Protein Kinase C β/Early Growth Response-1 Pathway
A Key Player in Ischemia, Atherosclerosis, and Restenosis

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Atherosclerosis, restenosis, and the consequences of ischemia are the major causes of morbidity and mortality worldwide. Elucidation of key contributing pathways in animal models of ischemia-reperfusion injury, atherosclerosis, and restenosis consequent to vascular injury may lead to great interest in determining if blocking these pathways could prevent vascular disease in human subjects. This review details the evidence that the protein kinase C (PKC) β/early growth response-1 axis plays a central role in the response to both acute and chronic vascular stresses in animal models and also indicates the clinical implications of a specific inhibitor of PKCβ, ruboxistaurin (LY333531). (J Am Coll Cardiol 2006;48:A47–55) © 2006 by the American College of Cardiology Foundation

Atherosclerosis, restenosis, and the consequences of ischemia are the key disease processes underlying vascular diseases, which are major causes of morbidity and mortality in both men and women worldwide. Although vascular injury is thought to be an important stimulus to atherosclerosis, restenosis, myocardial ischemia, lung ischemia, and so on, the mechanisms that mediate the aberrant response to injury remain to be fully clarified. Advances in molecular genetics have made it possible to remove or insert genes in animal models and, thereby, to determine the roles of their products in models of disease (1). Animal models of ischemia/reperfusion injury, atherosclerosis, and restenosis after vascular injury are crucial in studying the cellular and molecular mechanisms underlying vascular stresses. In this review, we will detail the evidence that the protein kinase C (PKC) β/early growth response-1 (Egr-1) axis plays a central role in experimental ischemia-reperfusion injury, atherosclerosis, and restenosis. These findings suggest the possibility that blockade of PKCβ/Egr-1 pathway by a specific inhibitor of PKCβ, for example, ruboxistaurin (LY333531), may have important clinical implications for diabetic and non-diabetic patients with vascular disorders.

EGR-1 AND PKCβII

The early growth response gene product (Egr-1), also known as Zif268, NGF1-A, Krox24, or TIS8, is a zinc finger transcription factor first identified due to its characteristic pattern of expression after exposure of cells to mediators associated with growth and differentiation (2,3). It has been assigned to the group of "immediate early genes" based on its rapid induction within minutes of a stimulus, and its rapid decay, often within hours. Although studies

limited to the in vitro milieu suggested that Egr-1 had a critical role in promoting cellular differentiation along a macrophage lineage (4–6), experiments in Egr-1–null mice indicated that the transcription factor was not essential for effective macrophage differentiation (5), because Egr-1-null mice were viable and developed and grew normally. The apparent absence of a life-threatening phenotype in Egr-1–null mice in homeostasis underscored the possibility that the biological impact of Egr-1 was likely relevant in induced stresses. Strongly supportive of this concept was the observation, in vitro, that Egr-1 induced a number of gene products linked to cellular perturbation, especially in the vasculature, including tumor necrosis factor (TNF)-alpha, intercellular adhesion molecule-1 (ICAM-1), CD44, platelet-derived growth factor A/B chain, basic fibroblast growth factor, transforming growth factor (TGF)-β, and macrophage colony stimulating factor (M-CSF) (7–9). Evidence from our laboratory and other investigators has accumulated linking activation of Egr-1 to both acute and chronic vascular stress, such as hypoxia (10–12), ischemia/reperfusion (13,14), and mechanical stress (15), shear stress (16,17), emphysema (18), atherosclerosis (19–21), and acute vascular injury (22–25).

Our laboratory has elucidated key roles for PKCβ in regulation of Egr-1 in vascular stress. The PKC family is a family of multifunctional isoenzymes that play a central role in signal transduction and intracellular crosstalk by phosphorylating at serine/threonine residues an array of substrates, including cell surface receptors, enzymes, contractile proteins, transcription factors, and other kinases (26). Based on their structure and cofactor regulation, a total of 12 isoforms of PKC have been classified into 3 groups: the diacylglycerol (DAG) and Ca^{2+}-dependent conventional or classical PKC isoforms (α, βI, βII, γ); the DAG-dependent, but Ca^{2+}-independent, novel PKC isoforms (δ, ϵ, η, θ, and μ); and the DAG- and Ca^{2+}-independent atypical PKC isoforms (ι, λ, and ζ) (the mouse and rat homologue of human PKCι is named PKCλ) (27). Protein kinase CβI and PKCβII are generated by alternative splic-

Abbreviations and Acronyms

apoE	=	apolipoprotein E
Egr-1	=	early growth response-1
ERK1/2	=	extracellular-signal-regulated protein kinase
JNK	=	c-Jun N-terminal kinase
OxLDL	=	oxidized low-density lipoprotein
PKC	=	protein kinase C

ing from a single gene, but they differ at their C-terminal 50 (βI) or 52 (βII) residues (27). Activation of PKC occurs in response to transient increases in DAG or exposure to phorbol ester (28) or hypoxia/ischemia (12,14). Other studies suggest that oxidant stress and hyperglycemia are triggers to activation of PKCβII (29–30). Hyperglycemia-induced DAG preferentially activates one or more PKC isoenzymes in the tissues; in heart and aorta, PKCβII is preferentially activated; this includes both smooth muscle cells (SMC) and endothelial cells (31,32). Links between PKCβ activation and endothelial dysfunction in diabetes have been elucidated, such as impaired nitric-oxide–mediated vasodilatation, increased release of endothelin-1, increased expression of inducible nitric oxide synthase, and ICAM-1, and enhanced monocyte adhesion to the vessel wall (33–38). In studies from our laboratory, we examined the role of PKCβ in the response to hypoxia, ischemia/reperfusion, acute vascular injury, and hyperlipidemia and in modulating expression of Egr-1 (12,14,25,39,40) by employing PKCβ-null mice (41–43). These mice and pharmacologic inhibition of PKCβ provide key strategies to test the impact of PKCβ in vivo. We demonstrated that activated PKC, especially βII isoform, is a critical upstream regulator of Egr-1 (12,14,25,39,40). Early growth response-1, in turn, functions as a master switch orchestrating the expression of diverse gene families to elicit a pathological response to hypoxia, ischemia/reperfusion, and vascular stress (10–14,21).

PKCβ/EGR-1 AXIS AND ISCHEMIA

Oxygen deprivation and subsequent restoration of blood flow to ischemic tissue somewhat paradoxically leads to reperfusion injury. Because of their rich vascular network, the lungs of both Egr-1–null (5) and PKCβ-null (41) mice provided ideal model systems to assess the contribution of PKCβ/Egr-1 axis in susceptibility to hypoxia or ischemia/reperfusion-induced vascular dysfunction, such as hypercoagulability and inflammation. We employed two animal models to simulate acute vascular stress: one is global hypoxia in which mice were placed in a hypoxic chamber and allowed free access to food and water, and the system parameters were adjusted to a final oxygen concentration of 5.8% to 6.2% (44); the other is murine lung ischemia/reperfusion in which blood flow to the left lung was blocked for up to 60 min (ischemic period) followed by reperfusion for 3 h (45).

Cells respond to oxygen deprivation by recruiting a number of central pathways such as hypoxia-inducible factor (HIF)-1 (46–49), nuclear factor κ binding (NFκB) (50–52), and Egr-1 (10,11,13). In an oxygen-scarce environment, HIF-1α promotes activation of the non–insulin-dependent glucose transporter and expression of erythropoietin and vascular endothelial growth factor (VEGF) (46–49). However, tissue-ischemia- or hypoxia-triggered pathways involving Egr-1–mediated expression of tissue factor (TF) and deposition of fibrin in the oxygen-deprived vasculature occurred in an HIF-1–independent manner (10,11). Hypoxia or ischemia/reperfusion leads to the generation of fibrin deposition in the lung (10,12–14), which is a critical underlying mechanism in the induction of TF. Analysis of the promoter of the gene encoding TF indicated the presence of binding elements for the transcription factor Egr-1 (53,54). We found that in cultured macrophages and HeLa cells, induction of hypoxia triggered increased transcription, translation, and nuclear translocation of Egr-1 (10,11). When homozygous Egr-1–null mice were subjected to hypoxia or ischemia/reperfusion, they displayed decreased up-regulation of TF and fibrin deposition in the lung compared with wild-type mice (10,13). These data supported the essential role of Egr-1 in mediating hypoxia- or ischemia/reperfusion-stimulated transcription of TF.

To begin to dissect the mechanisms underlying expression and activation of Egr-1 when oxygen levels decline, our studies delineated a previously unrecognized pathway involving rapid activation of PKCβII. This kinase initiates a signaling cascade that, via a series of steps including activation of raf, extracellular-signal-regulated protein kinase-1 and 2 (ERK1/2), and transcription factor Elk-1, leads to up-regulation of Egr-1, particularly in macrophages in the lung (12,14,55) and in vitro (11). In addition to phosphorylation of ERK1/2, PKCβ is also an early and key trigger to the activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein MAP kinase (p38 MAPK) that occurs in response to ischemia/reperfusion (14). Consistent with our findings, it has been reported that activation of JNK and/or p38 MAPK appeared in the heart or kidney exposed to ischemia/reperfusion and in cardiac myocytes subjected to hypoxia/reoxygenation (56–63). It is highly likely that the pattern and time course of ERK1/2, JNK, and p38 MAPK activation are tissue-specific (58,59,63,64). In addition, to precisely link PKCβ-dependent activation of these multifaceted signaling pathways to recruitment/stimulation of downstream targets in ischemia/reperfusion, we have provided the evidence that suppressed expression of Egr-1 in vitro in hypoxia/reoxygenation was achieved by preincubation of alveolar macrophages with inhibitors of PKCβ (LY379196) (31,32), ERK1/2 (PD98059) (65), and JNK (SP600125) (66) before exposure to hypoxia/reoxygenation, but not by an inhibitor of p38 MAPK (SB203580) (67). It is difficult to directly assess the impact of these kinases on downstream targets in vivo because of

the lack of orally administered inhibitors or ready-made chow containing such inhibitors of these 3 MAPKs (ERK1/2, JNK, or p38). Furthermore, the activated principal isoform of PKC relevant to hypoxic or ischemia/reperfusion lung injury was specifically PKCβII, not PKCβI, PKCδ, PKC, or PKCα (12,14).

The critical dependence of PKCβ on mechanisms linked to Egr-1 and procoagulant sequelae in hypoxia or ischemia/reperfusion was demonstrated by suppression of hypoxia- or ischemia/reperfusion-stimulated transcription, nuclear translocation, and translation of Egr-1 in PKCβ-null mice versus wild-type controls (12,14). In-depth studies revealed that, in addition to TF, activation of PKCβ and Egr-1 in ischemia/reperfusion led to up-regulation of a diverse class of proinflammatory cytokines (interleukin 1β), chemokines (macrophage inflammatory protein-2 [MIP-2], monocyte chemotactic protein 1 [MCP-1]), ICAM-1, VEGF, and procoagulant molecule plasminogen activator inhibitor-1 (PAI-1) (13,14). These initial events are well correlated with subsequent inflammation and thrombosis, which is associated with the development of vascular diseases. In contrast, Egr-1–null and PKCβ-null mice did not display enhanced expression of these proinflammatory and prothrombotic genes in hypoxia or ischemia/reperfusion. Moreover, in concordance with the central role for PKCβ and Egr-1 in ischemic stress, Egr-1–null and PKCβ-null mice were virtually protected from the deleterious impact of ischemia/reperfusion injury in the lung with enhanced animal survival and organ function (13,14).

In addition to mice deficient in PKCβ, pharmacologic inhibition of PKCβ provides an additional means to suppress the effects of PKCβ in vivo. Previous studies by others have employed the PKCβ inhibitor ruboxistaurin in a porcine model of ischemia-induced pre-retinal neovascularization (68) and in diabetic rats (69,70) to ameliorate, at least in part, early retinal and renal dysfunction (71). Our studies have provided the first evidence that administration of ruboxistaurin before ischemia/reperfusion suppressed up-regulation of Egr-1 transcripts in lung upon ischemia/reperfusion and demonstrated that pharmacologic blockade of PKCβ confers striking protection by enhancing animal survival from ischemia/reperfusion injury (14).

Based on the highly significant impact of PKCβ/Egr-1 in mediating lung injury after ischemia/reperfusion, we performed preliminary experiments in the heart. Compared with wild-type mice, PKCβ-null mice or wild-type mice treated with ruboxistaurin displayed decreased infarct volume after transient occlusion/reperfusion of the left anterior descending coronary artery (72).

Taken together, these findings delineate novel roles for PKCβ/Egr-1 axis in hypoxia or ischemia/reperfusion injury. The observation that activation of PKCβ-dependent signaling regulates recruitment of proinflammatory and prothrombotic mechanisms highlights the importance of blockade of the PKCβ/Egr-1 pathway by employing the PKCβ inhibitor ruboxistaurin for the prevention of organ dysfunc-

tion and damage in disorders characterized by hypoxia or ischemia/reperfusion injury.

PKCβ/EGR-1 AXIS AND ATHEROSCLEROSIS

Atherosclerosis, the cause of ischemic heart disease, myocardial infarction, stroke, and peripheral arterial disease, is characterized by chronic inflammation in the artery wall (73–76). Because accumulation of modified lipoproteins occurs in atherosclerosis, the mechanism by which lipoproteins or modified lipoproteins modulate signaling pathways and gene transcription and expression in different cell types has been the subject of intense recent investigation. To test the potential impact of modified lipoproteins on Egr-1 expression, our data have shown that oxidized lipoproteins such as oxidized low-density lipoprotein (oxLDL) induce expression of Egr-1 in RAW264.7 mouse macrophages in a dose-dependent manner (21). These findings suggest that, in early atherosclerosis, oxLDLs use a range of molecular pathways to initiate vascular perturbation.

We tested the impact of oxLDL on activation of MAP kinases, because these pathways have been linked to regulation of Egr-1. Oxidized low-density lipoprotein activated phospho-p44/42 (ERK1/2) MAP kinase in mouse macrophages with peak effect observed at 15 to 30 min (21). To determine if the mitogen-activated protein kinase (MEK)-ERK1/2 MAP kinase pathway was linked to oxLDL-mediated regulation of Egr-1, RAW cells were pre-treated with PD98059 (10 µmol/l) before incubation with oxLDL. Compared with cells treated with oxLDL alone, RAW mouse macrophages exposed to PD98059 and oxLDL displayed a significantly reduced expression of Egr-1 antigen by Western blotting (by 75%) (21). These data were the first to show that the MEK-ERK1/2 MAP kinase pathway importantly contributes to oxLDL-mediated induction of Egr-1 expression in macrophages, cells importantly involved in atherogenesis and lesion progression.

Pivotal studies in the discovery of the biological impact of Egr-1 in atherosclerosis came from experiments by the laboratories of McCaffrey et al. (19), who showed that transcripts for Egr-1 were up-regulated in human atherosclerotic lesions and in the lesions of mice deficient in the LDL receptor fed a high-fat diet. Moreover, increased Egr-1 expression in the human lesion was associated with an elevation in the expression of several known Egr-1 target genes, such as TNF, ICAM-1, and M-CSF, suggesting that Egr-1 is transcriptionally active in human atheroma (19). Immunohistochemistry localized Egr-1 largely to SMC, macrophages, and to a lesser extent, endothelial cells within the lesions (19). In a murine model of mice deficient in the LDL-receptor, upon introduction of a high-fat diet, a progressive increase in expression of Egr-1 was noted in the aorta, especially within SMC (19). Other investigators reported that Egr-1 is expressed primarily in SMC in the fibrous cap, as well as in the areas of macrophage infiltration and in endothelial cells (20). These observations led us to

hypothesize that activation of Egr-1 might be implicated in the initiation and/or progression of atherosclerosis, which represents the convergence of a range of complex processes in multiple cells, such as, but not limited to, endothelial cells, vascular SMC, macrophages, and lymphocytes. Consistent with this hypothesis, quantitative polymerase chain reaction (PCR) revealed an age-dependent increase in transcripts for Egr-1 in the aorta of apolipoprotein E (apoE)-null mice versus wild-type C57BL/6 control mice (21). Immunohistochemistry revealed that the principal Egr-1–expressing cells in the atherosclerotic lesions of apoE-null mice were macrophages (as demonstrated by colocalization of Egr-1 with F4/80 epitopes) and vascular SMC (as demonstrated by colocalization of Egr-1 with alpha-smooth muscle actin) (21). These data suggested that, in the hyperlipidemic environment triggered by genetic deletion of apoE, a steady increase in Egr-1 potentially reflects a response to vascular accumulation of modified lipoproteins.

Based on the observations of others implicating roles for Egr-1 in modulation of vascular properties linked to atherosclerosis, it has been shown that Egr-1 contributes, in part, to CD40-ligand-induced expression of TF in human endothelial cells (77). Other studies suggest a potential role for Egr-1 in transcriptional activation of peroxisome proliferator-activated receptor gamma 1 in vascular SMC (78); elevated Egr-1 in human atherosclerotic cells transcriptionally represses TGF type II receptor, thus providing a mechanism to suppress vascular repair pathways (79). Oxidized phospholipids such as oxidized L-alpha-palmitoyl-2-arachidonoyl-sn-glycerol-3-phosphorylcholin induce expression of Egr-1 in human umbilical vein endothelial cells (80), and infectious agents (*Chlamydia pneumoniae*) linked to acceleration of atherosclerosis up-regulate expression of Egr-1 (and, in turn, TF) in macrophages (81,82).

To establish cause-effect relationships between Egr-1 and atherogenesis, we tested the hypothesis that genetic deletion of Egr-1 in the apoE-null background would modify the course of atherosclerosis. We thus generated mice deficient in both Egr-1 and apoE and tested the impact on atherosclerosis. Compared with mice solely deficient in apo E, mice deficient in both apo E and Egr-1 displayed a striking reduction in the atherosclerotic lesion area and complexity at age 24 weeks on a normal chow diet (21). The distinctions between double- and single-null animals at age 14 weeks were also significant. In parallel, quantitative PCR revealed that the transcripts for key mediators of inflammation and the procoagulant response, the murine homologue of monocyte chemoattractant protein 1 (JE/MCP-1), IL-1β, TF, PAI-1, vascular cell adhesion molecule-1, and ICAM-1 were reduced in the aorta of doubly apoE- and Egr-1-null mice versus apoE-null mice at age 24 weeks (21). Importantly, levels of plasma cholesterol and triglycerides did not differ between mice doubly deficient in Egr-1 and apoE compared with apoE-null mice (21).

Furthermore, to test the hypothesis that PKCβ is a central upstream regulator of Egr-1 in atherogenesis in a hyperlipidemic background, we bred PKCβ-null mice into apoE-null background and tested the impact on atherosclerosis. Our preliminary data showed that mice lacking both apoE and PKCβ displayed significantly decreased atherosclerosis compared with apoE-null mice (39,40). Further, apoE-null mice fed chow containing the PKCβ inhibitor, ruboxistaurin, displayed significantly decreased atherosclerosis compared with the mice fed chow containing vehicle as a control (40).

Taken together, these findings provide definitive mechanistic support for the link between PKCβ/Egr-1 axis and the pathogenesis of atherosclerosis and suggest important implications of blockade of this pathway by employing the PKCβ inhibitor ruboxistaurin for the management of this disorder.

PKCβ/EGR-1 AXIS AND RESTENOSIS

Restenosis remains a challenge after angioplasty and stenting (83–93), especially in subsets of human subjects, such as diabetic patients. In addition to paclitaxel or sirolimus-coated stents and glycoprotein IIb/IIIa inhibitors (94–97), efforts to identify new and powerful adjunctive therapies will accelerate effective therapeutic intervention strategies, particularly in diabetic subjects. In this context, studies from our laboratory provided insights into a likely mechanism by which Egr-1 was up-regulated and its key upstream regulator, PKCβ, activated in the response to acute arterial injury.

Key evidence for the inducibility of Egr-1 under stress conditions of acute arterial injury first emerged from studies in denuding arterial injury in the rat aorta. Early growth response-1 and a number of its target genes were induced at the wound margins (22,23). The first in-vivo evidence of a specific role for Egr-1, and not only an association, in the response to arterial injury came from experiments using a DNA enzyme that specifically cleaves Egr-1 mRNA. Application of this technology in rats subjected to carotid artery injury blocked neointima formation (24). The recent successful application of this strategy in preventing in-stent restenosis in a porcine model (98) suggested that this factor might have an impact on restenosis in human subjects (99,100). However, these studies did not elucidate the pathway by which Egr-1 is up-regulated after vascular injury, nor have they highlighted the downstream implications of this axis on vascular repair.

Our previous observations of the striking up-regulation of Egr-1 in vascular SMC in atherosclerosis led us to test the hypothesis that the PKCβ/Egr-1 pathway might be involved in the vascular response to arterial injury, as neointimal expansion commonly accompanies both chronic vascular stress (atherosclerosis) and the response to acute arterial injury, such as that induced by angioplasty. In a murine model of acute endothelial denudation of the fem-

oral artery, a time-dependent increase in transcripts for Egr-1 was observed by quantitative PCR (25). When Egr-1 null mice were subjected to acute femoral artery endothelial denudation injury, a significant decrease in neointimal expansion was observed on day 28 versus that observed in wild-type mice. Immunohistochemistry revealed that the principal Egr-1-expressing cells in the expanding neointima were vascular SMC, as demonstrated by colocalization of Egr-1 and alpha-smooth muscle actin (25).

Consistent with an important role for PKCβ in these processes, in wild-type mice, a time-dependent increase in activation of PKCβII manifested by antigen in the membranous fraction by approximately 7.5-fold was observed from injured femoral artery compared with sham control, with the peak occurring at 30 min after acute denuding injury (25). In contrast, immunoblotting with an antibody specific to the PKCβI isoform showed no change between membranous fractions in injured versus sham-treated vessel segments. By 30 min after denudation, no changes in PKCα, PKCδ, and PKCι isoforms were detected in the membranous fractions in injured wild-type and PKCβ-null mice versus sham (25). Based on these considerations, we subjected homozygous PKCβ-null mice to acute arterial injury. Compared with wild-type mice, PKCβ-null animals displayed significantly lower intima/media ratio on day 28 after injury (25). In parallel, transcripts for Egr-1 were dramatically reduced in the injured femoral arteries of PKCβ-null versus wild-type mice (25). The principal cells forming the expanding neointima in wild-type mice were SMCs identified by an antibody to alpha-smooth muscle actin (25).

To address the mechanisms by which PKCβ modulates neointimal expansion, we assessed the expanding neointima at an early time after injury, at which point SMC proliferation was previously found to be accelerated (101). Incorporation of bromodeoxyuridine (BrdU) was significantly decreased in SMCs of the expanding neointima in PKCβ-null versus wild-type mice on day 7 after acute injury. In vitro, we examined the impact of PKCβ on 2 central functional properties of SMCs, proliferation and migration, using a prototypic stimulus for PKCβ, phorbol myristate acetate (PMA), and the PKCβ inhibitor LY379196. PMA-triggered incorporation of titrated thymidine was suppressed in human and murine aortic vascular smooth muscle cells by LY379196 (25). Further, we studied the role of PKCβ in mediating cellular migration, a key property of SMC in the expanding neointima after injury. PMA-triggered prominently increased numbers of migrating primary murine aortic SMC was significantly suppressed by LY379196; in parallel, in primary cultures of human and murine aortic SMC, stimulation with PMA-triggered increased expression of Egr-1 in a manner inhibited by the PKCβ inhibitor LY379196 (25). These findings established that Egr-1 was a downstream target of PKCβ in acute arterial injury and that proliferation and migration of SMC were modulated, at least in part, via PKCβ.

To dissect the precise mechanisms by which the PKCβ/Egr-1 axis plays a key role in neointimal expansion after acute arterial injury, we studied signal transduction pathways, including MAPK pathway, especially ERK1/2, JNK, Janus kinase (Jak) 2, and signal transducer and activator of transcription (Stat) 3, in the response to arterial injury in SMC (102,103). Our data revealed that markedly increased phosphorylated ERK1/2 and phospho-JNK in the injured arterial segments from wild-type mice were significantly suppressed in PKCβ-null mice. However, there were no significant differences in elevated phospho-Jak2 or phospho-Stat3 between wild-type and PKCβ-null mice on day 7 after injury versus sham-treated mice (25). In addition, to precisely link PKCβ-dependent activation of MAP kinase signaling pathways to recruitment/stimulation of downstream targets after acute arterial injury, we dissected these pathways in vitro using primary cultures of murine aortic SMC. Our studies demonstrated that PMA triggered increased phosphorylation of ERK1/2, and JNK was suppressed by PKCβ inhibitor LY379196. In parallel, PMA triggered a significant increase in expression of Egr-1 transcripts in murine aortic SMC in a manner suppressed by the ERK1/2 inhibitor PD98059, but not by the JNK inhibitor SP600125 (25). These findings suggested that PKCβ-mediated regulation of Egr-1 was due, at least in part, to phosphorylation of ERK1/2, but not via phosphorylation of JNK. Moreover, we examined the impact of PKCβ on proliferation of murine aortic SMC. Although PMA caused a significant increase in titrated thymidine incorporation in wild-type SMC, pre-treatment with PD98059 strikingly suppressed this effect, whereas SP600125 caused a statistically significant attenuation in proliferation, albeit to a degree less than that observed by blockade of ERK1/2 MAP kinase in murine aortic SMC (25).

Furthermore, we used pharmacologic inhibition of PKCβ to suppress its effects in vivo. In wild-type mice subjected to femoral artery injury, administration of ruboxistaurin resulted in decreased incorporation of BrdU on day 7 after injury and decreased neointimal expansion on day 28 after injury compared with vehicle-treated control mice (25).

Taken together, these findings highlight novel roles for PKCβ/Egr-1 axis in the SMC response to acute vascular injury and the development of pathological neointimal expansion and suggest that blockade of the PKCβ/Egr-1 pathway by employing the PKCβ inhibitor ruboxistaurin may have an important benefit in management of complications of angioplasty and stenting in human subjects.

EGR-1 AND PKCβII AND VASCULAR INJURY: UNIFYING HYPOTHESES

Taken together, these data support the premise that PKCβII and a key downstream target in the vasculature,

Egr-1, respond acutely to vascular stress. Thus, although seemingly disparate, atherosclerosis, restenosis, and ischemia/reperfusion injury appear to be linked by common mechanisms that, by activation of endothelium, SMC, and inflammatory cells contribute critically to vascular injury in these settings. Future studies must dissect the common, or perhaps in part distinct biochemical, species that activate PKC*β*II in the perturbed vessel wall. Interestingly, the acuteness (ischemia/reperfusion injury and restenosis) or chronicity (atherosclerosis) of the triggering stimulus does not discriminate in terms of ability to recruit PKC*β*II and downstream, Egr-1. In this context, clues may be deduced, at least in part, from the biochemical species generated in the hyperglycemic environment that activate PKC*β*II, such as DAG. How this or distinct species are generated that signal via PKC*β*II in atherosclerosis, restenosis, or ischemia/reperfusion holds the key to understanding the fascinating biology of this axis.

In the meantime, however, valuable insights into PKC*β*II as a therapeutic target in diabetes and vascular stress have been uncovered by the development and pre-clinical and clinical testing of ruboxistaurin. In the section to follow, we conclude with a review of the major published studies on this agent.

PKC*β* AND CLINICAL IMPLICATIONS

Ruboxistaurin mesylate is a bisindolylmaleimide that shows a high degree of specificity for inhibiting PKC*β* isoform (104). It is currently undergoing phase 3 clinical trials. A growing body of evidence from animal and human studies indicates that inhibition of PKC*β* may have renal, retinal and vascular protective effects.

Pre-clinical studies of the impact of ruboxistaurin on progression of diabetic nephropathy have been completed in 3 animal models of diabetic nephropathy: 1) the streptozotocin (STZ) rat resembles type 1 diabetes mellitus in humans; 2) Lepr^{db}/Lepr^{db} (formerly known as the db/db) mouse is a genetically obese mouse model, resembling type 2 diabetes mellitus in humans; and 3) the STZ-Ren2-rat is a hypertensive rat model made diabetic with STZ. In these diabetic animal models, treatment with ruboxistaurin normalized glomerular hyperfiltration, decreased urinary albumin excretion, and reduced glomerular TGF*β*1 levels and extracellular matrix protein production, thus reducing mesangial expansion, glomerulosclerosis, tubulointerstitial fibrosis, and loss of renal function (105).

Diabetes also induces early functional abnormalities in various vascular beds. Mean retinal circulation time and
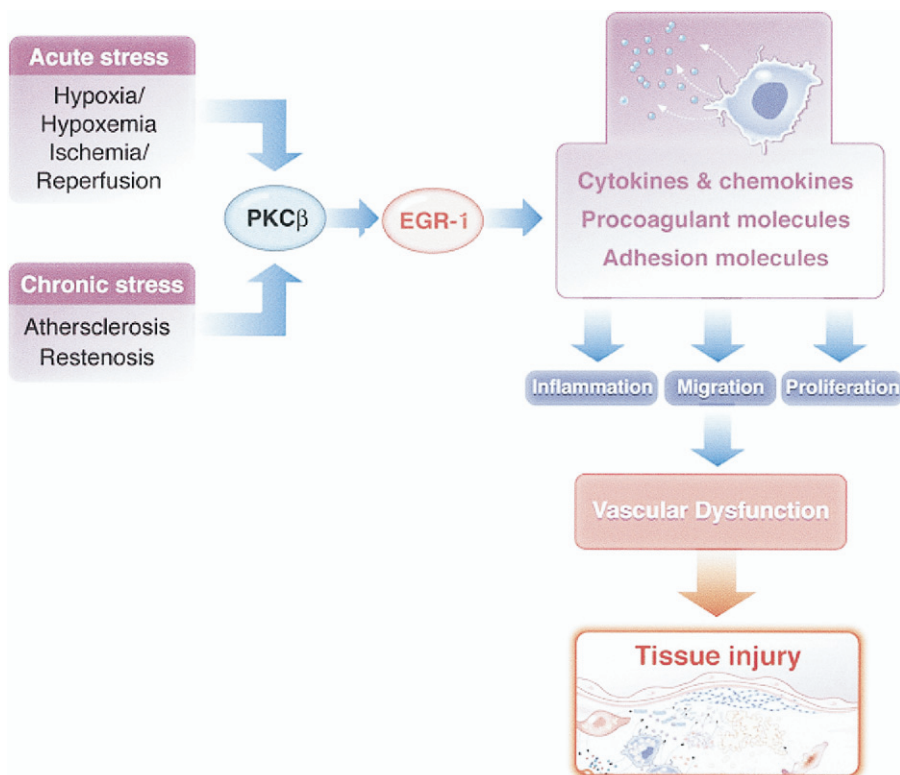

Figure 1. Protein kinase C*β*-early growth response-1 (PKC*β*-Egr-1): implications for vascular stress. In this review, we provided the experimental evidence linking PKC*β* and Egr-1 to the pathogenesis of tissue injury. Studies support the premise that both acute stresses, such as that induced by hypoxia/hypoxemia and ischemia/reperfusion, as well as chronic stresses, such as that which occurs in atherosclerosis and restenosis, activate PKC, particularly the *β* isoform. PKC*β*-mediated up-regulation of Egr-1 results in expression of cytokines, chemokines, procoagulant, and adhesion molecules; processes that lead to amplification of vascular inflammation, migration, and proliferation. Once set in motion, such processes, dependent on PKC*β*-Egr-1, lead to chronic vascular dysfunction and, ultimately, if left unchecked, tissue injury. Novel inhibitors of PKC*β* in clinical trials may hold promise for the treatment of these acute and chronic vascular diseases.

glomerular filtration rate (GFR) were increased in rats rendered diabetic for 2 weeks by STZ (69). Oral administration of ruboxistaurin in the chow (1 to 10 mg/kg/day for a 4-week period) normalized retinal blood flow and reduced GFR in diabetic rats (69). In a porcine model, ruboxistaurin reduced ischemia-induced pre-retinal neovascularization (68). A similar normalization of retinal blood flow and amelioration of diabetes-induced retinal hemodynamic abnormalities were observed in patients with diabetes who had been treated for 28 days with 32 mg (16 mg twice a day) of ruboxistaurin compared with those who received placebo (106,107).

The development of cardiovascular disease and nephropathy are closely linked in diabetes. However, studies of the general population have shown that increased risk for cardiovascular disease is associated with hyperglycemia regardless of whether overt diabetes has been diagnosed previously (108). Hyperglycemia-induced DAG preferentially activates PKCβ and also is associated with changes in endothelial cell function that precedes the development of atherosclerosis. The evidence suggested that hyperglycemia impairs endothelial function by a process dependent on PKCβ activation, and this impairment can be prevented by treatment with ruboxistaurin (109).

Taken together, to date, blockade of the PKCβ signaling pathway by employing the PKCβ inhibitor ruboxistaurin in diabetic animal models and clinical trials in patients with diabetes holds promise as a novel strategy in the treatment of diabetes-related microvascular complications such as eye disease and kidney disease. Our findings in euglycemia support testing this agent in non-diabetic vascular stress.

CONCLUSIONS

These data provide the first evidence that key species and stresses implicated in vascular injury, such as modified lipoproteins, glucose and DAG, acute physical stress, and hypoxia and ischemia/reperfusion transduce their key pathogenic effects, at least in part, via rapid and, in certain settings, chronic recruitment of the PKCβ/Egr-1 axis (Fig. 1). This work raises the possibility that blockade of the PKCβ/Egr-1 axis by a pharmacologic inhibitor of PKCβ, ruboxistaurin, may attenuate neointimal expansion or organ dysfunction and damage triggered by acute mechanical injury, chronic atherosclerosis, or ischemia-reperfusion stress. In addition, based on the bioavailability and tolerability of ruboxistaurin in diabetes and our findings in euglycemia, we speculate that blockade of PKCβ signaling pathway may hold promise as a therapeutic intervention in treating macrovascular disease involving the heart and large vessels in both diabetes and non-diabetes, although this remains to be proven in clinical trials.

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APPENDIX

For a list of abbreviations used in this report, please see the online version of this article.