Rho-kinase and Insulin Signaling

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Abstract

Insulin's ability to acutely stimulate glucose uptake and metabolism in peripheral tissues is essential for normal glucose homeostasis. Resistance to this insulin effect is a major pathogenic feature of type 2 diabetes and obesity. Although many of the proximal steps in insulin signaling have been identified, the molecular mechanisms underlying insulin resistance under various metabolic states are still unclear. Recent study suggests that Rho-kinase is an important mediator of insulin signaling and glucose homeostasis. Specifically, Rho-kinase directly controls insulin receptor substrate-1, which plays an important role in regulating insulin action. Inhibition of Rho-kinase function results in a decreased insulin response, leading to insulin resistance. Thus, Rho-kinase is identified as a novel regulator of insulin action and glucose homeostasis, and a potential target for new diabetes drugs. **(J Kor Diabetes Assoc 30:237~245, 2006)**

Introduction

Impairment of the normal response to insulin (insulin resistance) is a major risk factor for type 2 diabetes and contributes to the morbidity of obesity. Insulin resistance even without overt hyperglycemia is associated with other common disorders, including hypertension, dyslipidemia, cardiovascular disease and cardiac death, as well as polycystic ovarian disease[1]. When insulin resistance cannot be compensated for by increased insulin secretion, frank hyperglycemia or diabetes occurs[2]. In insulin-resistant states, there are defects in multiple actions of insulin, including: 1) impaired stimulation of glucose uptake in skeletal muscle and adipocytes, 2) impaired inhibition of glucose production from the liver, and 3) alterations in lipid metabolism causing dyslipidemia[1]. The mechanisms underlying insulin resistance remain largely unknown although they are thought to result, at least in part, from impaired insulin-stimulated signal transduction.

Insulin action is mediated by a cascade of tyrosyl phosphorylation events, initiated by insulin binding to its cell surface receptor (an intrinsic receptor tyrosine kinase), followed by receptor autophosphorylation and activation of insulin receptor (IR) kinase activity[3]. The kinase activity of IR is essential for downstream activation of virtually all of insulin's metabolic and growth-promoting effects[4]. This has been shown using cells from patients with naturally occurring IR mutations as well as by site-directed mutagenesis of the IR[5]. The activated IR kinase phosphorylates insulin receptor substrates (IRSs) on multiple tyrosine residues. Phosphotyrosine residues on IRSs act as docking sites for many src homology-2 (SH2) domain-containing proteins, including the p85 regulatory subunit of phosphoinositide 3-kinase (PI 3-kinase). Upon binding to IRS proteins, PI 3-kinase is activated, leading to a number of insulin's metabolic effects. In muscle and adipocytes, activation of PI 3-kinase is necessary, although not fully sufficient, for stimulation of glucose transport[6,7]. Rho-kinase, a downstream target for Rho, has been shown to participate in the insulin signaling network by interacting with IRS-1[8,9]. We discuss here the role of the Rho-kinase pathway in IRS-1-mediated insulin signaling and glucose metabolism.

Regulation of small G-proteins

Small G-proteins of the Rho family are monomeric

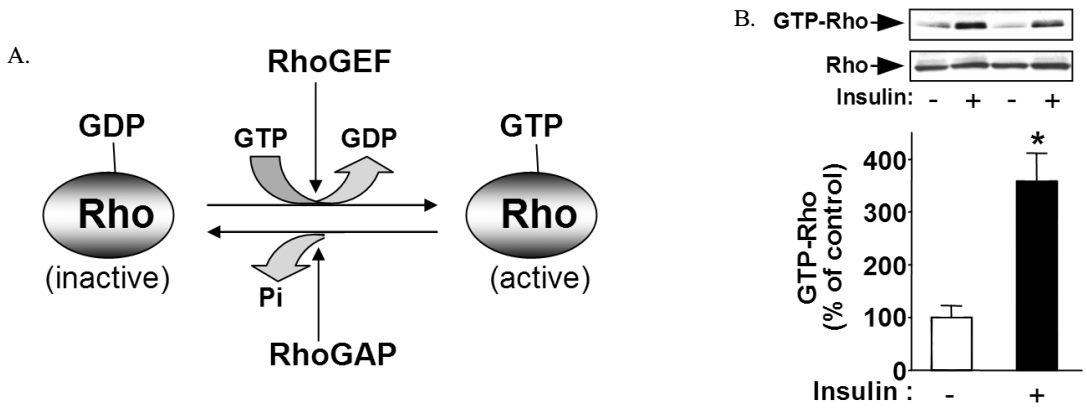

Fig. 1. Regulation of Rho.

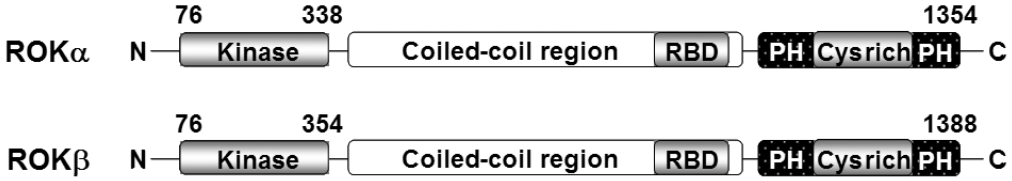

Fig. 2. The structure of Rho-kinase.

guanosine nucleotide-binding proteins of 25~35 kDa. Rho proteins are inactive when bound to GDP and activation occurs when GDP is released and replaced by GTP. This cycling is largely regulated by two classes of proteins, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs)[10,11] (Fig. 1). RhoGEFs function to promote the conversion of inactive Rho to active Rho, while RhoGAPs stimulate the intrinsic GTP hydrolysis activity of Rho protein, thereby converting it to a GDP-bound, inactive form[10,11] (Fig. 1A). Activation of Rho by various agonists requires translocation of inactive cytosolic Rho to membranes. Thus, appearance of Rho in the membrane fraction is indicative of Rho activation[12,13]. In isolated adipocytes, insulin stimulates Rho translocation to the membrane by a PI 3-kinase dependent mechanism [13,14]. Recent findings also demonstrate that insulin activates GTP-Rho activity in skeletal muscle *in vivo*[15] (Fig. 1B), suggesting that Rho signaling is involved in the insulin signaling pathway.

Rho-kinase structure

Rho-kinase is a Ser/Thr protein kinase identified as a GTP-Rho-binding protein[16]. There are two isoforms of Rho-kinase, ROKα (also known as ROCK-2)[17,18] and ROKβ (also known as ROCK-1)[18,19]. Rho-kinase has a kinase domain at its N-terminal domain, a coiled-coil domain in its middle portion, and a putative pleckstrin-homology (PH) domain at its C-terminal domain split by the insertion of a Cys-rich region. The Rho binding domain (RBD) of Rho-kinase is in the C-terminal portion of the coiled-coil domain[16] (Fig. 2). The lack of the C-terminal portion in Rho-kinase, either PH or PH with coiled-coil domains, makes Rho-kinase constitutively active. In contrast, the kinase activity deficient form, or the C-terminal fragments that lack the kinase domain, serves as the dominant-negative form of Rho-kinase in cells[16]. The amino acid sequences of the two Rho-kinase isoforms have 65% overall identity, and the proteins show the highest similarity to their kinase domains (92% identity).

Rho-kinase tissue expression and subcellular localization

Despite the striking similarity of the protein sequences of the two ROCK isoforms, significant differences regarding their respective tissue distribution have been reported, which indicates distinct functions of each isoform *in vivo*. ROCK-2 is preferentially expressed in

brain, whereas ROCK-1 shows the highest expression levels in non-neuronal tissues, including heart, lung and skeletal muscles. Both ROCK isoforms are also expressed in the adipose tissues of rodents. ROCK-2 in bovine brain is mainly expressed in the pyramidal neurons of the hippocampus and cerebral cortex, as well as in the Purkinje cells of the cerebellum[20]. Interestingly, during postnatal development of the mouse brain, ROCK-2 expression levels gradually increased[21]. These findings indicate that the proteins might have a special role in these cell/tissue types. Localization of ROCK-2 by immunofluorescence technique has shown that the protein is distributed mainly in the cytoplasma[16,17]. This is supported by cell-fractionation studies showing that the main portion of ROCK-2 is in the cytosolic fraction[17]. A small amount of ROCK-2 is also founded in the membrane fraction[16]. Indeed, ROCK-2 translocates from the cell cytosol to plasma membrane when active RhoA is overexpressed[16,17] or after stimulation of cells with factors that activate Rho[22,23]. The determination of ROCK-1 localization has proven to be more difficult, but a recent report has indicated that it might colocalize with centrosomes[24].

Regulation of Rho-kinase activity

Rho-kinase activity is enhanced by binding with RhoGTP through a Rho-binding domain[16]. In the inactive form, the PH domain and the Rho binding domain of Rho-kinase bind to the amino-terminal region of the protein, which forms an autoinhibitory loop[25]. Once GTP-bound Rho binds to the Rho binding domain of Rho-kinase, this results in an open confirmation of the kinase and increases catalytic activity. In addition, intracellular second messengers such as arachidonic acid and sphingosylphosphorylcholine, can activate Rho-kinase independently of Rho[26]. Interestingly, ROCK-1, but not ROCK-2, can be activated by caspase-3-mediated cleavage near the carboxyl terminus[27]. Moreover, recent studies demonstrate that Rho-kinase activity increases in response to insulin in insulin-target tissue of mice[15].

Rho-kinase function

Rho-kinase has been implicated in a variety of cellular functions, including actin cytoskeleton, the assembly of myosin-actin filaments and cell contractility[28]. Inhibition of Rho-kinase activity by a chemical inhibitor or expression of dominant negative Rho-kinase causes loss of actin stress fibers and focal adhesion complexes[29], whereas expression of wild type Rho-kinase induces the formation and contractility of actin stress fibers in several cell types[29,30]. This is thought to be mediated both by phosphorylating the myosin light chain and by phosphorylating the myosin-binding subunit of myosin phosphatase[31,32]. Thus Rho-kinase regulates cell polarity and migration through cellular contraction and focal adhesions. Treatment of a hypertensive animal with the Rho-kinase inhibitor results in decreased blood pressure[33], suggesting that the Rho-kinase inhibitor is a potential therapeutic target for many diseases that result from abnormally high smooth muscle contraction. Recently, Rho-kinase has been linked to the control of cell size by enhancing IGF-induced cAMP response element binding protein (CREB) phosphorylation[34]. Furthermore, Rho-kinase is identified as an essential modulator of IGF-1 signals that are involved in the regulation of adipogenesis[35].

The potential role of Rho/Rho-kinase in glucose transport

Rho proteins play an important role in many cellular processes, including signal transduction, vesicle trafficking from intracellular organelles, and cytoskeletal organization[36,37]. All of these processes are potentially involved in insulin-stimulated glucose transport in adipocytes and muscle cells[38-40]. In fact, another small G-protein, TC10 and cdc42, is involved in the regulation of insulin-stimulated Glut4 translocation via a PI-3-kinase independent pathway[41]. Insulin-stimulated glucose transport is decreased by treatment with C3 transferase, a Rho inhibitor, and is increased by treatment with GTPγS, an activator of Rho, in isolated adipocytes[13,14]. Overexpression of a wild type Rho stimulates glucose transport while a dominant negative form of Rho inhibits insulin-induced glucose transport in 3T3-L1 adipocytes and isolated adipocytes[14]. In addition, GTPγS activates membrane-associated PI 3-kinase in isolated adipocytes but this activation is blocked by a Rho inhibitor. Rho-kinase, one of the major Rho targets, is activated by binding with Rho[42,43]. Therefore, it is possible that Rho

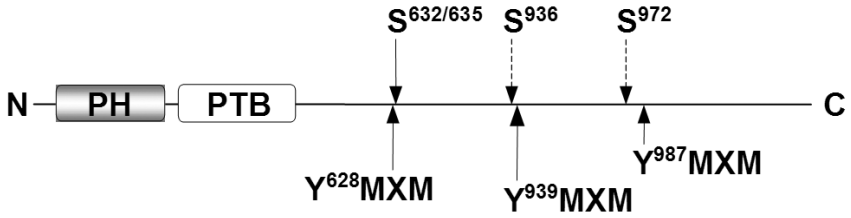

Fig. 3. Schematic diagram of IRS-1 serine phosphorylation sites by Rho-kinase. The relative positions of the pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains are indicated.

may require Rho-kinase activation to mediate its effects on insulin stimulation of glucose transport. Indeed, our recent findings demonstrate that inhibition of Rho-kinase decreases insulin-stimulated glucose transport in 3T3-L1 adipocytes and L6 muscle cells[15]. In addition, suppression of endogenous Rho-kinase expression by small interfering RNA causes a significant decrease in insulin-induced glucose transport in 3T3-L1 adipocytes. These effects are most likely due to decreased Glut4 translocation[15]. Collectively, these data support the hypothesis that the Rho/Rho-kinase pathway is necessary for insulin's metabolic action on glucose transport.

Does Rho-kinase regulate insulin signaling?

Studies suggest that Rho-kinase may play an important role in insulin signaling via a direct interaction with IRS-1[8,9]. However, it is unclear how Rho-kinase regulates insulin-stimulated glucose metabolism via IRS-1, and the molecular mechanism(s) by which Rho-kinase regulates insulin signaling have not been established. A recent study demonstrates that inhibition of Rho-kinase decreases insulin-stimulated IRS-1-associated PI 3-kinase activity in adipocytes and myotubes[15]. This is mainly due to decreased tyrosine phosphorylation of the YXXM motif in IRS-1, which can lead to reduced interaction of IRS-1 with the p85 subunit of PI 3-kinase[15]. Indeed, insulin-stimulated IRS-1 binding to the p85 regulatory subunit of PI 3-kinase is impaired in adipocytes expressing dominant negative Rho-kinase[15]. However, some studies indicate that Rho activation by the expression of constitutively active Rho or absence of Rho-inhibitory protein can impair the early steps of insulin/IGF signaling, including IRS-1 tyrosine phosphorylation in vascular smooth muscle cells[9] and fibroblasts derived from p190B RhoGAP null mice[34]. Since Rho has multiple target molecules including rhophilin, rhotekin, citron, and protein kinase N[44-47], it is unclear whether an inhibitory role of Rho-kinase on insulin signaling occurs through Rho-kinase itself or other downstream mediators. In this regard, it seems unlikely that the alteration in Rho signaling can simply explain all aspects of downstream pathways. This is supported by the fact that insulin activates MAPK in Xenopus oocytes through a Rho-kinase pathway that is independent of an upstream Rho pathway[48].

Rho-kinase and IRS-1 serine phosphorylation

It is clear that serine phosphorylation of IRS-1 protein in response to insulin has a dual role either to enhance or inhibit insulin signaling[49]. IRS-1 contains more than 100 potential serine phosphorylation sites[3] which are recognized by many kinases, including JNK[50], PKCs [51-54], mTOR[55], MAPK[56] and AMPK[57]. It is likely that the IRS-1 function is regulated in several ways, including the interaction between specific serine kinases and IRS-1, regulation of the associated kinase activities, and the selection of specific phosphorylation sites. Therefore, identifying the serine kinase that phosphorylates IRS-1 specific serine residues is important in understanding the mechanism behind the dysregulation of insulin action in altered metabolic states. A recent study finds that Rho-kinase directly phosphorylates serine 632/635, 936 and 972 on IRS-1 *in vitro*[15]. Interestingly, serine 632/635 is only four amino acids from tyrosine 628, serine 936 is only two amino acids from tyrosine 939 and serine 972 residue is close to tyrosine 987 (Fig. 3), all of which are in the YMXM motif that is involved in the binding of p85 regulatory subunit of PI 3-kinase[49]. Replacing these serines with alanine causes a significant inhibition of insulin-stimulated tyrosine phosphorylation of IRS-1 and

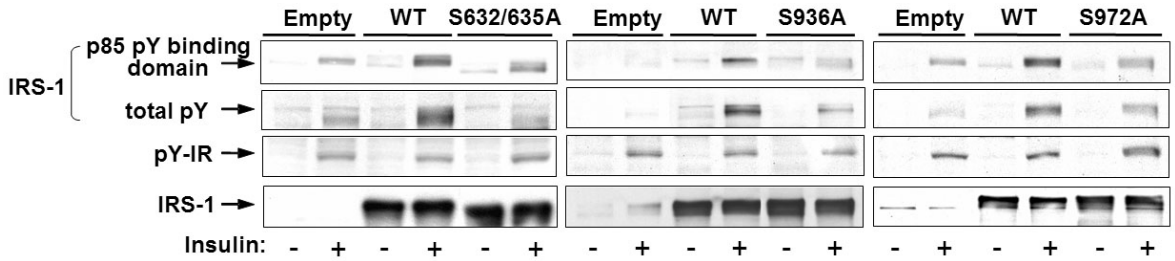

Fig. 4. Effects of IRS-1 serine phosphorylation on insulin signaling in CHO_{IR} cells.
CHO_{IR} cells were transfected with a WT-IRS-1 or a S632/635A-IRS-1 or a S936A-IRS-1 or a S972A-IRS-1cDNA. After 48 hours, cells were stimulated with insulin (10 nM) for 15 minutes. Cell lysates were subjected to immunoprecipitation with an IRS-1 antibody. p85 and IRS-1 were visualized by immunoblotting with a p85 antibody or an IRS-1 antibody. IRS-1 and IR were visualized by immunoblotting with a phosphotyrosine, a phospho-specific IRS-1 or a total IRS-1 antibody.

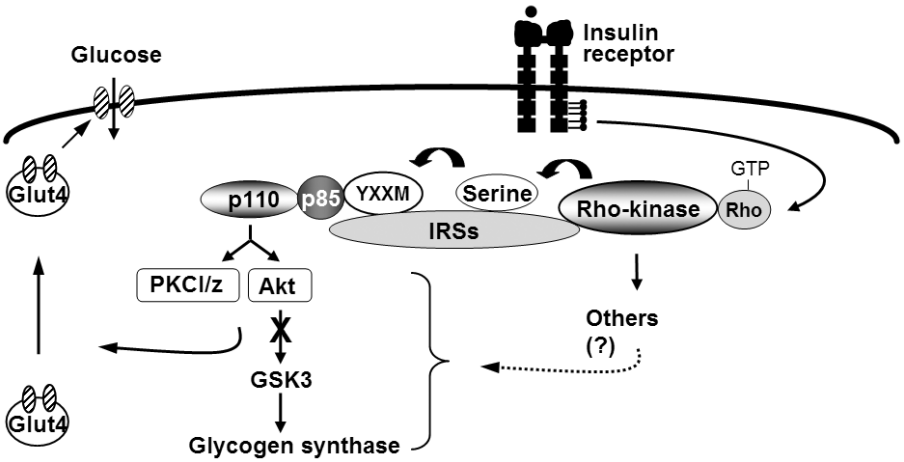

Fig. 5. A hypothetical mechanism by which Rho-kinase enhances insulin signaling and glucose metabolism. Upon insulin stimulation, Rho-kinase is activated and rapidly phosphorylates IRS-1 on serine residues, which in turn increases the ability of IRS-1 to activate PI 3-kinase, leading to enhanced glucose transport activity and other downstream pathways.

insulin-stimulated PI 3-kinase activity associated with IRS-1 (Fig. 4, YBK unpublished data). Thus, it is hypothesized that these serine phosphorylations positively regulate insulin action by facilitating the interaction of IRS-1 with the p85 subunit, leading to increased PI 3-kinase activity (Fig. 4).

Of note, some studies indicate that IRS-1 serine 632/635 phosphorylation is negatively regulated via an mTOR-p70S6K-dependent pathway[55,58]. A possible explanation for this discrepancy is that these sites in IRS-1 could have a dual role in serving as either a positive or negative modulator of insulin signaling under certain conditions. In fact, phosphorylation of serine 789 in IRS-1 can enhance insulin signaling in AICAR treated myotubes[57] while also attenuating insulin signaling in the liver of insulin-resistant animals[59].

Role of IRS-1 serine 632/635 in glucose transport

Given that serine 632/635 residue is adjacent to the YXXM motif domain in IRS-1[60], it is conceivable that the reduction in serine phosphorylation of IRS-1 could inhibit a conformational change in the phosphotyrosine -binding domain that enhances its affinity for PI 3-kinase. As a result, the ability of IRS-1 to bind to the p85 subunit is diminished, leading to reduced glucose transport. In fact, expression of S632/635A-IRS-1 inactive mutant significantly decreases insulin-stimulated glucose transport in 3T3-L1 adipocytes (YBK, unpublished data). Concurrently, insulin-induced PI 3-kinase activity associated with IRS-1 is decreased in these cells. These data suggest that IRS-1 serine 632/635 phosphorylation is required for Rho-kinase-mediated glucose transport, establishing a novel signaling mechanism for the

regulation of glucose transport and insulin signaling. However, these data cannot completely rule out the possibility that some Rho-kinase effects on insulin-stimulated IRS-1 serine phosphorylation might be mediated by other mechanisms, e.g., actin cytoskeleton.

In vivo role of Rho-kinase in glucose metabolism

It is important to know that Rho-kinase activation contributes to insulin resistance *in vivo*. Some data demonstrate that long-term Rho-kinase inhibition by 4 weeks treatment with fasudil (a Rho-kinase inhibitor) not only decreased blood pressure but improved glucose tolerance in Zucker obese rats[61]. However, a recent study finds that treating normal mice with the Rho-kinase inhibitor causes insulin resistance *in vivo* by reducing insulin-mediated glucose uptake in skeletal muscle and whole body[15]. Furthermore, our preliminary data show that insulin-stimulated Rho-kinase activity is impaired in the skeletal muscle of insulin-resistant mice with obesity and diabetes (YBK, unpublished data). Therefore, the exact role of Rho-kinase in glucose metabolism *in vivo* is unclear at this time.

Perspectives

Recent experiments have defined new functions of Rho-kinase in regulating insulin signaling and glucose transport in cultured cell lines. However, the physiological role of Rho-kinase in glucose homeostasis and insulin action *in vivo* has not been fully addressed. It is therefore important to investigate whether Rho-kinase activity is altered *in vivo* in insulin-target tissues under various pathophysiological conditions, and whether such alteration is related to changes in insulin action or sensitivity. In addition, to address the causal relationship between Rho-kinase activity and insulin action (and other phenotypes) *in vivo*, it is also important to examine the effects of selective Rho-kinase overexpression/deficiency in adipocytes and muscle on whole-body insulin sensitivity, glucose homeostasis, and leanness. Combined, these future studies will identify a novel role/mechanism of the Rho-kinase pathway in the regulation of insulin signaling in insulin-target tissues, which is critical for whole body insulin sensitivity and glucose homeostasis. Ultimately, these findings may help identify a novel anti-diabetes/obesity drug-target.

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