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Bis(maltolato)-oxovanadium (IV)-induced phosphorylation of PKB, GSK-3 and FOXO1 contributes to its glucoregulatory responses (Review)

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Abstract. Over the last several decades, a large body of evidence has accumulated to suggest that organo-vanadium compounds (OVC) are more potent than inorganic vanadium salts in regulating hyperglycemia and insulin-resistance in rodent models of both type I and type II diabetes. Among these OVC, vanadium (IV) oxo bis(maltolato) (BMOV) was the first to be investigated for its higher potency over inorganic vanadium salts in eliciting insulin-like properties in both *in vitro* and *in vivo* systems. While the precise molecular mechanism by which BMOV exerts its insulin-mimetic effects remains poorly defined, studies have shown that BMOV is a potent activator of several key components of the insulin signaling pathways, such as phosphatidyl-inositol 3-kinase (PI3-K), and its downstream effector, protein kinase B (PKB). In addition, BMOV-induced phosphorylation of PKB has also been associated with the enhanced phosphorylation of glycogen synthase kinase-3 (GSK-3) and forkhead box protein 1 (FOXO1). Since PKB is instrumental in mediating the effects of insulin on glucose transport, glycogen synthesis and gluconeogenesis, it is reasonable to suggest that activation of this pathway by BMOV serves as a mechanism for its insulin-like effects.

Contents

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1. Introduction

Recent decades have witnessed a dramatic surge in the incidence of diabetes, and according to the estimates of the World Health Organization (WHO), about 180 million people worldwide currently have this disease, which may double by 2030 (1). Diabetes is caused by an absolute or relative lack of insulin secretion or action. Insulin is a polypeptide hormone, synthesized and secreted by the ß-cells of the pancreas, which regulates carbohydrate metabolism. Two major forms of diabetes are: type 1, formerly known as insulin-dependent diabetes mellitus, and type 2, that is, non-insulin-dependent diabetes mellitus. In type 1 diabetes, the absolute lack of insulin secretion is due primarily to the destruction of ß-cells by autoimmune mechanisms. On the other hand, in type 2 diabetes, ß-cells are able to produce insulin, but their insulin secretory response and insulin action on target tissues are defective. About 90% of the total diabetic population falls into the type 2 category, while the remaining 10% are type 1.

Type 2 diabetes may be treated by diet control and/or by oral hypoglycemic agents, such as sulfonylurea, biguanides and thiazolidinediones. However, type 1 diabetics require regular daily injections of insulin to treat their diabetes, as well as some type 2 diabetics in the advanced stage of the disease. Although the availability of highly-purified insulin and the use of oral hypoglycemic drugs as monotherapy or in combination with other agents have greatly improved the management of diabetes, it still remains a major health concern for humans with its increased prevalence and associated secondary complications, such as cardiovascular and kidney diseases. Thus, new therapeutic approaches are needed to more efficiently treat and hopefully cure diabetes.

In this regard, several studies performed in the last 2 decades have demonstrated that vanadium compounds exert various insulin-mimetic and anti-diabetic effects *in vitro* and *in vivo*

(reviewed in refs. 2-4). These insulin-like effects of vanadium compounds include improved insulin sensitivity and glucose homeostasis in animal models of type 1 and 2 diabetes mellitus, as well as in a small number of diabetic human subjects (5-8). Although the precise mechanism by which vanadium compounds elicit their insulin-like effects is not clear, their ability to enhance glucose transport (9-13), glycogen synthesis (9,14,15,16), lipogenesis (17,18) and to inhibit lipolysis (12,18), as well as gluconeogenesis (9), has been suggested to play an important role in this process (2).

Initial studies employed inorganic vanadium salts, such as sodium orthovanadate (SOV) and vanadyl sulphate (VS), however, administration of these salts caused side effects such as gastrointestinal discomfort (19,20). To overcome the gastrointestinal side-effects and to enhance vanadium absorption through the gut, McNeill *et al* pioneered the use of the organo-vanadium complex (OVC) bis(maltolato) oxo-vanadium (BMOV), instead of VS or SOV (21). Encouraged by better tolerance of BMOV, several OVCs have been synthesised and tested for their ability to exert insulin-like effects. These include vanadium (IV) oxo bis (acetylacetonate) ($VO(acac)_2$/VAC) (22), vanadium (IV) oxo bis (3-ethylacetylacetone) (VET), vanadium (IV) oxo bis (ethylmaltolato) (BEOV), vanadium (IV) oxo bis (6-methylpicolinato), and L-glutamic acid δ-monohydroxamate-NaOV complex (23-31). These OVC have been shown to be better absorbed and less toxic than inorganic salts and exhibit a much higher glucose lowering effect in animal models of diabetes as compared to inorganic vanadium salts (reviewed in ref. 2). Our laboratory has been involved in elucidating the molecular mechanism by which OVCs, such as BMOV, exert a more potent insulin-like effect. Our focus has been to investigate the effects of these OVC on key elements of the insulin signaling pathways implicated in regulating glucose homeostasis. The objective of this review is to provide an overview of insulin signaling pathways and summarize some of our studies highlighting the modulation of these pathways by BMOV.

2. The insulin signaling pathway

Insulin is the primary glucoregulatory hormone which is secreted from the pancreatic ß-cells in response to elevated blood glucose levels. Insulin stimulates glucose uptake in muscle and fat tissues (32) and inhibits gluconeogenesis in the liver. The first event in insulin action involves the binding of insulin to its receptor. The insulin receptor (IR) is composed of two extracellular α-subunits and two transmembrane ß-subunits linked to each other by disulfide bonds (33). The α-subunit is located entirely outside of the cell and contains the insulin-binding site, while the ß-subunit possesses both extra-cellular and intracellular domains. The ß-subunits possess protein tyrosine kinase (PTK) activity required for insulin action.

Binding of insulin to the α-subunit of the IR causes a conformational change, leading to the activation of the intrinsic PTK activity of the IR-ß-subunit by multi-site tyrosine phosphorylation (34). The activated PTK of the IR-ß-subunits induce the tyrosine phosphorylation of several downstream substrates, including insulin receptor subtrates (IRSs), Src homology collagen (Shc) and adaptor protein with pleckstrin homology (PH) and Src homology 2 (SH2) domains (APS) (35). These tyrosine phosphorylated substrates function as scaffolds to bind various signaling intermediates responsible for triggering the activation of two main signaling pathways.

In one pathway, binding of growth factor receptor bound-2 (Grb-2) complexed with mammalian son of sevenless (mSOS) to IRS-1 results in the activation of the Ras and Raf signaling system (Fig. 1). Activated Raf phosphorylates MEK (MAPK kinase), which in turn phosphorylates extracellular signal-regulated kinase (ERK1/2) on threonine (Thr) and tyrosine (Tyr) residues located in the activation loop of the kinase. ERK1/2 then phosphorylates and activates a downstream ribosomal protein kinase, $p90^{rsk}$. Both ERK1/2 and $p90^{rsk}$ can be translocated to the nucleus, where they phosphorylate transcription factors, such as c-Jun, CHOP, CREB and MEF-2 (reviewed in ref. 36). The first component in this cascade is Ras, a member of the small GTP-binding protein family. Ras cycles between an active GTP-bound and an inactive GDP-bound state. Once activated, Ras binds to the membrane and recruits Raf, which in turn phosphorylates MEK in specific serine/threonine residues. MEK is a dual specificity protein kinase and phosphorylates ERK1/2 in Thr and Tyr residues. Activation of ERK1/2 leads to the phosphorylation of downstream cytosolic regulatory proteins, such as $p90^{rsk}$ which phosphorylates ribosomal proteins and participates in protein synthesis. ERK1/2 can also be translocated from the cytosol to the nucleus, where it phosphorylates many transcription factors which lead to activation of genes involved in growth and differentiation, and contribute to the mitogenic and growth-promoting effects of insulin (reviewed in ref. 37).

In the other pathway, the binding of the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) to IRS-1 activates the p110 catalytic subunit of PI3-K (Fig. 1). PI3-K catalyses the phosphorylation of phosphatidylinositol (PI) at position 3 of the inositol ring, and generates 3-phosphorylated forms of PI, such as phosphatidylinositol 3, 4, 5 triphosphate (PIP_3) (38). Thus, generated PIP3 contributes to the activation of protein kinase B (PKB) (38,39). PKB is activated by dual phosphorylation on threonine 308 and serine 473, which is catalyzed by PDK 1/2 (40,41). In recent years, PKB and its downstream substrates have emerged as key contributors in modulating glucoregulatory responses. For example, PKB is involved in translocation of glucose transporter-4 (GLUT4) vesicles to the surface of fat and muscle cells, with the resultant activation of glucose uptake and transport (42-45). PKB, through phosphorylation and thereby inactivation of glycogen synthase kinase-3 (GSK-3), participates in the stimulation of glycogen synthase activity and glycogen synthesis (46,47). PKB-induced inactivation of GSK-3 and forkhead box-containing protein (FOXO) transcription factor O1 (FOXO1) suppresses the transcription of phosphoenol-pyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), down-regulating gluconeogenesis (48,49).

3. BMOV, protein tyrosine phosphatases (PTPases) and insulin signaling

PTPases have emerged as important negative regulators of insulin action by virtue of their abilities to dephosphorylate

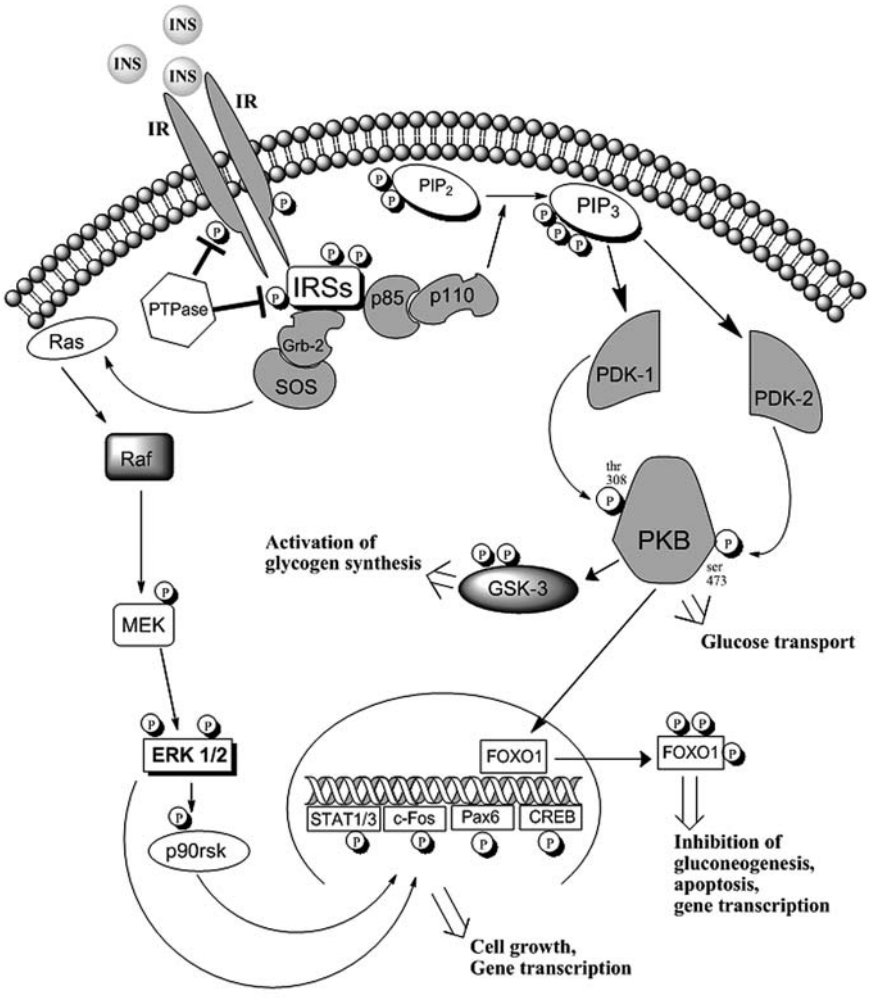

Figure 1. Activation of the IR signaling cascade. Upon insulin binding to the extracellular α-subunits of the IR, a conformational modification of the ß-subunits occurs, resulting in its autophosphorylation on several tyrosine residues. Activated IR phosphorylates several intracellular substrates, such as insulin receptor substrate-1 (IRS-1). Phosphorylated IRS-1 serves as a docking protein for binding several signaling molecules that activate two main signaling pathways. Binding of the Grb-2-SOS complex to IRS-1 contributes to the activation of Raf, MEK and ERK1/2 activation, the latter of which goes on to phosphorylate and activate $p90^{rsk}$. Both ERK1/2 and $p90^{rsk}$ can be translocated to the nucleus where they can activate transcription subsequent to the phosphorylation of several transcription factors, including STAT1/2, c-Fos, Pax6 and CREB. Phosphorylated IRS-1 also recruits the p85 subunit of PI3-K, activating its p110 catalytic subunit, catalyzing the conversion of PIP2 to PIP3. PIP3 recruits PKB, PDK-1 and PDK-2 to the plasma membrane, where PKB becomes phosphorylated and activated. PKB mediates a variety of cellular responses, such as glucose transport, glycogen synthesis, and gluconeogenesis, through several downstream substrates, such as GSK-3 and FOXO1.

and inactivate IR-ß subunits and IRS-1 (50). A dysregulation of PTP activity has been suggested to contribute to the pathogenesis of insulin resistance and diabetes, based on studies showing that suppression of PTP1B, LAR or LMW-PTP improves insulin resistance in animal models (51-54). Moreover, an increase in expression levels and the catalytic activity of PTP1B in obese/diabetic rodent models, as well as in human subjects, has also been documented (55-58). Thus, PTP inhibitors have been suggested as potential therapeutic agents in the treatment of type 2 diabetes in obesity (59). It was also shown that *in vivo* therapy of diabetic rodents with various vanadium compounds (60-62), as well as *in vitro* vanadium treatment of hepatocytes (63) and hepatoma cells (64) inhibited the activity of multiple PTPases, such as SH2 domain-containing PTPase (SHP2) (63), PTP1B (62,65) and total PTPase (22,60,61,63,64). Furthermore, as compared to inorganic vanadium salts, organo-vanadium compounds (OVC), such as BMOV and VAC, have been shown to be more potent than VS in inhibiting PTP activity (22). This

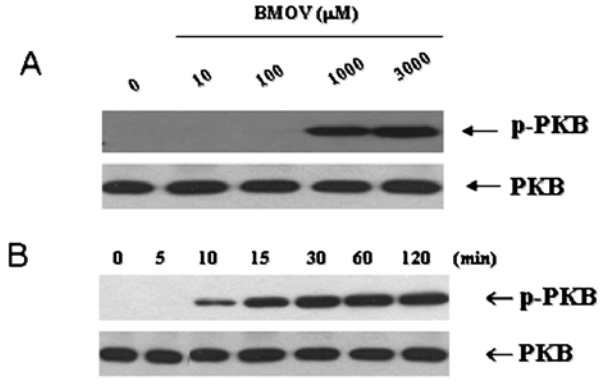

Figure 2. Dose- and time-dependent phosphorylation of PKB. Confluent, serum-starved HepG2 cells were incubated for 15 min with multiple concentrations of BMOV (A), or with 1 mM BMOV for the indicated time periods (B). The cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Ser 473) and total-PKB antibodies. [Reproduced from Mehdi *et al* (67), with permission from the American Chemical Society].

higher PTPase inhibitory potential of the OVC was associated with more robust tyrosine phosphorylation of several proteins, including the IR-ß and IRS-1 (22,65), and was associated with activation of several components of the insulin signaling pathways (66-70) (Fig. 1). Among these pathways, the PKB pathway has been suggested as an important transducer of glucoregulatory responses of BMOV.

4. BMOV activates PKB phosphorylation

BMOV has been reported to enhance the activation state of PKB as judged by its enhanced phosphorylation of Ser 473 (Fig. 2). This phosphorylation occurred in a dose-dependent fashion (Fig. 2A), and could be detected within 10 min of treatment of HepG2 cells with BMOV (Fig. 2B) (67). Moreover, BMOV-induced enhanced PKB phosphorylation required PI3-K, since pharmacological blockade of PI3-K with wortmannin in CHO cells overexpressing IR almost completely abrogated the BMOV response (Fig. 3) (22). In addition to BMOV, other OVCs, such as bis(acetylacetonato)-oxovanadium (IV), bis(picolinate)- oxovanadium (IV), bis(3-methylpicolinato) -oxovanadium (IV), bis(6-methylpicolinato) -oxovanadium (IV), and bis(allixinato)-oxovanadium (IV) ($VO(alx)_2$), were also shown to induce the Ser 473 phosphorylation of PKB in 3T3-L1 adipocytes (22,68-70). In fact, recent studies showed that daily oral administration of $VO(alx)_2$ enhanced PKB and GSK-3 phosphorylation and decreased elevated blood glucose levels in a streptozotocin (STZ)-induced diabetic mouse model (70), suggesting that PKB phosphorylation is a key step in the regulation of glucose homeostasis in response to OVCs (70).

5. BMOV enhances phosphorylation of GSK-3

As stated earlier, GSK-3 is a downstream substrate of PKB, which has a ubiquitous distribution and exists in two isozymic forms, GSK-3α and GSK-3ß (47,71,72,). In the basal state, GSK-3 remains constitutively active; however, PKB catalyzed phosphorylation of serine 21 in the case of GSK-3α and serine 9 in the case of GSK-3ß, renders it inactive (71,72). GSK-3 has been suggested to play an important role in the regulation of glycogen synthesis by its ability to induce serine phosphorylation, and thereby, inactivation of glycogen synthase (GS), the final and rate limiting enzyme involved in glycogen synthesis (72,71,47). In its basal state, GSK-3 suppresses GS through inhibitory phosphorylation, thereby inhibiting glycogen synthesis. Upon phosphorylation by PKB, GSK-3 activity is inhibited, resulting in the activation of GS and an enhancement of glycogen synthesis (73).

GSK-3 also plays a role in regulating the expression of PEPCK and G6Pase (43). PEPCK plays an important role in gluconeogenesis, catalyzing the conversion of oxaloacetate to phosphoenolpyruvate, and is found primarily in the liver, kidney, small intestine and adipose tissue. G6Pase, which is also found in the liver, kidney and small intestines, catalyzes the hydrolysis of glucose-6-phosphate to glucose, the final step in both glycogenolysis and gluconeogenesis. Both PEPCK and G6Pase levels have been shown to be elevated in diabetic states (74-76).

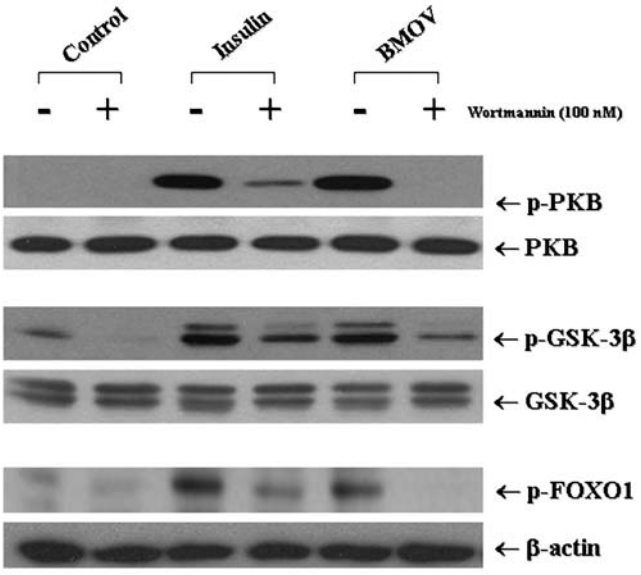

Figure 3. Effect of Wartmannin on BMOV and insulin-induced phosphorylation of PKB, GSK-3 and FOXO1. Confluent, serum-starved CHO-IR cells were incubated in the presence or absence of 100 nM Wortmannin for 30 min, followed by incubation with 100 nM of insulin for 5 min or 1 mM of BMOV for 10 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Ser 473) and total PKB antibodies, phospho-specific (Ser 9)-GSK-3ß and total GSK-3α/ß antibodies, phospho-specific (Ser 256) FOXO1 and total ß-actin antibodies. [Reproduced from Mehdi *et al* (67) with permission from the American Chemical Society].

BMOV-induced activation of PKB was associated with an enhanced phosphorylation of GSK-3 (Fig. 4). BMOV-induced GSK-3 phosphorylation was also shown to be stimulated in a dose- and time-dependant fashion (Fig. 4A and B). These observations may explain the potent effects of BMOV in glucose utilization and storage observed in BMOV-treated animals (24,77,78), since an increased GSK-3 phosphorylation will promote a greater glycogen synthesis via increased GS activity resulting in decreasing blood glucose, and suppress gluconeogenesis by inhibiting the exaggerated expression of gluconeogenic genes PEPCK and G6Pase (78).

6. BMOV enhances phosphorylation of FOXO1

Another important downstream target of PKB is FOXO1. FOXO1 is part of the Forkhead box-containing protein subtype O family of transcription factors, which consists of FOXO2 and FOXO3 and FOXO6 (79). It should be noted that only FOXO1, -3a, and -4 are substrates of PKB (80). PKB regulates FOXO activity through its phosphorylation in serine/threonine residues, resulting in its translocation from the nucleus to the cytoplasm (80). Similar to GSK-3, FOXO has also been shown to play an important role in regulating the expression of genes involved in gluconeogenesis, such as G6Pase and PEPCK (81-84).

Since BMOV induces PKB phosphorylation (22), we investigated if BMOV treatment of HepG2 cells also resulted in the phosphorylation of FOXO1. BMOV caused a dose- and time-dependant phosphorylation of FOXO1 in HepG2 cells (Fig. 5A and B). Another OVC, Bis(allixinato) oxovanadium (IV) (Valx), has similar effects on PKB and

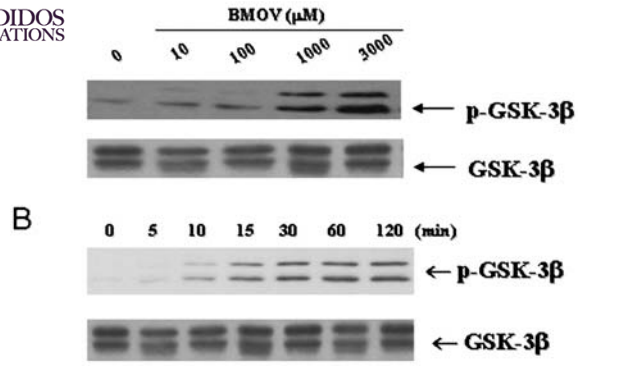

Figure 4. Dose-and time-dependent phosphorylation of GSK-3. Confluent, serum-starved HepG2 cells were incubated for 15 min with multiple concentrations of BMOV (A), or with 1 mM BMOV for the indicated time periods (B). The cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Ser9)-GSK-3ß and total-GSK-3α/ß antibodies. [Reproduced from Mehdi *et al* (67) with permission from the American Chemical Society].

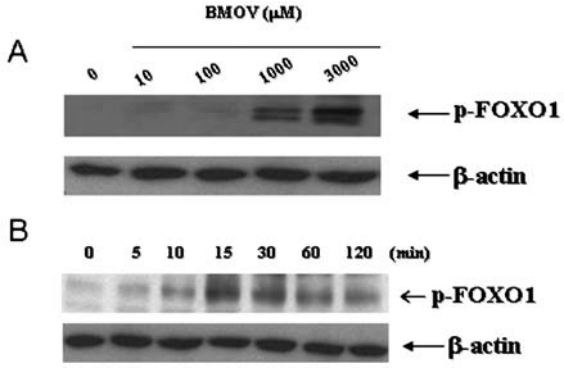

Figure 5. Dose and time-dependent phosphorylation of FOXO1. Confluent, serum-starved HepG2 cells were incubated for 15 min with multiple concentrations of BMOV (A), or with 1 mM BMOV for the indicated time periods (B). The cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Ser 256) FOXO1 and total ß-actin antibodies.

FOXO1 phosphorylation in 3T3-L1 adipocytes (69). It should be noted that lower concentrations of Valx were used in this study (69), as compared to the concentrations of BMOV used in previous studies, to yield similar results (22,67). We believe that this difference may be due to the varying response of the different cell types used in these studies, since different cell types may exhibit varying sensitivity to different compounds. Furthermore, oxidation of vanadium compounds due to long term storage, may also affect their potency. This notion is supported by our recent studies in which newly synthesized BMOV induced the phosphorylation of PKB and FOXO1 at concentrations as low as 100 μM (unpublished data).

7. BMOV and IR phosphorylation

It is well established that the tyrosine phosphorylation of the IR-ß subunit is critical in transducing the downstream effects of insulin, however, the contribution of the IR-ß subunit in BMOV action is not clear. Treatment of CHO-IR or HepG2 cells with either BMOV or VAc induced a robust phosphorylation of multiple proteins in tyrosine residues with molecular sizes ranging from 250 to 35 kDa (22). These proteins included both IR and IRS-1, suggesting that IR phosphorylation maybe an intermediate step in transducing the effect of BMOV on signaling events. Further proof for a role of IR-ß subunit in BMOV-induced effects was obtained by utilizing cells that overexpress an inactive form of IR-PTK (CHO-1018) (67). While it was observed that the level of insulin-induced phosphorylation of PKB was significantly reduced in CHO-1018 cells, BMOV-evoked increase in PKB phospho-rylation was unaltered (67). These data suggested that despite increasing the tyrosine phosphorylation of IR-ß subunit and IRS-1, BMOV-induced phosphorylation of PKB was independent of IR-PTK activity (67). To identify the putative PTK responsible for mediating BMOV-induced PKB phosphorylation, further studies using pharmacological inhibitors of several growth factor receptors, including platelet-derived growth factor receptor (PDGFR), insulin-like growth factor receptor (IGF-1R), and epidermal growth factor receptor (EGFR), were undertaken. It was found that only AG1024, a highly selective inhibitor of IGF-1R (85), was able to block BMOV-induced PKB phosphorylation (67). Use of Antisense Oligonucleotides (ASO) against IGF-1Rß provided additional evidence to support a predominant role of IGF-1R in transducing the signal of BMOV leading to PKB phosphorylation, since the ASO treatment resulted in decreased IGF-1Rß expression, as well as attenuation of BMOV-induced PKB phosphorylation (67).

8. Conclusions

Based on observations from a large number of studies using several different OVCs, it is clear that vanadium-based complexes have potent anti-diabetic and insulin-mimetic properties. Many of these compounds have been shown to be effective in controlling blood glucose levels in animal models of diabetes. Furthermore, significant advances have been made in elucidating the molecular mechanisms of action of vanadium compounds. Our studies have demonstrated that BMOV is more potent in inducing the phosphorylation of PKB, GSK-3 and FOXO1, the key components of insulin signaling pathways, than inorganic vanadium compounds. Furthermore, we have also shown that IGF-1R transactivation may be a key upstream transducer of BMOV signaling, which, through the activation of the PI3K pathway, mediates phosphorylation of PKB and its downstream targets that regulate glucose transport, glycogen synthesis and gluconeogenesis. Thus, the ability of BMOV to upregulate insulin signaling pathways appears to be an important mechanism responsible for its gluco-regulatory responses, and its insulin-like properties.

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