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INSULIN SIGNALING IN THE VASCULATURE

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1. ABSTRACT

An abnormal vasodilation is a major defect observed in the arteries of diabetic and hypertensive individuals. Myosin bound phosphatase (MBP) dephosphorylates myosin light chains which play a dominant role in vascular smooth muscle (VSM) contraction. Using two distinct approaches, we have demonstrated that insulin rapidly stimulates MBP and simultaneously inhibits RhoA/Rho kinase signaling via the nitric oxide (NO)/cGMP signaling pathway. Insulin activates MBP by decreasing Thr695 phosphorylation of myosin-bound subunit (MBS) via two different but cross-talking signaling pathways. Firstly, insulin inactivates Rho kinase by blocking RhoA activation and translocation to the membrane fraction via increased cGMP/cGK-1α mediated RhoA phosphorylation and decreased geranylgeranylation. Secondly, insulin induces iNOS expression via PI3-kinase signaling leading to generation of NO/cGMP which activates MBP via cGK-1α mediated inhibition of MBS^{Thr695} phosphorylation via Rho kinase inactivation. MBP activation prevents agonist induced MLC_{20} phosphorylation as well as VSMC contraction. VSMCs isolated from SHR and diabetic rats exhibit elevations in Rho kinase, which increases MBS^{Thr695} phosphorylation and inhibits MBP. The defects appear to be at the level of PI3-kinase activation due to impaired insulin-induced IRS-1 tyrosine phosphorylation because of increased association of active Rho kinase with the IRS-1 leading to increased IRS-1 serine phosphorylation, which interrupts with downstream insulin signaling.

2. INTRODUCTION

Vascular smooth muscle cells (VSMCs) are the major constituents of blood vessel walls responsible for the maintenance of vascular tone. Increased contractility of VSMCs, an abnormal vascular tone, and defective vasorelaxation are the earliest abnormalities observed in atherosclerosis, diabetes and hypertension (1-4). Insulin inhibits VSMC contraction, migration and growth in the normal vasculature (5-9) and insulin's failure to do so in insulin resistant states may contribute to enhanced atherosclerosis/restenosis in these clinical conditions. The exact mechanism of insulin inhibition of contraction in normal VSMCs is unknown. Smooth muscle (SM) contraction and relaxation is largely mediated by phosphorylation and dephosphorylation of MLC_{20} at serine-19 by myosin light chain kinase (MLCK) and myosin bound phosphatase (MBP, 10-11). Intracellular Ca^{2+} levels $[Ca^{2+}]i$ modulate the MLCK to MBP activity ratio and the degree of contractile force. However, calcium sensitivity of MLC phosphorylation is also dynamically modulated through a G-protein-coupled Ca^{2+} independent process, which inhibits MBP (12-14). Thus, MBP appears to be the critical phosphatase regulating smooth muscle contractility and is widely recognized to be the common target of signaling pathways that modulate smooth muscle tone. To date, the precise mechanism of *in vivo* MBP regulation is not fully understood.

MBP holoenzyme consists of three subunits (15-17), the 38 kDa catalytic subunit of protein phosphatase-1

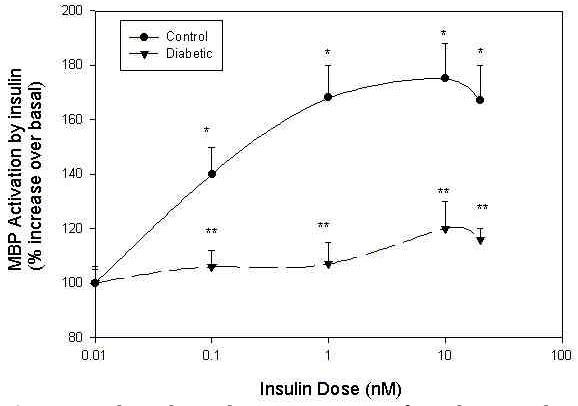

Figure 1. Physiological concentrations of insulin stimulate MBP in a dose-dependent manner. Diabetes is accompanied by impaired MBP activation by insulin.

(PP-1C), a large 110-130 kDa regulatory subunit (MBS), and a small 20 kDa subunit. The 130 kDa subunit (MBS) binds to myosin and regulates the catalytic activity of the phosphatase (18-19). Phosphorylation of MBS by an associated kinase results in an inhibition of phosphatase activity (18). Furthermore, the active GTP-bound Rho, the small guanosine triphosphate, specifically interacts with MBS (18). The Rho-associated kinase directly phosphorylates MLC_{20} (20), CPI-17 (21), and MBS, and consequently inactivates MBP (18), resulting in an increase in MLC_{20} phosphorylation and SM contraction.

Recent evidence suggest that cGMP dependent protein kinase 1a (cGK Ia) is targeted to SMC contractile apparatus by a leucine zipper interaction with the MBS and this interaction is essential to the regulation of VSM tone (22). Thus, MBS assembles a multienzyme complex, tethering a phosphatase and at least three distinct kinases (Rho kinase, cGK I and Raf-1 (23) with counter-regulatory effects on MBP activity. Additionally, insulin-stimulated NO/cGMP signaling may regulate the activation status of Rho kinase at the level of Rho and cause reductions in MBS phosphorylation leading to MBP activation (24).

Increased Ang II action in the vasculature has been strongly implicated in the pathogenesis of macrovascular disease (25-26). Therefore, interactions between Ang II and insulin signaling may have an important role in the regulation of vascular physiology and development of atherosclerosis. Ang II type 1 receptor, AT_1 mediates most of the cardiovascular and growth effects of Ang II via the MAP kinase signaling pathway (27-28), while the AT_2 receptor may attenuate the effects of AT_1 on blood pressure regulation, cardiac and vascular cell growth (29,30-32). Therefore, the possibility exists that insulin's vasodilatory effects may be mediated via AT_2 receptor signaling by preventing Rho kinase activation.

In this review, we summarize our current knowledge of the regulation of insulin signaling pathways which mediate vasorelaxation under normal as well as pathophysiologic conditions and their cross-talk with pathways initiated by the vasoconstrictor agents.

3. MOLECULAR BASIS OF INSULIN-INDUCED VASORELAXATION

Insulin causes relaxation of precontracted aortic segments in both intact and endothelium denuded aortic preparations as well as mesenteric arteries (33-34). However, higher concentration of insulin are needed to elicit relaxation in endothelium denuded aortic rings. The above effect of insulin was accompanied by dephosphorylation of 20 kDa myosin light chains suggesting that insulin may be activating a smooth muscle phosphatase to mediate its vasodilatory effects.

3.1. Role of myosin bound phosphatase

To examine whether the inhibitory effect of insulin on phenylephrine -induced contraction of aortic medial segments was due to an increase in MBP activity. MBP activity was assayed in myosin-enriched fractions using [^{32}P]-labeled myosin light chain (MLC) and [^{32}P]-labeled phosphorylase a as substrates (35-36). Insulin rapidly increased MBP activity by 80% in a time and dose-dependent manner, which correlated with the kinetics of MLC_{20} dephosphorylation (37-38). Conditions associated with insulin resistance such as diabetes resulted in impaired MBP activation by insulin (Figure 1). These observations prompted detailed studies on molecular basis of regulation of MBP activation by insulin.

3.2. Regulation of myosin phosphatase by its targeting subunit, MBS

Recent studies have identified two major inhibitory phosphorylation sites on MBS, which appear to profoundly influence MBP enzymatic activity (39-40). For example, in Swiss 3T3 cells, LPA treatment was accompanied by an increase in Thr695 phosphorylation on MBS, and a Rho kinase inhibitor, Y-27632 (39), blocked this effect. To examine whether insulin affects MBS phosphorylation status, metabolic labeling and immunoprecipitation studies were performed. Insulin caused a rapid 53-70% decrease in [^{32}P] incorporation into MBS, which was sustained for 20 min time period, studied. In addition, insulin prevented thrombin-induced increase in MBS phosphorylation and restored MBP activity to levels observed with insulin alone (37). Further studies with site and phosphorylation specific anti-MBS antibodies revealed that insulin specifically decreases basal and thrombin-induced MBS^{Thr695} phosphorylation (Figure 2). The effect of insulin on MBS phosphorylation was prevented by 1 nM okadaic acid suggesting the possibility that activated MBP may autodephosphorylate MBS. Thus, it appears that insulin increases MBP activity in part by reducing MBS^{Thr695} phosphorylation.

3.3. Role of Nitric Oxide/cGMP signaling in myosin phosphatase activation via MBS

Insulin is known to promote its vasodilatory effects via NO generation by activating endothelial nitric oxide synthase (eNOS, 41-44). Since iNOS is the predominant isoform in VSMCs and insulin rapidly stimulates the induction of iNOS protein (45-47), we examined the contribution of iNOS and cGMP signaling pathways in insulin-mediated MBS dephosphorylation, MBP activation

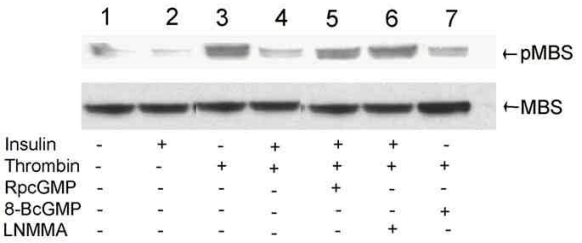

Figure 2. Insulin inhibits thrombin-induced MBSThr695 phosphorylation via the activation of NO/cGMP signaling pathway.

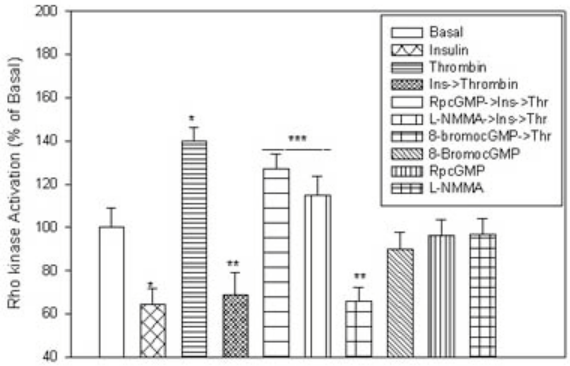

Figure 3. Insulin inhibits Rho kinase activation by thrombin via the NO/cGMP signaling pathway

and vasorelaxation. Pretreatment with L-NMMA and RpcGMP prevented insulin-mediated decrease in MBS^{Thr695} phosphorylation and restored thrombin-mediated increase in MBS phosphorylation and inactivated MBP (Figure 2). In contrast, pretreatment with 8-bromo cGMP, a cyclic GMP agonist prevented thrombin-mediated MBS^{Thr695} phosphorylation and activated MBP in a manner comparable to insulin. These results clearly indicate that NO/cGMP signaling pathway mediates insulin's inhibitory effects on MBS phosphorylation to cause MBP activation. Earlier studies have shown that cGK Ia, the downstream effector of NO/cGMP signaling associates with MBS (22-23).

3.4. Alterations in myosin phosphatase activation in insulin resistant states

To further understand the importance of MBP in vascular function, we examined the activity of this enzyme in VSMCs isolated from diabetic GK rats, a model for Type II diabetes (48). Diabetes and hypertension resulted in a 43% decrease in basal MBP activity along with a 75% reduction in insulin-stimulated MBP activation. The above effect of diabetes on MBP activation status was accompanied by failure of insulin to cause dephosphorylation of MBS (35).

4. REGULATION OF VASCULAR SMOOTH MUSCLE CELL CONTRACTION BY RHO SIGNALING PATHWAY

Rho family of small GTPases are the well known intracellular signaling proteins which act as molecular switches to control actin cytoskeleton organization in many cell types including smooth muscle (49-52). Recent evidence suggest that RhoA dependent signaling pathway can control many of the functions of vascular smooth muscle cells (VSMCs) such as contraction, migration and proliferation (53-54). In VSMCs, the contracting effect of RhoA result from the activation of one of its downstream targets, Rho-dependent kinase (ROK-∀), which phosphorylates the regulatory subunit of myosin light chain phosphatase (MBS) leading to the inhibition of its function by reductions in the phosphatase activity (55-56), thus allowing an increase in the level of phosphorylated myosin light chain and contraction at a constant intracellular calcium level $[Ca^{2+}]i$ (57), a phenomenon defined as Ca^{2+} sensitization (58). Numerous reports (59-60) suggest that Rho signaling is upregulated upon stimulation with agonists such as thrombin or angiotensin II (Ang II).

4.1. Effect of insulin on agonist-induced RhoA translocation and Rho kinase activation.

We tested the possibility that insulin may be inhibiting Rho kinase activity and, thereby, decreasing MBS phosphorylation. Rho kinase activity was assayed in anti-ROK-alpha immunoprecipitates using MBP as a substrate. Insulin decreased basal Rho kinase activity and effectively prevented thrombin-mediated increase in Rho kinase activation (Figure 3). The effect of insulin on Rho kinase inactivation was accompanied by inhibition of thrombin-mediated translocation of RhoA from cytosol to membrane fraction.

4.2. Role of NO/cGMP signaling in insulin inactivation of Rho/Rho kinase

To test the role of NO/cGMP on Rho/Rho kinase signaling, VSMCS were pretreated with L-NMMA and RpcGMP followed by insulin and examined for thrombin-induced RhoA translocation. L-NMMA and RpcGMP prevented insulin inhibition of RhoA translocation and restored Rho kinase activation by thrombin (Figure 3). These observations indicate that NO/cGMP signaling exerts a profound inhibitory effect on RhoA translocation and Rho kinase activity to cause reductions in MBS phosphorylation leading to MBP activation.

4.3. Mechanism of Rho inactivation by insulin

Recent studies indicate that phosphorylation of RhoA by cAMP dependent protein kinase A as well as cGMP dependent protein kinases impairs its biological activity (61-62) while geranylgeranylation of RhoA by GGTases is required for its activation by agonists (63-64). To explore the possibility that insulin may be affecting these two major processes of post-translational modification to cause Rho inactivation, we examined RhoA phosphorylation status and geranylgeranyl transferase-I (GGTase I) activity and examined the effect of NO/cGMP signaling pathway on these two processes. Insulin increased RhoA phosphorylation by 2-fold which was prevented by the inhibitors of PI3-kinase, NOS and cGMP signaling pathway while cGMP agonist mimicked insulin effect by increasing RhoA phosphorylation (65). Further studies using rhotekin binding assays revealed that RhoA is inactive after insulin treatment as it did not bind to GST-rhotekin beads (65). In addition, we observed that insulin

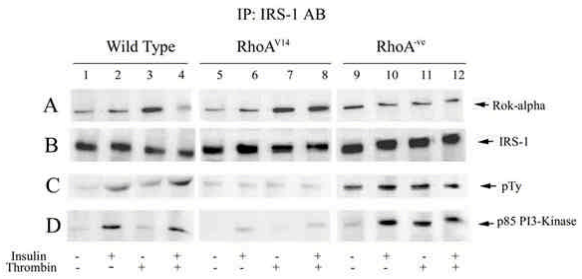

Figure 4. Rho kinase associates with IRS-1 and modulates insulin signaling. Expression of activated RhoA increases Rho kinase/IRS-1 association and prevents insulin-stimulated IRS-1 tyrosine phosphorylation and p85PI3-kinase/IRS-1 asociation.

also inhibits geranylgeranylation of RhoA by inhibiting GGTase I in VSMCs and this effect of insulin could be prevented by L-NMMA and RpcGMP (65). These results suggest that insulin inhibits posttranslational modification of RhoA via NO/cGMP signaling pathway presumably by activating cGK 1 a, the downstream effector of NO/cGMP signaling which in turn phosphorylates RhoA and inactivates Rho signaling via Rho kinase.

5. CROSS-TALK BETWEEN INSULIN SIGNALING AND RHO SIGNALING PATHWAYS

ROK-a 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 carboxy-terminal pleckstrin homology (PH) domain which is split by a cysteine-rich region (66-67). Insulin receptor substrate proteins (IRS) also contain an amino terminal PH domain and phosphotyrosine binding (PTB) domain. The PH domain is required for efficient phosphorylation of IRS-1 by the insulin receptor (68-69). A previous study by Farah *et al* (70) have shown that in Xenopus oocytes the carboxyl terminus of xROK-a (xROK-a-C) associated with PTB xIRS-1 domain and this association was further increased by $RhoA^{V14.}$ Microinjection of xROK-C mRNA into Xenopus oocytes selectively inhibited insulin-induced mitogen-activated protein kinase activation (MAPK) with a concomitant inhibition of oocyte maturation (71). We examined interaction between ROK-a and IRS-1 and its impact on downstream insulin signaling in VSMCs infected with dominant negative and constitutively active RhoA after exposure to insulin and thrombin.

5.1. Interaction between IRS-1 and Rho kinase and its impact on downstream insulin signaling

Potential interaction between ROK-a and IRS-1 investigated in VSMCs by co-immunoprecipitation studies revealed a significant amount of ROK-a in association with the IRS-1 protein (Figure 4A). Thrombin increased ROK-alpha/IRS-1 association which was prevented by insulin. Basal ROK -a/IRS-1 association was increased in VSMCs transfected with constitutively $RhoA^{V14}$. Thrombin stimulation further increased ROK-a/IRS-1 association in $RhoA^{V14}$ expressing cells which was not prevented by insulin. In contrast, VSMCs expressing dominant negative $RhoA^{N19}$ exhibited lack of thrombin-induced increase in ROK-alpha/ IRS-1 association. An examination of the activation status of Rho kinase bound to IRS-1 revealed that insulin decreased IRS-1 associated Rho kinase activity and prevented thrombin-induced elevations in Rho kinase. Expression of $RhoA^{V14}$ increased IRS-1 associated Rho kinase activity while dominant negative $RhoA^{N19}$ expressing cells revealed lack of thrombin-induced increase in Rho kinase activity in the IRS-1 immunoprecipitates. Insulin decreased Rho kinase activity in thrombin-treated cells below basal values. Thus, it appears that insulin is more effective in inhibiting ROK-a when cells were exposed to thrombin in VSMCs expressing $RhoA^{N19}$. Potential impact of ROK-a/IRS-1 association on insulin-induced IRS-1 tyrosine phosphorylation was examined n VSMCs expressing the active and inactive forms of RhoA. In control VSMCs, thrombin did not interfere with insulin-stimulated IRS-1 tyrosine phosphorylation. In contrast, expression of activated $RhoA^{V14}$ markedly reduced insulin stimulated IRS-1 tyrosine phosphorylation (Figure 4B). In contrast, VSMCs expressing dominant negative $RhoA^{N19}$ exhibited a 5-fold increase in IRS-1 tyrosine phosphorylation in the basal state, which was not affected by thrombin treatment. Insulin-stimulated IRS-1 tyrosine phosphorylation was accompanied by a 11-fold increase in p85 PI3-kinase association with IRS-1 (Figure 4C). VSMCs expressing activated $RhoA^{V14}$ exhibited 80% reduction in insulin-induced p85 $^{PI3\text{-}kinase}$ association with the IRS-1 in comparison with control VSMCs expressing vector alone. In contrast, VSMCs expressing dominant negative $RhoA^{N19}$ exhibited a 3-fold increase in basal p85/IRS-1 association and an approximately 10-fold increase in insulin-mediated p85 $^{PI3\text{-}kinase}$/IRS-1 association. Basal and insulin-stimulated P85 association with IRS-1 correlated very well with IRS-1 tyrosine phosphorylation in controls and to a lesser in $RhoA^{N19}$ expressing cells.

The observed reductions in insulin-induced IRS-1 tyrosine phosphorylation and p85 PI3-kinase/IRS-1 association in cells expressing $RhoA^{V14}$ were accompanied by a marked decrease in PI3-kinase enzymatic activity in the IRS-1 immunoprecipitates (72). In contrast, VSMCs expressing dominant negative $RhoA^{N19}$ exhibited insulin-induced increase in PI3-kinase activation, which was greater than that of control VSMCs.

Several studies have indicated that serine phosphorylation of IRS-1 inhibits its tyrosine phosphorylation and ability to associate with p85 subunit of PI3-kinase, thereby rendering cells resistant to insulin (73-74). To understand the mechanism whereby activated RhoA inhibits tyrosine phosphorylation of IRS-1 and its association with PI3-kinase, serine phosphorylation status of IRS-1 was examined. As seen in Figure 5, VSMCs expressing activated $RhoA^{V14}$ exhibit a 3-fold increase in basal IRS-1 serine phosphorylation in the IRS-1 immunoprecipitates which remained elevated upon treatment with insulin when compared to control VSMCs. In control VSMCs, insulin treatment decreased phosphoserine content of IRS-1 and prevented thrombin-induced increase in IRS-1 serine phosphorylation.

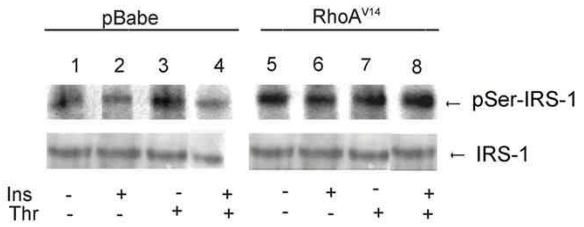

Figure 5. Rho kinase phosphorylates IRS-1 at serine residues.

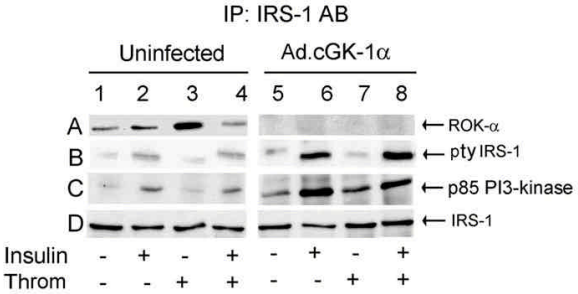

Figure 6. Expression of cGK-1a prevents Rho kinase/IRS-1 association and potentiates insulin-stimulated IRS-1tyrosine phosphorylation and its association with p85PI3-kinase.

5.2. Regulation of IRS-1 and Rho kinase interaction by cGK1a

Recent studies 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 (61). These observations together with our recent results demonstrating that Rho kinase inactivation by insulin could be reversed by inhibitors of NOS and cGMP signaling pathway suggested that Rho kinase activation status may be regulated by cGMP signaling (38). Therefore, we examined the activation status of ROK-a in VSMCs infected with adenoviral cGK Ia, the downstream effector of cGMP, and tested whether inactivation of Rho kinase by cGK Ia affects ROK-a association with IRS-1 and insulin signaling. Infection of VSMCs with Ad5.cGK Ia increased cGK Ia protein expression by > 10 fold over that of non-infected VSMCs, and increased basal cGK Ia enzymatic activity in the absence of cGMP by 3-fold. cGMP treatment of Ad5.cGK Ia cells produced a 4-fold increase in cGK I activity. Insulin treatment resulted in a 2-fold increase in cGMP-independent cGK 1 activity over basal presumably due to endogenous production of cGMP by insulin (72). Thrombin treatment did not alter cGK-I activity when present alone nor did it interfere with insulin's effect 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-a immunoprecipitates. ROK-a inactivation by cGK Ia was accompanied by a marked decrease in ROK-a association with IRS-1 in comparison to uninfected VSMCs (Figure 6A). In addition, cGK Ia infection increased insulin-stimulated IRS-1 tyrosine phosphorylation (Figure 6B) by 10-fold in comparison to non-infected VSMCs. This was accompanied by increased insulin-mediated p85/IRS-1 association (*Figure* 6C) resulting in a 2 -fold increase in PI3-kinase activity in the IRS-1 immunoprecipitates of insulin-stimulated Ad5.cGK Ia cells.

5.3. Impact of hypertension and diabetes on regulation of IRS-1 and Rho kinase interaction

Our earlier studies have shown that VSMCs isolated from spontaneously hypertensive rats (SHR) exhibit insulin resistance in terms of PI3-kinase activation, iNOS induction, as well as MBP activation when compared to WKY (46). In contrast, the growth mediating effects of insulin were enhanced in these cells due to sustained MAPK activation (47). To further investigate the pathophysiological relevance of the interactions we observed between ROK-a and IRS-1, we examined VSMCs isolated from SHR for potential changes in IRS-1/ROK-alpha association in response to AII as these animals exhibit hypersensitivity to AII. ROK-alpha association with IRS-1 was 2-fold higher in the basal state of SHR compared to that of WKY. Whereas insulin pretreatment decreased AII-induced ROK/ IRS-1 association in WKY, it failed to reduce the basal as well as AII-mediated ROK/IRS-1 association in SHR. Increased ROK/IRS-1 association in SHR was also accompanied by marked reductions in insulin-induced association of $p85^{PI3\text{-}kinase}$ with IRS-1 as well as IRS-1 tyrosine phosphorylation.

6. SUMMARY AND PERSPECTIVE

An abnormal vasodilation is a major defect observed in the arteries of diabetic and hypertensive individuals. While almost all previous studies have focused on the role of endothelial dysfunction in the development of cardiovascular complications in diabetes, our studies address an important, yet understudied, aspect of insulin signaling-the molecular basis of regulation of myosin associated phosphatase by insulin. Myosin phosphatase is responsible for dephosphorylation of myosin light chains, which play a dominant role in vascular smooth muscle contraction. Using two distinct approaches to elucidate the exact role of Rho signaling in insulin activation of myosin phosphatase, we have provided evidence that insulin inhibits Rho kinase and site-specific phosphorylation of a myosin-bound subunit, MBS in rat vascular smooth muscle cell cultures to cause the activation of myosin-associated phosphatase. Furthermore, insulin inhibits Rho translocation and Rho kinase activation via NO/cGMP signaling pathway. Thus, we have established the interphase of these two signaling pathways in regulation of VSMC contraction via MBS. Thus, in a therapeutic context, the activation of myosin phosphatase could be of value in preventing excessive contractility of VSMCs. A putative model of insulin induced VSM relaxation is presented in Figure 7.

7. ACKNOWLEDGEMENT

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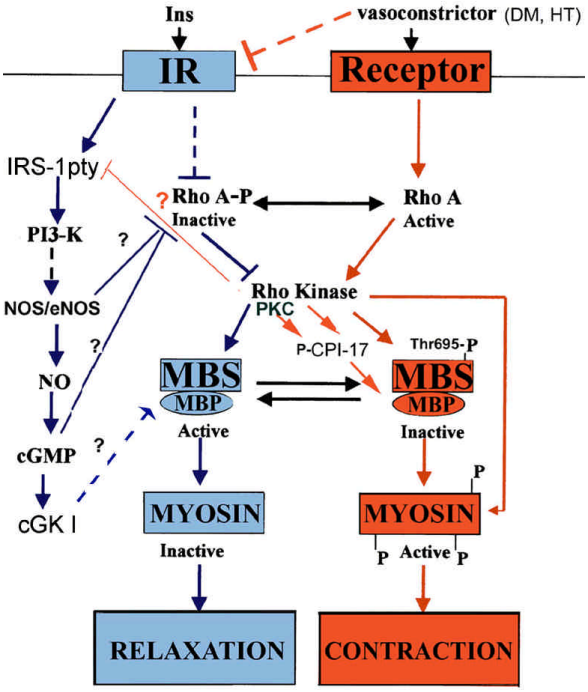

Figure 7. A putative model of insulin-induced vasorelaxation.

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