

Protein Kinase C- β Contributes to Impaired Endothelial Insulin Signaling in Humans With Diabetes Mellitus

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Background—Abnormal endothelial function promotes atherosclerotic vascular disease in diabetes. Experimental studies indicate that disruption of endothelial insulin signaling, through the activity of protein kinase C- β (PKC β) and nuclear factor κ B, reduces nitric oxide availability. We sought to establish whether similar mechanisms operate in the endothelium in human diabetes mellitus.

Methods and Results—We measured protein expression and insulin response in freshly isolated endothelial cells from patients with type 2 diabetes mellitus (n=40) and nondiabetic controls (n=36). Unexpectedly, we observed 1.7-fold higher basal endothelial nitric oxide synthase (eNOS) phosphorylation at serine 1177 in patients with diabetes mellitus ($P=0.007$) without a difference in total eNOS expression. Insulin stimulation increased eNOS phosphorylation in nondiabetic subjects but not in diabetic patients ($P=0.003$), consistent with endothelial insulin resistance. Nitrotyrosine levels were higher in diabetic patients, indicating endothelial oxidative stress. PKC β expression was higher in diabetic patients and was associated with lower flow-mediated dilation ($r=-0.541$, $P=0.02$). Inhibition of PKC β with LY379196 reduced basal eNOS phosphorylation and improved insulin-mediated eNOS activation in patients with diabetes mellitus. Endothelial nuclear factor κ B activation was higher in diabetes mellitus and was reduced with PKC β inhibition.

Conclusions—We provide evidence for the presence of altered eNOS activation, reduced insulin action, and inflammatory activation in the endothelium of patients with diabetes mellitus. Our findings implicate PKC β activity in endothelial insulin resistance. (*Circulation*. 2013;127:86-95.)

Key Words: diabetes mellitus ■ endothelium ■ insulin resistance ■ nitric oxide

Type 2 diabetes mellitus affects a rapidly growing portion of the global population and is associated with high cardiovascular risk.^{1,2} Abnormal endothelial function contributes to the development of atherosclerosis and clinical vascular events in diabetes mellitus.^{3,4} Novel therapeutic strategies that restore endothelial function hold promise as interventions to lower cardiovascular risk in diabetes mellitus.

Endothelial inflammatory activation via nuclear factor kappa B (NF κ B) may perturb insulin-mediated signaling pathways, thereby reducing nitric oxide bioactivity and increasing cytokine release.^{8,9}

Previous studies link increased activity of protein kinase C-beta (PKC β) to endothelial dysregulation in diabetes mellitus.¹⁰ In the diabetic state, excess nutrients stimulate PKC β through increased formation of lipid diacylglycerol (DAG), a byproduct of glucose and lipid metabolism.¹¹ In cultured endothelial cells and animal models, PKC β activation interrupts insulin-induced eNOS stimulation, and PKC β inhibition restores vascular function.^{12,13} Increased PKC β activity has also been implicated in endothelial cell inflammatory activation.¹⁴

Endothelial dysfunction is a prominent feature of clinical diabetes mellitus. Previous studies by our group and others have demonstrated reduced endothelium-dependent vasodilation in patients with diabetes mellitus.^{4,15} The consequences of

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Experimental studies link the metabolic disturbances in diabetes mellitus to altered endothelial phenotype. In cultured endothelial cells, insulin activates endothelial nitric oxide synthase (eNOS) through phosphorylation at serine 1177.⁵ In animal models relevant to diabetes mellitus, endothelial insulin resistance impairs eNOS activation, reduces endothelium-dependent vasodilation, and promotes atherogenesis.^{6,7}

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diabetes mellitus on endothelial insulin signaling and endothelial inflammatory activation remain incompletely characterized in the human vasculature. The present study sought to investigate endothelial insulin signaling, inflammation, and the contribution of PKC β activation to altered endothelial phenotype in human diabetes mellitus.

Methods

Study Subjects

We enrolled adults with type 2 diabetes mellitus defined as fasting serum glucose ≥ 126 mg/dL or ongoing treatment for type 2 diabetes mellitus at Boston Medical Center and control individuals without diabetes mellitus defined as fasting glucose < 100 mg/dL. All subjects were studied in the fasting state, and a blood sample was taken for measurement of lipid levels and glucose levels in the Boston Medical Center Clinical Laboratory. Vasoactive medications were held on the morning of the study (last dose 24 hours before testing). The study protocol was approved by the Boston Medical Center Institutional Review Board, and all participants provided written informed consent.

Vascular Function Testing

We measured brachial artery flow-mediated dilation as described.¹⁶ Briefly, high-resolution ultrasound was used to measure brachial artery diameter before and 1 minute after a 5-minute cuff occlusion of the upper arm (proximal to the imaged portion of the artery) designed to induce a hyperemic response. Doppler flow signals were measured in the resting state and immediately after cuff release.

Peripheral Endothelial Cell Collection

Peripheral venous endothelial cell biopsy was performed as previously described.^{17,18} Briefly, a 20-gauge intravenous catheter was inserted into a superficial forearm vein under aseptic technique. An 0.018-in J-wire (Arrow International, Reading PA) was introduced through the catheter, and endothelial cells were collected by gentle abrasion of the vessel wall. Endothelial cells were recovered from the wire tip by centrifugation in a dissociation buffer and plated on poly-L-lysine coated microscope slides (Sigma, St. Louis, MO). Once plated, cells were either fixed immediately in 4% paraformaldehyde or stimulated with insulin (described below) before fixation. Slides were washed in PBS, dried, and stored at -80°C until further processing.

Insulin-Stimulation of Freshly Isolated Human Endothelial Cells

To select the optimal time point for evaluating insulin-mediated eNOS phosphorylation in endothelial cells, we performed a time course evaluation using commercially available cultured human aortic endothelial cells (HAECs; Lonza Inc, Walkersville, MD). After overnight starvation, HAECs were treated with insulin 10 nmol/L in endothelial growth medium-2 medium devoid of growth factors at 37°C and fixed at multiple time points up to 60 minutes. With insulin treatment, eNOS phosphorylation increased transiently at 5 minutes and then was sustained at 30 through 60 minutes (Figure 1 in the online-only Data Supplement). Thus, for our studies of freshly isolated human cells, we measured insulin-mediated eNOS phosphorylation at 30 minutes.

Freshly isolated endothelial cell samples were split immediately after isolation and before fixation with paraformaldehyde and incubated with either 0 or 10 nmol/L insulin in endothelial growth medium-2 Bullet Kit medium (Lonza Inc), devoid of growth factors, at 37°C for 30 minutes. Cells were then washed in PBS, fixed, dried, and frozen as above. To test the hypothesis that PKC β inhibition improves endothelial insulin signaling in diabetes mellitus, cells were similarly incubated with 0 or 10 nmol/L insulin concurrently with LY379196, a PKC β -specific inhibitor (kindly supplied by Eli Lilly and Company, Indianapolis, IN), at a concentration of 0 or 30

nmol/L for 30 minutes. The dose of LY379196 was based on previous reports evaluating commercially available cultured endothelial cells.¹⁹ Cells were processed as described above.

Assessment of Protein Expression by Quantitative Immunofluorescence

Fixed samples were thawed and rehydrated with PBS containing 50 mmol/L glycine (Sigma) for 10 minutes. Cells were permeabilized with 0.1% Triton X-100, and nonspecific binding sites were blocked with 0.5% bovine serum albumin (BSA). Slides were incubated for 1 hour at 37°C with primary antibodies against 1 of the following targets: eNOS (1:100 dilution; BD Biosciences, San Diego, CA), phosphorylated eNOS at serine 1177 (1:200 dilution; Millipore, Billerica, MA) and threonine 495 (1:100 dilution; Cell Signaling, Danvers, MA), Akt (1:100 dilution; Cell Signaling), PKC β (1:100 dilution; Abcam, Cambridge, MA), phosphatase and tensin homolog (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), nitrotyrosine (1:1000 dilution; Millipore, Billerica, MA), intercellular adhesion molecule (1:150 dilution; Santa Cruz Biotechnology), I κ B α (1:100 dilution; Novus, Littleton, CO), or p65 (1:375 dilution; Novus). All cells were also stained with an anti-von Willebrand Factor (vWF) antibody (1:300 dilution; Dako, Carpinteria, CA) to aid in endothelial cell identification. After incubation with the primary antibodies, the slides were washed and incubated with corresponding Alexa Fluor-488 and Alexa Fluor-594 antibodies (1:200 dilution; Invitrogen, Carlsbad, CA) and mounted under glass coverslips with Vectashield containing DAPI for nuclear identification (Vector Laboratories, Burlingame, CA). For each batch of patient-derived cells, we stained a control slide of cultured HAECs, maintained in endothelial growth medium-2 Bullet Kit medium (Lonza) at 37°C with 5% CO_2 and taken from a single index passage.

Slides were imaged with a fluorescence microscope (Nikon Eclipse TE2000-E) at $\times 20$ magnification, and digital images of the cells were captured using a Photometric CoolSnap HQ2 Camera (Photometrics, Tucson, AZ). Exposure time was held constant, and image intensity was corrected for background fluorescence. Fluorescent intensity was quantified by NIS Elements AR Software (Nikon Instruments Inc, Melville, NY). For each protein of interest, fluorescent intensity was quantified in 20 cells from each patient and averaged. To minimize batch-to-batch variability in staining intensity, fluorescence intensity for each patient sample was normalized to the intensity of HAEC staining performed simultaneously. Intensity is expressed in arbitrary units (au) calculated by dividing the average fluorescence intensity from the patient sample by the average fluorescence intensity of the HAEC sample and multiplying by 100. Intensity quantification was performed blinded to subject identity and diabetes mellitus status, and each batch included patients with and without diabetes mellitus.

Statistical Analyses

Statistical analyses were performed using SPSS version 19.0. The distribution of continuous clinical characteristics and vascular function measures were evaluated by examining a histogram and the Shapiro-Wilk test and compared between diabetic patients and nondiabetic controls using the independent samples *t* test or Mann-Whitney *U* test as appropriate. Categorical clinical characteristics were compared using χ^2 testing. Baseline endothelial cell protein expression, and change in eNOS phosphorylation with insulin, were compared between diabetic patients and nondiabetic controls using Mann-Whitney *U* test given the sample size. We compared flow-mediated dilation between patients with diabetes mellitus and nondiabetic controls adjusting for race, body mass index, total cholesterol, high-density lipoprotein cholesterol, and systolic blood pressure using a general linear model. We evaluated the effect of LY379196 on eNOS phosphorylation, the response to insulin, and I κ B α expression with related samples Wilcoxon Signed Rank Test. We compared clinical characteristics, vascular measures, and eNOS phosphorylation responses by evaluating correlation coefficients. A 2-sided *P* value < 0.05 was considered statistically significant.

Results

Study Subjects and Vascular Function

We enrolled 40 patients with diabetes mellitus and 36 nondiabetic control participants. As shown in the Table, patients with diabetes mellitus had metabolic abnormalities, including higher body mass index, systolic blood pressure, fasting serum glucose, and glycosylated hemoglobin A1c, and lower high-density lipoprotein cholesterol. Patients with diabetes mellitus had lower low-density lipoprotein cholesterol, likely related to concomitant lipid-lowering therapies. Flow-mediated dilation of the brachial artery was lower in the patients with diabetes mellitus as compared with the nondiabetic controls, consistent with the presence of endothelial dysfunction. In a multivariable model adjusting for race, total cholesterol, high-density lipoprotein cholesterol, and systolic blood pressure, patients with diabetes mellitus had lower flow-mediated dilation than nondiabetic controls (least square mean \pm SE, 6.4 \pm 0.7 versus 9.7 \pm 0.7; $P=0.003$). Nitroglycerin-mediated dilation was lower in the patients with diabetes mellitus,

Table. Clinical Characteristics and Vascular Function

| | Nondiabetic (n=36) | Diabetic (n=40) |
|------------------------------------|--------------------|------------------|
| Clinical Characteristics | | |
| Age, y | 51 \pm 10 | 55 \pm 7 |
| Female sex, % | 33 | 45 |
| Black race, % | 36 | 57 |
| Body mass index, kg/m ² | 27.4 \pm 4.8 | 33.5 \pm 7.7* |
| Total cholesterol, mg/dL | 198 \pm 35 | 172 \pm 48* |
| LDL cholesterol, mg/dL | 121 \pm 26 | 96 \pm 34* |
| HDL cholesterol, mg/dL | 54 \pm 18 | 45 \pm 13* |
| Triglycerides, mg/dL | 118 \pm 77 | 163 \pm 125 |
| Fasting glucose, mg/dL | 89 \pm 11 | 147 \pm 77* |
| Hemoglobin A1c, % | 5.4 \pm 0.5 | 8.9 \pm 5.5* |
| Systolic blood pressure, mm Hg | 126 \pm 12 | 138 \pm 20* |
| Diastolic blood pressure, mm Hg | 73 \pm 7 | 77 \pm 12 |
| Antiplatelet therapy, % | 3 | 41* |
| Lipid lowering therapy, % | 8 | 49* |
| ACE inhibitor or ARB therapy, % | 3 | 61* |
| Metformin, % | 0 | 67* |
| Sulfonylureas, % | 0 | 18* |
| Thiazolidinediones, % | 0 | 5* |
| Insulin therapy, % | 0 | 43* |
| Vascular function | | |
| Baseline diameter, mm | 4.19 \pm 0.72 | 4.33 \pm 0.77 |
| Baseline flow, ml/min | 119 \pm 68 | 133 \pm 75 |
| Hyperemic flow, ml/min | 1018 \pm 438 | 892 \pm 469 |
| Flow-mediated dilation, % | 10.0 \pm 4.0 | 6.5 \pm 4.3* |
| Flow-mediated dilation, mm | 0.40 \pm 0.13 | 0.26 \pm 0.16* |
| Nitroglycerin-mediated dilation† | 13.6 \pm 5.2 | 10.1 \pm 4.6* |

Data are expressed as mean \pm SD. ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

* $P<0.05$.

†n=18 nondiabetic, 26 diabetic.

suggesting the presence of smooth muscle dysfunction or inactivation of nitric oxide by reactive oxygen species as well as impaired endothelium-dependent vasodilation. There were no differences in arterial diameter or resting or hyperemic flow.

Endothelial Nitric Oxide Synthase Activation and Response to Insulin in Freshly Isolated Endothelial Cells

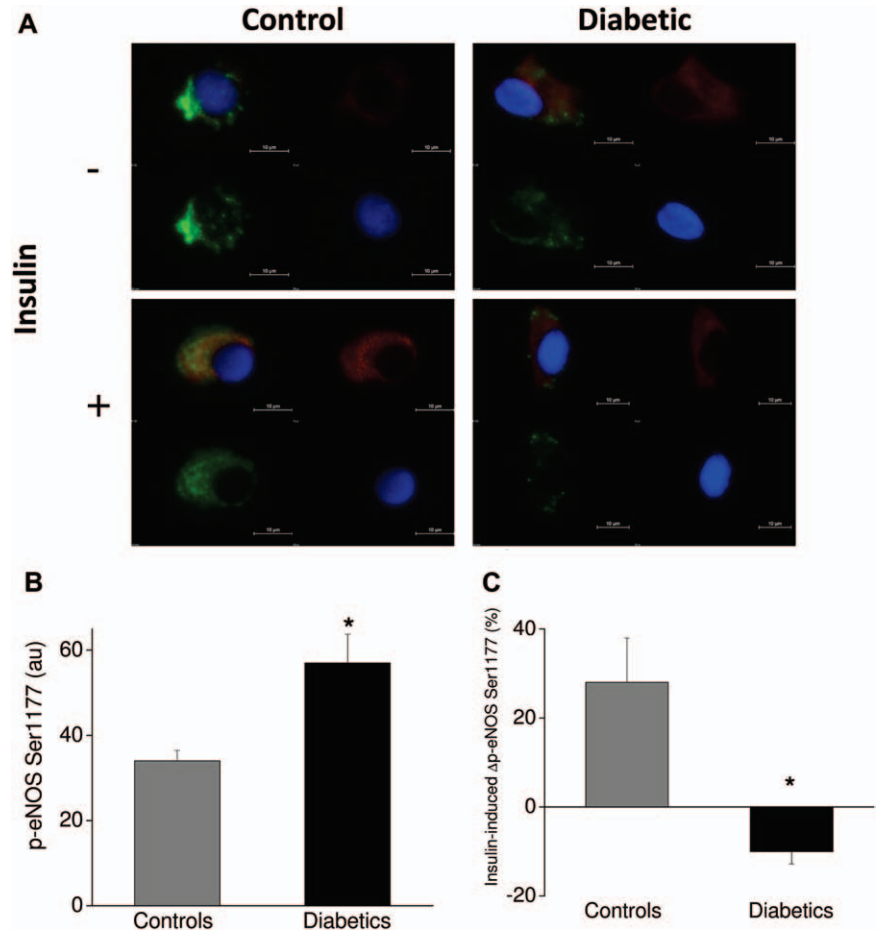
To evaluate activation of eNOS, we quantified levels of eNOS phosphorylation at serine 1177 and threonine 495 in diabetic individuals. Notably, diabetic individuals had higher basal levels of activated eNOS at serine 1177 compared with controls (Figure 1, Figure II in the online-only Data Supplement). To measure insulin-induced eNOS phosphorylation, we exposed cells from diabetic and nondiabetic subjects to 0 or 10 nmol/L insulin for 30 minutes as shown in Figure 1 and Figure II in the online-only Data Supplement. With insulin stimulation, eNOS phosphorylation at serine 1177 increased 38 \pm 30% in cells from nondiabetic subjects (n=9, $P=0.04$). In contrast, eNOS phosphorylation at serine 1177 decreased 10 \pm 9% with insulin stimulation in cells from diabetic patients (n=12, $P=0.006$). The difference in overall insulin-induced eNOS phosphorylation at serine 1177 between the diabetic and nondiabetic groups was highly significant ($P=0.003$). Among the patients with diabetes mellitus, there were no differences in basal or insulin-stimulated eNOS phosphorylation at serine 1177 based on use of statins, angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, metformin, or insulin (Table I in the online-only Data Supplement).

There was no difference in basal eNOS phosphorylation at the inhibitory site threonine 495 (Figure 2 and Figure II in the online-only Data Supplement). In response to insulin stimulation, the change in inhibitory eNOS phosphorylation at threonine 495 differed between diabetic patients (18 \pm 9%) and nondiabetic controls (−18 \pm 5%) (Figure 2, $P=0.002$).

Overall, a greater increase in eNOS phosphorylation with insulin stimulation was associated with higher flow-mediated dilation ($r=0.523$, $P=0.04$; Figure III in the online-only Data Supplement). Among the diabetic patients, higher body mass index was associated with lower insulin-stimulated eNOS phosphorylation ($r=-0.600$, $P=0.04$). Fasting glucose and hemoglobin A1c were not associated with insulin-stimulated eNOS phosphorylation.

As shown in Figure 3, total eNOS and Akt expression were similar in endothelial cells from patients with diabetes compared with control subjects. Expression of phosphatase and tensin homolog, a phosphatase that opposes PI3 kinase action and inhibits Akt activation, was similar in diabetic patients and nondiabetic controls (Figure 3 and Figure IV in the online-only Data Supplement). These results suggest alterations in eNOS activation with comparable eNOS expression in patients with diabetes mellitus. As shown in Figure 3, expression of nitrotyrosine, a marker of oxidative stress, was higher in patients with diabetes mellitus than in nondiabetic controls. Basal eNOS phosphorylation at serine 1177, total

Figure 1. Diabetes mellitus is associated with altered endothelial nitric oxide synthase (eNOS) activation and insulin resistance in endothelial cells. As described in Methods, freshly isolated endothelial cells identified by von Willebrand factor (vWF) staining (green) and nuclear morphology (DAPI in blue) were obtained from patients with diabetes mellitus (n=12) and nondiabetic controls (n=9). Cells were incubated with 0 nmol/L or 10 nmol/L insulin for 30 minutes and then fixed. eNOS activation was evaluated as eNOS phosphorylation at serine 1177 (red). Protein levels were quantified by evaluating 20 cells for each patient under each condition. **A**, Representative cells from a patient with diabetes mellitus (right) show higher basal eNOS phosphorylation at serine 1177 (**top**, red) compared with the nondiabetic control (**left top**, red). Insulin increased eNOS phosphorylation at serine 1177 in the nondiabetic control (**left top**, red vs **left bottom**, red) but not in the diabetic patient (**right top**, red vs **right bottom**, red). **B**, Pooled data show that basal eNOS phosphorylation at serine 1177 was 67% higher in endothelial cells from patients with diabetes mellitus compared with nondiabetic controls (* $P<0.01$). **C**, Pooled data demonstrate that insulin increased eNOS phosphorylation at serine 1177 in endothelial cells from nondiabetic controls but not in endothelial cells from patients with diabetes mellitus (* $P=0.003$).



eNOS expression, and nitrotyrosine level were not associated significantly with flow-mediated dilation.

PKC β Inhibition Restores Endothelial Nitric Oxide Synthase Activation and Insulin Signaling in Diabetes Mellitus

To investigate PKC β activation in the diabetic endothelium, we measured total PKC β expression. As shown in Figure 4 and Figure V in the online-only Data Supplement, PKC β expression was higher in patients with diabetes mellitus compared with nondiabetic controls. Higher PKC β expression was associated with lower flow-mediated dilation of the brachial artery in the overall sample (Figure 4) and among the patients with diabetes mellitus ($r=-0.674$, $P=0.03$).

To evaluate the role of PKC β activation in the impairment of eNOS signaling in diabetes mellitus, we treated freshly isolated endothelial cells with LY379196, a pharmacological inhibitor of PKC β .¹⁹ As shown in Figure 5 and Figure VI in the online-only Data Supplement, PKC β inhibition decreased the basal levels of eNOS phosphorylation at serine 1177 in endothelial cells from patients with diabetes mellitus. The change in basal eNOS phosphorylation with PKC β inhibition was not significantly correlated with flow-mediated dilation.

Treatment with LY379196 improved the insulin-mediated change in eNOS phosphorylation at serine 1177 in endothelial cells from diabetic patients, suggesting that PKC β activity impedes insulin-mediated activation of eNOS in diabetes

mellitus (Figure 5). PKC β inhibition did not change in basal eNOS phosphorylation at threonine 495 in patients with diabetes mellitus (Figure VII in the online-only Data Supplement). Treatment with LY379196 did not change significantly the insulin-mediated levels of eNOS phosphorylation at threonine 495 in patients with diabetes mellitus (Figure VII in the online-only Data Supplement).

NF κ B Activation in Freshly Isolated Endothelial Cells in Diabetes

To evaluate endothelial inflammatory activation, we examined the activity of NF κ B by quantification of subunit p65, the inhibitory regulator I κ B α , and intercellular adhesion molecule-1 (Figure 6 and Figure V in the online-only Data Supplement).²⁰ Endothelial cells from diabetic patients showed a trend toward higher levels of p65 when compared with nondiabetic controls. Expression of the negative regulator of NF κ B, I κ B α , was lower in endothelial cells from diabetic patients than in nondiabetic controls. Further, expression of intercellular adhesion molecule-1, a protein regulated by NF κ B, trended toward higher in endothelial cells from patients with diabetes mellitus than in nondiabetic controls. Endothelial p65, intercellular adhesion molecule-1, and I κ B α expression levels were not associated significantly with flow-mediated dilation.

To evaluate the role of PKC β in the NF κ B activation in diabetes mellitus, we measured I κ B α expression before and after

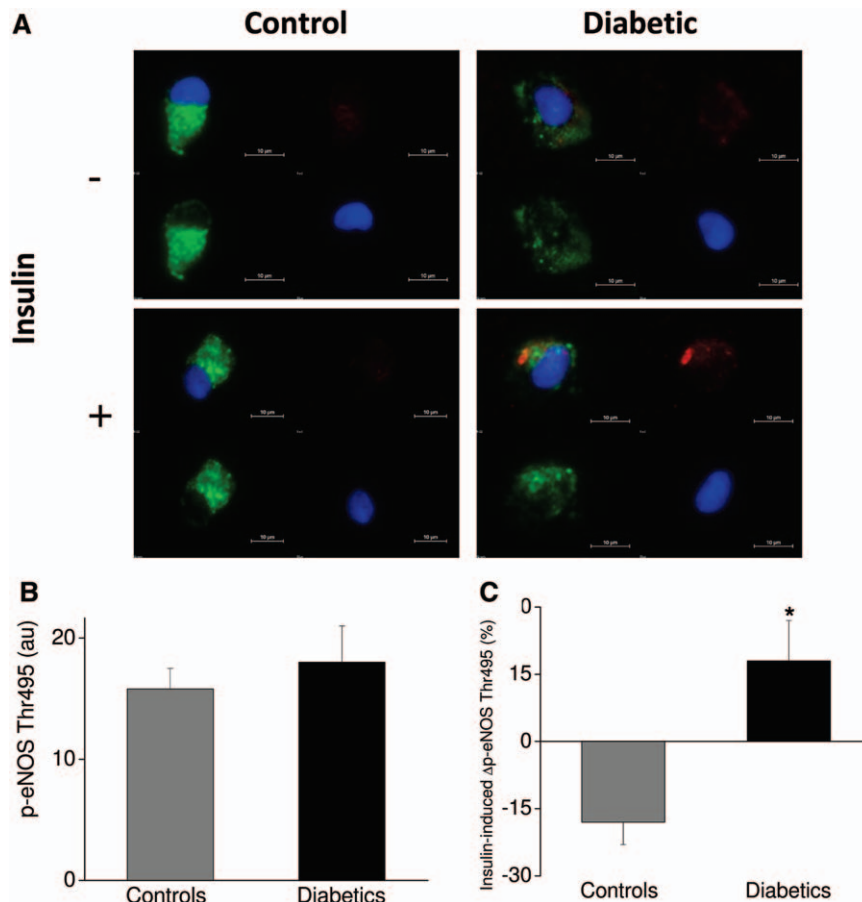


Figure 2. Diabetes mellitus and inhibitory eNOS phosphorylation. As described in Methods, freshly isolated endothelial cells identified by von Willebrand factor (vWF) staining (green) and nuclear morphology (DAPI in blue) were obtained from patients with diabetes mellitus ($n=9$) and nondiabetic controls ($n=6$). Cells were incubated with 0 nmol/L or 10 nmol/L insulin for 30 minutes and then fixed. Endothelial nitric oxide synthase (eNOS) phosphorylation at threonine 495 (red) was quantified by evaluating 20 cells for each patient under each condition. **A**, Representative cells from a patient with diabetes (**right**) show similar basal eNOS phosphorylation at threonine 495 (**top**, red) compared with the nondiabetic control (**left top**, red). Insulin decreased eNOS phosphorylation at threonine 495 in the nondiabetic control (**left top**, red vs **left bottom**, red) and increased phosphorylation at this site in the diabetic patient (**right top**, red vs **right bottom**, red). **B**, Pooled data show that basal eNOS phosphorylation at threonine 495 was similar in endothelial cells from patients with diabetes mellitus compared with nondiabetic controls ($P=0.95$). **C**, Pooled data demonstrate that insulin decreased eNOS phosphorylation at threonine 495 in endothelial cells from nondiabetic controls but increased in endothelial cells from patients with diabetes ($*P=0.009$).

treatment with LY379196 for 30 minutes. In endothelial cells from diabetic patients, inhibition of PKC β increased I κ B α expression, suggesting a link between PKC β activity and activation of NF κ B (Figure 6).

Discussion

In the present study, we provide direct evidence that signaling pathways responsible for eNOS activation are altered in endothelial cells from patients with diabetes mellitus. Interestingly, we found elevated levels of activated eNOS in conjunction with reduced flow-mediated vasodilation in diabetic patients compared with control individuals. Basal expression of total eNOS, Akt, and phosphatase and tensin homolog were similar in diabetes mellitus, suggesting that abnormal endothelial function is not attributable to altered levels of these proteins. Activation of eNOS by insulin was severely blunted in endothelial cells from diabetic patients, indicating the presence of endothelial insulin resistance. Inhibitory eNOS phosphorylation was increased by insulin in patients with diabetes mellitus but decreased in nondiabetic controls. In addition, we observed endothelial inflammatory activation and oxidative stress, evidenced by increased markers of NF κ B activity and increased nitrotyrosine expression in endothelial cells from diabetic patients.

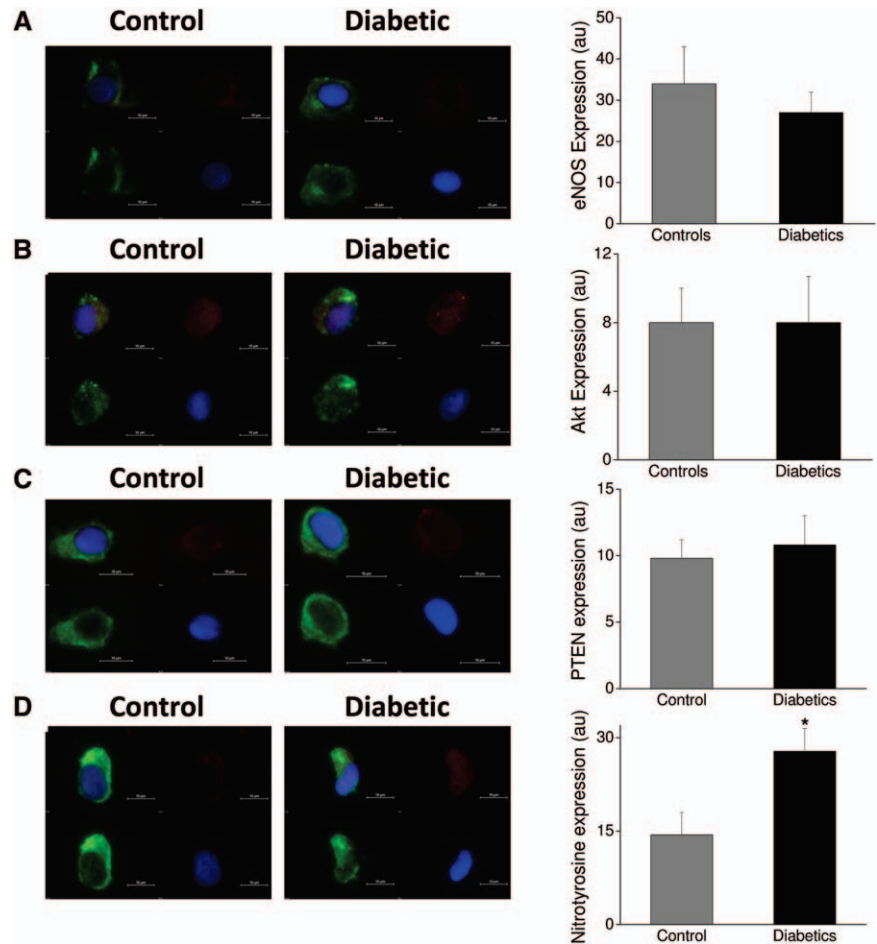
Inhibition of PKC β with LY379196 blunted the diabetes-associated endothelial abnormalities in basal and insulin-stimulated eNOS phosphorylation state. Further, PKC β inhibition increased I κ B α expression suggesting that PKC β inhibition

reduced NF κ B activation in endothelial cells from patients with diabetes mellitus. Taken together, our findings suggest that PKC β activity contributes to the abnormal endothelial phenotype characterized by impaired insulin-mediated eNOS activation in diabetes mellitus.

Experimental studies have identified insulin as a regulator of endothelial nitric oxide production. In commercially-available cultured endothelial cells, insulin enhances eNOS activity through phosphatidylinositol-3 kinase (PI3K)–Akt mediated phosphorylation at serine 1177.⁵ Relevant to our study of flow-mediated dilation, shear stress also activates eNOS via the PI3K–Akt signaling axis.²¹ In animal models, diminished vascular insulin signaling is associated with reduced nitric oxide bioavailability.^{22,23} Further, endothelial-specific elimination of the insulin receptor leads to reduced insulin-mediated eNOS activation, endothelial vasomotor dysfunction, inflammatory activation, and accelerated plaque formation.⁷ These studies support the concept that endothelial insulin resistance contributes to the development of endothelial dysfunction and atherosclerosis; however, the relevance to human diabetes mellitus has not been established previously.

Our data provide insight into the consequences of human diabetes mellitus on pathways that modulate eNOS activity under basal and stimulated conditions. We observed excessive basal eNOS activation and reduced flow-mediated dilation in diabetic patients. The differences in resting eNOS phosphorylation are concordant with a previous report describing high

Figure 3. Abnormal endothelial nitric oxide synthase (eNOS) activation is not attributable to differential eNOS, Akt, or phosphatase and tensin homolog (PTEN) expression in diabetes mellitus. Diabetes mellitus is associated with oxidative stress in endothelial cells. Freshly isolated endothelial cells collected from patients with diabetes mellitus and nondiabetic controls were fixed and stained (20 cells per patient for each protein). **A**, Representative cell from a diabetic patient (**right**) shows similar eNOS expression (red) compared with a cell from a nondiabetic control (**left**, red). Pooled data show that eNOS levels were comparable in the nondiabetic controls ($n=8$) and diabetics ($n=10$, $P=0.46$). **B**, Representative cell from a diabetic patient (**right**) shows similar Akt expression (red) compared with a cell from a nondiabetic control (**left**, red). Pooled data show that Akt levels were comparable in the nondiabetic controls ($n=6$) and diabetics ($n=10$, $P=0.79$). **C**, Representative cell from a diabetic patient (**right**) shows similar PTEN expression (red) compared with a cell from a nondiabetic control (**left**, red). Pooled data show that PTEN levels were comparable in the nondiabetic controls ($n=8$) and diabetics ($n=10$, $P=1.0$). **D**, Representative cell from a diabetic patient (**right**) shows higher nitrotyrosine expression (red) compared with a cell from a nondiabetic control (**left**, red). Pooled data show that nitrotyrosine levels were higher in diabetic patients ($n=5$) compared with the nondiabetic controls ($n=5$, $*P=0.03$).



basal eNOS phosphorylation in obese subjects.²⁴ Complex processes determine nitric oxide bioavailability, including the amount of nitric oxide synthesized and the extent of nitric oxide destruction by reactive oxygen species.²⁵ Higher eNOS activation may represent an adaptive response to high oxidative stress in diabetes.²⁶ Consistent with this possibility, we observed higher nitrotyrosine levels in the endothelial cells from diabetic patients.^{27,28} Under pathological conditions including diabetes mellitus, eNOS may become uncoupled and produce superoxide, rather than NO.²⁹ It is plausible that elevated eNOS phosphorylation in diabetes mellitus reflects a compensatory alteration to chronically reduced nitric oxide levels. The higher basal Ser1177 phosphorylation in endothelial cells from diabetic subjects raises the possibility that the blunted response to insulin stimulation represents a ceiling effect that limits the amount of additional phosphorylation that can occur.

Our study extends our understanding of nitric oxide regulation in diabetic endothelial cells by examining the functional response to insulin. We demonstrated that insulin promotes eNOS activity by increasing activating (Ser1177) and decreasing inhibitory (Thr495) phosphorylation in endothelial cells from nondiabetic control individuals. In contrast, endothelial cells isolated from diabetic patients displayed a marked abnormality of endothelial insulin action. These findings suggest that modulation of eNOS activation through the PI3K-Akt

pathway is disrupted in human diabetes mellitus. Further, insulin augments inhibitory phosphorylation of eNOS that may contribute to uncoupling and oxidative stress.³⁰ The inability to upregulate eNOS activity in response to acute insulin administration was accompanied by abnormal shear stress-mediated vasodilator responses. Our findings support the possibility that insulin resistance in the endothelium contributes to endothelial dysfunction in humans with diabetes mellitus.

The present study suggests that PKC β activity is an important determinant of abnormal eNOS activation in diabetes mellitus. In animal models of diabetes mellitus, PKC β inhibition improved insulin-mediated PI3K/Akt signaling in parallel with endothelium-mediated vasodilation.¹² In human subjects, PKC β inhibition with ruboxistaurin treatment prevented the development of endothelial dysfunction induced by acute hyperglycemia and improved flow-mediated dilation in diabetic patients.^{31,32} Consistent with these studies, we observed higher PKC β levels in endothelial cells from patients with diabetes mellitus. Higher PKC β expression was associated with reduced endothelial function evidenced by lower flow-mediated dilation. Inhibiting PKC β activity improved the responsiveness to insulin in endothelial cells and reduced basal eNOS phosphorylation in diabetes. In cell culture, PKC β has been described as reducing eNOS activity through phosphorylation at the inhibitory site Thr495. We did

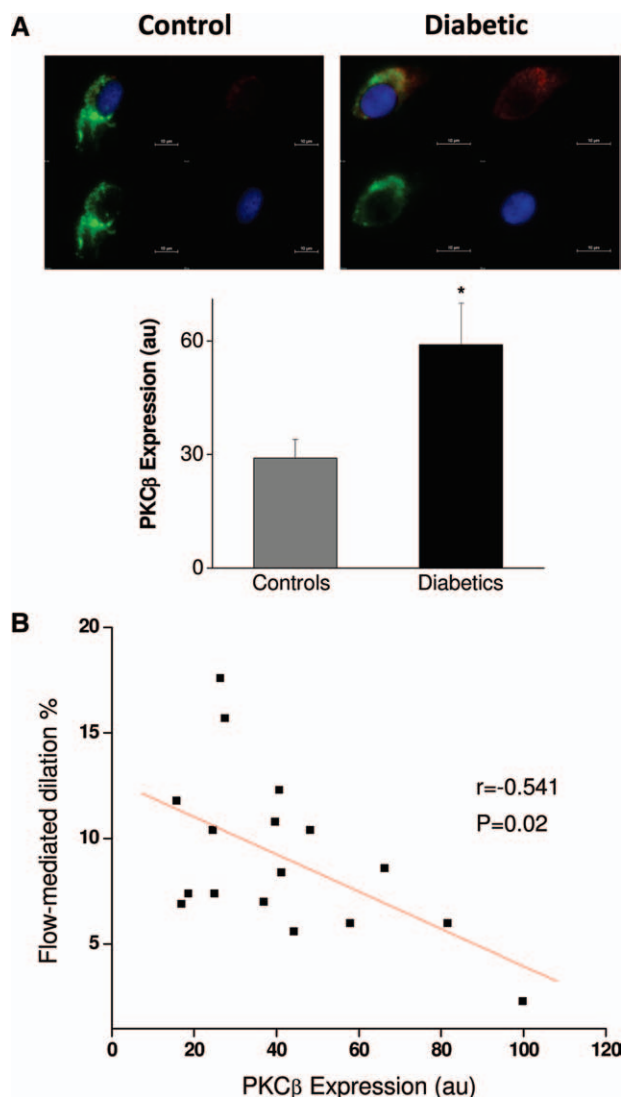


Figure 4. Protein kinase C- β (PKC β) and endothelial function. **A**, Representative cell from a diabetic patient (right) shows higher PKC β expression (red) compared with a cell from a nondiabetic control (left, red). Pooled data (20 cells analyzed per patient) show that PKC β levels were higher in diabetic patients ($n=11$) compared with the nondiabetic controls ($n=7$, $*P=0.03$). **B**, Higher endothelial PKC β expression levels were associated with lower flow-mediated dilation.

not observe differences in basal or insulin-mediated eNOS phosphorylation at threonine 495 with PKC β inhibition. Collectively, our findings support the concept that PKC β activity impedes regulation of eNOS activation in diabetes mellitus.

Previous investigations of eNOS expression in diabetic conditions have yielded conflicting results. In cultured endothelial cells, hyperglycemia has been shown to decrease total eNOS protein levels; however, similar glucose conditions were reported to increase eNOS expression along with increased superoxide production and reduced NO bioactivity.^{33,34} Increased vascular eNOS expression has also been observed in the aortas of insulin resistant rats, and eNOS expression was normalized after treatment with a PKC

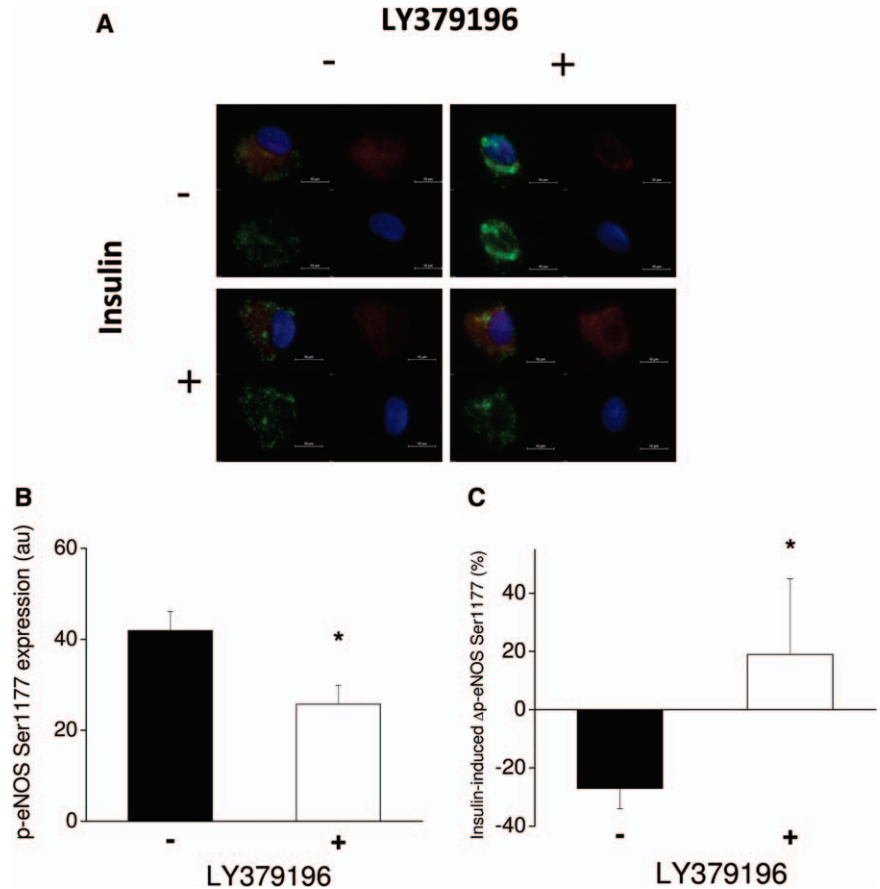
inhibitor.¹² In patients undergoing coronary artery bypass grafting, expression of eNOS was lower in the internal mammary arteries in the presence of diabetes.³⁵ However, eNOS levels were similar in freshly isolated endothelial cells from obese and lean individuals.²⁴ In the current study, endothelial eNOS expression was comparable in diabetic patients and controls, suggesting that eNOS deficiency is not an important mechanism for endothelial dysfunction in diabetes mellitus.

Inflammatory activation may contribute to endothelial dysfunction in diabetes mellitus. Experimental data link NF κ B activity to vascular insulin resistance and endothelial dysfunction.³⁶ Previous human data in older and obese individuals support a proinflammatory phenotype in endothelial cells characterized by increased NF κ B activation.^{24,37} Similarly, we found evidence of higher NF κ B activation in endothelial cells of patients with diabetes mellitus in conjunction with abnormal eNOS signaling. Our findings suggest the possibility that endothelial inflammatory activation through NF κ B contributes to vascular insulin resistance. PKC β inhibition increased I κ B α expression in endothelial cells from patients with diabetes mellitus, consistent with a link between PKC β activity and endothelial inflammation.¹⁴ Additional studies are needed to evaluate whether NF κ B inhibitors have a beneficial effect on endothelial signaling in human diabetes mellitus.

Several limitations of our study should be noted. The studies were performed using venous and not arterial endothelial cells, which may be more directly relevant to atherogenesis. However, venous endothelial cells are exposed to the systemic metabolic disturbances present in diabetes mellitus. Further, previous reports indicate correlations between protein expression in venous and arterial endothelial cells obtained by similar biopsy methodology.^{18,38} The relatively modest sample size for the endothelial cell evaluations precludes multivariable adjustment for confounders. Dysregulation of eNOS activation in diabetes mellitus is likely complex and multifactorial.⁵ Based on animal studies, we prospectively sought to investigate the contribution of PKC β activity.¹⁰ Further studies are warranted to evaluate additional mechanisms for altered eNOS signaling in diabetes mellitus. Additional phosphorylation sites have been described that regulate eNOS activity, which we have not evaluated in the present study.²⁵ As has been described previously, we observed lower flow-mediated and nitroglycerin-mediated vasodilation in patients with diabetes mellitus, indicating multilevel abnormalities in vasomotor function.^{39–42} These limitations are counterbalanced by the novel functional demonstration of insulin action in endothelial cells from patients and evidence supporting a role for PKC β in abnormal endothelial signaling in humans with diabetes mellitus.

In summary, we have demonstrated the presence of endothelial insulin resistance in diabetic patients with endothelial dysfunction that is alleviated by PKC β inhibition. The emergent diabetes mellitus epidemic necessitates the identification of novel therapeutic interventions for cardiovascular disease reduction. Pharmaceutical agents that reduce PKC β

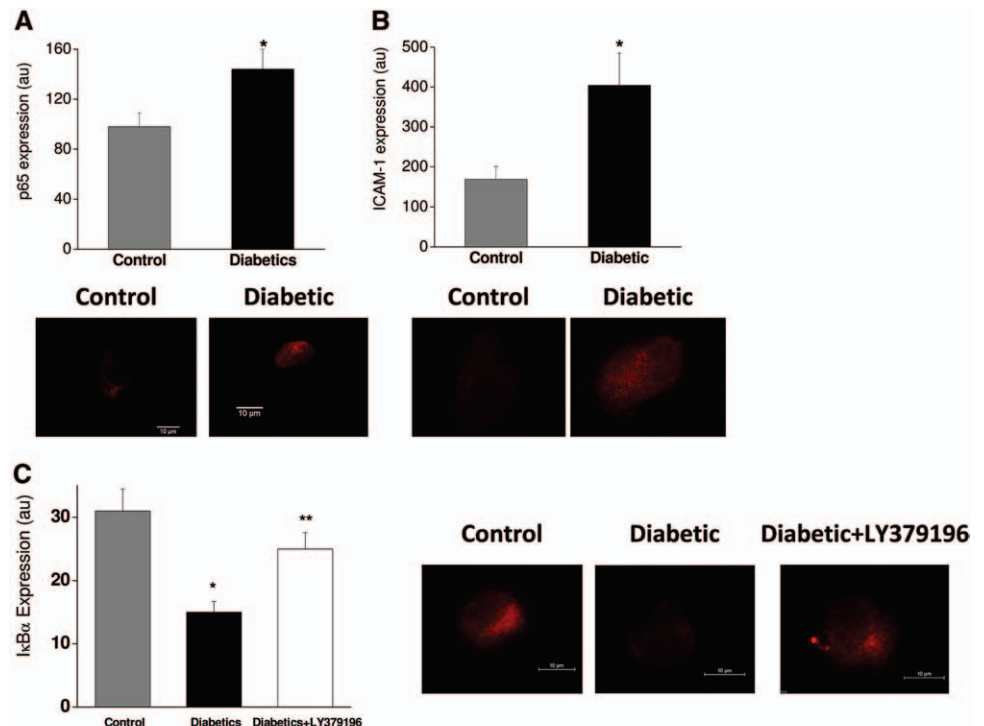
Figure 5. Inhibition of protein kinase C- β (PKC β) alters endothelial nitric oxide synthase (eNOS) phosphorylation and insulin response in endothelial cells from diabetic patients. As described in Methods, freshly isolated endothelial cells from patients with diabetes mellitus ($n=7$) were treated with 0 or 30 nmol/L LY379196, a pharmacological inhibitor of PKC β , along with 0 or 10 nmol/L insulin for 30 minutes, and eNOS phosphorylation at serine 1177 was measured (20 cells per patient in each condition). **A**, Representative cells from a patient with diabetes mellitus treated with LY379196 (**right**) shows lower basal eNOS phosphorylation at serine 1177 (**top**, red) compared with control condition (**left top**, red). Treatment with LY379196 improved the insulin-mediated change in eNOS phosphorylation at serine 1177 (**right top**, red vs **right bottom**, red) compared with control condition (**left top**, red vs **left bottom**, red). **B**, Basal eNOS phosphorylation at serine 1177 was reduced in endothelial cells from patients with diabetes mellitus treated with LY379196 ($*P=0.02$). **C**, Treatment with LY379196 improved insulin-mediated eNOS phosphorylation at serine 1177 in endothelial cells from patients with diabetes mellitus ($*P=0.04$).



activity have shown potential in selected clinical trials to reduce microvascular complications in diabetes mellitus.¹⁰ Our findings provide support for developing interventions that

restore insulin action in the endothelium, potentially by inhibiting PKC β , as a strategy to lower vascular disease burden in patients with diabetes mellitus.

Figure 6. Protein kinase C- β (PKC β) and endothelial inflammatory activation in diabetes mellitus. **A**, There was a trend for higher expression of nuclear factor kappa B (NF κ B) subunit p65 in endothelial cells from patients with diabetes mellitus ($n=4$ diabetic and 5 nondiabetic, $*P=0.06$). **B**, There was a trend for higher expression of intercellular adhesion molecule-1, a cellular adhesion molecule regulated by NF κ B, in endothelial cells from patients with diabetes mellitus ($n=4$ diabetic and 5 nondiabetic, $*P=0.06$). **C**, Expression of I κ B α , an inhibitor of NF κ B, was lower in endothelial cells from patients with diabetes ($n=6$ diabetic and 5 nondiabetic, $*P=0.004$). Treatment with LY379196 for 30 minutes increased I κ B α expression in patients with diabetes mellitus ($**P=0.03$).



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Disclosures

None.

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CLINICAL PERSPECTIVE

Atherosclerotic vascular disease in patients with type 2 diabetes mellitus is a pressing health problem. Abnormal endothelial function contributes to cardiovascular disease development and manifestation in diabetic patients. Experimental studies link insulin resistance and endothelial dysfunction. We demonstrated that endothelial cells collected directly from patients with diabetes mellitus have impaired activation of endothelial nitric oxide synthase in response to insulin. Further, we observed evidence of increased oxidative stress and inflammatory activation in the endothelial cells from diabetic patients. We found evidence of higher levels of PKC β in the endothelial cells from diabetic patients that were associated with lower endothelial function measured by brachial artery flow-mediated dilation. Treatment with a PKC β inhibitor improved insulin signaling and reduced inflammatory activation in the endothelial cells from patients with diabetes mellitus. This study provides evidence that patients with diabetes mellitus have endothelial insulin resistance and supports the possibility that treatments aimed at restoring insulin action in the endothelium may be a novel strategy to reduce vascular disease burden in this high risk group.

Protein Kinase C- β Contributes to Impaired Endothelial Insulin Signaling in Humans With Diabetes Mellitus

Corey E. Tabit, Sherene M. Shenouda, Monica Holbrook, Jessica L. Fetterman, Soroosh Kiani, Alissa A. Frame, Matthew A. Kluge, Aaron Held, Mustali M. Dohadwala, Noyan Gokce, Melissa G. Farb, James Rosenzweig, Neil Ruderman, Joseph A. Vita and Naomi M. Hamburg

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SUPPLEMENTAL MATERIAL

Supplemental Figure 1. Time course of insulin-mediated eNOS phosphorylation at serine 1177 in HAEC's. HAEC's were treated with 10nM insulin and fixed at time points up to 60minutes. eNOS phosphorylation was quantified using immunofluorescence with 20 cells assessed per time point. There was a transient increase in eNOS phosphorylation at 5 minutes and a sustained increase at 30 minutes.

Supplemental Figure 2. Box and whisker plots showing eNOS activation and insulin resistance in endothelial cells. Top panels show baseline eNOS phosphorylation at serine 1177 and response to insulin comparing diabetic patients and non-diabetic controls. Lower panels show baseline eNOS phosphorylation at threonine 495 and response to insulin comparing diabetic patients and non-diabetic controls.

Supplemental Figure 3. Association of insulin-mediated change in eNOS activation and flow-mediated dilation.

Supplemental Figure 4. Box and whisker plots showing endothelial cell expression of eNOS, Akt, PTEN, and nitrotyrosine in diabetic patients and non-diabetic controls.

Supplemental Figure 5. Box and whisker plots showing endothelial cell expression of PKC β , p65, and ICAM in diabetic patients and non-diabetic controls. Expression of I κ B α in non-diabetic controls, and patients with diabetes before and after treatment with LY379196.

Supplemental Figure 6. Box and whisker plots showing endothelial expression of eNOS phosphorylated at serine 1177 and response to insulin in patients with diabetes before and after treatment with LY379196.

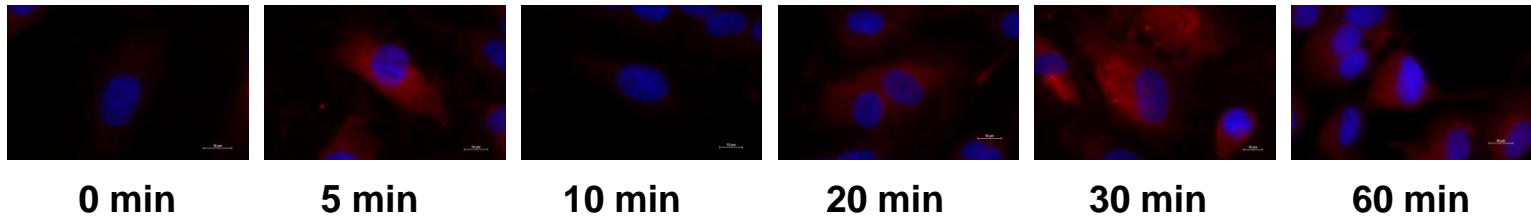
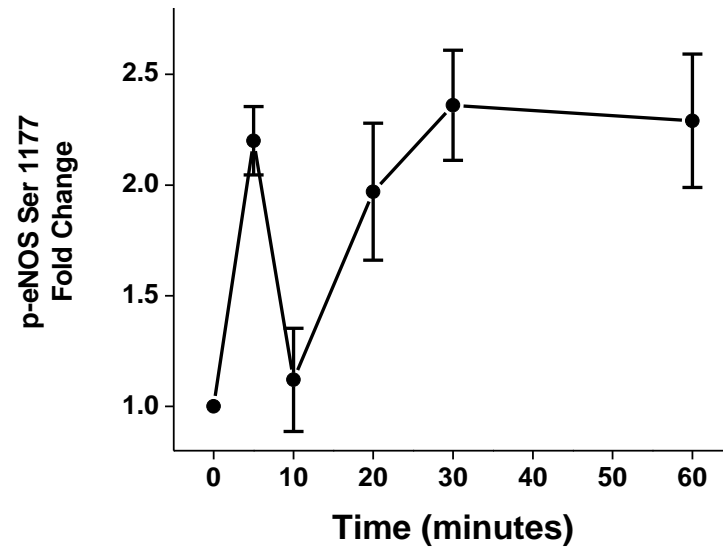
Supplemental Figure 7. Inhibition of PKC β and inhibitory eNOS phosphorylation. As described in Methods, freshly isolated endothelial cells from patients with diabetes (n=9) were treated with 0 or 30nM LY379196, a pharmacologic inhibitor of PKC β , along with 0 or 10nM insulin for 30 minutes and eNOS phosphorylation at threonine 495 was measured (20 cells per patient in each condition). **A.** Representative cells from a patient with diabetes treated with LY379196 (right) shows similar basal eNOS phosphorylation at threonine 495 (top, red) compared to control condition (left top, red). Treatment with LY379196 did not change the insulin-mediated increase in eNOS phosphorylation at threonine 495 compared to control condition. **B.** Basal eNOS phosphorylation at threonine 495 was similar in endothelial cells from patients with diabetes treated with LY379196 (P=0.95). **C.** Treatment with LY379196 did not alter eNOS phosphorylation at threonine 495 in response to insulin in endothelial cells from patients with diabetes (P=0.07).

Supplemental Table 1. Medication use and eNOS activation in patients with diabetes

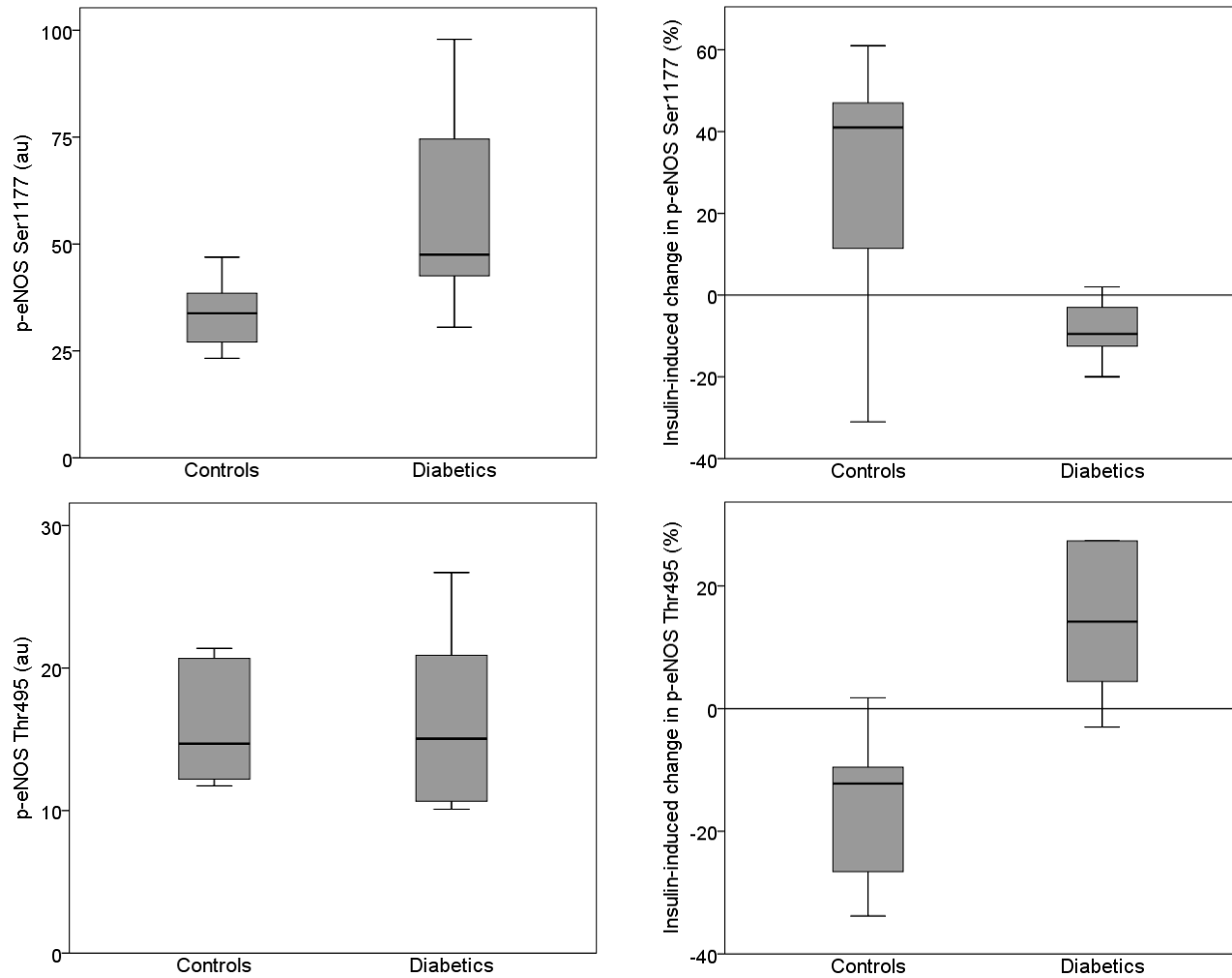
| | Statin | | | ACEI or ARB | | | Metformin | | | Insulin | | |
|--|---------------|------------|------|--------------------|------------|------|------------------|------------|------|----------------|------------|------|
| | No N=5 | Yes N=7 | P | No N=4 | Yes N=8 | P | No N=4 | Yes N=8 | P | No N=6 | Yes N=6 | P |
| p-eNOS Ser1177 (au) | 49±24 | 62±23 | 0.37 | 41±12 | 64±24 | 0.10 | 53±7 | 59±29 | 0.69 | 51±25 | 63±22 | 0.39 |
| Insulin-induced Δp-eNOS Ser1177 (%) | -13±13 | -8±6 | 0.41 | -6±4 | -12±11 | 0.36 | -12±7 | -9±11 | 0.65 | -11±14 | -9±4 | 0.72 |

mean±SD

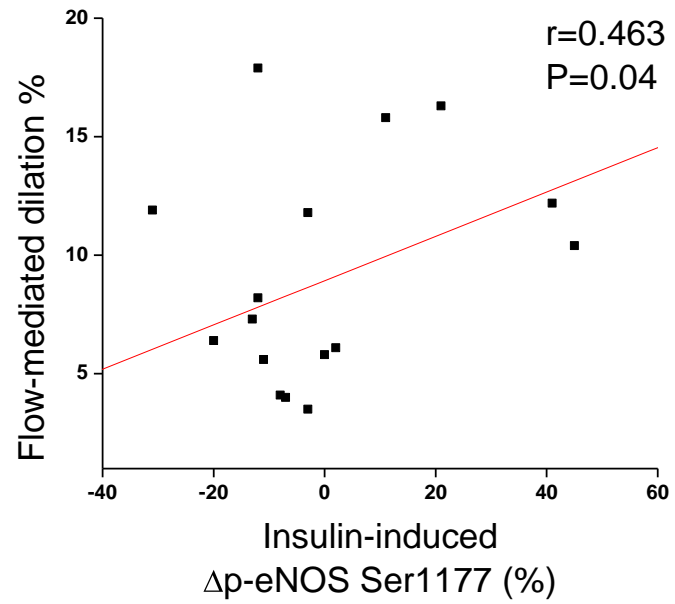
ACEI: Angiotensin converting enzyme inhibitor; ARB: Angiotensin receptor blocker; eNOS: endothelial nitric oxide synthase



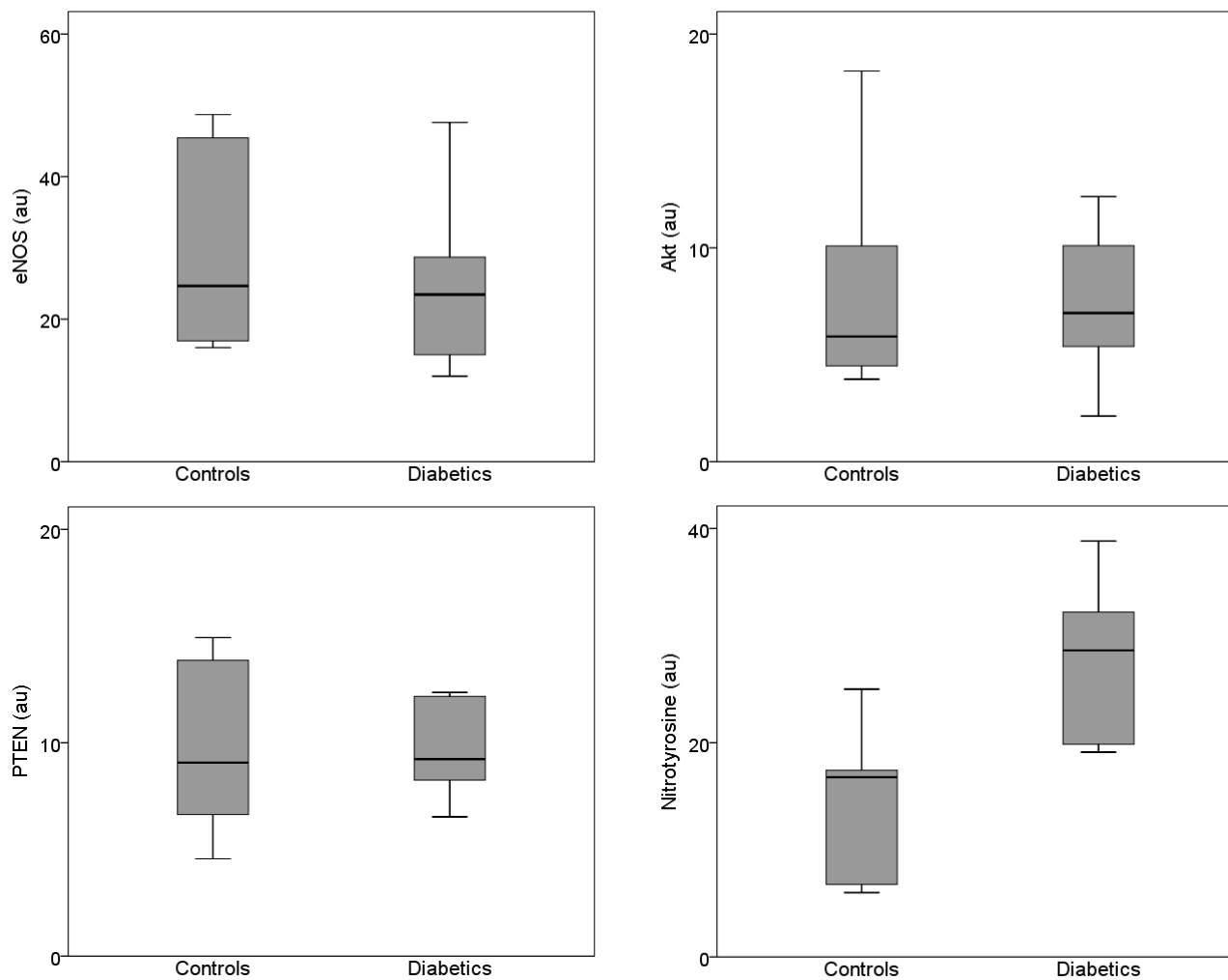
Supplementary Figure 1



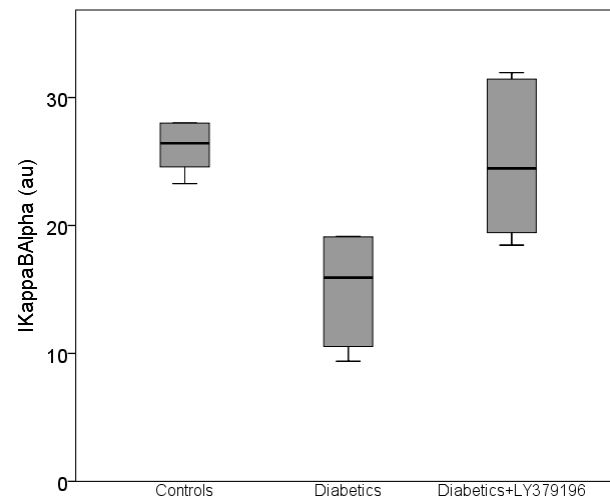
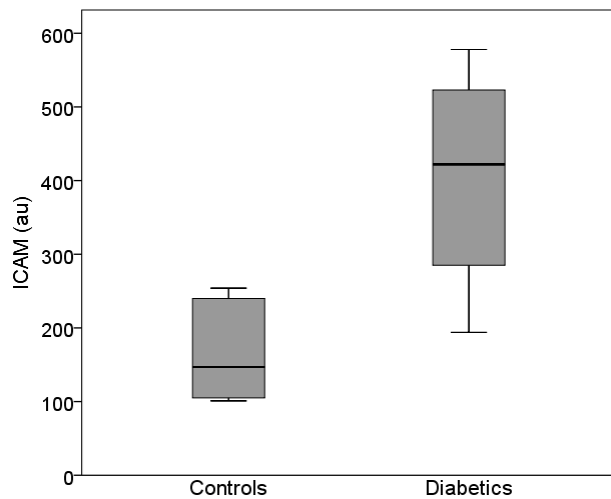
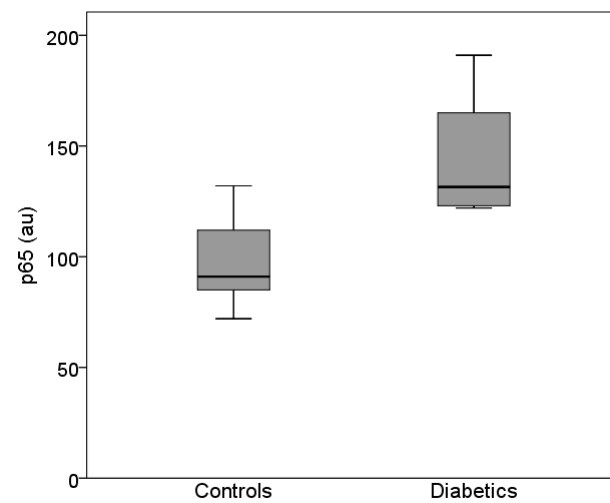
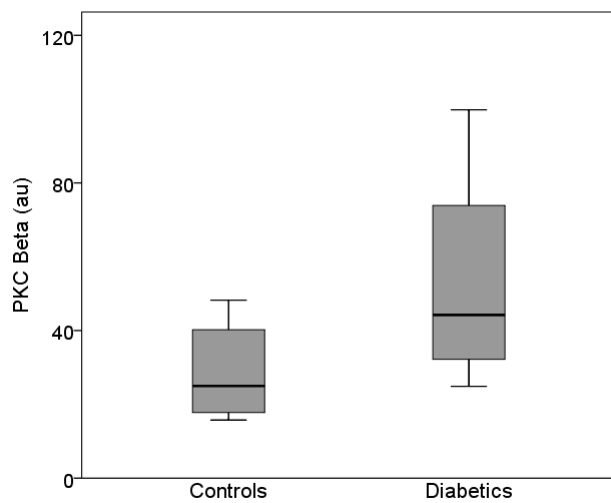
Supplementary Figure 2



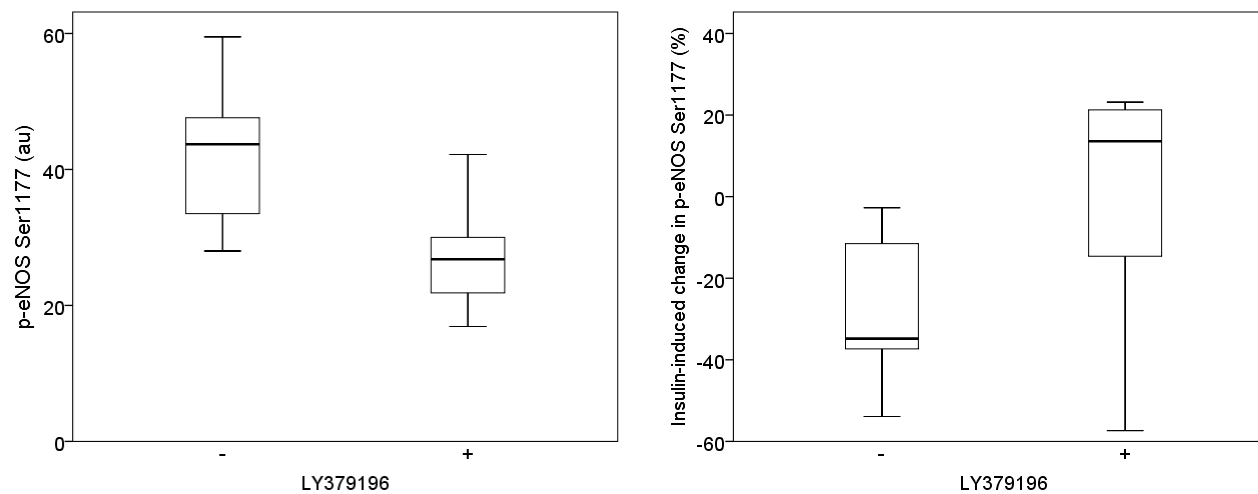
Supplementary Figure 3



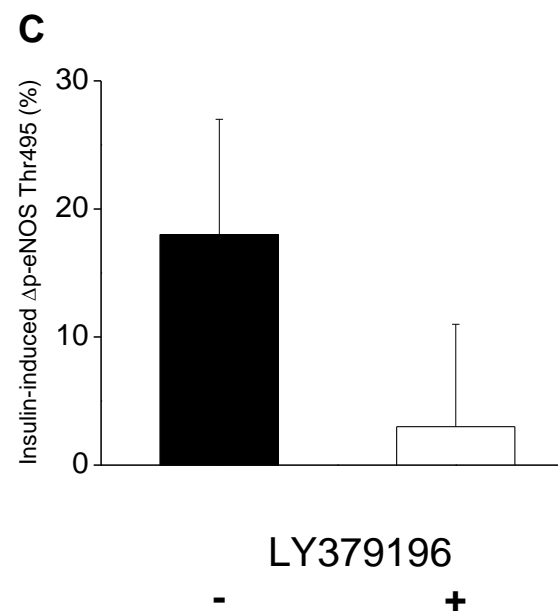
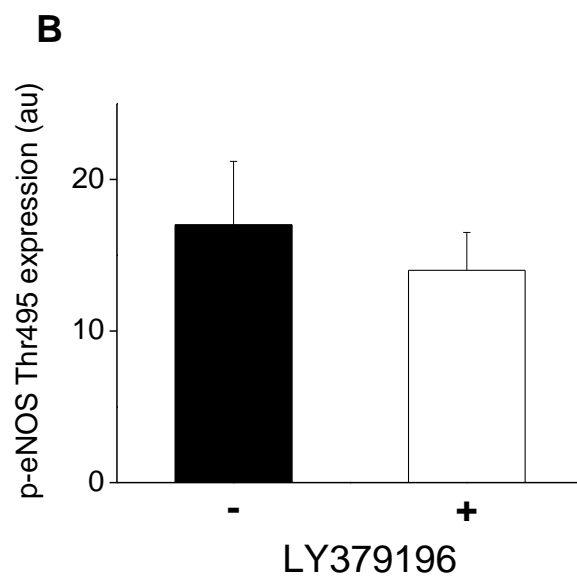
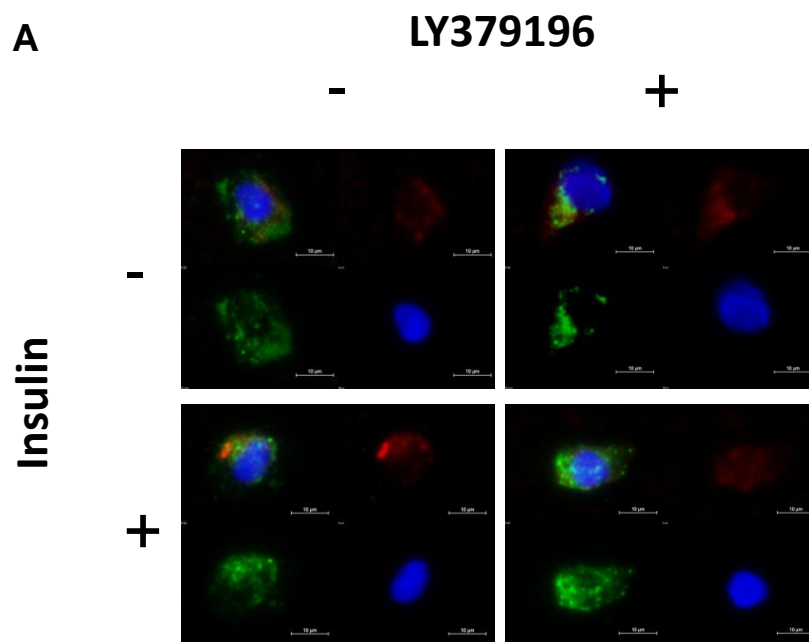
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Supplementary Figure 7