. ERK transmits mitogenic signals by translocation to the nucleus where multiple genes implicated in the pathogenesis of atherosclerosis and restenosis are induced.

Here, we showed that the εPKC inhibitor εV1–2 did not affect early PDGF-induced ERK and Akt activation, suggesting that εPKC is probably not an immediate upstream regulator of these enzymes. However, after 6 hours of PDGF stimulation, ERK levels were significantly decreased following εV1–2 treatment, which resulted in decreased amounts of active ERKs in PDGF-stimulated VSMC. εPKC activation may also control ERK activity by reducing protein stability and/or decreasing its expression in VSMC. Saito et al. reported involvement of εPKC in the activation of Grb2-associated binder-1, an adapter protein related to the insulin receptor substrate family. Because ERK2 is downstream of Grb2-associated binder-1, the εPKC-mediated PDGF effect that we observed may be due to inhibition of the εPKC-mediated PDGF-Grb2-associated binder-1 pathway activation. Although we only found mild decreases of Akt levels by εPKC inhibition in vivo, Akt phosphorylation levels were significantly decreased in vitro by εV1–2 treatment when determined 6 hours after PDGF treatment. These data suggest that εPKC also controls Akt activity by direct or indirect phosphorylation in VSMC. However, the moderate (~12%) versus saline to 29% versus TAT47–57) reduction of Akt activity observed in our εV1–2-treated balloon-denuded aortas failed to reach significance.

εPKC inhibition may also attenuate VSMC chemotaxis following stimulation with PDGF. ERK activation is required for PDGF-induced VSMC chemotaxis as well as for hyperglycemia-induced chemotaxis of VSMC and cross-talk between ERK and Akt signals. Therefore, although not.

**Figure 5.** Intimal hyperplasia in stented aortas. Histological cross-sections of stented aortas are shown 6 weeks after implantation subsequent to mechanical injury (n = 6 animals per group) (A). εV1–2 treatment significantly reduced neointimal plaque development (B). Luminal obliteration, quantified by OCT (C), was also significantly reduced in εV1–2-treated animals (D).
assessed directly in this study, inhibition of chemotaxis of VSMC may also contribute to the benefit of eV1–2 treatment on neointimal hyperplasia.

ePKC is the only PKC isozyme that has been considered an oncogene, because it collaborates with Ras/Raf/ERK and Akt pathways to regulate cell survival and death.28 Reverse, cytosolic translocation of ePKC was shown to correlate with ceramide-induced apoptosis in leukemia cells.29 ePKC knockout mice exhibited significantly decreased survival,30 whereas overexpression of ePKC in rat embryo fibroblasts inhibited apoptosis induced by cisplatin.31 Antiapoptotic ePKC further enhances antiapoptotic Bcl-2 members while inhibiting the proapoptotic factors. Therefore, ePKC in advanced plaques may represent an attempt to inhibit apoptosis and thus maintain plaque stability.27 Our results reveal mild antiapoptotic effects of ePKC activation in VSMC and demonstrate that ePKC inhibition by eV1–2 significantly increases apoptosis. In the context of postangioplasty restenosis, the inhibition of VSMC proliferation and the induction of VSMC apoptosis is appealing and has led to regression of experimental vascular lesions.33 However, VSMC apoptosis in advanced plaques may also contribute to fibrous cap weakening and thereby promote plaque rupture and vessel thrombosis.

**ePKC Inhibition for the Prevention of Restenosis After Stent Placement**

Driven by the success of reducing arterial restenosis with ePKC inhibition, we next determined whether the effect would reproduce in a model of restenosis following stent placement. Because the ePKC inhibitor eV1–2 is a peptide, we could not use it for stent coating, and the drug delivery again was performed systemically via SC pumps. eV1–2 significantly reduced both neointimal plaque area as determined by histology and luminal diameter.34 However, ePKC apoptosis in advanced plaques may represent an attempt to inhibit apoptosis and thus maintain plaque stability.27 Our results reveal mild antiapoptotic effects of ePKC activation in VSMC and demonstrate that ePKC inhibition by eV1–2 significantly increases apoptosis. In the context of postangioplasty restenosis, the inhibition of VSMC proliferation and the induction of VSMC apoptosis is appealing and has led to regression of experimental vascular lesions.33 However, VSMC apoptosis in advanced plaques may also contribute to fibrous cap weakening and thereby promote plaque rupture and vessel thrombosis.

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**Disclosures**

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**References**


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Sustained inhibition of epsilon protein kinase C inhibits vascular restenosis after balloon injury and stenting

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SUPPLEMENTAL MATERIAL

Detailed Methods:
The rat aortic denudation model
Male Sprague-Dawley rats weighing 550-600g were used. The animals were anesthetized with isoflurane (2%) and ketamine (25mg/kg). Under microscopic view, an upper median mini-laparotomy was performed to expose the infrarenal aorta. The aortic endothelium was denuded by the passage of a 2-french Fogarty arterial embolectomy catheter (Baxter Healthcare, Deerfield, IL).

The rat aortic stenting model
After denudation, Yukon Plus stents (2.5mm diameter, 12mm length) were deployment (Translumina GmbH, Hechingen, Germany). The small aortic incision was closed with 8-0 Prolene sutures (Ethicon, Norderstedt, Germany).

Optical coherence tomography (OCT) imaging
OCT images were obtained with the M2 OCT imaging system (LightLab Imaging, Inc., Westford, MA). ImageWire™ is an imaging probe to deliver the light to the tissue and collect the signals. The ImageWire™ consists of 0.006” (0.15mm) fiber-optic core, inside a sheath with a maximum O.D. of 0.019” (0.48mm). Motorized pullback OCT imaging was performed at a pullback rate of 1.0mm per second. Images were acquired at 15 frames per second, displayed with a color look-up table and digitally archived. OCT measurements were performed using the LightLab OCT imaging proprietary software with a mouse-based interface. The system was calibrated to the reflection of the OCT imaging wire, which is the standard calibration technique for this system, then, lumen and stent cross sectional areas (CSA) were manually traced at each 1.0mm intervals. Plaque cross sectional area was calculated as stent CSA subtracted from the lumen CSA. Percent plaque area was calculated as plaque CSA divided by stent CSA (%). Average percent plaque areas were calculated.

Processing of stented aortas
The specimens were fixed in 4% formalin, dehydrated in a graded series of alcohol, and infiltrated in a mixture (MMA I) of 80% methylmethacrylate and 20% dibutylphthalate for 1 day, MMA I with 1% dry benzooyl peroxide for 1 day, and MMA I with 3% dry benzooyl peroxide (MMA III) for 1-2 days at 4°C. Thereafter, the specimens were polymerized in fresh MMA III in glass vials in a water bath on a pre-polymerized base. Slow polymerization was achieved by keeping the vials at 26°C overnight, increasing the temperature to 28°C the next morning, and then increasing the temperature gradually by 0.5°C over 12 hours until polymerization
occurred. The polymerized blocks were sectioned at 5µm thickness using a MICROM HM 360 microtome equipped with a tungsten carbide knife. Sections were stained with hematoxylin and eosin (H&E).

**Determination of neointimal fibrosis and cell density**
The fractional area of fibrosis (light blue in Masson-Goldner trichrome staining) was determined morphometrically using ImageJ 1.43s (Bethesda, MD). The amount of VSMC per 100 µm² of neointimal cross-sectional area was determined using ImageJ 1.43s.

**In vitro VSMC and EC culture assays**
Rat aorta VSMC cultures were kindly provided by Drs. Alec Glassford and Phil Tsao from Department of Cardiovascular Medicine, Stanford University after isolation from Sprague Dawley rats by enzymatic digestion and used at passages less than 10. Rat EC were isolated by collagenase digestion. Briefly, after sterile aortic harvest, segments of 2 or 3 cm were flushed with collagenase (Gibco, Germany) and held under pressure until RAEC were washed out after 2 hours. Harvested cells were purified using Magnetic Cell Sorting (Miltenyi, Germany) and cultured. VSMC were cultured in low glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 100 units/mL penicillin and 100 µg/ml streptomycin. EC were cultured in EC media (Promocell, Germany). VSMC and EC were serum starved for 24 hours and then TAT₄₇-₅₇ (control), ψεRACK or εV₁-₂ (1 µM each) was added to the culture media. 15 min after peptide addition, PDGF at 25 ng/ml (for VSMC) or VEGF at 10 ng/ml (for EC) was added to the cells. Peptides were continuously supplied every 8 hours for 48 hours. VSMC and EC proliferation was quantified using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI). Absorbance at 490 nm was measured with the Magellan ELISA Reader and Software (Tecan Systems Inc., San Jose, CA). EC were stained with Alexa Fluor 633 Phalloidin and DAPI (Invitrogen) for immunofluorescence microscopy. Mitogen-stimulated VSMC and EC proliferation is expressed as n-fold compared to unstimulated cell cultures. To assess an effect of the εPKC modulators on apoptosis, the TUNEL test (Invitrogen) was performed on VSMC cultures according to the manufacturer’s protocol.

**In vitro SMC migration assay**
A standard scratch was made across the culture slides of confluent SMC monolayers using a sterile cell scratcher. Cells were then incubated with εPKC modulators for 4 more days. Cell nuclei were stained with DAPI (Invitrogen) for immunofluorescence microscopy. The amount of cells that had migrated into 1080 µm long segments of the 600 µm wide scratch were counted using ImageJ 1.43s.

**In vitro PKC translocation assay and western blotting**
Levels of active εPKC in VSMC were determined by cell fractionation and western blot analysis. Cells were serum-starved for 2 days and then pre-treated with εV₁-₂ or TAT₄₇-₅₇ for 10 min. Five min after addition of 25 ng/mL PDGF, cells were collected, homogenized in homogenization buffer (20 mM Tris-HCl, pH 7.5, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA) containing a cocktail of protease and phosphatase inhibitors (Sigma) using 25G syringe needles. Cell homogenates were spun at 100,000g for 30 min at 4°C and supernatants were collected as soluble samples. The pellets were homogenized with homogenization buffer containing 1% Triton X-100 and were spun at 100,000g for 30 min at 4°C. The supernatants, which are Triton soluble fractions, contain activated εPKC. All samples were separated by 10% SDS-polyacrylamide gel electrophoresis and the proteins were transferred to Immobilon-P transfer membranes (Millipore). Immunoblotting was performed using anti-εPKC rabbit polyclonal antibodies (Santa Cruz Biotechnology) followed by HRP-conjugated goat anti-rabbit IgG antibodies. Akt, ERK, phosphorylated Akt and ERK levels were measured as described above.