Impaired insulin-mediated vasorelaxation in diabetic Goto-Kakizaki rats is caused by impaired Akt phosphorylation

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Lee JH, Palaia T, Ragolia L. Impaired insulin-mediated vasorelaxation in diabetic Goto-Kakizaki rats is caused by impaired Akt phosphorylation. Am J Physiol Cell Physiol 296: C327–C338, 2009.—Insulin resistance associated with Type 2 diabetes contributes to impaired vasorelaxation. Previously, we showed the phosphorylation of myosin-bound phosphatase substrate MYPT1, a marker of the vascular smooth muscle cell (VSMC) contraction, was negatively regulated by Akt (protein kinase B) phosphorylation in response to insulin stimulation. In this study we examined the role of Akt phosphorylation on impaired insulin-induced vasodilation in the Goto-Kakizaki (GK) rat model of Type 2 diabetes. GK VSMCs had impaired basal and insulin-activated Akt phosphorylation as well as increases in basal MYPT1 phosphorylation, inducible nitric oxide synthase (iNOS) expression, and nitrite/nitrate production compared with Wistar-Kyoto controls. Both iNOS expression and the inhibition of angiotensin (ANG) II-induced MYPT1 phosphorylation were resistant to the effects of insulin in diabetic GK VSMC. We also measured the isometric tension of intact and denuded GK aorta using a myograph and observed significantly impaired insulin-induced vasodilation. Adenovirus-mediated overexpression of constitutively active Akt in GK VSMC led to significantly improved insulin sensitivity in terms of counteracting ANG II-induced contractile signaling via MYPT1, myosin light chain dephosphorylation, and reduced iNOS expression, S-nitrosylation and survivin expression. We demonstrated for the first time the presence of Akt-independent iNOS expression in the GK diabetic model and that the defective insulin-induced vasodilation observed in the diabetic vasculature can be restored by the overexpression of active Akt, which advocates a novel therapeutic strategy for treating diabetes.

VASCULAR DYSFUNCTION characterized by increased contractility of vascular smooth muscle cells (VSMCs), abnormal vascular tone (60), and defective vasorelaxation (52) are the common abnormalities observed in atherosclerosis, diabetes, and hypertension (77). Insulin resistance often coexists in these diseases and is a well-known factor in the development of Type 2 diabetes (65). One important mechanism responsible for the defective vasorelaxation in diabetes has been impaired insulin-mediated relaxation of vasculature due to insulin resistance (38, 52).

Smooth muscle contraction and relaxation are tightly coupled to the phosphorylation and dephosphorylation, respectively, of the regulatory myosin light chain (MLC20) (32). MLC phosphorylation state is determined by the relative activities of myosin light chain kinase (MLCK) and myosin-bound phosphatase (MBP) (39). MLCK phosphorylates MLC leading to contraction (39), and MBP dephosphorylates MLC, leading to relaxation (3). Contractile agents such as angiotensin II (ANG II) activate the small GTPase, RhoA, and Rho-associated kinase α (ROKα), which then cause the phosphorylation of MYPT substrate (MYPT1) at threonine-695 and the inactivation of MBP in a calcium-independent manner (27, 44, 48). The MBP inactivation, via phosphorylation of MYPT1, results in the phosphorylation of MLC20 at serine-19 and threonine-20 leading to calcium-independent cell contraction (32–34). Insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation, in response to insulin, activates phosphatidylinositol 3-kinase (PI3-K)/Akt (protein kinase B) and the expression of inducible nitric oxide (NO) synthase (iNOS) (8, 38, 48, 73). The vasodilatory effects of insulin are mediated by NO (78) produced by iNOS (8, 38, 48, 73), which then activates cGK1α and results in the dephosphorylation of threonine-696 on a MYPT1 and inactivation of RhoA and ROKα (10, 11, 27, 48, 73). Akt activates endothelial NO synthase (eNOS) by serine-1177/1179 phosphorylation that facilitates association of the enzyme with calmodulin reducing its inhibitory interaction with caveolin-1 (53), causing NO-dependent endothelial vasodilation. Previously, we showed that insulin-induced Akt phosphorylation is essential for the dephosphorylation of ANG II-induced MYPT1 phosphorylation, resulting in VSMC relaxation via iNOS expression (48) (see depicted hypothesized signaling pathway in Fig. 7).

Studies involving the role of insulin in glucose metabolism have suggested that maintaining precise physiological levels of Akt/PKB may be critical to avoid insulin resistance. This is evidenced by data linking impaired Akt expression and activity with Type 2 diabetes (46, 47) and the increased activity observed in the renal cortex of db/db mice (26). In addition, Akt2 null mice exhibit both fasting hyperglycemia and glucose intolerance (30). Akt2/PKBβ−/− adipocytes have a reduction in insulin-induced hexose uptake and lower glucose transporter 4 (GLUT4) translocation (7). Collectively, these studies demonstrated that the absence of Akt2/PKBβ could mimic the insulin-resistant state. Given our data as well as others demonstrating that insulin causes vasodilation on VSMC via the PI3K/Akt pathway (8, 38, 48, 73), it is interesting to examine whether any abnormalities in Akt signaling might cause insulin resistance in insulin-induced vasodilation. Indeed, no studies have been conducted examining the role of Akt on insulin-induced vasorelaxation in the diabetic aorta.

We hypothesized that abnormalities in Akt activation may cause the insulin-induced vasodilation defects observed in
diabetes. In this study, we used nonobese insulin-resistant Goto-Kakizaki (GK) rats, a highly inbred strain of Wistar-Kyoto (WKY) rats that spontaneously develop Type 2 diabetes (31), to dissect the pathogenesis of insulin resistance. We explored the correlation between insulin resistance, defective Akt activation, insulin-resistant iNOS expression, and impaired insulin-induced vasodilation. Using a myograph to measure isometric tension, we demonstrated impaired insulin-induced vasodilation and furthermore attempted to restore the insulin sensitivity in insulin-induced vasodilation in diabetic GK VSMC by overexpressing constitutively active Akt. This is the first study that demonstrates the role Akt phosphorylation has in insulin-resistant vasodilation using a rat model of Type 2 diabetes.

MATERIALS AND METHODS

Human insulin (recombinant DNA origin) was from Novo Nordisk Pharmaceuticals (Princeton, NJ). Synthetic human ANG II, sodium orthovanadate, bovine serum albumin, and antibodies against β-actin and Flag M2 were purchased from Sigma-Aldrich (St. Louis, MO). Anti-iNOS antibody was from Transduction Laboratories (Lexington, KY). Primocin (anti-mycoplasma), a transfection reagent specific for smooth muscle, was purchased from Amaxa biosystems (Cologne, Germany). siCONTROL nontargeting small interfering RNA (siRNA) and custom siRNA were purchased from Drimagen (Lafayette, CO). Enhanced chemiluminescence (ECL), anti-rabbit IgG, anti-mouse IgG (horseradish peroxide linked) were from Amersham Biosciences (Buckinghamshire, UK). Specific antibody targeting MYPT1 and phosphorylated MYPT1 on threonine-696 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific for survivin (specific for Akt, phospho-Akt (Ser-473), phospho-myosin light chain 20 (Thr-18/Ser-19) were purchased from Cell Signaling Technology (Beverly, MA). Western blot reagents were from Bio-Rad Laboratories (Hercules, CA). S-Nitrosylated protein detection kit was purchased from Cayman Chemicals (Ann Arbor, MI).

Culture of VSMCs and treatment with insulin. Primary cultures of VSMCs in primary culture were obtained by enzymatic digestion of the aortic media of male WKY and GK rats with body weights of 200–220 g, as described in our recent publications (9, 49). Procedures involving animals and their care were conducted in conformity with institutional guidelines in compliance with international laws and policies (National Institutes of Health Guide for the Care and Use of Laboratory Animals, 1996, 7th ed.). Unless otherwise indicated, primary cultures of VSMCs were maintained in α-minimal essential medium (MEM) containing 10% FBS and 1% antibiotic-antimycotic and antimycoplasmic mixture. VSMCs isolated from diabetic GK rats were maintained in medium containing 20 mmol/l glucose to mimic a hyperglycemic condition. Subcultures of VSMCs at passage 5 were used in all experiments. At 24 h after plating, cells were serum starved for 24 h in serum-free α-MEM containing 1% antibiotics and 5.5 mmol/l glucose for WKY VSMC and 20 mmol/l glucose for GK VSMC. The next day cells were exposed to insulin (0–100 nM) for 10 min, ANG II (100 nM) for 15 min, or ANG II (5 min) followed by insulin.

Animal and tissue preparation. A colony of type II GK rats was established at this institute, originally supplied by Dr. Robert V. Farese (BVA Hospital, Tampa, FL) as detailed earlier (9, 49). WKY rats were purchased from Taconic Farms (Germantown, NY). Both WKY and GK rats were euthanized at 7–8 wk of age with 95% CO2 inhalation, which was approved by Institutional Laboratory Animal Care and Use Committee of Winthrop University Hospital. The thoracic aorta of rats were rapidly and carefully excised and placed in ice-cold physiological saline solution (PSS). The fat and connective tissues were removed, and the aorta was cut into 3-mm long rings using multisecting tool. Four rings with intact or denuded endothelium were mounted simultaneously in a Multi-Chamber myograph (model DMT 610M, AD Instruments) filled with PSS (7 ml) of the following composition (in mmol/l): 119.0 NaCl, 24 NaHCO3, 4.7 KCl, 1.6 CaCl2·2H2O, 1.17 MgSO4·7H2O, 5.5 glucose, 0.026 EDTA, and 1.18 KH2PO4. In the case of GK tissue, PSS contained 20 mmol/l of glucose to mimic hyperglycemic condition. The solution in the baths was constantly aerated with 95% O2-5% CO2 and kept at 37°C (pH 7.4).

Isometric force measurement. Contractile force, measured with isometric transducers built into the Multi-Chamber Myograph (DMT610M, AD Instruments), was stored with a data acquisition system (PowerLab 8SP, AD Instruments) and analyzed by computer with Chart 5 (AD Instruments). As described in our previous study (49), after 45 min of equilibration with a resting tension of 2 g, aorta rings either intact or demuced, were primed by exposure to an activation solution that substitutes equal molar concentration of NaCl with KCl, until the contractile response reaches a plateau. The ring segments were then incubated with 1 μmol/l phenylephrine (PE) to get the maximal contractile response. The PE responses from each bath were used as 100% to compare each agonist-induced contractile response.

Overexpression of Akt with adenovirus (Ad-myr-AKT-Flag) treatment in VSMC-adenovirus constructed with myr-AKT-Flag were made at the Gene Transfer Vector Core (University of Iowa) as described earlier (6). Constitutively active forms of Akt have been obtained by fusion of NH2-terminal c-Src myristilation residues to Akt. VSMCs were grown to 80% confluency, and cells were washed with serum-free media and then treated with Ad-β-gal or Ad-myr-AKT-Flag for 4 h with agitation once per every hour. After 4 h, serum was added to the cells and incubated overnight. The next day cells were washed with fresh media and kept for another 24 h. Cells were serum starved for 24 h before the experiments with insulin or ANG II.

Transfection of VSMC with siAKTc. The sequence of siRNA targeting Akt (siAKTc) targets the homologous site of rat Akt1 (1040–1058) and rat Akt2 (1043–1061) and has been shown to abolish Akt 1 and Akt 2 expression (42, 48). This site is common in rats and human. siCONTROL, which is nontargeting siRNA no. 1 from Drimagen, was used to show the nonspecific effect of siRNA transfection. VSMCs were transfected with Amaxa Nucleofector by electroporation with siCON or siAKTc following the manufacturer’s instructions. Forty eight hours after the transfection, cells were serum starved for 24 h and experiments were done as described above.

Western blot analysis. Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 100 mM NaCl, 0.5% SDS, 0.75% deoxycholate, 100 mM NaCl, 100 mM NaF, 50 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 2 μM microcystin, 50 mM β-glycerophosphate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 10 μg/ml leupeptin, and 10 μg/ml aprotinin with phosphatase inhibitors. Lysates were spun down for 30 min at 14,000 g. Equal amounts of proteins were heated with sample buffer containing 2% SDS, 0.2 M Tris-HCl (pH 7.5), 20 mM EDTA, 10% glycrol, and bromophenol blue for 5 min at 95°C and then loaded on SDS-PAGE. The separated proteins were transferred to nitrocellulose membrane and probed with specific antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies and detected by ECL. The extent of each protein was quantitated by dividing the intensity of total specific antibodies or GAPDH by the intensity of each protein. The intensity of each protein phosphorylation was normalized to the total protein amount of target protein.

Quantitative real-time PCR. To amplify iNOS and GAPDH cDNA, sense and antisense oligonucleotide primers were designed based on the published cDNA sequences (66). Oligonucleotides were obtained from Sigma-Genosys (St. Louis, MO). Sequences for the real-time PCR are as follows: iNOS sense 5’-AGAGCAGGAAAGGCGGAATGGT-3’ and iNOS antisense 5’-AGGACCAAGCGATATATGG-3’; GAPDH sense GGAGAAGACTGGCAATGTAG-3’, and GAPDH antisense 5’-AGAGGCAAGCGAGGCGGCGG-3’.
CCCTGTGGCTGTAAGCCATT-3'. Total RNA was isolated using an RNA isolation kit (QIAGEN, Valencia, CA) from WKY and GK VSMC. cDNA was synthesized with the first-strand cDNA synthesis Kit for RT-PCR (Roche Applied Science, Indianapolis, IN) for the first-strand synthesis of single-stranded cDNA from RNA for use as a PCR template using 2–3 μg total RNA in a 20-μl reaction volume. For real-time PCR, the cDNA was amplified using LightCycler 480 SYBR Green I Master for PCR and the LightCycler 480 System (Roche Applied Science, Indianapolis, IN). The double-strand DNA-specific dye SYBR Green I incorporated in the PCR reaction buffer LightCycler 480 to allow for quantitative detection of the PCR product in a 25-μl reaction volume. The temperature profile of the reaction was 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 45 s, and extension at 72°C for 60 s. An internal housekeeping gene control GAPDH was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the RT. The size of the PCR product was verified on a 1.5% agarose gel, followed by melting-curve analysis thereafter.

**Nitrite measurement.** Similar number of confluent WKY and GK VSMC cells were serum starved and changed to a phenol red free α-MEM and left for 24 h before the media were collected to determine the production of NOx. Total nitrite and nitrate production was measured with a Greiss Reagents kit purchased from Calbiochem (San Diego, CA) after the conversion of nitrate to nitrite with nitrate reductase as instructed by company.

**Biotin-switch assay for detection of S-nitrosylated Akt after immunoprecipitation with anti-Akt.** VSMCs were infected with either Ad-β-gal or Ad-myr-Akt to overexpress constitutively active Akt. Forty-eight hours after the transfection, cells were serum starved for 24 h. Other sets of quiescent VSMC cells were serum starved and treated with insulin. The Biotin switch assay was performed using an S-nitrosylated detection kit from Cayman Chemicals on the lysates that were immunoprecipitated with anti-Akt, following the manufacturer's instructions.

**Statistics.** The results are presented as means ± SE of four to six independent experiments. Paired Student's t-test was used to compare CCCTGTGGCTGTAAGCCATT-3'. Total RNA was isolated using an RNA isolation kit (QIAGEN, Valencia, CA) from WKY and GK VSMC. cDNA was synthesized with the first-strand cDNA synthesis Kit for RT-PCR (Roche Applied Science, Indianapolis, IN) for the first-strand synthesis of single-stranded cDNA from RNA for use as a PCR template using 2–3 μg total RNA in a 20-μl reaction volume. For real-time PCR, the cDNA was amplified using LightCycler 480 SYBR Green I Master for PCR and the LightCycler 480 System (Roche Applied Science, Indianapolis, IN). The double-strand DNA-specific dye SYBR Green I incorporated in the PCR reaction buffer LightCycler 480 to allow for quantitative detection of the PCR product in a 25-μl reaction volume. The temperature profile of the reaction was 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 45 s, and extension at 72°C for 60 s. An internal housekeeping gene control GAPDH was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the RT. The size of the PCR product was verified on a 1.5% agarose gel, followed by melting-curve analysis thereafter.

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**Statistics.** The results are presented as means ± SE of four to six independent experiments. Paired Student's t-test was used to compare the basal versus insulin-treated preparations. Unpaired t-test or ANOVA was used to compare the mean values between treatments. A P value of <0.05 was considered statistically significant.

**RESULTS**

**Impaired Akt basal phosphorylation and augmented MYPT1 basal phosphorylation in GK VSMC.** Akt phosphorylation at serine-473 is known to be important for full Akt activity (25), and recently our laboratory showed that the phosphorylation and activation of Akt by insulin plays an important role in reducing ANG II-induced contractile responses such as MYPT1 phosphorylation and MLC20 (48), resulting in the increase of insulin-induced vasodilatory MBP activity in VSMC. To better understand the defects in vascular tone regulation in diabetes, we determined the basal phosphorylation status of Akt and MYPT1. In GK VSMC, basal Akt phosphorylation was reduced to 85% of WKY (Fig. 1, A and B), whereas the total amount of Akt was similar in WKY and GK VSMC (Fig. 1A). The basal phosphorylation of MYPT1 was increased to 178% in GK VSMC (Fig. 1, A and B), demonstrating that defective Akt phosphorylation may have increased MYPT1 phosphorylation in GK VSMC.

We also looked at the basal insulin receptor (IR) and insulin-like growth factor receptor (IGF-R) in GK VSMC to determine the basal insulin-signaling component. IR expression was similar in WKY and GK, but determined as similar in previously published work by Sandu et al. (74). Unlike IGF-1 receptor, IGF-IIR does not potentiate the signaling of IGF-1 or IGF-II and is characterized as a tumor suppressor (45). Therefore, we determined whether there is any difference in the IGF-1 receptor (α and β) as shown in Fig. 1, C and D. We found that IGF-1 receptor expression was similar in WKY and GK VSMC; and
thus the defective insulin-induced vasodilation is not due to the difference in IR/IGF-R expression but rather due to the changes in the insulin-signaling pathways.

**Impaired insulin-induced Akt phosphorylation and Akt-independent iNOS expression in GK VSMC.** To investigate the role of Akt on insulin-dependent iNOS expression, the insulin-induced Akt phosphorylation and iNOS expression were compared in WKY control and GK diabetic VSMC. The basal iNOS expression was 2.5-fold higher than that in the WKY control (Fig. 2, A and B). Akt is phosphorylated by insulin (1, 10, and 100 nM insulin) in a dose-dependent manner in WKY and GK VSMC (Fig. 2, C and D). Insulin-induced Akt phosphorylation (10 and 100 nM) was impaired in GK VSMC to 46% and 65% compared with WKY, respectively (Fig. 2C; lanes 7 and 8; Fig. 2D). Insulin dose-response curve for pAkt showed that the WKY showed a higher slope than that of GK (Fig. 2D). Insulin (10 and 100 nM) induced iNOS to 2- and 5-fold higher over the basal in WKY VSMC (Fig. 2C; lanes 3 and 4; Fig. 2E), respectively. Whereas insulin induced iNOS expression in a dose-dependent manner in control WKY VSMC, the iNOS expression was insulin resistant and not correlated with insulin dose in GK VSMC (Fig. 2E). Insulin at 100 nM significantly decreased the iNOS expression in GK (Fig. 2C; lane 8; Fig. 2E), opposing the insulin effect in WKY control.

**Impaired insulin-induced Akt phosphorylation caused an insulin-resistant reduction of ANG II-induced contraction in GK aorta and phosphorylation of MYPT1 in GK VSMC.** Our laboratory recently showed that insulin inhibits ANG II-induced contractile response via phosphorylation of Akt and the dephosphorylation of MYPT1 and MLC20 in VSMC (17). Thus the impaired Akt phosphorylation (Figs. 1 and 2) may have caused impaired inhibition of contractile signaling in GK diabetic VSMC. To determine the effect of defective Akt activation on insulin-induced vasodilatation and on the contractile response, such as MYPT1 in GK VSMC, the phosphorylations of Akt at serine-473 and MYPT1 were analyzed. Pretreatment of ANG II (100 nM) reduced insulin-induced Akt phosphorylation in both GK and WKY. ANG II (100 nM) completely abolished insulin (100 nM)-induced Akt phosphorylation in GK VSMC, whereas in WKY VSMC, ANG II (100 nM) only reduced insulin-induced Akt phosphorylation to 50% (Fig. 3, A and B; lane 5 vs. 10). GK VSMC showed insulin resistance when reducing the ANG II-induced MYPT1 phosphorylation.

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**Fig. 2.** Impaired insulin (Ins)-induced Akt phosphorylation accompanied with abnormal inducible nitric oxide synthase (iNOS) expression in GK VSMC. Equal amounts of protein from WKY and GK cell lysate sample were resolved by SDS-PAGE and transferred to nitrocellulose membrane. A: Western blot analysis for protein expression. B: intensities of iNOS protein were quantitated by densitometric analysis and normalized to the abundance of total β-actin. Quiescent VSMCs at day 9 were stimulated with insulin (1, 10, and 100 nM) for 10 min (n = 3). C: equal amounts of protein from each cell lysate sample were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and the total and phosphorylated levels of Akt and iNOS expression measured by Western blot analysis. Intensities of phosphorylated protein were quantitated by densitometric analysis and normalized to the abundance of total protein for Akt and to β-actin for iNOS. D: insulin dose-response curve versus Akt phosphorylation were plotted as fold increase. E: insulin dose-response curve versus iNOS expression were plotted. *P < 0.05 WKY vs. GK.
phosphorylation. Insulin (1, 10, and 100 nM) reduced the ANG II-induced MYPT1 phosphorylation by 67%, 56%, and 48% (Fig. 3C: Fig. 3A; lanes 3–5), respectively. In contrast, the GK MYPT1 phosphorylation responses were reduced only to 95%, 80%, and 79% (Fig. 3C: Fig. 3A; lanes 8–10), respectively. Insulin-induced dephosphorylation of MYPT1 was impaired in GK VSMC, implying insulin-resistant vasorelaxation through MBP activation in diabetic GK.

We then measured the isometric tension of impaired contraction with denuded aorta using the myograph. ANG II caused the contraction in WKY and GK denuded aorta, and then insulin (1, 10, and 100 nM) relaxed the ANG II-induced contraction by 26.7%, 46.2% and 64% in WKY aorta (Fig. 3, D and E), respectively. In contrast, the insulin-induced vasodilation was only by −5%, 5%, and 19% (Fig. 3, D and E) in GK, respectively. These data show the insulin-resistant vasodilation in GK-denuded aorta.

To understand the endothelial effect on insulin-induced vasodilation, we compared the degree of insulin-induced relaxation in GK and WKY using both intact and denuded aorta. In WKY, insulin (10 nM) relaxed the ANG II-induced contraction to 63.5% in intact aorta, whereas it was only to 36% in denuded aorta (Fig. 3F). Similarly, in GK aorta, intact aorta responded better to insulin to cause relaxation on ANG II-induced contraction to 19.9% and 6.5%, respectively (Fig. 3F). Thus insulin also causes relaxation in an endothelium-dependent manner in both GK and WKY aorta.

Constitutively active myristilated Ad-myr-AKT caused Akt overexpression and restored the impaired GK Akt phosphory-
lation. Since the impaired insulin-induced Akt phosphorylation seemed to cause the defective inhibition of ANG II-induced contraction, Ad-myr-AKT was used to overexpress constitutively active Akt in GK VSMC and determine whether the increased Akt phosphorylation restored the insulin-induced vasodilation in GK diabetic VSMC. We used a mutant Akt with a myristilated signal at the COOH-terminus as described in our earlier publication (48). Ad-myr-AKT induced the overexpression of active Akt in GK VSMC to 5.3-fold over Ad-β-gal (Fig. 4A; lanes 9–12). Basal Akt phosphorylation was increased threefold by Ad-myr-AKT in GK VSMC over Ad-β-gal control (Fig. 4A; lane 5 vs. 9; Fig. 4B). Insulin-induced Akt phosphorylation was also increased twofold compared with that in Ad-β-gal-treated GK VSMC, respectively (Fig. 4A; lane 7 vs. 11). Ad-myr-Akt restored the basal and insulin-induced Akt phosphorylation to WKY Ad-β-gal levels (Fig. 4A; lanes 1–4 vs. 9–12).

To confirm the effect of increased Akt phosphorylation on reducing contractile responses such as MYPT1 and MLC20 phosphorylation in GK diabetic VSMC, the effects of Ad-myr-AKT on phosphorylation of MYPT1 and MLC20 were examined. The phosphorylation of MLC20 was examined as the marker of the contractile status of VSMC. Basal phosphorylation of MYPT1 was significantly reduced to 60% of the GK control (Fig. 4A; lane 5 vs. 9; Fig. 4C). Impaired insulin dephosphorylation on ANG II-induced MYPT1 phosphorylation was significantly improved to 73% of that of Ad-β-gal (Fig. 4A; lane 8 vs. 12; Fig. 4C), implying that the increased Akt phosphorylation and activity restored the insulin-induced dephosphorylation of MYPT1. Whereas basal MLC20 phosphorylation was not changed (Fig. 4A; lane 5 vs. 9; Fig. 4D), ANG II-induced MLC20 phosphorylation was completely abolished by overexpression of constitutively active Akt in GK diabetic VSMC (Fig. 4A; lane 7 vs. 11; Fig. 4D). The impaired insulin-induced dephosphorylation of ANG II-induced MLC20 phosphorylation was abolished by overexpression of constitutively active Akt (Fig. 4A; lane 8 vs. 12; Fig. 4D). These results demonstrate that the defective vasodilation can be restored by addition of active Akt in GK diabetic vasculature.

Upregulation of iNOS and opposite role of Akt on iNOS expression in GK diabetic VSMC. Since we found that the insulin-induced phosphorylation of Akt is essential to iNOS expression in WKY VSMC (48), we determined any abnormalities in iNOS expression in GK diabetic VSMC. First, we determined the basal expression of iNOS and then determined whether high glucose stimulated its induction. iNOS gene expression was significantly higher in GK at both high glucose (4.4-fold) and low glucose (3-fold; Fig. 5A). The production of nitrite was also twofold higher in GK VSMC (Fig. 5B). There were no significant differences between low glucose and high glucose stimulation in iNOS gene expression as well as nitrite production in GK VSMC, implying that the chronic glucose

![Fig. 4. Overexpression of constitutively active Akt restores insulin-induced MYPT1 and MLC20 dephosphorylation in GK VSMC. Quiescent VSMCs infected with Ad-β-gal or Ad-myr-AKT were serum starved and pulsed with insulin (10 nM) for 10 min, ANG II (100 nM) for 15 min, or ANG II (5 min) followed by insulin (10 nM). Equal amounts of protein from each cell lysate sample were resolved by SDS-PAGE and transferred to nitrocellulose membrane. Levels of pAkt and Akt (A and B); pMYPT1 and MYPT1 (A and C), and pMLC20 compared with β-actin as an internal control (A and D) were measured by Western blot analysis. Intensities of phosphorylated protein were quantitated by densitometric analysis and normalized to the abundance of total protein for Akt and MYPT1. Densitometric analyses of four separate experiments are given below each graph. *P < 0.05 WKY vs. GK; §P < 0.05 vs. corresponding value of Ad-β-gal; ‡P < 0.05 vs. insulin; #P < 0.05 vs. ANG II.](http://ajpcell.physiology.org/)
exposure may be needed to determine the effect of high glucose. There was no significant effect of high glucose on WKY VSMC compared with that of low glucose (data not shown). To determine the role of Akt on iNOS expression in GK diabetic VSMC, we used siAKTc (Fig. 5C) and constitutively active Ad-myr-Akt (Fig. 5D). siAKTc significantly increased the iNOS expression to 2.4-fold in GK diabetic VSMC (Fig. 6C), whereas in WKY, siAKTc completely abolished insulin-induced iNOS (Fig. 5C) (48). Interestingly also, the Ad-myr-Akt decreased the iNOS expression to 60% of the Ad-β-gal control (Fig. 5D). The insulin-induced iNOS expression was not significantly different by using siAKTc or Ad-myr-Akt compared with their each control, siControl, or Ad-β-gal. Thus the abnormal upregulation of iNOS and the opposite effect of Akt on iNOS expression may be linked to the abnormalities in insulin-induced vasodilatation in GK VSMC.

Ad-myr-Akt reduced the upregulation of anti-apoptotic factors, iNOS, and survivin and decreased the S-nitrosylation in GK VSMC. We speculated that the Akt-independent iNOS upregulation in the current study may have caused insulin resistance via S-nitrosylation of Akt induced by the excessive production of NO by overexpressed iNOS. Thus we measured the S-nitrosylation of Akt in WKY and GK VSMC. The basal S-nitrosylation in GK VSMC is 5.3-fold higher than that of WKY VSMC (Fig. 6A, lane 1 vs. 5; Fig. 6B, C, and D), correlating with the basal iNOS and nitrite production. Whereas insulin increased the S-nitrosylation of Akt in a dose-dependent manner in WKY VSMC, S-nitrosylation was not insulin-dependent in GK diabetic VSMC (Fig. 6A and B). Interestingly, constitutively active Akt increased the S-nitrosylation in WKY VSMC (Fig. 6C, lane 2; Fig. 6D), whereas overexpression of constitutively active Akt decreased the basal upregulated S-nitrosylation in GK VSMC, correlating with the iNOS expression (Fig. 6C, lane 4; Fig. 6D). As a result, the lowered basal and insulin-stimulated Akt phosphorylation is possibly due to the inactivation of Akt by S-nitrosylation and may contribute to the impaired insulin-induced vasorelaxation in GK VSMC.

Survivin (SVV), a member of the “inhibitor of apoptosis” family, functions as a key regulator of mitosis and programmed cell death (56). Recently, in vitro and in vivo studies have demonstrated an important role for SVV in the regulation of endothelial cell (EC) and SMC survival and suggest that it may be involved in the vascular injury response (12), in addition to its role in cancer biology (24). In GK diabetic VSMC, we previously demonstrated that basal apoptosis is nearly twofold higher than that of WKY (64), which correlates well with the current finding of decreased Akt activity. Thus, given the increased inflammation and apoptosis observed in GK VSMC, iNOS may be overexpressed as an anti-apoptotic factor (19). In addition, transforming growth factor (TGF)-β induces apoptosis through repressing the PI-3 kinase/Akt (85), and PI-3 kinase/Akt in turn regulates inhibition of apoptosis via the expression of SVV (59). It appears that the expression of iNOS

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Fig. 5. Opposite role of Akt on iNOS expression in GK diabetic VSMC. Equal amounts of total RNA from WKY and GK VSMC post to stimulation with high glucose or low glucose for 24 h were lysed and subject to quantitative real-time PCR. A: basal iNOS gene expression were measured by real time PCR for gene expression. B: nitrite/nitrate production from the culture media of WKY and GK VSMC were measured by the Greiss methods as described in MATERIALS AND METHODS. WKY and GK VSMC cells transfected with siAKTc (C) or Ad-myr-Akt (D) for 48 h were serum starved and pulsed with insulin (10 nM) for 10 min, ANG II (100 nM) for 15 min, or ANG II (5 min) followed by insulin (10 nM). Equal amounts of protein from each cell lysate were resolved by SDS-PAGE and transferred to nitrocellulose membrane, and the levels of iNOS, pAkt, and Akt compared with β-actin as an internal control. pAkt level was determined after total Akt amount adjusted. Denisometric analyses of four separate experiments with GK VSMC are given below each graph. *P < 0.05 siCON vs. siAKTc (C); *P < 0.05 β-gal vs. Ad-myr-Akt (D).
and SVV are linked to each other (35, 68) and also share PI-3 kinase/Akt as an upstream regulator of anti-apoptosis in the vasculature (59, 85). Therefore, we decided to determine whether the level of SVV correlated with iNOS expression and apoptosis in GK VSMC. As shown in Fig. 6, C and E, we found that the basal SVV protein expression in GK VSMC is upregulated twofold over WKY and correlates well with the exaggerated proliferation found in diabetic GK VSMC (64). In addition, we found that SVV and iNOS expression were increased by upstream Akt overexpression via Ad-myr-Akt in WKY control VSMC (Fig. 6C). However, we hypothesized that the overexpression of Akt would decrease the basal iNOS and SVV due to a decrease in apoptosis and exaggerated proliferation in GK VSMC, since Akt plays as survival signal in the cell (4). We found that Ad-myr-Akt caused a decrease in both iNOS and SVV protein expression, possibly due to the inhibition of apoptosis by constitutively active Akt. Therefore, Akt-independent iNOS and SVV upregulation in GK may function as the anti-apoptotic factors when GK VSMC suffer from increased basal apoptosis (64). The restoration of Akt phosphorylation and activation by Ad-myr-Akt stabilizes GK cells by increasing the survival signal, as indicated by a decrease in iNOS and SVV (Fig. 6C).

DISCUSSION

In this study we examined defects in insulin-induced vasodilatory signaling in the Type 2 diabetic GK vasculature and found that while Akt expression was unaltered, basal and insulin-induced Akt phosphorylations were impaired (Fig. 1A and Fig. 3, C and D). Correspondingly, Akt expression and insulin stimulated activity were found to be impaired in skeletal muscle of GK rats (46), muscle biopsies from Type 2 diabetic patients (46, 47), and in insulin-resistant human adipocytes (69). Furthermore, Akt2 null mice exhibited fasting hyperglycemia and glucose intolerance (30), providing evidence for the relationship between defects in Akt and insulin resistance. Thus defects in Akt may also contribute to impaired insulin-induced vasorelaxation.

We observed increased basal MYPT1 phosphorylation (Fig. 1, A and B), which is known to be negatively regulated by Akt activation. Ad-myr-AKT restored impaired Akt phosphorylation in GK VSMC back to the level of WKY VSMC (Fig. 4, A and B), suggesting that insulin-resistant Akt phosphorylation can be restored by decreasing phosphorylation of MYPT1 and MLCK20. There is, however, a slight discrepancy associated with this observation. Akt expression and activity increased in the renal cortex of db/db mice (26), a genetic model of Type 2 diabetes. Moreover, studies performed on the skeletal muscle of Type 2 diabetic patients and subjects with impaired glucose tolerance suggest insulin-induced defects are found in IRS and PI3K, while Akt activation is normal (43). Similarly, dissociation of stimulated PI3K and Akt activities were reported in adipocytes of male insulin-resistant BtB6 mice (57). Thus defects in Akt expression, phosphorylation, and activity do depend on the type of tissue and animal model being studied.

We demonstrate for the first time the presence of PI3K/Akt-independent iNOS expression in the nonobese diabetic model. In WKY VSMC, Akt is upstream of the iNOS expression, cGK1α, and MBP activity (48). iNOS is known to be expressed even after a 10-min stimulation as we and others described previously (36, 48, 74). Other studies also showed that iNOS is expressed and activated by insulin and IGF-1 after 10 min, and that pretreatment with ANG II reduced IGF-1 induced iNOS expression and activity (36). Excessive islet NO generation was reported recently in Type 2 diabetic GK rats (71), which is a similar finding to ours. We also reported that isolated aorta expressed increased iNOS in GK (49). The impaired Akt phosphorylation in vivo using GK aorta was...
recently reported (22). This is the first study to demonstrate iNOS might be implicated in the development of nonimmuno-
genetic Type 2 diabetes in vascular dysfunction.

Although Akt phosphorylation was impaired in GK VSMC, the downstream event, iNOS expression, was upregulated (Fig. 2, A–C and E). Basal gene expression is upregulated in GK regardless of glucose concentration (Fig. 5A). Since the aim of the study is more to determine the abnormalities in GK diabetic VSMC than the mechanism of how high glucose caused the abnormalities, we did not investigate further the mechanism beneath the effect of high glucose in WKY and GK VSMC. In addition, nitrite/nitrate production is also increased due to the upregulation of iNOS expression (Fig. 5B). Similar to our finding, NO overproduction by iNOS was shown to be independent of PI3K/Akt activation (62, 88), and iNOS induction in obese wild-type mice skeletal muscle was associated with the impaired activation of PI-3K and Akt by insulin (62). We also detected that the increased Akt activation, by overexpression of active Akt, reduced the iNOS expression in GK diabetic VSMC (Fig. 5D). It is difficult to explain the opposite role of Akt on iNOS expression in GK diabetic VSMC. We speculated that iNOS overexpression may protect against exaggerated contraction and reflect the increased inflammation in diabetic vasculature in our recent publication (49). However, despite the host-protective role of iNOS, excess production of NO appears to be linked to tissue damage and organ dysfunction (75). Thus, in a critical situation like the increased inflammation in diabetic vasculature, it may be possible that Akt functions as a survival signal to protect cells from apoptosis (5). Therefore, overexpression of Akt resulting in decreased expression of iNOS may be due to the reduction of the apoptosis or damage (Fig. 5D), which needs further investigation.

Strengthening our findings, diabetes and atherosclerosis accompany an increased inflammatory response due to vascular injury as evidenced by the overexpression of iNOS, reactive oxygen species (ROS), and inflammatory mediators in the diabetic population (82). Chronic low-grade inflammation has been proposed to be involved in the pathogenesis of obesity-related insulin resistance and Type 2 diabetes. The expression of pro-inflammatory cytokines, including TNF (1), IL-6 (2), and IL-1β (54), are upregulated in animal models of and patients with Type 2 diabetes. Knowing that increased inflammatory action may play an important role in the pathophysiology of cardiovascular (CV) abnormalities in hypertension, atherosclerosis, or diabetes, iNOS may be an important mediator of CV disease. Expression of iNOS is upregulated by most, if not all, inducers of insulin resistance, including pro-inflammatory cytokines (75), obesity (23, 76), free fatty acids (76), hyperglycemia (16), endotoxins (55, 79), and oxidative stress. In fact, elevated expression of iNOS was observed in skeletal muscle of mice fed a high-fat diet (62), in the hearts of Zucker diabetic rats (90), and in skeletal muscle (82), retina (14), and platelets of patients with Type 2 diabetes (81). Moreover, iNOS deficiency protects from high-fat diet-induced insulin resistance (62) in the obese Zucker rat model. These studies clearly demonstrate the important role of iNOS in insulin resistance. Thus overexpression of iNOS seen in the current study is at least partly due to enhanced inflammatory responses and insulin resistance in the GK diabetic rat. Although the mechanism of how iNOS causes or exacerbates insulin resistance remains largely unknown, it has been shown that the overproduction of NO causes S-nitrosylation of insulin-responsive molecules such as Akt, insulin receptor-β, and insulin receptor substrate-1 in ob/ob diabetic mice, resulting in insulin resistance (15, 87), and has been shown to be independent of cGMP mobilization (40). Therefore, reducing iNOS expression by knocking out the iNOS gene could improve insulin-induced glucose transport and hexose uptake (61). As seen in Fig. 6, A–D, the impaired Akt activation and insulin-induced vasodilation is partly due to S-nitrosylation of Akt in GK VSMC.

ANG II, in contrast to other contractile agents, generates ROS, a recognized player in the pathogenesis of vascular dysfunction associated with insulin resistance and non-insulin-dependent diabetes mellitus (16, 41, 77, 89). ROS such as superoxide and its reactive nitrogen derivative, peroxynitrite, are known vasoconstrictors in many vascular beds (58, 67, 84). We speculate that one possible mechanism of insulin-resistant vasorelaxation is that excessive NO together with enhanced ROS may produce peroxynitrite (ONOO−) (75, 86), an intermediate formed from the equimolar interaction of NO and superoxide known to cause impaired vasodilation by reducing NO bioavailability.

ANG II- and insulin-induced Akt activation may be two distinct and separate signaling mechanisms. ANG II is known to activate Akt both via endothelial growth factor receptor (EGFR) transactivation pathways via AT 1 receptor activation, as well as via nonreceptor tyrosine kinases, such as Src in VSMC (20, 21, 51, 80). ANG II-activated Akt activation is mediated by metabolites of arachidonic acid generated via a calmodulin-dependent kinase II (CaMKII)-stimulated Ca2+-dependent phospholipase A2 (50) and phospholipase D2 (51). Akt can be activated via a PI3-K-independent mechanism.

Fig. 7. Impaired insulin-mediated Akt activation and upregulation of iNOS/NO causes impaired insulin-mediated vasorelaxation in the GK diabetic vasculature. In GK vasculature, insulin fails to cause relaxation. Whereas insulin receptor (IR) content and tyrosine phosphorylation were similar in GK and WKY VSMC, insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI3-K) activities were impaired. Therefore, phosphorylation and activation of Akt, downstream of IRS-1 and PI3-K, were impaired in GK VSMC. However, the expression of iNOS, downstream of Akt, was upregulated, due to increased inflammation, oxidative stress, and high glucose. In turn, the basal NO and nitrite production were increased, causing S-nitrosylation of Akt, possibly resulting in impaired Akt activation.

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through PKA (70) and Ca\(^{2+}\)/calmodulin-dependent kinase activation (50). ANG II-induced Akt phosphorylation is inhibited by diphenylene iodonium or overexpression of catalase, suggesting a role for NAD(P)H oxidase-derived ROS in ANG II-induced Akt activation via p38 MAP kinase pathway (80). Even though ANG II itself can phosphorylate and activate Akt similar to insulin (Fig. 3A, lane 6 vs. 7) in GK VSMC, ANG II is known to inhibit insulin signaling at multiple steps, acting through JAK-2/IRS-1/p38 kinase, JNK, and ERK (83). ANG II increases serine phosphorylation of the insulin receptor (IR), IRS-1, and the p85 subunit of the PI3-K. At the same time, ANG II inhibits insulin-stimulated tyrosine phosphorylation of IRS-1, preventing the docking between IRS-1 and PI3-K (28, 29, 83). Thus insulin-induced Akt phosphorylation is inhibited by ANG II via inhibition of upstream of Akt, such as IR, IRS-1, and p85 subunit of the PI3-kine (28), whereas ANG II-induced Akt phosphorylation is ROS dependent. Akt activation results in the stimulation of a variety of proteins, such as p70 ribosomal S6 kinase (RSK), glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)), Bad, and mammalian target of rapamycin (mTOR), which are specific for different functions of insulin, which include glucose uptake, glycogen synthesis, protein synthesis, and inhibition of apoptosis (25). Therefore, we believe that Akt activation by ANG II and insulin mediate distinct functions via different signaling pathways in VSMC.

As shown in our result in Fig. 3F, insulin-induced relaxation is impaired in intact aorta, implying endothelial dysfunction in GK aorta. Endothelium-dependent vascular relaxation in response to acetylcholine (ACh) and endothelium-independent vascular relaxation in response to sodium nitroprusside (SNP) were impaired in GK rats (18). Some of our studies were performed with denuded vessels to remove the effect of the endothelium in these studies. Vascular endothelial cell dysfunction lead to reduction in endothelium-derived relaxing factors such as nitric oxide via eNOS, prostacyclin, and endothelium-derived hyperpolarizing factor, or increased production of contracting factors such as endothelin-1 (ET-1) and thromboxane A2 (13). Insulin resistance in spontaneously hypertensive rats is associated with endothelial dysfunction characterized by the imbalance between NO and ET-1 production, suggesting that ET-1 acts as the vasoconstrictive component that is released from the endothelium to change the contractility of smooth muscle cells (63). The overall vasodilation response to insulin in intact aorta was different compared with that in the denuded aorta of GK diabetic rats, even though the presence of the endothelial dysfunction. Thus the insulin-induced relaxation may be the sum of the impaired endothelium-derived relaxing factors and increased vasoconstriction factors.

Diabetes is accompanied by alterations in MBP activation and its downstream signaling pathways (72). GK diabetic VSMC exhibited marked impairment in MBP activation by insulin, which was accompanied by failure of insulin to decrease the phosphorylation of MYPT1 and inhibit Rho kinase activity, resulting in increased MLC20 phosphorylation and VSMC contraction (8). Although GK diabetes does not affect insulin-stimulated tyrosine phosphorylation of the insulin receptor or its content, insulin-stimulated IRS-1 tyrosine phosphorylation was severely impaired. This was accompanied by marked reductions in IRS-1-associated PI3-K activity (72). Thus the impaired Akt activation (Figs. 1A and 2B), an effector of PI3-K, is well correlated with this previous finding. Even though iNOS expression is increased, as iNOS is independent from PI3K/Akt activation, downstream cGK1\(\alpha\) is not affected by iNOS/NO signaling, given previous studies from our laboratory demonstrating lower cGK1\(\alpha\) protein expression in GK diabetic VSMC (37). Since ROK is the upstream of MYPT1, the increased phosphorylation of MYPT1 represents the ROK activity (27, 44). As shown in Fig. 1, A and B, the increased basal and ANG-II-induced MYPT1 phosphorylation is higher than that in control VSMC, representing the increased ROK activity in GK diabetic VSMC. Thus our study compliments our previous finding that the basal and ANG II-induced ROK activity is increased in GK diabetic VSMC and denuded aorta (49, 72). Even as pMYPT1 was increased due to impaired Akt activation in GK VSMC, basal pMLC20 was lower (Fig. 3, A and D). Since the regulation of pMLC20 is also dependent on the MLCK activation, which reverses the effect of MBP, the decreased basal pMLC20 may be due to the change in the calcium-dependent contractile signaling pathway. Therefore, the multiple pathways involved with the regulation of MBP and the impact on MLC20 can be further investigated to clarify the dissociation of the increased pMYPT1 and decreased basal pMLC20 in GK VSMC. Also, further study needs to investigate any abnormalities in calcium-dependent contractile signaling in GK diabetic vasculature.

In conclusion, we demonstrate that defective Akt activation in the GK diabetic vasculature causes impaired insulin-induced vasodilation, which is rescued by the overexpression of constitutively active Akt. Upregulation of iNOS may be a protective mechanism against excessive contraction, abnormal signaling resulting from oxidative stress, and due to enhanced inflammation in the diabetic vasculature (Fig. 7), which needs further investigation to clarify. Collectively, these data advocate a novel strategy to correct defective vasodilation in diabetes.

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