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AKT Phosphorylation Is Essential For Insulin-induced Relaxation of Rat Vascular Smooth Muscle Cells

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Abstract

Insulin resistance, a major factor in the development of type 2 diabetes, is known to be associated with defects in blood vessel relaxation. The role of Akt on insulin-induced relaxation of vascular smooth muscle cell (VSMC) was investigated using siRNA targeting Akt (siAKTc) and adenovirus constructing myristilated Akt (ad-myr-AKT) to either suppress endogenous Akt or overexpress constitutively active Akt, respectively. siAKTc decreased both basal and insulin-induced phosphorylations of Akt and glycogen synthase kinase (GSK) 3 β , abolishing insulin-induced nitric oxide synthase (iNOS) expression. cGMP dependent kinase 1 α (cGK1 α) and myosin bound phosphatase (MBP) activities, both downstream of iNOS, were also decreased. siAKTc treatment resulted in increased insulin and angiotensin II (AT II)-stimulated phosphorylation of contractile apparatus, such as MBP substrate (MYPT1) and myosin light chain (MLC20), accompanied by increased Rho associated kinase α (ROK α) activity, demonstrating the requirement of Akt for insulin-induced vasorelaxation. Corroborating these results, constitutively active Akt up-regulated the signaling molecules involved in insulin-induced relaxation such as iNOS, cGK1 α and MBP activity, even in the absence of insulin stimulation. On the contrary, the contractile response involving the phosphorylation of MYPT1, MLC20 and increased ROK α activity stimulated by AT II, were all abolished by overexpressing active Akt. In conclusion, we demonstrated here that insulin-induced VSMC relaxation is dependent on Akt activation via iNOS, cGK1 α and MBP activation, as well as the decreased phosphorylations of MYPT1 and MLC20, and decreased ROK α activity.

Keywords

angiotensin II; myosin bound phosphatase substrate; inducible nitric oxide synthase; cGMP dependent kinase 1 α ; Rho associated kinase α

Introduction

Defects in the relaxation mechanisms of smooth muscle within the blood vessels of patients with hypertension, non-insulin-dependent diabetes, and obesity have been reported in many studies (2,40,51,52,66). Insulin is known to induce relaxation of VSMC contraction *in vivo* and *in vitro* (40,41). Although insulin resistance has been shown to impair insulin-induced relaxation in the vasculature, the precise signaling mechanisms involved are poorly understood, despite the pathophysiological importance.

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Myosin bound phosphatase (MBP) is a heterotrimer consisting of a protein phosphatase-1 catalytic subunit (PP1C), a 130-kDa regulatory targeting subunit (MYPT1), and a 20-kDa subunit (M20) of unknown function (32). MYPT1 is phosphorylated at threonine 695 by Rho associated kinase α (ROK α) activated by the small GTPase, RhoA, which leads to the inactivation of MBP (25,46). Since MBP dephosphorylates myosin light chain (MLC20) and induces the relaxation of vascular smooth muscle cells (VSMC) without changing intracellular Ca^{2+} (34), Rho can increase the sensitivity of VSMC contraction to a given intracellular Ca^{2+} concentration (50). Myosin light chain kinase is activated by intracellular calcium and phosphorylates MLC20 at serine 19 and threonine 20, leading to cell contraction (31,35,36). Several downstream signaling pathways that inhibit MBP activity have been discovered recently, including RhoA/ROK α (46), protein kinase C activation of the inhibitory phosphoprotein CPI-17 (22), and arachidonic acid (30). ROK α also can phosphorylate the CPI-17 at threonine 38, which thereby becomes a potent inhibitor of MBP (72). MBP binds PP1 and MLC20 at its amino terminus and the M20 subunit and cGMP-dependent protein kinase 1 α (cGK1 α) at its carboxyl terminus. The MBP-cGK1 α interaction is necessary for nitric oxide (NO) /cGMP-mediated activation of MBP (71).

Insulin-induced IRS-1 tyrosine phosphorylation activates phosphatidylinositol 3-kinase (PI3-K) and the expression of inducible nitric oxide synthase (iNOS) (8,41,62), NO and cGK1 α , resulting in the dephosphorylation of threonine 695 on MYPT1 and inactivation of RhoA and ROK α (10,11,25,62).

Angiotensin II (AT II) plays an important role in the contraction process, remodeling cardiovascular structure and tone via AT₁ / AT₂ receptor activation (27,33,78). It has been reported that AT II inhibits insulin signaling and induces insulin resistance. Recent studies have revealed that an AT II excess may produce a vascular resistance to insulin via the attenuation of insulin signaling at the level of IRS-1 and PI3-K (27,78). Therefore it is important to understand the cross-talk between AT II and insulin as it pertains to contraction and relaxation in both normal and insulin resistant states.

Akt protein is a serine/threonine kinase and a downstream effector of PI3-K. Akt plays a central role in the metabolic actions of insulin including glucose transport, and the synthesis of glycogen (18,23). Mammalian genomes contain three Akt genes, Akt1, Akt2, and Akt3 that encode three widely expressed isoforms of Akt kinase. Akt can be activated by a wide variety of stimuli such as insulin, insulin-like growth factor-1 (IGF-1), AT II, reactive oxygen species (ROS), etc...(81). Glycogen synthase kinase 3-beta (GSK3 β) has been identified as a physiological substrate for Akt. Phosphorylation of GSK3 β by Akt subsequently promotes glycogen synthesis (18). Akt activation by insulin is mediated via tyrosine kinase activity of the insulin receptor, (IR), IRS-1 and IRS-2 (69,70). Tyrosyl phosphorylation of IRS-1 and -2 provides binding sites for specific proteins containing SH2 domains including the 85 kDa regulatory subunit of PI3-K (15). Phosphatidylinositol 3-phosphate (PIP3) produced by the catalytic subunit of PI3-K activates Akt by binding to the PH domain of Akt kinases (12), causing Akt translocation to the plasma membrane. PIP3-dependent kinases (PDKs), as well as undefined kinases activate Akt upon its membrane translocation by phosphorylation on threonine 308 (1) and serine 473 (12).

Many studies report a discrepancy between PI3-K and Akt activation (5,14,28,60). Although Akt is involved in glucose transport, the atypical protein kinase C family (ζ and λ), both of which are the downstream effectors of PI3-K (54), are involved in glucose transport in a different manner (28). While PI3-K and the atypical protein kinase C family (ζ and λ) are responsible for insulin-induced Glut4 translocation in 3T3-L1 adipocytes and L6 myocytes, Akt1 and Akt2 activity may be responsible for activating glycogen synthase (28). This study demonstrates the distinctive separated signaling pathways from the upstream event, PI3-K

activation, to activation of Akt and atypical form of PKC in insulin signaling. Moreover, there is a report that wortmannin sensitive PI3-K C2 type which is responsive to calcium and its role in actin rearrangement and contraction (5,14). Akt can be activated also in a PI3-K independent mechanism through PKA (60) and Ca^{2+} /calmodulin dependent kinase (80) activation.

The aim of this study was to examine the role of the serine/threonine kinase, Akt, signaling pathway in regulation of insulin-induced vasodilation in relation to the insulin mediating interactions between AT II in VSMCs. To demonstrate this, siAKTc and ad-myr-AKT were used to suppress the endogenous Akt or overexpress the constitutively active Akt in VSMC, respectively. Although previous studies have demonstrated a role for PI3-K in insulin-induced VSMC relaxation, it is important to investigate the involvement of Akt in insulin-induced relaxation. This is the first report to demonstrate a mechanism of insulin-induced relaxation signaling pathway in relation to Akt. Here, we show that Akt regulates insulin-stimulated expression of iNOS, resulting in an increase in NO. This caused the activation of cGK1 α and MBP, which were both responsible for the decrease in the phosphorylation status of MYPT1 and MLC20, and the accompanied reduction of ROK α activity, causing VSMC relaxation.

Materials and Methods

Human Insulin (recombinant DNA origin) was from Novo Nordisk Pharmaceuticals, Inc. (Princeton, NJ). Synthetic human Angiotensin II, sodium orthovanadate, bovine serum albumin, calmodulin, and antibodies against β -actin and Flag M2 were purchased from Sigma-Aldrich (St. Louis, MO). Anti-iNOS Antibody was from Transduction Laboratories, Inc. (Lexington, KY). Rho kinase II/ROK α positive control, cGK1 α positive control, Rho-Kinase and Cyclic GMP dependent protein kinase (cGK) assay kit were all purchased from Cyclex (Ina Nagano, Japan). Primocin (anti-mycoplasmic), transfection reagent specific for smooth muscle were purchased from Amaxa biosystems (Cologne, Germany). siCONTROL non-targeting siRNA and custom siRNA were purchased from Dharmacon, Inc. (Lafayette, CO). Enhanced Chemiluminescence (ECL), anti-rabbit IgG, anti-mouse IgG (HRP-linked) were from Amersham Biosciences, (Buckinghamshire, England). Specific antibody targeting MYPT1 and phosphorylated MYPT1 on threonine 696 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific for Akt, phospho-Akt (Ser 473), phospho-Myosin Light Chain 20 (Thr18/Ser19) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Western blot reagents were from Bio-Rad Laboratories, Inc. (Hercules, CA). Myosin light chains were prepared from chicken gizzards according to the published protocol (26). [γ - ^{33}P]-ATP (specific activity 3000 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Okadaic acid was from Moana Bioproducts (Honolulu, Hawaii).

Culture of VSMCs and Treatment with Insulin

VSMCs in primary culture were obtained by enzymatic digestion of the aortic media of male Wistar Kyoto (WKY) rats with body weights of 200–220 g as described in our recent publication (8,9). Unless otherwise indicated, primary cultures of VSMCs were maintained in α -MEM containing 10% FBS, and 1% antibiotic/antimycotic and antimycoplasmic mixture. Subcultures of VSMCs at passage 5 were used in all experiments. All experiments on MBP activation, Akt, MYPT1, GSK3 β and MLC20 phosphorylation, and Rho kinase and cGK1 α were performed on highly confluent cells at identical passages. Prior to each experiment, cells were serum-starved for 24 h in serum-free α -MEM containing 5.5 mM glucose and 1% antibiotics. The next day, cells were exposed to insulin (0–100 nM) for 10 min, AT II (100 nM) for 15 min or AT II followed by insulin.

Overexpression of Akt with Adenovirus (ad-myr-AKT) treatment in VSMC

Adenovirus constructed with myr-AKT-Flag were made at Gene transfer vector core (University of Iowa) as described previously (3). VSMCs were grown to 80% confluency and cells were washed with serum-free media then treated with ad- β -gal or ad-myr-AKT for 4h with agitation once per every hour. After 4h, serum was added to the cells and incubated overnight. Next day, cells were washed with fresh media and kept for another 24h. Cells were serum-starved for 24h, before the experiments were performed with insulin or AT II.

Transfection of VSMC with siAKTc

The sequence of siRNA targeting Akt (siAKTc) targets the homologue site of rat Akt 1 (1040-1058) and rat Akt 2 (1043-1061) which have been shown to abolish Akt 1 and Akt 2 expression (45). This site is common in rats and humans. siCONTROL which is non-targeting siRNA #1 from Dharmacon was used to illustrate the non specific effect of siRNA transfection. VSMCs were transfected with Amaxa Nucleofector by electroporation with siCON or siAKTc following the manufacturer's instructions. 48hrs after the transfection, cells were serum-starved for 24h and experiments were done as described above.

Preparation of Myosin-Enriched Fractions

Myosin-enriched fractions of VSMCs were prepared by extraction with a high-salt buffer as described previously (38,67). Okadaic acid at a 1.0 nmol/l concentration was included during the enzyme assay to inhibit any residual protein phosphatase 2A activity (16,67).

Measurement of Myosin-Bound Phosphatase Activity

Phosphatase activity in myosin-enriched fractions was assayed using [^{33}P]-labeled phosphorylase 'a' and [^{33}P]-labeled MLC20 as substrates (16). Briefly, equal amounts of proteins (1 μg) were diluted with assay buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 28 mM β -mercaptoethanol, and 30 mM KCl). The reaction was initiated by the addition of [^{33}P]-labeled substrates and stopped after 10 min incubation at 30 °C by the addition of 20% trichloroacetic acid (TCA). The radioactivity released in the TCA supernatants was counted as detailed in our recent publications (67). [^{33}P]-labeled phosphorylase 'a' was prepared by incubating [γ - ^{33}P]-ATP with purified phosphorylase kinase and phosphorylase 'b' (16). [γ - ^{33}P]-labeled MLC20 was prepared according to the published protocol (38) by incubating MLC20 (0.8 mg/ml) with purified MLCK (50 $\mu\text{g/ml}$), 0.1 mg/ml calmodulin, and 50 $\mu\text{mol/l}$ [γ - ^{33}P]ATP.

Western blotting

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 AEBSF, 10 $\mu\text{g/ml}$ leupeptin and 10 $\mu\text{g/ml}$ aprotinin with phosphatase inhibitors. Lysates were spun down for 30 min at 14,000xg. Equal amounts of proteins were heated with sample buffer, containing 2% SDS, 0.2 M Tris-HCl (pH 7.5), 20mM EDTA, 10% glycerol 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 HRP-conjugated secondary antibodies and detected by enhanced chemiluminescence (ECL). The extent of each protein was quantitated by dividing the intensity of β -actin. In some cases, the intensity of each protein phosphorylation was normalized to the total protein amount of target protein.

Measurement of Rho Kinase and cGK1 α activity

Rho Kinase and cGK1 α activities were performed as per the manufacturer's instructions (Cyclex, Ina Nagano, Japan). Rho Kinase activity was measured based on the phosphorylation of threonine 695 of MYPT1 with cell lysates. Briefly, equal amounts of cell lysates were incubated with each substrate pre-coated on plates. Phospho-specific antibody labeled with HRP then incubated for 1h prior to the addition of TMB substrate. The absorption was measured at 450nm with a reference wavelength of 550nm. Rho kinase II/ROK α and cGK1 α positive control were used as positive controls for each experiment. Enzyme concentration and time of incubation were adjusted to ensure first-order kinetics.

Statistics

The results are presented as means $\pm$ SEM of four to six independent experiments each performed in duplicate at different times. Paired Student's *t* test was used to compare the basal vs. 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

AT II inhibits insulin-stimulated Akt phosphorylation and induces the phosphorylation of both MYPT1 and MLC20

Akt is phosphorylated at serine 473, known to be important for full Akt activity (81). Contraction of VSMC is accompanied by the dephosphorylation of MYPT1 and phosphorylation of MLC20 (32). To understand the role of Akt on insulin-induced vasodilatation and the effects on the contractile apparatus, such as MYPT1 and MLC20 in VSMC, the phosphorylations of Akt at serine 473, MYPT1, and MLC20 were analyzed in response to various doses of insulin in the presence and absence of AT II. Insulin, at 1 nM, 10 nM, and 100 nM, stimulated Akt phosphorylation in a dose dependent manner by 1.3-, 2.7-, and 8.3-fold over basal, respectively (Fig. 1A, lanes 3-5). Pre-incubation of cells with AT II (100nM) reduced insulin-stimulated Akt phosphorylation to 0.7-, 1.7- and 3.7-fold over basal, respectively (Fig. 1A, lanes 6-8). Noteworthy is the fact that AT II alone induced Akt phosphorylation to 1.6 fold over basal level (Fig. 1A, lane 2), as reported by others (55,74). AT II incubation increased the phosphorylations of MYPT1 and MLC20 to 1.9-fold and 2.9-fold over basal levels, respectively (Figs. 1B and 1C, lane 2 vs. lane 1). Insulin, at 1nM, 10nM, and 100 nM, reduced the phosphorylation of MYPT1 by AT II down to 52 %, 56 %, and 41 % of levels with AT II alone (Fig. 1B, lanes 6-8 vs. lane 2). Insulin also decreased MLC20 phosphorylation to 118 %, 73 % and 25 % of AT II alone (Fig. 1C, lanes 6-8 vs. lane 2).

Suppression of endogenous Akt by siRNA reduced Akt-GSK3 β phosphorylation and iNOS expression and increased insulin-stimulated phosphorylation of MYPT1 and MLC20

In order to investigate the role of Akt on insulin-dependent iNOS expression and inhibition of vaso-contractile machinery, such as MYPT1 and MLC20, we used the siRNA targeting constitutive Akt (siAKTc). Control (siCON) RNA, which is non-targeting siRNA #1 from Dharmacon (Boulder, CO), was used to demonstrate the non specific effect of siRNA transfection. siAKTc decreased Akt expression to 82% (Fig. 2A, lanes 5-8) and in turn caused a decrease in the phosphorylation of Akt and GSK3 β (Fig. 2A and 2B, lanes 5-8), downstream of Akt. The basal level of Akt phosphorylation was reduced to 60% (Fig. 2A lane 1 vs. 5) and insulin-induced phosphorylation significantly decreased to 46% (Fig. 2A lane 3 vs. 7). AT II-induced Akt phosphorylation was also reduced by siAKTc to 13% (Fig. 2A lane 2 vs. 6). To confirm the specificity of siAKTc on the effect of Akt phosphorylation, phosphorylation of ERK 1/2 were also determined. siAKTc did not change the status of ERK 1/2 phosphorylation stimulated by AT II, insulin or in combination of both in VSMC and almost identical to the

response in siCON, demonstrating the specific effect of siAKTc on Akt (Fig. 2A, lanes 1-4 vs. 5-8). GSK3 β is a well known down stream effector for insulin to regulate glycogen synthesis (18) and cell cycle (20). To confirm the effect of siAKTc in VSMC, the phosphorylation of GSK3 β was determined as a direct Akt substrate. The phosphorylation of GSK3 β by insulin also was decreased by siAKTc in a similar manner (Fig. 2B, lanes 1-4 vs., 5-8). Using siAKTc, the role of Akt on MYPT1 phosphorylation was determined. siAKTc did not significantly change the basal level of phosphorylation of MYPT1 (Fig. 2B). siAKTc treatment increased the phosphorylation of MYPT1 by insulin and AT II followed by insulin by 2.3 and 4 fold, respectively (Fig. 2B, lanes 7 & 8). This result demonstrates the involvement of Akt on insulin-stimulated decrease in phosphorylation of MYPT1 (Fig. 2B).

iNOS expression, induced by insulin, is related to vasodilatation by the enhanced production of nitric oxide in VSMCs. To show the involvement of Akt on insulin-induced iNOS expression, VSMCs were treated with siAKTc in order to determine whether the regulation of iNOS expression is downstream of Akt. As shown in Fig 2C, insulin induced a 2.6-fold increase in iNOS expression over the basal level (lane 3) that was inhibited by AT II by 42% (lane 4). siAKTc treatment abolished basal as well as insulin-induced iNOS expression (Fig. 2C, lanes 5-8). MLC20 phosphorylation and dephosphorylation is recognized as the marker of contraction and relaxation, respectively. The phosphorylation of MLC20 by control, insulin and AT II-insulin were increased to 143%, 124% and 142% by siAKTc, respectively (Fig. 2C, lane 5 & 7-8), implying increased contraction via the decrease in Akt expression. While siAKTc treatment caused a decrease in iNOS expression (Fig. 2C, lanes 5-8), phosphorylation of MLC20 and MYPT1 were significantly increased (Fig. 2B and C, lanes 5-8), implying that Akt plays an important role in iNOS-dependent vasodilatation.

Akt overexpression via adenovirus constructed myristilated Akt (ad-myr-AKT) increased the phosphorylations of Akt and GSK3 β , as well as the expression of iNOS, while reducing the basal and AT II-induced phosphorylation of MYPT1 and MLC20

To confirm the role of Akt on insulin-induced vasodilatation and its inhibitory action on AT II-induced contraction, admyr-AKT was used to overexpress constitutively active Akt. We used a mutant Akt with a myristilated signal at the C-terminus. This mutation targets Akt permanently to the cell membrane, rendering it continuously susceptible to PDK phosphorylation (59). In cells infected with myr-AKT, the basal level and AT II-induced phosphorylation of Akt were increased to 4- and 3.5-fold over controls, respectively (Fig. 3A, lanes 1 & 2 vs. 4 & 5). Insulin and AT II-insulin-stimulated Akt phosphorylation was also higher than cells infected with ad- β -gal by 1.5- and 2.5- fold over controls (Fig. 3A, lanes 3&4 vs. 7 & 8). The phosphorylation of Akt as well as GSK3 β increased in parallel with the extent of Akt overexpression (Figs. 3A and 3B). The direct down stream effector, GSK3 β , was phosphorylated in response to insulin to 1.7-fold over basal (Fig. 3B, lane 3). The overexpression of Akt caused a 2.7-fold increase of basal GSK3 β phosphorylation and 2.2-fold increase over basal by AT II (Fig. 3B; lanes 5-6). This result implies that Akt over-stimulation also caused the enhanced GSK3 β phosphorylation, directly downstream of Akt. To determine the inhibitory role Akt had on the contractile machinery, the effect of ad-myr-AKT on MYPT1 phosphorylation was examined. Ad-myr-AKT decreased basal level of phosphorylation of MYPT1, compared to that of ad- β -gal treatment as a control (Fig. 3B, lanes 1 vs. 5). AT II-induced phosphorylation of MYPT1 also decreased by the over-expression of Akt to basal level, demonstrating that the overexpression of Akt leads to dephosphorylating MYPT1 and enhancing the relaxation mechanism (Fig. 3B, lanes 2 vs. 6).

To confirm that the signaling pathway leading to Akt-dependent vasodilatation is via iNOS expression in VSMCs, ad-myr-AKT was employed to overexpress Akt protein. Western blotting with specific antibody targeting iNOS showed that the overexpression of Akt enhanced

the basal and AT II-induced expression of iNOS to more than 2-fold over those of ad- β -gal treated cells (Fig. 3C, lanes 1-2 vs. 5-6). Insulin-induced iNOS expression was not further increased when compared to the expression level by same treatment in ad- β -gal treated cells (Fig. 3C, lanes 3 vs. 7). This implies that 10nM insulin may have induced maximal level of iNOS protein expression after the given time. These results, along with the results obtained from the siAKTc treatment, strongly demonstrate that iNOS expression is downstream of Akt. Similarly, the effects were observed with MYPT1 phosphorylation (Fig. 3B), where the basal level, as well as AT II-induced phosphorylation of MLC20, were completely abolished by the overexpression of Akt; confirming the role of Akt on relaxation detected by the dephosphorylation of MLC20 (Fig. 3C, lanes 1-2 vs. 5-6). Insulin-induced dephosphorylation of MLC20 was further enhanced by Akt overexpression (Fig. 3C, lanes 3 vs. 7). These results demonstrate the involvement of Akt on the insulin-stimulated decreased phosphorylations of MYPT1 and MLC20 (Fig. 3B and 3C). Thus, the insulin-induced relaxation of AT II-induced contraction in VSMC is dependent on Akt-iNOS expression, which in turn caused the dephosphorylation of MYPT1 and MLC20, resulting in VSMC relaxation.

Akt is responsible for insulin-induced myosin-bound phosphatase (MBP) activity

It is recognized that insulin causes MBP activation via the dephosphorylation of MYPT1. In order to speculate on the involvement of Akt on insulin-induced MBP activation, an MBP activity assay was performed using either siAKTc- or ad-myr-AKT-treated VSMCs. The basal level of inorganic phosphate (Pi) released was 3.2 ± 0.1 nmoles/min/mg protein and the insulin-induced level was 8.5 ± 0.1 nmoles/min/mg protein (Fig. 4A). Insulin induced a 2-fold increase of MBP activity over the control and AT II decreased it by 20% in VSMC (Fig. 4B). siAKTc, expressing low levels of Akt protein and an accompanying decreased phosphorylation (see Fig 2A, lanes 1-4 vs. 5-8), eliminated insulin-induced MBP activity and returned levels back to near basal; demonstrating a critical role of Akt on insulin-induced MBP activation (Fig. 4B). In contrast, admyr-AKT treatment of VSMCs yielded basal and AT II-induced MBP activities between 2- to 1.7-fold over the ad- β -gal control, confirming the role of Akt on insulin-induced MBP activity (Fig. 4B).

Akt is responsible for insulin-stimulated cGK1 α activity and induces the vasodilatation via the inhibition of ROK α activity

NO produced by iNOS has been shown to increase cGMP, which in turn increases cGK1 α activity (39). In order to delineate the signaling pathway from Akt to cGK1 α by insulin, deletion and overexpression of constitutively active Akt, using siAKTc and ad-myr-AKT, were employed and the effects on cGK1 α and ROK α activity measured. Insulin induced a 1.8-fold increase of cGK1 α over the control which was inhibited by AT II back to the basal level (Fig. 5A). siAKTc also abolished the insulin-induced cGK1 α activity and returned levels back to basal (Fig. 5A). In contrast, overexpression of Akt increased both the basal level and AT II-induced cGK1 α 2-fold when compared to the results in controls (ad- β -gal) of same treatment. However, insulin induced cGK1 α activity was not further increased by increased active Akt protein overexpression (Fig. 5A). These results demonstrate that Akt plays an important role in induction of cGK1 α activity in response to insulin, causing vasodilatation.

Many contractile reagents activate RhoA, causing translocation of RhoA to the membrane and thus ROK α activation (46). Activated ROK α then phosphorylates MYPT1 (25,46), and subsequent VSMC contraction. Insulin is known to inhibit ROK α activity and induce vasodilation (11,62). To more clearly define the role of Akt on inhibition of ROK α activity, VSMCs were treated with siAKTc and ad-myr-AKT. AT II induced a 2-fold increase in ROK α activity over the control (Fig. 5B). While insulin alone did not cause any significant increase in ROK α activity, it did inhibit AT II-induced ROK α activity to 32% (Fig. 5B). With the decreased Akt protein levels obtained by siAKTc treatment, ROK α activity is increased to

2-fold over the basal levels. ROK α activity by insulin or AT II followed by insulin also significantly increased to 2.3- to 2.8-fold of similar treatment in the controls (siCON) (Fig. 5B), suggesting that the absence of the inhibition of ROK α activation by Akt resulted in enhanced ROK α activity. Furthermore, when Akt is overexpressed by ad-myr-AKT, AT II-induced ROK α activity is decreased to basal levels, demonstrating Akt was responsible for the inhibition of ROK α activation by contractile agent (Fig 5B). These data demonstrated the Akt phosphorylation induced by insulin was responsible for iNOS expression-cGK1 α activation-MBP activation, resulting relaxation of VSMC by lowering ROK α activity and dephosphorylating MYPT1 and MLC20.

Discussion

This is the first study that provides direct evidence for the role of Akt on insulin-induced vasodilatation in VSMCs. Our main finding is that Akt is required for the insulin-induced relaxation of VSMCs via the induction of iNOS and the activation of cGK1 α and MBP. Akt is also necessary for the activation of MBP activity through the dephosphorylation of MYPT1 (T695) and results in the dephosphorylation of MLC20, a marker of VSMC relaxation. The changes observed in insulin-stimulated Akt phosphorylation correlate well with the extent of MYPT1 and MLC20 dephosphorylation (Fig. 1), the increase in MBP and cGK1 α enzymatic activity (Fig 4) and the decrease in ROK α activity (Fig 5), therefore cause vasodilatation. Pretreatment of cells with AT II caused the inhibition of insulin-stimulated Akt phosphorylation and the subsequent signaling thereafter. Evidence from either siAKTc or ad-myr-AKT treatment of VSMCs demonstrated that the insulin-induced Akt activation is critical and responsible for the downstream signaling molecules, such as iNOS-cGK1 α -MBP, which are known vasodilatory signals. Also, insulin-induced Akt phosphorylation inhibits the contractile response stimulated by AT II, therefore causes the dephosphorylation of MYPT1 and MLC20 and inhibition of ROK α activity, resulting in the inhibition of contraction. Thus, current study showed the strong evidence of the Akt role in stimulation of vasorelaxation mechanism, causing inhibition on vasoconstriction.

The current study supports the importance of insulin-induced VSMC vasorelaxation on the regulation of vascular tone via Akt-mediated iNOS expression and the inhibition of contraction. Numerous studies examining endothelium-dependent vasodilatation have not demonstrated the vasorelaxation mechanism of insulin in VSMCs. Endothelium dependent vasorelaxation plays important role in regulating the vascular tone, since endothelial NOS (eNOS)/neuronal NOS (nNOS) are constitutively expressed in endothelium (49,63). Akt is also known to be important in regulation of endothelium-dependent vasodilation by activating eNOS which is phosphorylated on Ser-1177/1179 then facilitates association of the enzyme with calmodulin reducing its inhibitory interaction with caveolin-1 (57,58). In VSMCs, Ca²⁺ dependent NOS, such as eNOS or nNOS, is not present, however, iNOS is known to be expressed (9,37,63). Even though iNOS is induced by inflammatory cytokines such as interleukin-11 β , tumor necrosis factor, interferon γ , and endotoxin, as well as lipopolysaccharide (LPS), it is also known that the iNOS is expressed without these stimulants (63). Acute treatment with insulin for 10min showed the increase in iNOS expression in the present study. In our study, we found that the induction of iNOS by insulin is dependent on Akt, since siAKTc suppressed iNOS activation and ad-myr-AKT could induce enhanced iNOS expression. Considering that the production of NO by iNOS is 1000-fold greater than that of eNOS and nNOS (29,49,79), insulin may have affected the vasoconstriction of the vasculature directly and more effectively in VSMC than endothelium, since it is where the contraction response occurs. Similar findings are found in that iNOS is expressed and activated by insulin and IGF-1 in 10 min and pretreatment of AT II reduced IGF-1 induced iNOS expression and activity (37). Here, we have shown direct evidence of the role of Akt in iNOS expression.

Maintaining precise physiological levels of Akt/PKB may be critical in order to avoid insulin resistance. This is evidenced by studies linking impaired Akt expression and activity with *type 2* diabetes (47,48), or the increased activity observed in the renal cortex of *db/db* mice (24). MBP and cGK1 α activities, as well as phosphorylation of MYPT1 and MLC20, are resistant to insulin after siAKTc treatment (Figs. 4 and 5). Thus, it is interesting to investigate whether the defects in Akt may have caused the insulin resistance observed in the vasorelaxation of the diabetic rodent model. In support of this, Akt2null mice exhibited both fasting hyperglycemia and glucose intolerance (29).

The inhibitory effect of insulin on contraction may be conditional and dependent upon the contractile response. Indeed, the order of insulin treatment in combination with the contractile agent may well be critical for the inhibitory action of insulin on contraction. For example, it has been reported that induction of cGMP by insulin, which mediates vasorelaxation, was conditional to the stimulation of contractile agent, serotonin, but not on insulin alone (41). Likewise, we have also found that pre-incubation with the contractile agent, AT II, prior to insulin exposure only primed for insulin to inhibit AT II-induced contraction. A similar finding was presented in a study with AT II inhibition of IGF-1-induced iNOS expression (37). Thus, insulin may have biphasic effects in terms of its inhibition of contraction, a mechanism which needs further investigation. There was difference in A-I response in Fig. 5B between siRNA and ad-virus controls. If there were "off target" effects from siCON, other stimulation such as AT II or insulin may respond in a different manner like A-I in siCON, compared to those in Ad- β -gal treated cells. However, only A-I response was different, while AT II and insulin responses are very identical. Thus, it is only the difference in insulin potency to reduce AT II-induced ROK α activity (Fig 5B).

The critical role of RhoA / ROK α on vascular contraction and hypertension has been demonstrated in many studies (10,43,72,76). Our present study demonstrated increased ROK α activity by contractile AT II and the acute effect of insulin on the inhibition of ROK α activity causing vasodilatation. Similarly, *in vivo*, ROK α inhibitors restore normal blood pressure in several hypertensive rat models, demonstrating their role in contraction (72). Inhibition of ROK α with a specific inhibitor improved the insulin resistance and hypertension observed in obese Zucker rats (43) by reducing blood pressure, and the improvement in serine phosphorylation of IRS-1 and insulin signaling in skeletal muscle (43).

Knowing the inhibitory role of insulin on contraction via inhibition of RhoA/ROK α activation, the insulin dependency on RhoA/ROK α activation on glucose metabolism may be cell type specific. Despite the well known role of RhoA/ROK α in vascular contraction (10,72,76), several studies have demonstrated that the known vasodilator, insulin, induces the activation of RhoA /ROK α as well as its role on glucose homeostasis and insulin resistance in C2C12 skeletal muscle cells and adipocytes (28,43,44,68). It is also known that insulin translocates Rho by a PI3-K dependent mechanism (23). Given these observation, ROK α activation has been shown to be crucial for insulin activation on glucose homeostasis in C2C12 skeletal muscle cells and adipocytes. Inactivation of ROK α also reduces insulin-stimulated IRS-1 tyrosine phosphorylation and PI3-K activity. Moreover, inhibition of ROK α activity in mice causes insulin resistance by reducing insulin-stimulated glucose uptake in skeletal muscle *in vivo*. Thus, ROK α activation by insulin is an important regulator of insulin signaling, specifically in glucose metabolism in skeletal muscle cells and adipocytes (28). However, the role of RhoA/ROK α activation on insulin responsive glucose metabolism is still controversial. For example, the C3 toxin from *Clostridium botulinum* which inhibits RhoA function has been reported to mimic (13), inhibit (68), as well as have no effect (75) on insulin-stimulated glucose uptake and GLUT4 translocation. Moreover, in the vasculature, Rho has been shown to be implicated in the migration (43) and proliferation of VSMCs (64), and the suppression of eNOS expression in vascular endothelial cells (53). Considering the MYPT1 phosphorylation as a

direct downstream effector of ROK α , we also observed the slight increase of MYPT1 phosphorylation by acute insulin (100nM) in VSMCs (Fig. 1B). Similarly, phosphorylation of MYPT1 was also reported by chronic stimulation of insulin in VSMCs (43). However, the extent of the stimulation was very low compared to that of AT II observed in the current study and insulin did not cause VSMC contraction. Also, there is no report of insulin's direct role in contraction but inhibition of AT II-induced contraction in VSMC. Thus, ROK α activation by insulin does not play any direct role in contraction of VSMC. Therefore, insulin induced RhoA/ROK α activation may have been related to the migration and proliferation signaling in VSMCs rather than insulin-induced relaxation.

Recently, the novel signaling pathway of AT II-induced Akt activation has been proposed in several studies (55,60,73,74). We found that in addition to the vasodilator insulin, the contractile agent AT II can also induce Akt phosphorylation (Fig. 2B). The exact role of AT II-induced Akt phosphorylation, however, is still not fully understood. Compared to insulin-induced Akt activation, the extent of AT-II induced Akt phosphorylation was very low (1.6 versus 8-fold). Indeed, there might be different signaling pathway involved in AT II-induced Akt phosphorylation compared to that by insulin. AT II is known to activate Akt both via EGFR transactivation pathways, as well as via non-receptor tyrosine kinases, such as Src in VSMC (21,56,77). AT II-induced Akt activation is mediated by metabolites of arachidonic acid generated via a calmodulin-dependent kinase II (CaMKII)-stimulated Ca²⁺-dependent phospholipase A2 (55) and phospholipase D2 (56). Akt can be activated via a PI3-K-independent mechanism through PKA (60) and Ca²⁺/calmodulin dependent kinase activation (55). Moreover, wortmannin sensitive PI3-K C2 type, which is responsive to calcium, has been shown to play an important role in actin rearrangement and contraction (5,14). The role of reactive oxygen species (ROS)-dependent p38 MAPK and MAP kinase activated protein kinase 2 (MAPKAPK-2) has also been proposed in AT II-induced Akt activation in VSMCs (73,74). Therefore, suppression of AT II-induced contraction by siAKTc (AT II 5 min stimulation, data not shown) may imply a role for Akt in contraction via a PI3-K-independent or calcium responsive PI3-K C2 type-dependent and ROS-dependent mechanism, which is separate from the insulin-induced PI3-K-dependent vasodilatation pathway in VSMCs. Supporting the role of calcium-dependent Akt signaling on contractility, constitutively active Akt enhanced myocardial contractility *in vivo* in transgenic mice overexpressing Akt via altered sarcoplasmic reticulum Ca²⁺ release (17). Overexpression of active Akt in our study, however, suppressed the phosphorylation of MYPT1 and MLC20 (Fig. 2) as well as ROK α activity (Fig. 5B), confirming the inhibitory effect of Akt on contraction, and demonstrating a major Akt pathway being PI3-K-dependent vasodilatation.

The importance of AT II and insulin interaction on insulin resistance has been demonstrated in many studies (4,27,65,66,78). The mechanism by which AT II inhibits insulin signaling has shown that AT II increases serine phosphorylation of the insulin receptor, IRS-1 as well as the p85 subunit of the PI3-K. At the same time, AT II inhibits insulin-stimulated tyrosine phosphorylation of IRS-1 preventing the docking between IRS-1 and PI3-K (27,78). In the current study, insulin activates MBP and caused MLC20 dephosphorylation, resulting vasodilation via Ca²⁺ sensitization but not actual Ca²⁺ transient change (7,62). However, many studies also report that insulin inhibits the contractile response by reducing the Ca²⁺ transients in VSMC. In these studies, the mechanism by which insulin inhibits Ca²⁺ transients were via alteration of inositol 1,4,5-triphosphate releasable Ca²⁺ store (61), insulin-stimulated glucose transport (42) and via the cGMP and NOS dependent manner (41). The latter may have also influenced the Ca²⁺ sensitization via MBP activation, as recent studies demonstrated (7,62). Our study also found that iNOS expression, MBP and cGK1 α activity by insulin are inhibited (Fig. 2C,3C,4 and 5A) by AT II pretreatment, while AT II-induced phosphorylation of MYPT1 and MLC20 (Fig. 1B and C) and activation of ROK α (Fig. 5B) are inhibited by insulin. The AT II-induced inhibition of insulin-induced Akt phosphorylation has been shown in PC12W

cells (19). It has been reported that the production of renal AT II is increased in the streptozotocin-induced diabetes rat model (6), implying a relationship between AT II and insulin resistance. Therefore, chronic interaction of hyper-AT II and hyper-insulin in diabetic subjects may be involved in insulin resistance, which may have an important role in the regulation of vascular physiology and the development of hypertension and diabetes.

The current study demonstrates for the first time the essential involvement of Akt in insulin responsive signaling proteins such as iNOS-cGK1 α -MBP, related to VSMC relaxation, which was evidenced from either siAKTc or ad-myr-AKT treatment of VSMCs. The contractile response stimulated by AT II is inhibited by insulin-induced Akt phosphorylation, leading to the dephosphorylation of MYPT1 and MLC20 and inhibition of ROK α activity, therefore causing the inhibition of contraction. The current study demonstrates the importance of vascular tone regulation by insulin-induced Akt signaling in VSMC. Additional studies focusing on the role of Akt plays in defective vasorelaxation among diabetic subjects is indicated.

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Fig. 1.

AT II inhibits insulin-stimulated Akt phosphorylation and induces the phosphorylation of both MYPT1 and MLC20. Quiescent VSMCs at day 9 were stimulated with insulin (1, 10 and 100 nM) for 10 min alone or following pre-treatment with AT II (100 nM) (n=3). Equal amounts of protein from each cell lysate sample were resolved by SDS-PAGE and transferred to nitrocellulose membranes and the total and phosphorylated levels of (A) Akt; (B) MYPT1; and (C) MLC20 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, and to β -actin for MLC20. * $P < 0.05$ versus basal control; # $P < 0.05$ versus corresponding insulin concentration; \$ $P < 0.05$ versus AT II.

Fig. 2.**Akt phosphorylation is required for iNOS expression and the decrease in**

phosphorylation of MYPT1 and MLC20. Quiescent VSMCs transfected with siCON or siAKTc were starved and treated with insulin (10 nM) for 10 min, AT II (100 nM) for 15 min or AT II for 5 min followed by insulin (10 nM). Equal amounts of protein from each cell lysate sample were resolved by SDS-PAGE, transferred to nitrocellulose membranes and the levels of (A) pAkt/Akt and phospho-ERK1/2 protein expression compared to β-actin as an internal control; (B) GSK3β and MYPT1 phosphorylation; and (C) the expression of iNOS and MLC20 phosphorylation were measured by western blot analysis. Densitometric analyses of four

separate experiments are given below each graph. * $P < 0.05$ vs. basal siCON; ** $P < 0.05$ vs. corresponding value of siCON; \$ $P < 0.05$ vs. AT II in siCON; # $P < 0.05$ vs. Insulin in siCON.

Fig. 3.
Overexpression of constitutively active Akt increased iNOS expression and decreased phosphorylation of MYPT1 and MLC20. Quiescent VSMCs infected with ad-β-gal or ad-myr-AKT were starved and treated with insulin (10 nM) for 10 min, AT II (100 nM) for 15 min or AT II for 5 min followed by insulin (10 nM). Equal amounts of protein from each cell lysate sample were resolved by SDS-PAGE, transferred to nitrocellulose membranes and the levels of (A) pAkt/Akt protein expression compared to β-actin as an internal control; (B) GSK3β and MYPT1 phosphorylation normalized to abundance of total GSK3β or MYPT1; and (C) expression of iNOS and MLC20 phosphorylation were measured by western blot analysis. Densitometric analyses of four separate experiments are given below each graph.

* $P < 0.05$ vs. basal ad- β -gal; ** $P < 0.05$ vs. corresponding value of ad- β -gal; \$ $P < 0.05$ vs. AT II in ad- β -gal; # $P < 0.05$ vs. Insulin in ad- β -gal.

Fig. 4.

The effects of siAKTc and ad-myr-AKT on MBP activity in VSMC. Quiescent VSMCs infected with siAKTc or ad-myr-AKT were treated with insulin (10 nM) for 10 min, AT II (100 nM) for 15 min or AT II for 5 min followed by insulin (10 nM). *Panel A* represents MBP activity measured as Pi released / min / mg protein in myosin enriched fractions. *Panel B* represents MBP activity assayed in myosin-enriched fractions of siAKTc and ad-myr-AKT infected cells using ^{33}P -labelled MLC as a substrate. The method delivered Akt for siRNA was siAKTc and for ad-virus was ad-myr-AKT. In the case of siRNA, control was siCON and control for ad-virus was ad- β -gal. Results are the Mean $\pm$ SE of four different experiments performed in duplicate. * $P < 0.05$ vs. basal siCON or ad- β -gal; ** $P < 0.05$ vs. corresponding value of siCON or ad- β -gal; \$ $P < 0.05$ vs. AT II in siCON or ad- β -gal; # $P < 0.05$ vs. Insulin in siCON or ad- β -gal.

Fig. 5.

Effects of siAKTc and ad-myr-AKT on cGK1 α and ROK α activity. Quiescent VSMCs transfected with either siAKTc or ad-myr-AKT were treated with insulin (10 nM) for 10 min, AT II (100 nM) for 15 min or AT II for 5 min followed by insulin (10 nM). *Panel A* represents insulin-induced cGK1 α activity from equal amounts of protein incubated with cGK1 α substrate and detected by ELISA. *Panel B* represents ROK α activity. The method delivered Akt for siRNA was siAKTc and for ad-virus was ad-myr-AKT. In the case of siRNA, control was siCON and control for Ad-virus was ad- β -gal. Results are the Mean $\pm$ SE of four different experiments performed in duplicate. * $P < 0.05$ vs. basal siCON or ad- β -gal; ** $P < 0.05$ vs. corresponding value of siCON or ad- β -gal; \$ $P < 0.05$ vs. AT II in siCON or ad- β -gal; # $P < 0.05$ vs. Insulin in siCON or ad- β -gal.