

Diabetes in the Goto-Kakizaki Rat Is Accompanied by Impaired Insulin-Mediated Myosin-Bound Phosphatase Activation and Vascular Smooth Muscle Cell Relaxation

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Our laboratory has demonstrated that insulin rapidly stimulates myosin-bound phosphatase (MBP) activity in vascular smooth muscle cells (VSMCs). In this study, we examined whether diabetes is accompanied by alterations in MBP activation and elucidated the components of the signaling pathway that mediate the effects of diabetes. VSMCs isolated from Goto-Kakizaki (GK) diabetic rats (a model for type 2 diabetes) exhibited marked impairment in MBP activation by insulin that was accompanied by failure of insulin to decrease the phosphorylation of a regulatory myosin-bound subunit (MBS) of MBP and inhibit Rho kinase activity resulting in increased myosin light-chain (MLC)₂₀ phosphorylation and VSMC contraction. In VSMCs isolated from control rats, insulin inactivates Rho kinase and decreases MBS phosphorylation, leading to MBP activation. In addition to this pathway, insulin also appears to activate MBP by stimulating the phosphatidylinositol (PI) 3-kinase/nitric oxide (NO)/cGMP signaling pathway because treatment with the synthetic inhibitors of PI 3-kinase, NO synthase (NOS), and cGMP all blocked insulin's effect on MBP activation, whereas cGMP agonists and sodium nitroprusside (SNP) mimicked insulin's effect on MBP activation. VSMCs from diabetic GK rats exhibit reductions in insulin-mediated induction of inducible NOS protein expression and cGMP generation but normal MBP activation in response to SNP and cGMP agonist. This observation led us to examine the effect of diabetes on the activation status of the upstream insulin-signaling components. Although GK diabetes did not affect insulin-stimulated tyrosine phosphorylation of the insulin receptor or its content, insulin-stimulated insulin receptor substrate (IRS)-1 tyrosine phosphorylation was severely impaired. This was accompanied

by marked reductions in IRS-1-associated PI 3-kinase activity. We conclude that insulin stimulates MBP via its regulatory subunit, MBS, by inactivating Rho kinase and stimulating NO/cGMP signaling via PI 3-kinase as part of a complex signaling network that controls MLC₂₀ phosphorylation and VSMC contraction. Defective signaling via Rho kinase and the IRS-1/PI 3-kinase/NOS/cGMP pathway may mediate the inhibitory effects of hyperglycemia and diabetes on MBP activation in this experimental model. *Diabetes* 49:2178–2189, 2000

Diabetes and obesity are associated with an increased risk of hypertension, atherosclerosis, and cardiovascular disease. Numerous epidemiologic studies indicate that insulin resistance as well as hyperinsulinemia associated with type 2 diabetes contribute largely to the development of hypertension and atherosclerotic lesions (1–4). The mechanisms linking these diseases, however, are not well understood. Vascular smooth muscle cells (VSMCs) are the major constituent of blood vessel walls responsible for the maintenance of vascular tone. These cells also play an important role in the pathogenesis of type 2 diabetes, hypertension, and cardiovascular diseases. Increased contractility of VSMCs, an abnormal vascular tone, and defective vasorelaxation are earliest abnormalities observed in these disease states (1–4). Insulin can inhibit VSMC contraction, migration, and growth in the normal vasculature (5–7), and insulin's failure to do so in insulin-resistant states may contribute to enhanced atherosclerosis/restenosis in these clinical conditions. Studies in human subjects indicate that hypertension and type 2 diabetes are associated with an impaired vasodilatory response to acetylcholine and sodium nitroprusside (SNP), suggesting impaired VSMC responsiveness to endothelium-derived nitric oxide (EDNO) (8–10). However, these studies do not distinguish between hyperglycemia, insulin deficiency, or insulin resistance as possible causes of any EDNO abnormalities observed. Given the importance of EDNO as a regulator of vascular tone (11), platelet adhesiveness (12), thrombosis (13), and VSMC mitogenesis (14), it appears that insulin resistance at the level of endothelium may have profound implications with respect to the increased risk of macrovascular disease seen in insulin-resistant humans. An understanding of the exact mechanism of insulin's inhibition of contraction in normal VSMCs is necessary to explore the

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All, angiotensin II; ECL, enhanced chemiluminescence; ENDO, endothelium-derived nitric oxide; HRP, horseradish peroxidase; iNOS, inducible NOS; IR, insulin receptor; IRS, IR substrate; L-NMMA, N^G-monomethyl-L-arginine; MAPK, mitogen-activated protein kinase; MBP, myosin-bound phosphatase; MBS, myosin-bound subunit; MLC, myosin light-chain; MLCK, MLC kinase; NO, nitric oxide; NOS, NO synthase; PI, phosphatidylinositol; PKC, protein kinase C; PP-1C δ , δ -isoform of the catalytic subunit of protein phosphatase 1; PVDF, polyvinylidene difluoride; SNP, sodium nitroprusside; VSMC, vascular smooth muscle cell.

pathophysiology of the vascular complications associated with diabetes.

Smooth muscle contraction is regulated by the level of myosin phosphorylation that is catalyzed by myosin light-chain kinase (MLCK) and the dephosphorylation by a myosin-bound phosphatase (MBP) (7). MBP holoenzyme purified from smooth muscle is a heterotrimer consisting of 130-, 38-, and 20-kDa subunits (15,16). The 38-kDa subunit is identified as a δ -isoform of the catalytic subunit of protein phosphatase 1 (PP-1C δ). The 130-kDa subunit has been shown to bind myosin and also interact with the catalytic subunit (15). The holoenzyme shows a higher activity toward myosin when compared with the catalytic subunit alone (15). These findings showed that the 130-kDa subunit is a regulatory and myosin-targeting myosin-bound subunit (MBS) of MBP. Recently, MBS has been shown to be located downstream of the small GTP protein Rho (17). Rho kinase, which is activated by GTP-RhoA (17), inhibits MBP activity by phosphorylation of MBS (18), resulting in an increase in 20-kDa myosin light-chain (MLC)₂₀ phosphorylation and contraction of smooth muscle. Although these results clearly explain how activated Rho increases Ca²⁺ sensitivity of both MLC₂₀ phosphorylation and contraction (the pathway that leads from receptor, to G proteins, and then to the ultimate inhibition of the phosphatase) is not known. Arachidonic acid has been suggested as a possible secondary messenger because it dissociates the trimeric structure of the phosphatase leading to its inhibition (19). In addition, Ikebe and Brozovich (20) reported that agonist-induced activation of protein kinase C can also increase VSMC contraction via an inhibition of MBP.

Given that insulin's vasodilatory effects are mediated via nitric oxide (NO) (21) and cGMP inhibits MLC₂₀ phosphorylation (22), it can be hypothesized that insulin may inhibit VSMC contraction by activating MBP via a NO/cGMP signaling pathway. Whether or not dephosphorylation of MBS via agonist-induced signal transduction pathways causes activation of MBP remains unknown.

We have recently demonstrated that insulin causes a rapid activation of MBP in primary cultures of VSMCs isolated from control rats. The present study was undertaken to investigate whether hyperglycemia associated with diabetes is accompanied by alterations in MBP activation and to elucidate the components of the signaling pathway that mediate the effects of hyperglycemia using VSMCs isolated from the aortas of Goto-Kakizaki (GK) diabetic rats, a model for type 2 diabetes (23).

Nonobese insulin-resistant GK rats are a highly inbred strain of Wistar (WKY) rats that spontaneously develop type 2 diabetes (24). This genetic rat model is particularly relevant to understanding human type 2 diabetes because the defects in glucose-stimulated insulin secretion, peripheral insulin resistance, hyperinsulinemia, and hyperglycemia are seen as early as 4 weeks after birth (24). Therefore, this model provides a valuable tool for dissecting the pathogenesis of insulin resistance.

The results of this study indicate that hyperglycemia associated with diabetes is accompanied by marked impairment of MBP activation by insulin. Furthermore, insulin resistance of MBP activation is accompanied by increased MBS phosphorylation and Rho kinase activation and a decrease in insulin receptor substrate (IRS)-1-associated phosphatidylinositol (PI) 3-kinase activity leading to impaired NO/cGMP signaling and MBP activation. Inhibition of MBP seems to be

coordinated with enhanced VSMC contraction because of an increase in MLC₂₀ phosphorylation in VSMCs isolated from diabetic GK rats.

RESEARCH DESIGN AND METHODS

Cell culture reagents, fetal bovine serum, phosphorylase kinase, and phosphorylase b were purchased from Life Technologies (Grand Island, NY). [γ -³²P]ATP (specific activity $\geq 3,000$ Ci/mmol) and ³²P-orthophosphoric acid were purchased from Dupont/New England Nuclear (Boston, MA). 8-bromo-cGMP, N^G-monomethyl-L-arginine (L-NMMA), wortmannin, and Rp-guanosine-3',5'-cyclic monophosphorothioate (RpGMP) were purchased from Biomol Research (Plymouth Meeting, PA). Electrophoresis and protein assay reagents were from Bio-Rad (Richmond, CA). Okadaic acid was from Moana Bioproducts (Honolulu, HI). Porcine insulin was a gift from Eli Lilly (Indianapolis, IN). Type-1 collagenase was from Worthington Biochemical (Freehold, NJ). Antibody against the 160-kDa rho kinase (ROK)- α was purchased from Transduction Laboratories (San Diego, CA). Antibodies against insulin receptor (IR) and Rho A were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against the pleckstrin homology domain of IRS-1 and p85 PI 3-kinase were obtained from Upstate Biotechnology (Lake Placid, NY). Monoclonal phosphotyrosine antibody was purchased from Zymed (San Francisco, CA). MLC₂₀ antibody, anti-mouse IgG-Agarose, protein A-Sepharose CL-4B, protease inhibitors, calmodulin, sodium orthovanadate, angiotensin II (AII), thrombin, and all other reagents were purchased from Sigma (St. Louis, MO). Anti-MBS antibody and MLCK were a gift from Dr. Hartshorne (Tucson, AZ). MLCs were prepared from chicken gizzards according to the published protocol (25).

Culture of VSMCs and treatment with insulin. A colony of type II diabetic GK rats was established at this institute with the animals supplied by Dr. Robert V. Farese (VA Hospital, Tampa, FL) as detailed earlier (23). VSMCs in primary culture were obtained by enzymatic digestion of the aortic media isolated from 7- to 8-week-old GK and WKY male rats as described in our recent publications (26,27). Primary VSMC cultures were maintained in α -minimal essential medium containing 10% fetal bovine serum and 1% antibiotic/antimycotic mixture. VSMCs isolated from diabetic GK rats were maintained in medium containing 20 mmol/l glucose to mimic a hyperglycemic condition. Initial studies established that VSMCs from diabetic GK rats maintain their phenotype up to the sixth passage. Therefore, subcultures of VSMCs at passage five were used in this study. Experiments were performed on highly confluent 9- to 11-day cells. Before each experiment, cells were serum starved for 24 h in serum-free α -minimal essential medium containing 1% antibiotics and 5.5 mmol/l glucose for WKY rats and 20 mmol/l glucose for GK rats. The next day, cells were exposed to insulin (0–100 nmol/l) for 0–30 min. In some experiments, VSMCs were pretreated with various inhibitors for 30 min followed by exposure to insulin, as detailed in the figure legends.

Preparation of myosin-enriched fractions. Myosin-enriched fractions of VSMCs were prepared as described previously (28). Okadaic acid at a 1 nmol/l concentration was included during the enzyme assay to inhibit any residual protein phosphatase 2A activity (29,30).

Measurement of MBP activity. Phosphatase activity in myosin-enriched fractions was assayed using [³²P]-labeled phosphorylase as well as [³²P]-labeled MLCs as substrates (31). [³²P]-labeled phosphorylase a was prepared by incubating [γ -³²P]ATP with purified phosphorylase kinase and phosphorylase b (32). [³²P]-labeled MLC was prepared according to the published protocol (33) by incubating MLC (0.8 mg/ml) with purified MLCK (50 μ g/ml), 0.1 mg/ml calmodulin, and 50 μ mol/l [γ -³²P]ATP.

Metabolic labeling of VSMCs and measurement of MBS phosphorylation by immunoprecipitation and Western blot analyses. Serum-starved VSMCs were metabolically labeled with ³²P-orthophosphoric acid (0.3 mCi/ml) for 4 h and exposed to various agonists, followed by insulin, as detailed in the figure legends. Equal amounts of precleared lysate proteins (0.25 mg) with 5 μ l anti-MBS antibody were prebound to 100 μ l protein A-Sepharose (29). The immunoprecipitates were washed exhaustively with the lysis buffer, separated by SDS-PAGE, and transferred to the polyvinylidene difluoride (PVDF) membrane followed by autoradiography. The level of phosphorylation was measured by densitometric scanning of the autoradiograms. To overcome variations in proteins due to immunoprecipitation, the membranes were probed with an anti-MBS antibody followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies and detection by enhanced chemiluminescence (ECL). The extent of MBS phosphorylation was quantitated by dividing the intensity of the radioactive signal with the protein signal.

Immunoprecipitation and in vitro assay of Rho kinase activity in the immunocomplexes. Equal amounts of precleared lysate proteins (100 μ g) were immunoprecipitated overnight at 4°C with the anti-ROK- α antibody (6 μ g/tube) with

constant shaking. Rho kinase activity in the immunoprecipitates was assayed according to the published protocol with slight modifications (34) using a myosin-enriched fraction isolated from control cells or purified myosin as a substrate in the presence of 100 $\mu\text{mol/l}$ [γ - ^{32}P]ATP. The reaction was stopped by transferring 25- μl aliquots of the reaction mixture to phosphocellulose paper that was washed extensively with 75 mmol/l orthophosphoric acid and air dried, and ^{32}P incorporation was determined by liquid scintillation chromatography.

Analyses of agonist-induced Rho translocation to the plasma membranes. Membrane and cytosolic fractions were prepared according to the published protocol (35). Equal amounts of proteins from membrane fractions were subjected to SDS-PAGE, transferred to PVDF membrane, and probed with mouse anti-Rho A antibody followed by incubation with HRP-labeled secondary antibody and subsequent detection with ECL.

Immunoprecipitation of IR and IRS-1 and Western blot analyses. VSMCs were treated with and without insulin (100 nmol/l) for 10 min. Cell lysates were precleared as detailed (29) and equal amounts of lysate proteins (4 mg) were immunoprecipitated with anti-insulin receptor antibody (10 μg) and anti-IRS-1 antibody (10 μg) overnight. The immunoprecipitates were separated on 7.0% SDS polyacrylamide gel followed by Western blot analyses with the antiphosphotyrosine antibody. To overcome variations in proteins due to immunoprecipitation, blots were stripped and reprobed with anti-IR and anti-IRS-1 antibody. The extent of tyrosine phosphorylation of IR and IRS-1 was quantitated by dividing the intensity of the phosphotyrosine signal with the protein signal.

Immunoprecipitation and in vitro assay of PI 3-kinase activity in the IRS-1 immunoprecipitates. Equal amounts of precleared lysate proteins (100 μg) were immunoprecipitated with rabbit anti-IRS-1 antibody. PI 3-kinase activity was assayed in the IRS-1 immunoprecipitates as detailed in our recent publication (24).

MLC phosphorylation. MLC₂₀ phosphorylation was analyzed by urea and by glycerol-PAGE separation of the mono- and diphosphorylated forms of MLC₂₀ as detailed previously (36). MLC₂₀ was detected by immunoblot analysis with MLC₂₀ antibody followed by treatment with HRP-conjugated secondary antibodies and detection by ECL. The autoradiograms were scanned and quantified, and the percent maximal MLC₂₀ phosphorylation was determined by dividing the sum of fast migrating diphosphorylated MLC₂₀ area and the monophosphorylated MLC₂₀ area by the total of phosphorylated and non-phosphorylated areas (36).

Measurement of VSMC contraction. VSMC contraction was measured by analyzing the insulin-mediated decrease in thrombin-stimulated transvascular HRP diffusion according to the published protocol (37). Briefly, VSMCs were plated on collagen-coated polyethylene terephthalate cell culture inserts (3- μm pore size, Becton Dickinson). Serum-starved VSMCs were treated in quadruplicate with and without insulin (100 nmol/l) for 30 min followed by the addition of 500 μl serum-free medium containing thrombin (0.5 U/ml). For controls, thrombin and insulin were omitted. After 15 min, the lower compartment was filled with 500 μl serum-free medium, and the medium in the upper compartment was replaced with fresh medium containing HRP (0.34 mg/ml). After 1 min, 60 μl of medium from the lower compartment was transferred to a tube and mixed with 860 μl of reaction buffer containing 50 mmol/l NaH_2PO_4 , 5 mmol/l guaiacol, and 0.6 mmol/l of freshly made H_2O_2 . The absorbance was measured at 470 nm after a 15-min incubation at room temperature.

Protein assay. Proteins in the cellular extracts and lysates were quantitated by the bicinchoninic acid (38) or by the Bradford technique (39).

Statistical analysis. The results are presented as means \pm SE of four to six independent experiments, each performed in triplicate at different times. Paired Student's *t* test was used to compare the basal and insulin-treated preparations. Unpaired *t* test or analysis of variance was used to compare the mean values between treatments. A *P* value of <0.05 was considered statistically significant.

RESULTS

Characteristics of diabetic GK rats. As reported previously by us and others (23,24,40), diabetic GK rats exhibit a twofold increase in postprandial plasma glucose and insulin levels when compared with WKY controls (plasma glucose 19.5 \pm 2.6 vs. 8.9 \pm 2.8 mmol/l; plasma insulin 1.3 \pm 0.1 vs. 0.7 \pm 0.1 nmol/l, blood collected at the time of death at 8:00 A.M.). In contrast, body weight was comparable between the age-matched WKY control and GK rats (193 \pm 13.7 vs. 185 \pm 9.2 g). Similar results were obtained by other groups (40). Mean fasting plasma glucose and insulin levels were not measured

in this study, but other studies (41) have reported elevated levels of fasting plasma glucose (10.1 \pm 0.21 vs. 6.7 \pm 0.1 mmol/l) and insulin (1,100 \pm 65 vs. 675 \pm 43) in this rat model starting from 6 weeks after birth.

Diabetes causes reductions in the basal and insulin-stimulated MBP activation. Our recent studies have shown that physiological concentrations of insulin rapidly increase MBP activity in myosin-enriched fractions isolated from control WKY rats. To further understand the importance of MBP in VSMC function, we examined the activity of this enzyme in VSMCs isolated from diabetic GK rats. Diabetes resulted in a 43% decrease in basal MBP activity when compared with control VSMCs isolated from WKY rats (Fig. 1A). Whereas incubation with 100 nmol/l insulin for 10 min caused a 63% increase in MBP activity in WKY rats, VSMCs from diabetic GK rats exhibited only a 15% increase in MBP activity over the basal values (Fig. 1A).

In WKY rats, 1 nmol/l insulin caused a half-maximal stimulation of MBP, with a maximum response seen with 10 nmol/l insulin after 10 min (Fig. 1B). The observed reductions in MBP activation by insulin in VSMCs from diabetic rats were due to a marked decrease in insulin responsiveness at all concentrations of insulin (Fig. 1B). Maintenance of GK VSMCs in medium containing normal concentrations of glucose did not restore cellular responsiveness to insulin in terms of MBP activation, whereas incubation of VSMCs isolated from WKY rats in medium containing high glucose did result in the inhibition of insulin's effect on MBP activation (data not shown). This observation suggests that defective MBP activation seen in diabetic GK rat VSMCs may be due to a genetic difference between diabetic GK and WKY rather than hyperglycemia or other metabolic defects secondary to diabetes. Furthermore, the observed effect of high glucose on MBP inhibition was not due to changes in osmolality because treatment with mannitol did not inhibit insulin's effect on MBP activation in WKY rats.

Western blot analysis indicated that GK diabetes did not alter the contents of either MBS or PP-1C δ examined in the myosin-enriched fraction (Fig. 1C) as well as the MBS immunoprecipitates (Fig. 1D). Furthermore, insulin treatment did not increase the amount of PP-1C δ bound to MBS in WKY rats. This observation suggests that insulin may be activating the enzyme already bound to the myosin regulatory subunit (MBS), and the observed reductions in MBP activity in VSMCs isolated from diabetic GK rats may be due to impaired activation of MBP bound to MBS.

Insulin fails to decrease MBS phosphorylation in VSMCs isolated from diabetic GK rats. Recent studies suggest that the large 110/130-kDa MBS of MBP gets phosphorylated by agonists that stimulate smooth muscle contraction (17,18). Furthermore, phosphorylation of MBS results in inactivation of MBP resulting in a decrease in MBP activity (18). Our observations that physiological concentrations of insulin rapidly stimulate MBP suggest that insulin may be activating the phosphatase either by altering MBS phosphorylation status and/or activating the catalytic subunit bound to MBS via another mechanism. Therefore, we examined the effect of insulin on MBS phosphorylation status in VSMCs isolated from control rats as well as diabetic GK rats. Serum-starved VSMCs were labeled with ^{32}P -orthophosphoric acid (0.3 mCi/ml) for 4 h to attain steady-state equilibrium (29) followed by the addition of insulin for 0–30 min. Cell lysates were immuno-

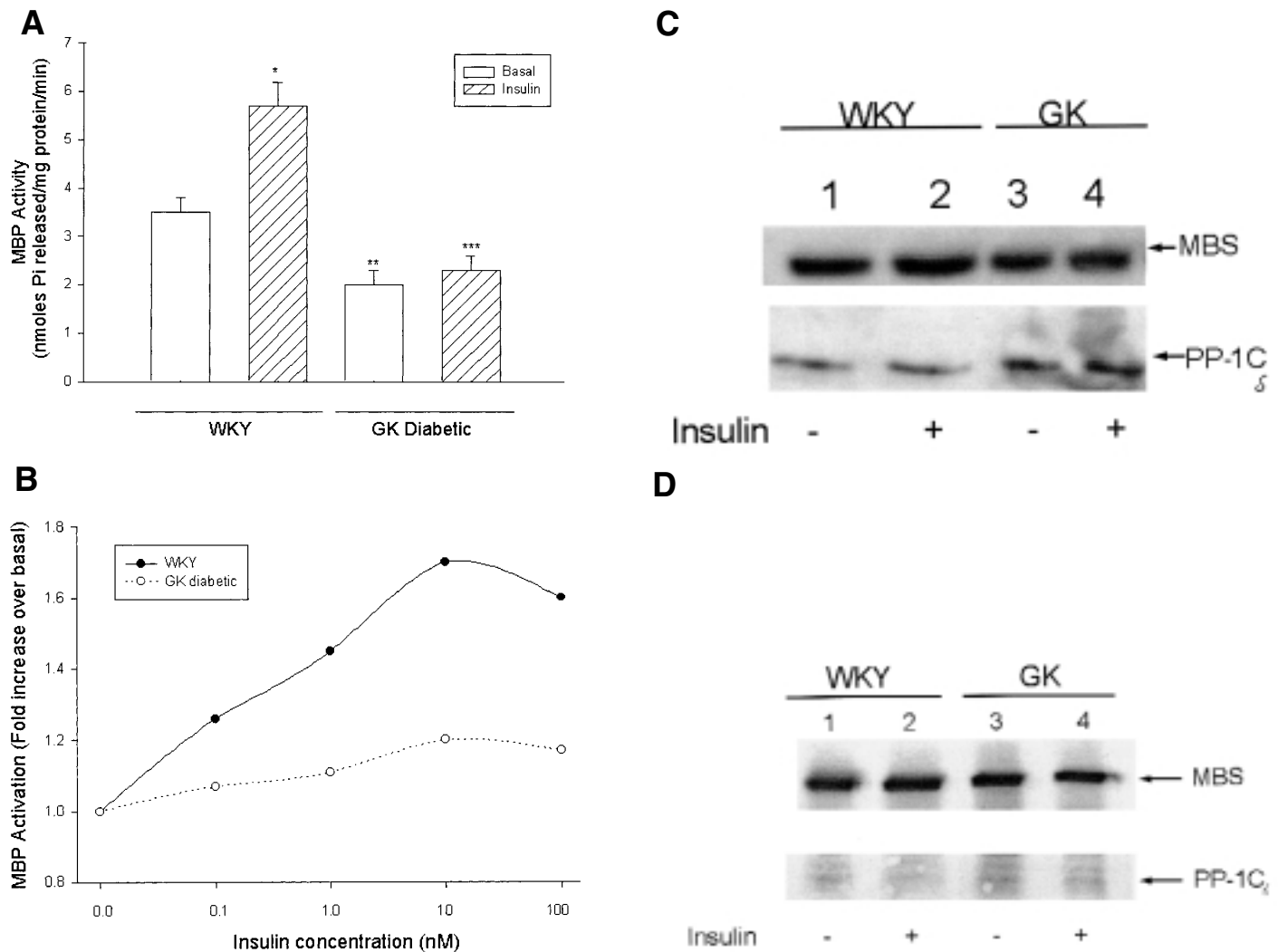


FIG. 1. A: GK diabetes causes impairment of MBP activation by insulin. VSMCs isolated from control WKY rats and diabetic GK rats were grown to confluence and treated with and without insulin (100 nmol/l) for 10 min. MBP activity was assayed in myosin-enriched fractions using ^{32}P -labeled MLC as a substrate. Results are the mean \pm SE of four different experiments performed in duplicate. * $P < 0.05$ vs. WKY basal; ** $P < 0.05$ vs. WKY basal; *** $P < 0.05$ vs. insulin-stimulated WKY. **B:** Dose response of MBP activation by insulin in VSMCs from WKY and diabetic GK rats. VSMCs were exposed to various concentrations of insulin for 10 min and assayed for MBP activity as detailed in A. Results are the mean \pm SE of two separate experiments performed in duplicate. **C:** Diabetes does not alter the contents of MBS and PP-1C δ in the myosin-enriched fractions. VSMCs from WKY and diabetic GK rats were grown to confluence and treated with insulin (100 nmol/l \times 10 min). Equal amounts of proteins (20 μg) from myosin-enriched fractions were separated on a 7.5% SDS polyacrylamide gel. The proteins were transferred to the PVDF membrane. The membrane was cut into two halves across the 50-kDa marker. The top portion was probed with anti-MBS antibody, and the bottom portion was used to analyze PP-1C δ content. A representative immunoblot is shown. Similar results were obtained in multiple experiments. **D:** Insulin does not increase the association of PP-1C δ with the MBS. Equal amounts of proteins from control and insulin-treated VSMC lysates were immunoprecipitated with the anti-MBS antibody as detailed in the text. The immunoprecipitates were separated by SDS-PAGE followed by Western blot analysis as detailed in C. A representative Western blot is shown.

precipitated with anti-MBS antibody and examined for ^{32}P incorporation into MBS by SDS-PAGE and autoradiography. The intensity of the ^{32}P signal was quantitated by densitometric scanning and corrected for any variations in MBS protein by dividing the ^{32}P signal with the protein signal. As shown in Fig. 2A, treatment of VSMCs isolated from control WKY rats with 100 nmol/l insulin for 2 min caused a rapid 53% decrease in MBS phosphorylation (Fig. 2A, compare lane 2 vs. lane 1 and Fig. 2C). A 72% decrease in ^{32}P incorporation into MBS was observed after a 5-min incubation with insulin (Fig. 2A, compare lane 3 vs. lane 1 and Fig. 2C). The observed decrease in MBS phosphorylation was sustained for 20 min of insulin treatment (Fig. 2A, compare lane 5 vs. lane 1). In con-

trast, insulin treatment did not decrease MBS phosphorylation in VSMCs isolated from diabetic GK rats at all time points studied (Fig. 2B, compare lane 1 vs. lanes 2–5 and Fig. 2C).

Insulin inhibits Rho kinase activity in control VSMCs: GK diabetes is accompanied by impaired inhibition of Rho kinase by insulin. Numerous reports (17,18) suggest that MBS is phosphorylated by Rho kinase, which is activated on stimulation with agonists such as thrombin or AII. We tested the possibility that insulin may be inhibiting Rho kinase activity and thereby decreasing MBS phosphorylation in VSMCs isolated from control rats. Diabetes may be accompanied by failure of insulin to effectively inhibit Rho kinase activity. To test this hypothesis, Rho kinase was

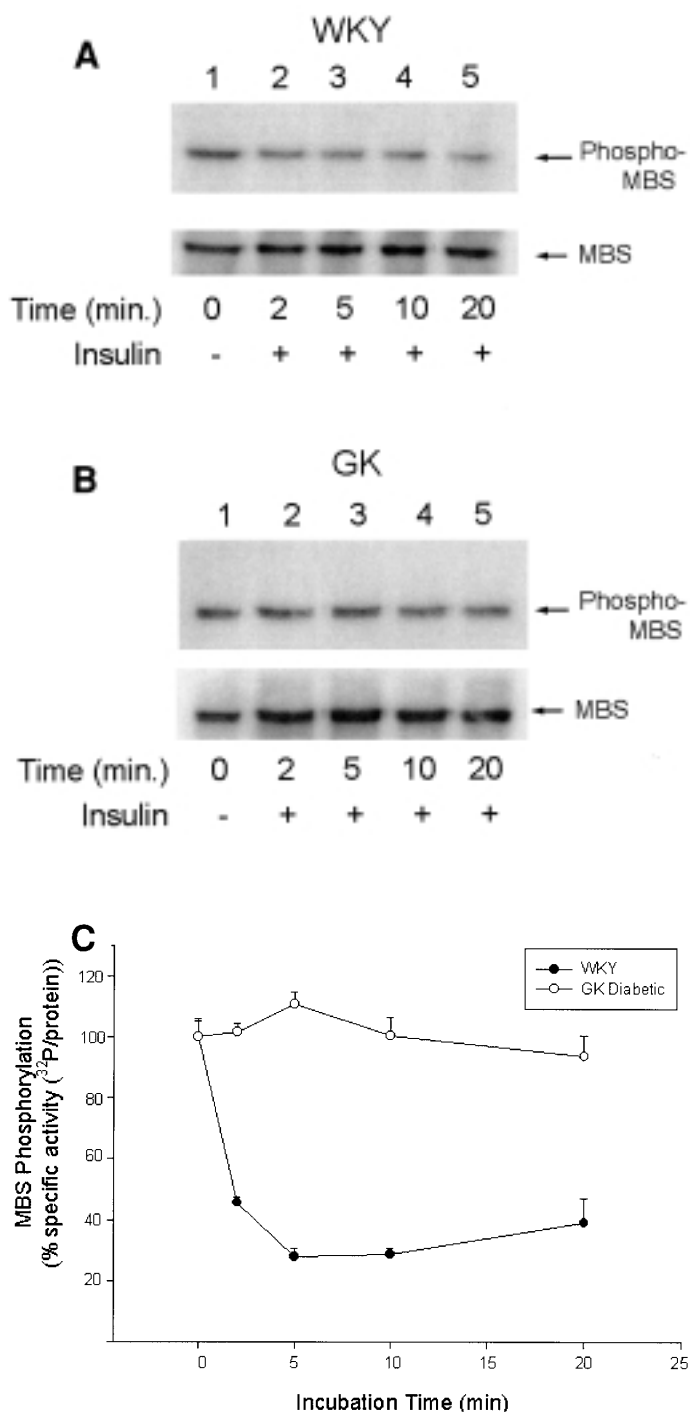


FIG. 2. A and B: Insulin decreases the phosphorylation status of MBS. GK diabetes is accompanied by lack of insulin-mediated decrease in MBS phosphorylation. ^{32}P -labeled VSMCs were treated with 100 nmol/l insulin for 2–30 min. Equal amounts of cell lysate proteins (250 μg) were immunoprecipitated with anti-MBS antibody. The immunoprecipitates were subjected to SDS-PAGE and transferred to PVDF followed by autoradiography. The blot was probed with anti-MBS antibody to estimate the quantity of MBS protein immunoprecipitated. A representative autoradiogram is shown. Similar results were obtained in three separate experiments. **C:** Quantitation of MBS phosphorylation in WKY and diabetic GK VSMCs. Data from the autoradiograms were quantitated by densitometric scanning and normalized for variations in proteins by dividing the intensity of the ^{32}P signal with that of the protein signal. The ^{32}P -specific activity of MBS in the basal state was assigned a value of 100%, and the insulin effect was calculated relative to the control. Results are the mean of two independent experiments.

immunoprecipitated from equal amounts of lysate proteins prepared from control and insulin-treated VSMCs isolated from WKY and diabetic GK rats. The immunoprecipitates were assayed for kinase activity by an immunocomplex kinase assay using MBP as a substrate. As shown in Fig. 3A, insulin treatment of control VSMCs for 10 min caused a 40% decrease in Rho kinase activity when compared with untreated cell lysates. More importantly, pretreatment with insulin effectively prevented the thrombin-mediated increase in Rho kinase activity in WKY rats (Fig. 3A). In contrast, VSMCs isolated from diabetic GK rats exhibited a 25% increase in basal Rho kinase activity when compared those from WKY rats. Thrombin caused a very small increase in GK VSMCs. Insulin treatment did not decrease Rho kinase activity in VSMCs from diabetic GK rats.

In WKY rats, insulin caused a rapid 40% decrease in Rho kinase activity after 2 min (Fig. 3B), which was sustained for the entire 20-min time period examined. In contrast, VSMCs isolated from diabetic GK rats exhibited a transient increase in Rho kinase activity upon insulin treatment up to 10 min, with a return to basal levels after a 20-min period. The failure of insulin to inhibit Rho kinase activity in VSMCs isolated from diabetic GK rats correlates directly with the increase observed in MBS phosphorylation and the inability of insulin to decrease MBS phosphorylation.

The effect of insulin on Rho kinase inactivation in WKY rats was accompanied by a partial inhibition of thrombin-mediated translocation of Rho from the cytosol to the membrane fraction (Fig. 3C, compare lane 4 vs. lane 3). Thrombin alone caused a threefold increase in Rho content in the membrane fraction. Concomitant decreases in cytosolic Rho were not consistently observed (data not shown), probably because a large fraction of the total Rho protein still remained cytosolic. Insulin alone caused very little change in the amount of Rho in the membrane fraction (Fig. 3C, lane 2). In contrast, VSMCs from diabetic GK rats exhibited an increase in the amounts of Rho in the membrane fraction in the basal state as well as in insulin-stimulated conditions. Furthermore, treatment with thrombin caused a threefold increase in the membrane Rho content. Insulin failed to decrease thrombin-induced Rho translocation in diabetic GK VSMCs (Fig. 3C, bottom panel, compare lane 4 vs. lane 3).

Role of NO synthase signaling in insulin-mediated MBP activation: GK diabetes is accompanied by inhibition of NO synthase signaling. Given that insulin's vasodilatory effects are mediated via NO (8), we next examined whether the inhibitors of the NO synthase (NOS) and cGMP signaling pathways affect insulin-mediated MBP activation in control VSMCs and whether diabetes is accompanied by reductions in NOS and cGMP levels in VSMCs isolated from diabetic GK rats. As shown in Fig. 4, pretreatment with L-NMMA (a NOS inhibitor) and RpcGMP (a cGMP antagonist) blocked insulin's effect on MBP activation in WKY rats. Furthermore, SNP (an NO donor) and 8-bromo cGMP (a cGMP agonist) both mimicked insulin's effects on MBP activation (Fig. 4). Combined treatment with insulin and SNP or 8-bromo cGMP did not further increase MBP activation (data not shown). More importantly, VSMCs isolated from diabetic GK rats exhibit normal responsiveness to SNP and 8-bromo cGMP in terms of MBP activation (Fig. 4). These results suggest that the insulin-induced NOS/cGMP signaling pathway may participate in insulin-mediated MBP activation, and an abnormal

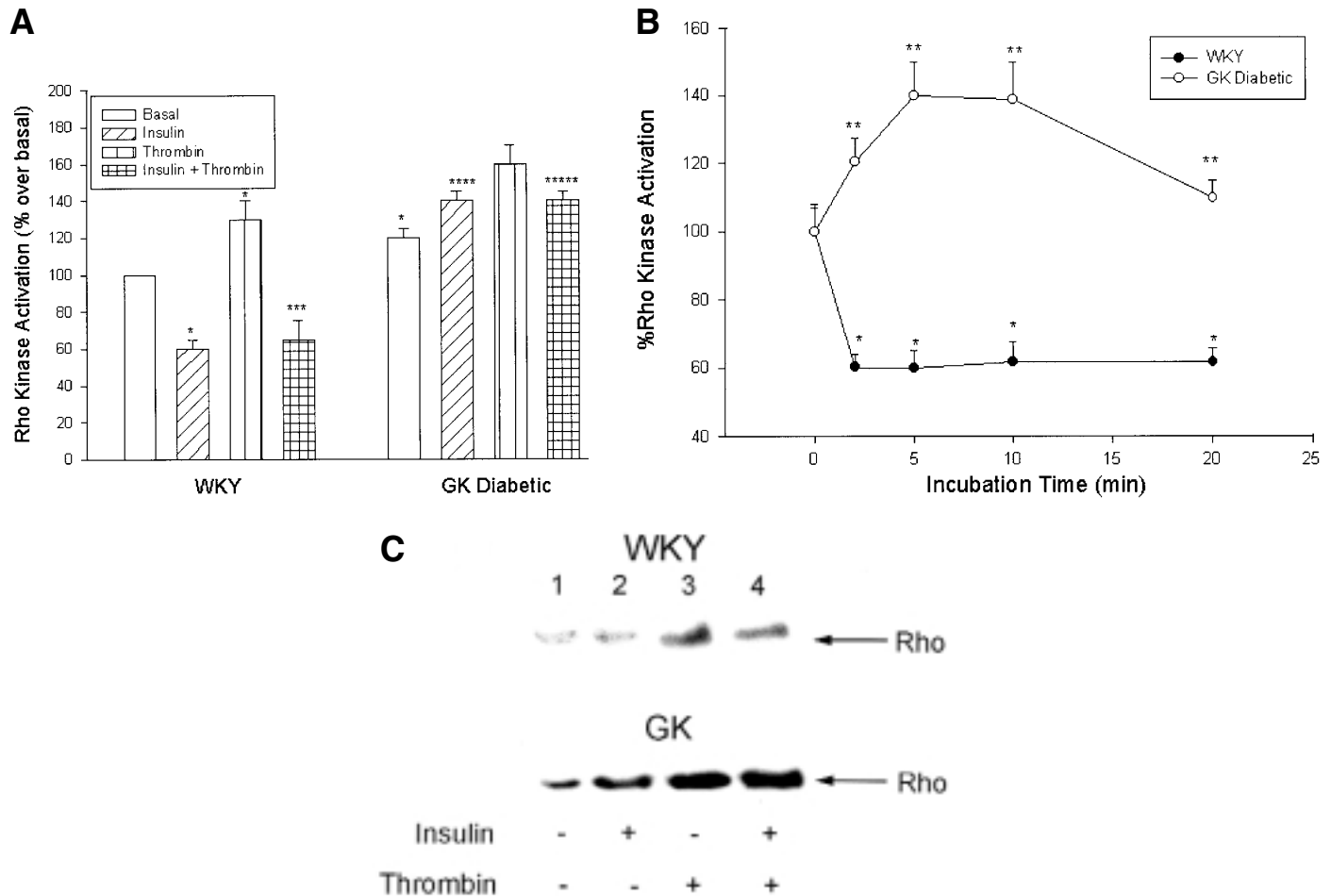


FIG. 3. A: Insulin inhibits Rho kinase activity and prevents a thrombin-mediated increase in Rho kinase activation. GK diabetes is accompanied by upregulation of Rho kinase activity. VSMCs from WKY and diabetic GK rats were treated with insulin (100 nmol/l) for 10 min followed by 1 U/ml thrombin for 5 min. Equal amounts of cell lysate proteins (100 μ g) were immunoprecipitated with anti-ROK- α antibody. Rho kinase activity in the immunoprecipitates was assayed using MBP as a substrate along with γ -[32 P]ATP. Results are the mean \pm SE of four separate experiments performed. * P < 0.05 vs. WKY basal; *** P < 0.05 vs. WKY thrombin; **** P < 0.05 vs. insulin-treated WKY; ***** P < 0.05 vs. insulin plus thrombin-treated WKY. **B:** Kinetics of insulin-mediated inactivation of Rho kinase in WKY. Insulin activates Rho kinase in VSMCs isolated from diabetic GK rats. VSMCs were treated with 100 nmol/l insulin for 0–20 min. Equal amounts of proteins were immunoprecipitated as detailed in A followed by assay of Rho kinase activity. Results are expressed as percent of basal activity. * P < 0.05 vs. WKY basal; ** P < 0.05 vs. insulin-treated WKY. **C:** Diabetes is accompanied by the lack of insulin-mediated inhibition of thrombin-induced translocation of Rho to the membrane fraction. VSMCs from WKY and diabetic GK rats were exposed to insulin and thrombin as detailed in A. Equal amounts of membrane proteins were subjected to SDS-PAGE followed by Western blot analysis with anti-Rho A antibody. A representative autoradiogram is shown. Similar results were obtained in four different experiments.

induction of the NOS/cGMP signaling pathway may contribute to impaired MBP activation in conjunction with defective Rho kinase signaling.

To further confirm that the impaired MBP activation observed in VSMCs isolated from diabetic GK rats is indeed due to defective NOS signaling, VSMCs were exposed to insulin and examined for insulin-induced inducible NOS (iNOS) protein expression and cGMP generation by Western blot analysis and radioimmunoassay, respectively (27). As shown in Fig. 5A, insulin caused a rapid iNOS protein expression in VSMCs isolated from WKY rats. In contrast, insulin-induced iNOS protein expression was markedly decreased in diabetic GK rat VSMCs. The decrease in iNOS protein expression was accompanied by marked reductions in cGMP generation in VSMCs isolated from diabetic GK rats (Fig. 5B). Furthermore, pretreatment with wortmannin (a PI 3-kinase inhibitor) and L-NMMA (a NOS inhibitor) blocked insulin-mediated cGMP

generation in WKY rats (Fig. 5C). SNP, which stimulates guanylyl cyclase directly via NO, caused a comparable increase in GMP generation in both WKY and diabetic GK rat VSMCs. These observations suggest that the defects observed in GK diabetes are upstream of guanylyl cyclase at the level of NOS induction. In our earlier studies, we have demonstrated that insulin rapidly induces iNOS protein expression and cGMP generation in VSMCs from WKY, whereas VSMCs isolated from spontaneous hypertensive rats exhibit resistance to insulin in terms of iNOS protein induction and cGMP generation (27).

GK diabetes is accompanied by marked reductions in insulin-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase activity in the IRS-1 immunoprecipitates but has normal insulin receptor tyrosine phosphorylation. Recent studies by Jiang et al. (42) have shown that diabetes in the obese Zucker rat (*fa/fa*) was accompanied by marked reductions in IRS-1 tyrosine phosphorylation leading to a

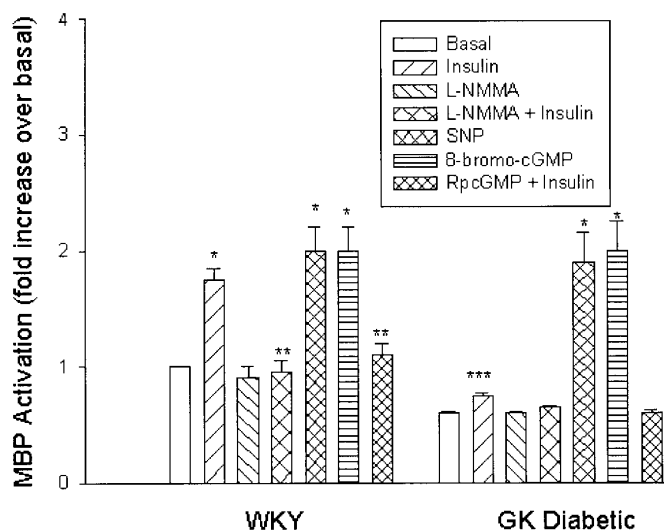


FIG. 4. Inhibitors of NOS and cGMP signaling block insulin-mediated MBP activation in WKY VSMCs. Diabetic GK VSMCs exhibit normal responsiveness to SNP and cGMP agonists. Serum-starved VSMCs were treated with L-NMMA (1 mmol/l) and RpcGMP (0.1 mmol/l) for 30 min followed by 100 nmol/l insulin for 10 min or treated with 100 nmol/l SNP or 8-bromo cGMP (1 mmol/l) for 10 min. MBP activity was measured in myosin-enriched pellets using MLC as a substrate. Results are the mean \pm SE of four independent experiments performed in duplicate. * $P < 0.05$ vs. basal; ** $P < 0.05$ vs. insulin; *** $P < 0.05$ vs. insulin-treated WKY.

selective decrease in PI 3-kinase activation by insulin in vascular tissue.

Given that MBP activation by insulin could be blocked by wortmannin, a PI 3-kinase inhibitor, we next examined whether GK diabetes is accompanied by alterations in IR and its upstream signaling components leading to impaired MBP activation. As shown in Fig. 6A, both the IR content and insulin-induced tyrosine phosphorylation of IR was comparable between VSMCs isolated from diabetic GK rats and control rats. In contrast, basal and insulin-stimulated IRS-1 tyrosine phosphorylation was markedly impaired in VSMCs isolated from diabetic GK rats (60% decrease when compared with VSMCs isolated from control rats; Fig. 6B). These changes in IRS-1 tyrosine phosphorylation were accompanied by >60% reductions in basal and insulin-stimulated PI 3-kinase activity assayed in the IRS-1 immunoprecipitates (Fig. 6C). Western blot analysis of the IRS-1 immunoprecipitates revealed that the observed reductions in PI 3-kinase activity in the IRS-1 immunoprecipitates from GK rats were due to a 70% decrease in the association of the p85 regulatory subunit of PI 3-kinase with IRS-1 (Fig. 6D).

GK diabetes is accompanied by increased thrombin-induced VSMC contraction and MLC₂₀ phosphorylation: insulin fails to inhibit thrombin effect. We next examined whether impaired MBP activation results in increased contractility of diabetic GK rat VSMCs. In these experiments, the ability of insulin to inhibit thrombin-induced transvascular diffusion of HRP due to a contraction-induced increase in cell permeability was determined spectrophotometrically as detailed in RESEARCH DESIGN AND METHODS. Pretreatment with insulin caused a 60% decrease in thrombin-induced contractions in VSMCs isolated from WKY rats (Fig. 7). In contrast, insulin pretreatment did not prevent

thrombin-mediated increase in cell permeability in GK rat VSMCs (Fig. 7).

Increased VSMC contraction in diabetic GK rat VSMCs was accompanied by a marked increase in AII-mediated MLC₂₀ phosphorylation (Fig. 8). In control VSMCs, pretreatment with 100 nmol/l insulin for 10 min prevented AII-mediated MLC₂₀ phosphorylation as seen by reduction in the contents of mono- and diphosphorylated MLCs by Western blot analysis of TCA precipitates separated on a urea-glycerol gel.

DISCUSSION

In confirmation with the recent studies of Jiang et al. (42), we also observed that VSMCs isolated from diabetic GK rats exhibit marked resistance to insulin-mediated upstream signaling via the IRS-1/PI 3-kinase pathway. This resistance results in marked impairment in downstream MBP activation, which is accompanied by increased MLC₂₀ phosphorylation and VSMC contraction. In addition, diabetes causes elevations in Rho kinase activity in VSMCs leading to MBS phosphorylation, which further inactivates MBP causing excessive contractility of VSMCs.

In contrast with its effect in diabetic GK rats, insulin rapidly stimulates MBP in VSMCs isolated from control WKY rats. The kinetics of MBP activation by insulin parallels the time course of the insulin-mediated decrease in MBS phosphorylation as well as Rho kinase activity. Thus, insulin stimulates MBP in part by reducing the phosphorylation of its regulatory subunit, MBS, via Rho kinase inhibition. Insulin activation of MBP is accompanied by the inhibition of MLC₂₀ phosphorylation as well as the inhibition of VSMC contraction (Fig. 9).

Several lines of evidence presented in this study suggest that impaired MBP activation by insulin in VSMCs from diabetic GK rats is due to defective signaling from the Rho kinase and PI 3-kinase/NO/cGMP signaling pathways. First, basal and thrombin-stimulated Rho kinase activity is higher in VSMCs isolated from diabetic GK rats, and insulin fails to inactivate Rho kinase and decrease MBS phosphorylation in these VSMCs. This is in opposition to VSMCs isolated from control WKY rats that exhibit a rapid insulin-mediated Rho kinase inactivation that is accompanied by a marked time-dependent reduction in MBS phosphorylation. Second, insulin pretreatment does not prevent a thrombin-mediated increase in translocation of Rho to the membrane fraction, and this further contributes to the sustained Rho kinase activation observed in VSMCs isolated from diabetic GK rats. Third, VSMCs from diabetic GK rats exhibit marked impairment in insulin-mediated activation of PI 3-kinase due to reductions in IRS-1 tyrosine phosphorylation and the association of the p85 subunit of PI-3 kinase with IRS-1. Our observations coincide with the recent studies by Jiang et al. (42). Using another rat model of diabetes (the obese Zucker rat), these authors demonstrated a selective decrease in IRS-1/IRS-2-associated PI 3-kinase activation in vascular tissues while preserving insulin signaling via the mitogen-activated protein kinase (MAPK) pathway. Thus, an imbalance between PI 3-kinase and MAPK signaling pathways in vascular tissues may lead to the development of cardiovascular abnormalities observed in insulin-resistant states.

In the present study, we have shown that defective PI 3-kinase signaling results in inhibition of induction of iNOS protein expression and cGMP generation but normal responsiveness to SNP and cGMP agonists. Thus, defective iNOS protein expression and cGMP generation may contribute to the

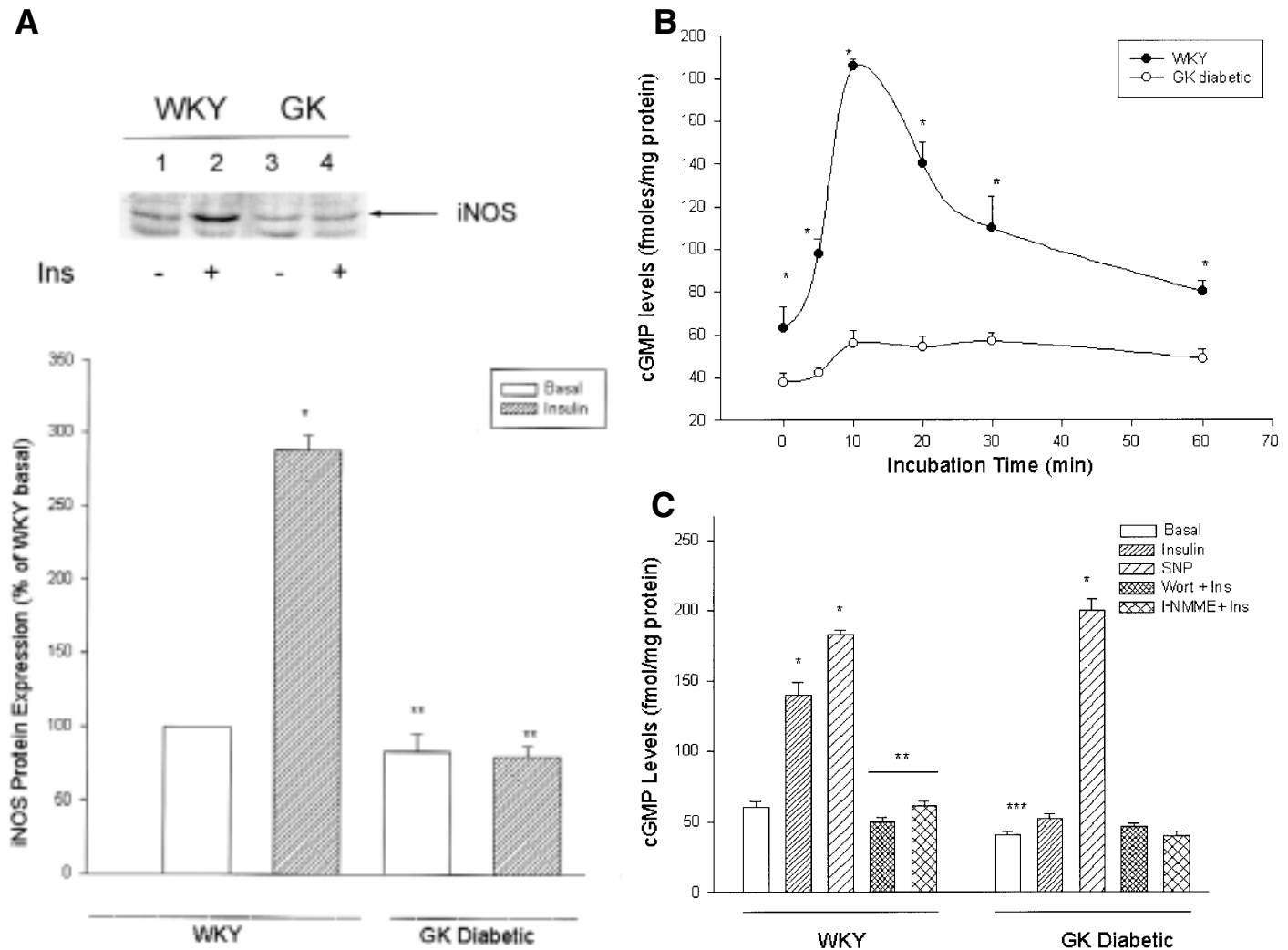


FIG. 5. A: GK diabetes is accompanied by impaired insulin-induced iNOS protein expression. VSMCs were stimulated with and without insulin (100 nmol/l \times 10 min). Equal amounts of proteins (50 μ g) isolated from WKY and diabetic GK VSMCs were subjected to SDS-PAGE followed by Western blot analysis with anti-iNOS antibody and detection with ECL. The intensity of signal in each lane was quantitated by densitometric analysis. Results are the mean \pm SE of four separate experiments. * P < 0.05 vs. WKY basal; ** P < 0.05 vs. WKY basal and insulin-treated. **B:** GK diabetes is accompanied by marked impairment in insulin-stimulated cGMP generation. VSMCs were stimulated with insulin (100 nmol/l) for the indicated times followed by the extraction with 70% ethanol. The ethanol extracts were analyzed for cGMP content by radioimmunoassay as detailed in RESEARCH DESIGN AND METHODS. Results are the mean \pm SE of three separate experiments. * P < 0.05 vs. WKY. **C:** Wortmannin (Wort) and L-NMMA block insulin's effect on GMP generation. Diabetic GK VSMCs exhibit normal responsiveness to SNP in terms of cGMP production. VSMCs were treated with wortmannin (100 nmol/l) and L-NMMA (1 mmol/l) for 30 min before treatment with insulin (Ins) or treated with SNP (100 nmol/l) as detailed in Fig. 4. cGMP levels were measured in ethanol supernatants. Results are the mean \pm SE of three separate experiments. * P < 0.05 vs. basal; ** P < 0.05 vs. insulin; *** P < 0.05 vs. WKY basal.

impaired MBP activation observed in VSMCs isolated from diabetic GK rats. We have recently demonstrated that inhibition of PI 3-kinase with wortmannin blocks insulin-mediated iNOS induction (27), cGMP generation (27), and MBP activation in VSMCs isolated from WKY rats. Furthermore, inhibition of NOS activity with L-NMMA and blockade of cGMP signaling with RpcGMP attenuates the effect of insulin on MBP activation in WKY rats. The fact that SNP and cGMP agonists stimulate MBP activation in VSMCs isolated from diabetic GK rats to levels seen in WKY rats suggests that the signaling defects in diabetic GK rat VSMCs lie at the level of NOS due to an inhibition of PI 3-kinase activation that mediates the induction of iNOS in VSMCs.

Figure 9 depicts the putative signaling pathways involved in MBP activation by insulin in normal VSMCs. Our results

suggest that defects in both the Rho kinase and the PI 3-kinase-mediated NOS signaling pathways contribute to the inhibitory effects of hyperglycemia and diabetes on MBP activation. To our knowledge, this is the first study to demonstrate MBP activation by insulin in VSMCs in vivo via reductions in MBS phosphorylation status and its abnormal regulation in the insulin-resistant state associated with diabetes. Other studies have demonstrated an inhibitory regulation of MBP by a G-protein-dependent mechanism (22). Thus, an inhibition of MBP activity by thrombin (37), arachidonic acid (19), protein kinase C (PKC) (20), and guanosine-5'-O-(2-thiotriphosphate), tetralithium salt (GTP γ S) (15–17) has been reported. This study adds a new dimension to the above observations by demonstrating that insulin's stimulatory effect on MBP activation is in part due to a reduction in the

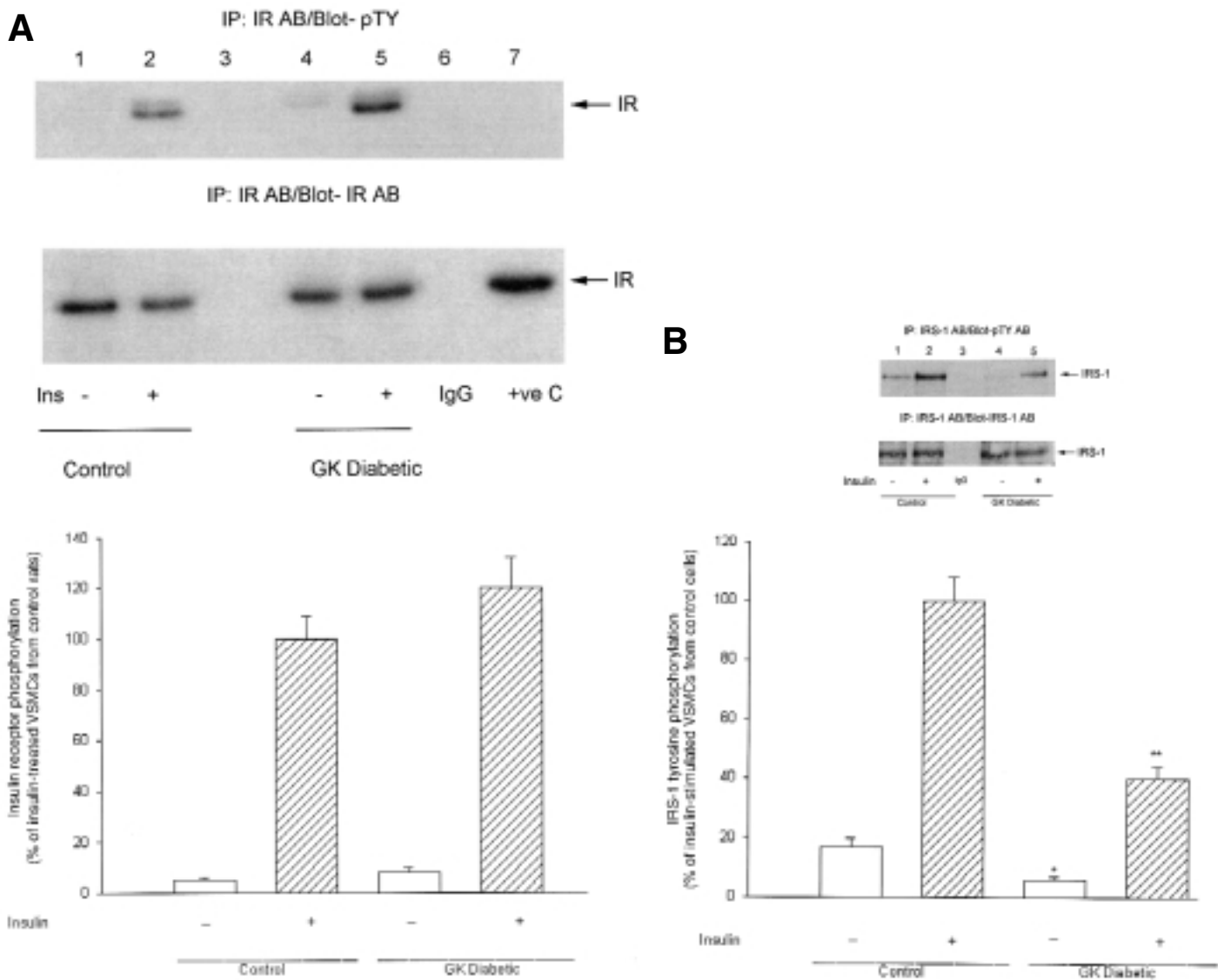


FIG. 6. A and B: Diabetes is accompanied by impaired insulin-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase activation but normal insulin receptor activation in VSMCs isolated from GK rats. **A:** VSMCs were treated with and without insulin for 10 min. Equal amounts of precleared protein lysates (4 mg) were immunoprecipitated with the insulin receptor antibody (IR AB) (2 µg) or IgG followed by Western blot analyses with phosphotyrosine (pTY) antibody (top panel). The blots were stripped and reprobed with the IR antibody (middle panel). Data from three separate experiments were quantitated by densitometric analyses of the autoradiograms and corrected for variations in IR protein by dividing the phosphotyrosine signal with the signal obtained with the IR antibody (specific activity). Results are expressed as percent of insulin-stimulated VSMCs isolated from control rats, assigning a value of 100% to the mean specific activity (bottom panel). **B:** Pre-cleared protein lysates (4 mg) from control and insulin-treated VSMCs were immunoprecipitated with the IRS-1 antibody (IRS-1 AB) (10 µg) followed by Western blot analyses with phosphotyrosine (pTY) antibody (top panel). Blots were stripped and reprobed with IRS-1 antibody (middle panel). Data from three separate experiments were quantitated by densitometric analyses of the autoradiograms and corrected for variations in IRS-1 protein as detailed in **A**. Results are expressed as percent of insulin-stimulated VSMCs isolated from control rats, assigning a value of 100% to the mean specific activity (bottom panel). **P* < 0.05 vs. untreated control; ***P* < 0.05 vs. insulin-treated VSMCs from control rats. +ve C, positive control; Ins, insulin; IP, immunoprecipitation.

MBS phosphorylation state as well as because of the concomitant activation of the enzyme bound to MBS via cGMP signaling, thereby causing MLC₂₀ dephosphorylation and insulin-mediated vasorelaxation. Thus, the impaired vasorelaxation observed in patients with diabetes may be due to inherent reductions in MBP activity resulting from defective regulation of MBP activation in response to insulin. Given the knowledge that PKC levels are elevated in VSMCs isolated from diabetic rat aortas (43) and the fact that PKC can activate Rho and inhibit MBP, it is tempting to speculate that the exaggerated Rho kinase signaling and impaired insulin acti-

vation of MBP observed in diabetes may be due to an elevation in PKC activity via excessive release of arachidonic acid by phospholipase A2 (44). Arachidonic acid could increase Rho kinase activity as well as interact directly with MBS, causing dissociation of the holoenzyme and thereby reducing MBP activity, or it could activate a kinase that phosphorylates MBS and inhibits MBP. Alternatively, the PI 3-kinase pathway may regulate Rho kinase activation. However, in our preliminary studies, we did not observe a blockade of insulin's inhibitory effects on Rho kinase with wortmannin, but L-NMMA and RpcGMP did prevent insulin-mediated Rho kinase

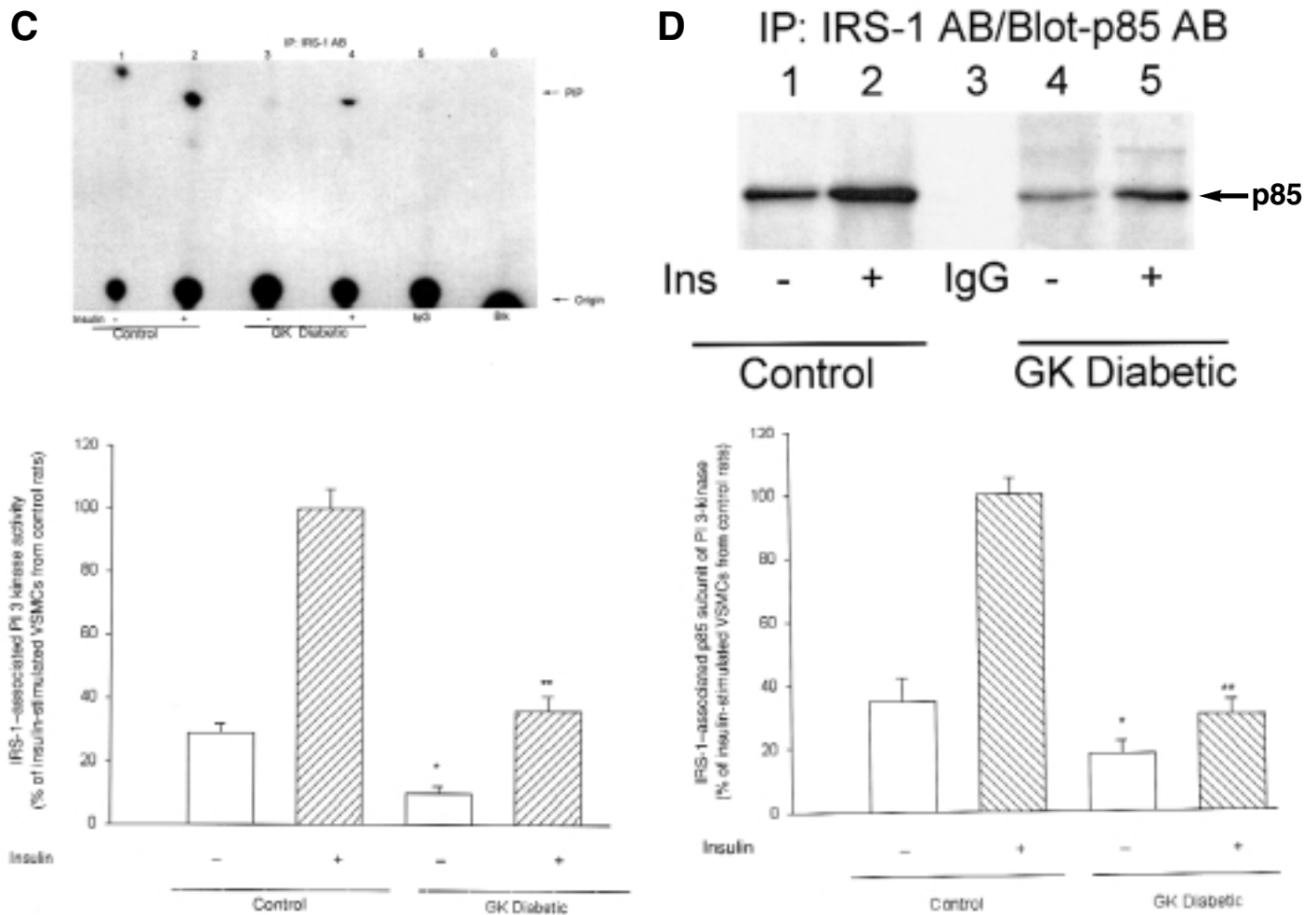


FIG. 6. C and D: Diabetes is accompanied by impaired insulin-stimulated IRS-1 tyrosine phosphorylation and PI 3-kinase activation but normal insulin receptor activation in VSMCs isolated from GK rats. **C:** Equal amounts of precleared protein lysates (100 μ g) were immunoprecipitated with the IRS-1 antibody (IRS-1 AB) followed by assay of PI 3-kinase activity in the immunoprecipitates. The products of PI 3-kinase reaction were separated by thin-layer chromatography. A representative autoradiogram of thin-layer chromatography is shown in the top panel. The bottom panel shows radioactivity incorporated into phosphatidylinositol phosphate (PIP) that was quantitated by the cut and count technique. Results are the mean \pm SE of three separate experiments. * $P < 0.05$ vs. untreated control; ** $P < 0.05$ vs. insulin-treated VSMCs from control rats. **D:** The IRS-1 immunoprecipitates were examined for p85 PI 3-kinase association by Western blot analyses with p85 antibody (p85 AB) as detailed in **B**. * $P < 0.05$ vs. untreated control; ** $P < 0.05$ vs. insulin-treated VSMCs from control rats. Blk, blank; Ins, insulin; IP, immunoprecipitation.

inactivation in VSMCs isolated from WKY rats, suggesting a complex cross-talk between the PI 3-kinase/NOS/cGMP signaling and Rho signaling pathways.

Regarding the role of the NO/cGMP signaling pathway in insulin-mediated MBP activation, studies by others (45) as well as our laboratory (27) have shown that insulin stimulates the induction of iNOS protein expression leading to the generation of NO and causes an elevation in cGMP levels in human endothelial cells as well as VSMCs. Also, it is well known that the cGMP signaling pathway inhibits contraction of smooth muscle by activating MBP (22). It is not exactly known how cGMP activates MBP. Recent studies have excluded the possibility that phosphorylation of MBS by cGMP-dependent protein kinase G affects the phosphatase activity toward MLC₂₀ (46). cGMP agonist 8-bromo cGMP does mimic insulin's inhibitory effect on Rho kinase activity (data not shown). In addition, recent studies have shown that MBP activity may be regulated by heat-stable

endogenous inhibitors, for example, CP17 (47). Phosphorylation of these inhibitors by PKC causes MBP inhibition. It is plausible that the elevated PKC levels observed in the VSMCs of diabetic rats may cause elevations in the levels of endogenous MBP inhibitors leading to impaired MBP activation.

In summary, we have demonstrated that insulin resistance of GK diabetes is accompanied by abnormal regulation of MBP activity due to multiple defects in Rho kinase and PI 3-kinase-mediated iNOS/cGMP signaling—the two major signaling pathways that participate in insulin-mediated MBP activation and vasorelaxation.

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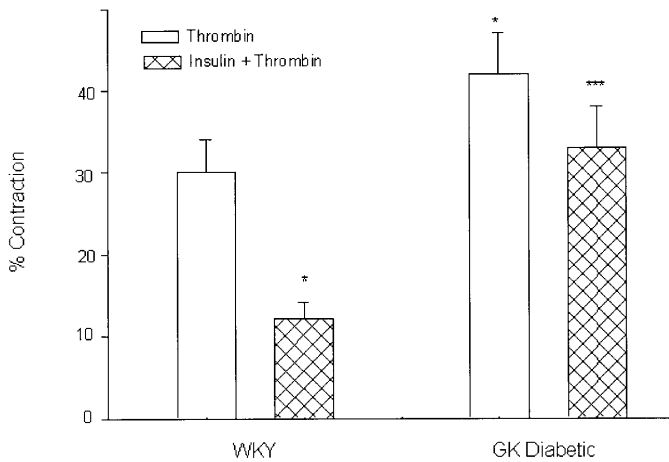


FIG. 7. Insulin fails to inhibit thrombin-induced VSMC contraction in diabetic GK VSMCs. Serum-starved VSMCs were treated with and without insulin (100 nmol/l) for 30 min followed by treatment with thrombin (0.5 U/ml × 15). Cell contraction was measured as detailed in the text. Results are the mean ± SE of three experiments. **P* < 0.05 vs. thrombin-treated WKY; ****P* < 0.05 vs. insulin plus thrombin-treated WKY.

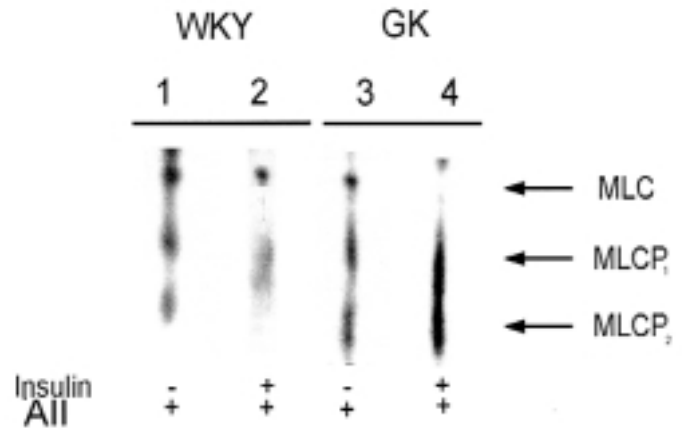


FIG. 8. GK diabetes is accompanied by failure of insulin to inhibit AII-mediated MLC₂₀ phosphorylation (MLCP). VSMCs were treated with and without insulin (10 nmol/l) for 10 min followed by the addition of AII (100 nmol/l) for 5 min followed by glycerol PAGE and immunoblotting with anti-MLC₂₀ antibody. A representative autoradiogram is shown.

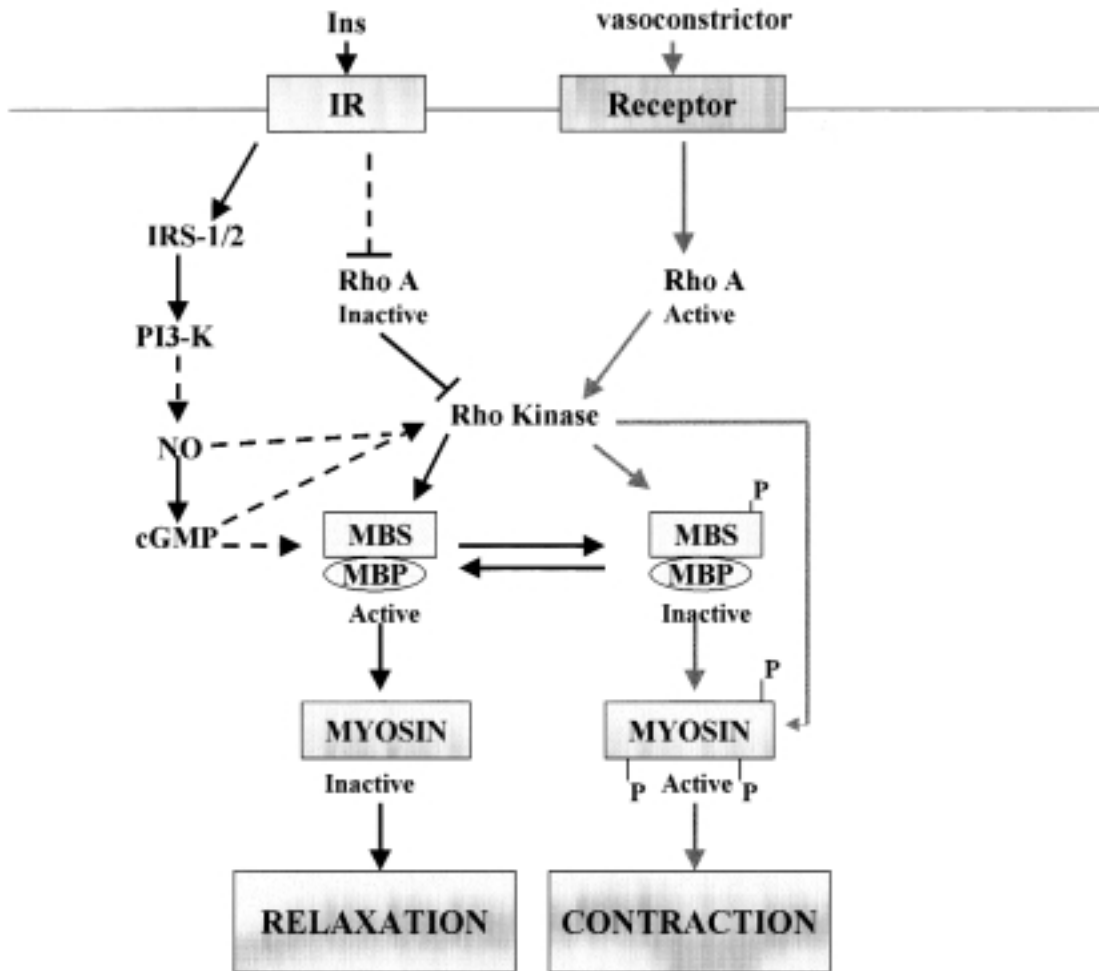


FIG. 9. Schematic representation of the proposed pathways for insulin activation of MBP. Insulin-stimulated receptor tyrosine kinase mediates the activation of IRS-1/IRS-2/PI 3-kinase and the NO/cGMP signaling pathway, which activates MBP in part by decreasing MBS phosphorylation. In addition, insulin inhibits Rho kinase activation, thereby preventing MBP inactivation due to MBS phosphorylation by Rho kinase. The NOS/cGMP signaling pathway may regulate Rho kinase activation as well. GK diabetes is accompanied by upregulation of Rho kinase activity as well as inhibition of PI 3-kinase activation leading to decreased generation of NO/cGMP, which causes impaired MBP activation partly due to excessive MBS phosphorylation by Rho kinase.

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