Original Research *Feature article*

Inhibition of protein kinase C beta phosphorylation activates nuclear factor-kappa B and improves postischemic recovery in type 1 diabetes

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Impact statement

Diabetes worsens the outcomes of peripheral arterial disease (PAD) likely in part through inducing chronic inflammation. However, in PAD, recovery requires the nuclear factor-kappa B (NF-κB) activation, a known contributor to inflammation. Our study shows that individually, both ischemia and high glucose activate the canonical and non-canonical arms of the NF-κB pathways. We show for the first time that prolonged high glucose specifically impairs ischemia-induced activation of the canonical NF-κB pathway through activation of protein kinase C beta (PKCβ). Accordingly, inhibition of PKCβ restores the ischemia-induced NF-κB activity both *in vitro* in endothelial cells and *in vivo* in hind limbs of type 1 diabetic mice and improves perfusion recovery after experimental PAD. Thus, this study provides a mechanistic insight into how diabetes contributes to poor outcomes in PAD and a potential translational approach to improve PAD outcomes.

Abstract

Peripheral artery disease (PAD) is a major health problem and is caused by atherosclerosis in arteries outside the heart leading to impaired blood flow. The presence of diabetes significantly increases the likelihood of having worse outcomes in PAD, and the molecular mechanisms involved are poorly understood. Hyperglycemia in diabetes activates the nuclear factor-kappa B (NF-κB) pathway, and chronic inflammation in diabetes is associated with vascular complications. Ischemia also activates NF-κB signaling that is important for perfusion recovery in experimental PAD. We hypothesized that prolonged exposure of endothelial cells to high glucose in diabetes impairs ischemic activation of the NF-κB pathway and contributes to poor perfusion recovery in experimental PAD. We assessed the effect of high glucose and ischemia on canonical and non-canonical NF-κB activation in endothelial cells and found both conditions activate both pathways. However, exposure of endothelial cells to high glucose impairs ischemia-induced activation of the canonical NF-κB pathway but not the non-canonical pathway. We probed an array of antibodies against signaling proteins in the NF-κB pathway to identify proteins whose phosphorylation status are altered in endothelial cells exposed to high glucose. Protein kinase C beta (PKCβ) was among the proteins identified, and its role in impaired ischemia-induced activation of NF-κB during hyperglycemia has not been previously described. Inhibition of PKCβ improves ischemia-induced NF-κB activation *in vitro* and *in vivo*. It also improves perfusion recovery in diabetic mice following experimental PAD. Thus, in diabetes, PKCβ phosphorylation contributes to impaired ischemic activation of NF-κB and likely a mechanism contributing to poor PAD outcomes.

Keywords: Nuclear factor-kappa B, ischemia, hyperglycemia, diabetes, protein kinase C beta, peripheral arterial disease

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Introduction

Peripheral artery disease (PAD) is a chronic disease of blood vessels that affects approximately 12 million people in the United States and over 200 million people worldwide.[1] There are two classic clinical presentations of PAD, intermittent claudication (lower extremity pain with ambulation relieved by rest) and critical limb ischemia (CLI, pain at rest that may be associated with ulceration or gangrene). Risk factors for development of PAD are the same for development of coronary artery disease and include smoking, diabetes, hypertension, and hyperlipidemia.[2] However, diabetes and smoking account for 80% of the risk of developing PAD. Moreover, individuals with PAD and diabetes are five times more likely to develop CLI.[2]

Prior studies by our lab and others have shown that in preclinical models of PAD (experimental PAD), diabetes impairs postischemic angiogenesis and perfusion recovery.[3] Although some studies have provided mechanistic insight into how diabetes may impair perfusion recovery in experimental PAD, the molecular mechanisms involved remains poorly understood.[3] Hyperglycemia is a key metabolic derangement in diabetes. It is also known that inflammation plays a key role in many of the pathologic processes associated with diabetes complications.[4] NF-κB is a key transcription factor involved in inflammation,[4] and studies have shown that high glucose can activate NF-κB signaling.[5,6] The NF-κB transcription factors bind to their target DNA sequences as dimers composed of the members of the NF-κB family. These members include RelA (also named p65), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100), which form various combinations of dimers to transactivate target genes. The p105 and p100 proteins are precursors to p50 and p52 subunits, respectively.[7,8] Inducer-mediated canonical activation of NF-κB is accomplished by phosphorylation and degradation of Iκ-Bα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) leading to nuclear localization of the NF-κB complex that binds to the IκB sequence of the target genes.[9] In a non-canonical activation, RelB/p52 dimer complex is activated.[10] The p100 protein functions in similar manner as IκBα to inhibit RelB nuclear translocation. An inducible processing of p100 whereby the C-terminal of the p100 is proteolyzed yields the p52 subunit, and the RelB/p52 dimers translocate to the nucleus to function as transcription factors.

Therefore, it is possible that the NF-κB pathway may be involved in the impaired perfusion recovery observed following experimental PAD in the context of diabetes. Interestingly, studies have also shown that ischemia activates NF-κB,[11,12] and this signaling pathway is a key regulator of arteriogenesis, collateral formation as well as tissue perfusion following experimental PAD.[13] However, how high glucose activation of the NF-κB pathway in diabetes impacts activation of the same pathway by ischemia is not clear. Moreover, whether the canonical and non-canonical pathways play differing roles in postischemic perfusion recovery is not known. We hypothesized that prolonged exposure of endothelial cells to high glucose in diabetes leads to chronic activation of the NF-κB pathway that impairs ischemia-induced activation of the pathway and contributes to poor perfusion recovery following experimental PAD. We conducted a series of *in vitro* and *in vivo* studies to test this hypothesis.

Materials and methods

Animal studies

All experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Sciences Center. Mice (C57BL/6J, Catalog # 000664, and C57BL/6J-Ins2Akita/J, Catalog # 003548) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The Ins2Akita is a well-studied model of type 1 diabetes.[14,15] Mice had free access to water and standard rodent chow. The PKCβ inhibitor, ruboxistaurin (Rbx; LY333531, Cat# HY-10195B, MedChemExpress, Princeton, NJ, USA), was administered to mice at 1 mg/kg/day by oral gavage starting 5 days prior to induction of hind limb ischemia (HLI) and continued through the study.[16]

HLI or experimental PAD

HLI was induced by unilateral ligation and excision of the femoral artery as previously described.[17] Control mice were matched for strain, sex, and age. Diabetes was confirmed in mice by Hba1c measurement (Hba1c >8) prior to the experiments. Both experimental and control groups had similar glycemic exposure as determined by HbA1c. Ischemia was confirmed by monitoring postsurgical blood flow in the ischemic and contralateral non-ischemic limbs by Laser Speckle Contrast Imaging (Perimed AB, Sweden) as described previously.[18,19] Measurements of perfusion recovery were performed immediately after HLI surgery, (day 0) and then on day 3, and once weekly for 2 weeks.

Cell cultures

Pooled human vascular endothelial cells (HUVECs) between passage 4 to 7 were used (Cell Applications, Cat # 200p-05n, San Diego, CA, USA). Cells were grown in endothelial cell growth medium (ECGM, Cell Applications, Inc., San Diego, CA, USA) supplemented with 10% fetal bovine serum (FBS, Cat # 10437028, Gibco-Thermo Fisher Scientific, Waltham, MA, USA) under standard culture conditions (20% O_2) or 24 h in ischemic conditions (2% O_2 without FBS, Supplemental Material). Ischemia was simulated *in vitro* as previously described by culturing cells in ECGM without serum and in 2% oxygen (BioSpherix, Lacona).[20] Hyperglycemic conditions were created by supplementing the ECGM with 25 mM D-glucose (normal glucose concentration 5 mM D-glucose). Inhibition of PKCβ was achieved by addition of Rbx at 20 nm/L, 30 min before adding high D-glucose to ECGM.[16]

Protein analysis

Cell and tissue lysates were prepared as described previously.[21,22] Nuclear extracts were prepared using a commercially available kit (NE-PER, ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Protein expression was measured by Western blot analysis using anti-IκBα (Cat#9242 CST), anti-phospho-IκB (Cat#9246, CST), anti-phospho-p65 (Cat#3036, CST), anti-p65 (Cat# 3033 CST), anti-RelB antibody (Cat#4922 CST), anti-cRel antibody (Cat#12707 CST), anti-pSer661-PKCβ (Cat# DBOA15543, Vita Scientific), and anti-PKCβ1/2 Cat# 46809 (Cell Signaling Technology, Danvers, MA, USA) primary antibodies. Western blot gel images were captured by Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Quantification of the protein bands on blots were performed by Scion Image software (Torrance, CA, USA) and

Image Studio Lite version 5.2 (Li-COR Biotechnology, Lincon, Nebraska). Abundance of phosphoproteins was determined as the ratio of band intensity of phosphoprotein to the total protein. For loading control, antibody to β-actin was used. For blots involving non-ischemic and ischemic samples, Ponceau S-stained protein bands in the respective lanes were used as loading controls since ischemia alters expression of β-actin and many typical loading controls.

Phosphoprotein array

A high throughput platform of antibodies array was used to detect differences in phosphorylation of signaling molecules involved in the NF-κB pathway (NF-κB Phospho Antibody Array, Cat# PNK215, Full Moon Biosystems, Sunnyvale, CA, USA). Cell extracts from HUVEC exposed to either 25 mM L-glucose or 25 mM D-glucose were analyzed for the phosphoprotein levels according to the manufacturer's suggested protocol. Fluorescence signals were recorded and digitized using a microarray scanner and analyzed by manufacturer's recommendations using ImageJ.[23] Following background corrections, the signals were normalized against the median intensity of all the experimental spots on the array. The fold change in phosphorylation was calculated by dividing the intensity of the phosphorylated spot by the signal intensity of corresponding non-phosphorylated spot for each protein. Differential expression between the 25 mM L-glucose and 25 mM D-glucose samples was calculated by dividing the phosphorylation ratio of the 25 mM D-glucose with that of the 25 mM L-glucose control. Significant expression was taken as greater than 1.4- or less than 0.6-fold. This experiment was carried out with three samples each per experimental group, and the array contained six spots for each antibody.

Statistical analysis

All measurements are expressed as mean ± SEM. Statistical comparisons between two groups (e.g. treated vs. untreated) were performed by *t*-test; where more than two groups were involved, we used one-way analysis of variance. A P value of <0.05 was considered statistically significant.

Data and resource availability

The data-sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Results

Ischemia activates canonical and non-canonical pathways of NF-κB in endothelial cells

We first determined whether ischemia induces the canonical or non-canonical pathway of NF-κB activation in cultured HUVEC. Cultured cells were exposed for 24 h to either normal or ischemic conditions followed by preparation of cytoplasmic and nuclear fractions. Western blot analyses of the cytoplasmic fraction showed that compared to the cells grown under normal conditions, the cells exposed to ischemia had a significant decrease in IκBα protein, an indicator of the activated canonical NF-κB pathway (Figure 1(a) and (b); fold change normoxic to ischemic; 1.0 ± 0.02 vs. 0.67 ± 0.03, $n = 6$, $P < 0.05$). Analyses of the

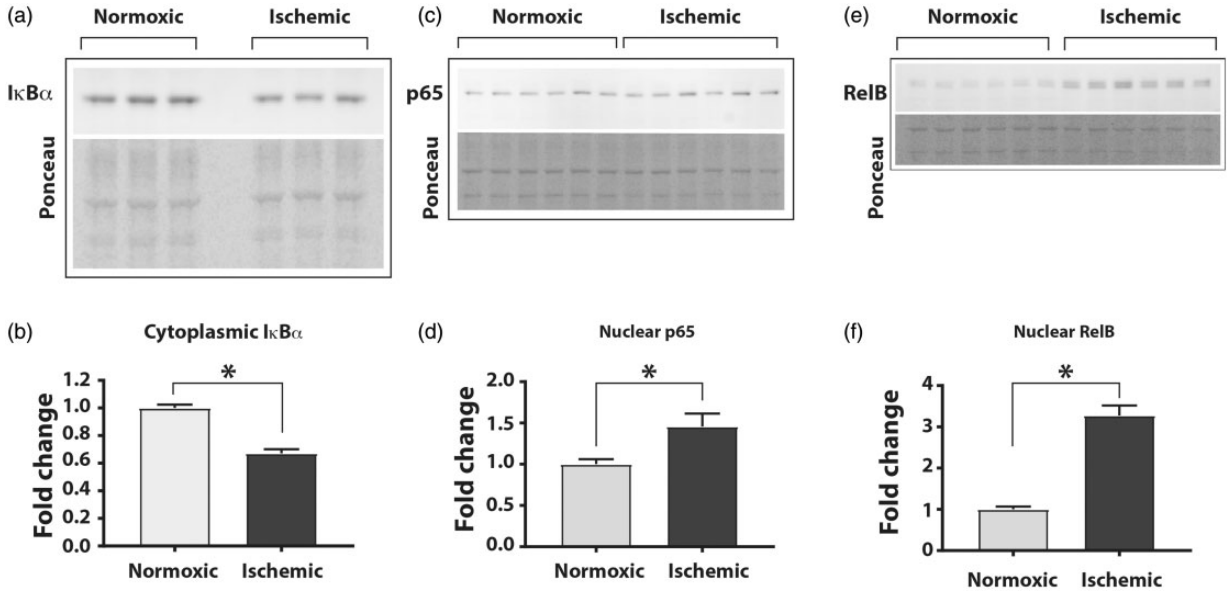

Figure 1. Ischemia activates the canonical and non-canonical NF-κB pathways in HUVEC. Cells were exposed to either normoxia (21% O_2) or ischemia (2% oxygen and nutrient starvation) for 24 h followed by isolation of cytoplasmic and nuclear fractions. (a) The cytoplasmic fractions were analyzed for expression of IκBα by Western blots using specific antibody, and (b) signals were quantified using densitometry by measuring the ratio of band intensity to total protein in the corresponding lanes of the Ponceau S-stained blots. (c) Western blots of the nuclear fractions were probed with anti-p65/RelA antibody and (d) signal quantified by ratio of p65 band intensity to total protein in the lanes, (e) Western blot for non-canonical pathway using an anti-RelB antibody and (f) quantitation of the signal intensity. The total protein from the respective lanes of Ponceau S-stained blots was used. Unpaired *t*-test with Welch's correction and two-tailed distribution was used for statistical calculations. Asterisks denote $P < 0.05$, and $n =$ 6 to 12 in each group.

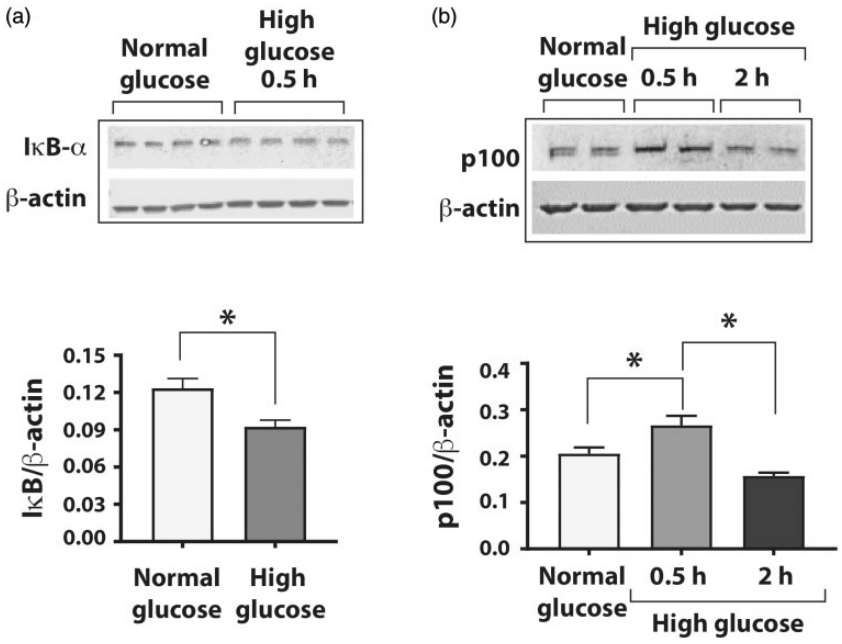

Figure 2. High glucose activates both canonical and non-canonical NF-κB pathways in HUVEC. Cells were exposed to normal D-glucose (5 mM), high D-glucose (25 mM), or high L-glucose (25 mM) for varying durations, and cell lysates were prepared for Western blot analyses for either degradation of IκBα (the canonical pathway) or of p100 (non-canonical pathway) using specific antibodies. Signals generated by antibody to β-actin were used for normalizing the results. (a) Western blot of total lysates from endothelial cells exposed to normal D-glucose or high D-glucose for 0.5 h showing signals for IκBα and β-actin. Changes in protein levels of IκBα in cells exposed to high D-glucose are presented as ratio of IκBα/β-actin. (b) High glucose exposure induced the non-canonical NF-κB pathway by degradation of p100 subunit after 2 h of high D-glucose exposure. The graph shows reduced p100 on the Western blot after 2 h of high D-glucose exposure. Asterisks denote $P < 0.05$, and $n =$ 5 to 14 in each group.

cell lysates from cells exposed to ischemia showed no change in p65 expression (Supplemental Figure 1a and b) but a significant increase in nuclear abundance of p65 subunit of the NF-κB (Figure 1(c) and (d); fold change normoxic to ischemic 1.0 ± 0.06 vs. 1.45 ± 0.15, $n = 6$, $P <$ 0.05). Additionally, expression of total RelB, a component of the non-canonical NF-κB pathway, was significantly increased in cell lysates (Supplemental Figure 1a and b, bottom left panel) and also in the nuclear fractions of the cells exposed to ischemia (Figure 1(e) and (f); fold change normoxic to ischemic 1.0 ± 0.16 vs. 3.27 ± 0.5, $n = 6$, $P < 0.01$). However, the total level of c-Rel was not changed in ischemic cells (Supplemental Figure 1a and b). Thus, ischemia induced activation of both canonical and the non-canonical pathways of the NF-κB in cultured endothelial cells.

Short-term exposure to high glucose activates both canonical and non-canonical NF-κB pathways

Hyperglycemia is known to cause inflammatory changes in endothelial cells through activation of the NF-κB pathways.[24] However, the duration of exposure necessary for activation varies in different studies.[25,26] We assessed the effects of short and prolonged exposures of HUVECs to high glucose on the activation of canonical and non-canonical NF-κB pathways. Western blot analyses showed that compared to the HUVEC grown in normal glucose, the cells grown in high glucose concentration for 0.5 h had significantly decreased IκBα levels (Figure 2(a); IκBα/β-actin 0.120 ± 0.004 vs. 0.100 ± 0.003, $n = 4$, $P < 0.05$), which was restored after 2 h (Supplemental Figure 2). Exposure of HUVEC to high glucose decreased the p100 processing (a measure of non-canonical NF-kB pathway activation) at 0.5 h but significantly increased the degradation of p100 at 2 h compared to the cells in normal glucose (Figure 2(b); p100/β-actin 0.20 ± 0.01 in normal glucose vs. 0.27 ± 0.01 in high D-glucose at 0.5 h vs. 0.16 ± 0.00 in high D-glucose at 2 h, $n = 4$, $P < 0.05$). Thus, high glucose activates both canonical and non-canonical NF-κB pathway with the activation of the canonical pathway preceding the non-canonical pathway.

Prolonged high glucose stimulation impairs ischemia-induced IκBα phosphorylation and degradation in endothelial cells

To determine the impact of high glucose on ischemia-induced NF-κB activation, we assessed the extent of IκBα phosphorylation and degradation. HUVEC cultures were grown for three days in normal D-glucose, high D-glucose, or in metabolically inert high L-glucose and subjected to ischemia for 24 hours. Analyses of cellular lysates by Western blots showed a significant increase in the phosphorylation of IκBα protein (pIκBα) from ischemic cells cultured in normal glucose and those in high L-glucose but not in the lysates from the ischemic cells cultured in high D-glucose (Figure 3(a) and (b); pIκBα/IκBα in normal glucose 1.59 ± 0.08; high L-glucose 1.36 ± 0.17, high D-glucose 1.0 ± 0.05, $n = 4$, $P < 0.05$).

Consistent with the above findings, IκBα levels decreased in ischemic cells grown in normal D-glucose and high L-glucose but not in high D-glucose (Figure 3(c) and (d); fold change normoxic vs. ischemic 0.68 ± 0.06 in

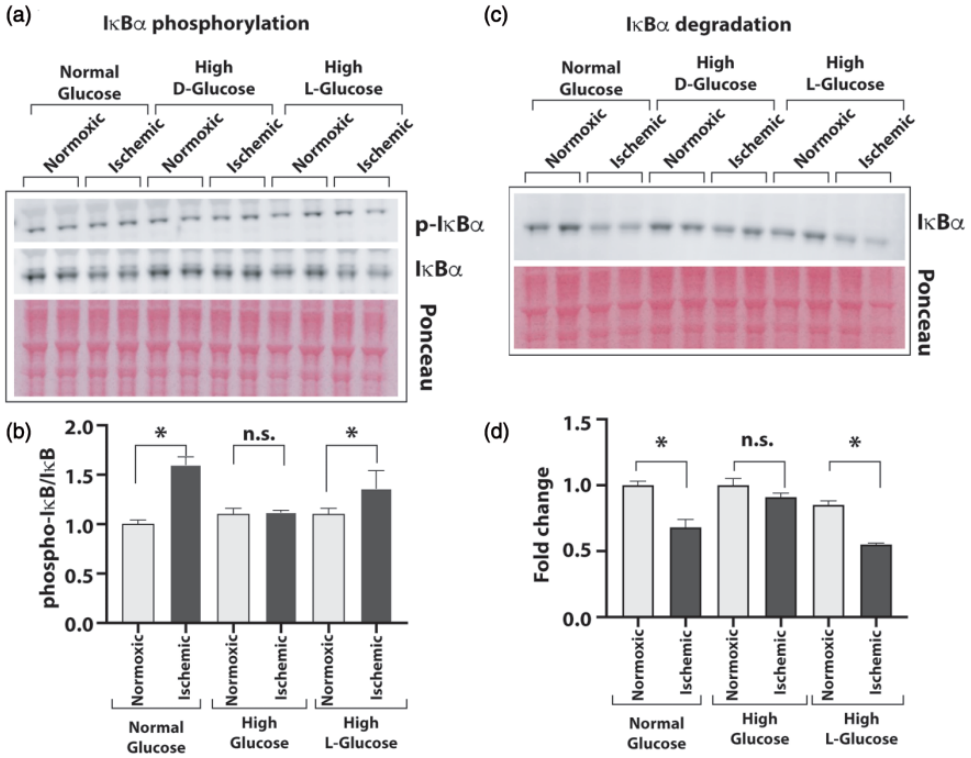

Figure 3. High glucose exposure attenuates the ischemia-induced NF-κB activation. HUVEC were grown in normal (5 mM) or high D-glucose (25 mM) containing culture medium before exposing them to ischemia and analyzing cell lysates for IκBα phosphorylation and degradation to measure the activation of NF-κB. Control cells were also grown in high concentration of metabolically inert L-glucose (25 mM) in these experiments. (a) Western blots of phosphorylated (p-IκBα) and unphosphorylated (IκBα) forms of IκBα protein from cells grown in normal, high D-glucose, or high L-glucose with normal (normoxic) or ischemic treatments. (b) Intensity of protein bands on Ponceau S-stained blots was used for loading control. Protein quantification of IκBα was done by densitometry, and results are presented in the bar graph as the ratio of p-IκBα to unphosphorylated form. (c) Degradation of IκBα was tested by Western blot of the HUVEC cultures in normal or high glucose conditions using specific antibody. Signals from Ponceau S-stained the blot were used for loading controls. (d) The ratio of IκBα to total protein is presented in the bar graph. Pair-wise comparisons were done between normoxic and ischemic samples in each group for statistical analyses. Asterisks denote $P < 0.05$, and n.s. denotes not significant; $n = 5$ to 14 in each group. (A color version of this figure is available in the online journal.)

normal glucose, 0.55 ± 0.03 in high L-glucose and 0.91 ± 0.03 in high D-glucose, $n = 3$–4, $P < 0.05$). These results suggest that hyperglycemic condition impairs the phosphorylation and degradation of IκBα, thus impairing the ischemia-induced activation of the NF-κB pathway in endothelial cells.

Prolonged high glucose stimulation impairs canonical NF-κB pathway activity but not the non-canonical NF-κB pathway activity in endothelial cells

We next examined whether prolonged high glucose exposure dampens the activation of both the canonical and the non-canonical NF-κB pathway. Western blots analyses showed significant increase of p65 subunit (the canonical pathway) in the nuclear extracts from ischemic cells grown either in normal D-glucose or in high L-glucose but not in high D-glucose (Figure 4(a); nuclear p65/Ponceau: ischemic to non-ischemic 1.81 ± 0.19 vs. 1.00 ± 0.05 in normal D-glucose, 2.11 ± 0.10 vs. 1.11 ± 0.08 in high L-glucose and 1.31 ± 0.09 vs. 1.08 ± 0.06 in high D-glucose, $n = 6$–8, $P < 0.05$). However, analysis of nuclear RelB subunit (the non-canonical pathway) was increased in all three condition (Figure 4(b); nuclear RelB/Ponceau: ischemic to non-ischemic 1.61 ± 0.15 vs. 1.00 ± 0.05 in normal D-glucose, 1.62 ± 0.17 to 0.96 ± 0.05 in high D-glucose, 1.88 ± 0.23 vs. 1.07 ± 0.11 in high L-glucose, $n = 6$–7, $P < 0.05$). Thus, hyperglycemic conditions impair ischemia-induced activation of the canonical NF-κB pathway but not the non-canonical pathway in endothelial cells.

High glucose exposure causes hyperphosphorylation of PKCβ in non-ischemic and ischemic endothelial cells

To further understand how hyperglycemia attenuates ischemia-induced NF-κB activation in HUVECs, we used a high-density array of 215 antibodies to compare the phosphorylation states of proteins involved in NF-κB activation. We used lysates from HUVECs that were grown in either high L-glucose or high D-glucose for three days. Comparison of the protein phosphorylation showed that phosphorylation of several signaling proteins was increased in the high D-glucose-grown cells, while phosphorylation of several other proteins was decreased (Figure 5(a)). Histone deacetylase 5 (HDAC5^{pSer259}), protein kinase B (AKT^{pThr308}), and PKCβ^{pSer661} ranked among the highest phosphorylated proteins under the high D-glucose conditions. A literature-based analysis identified PKCβ to be implicated in hyperglycemic toxicity and endothelial dysfunction.[27,28] Hence, we selected PKCβ for a detailed study.

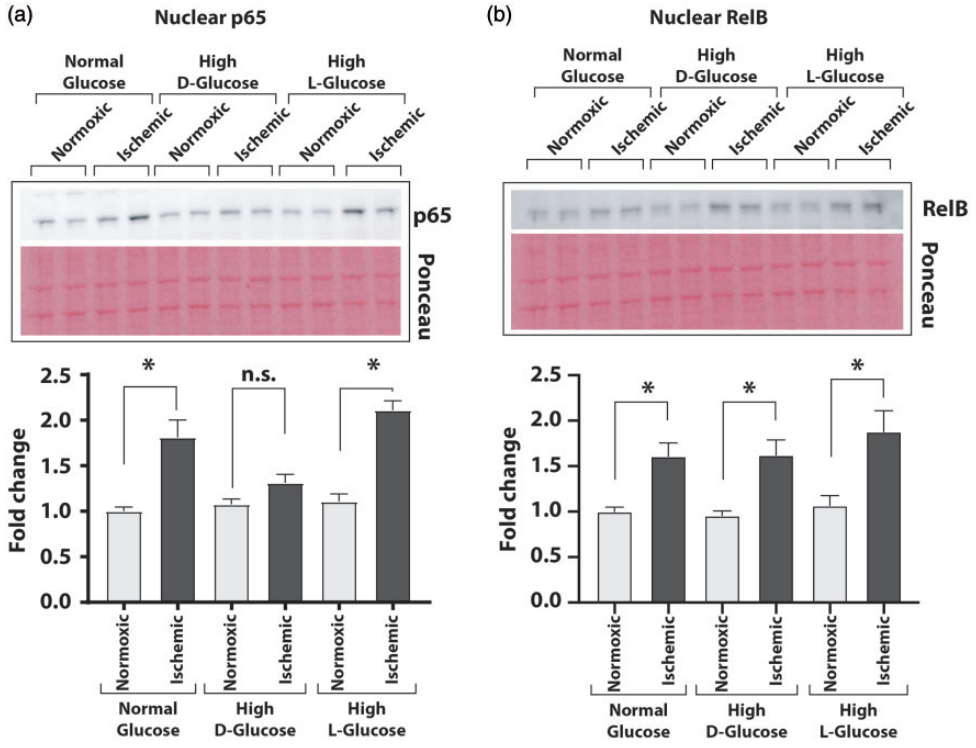

Figure 4. High D-glucose condition impairs the canonical pathway of ischemia-induced NF-κB activation. Nuclear fractions from HUVEC grown in normal or high D-glucose containing medium were analyzed by Western blotting for enrichment of NF-κB subunits, and quantified results of densitometry are presented in bar graphs. (a) Compared to the nuclear extracts of the normoxic cells, the nuclear fractions of the ischemic cells contained significantly greater amounts of p65 subunit of the NF-κB when grown in normal D-glucose. The p65 subunit did not accumulate in the nuclear fractions of ischemic cells grown in high D-glucose. Nuclear enrichment of p65 was also seen in the ischemic cells grown in high L-glucose-containing medium. (b) RelB subunit was significantly more enriched in the nuclear fraction of the ischemic HUVEC grown in normal D-glucose, high L-glucose, or high D-glucose than in the nuclear fractions of the normoxic cells. Results in the graphs are from densitometry of Western blot signals and are presented as the ratio of target protein signal to Ponceau S-stained total protein signal on blots. Asterisks denote $P < 0.05$, and n.s. denotes not significant; $n = 4$ to 6 per group. (A color version of this figure is available in the online journal.)

Western blot comparisons of PKCβ^{pSer661} in the lysates from HUVECs confirmed increased phosphorylation of PKCβ under high D-glucose growth condition (Figure 5(b) and (c); PKCβ^{pSer661}/PKCβ in normal glucose vs. high glucose 0.85 ± 0.16 vs. 8.76 ± 1.85, $n = 4$, $P < 0.01$). This increase in PKCβ^{pSer661} coincided with increased IκBα protein (Figure 5(b) and (d); IκBα/β-actin, 0.28 ± 0.05 in normal D-glucose vs. 0.69 ± 0.04 in high D-glucose, $n = 4$, $P < 0.01$) likely because of reduced degradation of IκBα protein.

We next analyzed whether hyperphosphorylation of PKCβ^{pSer661} is associated with impaired ischemia-induced NF-κB activation in endothelial cells following prolonged high glucose stimulation. Lysates from HUVECs exposed to ischemia in the presence of normal D-glucose, high D-glucose, or high L-glucose were analyzed by Western blotting. There was increased PKCβ^{pSer661} in the high D-glucose-treated cells compared to the lysates from normal D-glucose or high L-glucose-treated cells (Figure 5 (e) and (f); PKCβ^{pSer661}/PKCβ, 4.85 ± 1.06 in normal glucose vs. 11.07 ± 0.72 in high D-glucose and 5.03 ± 0.71 in high L-glucose, $n = 3$–4, $P < 0.05$). Moreover, this was associated with higher levels of IκBα, consistent with impaired activation of the NF-κB pathway (Figure 5(e) and (g); IκBα/β-actin, 18.21 ± 1.03 in normal glucose vs. 26.47 ± 2.46 in high D-glucose, and 20.02 ± 1.78 in high L-glucose, $n = 3$–4, $P < 0.05$). These results suggest that high D-glucose induced phosphorylation of PKCβ on the Ser661 residue and impaired ischemia-induced NF-κB activation.

Inhibition of PKC$\beta^{pSer661}$ restores ischemic activation of NF-κB under hyperglycemia

We hypothesized that inhibiting the phosphorylation of PKCβ would restore impaired IκBα degradation and augment NF-κB activation. To test this hypothesis, we cultured HUVEC in normal D-glucose or high D-glucose medium in the presence or absence of a specific inhibitor of PKCβ, Rbx (20 nM Rbx)[29] and subjected the cells to ischemia. Compared to the cells grown in normal D-glucose plus ischemia, cells grown in high D-glucose plus ischemia showed significantly increased PKCβ^{pSer661} (Figure 6(a) and (b); PKCβ^{pSer661}/PKCβ 4.40 ± 0.45 in normal D-glucose plus ischemia vs. 10.16 ± 1.56, $P = 0.0001$ in high D-glucose plus ischemia, $n = 3$–4, $P < 0.05$). The PKCβ^{pSer661} levels in high L-glucose plus ischemia were not different from those in normal glucose plus ischemia (data not shown). However, in cells grown in high D-glucose medium in the presence of Rbx, the PKCβ^{pSer661} levels reverted to normal levels (Figure 6(a) and (b), PKCβ^{pSer661}/PKCβ 4.40 ± 0.45 in normal D-glucose plus ischemia vs. 4.00 ± 0.80 in high D-glucose plus ischemia and Rbx, $n = 3$–4, $P < 0.05$).

We also analyzed the activation of the NF-κB pathway by measuring abundance of IκBα protein in these lysates.

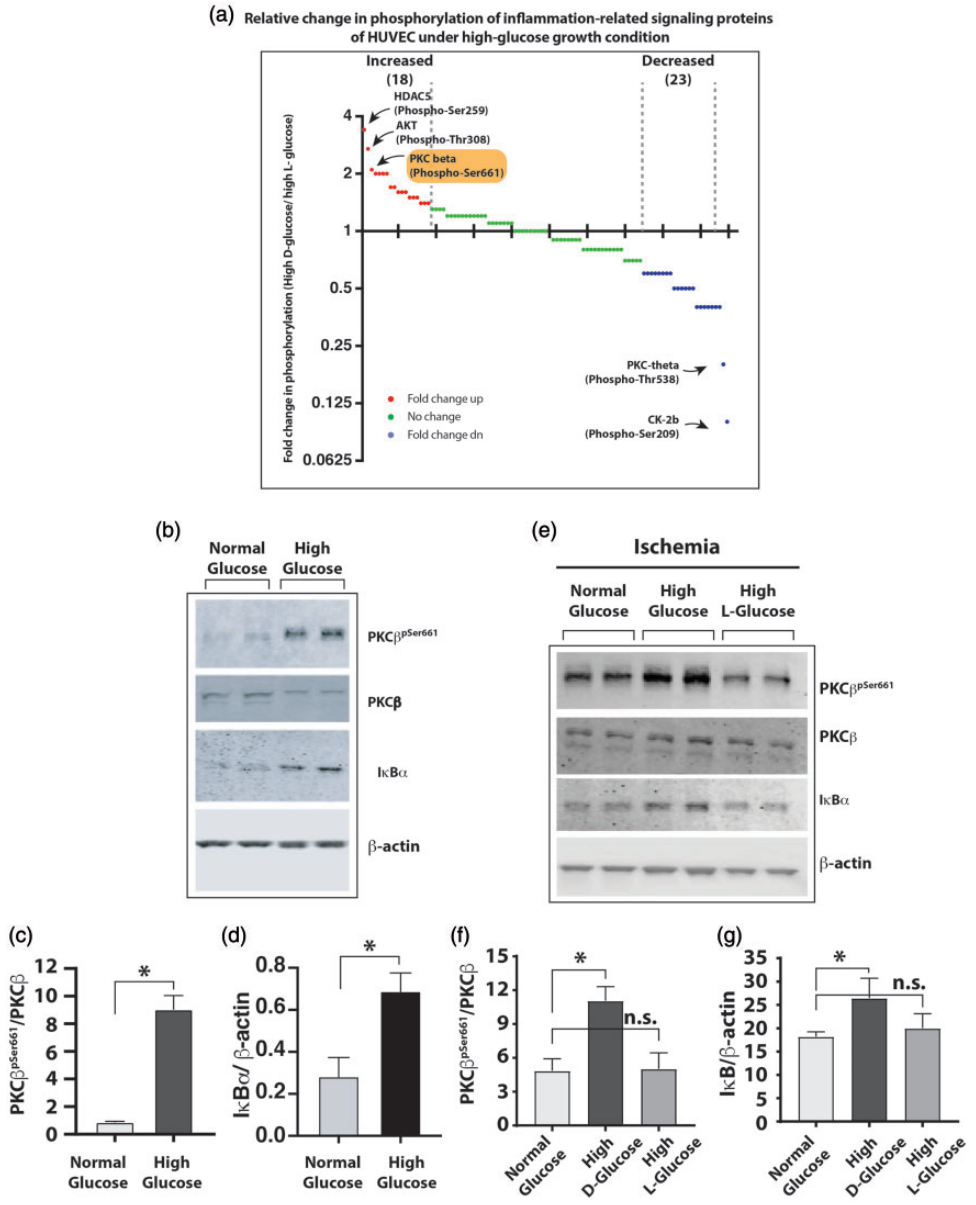

Figure 5. (a) Antibody array-based screening of relative change in phosphorylation state of proteins involved in the NF-κB activation pathway. Glass slides with printed panel of 215 antibodies were used to detect the relative abundance of total and phosphorylated proteins in HUVEC grown in either high (25 mM) L-glucose or D-glucose for 3 days. Cell lysates were overlaid on antibody panels and signals were detected using fluorescent-tagged secondary antibody. Digitized signals were analyzed as described in Materials and methods section. The graph shows fold change in the phosphorylated protein signals as a ratio of phosphorylated/total signal from high D-glucose- to high L-glucose-grown cells. The top three greatest increase in phosphorylation is labeled on the graph. PKCβ (phospho-Ser661) signal was among the highest increase signals. (b) Western blots of HUVEC grown in high D-glucose and normal D-glucose analyzed for phosphorylated PKCβ (PKC$\beta^{pSer661}$) and accumulation of IκBα. (c) High glucose enhances phosphorylation of Serine-661 residue of PKCβ as measured by the ratio of PKC$\beta^{pSer661}$ to PKCβ. (d) High glucose impairs degradation of IκBα; the IκBα protein was significantly increased in high D-glucose-grown cells. (e) Western blots showing phosphorylated and total PKCβ and IκBα in high D-glucose-grown HUVEC subjected to ischemia. (f) PKCβ was increased in high D-glucose-grown cells with ischemia as measured by PKC$\beta^{pSer661}$/PKCβ ratio. (g) The IκBα levels also were significantly increased in ischemic cells grown in high D-glucose. Asterisks denote $P < 0.05$, and n.s. denotes not significant; $n = 4$ to 6 per group. (A color version of this figure is available in the online journal.)
HUVEC: human vascular endothelial cell; HDAC5: histone deacetylase 5; PKC: protein kinase C; AKT: protein kinase B.

Although IκBα protein levels were increased in cells grown in high D-glucose plus ischemia, treatment of cells with Rbx significantly reduced the accumulation of IκBα in cells grown in high D-glucose plus Rbx to the same levels as normal glucose plus ischemia or high L-glucose plus ischemia (Figure 6(c); IκBα/β-actin, 21.57 ± 2.86 in normal glucose plus ischemia vs. 27.93 ± 1.63 in high D-glucose plus ischemia and 19.34 ± 1.35 in high D-glucose plus ischemia plus Rbx, $n = 3$, $P < 0.05$). Thus, PKCβ inhibitor Rbx decreases hyperglycemia-induced PKCβ^{pSer661} phosphorylation and restores the activation of NF-κB through the canonical IκBα degradation. These results suggest that hyperphosphorylation of PKCβ^{pSer661} under hyperglycemic conditions impairs the activation of canonical NF-κB.

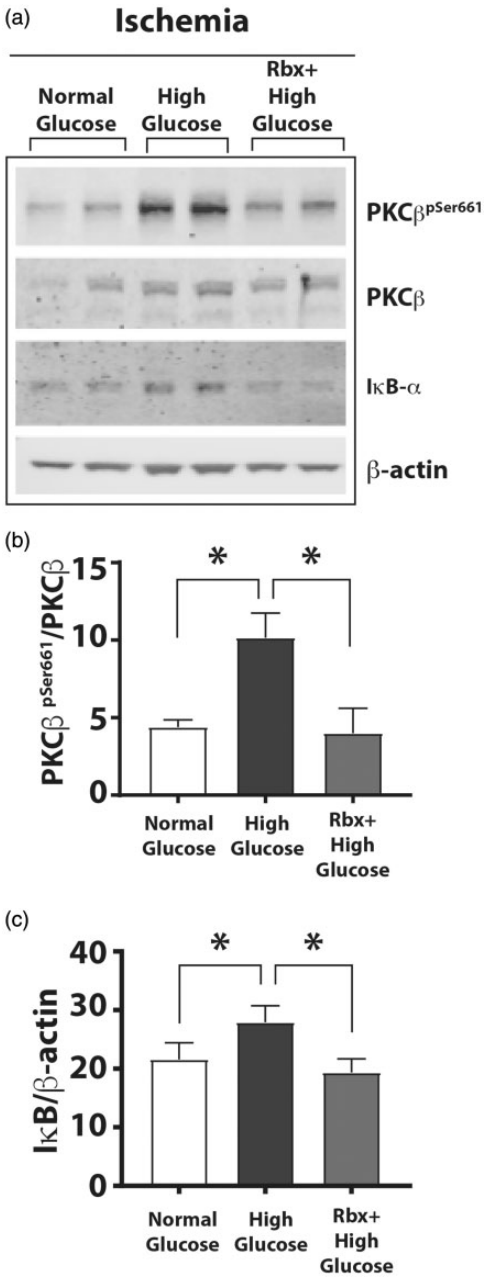

Figure 6. A PKCβ inhibitor Rbx decreased phosphorylation of Ser661 residue of PKCβ and increased IκBα degradation. HUVEC were grown in normal, high D-glucose, or high D-glucose + Rbx with ischemia for 24 h. (a) Western blots of HUVEC grown in different glucose concentrations with ischemia. (b) Autophosphorylated PKC$\beta^{pSer661}$ band showed greater intensity in cell lysates with high D-glucose that was decreased in the cells grown in the high D-glucose + Rbx (20 nM). (c) The IκBα levels (IκBα/β-actin) in the corresponding samples were increased in high D-glucose but were significantly reduced in the cells treated with Rbx. Asterisks denote $P < 0.05$, and n.s. denotes not significant; n= 4 to 6 per group.
PKCβ: protein kinase C beta; Rbx: ruboxistaurin.

Inhibition of PKCβ phosphorylation on Ser661 induces activation of NF-κB and improves perfusion recovery in mice with type 1 diabetes following experimental PAD

Given our *in vitro* findings, we tested whether the mechanism of impaired NF-κB activation from increased PKCβ^{pSer661} levels under hyperglycemic conditions was also operative *in vivo* and whether inhibition of PKCβ by Rbx would ameliorate the impaired NF-κB activation and improve perfusion recovery in hyperglycemic diabetic mice following experimental PAD. We first compared PKCβ phosphorylation status in the gastrocnemius muscle from non-ischemic hind limbs of non-diabetic and diabetic mice. The Akita mice with type 1 diabetes have increased PKCβ^{pSer661}, and Rbx administration decreased this phosphorylation of PKCβ (Figure 7(a) and (b); PKCβ^{pSer661}/PKCβ, 0.67 ± 0.03 in non-diabetic C57BL/6J vs. 1.00 ± 0.06 in diabetic Akita mice and 0.56 ± 0.02 in Akita treated with Rbx, $n = 5$, $P < 0.01$).

Next, experimental PAD was induced in diabetic Akita mice treated with Rbx or vehicle daily five days prior to the surgery. Western blot analyses of the ischemic gastrocnemius muscle of the untreated and the treated mice showed that mice treated with Rbx had decreased PKCβ^{pSer661} (Figure 7(c) and (d), PKCβ^{pSer661}/PKCβ, 1.10 ± 0.06 in ischemia untreated vs. 0.39 ± 0.02 in ischemia Rbx, treated, $n = 5$, $P < 0.05$). Moreover, the inhibition of PKCβ^{pSer661} by Rbx also reduced the abundance of IκBα (Figure 7(e) and (f), IκBα/glyceraldehyde 3-phosphate dehydrogenase, 0.10 ± 0.02 in ischemic untreated vs. 0.05 ± 0.01 ischemic Rbx treated, $n = 5$–6, $P < 0.05$), a sign of restoring the NF-κB activity in the hind limb muscles. Thus, similar to our *in vitro* results with cultured HUVEC, inhibition of PKCβ phosphorylation (PKCβ^{pSer661}) by Rbx induces NF-κB pathway (IκBα degradation) in the hind limb tissues of hyperglycemic Akita mice.

Inhibition of PKCβ enhances postischemic perfusion in PAD

We measured the effect of inhibition of PKCβ by Rbx on the perfusion recovery in ischemic limbs of diabetic mice. Perfusion recovery was compared to that of non-diabetic mice and diabetic mice treated with vehicle. Serial monitoring of perfusion over two-week period by laser speckle contrast imaging showed no difference in perfusion immediately postsurgery between the control non-diabetic, the diabetic Akita treated with vehicle, and the Akitas treated with Rbx (Figure 7(g) and (h), % perfusion recovery in control non-diabetic $15.31 \pm 1.55\%$ vs. $13.36 \pm 2.85\%$ in vehicle-treated Akitas vs. $15.48 \pm 2.52\%$ in Rbx-treated Akitas, $n = 5$–8, $P > 0.05$). Analysis of perfusion recovery over two weeks showed vehicle-treated Akita as expect had impaired perfusion recovery compared to the non-diabetic C57BL/6J controls; however, Rbx-treated Akitas showed comparable perfusion recovery to the non-diabetic C57BL/6J mice (Figure 7(g) and (h); % perfusion recovery at week 2, $80.11 \pm 10.29\%$ non-diabetic C57BL/6J vs. $55 \pm 10.17\%$ in vehicle-treated Akitas and $96.35 \pm 2.30\%$ in Akitas treated with Rbx, $n = 5$–8 and $P < 0.05$).

Together, these results suggest that during limb ischemia, the NF-κB is induced by activation of both canonical and non-canonical pathway. However, hyperglycemia impairs the activation of the canonical pathway through increased phosphorylation of PKCβ. Inhibition of PKCβ improves the impairment of the canonical pathway and improves the postischemic limb perfusion in diabetic mice following experimental PAD. Thus, PKCβ is a critical

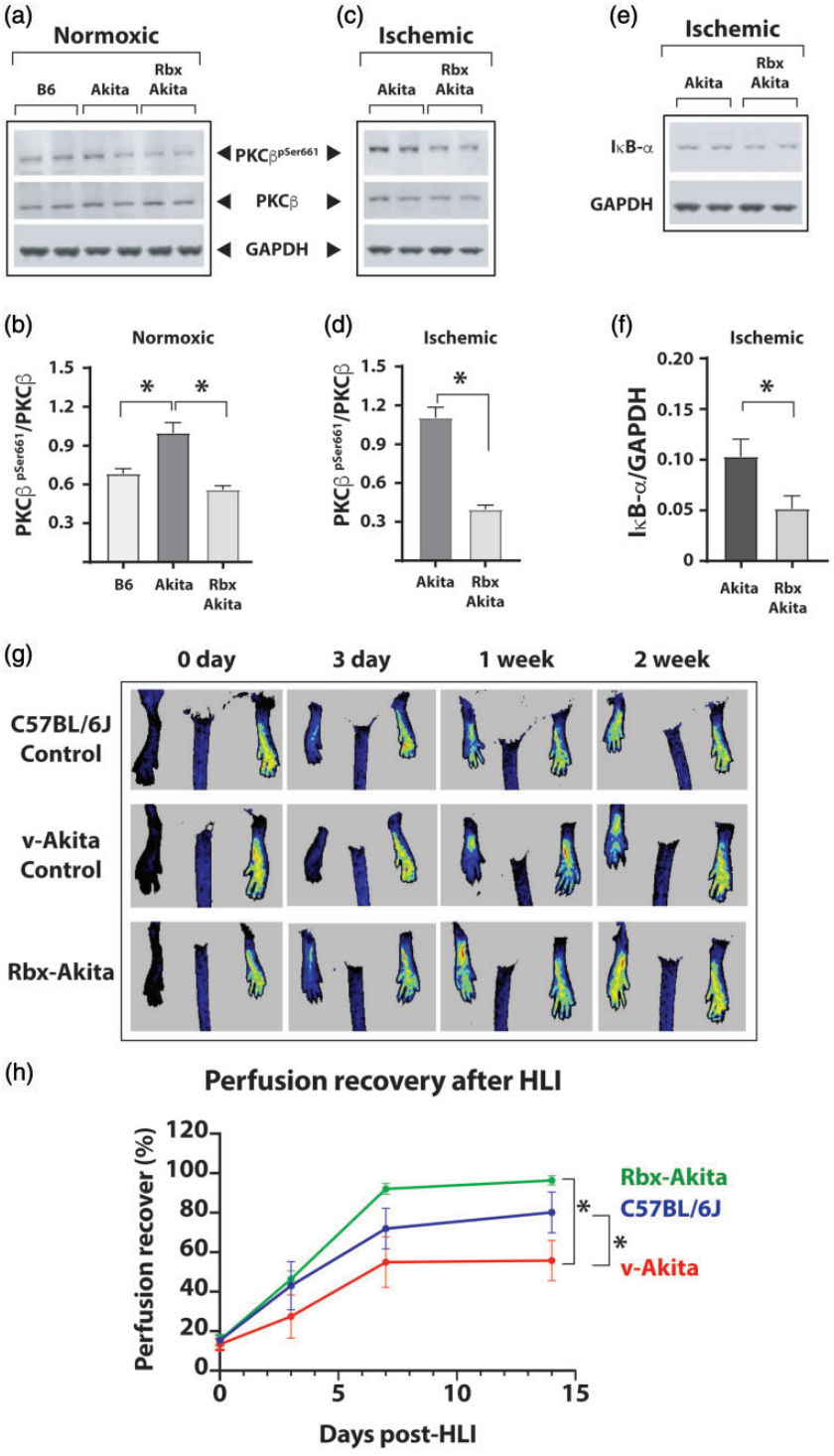

Figure 7. PKCβ inhibition restores NF-κB activation in hyperglycemic diabetic Akita mice after ischemia. (a) Following an HLI (three days postsurgery), Western blots of the gastrocnemius muscle homogenates from the contralateral non-ischemic hind limb were performed. (b) Quantification of the protein bands showed increased basal levels of PKC$\beta^{pSer661}$ in untreated Akita mice compared to the WT C57BL/6J mice. Homogenates from Rbx-treated Akita mice showed significantly decreased PKC$\beta^{pSer661}$ levels. (c) Western blots of ischemic limb of untreated and Rbx-treated Akita mice. (d) The protein levels of phosphorylated PKCβ (PKC$\beta^{pSer661}$) were similar to those of non-ischemic limb samples but significantly decreased in the ischemic limb of Rbx-treated Akita mice. (e) Activation of the NF-κB canonical pathway was measured by Western blots of IκBα protein in ischemic hind limbs of untreated Akita and Rbx-treated Akita mice. (f) The IκBα levels were significantly greater in samples from Akita mice without treatments. In Rbx-treated ischemic limbs of Akita mice, the IκBα levels significantly dropped similar to the PKC$\beta^{pSer661}$. Asterisks denote $P < 0.05$, and n.s. denotes not significant; $n = 4$ to 6 per group. (g) Recovery of blood perfusion after induced HLI in WT (C57BL/6J), vehicle-treated Akita (v-Akita), and Rbx-treated Akita (Rbx-Akita) mice viewed by laser speckle imaging immediately after surgery (day 0), day 3, 1 week, and 2 weeks after surgery. (h) Blood flow in the hind limb was measured in each mouse at multiple time points after induction of ischemia. The extent of perfusion in the operated limb is compared to that of the perfusion in the unoperated limb in the same mouse and expressed as a percentage as shown. Zero on X-axis indicates perfusion immediately after surgery, and $n = 5$ to 8 mice in each group; asterisks denote $P < 0.05$. (A color version of this figure is available in the online journal.)
PKCβ: protein kinase C beta; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; Rbx: ruboxistaurin; HLI: hind limb ischemia.

factor in regulating the postischemic NF-κB activity in diabetes.

Discussion

Diabetes contributes to poor outcomes in peripheral arterial disease (PAD),[22,30,31] but the molecular mechanisms involved are not well understood. Chronic activation of NF-κB signaling is a key inflammatory process in diabetes.[32–35] NF-κB signaling also plays a key role in normal cellular functions including survival.[36] Mice with genetic deficiency in NF-κB activation in the vascular bed show aberrant endothelial function and poor postischemic perfusion recovery.[13] However, how hyperglycemia and chronic activation of NF-κB in diabetes affects the beneficial NF-κB signaling required for postischemic perfusion recovery in experimental PAD has not been studied. In this study, we have shown that in the endothelial cells, hyperglycemic conditions impair ischemia-induced NF-κB activation likely through enhancing the phosphorylation of Ser661 residue on PKCβ. Inhibition of PKCβ by Rbx restored ischemia-induced NF-κB activity both *in vitro* and *in vivo* and improved the perfusion recovery in diabetic mice after experimental PAD.

The NF-κB pathway may be activated via two major pathways termed the canonical and the non-canonical pathways that typically enhance the nuclear localization of p65-p50 or RelB/p52 dimer subunits, respectively. Analyses of phosphorylation and degradation of IκBα in the cytoplasmic compartment as well as enrichment of both p65 and RelB subunits in the nuclear fractions suggested that both the canonical and non-canonical pathways were activated in ischemic HUVEC. Moreover, upon short-term exposure of HUVECs to high D-glucose, both canonical and non-canonical pathways of NF-κB were also activated. Although the high glucose-mediated activation of the NF-κB pathway has been known,[37] our study reveals a novel finding that ischemia-induced NF-κB activation was attenuated *in vitro* and *in vivo* in the context of prolonged high glucose exposure. This finding was unexpected since either ischemia or high glucose conditions are capable of inducing the canonical and non-canonical pathways. Although the canonical pathway is activated by inflammatory signals and the non-canonical pathway by developmental signals, a great degree of cross-talk between these pathways has been reported[38] whereby these pathways can exert mutual restraint on each other. Thus, in our studies, prior activation of the NF-κB pathways by high D-glucose appears to asymmetrically attenuate ischemic activation of the canonical NF-κB pathway (Figure 8).

Hyperglycemia in diabetes contributes to endothelial cells dysfunction through various mechanisms including generation of reactive oxygen species, advanced glycation end products, and activation of PKC.[39,40] Activation of the NF-κB pathway entails a cascade of phosphorylation events performed by several protein kinases. A comparison of phosphorylated proteins from HUVEC grown either in normal or high D-glucose using a phosphoantibody arrays identified increased phosphorylation of a number of protein and HDAC5^{pSer259}, AKT^{pThr308}, and PKCβ^{pSer661} ranked among the highest phosphorylated proteins. HDAC5 binds to myocyte enhancer factor-2 transcription factor to repress genes involved in cardiac myocyte hypertrophy. Our review of the literature showed HDAC5^{pSer259} is downstream of p65 activation in NF-κB signaling[41] thus less likely to explain the impaired NF-kB activation we are studying. Additionally, although there is evidence that AKT activation could be upstream of NF-κB signaling, AKT activation has been associated with anti-apoptotic and pro-proliferation effects on endothelial cells[42] and, therefore, less likely to be the mechanism contributing to

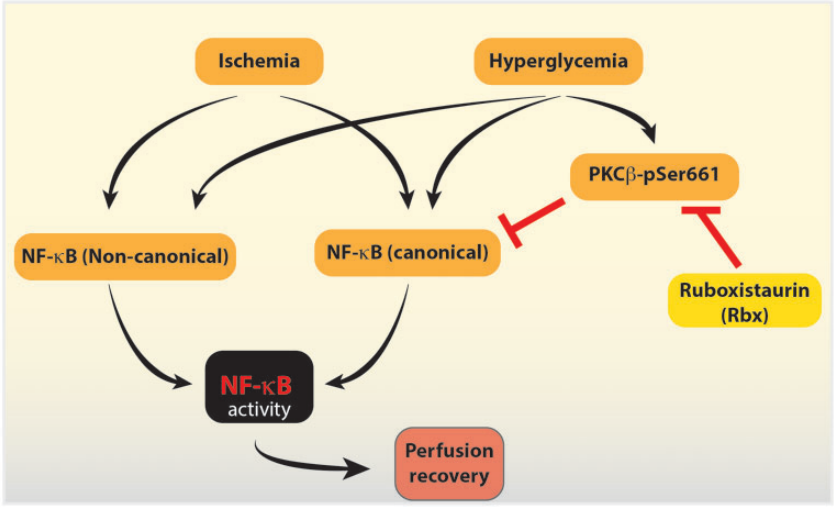

Figure 8. Interaction between ischemia and hyperglycemia-induced signaling in NF-κB activation and perfusion recovery in PAD. Both ischemia and hyperglycemia induce signaling to activate NF-κB that is required for perfusion recovery. However, chronic hyperglycemic conditions attenuate the ischemia-induced activation of the canonical NF-κB pathway and contribute to poor perfusion recovery, through the effects of increased phosphorylation of PKCβ (PKCβ-pSer661). Inhibition of PKCβ activity by the specific inhibitor ruboxistaurin (Rbx) restores the canonical NF-κB pathway and improves perfusion recovery in diabetic mice after PAD. (A color version of this figure is available in the online journal.)
PKCβ: protein kinase C beta; NF-κB: nuclear factor-kappa B.

impaired perfusion recovery. Lastly, in high glucose, activation of PKCα and PKCβ isoforms is associated with adverse effects on recovery from ischemic cardiac injury[28] and increased apoptosis of brain microvascular endothelial cells.[43] PKC is a serine/threonine kinase that can modulate the functions of other proteins by phosphorylating them.[44] High glucose is known to increase expression and activation of PKCβ and PKCδ isoforms.[45] The PKCβ1 isoform contains a conserved Serine-661 residue in the C-terminal region that is autophosphorylated and determines the subcellular localization of the kinase.[46] Prolonged activation of PKCβ in cancer cells is associated with impaired NF-κB activation by TNF.[47] Administration of the inhibitors of PKCβ in diabetic mice ameliorates diabetic complications in the retinal and kidney.[48,49] Hence, assessment of the role of PKCβ phosphorylation in impaired ischemia-induced NF-κB activation was favored. The increased PKCβ^{pSer661} is associated with impaired activation of NF-κB in HUVEC and in ischemic hind limbs of diabetic mice. However, treatment with Rbx a specific inhibitor of PKCβ[50] reduced the phosphorylation of Serine-661 of PKCβ, and this was associated with restored ischemia-induced NF-κB activation in the endothelial cells as well as in the hind limbs of treated diabetic mice. Moreover, compared to the wild-type (WT) mice, the untreated Akita mice showed an impaired perfusion in ischemic limbs, and this was improved in Rbx-treated Akita mice. Interestingly, Rbx has been investigated as potential therapy in several disease conditions resulting from diabetes complications including diabetic peripheral neuropathy, retinopathy, nephropathy, and macular edema.[51,52] Recent large animal studies also show that Rbx may have therapeutic efficacy in preclinical diseases models not resulting from diabetes complications.[53] Our result showing that postischemic perfusion recovery in diabetic mice treated with Rbx was superior to that of non-diabetic mice is consistent with possible effects of Rbx beyond diabetes-related complications.

Lastly, although ischemia activates both the canonical and non-canonical NF-κB pathways, only activation of the canonical pathway is impaired in diabetes/hyperglycemia; this suggests that the canonical NF-κB pathway and not the non-canonical pathway plays a key role in postischemic perfusion recovery. Thus, hyperglycemia in diabetes contributes to poorer experimental PAD outcomes by hyperphosphorylating PKCβ that results in impairment in ischemia-induced activation of the canonical NF-κB signaling pathway.

Authors' contributions: SA and TW performed experiments and analyzed results. MVS wrote the manuscript, researched data, and contributed to discussion, and AOD designed the studies and reviewed/edited the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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SUPPLEMENTAL MATERIAL

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