

# Activation of Vascular Protein Kinase C- $\beta$ Inhibits Akt-Dependent Endothelial Nitric Oxide Synthase Function in Obesity-Associated Insulin Resistance

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Activation of protein kinase C (PKC) in vascular tissue is associated with endothelial dysfunction and insulin resistance. However, the effect of vascular PKC activation on insulin-stimulated endothelial nitric oxide (NO) synthase (eNOS) regulation has not been characterized in obesity-associated insulin resistance. Diacylglycerol (DAG) concentration and PKC activity were increased in the aorta of Zucker fatty compared with Zucker lean rats. Insulin-stimulated increases in Akt phosphorylation and cGMP concentration (a measure of NO bioavailability) after euglycemic-hyperinsulinemic clamp were blunted in the aorta of fatty compared with lean rats but were partly normalized after 2 weeks of treatment with the PKC $\beta$  inhibitor ruboxistaurin (LY333531). In endothelial cell culture, overexpression of PKC $\beta$ 1 and - $\beta$ 2, but not PKC $\alpha$ , - $\delta$ , or - $\zeta$ , decreased insulin-stimulated Akt phosphorylation and eNOS expression. Overexpression of PKC $\beta$ 1 and - $\beta$ 2, but not PKC $\alpha$  or - $\delta$ , also decreased Akt phosphorylation stimulated by vascular endothelial growth factor (VEGF). In microvessels isolated from transgenic mice overexpressing PKC $\beta$ 2 only in vascular cells, Akt phosphorylation stimulated by insulin was decreased compared with wild-type mice. Thus, activation of PKC $\beta$  in endothelial cells and vascular tissue inhibits Akt activation by insulin and VEGF, inhibits Akt-dependent eNOS regulation by insulin, and causes endothelial dysfunction in obesity-associated insulin resistance. *Diabetes* 55:691–698, 2006

The development of atherosclerosis is preceded by, and partly results from, decreased production of endothelium-derived nitric oxide (NO) (1). Insulin resistance, an independent risk factor for cardiovascular disease (2), is associated with endothelial dysfunction and a loss of the vasodilatory

effect of insulin (3). Insulin increases endothelial NO synthase (eNOS) catalytic activity (4) as well as eNOS gene expression (5–8), but both eNOS activity (9) and expression (5) are decreased in insulin-resistant animals. Therefore, the compromised effectiveness of insulin in regulating eNOS is believed to cause endothelial dysfunction in insulin resistance and in diabetes.

Both insulin-stimulated eNOS activation (4) and induction of eNOS gene expression (5,7) are dependent on phosphatidylinositol (PI) 3-kinase, and insulin-stimulated eNOS activation is mediated by a downstream effector of PI 3-kinase signaling, protein kinase Akt, which directly phosphorylates eNOS on Ser1177 (10). We have shown selective impairment of vascular activation of PI 3-kinase and Akt as well as insulin signaling upstream of PI 3-kinase in the Zucker fatty rat (11). Besides insulin, other stimuli signal through the PI 3-kinase/Akt pathway during activation and induction of eNOS, among them estrogen and other hormones, growth factors like vascular endothelial growth factor (VEGF), and shear stress (12). Therefore, compromised Akt signaling in the insulin-resistant state may affect eNOS regulation by several stimuli in addition to insulin. Thus, insulin resistance may have a greater impact on eNOS regulation than can be accounted for by inhibition of the effects of insulin alone.

Many factors in insulin resistance may cause endothelial dysfunction (13), among them postprandial hyperglycemia, elevated concentrations of free fatty acids (FFAs), activation of the renin-angiotensin system, oxidative stress, and proinflammatory cytokines. Our laboratory has suggested that activation of protein kinase C (PKC) plays an important role in vascular complications of diabetes. Diabetes or high glucose concentrations will activate certain PKC isoforms in vascular cells, including the PKC $\beta$ 1 and - $\beta$ 2 isoforms, likely by de novo synthesis of diacylglycerol (DAG) from glucose or FFAs (13). Ruboxistaurin (LY333531), a selective PKC $\beta$  inhibitor, improves the decreased retinal blood flow seen in diabetic rats (14), the decreased endothelium-dependent vasodilation observed in humans during hyperglycemia (15), and the decreased vascular NO production seen in Zucker fatty rats (9). One mechanism that may explain these changes is that PKC activation selectively inhibits activation of PI 3-kinase/Akt in the vasculature (5,11). However, to date there has been no systematic demonstration of which PKC isoforms are responsible for decreased PI 3-kinase/Akt-dependent eNOS regulation in vascular cells. In the current study, we characterize the interaction between

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BAEC, bovine aortic endothelial cell; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; eNOS, endothelial nitric oxide synthase; FFA, free fatty acid; GFP, green fluorescent protein; PI, phosphatidylinositol; PKC, protein kinase C; VEGF, vascular endothelial growth factor.

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TABLE 1  
Physiological parameters during hyperinsulinemic clamp in Zucker rats

	RBX	n	Body mass (g)	Plasma insulin concentration (nmol/l)		GDR (mmol · kg <sup>-1</sup> · min <sup>-1</sup> )
				Before clamp	End of clamp	
Lean	–	10	368.3 ± 4.2	0.12 ± 0.07	1.38 ± 0.27	228.6 ± 19.9
	+	6	367.5 ± 7.5	0.11 ± 0.09	1.79 ± 0.43	278.5 ± 8.3*
Fatty	–	10	567.0 ± 8.2†	1.21 ± 0.24	2.93 ± 0.5	52.1 ± 14.4
	+	6	565.0 ± 10.6	1.59 ± 0.15	3.23 ± 0.41	64.9 ± 18.8‡

Data are means ± SE. \*NS compared with lean without ruboxistaurin (RBX) treatment; † $P < 0.01$  compared with lean without ruboxistaurin treatment; ‡NS compared with fatty without ruboxistaurin treatment. GDR, glucose disposal rate.

isoform-specific PKC activation and the PI 3-kinase/Akt/eNOS signaling pathway in cell culture and in animal models.

## RESEARCH DESIGN AND METHODS

All protocols for animal use and euthanasia were reviewed and approved by the animal care committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines. Male Zucker fatty rats and control Zucker lean rats (Harlan Sprague Dawley, Indianapolis, IN) at 14 weeks of age were allocated to either of two treatment regimens for 2 weeks: normal chow ad libitum or chow containing 0.062% (wt/wt) of the PKC $\beta$ -specific inhibitor ruboxistaurin (LY333531) (Lilly Research Laboratories, Indianapolis, IN), corresponding to a dose of 30 mg/kg body wt. Physiological parameters of the Zucker obese and lean rats are described in Table 1.

**Transgenic mice overexpressing vascular PKC $\beta$ 2.** The creation of FVB mice with a PKC $\beta$ 2 transgene under control of the prepro-endothelin promoter (PKC $\beta$ 2Tg), resulting in overexpression of PKC $\beta$ 2 in endothelial cells and vascular smooth muscle cells, has been described previously (16). The mice used in the current study were bred from the same colony. Incorporation of the transgene was demonstrated by Southern blotting, and transgene expression was confirmed by Northern blotting and immunoblotting from preparations of heart, aorta, and retina. Male mice were studied at 20 weeks of age using wild-type nonlittermate FVB mice as controls.

**Isolation of microvessels from adipose tissue.** Isolation of microvessels from epididymal fat pads by collagenase digestion and centrifugation was performed with a previously validated method (5,11). Fat pads were excised from transgenic mice overexpressing vascular PKC $\beta$ 2 or from control wild-type FVB mice at 20 weeks of age. Isolated microvessels were resuspended in Dulbecco's modified Eagle's medium (DMEM) with 0.1% BSA and left for 30 min at 37°C or stimulated for the same duration by adding insulin to the suspension.

**Euglycemic-hyperinsulinemic clamp.** Measurement of insulin-stimulated vascular cGMP production was used to assess NO bioavailability in vivo (see below). Because cGMP produced as a result of insulin stimulation would not be stable during isolation of microvessels as described above, we chose to measure cGMP concentration before and after insulin stimulation as part of a euglycemic-hyperinsulinemic clamp in Zucker rats, performed as described previously (11).

**Preparation of aorta and heart.** Immediately after killing each rat, the aorta and heart were isolated, frozen in liquid nitrogen, and stored at –80°C for subsequent analysis. Frozen tissue was crushed in liquid nitrogen, and the powdered aorta was divided into two parts for Western blot analysis and cGMP assay, respectively.

**Measurement of DAG concentrations and PKC activity.** As described previously, DAG in the aorta was determined by a DAG kinase assay (17), and PKC activity was measured in cytosol and membrane fractions of tissue lysate as <sup>32</sup>P-incorporation into the peptide substrate RKRTLRL (single-letter amino acid abbreviations) (17).

**Measurement of vascular cGMP concentration.** Vascular cGMP concentration was measured using a commercially available enzyme immunoassay (Amersham Biosciences). Aortas were homogenized in 6% (vol/vol) trichloroacetic acid at 4°C, thus precipitating sample protein. After centrifugation (2,000 × *g* for 15 min at 4°C), the pellet was stored for subsequent measurement of protein content, and the supernatant was used for the determination of cGMP concentration by enzyme immunoassay according to the manufacturer's instructions.

**Separation of cytosol and membrane fractions of aorta lysate.** Cytosol and membrane fractions of lysate from aorta were fractionated by a method involving ultracentrifugation steps as described in detail previously (17).

**Insulin receptor substrate 2-associated PI 3-kinase activity.** PI 3-kinase activity was assayed in insulin receptor substrate 2 immunoprecipitates followed by separation of <sup>32</sup>P-labeled phospholipids with thin-layer chromatography as described previously (5).

**Cell culture.** Bovine aortic endothelial cells (BAECs) were isolated by scraping the luminal side of calf aorta with a cover glass. Cells were cultured in DMEM containing 5.5 mmol/l glucose and 10% platelet-poor horse serum (Sigma, St. Louis, MO) and used between passages 2 and 7. Subconfluent cells were starved in DMEM containing 0.1% BSA for 24 h before stimulation with insulin or VEGF. In experiments with adenovirus infection, serum starvation was begun 24 h after the start of virus incubation.

**Recombinant adenovirus.** We have previously described construction of recombinant adenovirus (18), including those encoding a constitutively active Akt (gag fused to the NH<sub>2</sub>-terminal of Akt); an Akt mutant acting as a dominant-negative active Akt (Akt T308A/S473A); enhanced green fluorescent protein (GFP) gene together with PKC $\alpha$ , - $\beta$ 2, - $\delta$ , or - $\zeta$ ; GFP together with a PKC $\zeta$  K273W dominant-negative mutant; and control adenovirus encoding either gag or GFP. An adenovirus encoding PKC $\beta$ 1 was made with similar techniques. We have previously shown that BAECs have endogenous expression of PKC $\alpha$ , - $\beta$ 1, - $\beta$ 2, - $\delta$ , - $\epsilon$ , and - $\zeta$  (19).

BAECs were infected with one of the adenovirus constructs described above or with control adenovirus containing the same parental genome but being a vector only for gag or for GFP. Cell cultures were incubated with 0.5 × 10<sup>8</sup> plaque-forming units for 1 h. The virus solution was then diluted fourfold in growth medium, and the cultures were incubated for an additional 24 h. Expression of each recombinant protein was confirmed by Western blotting. The PKC constructs caused expression of the isoform in question to increase ~10-fold compared with cells infected with the control adenovirus.

**Western blotting.** Cells, isolated microvessels, or frozen, powdered aorta were solubilized in lysis buffer as described previously (5). In the case of aorta, lysis was promoted by treatment in a glass Dounce homogenizer on ice. Lysate was assayed with Western blotting as described previously (5), using antibodies to phospho-Akt (Ser473), Akt (both from Cell Signaling Technology, Beverly, MA), and eNOS (BD Biosciences Pharmingen, San Diego, CA).

**RNA isolation and Northern blot analysis.** Total RNA from BAECs was isolated, and eNOS mRNA was assayed by Northern blotting and normalized to 36B4 mRNA as described previously (5).

**Statistical analysis.** Data are presented as the means ± SE. Results were analyzed with the Kruskal-Wallis one-way ANOVA, using post hoc comparisons with paired or unpaired *t* test as appropriate. *P* values <0.05 were considered statistically significant.

## RESULTS

**Vascular insulin signaling is decreased in Zucker fatty rats.** To study obesity-associated changes in vascular insulin signaling, we used the model of nondiabetic insulin resistance offered by the Zucker fatty rat (20). As expected, Zucker fatty rats had elevated fasting plasma insulin, 10.1 ± 2.0-fold higher than Zucker lean rats (Table 1). Fasting blood glucose was 98 ± 5 mg/dl (5.4 ± 0.3 mmol/l) in Zucker lean rats and 124 ± 8 mg/dl (6.9 ± 0.4 mmol/l) in Zucker fatty rats ( $P = 0.04$ ). During euglycemic-hyperinsulinemic clamp, insulin-stimulated glucose disposal rate was reduced by 78 ± 5% in Zucker fatty rats compared with lean rats ( $P < 0.001$ , Table 1).

In the aorta of Zucker fatty rats, total DAG concentration was 46% higher than in lean rats (Fig. 1A,  $P < 0.05$ ). PKC activity was not different in the cytosol fraction in

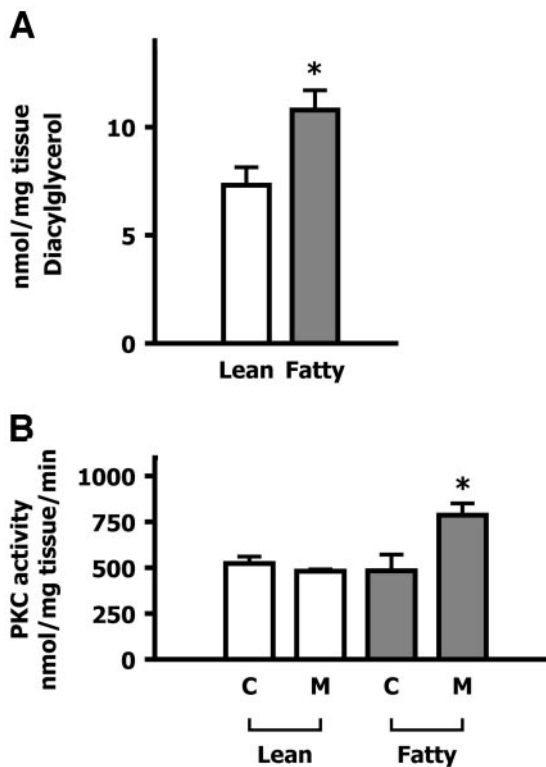


FIG. 1. Vascular diacylglycerol concentrations and PKC activity in aorta of Zucker lean and fatty rats. Aortas were isolated from Zucker lean and Zucker fatty rats. **A:** DAG concentration in aorta ( $n = 6$ ;  $*P < 0.05$ ). **B:** PKC activity in cytosol (C) and membrane (M) fractions of lysate from aorta ( $n = 6$ ;  $*P < 0.05$ ).

fatty and lean rats (Fig. 1B), but membrane-associated PKC activity was 63% higher in fatty rats (Fig. 1B,  $P < 0.05$ ). We have previously found, by using antibodies against PKC $\alpha$ , - $\beta$ 1, - $\beta$ 2, and - $\gamma$ , that only PKC $\alpha$  and - $\beta$ 2 were detectable in the aorta of rats with streptozotocin-induced diabetes and that only PKC $\beta$ 2 expression was increased in the membrane fraction (17). To examine PKC isoform expression in the lean and fatty Zucker rats, we immunoblotted PKC $\alpha$ , - $\beta$ 2, - $\delta$ , and - $\epsilon$  in the cytosol and membrane fractions of lysate from aorta. The membrane-associated expression was increased for all of these isoforms (Fig. 2A), whereas cytosol-associated expression was unchanged (Fig. 2A; summarized data not shown). Furthermore, protein expression of eNOS was decreased by 64% in the heart of fatty rats compared with lean rats ( $P < 0.05$ , Fig. 2B), although it was unchanged in aorta ( $120 \pm 36\%$  of the levels in lean rats, NS). In microvessels isolated from fat pads, Akt protein expression was not significantly different between lean and fatty Zucker rats ( $94 \pm 25\%$  in lean rats relative to fatty rats,  $P = 0.6$ ; results not shown).

In lean rats, hyperinsulinemic clamp stimulated Akt phosphorylation by  $3.0 \pm 1.0$ -fold compared with saline infusion, but hyperinsulinemia had no effect on Akt phosphorylation in fatty rats (Fig. 3A). NO production in the aorta was measured as cGMP production. Hyperinsulinemic clamp increased cGMP concentration in the aorta by  $2.9 \pm 0.7$ -fold in lean rats ( $P < 0.05$ ) but had no effect in fatty rats (Fig. 3B).

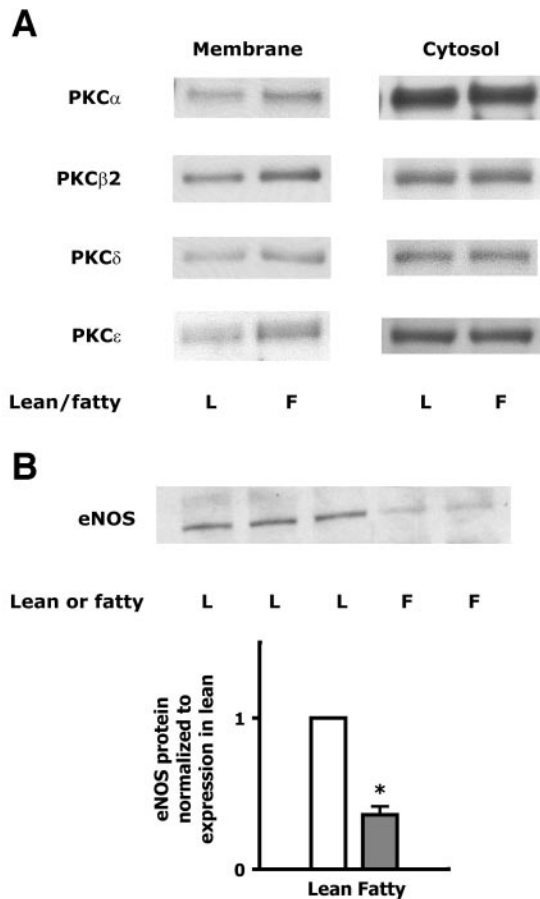
We have previously shown that activation of PKC with phorbol ester or overexpression of PKC $\beta$ 1 inhibits insulin-stimulated eNOS expression in endothelial cell culture (5).

We therefore evaluated whether vascular insulin resistance in vivo can be reversed by inhibition of PKC $\beta$ . A separate group of Zucker lean and fatty rats was treated for 2 weeks with the selective PKC $\beta$  inhibitor ruboxistaurin administered in chow before being studied by a euglycemic-hyperinsulinemic clamp. Glucose disposal rate was not statistically different between ruboxistaurin-treated and untreated rats in either lean or fatty rats (Table 1). Insulin-stimulated Akt phosphorylation in aorta was not different in ruboxistaurin-treated lean rats compared with untreated lean rats, but ruboxistaurin treatment partly restored insulin-stimulated Akt phosphorylation in fatty rats (no increase compared with saline infusion in untreated fatty rats;  $2.1 \pm 0.2$ -fold increase in ruboxistaurin-treated fatty rats;  $P < 0.01$ , Fig. 3A). Similar results were obtained for cGMP production. Thus, ruboxistaurin treatment did not change insulin-stimulated cGMP production in aorta of lean rats but completely normalized insulin-stimulated cGMP production in fatty rats (no increase in cGMP concentration compared with saline infusion in untreated fatty rats;  $3.4 \pm 0.8$ -fold increase in ruboxistaurin-treated fatty rats;  $P < 0.05$ , Fig. 3B).

**Insulin-stimulated induction of eNOS expression is dependent on Akt.** Akt, activated by PI 3-kinase, directly phosphorylates eNOS on Ser1177 after stimulation of endothelial cells with insulin, growth factors, shear stress, or other factors (12). We have previously shown that insulin-stimulated induction of eNOS gene expression is dependent on PI 3-kinase (5). For the present study, we wished to evaluate whether insulin-stimulated eNOS gene induction is dependent on Akt or PKC $\zeta$ , both of which are downstream of PI 3-kinase during insulin signaling. In BAECs, dominant-negative Akt expression significantly decreased eNOS mRNA compared with the control condition (Fig. 4). After infection with GFP or gag control adenovirus, 6 h of insulin stimulation increased eNOS mRNA expression (by  $1.7 \pm 0.3$ - and  $1.7 \pm 0.1$ -fold compared with GFP control and gag control, respectively; Fig. 4). Expression of dominant-negative Akt completely inhibited insulin-stimulated eNOS induction, whereas dominant-negative PKC $\zeta$  had no effect (Fig. 4).

**PKC $\beta$  isoforms inhibit insulin-stimulated Akt activation and induction of eNOS expression.** As mentioned above, we have previously shown that activation of PKC with phorbol ester or overexpression of PKC $\beta$ 1 inhibit insulin-stimulated eNOS expression (5). We next evaluated whether effects on insulin-stimulated Akt-mediated eNOS induction are limited to the PKC $\beta$  isoforms. In BAECs, adenovirus-mediated overexpression of PKC $\alpha$ , - $\beta$ 1, - $\beta$ 2, - $\delta$ , or - $\zeta$  did not change basal Akt phosphorylation compared with control adenovirus infection. In cultures infected with control adenovirus, insulin stimulation for 30 min increased Akt phosphorylation by 3.6-fold, and this stimulation was inhibited by overexpression of PKC $\beta$ 1 and - $\beta$ 2 (by 50 and 51%, respectively,  $P < 0.01$ , Fig. 5A). Overexpression of PKC $\alpha$ , - $\delta$ , and - $\zeta$  inhibited insulin-stimulated Akt phosphorylation to a lesser degree (by 27–37%), but these differences were not statistically significant.

A similar pattern was observed with regard to eNOS mRNA expression. Overexpression of PKC $\alpha$ , - $\beta$ 1, - $\beta$ 2, - $\delta$ , or - $\zeta$  did not change basal eNOS mRNA. Insulin stimulation for 6 h increased eNOS mRNA 2.2-fold, and this stimulation was significantly inhibited by overexpression of PKC $\beta$ 1 and - $\beta$ 2. Inhibition was also observed for PKC $\alpha$ , - $\delta$ , and - $\zeta$ , although these changes were not statistically



**PKC isoform expression in membrane fraction: fold difference between Zucker lean and fatty rats.**

	Lean	Fatty	<i>p</i>
PKC $\alpha$	1.00 $\pm$ 0.07	1.35 $\pm$ 0.19	0.01
PKC $\beta$ 2	1.00 $\pm$ 0.08	1.44 $\pm$ 0.07	0.0002
PKC $\delta$	1.00 $\pm$ 0.08	1.35 $\pm$ 0.22	0.03
PKC $\zeta$	1.00 $\pm$ 0.10	1.37 $\pm$ 0.22	0.02

Data normalized to mean of all isoforms in lean rats and expressed as mean  $\pm$  SD.

**FIG. 2.** PKC and eNOS expression in aorta and heart from Zucker lean and fatty rats. Aorta and heart were isolated from Zucker lean and fatty rats. Representative immunoblots are shown along with summarized data. **A:** Expression of PKC isoforms was measured by Western blotting in cytosol and membrane fractions of aorta lysate ( $n = 4$ ). **B:** Expression of eNOS was measured by Western blotting in whole lysate from heart ( $*P < 0.05$ ).

significant (Fig. 5B). Thus, the inhibitory effect of PKC on eNOS induction stimulated by insulin and mediated by Akt is preferentially due to activation of the PKC $\beta$  isoforms.

**PKC activation inhibits Akt activation stimulated by VEGF.** As previously stated, several hormones and growth factors besides insulin are known to regulate eNOS activation through the PI 3-kinase/Akt pathway (12). To determine whether Akt activation caused by stimuli other than insulin is also inhibited by PKC, BAECs overexpressing PKC $\alpha$ , - $\beta$ 1, - $\beta$ 2, and - $\delta$  were stimulated with VEGF. Adenovirus-mediated overexpression of PKC $\beta$ 1 and - $\beta$ 2 inhibited VEGF-stimulated Akt phosphorylation by 98  $\pm$  21 and 95  $\pm$  15%, respectively, compared with control adenovirus infection ( $P < 0.01$ , Fig. 6). In contrast, overexpression of PKC $\alpha$ , - $\delta$ , and - $\zeta$  did not significantly inhibit VEGF-stimulated Akt phosphorylation. Thus, the selective ability of the PKC $\beta$  isoforms to inhibit Akt signaling is not limited to insulin signaling.

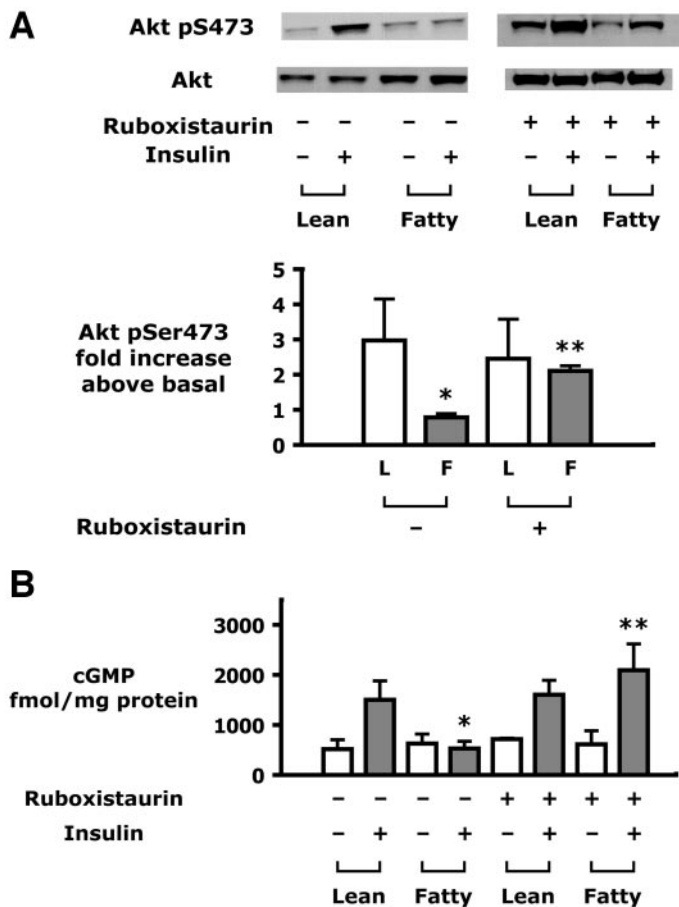
**Insulin signaling is impaired in microvessels from transgenic mice overexpressing PKC $\beta$ 2 in vascular tissue.** To confirm whether the PKC $\beta$  isoforms can inhibit vascular insulin signaling in vivo, we studied mice overexpressing PKC $\beta$ 2 only in vascular tissue (PKC $\beta$ 2Tg). In microvessels isolated from epididymal fat from PKC $\beta$ 2Tg mice, protein expression of PKC $\beta$ 2 and PKC activity were both increased by twofold compared with wild-type mice (data not shown). There was no significant difference in body weight or fasting plasma glucose concentrations between transgenic and wild-type mice (data also not shown). mRNA expression of eNOS in microvessels was

decreased by 42  $\pm$  7% in PKC $\beta$ 2Tg mice compared with wild-type mice (data not shown;  $P < 0.05$ ). Akt phosphorylation in response to stimulation of microvessels with insulin ex vivo was decreased by 60  $\pm$  12% ( $P < 0.05$ , Fig. 7).

**DISCUSSION**

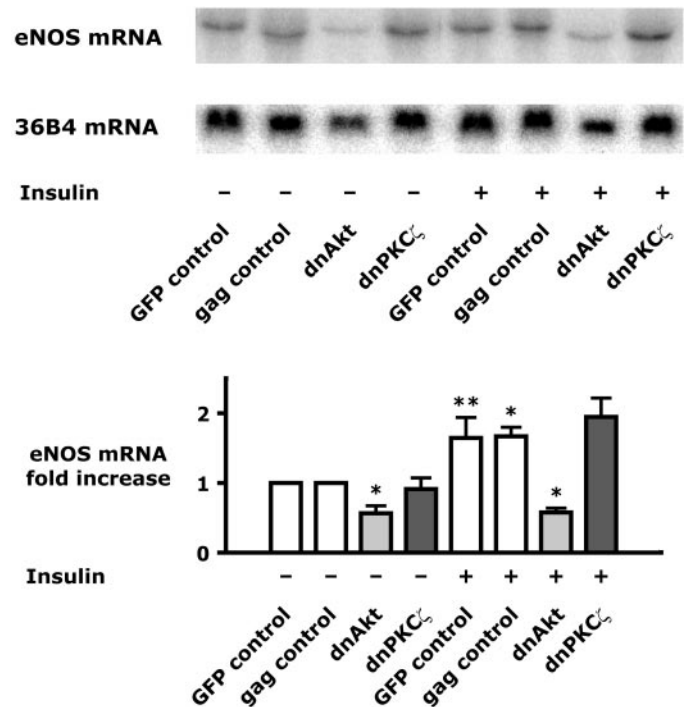
This study has provided evidence that activation of PKC, in particular the  $\beta$ -isoforms, can induce insulin resistance in endothelial cells and vascular tissue and cause endothelial dysfunction both in cultured cells and in vivo. More specifically, overexpression of PKC $\alpha$ , - $\beta$ 1, - $\beta$ 2, - $\delta$ , and - $\zeta$  in endothelial cell culture showed that insulin-stimulated, Akt-dependent eNOS gene expression is preferentially inhibited by the PKC $\beta$  isoforms. Furthermore, studies in mice overexpressing PKC $\beta$ 2 in the vasculature and in Zucker fatty rats showed that in vivo, PKC $\beta$ 2 activation is sufficient to inhibit vascular insulin-stimulated Akt signaling and that the impaired insulin-stimulated vascular NO production in obesity-associated insulin resistance can be partially normalized with the PKC $\beta$ -selective inhibitor ruboxistaurin.

It is well-established that chronic activation of vascular PKC in diabetes is due to de novo synthesis of DAG from glucose (13). We provide the first evidence that DAG concentrations and PKC activity are increased in blood vessels in insulin resistance associated with obesity. In the Zucker fatty rat, vascular PKC activation may be the result of increased plasma concentrations of FFAs (21). Elevated



**FIG. 3.** Akt phosphorylation and cGMP production in aorta of Zucker lean and fatty rats after an euglycemic-hyperinsulinemic clamp. Zucker lean or Zucker fatty rats were fed chow with or without the PKC $\beta$ 2 inhibitor ruboxistaurin for 2 weeks. Animals were subjected to saline infusion or to insulin infusion during a euglycemic-hyperinsulinemic clamp for 1 h. **A:** Phosphorylation of Akt Ser473 was measured in lysate of aorta. Representative immunoblots are shown above summarized data ( $n = 4$ ;  $*P < 0.05$  vs. insulin-stimulated Akt phosphorylation in lean rats without ruboxistaurin treatment;  $**P < 0.01$  vs. insulin-stimulated Akt phosphorylation in fatty rats without ruboxistaurin treatment). **B:** cGMP concentration was measured in lysate of aorta ( $n = 4$ ;  $*P < 0.05$  vs. lean rats without ruboxistaurin treatment;  $**P < 0.05$  vs. fatty rats without ruboxistaurin treatment).

FFAs have been reported to activate PKC in skeletal muscle (22–24). Decreased insulin action is associated with activation of PKC $\beta$ 2 and  $-\delta$  in humans (22), as opposed to the PKC $\theta$  isoform in rats (23) and mice (24), suggesting that the PKC isoform involved may be species dependent. A tissue-specific activation of PKC isoforms may also exist: because the PKC $\beta$ -specific inhibitor ruboxistaurin improved vascular insulin signaling in the Zucker fatty rat in our study, but had no effect on whole-body insulin sensitivity, it is possible that different PKC isoforms inhibit insulin signaling in blood vessels than in other tissues. We performed experiments in rats and bovine cell culture because we have extensive experience with insulin clamp and ruboxistaurin treatment in rats and with insulin signaling and adenovirus-mediated overexpression of PKC isoforms in BAECs. Furthermore, for in vivo overexpressing of PKC $\beta$ 2, we used mice in which transgenic techniques are standard. Even though the conclusions regarding the isoform or isoforms activated in vascular tissue are limited by the use of several species in the current study, we have previously shown

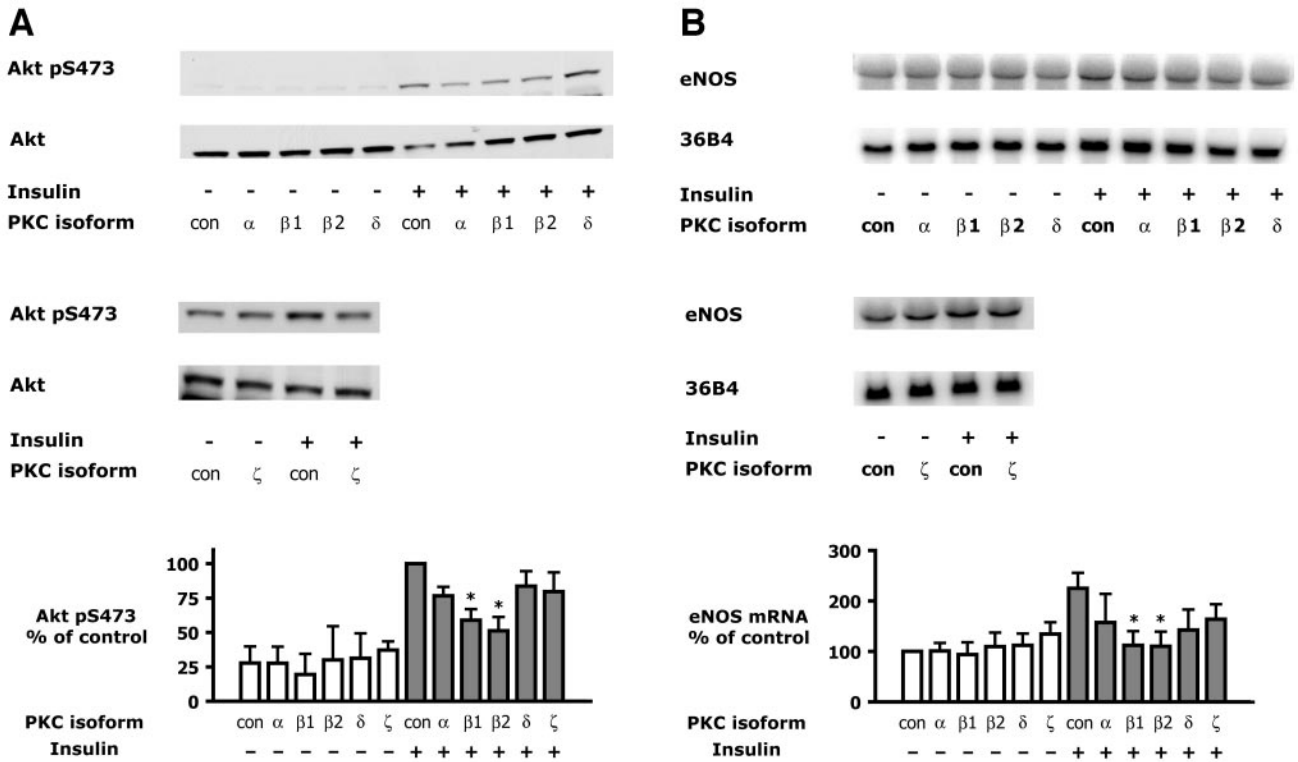


**FIG. 4.** Insulin-stimulated eNOS mRNA expression in BAECs expressing dominant-negative Akt or PKC $\zeta$ . BAECs were infected with control adenovirus expressing only GFP or gag or with adenovirus expressing dominant-negative Akt (dnAkt) or dominant-negative PKC $\zeta$  (dnPKC $\zeta$ ). Cell cultures were stimulated with insulin (100 nmol/l, 30 min) as indicated, and eNOS mRNA was measured by Northern blotting. Summarized data are shown below data from one representative experiment ( $n = 3$ ;  $*P < 0.01$  vs. gag control;  $**P < 0.05$  vs. GFP control).

that the  $\beta$ 2 isoform of PKC is selectively activated by diabetes and high glucose concentrations in vascular tissue of rats (17) and mice (25) as well as in bovine vascular cells (26). Furthermore, the observation that ruboxistaurin prevented impairment of endothelium-dependent vasodilation in humans during hyperglycemia is consistent with activation of PKC $\beta$  in human vasculature (15).

Insulin-stimulated cGMP production in the aorta was decreased in parallel with Akt phosphorylation in Zucker fatty rats compared with lean rats and increased after ruboxistaurin treatment compared with no treatment. cGMP is a second messenger for NO. A limitation of cGMP as a marker of NO production is that it only indicates NO bioavailability. Thus, differences in insulin-stimulated cGMP production may reflect differences in eNOS expression, eNOS phosphorylation, eNOS regulation by intracellular  $Ca^{2+}$  concentrations, or NO stability. Furthermore, cGMP may also be produced in response to stimulation of the transmembrane form of guanylate cyclase by hormones such as atrial natriuretic factor.

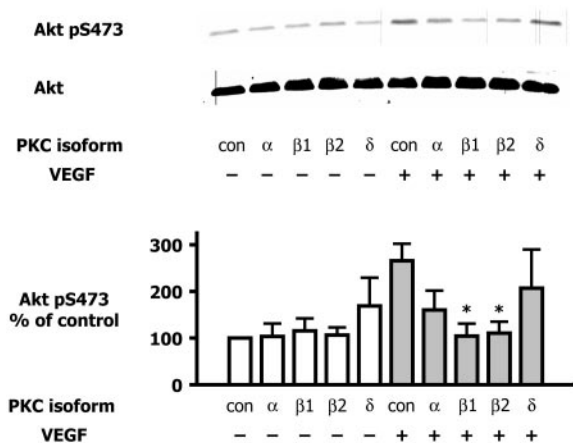
In endothelial cell culture, we observed that insulin-stimulated induction of eNOS expression is dependent on Akt. Thus, insulin stimulation of acute activation of eNOS (4) as well as upregulation of eNOS expression is mediated by Akt. Furthermore, we observed that PKC $\beta$ 1 and  $-\beta$ 2, but not PKC $\alpha$ ,  $-\delta$ , or  $-\zeta$ , decreased insulin-stimulated Akt-dependent induction of eNOS gene expression. The comparison of different PKC isoforms overexpressed in BAECs is limited by the fact that similar overexpression levels of PKC isoforms in whole-cell lysate may not result in the same extent of PKC activation for each isoform. There-



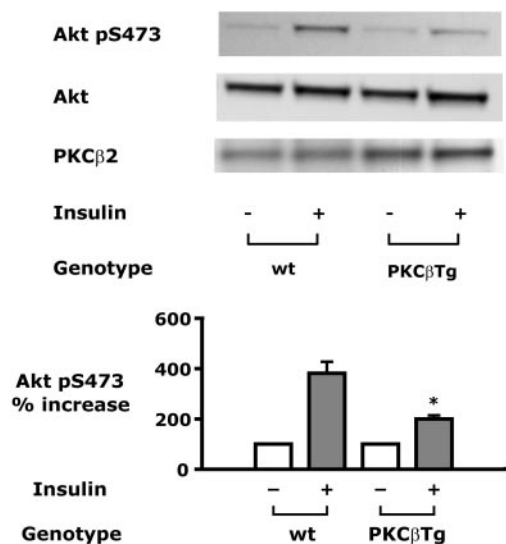
**FIG. 5.** Insulin-stimulated Akt phosphorylation and eNOS mRNA expression in BAECs overexpressing various PKC isoforms. BAECs were infected with control adenovirus expressing only GFP or gag or with adenovirus expressing one of several PKC isoforms ( $\alpha$ ,  $\beta$ 1,  $\beta$ 2,  $\delta$ , or  $\zeta$ ). Cell cultures were stimulated with insulin (100 nmol/l, 30 min) as indicated. Summarized data are shown below data from one representative experiment. **A:** Phosphorylation of Akt Ser473 was measured in whole-cell lysate ( $n = 3$ ;  $*P < 0.01$ ). **B:** eNOS mRNA was measured by Northern blotting ( $n = 4$ ;  $*P < 0.05$ ).

fore, we cannot exclude the possibility that isoforms other than PKC $\beta$ 2 may also interfere with Akt/eNOS signaling. We did not attempt to measure activity of individual overexpressed PKC isoforms, because immunoprecipitated activity from the membrane fraction of cell lysate would not only be technically challenging but also inherently flawed because it would depend on variable affinity in antibody association with each PKC isoform. Nonetheless, the importance of PKC $\beta$  in modulating this pathway was further supported by the finding that insulin-stimu-

lated Akt activation was blunted in PKC $\beta$ 2Tg mice and that insulin-stimulated Akt activation and NO bioavailability could be partly normalized by pharmacological PKC $\beta$  inhibition in Zucker fatty rats. Importantly, in PKC $\beta$ 2Tg mice, in which the prepro-endothelin promoter was used to target PKC $\beta$ 2 overexpression to vascular cells, we



**FIG. 6.** VEGF-stimulated Akt phosphorylation in BAECs overexpressing various PKC isoforms. BAECs were infected with adenovirus expressing various GFP (con) or expressing GFP as well as one of several PKC isoforms ( $\alpha$ ,  $\beta$ 1,  $\beta$ 2, or  $\delta$ ). Cell cultures were stimulated with VEGF (25 ng/ml, 15 min) as indicated ( $n = 3$ ;  $*P < 0.01$  vs. GFP expression with insulin stimulation).



**FIG. 7.** Akt phosphorylation in microvessels isolated from fat pads from vascular-specific PKC $\beta$ 2 transgenic mice. Microvessels were isolated from epididymal fat pads from wild-type mice (wt) or transgenic mice with PKC $\beta$ 2 driven by the prepro-endothelin promoter (PKC $\beta$ 2Tg). Isolated vessels were stimulated with insulin (100 nmol/l, 30 min), and phosphorylation of Akt Ser473 was measured in vessel lysate ( $n = 4$ ;  $*P < 0.05$ ).

demonstrated a reduction of eNOS expression by >40% caused by vascular PKC $\beta$ 2 activation without systemic signs of metabolic abnormalities. These findings are in agreement with findings in mice with knockout of endothelial insulin receptors, in which loss of endothelial insulin signaling and reduced vascular eNOS expression did not result in abnormal glucose tolerance (27).

In cultured endothelial cells, we also demonstrated that activation of PKC can inhibit Akt activation stimulated not only by insulin but also by VEGF. This finding has important implications. First, impaired Akt signaling in insulin resistance may compromise enzyme activation and gene expression of eNOS stimulated by several factors, not only insulin. Second, decreased insulin-stimulated eNOS gene expression may limit eNOS enzyme activity stimulated by several factors besides insulin, such as VEGF.

In the present study, eNOS protein expression was decreased in the heart of Zucker fatty rats compared with lean rats but not in the aorta. We have previously published that in microvessels isolated from Zucker fatty rats, vascular eNOS mRNA expression is reduced to 29% of the level in control Zucker lean rats (5). Biological effects in microvascular cells are much more sensitive to insulin than in cells from the aorta, as we have previously published (28). To support this idea, we have recently studied the effect of subcutaneous insulin implants on eNOS mRNA expression in microvessels from lean and fatty rats after a treatment duration of 2 weeks. The results showed that the effect of insulin on eNOS expression was reduced in Zucker fatty rats compared with lean rats (data not shown). The concept that eNOS expression decreases as a result of impaired insulin signaling in vascular endothelium was supported in the current study by demonstrating that eNOS expression is decreased in PKC $\beta$ 2Tg mice. These findings are consistent with the 62% reduction of eNOS mRNA in the aorta found in mice with knockout of endothelial insulin receptors (27).

In summary, the current study shows that activation of PKC, in particular the  $\beta$ -isoforms, is present in vascular tissue in insulin resistance associated with obesity and causes inhibition of Akt-dependent eNOS regulation. We propose that these mechanisms may be part of the explanation for why the atherosclerotic process is accelerated in people with obesity-associated insulin resistance and in patients with diabetes. Further studies will be needed to show whether PKC $\beta$  activation enhances atherosclerosis or restenosis and not just endothelial cell dysfunction. Clinical trials have shown that the PKC $\beta$  isoform inhibitor ruboxistaurin is safe during long-term treatment in humans, and this will allow testing of the hypothesis that PKC $\beta$  activation causes endothelial dysfunction and accelerates atherosclerosis in insulin resistance and diabetes.

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