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THERAPEUTIC POTENTIAL FOR PROTEIN KINASE C INHIBITOR IN VASCULAR RESTENOSIS

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Abstract

Vascular restenosis, an overreaction of biological response to injury, is initialized by thrombosis and inflammation. This response is characterized by increased smooth muscle cell migration and proliferation. Available pharmacological treatments include anticoagulants, antiplatelet agents, immunosuppressants and antiproliferation agents. Protein kinase C (PKC), a large family of serine/threonine kinases, has been shown to participate in various pathological stages of restenosis. Consequently, PKC inhibitors are expected to exert a wide range of pharmacological activities therapeutically beneficial for restenosis. In this review, the roles of PKC isozymes in platelets, leukocytes, endothelial cells and smooth muscle cells are discussed, with emphasis given to smooth muscle cells. We will describe cellular and animal studies assessing prevention of restenosis with PKC inhibitors, particularly targeting -alpha, -beta, -delta and -zeta isozymes. The delivery strategy, efficacy and safety of such PKC regulators will also be discussed.

Keywords

protein kinase C; smooth muscle cell; migration; proliferation; vascular restenosis

Introduction

Vascular restenosis is the renarrowing of a blood vessel after surgical and mechanical intervention in patients with occlusive vascular diseases. Vessel renarrowing can be caused by constrictive wall recoiling and intimal hyperplasia.^{1,2} Recoil may be minimized by stent placement, but intimal hyperplasia still leads to in-stent restenosis at an inherent rate of approximately 25%. Although restenosis is a multiple-staged proliferative disease, it is known that vascular smooth muscle cell (VSMC) migration and proliferation play a critical role in its formation.³⁻⁵ Protein kinase C (PKC), a large family of serine/threonine kinases, plays multiple essential (patho)physiological roles in the cardiovascular system by governing various signaling cascades.^{6,7} Emerging evidence has shown that PKC is

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involved in the formation of intimal hyperplasia. In this review, we will first introduce the basic knowledge of PKC biology and then elaborate on the roles of PKC isozymes and effects of PKC inhibitors in the progression of restenosis, particularly in SMC migration and proliferation, and finally discuss the delivery strategy, efficacy and safety of PKC regulators.

PKC Biology

The PKC family includes 10 homologous protein kinases, consisting of a regulatory domain and a catalytic domain. The catalytic region is highly conserved and binds ATP and protein substrates; the regulatory region contains motifs that bind the second messengers diacylglycerol (DAG) and calcium in the presence of phosphatidylserine, and is less conserved. Depending on the regulatory region, PKCs can be categorized into three groups: the conventional PKCs (-alpha, -betaI, -betaII and -gamma) have DAG- and Ca^{2+} -binding domains; the novel PKCs (-delta, -epsilon, -eta, -theta) have DAG- but not Ca^{2+} -binding domains; the atypical PKCs (-lambda/iota, and -zeta) have neither Ca^{2+} - nor DAG-binding domains. The distribution and abundance of each isozyme varies by cell subtype. For example, the levels of PKC-alpha, -beta, -delta and -epsilon, but not PKC-zeta, are relatively high in human smooth muscle cells.⁸ In endothelial cells, the level of PKC-delta is lower than PKC-zeta.^{9,10} Generally, PKC is activated by G-protein coupled receptors and translocates from the cytosol to the plasma/nuclear membrane or cytoskeleton. Mitogen-activated protein kinases, such as ERK, p38 and JNK, are common downstream effectors of PKC.¹¹⁻¹³ Akt signaling is also modulated by PKC.¹⁴ Cell migration-related molecules, such as focal adhesion kinase,¹⁵⁻¹⁷ paxillin,¹⁷⁻¹⁹ and vinculin,¹⁹ also can be activated by PKC. It is likely that different PKCs preferentially activate different substrates/effectors, yet relatively few isozyme-specific substrates have been identified.

PKC and Restenosis

Therapeutic intervention of vascular diseases usually initiates endothelial denudation, artery stretching and plaque compression, triggering a substantial local thrombotic and inflammatory reaction. Platelets, clotting proteins, and inflammatory cells adhere to the exposed subendothelium forming a thin carpet of thrombus. Chemocytokines released from platelets and inflammatory cells recruit more circulating leukocytes to the injury site. Inflammatory cells infiltrate the blood vessel, releasing cytokines, such as tumor necrosis factor-alpha (TNF- α), monocyte chemotactic protein-1 (MCP-1) and growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-1), and basic fibroblast growth factor (bFGF). These bioactive molecules stimulate medial SMCs to migrate to and proliferate in neointima, leading to intimal hyperplasia. In this section, we will discuss the roles of PKC in multiple stages of restenosis, with an emphasis on SMC migration and proliferation, which are currently regarded as key determinants in the etiology of restenosis.

Role of PKC in restenosis initiation

PKC and platelet activation—Both experimental²⁰ and clinical²¹ studies showed acute accumulation of platelets surrounding the stent struts. Platelet-derived growth factor is an important stimulus for VSMC migration and proliferation. Four major platelet-expressed isozymes, PKC-alpha, -beta, -delta and -theta, play distinct roles in its aggregation and secretion. It seems that conventional PKCs promote while novel PKCs inhibit platelet secretion and thrombus formation.^{22,23} Blocking PKC-alpha by gene deletion, immunodepletion or drug inhibitor (Go6850 and Go6976) abrogated platelet secretion in both mice²⁴ and humans.²⁵⁻²⁷ PKC -delta and -theta were also shown to modulate platelet secretion but in a more complicated manner. One set of studies suggested that PKC-delta^{28,29} and -theta³⁰ play negative roles in platelet activation. Pharmacological inhibition

by rottlerin potentiated human platelet aggregation, which was initiated by alboaggregin A²⁸ or collagen²⁹. Knocking out either PKC-delta²⁹ or -theta³⁰ potentiated murine platelet aggregation. However, another set of studies implied that PKC-delta³¹ and -theta^{32,33} are required for platelet activation: Gene deficiency or selective inhibition (by deltaV1-2) of PKC-delta impaired protease-activated receptor 4-mediated platelet secretion in the absence and presence of thromboxane A₂.³¹ Deficiency of PKC-theta impaired murine platelet activation triggered by glycoprotein VI³¹ or thrombin.³² Inhibition of PKC-theta by selective antagonistic RACK peptide exerted a similar result.³²

PKC and inflammation—Besides platelets, leukocytes are another primary source of cytokines and growth factors for stimulating VSMC migration and proliferation. Leukocyte recruitment is facilitated by endothelial cell-expressed adhesion molecules. Activation of PKC-alpha,³⁴ -beta,³⁵ and -zeta,³⁶ as measured by protein phosphorylation³⁴, translocation^{35,36} or enzyme activity³⁶, were found to potentiate adhesion molecule expression. Monocyte chemotactic protein-1 (MCP-1) is another molecule which enlists leukocytes to endothelial layer. Transfection of endothelia with PKC-epsilon antisense oligonucleotide abolished shear stress-induced MCP-1 expression, suggesting that PKC-epsilon may regulate MCP-1 expression in the endothelial cells.³⁷ Instead of regulating endothelial cell function, PKC-zeta directly participates in leukocyte infiltration. Selective blockade of this isozyme by peptide inhibitor impaired interleukin 8-induced neutrophil migration.³⁸

Role of PKC in SMC migration

Smooth muscle cell migration and proliferation are crucial events in the pathogenesis of intimal hyperplasia. Under normal conditions, vascular medial SMCs are quiescent and exhibit low levels of migrating or proliferating activity. With the stimuli of cytokines/growth factors released in the injury area, medial SMCs migrate to the injury areas and proliferate at the site in an attempt to repair the wound. SMC migration involves intracellular actin polymerization, adhesion molecule expression at the cell surface, and extracellular activation of matrix metalloproteinase. With microchemotaxis chamber or morphological analysis, PKC-alpha,³⁹ -beta,⁴⁰ and -delta^{12,19,41-43} were reported to modulate SMC migration by either promoting actin polymerization or enhancing cell adhesion. It has been found that matrix metalloproteinases mediate adipokine resistin-induced VSMC migration.⁴⁴

Role of PKC in SMC proliferation

Several lines of evidence have established that certain PKCs regulate VSMC proliferation. Known PKCs include -alpha, -beta, -delta and -zeta isozymes.

PKC-alpha is one of the most prevalent isozymes in vascular SMCs.^{8,39,45} Marilley *et al*⁴⁶ observed that there is a positive correlation between cell proliferation and PKC alpha levels in human aortic vascular SMCs. Selective depletion of PKC-alpha with PKC-alpha antisense oligodeoxynucleotide caused 45-55% inhibition of ³H-thymidine incorporation into SMCs.⁴⁵ However, Sasaguri *et al*.⁴⁷ and Wang *et al*.⁴⁸ showed different results. Using cell cycle analysis or cell counting method, they found that, instead of promoting cell growth, PKC-alpha mediated SMC growth inhibition in porcine and rat VSMCs.

PKC-beta appears preferentially activated by high glucose⁴⁹⁻⁵¹ and low-density lipoprotein⁵² in human and rat aortic SMCs. Based on studies with [³H]thymidine incorporation assay, PKC-beta was also found to mediate synergistic proliferative effect of PDGF and high glucose level on human coronary SMCs.⁵³ Selective PKC-beta inhibitor LY-379196 attenuated DNA synthesis and cell growth. Further animal study showed that

PKC-beta null mice or mice treated with the PKC-beta inhibitor LY379196 experienced significantly decreased neointimal expansion.⁵⁴

PKC-delta mediates vascular SMC proliferation, triggered by PDGF, bFGF and IGF-I. Using [³H]thymidine incorporation assay, Yamaguchi *et al.*¹² reported that PDGF-induced SMC proliferation is mediated by PKC-delta.¹² Going one step further, Ginnan *et al.*¹³ showed that ERK1/2 is an effector molecule of PKC-delta after PDGF stimulus. Studies using [³H]thymidine incorporation, 5-bromo-2-deoxyuridine labeling and cell counting assays demonstrated that this kinase also mediated bFGF and IGF-I-stimulating VSMC growth.^{11,14,55} In contrast, Liu and his colleagues demonstrated the opposite results: over expression of PKC-delta inhibited PDGF-induced cell growth in rat vascular SMCs.⁴³ It should be noted that, PKC-delta also plays a role in regulating apoptotic progress and hence interferes with cell homeostasis. An exacerbated vein graft arteriosclerosis has been observed in PKC-delta-null mice.⁵⁶

Three research groups demonstrated that PKC-zeta is involved in SMC growth and intimal hyperplasia. However, the exact function of PKC-zeta varies in these studies. Using [³H]-thymidine incorporation assay, Zhao *et al.*⁵⁷ reported that angiotensin-induced cell proliferation was downregulated by PKC-zeta inhibitor in rat SMCs. Parmentier *et al.*⁵⁸ treated rats with selective PKC-zeta antisense oligodeoxyribonucleotide and found that neointimal formation was reduced significantly. These two studies suggested that PKC-zeta plays a favorable role for cell growth at least in rats. However, in rabbit aortic SMCs, activation of PKC-zeta by bFGF and interleukin is correlated with inhibition of cell proliferation, while inhibition of this kinase with antisense oligonucleotide stimulates cell proliferation.⁵⁹ In summary, alpha, beta, delta and zeta PKCs have been found to be involved in modulating VSMC proliferation. Among these isozymes, PKC-beta seems to play a consistent role in promoting cell growth while many conflicting results exist with other three PKCs. The conflicting results may reflect differences in between species, the use of different mitogens and the use of different and sometime isozyme-non-selective tools in the studies.

Overall, vascular restenosis is a multi-staged disease in which smooth muscle cell migration and proliferation play a central role (Figure 1). Different PKC isozymes seem preferentially involved in various stages of disease progression as summarized in Figure 1.

PKC Inhibitor and Restenosis Prevention

Restenosis is a multi-staged vascular disease. Drugs with anti-thrombotic, anti-inflammatory, and anti-proliferative activities could be effective in preventing this disease at different stages. Early attempts focused on anti-thrombotic agents, but clinical reports demonstrate a limited success.⁶⁰ SMC migration and proliferation are currently considered to play a key role. Emerging data have demonstrated that PKC inhibitors are effective in inhibiting SMC activation. Table 1 lists some common PKC inhibitors. These inhibitors belong to different structure classes and take effect through different mechanisms.

PKC inhibitors

Staurosporine, calphostin C and chelerythrine are known pan-PKC inhibitors. They are effective inhibitors of both conventional and novel PKC isozymes. Yang *et al* found that staurosporine inhibited oxidized low density lipoprotein (LDL)-induced rat VSMC growth;⁶¹ calphostin C and chelerythrine abolished lipoprotein lipase-induced human VSMC proliferation;⁶² chelerythrine also decreased phenylephrine-induced SMC proliferation.⁶³ In contrast to above pan-PKC inhibitors, some PKC regulators show a greater isozyme selectivity.

PKC-alpha inhibitor—Available PKC-alpha inhibitors include antisense oligonucleotides ISIS9606 and ISI3521. The ATP-binding site inhibitor, Go6976, also selectively inhibits PKC-alpha at lower concentrations (0.1-2nM). However, ATP binding site inhibitors are less selective. They can inhibit a number of other protein kinases at higher concentrations.⁶⁴ Although direct experiments examining the role of PKC-alpha inhibitors in restenosis are limited, based on the promoting role of PKC-alpha in SMC proliferation,^{45,46} it is worthwhile to test the potential suppressive effect of PKC-alpha inhibitor on VSMC growth. In addition to smooth muscle cells, this inhibitor might abrogate platelet activation²⁷ and reverse endothelial dysfunction^{34,65}, which also are therapeutically beneficial to treat restenosis.

PKC-beta inhibitor—Available ATP-binding site inhibitors of PKC-beta include LY317615, LY333531, LY379196 and CGP53353. They may have the same limitation as discussed for the PKC-alpha inhibitors.⁶⁴ Competitive inhibitors of localization of activated PKC, betaIV5-3 and betaIV5-3 have also been used, and they show a selective effect on the corresponding isozymes betaI and betaII PKC.⁶⁶ Compared to PKC-alpha inhibitor, more experimental evidence has been obtained with PKC-beta inhibitors in the vascular system. *In vitro* studies showed that PKC-beta inhibition attenuated SMC proliferation. Consistent results were obtained on cells isolated from rats,^{50,51,67} rabbits,⁶⁸⁻⁷⁰ pigs⁷¹ and humans.^{49,53,72} The suppressive effect on cell migration after PKC-beta deactivation was also reported.⁴⁰ Further *in vivo* data confirmed that mice fed with LY333531 displayed significantly decreased neointimal thickening in response to acute femoral artery injury.⁵⁴

PKC-delta inhibitor—Available inhibitors include the peptide inhibitors of anchoring of the active enzymes delta V1-1⁷³ aka KAI-9803⁷⁴ and a less selective, ATP-binding site competitive inhibitor, rottlerin.⁷⁵ The efficacy of PKC-delta inhibition against VSMC migration has been observed in mechanical stress- and drug-associated cellular models.^{19,41} Also, the effect of inhibition of PKC-delta on DNA synthesis and cell proliferation in human VSMCs has been reported. Genetic and pharmacological (by rottlerin) approaches brought about similar results.⁵⁵ Different from inhibiting VSMCs, suppressing PKC-delta was found to stimulate endothelial growth and angiogenesis,⁷⁶⁻⁷⁸ which has not been observed with other PKC isozymes. This is interesting because a differential effect between SMCs and endothelial cells is ideal for an anti-restenosis drug. As for animal tests, two *in vivo* studies showed protection by PKC-delta peptide inhibitor deltaV1-1 (combined with PKC-epsilon selective activator pseudo-epsilonRACK) against coronary stenosis both in mice⁷⁹ and rats.⁸⁰ In murine cardiac allografts, graft coronary artery narrowing related to ischemia-reperfusion injury was suppressed by a brief treatment with pseudo-epsilonRACK and deltaV1-1. The percentage of luminal narrowing and intima-media ratio were decreased by >60% at 30 days after heart transplantation.⁷⁹ In another graft coronary artery disease in rats, one early combination injection of pseudo-epsilonRACK and deltaV1-1 decreased the percentage of luminal narrowing by 78% and decreased the intima-media ratio by 58% at 90 days after cardiac injury.⁸⁰ One potential concern for PKC-delta inhibitors is that they might interfere with cellular apoptosis and even aggravate stenosis. An exacerbated vein graft arteriosclerosis has been observed in PKC-delta-null mice.⁵⁶ Another potential concern is that PKC-delta inhibitor, such as rottlerin, may stimulate platelet activation and accentuate thrombosis.^{22,23} However, an *in vivo* study demonstrated that a deficiency of PKC-delta does not stimulate thrombosis in mice.²⁷ Recent clinical trials showed that the selective peptide PKC-delta inhibitor inhibits some of the damage induced by myocardial infarction in patients without inducing any adverse effects.⁷⁴

PKC-zeta inhibitor—Spheciosterol sulfate C is a small molecule which can selectively inhibit PKC-zeta.⁸¹ However, due to the unclear role of PKC-zeta in VSMC growth, the efficacy of PKC-zeta inhibitor in restenosis is uncertain at present.

Because PKC isozymes have unique and sometimes opposing roles,^{22,23} the use of isozyme-selective tools is essential. The investigation of PKC inhibitors in vascular restenosis is just at its infancy. Current data of targeting PKC-beta and -delta are encouraging despite some discrepancy that may be attributed to the tools used and/or some species differences. Inhibitors for PKC-alpha and -zeta warrant more studies. Ideally, an anti-restenosis drug would be a potent and direct inhibitor of SMC activity with little or no effect on endothelial cells and would not exacerbate platelet aggregation or inflammation. Future studies of screening PKC inhibitors for restenosis ought to be conducted along this direction.

Drug Delivery: Systematically vs. Locally

Systemic administration of anti-platelet agents and anti-coagulants show limited success in preventing restenosis in the clinic.^{82,83} Similarly, limited results of success were obtained with oral administration of anti-inflammation,^{84,85} immunosuppression⁸⁶ and anti-migration/proliferation agents.⁸⁷ The lack of efficacy in clinical studies may be partly due to either an insufficient drug concentration at the injury site or a lack of chronically adequate dose. In addition, because PKC modulates many critical physiological functions. Unwanted drug effects may occur when non-selective PKC inhibitors are systemically administered. (It should be noted that sustained delivery of several peptide inhibitors of PKC for a couple of months were found to be safe in animals.)^{79,88} Nevertheless, local delivery of PKC inhibitors may be a better approach for preventing restenosis; coated onto stents or balloons, selective PKC inhibitors may be released directly into the injured area with a higher concentration. In fact, current drug-eluting stents/balloons provide a relatively high drug concentration at the site of injury and minimize systemic side effects.^{89,90} Both PKC-betaII inhibitor (e.g. BetaIV5-3) and PKC-delta inhibitor (e.g. DeltaV1-1) are promising candidates for coating stents or balloons as inhibitors for these two PKC isozymes exert satisfactory efficacy and safety in vitro and in animal trials.

Summary

PKC is involved in the transduction of many signals that participate in restenosis, including platelet activation, inflammation, smooth muscle cell migration/proliferation and endothelial growth. Therefore, inhibition of PKC activity by selective inhibitors is expected to be an effective means for restenotic prevention. Based on current data, PKC-beta and PKC-delta inhibitors showed some therapeutic promise both *in vitro* and *in vivo*. Benefit for other selective PKC inhibitors, such as those targeting -alpha, -epsilon and -zeta subtypes, await further study.

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Abbreviations

bFGF	basic fibroblast growth factor
DAG	diacylglycerol
IGF-1	insulin-like growth factor

MCP-1	monocyte chemotactic protein-1
PDGF	platelet-derived growth factor
PKC	protein kinase C
RACK	receptor for activated C kinase
SMC	smooth muscle cell
TNF-alpha	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor

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Figure 1.

Table 1

Currently available protein kinase C inhibitors.

Drug Name	Structure Class	Inhibiting Target	Inhibition Selectivity
Balanol	Benzophenone	ATP-binding site	Pan-PKC
Chelerythrine	Benzophenone	ATP-binding site	Pan-PKC
Calphostin c	Perylenequinone	Regulatory domain	Pan-PKC
Midostaurin (PKC412)	Indolocarbazole	ATP-binding site	Pan-PKC
Sotrastaurin (AEB071)	Indolocarbazole	ATP-binding site	Pan-PKC
Staurosporine	Indolocarbazole	ATP-binding site	Pan-PKC
Go6976	Indolocarbazole	ATP-binding site	Conventional PKCs
UCN01	Indolocarbazole	ATP-binding site	Conventional PKCs
Tamoxifen	Taxol	Regulatory domain	Conventional PKCs
Rottlerin (Mallotoxin)	Phenolic ketone	ATP-binding site	Novel PKCs
Isis9606	Antisense oligonucleotide	mRNA	PKC-alpha
Isis3521 (Aprinocarsen)	Antisense oligonucleotide	mRNA	PKC-alpha
LY317615 (Enzasautaurin, RO-31-8220, RO32-0432, GF109203x)	Indolocarbazole	ATP-binding site	PKC-beta
LY333531 (Ruboxistaurin, Bisindolymaleimide)	Indolocarbazole		PKC-beta
LY379196	Indolocarbazole	ATP-binding site	PKC-beta
CGP53353	Indolocarbazole	ATP-binding site	PKC-beta
BetaIV5-3	Short peptide	PKC translocation	PKC-betaI
BetaIIV5-3	Short peptide	PKC translocation	PKC-betaII
KAI-9803 (deltaV1-1)	Short peptide	PKC translocation	PKC-delta
KAI-1678 (epsilonV1-2)	Short peptide	PKC translocation	PKC-epsilon
KCe-12	Short peptide	Substrate-binding site	PKC-epsilon
KCe-16	Short peptide	Substrate-binding site	PKC-epsilon
5-vinyl-3-pyridinecarbonitriles	Carbonitrile	Catalytic domain	PKC-theta
2,4-Diamino-5-nitropyrimidines	Pyrimidine	Catalytic domain	PKC-theta
Spheciosterol sulfate C	Sterol	Catalytic domain	PKC-zeta

NOTE: Because the ATP-binding site in protein kinases is so similar, generating ATP-binding site inhibitors that are specific for one protein kinase was found to be a challenge. Many of these inhibitors, that were initially thought to be highly specific, were subsequently found to affect multiple kinases from a variety of protein kinase families.⁶⁴