Induction of Vascular Insulin Resistance and Endothelin-1 Expression and Acceleration of Atherosclerosis by the Overexpression of Protein Kinase C-β Isoform in the Endothelium

Qian Li,* Kyoungmin Park,* Chenzhong Li, Christian Rask-Madsen, Akira Mima, Weier Qi, Koji Mizutani, Paul Huang, George L. King

Rationale: Loss of insulin action in the endothelium can cause endothelial dysfunction and atherosclerosis. Hyperglycemia and elevated fatty acids induced by diabetes mellitus can activate protein kinase C-β isoforms and selectively inhibit insulin signaling via phosphatidylinositol 3-kinase/Akt pathway to inhibit the activation of endothelial nitric oxide synthase and metabolic actions.

Objective: To demonstrate that overexpressing protein kinase C-β2 isoform in endothelial cells can cause selective insulin resistance and exacerbate atherosclerosis in the aorta.

Methods and Results: Protein kinase C-β2 isoform was overexpressed in endothelial cells using a promoter of vascular endothelial cell cadherin. These mice were cross-bred with apoE−/− mice [Tg (Prkcb)apoE−/−]. On a Western diet, Tg(Prkcb)apoE−/− and apoE−/− mice did not differ in systemic insulin sensitivity, glucose tolerance, plasma lipid, or blood pressure. Insulin action in endothelial cells and femoral artery from Tg(Prkcb)apoE−/− mice was impaired by ≈40% with respect to Akt/endothelial nitric oxide synthase activation, and leukocyte-endothelial cell binding increased in cultured lung endothelial cells from Tg(Prkcb)apoE−/− mice compared with that from apoE−/− mice. Basal and angiotensin-stimulated big endothelin-1 levels were elevated in Tg(Prkcb)apoE−/− mice compared with apoE−/− mice. The severity of atherosclerosis in the aorta from Tg(Prkcb)apoE−/− mice increased by ≈70% as measured by en face fat staining and plaque content of the number of smooth muscle cells, macrophages, and extracellular matrix.

Conclusions: Specific protein kinase C-β2 activation in the endothelial cells caused dysfunction and accelerated atherosclerosis because of loss of insulin-stimulated Akt/endothelial nitric oxide synthase activation and angiotensin-induced increases in endothelin-1 expression. (Circ Res. 2013;113:418-427.)

Key Words: atherosclerosis ■ endothelium ■ insulin resistance ■ protein kinase C

Systemic metabolic abnormalities, such as hyperlipidemia, hyperglycemia, and hypertension, are important risk factors for cardiovascular disease associated with diabetes mellitus and insulin-resistant states. 1,2 Clinical studies, such as The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications, showed that intensive glycemic control lowers the risk for cardiovascular disease in type 1 diabetic patients; however, other studies, such as Action to Control Cardiovascular Risk in Diabetes and Action in Diabetes and Vascular Diseases: Preterax and Diamicron MR Controlled Evaluation, suggested that its modifying effects are not clear for type 2 diabetic patients, 3,5 indicating that other factors could be as important as glycemic control. One such metabolic or hormonal factor is insulin resistance on the vascular wall, which can cause endothelial dysfunction and contribute to the development of cardiovascular disease complications in type 2 diabetes mellitus. 1 The importance of insulin action on atherosclerosis was clearly established recently by findings that mice with double-knockout of apolipoprotein E (apoE) and insulin receptor on endothelial cells had development of atherosclerosis at 2-times to 3-times greater severity than apoE−/− mice. 6 The mechanism of antiatherogenic actions of insulin on the vascular endothelium is predominantly mediated via the insulin receptor substrate (IRS)/phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which has been shown to activate endothelial nitric oxide synthase (eNOS) 7,8 and to downregulate vascular cell adhesion...
molecule-1 (VCAM-1). Expression of eNOS and VCAM-1 can play an important role in the activation of endothelial cells, which is a key initial step for atherosclerosis. Nitric oxide (NO) produced by eNOS activation is the most important endothelium-derived relaxing factor, and it protects the artery from atherosclerosis by multiple mechanisms. VCAM-1 has an important role in regulating the binding of leucocytes to endothelial cells, and its reduction can attenuate the development of atherosclerosis. In insulin-resistant states, such as obesity and type 2 diabetes mellitus, selective inhibition of the IRS/PI3K/Akt pathway, but not mitogen-activated protein kinase (MAPK) cascade, is probably because of abnormal elevation of metabolites, such as free fatty acids and glucose. One mechanism causing the selective inhibition of insulin action is likely because of protein kinase C (PKC) activation, which has been shown to be a consistent feature in the endothelium in diabetes mellitus or insulin resistance. PKC activation, especially the PKC-β isoform, has been reported to selectively inhibit insulin activation of Akt and eNOS in endothelial cells of diabetic animals and patients by decreasing the phosphorylation of Akt at Ser473 and eNOS at Ser1177. We have characterized the effects of PKC-β activation on multiple steps of the insulin signaling cascade from the receptor to Akt phosphorylation in endothelial cells and have identified a novel phosphorylation site on p85 subunit of PI3K (threonine 86), which is partially responsive to PKC activation and blunts insulin activation of Akt/eNOS in endothelial cells. In addition, mice with whole-body PKC-β knockout exhibited decreased severity of atherosclerosis, further supporting its role in the atherosclerotic process. However, whether PKC-β activation in the endothelium can inhibit insulin action and accelerate atherosclerosis has not been reported. In this study, we have characterized the effects of overexpressing PKC-β2 isoform selectively to the endothelium on insulin signaling and action in the arteries and on the development of atherosclerosis.

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**Methods**

**Animals**

Mice with endothelial-specific overexpression of PKC-β2 isoform were generated as previously described. The transgene was injected into the embryos of pure C57/BL6J mice, and the background of the transgenic mice is C57/BL6J. This was crossed with apoE−/− mice that were purchased from the Jackson Laboratory (Bar Harbor, ME), also on C57/BL6J background. The following primers were used for PKC-β2 isoform genotyping: 5′-ATGGATTACAAAG GATGACGACGATAAG-3′ and 5′-AGTTGGCGTCAAGAAGTGGCCTGT-3′. Male mice were fed a Western diet (Harland; 42% calories from fat, containing 0.15% cholesterol) starting at 6 weeks of age and continued for 12 weeks. All protocols for animal use and euthanasia were reviewed and approved by the Animal Care Committee of the Joslin Diabetes Center. They are in accordance with National Institutes of Health guidelines following the standards established by the Animal Welfare Acts and by the documents entitled Principles for Use of Animals and Guide for the Care and Use of Laboratory Animals.

Endothelial cell culture, glucose tolerance tests, insulin tolerance tests, quantification of atherosclerotic lesion size in aorta, immuno-histochemistry, collagen staining, vascular reactivity studies, Western blotting, real-time polymerase chain reaction analysis, leukocyte endothelial cell adhesion, angiotensin II (Ang II) infusion, measurement of big endothelin-1 (ET-1), and ET-1 were performed as described in the Online Data Supplement.

**Statistical Analysis**

Comparisons of the 2 groups were made using paired or unpaired t test, as appropriate. Comparison among >2 groups was performed by 1-way ANOVA, followed by the post hoc analysis with paired or unpaired t test, to evaluate statistical significance between the 2 groups. Statistical significance was defined as P<0.05. In text and graphs, data are presented as mean±SD.

**Results**

**Insulin Signaling in the Endothelium**

Endothelial-targeted PKC-β2 transgenic/apoE−/− [Tg(Prkcb) apoE−/−] mice were generated by cross-breeding endothelial-specific PKC-β2 transgenic mice [Tg(Prkcb) with apoE−/− mice. Levels of PKC-β2 mRNA increased by 32±4-fold (P<0.01, data not shown) and PKC-β2 protein increased by 2-fold (Figure 1A and 1B) in cultured lung endothelial cells from Tg(Prkcb)apoE−/− mice compared with that from apoE−/− mice. Expressions of PKC-β2 mRNA levels in macrophages and smooth muscle cells (SMCs) were not different between apoE−/− and Tg(Prkcb)apoE−/− mice (data not shown). We have reported that the loss of insulin receptors on endothelial cells exacerbated atherosclerosis, which correlated to decreased phosphorylation of eNOS and increased expression of VCAM-1. Insulin receptor tyrosine phosphorylation after insulin stimulation was not different between the 2 groups of mice (Online Figure IA and IB). Insulin-induced Akt/eNOS activation was determined in primary cultured endothelial cells from apoE−/− and Tg(Prkcb)apoE−/− mice. Insulin increased Akt Ser473 phosphorylation (p-Akt) by 9.4±0.9-fold (Figure 1C and 1E) and increased eNOS Ser1176 (mouse site, equivalents of human Ser1177 and bovine Ser1179) phosphorylation (p-eNOS) by 4.3±0.8-fold (Figure 1C and 1D) in apoE−/− mice. However, insulin-stimulated p-Akt was attenuated by 42% (P<0.01) and eNOS Ser1176 phosphorylation was reduced by 39% (P<0.01) in Tg(Prkcb)apoE−/− mice compared with apoE−/− mice (Figure 1C–1E). Insulin signaling also was studied in vivo by intravenous infusion of insulin, and p-eNOS Ser1176 was measured in the femoral artery. Insulin increased p-eNOS Ser1176 by 1.7±0.5-fold in the femoral artery of apoE−/− mice (Figure 1F and 1G). The basal level of p-eNOS Ser1176 was 49% lower in the femoral artery of Tg(Prkcb)apoE−/− mice than in that of apoE−/− mice (P<0.05; Figure 1F and 1G). The effect of insulin on p-eNOS Ser1176 was impaired by 25% in
Phosphorylation of Akt and eNOS was stimulated by 100 nmol/L insulin in MLECs from apoE−/− or Tg(Prkcb)apoE−/− mice. Western blots show eNOS Ser1176 phosphorylation. 

Figure 1.

Protein kinase C (PKC)-β2 expression and its effects on insulin-induced Akt/endothelial nitric oxide synthase (eNOS) phosphorylation and P85α phosphorylation. 

A. PKC-β2 protein in cultured mouse lung endothelial cells (MLEC) from apolipoprotein E-deficient (apoE−/−) or Tg(Prkcb)apoE−/− mice. PKC-β2 protein was determined by Western blotting and normalized by actin. 

B. Representative Western blots show Akt Ser473 and eNOS Ser1176 phosphorylation. 

C. Mean values of eNOS Ser1176 (apoE−/−, n=3; apoE−/− plus insulin, n=5; Tg(Prkcb)apoE−/−, n=3; Tg(Prkcb)apoE−/− plus insulin, n=6). 

D. Akt Ser473 phosphorylation in cultured lung endothelial cells from apoE−/− or Tg(Prkcb)apoE−/− mice (apoE−/−, n=4; apoE−/− plus insulin, n=7; Tg(Prkcb)apoE−/−, n=4; Tg(Prkcb)apoE−/− plus insulin, n=7). 

E and G. The phosphorylation of eNOS stimulated by insulin in femoral artery of apoE−/− or Tg(Prkcb)apoE−/− mice. Insulin (10 mIU/g body weight) was injected and femoral artery was removed after 5 minutes. 

F and I. Phosphorylation of threonine 86 (Thr86) of P85α was determined by Western blotting and normalized by actin. 

Phosphorylation of threonine 86 of P85α (Thr86) and blunted insulin Akt/eNOS signaling. 

Activation of PKC by phorbol 12-myristate 13-acetate (PMA) increased P85 phosphorylation in primary cultured endothelial cells of both apoE−/− mice and Tg(Prkcb)apoE−/− mice. The P85 phosphorylation increased by 1.59±0.07-fold in endothelial cells from Tg(Prkcb)apoE−/− mice compared with that from apoE−/− mice (P<0.05; Figure 1G and 1H). We reported that PKC activation phosphorylated threonine 86 of P85α and blunted insulin Akt/eNOS signaling. 

Apoptosis induced by withdrawing growth factors increased DNA fragmentation in both groups of mice, but it was lower in endothelial cells from Tg(Prkcb)apoE−/− mice than that from apoE−/− mice (Online Figure IE). 

Systemic Metabolic and Vascular Functional Parameters

All mice were fed high-fat (42%) diet chow. The body weight did not differ between apoE−/− and Tg(Prkcb)apoE−/− mice after 12 weeks of high-fat feeding (30.7±5.5 vs 28.5±4.6 g; Online Figure IIA). Blood pressure, as measured by tail vein plethysmography, was similar for systolic blood pressure (112±6 vs 121±10 mm Hg) and diastolic blood pressure (94±6 vs 96±6 mm Hg) between apoE−/− and Tg(Prkcb)apoE−/− mice (Online Figure IIB). Levels of total cholesterol (462.5±161.6 vs 406.8±125.2 mg/dL) and triglycerides (151.0±82.2 vs 116.4±47.4 mg/dL) in plasma were not different after high-fat feeding (Online Figure IIC and IID). The lipoprotein profile of cholesterol also was characterized by fast protein liquid chromatography to assess very-low-density lipoprotein, low-density lipoprotein, and high-density lipoprotein properties, which did not differ between the 2 groups of animals (Online Figure III). 

Apoptosis in Endothelial Cells

Apoptosis induced by withdrawing growth factors was determined in endothelial cells from both groups of mice. Withdrawing growth factors increased DNA fragmentation in both groups of mice, but it was lower in endothelial cells from Tg(Prkcb)apoE−/− mice than that from apoE−/− mice (Online Figure IE).
in the endothelium did not change whole-body glucose tolerance, systemic insulin sensitivity, plasma lipids, or blood pressure.

**Arterial Responses to Acetylcholine and Sodium Nitroprusside**

Functionally, pressure myograph was used to determine whether impaired eNOS phosphorylation in Tg(Prkcb)apoE−/− mice affected arterial dilation. When arterial dilation induced by acetylcholine (Ach) was normalized by the effect of 10 μmol/L sodium nitroprusside (SNP), responses to multiple concentrations of Ach were impaired in Tg(Prkcb)apoE−/− mice compared with 89.9±6.0% in Tg(Prkcb)apoE−/− mice (P<0.05). At 1 μmol/L Ach, vasodilation was 100.6±4.6% of dilation induced by SNP in apoE−/− mice compared with 89.9±7.1% in Tg(Prkcb)apoE−/− mice (P<0.05). At 3 μmol/L Ach, the degree of vasodilation was 103.9±4.1% of dilation induced by SNP in apoE−/− mice compared with 91.2±5.2% in Tg(Prkcb)apoE−/− mice (P<0.05). At 10 μmol/L Ach, the degree of vasodilation was 103.9±4.1% of dilation induced by SNP in apoE−/− mice compared with 91.2±5.2% in Tg(Prkcb)apoE−/− mice (P<0.05). Ach at 3 μmol/L induced 102.3±5.5% of dilation similar to SNP in apoE−/− mice and was only 91.0±4.8% in Tg(Prkcb)apoE−/− mice (P<0.05). Even at 10 μmol/L Ach, the degree of vasodilation was 103.9±4.1% of dilation induced by SNP in apoE−/− mice compared with 91.2±5.2% in Tg(Prkcb)apoE−/− mice (P<0.05). The EC50 of Ach was not different between both groups of mice (apoE−/− vs Tg(Prkcb)apoE−/− mice: 18.3±5.7 vs 18.3±1.2 nmol/L). To study the direct responses of arterial SMCs to NO, the actions of SNP were characterized. Vasorelaxation to SNP at 10 nmol/L (73.2±14.4% vs 51.3±2.8%; P<0.05) and at 30 nmol/L (87.2±5.1% vs 73.3±0.8%; P<0.05) were increased in arteries from Tg(Prkcb)apoE−/− mice compared with that from apoE−/− mice (Figure 2B). However, responses to SNP at concentrations from 30 nmol/L to 10 μmol/L were not different between apoE−/− mice and Tg(Prkcb)apoE−/− mice.

**Expression of VCAM-1 and Leukocyte-Endothelial Cell Adhesion**

Because insulin can inhibit VCAM-1 expression in the endothelium via the IRS/Akt pathway, the basal levels of VCAM-1 mRNA were characterized and found to be increased by 3.5±2.4-fold (P<0.01) in cultured lung endothelial cells from Tg(Prkcb)apoE−/− mice compared with those from apoE−/− mice (Figure 3A). The addition of PMA, an activator of PKC, increased VCAM-1 mRNA by 4±2.6-fold and 14±4.5-fold in cultured lung endothelial cells from apoE−/− mice and Tg(Prkcb)apoE−/− mice, respectively (Figure 3A). However, protein expression of VCAM-1 was increased by 1.8±0.6-fold in cultured lung endothelial cells from Tg(Prkcb)apoE−/− mice versus apoE−/− mice at basal conditions (Figure 3B). Insulin inhibited VCAM-1 protein by 44±5% in cells from apoE−/− mice (Figures 3B and 3C). In contrast, the addition of insulin did not decrease VCAM-1 expression in endothelial cells from Tg(Prkcb)apoE−/− mice (Figures 3B and 3C).

Functionally, attachments of fluorescent-labeled RAW 264.7 macrophages to endothelial monolayer were determined by spectrometer. PMA increased leukocyte-endothelial cell binding by 2.1±0.2-fold compared with vehicle in cultured lung endothelial cells from apoE−/− mice (Figure 3D). Preincubation of cells with insulin inhibited PMA-induced leukocyte-endothelial cell binding by 36% (1.4±0.1-fold vs 2.1±0.2-fold; P<0.01; Figure 3D). Leukocyte-endothelial cell binding was increased by 2.0±0.1-fold (P<0.01) in cultured lung endothelial cells from Tg(Prkcb)apoE−/− mice compared with those from apoE−/− mice (Figure 3D). However, insulin was unable to decrease PMA-induced leukocyte-endothelial cell binding in cells from Tg(Prkcb)apoE−/− mice (Figure 3D). The increase in VCAM-1 protein after PMA stimulation was comparable with the effect of PMA on leukocyte binding to endothelium. Similarly, insulin decreased PMA-induced VCAM-1 protein, which is comparable with leukocyte binding to endothelium (Figures 3E and 3F). Basal VCAM-1 protein expression increased in Tg(Prkcb)apoE−/− mice. PMA increased VCAM-1 protein expression by 1.48±0.26-fold in apoE−/− mice, which was inhibited by insulin in endothelial cells from apoE−/− mice. However, the inhibitory effect of insulin on PMA-induced VCAM-1 expression was impaired in endothelial cells from Tg(Prkcb)apoE−/− mice (Figure 3E and 3F). Basal VCAM-1 protein expressions also were measured in aortic endothelial cells and were found to be 2.28±0.47-fold higher in Tg(Prkcb)apoE−/− mice than apoE−/− mice (P<0.05; Online Figure IIIC and IIID).

**Ang II Actions and ET-1 Expression**

PKC-β isoform activation also can cause insulin-independent effects. We studied the actions of Ang II on the expression of platelet-derived growth factor and ET-1 in the aorta because the actions of these cytokines have been shown to be increased in the vascular wall by diabetes mellitus or insulin-resistant states.20-23 Basal ET-1 mRNA expressions in the aorta of apoE−/− mice and Tg(Prkcb)apoE−/− mice were not different. Ang II infusion increased ET-1 mRNA levels by 1.9±0.4-fold compared with vehicle in the aorta of apoE−/− mice (Figure 4A). Interestingly, the actions of Ang II on ET-1 mRNA expression were greater in the aorta of Tg(Prkcb)apoE−/− mice compared with that in apoE−/− mice by

**Figure 2.** Artery relaxation induced by acetylcholine (Ach) or sodium nitroprusside (SNP). A, Ach induced common carotid artery relaxation. (apolipoprotein E–deficient [apoE−/−], n=4; Tg[Prkcb]apoE−/−, n=5; *P<0.05 vs apoE−/−). B, SNP induced common carotid artery relaxation (apoE−/−, n=4; Tg[Prkcb]apoE−/−, n=5; *P<0.05 vs apoE−/−).
The levels of ET-1 also were significantly higher by 3.6-fold in endothelial cells from Tg(Prkcb)apoE−/− mice compared with those from apoE−/− mice (data not shown). Because we have reported that the effect of Ang II specifically increases ET-1 expression in cultured lung endothelial cells. Lung endothelial cells were treated with or without 100 nmol/L PMA. VCAM-1 mRNA was measured (n=3, Tg(Prkcb)apoE−/− n=9, Tg(Prkcb)apoE−/−+PMA n=3). B and C, VCAM-1 protein expression in cultured lung endothelial cells. Lung endothelial cells were treated with or without 100 nmol/L insulin. VCAM-1 protein was determined by Western blotting and normalized by actin. B, Representative Western blots. C, Mean value of the ratio of VCAM-1 to actin (n=3 for each group). D, Leukocyte-endothelial binding. The binding of RAW 264.7 macrophages to monolayer lung endothelial cells from apoE−/− or Tg(Prkcb)apoE−/− mice was determined by real-time polymerase chain reaction and normalized by 36B4 (apolipoprotein E–deficient [apoE−/−], n=8; apoE−/− plus PMA (P<0.05; Figure 4B). In Tg(Prkcb)apoE−/− mice, the mean area covered by aortic atherosclerotic lesions, as evaluated by en face Sudan IV staining and expressed relative to the whole aortic area, was 1.7-fold higher than that in apoE−/− mice (P<0.05; Figure 5A and 5B). Atherosclerotic plaques increased by 2.16±0.178-fold in abdominal aorta from Tg(Prkcb)apoE−/− mice (P<0.05) but were not different in aortic root and aortic arch (Figure 5C–5E). Analysis of cross-sections of aortic plaque showed that the SMC content in the plaque of Tg(Prkcb)apoE−/− mice was more than 2-times greater than that in apoE−/− mice (10±4.8% vs 4.2±2.4%; P<0.05; Figure 6A and 6B). Absolute macrophage content, measured by macrophage galactospecific lectin-2 staining, was increased in Tg(Prkcb)apoE−/− mice compared with apoE−/− mice (162.657±71.689 vs 68.876.76±34.6419.5; P<0.05; Figure 6C and 6D). The content of collagen as measured by aniline blue staining was increased by 2-fold in Tg(Prkcb)apoE−/− mice compared with apoE−/− mice (8203.5±3160.9 vs 3899.7±2794.5; P<0.05; Figure 6E and 6F). Area of necrosis in the plaque was denoted by a lack of staining by trichrome, which indicated the absence of cells and collagen. The necrosis area was 1.99±0.55-times higher in Tg(Prkcb)apoE−/− mice compared with apoE−/− mice (11.0±3.1% vs 5.5±2.5%; P<0.05; Figure 6G and 6H).

Discussion

In the present study, we have directly demonstrated that overexpression of PKC-β2 in endothelial cells inhibits insulin signaling and insulin action and increases expression
of ET-1, resulting in endothelial dysfunction and accelerated atherosclerosis. These findings may help explain the elevated risk for atherosclerosis in diabetic and insulin-resistant states because PKC activation, especially the β-isoform, has been shown to be induced by hyperglycemia or elevated free fatty acids in many vascular tissues.26 The inhibiting role of PKC-β on insulin activation of eNOS clearly has been shown in endothelial cells from diabetic patients.15 At the cell signaling level, the vasotropic effects of insulin, such as the activation of eNOS, induction of heme oxygenase-1 and vascular endothelial growth factor, and downregulation of VCAM-1, are mediated through activation of the IRS/PI3K/Akt pathway.6,27,28 PKC activation, especially β-isoform, has been shown to selectively inhibit this pathway by serine phosphorylation of the p85 subunit of PI3K activation in the endothelial cells. Previous studies of the effects of PKC-β activation and atherosclerosis have used whole-body PKC-β knockout mice or systemic use of PKC-β selective inhibitor, which showed a reduction of atherosclerosis in apoE−/− mice.17 However, the systemic inhibition of PKC-β isoform cannot attribute its effects to the endothelium specifically because activation of PKC-β isoform by diabetes mellitus and its inhibition have been reported to affect many tissues and functions to regulate multiple metabolic pathways.29 The present study used mice, with overexpression of PKC-β2 targeted specifically to the endothelial cells by use of the vascular endothelial cell cadherin promoter. The Tg(Prkcb)apoE−/− mice have allowed us to test the idea that PKC-β activation specifically in the endothelial cells can cause vascular-selective insulin resistance, endothelial

![Figure 4](image-url)  
**Figure 4.** The effect of angiotensin II (Ang II) on endothelin-1 (ET-1) expression in the aorta. A and B, Ang II or vehicle was infused into apolipoprotein E-deficient (apoE−/−) or Tg(Prkcb)apoE−/− mice for 3 hours. A, ET-1 mRNA was determined by real-time polymerase chain reaction (PCR; apoE−/− infused with vehicle, n=3; apoE−/− infused with Ang II, n=5; Tg(Prkcb)apoE−/− infused with vehicle, n=3; Tg(Prkcb)apoE−/− infused with Ang II, n=7). B, Big ET-1 in aorta was measured by enzyme-linked immunosorbent assay (ELISA). (apoE−/− infused with vehicle, n=3; apoE−/− infused with Ang II, n=5; Tg(Prkcb)apoE−/− infused with vehicle, n=4; Tg(Prkcb)apoE−/− infused with Ang II, n=5). C, C, cultured mouse lung endothelial cells were treated with 100 nmol/L Ang II for 16 hours, and ET-1 mRNA was determined by real-time PCR and normalized by 36B4. (n=3 for each group). D, ET-1 protein in medium from cultured mouse lung endothelial cells was measured by ELISA and normalized by the total protein extracted from the cells growing in the same well (n=3 for each group). E and F, ERK phosphorylation. Phosphorylation of ERK in mouse lung endothelial cells. E, Representative Western blots show ERK phosphorylation. F, Quantitative analysis of ERK phosphorylation (n=8 for each group).
dysfunction, and accelerated atherosclerosis in the absence of systemic insulin resistance and its metabolic changes. Specific enhancement of PKC-β in the endothelium clearly decreased insulin signaling only in the vascular endothelium without affecting systemic insulin action, indicating that decreasing insulin action on the endothelium will not contribute to systemic insulin resistance, which has been suggested. We have previously identified threonine 86 of the p85 regulatory subunit of PI3K as a possible target of PKC-β activation in endothelial cells. This phosphorylation of p85 decreased the association of IRS1 with p85/PI3K after insulin stimulation.16

A common characteristic of endothelial dysfunction of diabetes mellitus is the impairment of NO bioavailability because of either decreased production or increased degradation by oxidants. Our new findings clearly showed that the increase in PKC-β expression in endothelial cells inhibited insulin activation of p-Akt/p-eNOS, resulting in decreased NO production. This is supported by the biological effect of the relaxation assay in which aorta from Tg(Prkcb)apoE−/− mice exhibited less response to Ach. Another interesting finding is the arterial response to SNP, which exhibited a hyperactive response in Tg(Prkcb)apoE−/− mice. It is possible that this alteration is a compensatory response to lower ambient levels of NO in Tg(Prkcb)apoE−/− mice. One direct effect of impaired insulin-stimulated activation of the PI3K/Akt pathway is the increased expression of VCAM-1, which is normally downregulated by insulin via the IRS/Akt/PI3K pathway. Previously, we have reported that VCAM-1 expression in endothelial cells or in aorta from mice with insulin receptors specifically deleted from the endothelial cells (endothelial insulin receptor and ApoE knockout mice) had upregulation of VCAM-1 and accelerated atherosclerosis.6 The increase in VCAM-1 expression in Tg(Prkcb)apoE−/− mice also had biological significance because it is associated with elevated binding of leukocytes. The increase in leukocyte binding to the endothelium was also observed in endothelial insulin receptor and ApoE knockout mice.6

The pathology of the atherosclerotic plaque in Tg(Prkcb)apoE−/− mice was different from endothelial insulin receptor and ApoE knockout mice regarding the increased numbers of SMC compared with apoE−/− mice. The increase in SMC numbers in atherosclerotic plaques could be a result of elevations in proliferation and migration because this is observed in patients with diabetes mellitus and
insulin resistance. In fact, some studies have suggested that hyperinsulinemia could be responsible for excessive SMC proliferation, although our recent studies did not support this idea. We also explored the possibility that PKC-β activation directly increased ET-1 expression in the endothelial cells and this effect was exacerbated by Ang II, whose actions are increased in diabetes mellitus. The results from Tg(Prkcb)apoE−/− mice suggest that PKC-β activation in the endothelial cells increased ET-1 expression in the aorta and other arteries from Tg(Prkcb)apoE−/− mice. Increase in the PKC activity has been reported to increase ET-1 expression in several cell types possibly via the MAPK pathway. The additive effect of Ang II in vivo is demonstrated by mRNA levels in the aorta of both genes. Big ET-1 was measured instead of ET-1, because the half-life of ET-1 protein is very short once secreted and protein levels of big ET-1 are much lower than small ET-1. So big ET-1 levels were measured in order to determine the effect of PKC-β activation on ET-1 expression.

The increases in atherosclerosis were observed mainly in the descending aorta and not in the root or arch in Tg(Prkcb)apoE−/− mice versus apoE−/− mice. This is comparable with autopsy studies in diabetic patients, which have identified that the extent of atherosclerosis is most significantly increased in the abdominal aorta compared with nondiabetic patients. In addition, the amount of atherosclerosis in the aortic root is not so common in the clinical setting, whereas the extent of atherosclerosis in the abdominal aorta correlates well clinically with atherosclerosis in the coronary artery. In the rodent model, atherosclerosis appears earlier in the aortic root and arch and is more severe than in other parts of the aorta. This has been attributed to differences in heart rate (550 bpm in mice compared with 70 bpm in the average human) and flow. The increase in atherosclerosis in the abdominal aorta in humans is thought to be the result of the turbulent flow. The induction of diabetes mellitus or overexpression of PKC in the endothelium may change the flow from laminar to turbulent in the abdominal aorta and increase atherosclerosis.

The collagen content increased in the plaque of Tg(Prkcb)apoE−/− mice, but they were mainly located around the necrotic core and not on the cap of the lesion. Thus, the findings show an increase in the necrotic cores and the location of the extracellular matrix at the lower part of the plaque near the adventitia, which suggest that the plaques of Tg(Prkcb)apoE−/− mice were not more stable than those of apoE mice.

The overexpression of PKC-β in the endothelium may not be the same as activation of endogenous PKC-β in effecting the function in the endothelium and atherosclerosis. Previously, we have shown that angiotensin activation can cause inhibition of IRS/Akt/eNOS activation in bovine aortic endothelial cells and can enhance MAPK activation. In addition, it targeted threonine 86 phosphorylation of p85/PI3 kinase as shown in Tg(Prkcb)apoE−/− mice (Figure 1H and 1I). Thus, we speculate that even without overexpression of PKC-β, the activation of endogenous PKC-β will also inhibit insulin activation of phospho-Akt and eNOS and the loss of inhibition of VCAM-1.

The change of phospho-eNOS in femoral artery after insulin stimulation was not significantly different between the 2 groups of mice; however, the maximum response to insulin was lower in Tg(Prkcb)apoE−/− mice compared with apoE−/− mice. We believe this difference is important because the data shown in Figure 3 regard insulin stimulation was not significantly different between the 2 groups of mice; however, the maximum response to insulin was lower in Tg(Prkcb)apoE−/− mice compared with apoE−/− mice. We believe this difference is important because the data shown in Figure 3 regard the expression of VCAM-1 indicated that the ability of insulin to inhibit VCAM-1 expression is significantly decreased and the basal level of VCAM-1 expression is increased in Tg(Prkcb)apoE−/− mice. Because the inhibitory effect of insulin on VCAM-1 expression is caused by PI3K/Akt pathway, this increased expression of VCAM-1 suggests that the maximum effect attained by insulin through the Akt pathway will have functional significance.

Overexpression of PKC-β seems to decrease apoptosis of the endothelial cells. This is not surprising because PKC-β activation is known to enhance MAPK (Online Figure ID) to decrease apoptosis and increase proliferation in many types of cells. The increase in atherosclerosis exhibited by Tg(Prkcb)apoE−/− mice is likely because of multiple factors, including the inhibition of insulin signaling to activate p-eNOS and lack of inhibition of VCAM-1 expression as shown in Figures 1C, 1D, 3C, and 3F. In addition, it is also likely that PKC-β activation can increase other atherogenic effects, independent of its inhibition of the activation of insulin of the IRS/Akt/eNOS pathway. This is exhibited by the enhanced basal MAPK phosphorylation, which is known to effect PKC activation. In addition, the effect of Ang II to increase ET-1 expression is also elevated independently of insulin. Multiple studies have shown that angiotensin activation can lead to PKC activation and result in actions, including increased expression of ET-1. Because ET-1 expression has been shown to accelerate or exacerbate atherosclerosis, this is likely an insulin signaling–independent pathway by which PKC-β activation enhances atherosclerosis.

In summary, this study provided definitive evidence that PKC-β activation in the endothelium will accelerate atherosclerosis. In particular, an increase in migration or proliferation of SMC across the internal elastic membrane results in increased production of extracellular matrix. There could be multiple mediators of PKC-β activation that accelerate
atherosclerosis, including selective loss of insulin action on p-Akt/eNOS, increase in VCAM-1 expression, elevation of ET-1 expression, and enhancing Ang II effects. These results improve our understanding of the molecular mechanisms of atherogenesis and point to inhibition of PKC-β as a potential strategy for preventing atherosclerosis in insulin resistance, type 2 diabetes mellitus, and other insulin resistance–related diseases.

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Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- Insulin has important actions in endothelial cells.
- Endothelial insulin receptor and apolipoprotein E double-knockout mice had development of more atherosclerosis than apolipoprotein E-null mice.
- Protein kinase C (PKC-β) activated in endothelial cells in diabetes mellitus selectively inhibits Akt and endothelial nitric oxide synthase activation by insulin in cultured endothelial cells and causes endothelial dysfunction.

**What New Information Does This Article Contribute?**

- Overexpression of PKC-β2 in endothelial cells inhibits the antiatherogenic actions of insulin, including activation of Akt–endothelial nitric oxide synthase and downregulation of vascular cell adhesion molecule-1.
- Endothelial overexpression of PKC-β2 produced more endothelin-1 at the basal level and after angiotensin II stimulation than wild-type mice.
- Overexpression of PKC-β2 in endothelial cells accelerated the development of atherosclerosis in apolipoprotein E-deficient mice.

Diabetes mellitus and states of insulin resistance accelerate atherogenesis. One risk factor for this acceleration of atherogenesis is insulin resistance in endothelial cells. PKC-β has been found to be activated in the endothelial cells of diabetic patients and has been found to selectively inhibit insulin-induced Akt and endothelial nitric oxide synthase activation. We examined the effects of overexpressing PKC-β2 isoform selectively to the endothelium on insulin signaling and action in the arteries and on the development of atherosclerosis in mice. We observed that PKC-β2 overexpression in endothelial cells accelerated the development of atherosclerosis by inhibiting the antithrombogenic actions of insulin, including activation of Akt–endothelial nitric oxide synthase, downregulation of vascular cell adhesion molecule-1, and elevation of endothelin-1 production. These findings suggest that PKC-β2 contributes to diabetic macrovascular complications and that inhibition of PKC-β may be a promising treatment for macrovascular complications in diabetic patients.
Induction of Vascular Insulin Resistance and Endothelin-1 Expression and Acceleration of Atherosclerosis by the Overexpression of Protein Kinase C-β Isoform in the Endothelium

Qian Li, Kyoungmin Park, Chenzhong Li, Christian Rask-Madsen, Akira Mima, Weier Qi, Koji Mizutani, Paul Huang and George L. King

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Supplemental Material

Supplemental methods

Anesthesia
Mice were anesthetized with Pentobarbital (50mg/kg, i.p.).

Endothelial cell culture
Lung tissue, excluding central bronchi and vessels, was removed and minced with scalpels. The minced tissues were incubated for 1 hour at 37°C in Dulbecco’s modified Eagle medium (DMEM) containing 0.2% (w/v) collagenase type 1 (Worthington Biochemical Corporation, Lakewood, NJ) and 0.1% BSA. The tissue digest was passed through a 40μm strainer and centrifuged at 200 × g. The cells were seeded on dishes coated with 0.1% (w/v) collagen I (BioCoat, BD BioSciences, Franklin Lakes, NJ) and were grown in DMEM with 10% (v/v) horse serum, 50µg/ml endothelial cell growth substance (Biomedical Technologies, Stoughton, MA), and 100µg/ml heparin. After trypsin treatment during the first and second passage, cells were sorted with Dynabeads conjugated with sheep anti-rat IgG (Dynal/Invitrogen, Grand Island, NY) and complexed to ICAM-2 rat anti-mouse monoclonal antibody (BD Pharmingen, San Jose, CA) in DMEM containing 0.1% BSA for 30 minutes at 4°C. Cells were used at passages 3-6 after serum starvation for 16 hours in DMEM containing 0.1% BSA. The aorta endothelial cells were cultured as described above.

Glucose and insulin tolerance tests
Mice were fasted for 6 hours and then glucose (2mg/g body weight) or insulin (0.75mIU/g body weight) was administered to mice via intraperitoneal injection as described previously. Blood was sampled from tail vein for glucose determination at 0, 30, 60,120 minutes after glucose or insulin injection.

Quantification of atherosclerotic lesion size in aorta
The mice were fed western diet (Harlan Laboratories, Madison, WI) which contained 0.15% cholesterol for 12 weeks. Then the aorta was harvested and stained for 5 minutes in 0.5% Sudan IV, and destained for 5 minutes in 70% ethanol. The stained aortas were placed on a glass slide and photographed with a QColor3 Digital Camera mounted on an Olympus stereo microscope. Lesion areas were measured by Photoshop and ImageJ as reported previously.

Immunohistochemistry
The abdominal aorta, from superior mesenteric artery to just below renal artery, was dissected and embedded in OCT medium and frozen on dry ice. The aorta containing plaque was sectioned with a 7μm thickness and 6-8 sections, each 70μm apart, were mounted on the slide. The slides were fixed in acetone in -20°C for 10 minutes and then incubated with antibodies as indicated below.

Trichrome staining
Collagen and necrosis area in the plaque of abdominal aorta was stained using Trichrome Stain kit (Sigma-Aldrich Inc., St. Louis, MO).

Vascular reactivity studies
All reagents were obtained from Sigma-Aldrich and dissolved in distilled and double filtered water. Vessel reactivity studies were performed in a pressure myograph system (Danish Myo Technologies, Denmark) as described previously. The left carotid arteries were isolated and mounted onto glass cannulas and maintained at 85mmHg pressure at 37°C in physiologic saline-HEPES buffer oxygenated with 95% O2 and 5% CO2. Vessels were constricted with 10⁻⁵ M phenylephrine followed by stepwise addition of ACh to generate vasodilator response curves. After basal NO production was blocked by
applying L-NAME (3×10⁻⁴ M, 30 minutes), vasodilator response curves were generated by stepwise addition of SNP.

Western blotting
Mouse lung endothelial cells were incubated with DMEM containing 0.1% BSA for 16 hours and then were stimulated with 100nM insulin for 5 minutes for detecting eNOS and Akt phosphorylation. The cells were incubated with 100nM insulin for 48 hours or 100nM PMA for 6 hours for detecting VCAM-1 protein. To observe the actions of insulin on eNOS phosphorylation in vivo, insulin (10mIU/g body weight) was administered to mice via intravenous injection and femoral arteries were harvested at 5 minutes after insulin injection. Protein extraction and immunoblot procedures were performed as reported previously. Protein samples were separated by electrophoresis in a 7% Tris-HCl polyacrylamide gel and transferred to a PVDF membrane, which was blocked with 5% non-fat dry milk in Tris-buffered saline-0.1% Tween-20 and incubated with primary antibody in 4°C overnight. Detection was carried out using an ECL Plus Western Blotting Detection kit (Thermo Scientific, Rockford, IL). Quantitative densitometry was performed using ImageJ. Antibody directed against human S1177 phospho-eNOS and eNOS was obtained from BD Transduction Laboratories.

Real-time PCR analysis
1μg mRNA was used to generate cDNA using high capacity cDNA reverse transcription kit (Applied Biosystems, Grand Island, NY). Gene expression level was normalized to the expression level of 36B4. PCR primers were: mouse PKCβ2 isoform, forward 5’-AGGGATTCCAGTGTCAAGTCTGCT-3’, reverse 5’-GGACTGGAGTGACGTGCTTGCT-3’, mouse ET-1, forward 5’-GTGTCTAATCTGACCACCTG-3’, reverse 5’-CCTGACTGACATCTAACTGCGCTG-3’, mouse VCAM-1, forward 5’-TCTGAACCCAAACAGAGGCAGAGT-3’, reverse 5’-AGCTGGTATCCCATCACTTGAGGA-3’, mouse 36B4, forward 5’-GCTCCAAGCAGATGCAGCA-3’, reverse 5’-CCGGATGTGAGGCAGCAGCAG-3’.

Leukocyte-endothelial cell adhesion
Mouse lung endothelial cells (10⁵ cell/well) were cultured for 48 hours and incubated with vehicle, PMA (100nM) and insulin (100nM) for 16 hours in DMEM (37°C, 5% CO2). After treatment, the medium was removed and endothelial cells were washed with PBS. Raw264.7 cells labeled with Leuko Tracker (Cell Biolabs Inc. San Diego, CA) were added using 10⁶/cells per well and allowed to adhere for 30 minutes. Non-adherent cells were removed by washing with PBS. After lysing cells, fluorescence was measured by a fluorescence plate reader at 480 nm/520 nm.

Angiotensin II infusion
In mice 8 to 10 weeks of age, a catheter was placed in the right jugular vein. The animals were allowed to recover from surgery for 3 days. Angiotensin II was infused at 1000ng/kg body weight/min for 3 hours. PBS was infused in vehicle group. The aorta was isolated for measurement of ET-1 and Big ET-1 expression.

Measurement of aorta Big ET-1
Aortas were homogenized with RIPA buffer and Big ET-1 in the lysis was measured with an ELISA kit (Biotang Inc., Waltham, MA).

Measurement of ET-1
Mouse lung endothelial cells were cultured in DMEM containing 0.1% BSA for 24 hours and then the medium was collected for the measurement of ET-1 with an ELISA kit (Enzo Life Sciences, Farmingdale, NY). The value of ET-1 was normalized by the total protein extracted from the cells growing in the same well.
Cell death ELISA

Mouse lung endothelial cells were with or without withdrawing growth factors for 24 hours and DNA fragmentation was determined by using Cell Death ELISA kits (Roche, Indianapolis, IN).
References


Supplemental legends

Supplemental Figure I. Insulin signaling and endothelial apoptosis.
(A-B), Tyrosine phosphorylation of insulin receptor β was stimulated by 100 nM insulin in MLECs from ApoE⁻/⁻ or Tg(Prkcb)ApoE⁻/⁻ mice. A. Representative western blots show tyrosine phosphorylation of insulin receptor β. B, mean values of tyrosine phosphorylation of insulin receptor β (n=3 for each group).
(C-D), ERK phosphorylation was stimulated by 100nM insulin in MLECs from ApoE⁻/⁻ or Tg(Prkcb)ApoE⁻/⁻ mice. C. Representative western blots show ERK phosphorylation. D, mean values of phosphorylation of ERK (n=8 for each group). E, Apoptosis induced by withdrawing growth factors were determined by Cell Death ELISA (n=3 for each group).

Supplemental figure II, Characteristics of Tg(Prkcb)ApoE⁻/⁻ mice.

Supplemental Figure III. Insulin-induced eNOS phosphorylation and VCAM-1 expression in aorta endothelial cells.
(A-B) aorta endothelial cells were stimulated with 100nM insulin and eNOS phosphorylation at Ser1176 was determined by western blotting (ApoE⁻/⁻ unstimulated n=8, ApoE⁻/⁻ insulin-stimulated n=11, Tg(Prkcb)ApoE⁻/⁻ unstimulated n=5, Tg(Prkcb)ApoE⁻/⁻ insulin stimulated n=9). (C-D), VCAM-1 expression in aorta endothelial cells. VCAM-1 expression in aorta endothelial cells was determined by western blotting. C, Representative western blots show VCAM-1 and actin expression. D, mean values of the ratio of VCAM-1 to actin (n=3 for each group).
Supplemental Figure II

A. Body weight (gram)

B. Blood pressure (mmHg)

C. Cholesterol (mg/dl)

D. Triglyceride (mg/dl)

E. Cholesterol (mg/fraction) vs. Fraction Number

F. Glucose mg/dl vs. Time (min)

G. Glucose mg/dl vs. Time (min)
Supplemental Figure III

A

p-eNOS

eNOS

Insulin

- + + + - + + +

ApoE<sup>-/-</sup> Tg(Prkcb)ApoE<sup>-/-</sup>

B

p<0.05 p<0.05

p-eNOS/eNOS

Insulin

- + - +

ApoE<sup>-/-</sup> Tg(Prkcb)ApoE<sup>-/-</sup>

C

VCAM-1

actin

ApoE<sup>-/-</sup> Tg(Prkcb)ApoE<sup>-/-</sup>

D

p<0.05

VCAM-1/actin

ApoE<sup>-/-</sup> Tg(Prkcb)ApoE<sup>-/-</sup>