Université de Montréal

Molecular Mechanism of insulin-enhancing and –mimetic action of Vanadium Compounds

par

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Molecular Mechanism of insulin-enhancing and –mimetic action of Vanadium Compounds

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Abstract

Vanadium has emerged as an extremely potent agent with insulin-like properties. These insulin-like properties have been demonstrated in isolated cells, tissues, different animal models of type 1 and type 2 diabetes as well as in a limited number of human subjects. Vanadium treatment has been found to improve abnormalities of carbohydrate and lipid metabolism and of gene expression in rodent models of diabetes. In isolated cells, it enhances glucose transport, glycogen and lipid synthesis, and inhibits gluconeogenesis and lipolysis. The molecular mechanisms responsible for the insulin-like effects of vanadium compounds have been shown to involve the activation of several key components of insulin signaling pathways, which include the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI3-K) / protein kinase B (PKB). It is interesting that the vanadium effect is associated with enhanced tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) while the requirement for insulin receptor (IR) protein tyrosine kinase (PTK) activity in vanadium-mediated effects is still controversial. Since the MAPK and PI3-K/PKB pathways are implicated in mediating the mitogenic and metabolic effects of insulin respectively, it is plausible that vanadium-induced activation of these pathways serves as a mechanism for its insulin-like responses. The work presented in this thesis has been designed to explore the cellular mechanisms of action of vanadium compounds as insulin-enhancing and -mimicking agents.

In the first part of our studies, we have investigated the molecular mechanism by which vanadyl sulfate (VS) prolongs and enhances insulin action. We demonstrated that VS enhances the magnitude and duration of insulin-induced ERK1/2 and PI3-K activities, which is associated with prolonged interaction between IRS-1 and the $p^{85\alpha}$ subunit of PI3-K. Since ERK1/2 and PI3-K are key components of the insulin signaling pathway, these

studies have suggested that prolonged activation of these two kinases contribute to the molecular mechanisms of the insulin-enhancing effect of VS.

In the second part, we have investigated the mechanism responsible for a stronger insulinomimetic effect of organo-vanadium compounds (OVC) over inorganic vanadium salts. Our studies have demonstrated that OVC are stronger inhibitors of protein tyrosine phosphatases (PTPases) and this effect is associated with robust tyrosine phophorylation of several cellular proteins, including IRβ-subunit and IRS-1. In addition, the OVC are superior to VS in augmenting the association between IRS-1 and p^{85}, leading to a potent activation of PKB/glycogen synthase kinase- 3β (GSK-3β) phosphorylation. Taken together, these studies indicate that the high PTPase inhibitory potential of OVC translates into greater phosphorylation of PKB and GSK-3β, which may in turn contribute to greater effectiveness of OVC in ameliorating glucose homeostasis and insulin sensitivity in rodent models of diabetes mellitus.

In the third part of our studies, we have attempted to identify the putative protein tyrosine kinase (PTK) as an upstream modulator of vanadium (IV) oxo bis (maltolato) (BMOV) (an OVC which has been shown to be a more potent insulino-mimetic/antidiabetic agent than inorganic vanadium salts)-induced PKB phosphorylation. These studies have revealed that among several receptor PTKs, the activation of insulin-like growth factor receptor type 1 (IGF-1R)-PTK plays an important role in provoking BMOV-induced PKB phosphorylation. We have also demonstrated an involvement of PKCδ as a mediator in BMOV-induced PKB phosphorylation by using pharmacological isozyme-specific inhibitors of PKC.

Hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS), has been shown to mimic insulin action, and many studies have indicated that vanadium treatment of cells results in the generation of ROS such as H_2O_2. Vanadium-induced production of ROS has been reported to mediate its cellular effects in many cells. Therefore, *in the last part* of these studies, we have examined if the phosphorylation of PKB and ERK1/2 induced by H_2O_2 shares some common features with vanadium with regard to a role for IR-PTK as an

upstream modulator of this effect. These studies have demonstrated that in contrast to vanadium compounds, H_2O_2-induced activation of ERK1/2 and PKB requires both tyrosine kinase activity of IR as well as c-Src, but the role of c-Src-PTK is more dominant in this process.

In conclusion, the studies presented in this thesis have identified that vanadium induced prolonged interaction of IRS-1/p^{85}, resulting in sustained activation of ERK1/2/PI3-K is important in enhancing insulin action. We have also demonstrated that, compared to inorganic vanadium salts the OVC are more potent inhibitors of PTPases and stronger activators of PI3-K/PKB. This observation may form the basis for the high efficacy of OVC in regulating glucose homeostasis in diabetic rodents. An important role for IGF-1R activation as an upstream PTK to evoke BMOV-induced PKB phosphorylation was also identified in these studies. Finally, we have established that H_2O_2, another insulin-mimetic agent, utilizes the IR and c-Src-PTK to initiate PI3-K/PKB and ERK1/2 activation.

Keywords: Diabetes; Vanadium; Insulin Signaling; Insulin Receptor; Protein Tyrosine Kinase; Protein Kinase B; Extracellular Signal-Regulated Kinase1/2; Protein Tyrosine Phosphatase

Résumé

Le vanadium se comporte comme un agent extrêmement efficace ayant des propriétés comparables à celles de l'insuline. Ces propriétés comparables à celles de l'insuline ont été démontrées dans des cellules isolées, des tissus, chez différents types d'animaux ayant un diabète de type 1 et de type 2, aussi bien que chez un nombre limité de personnes humaines. Le traitement au vanadium améliore les anomalies du métabolisme des glucides et des lipides et de l'expression des gènes chez les modèles animaux diabétiques. Dans les cellules isolées, il rehausse le transport du glucose, la synthèse du glycogène et des lipides, et empêche la gluconéogenèse et la lipolyse. Il a été démontré que les mécanismes moléculaires responsables des effets des composés de vanadium comparables à ceux induits par l'insuline impliquent l'activation de plusieurs composantes clés de la voie de signalisation de l'insuline. Celles-ci incluent les protéines mitogen-activated-protein kinases (MAPKs), les extracellular signal-regulated kinase1/2 (ERK1/2) et le phosphatidylinositol 3-kinase (PI3-K) / la protéine kinase B (PKB). Il est intéressant de constater que l'effet du vanadium est associé à la phosphorylation de la tyrosine du substrat du récepteur d'insuline de type 1 (IRS-1) tandis que le recours à l'activité de la protéine kinase de la tyrosine (PTK) du récepteur de l'insuline (IR) pour les effets du vanadium est sujet de controverse. Puisque les voies de la MAPK et de la PI3-K / PKB sont impliquées dans la médiation des effets mitogènes et métaboliques de l'insuline respectivement, il est plausible que l'activation de ces voies par le vanadium serve de mécanisme pour ces réponses. Le travail présenté dans cette thèse a été conçu pour explorer les mécanisme cellulaires de l'action des composés du vanadium comme agents de rehaussement et d'imitation de l'insuline.

Dans la première partie de notre étude, nous avons étudié le mécanisme moléculaire par lequel le sulfate de vanadyl (VS) prolonge et rehausse l'action de l'insuline. Nous avons démontré que le VS rehausse l'ampleur et la durée des ERK1/2 et l'activité de

la PI3-K induites par l'insuline, associées à l'interaction prolongée entre l'IRS-1 et la sous-unité $p^{85\alpha}$ de la PI3-K. Puisque les ERK1/2 et la PI3-K sont des composantes clés de la voie de signalisation de l'insuline, ces études ont suggéré qu'une activation prolongée de ces deux kinases contribue au mécanismes moléculaires de l'effet de rehaussement de l'insuline par le VS.

Dans la deuxième partie, nous avons étudié le mécanisme responsable d'un effet insulino-mimétique plus fort des composés organiques de vanadium (COV) versus les sels inorganiques de vanadium. Nos études ont démontré que les COV sont des inhibiteurs plus puissants des protéines phosphatases de la tyrosine (PTPase) et que cet effet est associé à une phosphorylation plus robuste de la tyrosine de plusieurs protéines cellulaires, y compris la sous-unité IRβ et l'IRS-1. En outre, les COV sont supérieurs au VS pour augmenter l'association entre l'IRS-1 et la p^{85} menant à une activation plus efficace de la phosphorylation de la PKB et de la glycogen synthase kinase 3β (GSK-3β). Dans son ensemble, cette étude a indiqué que l'inhibition plus marquée de la PTPase par les CVO se traduit par une plus grande phosphorylation de la PKB et de la GSK-3β, qui, à tour de rôle, peuvent contribuer à une plus grande efficacité des COV en améliorant l'homéostasie du glucose et la sensibilité à l'insuline chez les animaux diabétiques.

Dans la troisième partie de notre étude, nous avons essayé d'identifier la protéine kinase de la tyrosine (PTK) putative comme modulateur de la phosphorylation de la PKB induite par le vanadium (IV) *oxo bis maltolato* (BMOV) (un COV qui s'est avéré un agent insulino-mimétique / antidiabétique plus efficace en comparaison avec les sels inorganiques de vanadium) en induisant la. Ces études ont indiqué que, parmi plusieurs récepteurs PTKs, l'activation du récepteur de l'insulin-like growth factor type 1 (IGF-1R) joue un rôle important en provoquant la phosphorylation de la PKB induite par le BMOV. Nous avons également observé une participation de la PKCδ comme médiateur dans la phosphorylation de la PKB induite par le BMOV en utilisant des inhibiteurs pharmacologiques spécifiques d'isozymes de la PKC.

Le peroxyde d'hydogène (H_2O_2), une espèce réactive oxygénée (ROS), a démontré une action imitatrice de l'insuline, et plusieurs d'études ont indiqué que le traitement des cellules

au vanadium entraîne la génération de ROS , telle que le H_2O_2. Il a été démontré que la production de ROS par le vanadium est impliquée dans la médiation de ses effets cellulaires. Par conséquent, *dans la dernière partie* de cette étude, nous avons examiné si la phosphorylation de la PKB et des ERK1/2 induites par le H_2O_2 partage quelques caractéristiques communes avec le vanadium quant au rôle du récepteur de l'insuline (IR)-PTK comme modulateur de cet effet. Ces études ont démontré que, contrairement au vanadium, l'activation des ERK1/2 et de la PKB par le H_2O_2 requiert l'activité de la kinase de la tyrosine du IR, et celle du c-Src, mais la PTK du c-Src joue un rôle plus dominant dans ce processus.

En conclusion, les études présentées dans cette thèse ont identifié que le vanadium potentialise l'action de l'insuline via une interaction prolongée entre l'IRS-1 et le p^{85} ayant pour résultat une activation soutenue des ERK1/2/ PI3-K. Nous avons également démontré que, par rapport aux sels inorganiques de vanadium, les COV sont des inhibiteurs plus efficaces des PTPases et des activateurs forts de la PI3-K / PKB. Cette observation peut servir de base pour interpréter l'efficacité plus marquée des COV pour maintenir l'homéostasie du glucose chez les animaux diabétiques. Un rôle important de l'activation de l'IGF-1R agissant comme PTK pour évoquer la phosphorylation de la PKB induite par le BMOV a également été identifié dans ces études. Nous avons également établi que le H_2O_2, un autre agent insulino-mimétique, utilise la PTK du récepteur de l'insuline et du c-Src pour induire l'activation de la PI3-K/ PKB et des ERK1/2.

Mots-clés : diabète, vanadium, signalisation de l'insuline, récepteur de l'insuline, protéine kinase de la tyrosine, protéine kinase B, extracellular signal-regulated kinase1/2, protéine phosphatase de la tyrosine

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List of abbreviations

AMP	adenosine monophosphate
Ang II	Angiotensin II
ASO	antisense oligonucleotide
ATP	adenosine triphosphate
BMOV	vanadium (IV) oxo bis(maltolato)
cAMP	cyclic adenosine monophosphate
CHO-HIR	chinese hamster ovary cells overexpressing human insulin receptor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
FKHR	forkhead transcription factor
FA	fatty acid
FAS	FA synthase
G6Pase	glucose-6-phosphatase
GDP	guanosine diphosphate
GLUT-4	glucose transporter protein type 4
GPCR	G-protein-coupled receptor
GTP	guanosine triphosphate
Grb-2	growth factor receptor binder-2
GS	glycogen synthase
GSK-3	glycogen synthase kinase- 3
H_2O_2	hydrogen peroxide
HepG2	human hepatoma cell
HSL	hormone sensitive lipase
IGF-1R	insulin-like growth factor type 1 receptor
IR	insulin receptor
IRS	insulin receptor substrate

Jak	Janus tyrosine kinase
JNK	Jun N-terminal kinase
kDa	kiloDalton
LAR	Leucocyte Antigen Receptor
MAPK	mitogen activated protein kinase
MEK	mitogen extracellular regulated kinase
MKP	mitogen activated protein kinase phosphatases
mTOR	mammalian target of rapamycin
$NaVO_3$, NaMV	sodium metavanadate
Na_3VO_4, NaOV	sodium orthovanadate
OVC	organo-vanadium compounds
$p70^{s6k}$	p70 ribosomal S6 kinase
$p90^{rsk}$	p90 ribosomal kinase
PDGFR	Platelet derived growth factor receptor
PDK	phosphoinositide-dependent kinase
PEPCK	phosphoenolpyruvate carboxykinase
PH	pleckstrin homology
PI3-K	phosphatidylinositol 3-kinase
PI	phosphatidylinositol
$PI4,5P_2$	phosphatidylinositol 4, 5 triphosphate
$PI3, 4,5P_3$	phosphatidylinositol 3, 4, 5 triphosphate
PKB	protein kinase B
PKC	protein kinase C
PTB	phosphotyrosine binding
PTEN	phosphatase and tension homologue deleted on chromosome 10
PTK	protein tyrosine kinase
PTP1B	protein tyrosine phosphatase-1B
PTPase	protein tyrosine phosphatase
ROS	reactive oxygen species

SAPK	stress-activated protein kinase
SH2	src homology 2
SHC	src homology collagen
SHP2	SH2 domain-containing tyrosine phosphatase-2
SOS	son of seven less
STZ	streptozotocin
TNF-α	tumor necrosis factor-α
VAC	vanadium (IV) oxo bis (acetylacetone)
VET	vanadium (IV) oxo bis (3-ethylacetylacetone)
VS	vanadyl sulphate

Dedication

To the exceptional writer and the great philosopher!
To the One who illuminated our materialistic world
With a ray of light from the spheres of the Eternal Spirit!
To the Lord of purity, immaculateness and love!
To the spirit of my Teacher, my Guide and my Beloved Prophet!
To Dr. Dahesh[*]*,*
I dedicate this thesis!

..."In this day, I ran up to the Creative Force,
Asking for true strength of will and firm resolve.
In this day, I put all my trust in God,
Laying to rest the past, together with its good and evil!
In this day! I turned my eyes toward the (High Ideals):
Virtue, truth, love, eternal life, justice and beauty!
In this day, my heart turned toward heaven and to God!"

Dr Dahesh
Jerusalem, December 8th, 1935
A selection from his book *The Broken Heart*.

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[*] Dr. Dahesh (1909-1984) is a Lebanese author and philosopher. His writings consist of 150 works ranging over different literary genres. He proclaimed his doctrine, known as Daheshism, in Beirut on March 23rd, 1942. Daheshism expresses a belief in the essential Unity of religions and human brotherhood and in the necessity of renouncing violence and detestable sectarian and religious fanaticism.

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CHAPTER 1

Introduction

1.1-HISTORICAL ASPECT OF VANADIUM

Vanadium, a group V trace element that belongs to the first transition series of elements, is ubiquitously distributed in the biosphere as well as in mammals and represents the 21st most abundant element (about 0.02%) in the earth's crust (1). Pure vanadium is a bright silver-white, soft, ductile metal. Andreas Manuel Del Rio was the first chemist to postulate the existence of this new element in 1801, but Nils Gabriel Sefstrom, a Swedish chemist, has been credited for its discovery in 1831 (2). It was named after Vanadis, the Norse goddess of beauty, youth and lustre, because its salts possess beautiful colours (2). Vanadium has become the subject of interest among nutritionists since the discovery of this metal as an essential element in various species (3;4). While the vanadium requirement in lower organisms has been established, its essential value in humans remains to be proven (5;6). Although most foods contain low amounts of vanadium (<1 ng/g), they are a major source of exposure to vanadium for the general population (7). Many cereals, fish, fresh fruits and vegetables contain the element: more than 40 mg per g. Daily vanadium intake has been estimated to be 10-160 μg, and its main food sources are black pepper, dill seeds, mushrooms, parsley, shellfish and spinach, which contain between 0.05 to 1.8 μg vanadium per g (7;8). Analysis of body fluids, organs and tissues has estimated that the total body pool of vanadium in humans is between 100 and 200 μg (6;7), and it ranges from 0.014 to 7.2 μM in mammalian cells (1;8).

1.2-CHEMISTRY OF VANADIUM

1.2.1-Vanadium salts: Inorganic compounds

The vanadium element can exist in four valency states, 2, 3, 4 and 5, and, thus, its chemistry is complex (2;9). Vanadium occurs as vanadyl (VO^{2+}) below pH 3.5 and in basic solutions, its predominant form is orthovanadate (VO_4^{-3}), which is chemically similar to the phosphates (PO_4^{-3}) (2;9). Vanadium presents as $H_2VO_4^-$ in neutral solutions (7). Metavanadate (VO^{-3}), the predominant species in body fluids (e.g. plasma), enters cells by an anion transport system and is reduced by glutathione (GSH) to the vanadyl state (VO2+ form. Exogenously-administered vanadyl sulphate (VS) and ammonium vanadate have been found to tightly bind serum transferrin, indicating that this protein may serve as a vanadium transporter (10). As vanadyl, it can interact potently with the phosphatases, and inhibition is attributed to a five-coordinate vanadate complex which mimics the transition state of the phosphate ester hydrolysis reaction. Chemical structures of these vanadium salts are shown in Fig. 1.

1.2.2-Vanadium complexes: Pervanadate and organovanadium compounds

In an attempt to improve potential therapeutic efficacy, several vanadium complexes were synthesized by many laboratories. Such complexes include peroxovanadate generated upon mixing of vanadate with hydrogen peroxide (H_2O_2) (11). These compounds, similar to vanadate, also have structural resemblance to the phosphate. Interestingly, adding peroxo group(s) sequentially causes an increase in their potency to inhibit protein tyrosine phosphatases (PTPases). This may be due to their enhanced ability to irreversibly oxidize the bound thiol groups on PTPase.

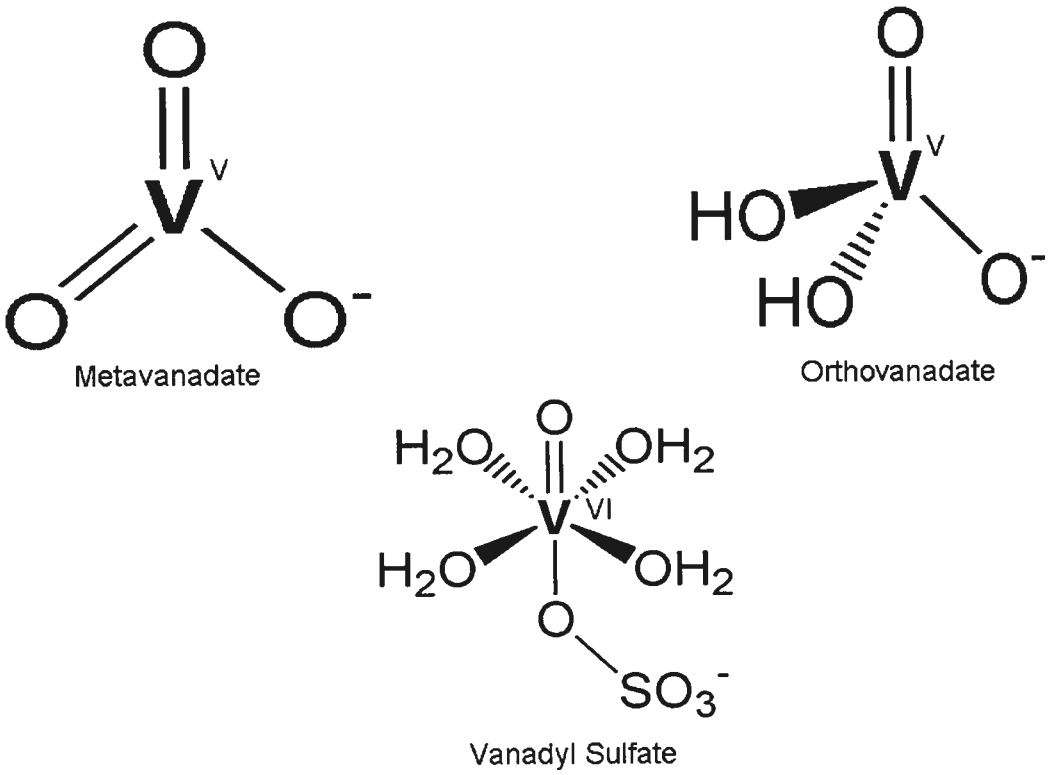

Figure 1: Chemical structure of inorganic vanadium compounds

Peroxovanadate exerts redox activities (oxidation-reduction) which arise due to the formation of a complex upon reaction with H_2O_2. It has been reported that in the acidic, basic and neutral pH ranges, vanadate forms mono-, tri- and diperoxo-vanadium complexes, respectively. In this regard, vanadate can act as an antioxidant because it removes H_2O_2 from the surrounding upon chemical reaction. Diperoxovanadate, at physiological pH, is stable for several hours and degraded at a slower rate by catalase compared to free H_2O_2.

Other types of vanadium complexes, named organovanadium compounds (OVC), are synthesized by complexing vanadyl to organic ligands. These include vanadium (IV) oxo bis(maltolato) (BMOV), vanadium (IV) oxo bis (acetylacetonate) ($VO(acac)_2$/VAC), 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 (12-18). The chemical structures of some of these OVC are shown in Fig. 2. These OVC have been shown to be more absorbed and less toxic than inorganic salts (12;19-21). Subsequently, they were found to exhibit significantly enhanced insulin-mimetic activity in diabetic laboratory animals compared to inorganic VO^{2+} introduced as VS.

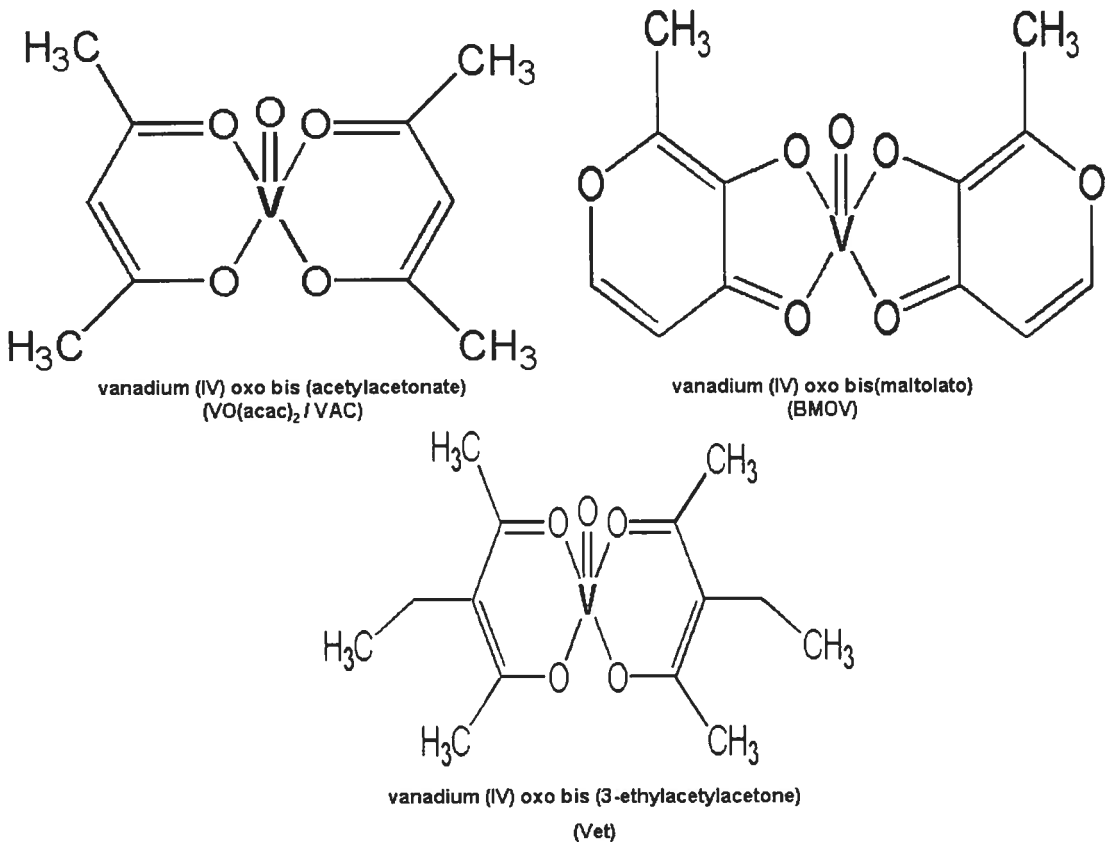

Figure 2: Chemical structure of some organovanadium compounds

1.3-DIABETES AND INSULIN-MIMETIC EFFECTS OF VANADIUM COMPOUNDS

Diabetes has been known to afflict human populations since prehistoric times. Susruta, an Indian physician, described the diabetic syndrome in 400 B.C., calling it "Madhumeh" or "honeyed urine" (22;23). Our modern era has witnessed a surge in the incidence of diabetes, and it is estimated that at present approximately 150 million people world wide have the disease (24;25). According to the World Health Organisation (WHO), this number might double by 2025 (25).

Diabetes is caused by an absolute or relative lack of insulin secretion or action, and has been classified into two major forms: type I, formerly known as insulin-dependent diabetes mellitus, and type II, that is, non-insulin-dependent diabetes mellitus. In type I diabetes, the absolute lack of insulin secretion is due primarily to the destruction of β-cells by autoimmune mechanisms. On the other hand, in type 2 diabetes, β-cells are able to produce insulin, but their insulin secretory response and insulin action on target tissues are defective, which results in hyperinsulinemia and insulin resistance (26). About 90% of the total diabetic population falls into the type II category, while the remaining 10% are type I.

Type II diabetes may be treated by diet control or by oral hypoglycemic agents, such as sulfonylurea, biguanides and thiazolidinediones (27-30). However, type I diabetics require regular daily injections of insulin to treat their diabetes, and some type II diabetics in the advanced stage of the disease also need insulin. Although the availability of highly-purified insulin and the use of oral hypoglycaemic drugs as monotherapy or in combination with other agents (27;28) have greatly improved the management of diabetes, it still remains a major health concern for humans with its prevalence increasing out of control (31). Thus, new therapeutic approaches are needed to more efficiently treat and hopefully cure diabetes.

In this regard, several studies performed in the last 2 decade have suggested that compounds of the trace element vanadium exert various insulin-mimetic and anti-diabetic effects in vitro and in vivo (32). The earliest documented evidence of the insulin-like effects of the inorganic vanadium salt, sodium orthovanadate (Na3VO4), was published by Lyonnet et al. (33) in 1899, 22 years before the discovery of insulin. This group observed that oral Na3VO4 administration decreased glucosuria in 2 out of 3 diabetic patients (33). Their study went unnoticed for a long time, but the demonstration of an in vitro insulin-mimetic effect of vanadium salts by Tolman et al. (34) in 1979 sparked further interest. The latter group (34) showed that several inorganic vanadium compounds, similarly to insulin, stimulated glucose transport and oxidation in adipocytes, increased glycogen synthesis in the rat diaphragm and hepatocytes, and inhibited gluconeogenesis in liver cells. Since then, numerous studies have revealed various insulin-mimetic effects of vanadium compounds in vitro and in vivo, including the stimulation of glucose transport and glucose oxidation (34-38), glycogen synthesis (34;39-41), and lipogenesis (42;43) as well as the inhibition of lipolysis (37;43) and gluconeogenesis (34).

Among the in vivo actions of vanadium, the discovery that attracted the attention of diabetologists and endocrinologists was the seminal work of Heyliger et al. (19) which showed that Na_3VO_4 normalized hyperglycemia in an animal model of diabetes mellitus. Since then, these findings were followed by those of several groups who confirmed and extended them to animal models of type 1 and type 2 diabetes mellitus as well as humans. Both inorganic salts, such as sodium orthovanadate (Na_3VO_4, NaOV), Vanadyl sulfate ($VOSO_4$ $3H_2O$ / $4H_2O$, VS), sodium metavanadate ($NaVO_3$, NaMV) as well as OVC such as BMOV, VAC, VET, or bis (6-methylpicolinato) oxo vanadium were tested in most of these studies. Following section will summarizes some of these studies.

1.3.1-Effect on animal models of type I diabetes mellitus

1.3.1.1-Effect of inorganic vanadium salts

The studies of Heyliger et al. (19) were performed in female rats rendered diabetic by injection of streptozotocin (STZ), a compound that specifically destroys insulin-producing β cells of the pancreas (44). STZ-diabetic rats are generally considered a model of type 1 diabetes mellitus (45). Orally-administered NaOV in drinking water (0.8 mg/ml), over a period of 42 days, was shown to decrease plasma glucose from 20 mM to 8.7 mM (19). Interestingly, in these studies, vanadium was not associated with an increase in plasma insulin. In fact, NaOV treatment lowered insulin levels even in normal rats without significantly altering plasma glucose (19). It was, therefore, suggested that the vanadium-induced reduction of glycemia was independent of its effect on insulin secretion. These investigators also noted severe diarrhoea in some STZ-diabetic rats. This condition, however, was corrected by administering NaOV in 0.5% NaCl solution (19).

The studies of Heyliger et al. (19) were confirmed and expanded by Meyerovitch et al. (20) who demonstrated that NaMV (0.8 mg/ml in drinking water) decreased plasma glucose in STZ-diabetic male Wistar rats to almost normoglycemic (in some cases, hypoglycaemic) levels within 2 to 4 days of treatment (20). Subsequently, the glucose-lowering effects of vanadium compounds were validated by several other investigators. In all these studies, hyperglycaemia was significantly reduced and, in many cases, virtually normalised by oral administration of vanadium compounds to diabetic rodents. The dose of vanadium salts required for exerting a maximum glucose-lowering effect varied between studies, but a median concentration of 0.5 mg/ml in drinking water appeared to be sufficient. The glucose-lowering action was maintained during the course of therapy, but the animals became hyperglycaemic within 2-4 days after its cessation (20). In 1 experiment, however, where STZ-diabetic rats were given VS for 3 weeks, normoglycaemia was sustained for 13 weeks after withdrawal of treatment (46).

A comparative study of NaOV, VS and NaMV showed that regardless of the type of vanadium salts used, the decrease in plasma glucose levels, glucosuria and urinary volume and the improvement in oral glucose tolerance were similar in all cases (47). These investigators also observed that although plasma glucose levels increased rapidly after treatment withdrawal, they remained significantly lower than those found in non-treated STZ-diabetics for at least an additional 4 weeks (47).

1.3.1.2-Effect of organic vanadium compounds

To overcome the gastrointestinal side-effects of vanadium and to enhance its absorption through the gut, McNeill et al. (12) pioneered the use of OVC in rat models of diabetes. Administration of a VS-maltol complex (BMOV, 1.58 mM) to STZ-diabetic rats in drinking water for 4 weeks reduced plasma glucose from 13.9 to 7.0 mM and corrected the polydypsia associated with diabetes (12). BMOV elicited the glucose-lowering response within 1 day of treatment, decreasing plasma glucose from 20 to 8 mM (21), in contrast to the use of inorganic vanadium salts, which required about 4-7 days for a similar glucose-lowering outcome (19;20). Intraperitoneal injection of BMOV was even more effective than BMOV administration in drinking water because it decreased blood glucose to less than 9 mM within 8 hours (21). The ED50 of BMOV was found to be 3 times lower than that of VS (0.08 versus 0.22 mmol/kg) and thus, it was 3 times more potent (21). In addition, BMOV was better tolerated, as evidenced by a lack of gastrointestinal toxicity (diarrhoea), and no mortality (48).

Subsequently, several new OVC were synthesised to test their antidiabetic potential (14-17). Among these complexes, the anti-diabetic potential of BMOV has been investigated most extensively (12;19-21).

Reul et al. (13) recently compared the effects of 3 different OVC, BMOV, VAC, and VET to inorganic VS on glucose metabolism in STZ-diabetic rats. Their studies showed that all 3 OVC were more potent than VS in eliciting the hypoglycaemic response, and VAC appeared to be superior to VET and BMOV (13). The superior effect of VAC may be due

to its better intestinal absorption since a higher plasma vanadium level was achieved at 1 and 3 weeks in comparison to either BMOV or VET (13). Moreover, vanadium therapy of diabetic rats with OVC did not evoke any marked toxicity on hepatic and renal functions (13). Thus, it appears that these complexes have an advantage over the inorganic salts as potential anti-diabetic agents.

In addition to inorganic and organic vanadium compounds, peroxovanadium compounds have been tested as insulin-mimetic agents (11;49). Intraperitoneal or intrajugular injection of bis-peroxovanadium into STZ-diabetic and biobreeder rats markedly reduces hyperglycaemia within 30 min (49;50). However, unlike other vanadium compounds, not many studies have been performed with peroxovanadium compounds.

1.3.2-Effect on animal models of type II diabetes mellitus

The anti-diabetic potential of various vanadium compounds has also been examined in animal models of type 2 diabetes mellitus. Three well-characterised models, genetically-obese, fatty (fa/fa) Zucker rats, genetically-diabetic C57 BL/KsJ-db/db (db/db) mice, and genetically-diabetic ob/ob mice (45), have been investigated. Administration to fa/fa rats of NaOV 0.5 mg/ml in drinking water and up to 0.25 mg/g in food for 3 months considerably improved their glucose homeostasis as well as oral glucose tolerance (51). In addition, during an intravenous glucose tolerance test, the glucose disappearance rate, an index of glucose utilisation, was 50% higher in treated rats compared to the controls, whereas plasma insulin levels were reduced by 50% (51). In db/db mice, 12-week therapy with NaOV 0.6 mg/ml decreased plasma glucose from 24 to 7 mM (52), and only 0.25 mg/ml was sufficient to lower blood glucose from 15 to 8 mM (52). A similar response was exhibited in ob/ob mice where the glucose-lowering effect was evident within a week, reaching a maximum after 16 days of treatment (53).

The impact of a diet mixed with NaOV (1.5 mg/g) (rather than in drinking water) was also investigated, in a high-sucrose diet-induced hyperinsulinemic insulin-resistant rat model, and it was found that hyperglycaemia as well as hyperinsulinemia (54) were significantly improved. In addition to NaOV, the organo-vanadium compound BMOV was found to reduce hyperinsulinemia, insulin resistance and glucose intolerance in fa/fa Zucker rats (55-57). On the other hand, the effect of peroxovanadium compounds on the above rodent models of type 2 diabetes mellitus has not yet been examined.

VS has been investigated in Psammomys obesus, a gerbil (nicknamed the "sand rat") that represents a nutritionally-induced model of diabetes and insulin resistance (58). Psammomys obesus receiving a high-energy diet become hyperglycaemic and hyperinsulinemic (59). VS at a dose of 5 mg/kg body weight for 5 days resulted in prolonged restoration of normoglycaemia and normoinsulinemia in this model (58). In addition, these changes were associated with a normal glucose tolerance test and a decreased level of the hepatic gluconeogenic enzyme phosphoenol pyruvate carboxykinase (PEPCK) (58). Interestingly, VS was ineffective when administered to sand rats that had completely lost their insulin secretory capacity, indicating a requirement of low-level insulin for vanadium to work in these animals. Thus, vanadium appeared to be an insulin potentiator/enhancer rather than mimicker in improving insulin resistance in sand rats (58).

1.3.3-Clinical studies in human diabetes mellitus

The demonstration of a beneficial action of vanadium compounds in both type 1 and type 2 animal models of diabetes mellitus encouraged several investigators to embark on research in human diabetics. In earlier studies, small doses (50-125 mg/day) of NaMV or VS were administered orally to a limited number of type 1 or type 2 diabetic subjects for periods ranging from 2 to 4 weeks (60-62). In type 1 diabetics, NaMV (125 mg/day) for 2 weeks had no effect on fasting plasma glucose levels, but caused a small yet significant decline in daily insulin requirements and improved glucose utilisation in 2 out of 5 patients (60). In type 2 diabetics, NaMV resulted in increased insulin sensitivity due to enhanced non-

oxidative glucose disposal (60). Similarly, VS improved insulin resistance in type 2 diabetics accompanied by a slight decrease in fasting plasma glucose and glycosylated haemoglobin (HbA_{1c}), enhanced insulin-mediated glucose uptake and suppressed hepatic glucose production (HGP) (61;62).

Since only a limited number of subjects were treated with low doses of vanadium for a short duration in these early studies, 2 independent groups recently investigated the effect of long-term treatment with higher VS doses in a larger type 2 diabetic population (63;64). Goldfine et al. (63) treated 16 patients with VS doses varying from 75 to 300 mg/day for 6 weeks and observed that fasting glucose declined significantly only in the 300-mg VS group whereas HbA_{1c} decreased in both the 150- and 300-mg groups. Interestingly, these treatments had no effect on either basal HGP or on the suppression of HGP in response to insulin. A similar study conducted by Cusi et al. (64), who treated 11 type 2 diabetics with VS at a dose level of 150 mg/day for 6 weeks, found that both HbA_{1c} and fasting plasma glucose were significantly decreased. In these investigations, VS reduced endogenous glucose production and increased insulin-mediated glucose disposal. However, none of the clinical research undertaken so far demonstrated complete normalisation of hyperglycaemia, as has been the case in animal models of diabetes mellitus. The difference may be attributed to a much lower blood vanadium level reached in patients (1-10 μM) than in animals as well as the duration of therapy (63).

1.4-MECHANISM OF THE HYPOGLYCAEMIC EFFECT OF VANADIUM

The precise mechanism by which vanadium compounds improve hyperglycaemia and glucose homeostasis in diabetes remains unclear. Vanadium therapy in a type 1 model of diabetes mellitus slightly but insignificantly increased plasma insulin (19;20), whereas a significant, up to 50% decrease in plasma insulin was observed in type 2 models (51-53;65;66). Clearly, this alteration in insulin levels can not be attributed to the anti-diabetic effects of vanadium compounds in type 1 diabetics, but may be beneficial for type 2

diabetics. The hypophagic influence of vanadium compounds might contribute to their hypoglycaemic outcome (67), but studies showing that organic or peroxovanadium compounds exert acute glucose-lowering actions within a few minutes to hours after their administration (not sufficient to suppress feeding) (11;12;21;48-50) do not support this hypothesis. Furthermore, both in vitro and in vivo, vanadium compounds modify glucose and lipid metabolism in adipose tissue, muscle, liver and several cultured cell lines (32;68), which may serve as a more plausible mechanism for the glucose regulatory effects of vanadium.

1.4.1-Effect on glucose transport

The stimulatory effect of NaOV on glucose transport has been observed in rat adipocytes (36;38;69), rat skeletal muscle (40;70), human skeletal muscle (71;72), 3T3-L1 adipocytes (73), and L-6 myotubes (74;75). In most of these studies, a high (mM) vanadium concentration was required to enhance glucose transport. However, Dubyak and Kleinzeller (36) reported that long-term (3-hour) treatment with 300 μM NaOV stimulated glucose transport, which was 80% the value achieved with 17 nM insulin. Thus, longer incubation with low vanadium concentrations may be sufficient to evoke a physiologically-relevant increase in glucose transport.

Since insulin-stimulated glucose transport is mediated by an insulin-sensitive glucose transporter protein type 4, GLUT-4 (76;77), the effect of NaOV, VS or BMOV on GLUT-4 was investigated in diabetic rodent models (58;78-82). In the basal state, GLUT-4 is localised in intracellular sites, and upon insulin stimulation, it is translocated to the cell surface to facilitate glucose uptake by target cells (76). Vanadium exposure of STZ-diabetic rats has been shown to restore the expression and/or cell surface translocation of GLUT-4 in skeletal muscle (78;79), and heart (80;81). In addition, VS treatment of a nutritionally-induced, insulin-resistant model of Psammomys obesus, has been found to restore membrane-associated as well as total protein and mRNA content of GLUT-4 in the gastrocnemius muscle (58). On the other hand, in contrast to STZ-diabetic or sand rats,

NaOV in insulin-resistant fa/fa rats increased insulin-mediated glucose utilisation in muscle without any effect on either GLUT-4 protein or mRNA (82). The discrepancy between the vanadium effect in STZ-diabetic or sand rat and insulin-resistant fa/fa rodents may be attributed to the fact that GLUT-4 content remains unaltered in insulin-resistant models (83;84) whereas STZ-diabetic rats exhibit decreased GLUT-4 expression (78;85;86). Thus, it is difficult to establish a simple correlation between GLUT-4 content and glucose uptake and utilisation in vivo (82). However, an increase in the intrinsic activity or enhanced targeting of GLUT-4 to the cell surface might contribute to improved glucose uptake by vanadium (82). In isolated rat adipocytes (87) and in cultured L-6 myotubes (75), NaOV has been shown to stimulate GLUT-4 translocation to the cell surface. Thus, vanadium compounds are capable of stimulating glucose transport through an effect on GLUT-4.

1.4.2-Effect on glycogen metabolism

Another physiological response modulated by vanadium is mediated by its action on glycogen synthesis. NaOV and VS have been found to stimulate glycogen synthesis in in vitro systems, including the mouse diaphragm (41), rat hepatocytes and diaphragm (34;88), rat adipocytes (39;89), Chinese hamster ovary cells over-expressing insulin receptor (CHO-HIR) (90) and 3T3-L1 adipocytes (73). These compounds also improve depressed glycogen levels in the liver of STZ-diabetic (91;92) as well as in other insulin-resistant rodent models (52;93). In addition, NaOV has been demonstrated to restore the glycogen synthetic rate in 90% pancreatectomized, diabetic rats (94). On the other hand, treatment of 7-week STZ-diabetic rats to BMOV failed to improve insulin-stimulated glycogen synthase activation in skeletal muscle (95) whereas similar treatment enhanced it in fa/fa Zucker rats (96). The reason for these discrepancies is not clear, but it is possible that the basal level of glycogen synthase activity is modulated differentially in a tissue-specific manner in various models of diabetes (93).

Since vanadate treatment normalises the decreased activity of the active glycogen synthase form in the liver of STZ-diabetic rats (91), and activates glycogen synthase in rat adipocytes (89), it has been suggested that this may be one of the mechanisms by which vanadium compounds enhance glycogen synthesis (89;91). Vanadate has also been found to increase glucose-6-phosphate (G6P) by inhibiting glucose-6-phosphatase (G6Pase) activity in rat adipocytes (97;98). Moreover, vanadate restores the depressed levels of G6P in the adipose tissue, liver and muscle of STZ-diabetic rats (97). Since G6P is an allosteric modulator of glycogen synthase activity (99), alteration of its levels may modify the activation status of glycogen synthase and, eventually, glycogen synthesis. Similarly to the effects in animal models of diabetes, 3-week VS therapy (100 mg/day) in type 2 diabetic patients has been associated with an increase in insulin-stimulated glycogen synthesis (61). However, in a more recent study, VS treatment (150 mg/day) for 6 weeks in humans, although it caused a 1.5-fold enhancement of glycogen synthase fractional velocity (an indicator of heightened glycogen synthase activation), failed to elicit any significant change in either basal or insulin-stimulated glycogen synthase activity (63). Thus, the impact of VS therapy on glycogen synthase activity in humans remains controversial, and needs to be examined in detail.

1.4.3-Effect on gluconeogenesis

In addition to the stimulatory action on glucose uptake and utilisation, vanadium-induced suppression of hepatic glucose output can also contribute to improved glucose homeostasis. In fact, decreased hepatic glucose production has been noted in diabetic animals (94;100;101) and humans after vanadium therapy (61;62;64;101), but some studies have failed to obtain this result (60;63). The reason for these discrepant findings is not clear at present, but the study model, duration of therapy, and vanadium dose might have been determining factors. An inhibitory action of NaMV on glucose output has been documented in the isolated, perfused rat liver (102), and VO(acac)2 has been shown to suppress gluconeogenesis in isolated hepatocytes and kidney cortex tubules from control and diabetic rabbits (103). Consistent with these data on gluconeogenesis, vanadium treatment

decreased the heightened expression of the gluconeogenic enzymes PEPCK and G6Pase (13;98;104-109).

1.4.4-Effect on lipogenesis and lipolysis

In addition to their action on glucose metabolism, vanadium compounds have been reported to modulate lipid metabolism both in vivo and in vitro. NaOV treatment of insulin-resistant, sucrose-fed diabetic rats and fa/fa Zucker rats significantly lowered plasma triglycerols (93). Furthermore, NaMV and VS decreased plasma cholesterol levels in humans (63;64;110), and this effect was associated with a decline of low- as well as high-density lipoproteins (64); however, no change in either plasma free fatty acid or triglyceride fractions was noted (62;63). Vanadate has also been shown to reduce total and free cholesterol levels in normal subjects (111), which may be due to inhibition of the steps involved in cholesterol biosynthesis (112;113). In isolated hepatocytes (114) and adipocytes (37), NaOV modulated lipid metabolism by stimulating lipogenesis and suppressing lipolytic activity.

1.5-VANADIUM TOXICITY

Despite their impressive anti-diabetic properties, vanadium compounds have been associated with several toxic effects (115;116). The most common toxic effects are diarrhoea, decreased fluid and food uptake, dehydration and reduced body weight gain (20;67;78;91;94;106;117) which can, however, be corrected by adding NaCl to the drinking water, adjusting the pH of the solution to neutrality, and by gradually increasing the dose of vanadium (20;41). Organic vanadium compounds are much safer as anti-diabetic agents than inorganic vanadium salts, and diabetic rats receiving organic compounds did not show any gastrointestinal side-effects and did not develop diarrhoea (13). Administration of the chelater tiron was also found to minimise vanadate toxicity without altering its anti-diabetic actions (115).

In addition to gastrointestinal discomfort, other toxic effects of vanadium salts have been documented in the literature, which include hepatotoxicity, nephrotoxicity and teratogenicity as well as developmental/reproductive toxicity (115;116). They stimulate mitogenesis and cell proliferation in cultured cells (118) and, thus, have the potential to exert tumorigenic/carcinogenic activity. In contrast, recent work has demonstrated that vanadium compounds inhibited serum- and growth factor-stimulated mitogenesis (119;120) and possess anti-tumor activity (121;122). Many other studies have, however, failed to detect any change in the levels of urea, creatinine, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase (123), indices of kidney and liver functions. Moreover, no significant changes in the histopathology of several tissues, including the liver, spleen, stomach, heart and lung, have been observed among control and VS-treated animals (124). Electron microscopic examination of ob/ob mice treated with NaOV for 47 days did not reveal any sign of hepatotoxicity (20). Some reports have indicated that vanadium compounds cause behavioural changes in rats exposed to NaMV (125). In patients treated with vanadium salts, gastrointestinal discomfort was the most common toxic effect, which could be corrected by decreasing the dose level (60;61). Moreover, clinical studies have been of short duration (up to 6 weeks) and have utilised much lower doses than those administered in animal experiments; thus, the long-term toxicity of vanadium in humans remains to be explored. Clearly, at present, there is no consensus on the toxic effects of vanadium compounds, and detailed and systematic investigations are needed to evaluate the toxicity of various vanadium compounds before undertaking long-term clinical trials in humans.

1.6-MOLECULAR MECHANISM OF VANADIUM ACTION

At the molecular level, the precise mechanism by which vanadium elicits insulin-like and anti-diabetic effects remains poorly characterised. Since vanadate is a potent PTPases inhibitor, and insulin receptor (IR) activation requires increased tyrosine phosphorylation of its β subunit (126;127), it was believed that vanadium caused an activation of IR by

preventing dephosphorylation of the IR-β-subunit. The insulin like effects of vanadium salts were therefore attributed to be secondary to PTPase inhibition. In this manner one or more tyrosine kinases that participate in the insulin signaling cascade may be indirectly influenced. Thus, it is primordial to dissect the signal transduction pathways stimulated by insulin for better understanding of the mechanism of vanadium action at the cellular level.

1.6.1- The insulin signaling cascade

Insulin, the primary hormone implicated in blood glucose control, acts by stimulating glucose influx and metabolism in adipocytes and muscle and by inhibiting gluconeogenesis by the liver. The insulin action in target cells is propagated by binding to its receptor on cell membranes (Fig. 3). The IR consists of a heterodimeric α_2, β_2 structure. Insulin binding to the IR-α-subunit results in conformational changes, leading to enhanced intrinsic protein tyrosine kinase (PTK) activities of the β-subunit by multi-site tyrosine phosphorylation. Once activated, IR-PTK can phosphorylate several cytosolic IR substrates, including insulin receptor subtrates (IRSs), Src homology collagen (Shc) and adaptor protein with pleckstrin homology (PH) and Src homology 2 (SH2) domains (APS). The phosphorylated proteins dock downstream effector molecules that contain the SH2 domain (128), which are then able to activate two key signaling pathways. In one pathway, the association of IRS type 1 (IRS-1) with growth factor receptor binder-2 (Grb-2) complexed with mammalian son of seven less (mSOS) results in subsequent activation of the Ras, Raf, MEK and extracellular signal-regulated kinase (ERK) pathway. Activated ERK1/2 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, and contribute to the mitogenic and growth-promoting effects of insulin (128;129).

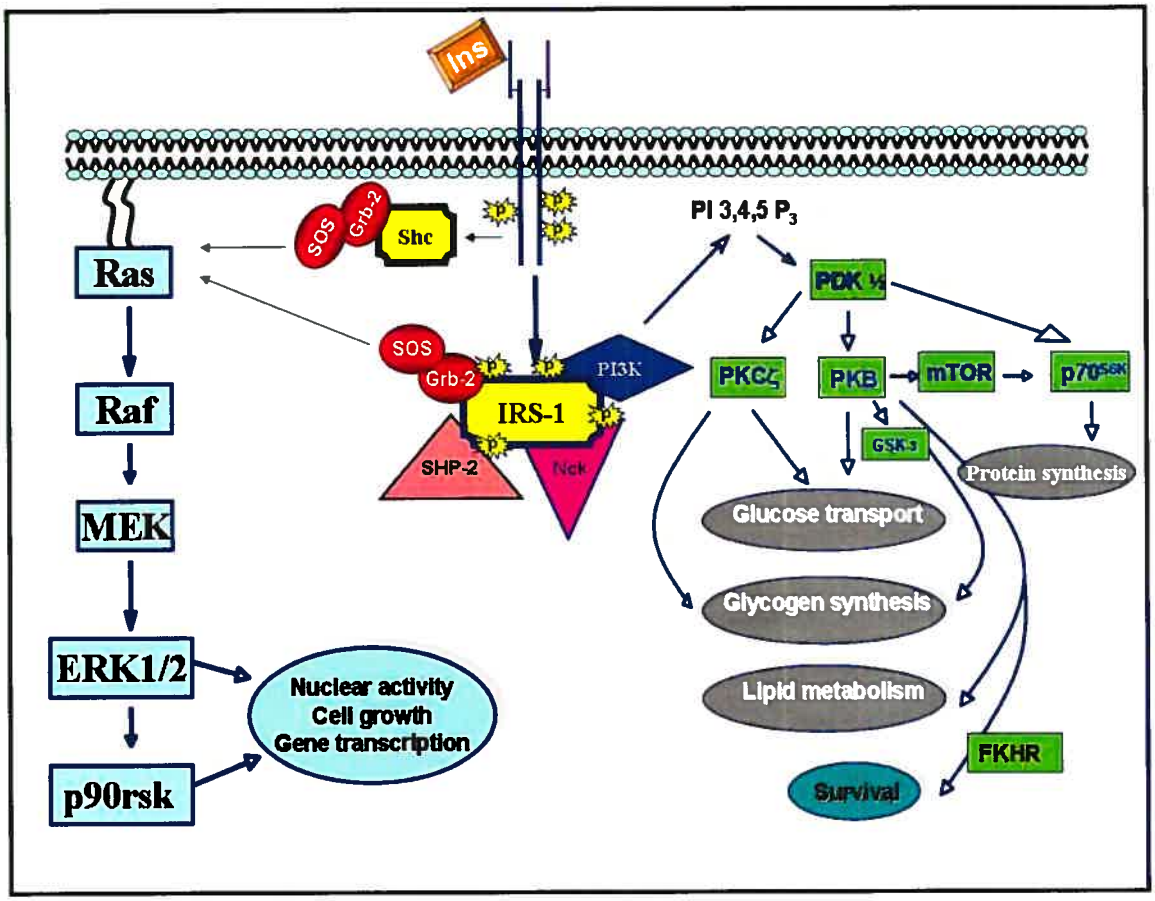

Figure 3: Schematic model showing key elements of the insulin-signaling cascade

Insulin (Ins) -induced tyrosine phosphorylation and activation of insulin receptor substrates (IRSs) by the protein tyrosine kinase (PTK) of β-subunits lead to recruitment of Src homology (SH-2) domain-containing signaling proteins, such as Grb-2-SOS, the p85 regulatory subunit of PI3-K, a lipid and protein kinase. The IRS-associated complex initiates 2 signaling pathways. One pathway, known as the MAPK pathway, consists of Ras/Raf/MEK/ERK1/2 and p90rsk. Both ERK1/2 and p90rsk can be translocated to the nucleus where they can activate transcription subsequent to the phosphorylation of several transcription factors. Another pathway involves PI3-K activation, which results in the generation of PIP3. PIP3 activates a variety of downstream signaling components, including PDKs, PKB, mTOR, p70s6k, PKC, GSK-3 and FKHR. PKB and PKC are involved in a glucose transport system. mTOR regulates protein synthesis via p70s6k. Glycogen synthesis is regulated by PKC directly and by PKB via GSK-3. PKB-mediated phosphorylation of FKHR inhibits its effect on transcriptional activity. (Abbreviations are defined in the list of abbreviations).

* Adapted/Modified from (128;129;301).

The second pathway that radiates from the IRS complex upon insulin stimulation involves phosphatidylinositol 3-kinase (PI3-K) activation (127;129) (Fig. 3). PI3-K phosphorylates phosphatidylinositol (PI) lipids at position 3 of the inositol ring, and generates 3-phosphorylated forms of PI, such as phosphatidylinositol 3, 4, 5 triphosphate (PIP_3) (130), which is implicated in the activation of phosphoinositide-dependent kinase (PDK) and related serine/threonine protein kinases responsible for the phosphorylation and stimulation of several downstream signaling protein kinases, such as protein kinase B (PKB) (also known as Akt), p70 ribosomal S6 kinase ($p70^{s6k}$)(131), and protein kinase C (PKC)-zeta (132). Activation of these protein serine/threonine kinases has been hypothesized to mediate the metabolic effects of insulin at the level of glucose transport, GLUT-4 translocation, glycogen and protein synthesis (129). In addition to the PI3-K-dependent pathway of insulin-induced glucose transport, recent studies have suggested a potential role of an alternate PI3-K-independent mechanism of glucose transport (133). This mechanism involves insulin-induced tyrosine phosphorylation of the protooncogene Cbl (133). Cbl associated protein (CAP) and APS are important components of this mechanism and contribute in the membrane targeting of tyrosine phosphorylated Cbl to lipid rafts which eventually activates a small 9 guanosine triphosphate (GTP) binding protein, TC 10 (134). The activation of TC 10 has been suggested to be a crucial step in mediating insulin-induced GLUT-4 translocation (134;135).

The following sections provide a full description of some of the key components of the insulin signaling cascade.

1.6.1.1- The insulin receptor

The IR is present in all vertebrate tissues with varying degrees of expression. Its concentration ranges from 40 receptors on circulating erythrocytes to more than 200,000 receptors on myocytes, adipocytes and hepatocytes (128). The IR gene is present on the short arm of human chromosome 19, is more than 150 kilobases, and contains 22 exons, which encode 4.2-kb cDNA (4). The IR is composed of two extracellular α-subunits and two transmembrane β-subunits linked to each other by disulfide bonds (128) (Fig. 4). Both

the α- and β- subunits are derived from a single proreceptor by proteolytic processing at a cleavage site consisting of four basic amino acids. Alternative splicing surrounding exon 11 results in two receptor isoforms differing by 12 amino acids close to the COOH terminus of the α-subunits. The α-subunit and β subunits weigh 135 and 95 kilodalton (kDa) respectively. The α-subunit is located entirely outside of the cell and contains the insulin-binding site with stoichiometry between 1 and 2 insulin molecules/receptor, whereas the intracellular section of the β-subunit possesses intrinsic insulin-regulated tyrosine kinase activity. Different biochemical approaches have found that the distinct regions of the first 500 amino acids of the α-subunits contain ligand-binding determinants (136). Therefore, insulin-binding affinity is increased by replacing amino acids 64-137 of the insulin-like growth factor type 1 receptor (IGF-1R) α-subunits by corresponding residues from the IR. In addition, replacing amino acids of residues 325-524 from the IR in this chimera enhances insulin-binding (136;137). The α-subunit of IR can also act as a regulatory subunit of the tetrameric receptor because the unoccupied IR α-subunits inhibit the tyrosine kinase activity of the β-subunits. Depletion of the α-subunits by deletion mutagenesis or proteolytic cleavage or certain point mutations in the α-subunits (Arg86 → Pro) abrogate this inhibition (138). The carboxy-terminal of the α-subunits has four Cys residues, 647, 682, 683 and 684, which are involved in subunit disulfide linkage. Cys 647 to Ser 647 mutation did not cause any change in insulin binding, but these mutants were defective in insulin-stimulated kinase activity. Therefore, insulin-stimulated signal transmission from ligand binding to kinase activation requires proper covalent interaction between the α- and β- subunits for normal subunit communication (139;140).

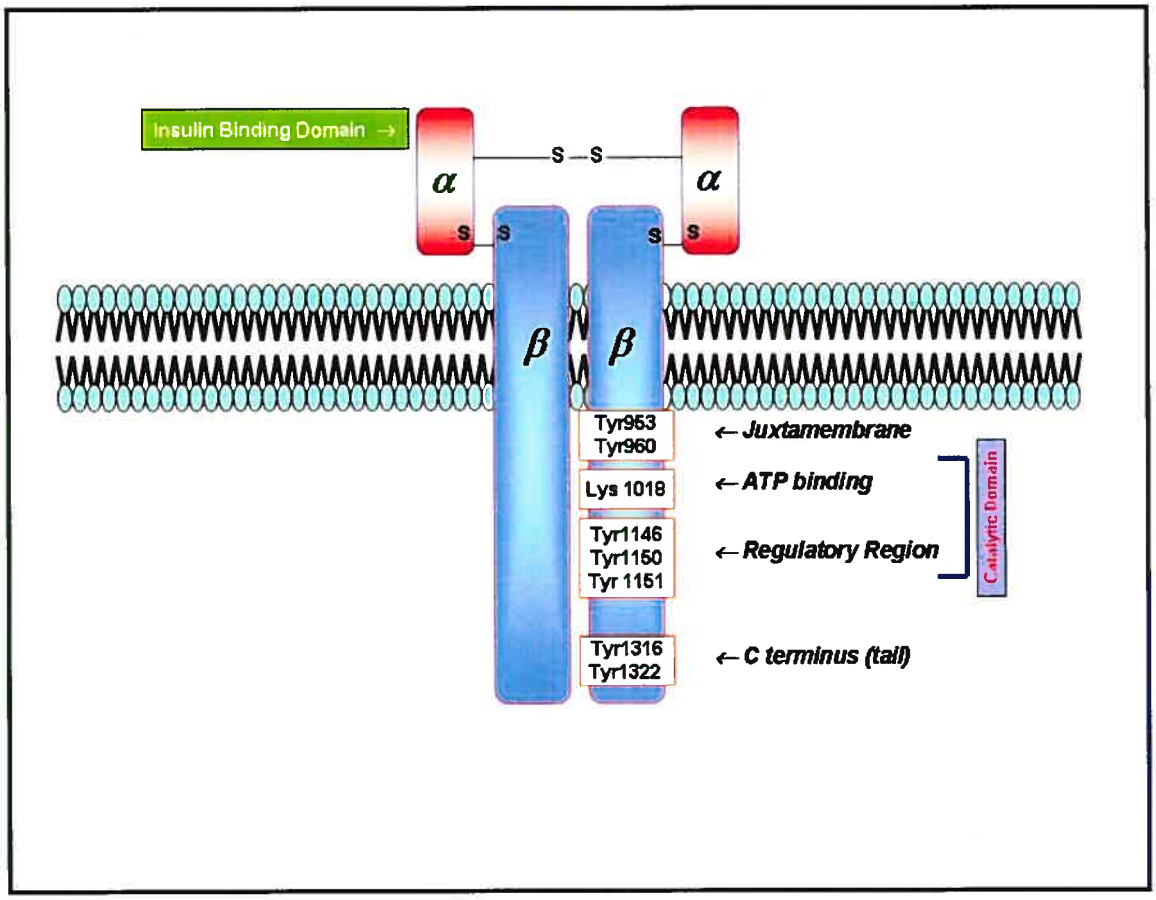

Figure 4: Structure of the insulin receptor

The insulin receptor is composed of two α-subunits and two β-subunits linked by disulfide bonds. The α-subunits are entirely extracellular and contain the insulin binding domains, while the β-subunits penetrate through the plasma membrane into the cytoplasm. The β-subunit has several tyrosine autophosphorylation sites, ATP binding domain and regulatory region necessary for the activation of its intrinsic protein tyrosine kinase activity.
* Adapted/Modified from (127).

The IR-β subunits consist of short extracellular sites for glycosylation, a transmembrane domain of 23 amino acids and an intracellular region which has protein tyrosine kinase activity required for insulin action (Fig. 4). The IR-β-subunits possesses different functional regions, including the adenosine triphosphate (ATP)-binding domain and autophosphorylation sites in the intracellular juxtamembrane region (Tyr 960, and possibly Tyr 953 and Tyr 972), a regulatory region (Tyr 1146, Tyr 1150 and Tyr 1151), and the C terminus (Tyr 1316 and Tyr 1322). In vitro mutagenesis experiments have provided evidence that a key lysine residue at position 1030 (Lys1030), which is an ATP-binding site, when replaced by other amino acids, causes complete loss of kinase activity of the β subunit although insulin-binding is unaffected (141;142). A similar function for a lysine residue at position 1018 has been found (143;144). The discrepancy in the position of lysine between these two studies is due to differences in the approach used in numbering these residues.

Tyrosine phosphorylation occurs through a transmechansim in which insulin binds to the α-subunit of one αβ-dimer and thus stimulates phosphorylation of the adjacent covalently-linked β-subunit (145). Autophosphorylation of all three tyrosine residues of the regulatory region increases tyrosine kinase activity of the IR. Deletion of 1 or all tyrosine residues in this region by mutation reduces insulin-stimulated kinase activity (146;147).

The juxtamembrane region of the IR-β-subunit containing Tyr 960 within NPXY (Asn-Pro-any amino acid-Tyr) motifs plays a role in signal propagation. Replacement of Tyr 960 by phenylalanine or alanine inhibits signal transmission without affecting autophosphorylation in the other regions and kinase activity in vitro (148;149). Analysis of Tyr960 point mutation shows a type of receptor that is impaired in internalization in response to insulin (150). In addition, Tyr960 to Phe point mutation is unable to phosphorylate IRS-1, a crucial IR substrate, which results in decreased PI3-K activation and blocks glycogen and DNA synthesis (151). Thus, the juxtamembrane region is a critical domain required for

both insulin-stimulated signaling via IRS-1 phosphorylation as well as receptor internalization.

The C terminus of the IR contains two autophosphorylation sites, Tyr 1316 and Tyr 1322, which may not be essential for kinase activation (152). In contrast, the C terminus has serine and threonine residues phosphorylated in response to phorbol esters, cyclic adenosine monophosphate (cAMP) analogues, and insulin itself, thus leading to decreased insulin-stimulated tyrosine kinase activity (153-155). Thus, the C terminus plays a regulatory role that is essential for signaling.

1.6.1.2-The insulin receptor substrates

As stated earlier, activated IR-PTK mediates the insulin response through tyrosine phosphorylation of IRS proteins which serve as docking sites for effector molecules responsible for propagating the insulin signal (156). At least four IRS proteins have been identified in mammals. While IRS-1 and IRS-2 are widely expressed in all tissues, IRS-4 is only expressed in the brain, thymus, kidney and β-cells and IRS-3 is present in rodents and highly restricted to adipose tissue (157). Two other small proteins called, Gab and $p62^{dok}$ have been shown to be substrates of the IR and are considered to be IRS proteins (156).

IRS proteins have several common features and are composed of a NH2-terminal PH and/or phosphotyrosine binding (PTB) domain followed by a COOH-terminal tail comprised of multiple tyrosine and serine/threonine phosphorylation site residues. The general structure of IRS-1 is presented in Fig. 5.

The role of the PH domain in IRS is not known because physiologically-relevant binding partners are undefined; however, phospholipids, acidic peptides, or specific proteins such as PH domain interacting protein (PHIP) are defined as PH domain-binding partners (158), but the mechanism of this coupling is unknown.

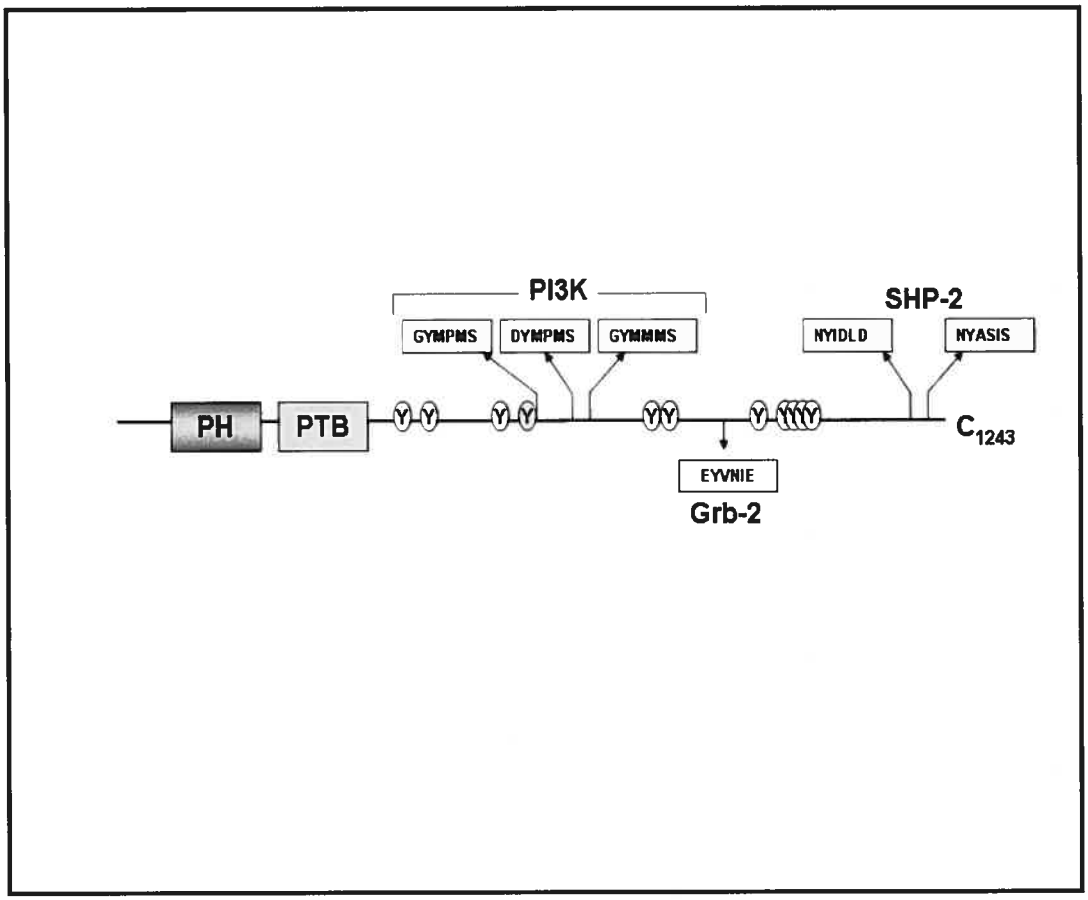

Figure 5: Structure of the Insulin Receptor Substrates-1 (IRS-1)

IRS-1 serves as a type of docking protein for recruitment and activation of other enzymes that ultimately mediate insulin effects. The relative positions of the pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains are indicated. Potential tyrosine phosphorylation sites are indicated by Y while known phosphorylation motifs are enclosed in boxes below potential binding partners, including PI3-K, Grb-2 and SHP-2. (Abbreviations are defined in the list of abbreviations)

* Adapted/Modified from (156).

The PTB domain was found to mediate interactions of IRSs with the IR, insulin-like growth factor type 1 receptor (IGF-1R) and interleukin-4 (IL-4) receptors through phosphorylated-NPXY motifs located in these receptors.

The tyrosine phosphorylated residues in the COOH-terminal serve as docking sites for SH2 domain-containing signal transducers, including p^{85}, a regulatory subunit of PI3-K, SH2 domain-containing tyrosine phosphatase (SHP2), Src-like kinase Fyn or adapter molecules, such as Grb-2, nck, Crk, SHB and others that mediate downstream signals (129). Moreover, other partners including SV40 large T antigen, $\alpha_v\beta_3$ and 14-3-3 bind to IRS proteins through an unknown tryosine phosphorylation-independent mechanism (129).

The role of serine/threonine phosphorylation sites has not been fully unravelled, but several studies have shown that factors, known to induce insulin resistance, such as free fatty acids (159), tumor necrosis factor-α (TNF-α) (160), angiotensin II (Ang II) (161), endothelin-1 (162) and chronic insulin treatment (163), lead to increased serine/threonine phosphorylation of IRS-1 and consequently to impairment of insulin signal transduction. Many Ser/Thr-kinases which phosphorylate IRS-1 are identified including Raf, MEK, mitogen-activated protein kinase (MAPK), $p90^{rsk}$, Rho kinase-α (ROK-α), and PKC isoforms and kinases downstream of the PI3-K cascade such as PDK-1, PKB, mammalian target of rapamycin (mTOR), $p70^{S6K}$ and glycogen synthase kinase-3β (GSK-3β) (164). Specific Ser/Thr phosphorylation sites in IRS-1 identified in vitro include Ser-302, Ser-307, Ser-612, Ser-636, Ser-639, Ser-731 and Ser-789. Of these, Ser-307 phosphorylation has been studied most intensively as a mechanism for disrupting IR/IRS-1 interactions (164). Importantly, a high level of phosphorylated Ser-307 has been detected in vivo in human skeletal muscles (165) and in insulin-resistant rodent models (166). Serine phosphorylation of IRS-1 by some of these kinases has been shown to impair the ability of insulin to enhance IRS-1 tyrosine phosphorylation (167) and lead to proteasome-mediated degradation (168). Taken together, Ser/Thr phosphorylation of IRS-1 could represent one

of the molecular mechanisms by which diabetogenic factors might induce insulin resistance.

1.6.1.3-Phosphatidylinositol 3-Kinase (PI3-K)

As discussed earlier, IRS proteins serve to couple IR-PTK to the PI3-K cascade. PI3-Ks are a family of lipid kinases that phosphorylate the D3 hydroxyl group (3'-OH) of the inositol ring in phosphatidyl inositol (PI). Products of the PI3-K reaction include PI3P, PI3, $4P_2$ and PI3, $4,5P_3$.

PI3-Ks have been divided into three classes (I, II and III) based on sequence homology and substrate preference. Class I PI3-Ks are heterodimers composed of a catalytic subunit with molecular mass 110 (p110) and an adapter/regulatory subunit. This class is further divided into classes IA and IB. While class IA is activated by receptor PTK (RPTK) and Ras, class IB is activated by heterotrimeric G protein-coupled receptor (GPCR) and Ras. The preferred in vivo substrate of this class I enzyme is $PI(4,5)P_2$. Nonetheless, in vitro this class can also phosphorylate PI, PI4P, and PI5P.

Class IA has three isoforms, α, β and δ, of the catalytic p110 subunit and several forms of regulatory subunits ($p^{85\alpha}$, $p^{55\alpha}$, $p^{50\alpha}$, $p^{85\beta}$ and $p^{55\gamma}$) generated by the expression and alternative splicing of three genes, $p^{85\alpha}$, $p^{85\beta}$ and $p^{55\gamma}$. Activation of class IA occurs since the catalytic p110 subunit associates with a p^{85} regulatory subunit which contains two SH2 domains that specifically recognize phosphorylated tyrosine in pYMXM (Tyr-Met-any amino acid-Met) motifs present on activated growth factor receptors or their substrates. The principal role played by this class seems to be the regulation of cell growth and proliferation (169;170).

On the other hand, Class IB has only one member of the catalytic subunit called p110γ and one form of the regulatory subunit p101. The p110γ/p101 complex is translocated to the membrane and activated by the β-subunits of trimeric G proteins at the membrane (169;170). Since p110γ is expressed at a low level in cardiomyocytes, endothelial and

vascular smooth muscle, it could be the isoform that plays a role by controlling vascular tone and heart contractility (171). In addition, p110γ is highly expressed in white blood cells throughout the hematopoietic system that controls processes in inflammation and allergy (172). Furthermore, both classes IA and IB can bind Ras.GTP to a N-terminal region in p110, leading to PI3-K activation (173). The ability of Ras to regulate PI3-K may be important in both Ras-mediated cellular morphology and DNA synthesis (174).

Class II PI3-Ks consists of two major mammalian subclasses, α and β, and by definition they contain a carboxy-terminal C2 domain, a protein module originally observed in PKC molecules, where it confers phospholipid binding. There are no known regulatory subunits in this class, which may not be required, since the size of the catalytic subunit of class II is about twice the size of classes I and III. This family contains several members, including m-cpk/p170, and its human homologue PI3-K-C2α, as well as human PI3-K-C2β or HsC2PI3-K and PI3-K-68_D/cpk identified in Drosophila (172). In vitro studies have shown that PI3-K-C2α prefers PtdIns as its substrates but it also phosphorylates PI4P and to a lesser degree, $PI(4,5)P_2$. On the other hand, PI3-K-C2β highly prefers PtdIns, poorly phosphorylates PI4P and has no effect on $PI(4,5)P_2$. Although Class II PI3-Ks have not been studied extensively, there is evidence that PI3-K-C2β can be stimulated via activated integrin in platelets (172).

Class III PI3-Ks is the only class of enzymes present in yeast; therefore, it is thought to represent the primordial PI3-K that gave to the other class. PI is the only substrate recognized by this class and is phosphorylated to generate PI3P (172). The catalytic subunit size of this Class III PI3-K is similar to that of class I. The structure of the catalytic subunit Vps34p, present in yeast, and its human homologue contains a carboxy-terminal catalytic phosphatidylinositolkinase (PIK) domain as well as a regulatory binding domain. The regulatory subunit of this class, Vps15p/p150, contains an amino-terminal myristylation signal, a Ser-Thr kinase domain, a series of Leu-rich repeats, and carboxy - terminal WD motifs. Based on several studies in yeast, Class III PI3-K is considered to play a role in vesicular trafficking, osmoregulation and endocytosis (175).

In addition to its lipid kinase activity, PI3-K has protein kinase activity and has been shown to phosphorylate the regulatory subunit $p^{85\alpha}$ at Ser 608 in the inter-SH2 domain (176) as well as insulin response sequence-1 (169). Recently it was found that the p110γ/TOR hybrid molecule that lacks lipid kinase activity activated the MAPK cascade, but failed to induce PKB activation, and suggesting that the protein kinase activity of PI3-K is linked to MAPK activation while its lipid kinase activity is crucial to activate PKB (169).

The effects of phospholipids generated by PI3-K in cells are mediated through their specific binding to at least two lipid-binding protein domains, the FYVE (Fab1, YGLO23, Vps27, and EEA1) and PH domains. While FYVE domains interact selectively with PI3P, the PH domains bind $PI3,4P_2$ and $PI3,4,5P_3$. Phosphoinositide-binding PH domains are found in numerous proteins, including PDK and PKB, which are mediators of class IA PI3-K signalling (Fig. 6) (177).

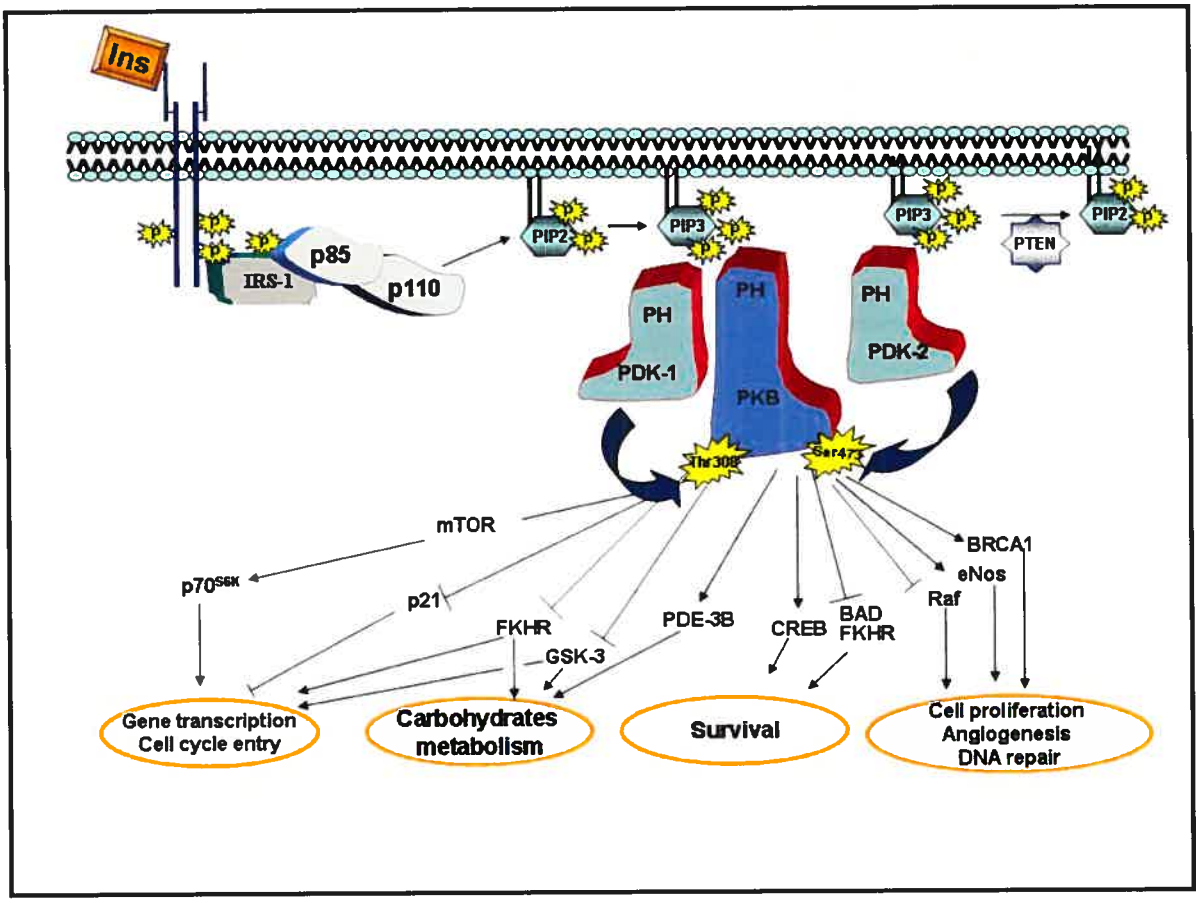

Figure 6: Schematic representation of PKB activation and its physiological role
PI3-K is activated while it's recruited to phosphorylated-IRS-1. PI3-K catalyzes the phosphorylation of phosphatidylinositol 4, 5 biphosphate (PIP2) leading to the formation of phosphatidylinositol 3, 4, 5 triphosphate (PIP3). PIP3 recruits PH domain containing proteins to the plasma membrane including PKB, PDK-1 and probably, the putative PDK-2, where PKB becomes phosphorylated and activated. PKB contribute to a variety of cellular responses, including cell growth, cell survival and metabolism by targeting different substrates. PTEN, a lipid phosphatase, dephosphorylates PIP3 to PIP2 and thus inhibits activation of PKB. (Abbreviations are defined in the list of abbreviations)
* Adapted/Modified from (169;182;186;301).

1.6.1.4-Protein kinase B (PKB)

PKB was originally named Akt because it was the product of the acute transforming retrovirus (Akt-8) gene isolated from an AKR thymoma (178). Sequence analysis of the viral oncogene and its cellular homologue revealed that it encodes a Ser/Thr kinase (179). Akt was also cloned by two different groups and was named RAC (Related to A and C kinase) by Coffer et al. (180) and PKB by Jones et al. (181). To date, the protein is most commonly referred to as Akt or PKB.

PKB, a 57 kDa protein, was so called because of its high homology with protein kinase A (PKA) and PKC. It is known to exist as three isoforms, α, β and γ (Akt1, Akt2 and Akt3, respectively) widely expressed in most mammalian cells (182;183). Each isoform possesses three functional distinct regions: an amino-terminal PH domain, a central catalytic Ser/Thr kinase domain and a carboxy-terminal regulatory domain that contains the hydrophobic motif (HM) (169). The kinase domain of PKB, located in the central region of the enzyme, has high similarity with other AGC kinases such as PKA, PKC, $p70^{S6K}$ and $p90^{rsk}$, and contains the conserved Thr residue whose phosphorylation is required for enzymatic activation. This threonine residue is flanked between highly conserved DFG and APE motifs in the activation loop, which lies in the C-lobe of the kinase domain. The PH domain of PKB interacts with similar affinity with membrane lipid products such as PI(3, 4)P_2 and PI(3,4,5)P_3 produced by PI3-K. Point mutation in the PH domain of PKB reduces its interactions with phosphoinositides as well as its activation by its upstream activator, PDK-1. These studies suggest that the PH domain of PKB plays a significant regulatory role in both membrane translocation and recognition by upstream kinases.

The second important regulatory domain of PKB is a carboxy-terminal of around 40 amino acids. This region possesses the F-X-X-F/Y-S/T-Y/F (HM) (where X is any amino acid) that is characteristic of many other AGC kinases. Phosphorylation of the Ser or Thr residue in this HM is necessary for full activation. In all mammalian PKB isoforms, this motif is identical, and is very important, because a deletion mutant motif completely abolishes PKB enzymatic activity. Interestingly, rat PKBγ/Akt3 and the human PKBγ-1 splice variant do

not have this motif indicating that activation of these variant kinases occurrs via an alternative mechanism independent of Ser/Thr residues in the HM. Further recent evidence shows that the HM has a dual role in regulating kinase activity. In addition, based on the biochemical and crystal structure PKB, it is suggested that the HM serves as an allosteric regulator of catalytic activity. Thus, the HM provides stability to the catalytic activity core due to intramolecular interaction between the phosphorylated HM and its acceptor structure, called the hydrophobic groove formed by αB- and αC- helices. In addition, the activation loop becomes ordered due to interactions with the αC helix.

Phosphorylation site mapping of PKB from quiescent or IGF-1 revealed that while Ser 124 and Thr 450 are constitutively phosphorylated and seem to contribute to stabilization of the proteins, phosphorylation of Th308 in the activation loop and Ser 473 in the carboxy-terminal site is detected in stimulated cells (169). The C terminal PH domain in PDK-1 binds phospholipids with 10-fold higher affinity than the PKB PH domain, which probably explains the constitutive localization of PDK-1 at the plasma membrane. Therefore, the following scenario for PKB activation has been proposed (Fig. 6): PI3-K activation leads to the production of PI(3, 4)P_2 and PI(3,4,5)P_3 at the inner leaflet of the membrane. These phospholipids interact with PKB and cause its translocation to the inner membrane where PDK-1 is located. Furthermore, it is thought that binding of 3'-phosphoinositides to PKB changes its conformational status, making the regulatory residues more accessible to phosphorylation. The PH domains might also mediate protein proximity between PKB and PDK-1 through homodimerization. PDK-1, believed to be constitutively active, subsequently phosphorylates Thr308 in PKB (184), which stabilizes the activation loop in an active conformational form. Phosphorylation of Thr 308 is a prerequisite for kinase activation, but phosphorylation of Ser 473 is required as well for full PKB activation. The putative Ser 473-PKB kinase called PDK-2, remains to be identified.

At least 13 substrates have been identified so far in mammalian cells (169;170). All identified substrates possess the same basic motif, RXRXXS/T, where X is any amino acid,

and S/T is the phosphorylation site. This consensus sequence is based on the sequence around the phosphorylation on GSK-3β, the first identified substrate.

PKB contributes to a variety of cellular responses, including cell growth, cell survival and metabolism (Fig. 6 and 7) (170;182;183). The downstream targets of PKB involved in cell death regulation include the forkhead transcription factors, the pro-apoptotic Bcl-2 family member BAD, and the cyclic AMP response element-binding protein (CREB). GSK-3β, phosphodiesterase-3B (PDE-3B), mTOR, the forkhead member FKHR, the cyclin-dependent kinase inhibitor p21 and possibly Raf-1 are targets involved in mediating carbohydrate metabolism and cell cycle regulation. In addition, phosphorylation of endothelial nitric oxide (eNOS) and breast cancer susceptibility-1 (BRCA1) by PKB might mediate angiogenesis and DNA repair.

In the following sections, we focus on the role of PKB in the regulation of carbohydrates (Fig. 7).

1.6.1.4.1-Role of PKB in glucose transport

Accumulating evidence implicates PKB in enhancing insulin-induced glucose transport through its effect on insulin sensitive glucose transporter isoform (GLUT-4). In the basal state, GLUT-4 is located in the intracellular vesicles which are translocated to the plasma membrane in response to insulin, thereby increasing the number of transporters available and thus glucose uptake (Fig. 7) (185;186). Different molecular approaches suggest that PKB is a mediator of insulin action in this process (186). For example, PKB rendered constitutively active by membrane-targeting mimics insulin by increasing the rate of GLUT-4 translocation and glucose transport in the absence of insulin (187) whereas PKB inhibition by microinjection of a PKB substrate peptide or an antibody to PKB suppresses insulin-stimulated GLUT-4 translocation to the plasma membrane in muscle and fat cells (188;189).

1.6.1.4.2-Role of PKB in glycogen synthesis

Glucose uptake activated by insulin is ultimately stored as glycogen in skeletal muscle. Glycogen synthase (GS) plays a pivotal role in this processes by catalyzing the final step in glycogen synthesis. The regulation of GS is complex and involves allosteric activators, translocation to the plasma membrane in the presence of glucose metabolites and insulin, inhibitory phosphorylation on Ser residues by different kinases (PKA, PKC and GSK-3), and activation of dephosphorylation by the Ser-Thr phosphatase PP1. Therefore, a model by which GS will be active and leads to glycogen synthesis is via phosphorylation of GSK-3 by PKB on Ser 9 which results in the inhibition of its catalytic activity (190) (Fig. 7). GSK-3 phosphorylates four Ser residues on GS, and PKB-mediated inactivation of GSK-3 contributes to a reduction of GS phosporylation and increase in GS and glycogen synthesis activation (191). In adipocytes, mutation of GSK-3 as a PKB-insensitive form results in suppression of insulin-stimulated GS activity. In addition, adipocytes transfected with constitutively active PKB significantly inhibit GSK-3 activity and induce GS activation whereas the same cells transfected with dominant inhibitory PKB show 50% inhibition of insulin-stimulated GS activation. Nonetheless, further work is needed to suggest the absolute contribution of PKB in the regulation of GS because several signaling molecules that are influenced by PKB modulate this process.

1.6.1.4.3-Role of PKB in gluconeogenesis

De novo synthesis of glucose in the liver from precursors such as lactate, gluconeogenic amino acids, and glycerol is a central mechanism that provides the organism with glucose in times of starvation. On the other hand, when glucose is directly available from external resources, gluconeogenesis needs to be shut off. PEPCK and G6Pase are implicated in regulation of the early and terminal steps of gluconeogenesis, respectively. Indeed, PEPCK and G6Pase appear to be positively regulated at the level of transcription (no allosteric modification) by cAMP or glucocorticoid signaling. Actually, insulin can suppress glucocorticoid and cAMP signaling-stimulated glucose production by downregulating the

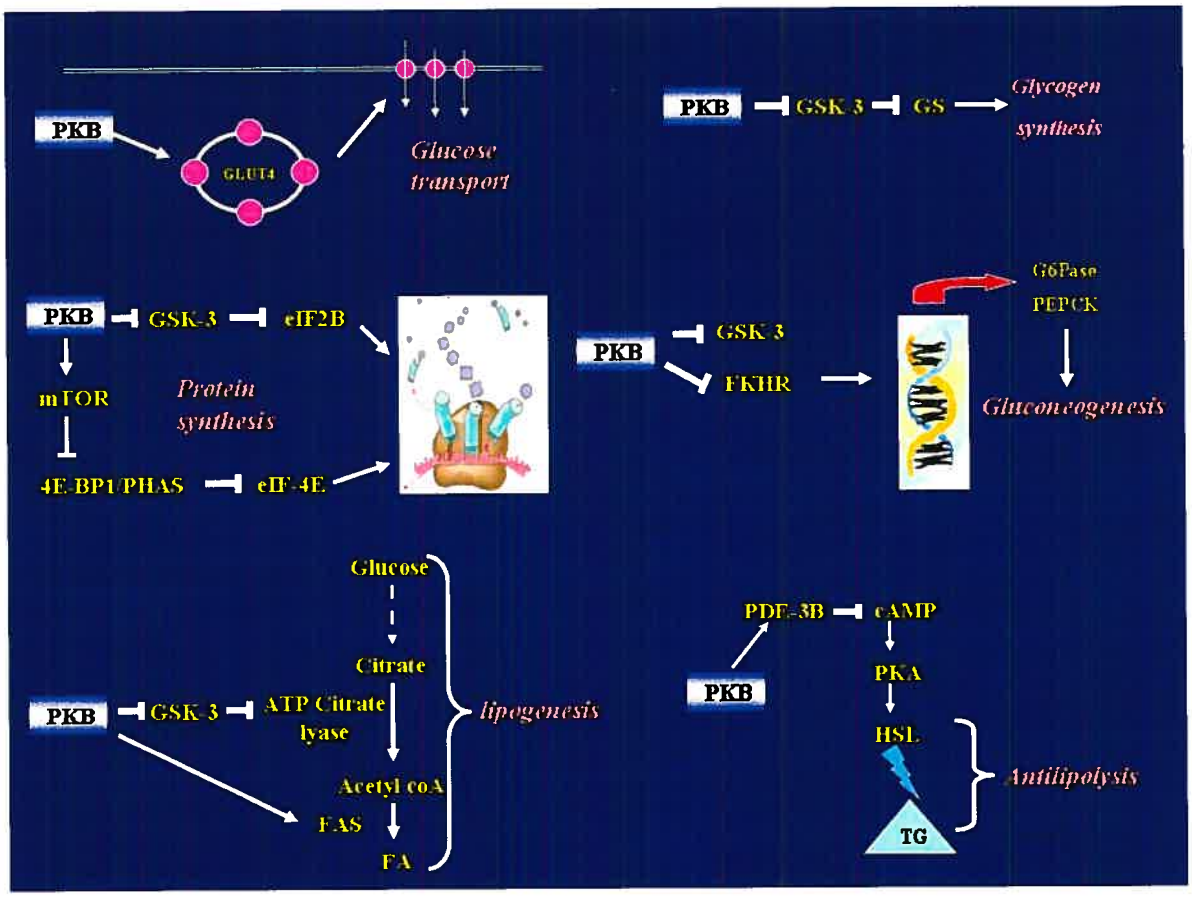

Figure 7: Recapitulative schema showing a role of PKB in the regulation of carbohydrate metabolism

(Abbreviations are defined in the list of abbreviations)
* Adapted/Modified from (182;186).

transcription of PEPCK and G6Pase, and PKB has been proposed as mediator of insulin-regulated gluconeogenesis (192). G6Pase and PEPCK promoters are known to contain so-called insulin-responsive element (IRE), but the transcription factors that bind to these regions have not been identified. In mammalian cells, the DAF-16 homologue Foxo1 (known as Forkhead trasncription factor, FKHR) have been found to be phosphorylated by PKB at three conserved Ser or Thr residues within the PKB consensus phosphorylation sites (RXRXXS/T), namely Thr^{24}, Ser^{256}, and Ser^{319} (Fig. 7) (193). Phosphorylation in these three places results in transcriptional inactivation and nuclear export of FKHR (194). Electrophoretic mobility shift assays have provided evidence of FKHR binding to insulin-responsive sequences in the G6Pase promoter, and reporter gene analysis has demonstrated transactivation of the PEPCK and G6Pase promoter in vitro (195). In addition, overexpression of FKHR markedly increases the expression of the endogenous G6Pase transcript in H4 rat hepatoma and LLC kidney epithelial cells. The overexpression of dominant negative FKHR mutants that are not regulated by PKB impairs the suppression of G6Pase gene transcription by insulin. In addition, the overexpression of a constitutively active FKHR mutant, which is resistant to inactivation by insulin, increases PEPCK and G6Pase expression and results in elevated fasting blood glucose concentrations (192).

A potential role for GSK-3 in the regulation of gluconeogenesis has been suggested from the use of specific inhibitors (Fig. 7). These compounds decrease hepatic glucose production in fasted fa/fa rats and induced hepatic glycogen synthesis (196). The decrease in hepatic glucose production in vivo after inhibition of GSK-3 might be due to suppression of PEPCK and G6Pase gene expression. It should be noted that pharmacological inhibition of GSK-3 activity in hepatoma cells was found to reduce gluconeogenesis and hepatic glucose output (197). Thus, the molecular events underlying the regulation of PEPCK and G6Pase gene expression by GSK-3 are unclear. PKB-mediated GSK-3 phosphorylation and inhibition could be one model by which PKB regulates glucose output.

1.6.1.4.4-Role of PKB in protein synthesis

Muscle and fat are two primary sites of insulin-dependent protein synthesis. Insulin regulates protein synthesis by stimulating initiation and elongation steps. Several studies have shown that PKB plays a crucial role in modulating key-components implicated in translational activity (Fig. 7) (182). In 3T3-L1 adipocytes, putative dominant-inhibitory PKB mutant protein blocks insulin-stimulated protein synthesis. In contrast, a constitutive active PKB increases protein synthesis in the absence of insulin in L-6 muscle cells and 3T3-L1 adipocytes. eIF-4E, a limiting initiation factor, binds to the 7-methylguanosine cap and directs mRNA to the 40S ribosomal subunit. In quiescent cells, eIF-4E is sequestered by 4E-binding protein (4E-BP1)/PHAS-I. Activation of mTOR, a 4E-BP1 kinase, deactivates 4E-BP1, releases eIF-4E, and promotes translation initiation. Therefore, one model posits that PKB activates mTOR by the phosphorylation of Ser 2448. In another model, PKB indirectly regulates the activity of eIF-2B, the guanine nucleotide exchange factor for eIF-2, through its downstream target GSK-3. GSK-3 inhibits eIF-2B by its phosphorylation on Ser 540 while PKB-mediated phosphorylation and inactivation of GSK-3 on Ser 23 and Ser 9 increase the exchange activity of eIF-2B and promote the recruitment of initiator tRNA to the 40S ribosome.

The phosphorylation of ribosomal S6 protein by $p70^{S6K}$ enhances translation by increasing the interaction of the 40S ribosomal subunit with a select population of mRNA containing 5'-TOP (terminal oligopyrimidine). A constitutively active PKB leads to activation of $p70^{S6K}$ and an increase in ribosomal S6 protein phosphorylation, but this hypothesis is not well- established because genetic experiments in Drosophila have shown that the activity of S6K is independent of the presence of a functional PKB gene.

1.6.1.4.5-Role of PKB in antilipolysis

The role of insulin in adipocytes is primordial for the regulation of lipolysis (Fig. 7). Hormone-sensitive lipase (HSL) which controls triglyceride (TG) breakdown in adipose tissue, is activated by PKA-mediated phosphorylation, and PKA activity is increased by cAMP. The cyclic nucleotide phosphodiesterase PDE-3B, phosphorylated and activated on

Ser 273 by PKB, reduces cAMP concentrations and decreases HSL activity (198). In contrast, another report demonstrates that 1-(5 chloronaphthalenesulfonyl)-1H-hexahydro-1, 4-diazepine (ML-9), a powerful inhibitor of PKB activity in different cells as well as of recombinant PKB, prevents the stimulatory effect of insulin on glucose transport and protein translocation but not the antilipolytic effect in rat adipocytes (199).

1.6.1.4.6-Role of PKB in lipogenesis

Lipogenesis, the conversion of incoming glucose to fatty acid, is regulated by insulin. Several works have demonstrated a role for PKB in insulin-induced lipogenesis (Fig. 7) (186). Inhibition of GSK-3 by PKB might be involved in this effect. GSK-3 phosphorylates ATP citrate lyase on Thr 446 and Ser 450 and decreases its activity (200). This enzyme catalyzes the conversion of citrate to acetyl CoA in the cytosol. In addition, a study by Wang et al. revealed that insulin stimulation of the fatty acid synthase (FAS) promoter is mediated by the PI3-K pathway and that PKB/Akt a downstream effector (201). FAS catalyzes several steps in the conversion of malonyl-CoA and acetyl-CoA to long chain fatty acids. Furthermore, recent studies have shown that PKB induces transcription of enzyme implicated in cholesterol and fatty acid biosynthesis via activation of a major transcription factors called sterol-regulatory element binding proteins (SREBPs). These SREBPs regulates genes involved in fatty acid and cholesterol synthesis (202).

1.6.1.5- The Mitogen Activated Protein kinase (MAPK) pathway

MAPKs a family of Ser/Thr protein kinases widely conserved among eukaryotes, are involved in many cellular responses such as cell proliferation, cell differentiation, cell movement and cell death (203;204) . In mammalian cells, five MAPK families have been identified, including ERK1/2, Jun N-terminal kinase 1, 2 and 3 (JNK1/2/3) also called stress-activated protein kinase (SAPK), p38α/β/γ/δ, ERK5 and ERK7. These pathways are characterized by the following general system: Stimulus → MAPKKK → MAPKK→ MAPK→ responses. The MAPKKKs, which are Ser/Thr kinases, are often activated through phosphorylation and/or as a result of their interaction with a small GTP-binding

protein of the Ras/Rho family in response to extracellular stimuli. Activated-MAPKKKs phosphorylate and activate MAPKK, which then stimulates MAPK activity through dual phosphorylation on Thr and Tyr residues located in the activation loop of the kinases. Once activated, MAPKs phosphorylate target substrates on Ser or Thr residues followed by a proline; however, substrate selectivity is often conferred by specific interaction motifs located on physiological substrates. Furthermore, MAPK cascade specificity is also mediated through interaction with scaffolding proteins which organize pathways in specific modules through simultaneous binding of several components.

The most extensively studied groups of vertebrate MAPKs to date are the ERK1/2, JNKs, and p38 kinases (203;204). ERK1/2 are stimulated by mitogens such as polypeptide growth factors (PDGF, CSF-1, IGF-1, etc.) as well as insulin and phorbol 12-myristate 13-acetate (PMA). In contrast, SAPKs and p38 MAPK are potently induced by a wide variety of stresses, including UV irradiation, gamma irradiation, anisomycin heat shock, chemotherapeutic drugs, etc. but not by mitogens. These two pathways are also activated by ischemia or reperfusion following ischemia and by inflammatory cytokines. Interestingly, epidermal growth factor (EGF) can induce all three pathways, although its potency is variable. In addition, GPCR can stimulate all MAPK families.

As discussed above, ERK1/2 is the principal MAPK pathway activated by insulin; therefore, the following section will provide a detailed account of its mechanism of activation (Fig. 8).

The mammalian ERK1/2 pathway is constituted of the MAPKKKs A-Raf, B-Raf and Raf-1, the MAPKKs mitogen extracellular regulated kinase 1 and 2 (MEK1/2), and the MAPKs ERK-1 and ERK-2. Other MAPKKKs, called MEKK1/2/3 and c mos, have also been identified in this pathway, but their role in activation of the ERK1/2 pathway is limited (205). The mechanism of ERK1/2 activation starts whith signals derived from activated receptor tyrosine kinase (RTK) or GPCR to Raf/MEK/ERK through different isoforms of the small GTP-binding protein Ras. Ras is a 21 kDa molecular weight protein attached to the cytoplasmic face of the plasma membrane by a farnesyl group (206). The protein

serves as a key "molecular switch" in cytoplasmic signalling pathways triggered by activation of a variety of membrane receptors. In its inactive state, in unstimulated cells, Ras is found to be associated with guanosine diphosphate (GDP) (Ras-GDP). As discussed earlier, following activation of the IR and its substrates (e.g. Shc, IRS-1/2), Grb-2 associates with guanine nucleotide exchange factors such as SOS, and this causes the release of GDP, and binding of GTP to Ras (forming Ras-GTP) (Fig. 8). The exchange is accompanied by a conformation change in Ras, allowing it to bind to a wide range of downstream effector proteins, including isoforms of the Ser/Thr kinase Raf. In fact, the exact mechanism by which Raf is activated is still not completely clear, but it appears that Ras binding as well as multiple phosphorylation events at the membrane are required. Activated Raf binds to and phosphorylates the dual specificity kinases MEK-1 and -2, which in turn phosphorylate ERK1/2 within a conserved Thr-Glu-Tyr (TEY) motif in their activation loop. Once activated, ERK1/2 can activate a number of cytosolic proteins, such as $p90^{rsk}$ through its praline directed Ser/Thr kinase activity or it can translocate to the nucleus where it can phosphorylate and activate a number of transcription factors involved in immediate early gene (Fig. 8).

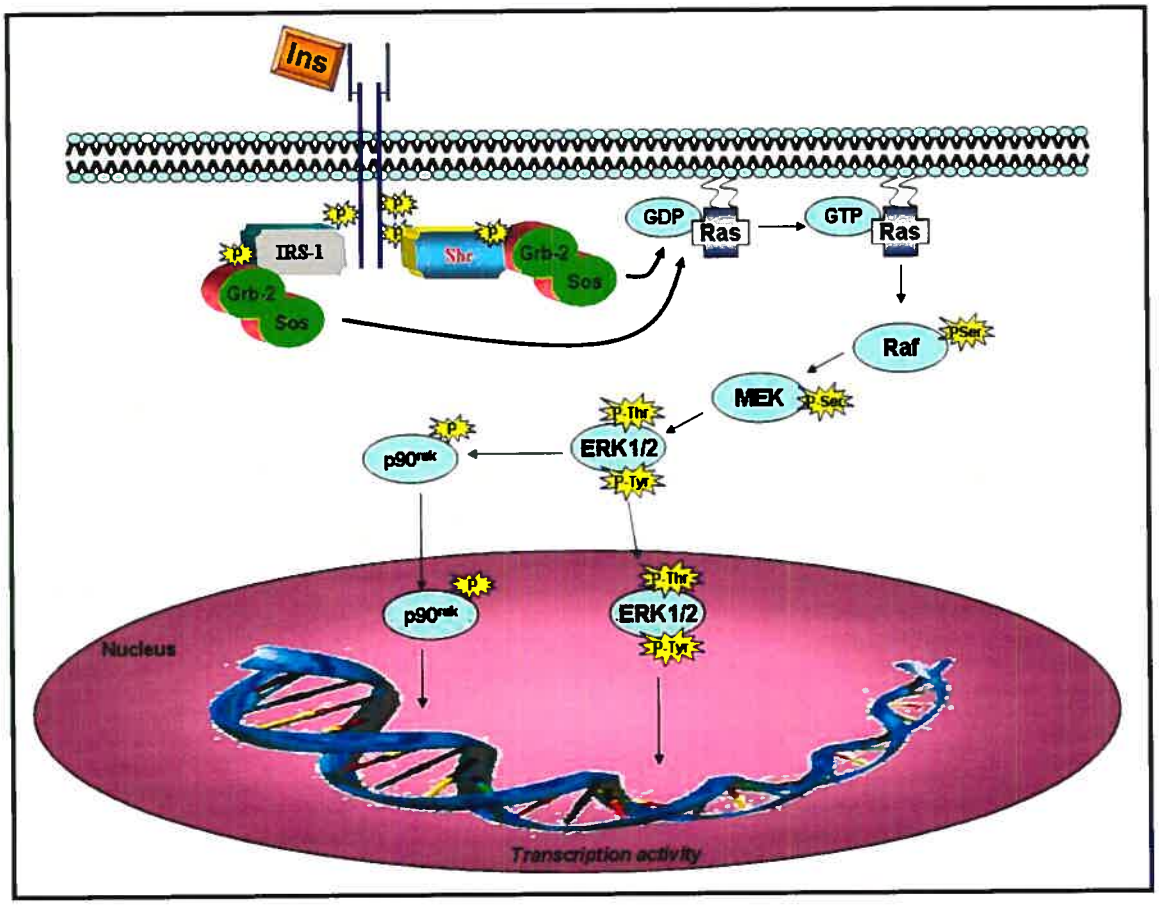

Figure 8: Schematic diagram showing key steps involved in insulin-induced activation of ERK1/2

In response to insulin, Shc or -IRS-1 become phosphorylated and binds Grb-2-SOS complex leading to p21ras stimulation by GTP loading with subsequent activation of Raf, MEK, and two isozymic forms of MAPK, ERK-1 and ERK 2. Activated MAPK phosphorylates and activates a downstream ribosomal protein kinase, $p90^{rsk}$. 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. (Abbreviations are defined in the list of abbreviations)

* Adapted/Modified from (203;204;301).

1.6.1.6-Potential protein tyrosine phosphatases (PTPases) implicated in the insulin signaling cascade

PTPases serve as important regulators of the insulin signaling pathway by their ability to catalyze the rapid dephosphorylation and inactivation of IR-β subunit and IR substrates (207).

PTPases can be divided into four classes: 1) classical receptor PTPases (R-PTPases), including leucocyte antigen receptor (LAR), PTPσ, PTPδ (type IIa); PTPμ, PTPκ, PTPλ/ψ/o (type IIb); PTPφ; PTPα, PTPε (type IV); PTPζ/β, PTPγ (type V); 2) classical non-receptor PTPases (NR-PTPases) including PTP1B, SHP-1, SHP2, PTP-PEST; 3) dual specificity PTPases (DS-PTPases); and 4) low molecular weight PTPases (208;209). While R-PTPases and NR-PTPases have specificity to phosphotyrosine residues, DS-PTPases recognize phosphotyrosine, phosphopthreonine and phosphoserine residues. R-PTPases and NR-PTPases fall into several subtypes based on their non-catalytic domain structures. R-PTPases, which have an extracellular domain, a single transmembrane segment and one or two tandemly-conserved PTPase catalytic domains are predominantly found in the plasma membrane, whereas NR-PTPases have a single PTPase domain with additional protein segments and are localized to a variety of intracellular compartments, including the cytosol, plasma membrane and endoplasmic reticulum (ER). All PTPases contain at least one conserved core of 230-250 amino acids that represent their catalytic domain. The PTPase signature motif, VIHCSAGXGRXG, i.e (Val/Ile) His-Gly-Arg-(Ser/Thr)Gly respectively, possesses an invariant cysteine residue that is critical for PTPase activity. The cysteine residues is essential to catalyze the hydrolysis of protein phosphotyrosine residue by forming a cysteinyl-phosphate intermediate.

The following sections briefly describe the involvement of major PTPases implicated in modulation of the insulin-signaling pathway.

1.6.1.6.1-PTP1B

PTP1B was the first PTPase discovered in the human placenta (210). Expressed ubiquitously, it is a type of NR-PTPase. PTP1B has a molecular size of 50 kDa and localizes predominantly in the ER through a cleavable C-terminal domain. Genetic analyses have revealed that PTP1B has a single-copy gene map to the long arm of human chromosome 20 in the region q13.1 to q13.2 that has been linked to a quantitative trait locus for obesity and insulin (209). PTP1B is believed to be a negative regulator of insulin action because decrease in its expression level by a variety of strategies resulted in increased insulin signaling and action. For example, osmotic loading of neutralizing PTP1B antibodies in rat hepatoma cells increased insulin-induced IRS-1 phosphorylation, PI3-K activity and DNA synthesis (212). Moreover, glucose transport and GLUT-4 translocation are decreased in PTP1B-overexpressing cells (213;214). Reduction of PTP1B expression in FAO cells by using antisense oligonucleotide (ASO) approach also caused an increase in insulin-stimulated phosphorylation of PKB and GSK3 (215). A role of PTPases in insulin action and in the pathogenesis of diabetes has been postulated on the basis of studies showing that vanadium-based or other small molecule inhibitors of PTPases can improve glucose homeostasis in rodent models of type I and II diabetes mellitus and in a small number of type II diabetic human subjects (19;60;123;216). In this regard, it is noteworthy that the expression level of PTPases has been shown to be impaired in rodent models of type 1 and type 2 diabetes mellitus as well as in human subjects (217-220). Further support for the involvement of PTPase in insulin's action comes from recent studies demonstrating that reduction of the PTPase PTP-1B, by using antisense oligonucleotides (ASO), normalizes blood glucose and improves insulin sensitivity in diabetic mice (221). Further proof for a role of PTP1B in insulin action comes from the studies using PTP1B knock out mice that exhibit a heightened insulin signaling sensitivity, enhanced glucose tolerance and resistance to high fat diet-induced obesity. The enhanced insulin responsiveness in the mice was due to an enhanced IR-phosphorylation(222;223).

1.6.1.6.2-LAR

LAR, a type of R-PTPases, is widely expressed in insulin-sensitive tissues (224). LAR possesses two cytoplasmic catalytic domains linked by a unique hydrophobic transmembrane stretching to a large extracellular segment. This extracellular segment contains three immunoglobulin-like repeats and four to eight type III fibronectin repeats. Treatment of intact cells with insulin results in increasing association between LAR and the IR. In addition, high LAR expression level is detected in the fat tissue of obese patients and in 3T3-L1 adipocytes pre-incubated with high glucose. LAR downregulation with ASO enhances and prolongs insulin signaling in hepatoma cells, while LAR overexpression suppresses insulin action. Furthermore, engineered LAR overexpression in mouse skeletal muscle suppresses IR signaling and causes insulin resistance. Also, in LAR-deficient mice, insulin-sensitivity is increased in the fasting state, but there are also unexpected defects in glucose homeostasis after insulin treatment that are more consistent with insulin resistance (209).

1.6.1.6.3-RPTPα (LRP) and RPTPε

PTPα (LRP) and its closely related enzyme PTPε are expressed in insulin-sensitive tissues (225). These phosphatases contain two cytoplasmic catalytic domains: a single transmembrane domain and a short, highly-glycosylated extracellular segment. Overexpression of the two PTPases in baby hamster kidneys (BHM) cells causes IR dephosphorylation indicating that they are negative regulators of IR (209). Moreover, overexpression of wild type PTPα in rat adipocytes diminishes insulin-induced GLUT-4 translocation. In contrast, some other studies have shown that PTPα fails to play a regulatory role in IR signaling. For example, blocking PTPα expression by ASO in 3T3-L1 adipocytes does not affect insulin-induced MAPK activation or DNA synthesisn and PTPα-deficient mice do not incur any glucose homeostasis dysregulation.

1.6.1.6.4 SHP2

SHP2 (also called PTP1D, SH-PTP2, SH-PTP3, Syp and PTP2C) is a ubiquitously-expressed cytosolic PTPase (226). SHP2 is composed of two amino-terminal SH2 domains, a central phosphatase domain and a carboxy-terminal tail containing two tyrosyl phosphorylation sites (208). In cell culture overexpression and yeast two hybrid system studies, SHP2 binds to the IR and IRS-1, and IRS-1 is also likely to be a direct substrate. In contrast, other investigators have failed to show a similar interaction between the IR and wild type SHP2, whereas such interaction occurred between the IR and catalytically inactive-SHP2. This suggests that the IR is also a direct substrate. However, another study indicates that the IR is not a substrate, but that SHP2 acts as an adaptor protein to link the IR and IRS-1. There is a lack of consensus about a positive and/or negative role of SHP2 in regulating insulin signaling. Overexpression of dominant negative SHP2 blocks insulin-stimulated mitosis in 3T3-L1 adipocytes and insulin-induced Ras activation. Furthermore, overexpression of dominant negative SHP2 in rat adipocytes modestly impairs insulin-induced GLUT-4 translocation. In contrast, disrupting binding between IRS-1 and SHP2 by mutating Tyr phosphorylation residues on IRS-1 results in increased the Tyr phosphorylation of IRS-1 and heightened insulin-dependent activation of PI3-K as well as protein synthesis but this mutation fails to alter MAPK activity and cell proliferation. Transgenic analyses tools have been used to explore SHP2 function. While SHP2(-/-) homozygous null mice do not survive, SHP2(+/-) heterozygotes appear to be normal with respect to glucose homeostasis, and IR and IRS-1 phosphorylation. In other studies, transgenic mice overexpressing dominant-negative SHP2 exhibited impairment of insulin sensitivity and reduced in IRS-1 phosphorylation as well as PI3-K and PKB activity. Thus, further work is needed to test whether SHP2 plays a major physiological role in insulin signaling.

1.6.1.6.5-Mitogen-activated protein kinase phosphatases (MKPs)

MKPs are dual specifity phosphatases because they dephosphorylate MAPKs at both the Tyr and Thr residues necessary for enzymatic activity. This family of phosphatases contains at least nine members, including MKP-1 (also termed CL100, 3CH134, Erp, and hVH-1), MKP-2 (also known as hVH-2 and TYP-1), PAC-1, B23 (also termed hVH-3), hVH-5, MKP-3 (same as rVH6), MKP-4 and MKP-5. However, previous studies have revealed that MKP-1, MKP-2, MKP-3 and PAC-1 are all able to inactivate ERK1/2 (227). These MKPs are not expressed or present at low levels in quiescent cells, but they are rapidly induced upon stimulation with agonists that include insulin, growth factors, oxidative stress, heat shock, and UV irradiation and are thus defined as immediate-early genes. Consistently, MKP-1 expression induced by insulin blocks both insulin-stimulated ERK1/2 activity and ERK1/2-dependent gene transcription (228). In addition, MKP-1 and MKP-4 have been shown to have a role in regulating adipocyte differentiation and insulin-stimulated glucose uptake in adipocytes (229;230). Furthermore, recent studies have suggested that MKP-3 is highly expressed in the livers of insulin-resistant and diabetic animals and it plays a role in regulating gluconeogenic gene expression and hepatic gluconeogenesis and thus, dysregulation of MKP-3 expression and/or function in liver may contribute to the pathogenesis of insulin resistance and type II diabetes (231). Taken together, these results indicate that MKPs are negatively implicated in the regulation of insulin signaling and, consequently, may contribute to the pathogenesis of insulin resistance.

1.6.1.6.6-PTEN

PTEN (phosphatase and tension homologue deleted on chromosome 10) contains the sequence motif that is highly conserved in members of the PTPase family (232). PTEN has been shown in vitro to possess phosphatase activity on phosphotyrosyl and phosphothreonyl-containing substrates as well as PIP3, a product of PI3-K. Thus, PTEN dephosphorylates the 3'-OH position on the inositol ring in PIP_3. Consequently, the hydrolysis of generated PIP3 inhibits the activation of PKB, a key mediator of insulin

action. Consistent with this, hyperactivation of the PI3-K/PKB pathway also appears to be the main result of PTEN deletion in mammalian systems. Specific inhibition of PTEN expression by ASO results in the normalization of blood glucose in db/db and ob/ob mice (233). Furthermore, deletion of PTEN in the liver leads to increased insulin sensitivity and improved overall glucose tolerance (234). In addition; mice lacking PTEN in adipose has been shown to be hypersensitive to insulin and resistant to STZ-induced diabetes (235). These studies suggest that PTEN has an important role in the insulin-signaling pathway.

1.6.2-Effect of vanadium on IR and IRS-1 phosphorylation

Since vanadium salts are potent inhibitors of PTPases (236), and increased tyrosine phosphorylation of the IR-β-subunit is critical for its activation (127;129), vanadium has been suggested to cause IR-PTK activation by preventing dephosphorylation of the IR-β-subunit. In fact, an earlier study showed that vanadate stimulated tyrosine phosphorylation of the IR-β-subunit in rat adipocytes (39), and it was also demonstrated that NaOV treatment partially reversed the decreased tyrosine phosphorylation of the IR-β subunit in STZ -diabetic rats without significantly affecting IR-PTK activity in the liver (237). However, subsequent experiments in adipocytes (42) and IR-overexpressing Chinese Hamster Ovary cells (CHO-HIR) (90;238) did not detect any change in IR-β-subunit tyrosine phosphorylation in response to either NaOV or VS (90;238). Similar observations were made in the rat diaphragm where NaOV did not stimulate tyrosine phosphorylation of the IR-β-subunit (41). These and other studies (101;117;239) indicated that tyrosine phosphorylation of the IR-β-subunit and IR-PTK activation may not be early events leading to the insulin-like effects of inorganic vanadium salts (32;240).

Further evidence for the lack of an involvement of IR-PTK in mediating the effect of VS on the phosphorylation of various components of the insulin-signaling cascade has been obtained in CHO cells overexpressing an inactive form of IR-PTK (CHO-1018). The PTK-inactive IR was generated by the mutation of lysine 1018 in the ATP-binding domain of the IR-β-subunit to alanine, which inactivated its ATP-binding ability and therefore PTK

activity (143). Both insulin and VS enhanced the phosphorylation of ERK1/2, PKB and GSK-3β in CHO-HIR overexpressing wild type human IR. However, in CHO-1018 cells, only the insulin response was significantly attenuated, whereas no alteration in VS-induced phosphorylation of these signaling molecules was detected. These data further confirmed that the VS effect was independent of IR-PTK activity. It should be noted that, unlike the inorganic salts of vanadium, pervanadate- or peroxovanadium-mediated insulin-mimetic actions have been associated with IR-PTK activation and enhanced tyrosine phosphorylation of the IR-β subunit (11;42). Similarly, BMOV was found to increase the tyrosine phosphorylation of the IR-β subunit (241). In rat adipocytes, the possible role of a NaOV–stimulated, staurosporine-inhibitable cytosolic and particulate form of PTK in vanadium actions has been suggested (242;243). This PTK has not been characterized in detail, and we do not know if it is unique to adipocytes, or if a similar PTK is expressed in other cell types.

Despite no effect on tyrosine phosphorylation of the IR-β-subunit by vanadium salts, several studies have reported a stimulatory action of VS and NaOV on the tyrosine phosphorylation of IRS-1 (90) and activation of its associated PI3-K activity (87;90). The putative PTK responsible for IRS-1 phosphorylation in response to VS remains unknown at present. However, some authors have reported that vanadium compounds could activate EGF receptor (EGFR) (244;245) and non-receptor PTKs, such as focal adhesion kinase (246), which may contribute to IRS-1 phosphorylation.

1.6.3-Effect of vanadium on MAPK pathway

As stated earlier, NaOV and VS have been shown to stimulate phosphorylation and activation of the MAPK family member ERK1/2 in several cell types, including CHO-HIR (247) and adipocytes (248). VS-induced ERK1/2 activation was associated with sequential stimulation of upstream components of the ERK1/2 signaling pathway, namely, MEK-1, c-Raf and c-Ras (247;248). Interestingly, in these studies, wortmannin and LY294002, both pharmacological inhibitors of PI3-K activity, completely attenuated VS stimulation of the

ERK1/2 pathway (247;248), indicating an essential role of this lipid kinase in propagating VS-induced responses in the Ras/Raf/MEK/ERK1/2 pathway. Recently, the involvement of p38 MAPK, another member of the MAPK family, was demonstrated in vanadium-induced glucose uptake in cardiomyocytes (249).

1.6.4-Effect of vanadium on the PI3-K pathway

A critical role of vanadium-induced PI3-K activation in mediating VS- and NaOV-induced stimulation of glucose uptake (74;250), GLUT-4 translocation(87;251) and glycogen synthesis (89;247) has been suggested in several cell types by using PI3-K inhibitors. Consistent with this suggestion, both VS and NaOV were found to increase the IRS/ p^{85} association, leading to activation of PI3-K activity in CHO-HIR and adipocytes (87;90;250). PKB was also shown to be activated in response to VS in CHO-IR (247), and to NaOV in adipocytes (250), L-6 myotubes (75) and adult cardiomyocytes (251). In CHO-HIR cells, VS enhanced PKB phosphorylation at Ser 473 and Thr 308 residues. Since PKB has been implicated in mediating the physiological response of insulin on glucose uptake and glycogen synthesis, it is possible that vanadium-induced activation of the PI3-K/PKB signaling system is one of the mechanisms that contributes to the insulin-like effects of vanadium compounds. In contrast, some studies have indicated the involvement of a PI3-K-independent pathway in vanadium-induced glucose transport (74;75). The nature of this PI3-K-independent pathway remains elusive at present, but may perhaps involve the CAP/APS/Cbl/TC10-dependent mechanism (134). However, to date vanadium-induced tyrosine phosphorylation of Cbl has not been demonstrated.

As discussed in the above sections, PKB-catalyzed phosphorylation of its downstream substrates, such as FKHR and its orthologs (193;252),as well as GSK-3 (190), results in their inactivation (190;193;252). Since FKHR and GSK-3 inactivation reduces the gene expression of two key gluconeogenic enzymes, PEPCK (197) and G6Pase (253), it may be postulated that the vanadium-induced effect on this pathway contributes to the suppression of gluconeogenesis observed in response to vanadium (55;98;104;105;107-109). Attempts

have also been made to establish correlations between the glucoregulatory response of vanadium and its actions on various components of the insulin-signaling system under in vivo conditions. VS therapy of type 2 diabetic patients was found to modify several components involved in insulin signaling, which included increases in basal IR and IRS-1 phosphorylation, and PI3-K activation in muscle homogenates (63). Similarly, NaMV treatment of diabetic subjects stimulated basal $p70^{s6k}$ and MAPK activity in circulating mononuclear monocytes (110). However, in contrast to these clinical studies, treatment of STZ-diabetic or fa/fa Zucker rats with BMOV was not associated with any significant alteration in the activation status of PI3-K (254), PKB (255) or GSK-3 (96) in skeletal muscle. Such apparent discrepancies between the in vitro and in vivo effects of vanadium on insulin-signaling pathways and the divergent responses observed in rodent versus human investigations into the role of various components of the signaling system in its glucoregulatory capacity remain to be clarified. It may, however, be possible that in vivo and in vitro effects of vanadium are different, and that additional or alternative mechanisms contribute to these actions of vanadium.

1.6.5-PTPases as targets of vanadium action

The precise mechanism by which vanadium stimulates the tyrosine phosphorylation of IRS-1, ultimately leading to activation of the Ras/Raf/MEK/ERK1/2 and PI3-K/PKB signaling systems, is also not known. However, it has been suggested that vanadium salts, by inhibiting PTPase activity, can cause an increase in the phosphotyrosine content of IRS-1 (87;90), and thus stimulate the insulin-signaling pathway (Fig. 9). Support for this notion comes from studies in which in vitro treatment of hepatocytes (256) and hepatoma cells (257) and in vivo therapy of diabetic rodents with various vanadium compounds (239;258;259) were found to inhibit the activities of several PTPase forms, including SH2 domain-containing PTPase (SHP2) (256), PTP1B (259) and total PTPase (239;256-258). In contrast to the effects on isolated cells and diabetic animal models, no significant influence on total PTPase activity was noted in muscle homogenates from VS-treated human subjects (63). The reason for this discrepancy is not clear, but it is possible that

only some specific PTPases are targets for inhibition by vanadium, which may not be detectable in crude tissue extracts containing many different forms of PTPases. Since the expression levels of PTPases, including PTP1B, are impaired in rodent models of diabetes (207), and the reduction of PTP1B by ASO has been shown to improve insulin sensitivity in ob/ob and db/db mice (221), the ability of vanadium to inhibit PTPase activity may be one of the mechanisms by which it exerts insulin-mimetic and anti-diabetic actions. Since PTEN demonstrated to be a potent negative regulator of insulin signaling and insulin sensitivity (233-235), another target of vanadium could be PTEN.

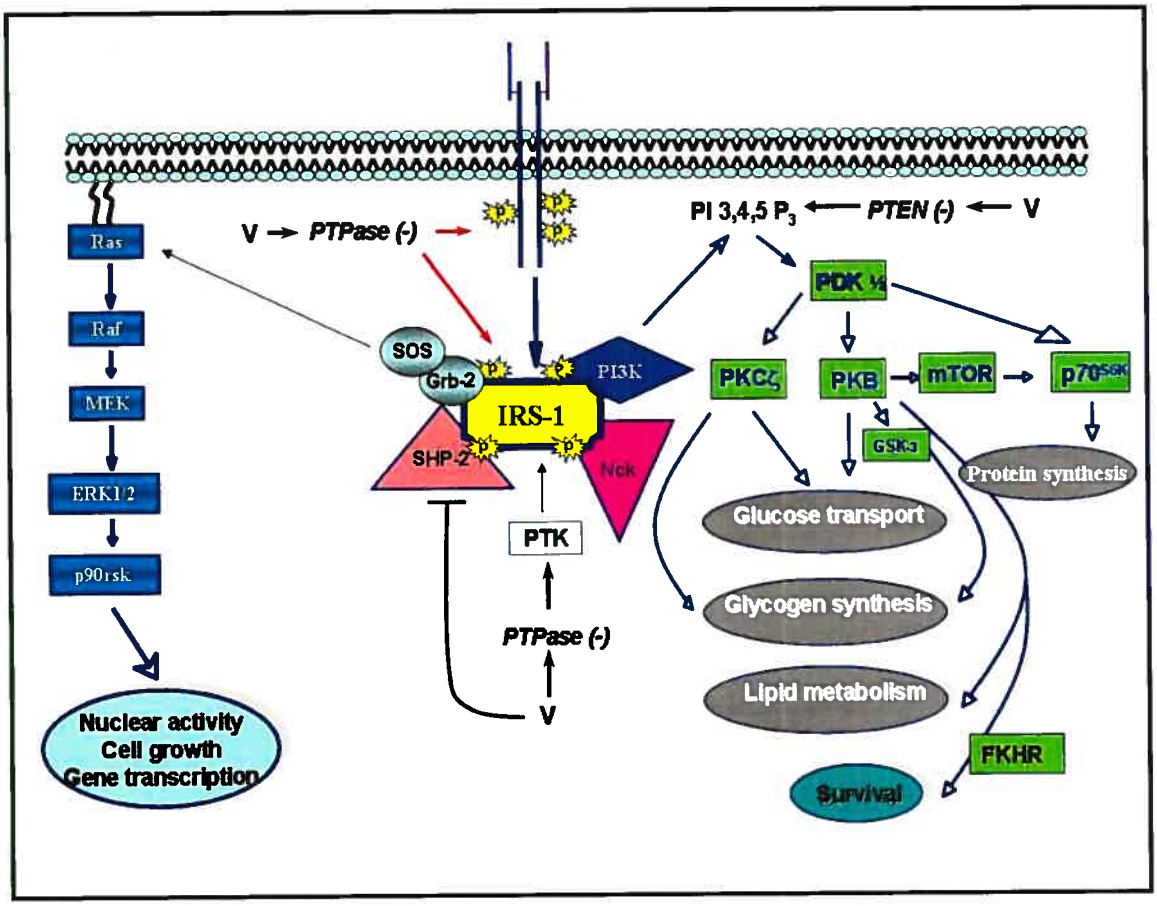

Figure 9: Schematic model showing potential targets of vanadium (V) action in relation to the insulin-signaling cascade

Protein Tyrosine Phosphatases (PTPases) or lipid phosphatase (PTEN) is (are) possible potential targets of vanadium (V). PTPase (e.g. PTP-1B or SHP-2) inhibition is capable of preventing the dephosphorylation of IRS, and thereby increases the tyrosine phosphorylation level. PTEN inhibition could prevent the dephosphorylation of PIP_3, which is important for the activation of PDK1/2 and PKB. (Abbreviations are defined in the list of abbreviations)

* Adapted/Modified from (301).

1.6.6-Reactive oxygen species (ROS) as a potential mediator of vanadium action

Accumulating evidence indicates that reactive oxygen species (ROS; superoxide anion radical $O_2^{\bullet-}$, H_2O_2, and hydroxyl radical $^{\bullet}OH$) may function as intracellular messengers to modulate signaling pathways. The changes of intracellular ROS have been detected in a variety of cells stimulated with cytokines, growth factors, and insulin (260-265). Furthermore, insulin stimulation has been shown to elicit a rapid production of H_2O_2, an important redox molecule that enhances the tyrosine phosphorylation of IR and IRS-1 (264). Exogenous H_2O_2 has also been shown to mimic many physiological effects of insulin such as glucose transport (266), lipogenesis (267) and lipolysis (268). The precise mechanism by which H_2O_2 exerts its insulin-like effects remains unclear; however, its ability to inhibit the activities of many PTPases, such as PTP1B (269), SHP-2 (270) and PTEN (271;272) may contribute as a potential mechanism. Similar to insulin, treatment of cells with vanadium results in the generation of ROS such as $O_2^{\bullet-}$ and H_2O_2 (273;274). The mechanism by which vanadium mediates generation of ROS involved the reduction of molecular oxygen to $O^{\bullet-}_2$ by both a flavoenzyme-containing NADPH complex and the mitochondria electron transport chain during the one-electron vanadate (V(V)) reduction process. The $O^{\bullet-}_2$ in turn generated H_2O_2, which reacted with vanadyl (V(IV)) to generate ·OH radical through a Fenton-type reaction ($V(IV) + H_2O_2 \rightarrow V(V) + \cdot OH + OH^-$) (273;274) (Fig. II /Annexe). A role of ROS as a mediator of vanadium-induced MAPKs phosphorylation in human lung (275) and in rat lung myofibroblasts (276) has been shown by utilisation antioxydants such as N-acety-L-cyteine (NAC) (a general antioxidant) and catalase (a specific H_2O_2 inhibitor). In addition, we have shown that in both CHO-IR and HepG2 cells, Diphenyleniodonium Chloride (DPI), an NADPH oxidase inhibitor, significanlty bolcked VAC-, BMOV- and VS- induced PKB phosphorylation (Fig. I/ Annexe). Moreover, ROS was also shown to contribute to of nuclear factor of activated T cells (NFAT) activation in response to vanadate (277). Therefore, ROS generation induced by vanadium may serve as a potential mechanism for its insulin-like effects.

1.6.7-Other PTKs than the IR as potential targets of vanadium

Evidence has also accumulated to indicate that the activation of many signaling processes requires inter-receptor communication. It is well-established that the stimulation of GPCRs can activate signaling from receptor PTK (R-PTK) by a process termed transactivation. Indeed, in recent years, it has become apparent that transactivation is a general phenomenon that has been demonstrated for many unrelated GPCRs and R-PTKs. For example, the EGFR is a well-characterized target of receptor transactivation, receiving signals from GPCRs, cytokine receptors, and other RTKs (278). Using the human liver cell line HepG2, tyrosine phosphorylation of the EGFR was increased 3-fold upon insulin treatment, most likely due to transactivation by the activated IR and IGF-1R heterodimer complex (279). Thus, it is possible that vanadium-induced signaling responses may be mediated through the activation of other growth factor receptor-PTKs.

In the following sections we will describe some of these PTKs.

1.6.7.1-Epidermal growth factor receptor (EGFR)

The EGFR is a receptor tyrosine kinase that is ubiquitously expressed in different cell types, with the most abundant expression in epithelial cells and many cancer cells. The EGFR belongs to a family containing three other members (ErbB2, ErbB3, and ErbB4) that undergo homodimerization or heterodimerization to induce autophosphorylation and receptor tyrosine kinase activation in response to ligand binding (280). Since Daub et al (281), identified the EGFR to be essential for endothelin-1, thrombin and lysophosphatidic acid -induced MAPK activation and c-fos gene transcription in 1996, several studies have demonstrated that the GPCR mediates EGFR transactivation. In addition to GPCRs, members of the cytokine superfamily have been reported to increase EGFR tyrosine phosphorylation and the EGFR-Grb2 association in the mouse liver in vivo. In this case, the EGFR was directly phosphorylated by Janus tyrosine kinase 2 (Jak2), which couples cytokine stimulation to MAPK activation and c-fos gene transcription via the transactivated EGFR. In addition, several receptor PTKs including platelet-derived growth factor receptor

(PDGFR), IGF-1R and integrins as well as non receptor PTK such as c-Src (282;283) have been shown to transactivate the EGFR (278).

1.6.7.2-Platelet growth factor receptor (PDGFR)

PDGFR is a receptor with intrinsic tyrosine kinase activity that regulates several functions in normal cells and is widely expressed in various malignancies. PDGFRs are expressed on erythroid and myeloid precursors in bone marrow as well as in monocytes, megakaryocytes, fibroblasts, endothelial cells, osteoblasts, and glial cells in (284). PDGFR has two isoforms, PDGFR-α and PDGFR-β, that differentially bind different PDGF members. Ligand binding induces receptor homo- or hetero-dimerization, activation and autophosphorylation of the tyrosine kinase domain (284). PDGFR-α and PDGFR-β also dimerize with EGFR, which can be stimulated by PDGF, and EGF stimulation has been shown to increase the tyrosine phosphorylation of PDGFR-β and subsequent recruitment of PI3-K to the PDGFR.

1.6.7.3-Insulin-like growth factor 1 receptor

IGF-1R is a tyrosine kinase receptor that is structurally very similar to the IR. In fact, in cells that express both receptors, hybrid receptors appear to form readily, although the biological consequence of these hybrids is not clear (285). In response to IGF-1, IGF-1R activation mediates tumor cell proliferation, motility, and protection from apoptosis. Recent work has suggested a role of IGF-1R transactivation in mediating Ang II responses (286;287).

1.6.7.4-c-Src

c-Src protein was the first tyrosine kinase to be discovered (288). c-Src kinase has a structure composed of two binding domains, SH2 and SH3, in addition to a catalytic kinase domain. The N-terminal region is myristylated, so that the protein is associated with the cell membrane. c-Src kinases are normally inactive by an autoinhibitory interaction between the SH2 domain, before the catalytic kinase domain, and a C-terminal phosphotyrosine residue (Tyr 527), while the SH3 domain interacts with the linker region between the SH2

domain and the N-terminal kinase lobe. SH2 and SH3 intramolecular interactions repress kinase activity by displacing the Cα helix in the N-terminal lobe and by positioning the activation loop to block access to the active site. c-Src activation of requires dephosphorylation of phosphorylated Tyr 527 followed by binding through its SH2 domain to specific tyrosine autophosphorylation sites in ligand-stimulated R-PTK, resulting in SH2 displacement from phosphorylated Tyr 527 or by binding of the SH3 domain to Pro-X-X-Pro motifs in target proteins. This actions results in autophosphorylation in trans of the conserved activation loop Tyr 416 and stabilization of the active conformation (288). c-Src can be activated by various extracellular signals through R-PTK, integrins, GPCRs, and cytokine receptors (288). It can modulate a variety of cellular functions, including proliferation, differentiation, survival, adhesion, morphological changes, and migration. c-Src also plays a primordial role as mediator of GPCR-induced EGFR transactivation as well as in cross-talk between different signaling pathways (278). For example, it mediates EGFR transctivation in response to H_2O_2 (282) and PMA (283) and transactivation IGF-1R in response to AngII (287).

1.7-OBJECTIVES OF THE PRESENT STUDY

As discussed above, vanadium compounds have been found to exert anti-diabetic effects in rodent models of type I and type II diabetes mellitus as well as in a limited number of studies in human diabetic subjects. These compounds have been shown to enhance and/or mimic insulin action, which includes increasing glucose transport, glycogen synthesis and lipogenesis as well as inhibition of gluconeogensis and lipolysis. In cellular in vitro system, the molecular mechanism of vanadium compounds correlates with the activation of different components of the insulin signaling pathway, such as PI3-K and MAPK. However, the precise mechanism by which vanadium compounds enhance/mimic the insulin-like effects is still poorly characterized. Thus, the purpose of the present studies was to elucidate the molecular mechanism of vanadium in different circumstances:

1) VS have been demonstrated to enhance insulin action, but the molecular mechanism of the insulin-enhanced effects of these salts is not known. Therefore, the first objective of the present studies was to examine if VS-induced effects on insulin action are associated with enhancement or augmentation in the activation state of key components of the insulin signaling pathway.

2) As discussed in the introduction, the superiority of OVC as antidiabetic agents versus inorganic vanadium compounds is well-documented but the molecular mechanism remains elusive. Therefore, delineating the mechanism of the higher efficiency of OVC over inorganic vanadium compounds was the second objective of our investigations.

3) The third objective was to define the upstream events responsible for activating the insulin signaling pathway by BMOV a widely-studied insulino-mimetic OVC.

4) As mentioned earlier, vanadium-induced production of ROS such as H2O2 has been shown to mediate its cellular effects in many cells. Therefore, the fourth objective

of this study is to examine if H_2O_2 shares some common features with vanadium, in regards to the role of protein tyrosine kinases as modulators of its effect.

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CHAPTER 2

Prolongation of insulin-induced activation of mitogen-activated protein kinases ERK 1/2 and phosphatidylinositol 3-kinase by vanadyl sulfate, a protein tyrosine phosphatase inhibitor [1].

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Subject area : Membrane Biochemistry

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2.1 ABSTRACT

Vanadium salts such as vanadyl sulfate (VS), potent inhibitors of protein tyrosine phosphatases, have been shown to mimic, augment and prolong insulin's action. However, the molecular mechanism of responses to these salts is not clear. In the present studies, we examined if VS-induced effects on insulin action are associated with enhancement or augmentation in the activation state of key components of the insulin signaling pathway. Treatment of insulin receptor-overexpressing cells with insulin or VS resulted in a time-dependent transient increase in phosphorylation and activation of extracellular signal-regulated kinases 1 and 2 (ERK 1/2) that peaked at about 5 min, then declined rapidly to about baseline within 30 min. However, when the cells were treated with VS before stimulation with insulin, sustained ERK 1/2 phosphorylation and activation were observed well beyond 60 min. VS treatment also prolonged the insulin-stimulated activation of phosphatidylinositol 3-kinase (PI3-K), which was associated with sustained interaction between insulin receptor substrate-1 (IRS-1) and the $P^{85\alpha}$ subunit of phosphatidylinositol 3-kinase (PI3-K) in response to insulin. These data indicate that prolongation of insulin-stimulated ERK 1/2 and PI3-K activation by VS is due to a more stable complex formation of IRS-1 and the $P^{85\alpha}$ subunit which may, in turn, be responsible for its ability to enhance and extend the biological effects of insulin.

Keywords: Insulin, Vanadium, Insulino-mimesis, Phosphatidylinositol 3-Kinase, Mitogen-activated protein kinases, Protein tyrosine phosphatases.

2.2-INTRODUCTION

Insulin is the primary hormone involved in glucose homeostasis, and an absolute or relative lack of its secretion or action leads to aberrant glucose metabolism and diabetes. Insulin' action is triggered by binding to its receptor on the cell membrane of target tissues. Insulin binding to the α-subunit of the insulin receptor (IR) results in conformational changes that activate intrinsic protein tyrosine kinase (PTK) activity of the β-subunit by its autophosphorylation in multiple tyrosine residues [1,2]. Once activated, the IR can phosphorylate several cytosolic insulin receptor substrates (IRSs) in tyrosine residues which function as docking proteins for src homology 2 domain-containing signaling proteins (reviewed in [3]). These proteins include growth factor receptor binder 2 (Grb-2)-mammalian son of sevenless (SOS) complex [4], with Grb-2 being growth factor-binding protein, SOS being a guanine nucleotide exchange factor for guanosine triphosphate (GTP)-binding protein, and the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (PI3-K), a lipid and protein kinase [5].

The IRS-associated complex initiates two signaling pathways. In one pathway, binding of the Grb-2-SOS complex to IRS-1 leads to p21ras stimulation by GTP loading with subsequent activation of Raf, mitogen-activated protein kinase kinase (MEK) and two isozymic forms of mitogen-activated protein kinase (MAPK), $p44^{mapk}$ (extracellular signal-regulated kinase-1, ERK-1) and $p42^{mapk}$ (ERK 2) [2,4]. Activated MAPK phosphorylates and activates a downstream ribosomal protein kinase, $p90^{rsk}$ [6].

Another pathway that radiates from the IRS complex upon insulin stimulation involves PI3-K activation [2]. PI3-K phosphorylates phosphatidylinositol lipids at the 3 position of the inositol ring and generates 3-phosphorylated forms of PI such as phosphatidylinositol 3, 4, 5 (PIP_3) [7] which are involved in the activation of PIP_3-dependent kinase (PDK) and related serine/threonine protein kinases responsible for the phosphorylation and stimulation of several downstream signaling protein kinases such as protein kinase B (PKB), $p70^{s6k}$ [8],

protein kinase-zeta [9] and glucocorticoid-inducible kinase [10]. Activation of these serine/threonine protein kinases has been implicated in many of the physiological responses of insulin at the level of glucose transport, glycogen synthesis and protein synthesis [11-17].

Enhanced tyrosine phosphorylation of IR and IRSs is critical for the initiation of insulin signaling, whereas tyrosine dephosphorylation of IR and its substrates can attenuate and terminate insulin's action (reviewed in [18,19]). IR and IRS-1 dephosphorylation is catalyzed in vitro by several protein tyrosine phosphatases (PTPases) that consist of cytoplasmic and transmembrane receptor-type PTPases [19]. A possible role of PTPases in insulin action and in the pathogenesis of diabetes has been postulated on the basis of studies showing that vanadium-based or other small molecule inhibitors of PTPases can improve glucose homeostasis in rodent models of type I and II diabetes mellitus and in a small number of type 2 diabetic human subjects [20-23]. In this regard, it is noteworthy that the expression level of PTPases has been shown to be impaired in rodent models of type 1 and type 2 diabetes mellitus as well as in human subjects [24-27]. Further support for the involvement of PTPase in insulin's action comes from recent studies demonstrating that reduction of the PTPase PTP-1B, by using antisense oligonucleotides, normalizes blood glucose and improves insulin sensitivity in diabetic mice [28]. In addition, the ability of vanadium salts, potent PTPase inhibitors, in lowering hyperglycemia and improving insulin sensitivity in animal models of diabetes mellitus and in human subjects [20,22,29] has generated interest in exploring the molecular mechanisms of vanadium action.

We have shown earlier that vanadium salts, such as sodium orthovanadate and vanadyl sulfate (VS), mimic insulin and activate several key components, such as PI3-K and ras/MAPK, of the insulin signaling pathway [15,30-32]. Sodium orthovanadate has also been reported to enhance and prolong insulin-stimulated activation of IR-PTK and lipogenesis in rat adipocytes [33]. However, the precise signaling steps responsible for these responses to vanadium remain poorly characterized. Therefore, in the present studies, we investigated if the vanadium-induced effects on insulin action are associated with an

enhancement or augmentation in the activation state of ERK 1/2 and PI3-K-two key elements of the insulin signaling pathway.

2.3-MATERIALS AND METHODS

Materials

Chinese hamster ovary cells overexpressing human insulin receptor (CHO-HIR cells) were a kind gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA). Insulin was obtained from Eli Lilly Co. (Indianapolis, IN), VS from Aldrich Chemical Co. (Milwaukee, WI), and polyclonal ERK-2 antibody, monoclonal antiphosphotyrosine antibody (4G10), IR and IRS-1 antibodies from Upstate Biotechnology (Lake Placid, NY). Phospho-specific p44/p42 antibody was from New England BioLabs (Beverly, MA), protein A sepharose beads from Pharmacia Biotech Inc. (Mississauga, Ontario, Canada), and the enhanced chemiluminescense (ECL) detection system kit was from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada). MAPK phophatase-1 (MKP-1) and p^{85} antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Silica gel-60 plates were obtained from Merck & Co. Inc. (Rahway, NJ).

Methods

Cell culture

Cells were maintained in HAM's F-12 medium containing 10% fetal bovine serum. They were grown to confluence in 100 mm plates and incubated in serum-free F-12 medium for 16 h prior to the experiment [15].

ERK activity

ERK catalytic activity was measured as described earlier [31]. Briefly, cells subjected to various experimental treatments were lysed in buffer A (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM Na orthovanadate, 10 mM Na fluoride, 10 mM Na pyrophosphate, 20 nM okadaic acid, 0.5 mM ethylenebis-(oxyethylenenitrilo)-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1% Triton X-100, and the lysates were clarified by centrifugation to remove insoluble material. The clarified lysates,

normalized to contain equal amounts of protein (100 g), were incubated for 4 h at 4°C with 5 µg of ERK-2 antibody pre-adsorbed to protein A-Sepharose beads. The immune complex was collected by centrifugation, followed by washing 3 times with buffer A and once with buffer B (20 mM Hepes (N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid), pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 mM -glycerophosphate), and 40 µl of kinase buffer (25 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 1 M staurosporine, 0.5 mM EGTA and 10 µl myelin basic protein (MBP) (1 mg/ml)) were added. The reaction was initiated by adding 5 µl of 1 mM adenosine 5-triphosphate (ATP) containing 0.5 µCi [µ-^{32}P]-ATP (specific activity 3,000 Ci/mmol; Amersham). After 12 min at 30°C, the reaction was stopped by spotting an aliquot of the supernatant on P-81 filter paper which was washed in 0.5% phosphoric acid and counted for radioactivity [30].

PI3-K assay

The clarified cell lysates were subjected to immunoprecipitation with 2 µg of $p^{85\alpha}$ antibody for 2 h at 4°C, followed by incubation with protein A sepharose for an additional 2 h. The immunoprecipitates were washed and subjected to PI3-K assay, as described earlier [15]. The phosphorylated lipid products were extracted and separated by ascending thin layer chromatography [15]. The radioactivity in the spots corresponding to PI3-phosphate were quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation

The clarified cell lysates, normalized to contain equal amounts of protein (500 µg), were subjected to immunoprecipitation with 1 µg of IRS-1 antibody for 2 h at 4°C, followed by incubation with protein A sepharose for an additional 2 h. Immunoprecipitated IRS-1 was collected by centrifugation, washed 2 times with buffer A and once with phosphate-buffered saline. The IRS-1 immunoprecipitates were electrophoresed on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, transferred to PVDF membranes, and incubated with polyclonal $p^{85\alpha}$ antibody (1:4,000) or a polyclonal IRS-1 antibody (1:2,000). Proteins were detected by a horse

radish peroxidase-conjugated second antibody and visualized with an ECL detection kit [32].

Immunoblotting

The clarified cell lysates normalized to contain equal amounts of protein were electrophoresed on 12% SDS-PAGE under reducing conditions, transferred to PVDF membranes, and incubated with monoclonal antiphosphotyrosine antibody (1:1,000), a polyclonal phospho-specific p44/p42 ERK 1/2 antibody (1:2,000), a polyclonal ERK 1/2 antibody (1:8,000) or a polyclonal MKP-1 antibody (1:500), followed by incubation with a horse radish peroxidase-conjugated second antibody. The antigen–antibody complex was visualized with an ECL detection kit. The immunoblots were quantified by densitometric scanning using NIH ImageJ software [32].

IR and IRS-1 tyrosine phosphorylation

The clarified lysates, normalized to contain equal amounts of protein, were pre-cleared with protein A sepharose for 30 min and incubated with either IR or IRS-1 antibody for 4 h at 4oC. Immunoprecipitates were collected with protein A sepharose, washed 3 times with 50 mM Hepes buffer, pH 7.5, containing 0.1% Triton X-100 and 0.1% SDS, and 2 times with the above buffer without SDS. The immunoprecipitate was solubilized by boiling in 2 x sample buffer for 10 min, electrophoresed on 10% SDS-polyacrylamide gels, and immunoblotted with antiphosphotyrosine antibody (1:1,000) [15]. The blots were visualized with an ECL detection system [32].

2.4-RESULTS AND DISCUSSION

Effect of VS on insulin-stimulated ERK 1/2 activation

ERK 1/2 activation constitutes a key step in the insulin signaling pathway [2], we first examined if VS can prolong the effect of insulin on this signaling component. Therefore, insulin receptor over-expressing CHO-HIR cells were used in these studies, and the activation status of ERK 1/2 was assessed with a phospho-specific antibody of ERK 1/2. This antibody reacts with only phosphorylated (Thr202 and Tyr204) and activated forms of ERK 1/2. As shown in Figure 1A, treatment of CHO-HIR cells with 100 nM insulin resulted in transient activation of ERK 1/2, as judged by enhanced phosphorylation. ERK 1/2 phosphorylation was rapid peaking at 5 min and declining to just above baseline at 15 min, with almost complete dephosphorylation in 2 h. A similar transient increase ERK 1/2 phosphorylation was seen in CHO-HIR cells stimulated with 100 μM VS, except that the intensity of phosphorylation was slightly lower than in insulin-stimulated cells, and a second phase of enhanced phosphorylation was detected at 2 h (Fig. 1B). However, when the cells were pretreated with 100 μM VS for 15 min prior to stimulation with 100 nM insulin, a rise in ERK 1/2 phosphorylation was evident as early as 1 min after the addition of insulin, with a peak at 2 min (Fig. 1C). In these cells, the level of ERK 1/2 phosphorylation declined slightly at 15 min, but was restored to just below peak levels at the 30- to 120-min time points (Fig. 1C). It is noteworthy that at all the time points tested, the magnitude of ERK 1/2 phosphorylation was significantly more robust than that observed with either insulin (Fig. 1A) or VS alone (Fig. 1B). The reason for this biphasic response remains unexplained at this moments and would require additional work. As shown in the bottom panel of the immunoblots, none of the experimental conditions altered the total amount of ERK 1/2.

In contrast to CHO-HIR cells, the effect of vanadate on ERK 1/2 phosphorylation and activation in rat adipocytes was not transient but remained sustained for up to 20 min [34].

In these studies however, no attempts were made to investigate if at later time points this response was modified [34]. Furthermore, vanadate did not exert any enhancing effect on insulin-stimulated ERK 1/2 activation in adipocytes during the 20 min time period of the study [34]. The reason for these discrepancies are not clear but it may possible that vanadium exerts a cell-type specific response.

To determine if the pattern of ERK 1/2 phosphorylation observed in the experiments described above correlated with the catalytic phosphokinase activity of ERKs, lysates prepared from cells stimulated for the same time periods with insulin, VS and VS followed by insulin were assayed using MBP as exogenous substrate [30]. As seen in Figure 2, similar to the effect on ERK 1/2phosphorylation, ERK 1/2 catalytic activity in response to insulin, VS as well as VS plus insulin (VS followed by insulin treatment) peaked at 5 min, then declined to just above baseline in insulin- and VS-stimulated cells (Figs. 2A and 2B). It is noteworthy that in VS-stimulated cells, there was a gradual recovery after 60 min which reached about 80% of peak stimulation at 2 h (Fig. 2B). This recovery of ERK 1/2 activation correlated with the second phase of enhanced ERK 1/2 phosphorylation in response to VS (Fig. 1B). Thus, in contrast to insulin, VS exhibited a second phase of ERK 1/2 phosphorylation and activation. The reason for this second phase of activation is not clear but may be attributed to the generation of peroxovanadium due to the production of reactive oxygen species (ROS). Vanadyl is known to react spontaneously with oxygen to generate superoxide anion (O_2^-), a ROS [35] leading to the formation of peroxovanadyl complexes [36]. We have tested a potential role of ROS generation in VS action by utilizing diphenylene iodonium (DPI), an inhibitor of NADPH oxidase and ROS scavenger. Pretreatment of cells with DPI prior to stimulation with VS and insulin was found to attenuate ERK 1/2 phosphorylation (data not shown). Recent studies have demonstrated that ROS generation plays a critical role in activation of the insulin signaling pathway [37]. Therefore, it is possible that increased ROS generation after long incubation with VS might be responsible for the second phase of ERK 1/2 phosphorylation.

In VS + insulin-treated cells, the decline in ERK 1/2 catalytic activity was minimal (about 10%), and this level of activation (about 80-90% of the peak value) was sustained for up to 2 h of incubation (Fig. 2C). On the other hand, the level of ERK 1/2 activation in cells stimulated with insulin only declined after 15 min and remained at about baseline (Fig. 2A). Thus, pretreatment of cells with VS prolonged the duration of ERK 1/2 activation in response to insulin.

Effect of insulin on MKP-1 expression

MKP-1 is a dual specificity phosphatase which dephosphorylates MAPK at both the tyrosine and threonine residues necessary for ERK 1/2 activation [38,39]. Stimulation of fibroblasts overexpressing the human insulin receptor by insulin has been shown to increase MKP-1 mRNA and protein levels, resulting in the attenuation of MAPK activity [40,41]. Therefore, we investigated if VS was exerting its effect by altering MKP-1 expression in CHO-HIR cells. As shown in Figure 3A, MKP-1 protein expression was detectable even in unstimulated cells, and this level of expression was not changed significantly by insulin treatment up to 60 min. A similar pattern of MKP-1 protein expression was detected in cells treated with VS followed by insulin (Fig. 3B). It thus appears that alteration in MKP-1 expression may not contribute to the observed effects of VS on sustained ERK 1/2 phosphorylation in CHO-HIR cells by insulin. Since MKP-1 expression in response to insulin is transcriptionally regulated [40], we sought to determine if blocking MKP-1 transcription by actinomycin-D, an inhibitor of RNA synthesis, would modify the ERK 1/2 phosphorylation pattern in response to either insulin alone or VS plus insulin. Actinomycin-D pretreatment, however, did not affect the time course of ERK 1/2 phosphorylation (data not shown). A similar result was observed with cycloheximide, a protein synthesis inhibitor (data not shown). These results suggest that the rapid decline in ERK 1/2 phosphorylation by insulin was not transcriptionally or translationally regulated. In addition, our earlier studies demonstrated that the effect of VS on ERK 1/2 phosphorylation was mediated by activation of MEK, which is an upstream kinase responsible for phosphorylating the threonine and tyrosine residues of ERK 1/2 [32]. Thus,

it appears that in CHO-HIR cells, the outcome of VS on ERK 1/2 phosphorylation and activation is achieved by enhanced MEK phosphorylation and not by inhibiting its dephosphorylation by MKP-1. This notion is supported by the studies in which VS or vanadate was found to enhance MEK activation in CHO-HIR cells [32] and MEK phosphorylation in rat adipocytes [34].

Effect of VS on insulin-stimulated PI3-K activation

We have shown earlier that VS-induced stimulation of ERK 1/2 phosphorylation and activation is not due to decreased ERK 1/2 dephosphorylation, but is mediated by sequential stimulation of elements upstream of the ERK 1/2 activation cascade [32]. VS or vanadate has also been found to stimulate PI3-K activity in CHO-HIR cells [32] and in adipocytes [34,42,43] and the use of PI3-K inhibitors has indicated that PI3-K is an upstream regulator of the ERK 1/2 activation cascade in these cells [32]. We, therefore, investigated if prolongation of insulin-stimulated ERK 1/2 activation by VS is associated with sustained activation of PI3-K activity. PI3-K activity in cells treated for various time periods with insulin, VS and VS followed by insulin was measured in $p^{85\alpha}$ immunoprecipitates from cell lysates [32]. As shown in Figure 4A, insulin stimulated PI3-K activity in a time-dependent manner. PI3-K activation peaked within 1 min of exposure of cells to insulin, and gradually declined to just above baseline at 30 min. This low level of activity was maintained for up to 120 min. A similar pattern of PI3-K activation in response to VS was observed except that the peak was detected only after 5 min of treatment of cells with VS and was slightly less robust as compared to insulin alone (Fig. 4B). Furthermore, VS-induced activation of PI3-K was biphasic, declining to just above baseline at 30 min but showing reactivation at 60 and 120 min of incubation (Fig. 4B). It should be noted that the biphasic impact of VS on PI3-K activation was similar to its effect on ERK 1/2 phosphorylation and activation (Figs. 1B and 2B). In contrast to the transient action of insulin, and the biphasic outcome of VS, analysis of PI3-K activity in cells treated with VS prior to stimulation with insulin revealed that VS pretreatment prolonged the duration of insulin action, and resulted in sustained PI3-K activation (Fig. 4C). The

prolongation of insulin-stimulated PI3-K activation by VS (Fig. 4C) correlated well with the effect of VS on insulin-induced ERK 1/2 phosphorylation and activation (Figs. 1C and 2C). However, one notable difference was that despite a sustained activation of PI3-K observed in VS plus insulin treated cells. ERK 1/2 phosphorylation still declined slightly at 15 min (Fig. 1C). The precise reason for this decline is not clear but may be attributed to its rapid dephosphorylation by ERK 1/2-specific PTPases other than MKP-1. Alternatively a potential role of a PI3-K-independent pathway in this response may be suggested, however, it should be noted that pharmacological inhibition of PI3-K activity has been shown to completely abrogate ERK 1/2 activation and phosphorylation induced by insulin and VS [32,34].

Effect of VS on insulin-induced association of $p^{85\alpha}$ with IRS-1

Since PI3-K activation requires association of its $p^{85\alpha}$ regulatory subunit with IRS-1, we next determined if the sustained PI3-K activation by insulin in VS-pretreated cells was due to a prolonged association of $p^{85\alpha}$ with IRS-1. As shown in Fig. 5A, insulin induced an increase in the association of IRS-1 with $p^{85\alpha}$ within 5 min of treatment, which peaked at 15 min and then decreased to almost basal levels within 60 min. Similar to insulin, treatment of cells with VS alone resulted in complex formation between $p^{85\alpha}$ and IRS-1 which peaked at 5 min (Fig. 5B). However, as compared to insulin treated cells, in VS treated cells, the magnitude of $p^{85\alpha}$/IRS-1 association was less and, a biphasic effect was observed i.e. a slight decline in $p^{85\alpha}$/IRS-1 association at 15 min followed by an enhancement in $p^{85\alpha}$ binding to IRS-1 at 30 min and 60 min (Fig. 5B). On the other hand, in cells pretreated with VS, insulin–induced association of $p^{85\alpha}$ with IRS-1 was sustained for up to 60 min (Fig. 5C). Thus, it is possible that the $p^{85\alpha}$/IRS-1complex is more stable in the presence of VS and contributes to prolongation of PI3-K activity by insulin (Fig. 4C)

Effect of VS on insulin-stimulated tyrosine phosphorylation of IR and IRS-1

Since the insulin response is mediated through enhancement in the tyrosine phosphorylation of IR and IRS-1 [2,7], we examined the effect of VS on insulin-stimulated tyrosine phosphorylation of IR and IRS-1. Insulin treatment of cells resulted in a rapid increase in phosphotyrosine content of IR (Fig. 6A) and IRS-1 (Fig. 6C). Maximum IR and IRS-1 phosphorylation was detected within 1 min of insulin stimulation, and this level did not change significantly (Fig. 6) for up to 120 min. It is interesting that the decline in ERK 1/2 and PI3-K activation observed in insulin-stimulated cells at 15 min (Figs. 1A, 2A and 4A) was not associated with a similar decrease in the tyrosine phosphorylation of either IR or IRS-1 (Fig. 6). In fact, at this time point, the global tyrosine phosphorylation of IR or IRS-1 was almost the same as that occurring at 1 min after insulin stimulation. Thus, it appears that enhanced tyrosine phosphorylation of IR and IRS-1 is critical for initiating the insulin signal, but may not have a significant role in regulating the duration of the response. Since VS pretreatment of cells prior to stimulation with insulin failed to alter the global tyrosine phosphorylation status of IR (Fig. 6B) or IRS-1 (Fig. 6D), while prolonging the duration of insulin-induced ERK 1/2 and PI3-K activation, it is possible that VS exerts these effects by inhibiting the dephosphorylation of specific phosphotyrosines in IR or IRS-1 or other proteins acting upstream of PI3-K in the insulin signaling pathway. One consequence of this effect is the sustained association of IRS-1 with the $p^{85\alpha}$ subunit and PI3-K activation. In contrast to the work presented here, vanadate was found to augment insulin-stimulated IR-PTK activity in rat adipocytes [33]. The reason for this discrepancy is not clear but it is possible that IR overexpressing cells respond differently than normal adipocytes. Further work is needed to define the precise mechanism by which VS modifies insulin-induced signaling pathways.

In summary, an important finding of the present study is that VS, a PTPase inhibitor and insulinomimetic agent, increased the magnitude and duration of insulin-stimulated ERK 1/2 and PI3-K activation. This effect of VS on insulin response was associated with an enhanced interaction of IRS-1 with the $p^{85\alpha}$ subunit of PI3-K which appeared to be

responsible for a sustained activation of PI3-K. Since PI3-K activation has been implicated in insulin-induced activation of glucose transport and lipogenesis, the results of the present investigation provide a mechanistic basis for the observed augmentation of insulin-induced lipogenesis [33] and glucose transport [44] by vanadate. Taken together, the present work provides first evidence suggesting that prolonged activation of ERK 1/2 and PI3-K, key components of the insulin signaling pathway, contributes to the molecular mechanism of the insulin-enhancing effect of this vanadium.

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2.6-FIGURE LEGENDS

Figure 1: VS delays the dephosphorylation of ERK 1 and ERK 2 in insulin-stimulated cells. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin (A), 100 μM VS (B) or 100 μM VS for 15 min followed by 100 nM insulin (C) for the indicated times. The cells were lysed and lysates subjected to immunoblotting using phospho-specific (Thr202/Tyr204)-ERK 1/2 antibodies (upper immunoblot) or total ERK 1/2 (lower immunoblot). The arrows indicate the position of phosphorylated, p-ERK 1/2 or total ERK 1/2. The bottom panel shows the densitometric quantification of phospho-ERK 1/2 bands. The results are representative of 3 independent experiments and are expressed as a percentage of the maximum.

Figure 2: VS prolongs the duration of insulin-induced ERK 1/2 activation. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin (A), 100 μM VS (B) or 100 μM VS for 15 min followed by 100 nM insulin (C) for the indicated times. The cells were lysed and lysates were analyzed for the phosphokinase activity of ERKs using MBP as exogenous substrate. Parallel samples were analyzed by immunoblotting to determine the total amount of ERK 1/2 and a representative blot is shown in the bottom panel. The results are mean ± SE from 3 separate experiments and are expressed as a percentage of the maximum.

Figure 3: MKP-1 levels are not modified by insulin or VS plus insulin.. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin (A) or 100 μM VS for 15 min followed by 100 nM insulin (B) for the indicated times. The cells were lysed, and the lysates subjected to immunoblotting using MKP-1 antibody. A representative immunoblot from 3 independent experiments is shown.

Figure 4: VS prolongs the duration of insulin-induced activation of phosphatidylinositol 3-kinase (PI3-K). Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin (A), 100 μM VS (B) or 100 μM VS for 15 min followed by

100 nM insulin (C) for the indicated times. The cells were lysed, and the cell lysates were subjected to immunoprecipitation using $p^{85\alpha}$ antibodies. PI3-K activity was measured in $p^{85\alpha}$ immunoprecipitates with phosphatidylinositol (PI) as substrate and [γ-^{32}P] ATP as phosphoryl group donor. The arrow marks the position of PI3-P. Parallel samples were analyzed by immunoblotting to determine the total amount of $p^{85\alpha}$ and a representative blot is shown in the middle panel. The results are representative of 3 separate experiments and are expressed as a percentage of the maximum.

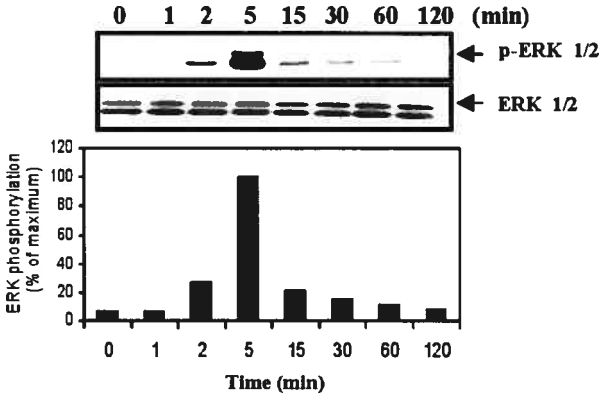
Figure 5: VS prolongs the duration of insulin-induced binding of $p^{85\alpha}$ to IRS-1. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin alone (A), 100 μM VS (B) or 100 μM VS for 15 min followed by 100 nM insulin (C) for the indicated times. The cells were lysed and equal amounts of clarified lysates subjected to immunoprecipitation using IRS-1 antibodies. IRS-1 immunoprecipitates (IP) were immunoblotted (IB) with either anti- $p^{85\alpha}$ (upper panel) (1:4,000) or with IRS-1 antibody (middle panel) (1:1,000). The bottom panel shows the densitometric quantification of $p^{85\alpha}$ bound to IRS-1. The results are representative of 3 independent experiments and are expressed as fold increase over controle (0 min) which was aribitrarily set set at 1.0

Figure 6: IR and IRS-1 phosphorylation on tyrosine are not altered by insulin or VS plus insulin. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin alone (A, C), or with 100 μM VS for 15 min followed by 100 nM insulin (B, D) for the indicated times. The cells were lysed and equal amounts of clarified lysates subjected to immunoprecipitation using IRS-1 or IR antibodies. The immunoprecipitates (IP) were immunoblotted with antiphosphotyrosine antibody (upper panels) or IR or IRS-1 antibodies (lower panels in each section). A representative immunoblot from 3 independent experiments is shown.

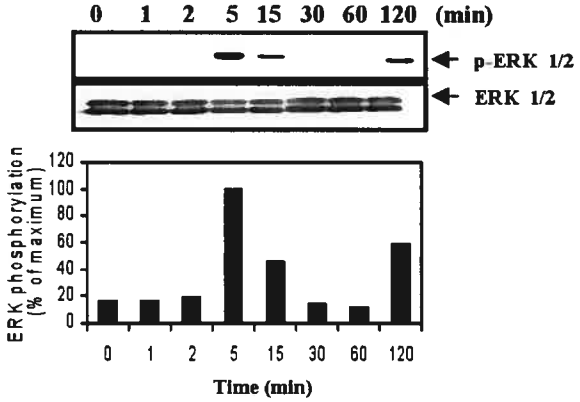
Abbreviations

ATP, adenosine 5-triphosphate; CHO-HIR, Chinese hamster ovary cells overexpressing human insulin receptor; DPI, diphenylene iodonium; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EGTA, ethylenebis-(oxyethylenenitrilo)-tetraacetic acid; ERK, extracellular signal-regulated kinases; Grb, growth factor receptor binder; GTP, guanosine triphosphate; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; IR, insulin receptor; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK kinase; MKP-1, MAPK phosphatase-1; PAGE, polyacrylamide gel electrophoresis; PI3-K, phosphatidylinositol 3-kinase; PTK, protein tyrosine kinase; PTPases, protein tyrosine phosphatases; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOS, mammalian son of sevenless; VS, vanadyl sulfate.

A


0 1 2 5 15 30 60 120 (min)
p-ERK 1/2
ERK 1/2
ERK phosphorylation (% of maximum)
120
100
80
60
40
20
0
0 1 2 5 15 30 60 120
Time (min)

B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p-ERK 1/2
ERK 1/2
ERK phosphorylation (% of maximum)
120
100
80
60
40
20
0
0 1 2 5 15 30 60 120
Time (min)

C

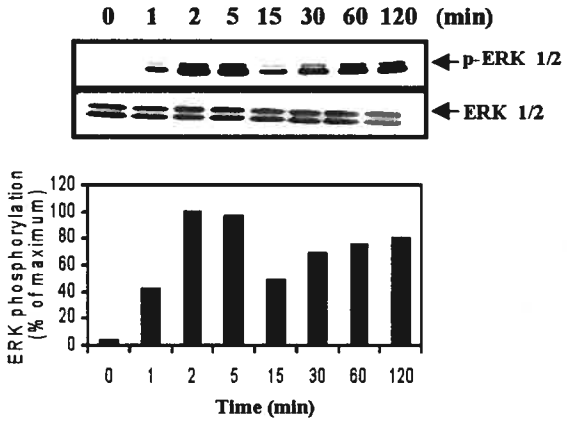
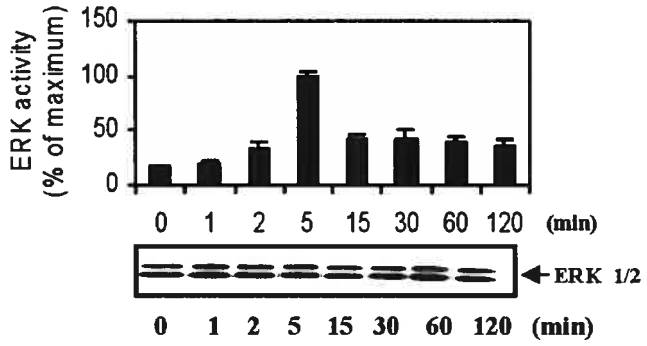
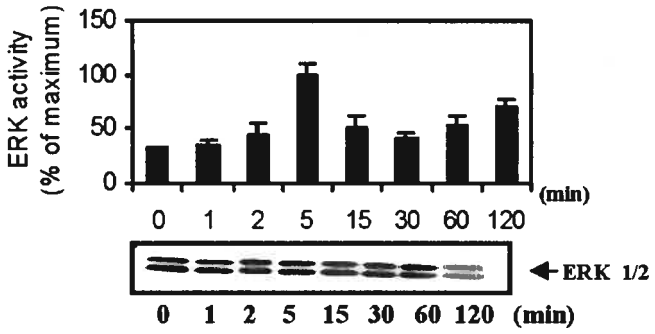

0 1 2 5 15 30 60 120 (min)
p-ERK 1/2
ERK 1/2
ERK phosphorylation (% of maximum)
120
100
80
60
40
20
0
0 1 2 5 15 30 60 120
Time (min)

Figure 1

A


ERK activity
(% of maximum)
150
100
50
0
0 1 2 5 15 30 60 120 (min)
ERK 1/2
0 1 2 5 15 30 60 120 (min)

B


ERK activity
(% of maximum)
150
100
50
0
0 1 2 5 15 30 60 120 (min)
ERK 1/2
0 1 2 5 15 30 60 120 (min)

C

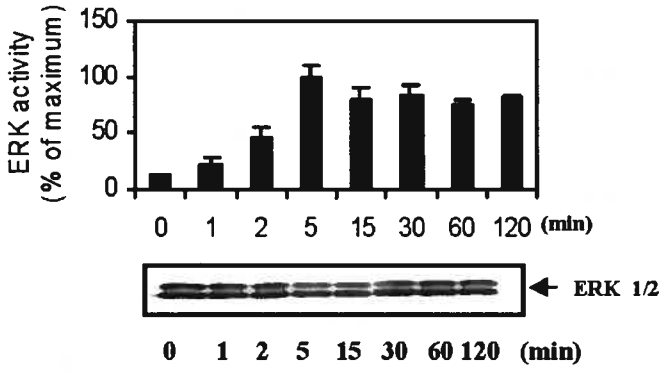

ERK activity
(% of maximum)
150
100
50
0
0 1 2 5 15 30 60 120 (min)
ERK 1/2
0 1 2 5 15 30 60 120 (min)

Figure 2

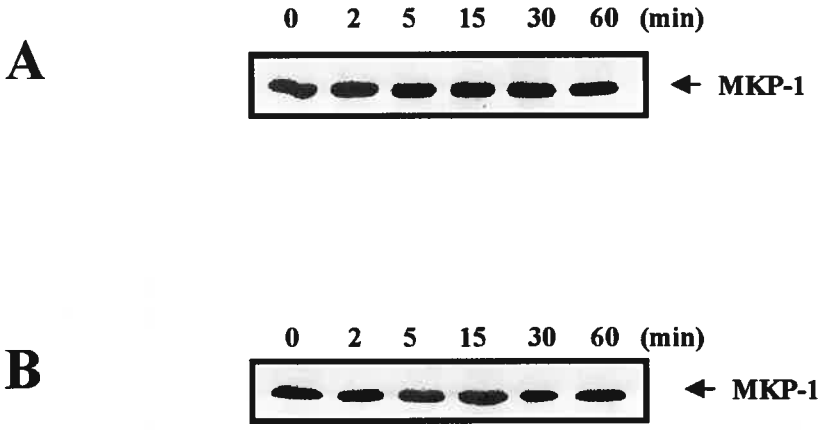


Figure 3

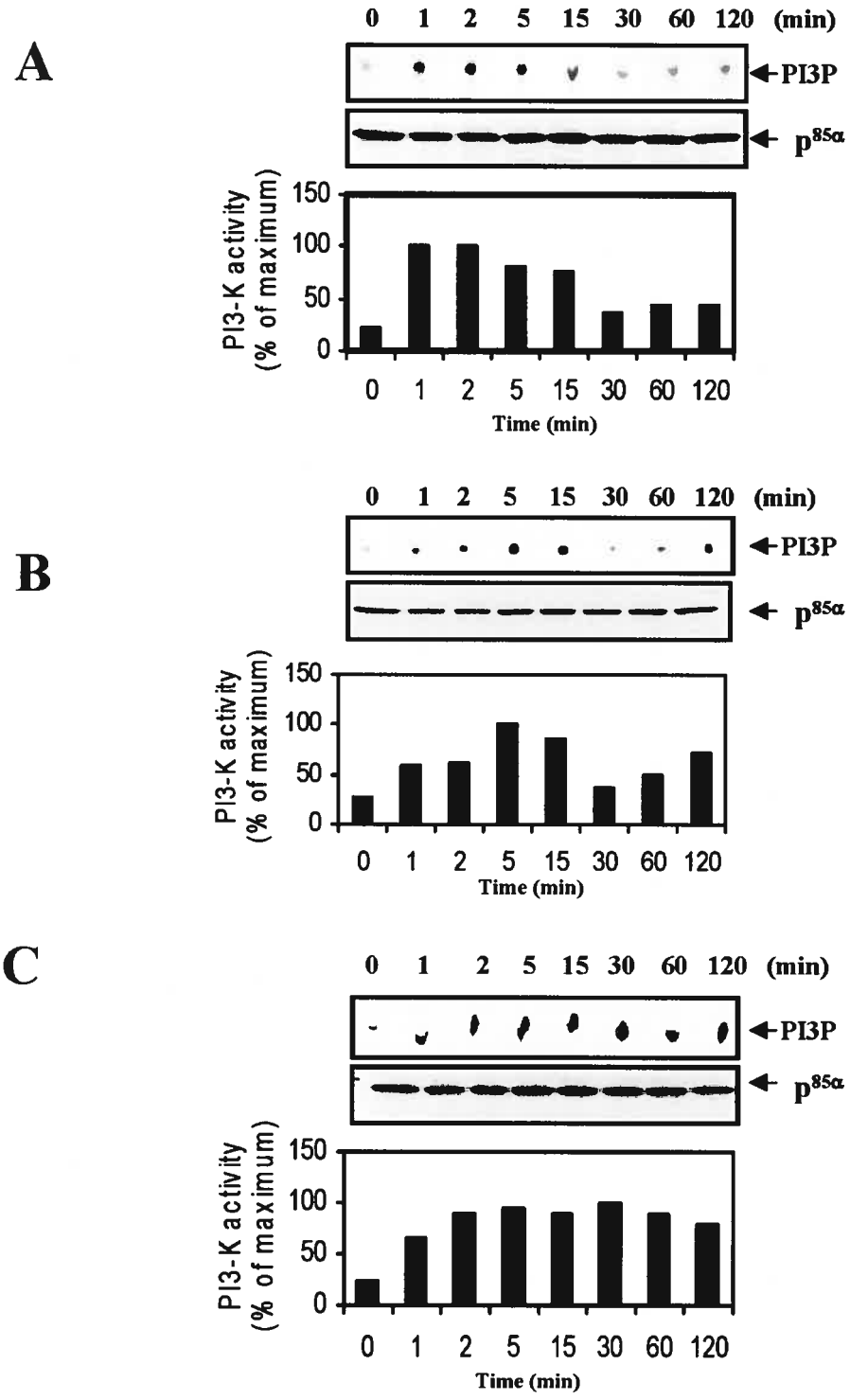

A
0 1 2 5 15 30 60 120 (min)
PI3P
p85α
PI3-K activity (% of maximum)
150
100
50
0
0 1 2 5 15 30 60 120
Time (min)
B
0 1 2 5 15 30 60 120 (min)
PI3P
p85α
PI3-K activity (% of maximum)
150
100
50
0
0 1 2 5 15 30 60 120
Time (min)
C
0 1 2 5 15 30 60 120 (min)
PI3P
p85α
PI3-K activity (% of maximum)
150
100
50
0
0 1 2 5 15 30 60 120
Time (min)

Figure 4

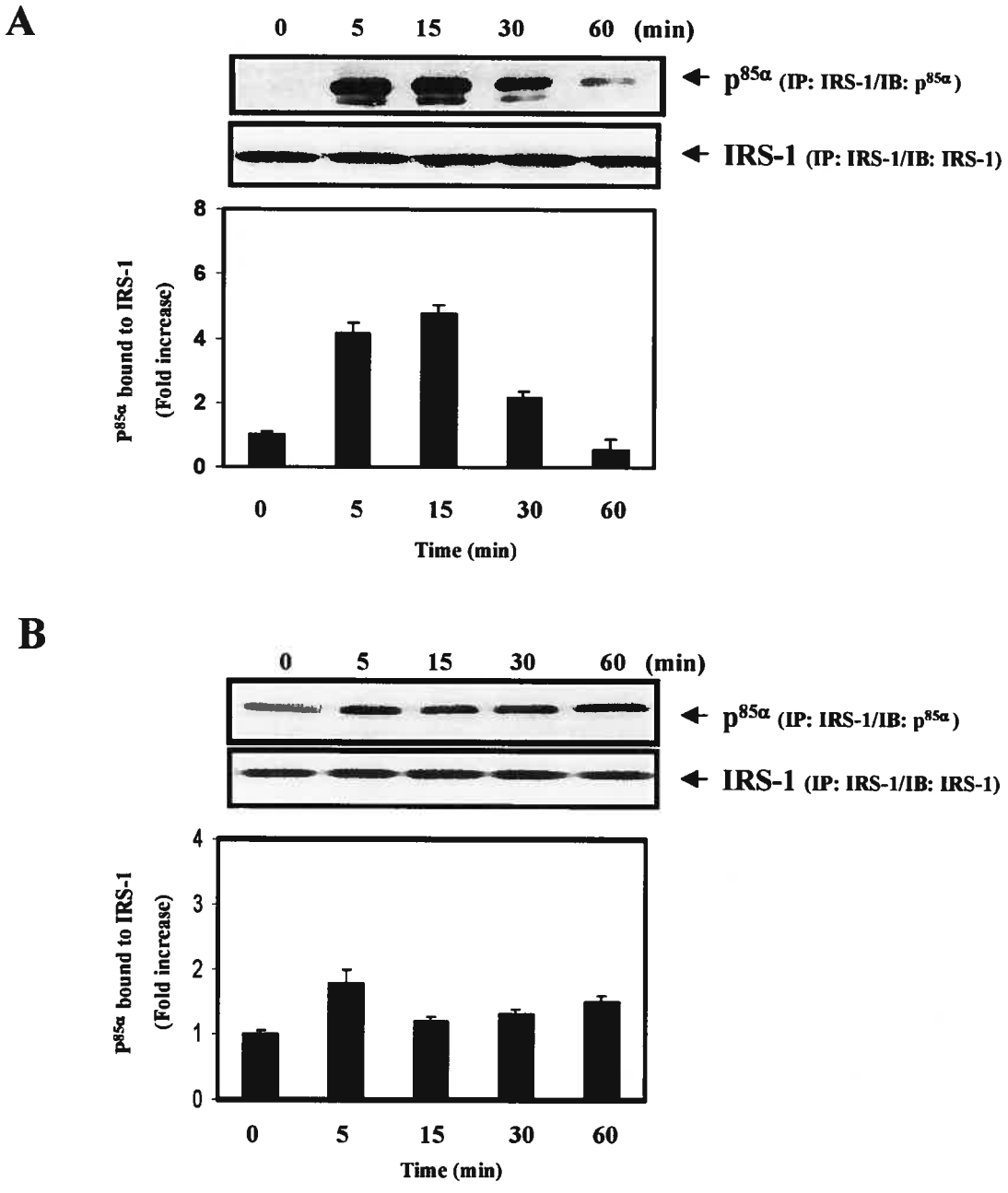

A
0 5 15 30 60 (min)
p85α (IP: IRS-1/IB: p85α)
IRS-1 (IP: IRS-1/IB: IRS-1)
p85α bound to IRS-1 (Fold increase)
0 2 4 6 8
0 5 15 30 60
Time (min)
B
0 5 15 30 60 (min)
p85α (IP: IRS-1/IB: p85α)
IRS-1 (IP: IRS-1/IB: IRS-1)
p85α bound to IRS-1 (Fold increase)
0 1 2 3 4
0 5 15 30 60
Time (min)

Figure 5

C

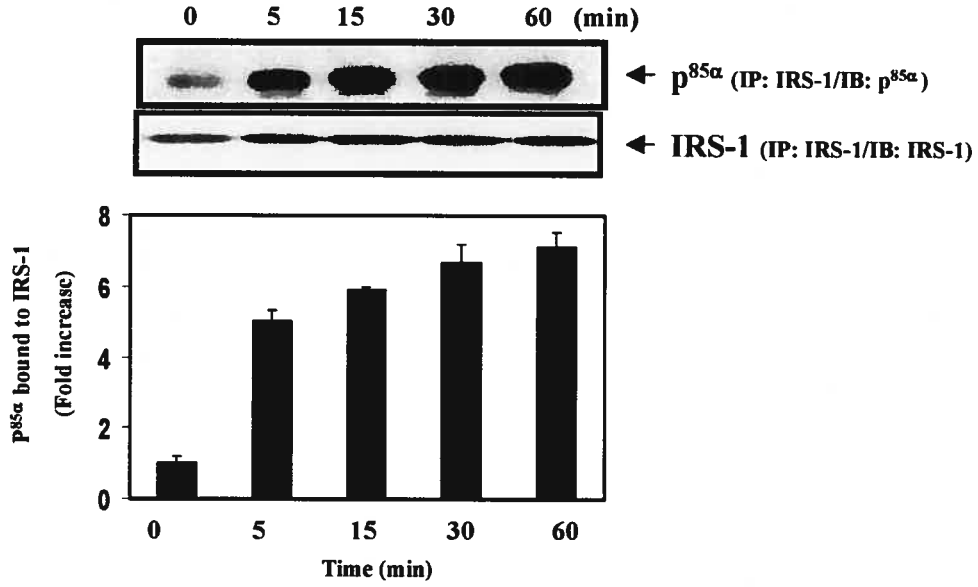

0 5 15 30 60 (min)
p85α (IP: IRS-1/IB: p85α)
IRS-1 (IP: IRS-1/IB: IRS-1)
p85α bound to IRS-1 (Fold increase)
0
2
4
6
8
0 5 15 30 60
Time (min)

Figure 5

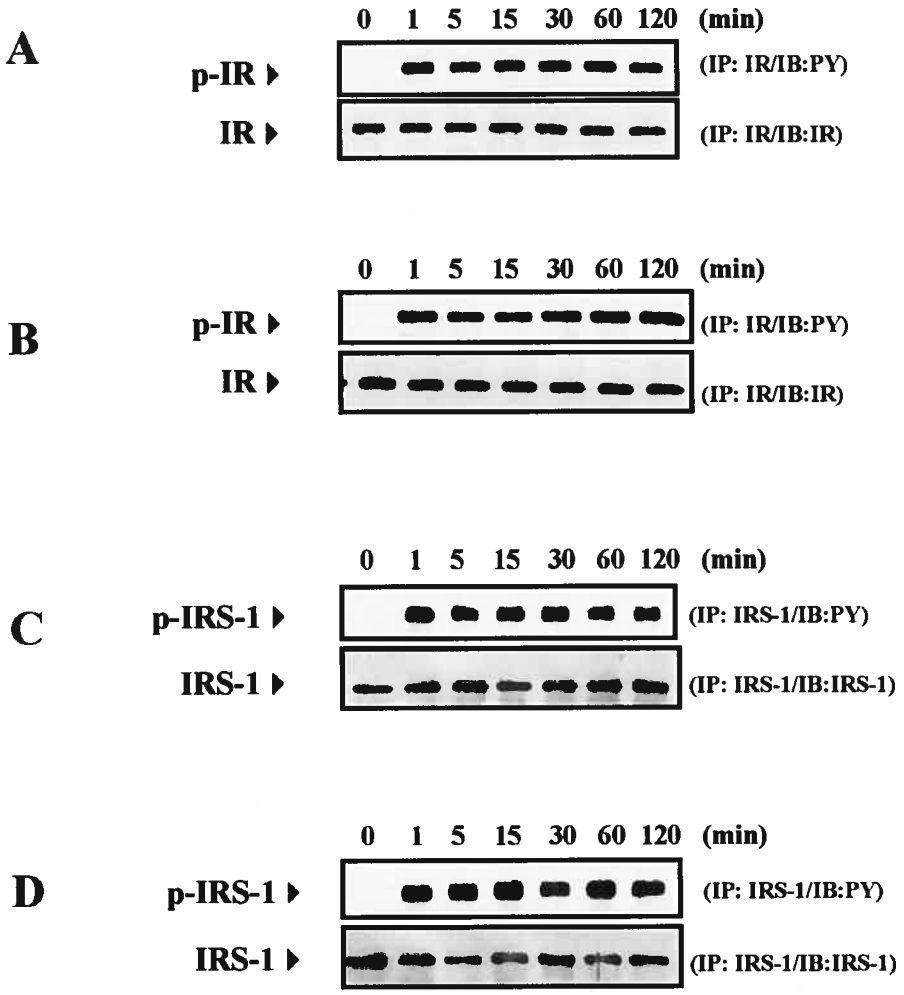

A
0 1 5 15 30 60 120 (min)
p-IR
(IP: IR/IB:PY)
IR
(IP: IR/IB:IR)
B
0 1 5 15 30 60 120 (min)
p-IR
(IP: IR/IB:PY)
IR
(IP: IR/IB:IR)
C
0 1 5 15 30 60 120 (min)
p-IRS-1
(IP: IRS-1/IB:PY)
IRS-1
(IP: IRS-1/IB:IRS-1)
D
0 1 5 15 30 60 120 (min)
p-IRS-1
(IP: IRS-1/IB:PY)
IRS-1
(IP: IRS-1/IB:IRS-1)

Figure 6

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CHAPTER 3

Organo-vanadium compounds are potent activators of the protein kinase B signaling pathway and protein tyrosine phosphorylation: Mechanism of insulinomimesis

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3.1-ABSTRACT

Organo-vanadium compounds (OVC) have been shown to be more effective than inorganic vanadium compounds in ameliorating glucose homeostasis and insulin resistance in rodent models of diabetes mellitus. However, the precise molecular mechanism OVC efficiency remains poorly defined. Since inorganic vanadium compounds have been found to activate several key components of the insulin signaling cascade, such as protein kinase B (PKB), the objective of the present study was to investigate if stimulation of PKB and its downstream targets glycogen synthase kinase-3 (GSK-3), are responsible for the more potent insulinomimetic effects of OVC. Among several vanadium compounds tested, vanadium (IV) oxo bis (acetylacetonate) and vanadium (IV) oxo bis(maltolato) markedly induced the phosphorylation of PKB as well as GSK-3β compared to vanadyl sulfate (VS), an inorganic vanadium salts in Chinese hamster ovary cells overexpressing the insulin receptor (IR). Furthermore, the OVC were stronger inhibitors of protein tyrosine phosphatase (PTPase) activity than VS. The higher PTPase inhibitory potential of the OVC was associated with more robust tyrosine phosphorylation of several cellular proteins, including the IRβ subunit and insulin receptor substrate-1 (IRS-1). In addition, greater IRS-1/$p^{85\alpha}$ interaction was elicited by the OVC than by VS. These data indicate that the higher PTPase inhibitory potential of OVC translates into greater phosphorylation of PKB, GSK-3β, which, in turn, may contribute to a more potent effect of OVC on glucose homeostasis.

Keywords

Diabetes; insulin; vanadium; protein kinase B; protein tyrosine phosphatase

Abbreviations

BMOV, vanadium (IV) oxo bis(maltolato); Crdipic, chromium (III) bis(2,6-pyridinecarboxylate); Co2dipic, cobalt (II) bis(2,6-pyridinecarboxylate) heptahydrate; CHO-HIR, Chinese hamster ovary cells overexpressing human insulin receptor; ECL, enhanced chemiluminescence; FKHR, forkhead transcription factor; GSK-3, glycogen synthase kinase-3; G6Pase, glucose-6-phosphatase; HircA, rat 1 fibroblast overexpressing human insulin receptor and PTP1B, IR; insulin receptor; IRS-1, insulin receptor substrate-1; NaMV, sodium metavanadate; NaOV, sodium orthovanadate; OVC, organo-vanadium compounds; PEPCK, phosphoenolpyruvate carboxykinase; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PTP1B, protein tyrosine phosphatase-1B; PY99, monoclonal antiphosphotyrosine antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STZ, streptozotocin; VAC, vanadium (IV) oxo bis (acetylacetonate); VS, vanadyl sulfate

3.2-INTRODUCTION

Compounds of the trace element vanadium have been demonstrated to exert a variety of insulin-like effects in in vitro and in vivo systems [1], including their ability to improve glucose homeostasis and insulin resistance in animal models of diabetes mellitus [1-3]. In addition, several reports have documented vanadium therapy-induced improvements in liver and muscle insulin sensitivity in a number of type II diabetic human subjects [4-8]. Vanadium compounds have also been shown to stimulate glucose uptake [9-11], glycogen synthesis [9, 12-15] and lipid synthesis [16, 17] in muscle, adipose and hepatic tissues. They inhibit gluconeogenesis, the activities of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in the liver and kidneys [9, 18-20] as well as lipolysis [17, 21] in fat cells. At the cellular level, vanadium activates key elements of the insulin signaling system: tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) [22] and activation of pathways such as extracellular signal-regulated kinase (ERK1/2) [23, 24], phosphatidylinositol 3-kinase (PI3-K) [22, 25] and proteine kinase B (PKB) [23, 26]. These effects are believed to be secondary to its ability to inhibit protein tyrosine phophatase (PTPase) activity [27].

PKB plays a critical role in insulin-induced glucose transport, glycogen synthesis, proliferation and cell survival [28]. It is phosphorylated on serine 473 and threonine 308 residues by PI3-K-dependent kinase [28, 29] resulting in its full activation. Activated PKB phosphorylates several downstream targets, such as glycogen synthase kinase-3 beta (GSK-3β) and forkhead transcription factor (FKHR). GSK-3β is phosphorylated by PKB on serine 9, and this phosphorylation leads to the inhibition of its catalytic activity [30]. GSK-3β plays an important role in the regulation of glycogen synthesis and regulates gluconeogenesis by altering the gene expression of PEPCK and G6Pase [31]. Vanadium treatment has been found to decrease the heightened expression and/or activity of PEPCK and G6Pase in isolated cells as well as in diabetic rats [18-20, 32, 33]. Recent studies have revealed that in comparison to inorganic vanadium compounds, such as vanadyl sulfate (VS), sodium metavanadate (NaMV) and sodium orthovanadate (NaOV), organo-

vanadium complexes (OVC) of vanadium such as vanadium (IV) oxo bis (acetylacetonate) (VAC) and vanadium (IV) oxo bis(maltolato) (BMOV) are more potent in lowering blood glucose in rodent models of diabetes mellitus [18, 34, 35]. However, the molecular mechanism responsible for the stronger insulinomimetic effect of OVC over inorganic vanadium salts remains unknown. Therefore, the aim of the present study was to investigate if the insulinomimetic and antidiabetic actions of OVC could be attributed to their ability to enhance PKB and GSK-3β phosphorylation.

3.3-MATERIALS AND METHODS

Materials

Chinese hamster ovary cells overexpressing human insulin receptor (CHO-HIR cells) were a generous gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA). Insulin was from Eli Lilly Co. (Indianapolis, IN), VS, NaOV and NaMV were from Aldrich Chemical Co. (Milwaukee, WI), and the OVC were kindly donated by Dr. Debbie Crans (Colorado State University, Fort Collins, CO). Polyclonal insulin receptor beta-(IRβ) subunit antibody, monoclonal antiphosphotyrosine antibody (PY99), polyclonal phospho-GSK-3β (Ser^{9}) antibody and polyclonal GSK-3β antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). Polyclonal phospho-PKB (Ser^{473}) and polyclonal PKB antibody were from Cell Signaling (Beverly, MA). Polyclonal IRS-1 antibody and polyclonal $p^{85\alpha}$ antibody were from Upstate Biotechnology (Lake Placid, NY). The enhanced chemiluminescense (ECL) detection system kit was from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada).

Methods

Cell culture

CHO-HIR cells were maintained in HAM's F-12 medium containing 10% fetal bovine serum, and rat 1 fibroblast overexpressing HIR and protein tyrosine phosphatase 1B (PTP1B) cells (HircA) were maintained in DMEM:F-12 medium containing 10% fetal bovine serum. The cells were grown to confluence in 60-mm plates and incubated in serum-free medium for 16 h prior to the experiment [23].

Immunoblotting

Cells subjected to various experimental treatments were lysed in buffer A (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM NaOV, 10 mM Na fluoride, 10 mM Na pyrophosphate,

20 nM okadaic acid, 0.5 mM ethylenebis-(oxyethylenenitrilo) tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1% Triton X-100, with the lysates being clarified by centrifugation to remove insoluble material. The clarified cell lysates normalized to contain equal amounts of protein were subjected to 7.5% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, transferred to PVDF membranes, and incubated with the indicated antibodies. Proteins were detected by a horse radish peroxidase-conjugated second antibody and visualized with an ECL detection kit [23].

Immunoprecipitation

The clarified cell lysates, normalized to contain equal amounts of protein (500 μg), were immunoprecipitated with 3 μg of IRS-1 or IRβ antibody overnight at 4 °C, followed by incubation with protein A sepharose for 2 h. Immunoprecipitated IRS-1 or IRβ was collected by centrifugation, washed 2 times with buffer A and once with phosphate-buffered saline containing PTPase and protease inhibitors. The immunoprecipitates were submitted to 7.5% SDS-PAGE under reducing conditions, transferred to PVDF membranes, and incubated with polyclonal p^{85} antibody (1:4,000) or PY99 (1:2,000). Proteins were detected by a horse radish peroxidase-conjugated second antibody and visualized with an ECL detection kit.

PTPase activity

Confluent, serum-starved HircA cells were incubated with 1 mM of vanadium compounds for 10 min. The cells were lysed in buffer A without NaOV and equal amounts of the clarified lysates were assayed for total PTPase activity as described elsewhere [36]. Briefly, lysates adjusted to contain the same amount of proteins were added to PTPase assay buffer containing 40 mM NaOAc/HCl, pH 5.00, 1 mM ethylenediamine tetraacetic acid, 1 mM DTT and 25 mM p-nitrophenyl phosphate.

Phosphatase reaction was carried out for 10 min at room temperature and stopped by the addition of 0.2 M NaOH. The amount of product (p-nitrophenol) produced was measured by monitoring the increase in absorbance at 405 nm optical density. The nonenzymatic hydrolysis of p-nitrophenol phosphate was corrected by measuring the increase in absorbance at 405 nm in the absence of enzyme.

3.4-RESULTS

Effect of different organic and inorganic vanadium compounds on PKB phosphorylation

To evaluate the potency of different vanadium compounds on PKB phosphorylation, CHO-HIR cells were treated with 1 mM of 2 OVC, VAC or BMOV or 3 inorganic vanadium compounds VS, NaMV or NaOV. PKB phosphorylation was measured by using a phospho-specific PKB antibody which detects only the phosphorylated and active form of PKB. As shown in Figure 1, the OVC BMOV and VAC were more potent than VS in enhancing PKB phosphorylation. In fact, the phosphorylation induced by VAC was virtually equipotent to that elicited by 100 nM insulin. However, other vanadium compounds or compounds of other metals as Cr and Co, failed to exert any effect on PKB phosphorylation.

To further compare the relative abilities of the most active vanadium compounds VAC, BMOV and VS on PKB phosphorylation, dose response analysis was performed. As shown in Figure 2A, VAC was more potent than either BMOV or VS in stimulating PKB phosphorylation, and caused a robust increase at 100 μM concentration.

Effect of different concentrations of VAC, BMOV and VS on GSK-3β phosphorylation

Since PKB elicits its response through the phosphorylation of several downstream targets such as GSK-3β [29], we investigated if vanadium compounds exerted any effect on the phosphorylation of this substrate. To test this possibility, lysates prepared from cells stimulated with different concentrations of VAC or BMOV and VS for 10 min were immunoblotted using phospho-specific antibodies for GSK-3β$^{(Ser9)}$. As shown in Figure 2B, all the vanadium compounds at the 2 concentrations increased the phosphorylation of GSK-3β. However, as was the case with PKB phosphorylation, VAC was the most potent compound, and was several fold more efficient than BMOV or VS. At 100 μM,

VAC was about 2 times more effective than BMOV and about 4 times stronger than VS in enhancing GSK-3β phosphorylation.

Effect of Wortmannin on VAC-, BMOV- and VS-induced phosphorylation of PKB and GSK-3β

PI3-K is an upstream regulator of the PKB signaling pathway in response to insulin [28, 29]. Therefore, by using Wortmannin a pharmacological PI3-K inhibitor [37] we investigated if PI3-K plays a role in vanadium compound–induced PKB and GSK-3β phosphorylation. As shown in Figure 3, Wortmannin pretreatment resulted in almost complete attenuation of insulin, as well as vanadium-induced PKB and GSK-3β phosphorylation.

Effect of VAC, BMOV and VS on PTPase activity and protein tyrosine phosphorylation

Vanadium compounds are potent PTPase inhibitors [1, 27, 38]. Therefore, we wished to examine if the higher capacity of VAC and BMOV vs VS on PKB and GSK-3β phosphorylation is reflected in their ability to differentially inhibit PTPase activity. As shown in Figure 4A, treatment of HircA cells overexpressing PTP1B with VAC or BMOV resulted in 50% inhibition whereas VS caused only 20% inhibition of total PTPase activity. Next, we examined if the higher potency of OVC to inhibit PTPase activity correlated with their ability to increase the tyrosine phosphorylation of cellular proteins. As shown in Figure 4B, treatment of CHO-HIR with 1mM of different vanadium compounds augmented the tyrosine phosphorylation of several proteins with varying degrees. The molecular sizes of these proteins ranged from 60 to 200 KDa. However, phosphorylation of protein was more robust with VAC than with either BMOV or VS.

Effect of VAC, BMOV and VS on IRβ and IRS-1 phosphorylation

Since the tyrosyl phosphorylation of IRβ is an early step in the insulin signaling cascade, we wished to determine if vanadium compounds enhanced the tyrosine phosphorylation of IRβ. As shown in Figure 5A, IRβ immunoprecipitation from CHO-HIR treated with 1mM of VAC, BMOV, VS or 100 nM of insulin, followed by immunoblotting with phosphotyrosine antibody, revealed that both VAC and BMOV induced an increase in tyrosyl phosphorylation of IRβ. However, under similar conditions, VS was ineffective and failed to enhance IRβ phosphorylation. We next assessed the effect of different vanadium compounds on the tyrosine phosphorylation of IRS-1. As shown in Figure 5B, insulin as well as all vanadium compounds enhanced IRS-1 tyrosine phosphorylation to varying degrees. BMOV- and VAC-induced IRS-1 tyrosine phosphorylation was identical to their effects on IRβ phosphorylation. VS which was unable to cause any detectable increase in IRβ tyrosine phosphorylation also enhanced IRS-1 phosphorylation, however, this was several times lower than that elicited by BMOV or VAC. Since PI3-K activation requires the association of its p85α regulatory subunit with the tyrosyl phosphorylated form of IRS-1, we next investigated the degree of association of p85α with IRS-1 in cells treated with various vanadium compounds. As shown in Figure 5B, all the agents tested enhanced the association of p85α with IRS-1, but insulin induced the highest level of association between IRS-1 and p85α, which was followed by VAC, BMOV and VS with decreasing order of potency. This degree of association is consistent with the level of IRS-1 as well as PKB phosphorylation achieved in response to insulin and vanadium compounds.

3.5-DISCUSSION

In the current study, we have demonstrated that OVC are more potent, than inorganic forms of vanadium in activating key components of the insulin signaling pathway. The OVC VAC and BMOV were more efficient than VS in stimulating PKB activity which was associated with an increase in GSK-3β phosphorylation. Our results also show that Wortmannin, a PI3-K inhibitor, decreased insulin- and vanadate-induced PKB and GSK3-β phosphorylation (Fig. 5) suggesting that PI3-K is an upstream component in vanadium-induced PKB and GSK-3β phosphorylation events. Although it is well-known that OVC are more potent than inorganic vanadium salts in improving glucose homeostasis in animal models of insulin resistance [18, 34, 35, 39, 40], the precise mechanism of this effect is unknown. Since activation of the PKB signaling system is believed to play a key role in mediating the metabolic responses of insulin [28], to the best of our knowledge, our data are the first to provide a molecular basis for the higher efficacy of OVC in improving glucose homeostasis compared to inorganic vanadium salts. More robust activation of PKB may also be responsible for several fold higher glucose transport observed in adipocytes in response to VAC [41] as well as the increased GLUT-4 translocation in strepzotocin (STZ)-diabetic rat skeletal muscle in vivo [42]. GSK-3β has been suggested to play an important role in decreasing the activity of glycogen synthase by catalyzing its serine phosphorylation [43] but PKB-induced phosphorylation of GSK-3β renders it inactive and allows the full activation of glycogen synthase [43]. Thus, the OVC-induced robust enhancement of GSK-3β phosphorylation observed in our study could serve as a potential mechanism for a more potent effect of these compounds on glucose utilization and storage seen in BMOV- and VAC-treated rodent models [18, 41].

GSK-3β has been implicated in regulating the gene expression of the key gluconeogenic enzymes PEPCK and G6Pase [28, 29, 31]. Vanadium compounds have also been shown to inhibit the heightened expression and/or activity of PEPCK and G6Pase in several diabetic rat models [18-20, 33, 44, 45]. Thus, it is possible that a greater ability of

BMOV and VAC to induce GSK-3β phosphorylation may contribute to the normalization of exaggerated PEPCK and G6Pase expression in the STZ-diabetic rat liver [20].

Our data also indicated that treatment of intact cells with OVC inhibited the activity of cellular PTPase more potently than VS. These data are in agreement with recent studies in which BMOV was reported to be a non-selective competitive inhibitor of several PTPases in vitro [39, 40, 46]. PTPases play an important role in the regulation of insulin signaling [47, 48], and it was shown recently that mice deficient in PTP1B, a cytoplasmic PTPase, exhibit increased insulin sensitivity with resistance to weight gain, even when put on a high-fat diet [49]. Moreover, several studies have documented an overexpression of PTPases in diabetes (reviewed in [47]), and BMOV treatment inhibits exaggerated PTP1B activity in the skeletal muscle of Zucker fatty rats, a model of insulin resistance [46]. Since enhanced tyrosine phosphorylation caused by both protein tyrosine kinase (PTK) activition and/or PTPase inhibition is primordial in triggering the insulin signaling cascade, it is possible that more potent inhibition of PTPase by OVC contributes to a heightened tyrosine phosphorylation of IRβ and IRS-1 by both VAC and BMOV. In confirmation with our data BMOV was recently shown to enhance the tyrosine phosphorylation of IRβ [39, 40]. In addition, the OVC were superior to VS in augmenting the tyrosine phosphorylation of the IRS-1 and IRS-1/$p^{85\alpha}$ association which might provide a molecular basis for the high impact of these compounds in activating PKB phosphorylation.

In summary, our results demonstrate that, compared to VS, OVC are more potent activators of PKB and GSK-3β phosphorylation in a PI3-K-dependent manner. A greater inhibitory effect of OVC on PTPases and increased protein tyrosine phosphorylation might serve as triggering mechanisms, leading to upregulation of the PKB signaling pathway and eventually contributing to a greater potency of OVC in improving glucose homeostasis.

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3.6-FIGURE LEGENDS

Figure 1: Vanadium compound-induced PKB phosphorylation in CHO-HIR cells

Confluent, serum-starved CHO-HIR cells were incubated with 100 nM of insulin for 5 min or 1 mM of different vanadium compounds or compounds of other metals for 10 min. The cell lysates were subjected to immunoblotting using phospho-specific (Ser 473)-PKB (upper immunoblot) or total PKB antibody (lower immunoblot) as described in the Materials and Methods section. The results are representative of 3 independent experiments. VAC, vanadium (IV) oxo bis (acetylacetonate); VS, vanadyl sulfate; BMOV, vanadium (IV) oxo bis(maltolato); Crdipic, chromium (III) bis(2,6-pyridinecarboxyylate); NaMV, sodium metavanadate; NaOV, sodium orthovanadate; Co2dipic, cobalt (II) bis(2,6-pyridinecarboxylate) heptahydrate.

Figure 2: VAC-, BMOV- and VS-induced PKB phosphorylation is dose-dependent in CHO-HIR cells

Confluent, serum-starved CHO-HIR cells were incubated with the indicated concentrations of VAC, BMOV or VS for 10 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Ser 473)-PKB antibody (upper immunoblot in A), total PKB antibody (lower immunoblot in A), phospho-specific (Ser 9)-GSK-3β (upper immunoblot in B) and total GSK-3β antibody (lower immuoblot in B). The results are representative of 3 independent experiments.

Figure 3: VAC-, BMOV- and VS-induced PKB and GSK-3β phosphorylation is dependent on PI3-K activity

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 VAC, BMOV or VS for 10 min. The cells were lysed, and the lysates

were subjected to immunoblotting, using phospho-specific (Ser 473)-PKB antibody (upper immunoblot in A), total PKB antibody (lower immunoblot in A), phospho-specific (Ser 9)-GSK-3β (upper immunoblot in B) and total GSK-3β antibody (lower immunoblot in B). The results are representative of 3 independent experiments.

Figure 4: VAC and BMOV are more potent than VS in inhibiting PTPase activity and in enhancing tyrosine phosphorylation

Confluent, serum-starved HircA (A) or CHO-HIR (B) cells were incubated with 1mM of VAC, BMOV or VS for 10 min. The cells were lysed, and the lysates were subjected to measure PTPase activity as described in the Materials and Methods section (A) or to immunoblotting, using antiphosphotyrosine antibody (PY99) (B). The results are representative of 3 independent experiments.

Figure 5: VAC and BMOV are more potent than VS in enhancing the tyrosine phosphorylation of IRβ, IRS-1 and IRS-1/p^{85} association

Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin for 5 min or with 1 mM of VAC, BMOV or VS for 10 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with an anti-IRβ (A) or IRS-1 (B) antibody. The immunoprecipitates were immunoblotted (IB) with the indicated antibodies. A representative immunoblot from 3 independent experiments is shown.

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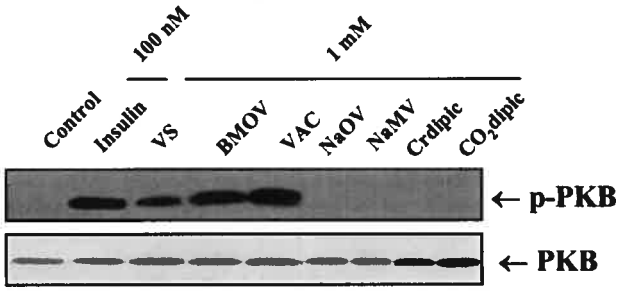
100 nM
1 mM
Control
Insulin
VS
BMOV
VAC
NaOV
NaMV
Crdiplc
CO2diplc
← p-PKB
← PKB

Figure 1

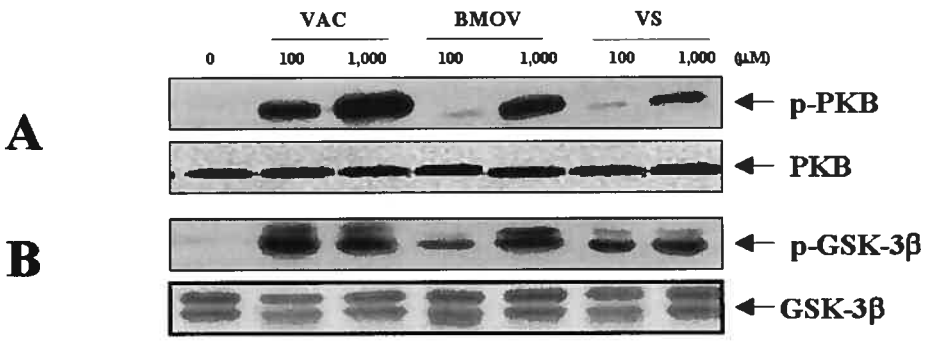
VAC
BMOV
VS
0
100
1,000
100
1,000
100
1,000
(μM)
A
p-PKB
PKB
B
p-GSK-3β
GSK-3β

Figure 2

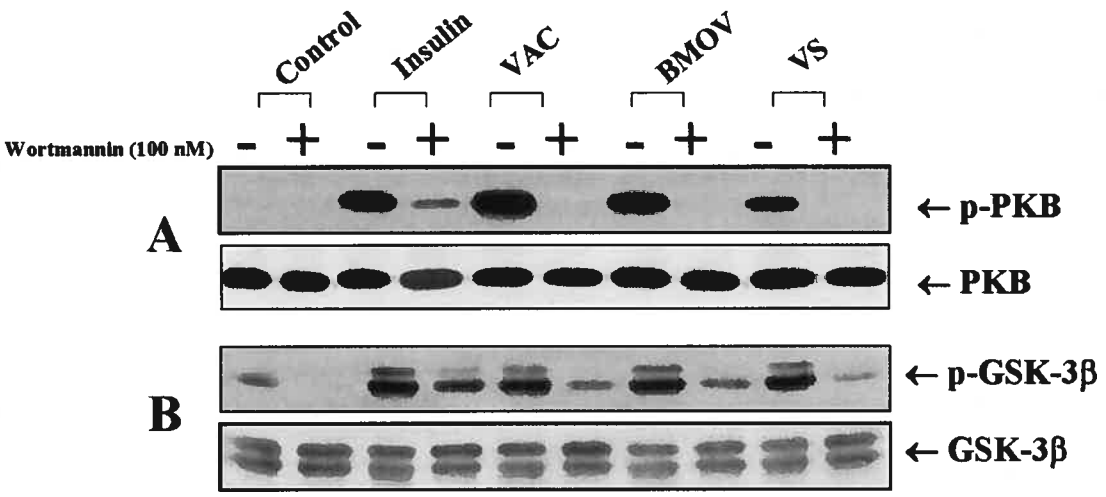
Control
Insulin
VAC
BMOV
VS
Wortmannin (100 nM) - + - + - + - + - +
A
← p-PKB
← PKB
B
← p-GSK-3β
← GSK-3β

Figure 3

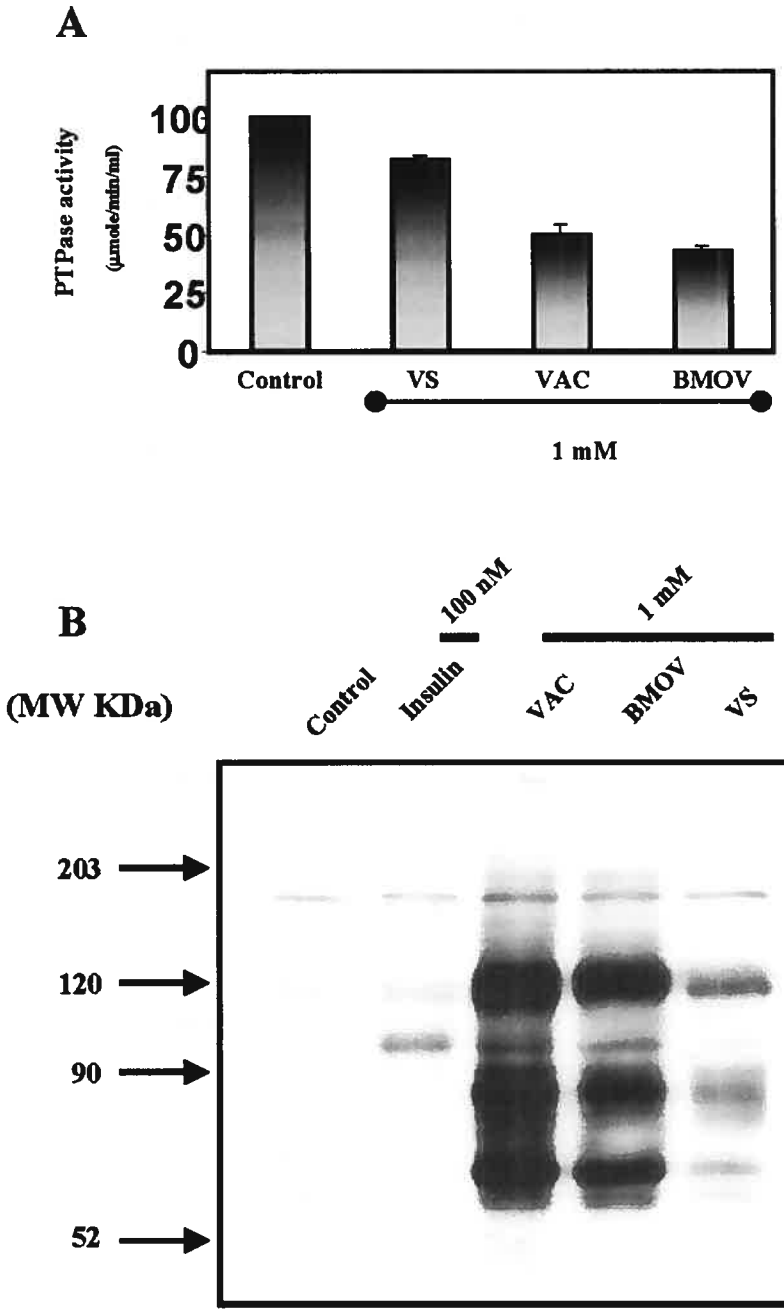
A
PTPase activity
(μmole/min/ml)
100
75
50
25
0
Control
VS
VAC
BMOV
1 mM
B
(MW KDa)
100 nM
1 mM
Control
Insulin
VAC
BMOV
VS
203
120
90
52

Figure 4

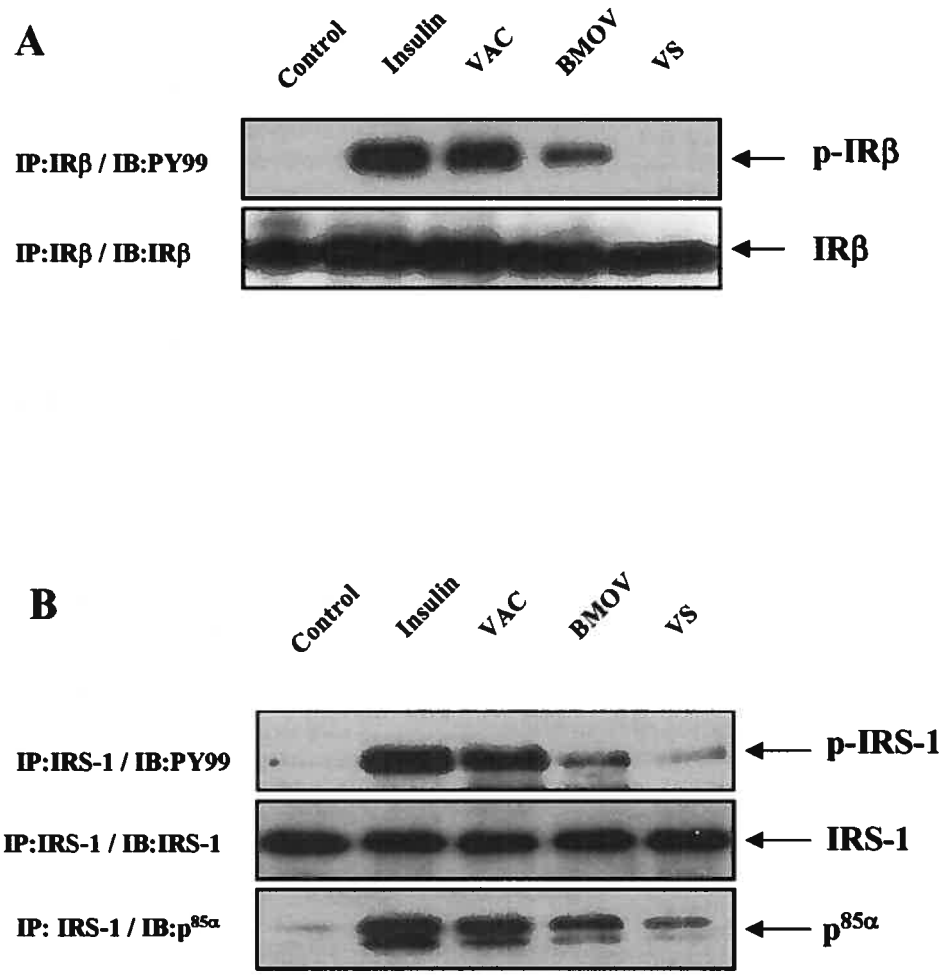
A
Control
Insulin
VAC
BMOV
VS
IP:IRβ / IB:PY99
p-IRβ
IP:IRβ / IB:IRβ
IRβ
B
Control
Insulin
VAC
BMOV
VS
IP:IRS-1 / IB:PY99
p-IRS-1
IP:IRS-1 / IB:IRS-1
IRS-1
IP: IRS-1 / IB:p85α
p85α

Figure 5

Biochemistry, 2006, In press

CHAPTER 4

Involvement of Insulin-Like Growth Factor type 1 Receptor and Protein Kinase C δ in Bis(maltolato)-oxovanadium (IV)-induced Phosphorylation of Protein kinase B in HepG2 cells

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Short title: BMOV-induced PKB activation

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Abbreviations

Keywords

Diabetes; insulin; vanadium; protein kinase B; protein tyrosine kinase; insulin-like growth factor receptor

Abbreviations

BMOV, vanadium (IV) oxo bis(maltolato); CHO-HIR, Chinese hamster ovary cells overexpressing human insulin receptor; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; GSK-3, glycogen synthase kinase-3; IGF-1R, insulin-like growth factor type 1 Receptor; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKCδ, protein kinase c delta; PMA, Phorbol-12-myristate-13-acetate; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PY99, monoclonal antiphosphotyrosine antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

4.1-ABSTRACT

Vanadium (IV) oxo bis(maltolato) (BMOV), an organo-vanadium compound, is a potent insulinomimetic agent and improves glucose homeostasis in various models of diabetes. We have shown earlier that BMOV stimulates the phosphorylation of PKB which may contribute as one of the mechanism for the insulinmimetic effect of this compound. However, the upstream mechanism of BMOV-induced PKB phosphorylation remains elusive. Therefore, in this study we have examined the upstream events leading to BMOV-induced PKB phosphorylation in HepG2 cells. Since BMOV is an inhibitor of Protein Tyrosine Phosphatases and through increased tyrosine phosphorylation may activate various Protein tyrosine kinases (PTK), we have investigated the potential role of different receptor or non receptor PTK in mediating BMOV-induced PKB phosphorylation. Among several pharmacological inhibitors tested, only AG1024, a selective inhibitor of IGF-1R-PTK almost completely blocked BMOV-stimulated phosphorylation of PKB. In contrast, AG1295 and AG1478, specific inhibitors of PDGFR and EGFR respectively were unable to block the BMOV response. BMOV-induced PKB phosphorylation was associated with an increased tyrosine phosphorylation of IRβ subunit. IRS-1 and $p^{85\alpha}$ subunit of PI3-Kinase. However, this response was independent of IR-PTK activity because in cells overexpressing a PTK-inactive form of IR, insulin response was attenuated while BMOV remained intact. A role of PKC in BMOV-induced response was also tested. Pharmacological inhibition with chelerythrine a non-selective PKC inhibitor or rottlerin a PKC_δ inhibitor as well as Chronic treatment with PMA attenuated BMOV-induced PKB phosphorylation. In contrast, GÖ6976 and RO 31-8220 PKCα/β selective inhibitor failed to alter BMOV effect. Taken together, these data suggest that IGF-1R and PKCδ are required to stimulate PKB phosphorylation in response to BMOV in HepG2 cells.

4.2-INTRODUCTION

Vanadium is a transition metal and its compounds have been shown to exert insulin-like properties both in vivo and in vitro systems (reviewed in (1;2)). Oral administration of vanadium leads to improved insulin resistance and lowers hyperglycemia in rodent models of diabetes mellitus as well as in limited studies with human subjects (1;3-5). Vanadium mimics many physiological effects of insulin including stimulation of glucose uptake, glycogen synthesis, and lipid synthesis in muscle, adipose, and hepatic tissues as well as inhibition of gluconeogenesis in the liver and kidneys and lipolysis in fat cells (1;2). In cultured cells, vanadium activates several key components of insulin signaling cascade implicated in mediating the physiological responses of insulin including the tyrosine phosphorylation of the insulin receptor substrate (IRS-1) (6;7) and activation of extracellular signal-regulated kinase (ERK1/2) (8-10) and phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) signaling cascade (6-8;10-12).

PKB , also known as Akt, is a 57 kDa serine/threonine kinase which has been implicated in the regulation of many physiological processes such as glucose transport, glycolysis, protein synthesis, lipogenesis, glycogen synthesis, suppression of gluconeogenesis, cell survival, determination of cell size and cell-cycle progression (reviewed in (13)). In most cell types, PKB is activated via a PI3-K-dependent mechanism through dual phosphorylation on serine 473 in the C-terminal regulatory region and threonine 308 within the catalytic loop. Phosphorylation of PKB on Thr 308 is catalyzed by phosphatidylinositol 3,4,5-triphosphate (PIP3)-dependent protein kinase 1 (PDK-1), but the kinase responsible for phosphorylation at Ser 473, the putative PDK-2, remains elusive (13;14). Activated PKB exerts its effect through the phosphorylation of several downstream targets, such as glycogen synthase kinase-3 β (GSK-3β), forkhead transcription factor (FKHR), Bcl-2 related protein (BAD), IkB Kinase , Mdm2, Caspase 9 and endothelial nitric-oxide synthase (eNOS) (13;14).

Recently, we have demonstrated that organo-vanadium compounds are more potent than inorganic vanadium salts in inhibiting the total PTPase activity and in increasing the total

protein tyrosine phosphorylation which was associated with a robust activation of PKB pathway (7). A much higher potency of organo-vanadium compounds, as compared to inorganic vanadium salts, to enhance the phosphorylation of PKB and its downstream substrates has been suggested as one of the mechanism to explain their greater antidiabetic effects (15-17). We have also shown that vanadium (IV) oxo bis(maltolato) (BMOV)-a well established antidiabetic and insulinmimetic (18-22) organo-vanadium compounds enhanced the tyrosine phosphorylation of the β -subunit of insulin receptor (IRβ) in Chinese hamster ovary cells overexpressing IR (CHO-IR) (7), suggesting an involvement of IR-protein tyrosine kinase (PTK) activity in provoking BMOV-response. However, a clear involvement of IR-PTK as an upstream inducer of vanadium action has not been established and a role of both IR-PTK-dependent (23-26) and –independent (6;11;27-29) events have been suggested to contribute to the insulin-like effects of vanadium. Therefore, in the present studies we have investigated a potential role of receptor PTKs in BMOV-induced PKB phosphorylation in human hepatoma (HepG2) cells.

4.3-MATERIALS AND METHODS

Materials

Insulin was from Eli Lilly Co. (Indianapolis, IN), Vanadium (IV) oxo bis(maltolato) (BMOV), was a kind gift from Dr. Debbie Crans (Colorado State University, Fort Collins, CO). Phorbol-12-myristate-13-acetate (PMA) was from Sigma Aldrich (St. Louis,MO). HepG2 cells (HB-8065) were obtained from American type culture collection (Rockville, MD). Human IGF-1 was from PeproTech Inc. (New Jersey, U.S.A.). Epidermal growth factor (EGF), platelet growth factor receptor (PDGF) and all pharmacological inhibitors were from Calbiochem (La Jolla, CA). Polyclonal insulin receptor-β subunit antibody, polyclonal insulin growth factor type 1 receptor-β subunit antibody, monoclonal antiphosphotyrosine antibody (PY99), polyclonal phospho-GSK-3β (Ser^{9}) antibody, polyclonal PKCδ and polyclonal GSK-3β antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). Polyclonal phospho-PKB (Ser^{473}), polyclonal PKB antibody and monoclonal phospho-inulin growth factor type 1 receptor (Tyr^{1131})/phospho-inulin receptor (Tyr^{1146}) receptor antibody were from cell signaling (Beverly, MA). Polyclonal insulin receptor substrate antibody and polyclonal $p^{85\alpha}$ antibody were from Upstate (Lake placid, NY). Phospho-PKCδ (Tyr^{311}) antibody was from Biosource (Camarillo, CA). Protein A sepharose beads and the enhanced chemiluminescense (ECL) detection system kit was from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada). All cell culture materials were from Invitrogen Corp. (Grand Island, NY).

Methods

Cell culture

HepG2 cells were maintained in DMEM medium containing 10 % fetal bovine serum. Chinese hamster ovary cells overexpressing either wild type human insulin receptor (CHO-IR) or the PTK mutant form (CHO-1018), a gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA), were maintained in HAM's F-12 medium containing 10% fetal bovine serum. They were grown to 80-90% confluence in 100-mm plates or 60 mm plates at 37°C in a humidified atmosphere of 5% CO_2. Prior to the experiment, cells were incubated in serum-free medium for 20 h (7;10).

Immunoblotting

Cells subjected to various experimental treatments were lysed in buffer A (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM Na orthovanadate, 10 mM Na fluoride, 10 mM Na pyrophosphate, 20 nM okadaic acid, 0.5 mM ethylenebis-(oxyethylenenitrilo) tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, and 1% Triton X-100, and the lysates were clarified by centrifugation to remove insoluble material. The clarified cell lysates normalized to contain equal amounts of protein were electrophoresed on 7.5 % or 10 % SDS-PAGE under reducing conditions, transferred to PVDF membranes, and incubated with indicated antibody. Proteins were detected by a horse radish peroxidase conjugated second antibody and visualized with an ECL detection kit (7;10).

Immunoprecipitation

The clarified cell lysates, normalized to contain equal amounts of protein (500 µg), were subjected to immunoprecipitation with 2 µg of various antibodies overnight at 4°C, followed by incubation with protein A sepharose for 2 h. Immunoprecipitated proteins were collected by centrifugation, washed 2 times with buffer A and once with phosphate buffered saline (PBS) containing PTPase and protease inhibitors. The

immunoprecipitates were electrophoresed on 7.5% SDS-PAGE under reducing conditions, transferred to PVDF membranes, and incubated with respective primary antibodies. Proteins were detected by a horse radish peroxidase conjugated second antibody and visualized with an ECL detection kit (7).

4.4-RESULTS

Effect of BMOV on PKB and GSK-3β phosphorylation in HepG2 cells

Our previous studies have demonstrated that organovanadium compounds such as BMOV are more potent activators of PKB pathway than inorganic vanadium compounds in CHO-IR cells (7). Therefore, in the present studies, we have first evaluated whether BMOV alters the activation of PKB and its downstream targets such as GSK-3β in HepG2 cells which has been used as a model to investigate insulin action in liver (30). Since increased phosphorylation in specific serine/threonine residues of both PKB and GSK-3β is critical for their activation state, we have utilized phospho-specific antibodies to monitor their activity. As shown in Fig. 1A, treatment of HepG2 cells for 15 min with escalating concentrations of BMOV enhanced PKB and GSK-3β phosphorylation in dose-dependent manner. BMOV elicited a robust phosphorylation of these two kinases at 1 mM. Next, we assessed the time-dependence of 1 mM BMOV response, as shown in Fig. 1B, 1 mM BMOV enhanced the phosphorylation of PKB and GSK-3β within 10 min which reached maximum in 30 min.

Effect of BMOV on tyrosine phosphorylation of total protein, insulin receptor (IR), IRS-1 and p^{85} in HepG2 cells

Since BMOV is a potent inhibitor of protein tyrosine phosphatases (PTPases), and an increase in the tyrosyl phosphorylation of several key proteins such as IRβ and IRS-1 is an early step in triggering the insulin signaling cascade, we wished to determine the effect of BMOV on tyrosine phosphorylation of the total proteins as well as on the phosphorylation of IRβ, IRS-1 and regulatory subunit of PI3-K (p^{85}) in HepG2 cells. As illustrated in Fig. 2A and 2B, BMOV increased the tyrosine phosphorylation of several proteins in a dose- and time-dependent fashion. The molecular size of these proteins

ranged between 35 and to 200 kDa and the increase in phosphorylation by BMOV could be detected within 10 min of treatment (Fig. 2B). To further analyze if some of these proteins were IR and its targets, phosphotyrosylproteins immunoprecipitates from BMOV treated cells were immunoblotted with antibodies to IRβ, IRS-1 and p^{85}. As shown in Fig. 2C, BMOV treatment resulted in a significant increase in the phosphorylation of IRβ, IRS-1 and p^{85}.

Role of IR-PTK on BMOV-induced PKB phsophorylation

BMOV-induced increased tyrsoine phosphorylation of IRβ suggested a possible role of IR-PTK in BMOV-induced effects on PKB phosphorylation. This possibility was probed further by utilizing CHO cells that overexpress an inactive form of IR-PTK (CHO-1018). The inactivation of IR-PTK in these cells was achieved by the mutation of lysine 1018 to alanine in the ATP-binding domain of the IRβ (31). As illustrated in Fig. 3, both insulin and BMOV enhanced the phosphorylation of PKB in CHO-IR cells overexpressing a normal IR. However, in CHO-1018 cells overxpressing PTK-inactive IR, the insulin-induced phosphorylation of PKB was almost completely attenuated, whereas the BMOV-evoked increase was not inhibited. These data suggested that in contrast to insulin, BMOV-induced phosphorylation of PKB was independent of IR-PTK activity.

Effect of receptor tyrosine kinase inhibitors on BMOV-induced PKB

A potential role of epidermal growth factor receptor (EGFR) transactivation in vanadyl sulfate (VS)-induced signaling has been demonstrated (32;33) and insulin-like growth factor type 1 receptor (IGF-1R) activation has also suggested to play a role in vasoactive peptide-induced signaling (34;35). Therefore, we wished to determine if IGF-1R, EGFR or platelet growth factor receptor (PGDFR) were the putative PTKs mediating BMOV-induced PKB phosphorylation. As depicted in Fig. 4A, AG1024 a highly specific inhibitor of IGF-1R-PTK (36) prevented the phosphorylation of PKB induced by BMOV as well as IGF-1. In contrast, while AG 1478, an EGFR-PTK inhibitor (37), completely blocked EGF-induced PKB phosphorylation, it failed to change the stimulatory effect of

BMOV on this event (Fig. 4B). Similarly, AG 1295, an inhibitor of PDGFR-PTK also had no effect on PKB phosphorylation induced by BMOV (38) (Fig. 4C). In fact, PKB phosphorylation was not detected in HepG2 cells in response to PDGF treatment suggesting that PDGFR may not be expressed in these cells.

Effect of different doses of AG1024 on BMOV-induced signaling

Since BMOV-induced PKB phosphorylation was altered solely by AG1024, we analyzed the effect of this inhibitor in more detail. As shown in Fig. 5A, treatment of cells with AG1024 inhibited the phosphorylation of PKB and GSK-3β in a dose-dependent fashion with almost complete attenuation observed at 1 μM. Since tyrosine phosphorylation of IGF-1R β-subunit (IGF-1Rβ) is primordial in increasing its PTK activity, we investigated the effect of BMOV on the tyrosine phosphorylation of IGF-1Rβ. As shown in Fig. 5B, tyrosine phosphorylation of IGF-1Rβ was increased by BMOV and pretreatment of cells with AG1024 prior to stimulation with BMOV resulted in a significant reduction in this response. IGF-1Rβ has three critical tyrosines at positions 1131, 1135, and 1136 in the kinase domain crucial to maintain its activity and to elicit all IGF-IR-dependent functions (39). Therefore, by using a phospho-specific antibody, which recognizes phosphorylation of the IGF-1Rβ at tyrosine 1131, we investigated the effect of BMOV on the phosphorylation of this site. As depicted in Fig. 5C, BMOV increased the phosphorylation of IGF-1Rβ on Tyr^{1131}, and similar to its effect on total tyrosine posphorylation of IGF-1Rβ, AG1024 markedly blocked this response (Fig. 5C). Cumulatively, these results suggested that BMOV-induced PKB phosphorylation was associated with an increased tyrosine phosphorylation of IGF-1Rβ.

Effect of AG1024 on the phosphorylation of IRS-1 and p^{85} induced by BMOV

Increased phosphorylation of IRS-1 and p^{85} regulatory subunit of PI3-K are critical intermediary steps to signal the activation of PKB signaling in response to IGF-1R activation. Therefore, we next assessed if BMOV-enhanced tyrosine phosphorylation of IRS-1 and p^{85} in HepG2 cells (Fig. 2C) was dependent on IGF-1R-PTK activity. The

results shown in Fig. 5D demonstrated that BMOV induced tyrosine phosphorylation of both IRS-1 and p^{85} which was suppressed by AG1024.

Requirement of PKCδ in BMOV-induced PKB phosphorylation

A potential role of PKC in mediating the responses of different stimuli such as PDGF (40), EGF (41;42), Vascular Endothelial Growth Factor (VEGF) (43;44), Angiotensin II (45), and H_2O_2 (46) have been suggested. Therefore, we sought to verify if PKC-dependent pathways were involved in BMOV-induced phosphorylation of PKB in HepG2 cells. PKCs are composed of three groups; the classical (cPKC) subtype (α, βI, βII, and γ) are activated by calcium, diacylglycerol (DAG), phosphatidylserine (PS) and phorbol esters; the novel (nPKCs) subtype (δ, ε, η, and θ) are activated by DAG, PS, phorbol esters and unsaturated fatty acids and the atypical (aPKCs) subtype (ζ and λ/ι) are insensitive to DAG, but are activated by PS and phopshatidylinositides (reviewed in (47)). The results shown in Fig. 6A demonstrate that GÖ 6976 or RO 31-8220, selective chemical inhibitors of cPKC and aPKC isoforms respectively, failed to inhibit BMOV-induced phosphorylation of PKB whereas chelerythrine chloride an isoform-non-selective inhibitor and rottlerin, a highly selective PKCδ inhibitor treatment significantly reduced PKB phosphorylation induced by BMOV. Involvement of PKC was further confirmed by downregulating the PKC activity in HepG2 cells by 24h treatment with Phorbol-12-myristate-13-acetate (PMA). As shown in Fig. 6B, PMA treated cells exhibited a significantly attenuated PKB phosphorylation in response to BMOV as compared to untreated cells. Immunoblotting of parallel samples showed that PMA treatment significantly reduced the amount of PKCδ protein in these cells whereas the total PKB levels remained unaltered by this treatment (Fig. 6B).

Effect of BMOV on tyrosine phosphorylation of PKCδ

Recent studies have identified tyrosine phosphorylation as a potential mechanism through which PKCδ activation is regulated (47). Therefore, we investigated the effect of

BMOV on tyrosine phosphorylation of PKCδ by immunoprecipitating PKCδ from lysates of cells treated with BMOV followed by immunoblot analysis with antiphosphotyrosine antibody. As depicted in Fig. 7A, BMOV treatment of HepG2 cells caused a significant increase in the tyrosine phopshorylation of PKCδ. We also assessed the effect of BMOV on the tyrosine phosphorylation of PKCδ at tyrosine 311 (Tyr^{311}). This tyrosine residue is flanked between the regulatory and catalytic domains and is critical for generating the active form of PKCδ in response to H_2O_2 and PMA (48-50). As shown in Fig. 7A, BMOV induced the Tyr^{311} phosphorylation of PKCδ. In addition, both total tyrosine and Tyr^{311} phosphorylation of PKCδ enhanced by BMOV was blocked by rottlerin (Fig. 7B). Taken together these data indicated that BMOV-induced phosphorylation of PKB is associated with an activation of PKCδ as judged by an increase in tyrosine phosphorylation residues.

Effect of AG1024 on BMOV-induced tyrosine phosphorylation of PKCδ

Since PKCδ has been shown to be tyrosine phosphorylated by various protein tyrosine kinases, including IGF-1R (51), PDGFR (52;53), EGFR (54), IR (53) and Src family kinases (49;53-55), we investigated if IGF-1R-PTK was responsible for triggering the phosphorylation of PKCδ in response to BMOV. As shown in Fig. 8A, treatment of HepG2 cells with AG1024 almost completely abolished BMOV-stimulated total tyrosine phosphorylation of PKCδ. In addition, AG1024 also inhibited phosphorylation of PKCδ in Tyr^{311} while AG1478, an EGFR inhibitor, failed to alter the phosphorylation of PKCδ on this site (Fig. 8B). Since PKCδ has been shown to be phosphorylated on Tyr^{311} in response to various agonists including PMA, we wished to determine if similar to BMOV, PMA-induced effects are also dependent on IGF-1R-PTK. As shown in Fig. 8C, in contrast to its inhibitory effect on PKCδ phosphorylation stimulated by BMOV, AG1024 was unable to block the effect of PMA suggesting that IGF-1R is an upstream regulator of PKCδ phosphorylation in response to BMOV and not PMA.

4.5-DISCUSSION

BMOV has been shown to exert glucoregulatory effects in many rodent models of diabetes (18-22), however, the precise mechanism through which this response is mediated remains largely unclear. We have shown earlier that BMOV activates PKB (7), a central player involved in carbohydrate metabolism (13), therefore, in this study we have studied the upstream mechanism responsible for this response and demonstrated that BMOV-enhanced phosphorylation of PKB in HepG2 cells was associated with an increased tyrosine phosphorylation of the total cellular proteins, IRβ, IGF-1Rβ, IRS-1 and p^{85} subunit of PI3K. We have also provided evidence that PTK activity of the IGF-1Rβ is responsible for BMOV-induced activation of the PKB pathways. This conclusion is based on the use of highly selective inhibitors of IGF-1R, EGFR and PDGFR-PTKs which showed that only the inhibition of IGF-1R-PTK by AG1024 blocked BMOV-induced PKB phosphorylation. Our results showing that BMOV treatment increased the total tyrosyl phosphorylation as well as tyr^{1131} phosphorylation of IGF-1Rβ which was specifically inhibited by AG1024 suggested that the PTK activity of IGF-1Rβ was indeed being stimulated in response to BMOV. A role of IGF-1Rβ in Angiotensin II and H_2O_2 (56;57) induced ERK1/2 phosphorylation, and in Angiotensin II and purinergic receptor-P_2Y_{12}-induced PI3-K/PKB signaling pathway has been reported earlier (35;58). Transactivation of other growth factor receptor PTKs has also been suggested to play critical intermediary role in response to agonists coupled to G-protein receptors (GPCR) (reviewed in (59)). However, the data presented here are the first to demonstrate that BMOV, a non GPCR-agent also signals through IGF-1R-PTK transactivation to activate PKB.

IR and IGF-1R show a high degree of homology and share several signaling features (reviewed in (60)) and despite having a higher IC_{50} for IGF-1R-PTK, AG1024 can also inhibit IR-PTK activity at high concentrations (36). Further, our results showing that BMOV treatment increased the tyrosine phosphorylation of IRβ might argue for a role of IR-PTK in BMOV-induced responses. However, our observations that in CHO-1018

cells insulin response was attenuated while BMOV effect was intact suggested an IR-PTK-independent mechanism of BMOV action. Earlier studies using inorganic vanadium compounds have also documented lack of a role of IR-PTK in inducing the vanadium response (27;61). In addition, several investigators have also reported that the insulin-like effects of vanadium were not associated with an increase in the tyrosine phosphorylation of IRβ in many systems (6;11;27-29). Thus,despite the fact that some studies have demonstarted an increase in IR tyrosine phosphorylation in vivo in response to vanadium treatment in diabetic animal models (18;62), our data support the notion that IGF-1R-PTK activation by BMOV may serve as an alternate mechanism to mediate the insulin-like effect of this compound.

PKCδ is a serine/threonine kinase, that plays a key role in many physiological responses as growth regulation (63;64), tissue remodeling (65;66), migration (67) and transformation (51). In response to insulin, PKCδ and PKCθ, two novel PKC isoforms, have been shown to convey insulin signal toward glucose transport (68;69), glycogen synthesis (70), and cell proliferation (71). In contrast, several studies have reported that PKCδ may down-regulate insulin signaling in response to high glucose concentrations (72) and other stimuli (73). Therefore, it appears that novel PKC isoforms may both mediate insulin stimulatory effects on glucose metabolism and inhibit insulin intracellular signals. Our data showing that PKB phosphorylation was blocked by chelerythrine and rottlerin and not by GÖ 6976 or RO 31-8220 suggested a role of PKCδ in BMOV action. Rottlerin has been extensively used as a selective inhibitor of PKCδ, however recent reports have suggested that it may have additional, non- PKCδ dependent-responses (74;75). Therefore, we directly tested the effect of BMOV on PKCδ activation by assessing increase in its tyrosine phosphorylation status and have shown that both, the total tyrosine phosphorylation as well as the phosphorylation of tyr^{311}, were enhanced by BMOV treatment of HepG2 cells. This is consistent with previous reports where pervanadate was shown to enhance the tyrosine phosphorylation and activation of PKCδ (49;55). Furthermore, our results showing that in cells chronically treated with PMA, the attenuated response of BMOV on PKB phosphorylation was associated with a decreased expression of PKCδ provided additional support for a role of this isoform of PKC in

BMOV action. To the best of our knowledge, our data are the first to demonstrate an involvement of PKC in vanadium-mediated PKB activation response. This is consistent with previous reports where an involvement of PKCs in mediating PKB activation was documented (43;76). In contrast however, a negative regulatory role of PKCδ in PKB activation has also been suggested (77;78). Because PKC expression and its action have been suggested to be tissue-specific (79), it is possible that PKCδ may elicit different responses depending on cell type. The attenuation of BMOV-induced PKCδ phosphorylation by AG1024 observed in our studies indicates that IGF-1R may be an upstream mediator of this event. A requirement of PKCδ in IGF-1R- induced signaling has also been described (51;80;81). However, the precise mechanism and intermediary steps responsible for PKCδ-mediated PKB activation in response to BMOV remains to be explored.

In summary, our studies have provided experimental evidence for a role of IGF-1R-PTK in BMOV-induced activation of PKB signaling pathway in HepG2 cells. We have also demonstrated that PKCδ activation plays an intermediary role to transduce IGF-1R signaling leading to PKB phosphorylation in response to BMOV. These data provide novel insight into the cellular mechanism of BMOV action.

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4.6-FIGURE LEGENDS

Figure 1: ***BMOV-induced PKB and GSK-3β phosphorylation is time- and dose-dependent in HepG2 cells***

Confluent, serum-starved HepG2 cells were incubated with different concentrations of BMOV for 15 min (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)-PKB antibodies (upper immunoblot in A and B) and phospho-specific (Ser 9)-GSK-3β antibodies (lower immunoblot in A and B). The results are representative of 3 independent experiments.

Figure 2: ***BMOV- enhanced tyrosine phosphorylation of total proteins and of IRβ, IRS-1 and p^{85}***

Confluent, serum-starved HepG2 cells were incubated with different concentrations of BMOV for 15 min (A) or with 1 mM BMOV for the indicated time periodes (B). The cells were lysed, and the lysates were subjected to immunoblotting, using antiphosphotyrosine antibodies (PY99) (A and B). In (C), cells treated without or with 1 mM of BMOV for 15 min were lysed and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with antiphosphotyrosine antibodies (PY99). The immunoprecipitates were immunoblotted (IB) with an anti-IRβ, -IRS-1 or p^{85} antibodies. A representative immunoblot from 3 independent experiments is shown.

Figure 3: ***BMOV-induced PKB phosphorylation is independent on IR-PTK activity in CHO cells***

Confluent, serum-starved CHO-IR and CHO-1018 cells were incubated in the absence or presence of 0.1 nM insulin (Ins) or with 1 mM of BMOV for 15 min. The cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Ser 473)-PKB antibodies. The results are representative of 3 independent experiments.

Figure 4: ***BMOV-induced PKB phosphorylation is inhibited by AG1024 but not by AG1478 or AG1295***

Confluent, serum-starved HepG2 cells were pretreated without (-) or with (+) 10 μM of AG1024 (A), AG1478 (B) or AG1295 (C) for 30 min followed by stimulation with 1mM of BMOV for 15 min, 10 ng/mL of IGF-1 or 10nM of EGF or 10 ng/mL PDGF for 10 min. The cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Ser 473)-PKB antibodies. The results are representative of 3 independent experiments.

Figure 5: ***AG1024 decreased BMOV-induced PKB phosphorylation in a dose dependent manner and reduced the tyrosine phosphorylation of IGF-1Rβ, IRS-1 and p^{85} subunit of PI3-K***

Confluent, serum-starved HepG2 cells were pretreated without (-) or with (+) different doses of AG1024 (A) or 1μM (B, C and D) for 30 min followed by stimulation with 1mM of BMOV for 15 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoblotting using phospho-specific (Ser 473)-PKB antibodies and phospho-specific (Ser 9)-GSK-3β antibodies (A) or phospho-IGF-1R (Tyr^{1131})/phospho-IR (Tyr^{1146}) (C) or to immunoprecipitation (IP) with antiphosphotyrosine antibodies (PY99) followed by immunoblotting with the indicated antibodies (B and D). The phosphorylation level of protein showed in A and C were quantitated by densitometric scanning using NIH ImageJ software and expressed as fold

increase over control cells (time = 0). A representative immunoblot from 3 independent experiments is shown.

Figure 6: ***chelerythine chloride, rottlerin and long-term PMA pretreatment inhibits BMOV-induced PKB phosphorylation***

Confluent, serum-starved HepG2 cells were pretreated without (-)or with (+) 5 μM of chelerythine chloride, GÖ 6976, RO 31-8220 or rottlerin for 30 min (A) or with 100 nM of PMA for 24hr (A) followed by stimulation with 1mM of BMOV for 15 min. The cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Ser 473)-PKB antibodies (upper immunoblot in A and B) or total PKCδ antibodies (lower immunoblot in B). The results are representative of 3 independent experiments.

Figure 7: ***rottlerin decreased BMOV-induced total tyrosine phosphorylation and Tyrosine^{311} of PKCδ***

Confluent, serum-starved HepG2 cells were pretreated with or without 5 μM of rottlerin for 30 min followed by stimulation in the absence or presence of 1mM of BMOV for 15 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with total PKCδ antibodies followed by immunoblotting with antiphosphotyrosine antibodies (PY99) or phospho-specific (Tyr 311) - PKCδ antibodies. A representative immunoblot from 3 independent experiments is shown.

Figure 8: ***AG 1024 decreased BMOV but not –PMA induced tyrosine phosphorylation of* PKCδ *on Tyrosine^{311}***

Confluent, serum-starved HepG2 cells were pretreated with or without 10 μM of AG1024 or AG 1478 (B) for 30 min followed by stimulation with 1mM of BMOV or 100nM of PMA for 15 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with total PKCδ antibodies

followed by immunoblotting with antiphosphotyrosine antibodies (PY99) (A) or were immunoblotted using phospho-specific (Tyr 311) - PKCδ antibodies (B and C). A representative immunoblot from 3 independent experiments is shown.

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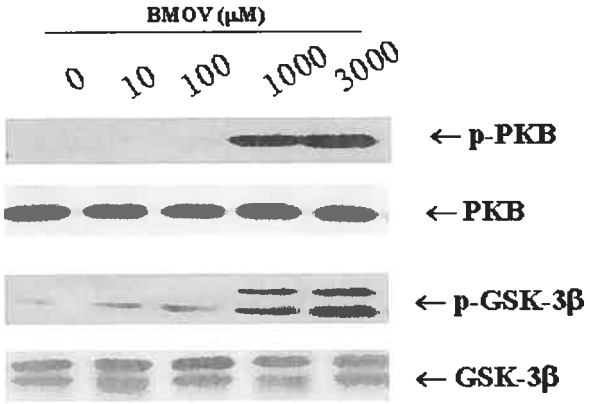
A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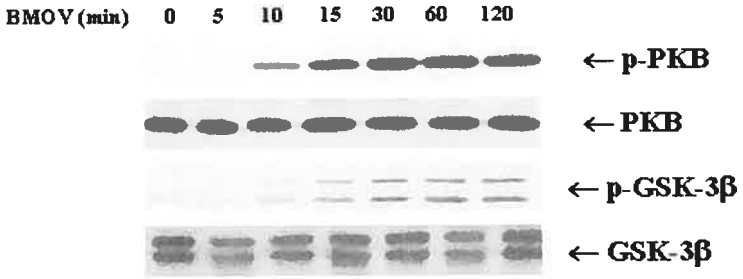
← p-GSK-3β
← GSK-3β

B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15
30
60
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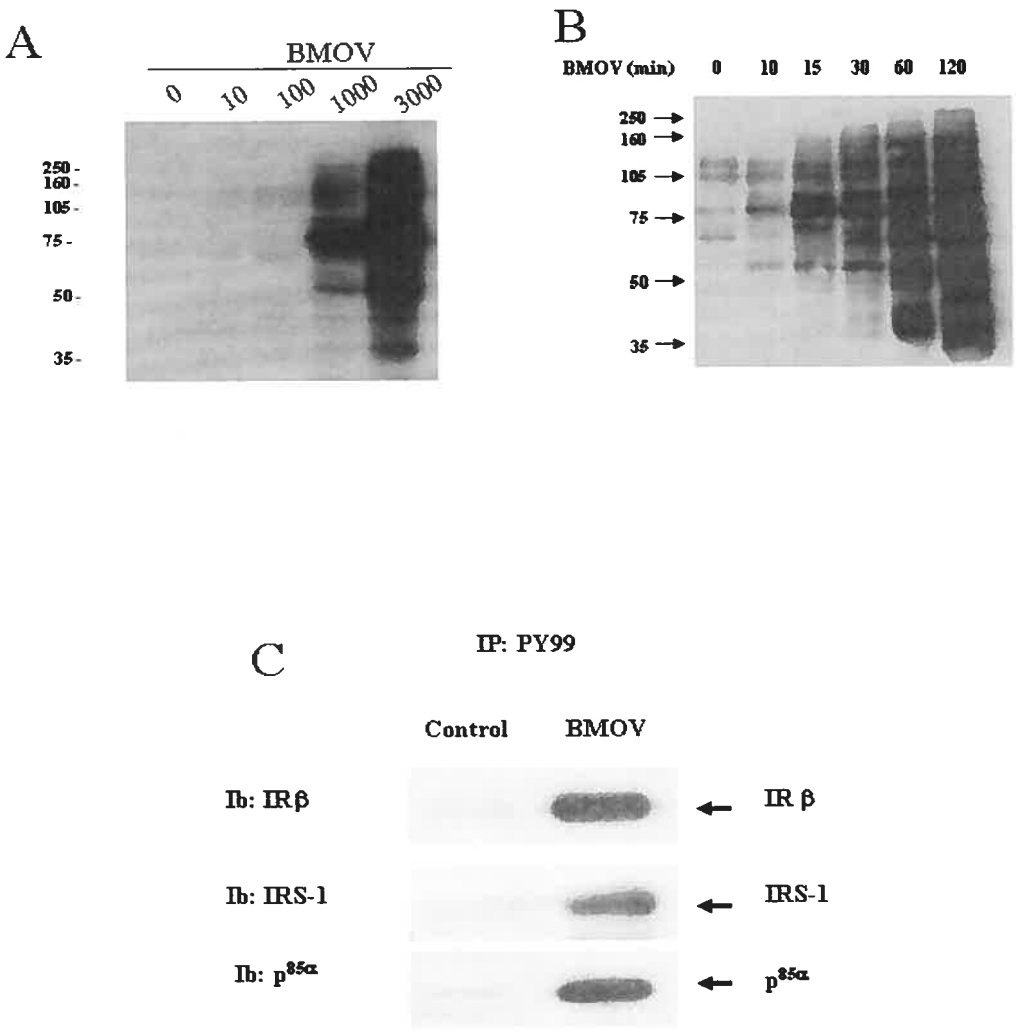
← PKB
← p-GSK-3β
← GSK-3β

Fig. 1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10
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1000
3000
250
160
105
75
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35
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BMOV (min)
0
10
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30
60
120
250
160
105
75
50
35
C
IP: PY99
Control
BMOV
Ib: IRβ
IR β
Ib: IRS-1
IRS-1
Ib: p85α
p85α

Fig. 2

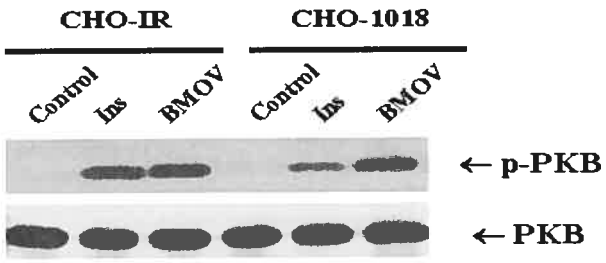
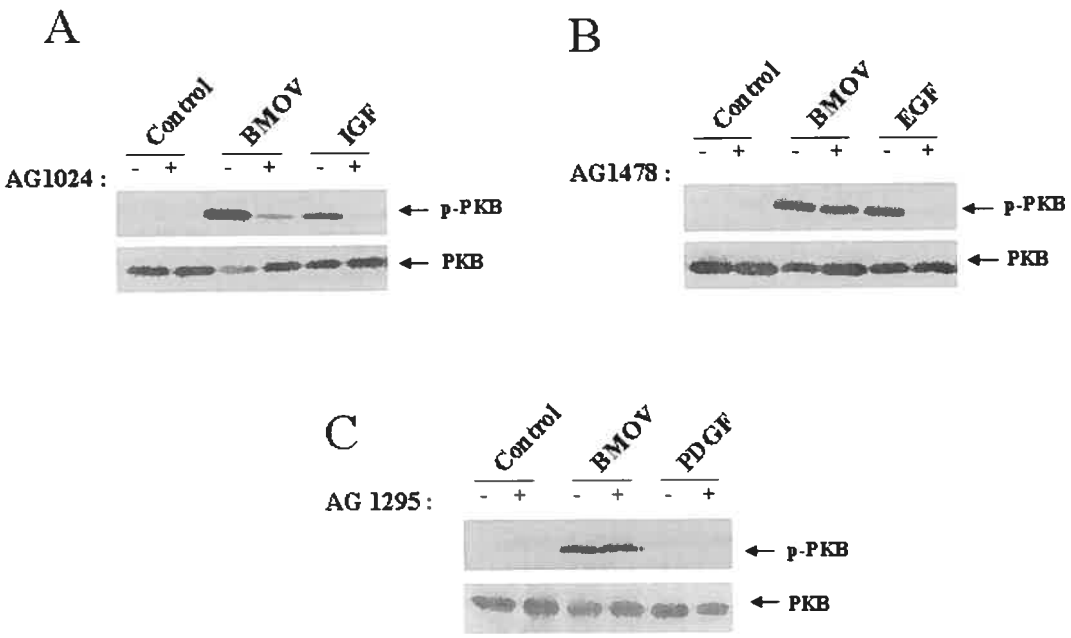


Fig. 3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Control
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IGF
AG1024 :
- + - + - +
p-PKB
PKB
B
Control
BMOV
EGF
AG1478 :
- + - + - +
p-PKB
PKB
C
Control
BMOV
PDGF
AG 1295 :
- + - + - +
p-PKB
PKB

Fig. 4

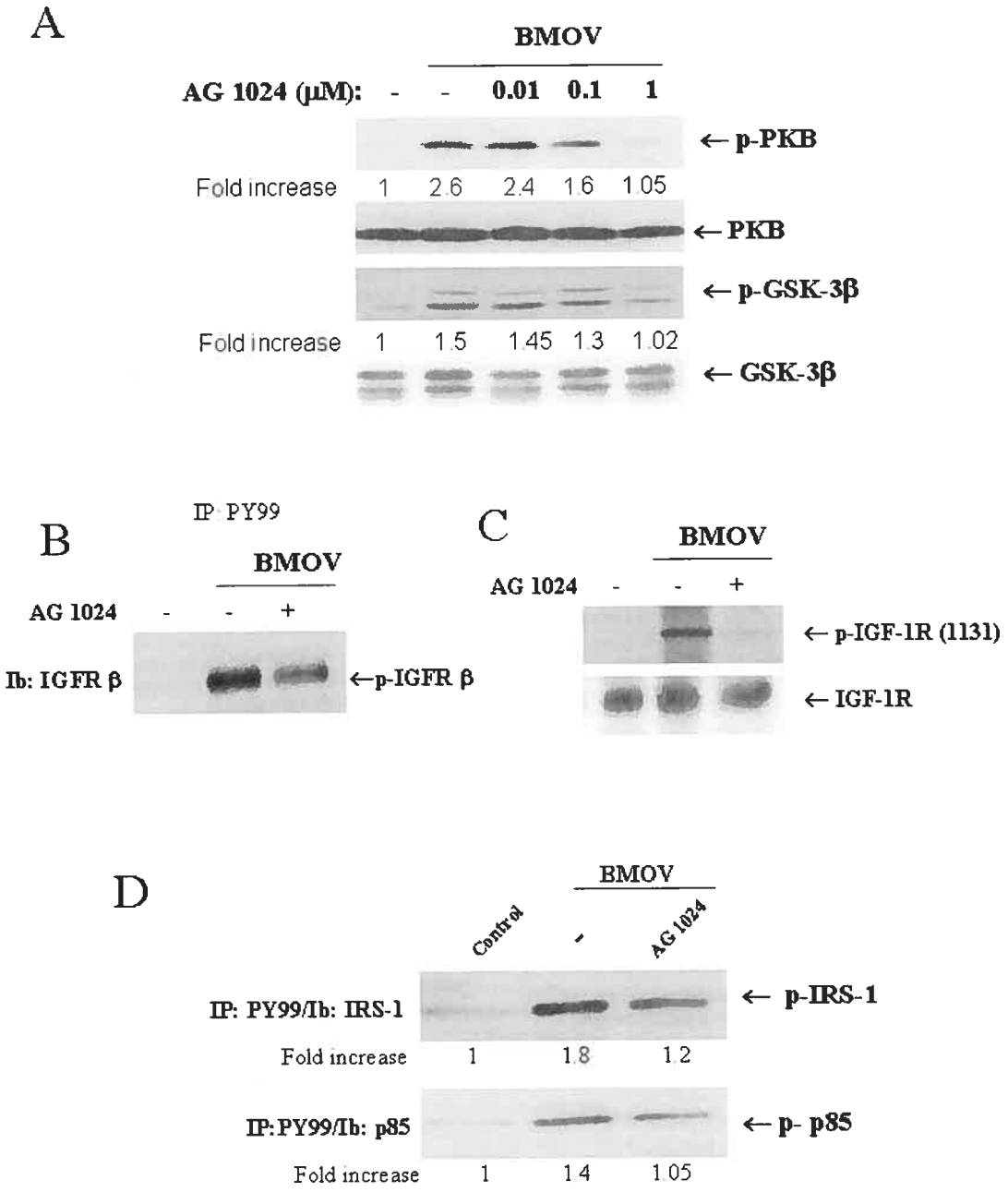
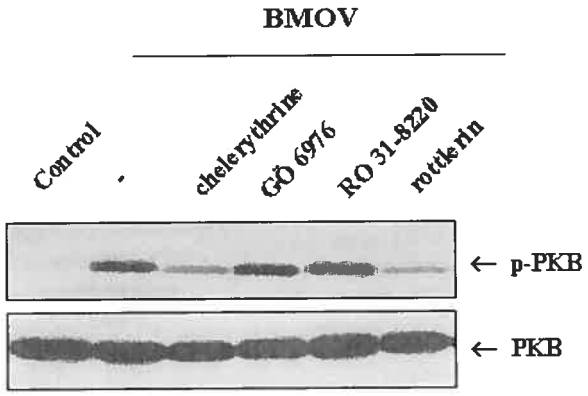

A
BMOV
AG 1024 (μM): - - 0.01 0.1 1
← p-PKB
Fold increase 1 2.6 2.4 1.6 1.05
← PKB
← p-GSK-3β
Fold increase 1 1.5 1.45 1.3 1.02
← GSK-3β
B
IP: PY99
BMOV
AG 1024 - - +
Ib: IGFR β
←p-IGFR β
C
BMOV
AG 1024 - - +
← p-IGF-1R (1131)
← IGF-1R
D
BMOV
Control
-
AG 1024
IP: PY99/Ib: IRS-1
← p-IRS-1
Fold increase 1 1.8 1.2
IP:PY99/Ib: p85
← p- p85
Fold increase 1 1.4 1.05

Fig. 5

A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Control
-
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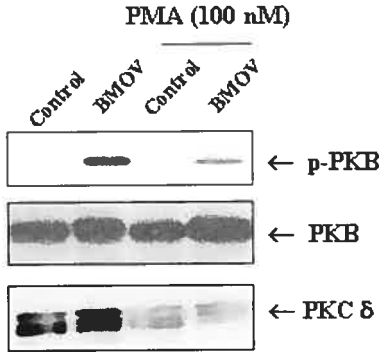
GÖ 6976
RO 31-8220
rottlerin
← p-PKB
← PKB

B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Control
BMOV
Control
BMOV
← p-PKB
← PKB
← PKC δ

Fig. 6

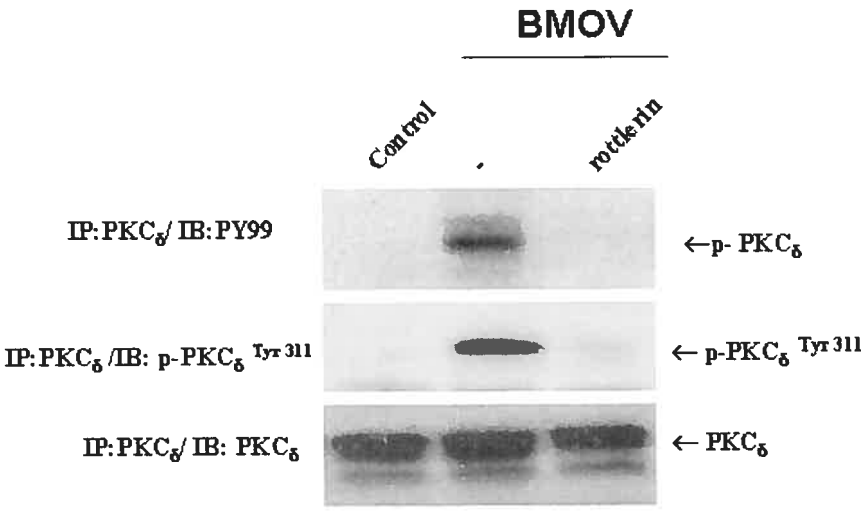
BMOV
Control
-
rottlerin
IP:PKCδ/ IB:PY99
←p- PKCδ
IP:PKCδ /IB: p-PKCδ Tyr 311
← p-PKCδ Tyr 311
IP:PKCδ/ IB: PKCδ
← PKCδ

Fig. 7

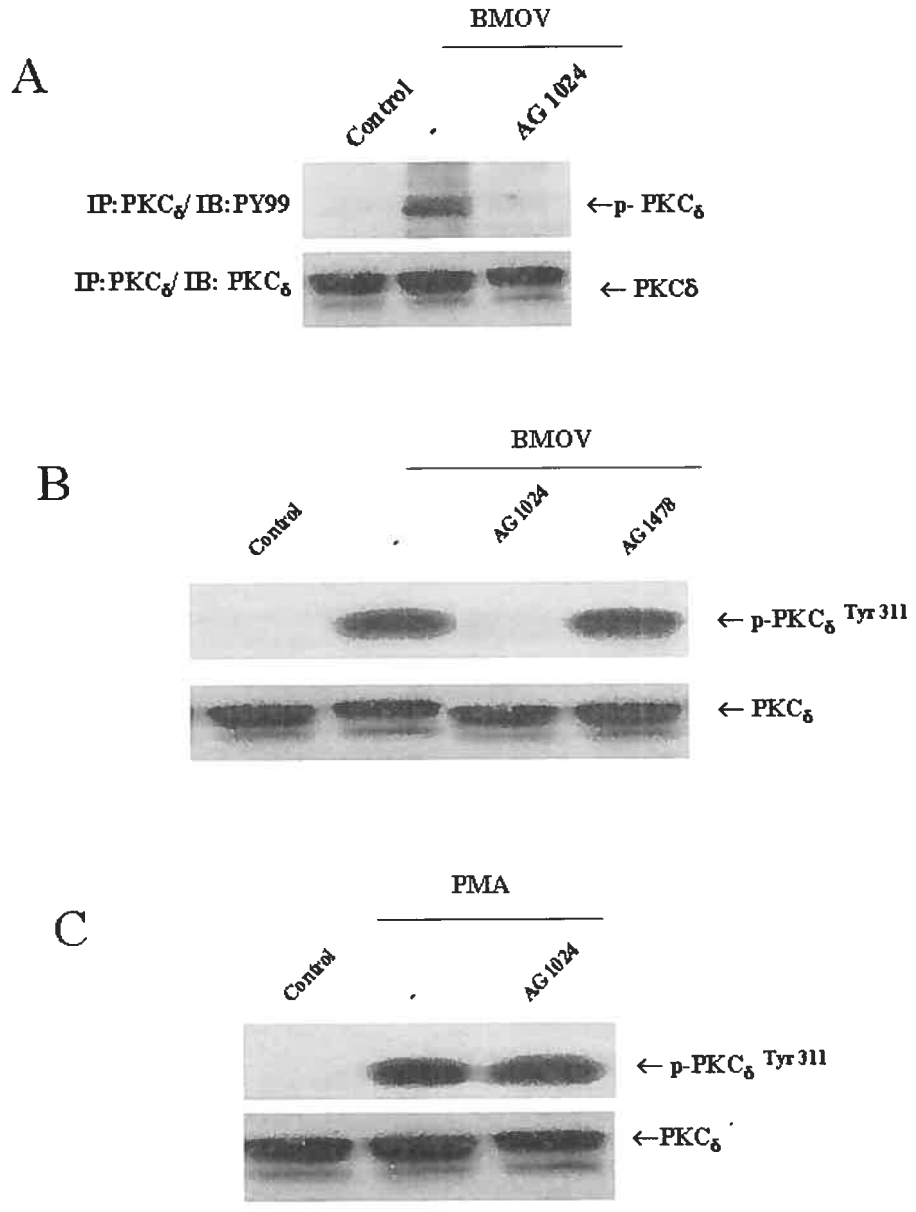
A
BMOV
Control
AG 1024
IP:PKCδ/ IB:PY99
←p- PKCδ
IP:PKCδ/ IB: PKCδ
← PKCδ
B
BMOV
Control
AG1024
AG1478
← p-PKCδ Tyr 311
← PKCδ
C
PMA
Control
AG1024
← p-PKCδ Tyr 311
←PKCδ

Fig. 8

CHAPTER 5

H_2O_2-induced phosphorylation of ERK1/2 and PKB requires tyrosine kinase activity of insulin receptor and c-Src

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Short title: Insulin receptor and Src tyrosine kinases in H_2O_2 signaling

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5.1-ABSTRACT

Hydrogen peroxide (H_2O_2) mimics many physiological responses of insulin, and increased H_2O_2 generation via the Nox-4 subunit of NAD(P)H oxidase was recently demonstrated to serve as a critical early step in the insulin signaling pathway. Exogenously-added H_2O_2 has also been shown to activate several key components of the insulin signaling cascade. H_2O_2-induced signaling responses have been found to be associated with the activation of receptor and non-receptor protein tyrosine kinases (PTK), including the insulin receptor (IR)-β subunit. Therefore, in the present studies on Chinese hamster ovary cells overexpressing wild type IR-PTK (CHO-IR) or a PTK-inactive form of IR (CHO-1018), we investigated whether IR-PTK plays a role in H_2O_2-induced signaling events. Treatment of CHO-IR cells with H_2O_2 increased the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase B (PKB) and glycogen synthase kinase-3 beta while enhancing tyrosine phosphorylation of the IR-β subunit and the p^{85} subunit of phosphatidylinositol 3-kinase (PI3K). Compared to CHO-IR cells, the stimulatory effect of H_2O_2 on ERK1/2 and PKB was partially reduced in CHO-1018 cells. However, pharmacological inhibition of Src family PTK by 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyramidine (PP-2) almost completely blocked H_2O_2-stimulated phosphorylation of the p^{85} subunit of PI3K, ERK 1/2 and PKB. Moreover, H_2O_2 but not insulin induced Tyr-418 phosphorylation of Src which was also suppressed by PP-2. Taken together, these data suggest that both IR-PTK and Src family PTKs contribute to H_2O_2-induced signaling in CHO-IR cells albeit IR-PTK has a less dominant role in this process.

5.2- INTRODUCTION

Reactive oxygen species have been suggested to serve as mediators of the action of several growth factors, cytokines and insulin (5,13,20,21,23,32). Insulin is the major hormone involved in glucose homeostasis, and its effect is initiated by binding to its receptor on cell membranes. The insulin receptor (IR) is a heterodimeric (α_2, β_2) protein, and insulin binding to the IR-α subunit results in conformational changes, leading to enhanced intrinsic protein tyrosine kinase (PTK) activity of the β subunit by multi-site tyrosine phosphorylation. Once activated, IR-PTK can phosphorylate several cytosolic IR substrates such as IRSs and Shc, which serve as docking sites for Src homology 2 (SH-2) domain-containing signaling molecules (37), triggering the activation of 2 key signaling pathways. In 1 pathway, the association of IRS-1 with Grb-2-SOS complex results in activation of the Ras, Raf, MEK and extracellular signal-regulated kinase (ERK) pathway (36,38). Activated ERK1/2 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, and contribute the mitogenic and growth-promoting effects of insulin (36,37). The second pathway that radiates from the IRS complex upon insulin stimulation involves phosphatidylinositol 3-kinase (PI3K) activation (36,38). PI3K phosphorylates phosphatidylinositol (PI) lipids at position 3 of the inositol ring, and generates 3-phosphorylated forms of PI, such as phosphatidylinositol 3, 4, and 5 (30), which are implicated in the activation of phospholipid-dependent kinase and related serine/threonine protein kinases. These activated kinases in turn are responsible for the phosphorylation and stimulation of several downstream signaling protein kinases, such as protein kinase B (PKB) (also known as Akt), glycogen synthase kinase 3 (GSK-3), $p70^{s6k}$ (11), and protein kinase C-zeta (PKCδ) (10). Activation of these protein serine/threonine kinases has been demonstrated to mediate the metabolic effects of insulin at the level of

glucose transport, glucose transporter (GLUT-4) translocation, glycogen and protein synthesis (36).

Recent studies have demonstrated that insulin stimulation of cells generates an hydrogen peroxide (H_2O_2) burst which enhances the tyrosine phosphorylation of IR and IRS-1 (23). An important role for the NAD(P)H oxidase catalytic subunit homolgue Nox-4 in enhancing H_2O_2 production in response to insulin has been suggested recently (22). In addition, exogenously-added H_2O_2 has been shown to mimic many physiological effects of insulin, including glucose transport (15), glycogen synthesis (17), lipogenesis (24), lipolysis (19) and phosphoenolpyruvate carboxykinase (PEPCK) gene expression (33). H_2O_2 has also been found to increase tyrosine phosphorylation of the IR-β subunit (15,16), and activation of the ERK1/2 and PI3K/PKB signaling pathways in several cell types (3,4,6,8,18,31,34,35). A potential role for epidermal growth factor (EGF) receptor transactivation (12,28,39) and the src family of PTK in H_2O_2-induced signaling has been postulated (1,2,12). However, despite the ability of H_2O_2 to enhance IR-PTK activity and IR-β subunit phosphorylation, the possible involvement of IR-PTK in H_2O_2-induced responses has not been characterized. Therefore, in the present studies, we investigated the possible requirement of IR-PTK in H_2O_2-induced phosphorylation of the ERK1/2 and PKB pathways.

5.3-MATERIALS AND METHODS

Materials

Insulin was obtained from the Eli Lilly Co. (Indianapolis, IN), H_2O_2 from the Sigma Chemicals Co. (St. Louis, MO), and IRS-1 and p^{85} antibodies from Upstate Biotechnology (Lake Placid, NY). Phospho-specific and total antibodies, against p44/p42 and GSK-3β, antiphosphotyrosine antibody (PY99), and IR antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). Phospho-specific and total PKB antibodies were procured from New England BioLabs (Beverly, MA), and phospho-specific c-Src antibody from Biosource (Camarillo, CA). Protein A sepharose beads and enhanced chemiluminescence (ECL) detection kits were from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada).

Methods

Cell culture

Chinese hamster ovary cells overexpressing either wild type human insulin receptor (CHO-IR) or the PTK mutant form (CHO-1018), a gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA), were maintained in HAM's F-12 medium containing 10% fetal bovine serum. They were grown to confluence in 100-mm plates and incubated in serum-free F-12 medium for 16 h prior to the experiment (26).

Cell lysis and immunoblotting

Cells subjected to various experimental treatments were washed twice with ice-cold phosphate-buffered saline and lysed in buffer A (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM Na orthovanadate, 10 mM Na fluoride, 10 mM Na pyrophosphate, 20 nM okadaic acid, 0.5 mM ethylenebis-(oxyethylenenitrilo)-tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1% Triton X-100). The lysates were clarified by

centrifugation to remove insoluble material. The clarified lysates, normalized to contain equal amounts of protein, were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, transferred to PVDF membranes, and incubated with respective primary antibodies, followed by incubation with a horse radish peroxidase-conjugated second antibody. The antigen-antibody complex was visualized with an ECL detection kit. The immunoblots were quantified by densitometric scanning with NIH ImageJ software (27).

Immunoprecipitation

The clarified cell lysates, normalized to contain equal amounts of protein (500 μg), were immunoprecipitated overnight with 1 μg of PY99 antibody at 4°C, followed by incubation with protein A sepharose for an additional 2 h. Immunoprecipitated phosphotyrosine proteins were collected by centrifugation, washed twice with buffer A and once with phosphate-buffered saline. The phosphotyrosine protein immunoprecipitates underwent 7.5% SDS-PAGE under reducing conditions, transferred to PVDF membranes, and incubated with respective primary antibodies. Proteins were detected by a horse radish peroxidase-conjugated second antibody and visualized with an ECL detection kit (27).

PI3K assay

The clarified cell lysates were subjected to immunoprecipitation with 2 μg of $p^{85\alpha}$ antibody for 2 h at 4°C, followed by incubation with protein A sepharose for an additional 2 h. The immunoprecipitates were washed before PI3K assay, as described earlier (26). The phosphorylated lipid products were extracted and separated by ascending thin layer chromatography (26). Radioactivity in the spots corresponding to PI3-phosphate was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

5.4-RESULTS

Effect of H_2O_2 on ERK1/2, PKB and GSK-3β phosphorylation in CHO-IR cells

We first evaluated whether H_2O_2 alters the activation of ERK 1/2, PKB and its downstream targets in CHO-IR cells. Since increased phosphorylation in specific serine/threonine residues of these molecules is associated with their activation, their activity was measured by using phospho-specific antibodies. As shown in Figure 1A, treatment of IR-overexpressing cells for 5 min with escalating concentrations of H_2O_2 induced ERK 1/2, PKB and GSK-3β phosphorylation. H_2O_2-increased ERK 1/2 phosphorylation at concentrations as low as 10 μM with maximal effect at 100 μM, whereas higher concentrations 500 μM to 1 mM, were required to elicit robust PKB phosphorylation. Next, we assessed the time-dependence of the H_2O_2 response. As seen in Figure 1B, 1 mM H_2O_2 rapidly enhanced the phosphorylation of ERK 1/2, PKB and GSK-3β. The increase, which declined slowly, occurred within 5 min to almost basal levels within 60 min of H_2O_2 treatment.

Effect of H_2O_2 on ERK1/2 and PKB phosphorylation in IR-PTK-deficient cells

To examine the role of IR-PTK in H_2O_2-induced effects on ERK 1/2 and PKB phosphorylation, we studied CHO-1018 cells that overexpress an inactive form of IR-PTK. The inactive form of IR was generated by the mutation of lysine 1018 to alanine in the ATP-binding domain of the IR-β subunit. This mutation results in the loss of ATP-binding activity and, thus, PTK function (7). As illustrated in Figure 2, both insulin and H_2O_2 enhanced the phosphorylation of ERK 1/2 and PKB in CHO-IR cells overexpressing a normal IR. However, in CHO-1018 cells, the insulin-induced phosphorylation of both ERK 1/2 and PKB was almost completely attenuated, whereas the H_2O_2-evoked increase was partially blocked in this cell type. These data suggested that, compared to insulin,

H_2O_2-induced phosphorylation of ERK 1/2 and PKB is exerted in part through IR-PTK activity.

Effect of H_2O_2 on IR, IRS-1 and p^{85} tyrosine phosphorylation

Since the insulin effects on ERK 1/2 and PKB are mediated through the enhanced tyrosine phosphorylation of IR and IRS-1, we next explored, if similarly to insulin, H_2O_2 increases the tyrosine phosphorylation of IR and IRS-1. As shown in Figure 3, both insulin and H_2O_2 augmented tyrosine phosphorylation of the IR-β subunit, but tyrosine phosphorylation of the IR-β subunit induced by H_2O_2 was significantly lower than that observed with insulin. Furthermore, whereas insulin enhanced the tyrosine phosphorylation of IRS-1, a similar effect of H_2O_2 on IRS-1 tyrosine phosphorylation was not detected in CHO-IR cells (Fig. 3). In contrast, both insulin and H_2O_2 treatment resulted in enhanced tyrosine phosphorylation of the p^{85} subunit of PI3K.

Requirement of c-Src in H_2O_2-induced ERK 1/2 and PKB phsophorylation

The results with CHO-1018 cells indicated that H_2O_2 effect on ERK 1/2 and PKB phosphorylation required additional signals besides IR-PTK. Since potential involvement of the Src family of PTKs in H_2O_2-induced signaling has been proposed in other cell types, we investigated if, in CHO cells also, Src-PTK was responsible for triggering the phosphorylation of various signaling components. We utilized PP-2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine), a selective inhibitor of Src-PTK, and PP-3 (4-amino-7-phenylpyrazol [3,4-d] pyrimidine), an inactive analogue of PP-2, to examine the contribution of Src in H_2O_2-induced increased phosphorylation events. As depicted in Figure 4, treatment of cells with PP-2 almost completely suppressed H_2O_2-stimulated phosphorylation of both ERK 1/2 and PKB. Since PP-2 is a potent and specific inhibitor of c-Src activity, and its ability to suppress H_2O_2-induced responses suggested a role of Src in this process, we directly assessed if H_2O_2 would enhance Src activity in CHO-IR cells. This was achieved by evaluating the increase in Tyr-418 phosphorylation in

the activation loop. As shown in Figure 4, H_2O_2 treatment enhanced the Tyr-418 phosphorylation of Src, which was almost completely blocked in PP-2-pretreated cells.

Effect of PP-2 on IR and p^{85} tyrosine phosphorylation and of wortmannin on ERK 1/2 and PKB phosphorylation induced by H_2O_2

To determine if c-Src is an upstream mediator of IR and p^{85} tyrosine phosphorylation, we evaluated the effect of PP-2 on H_2O_2-induced phosphorylation of these signaling components. As illustrated in Figure 5A, PP-2 treatment failed to block tyrosine phosphorylation of the IR-β subunit whereas it completely attenuated the tyrosine phosphorylation of the p^{85} regulatory subunit of PI3K. Next, we examined if p^{85} phosphorylation by H_2O_2 was associated with an increase in the PI3K activity of CHO-IR cells. As seen in Figure 5B, H_2O_2 treatment enhanced PI3K activity which was sensitive to inhibition by wortmannin, a specific PI3K inhibitor. Furthermore, both ERK 1/2 and PKB phosphorylation induced by H_2O_2 were almost completely attenuated by wortmannin pretreatment of cells.

5.5-DISCUSSIONS

In this study, we have shown that exogenously-added H_2O_2 enhanced the phosphorylation of ERK 1/2 and PKB signaling pathways in CHO-IR cells. The increased phosphorylation of ERK 1/2 and PKB was associated with heightened tyrosine phosphorylation of the IR-β subunit and p85 subunit of PI3K. We have also provided evidence that PTK activity of the IR-β subunit is partially responsible for H_2O_2-induced activation of the ERK 1/2 and PKB pathways. This contention is based on the results with the CHO cells that overexpress a PTK-inactive form of IR. Although earlier studies have demonstrated a stimulatory effect of H_2O_2 on tyrosine phosphorylation of the IR-β subunit in adipocytes, no attempts have been made to determine the contribution of IR-PTK to H_2O_2-inducing signaling events. Thus, to the best of our knowledge, this work provides the first evidence in support of the involvement of IR-PTK in H_2O_2-induced phosphorylation of ERK 1/2 and PKB.

H_2O_2 has recently emerged as an important redox molecule for the action of several growth factors, cytokines and insulin (5,13,20,21,23,32). Some of the effects of H_2O_2 on signaling events have been shown to be mediated by transactivation of EGF receptor PTK and the Src family of PTKs in several cell types (1,2,12,28,39). In CHO-IR cells H_2O_2 also enhanced Src activity, as judged by the increased phosphorylation of Tyr-418 in the activation loop of Src. The participation of Src in H_2O_2-induced signaling in CHO cells was evaluated by using PP-2, a highly specific inhibitor of Src PTK activity. PP-2 almost completely attenuated the H_2O_2-induced increase of phosphorylation of ERK 1/2, PKB and the p^{85} subunit of PI3K. This is in contrast to the partial inhibition elicited in PTK-deficient cells where the H_2O_2-induced effect was blocked by only 20-30% (Fig. 2). An important role of Src PTK in insulin-induced activation of PKCδ was demonstrated recently in primary cultures of skeletal myotubes (29). In these studies, insulin stimulated Src PTK activity which was blocked by PP-2 (29). Moreover, PP-2 also inhibited insulin-induced

IR-β tyrosine phosphorylation as well as glucose uptake in these myotubes. In the case of CHO-IR cells, however, neither insulin - nor H_2O_2-induced IR-β subunit phosphorylation was blocked by PP-2. Furthermore PP-2 was ineffective in inhibiting the increased phosphorylation of ERK 1/2 and PKB induced by insulin (data not shown), whereas it completely blocked the H_2O_2-evoked effect in these cells (Fig. 4), which suggested that Src-PTK-dependent pathways in CHO-IR cells mediate the H_2O_2 response and not that of insulin. Furthermore, in contrast to insulin, H_2O_2 treatment did not enhance IRS-1 tyrosine phosphorylation but increased tyrosine phosphorylation of the p^{85} subunit of PI3K which was sensitive to inhibition by PP-2. Src family PTK-catalyzed Tyr-688 phosphorylation of p^{85} was recently shown to activate PI3K in COS cells (9). The fact that H_2O_2 induced PI3K activation in these cells, and wortmannin, a specific inhibitor of PI3K, blocked H_2O_2-evoked activation of ERK 1/2 and PKB in these cells suggests that PI3K may be an upstream intermediate in H_2O_2 signaling events.

Recent studies have shown that insulin-induced generation of H_2O_2 via NAD(P)H oxidase activation serves as a trigger to initiate insulin signaling (22). In these experiments, ablation of Nox-4, one of the subunits of NAD(P)H oxidase complex, resulted in diminished H_2O_2 production, associated with decreased tyrosine phosphorylation of IR- and IRS-1 (22). The data indicated that endogenously-generated H_2O_2 in response to H_2O_2 is able to enhance IRS-1 phosphorylation in 3T3-L1 adipocytes (22), whereas the results with CHO-IR cells demonstrated that exogenous H_2O_2 fails to modify IRS-1 phosphorylation (Fig. 3). Thus, it appears that the intracellular upstream targets of endogenously generated and exogenously-added H_2O_2 may be different.

The precise mechanism by which H_2O_2 induces tyrosine phosphorylation of substrate proteins remains obscure; however, its ability to inhibit the activities of many protein tyrosine phosphatases (PTPases), such as PTP1B (18), and SHP-2 (25), has been suggested as a potential mechanism. PTPase inhibition by H_2O_2 is accomplished by oxidation of catalytically-essential cysteine residue in the active site of PTPase (14); this inhibition

shifts the equilibrium of the phosphorylation-dephosphorylation cycle, resulting in increased tyrosyl phosphorylation of substrate proteins, such as Src, the IR-β subunit and the p^{85} subunit of PI3K. These tyrosyl-phosphorylated proteins promote the assembly of signaling molecules responsible for activating various components of the mitogen-activated protein kinase and PI3K signaling pathways.

In summary, our studies have demonstrated that H_2O_2-induced activation of ERK 1/2 and PKB is associated with enhanced tyrosine phosphorylation of the IR-β subunit, the p^{85} subunit of PI3K and c-Src in CHO-IR cells. We have also provided evidence of the involvement of both IR-PTK- and Src-PTK-dependent upstream mechanisms as mediators of the H_2O_2 response.

Acknowledgements

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5.6-FIGURE LEGENDS

Figure 1: ***H_2O_2-induced ERK1/2, PKB and GSK-3β phosphorylation is time- and dose-dependent in CHO-IR cells***

Confluent, serum-starved CHO-IR were incubated with different concentrations of H_2O_2 for 5 min (A) or with 1 mM H_2O_2 for the indicated time periodes (B). The cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK1/2 antibodies (upper immunoblot in A and B), phospho-specific (Ser 473)-PKB antibodies (middle immunoblot in A and B) and phospho-specific (Ser 9)-GSK-3β antibodies (lower immunoblot in A and B). The results are representative of 3 independent experiments.

Figure 2: ***H_2O_2-induced ERK 1/2 and PKB phosphorylation is partially dependent on IR-PTK activity in CHO cells***

Confluent, serum-starved CHO-IR and CHO-1018 cells were incubated in the absence or presence of 0.1 nM insulin (Ins) (A) or with different concentrations of H_2O_2 (B) for 5 min. The cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK1/2 antibodies (upper immunoblot in A and B) and phospho-specific (Ser 473)-PKB antibodies (lower immunoblot in A and B). The results are representative of 3 independent experiments.

Figure 3: ***H_2O_2-induced tyrosine phosphorylation of IR and p^{85} but not IRS-1 in CHO-IR cells***

Confluent, serum-starved CHO-IR cells were incubated with 100 nM insulin (Ins) or with 1 mM H_2O_2 for 5 min. The cells were lysed, and equal amounts of total protein from the

clarified lysates were subjected to immunoprecipitation with an antiphosphotyrosine (PY99) antibody. The immunoprecipitates were immunoblotted with the indicated antibodies. A representative immunoblot from 3 independent experiments is shown.

Figure 4: ***H_2O_2-induced ERK1/2 and PKB phosphorylation is totally dependent on Src-PTK activity in CHO-IR cells***

Confluent, serum-starved CHO-IR cells were pretreated with or without 10 μM PP-2 or its inactive analogue PP-3 for 30 min, followed by incubation with 1 mM H_2O_2 or 100 nM insulin (Ins) for 5 min. The cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK 1/2 antibodies (upper immunoblot), phospho-specific (Ser 473)-PKB antibodies (middle immunoblot) and phospho-specific (Tyr-418)-Src antibodies (lower immunoblot). The results are representative of 3 independent experiments.

Figure 5: ***H_2O_2-induced p^{85} phosphorylation and ERK 1/2 and PKB phosphorylation are dependent on Src-PTK and PI3K, respectively***

Confluent, serum-starved CHO-IR cells were pretreated with 10 μM PP-2 or its inactive analogue PP-3 (A) or 100 nM wortmannin (W) (B and C) for 30 min, followed by incubation with 1 mM H_2O_2 for 5 min. In (A), the cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation with antiphosphotyrosine (PY99) antibody. The immunoprecipitates were immunoblotted with IR antibodies (upper immunoblot) and p^{85} antibodies (lower immunoblot). In (B), the cells were lysed, and the cell lysates were subjected to immunoprecipitation using $p^{85\alpha}$ antibodies. PI3K activity was measured in $p^{85\alpha}$ immunoprecipitates with phosphatidylinositol (PIP) as substrate, and [γ-^{32}P] ATP as phosphoryl group donor. The arrows mark the position of PI3-P. In (C), the cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK1/2 antibodies (upper

immunoblot) and phospho-specific (Ser 473)-PKB antibodies (lower immunoblot). The results are representative of 3 independent experiments.

Abbreviations

CHO-IR, Chinese hamster ovary cells overexpressing insulin receptor; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinases; GSK, glycogen synthase kinase; H_2O_2, hydrogen peroxide; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PAGE, polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKCδ, protein kinase C-zeta; PTK, protein tyrosine kinase; PTPases, protein tyrosine phosphatases; SDS, sodium dodecyl sulfate; PP-2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; PP-3, 4-amino-7-phenylpyrazol[3,4-d]pyrimidine; PY99, antiphosphotyrosine antibody.

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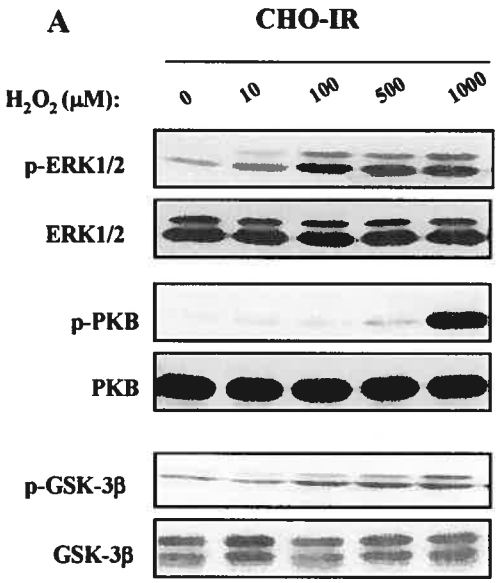
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CHO-IR
H2O2 (μM):
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100
500
1000
p-ERK1/2
ERK1/2
p-PKB
PKB
p-GSK-3β
GSK-3β

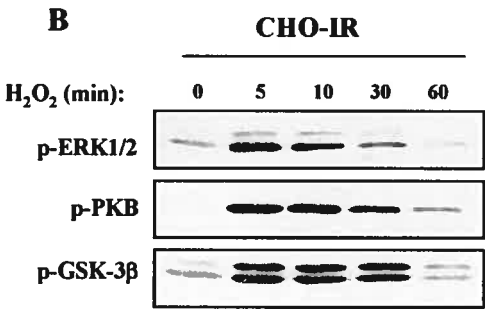
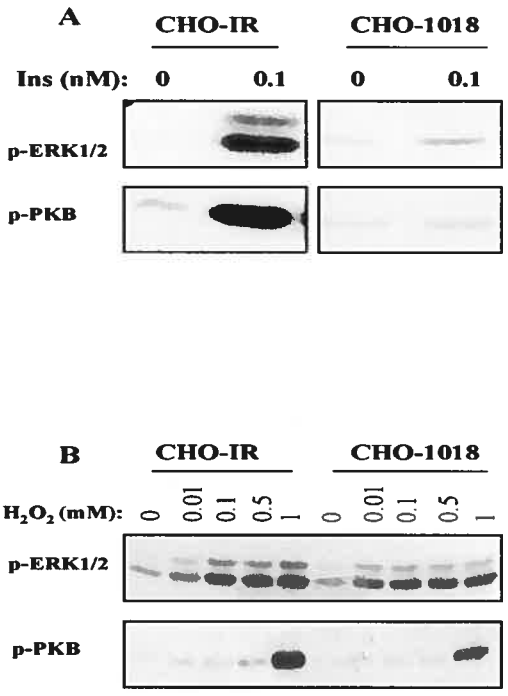

B
CHO-IR
H2O2 (min):
0
5
10
30
60
p-ERK1/2
p-PKB
p-GSK-3β

Fig. 1


A
CHO-IR
CHO-1018
Ins (nM): 0 0.1 0 0.1
p-ERK1/2
p-PKB
B
CHO-IR
CHO-1018
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p-ERK1/2
p-PKB

Fig. 2

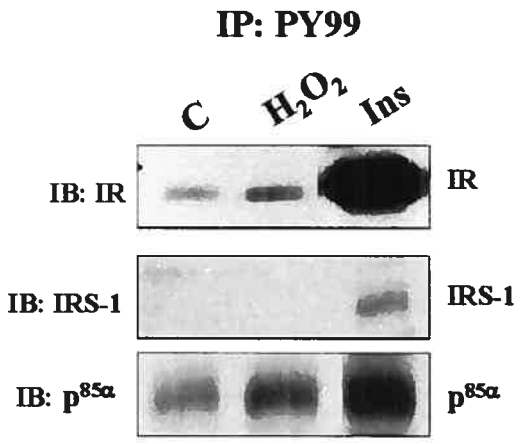


Fig. 3

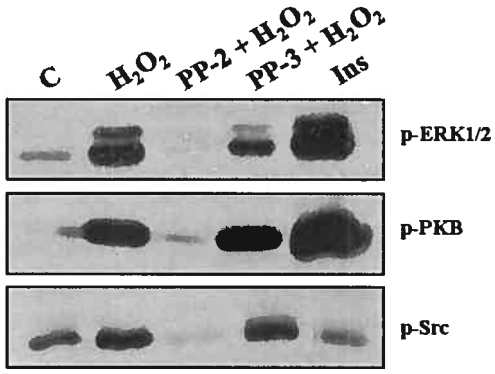
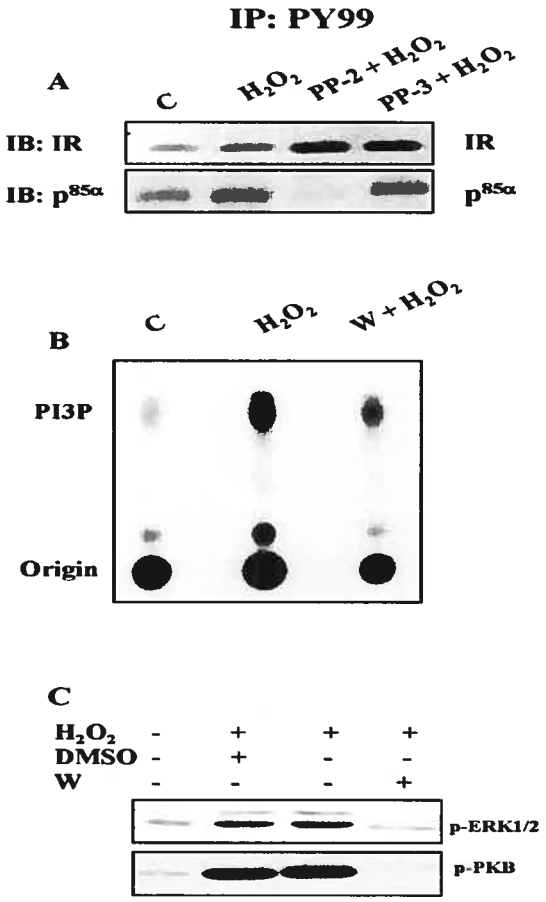


Fig. 4


IP: PY99
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IB: IR
IR
IB: $p^{85\alpha}$
$p^{85\alpha}$
B
C
H_2O_2
W + H_2O_2
PI3P
Origin
C
H_2O_2 - + + +
DMSO - + - -
W - - - +
p-ERK1/2
p-PKB

Fig. 5

CHAPTER 6

GENERAL DISCUSSION

Vanadium was first demonstrated to have insulin-like effect properties in vitro in 1979 (34) in adipocytes and hepatocytes and in vivo in 1985 (19). Since then, these findings were followed up by several groups who confirmed and extended them to animal models of type 1 and type 2 diabetes mellitus as well as in humans (68;240). The ability of vanadium compounds to stimulate glucose uptake, glycogen and lipid synthesis in muscle, adipose and hepatic tissues and to inhibit gluconeogenesis in the liver and kidney as well as lipolysis in fat cells contribute as a potential mechanism for their anti-diabetic insulin-like effects. At the cellular level, we have shown earlier that vanadium salts, such as VS and NaOV mimic insulin and activate several key elements of the insulin signal transduction pathway, such as the tyrosine phosphorylation of IRS-1, including PI3-K, PKB and Ras/MAPK.

6.1 POTENTIAL MECHANISM OF VS AS INSULIN-ENHANCER AGENT

In some of in vivo studies, the glucose-lowering effect of vanadium was shown to be dependent on the presence of endogenous insulin suggesting that vanadium does not act completely independently in vivo, but augments tissue sensitivity to low levels of plasma insulin (289). Moreover, in cell system, vanadate has been shown to enhance insulin-induced effects. For example, in isolated cardiomyocytes from obese Zucker rats, VS in combination with insulin potentiates insulin-induced glucose transport (290). In

adipocytes, sodium orthovanadate has also been reported to enhance and prolong insulin-stimulated activation of IR and lipogenesis (291). In addition, in combination with insulin, vanadium compounds prolong IR and IRS-1 phosphorylation in 32D mouse myeloid progenitor cells (292). However, the precise signaling steps by which vanadate enhances and/or prolongs insulin action remains poorly characterized. Since insulin mediates its physiological effects through PI3-K and ERK1/2 pathways, therefore, in the studies presented in chapter 2 (293), we investigated if vanadium-induced effects on insulin action are correlated with an enhancement or prolongation in the activation state of ERK1/2 and PI3-K signaling system. Thus, treatment of CHO-HIR cells with VS prior to insulin resulted in a sustained phosphoprylation of ERK1/2 and activity (293). Under these conditions, VS treatment had no effect on a dual-specificity protein phosphatase MKP-1 which is capable to dephosphorylate ERK1/2. In addition to ERK1/2 pathway; these studies have demonstrated that VS also prolonged the insulin-stimulated activation of PI3-K (293). Since PI3-K activation requires association between PI3-K via its $p^{85\alpha}$ subunits and phosporylated-IRS-1, our results demonstrated that sustained PI3-K activation by insulin in VS-pretreated cells was due to prolonged association of $p^{85\alpha}$ with IRS-1. Since vanadate-induced ERK1/2 activation is inhibited by PI3-K inhibitors and thus suggest an upstream role of PI3-K in the activation of ERK1/2 (87;247;248), we conclude that prolongation of insulin-stimulated ERK1/2 phosphorylation could be the result of sustained activation of PI3-K activity. Furthermore, we demonstrated in this study that the insulin-enhancer role of VS on ERK1/2 and PI3-K activation was independent of any alteration on the degree of the insulin-induced tyrosine phosphorylation of IRβ and IRS-1. Since vanadium salts are potent inhibitors of PTPases, it is possible that VS prolongs the duration of insulin-induced ERK1/2 and PI3-K activation through the inhibition of the dephosphorylation of specific tyrosines in IR or IRS-1 or other proteins acting upstream of PI3-K in the insulin signaling pathway. One consequence of this effect could be the sustained association of IRS-1 with $p^{85\alpha}$ and PI3-K activation. This data is confirmed by recent studies in which VS added to insulin-treated 3T3-L1 adipocytes did not alter the

effect of insulin on stimulating IR or IRS-1 tyrosine phosphorylation while VS enhanced insulin-induced glycogen synthesis (294). In contrast, vanadate was found to augment insulin-stimulated IR-PTK activity in rat adipocytes (291) and insulin-increased tyrosine phosphorylation of the insulin receptor and IRS-1 in cardiomyocytes (249) and in 32D mouse myeloid progenitor cells (292). The reason of these discrepant results is not clear but it could be due to the difference in the properties of cell lines used as well as the concentration and time of exposure with vanadium used in each of these studies. Taken together, our studies were the first to provide a molecular basis of the insulin-enhancer action of VS which could be reflected by prolongation and enhancing in the insulin-increased ERK1/2 and PI3-K activation responses. Since insulin signaling pathway is not precisely elucidated, we can not rule out that VS could enhance insulin action through additional putative mechanisms. Therefore, further work is needed to define the precise mechanism by which VS potentiates insulin-induced signaling pathways.

6.2 MECHANISM OF ACTION OF ORGANO-VANADIUM COMPOUNDS

Since relatively higher doses of inorganic vanadium compounds were needed to elicit a significant hypoglycemic response in diabetic models, it was felt, therefore, that the use of vanadium compounds with increased lipophilicity might lower the effective doses of vanadium needed to ameliorate hyperglycemia by improving gastrointestinal absorption. Therefore, several organo-vanadium compounds (OVC) were synthesized by conjugating vanadium element to organic ligand. These compounds were shown to be more potent than inorganic compounds in lowering hyperglycemia and improving insulin resistance in rodent models of diabetes mellitus (12-15;17;21;48;57). OVC was also more potent than inorganic vanadium in stimulating glucose transport (295), glycogen synthesis (296) as well as lipogenesis (297) in isolated cells. However, the molecular mechanism responsible for the stronger insulinmimetic effect of OVC over inorganic compounds remains poorly defined. Since our previous studies have found that inorganic vanadium compounds

activate several key components of the insulin signaling cascade including PI3-K, PKB and ERK1/2, we have examined here the possibility that the insulinmimetic and antidiabetic actions of OVC could be attributed to their ability to enhance the phosphorylation of PKB and its downstream target GSK-3β. Our results demonstrated that treatment of CHO-HIR by VAC or BMOV, two OVC, increased PKB and GSK-3β phosphorylation to a higher level than that detected in response to VS (298). As discussed above, PI3-K has been shown to be an upstream regulator of the PKB signaling pathway in response to insulin or inorganic vanadium compounds (90). Therefore, we investigated if OVC also activates PKB phosphorylation through PI3-K activity by using Wortmannin as a pharmacological PI3-K inhibitor. We found that PKB and GSK-3β phosphorylation induced by OVC was completely blocked by wortmannin suggesting that OVC also increased PKB phosphorylation through PI3-K. PKB pathway has been primordially implicated in the regulation of carbohydrate metabolism in response to insulin, therefore we have speculated that a more robust activation of PKB by OVC may explain their higher potency on increasing glucose transport in adipocytes (295), GLUT-4 translocation in diabetic rat skeletal muscle (80) as well as glucose utilization and storage (13;295;296). In addition, an important role of GSK-3β in the regulation of PEPCK and G6Pases has been shown (197) and vanadium compounds have been reported to inhibit the heightened expression and/or activity of PEPCK and G6Pase in several diabetic rat models (13;98;104-109;299), thus it could be possible that a greater potency of BMOV and VAC to induce GSK-3β phosphorylation may contribute to the correction of high PEPCK and G6Pase expression level in the diabetic rat liver.

Since vanadium compounds are potent protein tyrosine phosphatase (PTPase) inhibitors (236), the capacity of VAC, BMOV and VS on the inhibition of PTPase was also measured. Interestingly, the OVC were stronger inhibitors of PTPase activity than VS (298). Furthermore, the higher PTPase inhibitory potential of the OVC was associated with more robust tyrosine phosphorylation of several proteins, including the IRβ and IRS-1. In

addition, OVC exerted high efficiency in complexing IRS-1 and $p^{85\alpha}$ than VS. Since IRS-1/$p^{85\alpha}$ association is an important step leading to activate PI3-K, this data provide a molecular basis for the high impact of the OVC in activating PKB phosphorylation.

Among the two OVC tested in our studies (298), VAC has been shown to be more effective than BMOV or VS in increasing insulin signaling system. These observations are compatible with the idea that an upregulation of PI3-K/PKB signaling may be responsible for a potent effect of VAC in improving hyperglycemia and impaired hepatic gluconeogensis and glycolysis of diabetic rats (13) as well as in increasing glucose transport and lipogenesis in adipocytes (295;297). Our results on OVC-induced signaling have recently been confirmed by Mustafi et al. who demonstrated that VAC is more potent than VS in increasing PKB and GSK-3β phosphorylation in 3T3-L1 adipocytes in presence or absence of insulin (296).

In summary, the present findings indicate that, as compared to VS, OVC are more potent activators of PKB and GSK-3β phosphorylation. This response can be due to the higher PTPase inhibitory potential of OVC resulting in an increased phosphorylation of key tyrosine-phosphorylated substrates responsible to trigger the mechanism leading to upregulation of the PKB signaling pathway and eventually contributing to a greater potency of OVC in improving glucose homeostasis.

6.3 MECHANISM OF BMOV-INDUCED PKB PHOSPHORYLATION

BMOV is the most studied OVC and its insulin-like and antidiabetic properties of BMOV are well documented as discussed in chapter 1 and 3. However, the cellular mechanism of its insulin-mimetic effect is largely unclear. In CHO-HIR cells, BMOV was shown to be a potent activator of PKB and it was suggested that this might serve as a potential mechanism for the insulinmimetic and antidiabetic effect of this OVC (298). We have also

demonstrated that BMOV induced the tyrosine phosphorylation of IRβ and its substrat IRS-1 and increased the association between IRS-1 and p^{85} subunit of PI3-K. Since IRS-1, PI3-K and PKB are downstream targets of many receptors-PTKs; we attempted to identify the putative PTKs responsible for BMOV-induced effects on the activation of these signaling intermediates in human hepatoma (HepG2) cells (Chapter 4). The first finding in these studies was that BMOV, similarly to its effects on CHO-IR (298) activated PKB and GSK-3β phosphorylation as well as tyrosine phosphorylation of many proteins including IRβ, IRS-1 and p^{85} subunit of PI3-K in HepG2 cells (Chapter 4). To investigate if IR is an upstream PTK in mediating BMOV-induced phosphorylation of PKB, we used two types of CHO cells, the one overexpressing a normal IR (CHO-IR) and the other, overexpressing PTK-inactive form of IR (CHO-1018). While insulin failed to activate PKB phosphorylation in CHO-1018, BMOV increased PKB phosphorylation to same level in both cell lines. These data suggested that IR-PTK is not implicated in the phosphorylation of PKB in response to BMOV. To examine the involvement of other receptor-PTKs in the BMOV response, we have utilized a series of well characterized and widely used inhibitors of PTKs. Among these, AG1024, an IGF-1Rβ-PTK specific inhibitor (300), blocked the phosphorylation of PKB by BMOV. In contrast, AG1478 and AG1295, inhibitors of EGFR and PDGFR respectively, failed to change the activation of PKB in response to BMOV. These data suggested that IGF-1Rβ might play a role in BMOV action and allowed us to examine in more detail the implication of the IGF-1R-PTK in this event. We found that BMOV increased the tyrosine phosphorylation of IGF-1Rβ and this effect was inhibited by AG1024. In addition, AG1024 also suppressed the tyrosine phosphorylation of IRS-1 and p^{85} subunit of PI3-K further supporting the involvement of IGF-1Rβ in BMOV-induced PKB activation. Several earlier studies have shown that IR-PTK is not involved in mediating the effect of inorganic vanadium salts (238;301) and an increase in the tyrosine phosphorylation of IRβ in vanadium-mediated insulin-like effects was also not observed by several investigators (90;101;238), these studies have identified IGF-1R-PTK as an upstream regulator of PKB in response of BMOV.

In addition, these studies have also demonstrated that BMOV-induced PKB phosphorylation was decreased by Rottlerin, a PKCδ inhibitor, suggesting a role for this PKC isoform in BMOV signaling towards PKB (Chapter 4). Since PKCδ activity is associated with increase in its tyrosine phosphorylation, we investigated the effect of BMOV on the phosphorylation of total tyrosine and tyr311 of PKCδ. As speculated BMOV increased both total tyrosine as well as tyr311 phosphorylation. This effect was blocked by AG1024 pretreatment of HepG2 cells indicating that IGF-1R signals through PKCδ to activate PKB in resoponse to BMOV.

In summary, our studies show that BMOV-induced activation of PKB signaling pathway in HepG2 requires IGF-1R-PTK activity of and is independent of IR, PDGFR and EGFR. In addition, PKC_{δ} appears to mediate PKB activation in response to BMOV with evidence of an involvement of IGF-1R as an upstream modulator of PKCδ action in this response.

6.4 INVOLVEMENT OF INSULIN RECEPTOR (IR)- AND C-SRC-PTK(S) IN THE MECHANISM OF ACTION OF H_20_2

Insulin has been shown to mediate its cellular signalling and action through reactive oxygen species (ROS) generation such as H_2O_2. Nox-4, a homologue catalytic subunit of NADPH oxidase has been reported to be implicated in insulin-stimulated H_2O_2 production. Furthermore, treatment of cells with H_2O_2 has been demonstrated to mimic several physiological effects of insulin including stimulation of glucose transport, glycogen synthesis, lipogenesis as well as inhibition of lipolysis and PEPCK gene expression (302). In addition, H_2O_2 has also been found to mediate its insulin-like effects through activation of key insulin signalling components such as IRβ, PI3-K/PKB and ERK1/2. Furthermore, an involvement of ROS in vanadium-induced responses has also been demonstrated (273-275;277), and it was suggested that ROS generation by vanadium may serve as a potential mechanism for insulin-mimetic effects of these compounds.

Both H_2O_2 and vanadium have been shown to inhibit the activities of different PTPases which negatively regulates many PTKs including IR-PTK. Although the role of IR-PTK in mediating vanadium-induced cellular events has been extensively studied (42;87;90;101;117;237;238;303),the requirement of IR-PTK in H_2O_2–induced signalling has not been characterized. Therefore, we investigated if IR-PTK was involved in H_2O_2-evoked the activation of PKB and ERK1/2 pathways (304). We observed that H_2O_2 increased the phosphorylation of ERK1/2 and PKB in CHO-IR which was associated with an increased in the tyrosine phosphorylation of IRβ and $p^{85\alpha}$ subunit of PI3-K. By using the CHO-1018 cells that over express inactive form of IR, we found that H_2O_2-induced ERK1/2 and PKB was partially reduced while it was intact in CHO cells with an active IR-PTK which suggested that IR-PTK contributes partially in increasing the phosphorylation of PKB and ERK1/2. In contrast, the inhibition of the non receptor PTK c-Src by PP2, a pharmacological inhibitor of c-Src, suppressed completely H_2O_2-stimulated ERK1/2 and PKB activation, which suggested that c-Src activity has a more dominant role than IR in mediating the H_2O_2 responses. Consequently, we investigated the effect of PP2 on other components of insulin signalling pathway in response to H_2O_2. We demonstrated that c-Src inhibition by PP2 completely suppressed H_2O_2-induced tyrosine phosphorylation of $p^{85\alpha}$ whereas it failed to decrease its effect on IRβ phosphorylation suggesting that c-Src exerted its effect upstream of $p^{85\alpha}$ but downstream of IR. Cumulatively, these data suggested that the activity of both IR as well as c-Src is required to maintain full activation of ERK1/2 and PKB by H_2O_2.

Based on evidence that vanadium compounds induced the activation of PKB and ERK1/2 pathway in IR-PTK-independent manner (Chapter 4 and (301)) while H_2O_2 required at least partially an active IR-PTK to induce this events, we suggest that vanadium compounds and H_2O_2 exert insulin-like effects through different mechanism.

Conclusion and Perspectives

Overall, the work presented in this thesis (Fig. 10) demonstrates that vanadium-induced a prolonged interaction between IRS-1 and p^{85} resulting in a sustained activation of ERK1/2 and PI3-K could contribute as a potential mechanism of VS as an enhancer of insulin action. A more potent inhibition of protein tyrosine phosphatases (PTPases) by organo-vanadium compounds (OVC), resulting in a robust protein tyrosine phosphorylation of many proteins, including key components of insulin signaling pathway, as well as strong activation of PI3-K/PKB, may be responsible for a higher efficacy of the OVC versus inorganic vanadium salts as insulin-mimetic and antidiabetic agents. Further elaboration of the mechanism of action of BMOV, an OVC, has identified IGF-1R-PTK as an upstream mediator in the signaling events leading to PKB phosphorylation by BMOV. Finally, we have demonstrated that H_2O_2, another insulin-mimetic compound which is also generated in response to vanadium, induced PKB phosphorylation through both IR- and c-Src-PTK dependent pathway.

I believe that the results presented in this thesis have uncovered the mechanism(s) by which inorganic and organic vanadium compounds elicit their insulin-enhancing and -mimetic responses at the molecular level. The discovery that IGF-1R serves as a mediator of BMOV action is intriguing but requires further confirmation using IGF-1R deficient mice cells. Moreover, since many phosphotyrosylated proteins are detected in OVC-treated cells it will be important to identify the proteins by using proteomics approaches. Identification of these proteins will help to discover additional alternate new molecules involved in insulin action and glucose homeostasis.

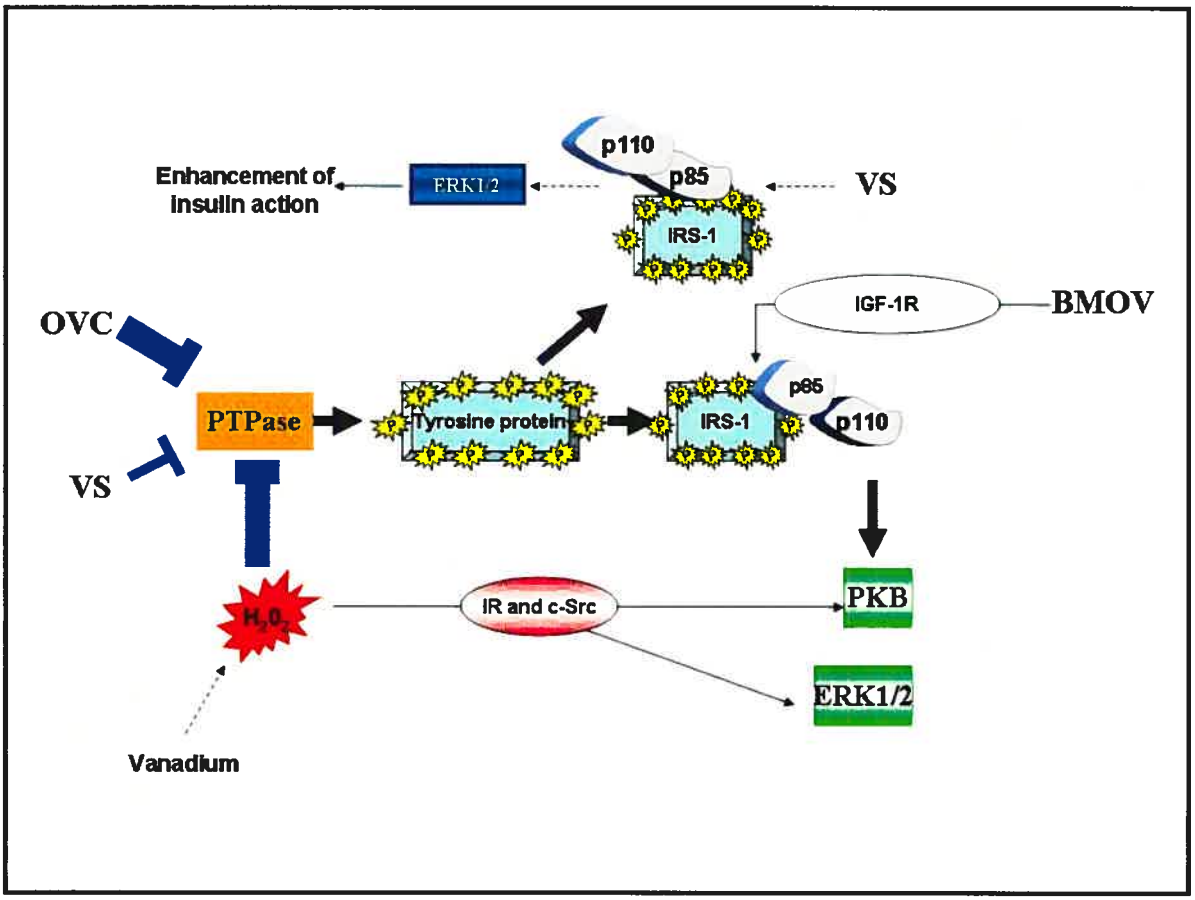

Figure 10: A model summarizing mechanism of insulin-enhancing and mimetic of vanadium compounds

The work presented in this thesis has demonstrated that a prolonged interaction of IRS-1/p^{85} in response to vanadyl sulfate (VS) resulted in a sustained activation of ERK1/2/PI3-K which might play an important role in enhancing the insulin action. We have also demonstrated that as compared to inorganic vanadium salts, the organo-vanadium compounds (OVC) are more potent inhibitors of protein tyrosine phospatases (PTPases) and strong activators of PI3-K/PKB signaling and thus may be responsible for a higher efficacy of OVC in regulating glucose homeostasis in diabetic models. An important role of IGF-1R activation as an upstream PTK to evoke BMOV-induced PKB phosphorylation was also identified in these studies. Finally, we have also established that H_2O_2, another insulin-mimetic agent which is also generated by vanadium utilized IR and c-Src-PTK to initiate the PI3-K/PKB and ERK1/2 activation. (Abbreviations are defined in the list of abbreviations)

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Annexe

- Figure I : Effect of diphenyliodonium chloride (DPI) on VAc-, BMOV- and VS-induced PKB phosphorylation in CHO-IR and HepG2 cells
- Figure II : Potential mechanism of Vanadium-evoked ROS generation
- Reprints of the published papers (Chapters 2, 3 and 5)

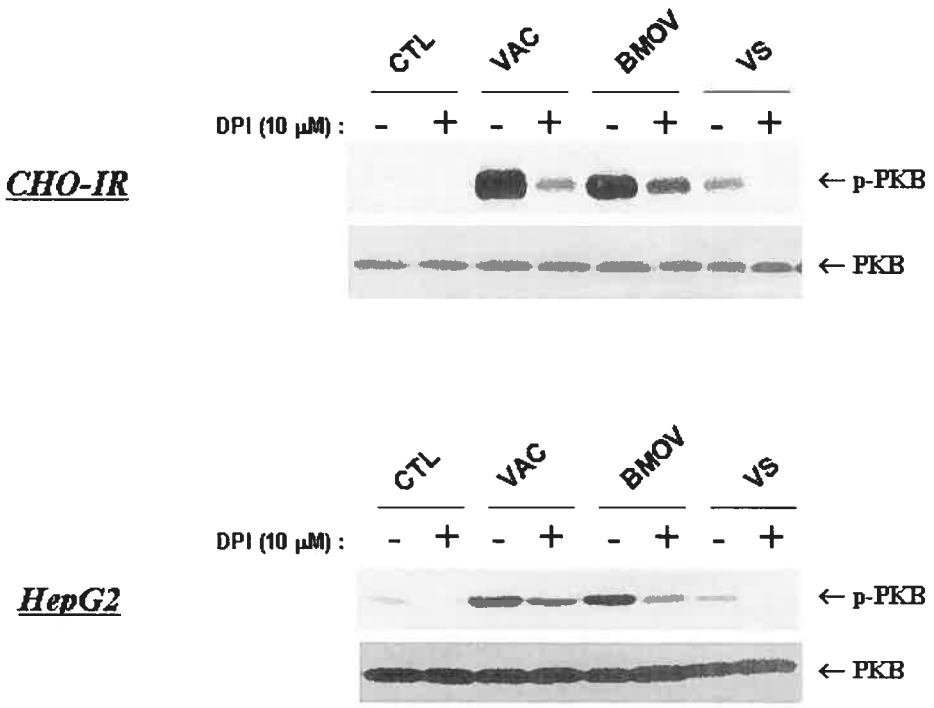

Figure I: Effect of diphenyliodonium chloride (DPI) on VAC-, BMOV- and VS-induced PKB phosphorylation in CHO-IR and HepG2 cells

Confluent, serum-starved CHO-IR cells or HepG2 cells were incubated in the presence or absence of 10μM DPI for 30 min, followed by incubation with 1 mM of VAC, BMOV or VS for 10 min. The cells were lysed, and the lysates were subjected to immunoblotting, using phospho-specific (Ser 473)-PKB antibody or total PKB antibody.

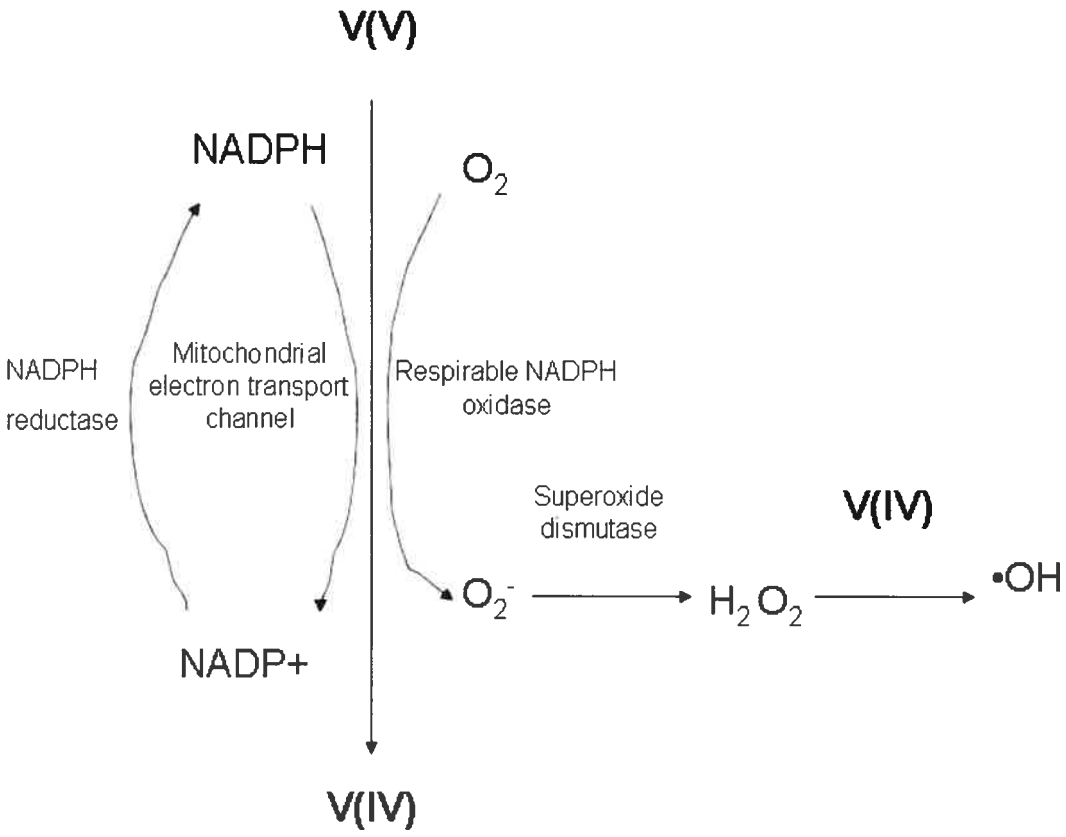

Figure II: Potential mechanism of Vanadium-evoked ROS generation

(Adapted from (273))

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Prolongation of insulin-induced activation of mitogen-activated protein kinases ERK 1/2 and phosphatidylinositol 3-kinase by vanadyl sulfate, a protein tyrosine phosphatase inhibitor☆

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Abstract

Vanadium salts such as vanadyl sulfate (VS), potent inhibitors of protein tyrosine phosphatases, have been shown to mimic, augment, and prolong insulin's action. However, the molecular mechanism of responses to these salts is not clear. In the present studies, we examined if VS-induced effects on insulin action are associated with enhancement or augmentation in the activation state of key components of the insulin signaling pathway. Treatment of insulin receptor-overexpressing cells with insulin or VS resulted in a time-dependent transient increase in phosphorylation and activation of extracellular signal-regulated kinases 1 and 2 (ERK 1/2) that peaked at about 5 min, then declined rapidly to about baseline within 30 min. However, when the cells were treated with VS before stimulation with insulin, sustained ERK 1/2 phosphorylation and activation were observed well beyond 60 min. VS treatment also prolonged the insulin-stimulated activation of phosphatidylinositol 3-kinase (PI3-K), which was associated with sustained interaction between insulin receptor substrate-1 (IRS-1) and the $p^{85\alpha}$ subunit of phosphatidylinositol 3-kinase (PI3-K) in response to insulin. These data indicate that prolongation of insulin-stimulated ERK 1/2 and PI3-K activation by VS is due to a more stable complex formation of IRS-1 with the $p^{85\alpha}$ subunit which may, in turn, be responsible for its ability to enhance and extend the biological effects of insulin.

Keywords: Insulin; Vanadium; Insulino-mimesis; Phosphatidylinositol 3-kinase; Mitogen-activated protein kinases; Protein tyrosine phosphatases

Insulin is the primary hormone involved in glucose homeostasis, and an absolute or relative lack of its secretion or action leads to aberrant glucose metabolism and diabetes. Insulin action is triggered by binding to its receptor on the cell membrane of target tissues. Insulin binding to the α-subunit of the insulin receptor (IR) results in conformational changes that activate intrinsic protein tyrosine kinase (PTK) activity of the β-subunit by its autophosphorylation in multiple tyrosine residues [1,2]. Once activated, the IR can phosphorylate several cytosolic insulin receptor substrates (IRSs) in tyrosine residues, which function as docking proteins for src homology 2 domain-containing signaling proteins (reviewed in [3]). These proteins include growth factor receptor binder 2 (Grb-2)-mammalian son of sevenless (SOS) complex [4], with Grb-2 being growth factor-binding protein, SOS being a guanine nucleotide exchange factor for guanosine triphosphate (GTP)-binding protein, and the 85-kDa regulatory subunit of phosphatidylinositol 3-kinase (PI3-K), a lipid and protein kinase [5].

The IRS-associated complex initiates two signaling pathways. In one pathway, binding of the Grb-2-SOS complex to IRS-1 leads to p21ras stimulation by GTP

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loading with subsequent activation of Raf, mitogen-activated protein kinase kinase (MEK), and two isozymic forms of mitogen-activated protein kinase (MAPK), $p44^{mapk}$ (extracellular signal-regulated kinase-1, ERK-1) and $p42^{mapk}$ (ERK 2) [2,4]. Activated MAPK phosphorylates and activates a downstream ribosomal protein kinase, $p90^{rsk}$ [6].

Another pathway that radiates from the IRS complex upon insulin stimulation involves PI3-K activation [2]. PI3-K phosphorylates phosphatidylinositol lipids at the 3 position of the inositol ring and generates 3-phosphorylated forms of PI such as phosphatidylinositol 3, 4, 5 (PIP_3) [7], which are involved in the activation of PIP_3-dependent kinase (PDK) and related serine/threonine protein kinases responsible for the phosphorylation and stimulation of several downstream signaling protein kinases such as protein kinase B (PKB), $p70^{s6k}$ [8], protein kinase-zeta [9], and glucocorticoid-inducible kinase [10]. Activation of these serine/threonine protein kinases has been implicated in many of the physiological responses of insulin at the level of glucose transport, glycogen synthesis, and protein synthesis [11–17].

Enhanced tyrosine phosphorylation of IR and IRSs is critical for the initiation of insulin signaling, whereas tyrosine dephosphorylation of IR and its substrates can attenuate and terminate insulin's action (reviewed in [18,19]). IR and IRS-1 dephosphorylation is catalyzed in vitro by several protein tyrosine phosphatases (PTPases) that consist of cytoplasmic and transmembrane receptor-type PTPases [19]. A possible role of PTPases in insulin action and in the pathogenesis of diabetes has been postulated on the basis of studies showing that vanadium-based or other small molecule inhibitors of PTPases can improve glucose homeostasis in rodent models of type I and II diabetes mellitus and in a small number of type 2 diabetic human subjects [20–23]. In this regard, it is noteworthy that the expression level of PTPases has been shown to be impaired in rodent models of type 1 and type 2 diabetes mellitus as well as in human subjects [24–27]. Further support for the involvement of PTPase in insulin's action comes from recent studies demonstrating that reduction of the PTPase PTP-1B, by using antisense oligonucleotides, normalizes blood glucose and improves insulin sensitivity in diabetic mice [28]. In addition, the ability of vanadium salts, potent PTPase inhibitors, in lowering hyperglycemia and improving insulin sensitivity in animal models of diabetes mellitus and in human subjects [20,22,29] has generated interest in exploring the molecular mechanisms of vanadium action.

We have shown earlier that vanadium salts, such as sodium orthovanadate and vanadyl sulfate (VS), mimic insulin and activate several key components, such as PI3-K and ras/MAPK, of the insulin signaling pathway [15,30–32]. Sodium orthovanadate has also been reported to enhance and prolong insulin-stimulated activation of IR-PTK and lipogenesis in rat adipocytes [33]. However, the precise signaling steps responsible for these responses to vanadium remain poorly characterized. Therefore, in the present studies, we investigated if the vanadium-induced effects on insulin action are associated with an enhancement or augmentation in the activation state of ERK 1/2 and PI3-K-two key elements of the insulin signaling pathway.

Materials and methods

Materials

Chinese hamster ovary cells overexpressing human insulin receptor (CHO-HIR cells) were a gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA). Insulin was obtained from Eli Lilly (Indianapolis, IN), VS was from Aldrich Chemical (Milwaukee, WI), and polyclonal ERK-2 antibody, monoclonal antiphosphotyrosine antibody (4G10), IR, and IRS-1 antibodies were from Upstate Biotechnology (Lake Placid, NY). Phospho-specific p44/p42 antibody was from New England BioLabs (Beverly, MA), protein A–Sepharose beads were from Pharmacia Biotech (Mississauga, Ont., Canada), and the enhanced chemiluminescense (ECL) detection system kit was from Amersham–Pharmacia Biotech (Baie d'Urfé, Que., Canada). MAPK phosphatase-1 (MKP-1) and p^{85} antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Silica gel-60 plates were obtained from Merck (Rahway, NJ).

Methods

Cell culture

Cells were maintained in HAM's F-12 medium containing 10% fetal bovine serum. They were grown to confluence in 100 mm plates and incubated in serum-free F-12 medium for 16 h prior to the experiment [15].

ERK activity

ERK catalytic activity was measured as described earlier [31]. Briefly, cells subjected to various experimental treatments were lysed in buffer A (25 mM Tris–HCl, pH 7.5, 25 mM NaCl, 1 mM Na orthovanadate, 10 mM Na fluoride, 10 mM Na pyrophosphate, 20 nM okadaic acid, 0.5 mM ethylenebis-(oxyethylenenitrilo)-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1% Triton X-100, and the lysates were clarified by centrifugation to remove insoluble material. The clarified lysates, normalized to contain equal amounts of protein (100 μg), were incubated for 4 h at 4 °C with 5 μg ERK-2 antibody preadsorbed to protein A–Sepharose beads. The immune complex was collected by centrifugation, followed by

washing three times with buffer A and once with buffer B (20 mM Hepes (*N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid), pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 10 mM β-glycerophosphate), and 40 μl of kinase buffer (25 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM DTT, 1 μM staurosporine, 0.5 mM EGTA, and 10 μl myelin basic protein (MBP) (1 mg/ml)) was added. The reaction was initiated by adding 5μl of 1 mM adenosine 5-triphosphate (ATP)[2] containing 0.5μCi [γ-^{32}P]ATP (specific activity 3,000 Ci/mmol; Amersham). After 12 min at 30 °C, the reaction was stopped by spotting an aliquot of the supernatant on P-81 filter paper, which was washed in 0.5% phosphoric acid and counted for radioactivity [30].

PI3-K assay

The clarified cell lysates were subjected to immunoprecipitation with 2 μg $p^{85\alpha}$ antibody for 2 h at 4 °C, followed by incubation with protein A–Sepharose for an additional 2 h. The immunoprecipitates were washed and subjected to PI3-K assay, as described earlier [15]. The phosphorylated lipid products were extracted and separated by ascending thin layer chromatography [15]. The radioactivity in the spots corresponding to PI3-phosphate was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunoprecipitation

The clarified cell lysates, normalized to contain equal amounts of protein (500 μg), were subjected to immunoprecipitation with 1 μg IRS-1 antibody for 2 h at 4 °C, followed by incubation with protein A–Sepharose for an additional 2 h. Immunoprecipitated IRS-1 was collected by centrifugation and washed two times with buffer A and once with phosphate-buffered saline. The IRS-1 immunoprecipitates were electrophoresed on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, transferred to PVDF membranes, and incubated with polyclonal $p^{85\alpha}$ antibody (1:4000) or a polyclonal IRS-1 antibody (1:2000). Proteins were detected by a horseradish peroxidase-conjugated second antibody and visualized with an ECL detection kit [32].

Immunoblotting

The clarified cell lysates normalized to contain equal amounts of protein were electrophoresed on 12% SDS–PAGE under reducing conditions, transferred to PVDF membranes, and incubated with monoclonal anti-phosphotyrosine antibody (1:1000), a polyclonal phospho-specific p44/p42 ERK 1/2 antibody (1:2000), a polyclonal ERK 1/2 antibody (1:8000) or a polyclonal MKP-1 antibody (1:500), followed by incubation with a horseradish peroxidase-conjugated second antibody. The antigen–antibody complex was visualized with an ECL detection kit. The immunoblots were quantified by densitometric scanning using NIH ImageJ software [32].

IR and IRS-1 tyrosine phosphorylation

The clarified lysates, normalized to contain equal amounts of protein, were pre-cleared with protein A–Sepharose for 30 min and incubated with either IR or IRS-1 antibody for 4 h at 4 °C. Immunoprecipitates were collected with protein A–Sepharose and washed 3 times with 50 mM Hepes buffer, pH 7.5, containing 0.1% Triton X-100 and 0.1% SDS, and two times with the above buffer without SDS. The immunoprecipitate was solubilized by boiling in 2× sample buffer for 10 min, electrophoresed on 10% SDS–polyacrylamide gels, and immunoblotted with antiphosphotyrosine antibody (1:1000) [15]. The blots were visualized with an ECL detection system [32].

Results and discussion

Effect of VS on insulin-stimulated ERK 1/2 activation

ERK 1/2 activation constitutes a key step in the insulin signaling pathway [2], we first examined if VS can prolong the effect of insulin on this signaling component. Therefore, insulin receptor over-expressing CHO-HIR cells were used in these studies, and the activation status of ERK 1/2 was assessed with a phospho-specific antibody of ERK 1/2. This antibody reacts with only phosphorylated (Thr202 and Tyr204) and activated forms of ERK 1/2.

As shown in Fig. 1A, treatment of CHO-HIR cells with 100 nM insulin resulted in transient activation of ERK 1/2, as judged by enhanced phosphorylation. ERK 1/2 phosphorylation was rapid peaking at 5 min and declining to just above baseline at 15 min, with almost complete dephosphorylation in 2 h. A similar transient increase in ERK 1/2 phosphorylation was seen in CHO-HIR cells stimulated with 100 μM VS, except that the intensity of phosphorylation was slightly lower than in insulin-stimulated cells, and a second phase of enhanced

[2] *Abbreviations used:* ATP, adenosine 5-triphosphate; CHO-HIR, Chinese hamster ovary cells overexpressing human insulin receptor; DPI, diphenylene iodonium; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EGTA, ethylenebis-(oxyethylenenitrilo)-tetraacetic acid; ERK, extracellular signal-regulated kinases; Grb, growth factor receptor binder; GTP, guanosine triphosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IR, insulin receptor; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAPK kinase; MKP-1, MAPK phosphatase-1; PAGE, polyacrylamide gel electrophoresis; PI3-K, phosphatidylinositol 3-kinase; PTK, protein tyrosine kinase; PTPases, protein tyrosine phosphatases; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOS, mammalian son of sevenless; VS, vanadyl sulfate.

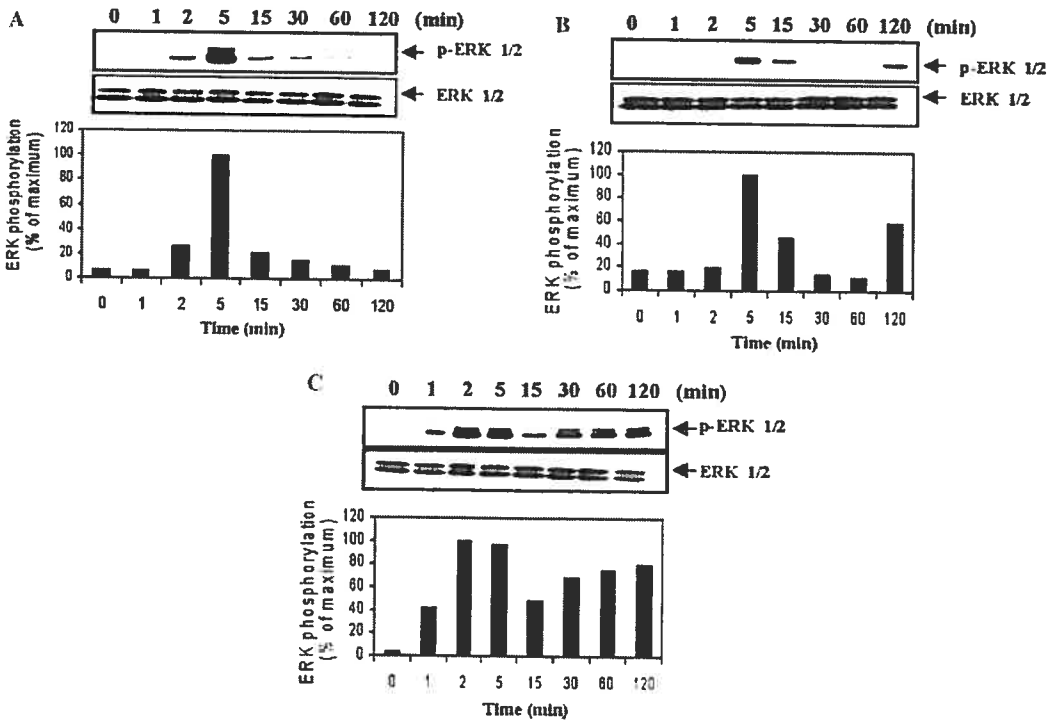

Fig. 1. VS delays the dephosphorylation of ERK 1 and ERK 2 in insulin-stimulated cells. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin (A), 100 μM VS (B) or 100 μM VS for 15 min followed by 100 nM insulin (C) for the indicated times. The cells were lysed and lysates were subjected to immunoblotting using phospho-specific (Thr202/Tyr204)-ERK 1/2 antibodies (upper immunoblot) or total ERK 1/2 (lower immunoblot). The arrows indicate the position of phosphorylated, p-ERK 1/2 or total ERK 1/2. The bottom panel shows the densitometric quantification of phospho-ERK 1/2 bands. The results are representative of three independent experiments and are expressed as a percentage of the maximum.

phosphorylation was detected at 2 h (Fig. 1B). However, when the cells were pretreated with 100 μM VS for 15 min prior to stimulation with 100 nM insulin, a rise in ERK 1/2 phosphorylation was evident as early as 1 min after the addition of insulin, with a peak at 2 min (Fig. 1C). In these cells, the level of ERK 1/2 phosphorylation declined slightly at 15 min, but was restored to just below peak levels at the 30- to 120-min time points (Fig. 1C). It is noteworthy that at all the time points tested, the magnitude of ERK 1/2 phosphorylation was significantly more robust than that observed with either insulin (Fig. 1A) or VS alone (Fig. 1B). The reason for this biphasic response remains unexplained at this moment and would require additional work. As shown in the bottom panel of the immunoblots, none of the experimental conditions altered the total amount of ERK 1/2.

In contrast to CHO-HIR cells, the effect of vanadate on ERK 1/2 phosphorylation and activation in rat adipocytes was not transient but remained sustained for up to 20 min [34]. In these studies however, no attempts were made to investigate if at later time points this response was modified [34]. Furthermore, vanadate did not exert any enhancing effect on insulin-stimulated ERK 1/2 activation in adipocytes during the 20 min time period of the study [34]. The reason for these discrepancies is not clear but it may be possible that vanadium exerts a cell-type specific response.

To determine if the pattern of ERK 1/2 phosphorylation observed in the experiments described above correlated with the catalytic phosphokinase activity of ERKs, lysates prepared from cells stimulated for the same time periods with insulin, VS and VS followed by insulin were assayed using MBP as exogenous substrate [30]. As seen in Fig. 2, similar to the effect on ERK 1/2 phosphorylation, ERK 1/2 catalytic activity in response to insulin, VS as well as VS plus insulin (VS followed by insulin treatment) peaked at 5 min, then declined to just above baseline in insulin- and VS-stimulated cells (Fig. 2A and B). It is noteworthy that in VS-stimulated cells, there was a gradual recovery after 60 min which reached about 80% of peak stimulation at 2 h (Fig. 2B). This recovery of ERK 1/2 activation correlated with the second phase of enhanced ERK 1/2 phosphorylation in response to VS (Fig. 1B). Thus, in contrast to insulin, VS exhibited a second phase of ERK 1/2 phosphorylation and activation. The reason for this second phase of activation is not clear but may be attributed to the generation of peroxovanadium due to the production of reactive oxygen species (ROS). Vanadyl is known to react spontaneously with oxygen to generate superoxide anion (O_2^-), a ROS [35] leading to the formation of

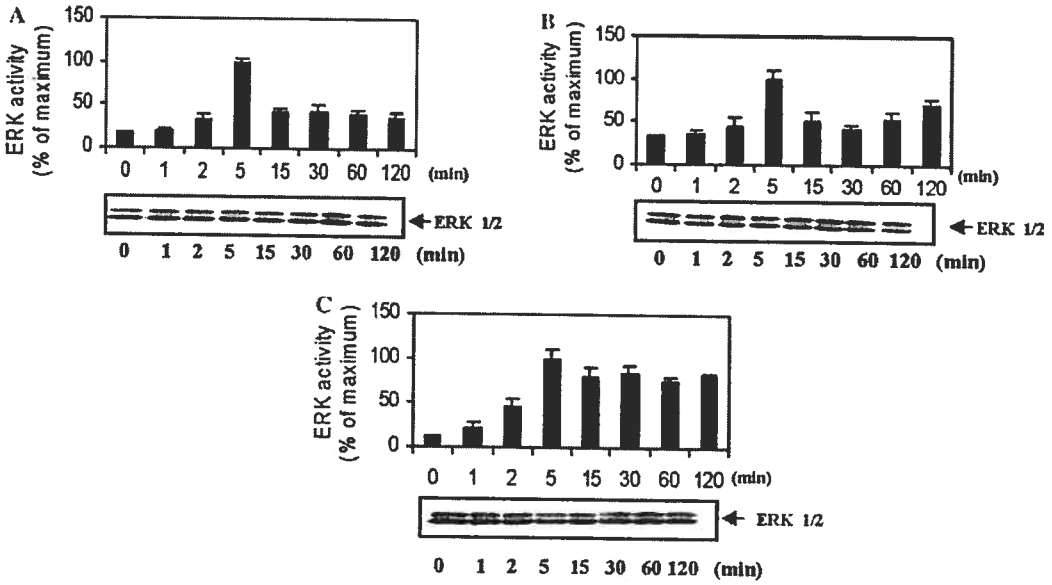

Fig. 2. VS prolongs the duration of insulin-induced ERK 1/2 activation. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin (A), 100 μM VS (B) or 100 μM VS for 15 min followed by 100nM insulin (C) for the indicated times. The cells were lysed and lysates were analyzed for the phosphokinase activity of ERKs using MBP as exogenous substrate. Parallel samples were analyzed by immunoblotting to determine the total amount of ERK 1/2 and a representative blot is shown in the bottom panel. The results are means ± SE from three separate experiments and are expressed as a percentage of the maximum.

peroxovanadyl complexes [36]. We have tested a potential role of ROS generation in VS action by utilizing diphenylene iodonium (DPI), an inhibitor of NADPH oxidase and ROS scavenger. Pretreatment of cells with DPI prior to stimulation with VS and insulin was found to attenuate ERK 1/2 phosphorylation (data not shown). Recent studies have demonstrated that ROS generation plays a critical role in activation of the insulin signaling pathway [37]. Therefore, it is possible that increased ROS generation after long incubation with VS might be responsible for the second phase of ERK 1/2 phosphorylation.

In VS + insulin-treated cells, the decline in ERK 1/2 catalytic activity was minimal (about 10%), and this level of activation (about 80–90% of the peak value) was sustained for up to 2 h of incubation (Fig. 2C). On the other hand, the level of ERK 1/2 activation in cells stimulated with insulin only declined after 15 min and remained at about baseline (Fig. 2A). Thus, pretreatment of cells with VS prolonged the duration of ERK 1/2 activation in response to insulin.

Effect of insulin on MKP-1 expression

MKP-1 is a dual specificity phosphatase which dephosphorylates MAPK at both the tyrosine and threonine residues necessary for ERK 1/2 activation of [38,39]. Stimulation of fibroblasts overexpressing the human insulin receptor by insulin has been shown to increase MKP-1 mRNA and protein levels, resulting in the attenuation of MAPK activity [40,41]. Therefore, we investigated if VS was exerting its effect by altering MKP-1 expression in CHO-HIR cells. As shown in Fig. 3A, MKP-1 protein expression was detectable even in unstimulated cells, and this level of expression was not changed significantly by insulin treatment up to 60 min. A similar pattern of MKP-1 protein expression was detected in cells treated with VS followed by insulin (Fig. 3B). It thus appears that alteration in MKP-1 expression may not contribute to the observed effects of VS on sustained ERK 1/2 phosphorylation in CHO-HIR cells by insulin. Since MKP-1 expression in response to insulin is transcriptionally regulated [40], we sought to determine if blocking MKP-1 transcription by actinomycin-D, an inhibitor of RNA synthesis, would modify the ERK 1/2 phosphorylation pattern in

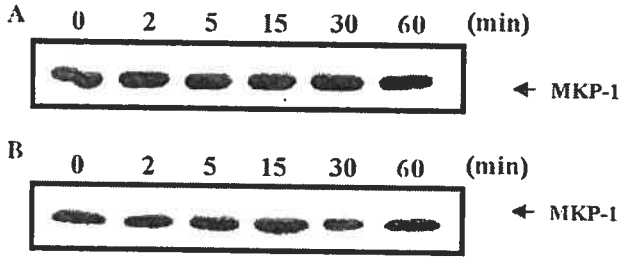

Fig. 3. MKP-1 levels are not modified by insulin or VS plus insulin. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin (A) or 100 μM VS for 15 min followed by 100 nM insulin (B) for the indicated times. The cells were lysed and the lysates were subjected to immunoblotting using MKP-1 antibody. A representative immunoblot from three independent experiments is shown.

response to either insulin alone or VS plus insulin. Actinomycin-D pretreatment, however, did not affect the time course of ERK 1/2 phosphorylation (data not shown). A similar result was observed with cycloheximide, a protein synthesis inhibitor (data not shown). These results suggest that the rapid decline in ERK 1/2 phosphorylation by insulin was not transcriptionally or translationally regulated. In addition, our earlier studies demonstrated that the effect of VS on ERK 1/2 phosphorylation was mediated by activation of MEK, which is an upstream kinase responsible for phosphorylating the threonine and tyrosine residues of ERK 1/2 [32]. Thus, it appears that in CHO-HIR cells, the outcome of VS on ERK 1/2 phosphorylation and activation is achieved by enhanced MEK phosphorylation and not by inhibiting its dephosphorylation by MKP-1. This notion is supported by the studies in which VS or vanadate was found to enhance MEK activation in CHO-HIR cells [32] and MEK phosphorylation in rat adipocytes [34].

Effect of VS on insulin-stimulated PI3-K activation

We have shown earlier that VS-induced stimulation of ERK 1/2 phosphorylation and activation is not due to decreased ERK 1/2 dephosphorylation, but is mediated by sequential stimulation of elements upstream of the ERK 1/2 activation cascade [32]. VS or vanadate has also been found to stimulate PI3-K activity in CHO-HIR cells [32] and in adipocytes [34,42,43] and the use of PI3-K inhibitors has indicated that PI3-K is an upstream regulator of the ERK 1/2 activation cascade in these cells [32]. We, therefore, investigated if prolongation of insulin-stimulated ERK 1/2 activation by VS is associated with sustained activation of PI3-K activity. PI3-K activity in cells treated for various time periods with insulin, VS and VS followed by insulin was measured in $p^{85\alpha}$ immunoprecipitates from cell lysates [32]. As shown in Fig. 4A, insulin-stimulated PI3-K activity in a time-dependent manner. PI3-K activation peaked within 1 min of exposure of cells to insulin and gradually declined to just above baseline at 30 min. This low level of activity was maintained for up to 120 min. A similar pattern of PI3-K activation in response to VS was observed except that the peak was detected only after 5 min of treatment of cells with VS and was slightly less robust as compared to insulin alone (Fig. 4B). Furthermore, VS-induced activation of PI3-K was biphasic, declining to just above baseline at 30 min but showing reactivation at 60 and 120 min of incubation (Fig. 4B). It should be

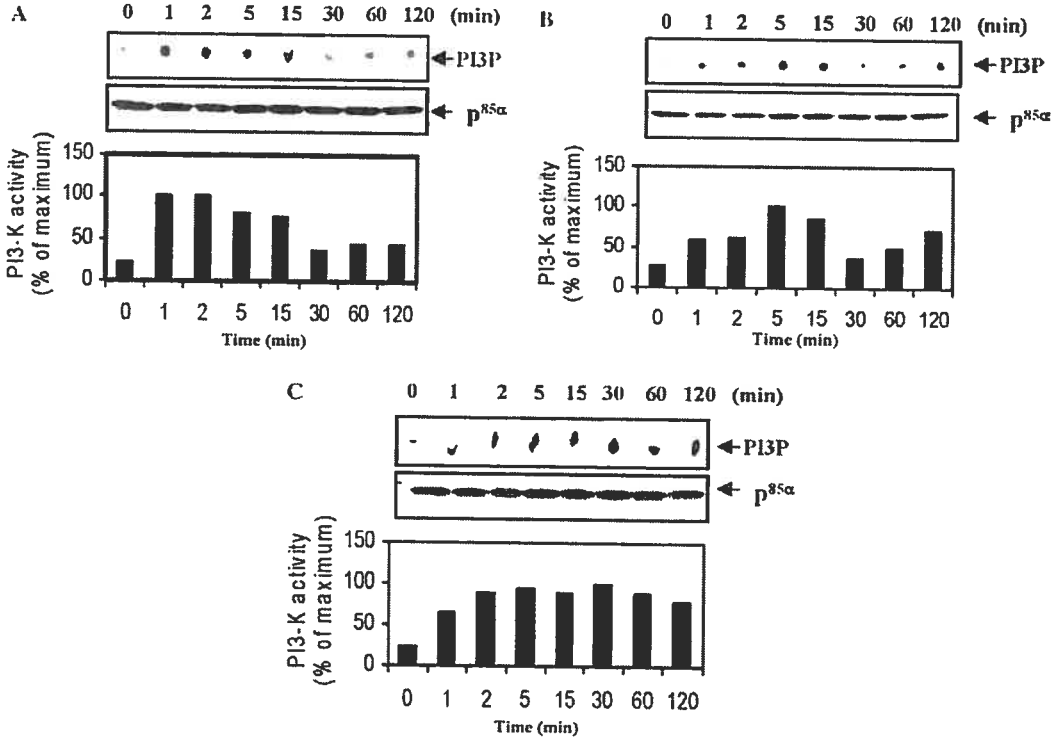

Fig. 4. VS prolongs the duration of insulin-induced activation of phosphatidylinositol 3-kinase (PI3-K). Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin (A), 100 μM VS (B) or 100 μM VS for 15 min followed by 100 nM insulin (C) for the indicated times. The cells were lysed and the cell lysates were subjected to immunoprecipitation using $p^{85\alpha}$ antibodies. PI3-K activity was measured in $p^{85\alpha}$ immunoprecipitates with phosphatidylinositol (PI) as substrate and [γ-^{32}P]ATP as phosphoryl group donor. The arrow marks the position of PI3-P. Parallel samples were analyzed by immunoblotting to determine the total amount of $p^{85\alpha}$ and a representative blot is shown in the middle panel. The results are representative of three separate experiments and are expressed as a percentage of the maximum.

noted that the biphasic impact of VS on PI3-K activation was similar to its effect on ERK 1/2 phosphorylation and activation (Figs. 1B and 2B). In contrast to the transient action of insulin, and the biphasic outcome of VS, analysis of PI3-K activity in cells treated with VS prior to stimulation with insulin revealed that VS pretreatment prolonged the duration of insulin action and resulted in sustained PI3-K activation (Fig. 4C). The prolongation of insulin-stimulated PI3-K activation by VS (Fig. 4C) correlated well with the effect of VS on insulin-induced ERK 1/2 phosphorylation and activation (Figs. 1C and 2C). However, one notable difference was that despite a sustained activation of PI3-K observed in VS plus insulin-treated cells, ERK 1/2 phosphorylation still declined slightly at 15 min (Fig. 1C). The precise reason for this decline is not clear but may be attributed to its rapid dephosphorylation by ERK 1/2-specific PTPases other than MKP-1. Alternatively a potential role of a PI3-K-independent pathway in this response may be suggested however, it should be noted that pharmacological inhibition of PI3-K activity has been shown to completely abrogate ERK 1/2 activation and phosphorylation induced by insulin and VS [32,34].

Effect of VS on insulin-induced association of $p^{85\alpha}$ with IRS-1

Since PI3-K activation requires association of its $p^{85\alpha}$ regulatory subunit with IRS-1, we next determined if the sustained PI3-K activation by insulin in VS-pretreated cells was due to a prolonged association of $p^{85\alpha}$ with IRS-1. As shown in Fig. 5A, insulin induced an increase in the association of IRS-1 with $p^{85\alpha}$ within 5 min of treatment, which peaked at 15 min and then decreased to almost basal levels within 60 min. Similar to insulin, treatment of cells with VS alone resulted in complex formation between $p^{85\alpha}$ and IRS-1 which peaked at 5 min (Fig. 5B). However, as compared to insulin treated cells, in VS treated cells, the magnitude of $p^{85\alpha}$/IRS-1 association was less and, a biphasic effect was observed, i.e., a slight decline in $p^{85\alpha}$/IRS-1 association at 15 min followed by an enhancement in $p^{85\alpha}$ binding to IRS-1 at 30 and 60 min (Fig. 5B). On the other hand, in cells pretreated with VS, insulin-induced association of $p^{85\alpha}$ with IRS-1 was sustained for up to 60 min (Fig. 5C). Thus, it is possible that the $p^{85\alpha}$/IRS-1 complex is more stable in the presence of VS and contributes to prolongation of PI3-K activity by insulin (Fig. 4C).

Effect of VS on insulin-stimulated tyrosine phosphorylation of IR and IRS-1

Since the insulin response is mediated through enhancement in the tyrosine phosphorylation of IR and IRS-1 [2,7], we examined the effect of VS on insulin-stimulated tyrosine phosphorylation of IR and IRS-1. Insulin treatment of cells resulted in a rapid increase in phosphotyrosine content of IR (Fig. 6A) and IRS-1

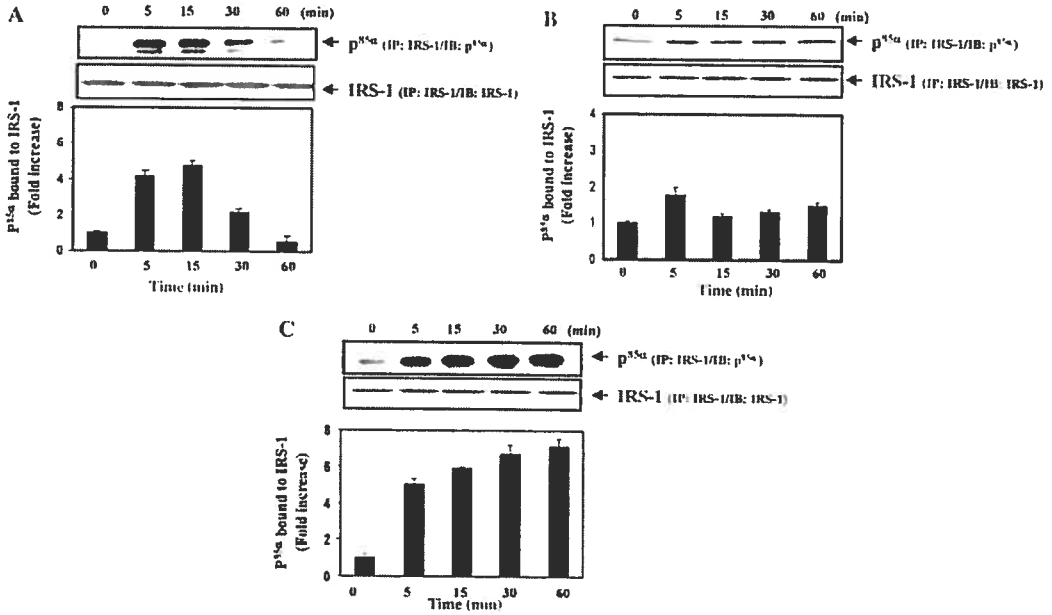

Fig. 5. VS prolongs the duration of insulin-induced binding of $p^{85\alpha}$ to IRS-1. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin alone (A), 100 μM VS (B) or 100 μM VS for 15 min followed by 100 nM insulin (C) for the indicated times. The cells were lysed and equal amounts of clarified lysates were subjected to immunoprecipitation using IRS-1 antibodies. IRS-1 immunoprecipitates (IP) were immunoblotted (IB) with either anti-$p^{85\alpha}$ (upper panel) (1:4000) or with IRS-1 antibody (middle panel) (1:1000). The bottom panel shows the densitometric quantification of $p^{85\alpha}$ bound to IRS-1. The results are representative of three independent experiments and are expressed as fold increase over control (0 min) which was arbitrarily set at 1.0.

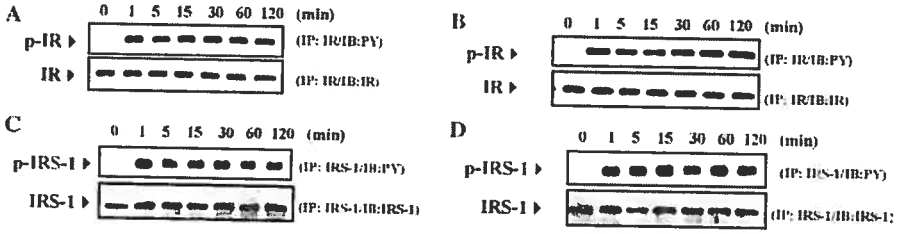

Fig. 6. Insulin-induced IR or IRS-1 phosphorylation on tyrosine is not altered by VS. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin alone (A, C), or with 100 μM VS for 15 min followed by 100 nM insulin (B, D) for the indicated times. The cells were lysed and equal amounts of clarified lysates were subjected to immunoprecipitation using IR antibodies (A,B) or IRS-1 antibodies (C,D). The immunoprecipitates (IP) were immunoblotted with antiphosphotyrosine antibody (upper panels) or IR or IRS-1 antibodies (lower panels in each section). A representative immunoblot from three independent experiments is shown.

(Fig. 6C). Maximum IR and IRS-1 phosphorylation was detected within 1 min of insulin stimulation and this level did not change significantly (Fig. 6) for up to 120 min. It is interesting that the decline in ERK 1/2 and PI3-K activation observed in insulin-stimulated cells at 15 min (Figs. 1A, 2A and 4A) was not associated with a similar decrease in the tyrosine phosphorylation of either IR or IRS-1 (Fig. 6). In fact, at this time point, the global tyrosine phosphorylation of IR or IRS-1 was almost the same as that occurring at 1 min after insulin stimulation. Thus, it appears that enhanced tyrosine phosphorylation of IR and IRS-1 is critical for initiating the insulin signal, but may not have a significant role in regulating the duration of the response. Since VS pretreatment of cells prior to stimulation with insulin failed to alter the global tyrosine phosphorylation status of IR (Fig. 6B) or IRS-1 (Fig. 6D) while prolonging the duration of insulin-induced ERK 1/2 and PI3-K activation, it is possible that VS exerts these effects by inhibiting the dephosphorylation of specific phosphotyrosines in IR or IRS-1 or other proteins acting upstream of PI3-K in the insulin signaling pathway. One consequence of this effect is the sustained association of IRS-1 with the $p^{85\alpha}$ subunit and PI3-K activation. In contrast to the work presented here, vanadate was found to augment insulin-stimulated IR-PTK activity in rat adipocytes [33]. The reason for this discrepancy is not clear but it is possible that IR overexpressing cells respond differently than normal adipocytes. Further work is needed to define the precise mechanism by which VS modifies insulin-induced signaling pathways.

In summary, an important finding of the present study is that VS, a PTPase inhibitor and insulinomimetic agent, increased the magnitude and duration of insulin-stimulated ERK 1/2 and PI3-K activation. This effect of VS on insulin response was associated with an enhanced interaction of IRS-1 with the $p^{85\alpha}$ subunit of PI3-K which appeared to be responsible for a sustained activation of PI3-K. Since PI3-K activation has been implicated in insulin-induced activation of glucose transport and lipogenesis, the results of the present investigation provide a mechanistic basis for the observed augmentation of insulin-induced lipogenesis [33] and glucose transport [44] by vanadate. Taken together, the present work provides first evidence suggesting that prolonged activation of ERK 1/2 and PI3-K, key components of the insulin signaling pathway, contributes to the molecular mechanism of the insulin-enhancing effect of this vanadium.

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Organo-vanadium compounds are potent activators of the protein kinase B signaling pathway and protein tyrosine phosphorylation: Mechanism of insulinomimesis

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Abstract

Organo-vanadium compounds (OVC) have been shown to be more effective than inorganic vanadium compounds in ameliorating glucose homeostasis and insulin resistance in rodent models of diabetes mellitus. However, the precise molecular mechanism of OVC efficiency remains poorly defined. Since inorganic vanadium compounds have been found to activate several key components of the insulin signaling cascade, such as protein kinase B (PKB), the objective of the present study was to investigate if stimulation of PKB and its downstream target glycogen synthase kinase-3 (GSK-3), are responsible for the more potent insulinomimetic effects of OVC. Among several vanadium compounds tested, vanadium (IV) oxo bis (acetylacetonate) and vanadium (IV) oxo bis(maltolato) markedly induced the phosphorylation of PKB as well as GSK-3β compared to vanadyl sulfate (VS), an inorganic vanadium salts in Chinese hamster ovary cells overexpressing the insulin receptor (IR). Furthermore, the OVC were stronger inhibitors of protein tyrosine phosphatase (PTPase) activity than VS. The higher PTPase inhibitory potential of the OVC was associated with more robust tyrosine phosphorylation of several cellular proteins, including the IRβ subunit and insulin receptor substrate-1 (IRS-1). In addition, greater IRS-1/$p^{85\alpha}$ interaction was elicited by the OVC than by VS. These data indicate that the higher PTPase inhibitory potential of OVC translates into greater phosphorylation of PKB and GSK-3β, which, in turn, may contribute to a more potent effect of OVC on glucose homeostasis.

Keywords: Diabetes; Insulin; Vanadium; Protein kinase B; Protein tyrosine phosphatase

Compounds of the trace element vanadium have been demonstrated to exert a variety of insulin-like effects in in vitro and in vivo systems [1], including their ability to improve glucose homeostasis and insulin resistance in animal models of diabetes mellitus [1–3]. In addition, several reports have documented vanadium therapy-induced improvements in liver and muscle insulin sensitivity in a number of type II diabetic human subjects [4–8]. Vanadium compounds have also been shown to stimulate glucose uptake [9–11], glycogen synthesis [9,12–15], and lipid synthesis [16,17] in muscle, adipose, and hepatic tissues. They inhibit gluconeogenesis, the activities of the gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) in the liver and kidneys [9,18–20] as well as lipolysis [17,21] in fat cells. At the cellular level, vanadium activates key elements of the insulin signaling system: tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) [22] and activation of pathways such as extracellular signal-regulated kinase (ERK1/2) [23,24], phosphatidylinositol 3-kinase (PI3-K)

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[22,25], and protein kinase B (PKB)[1] [23,26]. These effects are believed to be secondary to its ability to inhibit protein tyrosine phosphatase (PTPase) activity [27].

PKB plays a critical role in insulin-induced glucose transport, glycogen synthesis, proliferation, and cell survival [28]. It is phosphorylated on serine 473 and threonine 308 residues by PI3-K-dependent kinase [28,29] resulting in its full activation. Activated PKB phosphorylates several downstream targets, such as glycogen synthase kinase-3 β (GSK-3β) and forkhead transcription factor (FKHR). GSK-3β is phosphorylated by PKB on serine 9, and this phosphorylation leads to the inhibition of its catalytic activity [30]. GSK-3β plays an important role in the regulation of glycogen synthesis and regulates gluconeogenesis by altering the gene expression of PEPCK and G6Pase [31]. Vanadium treatment has been found to decrease the heightened expression and/or activity of PEPCK and G6Pase in isolated cells as well as in diabetic rats [18–20,32,33]. Recent studies have revealed that in comparison to inorganic vanadium compounds, such as vanadyl sulfate (VS), sodium metavanadate (NaMV), and sodium orthovanadate (NaOV), organo-vanadium complexes (OVC) of vanadium such as vanadium (IV) oxo bis (acetylacetonate) (VAC) and vanadium (IV) oxo bis(maltolato) (BMOV) are more potent in lowering blood glucose in rodent models of diabetes mellitus [18,34,35]. However, the molecular mechanism responsible for the stronger insulinomimetic effect of OVC over inorganic vanadium salts remains unknown. Therefore, the aim of the present study was to investigate if the insulinomimetic and antidiabetic actions of OVC could be attributed to their ability to enhance PKB and GSK-3β phosphorylation.

Materials and methods

Materials

Chinese hamster ovary cells overexpressing human insulin receptor (CHO-HIR cells) were a generous gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA). Insulin was from Eli Lilly (Indianapolis, IN), VS, NaOV, and NaMV were from Aldrich Chemical (Milwaukee, WI), and the OVC were kindly donated by Dr. Debbie Crans (Colorado State University, Fort Collins, CO). Polyclonal insulin receptor β (IRβ) subunit antibody, monoclonal antiphosphotyrosine antibody (PY99), polyclonal phospho-GSK-3β (Ser^9) antibody, and polyclonal GSK-3β antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). Polyclonal phospho-PKB (Ser^{473}) and polyclonal PKB antibody were from Cell Signaling (Beverly, MA). Polyclonal IRS-1 antibody and polyclonal $p^{85\alpha}$ antibody were from Upstate Biotechnology (Lake Placid, NY). The enhanced chemiluminescence (ECL) detection system kit was from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada).

Methods

Cell culture

CHO-HIR cells were maintained in Ham's F-12 medium containing 10% fetal bovine serum, and rat 1 fibroblast overexpressing HIR and protein tyrosine phosphatase 1B (PTP1B) cells (HircA) were maintained in DMEM–F-12 medium containing 10% fetal bovine serum. The cells were grown to confluence in 60-mm plates and incubated in serum-free medium for 16 h prior to the experiment [23].

Immunoblotting

Cells subjected to various experimental treatments were lysed in buffer A (25 mM Tris–HCl, pH 7.5, 25 mM NaCl, 1 mM NaOV, 10 mM Na fluoride, 10 mM Na pyrophosphate, 20 nM okadaic acid, 0.5 mM ethylenebis-(oxyethylenenitrilo) tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1% Triton X-100), with the lysates being clarified by centrifugation to remove insoluble material. The clarified cell lysates normalized to contain equal amounts of protein were subjected to 7.5 or 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, transferred to PVDF membranes, and incubated with the indicated antibodies. Proteins were detected by a horseradish peroxidase-conjugated second antibody and visualized with an ECL detection kit [23].

Immunoprecipitation

The clarified cell lysates, normalized to contain equal amounts of protein (500 μg), were immunoprecipitated with 3 μg of IRS-1 or IRβ antibody overnight at 4 °C, followed by incubation with protein A–Sepharose for 2 h. Immunoprecipitated IRS-1 or IRβ was collected by centrifugation, washed two times with buffer A and once with phosphate-buffered saline containing PTPase and protease inhibitors. The immunoprecipitates were submitted to 7.5% SDS–PAGE under reducing conditions, transferred

[1] *Abbreviations used:* PKB, protein kinase B; BMOV, vanadium (IV) oxo bis(maltolato); Crdipic, chromium (III) bis(2,6-pyridinecarboxylate); Co2dipic, cobalt (II) bis(2,6-pyridinecarboxylate) heptahydrate; CHO-HIR, Chinese hamster ovary cells overexpressing human insulin receptor; ECL, enhanced chemiluminescence; FKHR, forkhead transcription factor; GSK-3, glycogen synthase kinase-3; G6Pase, glucose-6-phosphatase; HircA, rat 1 fibroblast overexpressing human insulin receptor and PTP1B; IR, insulin receptor; IRS-1, insulin receptor substrate-1; NaMV, sodium metavanadate; NaOV, sodium orthovanadate; OVC, organo-vanadium compounds; PEPCK, phosphoenolpyruvate carboxykinase; PI3-K, phosphatidylinositol 3-kinase; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PTP1B, protein tyrosine phosphatase-1B; PY99, monoclonal antiphosphotyrosine antibody; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STZ, streptozotocin; VAC, vanadium (IV) oxo bis (acetylacetonate); VS, vanadyl sulfate.

to PVDF membranes, and incubated with polyclonal p^{85} antibody (1:4000) or PY99 (1:2000). Proteins were detected by a horseradish peroxidase-conjugated second antibody and visualized with an ECL detection kit.

PTPase activity

Confluent, serum-starved HircA cells were incubated with 1 mM of vanadium compounds for 10 min. The cells were lysed in buffer A without NaOV and equal amounts of the clarified lysates were assayed for total PTPase activity as described elsewhere [36]. Briefly, lysates adjusted to contain the same amount of proteins were added to PTPase assay buffer containing 40 mM NaOAc/HCl, pH 5.00, 1 mM ethylenediaminetetraacetic acid, 1 mM DTT, and 25 mM *p*-nitrophenyl phosphate. Phosphatase reaction was carried out for 10 min at room temperature and stopped by the addition of 0.2 M NaOH. The amount of product (*p*-nitrophenol) produced was measured by monitoring the increase in absorbance at 405 nm optical density. The non-enzymatic hydrolysis of *p*-nitrophenol phosphate was corrected by measuring the increase in absorbance at 405 nm in the absence of enzyme.

Results

Effect of different organic and inorganic vanadium compounds on PKB phosphorylation

To evaluate the potency of different vanadium compounds on PKB phosphorylation, CHO-HIR cells were treated with 1 mM of two OVC, VAC or BMOV or three inorganic vanadium compounds VS, NaMV or NaOV. PKB phosphorylation was measured by using a phospho-specific PKB antibody which detects only the phosphorylated and active form of PKB. As shown in Fig. 1, the OVC BMOV and VAC were more potent than VS in enhancing PKB phosphorylation. In fact, the phosphorylation induced by VAC was virtually equipotent to that elicited by 100 nM insulin. However, other vanadium compounds or compounds of other metals such as Cr and Co failed to exert any effect on PKB phosphorylation.

To further compare the relative abilities of the most active vanadium compounds VAC, BMOV, and VS on PKB phosphorylation, dose–response analysis was performed. As shown in Fig. 2A, VAC was more potent than either BMOV or VS in stimulating PKB phosphorylation and caused a robust increase at 100 μM concentration.

Effect of different concentrations of VAC, BMOV, and VS on GSK-3β phosphorylation

Since PKB elicits its response through the phosphorylation of several downstream targets such as GSK-3β [29], we investigated if vanadium compounds exerted any effect on the phosphorylation of this substrate. To test this possibility, lysates prepared from cells stimulated with different concentrations of VAC or BMOV and VS for 10 min were immunoblotted using phospho-specific antibodies for GSK-$3\beta^{(Ser9)}$. As shown in Fig. 2B, all the vanadium compounds at the two concentrations increased the phosphorylation of GSK-3β. However, as was the case with PKB phosphorylation, VAC was the most potent compound and was several fold more efficient than BMOV or VS. At 100 μM, VAC was about two times more effective than BMOV and about four times stronger than VS in enhancing GSK-3β phosphorylation.

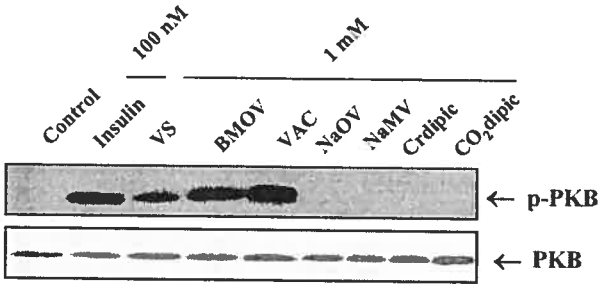

Fig. 1. Vanadium compound-induced PKB phosphorylation in CHO-HIR cells. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM of insulin for 5 min or 1 mM of different vanadium compounds or compounds of other metals for 10 min. The cell lysates were subjected to immunoblotting using phospho-specific (Ser 473)-PKB (upper immunoblot) or total PKB antibody (lower immunoblot) as described in Materials and methods. The results are representative of three independent experiments. VAC, vanadium (IV) oxo bis (acetylacetonate); VS, vanadyl sulfate; BMOV, vanadium (IV) oxo bis(maltolato); Crdipic, chromium (III) bis(2,6-pyridinecarboxyylate); NaMV, sodium metavanadate; NaOV, sodium orthovanadate; Co2dipic, cobalt (II) bis(2,6-pyridinecarboxylate) heptahydrate.

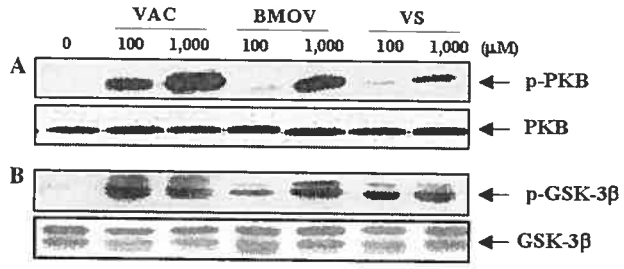

Fig. 2. VAC-, BMOV-, and VS-induced PKB phosphorylation is dose-dependent in CHO-HIR cells. Confluent, serum-starved CHO-HIR cells were incubated with the indicated concentrations of VAC, BMOV or VS for 10 min. The cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Ser 473)-PKB antibody (upper immunoblot in A), total PKB antibody (lower immunoblot in A), phospho-specific (Ser 9)-GSK-3β (upper immunoblot in B) and total GSK-3β antibody (lower immunoblot in B). The results are representative of three independent experiments.

Effect of Wortmannin on VAC-, BMOV-, and VS-induced phosphorylation of PKB and GSK-3β

PI3-K is an upstream regulator of the PKB signaling pathway in response to insulin [28,29]. Therefore, by

using Wortmannin as a pharmacological PI3-K inhibitor [37] we investigated if PI3-K plays a role in vanadium compound-induced PKB and GSK-3β phosphorylation. As shown in Fig. 3, Wortmannin pretreatment resulted in almost complete attenuation of insulin, as well as vanadium-induced PKB and GSK-3β phosphorylation.

Effect of VAC, BMOV, and VS on PTPase activity and protein tyrosine phosphorylation

Vanadium compounds are potent PTPase inhibitors [1,27,38]. Therefore, we wished to examine if the higher capacity of VAC and BMOV vs VS on PKB and GSK-3β phosphorylation is reflected in their ability to differentially inhibit PTPase activity. As shown in Fig. 4A, treatment of HircA cells overexpressing PTP1B with VAC or BMOV resulted in 50% inhibition, whereas VS caused only 20% inhibition of total PTPase activity. Next, we examined if the higher potency of OVC to inhibit PTPase activity correlated with their ability to increase the tyrosine phosphorylation of cellular proteins. As shown in Fig. 4B, treatment of CHO-HIR with 1 mM of different vanadium compounds augmented the tyrosine phosphorylation of several proteins with varying degrees. The molecular sizes of these proteins ranged from 60 to 200 kDa. However, phosphorylation of protein was more robust with VAC than with either BMOV or VS.

Effect of VAC, BMOV, and VS on IRβ and IRS-1 phosphorylation

Since the tyrosyl phosphorylation of IRβ is an early step in the insulin signaling cascade, we wished to determine if vanadium compounds enhanced the tyrosine phosphorylation of IRβ. As shown in Fig. 5A, IRβ immunoprecipitation from CHO-HIR treated with 1 mM of VAC, BMOV, and VS or 100 nM of insulin,

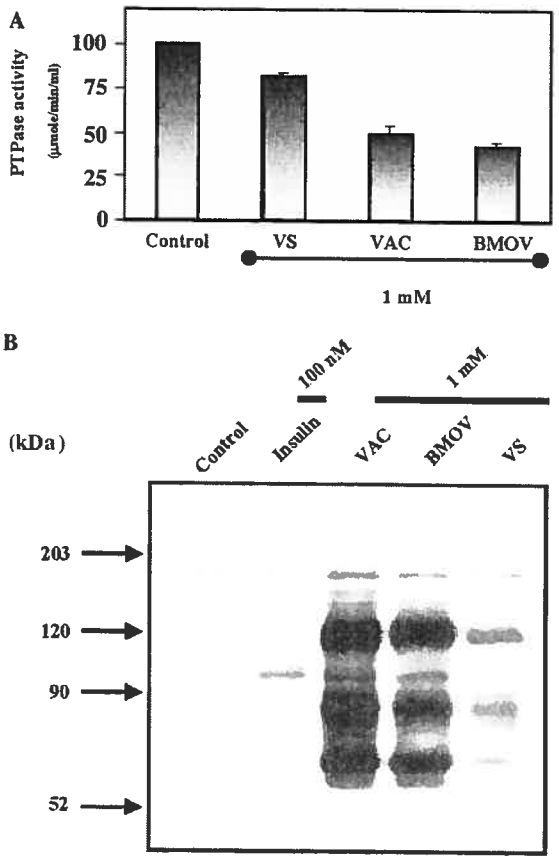

Fig. 4. VAC and BMOV are more potent than VS in inhibiting PTPase activity and in enhancing tyrosine phosphorylation. Confluent, serum-starved HircA (A) or CHO-HIR (B) cells were incubated with 1 mM of VAC, BMOV or VS for 10 min. The cells were lysed and the lysates were subjected to measure PTPase activity as described in Materials and methods (A) or to immunoblotting, using antiphosphotyrosine antibody (PY99) (B). The results are representative of three independent experiments.

followed by immunoblotting with phosphotyrosine antibody, revealed that both VAC and BMOV induced an increase in tyrosyl phosphorylation of IRβ. However,

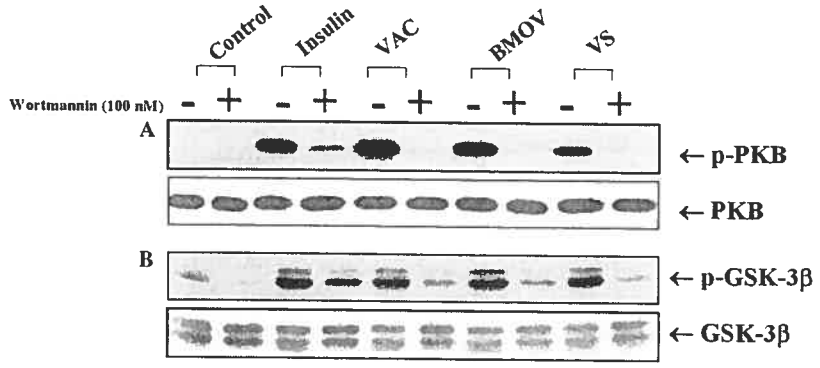

Fig. 3. VAC-, BMOV-, and VS-induced PKB and GSK-3β phosphorylation is dependent on PI3-K activity. Confluent, serum-starved CHO-IR cells were incubated in the presence or absence of 100 nM Wortmannin for 30 min, followed by incubation with 100 nM of insulin for 5 min or 1 mM of VAC, BMOV or VS for 10 min. The cells were lysed and the lysates were subjected to immunoblotting, using phospho-specific (Ser 473)-PKB antibody (upper immunoblot in A), total PKB antibody (lower immunoblot in (A)), phospho-specific (Ser 9)-GSK-3β (upper immunoblot in (B)), and total GSK-3β antibody (lower immunoblot in (B)). The results are representative of three independent experiments.

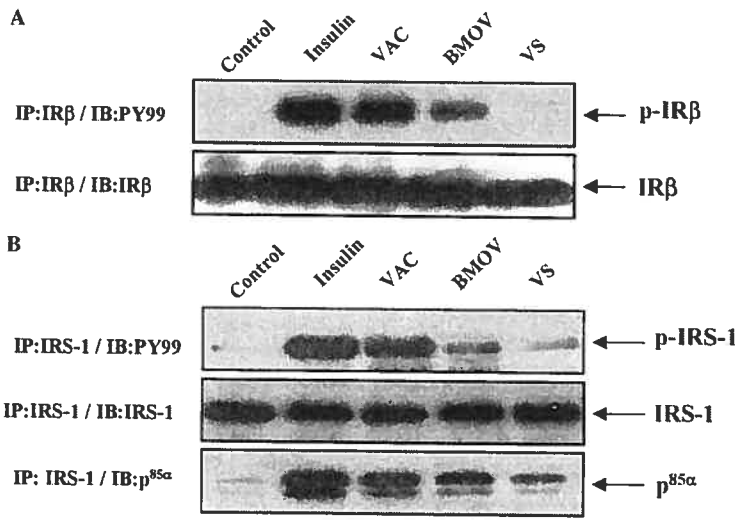

Fig. 5. VAC and BMOV are more potent than VS in enhancing the tyrosine phosphorylation of IRβ, IRS-1, and IRS-1/p^{85} association. Confluent, serum-starved CHO-HIR cells were incubated with 100 nM insulin for 5 min or with 1 mM of VAC, BMOV or VS for 10 min. The cells were lysed and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with an anti-IRβ (A) or IRS-1 (B) antibody. The immunoprecipitates were immunoblotted (IB) with the indicated antibodies. A representative immunoblot from three independent experiments is shown.

under similar conditions, VS was ineffective and failed to enhance IRβ phosphorylation. We next assessed the effect of different vanadium compounds on the tyrosine phosphorylation of IRS-1. As shown in Fig. 5B, insulin as well as all vanadium compounds enhanced IRS-1 tyrosine phosphorylation to varying degrees. BMOV- and VAC-induced IRS-1 tyrosine phosphorylation was identical to their effects on IR_β phosphorylation. VS which was unable to cause any detectable increase in IRβ tyrosine phosphorylation also enhanced IRS-1 phosphorylation, however, this was several times lower than that elicited by BMOV or VAC. Since PI3-K activation requires the association of its $p^{85\alpha}$ regulatory subunit with the tyrosyl phosphorylated form of IRS-1, we next investigated the degree of association of $p^{85\alpha}$ with IRS-1 in cells treated with various vanadium compounds. As shown in Fig. 5B, all the agents tested enhanced the association of $p^{85\alpha}$ with IRS-1, but insulin induced the highest level of association between IRS-1 and $p^{85\alpha}$, which was followed by VAC, BMOV, and VS with decreasing order of potency. This degree of association is consistent with the level of IRS-1 as well as PKB phosphorylation achieved in response to insulin and vanadium compounds.

Discussion

In the current study, we have demonstrated that OVC are more potent than inorganic forms of vanadium in activating key components of the insulin signaling pathway. The OVC VAC and BMOV were more efficient than VS in stimulating PKB activity which was associated with an increase in GSK-3β phosphorylation. Our results also show that Wortmannin, a PI3-K inhibitor, decreased insulin- and vanadate-induced PKB and GSK3-β phosphorylation (Fig. 5), suggesting that PI3-K is an upstream component in vanadium-induced PKB and GSK-3β phosphorylation events. Although it is well known that OVC are more potent than inorganic vanadium salts in improving glucose homeostasis in animal models of insulin resistance [18,34,35,39,40], the precise mechanism of this effect is unknown. Since activation of the PKB signaling system is believed to play a key role in mediating the metabolic responses of insulin [28], to the best of our knowledge, our data are the first to provide a molecular basis for the higher efficacy of OVC in improving glucose homeostasis compared to inorganic vanadium salts. More robust activation of PKB may also be responsible for several fold higher glucose transport observed in adipocytes in response to VAC [41] as well as the increased GLUT-4 translocation in streptozotocin (STZ)-diabetic rat skeletal muscle in vivo [42]. GSK-3β has been suggested to play an important role in decreasing the activity of glycogen synthase by catalyzing its serine phosphorylation [43] but PKB-induced phosphorylation of GSK-3β renders it inactive and allows the full activation of glycogen synthase [43]. Thus, the OVC-induced robust enhancement of GSK-3β phosphorylation observed in our study could serve as a potential mechanism for a more potent effect of these compounds on glucose utilization and storage seen in BMOV- and VAC-treated rodent models [18,41].

GSK-3β has been implicated in regulating the gene expression of the key gluconeogenic enzymes PEPCK and G6Pase [28,29.31]. Vanadium compounds have also been shown to inhibit the heightened expression and/or activity of PEPCK and G6Pase in several diabetic rat

models [18–20,33,44,45]. Thus, it is possible that a greater ability of BMOV and VAC to induce GSK-3β phosphorylation may contribute to the normalization of exaggerated PEPCK and G6Pase expression in the STZ-diabetic rat liver [20].

Our data also indicated that treatment of intact cells with OVC inhibited the activity of cellular PTPase more potently than VS. These data are in agreement with recent studies in which BMOV was reported to be a non-selective competitive inhibitor of several PTPases *in vitro* [39,40,46]. PTPases play an important role in the regulation of insulin signaling [47,48], and it was shown recently that mice deficient in PTP1B, a cytoplasmic PTPase, exhibit increased insulin sensitivity with resistance to weight gain, even when put on a high-fat diet [49]. Moreover, several studies have documented an overexpression of PTPases in diabetes (reviewed in [47]), and BMOV treatment inhibits exaggerated PTP1B activity in the skeletal muscle of Zucker fatty rats, a model of insulin resistance [46]. Since enhanced tyrosine phosphorylation caused by both protein tyrosine kinase (PTK) activation and/or PTPase inhibition is primordial in triggering the insulin signaling cascade, it is possible that more potent inhibition of PTPase by OVC contributes to a heightened tyrosine phosphorylation of IRβ and IRS-1 by both VAC and BMOV. In confirmation with our data BMOV was recently shown to enhance the tyrosine phosphorylation of IRβ [39,40]. In addition, the OVC were superior to VS in augmenting the tyrosine phosphorylation of the IRS-1 and IRS-1/$p^{85\alpha}$ association which might provide a molecular basis for the high impact of these compounds in activating PKB phosphorylation.

In summary, our results demonstrate that, compared to VS, OVC are more potent activators of PKB and GSK-3β phosphorylation in a PI3-K-dependent manner. A greater inhibitory effect of OVC on PTPases and increased protein tyrosine phosphorylation might serve as triggering mechanisms, leading to upregulation of the PKB signaling pathway and eventually contributing to a greater potency of OVC in improving glucose homeostasis.

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Involvement of Insulin-like Growth Factor Type 1 Receptor and Protein Kinase Cδ in Bis(maltolato)oxovanadium(IV)-Induced Phosphorylation of Protein Kinase B in HepG2 Cells†

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ABSTRACT: Vanadium(IV) oxo-bis(maltolato) (BMOV), an organovanadium compound, is a potent insulinomimetic agent and improves glucose homeostasis in various models of diabetes. We have shown previously that BMOV stimulates the phosphorylation of PKB which may contribute as one of the mechanisms for the insulinomimetic effect of this compound. However, the upstream mechanism of BMOV-induced PKB phosphorylation remains elusive. Therefore, in this study, we examine the upstream events leading to BMOV-induced PKB phosphorylation in HepG2 cells. Since BMOV is an inhibitor of protein tyrosine phosphatases and through enhanced tyrosine phosphorylation may activate various protein tyrosine kinases (PTK), we have investigated the potential role of different receptor or nonreceptor PTK in mediating BMOV-induced PKB phosphorylation. Among several pharmacological inhibitors that were tested, only AG1024, a selective inhibitor of IGF-1R-PTK, almost completely blocked BMOV-stimulated phosphorylation of PKB. In contrast, AG1295 and AG1478, specific inhibitors of PDGFR and EGFR, respectively, were unable to block the BMOV response. Moreover, efficient reduction of the level of IGF-1R protein expression by antisense oligonucleotides (ASO) attenuated BMOV-induced PKB phosphorylation. BMOV-induced PKB phosphorylation was associated with an increased level of tyrosine phosphorylation of the IRβ subunit, IGF-1Rβ subunit, IRS-1, and $p^{85\alpha}$ subunit of PI3-kinase. However, this response was independent of IR-PTK activity because in cells overexpressing a PTK-inactive form of IR, insulin response was attenuated while the effect of BMOV remained intact. A role of PKC in BMOV-induced response was also tested. Pharmacological inhibition with chelerythrine, a nonselective PKC inhibitor, or rottlerin, a PKCδ inhibitor, as well as chronic treatment with PMA attenuated BMOV-induced PKB phosphorylation. In contrast, GÖ6976 and RO31-8220 PKCα/β selective inhibitors failed to alter the BMOV effect. Taken together, these data suggest that IGF-1R and PKCδ are required to stimulate PKB phosphorylation in response to BMOV in HepG2 cells and provide new insights into the molecular mechanism by which this compound exerts its insulinomimetic effects.

Vanadium is a transition metal, and its compounds have been shown to exert insulin-like properties in both in vivo and in vitro systems (reviewed in ref *1*). Oral administration of vanadium leads to improved insulin resistance and lowers the incidence of hyperglycemia in rodent models of diabetes mellitus as well as in limited studies with human subjects (reviewed in ref *1*). Vanadium mimics many physiological effects of insulin, including stimulation of glucose uptake, glycogen synthesis, and lipid synthesis in muscle, adipose, and hepatic tissues as well as inhibition of gluconeogenesis in the liver and kidneys and lipolysis in fat cells (*1*). In cultured cells, vanadium activates several keys components of the insulin signaling cascade implicated in mediating the physiological responses of insulin, including the tyrosine phosphorylation of the insulin receptor substrate (IRS-1)[1] (*2*, *3*) and activation of extracellular signal-regulated kinase (ERK1/2) (*4*) and the phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) signaling cascade (*2*−*4*).

PKB, also known as Akt, is a 57 kDa serine/threonine kinase which has been implicated in the regulation of many physiological processes such as glucose transport, glycolysis,

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[1] Abbreviations: BMOV, vanadium(IV) oxo-bis(maltolato); CHO-HIR, Chinese hamster ovary cells overexpressing human insulin receptor; ECL, enhanced chemiluminescence; EGFR, epidermal growth factor receptor; GSK-3, glycogen synthase kinase-3; IGF-1R, insulin-like growth factor type 1 receptor; IR, insulin receptor; IRS-1, insulin receptor substrate-1; PDGFR, platelet-derived growth factor receptor; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKCδ, protein kinase Cδ; PMA, phorbol-12-myristate-13-acetate; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PY99, monoclonal antiphosphotyrosine antibody; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; VS, vanadyl sulfate.

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protein synthesis, lipogenesis, glycogen synthesis, suppression of gluconeogenesis, cell survival, determination of cell size, and cell-cycle progression (reviewed in ref *5*). In most cell types, PKB is activated via a PI3-K-dependent mechanism through dual phosphorylation of serine 473 in the C-terminal regulatory region and threonine 308 within the catalytic loop. Phosphorylation of PKB at Thr 308 is catalyzed by phosphatidylinositol 3,4,5-triphosphate (PIP3)-dependent protein kinase 1 (PDK-1), but the kinase responsible for phosphorylation at Ser 473, the putative PDK-2, remains elusive (*5*, *6*). Activated PKB exerts its effect through the phosphorylation of several downstream targets, such as glycogen synthase kinase-3 β (GSK-3β), forkhead transcription factor (FKHR), Bcl-2-associated death promoter (BAD), IkB kinase, Mdm2, caspase 9, and endothelial nitric oxide synthase (eNOS) (reviewed in refs *5* and *6*).

Recently, we have demonstrated that organovanadium compounds are more potent than inorganic vanadium salts in inhibiting the total protein tyrosine phosphatase (PTPase) activity and in increasing the level of total protein tyrosine phosphorylation which was associated with a robust activation of the PKB pathway (*3*). A much higher potency of organovanadium compounds, compared to that of inorganic vanadium salts, in enhancing the phosphorylation of PKB and its downstream substrates has been suggested as one of the mechanism to explain their greater antidiabetic effects (*7*−*9*). We have also shown that vanadium(IV) oxo-bis-(maltolato) (BMOV), a well-established antidiabetic and insulinmimetic organovanadium compound (*10*−*13*), enhanced the tyrosine phosphorylation of the β-subunit of the insulin receptor (IRβ) in Chinese hamster ovary cells overexpressing IR (CHO-IR) (*3*), suggesting the involvement of IR-protein tyrosine kinase (PTK) activity in provoking the BMOV response. However, the clear involvement of IR-PTK as an upstream inducer of vanadium action has not been established, and a role of both IR-PTK-dependent (*14*−*16*) and -independent (*2*, *17*−*20*) events has been suggested to contribute to the insulin-like effects of vanadium. Therefore, in these studies, we have investigated a potential role of receptor PTKs in BMOV-induced PKB phosphorylation in human hepatoma (HepG2) cells.

MATERIALS AND METHODS

Materials

Insulin was from Eli Lilly Co. (Indianapolis, IN). Vanadium(IV) oxo-bis(maltolato) (BMOV) was a kind gift from D. Crans (Colorado State University, Fort Collins, CO). Phorbol-12-myristate-13-acetate (PMA) and luciferase/luciferin were from Sigma Aldrich (St. Louis, MO). HepG2 cells (HB-8065) were obtained from American Type Culture Collection (Rockville, MD). Human IGF-1 was from PeproTech Inc. Epidermal growth factor (EGF), platelet growth factor receptor (PDGF), and all pharmacological inhibitors were from Calbiochem (La Jolla, CA). Polyclonal insulin receptor-β subunit antibody, polyclonal insulin-like growth factor type 1 receptor-β subunit antibody, monoclonal antiphosphotyrosine antibody (PY99), polyclonal phospho-GSK-3β (Ser^{9}) antibody, polyclonal PKCδ antibody, and polyclonal GSK-3β antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). Polyclonal phospho-PKB (Ser^{473}), polyclonal PKB, and monoclonal phospho-insulin-like growth factor type 1 receptor (Tyr^{1131})/phospho-insulin receptor (Tyr^{1146}) antibodies were from Cell Signaling (Beverly, MA). Polyclonal insulin receptor substrate and polyclonal p^{85} antibodies were from Upstate (Lake Placid, NY). Phospho-PKCδ (Tyr 311) antibody and phospho-insulin-like growth factor type 1 receptor (Tyr 1131/1135/1136)/phospho-insulin receptor (Tyr 1158/1162/1163) were from Biosource (Camarillo, CA). Protein A-Sepharose beads and the enhanced chemiluminescense (ECL) detection system kit were from Amersham Pharmacia Biotech (Baie d'Urfé, PQ). Lipofectamine and all cell culture materials were from Invitrogen Corp. (Grand Island, NY).

Methods

Cell Culture. HepG2 cells were maintained in DMEM containing 10% fetal bovine serum. Chinese hamster ovary cells overexpressing either wild-type human insulin receptor (CHO-IR) or the PTK mutant form (CHO-1018), a gift from M. F. White (Boston Children's Hospital, Boston, MA), were maintained in HAM's F-12 medium containing 10% fetal bovine serum. They were grown to 80−90% confluence in 100 mm plates or 60 mm plates at 37 °C in a humidified atmosphere of 5% CO_2. Prior to the experiment, cells were incubated in serum-free medium for 20 h (*3*).

Transfection with Oligonucleotides. HepG2 cells were cultured in a collagen-coated Petri dish overnight. The following day, cells were transfected with either IGF-1R antisense oligonucleotides (CGGCTTCTCCTCCATGGTCC) or scrambled oligonucleotides (TCTTCCGCGACTTGCTCCGC) using lipofectamine according to the manufacturer's instructions. Forty-eight hours after post-transfection, cells were serum starved overnight followed by stimulation with BMOV. Incubation was terminated by quickly washing the cells in cold PBS. Phosphorothioate oligonucleotides (*21*) were synthesized by Invitrogen Corp.

Immunoblotting. Cells subjected to various experimental treatments were lysed in buffer A [25 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 20 nM okadaic acid, 0.5 mM ethylenebis(oxyethylenenitrilo)-tetraacetic acid (EGTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/mL aprotinin, and 1% Triton X-100], and the lysates were clarified by centrifugation to remove insoluble material. The clarified cell lysates normalized to contain equal amounts of protein were electrophoresed via 7.5 or 10% SDS−PAGE under reducing conditions, transferred to PVDF membranes, and incubated with the indicated antibody. Proteins were detected by a horseradish peroxidase-conjugated second antibody and visualized with an ECL detection kit (*3*). In some cases, cells were directly lysed in sample buffer containing β-mercaptoethanol followed by Western blot analysis. The immunoblots were quantified by densitometric scanning using NIH ImageJ.

Immunoprecipitation. The clarified cell lysates, normalized to contain equal amounts of protein (500 μg), were subjected to immunoprecipitation with 2 μg of various antibodies overnight at 4 °C, followed by incubation with protein A-Sepharose for 2 h. Immunoprecipitated proteins were collected by centrifugation and washed two times with buffer A and once with phosphate-buffered saline (PBS) containing PTPase and protease inhibitors. The immunoprecipitates were

electrophoresed via 7.5% SDS−PAGE under reducing conditions, transferred to PVDF membranes, and incubated with the respective primary antibodies. Proteins were detected with a horseradish peroxidase-conjugated second antibody and visualized with an ECL detection kit (*3*).

Measurement of the Level of Intracellular ATP. The ATP levels was measured according to the procedure described previously (*22*) with minor modifications. HepG2 cells cultured in six-well plates were treated with increasing concentrations of rottlerin for 30 min. The cells were washed twice with cold PBS and lysed in a 0.1 M NaOH/0.5 mM EDTA mixture. Cell lysates were incubated at 60 °C for 20 min and frozen at −20 °C. The ATP levels was measured by adding 10 μL of the lysates to 50 μL of the luciferin/luciferase assay mix and measuring the light output for 15 s in a luminometer (Turner Designs). A standard curve was also constructed by using serial dilutions of ATP (from 10 nM to 1 μM).

Statistics. Statistical analysis was performed by one-way, repeated measures analysis of variance (ANOVA), followed by a Newman-Keuls post-test. All data are reported as means ± the standard error. The differences between means were considered significant when $P < 0.05$.

RESULTS

Effect of BMOV on PKB and GSK-3β Phosphorylation in HepG2 Cells. Our previous studies have demonstrated that organovanadium compounds such as BMOV are more potent activators of the PKB pathway than inorganic vanadium compounds in CHO-IR cells (*3*). Therefore, in these studies, we have first evaluated whether BMOV alters the activation of PKB and its downstream targets such as GSK-3β in HepG2 cells which has been used as a model to investigate insulin action in liver (*23*). Since enhanced phosphorylation of specific serine/threonine residues of both PKB and GSK-3β is critical for their activation state, we have utilized phospho-specific antibodies to monitor their activity. As shown in Figure 1A, treatment of HepG2 cells for 15 min with escalating concentrations of BMOV enhanced PKB and GSK-3β phosphorylation in a dose-dependent manner. BMOV elicited a robust phosphorylation of these two kinases at 1 mM. Next, we assessed the time dependence of the 1 mM BMOV response; as shown in Figure 1B, 1 mM BMOV enhanced the phosphorylation of PKB and GSK-3β within 10 min which reached a maximum in 30 min.

Effect of BMOV on Tyrosine Phosphorylation of Total Protein, Insulin Receptor (IR), IRS-1, and p^{85} in HepG2 Cells. Since BMOV is a potent inhibitor of PTPases, and an increase in the level of tyrosyl phosphorylation of several key proteins such as IR and IRS-1 is an early step in triggering the insulin signaling cascade, we determined the effect of BMOV on tyrosine phosphorylation of the total proteins as well as on the phosphorylation of IRβ, IRS-1, and p^{85}, the regulatory subunit of PI3-K in HepG2 cells. As illustrated in panels A and B of Figure 2, BMOV enhanced the tyrosine phosphorylation of several proteins in a dose- and time-dependent fashion. The molecular size of these proteins ranged between 35 and 200 kDa, and the increase in the level of phosphorylation caused by BMOV could be detected within 10 min of treatment (Figure 2B). To further analyze if some of these proteins were IR and its phospho-

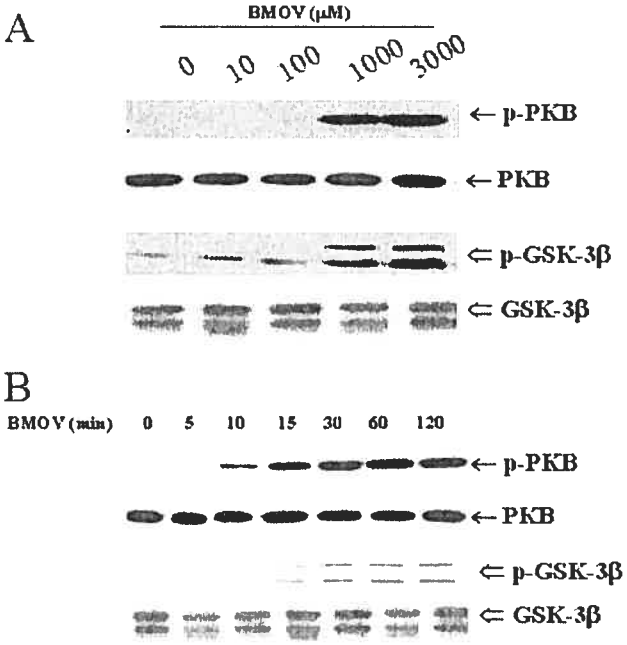

FIGURE 1: BMOV-induced PKB and GSK-3β phosphorylation is time- and dose-dependent in HepG2 cells. Confluent, serum-starved HepG2 cells were incubated with different concentrations of BMOV for 15 min (A) or with 1 mM BMOV for the indicated time periods (B). The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Ser 473) PKB antibodies (top immunoblot in panels A and B) and phospho-specific (Ser 9) GSK-3β antibodies (bottom immunoblot in panels A and B). The results are representative of three independent experiments.

tyrosyl-protein substrates, immunoprecipitates from BMOV-treated cells were immunoblotted with antibodies to IR, IRS-1, and p^{85}. As shown in Figure 2C, BMOV treatment resulted in a significant increase in the level of phosphorylation of IRβ, IRS-1, and p^{85}. To further confirm the identity of these proteins, IRβ or IRS-1 was immunoprecipitated from the lysates of the cells stimulated with or without BMOV followed by immunoblotting with anti-phosphotyrosine antibodies. As shown in Figure 2D, a similar enhancement of IRβ and IRS-1 tyrosine phosphorylation was observed under these conditions.

Role of IR-PTK on BMOV-Induced PKB Phosphorylation. BMOV-induced enhanced tyrosine phosphorylation of IRβ suggested a possible role of IR-PTK in BMOV-induced effects on PKB phosphorylation. This possibility was probed further by utilizing CHO cells that overexpress an inactive form of IR-PTK (CHO-1018). The inactivation of IR-PTK in these cells was achieved by the mutation of lysine 1018 to alanine in the ATP-binding domain of IRβ (*24*). As illustrated in Figure 3, both insulin and BMOV enhanced the phosphorylation of PKB in CHO-IR cells overexpressing a normal IR. However, in CHO-1018 cells overexpressing PTK-inactive IR, the level of insulin-induced phosphorylation of PKB was significantly reduced, whereas the BMOV-evoked increase was not affected. These data suggested that in contrast to insulin, BMOV-induced phosphorylation of PKB was independent of IR-PTK activity.

Effect of Receptor Tyrosine Kinase Inhibitors on BMOV-Induced PKB. A potential role of epidermal growth factor receptor (EGFR) transactivation in vanadyl sulfate (VS)-induced signaling has been demonstrated (*25*, *26*), and

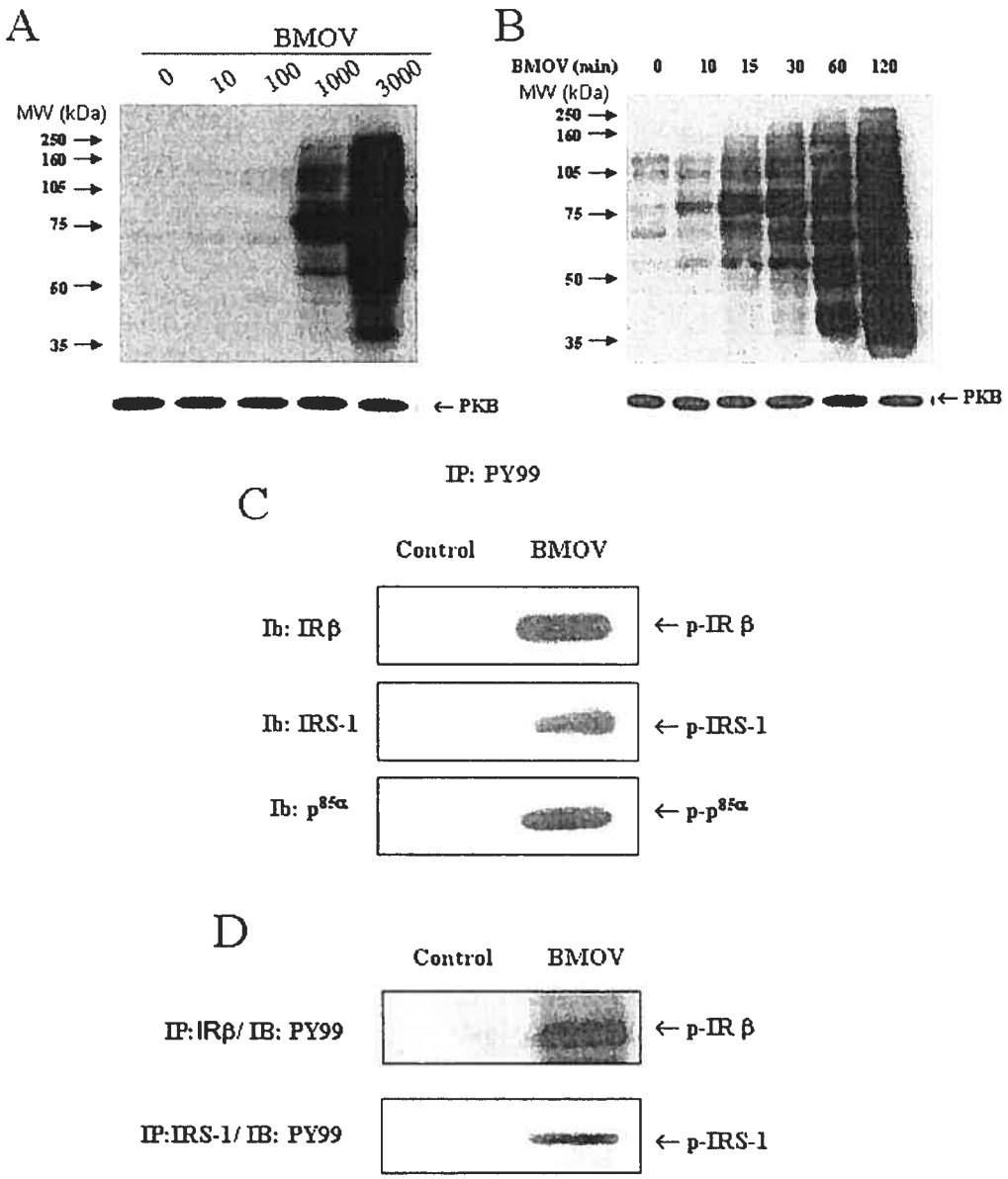

FIGURE 2: BMOV-enhanced tyrosine phosphorylation of total proteins and of IRβ, IRS-1, and p^{85}. Confluent, serum-starved HepG2 cells were incubated with different concentrations of BMOV for 15 min (A) or with 1 mM BMOV for the indicated time periodes (B). The cells were lysed, and the lysates were subjected to immunoblotting, using antiphosphotyrosine antibodies (PY99) (A and B). The blots shown in panels A and B were also probed with total PKB antibodies to control for equal loading. In panels C and D, cells treated without or with 1 mM BMOV for 15 min were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with antiphosphotyrosine antibodies (PY99) (C) or with anti-IRβ or -IRS-1 antibodies (D). The immunoprecipitates were immunoblotted (IB) with anti-IRβ, -IRS-1, or -p^{85} antibodies (C) or with antiphosphotyrosine antibodies (PY99) (D). A representative immunoblot from three independent experiments is shown.

insulin-like growth factor type 1 receptor (IGF-1R) activation has also been thought to play a role in vasoactive peptide-induced signaling (*27*, *28*). Therefore, we determined if IGF-1R, EGFR, or platelet growth factor receptor (PGDFR) was the putative PTK mediating BMOV-induced PKB phosphorylation. As depicted in Figure 4A, AG1024, a highly specific inhibitor of IGF-1R-PTK (*29*), prevented the phosphorylation of PKB induced by BMOV as well as IGF-1. In contrast, while AG1478, an EGFR-PTK inhibitor (*30*), completely blocked EGF-induced PKB phosphorylation, it failed to change the stimulatory effect of BMOV on this event (Figure 4B). Similarly, AG1295, an inhibitor of PDGFR-PTK (*31*), also had no effect on PKB phosphorylation induced by BMOV (Figure 4C). In fact, PKB phosphorylation was not detected in HepG2 cells in response to PDGF treatment, suggesting that PDGFR may be expressed at extremely low levels in these cells (*32*).

Effect of Different Doses of AG1024 on BMOV-Induced Signaling. Since BMOV-induced PKB phosphorylation was altered solely by AG1024, we analyzed the effect of this inhibitor in more detail. As shown in Figure 5A, treatment of cells with AG1024 inhibited the phosphorylation of PKB and GSK-3β in a dose-dependent fashion with almost complete attenuation observed at 1 μM. Since tyrosine phosphorylation of IGF-1R β-subunit (IGF-1Rβ) is primordial in increasing its PTK activity, we investigated the effect of BMOV on the tyrosine phosphorylation of IGF-1Rβ. As shown in Figure 5B, the level of tyrosine phosphorylation

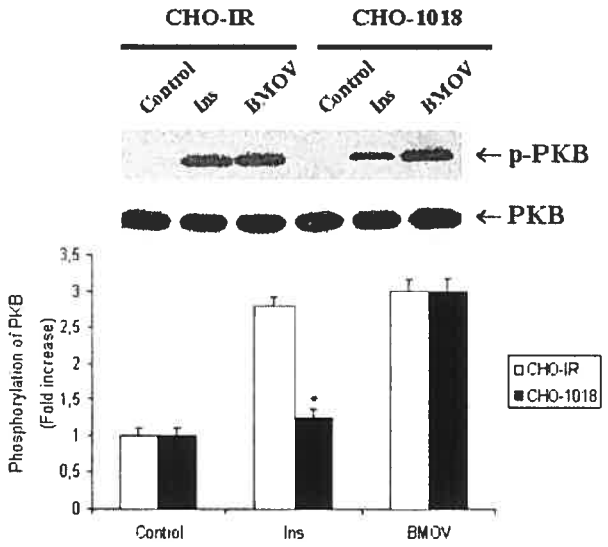

FIGURE 3: BMOV-induced PKB phosphorylation is independent of IR-PTK activity in CHO cells. Confluent, serum-starved CHO-IR and CHO-1018 cells were incubated in the absence or presence of 0.1 nM insulin (Ins) or with 1 mM BMOV for 15 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Ser 473) PKB antibodies. The phosphorylation level of PKB was quantified by densitometric scanning using NIH ImageJ and expressed as the fold increase over control cells. Values are means ± the standard error of at least three independent experiments. The asterisk indicates $P < 0.001$ vs CHO-IR cells stimulated with insulin (Ins).

of IGF-1 β was increased by BMOV and pretreatment of cells with AG1024 prior to stimulation with BMOV resulted in a significant reduction in this response. IGF-1Rβ has three critical tyrosines at positions 1131, 1135, and 1136 in the kinase domain crucial for maintaining its activity and for eliciting all IGF-1R-dependent functions (*33*). Therefore, by using two phospho-specific antibodies, one which recognizes phosphorylation of IGF-1Rβ at Tyr 1131, Tyr 1135, and Tyr 1136 Tyr 1131, we investigated the effect of BMOV on the phosphorylation of these sites. As depicted in Figure 5C, BMOV increased the level of phosphorylation of IGF-1Rβ on all three sites. Furthermore, like its effect on the total tyrosine phosphorylation of IGF-1Rβ, AG1024 markedly blocked this response (Figure 5C). These results suggested that BMOV-induced PKB phosphorylation was associated with an increased level of tyrosine phosphorylation of IGF-1Rβ.

Effect of AG1024 on the Phosphorylation of IRS-1 and p^{85} Induced by BMOV. Increased levels of phosphorylation of IRS-1 and p^{85} regulatory subunit of PI3-K are critical intermediary steps to signal the activation of PKB signaling in response to IGF-1R activation. Therefore, we next assessed if BMOV-enhanced tyrosine phosphorylation of IRS-1 and p^{85} in HepG2 cells (Figure 2C) was dependent on IGF-1R-PTK activity. The results shown in Figure 5D demonstrate that BMOV induced tyrosine phosphorylation of both IRS-1 and p^{85} which was significantly attenuated by AG1024.

Effect of IGF-1Rβ Antisense Oligonucleotides (ASO) on BMOV-Indcued PKB Phosphorylation. To further confirm the role of IGF-1R in BMOV-induced PKB phosphorylation, we utilized IGF-1Rβ-specific antisense oligonucleotides (ASO) which reduced the level of expression of IGF-1Rβ (*21*). Transfection of HepG2 cells was carried out with either scrambled (SCR) or increasing doses of IGF-1Rβ ASO. ASO treatment dose-dependently decreased the level of IGF-1Rβ expression (Figure 6A). When IGF-1Rβ ASO or SCR-treated cells were incubated with BMOV, the enhanced PKB phosphorylation was significantly diminished in the IGF-1Rβ ASO-treated group and not in the SCR group (Figure 6B). These data provide additional evidence to support a potential role of IGF-1R in mediating the BMOV response.

Requirement of PKCδ in BMOV-Induced PKB Phosphorylation. A potential role of PKC in mediating the responses of different stimuli such as PDGF (*34*), EGF (*35*, *36*), vascular endothelial growth factor (VEGF) (*37*, *38*), angiotensin II (*39*), and H_2O_2 (*40*) has been suggested. Therefore, we sought to verify if PKC-dependent pathways were involved in BMOV-induced phosphorylation of PKB in HepG2 cells. PKCs are composed of three groups. Members of the classical (cPKC) subtype (α, βI, βII, and γ) are activated by calcium, diacylglycerol (DAG), phosphatidylserine (PS), and phorbol esters; members of the the novel (nPKCs) subtype (δ, ϵ, η, and θ) are activated by DAG, PS, phorbol esters, and unsaturated fatty acids, and members of the atypical (aPKCs) subtype (ζ and λ/ι) are insensitive to DAG but are activated by PS and phosphatidylinositides

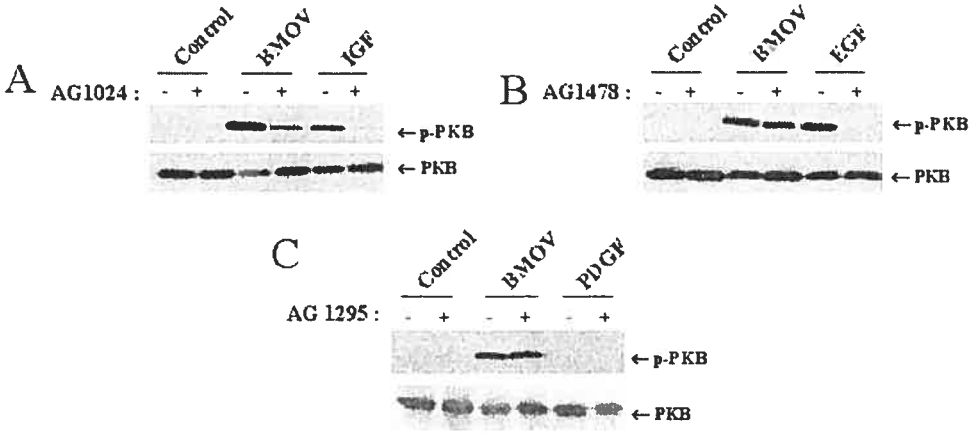

FIGURE 4: BMOV-induced PKB phosphorylation is inhibited by AG1024 but not by AG1478 or AG1295. Confluent, serum-starved HepG2 cells were pretreated without (−) or with (+) 10 μM AG1024 (A), AG1478 (B), or AG1295 (C) for 30 min followed by stimulation with 1 mM BMOV for 15 min or 10 ng/mL IGF-1, 10 nM EGF, or 10 ng/mL PDGF for 10 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Ser 473) PKB antibodies. The results are representative of three independent experiments.

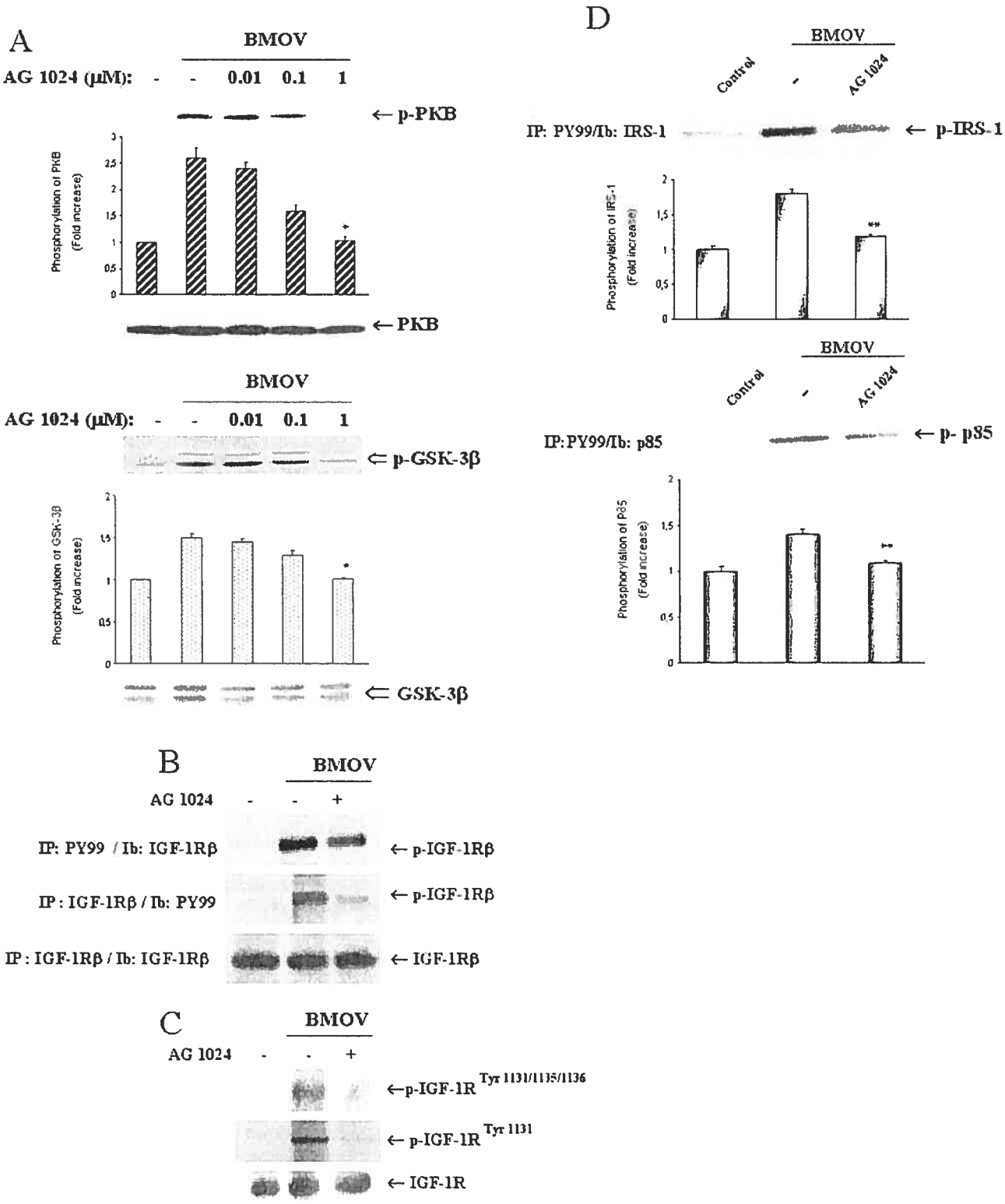

FIGURE 5: AG1024 decreased the level of BMOV-induced PKB phosphorylation in a dose-dependent manner and reduced the level of tyrosine phosphorylation of IGF-1Rβ, IRS-1, and p^{85} subunit of PI3-K. Confluent, serum-starved HepG2 cells were pretreated without (−) or with (+) different doses of AG1024 (A) or 1 μM AG1024 (B−D) for 30 min followed by stimulation with 1 mM BMOV for 15 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoblotting using phospho-specific (Ser 473)-PKB antibodies and phospho-specific (Ser 9)-GSK-3β antibodies (A) or phospho-IGF-1R ($Tyr^{1131/1135/1136}$)/phospho-IR ($Tyr^{1158/1162/1163}$) and phospho-IGF-1R (Tyr^{1131})/phospho-IR (Tyr^{1146}) (C) or to immunoprecipitation (IP) with antiphosphotyrosine antibodies (PY99) followed by immunoblotting with the indicated antibodies (B and D). The phosphorylation levels of the protein shown in panels A and D were quantified by densitometric scanning using NIH ImageJ software and expressed as fold increase over control cells. Values are means ± the standard error of at least three independent experiments. One asterisk indicates $P < 0.001$ vs BMOV alone, and two asterisks indicate $P < 0.01$ vs BMOV alone.

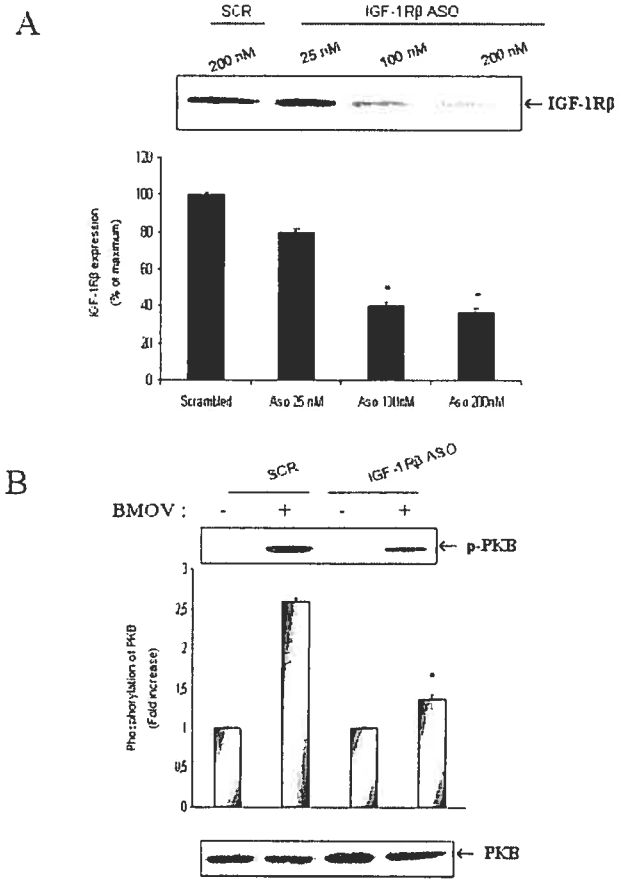

FIGURE 6: IGF-1Rβ ASO decreased BMOV-induced PKB phosphorylation. HepG2 cells were transfected with 25, 100, and 200 nM antisense oligonucleotides (ASO) to IGF-1Rβ or 200 nM scrambled oligonucleotides (SCR) (A) or with 100 nM ASO to IGF-1Rβ or 100 nM SCR (B). Unstimulated (A) or cells stimulated in the absence (−) or presence (+) of BMOV (B) were lysed, and the lysates were subjected to immunoblotting using total IGF-1Rβ antibodies (A) or phospho-specific (Ser 473)-PKB antibodies (B). Blot shown in top panel of (B) was reprobed for total PKB for equal loading and is shown in bottom. The results are representative of three independent experiments. The immunoblots were quantified by densitometric scanning using NIH ImageJ software. Values are means ± the standard error of at least three independent experiments. One asterisk indicates $P < 0.001$ and vs scrambled.

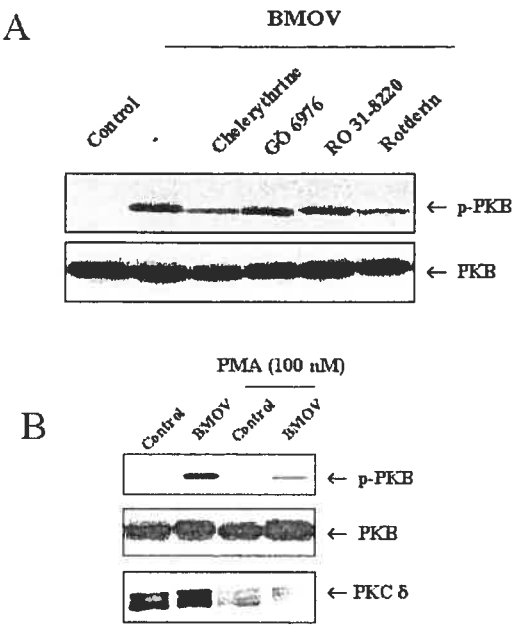

FIGURE 7: Chelerythine chloride, rottlerin, and long-term PMA pretreatment inhibits BMOV-induced PKB phosphorylation. Confluent, serum-starved HepG2 cells were pretreated without (−) or with (+) 5 μM chelerythine chloride, GÖ6976, RO31-8220, or rottlerin for 30 min (A) or with 100 nM PMA for 24 h (B) followed by stimulation with 1 mM BMOV for 15 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Ser 473) PKB antibodies (top immunoblot in panels A and B) or total PKCδ antibodies (bottom immunoblot in panel B). The results are representative of three independent experiments.

(reviewed in ref *41*). The results shown in Figure 7A demonstrate that GÖ6976 and RO31-8220, selective chemical inhibitors of cPKC and aPKC isoforms, respectively, failed to inhibit BMOV-induced phosphorylation of PKB, whereas chelerythrine chloride, an isoform-nonselective inhibitor, and rottlerin, a highly selective PKCδ inhibitor, treatment significantly reduced the level of PKB phosphorylation induced by BMOV. The involvement of PKC was further confirmed by downregulating the PKC activity in HepG2 cells by a 24 h treatment with phorbol-12-myristate-13-acetate (PMA). As shown in Figure 7B, PMA-treated cells exhibited significantly attenuated PKB phosphorylation in response to BMOV as compared to untreated cells. Immunoblotting of parallel samples showed that PMA treatment significantly reduced the amount of PKCδ protein in these cells, whereas the total PKB levels remained unaltered after this treatment (Figure 7B). Rottlerin has been suggested to exert inhibitory effects through depletion of ATP levels (*22*, *42*); in view of this, we determined if the rottlerin-induced decrease in ATP levels was contributing to its inhibitory effect on BMOV-stimulated PKB phosphorylation. It was found that up to 10 μM rottlerin was unable to lower the ATP levels in these cells (data not shown), suggesting that ATP depletion was not responsible for attenuating the BMOV effect in HepG2 cells.

Effect of BMOV on Tyrosine Phosphorylation of PKCδ. Recent studies have identified tyrosine phosphorylation as a potential mechanism through which PKCδ activation is regulated (*41*). Therefore, we investigated the effect of BMOV on tyrosine phosphorylation of PKCδ by immunoprecipitating PKCδ from lysates of cells treated with BMOV followed by immunoblot analysis with antiphosphotyrosine antibody. As depicted in Figure 8, BMOV treatment of HepG2 cells caused a significant increase in the level of tyrosine phosphorylation of PKCδ. We also assessed the effect of BMOV on the tyrosine phosphorylation of PKCδ at tyrosine 311 (Tyr 311). This tyrosine residue is flanked by the regulatory and catalytic domains and is critical for generating the active form of PKCδ in response to H_2O_2 and PMA (*43*, *44*). As shown in Figure 8A, BMOV induced the Tyr 311 phosphorylation of PKCδ. In addition, both total tyrosine and Tyr 311 phosphorylation of PKCδ enhanced by BMOV were blocked by rottlerin (Figure 8B). Taken together, these data indicated that BMOV-induced phosphorylation of PKB is associated with an activation of PKCδ as judged by an increase in the level of phosphorylation of key tyrosine residues in its catalytic domain.

Effect of AG1024 on BMOV-Induced Tyrosine Phosphorylation of PKCδ. Since PKCδ has been shown to be tyrosine-phosphorylated by various protein tyrosine kinases,

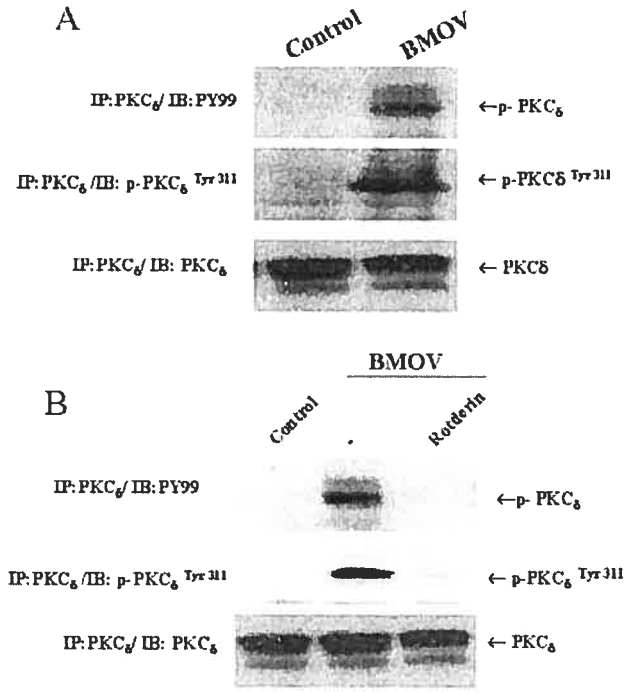

FIGURE 8: Rottlerin decreased the level of BMOV-induced total tyrosine phosphorylation at tyrosine 311 of PKCδ. Confluent, serum-starved HepG2 cells were pretreated with or without 5 μM rottlerin for 30 min followed by stimulation in the absence or presence of 1 mM BMOV for 15 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with total PKCδ antibodies followed by immunoblotting with antiphosphotyrosine antibodies (PY99) or phospho-specific (Tyr 311) PKCδ antibodies. A representative immunoblot from three independent experiments is shown.

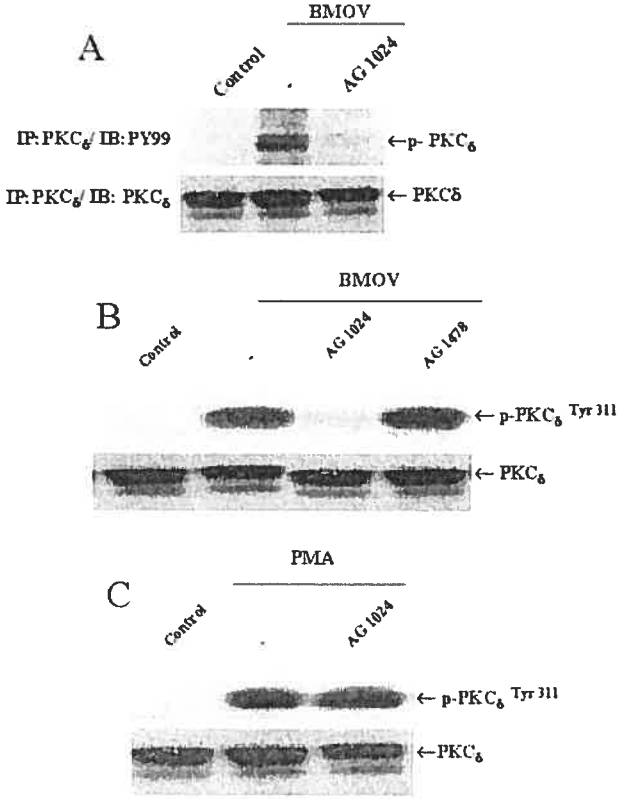

FIGURE 9: AG1024 decreased the level of BMOV- but not PMA-induced tyrosine phosphorylation of PKCδ at tyrosine 311. Confluent, serum-starved HepG2 cells were pretreated with or without 10 μM AG1024 or AG1478 (B) for 30 min followed by stimulation with 1 mM BMOV or 100 nM PMA for 15 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with total PKCδ antibodies followed by immunoblotting with antiphosphotyrosine antibodies (PY99) (A) or were immunoblotted using phospho-specific (Tyr 311) PKCδ antibodies (B and C). A representative immunoblot from three independent experiments is shown.

including IGF-1R (*45*, *46*), we investigated if IGF-1R-PTK was responsible for triggering the phosphorylation of PKCδ in response to BMOV. As shown in Figure 9A, treatment of HepG2 cells with AG1024 almost completely abolished BMOV-stimulated total tyrosine phosphorylation of PKC δ. In addition, AG1024 also inhibited phosphorylation of PKCδ at Tyr 311 while AG1478, an EGFR inhibitor, failed to alter the phosphorylation of PKCδ at this site (Figure 9B). Since PKCδ has been shown to be phosphorylated at Tyr 311 in response to various agonists, including PMA, we determined if like those of BMOV, PMA-induced effects are also dependent on IGF-1R-PTK. As shown in Figure 9C, in contrast to its inhibitory effect on PKCδ phosphorylation stimulated by BMOV, AG1024 was unable to block the effect of PMA, suggesting that IGF-1R is an upstream regulator of PKCδ phosphorylation in response to BMOV and not PMA.

DISCUSSION

BMOV has been shown to exert glucoregulatory effects in many rodent models of diabetes (*10*−*13*, *47*); however, the precise mechanism through which this response is mediated remains largely unclear. We have shown previously that BMOV activates PKB (*3*), a central player involved in carbohydrate metabolism (*5*). In the study presented here, we demonstrate that BMOV-enhanced phosphorylation of PKB in HepG2 cells is associated with an increased level of tyrosine phosphorylation of the total cellular proteins, IRβ, IGF-1Rβ, IRS-1, and p^{85} subunit of PI3K. We have also provided evidence that the PTK activity of the IGF-1Rβ is responsible for BMOV-induced activation of the PKB pathways. This conclusion is based on the use of highly selective inhibitors of IGF-1R, EGFR, and PDGFR-PTKs which showed that only the inhibition of IGF-1R-PTK by AG1024 blocked BMOV-induced PKB phosphorylation. Our results showing that BMOV treatment increased the level of total tyrosyl phosphorylation as well as Tyr 1131, 1135, and 1136 phosphorylation of IGF-1Rβ which was specifically inhibited by AG1024 suggested that the PTK activity of IGF-1Rβ was indeed being stimulated in response to BMOV. In addition, the data showing that antisense-induced reduction of IGF-1Rβ decreased the level of BMOV-stimulated PKB phosphorylation provided further proof for the participation of IGF-1R in mediating the effect of BMOV. A role of IGF-1Rβ in angiotensin II and H_2O_2 (*48*, *49*) induced ERK1/2 phosphorylation and in angiotensin II and purinergic receptor P_2Y_{12}-induced PI3-K/PKB signaling pathway has been reported previously (*28*, *50*). Transactivation of other growth factor receptor PTKs has also been suggested to play a critical intermediary role in response to agonists coupled to G-protein receptors (GPCR) (reviewed in ref *51*). However, the data presented here are the first to demonstrate that

BMOV, a non-GPCR agent, also signals through IGF-1R-PTK transactivation to activate PKB.

IR and IGF-1R exhibit a high degree of homology and share several common signaling features (reviewed in ref *52*), and despite having a higher IC_{50} for IGF-1R-PTK, AG1024 can also inhibit IR-PTK activity at high concentrations (*29*). Furthermore, our results showing that BMOV treatment increased the level of tyrosine phosphorylation of IRβ might support the idea of a role for IR-PTK in BMOV-induced responses. However, our observations that in CHO-1018 cells the extent of the insulin response was significantly decreased while the BMOV effect was intact suggested an IR-PTK-independent mechanism of BMOV action. Earlier studies using inorganic vanadium compounds have also documented the lack of a role of IR-PTK in inducing the vanadium response (*17*, *53*). In addition, several investigators have also reported that the insulin-like effects of vanadium were not associated with an increase in the level of tyrosine phosphorylation of IRβ in many systems (*2*, *17*−*20*). Thus, despite the fact that some studies have demonstrated an increase in the level of IR tyrosine phosphorylation in vivo in response to vanadium treatment in diabetic animal models (*10*, *54*), our data support the notion that IGF-1R-PTK activation by BMOV may serve as an alternate mechanism to mediate the insulin-like effect of this compound.

PKCδ is a serine/threonine kinase that plays a key role in many physiological responses such as growth regulation (*55*), tissue remodeling (*56*), migration (*57*), and transformation (*45*). In response to insulin, PKCδ and PKCθ, two novel PKC isoforms, have been shown to convey an insulin signal toward glucose transport (*58*, *59*), glycogen synthesis (*60*), and cell proliferation (*61*). In contrast, several studies have reported that PKCδ may downregulate insulin signaling in response to high glucose concentrations (*62*) and other stimuli (*63*). Therefore, it appears that novel PKC isoforms may both mediate stimulatory effects of insulin on glucose metabolism and inhibit the intracellular insulin signaling pathway. Our data showing that PKB phosphorylation was blocked by chelerythrine and rottlerin and not by GÖ6976 or RO31-8220 suggested a role for PKCδ in BMOV action. Since tyrosine phosphorylation of PKCδ has been shown to activate its catalytic activity (*43*, *44*), the results showing that both total tyrosine phosphorylation and phosphorylation of Tyr 311 in PKCδ were enhanced by BMOV provide additional support for the involvement of this isoform of PKC in mediating the BMOV response in HepG2 cells. Interestingly, like its effect on PKB phosphorylation, rottlerin pretreatment blocked the PKCδ tyrosine phosphorylation induced by BMOV. Rottlerin has been extensively used as a selective inhibitor of PKCδ; however, recent reports have suggested that it may have additional, non-PKCδ-dependent responses such as depletion of the intracellular ATP level (*22*, *42*, *64*). However, our data showing that rottlerin was unable to alter the ATP concentration in HepG2 cells suggested that rottlerin was exerting its effect via an ATP-independent mechanism in these cells.

Furthermore, our results showing that in cells chronically treated with PMA, the attenuated response of BMOV on PKB phosphorylation was associated with a decreased level of expression of PKCδ provided additional evidence of a role for PKCδ in BMOV action. To the best of our knowledge, these data are the first to demonstrate the involvement of PKC in vanadium-mediated PKB activation. This is consistent with previous reports where an involvement of PKCs in mediating PKB activation was documented (*37*, *66*). In contrast, however, a negative regulatory role of PKCδ in PKB activation has also been suggested (*67*, *68*). Because PKC expression and its action have been reported to be tissue-specific (*69*), it is possible that PKCδ may elicit different responses depending on cell type. The attenuation of BMOV-induced PKCδ phosphorylation by AG1024 observed in our studies indicates that IGF-1R may be an upstream mediator of this event. A requirement for PKCδ in IGF-1R-induced signaling has also been described (*45*, *70*, *71*). However, the precise mechanism and intermediary steps responsible for PKCδ-mediated PKB activation in response to BMOV remain to be explored. Moreover, additional studies using genetic approaches to silence or knock down PKCδ by siRNA and/or oligonucleotides will be needed to provide unequivocal evidence of a role for PKCδ in vanadium-induced responses on PKB in HepG2 cells.

In summary, our studies have provided experimental evidence of a role for IGF-1R-PTK in BMOV-induced activation of the PKB signaling pathway in HepG2 cells. We have also demonstrated that PKCδ activation plays an intermediary role in transducing IGF-1R signaling leading to PKB phosphorylation in response to BMOV. These data provide novel insights into the cellular mechanism of the insulinomimetic and glucoregulatory action of BMOV.

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H_2O_2-Induced Phosphorylation of ERK1/2 and PKB Requires Tyrosine Kinase Activity of Insulin Receptor and c-Src

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ABSTRACT

Hydrogen peroxide (H_2O_2) mimics many physiological responses of insulin, and increased H_2O_2 generation via the Nox-4 subunit of NAD(P)H oxidase was recently demonstrated to serve as a critical early step in the insulin signaling pathway. Exogenously added H_2O_2 has also been shown to activate several key components of the insulin signaling cascade. H_2O_2-induced signaling responses have been found to be associated with the activation of receptor and nonreceptor protein tyrosine kinases (PTK), including the insulin receptor (IR)-β subunit. Therefore, in the present studies on Chinese hamster ovary cells overexpressing wild-type IR-PTK (CHO-IR) or a PTK-inactive form of IR (CHO-1018), we investigated whether IR-PTK plays a role in H_2O_2-induced signaling events. Treatment of CHO-IR cells with H_2O_2 increased the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase B (PKB), and glycogen synthase kinase-3β while enhancing tyrosine phosphorylation of the IR-β subunit and the p85 subunit of phosphatidylinositol 3-kinase (PI3K). Compared with CHO-IR cells, the stimulatory effect of H_2O_2 on ERK1/2 and PKB was partially reduced in CHO-1018 cells. However, pharmacological inhibition of Src family PTK by 4-amino-5-(4-chlorophenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP-2) almost completely blocked H_2O_2-stimulated phosphorylation of the p85 subunit of PI3K, ERK1/2, and PKB. Moreover, H_2O_2, but not insulin, induced Tyr-418 phosphorylation of Src, which was also suppressed by PP-2. Taken together, these data suggest that both IR-PTK and Src family PTKs contribute to H_2O_2-induced signaling in CHO-IR cells albeit IR-PTK has a less dominant role in this process. *Antioxid. Redox Signal.* 7, 1014–1020.

INTRODUCTION

REACTIVE OXYGEN SPECIES have been suggested to serve as mediators of the action of several growth factors, cytokines, and insulin (5, 13, 20–22, 32). Insulin is the major hormone involved in glucose homeostasis, and its effect is initiated by binding to its receptor on cell membranes. The insulin receptor (IR) is a heterodimeric (α_2, β_2) protein, and insulin binding to the IR-α subunit results in conformational changes, leading to enhanced intrinsic protein tyrosine kinase (PTK) activity of the β subunit by multisite tyrosine phosphorylation. Once activated, IR-PTK can phosphorylate several cytosolic IR substrates such as IRSs and Shc, which serve as docking sites for Src homology 2 domain-containing signaling molecules (37), triggering the activation of two key signaling pathways. In one pathway, the association of IRS-1 with Grb-2-SOS complex results in activation of the Ras, Raf, MEK and extracellular signal-regulated kinase (ERK) pathway (36, 38). Activated ERK1/2 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, and contribute the mitogenic and growth-promoting effects of insulin (36, 37). The second pathway that radiates from the IRS complex upon insulin stimulation involves phosphatidylinositol 3-kinase (PI3K) activation (36, 38). PI3K phosphorylates phosphatidylinositol

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(PI) lipids at position 3 of the inositol ring, and generates 3-phosphorylated forms of PI, such as phosphatidylinositol 3,4,5-trisphosphate (30), which are implicated in the activation of phospholipid-dependent kinase and related serine/threonine protein kinases. These activated kinases in turn are responsible for the phosphorylation and stimulation of several downstream signaling protein kinases, such as protein kinase B (PKB) (also known as Akt), glycogen synthase kinase 3 (GSK-3), $p70^{s6k}$ (11), and protein kinase C-ζ (PKCζ) (10). Activation of these protein serine/threonine kinases has been demonstrated to mediate the metabolic effects of insulin at the level of glucose transport, glucose transporter (GLUT-4) translocation, glycogen and protein synthesis (36).

Recent studies have demonstrated that insulin stimulation of cells generates a hydrogen peroxide (H_2O_2) burst that enhances the tyrosine phosphorylation of IR and IRS-1 (22). An important role for the NAD(P)H oxidase catalytic subunit homologue Nox-4 in enhancing H_2O_2 production in response to insulin has been suggested recently (23). In addition, exogenously added H_2O_2 has been shown to mimic many physiological effects of insulin, including glucose transport (15), glycogen synthesis (17), lipogenesis (24), lipolysis (19), and phosphoenolpyruvate carboxykinase gene expression (33). H_2O_2 has also been found to increase tyrosine phosphorylation of the IR-β subunit (15, 16) and activation of the ERK1/2 and PI3K/PKB signaling pathways in several cell types (3, 4, 6, 8, 18, 31, 34, 35). A potential role for epidermal growth factor (EGF) receptor transactivation (12, 28, 39) and the src family of PTK in H_2O_2-induced signaling has been postulated (1, 2, 12). However, despite the ability of H_2O_2 to enhance IR-PTK activity and IR-β subunit phosphorylation, the possible involvement of IR-PTK in H_2O_2-induced responses has not been characterized. Therefore, in the present studies, we investigated the possible requirement of IR-PTK in H_2O_2-induced phosphorylation of the ERK1/2 and PKB pathways.

MATERIALS AND METHODS

Materials

Insulin was obtained from Eli Lilly Co. (Indianapolis, IN, U.S.A.), H_2O_2 from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and IRS-1 and p85 antibodies from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Phospho-specific and total antibodies, against ERK1/2 and GSK-3β, antiphosphotyrosine antibody (PY99), and IR antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Phospho-specific and total PKB antibodies were procured from New England BioLabs (Beverly, MA, U.S.A.), and phospho-specific c-Src antibody from Biosource (Camarillo, CA, U.S.A.). Protein A Sepharose beads and enhanced chemiluminescence (ECL) detection kits were from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada).

Cell culture

Chinese hamster ovary (CHO) cells overexpressing either wild-type human IR (CHO-IR) or the PTK mutant form (CHO-1018), a gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA, U.S.A.), were maintained in Ham's F-12 medium containing 10% fetal bovine serum. They were grown to confluence in 100-mm plates and incubated in serum-free F-12 medium for 16 h prior to the experiment (26).

Cell lysis and immunoblotting

Cells subjected to various experimental treatments were washed twice with ice-cold phosphate-buffered saline and lysed in buffer A [25 m*M* Tris-HCl, pH 7.5, 25 m*M* NaCl, 1 m*M* sodium orthovanadate, 10 m*M* sodium fluoride, 10 m*M* sodium pyrophosphate, 20 m*M* okadaic acid, 0.5 m*M* ethylene-bis(oxyethylenenitrilo)tetraacetic acid, 1 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1% Triton X-100]. The lysates were clarified by centrifugation to remove insoluble material. The clarified lysates, normalized to contain equal amounts of protein, were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, transferred to polyvinylidene difluoride (PVDF) membranes, and incubated with respective primary antibodies, followed by incubation with a horseradish peroxidase-conjugated second antibody. The antigen–antibody complex was visualized with an ECL detection kit. The immunoblots were quantified by densitometric scanning with NIH ImageJ software (27).

Immunoprecipitation

The clarified cell lysates, normalized to contain equal amounts of protein (500 μg), were immunoprecipitated overnight with 1 μg of PY99 antibody at 4°C, followed by incubation with protein A Sepharose for an additional 2 h. Immunoprecipitated phosphotyrosine proteins were collected by centrifugation, and washed twice with buffer A and once with phosphate-buffered saline. The phosphotyrosine protein immunoprecipitates were subjected to 7.5% SDS-PAGE under reducing conditions, transferred to PVDF membranes, and incubated with respective primary antibodies. Proteins were detected by a horseradish peroxidase-conjugated second antibody and visualized with an ECL detection kit (27).

PI3K assay

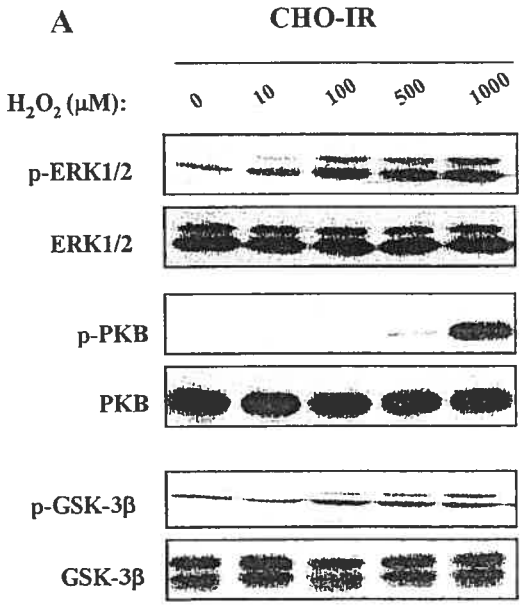
The clarified cell lysates were subjected to immunoprecipitation with 2 μg of p85α antibody for 2 h at 4°C, followed by incubation with protein A Sepharose for an additional 2 h. The immunoprecipitates were washed before PI3K assay, as described earlier (26). The phosphorylated lipid products were extracted and separated by ascending thin-layer chromatography (26). Radioactivity in the spots corresponding to PI 3-phosphate was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

Effect of H_2O_2 on ERK1/2, PKB, and GSK-3β phosphorylation in CHO-IR cells

We first evaluated whether H_2O_2 alters the activation of ERK1/2, PKB, and its downstream targets in CHO-IR cells. As increased phosphorylation in specific serine/threonine residues of these molecules is associated with their activa-

tion, their activity was measured by using phospho-specific antibodies. As shown in Fig. 1A, treatment of IR-overexpressing cells for 5 min with escalating concentrations of H_2O_2 induced ERK1/2, PKB, and GSK-3β phosphorylation. H_2O_2 increased ERK1/2 phosphorylation at concentrations as low as 10 μ*M* with maximal effect at 100 μ*M*, whereas higher concentrations (500 μ*M* to 1 μ*M*) were required to elicit robust PKB phosphorylation. Next, we assessed the time dependence of the H_2O_2 response. As seen in Fig. 1B, 1 m*M* H_2O_2 rapidly enhanced the phosphorylation of ERK1/2, PKB, and GSK-3β. The increase occurred within 5 min and then declined slowly to almost basal levels within 60 min of H_2O_2 treatment.



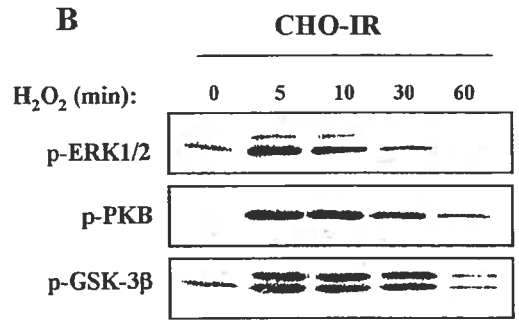


FIG. 1. H_2O_2-induced ERK1/2, PKB, and GSK-3β phosphorylation is time- and dose-dependent in CHO-IR cells. Confluent, serum-starved CHO-IR cells were incubated with different concentrations of H_2O_2 for 5 min (**A**) or with 1 m*M* H_2O_2 for the indicated time periods (**B**). The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK1/2 antibodies (upper immunoblot in A and B), phospho-specific (Ser-473)-PKB antibodies (middle immunoblot in A and B), and phospho-specific (Ser-9)-GSK-3β antibodies (lower immunoblot in A and B). The results are representative of three independent experiments.

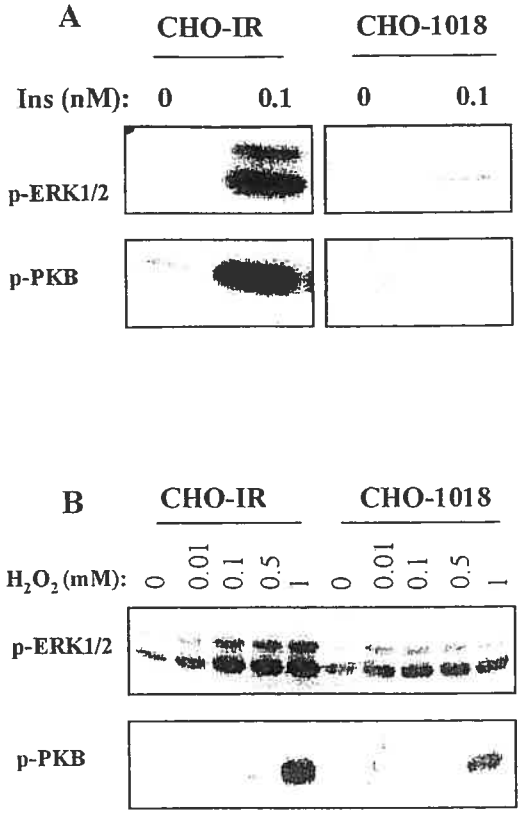


FIG. 2. H_2O_2-induced ERK1/2 and PKB phosphorylation is partially dependent on IR-PTK activity in CHO cells. Confluent, serum-starved CHO-IR and CHO-1018 cells were incubated in the absence or presence of 0.1 n*M* insulin (Ins) (**A**) or with different concentrations of H_2O_2 (**B**) for 5 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK1/2 antibodies (upper immunoblot in A and B) and phospho-specific (Ser-473)-PKB antibodies (lower immunoblot in A and B). The results are representative of three independent experiments.

Effect of H_2O_2 on ERK1/2 and PKB phosphorylation in IR-PTK-deficient cells

To examine the role of IR-PTK in H_2O_2-induced effects on ERK1/2 and PKB phosphorylation, we studied CHO-1018 cells that overexpress an inactive form of IR-PTK. The inactive form of IR was generated by the mutation of lysine 1018 to alanine in the ATP-binding domain of the IR-β subunit. This mutation results in the loss of ATP-binding activity and, thus, PTK function (7). As illustrated in Fig. 2, both insulin and H_2O_2 enhanced the phosphorylation of ERK1/2 and PKB in CHO-IR cells overexpressing a normal IR. However, in CHO-1018 cells, the insulin-induced phosphorylation of both ERK1/2 and PKB was almost completely attenuated, whereas the H_2O_2-evoked increase was partially blocked in this cell type. These data suggested that, compared with insulin, H_2O_2-induced phosphorylation of ERK1/2 and PKB is exerted in part through IR-PTK activity.

Effect of H_2O_2 on IR, IRS-1, and p85 tyrosine phosphorylation

As the insulin effects on ERK1/2 and PKB are mediated through the enhanced tyrosine phosphorylation of IR and IRS-1, we next explored if; similarly to insulin, H_2O_2 increases the tyrosine phosphorylation of IR and IRS-1. As shown in Fig. 3, both insulin and H_2O_2 augmented tyrosine phosphorylation of the IR-β subunit, but tyrosine phosphorylation of the IR-β subunit induced by H_2O_2 was significantly lower than that observed with insulin. Furthermore, whereas insulin enhanced the tyrosine phosphorylation of IRS-1, a similar effect of H_2O_2 on IRS-1 tyrosine phosphorylation was not detected in CHO-IR cells (Fig. 3). In contrast, both insulin and H_2O_2 treatment resulted in enhanced tyrosine phosphorylation of the p85 subunit of PI3K.

Requirement of c-Src in H_2O_2-induced ERK1/2 and PKB phosphorylation

The results with CHO-1018 cells indicated that the H_2O_2 effect on ERK1/2 and PKB phosphorylation required additional signals besides IR-PTK. As potential involvement of the Src family of PTKs in H_2O_2-induced signaling has been proposed in other cell types, we investigated if, in CHO cells also, Src-PTK was responsible for triggering the phosphorylation of various signaling components. We utilized PP-2 [4-amino-5-(4-chlorophenyl)-7-(*tert*-butyl)pyrazolo[3,4*d*] pyrimidine], a selective inhibitor of Src-PTK, and PP-3 (4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine), an inactive analogue of PP-2, to examine the contribution of Src in H_2O_2-induced increased phosphorylation events. As depicted in Fig. 4, treatment of cells with PP-2 almost completely suppressed H_2O_2-stimulated phosphorylation of both ERK1/2 and PKB. As

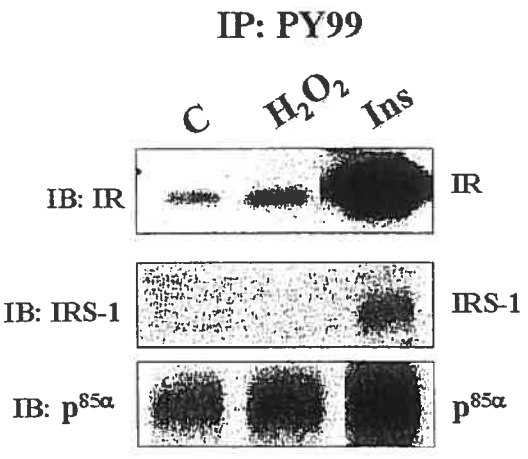


FIG. 3. H_2O_2-induced tyrosine phosphorylation of IR and p85, but not IRS-1, in CHO-IR cells. Confluent, serum-starved CHO-IR cells were incubated with 100 n*M* insulin (Ins) or with 1 m*M* H_2O_2 for 5 min. The cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with an antiphosphotyrosine (PY99) antibody. The immunoprecipitates were immunoblotted with the indicated antibodies. A representative immunoblot (IB) from three independent experiments is shown.

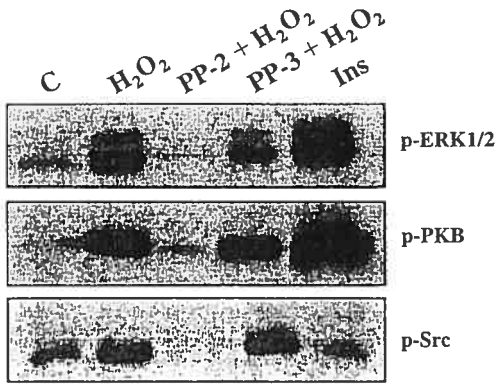


FIG. 4. H_2O_2-induced ERK1/2 and PKB phosphorylation is totally dependent on Src-PTK activity in CHO-IR cells. Confluent, serum-starved CHO-IR cells were pretreated with or without 10 μ*M* PP-2 or its inactive analogue PP-3 for 30 min, followed by incubation with 1 m*M* H_2O_2 or 100 n*M* insulin (Ins) for 5 min. The cells were lysed, and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK1/2 antibodies (upper immunoblot), phospho-specific (Ser-473)-PKB antibodies (middle immunoblot), and phospho-specific (Tyr-418)-Src antibodies (lower immunoblot). The results are representative of three independent experiments.

PP-2 is a potent and specific inhibitor of c-Src activity, and its ability to suppress H_2O_2-induced responses suggested a role of Src in this process, we directly assessed if H_2O_2 would enhance Src activity in CHO-IR cells. This was achieved by evaluating the increase in Tyr-418 phosphorylation in the activation loop of c-Src. As shown in Fig. 4, H_2O_2 treatment enhanced the Tyr-418 phosphorylation of Src, which was almost completely blocked in PP-2-pretreated cells.

Effect of PP-2 on IR and p85 tyrosine phosphorylation and of wortmannin on ERK1/2 and PKB phosphorylation induced by H_2O_2

To determine if c-Src is an upstream mediator of IR and p85 tyrosine phosphorylation, we evaluated the effect of PP-2 on H_2O_2-induced phosphorylation of these signaling components. As illustrated in Fig. 5A, PP-2 treatment failed to block tyrosine phosphorylation of the IR-β subunit, whereas it completely attenuated the tyrosine phosphorylation of the p85 regulatory subunit of PI3K. Next, we examined if p85 phosphorylation by H_2O_2 was associated with an increase in the PI3K activity of CHO-IR cells. As seen in Fig. 5B, H_2O_2 treatment enhanced PI3K activity, which was sensitive to inhibition by wortmannin, a specific PI3K inhibitor. Furthermore, both ERK1/2 and PKB phosphorylation induced by H_2O_2 were almost completely attenuated by wortmannin pretreatment of cells (Fig. 5C).

DISCUSSION

In this study, we have shown that exogenously added H_2O_2 enhanced the phosphorylation of ERK1/2 and PKB signaling pathways in CHO-IR cells. The increased phosphorylation of

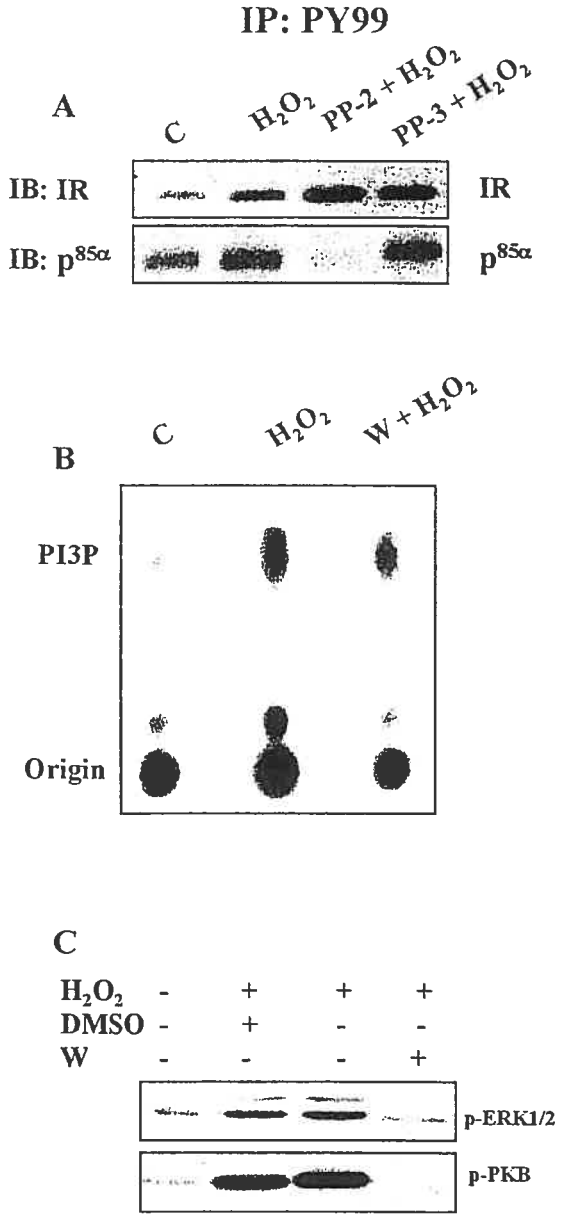


FIG. 5. H_2O_2-induced p85 phosphorylation and ERK1/2 and PKB phosphorylation are dependent on Src-PTK and PI3K, respectively. Confluent, serum-starved CHO-IR cells were pretreated with 10 μ*M* PP-2 or its inactive analogue PP-3 (**A**) or 100 n*M* wortmannin (W) (**B** and **C**) for 30 min, followed by incubation with 1 m*M* H_2O_2 for 5 min. In A, the cells were lysed, and equal amounts of total protein from the clarified lysates were subjected to immunoprecipitation (IP) with antiphosphotyrosine (PY99) antibody. The immunoprecipitates were immunoblotted (IB) with IR antibodies (upper immunoblot) and p85 antibodies (lower immunoblot). In B, the cells were lysed, and the cell lysates were subjected to immunoprecipitation using p85α antibodies. PI3K activity was measured in p85α immunoprecipitates with PI as substrate and [γ-^{32}P]ATP as phosphoryl group donor. The position of PI 3-phosphate (PI3P) is indicated. In C, the cells were lysed and the lysates were subjected to immunoblotting using phospho-specific (Tyr-204)-ERK1/2 antibodies (upper immunoblot) and phospho-specific (Ser-473)-PKB antibodies (lower immunoblot). DMSO, dimethyl sulfoxide. The results are representative of three independent experiments.

ERK1/2 and PKB was associated with heightened tyrosine phosphorylation of the IR-β subunit and p85 subunit of PI3K. We have also provided evidence that PTK activity of the IR-β subunit is partially responsible for H_2O_2-induced activation of the ERK1/2 and PKB pathways. This contention is based on the results with the CHO cells that overexpress a PTK-inactive form of IR. Although earlier studies have demonstrated a stimulatory effect of H_2O_2 on tyrosine phosphorylation of the IR-β subunit in adipocytes, no attempts have been made to determine the contribution of IR-PTK in H_2O_2-induced signaling events. Thus, to the best of our knowledge, this work provides the first evidence in support of the involvement of IR-PTK in H_2O_2-induced phosphorylation of ERK1/2 and PKB.

H_2O_2 has recently emerged as an important redox molecule for the action of several growth factors, cytokines, and insulin (5, 13, 20–22, 32). Some of the effects of H_2O_2 on signaling events have been shown to be mediated by transactivation of EGF receptor PTK and the Src family of PTKs in several cell types (1, 2, 12, 28, 39). In CHO-IR cells, H_2O_2 also enhanced Src activity, as judged by the increased phosphorylation of Tyr-418 in the activation loop of Src. The participation of Src in H_2O_2-induced signaling in CHO cells was evaluated by using PP-2, a highly specific inhibitor of Src PTK activity. PP-2 almost completely attenuated the H_2O_2-induced increase of phosphorylation of ERK1/2, PKB, and the p85 subunit of PI3K. This is in contrast to the partial inhibition elicited in IRPTK-deficient cells where the H_2O_2-induced effect was blocked by only 20–30% (Fig. 2). An important role of Src PTK in insulin-induced activation of PKCδ was demonstrated recently in primary cultures of skeletal myotubes (29). In these studies, insulin stimulated Src PTK activity, which was blocked by PP-2 (29). Moreover, PP-2 also inhibited insulin-induced IR-β tyrosine phosphorylation, as well as glucose uptake in these myotubes. In the case of CHO-IR cells, however, neither insulin- nor H_2O_2-induced IR-β subunit phosphorylation was blocked by PP-2. Furthermore, PP-2 was ineffective in inhibiting the increased phosphorylation of ERK1/2 and PKB induced by insulin (data not shown), whereas it completely blocked the H_2O_2-evoked effect in these cells (Fig. 4), which suggested that Src-PTK-dependent pathways in CHO-IR cells mediate the H_2O_2 response and not that of insulin. Furthermore, in contrast to insulin, H_2O_2 treatment did not enhance IRS-1 tyrosine phosphorylation, but increased tyrosine phosphorylation of the p85 subunit of PI3K, which was sensitive to inhibition by PP-2. Src family PTK-catalyzed Tyr-688 phosphorylation of p85 was recently shown to activate PI3K in COS cells (9). The fact that H_2O_2 induced PI3K activation in these cells, and wortmannin, a specific inhibitor of PI3K, blocked H_2O_2-evoked activation of ERK1/2 and PKB in these cells, suggests that PI3K may be an upstream intermediate in H_2O_2-induced signaling events.

Recent studies have shown that insulin-induced generation of H_2O_2 via NAD(P)H oxidase activation serves as a trigger to initiate insulin signaling (23). In these experiments, ablation of Nox-4, one of the subunits of NAD(P)H oxidase complex, resulted in diminished H_2O_2 production, associated with decreased tyrosine phosphorylation of IR and IRS-1 (23). The data indicated that endogenously generated H_2O_2 in response to insulin is able to enhance IRS-1 phosphorylation in 3T3-L1 adipocytes (23), whereas the results with CHO-IR cells dem-

onstrated that exogenous H_2O_2 fails to modify IRS-1 phosphorylation (Fig. 3). Thus, it appears that the intracellular upstream targets of endogenously generated and exogenously added H_2O_2 may be different.

The precise mechanism by which H_2O_2 induces tyrosine phosphorylation of substrate proteins remains obscure; however, its ability to inhibit the activities of many protein tyrosine phosphatases (PTPases), such as PTP1B (18), and SHP-2 (25), has been suggested as a potential mechanism. PTPase inhibition by H_2O_2 is accomplished by oxidation of catalytically essential cysteine residue in the active site of PTPase (14); this inhibition shifts the equilibrium of the phosphorylation–dephosphorylation cycle, resulting in increased tyrosyl phosphorylation of substrate proteins, such as Src, the IR-β subunit, and the p85 subunit of PI3K. These tyrosyl-phosphorylated proteins promote the assembly of signaling molecules responsible for activating various components of the mitogen-activated protein kinase and PI3K signaling pathways.

In summary, our studies have demonstrated that H_2O_2-induced activation of ERK1/2 and PKB is associated with enhanced tyrosine phosphorylation of the IR-β subunit, the p85 subunit of PI3K, and c-Src in CHO-IR cells. We have also provided evidence of the involvement of both IR-PTK- and Src-PTK-dependent upstream mechanisms as mediators of the H_2O_2 response.

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ABBREVIATIONS

CHO, Chinese hamster ovary; CHO-IR, Chinese hamster ovary cells overexpressing insulin receptor; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinases; GSK, glycogen synthase kinase; H_2O_2, hydrogen peroxide; IR, insulin receptor; IRS, insulin receptor substrate; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PP-2, 4-amino-5-(4-chlorophenyl)-7-(*tert*-butyl)pyrazolo[3,4-*d*]pyrimidine; PP-3, 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine; PTK, protein tyrosine kinase; PTPases, protein tyrosine phosphatases; PVDF, polyvinylidene difluoride; PY99, antiphosphotyrosine antibody; SDS, sodium dodecyl sulfate.

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