Published in final edited form as:

Atherosclerosis. 2010 September ; 212(1): 123–130. doi:10.1016/j.atherosclerosis.2010.05.039.

Reduction of PKC β II Activity in Smooth Muscle Cells Attenuates Acute Arterial Injury

Chun Huang, Jong Sun Chang, Yunlu Xu, Qing Li, Yu Shan Zou, and Shi-Fang Yan^{\$}

Division of Surgical Science, Department of Surgery, Columbia University Medical Center, New York, New York 10032

Abstract

Objective—The ubiquitous enzyme Protein Kinase C (PKC) has been linked to the pathogenesis of vascular injury, but the cell-specific and discrete functions of the β II isoform have yet to be discovered in this setting. Our previous findings demonstrated significantly increased PKC β II in the membrane fraction of injured femoral arteries in wild type (WT) mice and revealed reduction of neointimal expansion in PKC $\beta^{-/-}$ mice after acute vascular injury. As PKC $\beta^{-/-}$ mice are globally devoid of PKC β , we established novel transgenic (Tg) mice to test the hypothesis that the action of PKC β II specifically in smooth muscle cells (SMCs) mediates the formation of neointimal lesions in response to arterial injury.

Methods—Tg mice expressing SM22 α promoter-targeted mouse carboxyl-terminal deletion mutant PKC β II were produced using standard techniques, subjected to femoral artery injury and compared with littermate controls. Smooth muscle cells (SMC) were isolated from wild-type (WT) and Tg mice and exposed to a prototypic stimulus, tumor necrosis factor (TNF)- α . Multiple strategies were employed *in vivo* and *in vitro* to examine the molecular mechanisms underlying the specific effects of SMC PKC β II in neointimal expansion.

Results—*In vivo* and *in vitro* analyses demonstrated that PKC β II activity in SMCs was critical for neointimal expansion in response to arterial injury, at least in part via regulation of ERK1/2, Egr-1 and induction of MMP-9.

Conclusions—These data identify the SMC-specific regulatory role of PKC β II in neointimal expansion in response to acute arterial injury, and suggest that targeted inactivation of PKC β II may be beneficial in limiting restenosis via suppression of the neointima-mediating effects of Egr-1 and MMP-9.

Keywords

arterial injury; transgenic mouse; mutant PKC β II; signal transduction; SMC

© 2010 Elsevier Ireland Ltd. All rights reserved.

^{\$}Correspondence should be addressed to Dr. Shi-Fang Yan, Division of Surgical Science, Department of Surgery, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, New York 10032, tel 212 305 6030, fax 212 305 5337, sy18@columbia.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Alterations of vascular smooth muscle cell (VSMC) proliferation and migration are key properties of activated SMC in the injured vessel during the expansion of neointima, processes central to the pathogenesis of restenosis consequent to vascular injury¹⁻³. Prior studies reported that arterial injury induces expression of matrix metalloproteinase (MMP)-9 to degrade extracellular matrix, a critical prerequisite for VSMC proliferation and migration^{4,5}. Genetic deletion of MMP-9 resulted in smaller neointima in injured arteries of mice with reduced SMCs^{4,5}. However, as these mice were globally devoid of MMP-9, the cell type-specific signal transduction pathways and transcriptional mechanisms that regulate MMP-9 expression in response to arterial injury in cardiovascular diseases remain elusive.

The ubiquitous enzyme Protein Kinase C (PKC) has been linked to the pathogenesis of vascular injury, but the cell-specific and discrete functions of the β II isoform have yet to be discovered in this setting. Our previous findings demonstrated significantly increased PKC β II in the membrane fraction of injured femoral arteries in wild type (WT) mice and revealed reduction of neointimal expansion in PKC $\beta^{-/-}$ mice and in WT mice fed the PKC β inhibitor, ruboxistaurin, after acute vascular injury⁶. As PKC $\beta^{-/-}$ mice are globally devoid of PKC β which is expressed in multiple cell types, and ruboxistaurin targets the organism in a non-cell type specific manner, it was important to use a distinct strategy to analyze the molecular mechanisms governing SMC activation and neointimal expansion in acute arterial injury.

In this study, we tested the hypothesis that PKC β II activity in SMC mediates upregulation of Egr-1 and MMP-9 and contributes to the expansion of neointima in response to acute arterial injury. Transgenic mice expressing a mouse carboxyl-terminal deletion mutant PKC β II specifically in SMC, driven by SM22 α promoter^{7,8}, were generated and termed Tg-SM22-mtPKC β II mice or Tg mice. Femoral artery endothelial denudation injury was induced in Tg mice and WT littermates using a well-characterized model in which only the intima sustains mechanical injury⁹, and multiple strategies were employed *in vivo* and *in vitro* to delineate the SMC-specific regulatory role of PKC β II in upregulation of Egr-1 and MMP-9 and the coordinated response to injury in the vessel wall. These findings may provide the basis for the design of novel PKC isoform-specific inhibitory compounds that may achieve therapeutically useful changes in PKC β II signaling in cells.

Methods

Please refer to the data supplement for detailed methods.

Construction of Murine C-terminal Deletion Mutant PKC β II and the Generation of Transgenic Mice

Mouse PKC β II cDNA (wtPKC β II, provided by C.L. Ashendel)¹⁰ was truncated in its carboxy-terminus, by deletion of nine amino acids (EFLKPEVKS)¹¹, to produce mutant PKC β II (mtPKC β II). Tg mice were generated to drive expression of mtPKC β II in SMCs using a 445-bp prion of the SM22 α promoter^{7,8}.

Animal Studies and Induction of Vascular Injury

In our experiments, hemizygous Tg mice, homozygous Egr-1^{-/-}¹², PKC $\beta^{-/-}$ ¹³ mice and littermates (male, 8-12 weeks of age) were subjected to endothelial denudation injury to femoral arteries and sham control as previously described^{6,9}. Essentially, sham control mice were subjected to anesthesia and opening of the tissue layers surrounding the femoral artery, but no angioplasty guidewire-mediated endothelial denudation was performed. All

procedures were approved by Institutional Animal Care and Use Committee at Columbia. BrdU labeling was performed as previously described ^{6, 8}.

Histology and Immunohistochemistry

Elastic staining with van Giesson's staining kit (Sigma-Aldrich) was performed as previously published ^{6, 8}. Measurements of luminal area, media area and intimal area and calculations of the intima to media (I/M) ratio were performed as previously described ^{6, 9, 14}. Sections were stained with monoclonal anti-smooth muscle (SM)- α -actin IgG (1:100, Sigma-Aldrich) or antibody specific for Bromodeoxyuridine (BrdU) (Sigma) as previously described ^{6, 8}. Numbers of stained and total nuclei were counted, and BrdU labeling index was calculated (BrdU-labeled nuclei/total nuclei X 100%). Immunofluorescence double staining of sections was performed using primary antibodies to BrdU or MMP-9 and SM- α -actin followed by biotinylated secondary antibodies, and followed by incubation with fluorescein-avidin D or Texas Red-avidin D. The signals of images for antigen detection were performed using a LaserSharp 2000 scanning confocal microscope with epifluorescent illumination (BioRad Laboratories Inc.) (excitation wavelength 488 nm for fluorescein-avidin D, 568 nm for Texas Red-avidin D).

Murine Primary Smooth Muscle Cell and Endothelial Cell Culture

Three different lines of WT, Tg, PKC $\beta^{-/-}$ and Egr-1 $^{-/-}$ murine aortic smooth muscle cells (SMCs) were established from 3 individual mouse aortas using a modification of the procedure of Travo and Barrett ¹⁵. Three lines of WT and Tg murine aortic endothelial cells (MAECs) were established from 3 individual mouse aortas as described ¹⁶.

In Vitro Cell Migration Assays

Aortic SMCs were placed in the upper chamber of a 24-well transwell (Costar) pre-coated with 0.2% gelatin, and the medium containing either no chemoattractant (control) or TNF- α (100 ng/ml) was added to the lower chamber. Migrated cells on the lower surface of membranes were stained with Hematoxylin and Eosin, and then counted under a light microscope.

Western Blotting

Proteins were prepared from femoral artery segments, aortas, cells and cell supernatant. Those samples were subjected to Western blot using specific antibodies to phospho-PKC β II, PKC α , β I, β II, δ , ϵ , MMP-2 and MMP-9.

Zymography

Proteins prepared for detection of MMP-9 and MMP-2 activities were subjected to electrophoresis onto Gelatin-Zymogram gel (Invitrogen/Novex). After the suggested developing time, gels were stained with Coomassie Blue (BioRad). Images were obtained with an Alpha-Imager.

RNA Extraction and Real-Time PCR

Total RNA isolation, cDNA synthesis, and real time PCR analysis were performed as previously described ¹⁷. All PCR primers and TaqMan probes were designed using software PrimerExpress (Applied Biosystem) and published sequence data from the NCBI database.

Statistical Analysis

All data are expressed as the mean $\pm$ SEM. Statistical significance was analyzed by ANOVA using commercially available (Statview, version 5.0.1, Berkeley, California) software. *P*

values of less than 0.05 were considered statistically significant. All results shown here are from transgenic mouse line No.12 and similar results were observed in transgenic mouse line No. 9 (data not shown), unless data are noted to be specifically obtained from transgenic mouse line No. 9.

Results

Generation and Characterization of Transgenic Mice Expressing Mutant PKC β II Driven by SM22 α Promoter

Deletion of nine amino acids within the carboxyl-terminus of PKC β II (EFLKPEVK Δ S) was previously shown to markedly suppress PKC β II activity in the rabbit enzyme¹¹. To test this concept in mice, we generated murine mutant PKC β II by this strategy and sought to establish the specific action of PKC β II in SMCs in the response to arterial injury in mice *in vivo*. A transgene was created in which expression of mtPKC β II was placed under control of the SM22 α promoter^{7, 8} to direct expression to SMCs (termed Tg-SM22-mtPKC β II or Tg) (Figure S1A). Transgenic founders were identified by PCR and Southern blot analysis of genomic DNA from mouse tail tips (Figure S1B & S1C). Based on positive PCR results and a strong positive band shown in Southern blot, two founders, No. 9 and 12, were selected as breeders. Since they were generated by injection of the transgene into oocytes of B6CBAF1 mice, they were backcrossed ≥ 10 times into the C57BL/6 background. Their offspring were subjected to further studies.

The specificity and activity of PKC β II in aorta, aortic SMC and MAEC from Tg mice were evaluated by immunoblot analysis performed with specific antibodies to PKC α , β I, β II, δ , ϵ and phospho-PKC β II. First, the protein level of either PKC β II (Figure 1A) or phosphorylated PKC β II (Figure 1B) in the membranous fraction from Tg mouse aortas was lower than that from WT mouse aortas, whereas there were no differences in cytosolic fractions from Tg and WT aortas. As expected, PKC β II was undetectable in PKC $\beta^{-/-}$ mouse aortas (Figure 1A). Next, an immunoreactive band of either PKC β II (Figure 1C) or phosphorylated PKC β II (Figure 1D) in the membranous fraction from Tg SMCs was of very low intensity compared to that from WT SMCs. In contrast, immunoblotting with antibodies specific for 4 other PKC isoforms, including PKC α , PKC β I, PKC δ , and PKC ϵ (Figure 1E), revealed no differences between Tg and WT SMCs. Furthermore, we tested the protein level of PKC β II in membranous fraction from primary MAEC. Importantly, there were no differences in PKC β II antigen in the membranous fraction from Tg MAEC and WT MAEC, as would be anticipated with the SM22 α promoter (Figure 1E). Loading controls using anti-GAPDH IgG indicated identical protein loading (Figure 1A, 1B, 1C, 1D, & 1E). These data demonstrated that SMC-specific mtPKC β II dramatically reduced PKC β II activity by displaying decreased phosphorylated PKC β II in the membrane fraction accompanied by diminished membrane translocation of PKC β II in Tg mouse aortas, specifically in Tg SMC, not in Tg MAEC, and without alterations in other PKC isoform activities.

Reduction of PKC β II Activity in SMCs Attenuates Neointimal Expansion after Arterial Injury

Our previous findings demonstrated that genetic deletion or pharmacologic blockade of PKC β decreased the neointimal expansion consequent to arterial injury⁶. To establish the specific action of PKC β II in SMCs in the response to arterial injury, we performed endothelial denudation injury by angioplasty guide wire injury to the femoral artery in Tg mice. 28 days after denudation injury, we found that the mean intimal area was $7,777.68 \pm 1,893.71 \mu\text{m}^2$ in WT mice vs. $1,260.08 \pm 248.88 \mu\text{m}^2$ in Tg mice ($p < 0.001$); the mean medial area was $9,350.58 \pm 1,113.89 \mu\text{m}^2$ in WT mice vs. $10,881.16 \pm 792.83 \mu\text{m}^2$ in Tg mice ($p = \text{NS}$). This measurement translated into an intima/media (I/M) ratio of 0.96 ± 0.22 in WT mice vs. 0.11 ± 0.2 in Tg mice ($n \geq 9$ mice/group, $p < 0.001$; Figure 2A-C). In Figure 2D,

the adjacent section to that shown in Figure 2B was stained with an antibody to smooth muscle (SM)- α -actin. None of the sham-operated arteries had intimal proliferative lesions (data not shown), therefore, their I/M ratio was zero. These data demonstrated that smooth muscle cell PKC β II activity importantly contributes to neointimal expansion after vascular injury.

Reduction of PKC β II Activity in SMC Attenuates SMC Proliferation and Migration

To address the mechanisms by which mtPKC β II in SMCs resulted in reduced neointimal expansion, we assessed the expanding neointima in Tg mice compared to WT mice at an early time after injury, at which point SMC proliferation was previously found to be accelerated in WT mice^{6,9}. Immunohistochemical analysis for BrdU antigen demonstrated that there were very high BrdU labeling indices in the intimal proliferative lesions of injured artery walls from WT mice 7 days after denudation injury; in contrast, incorporation of BrdU into nuclei was significantly decreased in the expanding neointima in Tg mice vs. WT mice ($n \geq 7$ mice/group, 31 % versus 62%, $p < 0.001$) (Figure 3A-C). In addition, immunofluorescence double staining using antibodies to BrdU and SM- α -actin demonstrated colocalization of BrdU positive cells and SMC marker in proliferative SMCs of expanding neointima of WT mice (Figure 3D). Interestingly and importantly, these data confirmed that vascular SMCs were the main cellular component of intimal proliferative lesions impacted by PKC β II in response to vascular injury.

Coupled with *in vivo* studies, to thoroughly explore the impact of mtPKC β II on SMC migration, we studied the effect of mtPKC β II in SMCs on migration responses in primary cultured WT and Tg SMCs in the presence or absence of TNF- α , a known migration stimulatory factor for SMCs¹⁸. Addition of TNF- α (100 ng/ml) to the lower compartment significantly increased the number of migrating WT SMCs (≈ 3.3 -fold increase compared to untreated cells, $p < 0.001$; Figure 3E), a process significantly suppressed in TNF- α -stimulated Tg SMCs ($p < 0.05$, Figure 3E). These findings established that SMC PKC β II is critical for promoting increased SMC proliferation and migration upon injury.

Reduction of PKC β II Activity in SMC Attenuates Activation of MMP-9 Triggered by Acute Arterial Injury or TNF- α

To address the mechanisms linked to PKC β II-dependent SMC proliferation and migration after arterial injury, we tested the role of PKC β II in regulating expression of MMP-9 in WT, Tg, PKC $\beta^{-/-}$ and Egr-1 $^{-/-}$ mice upon denudation injury, as it is known that MMP-9 is a critical prerequisite for VSMC proliferation and migration,^{4,5} and that PKC β is an essential upstream regulator of the Egr-1⁶. Marked increases in MMP-9 gelatinolytic activity and antigen were observed within 4 hours after injury in WT mice (≈ 36 & ≈ 60 -fold compared to sham, $p < 0.05$ & $p < 0.001$), with a peak induction at 8 h (≥ 100 -fold compared to sham, $p < 0.0001$) (Figure 4A, 4B, S2A & S2B) and which were still detectable up to 24 hours after injury (Figure S2A & S2B). In contrast, MMP-2 was constitutively expressed in uninjured femoral arteries, and its activity did not increase within 24 hours after injury and was overall much less than that of MMP-9 after injury (data not shown). In addition, immunofluorescence double staining demonstrated colocalization of MMP-9 positive cells and SMC marker (using antibodies to MMP-9 and SM- α -actin, respectively) in SMCs of injured vessel walls of WT mice 8 h after injury (Figure 4C). In contrast, compared to WT mice, Tg, PKC $\beta^{-/-}$ and Egr-1 $^{-/-}$ mice displayed significantly less MMP-9 activity and antigen 8 h after injury (Figure 4A & 4B). However, MMP-2 activity remained unchanged in the injured vessel walls from WT, Tg, PKC $\beta^{-/-}$ and Egr-1 $^{-/-}$ mice at 8 h after denuding injury (Figure 4A). These data suggested that suppression of injury-induced MMP-9 expression by inactivation of PKC β II in vascular SMC in the injured vessel wall was highly prominent.

Next, to delineate the mechanisms by which activation of PKC β II in SMC resulted in upregulation of MMP-9 in response to arterial injury, we examined the impact of inactivation of PKC β II on MMP-9 expression *in vitro*. WT and Tg SMCs were exposed to TNF- α (100ng/ml) because of the known effects of TNF- α on induction of MMP-9 expression in vascular SMCs¹⁹. Conditioned media were concentrated and subjected to zymography/Western blot for MMP-9 activity/antigen. A significant induction of MMP-9 zymography activity and antigen was observed at 8 h after TNF- α treatment compared to that from untreated cells ($p<0.0001$; Figure S2C & 2D), with the maximum activity and antigen level at 24 h ($p<0.0001$; Figure 4D, 4E, S2C & 2D). In parallel, the induction of MMP-9 transcripts was increased within 4 h, with a peak expression at 12 h after TNF- α treatment compared to that in untreated cells ($p<0.0001$; Figure 4F & S2E). In contrast, Tg SMCs, as well as PKC $\beta^{-/-}$ and Egr-1 $^{-/-}$ SMCs, displayed diminished MMP-9 activity, antigen and transcripts stimulated by TNF- α compared to WT SMCs ($p<0.0001$; Figure 4D, 4E & 4F). However, MMP-2 activity remained unchanged in the presence or absence of TNF- α (data not shown). These data suggested that TNF- α induced activation of MMP-9 was regulated by PKC β II in SMCs.

Mechanisms Leading to Activation of MMP-9: *In Vivo* and *In Vitro* Analyses

In view of previous findings identifying significantly increased PKC β II antigen in the membranous fraction of injured femoral arteries⁶, and to examine the mechanisms underlying the potent effects of activation of PKC β II on mediating MMP-9 expression *in vitro*, we assessed membrane localization of PKC β II in SMCs after stimulation with TNF- α . Activation of PKC β II in WT SMCs after exposure to TNF- α was rapid; significantly increased PKC β II antigen was initiated at 15 min of TNF- α treatment ($p<0.0001$; Figure S3), reached an apparent maximum after 30 min ($p<0.0001$; Figure 5A & S3) and remained significantly activated up to 60 min of TNF- α treatment compared with untreated WT SMCs ($p<0.0001$; Figure S3). In contrast, Tg SMCs displayed an immunoreactive band of PKC β II in the membrane fraction of very low intensity compared to WT SMCs 30 min after exposure to TNF- α ($p<0.0001$; Figure 5A). Loading controls using anti-GAPDH IgG indicated identical protein loading (Figure 5A & S3). However, TNF- α stimulation did not affect other PKCs activation, such as PKC α , PKC β I, PKC δ , PKC ϵ , in Tg SMC compared to WT SMC (data not shown). These data demonstrated that activation of SMC PKC β II after TNF- α stimulation was suppressed by carboxyl-terminal deletion mutant PKC β II, mtPKC β II.

Based on the previous findings of PKC β -dependent regulation of phosphorylation of ERK1/2 after acute arterial injury⁶, we investigated the specific impact of mtPKC β II in SMCs on phosphorylation of ERK1/2 in response to acute arterial injury. Homogenates of injured vessels harvested 15 minutes after denudation in WT mice revealed markedly increased phosphorylated ERK1/2 compared with sham ($p<0.0001$; Figure 5B). In parallel, a significant increase in phosphorylation of ERK1/2 was observed in WT SMCs 30 min exposure to TNF- α compared to untreated cells ($p<0.0001$; Figure 5C). In contrast, the effects of injury or TNF- α induced ERK1/2 phosphorylation were significantly suppressed in Tg mice or Tg SMC ($p<0.0001$; Figure 5B & 5C). In addition, the patterns of reduced activation of phospho-ERK1/2 in Tg mice were similar to those in PKC $\beta^{-/-}$ mice (Figure 5B). However, total ERK1/2 was unchanged in each of these conditions (Figure 5B & 5C).

Next, to access downstream target genes of mtPKC β II after arterial injury, we tested its impact on Egr-1 mRNA expression induced by arterial injury or TNF α . Real-time PCR analysis revealed strikingly increased Egr-1 mRNA transcripts from vessels in WT mice harvested 1 h after injury compared to sham ($p<0.0001$; Figure 5D). In contrast, Tg mice showed significantly diminished Egr-1 transcripts compared to WT mice 1 h after injury ($p<0.0001$; Figure 5D). Consistent with these observations, Egr-1 transcripts were increased

by ≈ 73 -fold at 30 min after TNF- α treatment compared to untreated cells ($p < 0.0001$; Figure 5E). whereas, the induction of Egr-1 transcripts was significantly attenuated in Tg SMCs and PKC $\beta^{-/-}$ SMCs compared to WT SMCs 30 min after TNF- α treatment ($p < 0.0001$; Figure 5E).

Lastly, to explore the signal transduction pathway linked to upregulation of MMP-9 in response to cellular or vascular stress, we tested the role of PKC β and downstream MAP kinases in regulation of MMP-9 expression. Increased activity of MMP-9 in WT SMC stimulated with TNF- α at 24 h was reduced by inhibitors of PKC β (LY379196) ($p < 0.01$, Figure 5F) and ERK1/2 (U0126) ($p < 0.0001$, Figure 5F). In addition, our current findings demonstrated that vessel injury and TNF- α -stimulated activation of MMP-9 was suppressed in Egr-1 $^{-/-}$ mice and Egr-1 $^{-/-}$ SMCs (Figure 4A, 4B, 4D, 4E & 4F). Taken together, these findings suggested that PKC β -mediated regulation of MMP-9 expression was due, at least in part, to phosphorylation of ERK1/2 and upregulation of Egr-1.

Discussion

Vascular SMCs proliferate and migrate in response to injury²⁰. Our previous studies established the key role for PKC β in modulating Egr-1 expression linked to SMC activation and neointimal expansion in response to acute arterial injury⁶. Here, we determined the specific role of SMC PKC β II in neointimal expansion upon arterial injury by preparing Tg mice with targeted suppression of PKC β II activity in SMC using the SM22 α promoter. The sequences of those deleted 9 amino acids in the construct of murine mtPKC β II were the same as those reported in the previously published rabbit PKC β II C-terminal V5 deletion mutant M217¹¹. M217 has dramatically decreased catalytic activity and acts as in PKC β II-specific dominant-negative manner, inhibiting insulin-stimulated 2-deoxyglucose uptake in L6 myotubes; furthermore its autophosphorylation activity was 10-fold less than that of full length PKC β II¹¹. This construct was previously shown to block hypoxia-stimulated activation of Egr-1 and tissue factor (TF) in mouse macrophages²¹. Analogous to M217, SMC-specific mtPKC β II significantly reduced PKC β II activity by diminishing phospho-PKC β II and PKC β II antigen in membranous fraction in Tg aortas and Tg SMC with no effects on activities of other PKC isoforms. Furthermore, no changes in PKC β II activity were observed in aortic endothelial cells from Tg mice compared to WT mice, as would be expected since our construct was placed under control of the SM22 α promoter. Similarly, studies in Tg mice subjected to acute arterial injury revealed the prominent suppression of SMC activation and neointimal expansion compared to WT mice. The degree of suppression is consistent with our previous findings in global PKC $\beta^{-/-}$ mice or in WT mice fed the PKC β inhibitor, ruboxistaurin⁶. As PKC β is expressed in multiple cell types, and ruboxistaurin targets the organism in a non-cell type specific manner, our novel findings in these Tg mice underscore the primal action of PKC β II in SMCs upon the response to arterial injury. In addition, Tg SMCs exposed to TNF- α displayed marked reduction in SMC migration compared to that in WT SMCs. Contrary to protection against vascular injury in Tg mice or PKC $\beta^{-/-}$ mice, previous studies revealed that PKC $\delta^{-/-}$ mice displayed exacerbated vein graft arteriosclerosis and showed a significantly higher number of SMCs in arteriosclerotic lesions than that in PKC $\delta^{+/+}$ mice²². Thus, experiments using Tg mice in the present work confirm the central importance of the activity of PKC β II in SMC in triggering neointimal expansion.

A growing body of evidence has suggested that expression of MMP-9 is low in normal blood vessels, and that it is upregulated in injured vessels before any detectable SMC migration into the intima^{4, 5}. Consistent with these concepts, MMP-9 $^{-/-}$ mice have reduced SMC migration and proliferation and neointimal formation upon carotid artery injury^{4, 5}. Although basal levels of MMP-9 in SMC are usually low, MMP-9 expression may be

induced by treatment of vascular SMCs with TNF- α ¹⁹. Therefore, our observation of changes in SMC properties upon injury or TNF- α stimulation led us to consider whether MMP-9 expression and activity would be affected by mtPKC β II, genetic deletion of PKC β and Egr-1. The findings of the present studies revealed marked reduction in expression of MMP-9 and no alteration of MMP-2 in Tg-mtPKC β II, PKC β ^{-/-} and Egr-1^{-/-} mice compared to WT mice subjected to acute arterial injury. Indeed, in a manner somewhat analogous to our findings, one study showed that Egr-1 ablation decreased the MMP-9 expression and did not alter MMP-2 expression in IL-13-induced inflammation and alveolar remodeling response ²³. However, the mechanisms by which Egr-1 regulates MMP-9 expression have not been fully addressed and will be the subject of future investigation.

PKC has been described as being upstream to the activation (by phosphorylation) of MAPKs including ERK1/2 ²⁴⁻²⁷. In those studies, however, the specific role of the β II isoform of PKC was not addressed. Our previous findings demonstrated that PKC β is upstream to the activation of ERK1/2 and the consequent upregulation of Egr-1 after acute arterial injury ⁶, in ischemia/reperfusion (I/R)-triggered stress ²⁸ and in global hypoxemic stress *in vitro* ²¹ and *in vivo* ²⁹. The current results of attenuated phosphorylation of ERK1/2 in Tg mice triggered by vascular injury or TNF- α provided the new evidence that PKC β II plays a critical role in activation of ERK1/2 and its downstream targeted genes, such as Egr-1 and MMP-9, in response to stress stimuli.

Taken together, these studies delineate the SMC-specific regulatory role of PKC β II in neointimal expansion in response to acute arterial injury, at least in part via regulation of ERK1/2, Egr-1 and induction of MMP-9. Our data suggested that targeted inactivation of PKC β II may be beneficial in limiting restenosis via suppression of the neointima-mediating effects of Egr-1 and MMP-9. The lack of specificity, poor oral bioavailability, and potential side effects of currently available synthetic inhibitors of MMPs limits their use in the clinic. Thus, our findings may provide the basis for the design of novel PKC isoform-specific inhibitory compounds that may achieve therapeutically useful changes in PKC β II signaling in cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully acknowledge grant support provided by United States Public Health Service (National Heart, Lung, and Blood Institute Grant RO1 HL075529 and HL60901) and gratefully acknowledge Dr. Denise Cooper for her valuable guidance in these studies. We are grateful to Latoya Woods for her excellent assistance in the preparation of the manuscript.

References

1. Abedi H, Zachary Iss. Signalling mechanisms in the regulation of vascular cell migration. *Cardiovasc Res.* 1995; 30:544–556. [PubMed: 8575003]
2. Owens GK. Regulation of differentiation of vascular smooth muscle cells. *Physiol Rev.* 1995; 75:487–517. [PubMed: 7624392]
3. Schwartz SM, Heimark RL, Majesky MW. Developmental mechanisms underlying pathology of arteries. *Physiol Rev.* 1990; 70:1177–1209. [PubMed: 2217558]
4. Cho A, Reidy MA. Matrix metalloproteinase-9 is necessary for the regulation of smooth muscle cell replication and migration after arterial injury. *Circ Res.* 2002; 91:845–851. [PubMed: 12411400]

5. Galis ZS, Johnson C, Godin D, Magid R, Shipley JM, Senior RM, Ivan E. Targeted disruption of the matrix metalloproteinase-9 gene impairs smooth muscle cell migration and geometrical arterial remodeling. *Circ Res.* 2002; 91:852–859. [PubMed: 12411401]
6. Andrassy M, Belov D, Harja E, Zou YS, Leitges M, Katus HA, Nawroth PP, Yan SD, Schmidt AM, Yan SF. Central role of PKC β in neointimal expansion triggered by acute arterial injury. *Circ Res.* 2005; 96:476–483. [PubMed: 15662033]
7. Li L, Miano JM, Mercer B, Olson EN. Expression of the SM22 α promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *J Cell Biol.* 1996; 132:849–859. [PubMed: 8603917]
8. Sakaguchi T, Yan SF, Yan SD, Belov D, Rong LL, Sousa M, Andrassy M, Marso SP, Duda S, Arnold B, Liliensiek B, Nawroth PP, Stern DM, Schmidt AM, Naka Y. Central role of RAGE-dependent neointimal expansion in arterial restenosis. *J Clin Invest.* 2003; 111:959–972. [PubMed: 12671045]
9. Roque M, Fallon JT, Badimon JJ, Zhang WX, Taubman MB, Reis ED. Mouse model of femoral artery denudation injury associated with the rapid accumulation of adhesion molecules on the luminal surface and recruitment of neutrophils. *Arterioscler Thromb Vasc Biol.* 2000; 20:335–342. [PubMed: 10669628]
10. Tang YM, Ashendel CL. Isolation of cloned mouse protein kinase C β -II cDNA and its sequence. *Nucleic Acids Res.* 1990; 18:5310. [PubMed: 2402470]
11. Chalfant CE, Ohno S, Konno Y, Fisher AA, Bisnauth LD, Watson JE, Cooper DR. A carboxy-terminal deletion mutant of protein kinase C β II inhibits insulin-stimulated 2-deoxyglucose uptake in L6 rat skeletal muscle cells. *Mol Endocrinol.* 1996; 10:1273–1281. [PubMed: 9121494]
12. Lee SL, Wang Y, Milbrandt J. Unimpaired macrophage differentiation and activation in mice lacking the zinc finger transplantation factor NGFI-A (EGR1). *Mol Cell Biol.* 1996; 16:4566–4572. [PubMed: 8754857]
13. Leitges M, Schmedt C, Guinamard R, Davoust J, Schaal S, Stabel S, Tarakhosky A. Immunodeficiency in protein kinase C β deficient mice. *Science.* 1996; 273:788–791. [PubMed: 8670417]
14. Gallo R, Padurean A, Toschi V, Bichler J, Fallon JT, Chessbro JH, Fuster V, Badimon JJ. Prolonged thrombin inhibitor reduces restenosis after balloon angioplasty in porcine coronary arteries. *Circulation.* 1998; 97:581–588. [PubMed: 9494029]
15. Travo P, Barrett G, Burnstock G. Differences in proliferation of primary cultures of vascular smooth muscle cells taken from male and female rats. *Blood Vessels.* 1980; 17:110–116. [PubMed: 7362876]
16. Kobayashi M, Inoue K, Warabi E, Minutesami T, Kodama T. A simple method of isolating mouse aortic endothelial cells. *J Atheroscler Thromb.* 2004; 12:138–142. [PubMed: 16020913]
17. Harja E, Bucciarelli LG, Lu Y, Stern DM, Zou YS, Schmidt AM, Yan SF. Early growth response-1 promotes atherogenesis: mice deficient in early growth response-1 and apolipoprotein E display decreased atherosclerosis and vascular inflammation. *Circ Res.* 2004; 94:333–339. [PubMed: 14670837]
18. Jovinge S, Hultgardh-Nilsson A, Regnstrom J, Nilsson J. Tumor necrosis factor- α activates smooth muscle cell migration in culture and is expressed in the balloon-injured rat aorta. *Arterioscler Thromb Vasc Biol.* 1997; 17:490–497. [PubMed: 9102167]
19. Moon SK, Cho GO, Jung SY, Gal SW, Kwon TK, Lee YC, Madamanchi NR, Kim CH. Quercetin exerts multiple inhibitory effects on vascular smooth muscle cells: role of ERK1/2, cell-cycle regulation, and matrix metalloproteinase-9. *Biochem Biophys Res Commun.* 2003; 301:1069–1078. [PubMed: 12589822]
20. Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest.* 1983; 49:327–333. [PubMed: 6887785]
21. Yan SF, Lu J, Zou YS, Soh-Won J, Cohen DM, Buttrick PM, Cooper DR, Steinberg SF, Mackman N, Pinsky DJ, Stern DM. Hypoxia-associated induction of early growth response-1 gene expression. *J Biol Chem.* 1999; 274:15030–15040. [PubMed: 10329706]

22. Leitges M, Mayr M, Braun U, Mayr U, Li C, Pfister G, Ghaffari-Tabrizi N, Baier G, Hu Y, Xu Q. Exacerbated vein graft arteriosclerosis in protein kinase Cdelta-null mice. *J Clin Invest.* 2001; 108:1505–1512. [PubMed: 11714742]
23. Cho SJ, Kang MJ, Homer RJ, Kang HR, Zhang X, Lee PJ, Elias JA, Lee CG. Role of early growth response-1 (Egr-1) in interleukin-13-induced inflammation and remodeling. *J Biol Chem.* 2006; 281:8161–8168. [PubMed: 16439363]
24. Avdi NJ, Winston BW, Russel M, Young SK, Johnson GL, Worthen GS. Activation of MEKK by formyl-methionyl-leucyl-phenylalanine in human neutrophils. Mapping pathways for mitogen-activated protein kinase activation. *J Biol Chem.* 1996; 271:33598–33606. [PubMed: 8969228]
25. Grammer TC, Blenis J. Evidence for MEK-independent pathways regulating the prolonged activation of the ERK-MAP kinases. *Oncogene.* 1997; 14:1635–1642. [PubMed: 9135064]
26. Wei S, Liu JH, Epling-Burnette PK, Jiang K, Zhong B, Elkabani ME, Pearson EW, Djeu JY. IL-2 induces the association of IL-2Rbeta, lyn, and MAP kinase ERK-1 in human neutrophils. *Immunobiology.* 2000; 202:363–382. [PubMed: 11131153]
27. Crossthwaite AJ, Valli H, Williams RJ. Inhibiting Src family tyrosine kinase activity blocks glutamate signalling to ERK1/2 and Akt/PKB but not JNK in cultured striatal neurones. *J Neurochem.* 2004; 88:1127–1139. [PubMed: 15009668]
28. Fujita T, Asai T, Andrassy M, Stern DM, Pinsky DJ, Zou YS, Okada M, Naka Y, Schmidt AM, Yan SF. PKCbeta regulates ischemia/reperfusion injury in the lung. *J Clin Invest.* 2004; 113:1615–1623. [PubMed: 15173888]
29. Yan SF, Lu J, Zou YS, Kisiel W, Mackman N, Leitges M, Steinberg S, Pinsky D, Stern D. Protein kinase C-beta and oxygen deprivation. A novel Egr-1 dependent pathway for fibrin deposition in hypoxemic vasculature. *J Biol Chem.* 2000; 275:11921–11928. [PubMed: 10766820]

Figure 1. Characterization of Tg mice

(A) & (B) Cytosolic and membranous proteins from WT and Tg aortas were subjected to Western blotting with antibodies to PKC β II (A), phospho (P)-PKC β II (B) and GAPDH (A & B). (C) Membrane proteins from WT and Tg SMCs were subjected to Western blotting with anti-PKC β II, PKC α , PKC β I, PKC δ , PKC ϵ and GAPDH IgG. (D) Cytosolic and membranous proteins from WT and Tg SMCs were subjected to Western blotting with antibodies to P-PKC β II and GAPDH. (E) Membrane proteins from WT and Tg MAEC were subjected to Western blotting with anti-PKC β II and GAPDH IgG. Experiments were repeated three times and representative bands are shown.

Figure 2. Reduced neointimal expansion in Tg mice after acute arterial injury

(A) I/M ratio was determined on day 28 after denudation injury in WT (n=10) and Tg mice (n=9). (B and C), Histology. Van Giesson's elastic staining of midportion of femoral artery is shown from injured WT (n=10) (B) and Tg mice (n=9) (C) on day 28. (D), The section adjacent to that in B was immunostained with α -smooth muscle actin. Scale bar = 50 μ m.

Figure 3. Inhibition of SMC activation by mtPKC β II in Tg mice and cells

(A) BrdU labeling index was determined on day 7 after denudation injury followed by IP injection of BrdU. (B and C) Nuclei immunoreactive with BrdU are shown in the proliferative lesions of expanding neointima from WT (B) and Tg mice (C) (n=7_mice/group) on day 7 after denudation injury. Scale bar=50 μ m. Arrows indicated BrdU-labeled nuclei. (D) Sections of femoral artery segments were immunofluorescence double stained with an anti-BrdU antibody (red) and an anti-SMC- α -actin antibody (green); and the merged image is shown (mixed color). Scale bar=50 μ m. (E) Migration of WT and Tg SMCs was evaluated in the presence/absence of TNF- α (100 ng/ml) for 6 h.

Figure 5. Reduced phosphorylation of ERK1/2, upregulation of Egr-1 and PKCβII activity by mtPKCβII in Tg mice or cells

(A) Membrane protein (5 μg protein/lane) from WT and Tg SMCs incubated with TNF-α (100 ng/ml) was subjected to Western blot with anti-PKCβII or GAPDH IgG. (B and C), Homogenates of uninjured and injured vessel segments prepared from WT, PKCβ^{-/-} and Tg mice 15 minutes after denudation injury (B), or cell lysates from WT and Tg SMCs incubated with TNF-α (100 ng/ml) for 30 min (C) were subjected to Western blot for detection of Phospho (P)-ERK1/2 and Total (T)-ERK1/2. (D and E) Total RNA from vessel segments (D) or SMCs (E) was subjected to Real-time PCR analysis of Egr-1 mRNA expression. Data are represented as the relative expression of mRNA for Egr-1 normalized to 18S rRNA. N=9 mice/group. (F) WT SMCs were pre-incubated with or without inhibitors of PKCβ (LY379196, 30 nM) and ERK1/2 (U0126, 5 μM) for 1 h, respectively, followed by co-incubation with TNF-α (100 ng/ml) for 24 h. Conditioned media were concentrated and subjected to gelatin zymography for detection of MMP-9 activity. All experiments were repeated more than 3 times; representative bands are shown, and the mean ± SE is shown.