Active Rho Kinase (ROK-α) Associates with Insulin Receptor Substrate-1 and Inhibits Insulin Signaling in Vascular Smooth Muscle Cells*

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Recent studies from our laboratory have shown that insulin stimulates myosin-bound phosphatase (MBP) in vascular smooth muscle cells (VSMCs) by decreasing site-specific phosphorylation of the myosin-bound subunit (MBS) of MBP via nitric oxide/cGMP-mediated Rho/Rho kinase inactivation. Here we tested potential interactions between Rho kinase and insulin signaling pathways. In control VSMCs, insulin inactivates ROK-α, the major Rho kinase isoform in VSMCs, and inhibits thrombin-induced increase in ROK-α association with the insulin receptor substrate-1 (IRS-1). Hypertension (in spontaneous hypertensive rats) or expression of an active RhoA/V14 up-regulates Rho kinase activity and increases ROK-α/IRS-1 association resulting in IRS-1 serine phosphorylation that leads to inhibition of both insulin-induced IRS-1 tyrosine phosphorylation and phosphatidylinositol 3-kinase (PI3-kinase) activation. In contrast, expression of dominant negative RhoA or cGMP-dependent protein kinase type Iα inactivates Rho kinase, abolishes ROK-α/IRS-1 association, and potentiates insulin-induced tyrosine phosphorylation and PI3-kinase activation leading to decreased MBP phosphorylation and decreased MBP inhibition. Collectively, these results suggest a novel function for ROK-α in insulin signal transduction at the level of IRS-1 and potential cross-talk between cGMP-dependent protein kinase type Iα, Rho/Rho kinase signaling, and insulin signaling at the level of IRS-1/PI3-kinase.

Small GTPases of the Rho family are well known intracellular signaling proteins that act as molecular switches to control actin cytoskeleton organization in many cell types including smooth muscle (1–4). Recent studies indicate that RhoA-dependent signaling pathway controls vascular smooth muscle cell functions such as contraction, migration, and proliferation (5–6). In VSMCs,1 the contractile effect of RhoA results from the activation of Rho-dependent kinase (ROK-α), which phosphorylates the regulatory subunit of myosin light chain phosphatase (MBS) and thereby inhibits the phosphatase activity (7–8), thus allowing an increase in the level of phosphorylated myosin light chain and contraction at a constant intracellular calcium level [Ca2+]i (9). This phenomenon is defined as Ca2+-sensitization (10).

ROK-α and another isoform Rho kinase, ROCK1, are serine/threonine protein kinases that contain an amino-terminal catalytic kinase domain, a central coiled-coil domain in which Rho/GTP binds, and a carboxyl-terminal pleckstrin homology (PH) domain that is split by a cysteine-rich region (11–12). Insulin receptor substrate proteins (IRS) also contain an amino-terminal PH domain and phosphotyrosine binding domain domain. The PH domain is required for efficient phosphorylation of IRS-1 by the insulin receptor (13–15). In addition, IRS-1 also interacts with 14-3-3 proteins, a process apparently dependent on serine phosphorylation of IRS-1 (16).

Recent studies from our laboratory (17) have shown that insulin rapidly stimulates myosin-associated phosphatase (MBP) activity by causing a site-specific decrease in MBP phosphorylation by inactivating thrombin-stimulated Rho and one of its downstream effectors, Rho kinase. Furthermore, inhibition of PI3-kinase, nitric-oxide synthase (NOS), and cGMP signaling pathways abolished insulin-stimulated MBP activation suggesting the involvement of these signaling pathways in MBP activation (18). Thus, insulin stimulates MBP in VSMCs by activating the NO/cGMP signaling pathway that also inactivates Rho/Rho kinase (17). The effects of insulin on MBP activation and vasorelaxation were severely impaired in VSMCs isolated from diabetic Goto-Kakizaki rats and spontaneous hypertensive rats (SHR) due to defective IRS-1/PI3-kinase signaling as well as up-regulation of Rho kinase activity (18, 19). These observations prompted us to explore in detail potential interactions between Rho signaling and insulin signaling pathways.

In the present study, VSMCs were infected with an activated RhoAV14, dominant negative RhoAN19, and cGK Iα. We examined the effects of insulin and thrombin on ROK-α/IRS-1 association, IRS-1 tyrosine phosphorylation, IRS-1/p85PI3-kinase association, and PI3-kinase activation and its impact on MBP site-specific phosphorylation and MBP activation.

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‡ The abbreviations used are: VSMCs, vascular smooth muscle cells; ROK-α, Rho-dependent kinase; IRS-1, insulin receptor substrate-1; MBP, myosin-bound phosphatase; MBS, myosin-bound subunit; PI3-kinase, phosphatidylinositol 3-kinase; cGK Iα, cGMP-dependent protein kinase type Iα; PH, pleckstrin homology; MLC, myosin light chains; MAPK, mitogen-activated protein kinase; NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; SHR, spontaneous hypertensive rats; WKY, Wistar Kyoto rats; PIP, phosphatidylinositol phosphate.
The results of this study indicate that insulin inactivates ROK-α and inhibits thrombin-induced ROK-α/IRS-1 association in VSMCs. Hypertension or expression of activated RhoA V14 increases ROK-α activity and its association with IRS-1, leading to inhibition of downstream insulin signaling in VSMCs via IRS-1 serine phosphorylation that inhibits insulin-induced IRS-1 tyrosine phosphorylation and PI3-kinase. Expression of dominant negative RhoA or cGK Iα inactivates Rho kinase, abolishes ROK-α/IRS-1 association, and potentiates insulin-induced tyrosine phosphorylation and PI3-kinase activation leading to decreased MBP phosphorylation, thereby relieving MIP inhibition.

MATERIALS AND METHODS

Cell culture reagents, fetal bovine serum, and antibiotics were purchased from Invitrogen; [γ-32P]ATP (specific activity = 3000 Ci/mmol) and [32P]orthophosphoric acid were from PerkinElmer Life Sciences; 8-bromo-cGMP and N G-monomethyl-L-arginine acetate were from Bi- omol (Plymouth Meeting, PA). The electrophoresis and protein assay reagents were from Bio-Rad. Okadaic acid was from Moana Bioproducts (Honolulu, HI); type 1 collagenase was from Worthington. SDS-PAGE and Western blot reagents were from Bio-Rad. Antibody against the 160-kDa human Transducer Domain (San Diego, CA); monoclonal antibody against RhoA and polyclonal antibody against β-subunit antibody were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-IRS-1 antibody directed against the PH domain of IRS-1 and anti-p85 PI3-kinase antibody were from Upstate Biotechnology Inc.; anti-phosphotyrosine and anti-phosphoserine antibodies were from Zymed Laboratories Inc. Anti-MBS antibody was a kind gift from Dr. Hartshorne (Tucson, AZ). Anti-mouse IgG-agarose, protein A-Sepharose CL-4B, protease inhibitors, calmodulin, sodium orthovanadate, thrombin, and all other reagents were purchased from Sigma. Porcine insulin was a kind gift from Lilly.

Culture of VSMCs and Treatment with Insulin—VSMCs in primary culture were obtained by enzymatic digestion of the aortic media of male 200–220-g Wistar Kyoto (WKY) rats as described in our recent publications (17–20). Unless otherwise indicated, primary cultures of VSMCs were maintained in α-minimal essential medium containing 10% fetal bovine serum and 1% antibiotic/antimycotic mixture. Subcultures of VSMCs at passage five were used in all experiments. Measurements of Rho kinase activity, IRS-1/ROK-α association, MBP phosphorylation, and MBP activity were performed on highly confluent cultures of the same passage. Prior to each experiment, cells were serum-starved for 24 h in serum-free α-minimal essential medium containing 5.5 mM glucose and 1% antibiotics. The next day, cells were exposed to insulin (100 nM) alone for 10 min, thrombin (1 μg/ml) for 5 min, or insulin followed by thrombin (1 unit/ml) for 5 min. Immunoprecipitation and In Vitro Assay of Rho Kinase Activity in the Immune Complexes—Rho kinase was immunoprecipitated by incubating equal amounts of precleared lysate proteins (100 μg) with anti-ROK-α antibody (6 μg/tube) at 4 °C with constant shaking, and then kinase activity in the immunoprecipitates was assayed using [γ-32P]ATP, recombinant MBS, and GST-MYPT1 (667–1004) as a substrate (21). Enzyme concentration was adjusted to ensure first-order kinetics in which reaction rate was linear versus time. After incubation at 30 °C for 10 min, 25-μl aliquots of the reaction mixture were added to 20 μl of cold phosphocellulose and centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant and polybrene (8 μg/ml) in 2 ml of growth medium. The culture plates were centrifuged at 1,700 rpm in a Beckman Table Top Centrifuge (Allegra 6) for 1 h at room temperature, incubated overnight at 32 °C, and then shifted to 37 °C the next day. At the end of 48 h, VSMCs were trypsinized and plated into five 100-mm dishes containing 2 μg/ml puromycin to generate stably expressing clones. Pools of stable clones exhibiting constitutively active RhoA V14 or dominant negative RhoA N19 were amplified and used at the 5th passage for functional analyses to examine the effect of RhoA on the association between IRS-1 and Rho kinase and its downstream signaling components. Western blot analyses revealed a 3-fold increase in RhoA expression.

Construction of Adenoviral Vectors Expressing cGK Iα—The adenoviral vector for expressing cGK Iα was constructed by first cloning the cDNA of human cGMP-dependent protein kinase Iα into the multiple cloning site of the adenoviral vector, pBabe, carrying a puromycin resistance marker. The resulting clone was subcloned into pJM17, a plasmid containing the full-length genome of replication-deficient type 5 adenovirus (Microbix), into the Sal I and RI sites, respectively, of the cloning site of the adenoviral transfer plasmid pCMVI/cGKI. The adeno-retroviral vector, pBabe, carrying a puromycin resistance marker. The amount of ROK-α/IRS-1 association, and potentiates insulin-induced tyrosine phosphorylation and PI3-kinase activation leading to decreased MBP phosphorylation, thereby relieving MIP inhibition.

Measurement of cGK I Enzyme Activity in VSMCs—The biological activity of the cGK I in VSMCs infected with Ad5.cGKIα gene was assayed according to the method described by Francis et al. (23). Briefly, extracts prepared from uninfected and Ad5.cGKIα-infected VSMCs were assayed in a 50-μl reaction containing 20 μl Tris, pH 7.4, 200 μM MgCl2, 100 μM ATP, the synthetic peptide, Glasstide (Calbiochem), 20 mM sodium orthovanadate, 1 μM (R2)-cAMP, and 30,000 cpm/μl of [γ-32P]ATP. Assays were performed in the presence or absence of 10 μM cGMP at 30 °C for 10 min and were terminated by transferring samples onto phosphocellulose P-81 paper that was subsequently washed extensively in 75 mM phosphoric acid. Radioactivity bound to the phosphocellulose paper was counted by liquid scintillation spectrometrically.

Immunoprecipitation and Western Blot Analyses—VSMCs treated with and without insulin (100 nM) or thrombin; cell lysates were then prepared and precleared as detailed (19), and equal amounts of lysate proteins (1 mg) were immunoprecipitated with anti-IRS-1 antibody (10 μg) overnight. The immunoprecipitates were separated on 7% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane for Western blot analyses. The top portion of the membrane was probed with either ROK-α antibody or anti-phosphotyrosine antibody, and the bottom portion was probed for p85 subunit of PI3-kinase. To overcome any variations in proteins due to immunoprecipitation, blots were stripped and reprobed with anti-IRS-1 antibody that reacts with IRS-1 protein. The amount of ROK-α and p85 associated with IRS-1 as well as the extent of tyrosine phosphorylation of IRS-1 were measured by densitometric analysis of the ECL signals and quantitated by dividing the intensity of ROK-α, p85, and phosphotyrosine signals with the IRS-1 protein signal. To ensure that ECL signals were within the linear range, multiple exposures were taken during the short initial phase of ECL reaction. Only those signals that were in the linear range were used for quantitation.

Measurement of ROK-α Inhibits Insulin Signaling—Measurement of ROK-α Inhibits Insulin Signaling—Exposed to insulin (100 nM) alone for 10 min, thrombin (1 μg/ml) for 5 min, or insulin followed by thrombin (1 unit/ml) for 5 min. Immunoprecipitation and In Vitro Assay of Rho Kinase Activity in the Immune Complexes—Rho kinase was immunoprecipitated by incubating equal amounts of precleared lysate proteins (100 μg) with anti-ROK-α antibody (6 μg/tube) at 4 °C with constant shaking, and then kinase activity in the immunoprecipitates was assayed using [γ-32P]ATP, recombinant MBS, and GST-MYPT1 (667–1004) as a substrate (21). Enzyme concentration was adjusted to ensure first-order kinetics in which reaction rate was linear versus time. After incubation at 30 °C for 10 min, 25-μl aliquots of the reaction mixture were added to 20 μl of cold phosphocellulose and centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant and polybrene (8 μg/ml) in 2 ml of growth medium. The culture plates were centrifuged at 1,700 rpm in a Beckman Table Top Centrifuge (Allegra 6) for 1 h at room temperature, incubated overnight at 32 °C, and then shifted to 37 °C the next day. At the end of 48 h, VSMCs were trypsinized and plated into five 100-mm dishes containing 2 μg/ml puromycin to generate stably expressing clones. Pools of stable clones exhibiting constitutively active RhoA V14 or dominant negative RhoA N19 were amplified and used at the 5th passage for functional analyses to examine the effect of RhoA on the association between IRS-1 and Rho kinase and its downstream signaling components. Western blot analyses revealed a 3-fold increase in RhoA expression.

Construction of Adenoviral Vectors Expressing cGK Iα—The adenoviral vector for expressing cGK Iα was constructed by first cloning the cDNA of human cGMP-dependent protein kinase Iα into the multiple cloning site of the adenoviral vector, pBabe, carrying a puromycin resistance marker. The amount of ROK-α/IRS-1 association, and potentiates insulin-induced tyrosine phosphorylation and PI3-kinase activation leading to decreased MBP phosphorylation, thereby relieving MIP inhibition.
Measurement of Myosin-bound Phosphatase Activity—Myosin-enriched fractions of VSMCs were prepared as described previously (17–19). MBP activity in myosin-enriched fractions was assayed using 32P-labeled myosin light chains (MLC) as a substrate (17, 24). Okadaic acid at 1 nM concentration was included during the enzyme assay to inhibit any residual PP-2A activity (24, 25). 32P-labeled MLC was prepared according to the published protocol (26) by incubating MLC (0.8 mg/ml) with purified myosin light chain kinase (50 μg/ml), 0.1 mg/ml calmodulin, and 50 μM γ-[32P]ATP.

Protein Assay—Proteins in cellular extracts and lysates were quantitated by the bicinchoninic acid (27) or by the Bradford technique (28). Statistics—The results are presented as means ± S.E. of three to six independent experiments each performed in triplicate. Analysis of variance was used to compare mean basal values versus those after various treatments. A p value of <0.05 was considered statistically significant.

RESULTS

Insulin and Dominant Negative RhoA Abolish Thrombin-induced Increase in ROK-α/IRS-1 Association—Potential interaction between ROK-α and IRS-1 was investigated in VSMCs by immunoprecipitation of equal amounts of VSMC protein lysates with IRS-1 antibody. The immunoprecipitates were separated by SDS-PAGE followed by immunoblot analysis with anti-ROK-α antibody and anti-IRS-1 antibody, respectively (Fig. 1, A and B). As summarized in Fig. 1C, under basal conditions, a significant amount of ROK-α was found associated with the IRS-1 protein (Fig. 1A, lane 2, and C). Treatment with thrombin increased ROK-α/IRS-1 association >3-fold when compared with basal values (Fig. 1, A and C). Pre-exposure to insulin for 10 min prevented thrombin-induced increase in ROK-α content in the IRS-1 immunoprecipitates (Fig. 1A, compare lane 5 versus lane 4 and C). Insulin treatment alone did not alter ROK-α content in the IRS-1 immunoprecipitates (Fig. 1A, lane 3 and C). A 2-fold increase in basal ROK-α/IRS-1 association was observed in VSMCs expressing activated RhoV14 (Fig. 1A, lane 6 and C). Thrombin stimulation further increased ROK-α/IRS-1 association in RhoV14-expressing cells (Fig. 1A, lane 8 and C), which was not prevented by insulin (Fig. 1A, lane 9 and C). In contrast, VSMCs expressing dominant negative RhoAN19 exhibited no thrombin-induced increase in ROK-α/IRS-1 association (Fig. 1A, lane 12 and C); the amount of ROK-α associated with IRS-1 was more or less comparable for control, insulin, thrombin, and insulin + thrombin treatment (Fig. 1A, lanes 10–13, and C). Alterations in ROK-α/IRS-1 association were not due to variations in IRS-1 protein in the immunoprecipitates (Fig. 1B) as changes persisted when the data from all experiments were quantitated and normalized for IRS-1 proteins in the immunoprecipitates (Fig. 1C). Similar results were obtained when a reciprocal experiment was performed in which ROK-α immunoprecipitates were examined for IRS-1 association (data not shown). Total ROK-α levels were comparable between pBabe-, RhoA14, and RhoAN19-expressing VSMC lysates.

Measurement of Rho Kinase Activity in the IRS-1 Immunoprecipitates—Our recent studies (17–19) have shown that insulin inhibits basal as well as thrombin-stimulated Rho kinase activity assayed in the ROK-α immunoprecipitates. To examine the activation status of Rho kinase that is bound to IRS-1, we assayed Rho kinase activity in IRS-1 immunoprecipitates using recombinant MBS, GST-MYPT1 (667–1004), as a substrate. Insulin caused a 20% decrease in IRS-1-associated Rho kinase activity in VSMCs expressing the empty retroviral vector, pBabe (Fig. 2). Exposure to thrombin caused a 20% increase in Rho kinase activity over the basal value. Pretreatment with insulin decreased thrombin-induced Rho kinase activity below basal values and below the level of activity observed in cells treated with insulin alone (Fig. 2). Expression of an activated RhoV14 caused a 50% increase in basal Rho kinase activity that was further increased upon treatment with thrombin, presumably because of the presence of endogenous wild type RhoA. Insulin did not decrease Rho kinase activity in these cells (Fig. 2). In contrast, expression of dominant negative RhoAN19 caused a 20% decrease in basal Rho kinase activity when compared with VSMCs expressing pBabe vector (Fig. 2). Furthermore, thrombin treatment failed to increase Rho kinase activity in IRS-1 immunoprecipitates in these cells. More important, insulin decreased Rho kinase activity in thrombin-treated cells below basal values (Fig. 2). Thus, it appears that insulin is more effective in inhibiting ROK-α when cells were exposed to thrombin in VSMCs expressing RhoAN19. In preliminary experiments, ROK-α immunoprecipitated from thrombin-stimulated cells phosphorylated recombinant IRS-1 protein in an in vitro kinase assay (data not shown). This observation suggests that IRS-1 protein may be an in vivo substrate for ROK-α.

Expression of Activated RhoV14 Impairs Both Insulin-induced IRS-1 Tyrosine Phosphorylation and PI3-Kinase Activation—We next examined the potential impact of ROK-α/IRS-1 association on insulin-induced IRS-1 tyrosine phosphorylation in VSMCs expressing the active and inactive forms of RhoA.
Insulin caused a rapid 6-fold increase in IRS-1 tyrosine phosphorylation in control VSMCs infected with pBabe (Fig. 3A, lane 2 versus lane 1, and D). Thrombin alone caused a 2-fold increase in IRS-1 tyrosine phosphorylation. Pretreatment with insulin followed by thrombin further increased IRS-1 tyrosine phosphorylation (Fig. 3A, lane 4 versus lane 2, and D). In contrast, expression of activated RhoAV14 caused resistance to insulin, a 90% reduction of IRS-1 tyrosine phosphorylation in comparison to control VSMCs infected with pBabe (Fig. 3A, lanes 6 and 8 versus lanes 2 and 4, and D). More importantly, VSMCs expressing dominant negative RhoAN19 exhibited a 5-fold increase in IRS-1 tyrosine phosphorylation in the basal state (Fig. 3A, lane 9 versus lane 1, and D) that was not affected by thrombin treatment. Insulin treatment further increased IRS-1 tyrosine phosphorylation over the basal values (Fig. 3A, lane 10 versus lane 9, and D).

In control VSMCs, insulin-stimulated IRS-1 tyrosine phosphorylation was accompanied by a 110-fold increase in the association of p85 subunit of PI3-kinase with IRS-1 (Fig. 3B, lane 2 versus lane 1, and E). Alterations in IRS-1 tyrosine phosphorylation and p85/IRS-1 association were not due to variations in IRS-1 content (Fig. 3C) because changes persisted after the data were normalized for IRS-1 content between different treatments and experiments (see Fig. 3, D and E). Thrombin treatment caused a 2-fold increase in basal p85/IRS-1 association when present alone. Insulin-induced p85/IRS-1 association was not altered when thrombin was added after insulin pretreatment (Fig. 3B, lane 4 and E). VSMCs expressing activated RhoAV14 exhibited 80% reduction in insulin-induced p85/PLkinase association with the IRS-1 in comparison with control VSMCs expressing vector alone (Fig. 3B, lanes 6 and 8 versus lanes 2 and 4, and E). In contrast, VSMCs expressing dominant negative RhoAN19 exhibited a 3-fold increase in basal p85/IRS-1 association and an 10-fold increase in insulin-mediated p85/PLkinase/IRS-1 association compared with basal value (Fig. 3B, lanes 10 versus 9 and E). Thrombin alone increased p85/IRS-1 association. Thrombin treatment did not alter insulin-induced p85/IRS-1 association under these conditions (Fig. 3B, lane 12 and E). Basal and insulin-stimulated p85 association with IRS-1 correlated very well with IRS-1 tyrosine phosphorylation in pBabe and to a lesser in RhoAN19-expressing cells.

The observed reductions in insulin-induced IRS-1 tyrosine phosphorylation and p85 PI3-kinase/IRS-1 association in cells expressing RhoAV14 were accompanied by a marked decrease in PI3-kinase enzymatic activity in the IRS-1 immunoprecipitates (Fig. 3F, lanes 5–8, and G). In contrast, VSMCs expressing dominant negative RhoAN19 exhibited insulin-induced increase in PI3-kinase activation that was greater than that of control (pBabe) VSMCs (Fig. 3F, lanes 9–12 and G).

**Hypertension Is Accompanied by Increased ROK-α/IRS-1 Association and Inhibition of Insulin Signaling**—Our earlier studies have shown that VSMCs isolated from SHR exhibit insulin resistance in terms of PI3-kinase activation, iNOS induction, as well as MAPK activation when compared with Wistar Kyoto (WKY) (29). In contrast, the growth-mediating effects of insulin were enhanced in these cells due to sustained MAPK activation (30). To investigate further the pathophysiological relevance of the interactions we observed between ROK-α and IRS-1, we examined VSMCs isolated from SHR for potential changes in IRS-1/ROK-α association in response to AII because these animals exhibit hypersensitivity to AII. As seen in Fig. 4, A and B, ROK-α association with IRS-1 was 2-fold higher in the basal state of SHR compared with that of WKY (Fig. 4A, compare lane 1 versus lane 5, and B). Whereas insulin pretreatment decreased AII-induced ROK-α/IRS-1 association in WKY (Fig. 4A, compare lane 4 versus lane 3, and B), it failed to reduce the basal as well as AII-mediated ROK-α/IRS-1 association in SHR (Fig. 4A, lanes 5–8, and B). Increased ROK-α/IRS-1 association in SHR was also accompanied by marked reductions in insulin-induced association of p85 PI3-kinase with IRS-1 (Fig. 4A, lanes 6 and 8) as well as IRS-1 tyrosine phosphorylation (data not shown).

**Expression of RhoAV14 Increases IRS-1 Serine Phosphorylation**—Several studies (31, 32) have indicated that serine phosphorylation of IRS-1 inhibits its tyrosine phosphorylation and ability to associate with the p85 subunit of PI3-kinase, thereby rendering cells resistant to insulin. To understand the mechanism whereby activated RhoA inhibits tyrosine phosphorylation of IRS-1 and its association with PI3-kinase, we examined the serine phosphorylation status of IRS-1. As seen in Fig. 5, VSMCs expressing activated RhoAV14 exhibit a 3-fold increase in basal IRS-1 serine phosphorylation (Fig. 5, lane 5) in the IRS-1 immunoprecipitates that remained elevated upon treatment with insulin (Fig. 5, lane 6) when compared with control VSMCs. In control VSMCs, insulin treatment decreased phospho-serine content of IRS-1 and prevented a thrombin-induced increase in IRS-1 serine phosphorylation (Fig. 5, lanes 2 and 4).

**Expression of cGK Ia Inactivates Rho Kinase, Abolishes Thrombin-induced Increase in ROK-α/IRS-1 Association, and Enhances Both Insulin-induced IRS-1 Tyrosine Phosphorylation and PI3-KinaseActivation**—Recent studies (33) have shown that cGMP inactivates Rho signaling by promoting phosphorylation of RhoA via cGK Ia at serine 188, which interferes with the translocation and anchoring of RhoA at the plasma membrane surface. These observations together with our results, demonstrating that Rho kinase inactivation by insulin could be reversed by inhibitors of NOS and cGMP signaling pathways, suggested that Rho kinase activation status may be regulated by cGMP signaling (17). Therefore, we examined the activation status of ROK-α in VSMCs infected with adenoviral cGK Ia, the downstream effector of cGMP, and tested whether inactivation of Rho kinase by cGK Ia affects ROK-α association with IRS-1 and insulin signaling. As shown in Fig. 6A, infection of VSMCs with Ad5.cGK Ia increased...
FIG. 3. Increased ROK-α/IRS-1 association due to active RhoA\textsuperscript{V14} is accompanied by inhibition of insulin-stimulated IRS-1 tyrosine phosphorylation, IRS-1 recruitment, and activation of p85 PI3-kinase. VSMCs expressing pBabe, RhoA\textsuperscript{V14}, or RhoA\textsuperscript{N19} were treated with insulin (100 nM, 10 min) or thrombin (1 unit/ml, 5 min) or incubated with insulin followed by thrombin treatment. Equal amounts of lysate proteins were immunoprecipitated (IP) with IRS-1 antibody (AB) as detailed in Fig. 1. The immunoprecipitated proteins were subjected to SDS-PAGE followed by Western blot analysis using anti-phosphotyrosine antibody (p\text{tyIRS-1}) (A), anti-p85 PI3-kinase antibody (B), and anti-IRS-1 antibody (C). Autoradiograms from representative experiments are shown. D shows data from four different experiments that were quantitated by densitometry and then normalized for immunoprecipitated IRS-1 protein by dividing the intensity of phosphotyrosine and p85 PI3-kinase (E) signals with the IRS-1 signal. Results are expressed relative to untreated (control) VSMCs expressing pBabe which was assigned a value of 1. *, \( p < 0.05 \) versus pBabe control; **, \( p < 0.05 \) versus the respective pBabe control, thrombin, insulin, or insulin \( \rightarrow \) thrombin treatment; ***, \( p < 0.001 \) versus the respective RhoA\textsuperscript{V14} control, insulin, thrombin, or insulin \( \rightarrow \) thrombin treatment.

F shows that expression of activated RhoA\textsuperscript{V14} also inhibits insulin stimulation of PI3-kinase activity. VSMCs were exposed to insulin and thrombin, and equal amounts of cell lysate proteins (100 µg) were immunoprecipitated with anti-IRS-1 antibody as detailed in Fig. 1. PI3-kinase activity was assayed in IRS-1 immunoprecipitates using phosphatidylinositol as a substrate as detailed under “Materials and Methods.” The phospholipids were extracted and separated on a TLC plate that was analyzed by autoradiography. A representative autoradiorigram is shown. Similar results were obtained in four separate experiments. G shows quantitation of PI3-kinase activity. Radioactivity incorporated into PIP was quantitated by cutting out the PIP signal and counting radioactivity. Results are expressed as cpm incorporated into PIP. *, \( p < 0.05 \) versus pBabe control; **, \( p < 0.05 \) versus the respective pBabe control, thrombin, insulin, or insulin \( \rightarrow \) thrombin treatment; ***, \( p < 0.001 \) versus the respective RhoA\textsuperscript{V14} control, insulin, thrombin, or insulin \( \rightarrow \) thrombin treatment.
Hypertension is accompanied by increased ROK-α/IRS-1 association and decreased IRS-1 recruitment of p85 PI3-kinase. VSMCs isolated from WKY and SHR were exposed to AII (100 nM) for 10 min, with and without prior treatment with insulin (100 nM) for 10 min. Equal amounts of protein lysates (1 mg) were immunoprecipitated (IP) with anti-IRS-1 antibody (Ab) followed by Western blot analyses with anti-ROK-α and anti-p85 PI3-kinase antibodies, respectively, as detailed in Fig. 1. IRS-1 was immunoprecipitated (IP) and subjected to immunoblot analyses using anti-phosphoserine antibody. A, representative autoradiograms are shown. B, data from four experiments were quantitated by densitometry, normalized for immunoprecipitated IRS-1 protein by dividing the intensity of ROK-α with the IRS-1 signal and expressed relative to WKY control that was assigned a value of 1. *, p < 0.05 versus WKY control; **, p < 0.05 for each SHR sample versus the respective WKY samples.

cGK I protein expression by >10-fold over that of non-infected VSMCs and increased basal cGK I enzymatic activity in the absence of cGMP by 3-fold (Fig. 6B). cGMP treatment of Ad5.cGK Ia cells produced a 4-fold increase in cGK I activity over the basal values. Insulin treatment resulted in a 2-fold increase in cGMP-independent cGK I activity over basal (Fig. 6B), presumably due to endogenous production of cGMP by insulin (29, 30). Thrombin treatment did not alter cGK I activity when present alone nor did it interfere with the effect of insulin when added after insulin treatment. This may be explained by the observation that cGK Ia expression markedly inhibited basal as well as thrombin-induced increase in Rho kinase activity in ROK-α immunoprecipitates (Fig. 6C). ROK-α inactivation by cGK Ia was accompanied by a marked decrease in ROK-α association with IRS-1 in comparison to uninfected VSMCs (Fig. 7A, compare lanes 5–8 versus lanes 1–4). In addition, cGK Ia infection increased insulin-stimulated IRS-1 tyrosine phosphorylation (Fig. 7B, lanes 6 and 8) by 10-fold in comparison to non-infected VSMCs. This was accompanied by increased insulin-mediated p85/IRS-1 association (Fig. 7C, lanes 5–8) resulting in a 2-fold increase in PI3-kinase activity in the IRS-1 immunoprecipitates of insulin-stimulated Ad5.cGK Ia cells (Fig. 7E, lanes 6 and 8 versus lanes 2 and 4).

cGK IaExpression Inhibits MBS\textsuperscript{8905} Phosphorylation Leading to MBP Activation. Thrombin Fails to Inhibit the Insulin Increase in MBP in cGK Ia-infected VSMCs—Recent studies have identified inhibitory Rho kinase phosphorylation sites on MBS that appear to profoundly influence MBP enzymatic activity (34–35). For example, in Swiss 3T3 cells, Rho kinase activation by lysophosphatidic acid was accompanied by an increase in MBST695 phosphorylation, and this effect was blocked by a Rho kinase inhibitor, Y-27632 (34). In addition, insulin and 8-bromo-cGMP decreased MBST695 site-specific phosphorylation, and both effects could be prevented by treatment with either N\textsuperscript{6}-monomethyl-L-arginine acetate or (R\textsuperscript{-})-8-Br-cGMPS, suggesting that the NO/cGMP signaling pathway mediates insulin inhibition of MBS site-specific phosphorylation (17). In our earlier studies, we have shown that insulin rapidly increases cGMP levels in VSMCs (29) that could potentially activate cGK Ia to phosphorylate RhoA and thus inactivate Rho/Rho kinase (see Fig. 6C) to reduce MBS site-specific phosphorylation. Therefore, we examined MBS\textsuperscript{8905} phosphorylation in Ad5.cGK Ia-infected cells because cGK Ia is a downstream effector of cGMP signaling. As shown in Fig. 8A, insulin decreases basal and thrombin-stimulated MBS\textsuperscript{8905} phosphorylation in uninfected VSMCs. Infection with Ad5.cGK Ia resulted in a 60% decrease in basal MBS\textsuperscript{8905} phosphorylation and a lack of thrombin effect (Fig. 8A, lanes 5 and 7) versus control (Fig. 8A, lanes 1 and 3) VSMCs. This decrease was accompanied by a 45% increase in MBP activity in the basal state (Fig. 8B). Insulin treatment further increased MBP activity in cGK Ia-infected cells (Fig. 8B).
DISCUSSION

The results presented in this study indicate that insulin and thrombin negatively and positively modulate Rho kinase activity as well as its association with IRS-1 in VSMCs, respectively. Reciprocally, expression of activated RhoAV14 negatively regulates insulin signaling in VSMCs via increased activation and association of its downstream target, ROK-α with IRS-1. Increased ROK-α/IRS-1 association is accompanied by IRS-1 serine phosphorylation that results in inhibition of the following: 1) IRS-1 tyrosine phosphorylation, 2) association of p85 subunit of PI3-kinase with IRS-1, and 3) insulin stimulation of PI3-kinase enzymatic activity, ultimately resulting in inhibition of MBP activation by insulin. Thus, insulin signaling in VSMCs can be attenuated by Rho/Rho kinase binding and inhibition of IRS-1. This observation was further confirmed by expression of dominant negative RhoAN19 as well as cGK Iα, both of which inhibit Rho kinase activity, prevent thrombin-induced increase in ROK-α/IRS-1 association, restore IRS-1 tyrosine phosphorylation and PI3-kinase activation, and potentiate the downstream effects of insulin on MBP activity via inhibition of MBST695 site-specific phosphorylation (Fig. 9). To our knowledge, this is the first report demonstrating negative regulation of insulin signaling in VSMCs by activated Rho/Rho kinase via its association with IRS-1 and suppression of this by cGK Iα. It is unclear at present whether ROK-α without RhoA can bind IRS-1.

We have shown previously that insulin rapidly increases iNOS protein expression and cGMP generation via the PI3-kinase pathway in VSMCs (29). In addition, insulin inhibits thrombin-induced RhoA translocation to the membrane fraction via the NO/cGMP signaling pathway (17). Inhibition of RhoA translocation was accompanied by reductions in Rho kinase activity and decreased MBST695 site-specific phosphorylation. This results in stimulation of MBP and subsequent inhibition of actin cytoskeleton organization and VSMC contraction that may contribute to the well known vasodilator actions of insulin (36). These observations suggest that the inhibitory effects of insulin on Rho kinase are mediated via
Inactivation of RhoA. In addition, our preliminary data suggest that insulin may inhibit RhoA translocation by increasing RhoA phosphorylation as well as by impairing isoprenylation of RhoA via the NO/cGMP signaling pathway.

Several lines of direct evidence obtained in the present study suggest that cGK Iα, the downstream effector of the cGMP signaling pathway, reinforces insulin signaling by inhibiting ROK-α activity and ROK-α/IRS-1 association. First, adenoviral expression of cGK Iα markedly inhibits basal as well as thrombin-stimulated Rho kinase activity and further enhances the inhibitory effect of insulin on ROK-α activity in thrombin-stimulated VSMCs. Second, cGK Iα expression abolishes basal as well as thrombin-stimulated ROK-α/IRS-1 association, and this is accompanied by marked reductions in IRS-1 serine phosphorylation (data not shown) that may contribute to the observed increase in insulin-stimulated IRS-1 tyrosine phosphorylation, p85/IRS-1 association, and PI3-kinase activation. Most importantly, cGK Iα expression, similar to insulin, abolishes basal and thrombin-induced MBSThr695 site-specific phosphorylation and activates MBP. cGK Iα has been shown to associate directly with the MBS via a leucine zipper interaction (37), although cGK Iα could not stimulate MBP activity in vitro (38). cGK Iα has also been shown to phosphorylate and inactivate RhoA, blocking Rho kinase inhibition of MBP in VSMCs (33). Alternatively, cGK Iα may have an additional target.

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FIG. 8. cGK Iα expression abolishes basal and thrombin-mediated increase in MBSThr695 site-specific phosphorylation and increases MBP activation. VSMCs were infected with Ad.cGK Iα and exposed to insulin and thrombin as described in Fig. 6, and the myosin-enriched fractions were isolated in the presence of phosphatase inhibitors and examined for MBSThr695 site-specific phosphorylation by immunoblot analysis of equal amounts of lysate proteins with anti-MBST695 antibody (4). A duplicate membrane was probed with anti-MBS antibody for loading control. Representative autoradiograms are shown. B, myosin-enriched fractions prepared in the absence of phosphatase inhibitors were examined for MBP enzymatic activity using 32P-labeled MLC as a substrate. Results are the mean ± S.E. of three different experiments. *, p < 0.05 versus uninfected control; **, p < 0.05 versus thrombin (uninfected); ***, p < 0.05 versus the respective treatment of uninfected cells.

Fig. 8. Schematic representation of the proposed interaction between insulin signaling and Rho signaling pathways. Insulin-stimulated receptor tyrosine kinase mediates the activation of IRS-1/P13-kinase and the NO/cGMP/cGK Iα pathway that activates MBP in part by decreasing site-specific phosphorylation. The NO/cGMP/cGK Iα inhibits Rho signaling and prevents ROK-α activation and its association with IRS-1. Hypertension, expression of active RhoA(V14), and thrombin stimulation increase ROK-α/IRS-1 association and activate Rho kinase leading to increased IRS-1 serine phosphorylation that inhibits downstream insulin signaling by blocking IRS-1-tyrosine phosphorylation.

Chronic activation of RhoA by vasoconstrictors such as AII, as well as by disease states such as hypertension and diabetes, may inhibit iNOS/cGMP generation by specifically increasing ROK-α/IRS-1 association and thus block insulin signaling via the IRS-1/P13-kinase pathway (17–19). Previous studies have shown that AII pretreatment blocks insulin signaling in VSMCs by decreasing IRS-1-tyrosine phosphorylation due to an increase in IRS-1 serine/threonine phosphorylation (39). Our results provide a molecular basis for these observations by demonstrating that vasoconstrictors inhibit insulin signaling by activating Rho/ROK-α as well as by increasing ROK-α/IRS-1 association.

Most importantly, we have demonstrated that in addition to thrombin and AII, hypertension also increases ROK-α/IRS-1 association and inhibits insulin-stimulated IRS-1-tyrosine phosphorylation and recruitment and activation of p85 P13-kinase. Insulin pretreatment effectively blocks thrombin/AII-mediated increase in ROK-α/IRS-1 association in VSMCs isolated from control WKY rats, whereas it was ineffective in VSMCs expressing constitutively active RhoA(V14) and those isolated from SHR. Thus, hypertension and diabetes may be accompanied by up-regulation of Rho kinase activity resulting in an increase in ROK-α/IRS-1 association, inhibition of insulin signaling upstream of P13-kinase, iNOS induction, NO/cGMP generation, and MBP activation (18, 19). To our knowledge this is the first study demonstrating that hypertension is associated with ROK-α inhibition of insulin signaling. A previous study by Farah et al. (40) has shown that in Xenopus oocytes the carboxyl terminus of zROK-α (zROK-α-C) associated with the phosphotyrosine-binding xIRS-1 domain, and this association was further increased by RhoA(V14). Microinjection of zROK-C mRNA into Xenopus oocytes selectively inhibited insulin-induced mitogen-activated protein kinase (MAPK) activation with a concomitant inhibition of oocyte maturation (40). In

* N. Begum, O. A. Sandu, and M. Ito, unpublished data.
contrast, microinjection of full-length αRok−/− stimulated MAPK activation by insulin via Ras and promoted oocyte maturation (41). We have also shown that insulin differentially inhibits PI3-kinase and stimulates MAPK signaling pathways in VSMCs isolated from diabetic and hypertensive rats (29, 30). Thus, it appears that activated Rho/ROK−/− phosphorylation and up-regulates MAPKs by enhancing the ability of Ras. Further studies are in progress to understand whether Rho/ROK−/− associates with the other members of the IRS family as well as the other tyrosine-phosphorylated proteins, for example, Shc, Src, etc. In addition, other kinases, for example, Zip kinase, are postulated to mediate ROK−/− effects on MBS/MBP (42).

In summary, we have demonstrated that normally insulin inactivates Rho/ROK−/− and prevents thrombin and all-induced ROK−/−/IRS-1 association in order to preserve downstream PI3-kinase/Nos/cGMP/cGK I signaling leading to MBP activation. However, in diabetes a vicious cycle could occur in which impaired insulin signaling leads to increased vasostriction and hypertension that activates Rho and further depresses insulin signaling.

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Active Rho Kinase (ROK-α) Associates with Insulin Receptor Substrate-1 and Inhibits Insulin Signaling in Vascular Smooth Muscle Cells
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