PKCβ Promotes Vascular Inflammation and Acceleration of Atherosclerosis in Diabetic ApoE Null Mice

Linghua Kong, Xiaoping Shen, Lili Lin, Michael Leitges, Rosa Rosario, Yu Shan Zou, Shi Fang Yan

Objective—Subjects with diabetes mellitus are at high risk for developing atherosclerosis through a variety of mechanisms. Because the metabolism of glucose results in production of activators of protein kinase C (PKC)β, it was logical to investigate the role of PKCβ in modulation of atherosclerosis in diabetes mellitus.

Approach and Results—ApoE−/− and PKCβ−/−/ApoE−/− mice were rendered diabetic with streptozotocin. Quantification of atherosclerosis, gene expression profiling, or analysis of signaling molecules was performed on aortic sinus or aortas from diabetic mice. Diabetes mellitus–accelerated atherosclerosis increased the level of phosphorylated extracellular signal-regulated kinase 1/2 and Jun-N-terminus kinase mitogen–activated protein kinases and augmented vascular expression of inflammatory mediators, as well as increased monocyte/macrophage infiltration and CD11c+ cells accumulation in diabetic ApoE−/− mice, processes that were diminished in diabetic PKCβ−/−/ApoE−/− mice. In addition, pharmacological inhibition of PKCβ reduced atherosclerotic lesion size in diabetic ApoE−/− mice. In vitro, the inhibitors of PKCβ and extracellular signal-regulated kinase 1/2, as well as small interfering RNA to Egr-1, significantly decreased high-glucose–induced expression of CD11c (integrin, alpha X 9complement component 3 receptor 4 subunit), chemokine (C-C motif) ligand 2, and interleukin-1β in U937 macrophages.

Conclusions—These data link enhanced activation of PKCβ to accelerated diabetic atherosclerosis via a mechanism that includes modulation of gene transcription and signal transduction in the vascular wall, processes that contribute to acceleration of vascular inflammation and atherosclerosis in diabetes mellitus. Our results uncover a novel role for PKCβ in modulating CD11c expression and inflammatory response of macrophages in the development of diabetic atherosclerosis. These findings support PKCβ activation as a potential therapeutic target for prevention and treatment of diabetic atherosclerosis. (Arterioscler Thromb Vasc Biol. 2013;33:1779-1787.)

Key Words: antigens, CD11c ■ atherosclerosis ■ diabetes mellitus ■ inflammation ■ PKCβ

Subjects with diabetes mellitus are at 2- to 6-fold greater risk of developing atherosclerosis than nondiabetic individuals,1,2 constituting the main cause of morbidity and mortality.3,4 In diabetes mellitus, an emerging view is that events leading to accelerated atherosclerosis in affected subjects are underway before the diagnosis of the disease.5 Thus, an intensive search for new therapeutic targets in diabetic atherosclerosis is needed. These considerations led us to investigate molecular mechanisms by which diabetes mellitus accelerates atherosclerosis.

See accompanying article on page 1787

The ubiquitous enzyme protein kinase C (PKC), a family of ≥12 isoforms of serine and threonine kinases, has been linked to the pathogenesis of vascular injury. Although several PKC isoforms are expressed in vascular tissue, in the rodent model of diabetes mellitus, there is a preferential activation of PKCβII in the aorta and heart.6 Increasing evidence links hyperglycemia to diabetic microvascular complications via activation of PKCβ,7-9 but PKCβ-dependent mechanisms have yet to be elucidated in macrovascular complications of diabetes mellitus. We previously reported that PKCβ−/− ApoE−/− mice displayed less atherosclerosis than ApoE−/− mice and demonstrated links between activation of PKCβ and nondiabetic atherosclerosis.10 In parallel, our previous findings revealed that activated PKCβ, especially βII isoform, is a critical upstream regulator of early growth response 1 (Egr-1) in response to vascular stress, such as hypoxia,11,12 ischemia/reperfusion,13 and nondiabetic atherosclerosis.10 Egr-1, in turn, acts as a master switch coordinating expression of proinflammatory cytokines, chemokines, procoagulant molecules, cell adhesion molecules, and matrix metalloproteinase-2.10,14,15 However, macrovascular disease in diabetes mellitus is manifested by more accelerated and progressive atherosclerosis, which is more widely distributed. Because the metabolism of glucose results in production of activators of PKCβ, it was
logical to investigate the role of PKCβ in modulation of atherosclerosis in diabetes mellitus.

In this study, we hypothesized that in diabetes mellitus, enhanced activation of PKCβ is critical to augmented expression of a broad range of inflammatory mediators and increased phosphorylation of signaling molecules in the diabetic vascular wall, processes that contribute to accelerated atherosclerotic lesion initiation and progression in diabetes mellitus. Here, we tested these concepts in homozygous ApoE−/− and PKCβ−/−/ApoE−/− mice made diabetic with streptozotocin (stz). In addition, we tested the effects of a PKCβ inhibitor, ruboxistaurin, on atherosclerosis in diabetic ApoE−/− mice. Furthermore, in vitro, we tested the effects of small interfering RNA to Egr-1 or inhibitors of PKCβ, extracellular signal-regulated kinase 1/2 (ERK1/2), Jun-N-terminal kinase (JNK), and p38 mitogen-activated protein (MAP) kinase on high-glucose–induced pro-inflammatory gene expression in U937 macrophages.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Enhanced Activation of PKCβII in Aorta of Diabetic ApoE−/− Mice**

To dissect the contribution of PKCβ in the pathogenesis of diabetes mellitus–accelerated atherosclerosis, we first assessed the extent of activation of the PKCβII isoform in the aortas of diabetic ApoE−/− mice at age 10 weeks, a time point before the development of significant atherosclerosis. Compared with nondiabetic ApoE−/− mice, diabetic ApoE−/− mice displayed higher levels of phosphor-PKCβII in membranous fractions versus nondiabetic ApoE−/− mice (Figure 1; *P<0.0001*), although nondiabetic ApoE−/− mice showed an increase in phosphor-PKCβII in membranous fractions versus wild-type mice (Figure 1; *P<0.0001*). In contrast, no change in phosphor-PKCβ was detected in the membranous fraction from wild-type, nondiabetic ApoE−/−, and diabetic ApoE−/− mice. Loading controls using anti–β-actin IgG demonstrated identical protein loading (Figure 1). These observations indicated enhanced activation of PKCβII in diabetic ApoE−/− mice.

**Effects of PKCβ Deletion on Accelerated Atherosclerosis in Diabetic ApoE−/− Mice**

Mice were rendered diabetic at age 6 weeks and fed normal rodent chow. At age 14 weeks, mean atherosclerotic lesion area at the aortic sinus was 34362.17±7114.49 μm² (n=13) in nondiabetic ApoE−/− mice. Diabetes mellitus, as expected, led to an ≈1.91-fold increase in lesion area in diabetic ApoE−/− mice (65585.5±4345.13 μm²; n=14) compared with nondiabetic ApoE−/− mice; *P<0.001* (Figure 2A). In contrast, atherosclerotic lesion area at the aortic sinus was ≈38% lower in diabetic PKCβ−/−/ApoE−/− (40611.10±4767.72 μm²; n=15) compared with that in diabetic ApoE−/− mice; *P<0.001* (Figure 2A). Diabetic PKCβ−/−/ApoE−/− animals displayed no atherosclerotic lesions (not shown). The effects of PKCβ on diabetic atherosclerosis were sustained beyond the earliest stages of lesion formation and accelerated by aging. Specifically, at age 20 weeks, similar results were observed; however, the differences between double-null and single-null ApoE−/− mice were even more striking: mean atherosclerotic lesion area was ≈55% lower in diabetic PKCβ−/−/ApoE−/− mice (99259.62±11138.17 μm²; n=13) versus diabetic ApoE−/− animals (220952.86±14653.50 μm²; n=14); *P<0.0001* (Figure 2B). Sudan IV stain for aortic en face area, at age 20 weeks, also showed greater lesion area in diabetic ApoE−/− mice compared with nondiabetic ApoE−/− mice (*P<0.05*; Figure 2C), and this was reduced in diabetic PKCβ−/−/ApoE−/− mice compared with diabetic ApoE−/− animals (*P<0.05*; Figure 2C). In parallel with decreased atherosclerotic lesion area, the lesion complexity index also was significantly lower in diabetic PKCβ−/−/ApoE−/− mice versus diabetic ApoE−/− mice at age 20 weeks (0.562±0.05 versus 0.966±0.006; *P<0.0001*; Figure 2D).

**Effects of PKCβ Inhibition on Atherosclerosis in Diabetic ApoE−/− Mice**

If PKCβ activation contributed to accelerated atherosclerosis development, its inhibition should reduce lesions. We tested a selective inhibitor of this enzyme, ruboxistaurin. When ApoE−/− male mice expressing PKCβ were treated with ruboxistaurin from age 5 to 20 weeks, mean atherosclerotic lesion area at the aortic sinus was ≈59% lower in diabetic ApoE−/− male mice fed ruboxistaurin (106682.5±13626.72 μm²; n=13) versus diabetic ApoE−/− male mice fed vehicle chow (260267.86±22926.95 μm²; n=14; *P<0.0001*; Figure 3A). In parallel, the complexity index was significantly lower in diabetic ApoE−/− mice fed ruboxistaurin versus vehicle (0.487±0.052 versus 0.978±0.009; *P<0.0001*; Figure 3B).
Effects of PKCβ Deletion/Inhibition on Glycemia and Lipid in Diabetic ApoE−/− Mice

We examined factors that might account for the beneficial effects of PKCβ deletion or inhibition. Diabetic mice displayed a significantly higher plasma glucose level, cholesterol, and triglyceride than nondiabetic mice (Table I in the online-only Data Supplement). Importantly, no statistically significant differences were observed in levels of plasma glucose, cholesterol, and triglyceride in diabetic PKCβ−/−/ApoE−/− animals versus diabetic ApoE−/− mice or in diabetic ApoE−/− mice fed ruboxistaurin versus vehicle chow at age 14 weeks (data not shown) and at age 20 weeks (Table I in the online-only Data Supplement). Thus, the impact of deletion or blockade of PKCβ on accelerated atherosclerosis in diabetes mellitus was independent of modulation of other key risk factors for atherosclerosis.

Effects of PKCβ Deletion on Vascular Expression of Inflammatory Mediators and Activation of MAP Kinase in Diabetic ApoE−/− Mice

We sought to identify the specific mechanisms by which PKCβ contributed to early atherogenesis in diabetic ApoE−/− mice. Aortic RNA was prepared from nondiabetic and diabetic ApoE−/− and PKCβ−/−/ApoE−/− mice at age 10 weeks, a time point before atherosclerotic lesion development. The RNA samples were subjected to pathway-focused gene expression profiling using Mouse Atherosclerosis RT2 Profiler PCR Array. Our data revealed a progressive increase in expression of 14 of 84 stress-responsive genes (Table II in the online-only Data Supplement), including chemokines (Ccl2, Ccr1, and Ccr2), cytokines (IL1α, IL1b, IL1r2, and Spp1), transmembrane proteins (Itgαx and Itgb2), Matrix metallopeptidase (Mmp3), signal transducer (Msr1), and adhesion molecules (Sele, Sell, Selp, and Selplg) in diabetic ApoE−/− mice. In contrast, those gene transcripts were dramatically reduced in diabetic PKCβ−/−/ApoE−/− mice.

Next, to further validate a progressive increase in gene expression in diabetic ApoE−/− mice, primers were chosen for real-time polymerase chain reaction analysis if upregulation of the gene was ≥2-fold higher in aortas of diabetic ApoE−/− mice compared with that of nondiabetic ApoE−/− mice at age 10 weeks or ≤2-fold lower in aortas of diabetic PKCβ−/−/ApoE−/− mice compared with that of diabetic ApoE−/− mice. As indicated in Figure 4A, all of the above gene expression changes in aortas of diabetic ApoE−/− mice were verified; there was a significant increase in each of these genes in diabetic ApoE−/− aortas (P≤0.05, *P≤0.01, and ^P≤0.001) compared with nondiabetic ApoE−/− mice at age 10 weeks, whereas these
gene expression levels in diabetic aortas of PKCβ−/−/ApoE−/− mice were not significantly different compared with that of nondiabetic ApoE−/− mice at age 10 weeks. Furthermore, as indicated in Figure 4A, quantitative real-time polymerase chain reaction confirmed that the progressive increase in gene expression of those 14 inflammatory mediators in diabetic ApoE−/− mice was significantly reduced in diabetic PKCβ−/−/ApoE−/− mice (##P≤0.001, **P≤0.01, and ^^^P≤0.001). In contrast, as indicated in Figure 4B, some representative genes, such as Ace, Itga2, and PdgFrb, were not altered in diabetic mice and not affected by PKCβ deficiency.

In addition, we determined whether PKCβ modulated the expression of Egr-1 and activation of signaling mechanisms, such as ERK1/2 and JNK MAP kinases in the vessel wall of diabetic ApoE−/− mice at age 10 weeks. Real-time polymerase chain reaction revealed that transcripts for Egr-1 were significantly decreased in aortas of diabetic PKCβ−/−/ApoE−/− mice compared with nondiabetic ApoE−/− mice (P<0.01; Figure 4C). In contrast, transcripts for Egr-1 were significantly decreased in aortas of diabetic PKCβ−/−/ApoE−/− mice versus diabetic ApoE−/− mice (P<0.01; Figure 4C). Immunoblots displayed that Phosphor-ERK1/2 (P-ERK1/2; P<0.01; Figure 4D) and phosphor-JNK (P-JNK; P<0.01; Figure 4E) were significantly lower in the aortas of diabetic PKCβ−/−/ApoE−/− mice versus diabetic ApoE−/− mice. Loading controls using anti-total ERK1/2 (T-ERK) and anti-total JNK (T-JNK) IgG demonstrated identical protein loading (Figure 4D and 4E).

**Effects of PKCβ Deletion on Inflammatory-Appearing Lesions in Diabetic ApoE−/− Mice**

On the basis of the above findings that the increased expression of integrin, alpha X complement component 3 receptor 4 subunit (Itgax) transcripts was dependent on PKCβ activation in aortas of diabetic ApoE−/− mice, we sought to determine whether PKCβ-dependent Itgax expression contributes to inflammatory-appearing lesions in the development of diabetic atherosclerosis. CD11c (ITGAX), encoding integrin alpha X chain protein, expressed on monocytes/macrophages and dendritic cells (DCs).19 Immunofluorescence staining with a macrophage marker, anti-monocyte + macrophage antibody (MOMA-2) IgG, showed that infiltration of macrophages in atherosclerotic lesions of aortic sinus was significantly lower in diabetic PKCβ−/−/ApoE−/− mice (Figure 5A) compared with diabetic ApoE−/− mice (Figure 5B) at age 20 weeks (P<0.01; Figure 5D). Next, colocalization of CD11c and MOMA-2 revealed that the immunoreactivity for CD11c was predominantly present in the macrophages in the atherosclerotic lesions of diabetic ApoE−/− mice at age 20 weeks (Figure 5B and 5C). However, CD11c-expressing cells were significantly lower in diabetic PKCβ−/−/ApoE−/− mice (Figure 5A) compared with diabetic ApoE−/− mice (Figure 5B) at age 20 weeks (P<0.01; Figure 5E). Isotype control immunohistochemistry with isotype-matched hamster IgG and rat IgG showed no staining (Figure 5A–5C). These data suggest an important role of PKCβ in modulating CD11c expression in macrophages, which potentially contributed to the development of diabetic atherosclerosis.

**Effects of High Glucose on PKCβ-Dependent Signaling Pathways in U937 Macrophages**

Stimulated by the above findings, to further dissect the molecular mechanisms by which PKCβ activation upregulated gene expression in monocytes/macrophages in settings characterized by high levels of glucose, we used human U937 macrophages as an in vitro model system. Real-time polymerase chain reaction analysis of total RNA from U937 cells exposed to high glucose demonstrated an ∼3.72-, 2.88-, 2.95-, and 3.68-fold increase in Egr-1, CD11c, chemokine (C-C motif) ligand 2 (CCL2), and interleukin (IL)-1β transcripts, respectively, compared with U937 cells cultured in low glucose (P<0.0001; Figure 6A–6D), in a manner significantly suppressed by the inhibition of PKCβ (LY379196, 30 nmol/L;
PKCβ and Diabetic Atherosclerosis

P<0.0001 or P<0.001; Figure 6A–6D) and ERK1/2 (U0126; 5 µmol/L; P<0.0001; Figure 6A–6D). However, high-glucose–induced CD11c expression in U937 cells was not suppressed by the inhibitors of JNK (SP600125; 20 µmol/L) and p38 (SB203550; 20 µmol/L; Figure 6B). High-glucose–induced Egr-1 expression in U937 cells was suppressed by the inhibitor of JNK (P<0.0001; Figure 6A), not by an inhibitor of p38 (Figure 6A), and high-glucose–induced increase in CCL2 and IL-1β expression was suppressed by the inhibitors of JNK and p38 (P<0.0001; Figure 6C and 6D).

Next, we tested whether Egr-1, a downstream target of ERK1/2 and PKCβ in vascular stress, is required for high-glucose–induced expression of CD11c, CCL2, and IL-1β in U937 macrophages. Introduction of small interfering RNA to knockdown Egr-1 expression in U937 cells was not suppressed by the inhibitors of JNK (SP600125; 20 µmol/L) and p38 (SB203550; 20 µmol/L; Figure 6B). High-glucose–induced Egr-1 expression in U937 cells was suppressed by the inhibitor of JNK (P<0.0001; Figure 6A), not by an inhibitor of p38 (Figure 6A), and high-glucose–induced increase in CCL2 and IL-1β expression was suppressed by the inhibitors of JNK and p38 (P<0.0001; Figure 6C and 6D).

Discussion

We report an important role for PKCβ in acceleration of initiation and progression of atherosclerosis in diabetic ApoE−/− mice. Activation of PKCβII, indicated by translocation from the cytosol to the plasma membrane, has been shown in aortas of nondiabetic ApoE−/− mice versus wild-type C57BL/6 animals in our previous studies.10 Our present data demonstrated a significant increase in phosphor-PKCβII in the membrane fractions from aortas of diabetic ApoE−/− mice versus nondiabetic ApoE−/− mice at age 10 weeks, a time point before the development of significant atherosclerosis. These findings suggest that enhanced activation of PKCβII in a hyperglycemic and hyperlipidemic environment may reflect a response of the blood vessel wall to superimposed biochemical and metabolic stresses. Consistent with important roles for PKCβ in accelerated atherosclerosis in diabetes mellitus, our findings demonstrated that genetic deletion or pharmacological inhibition of PKCβ in diabetic ApoE−/− mice significantly diminished atherosclerotic lesion areas and complexity indices, but did not affect the levels of glucose, cholesterol, and triglyceride. These findings suggest that modulation of diabetic atherosclerosis by PKCβ deletion or blockade was independent of changes in glucose and lipids. Because the traditional therapeutic approaches by glycemic control lacks clearly an established benefit in macrovascular disease, our findings may suggest important implications for blockade of PKCβ signaling pathway by using the PKCβ inhibitor ruboxistaurin for the management of diabetic atherosclerosis.

Figure 4. Impact of diabetes mellitus and protein kinase C (PKC)β on the expression of inflammatory mediators and activation of MAP kinase. Gene expression profiling was validated by real-time PCR in aortic RNA of diabetic (D) ApoE−/− mice vs nondiabetic (ND) ApoE−/− mice (n≥3; #P≤0.05, **P≤0.01, and ^^P≤0.001) or D PKCβ−/−/ApoE−/− mice (n≥3; #P≤0.05, **P≤0.01, and ^^P≤0.001) at age 10 weeks (A). Shown are some representative genes not altered in D ApoE−/− or D PKCβ−/−/ApoE−/− mice (B). Aortic RNA was subjected to real-time PCR for detection of Egr-1 RNA (C). Aortic protein was subjected to Western blot for detection of phosphor (P)-extracellular signal-regulated kinase 1/2 (ERK1/2) and total (T)-ERK (D), P-Jun-N-terminus kinase (JNK) and T-JNK (E).
Both elevated glucose and lipids contribute to increased inflammation in diabetes mellitus.20,21 We assessed the mechanisms by which PKCβ plays a crucial role in the accelerated initiation and progression of atherosclerotic lesion areas in diabetes mellitus. Our data demonstrated that increased expression of inflammatory mediators and activation of signaling MAP kinases, including proinflammatory transcription factor Egr-1, chemokines (Ccl2, Ccr1, and Ccr2), cytokines (IL-1α, IL-1β, IL-1γ, and Spp1), transmembrane proteins (Itgax and Itgb2), matrix metallopeptidase (Mmp3), signal transducer (Msr1), adhesion molecules (Sele, Sell, Selplg, and Spp1), in early atherogenesis in the aortas of diabetic ApoE−/− mice is dependent on the state of glycemia and the state of PKCβ expression because genetic deletion of PKCβ in diabetic ApoE−/− mice dramatically attenuated expression of those inflammatory mediators and signaling molecules. Our findings reveal a critical role for PKCβ-dependent upregulation of inflammatory mediators and signaling molecules in initiating steps of atherogenesis in the diabetic vascular wall, leading to enhanced progression of atherosclerotic complications.

Given the inflammatory basis of atherosclerosis, macrophages have been considered to be important immune effector cells, which contribute to and promote the development of atherosclerotic plaques.22–24 An in vitro study showed that macrophages, when treated with oxidatively modified lipids, differentiate into foam cells and in the process acquire expression of DC marker.25 One of the DC markers, CD11c, which is not an exclusive marker of DCs but can also be found on macrophages,19 has been implicated as a functional integrin in human and mouse models.26,27 Our data revealed that the increased CD11c+ cells in atherosclerotic lesions in diabetic ApoE−/− mice are, at least in part, macrophages based on colocalization with MOMA-2. In concurrence with these findings, the cholesterol-rich diet–fed Ldlr−/− mice displayed foam cells expressing DC marker CD11c.28 Also, high-fat diet–fed ApoE−/− mice displayed increased CD11c+ cells, which were colocalized with MOMA-2 in atherosclerotic lesions.24,29 Interestingly, we provide the first evidence that the increase in gene expression of Itgax (CD11c) in diabetic ApoE−/− mice expressing PKCβ was suppressed in diabetic ApoE−/− mice devoid of PKCβ. In parallel, decreased % macrophages/total lesion area and % CD11c+ cells/total lesion area were evident in diabetic PKCβ−/−/ApoE−/− mice versus diabetic ApoE−/− mice. In addition, we demonstrated that PKCβ regulated increased expression of Egr-1 and phosphorylation of ERK1/2 and JNK MAP kinases in early atherogenesis in diabetes mellitus. Consistent with these observations, high-glucose–induced increase in Egr-1 transcripts in U937 macrophages was blunted by the inhibitors of PKCβ, ERK1/2, and JNK in the response to hypoxia,11,12,32 ischemia,13 acute vascular injury,33,34 and in non diabetic atherosclerosis,10 our current data suggest that PKCβ mediates upregulation of Egr-1, at least in part, via increased phosphorylation of ERK1/2 and JNK in early atherogenesis in diabetes mellitus. Furthermore, because there are 2 putative Egr-1–binding motifs in the

Figure 5. Impact of protein kinase C (PKCβ) on the inflammatory cells in atherosclerotic lesions in diabetes mellitus. The sections of aortic root from diabetic (D) PKCβ−/−/ApoE−/− mice (A; 10× magnification; scale bar, 200 µm) and D ApoE−/− mice (B; 10× magnification; scale bar, 200 µm; and C; 40× magnification; scale bar, 50 µm) at age 20 weeks were stained with anti-monocyte + macrophage antibody (MOMA-2) and anti-CD11c, or isotype-matched antibodies. Nuclei were counterstained with DAPI. The merging of MOMA-2 and CD11c or merging of isotype-matched antibodies is shown. To determine the relative amounts of MOMA-2 positive macrophages (D) or CD11c-expressing cells (E) in an atherosclerotic lesion, the positive area of MOMA-2 or CD11c was divided by the total lesion area of atherosclerosis.
proximal region of the CD11c (p150,95) promoter, which may participate in the regulation of CD11c expression based on our TRANSFAC database search, we examined the effects of high-glucose–stimulated PKCβ-dependent signaling pathway on CD11 gene expression in U937 macrophages. The knockdown of Egr-1 expression by small interfering RNA or inhibition of PKCβ and ERK1/2, but not JNK and p38, resulted in attenuation of high-glucose–induced CD11c expression in U937 macrophages. These data suggest that PKCβ regulates CD11 expression in the inflammatory macrophages in atherosclerosis associated with diabetes mellitus, at least in part, via activation of Egr-1 and ERK1/2 signaling pathway. Studies are underway to further probe the CD11c promoter sites by electrophoretic mobility shift and supershift assays and the chromatin immunoprecipitation assays to establish functionality.

Consistent with our other observations, we showed ample evidence of the involvement of other inflammatory components in the inflammatory and pathological processes of atherosclerosis. One of the chemoattractants implicated in early atherogenesis is monocyte chemotactant protein-1 (MCP-1/CCL2), which binds to C-C chemokine receptor 2 (CCR2). MCP-1/CCL2 is highly expressed in atherosclerotic plaques and mediates macrophage recruitment in the atheromatous lesion. Given the importance of MCP-1/CCR2 pathway in the process of atherosclerosis, our findings may have important implications in suppression of MCP-1/CCR2 pathway because we demonstrated that genetic deletion of PKCβ attenuated upregulation of MCP-1/CCL2 and CCR2 in vascular tissues of diabetic ApoE−/− mice at age 10 weeks, a time point linked to an early stage of atherogenesis. Because increased CD11c expression in adipose tissue and blood in both mice and humans was found as a consequence of elevated MCP-1 levels secreted by adipose tissue, correlations or interactions between CD11c and MCP-1 will be tested in future studies in vascular inflammation in diabetic atherosclerosis. In addition, a large number of proinflammatory cytokines has been shown to be expressed in human atherosclerotic lesions, particularly in association with infiltrating monocytes and macrophages. IL-1 has been implicated as a regulatory protein in the development and clinical sequelae of atherosclerosis. IL-1β is produced by activated macrophages and considered a master switch of inflammation. IL-1β and its related parent IL-1α have both been implicated in the pathogenesis of atherosclerosis in previous studies. Engagement of CD11c β2 integrin by antibodies was reported to induce IL-1β production on primary human monocytes. The mechanisms instigating and perpetuating inflammatory responses have been studied extensively; however, less is known about the mechanisms governing the resolution of inflammation. We demonstrated that all of the above inflammatory mediators were modulated by vascular activation of PKCβ because they were dramatically...
attenuated in diabetic ApoE−/− mice devoid of PKCβ. Based on our previous findings that MCP-1/CCL2 and IL-1β have been demonstrated as downstream target genes of Egr-1 in ath erosclerosis in euglycemia,15 and on our current in vitro data that the knockdown of Egr-1 expression by small interfering RNA or inhibition of PKCβ, ERK1/2, JNK, and p38 led to attenuation of high-glucose–induced CCL2 and IL-1β expression in U937 macrophages, we suggest that PKCβ regulates increased expression of CCL2 and IL-1β, at least in part, via activation of ERK1/2/Egr-1 and JNK/Egr-1 or p38 signaling pathways in atherosclerosis in diabetes mellitus. Although the exact mechanisms by which PKCβ regulates these inflammatory mediators in the development of diabetic atherosclerosis are also the subject of ongoing investigation, our present study may shed light on the effectiveness of PKCβ blockade as a strategy to protect the stressed vascular system from irreversible injury caused by inflammatory response in human atherosclerotic disease, especially in diabetes mellitus.

Although a growing body of evidence from animal and human studies indicates beneficial effects of PKCβ inhibition on microvascular parameters,6,9,19,20 our studies have examined the effects of PKCβ inhibitor, ruboxistaurin, on macrovascular disease in diabetes mellitus. Our data provide the first evidence that pharmacological blockade of PKCβ by the PKCβ inhibitor, ruboxistaurin, significantly attenuated atherosclerotic lesion areas and complexity indices in diabetic ApoE−/− mice. This work raises the possibility that blockade of the PKCβ by a pharmacological inhibitor, ruboxistaurin, may attenuate neointimal expansion, vascular injury, or damage triggered by inflammatory and pathological processes during the development of atherosclerosis in diabetes mellitus.

In summary, these data link enhanced activation of PKCβ to diabetes mellitus–accelerated atherosclerosis via a mechanism that includes modulation of vascular expression of inflammatory mediators and activation of signaling MAP kinases involved in inflammatory responses, processes that contribute to acceleration of vascular inflammation and atherosclerosis in diabetes mellitus. We have uncovered a novel role for PKCβ in modulating CD11c expression and inflammatory responses of macrophages in the development of diabetic atherosclerosis. Thus, a better understanding of the inflammatory mechanisms by which PKCβ contributes to such processes in the vessel wall could be relevant to devise preventive and therapeutic strategies for diabetic atherosclerosis. In addition, based on our demonstration that pharmacological inhibition of PKCβ reduced lesion size, we suggest that these studies may provide the basis for potential therapeutic application of PKCβ antagonism (ruboxistaurin) to diabetic atherosclerosis.

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Disclosures

None.

References

Significance

Our findings show a critical role for protein kinase C (PKCβ)-dependent upregulation of inflammatory mediators and signaling molecules in initiating steps of atherogenesis in the diabetic vascular wall, leading to enhanced progression of atherosclerotic complication. Interestingly, our data provide the first evidence that PKCβ modulates CD11c expression and inflammatory responses of macrophages in the development of diabetic atherosclerosis. Thus, a better understanding of PKCβ-dependent inflammatory mechanisms in the vessel wall could be relevant to devise preventive and therapeutic strategies for diabetic atherosclerosis. Furthermore, our demonstration that pharmacological inhibition of PKCβ reduced lesion size may provide the basis for potential therapeutic application of PKCβ antagonism (ruboxistaurin) to diabetic atherosclerosis.
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**Supplement Material**

**Table I. Levels of plasma glucose, cholesterol and triglyceride**

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<th>Cholesterol n</th>
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<th>p</th>
<th>Triglyceride n</th>
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<td>D ApoE&lt;sup&gt;−/−&lt;/sup&gt; and VEH</td>
<td>12</td>
<td>403.00 ± 15.11</td>
<td>0.47</td>
<td>12</td>
<td>819.01 ± 75.42</td>
<td>0.63</td>
<td>12</td>
<td>179.91 ± 2.71</td>
<td>0.75</td>
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<tr>
<td>D ApoE&lt;sup&gt;−/−&lt;/sup&gt; and RBX</td>
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<td>13</td>
<td>766.90 ± 73.66</td>
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<td>13</td>
<td>178.38 ± 3.26</td>
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**Table II. Gene expression profiling by PCR array in aortic RNA of Diabetic (D) ApoE<sup>−/−</sup> or diabetic (D) PKCβ<sup>−/−</sup>/ApoE<sup>−/−</sup> mice was compared to that of non-diabetic (ND) ApoE<sup>−/−</sup> mice (defined as 1.0-fold in each case) at age 10 weeks.**

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Mean of fold regulation in D ApoE&lt;sup&gt;−/−&lt;/sup&gt; vs. ND ApoE&lt;sup&gt;−/−&lt;/sup&gt; aortas</th>
<th>Mean of fold regulation in D PKCβ&lt;sup&gt;−/−&lt;/sup&gt;/ApoE&lt;sup&gt;−/−&lt;/sup&gt; vs. ND ApoE&lt;sup&gt;−/−&lt;/sup&gt; aortas</th>
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<td>Sele</td>
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<tr>
<td>Spp1</td>
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Materials and Methods

Animal studies.
Animal studies were carried out with the approval of the Institutional Animal Care and Use Committee of New York University and Columbia University. Homozygous ApoE−/− mice in the C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, Maine). Homozygous PKCβ−/−/ApoE−/− mice were generated in our laboratory as previously published. All of the mice were fed normal chow and rendered diabetic by intraperitoneal injections of streptozotocin (stz), 55 mg/kg in citrate buffer (0.05 mol/L; pH4.5) (Sigma) for five consecutive days at age 6 weeks. Control mice were treated with citrate buffer. Mice were sacrificed at age 10, 14 and 20 weeks. In other studies, ApoE−/− mice were fed chow containing the PKCβ inhibitor ruboxistaurin (LY-333531; 30 mg/kg daily) or vehicle chow without inhibitor from age 5 weeks to 14 or 20 weeks and rendered diabetic as above. Ruboxistaurin and vehicle chow were generously supplied by Dr. Louis Vignati (Eli Lilly, Indianapolis, IN), who provided specific instructions regarding the appropriate dose of ruboxistaurin.

Cell culture and treatments.
Human U937 histiocytes (resident macrophages)1, 2 were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA). RPMI 1640 medium containing 25 mM D-glucose was purchased from ATCC; RPMI 1640 medium without glucose was purchased from Life Technology (Grand Island, NY); RPMI 1640 containing 5 mM D-glucose was prepared by dilution of RPMI 1640 medium containing 25 mM D-glucose with glucose-free RPMI 1640. The cells were cultured in 5% CO2 in RPMI1640 medium containing 10% fetal bovine serum (FBS) and 100 μg/ml penicillin-streptomycin and 5 mM (low) or 25 mM (high) D-glucose. The medium was changed every 2-3 days. To assess the effect of increasing concentration of glucose and the effects of the specific inhibitors of signaling pathways on the stimulation of gene expression, cells were seeded and exposed to 5 mM or 25 mM glucose for 24 h in the absence or presence of inhibitors of PKCβ (LY379196, 30 nM), ERK1/2 (U0126, 5 μM), JNK (SP600125, 20 μM) and p38 (SB203580, 20 μM). Cells were harvested followed by RNA isolation. LY379196 was generously supplied by Dr. Matthew J Sheetz (Eli Lilly, Indianapolis, IN).

siRNA to knockdown Egr-1.
Human Egr-1 siRNA (ID No. s4538) and a scramble siRNA as a negative control (Cat. No. 4390846) were purchased from Life Technology. siRNA was transfected into U937 cells by electroporation according to the manufacturer’s instructions (Amaxa, Gaithersburg, MD). Briefly, 2 x 10^6 cells were electroporated in 100 µl Nucleofector solution (Amaxa Reagent C) containing 1 μg of siRNA using the preselected Amaxa Program W-001. siRNA transfected cells were seeded in a 60 mm dish with 4 ml supplemented RPMI-1640 medium containing high glucose. To control for off-target effects of siRNA, U937 cells was electroporated with a scramble siRNA. After 24 hours of incubation, cells were harvested followed by RNA isolation.

Biochemical analyses.
Levels of total cholesterol and triglyceride were determined in plasma of fasted mice using chromogenic assays (Thermo Electron Corporation). Glucose levels were determined from samples of tail vein blood using a glucometer (Abbott Diabetes Care Company).

Quantification of atherosclerotic lesion area.
Mice were fasted for 4 hours and then anesthetized. Atherosclerosis assays were performed at the indicated times of sacrifice. After fixation in 4% paraformaldehyde and cryopreservation in 15% sucrose, and then 30% sucrose, hearts were embedded in OCT compound (Tissue-Tek;
Sakura Finetek, Torrance, CA) and cryosectioned using a cryostat. The frozen sections from aortic roots were fixed in 10% buffered formalin. Six 6-µm sections were collected at 80-µm intervals starting at a 100-µm distance from the appearance of the aortic valves. The sections were stained with Oil Red O and counterstained with hematoxylin. Atherosclerotic lesion areas were quantified using a Zeiss microscope and image analysis system (AxioVision 4.8). Four serial sections each were placed on 6 slides (total 24 sections), and mean lesion areas were calculated by determining the mean lesion area of one section/slide for a total of 6 sections examined. The investigator was blinded to the experimental conditions.

En face preparation of aortas was performed as described previously. The aorta and its main branches were dissected from the aortic valve to the iliac bifurcation. The aortic tree was opened longitudinally with an extremely fine Vanna microscissor and pinned flat on a black wax surface in a dissecting pan with 0.2-mm-diameter stainless steel pins. The pinned aortas were stained with Sudan IV. Images of Sudan IV–stained aortas were captured with a standard digital camera and the atherosclerotic lesion area was measured by image analysis using Axiovision 4.8 software.

Pathway-focused gene expression profiling using real-time PCR (PCR array analysis). Total RNA was extracted from snap-frozen single aorta using RNeasy® fibrous tissue mini kit (Qiagen, Valencia, CA). RNA (1 µg) was converted to cDNA by reverse transcription (RT) and genomic DNA elimination using the RT2 First Strand Kit (SABiosciences, Frederick, MD, USA). Gene expression profiling was then performed according to the SABiosciences RT2 Profiler PCR Array System Kit (SA Biosciences, Frederick, MD, USA) using the RT2 SYBR Green/ROX qPCR Master Mix (SA Biosciences, Frederick, MD, USA) and Mouse Atherosclerosis RT2 Profiler™ PCR Array (SA Biosciences, Frederick, MD, USA). This array profiles the expression of 84 stress-responsive genes related to atherosclerosis. Each sample of cDNA was mixed with the PCR master mix according to the directions in the kit and 25 µl was loaded into each well of the same plate. Real-time PCR cycling was performed using the ABI PRISM 7900HT Sequence Detection System (Segment 1, 10 minutes at 95°C, followed by Segment 2, 15 seconds at 95°C, 1 minute at 60°C repeated for 40 cycles, followed by Segment 3, 1 minute at 95°C, 2 minutes at 65°C and 30 seconds at 95°C). Results from PCR array showed no contamination from genomic DNA given that the cycle threshold value in a kinetic PCR amplification curve in well H6 was always undetectable. Data analysis was performed using the PCR array data analysis web portal [http://www.superarray.com/pcrarraydataanalysis.php]. Transcriptional analyses were performed on aortic RNA isolated from nondiabetic and diabetic ApoE-/- and PKCβ-/-/ApoE-/-mice at 10 weeks of age.

Real-time PCR analysis.
Real-time PCR was carried out to validate the results of the PCR array. Total RNA (0.5 µg) was processed directly to cDNA synthesis using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems) according to the manufacturer’s protocol. Real time PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA). The primers and probe mixture used are listed in Table III. Real-time PCR analysis of Egr-1 expression was performed as previously published. Data were analyzed by the 2^−ΔΔCT method. Expression levels were normalized using TaqMan ribosomal RNA control reagents (18s rRNA, Applied Biosystems, Foster City, CA, USA). All reactions were performed in triplicate in ABI PRISM 7900HT Sequence Detection System.

Western Blotting.
Total protein and membrane protein fractions were prepared from aorta, and were subjected to Western blotting with rabbit anti-Egr-1, anti-Phosphor (P)-PKCβII, anti-P-PKCδ, anti-P-ERK1/2,
anti-total (T)-ERK1/2, anti-P-JNK and anti-T-JNK IgG (1:1000; Cell Signaling Technology Inc). To detect β-actin, blots were incubated with rabbit anti-β-actin (1:10,000, Sigma). HRP-conjugated donkey anti-rabbit IgG (1:2,000; Amersham Biosciences) secondary antibody was used to identify sites of binding of primary antibody.

**Immunofluorescence.**
Sections of aortic roots from diabetic ApoE−/− and diabetic PKCβ−/−/ApoE−/− mice at age 20 weeks were stained with a hamster monoclonal anti-CD11c IgG (1:100; Thermo Scientific), and then incubated with a biotinylated goat anti-hamster IgG (1:200; Vector Laboratories Inc., Burlingame, CA, USA), followed by incubation with fluorescein-avidin D. The sections were blocked with avidin/biotin blocking solution and then incubated with a rat monoclonal anti-MOMA-2 antibody (IgGb2; 1:100; abcam, Cambridge, MA USA). This was followed by incubation with a biotinylated rabbit anti-rat IgG (1:200; Vector Laboratories Inc.), followed by Texas Red-avidin D, and finally mounted with 4, 6-diamidino-2-phenylindole dihydrochloride (Vector Laboratories). Isotype-matched control antibodies, hamster IgG (1:100; AbD Serotec, Raleigh, NC, USA) and rat IgGb2 (1:100; AbD Serotec, Raleigh, NC, USA), were used as negative controls and a similar procedure was performed on the adjacent section. The signals of individual and merged images for antigen detection were performed using a Zeiss fluorescent microscope and Axiovision 4.8 software. To determine the relative amounts of MOMA-2 positive macrophages or CD11c-expressing cells in atherosclerotic lesions, the positive area of MOMA-2 or CD11c was divided by the total lesion area of atherosclerosis.

**Statistical Analysis.**
All data are reported as mean ± SEM. Data were analyzed by ANOVA using commercially available software (Statview, version 5.0.1, Berkeley, Calif). Probability values of <0.05 were considered statistically significant.

**References**


**Table III. Primers and probes used for real-time PCR analysis**

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