Université de Montréal

"Mechanism of Insulinomimesis by Vanadium Salts"

par

Sanjay Kumar Pandey
Département de Physiologie
Faculté de Médecine

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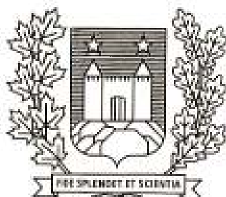

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"Mechanism of Insulinomimesis by Vanadium Salts"

presentée par

Sanjay Kumar Pandey

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Prèsident	Dr. Angelino Calderone
Directeur	Dr. Madhu B. Anand-Srivastava
Co-directieur	Dr. Ashok K. Srivastava
Membre du jury	Dr. Gérald Van de Werve
Examinàteur externe	Dr. Leonard Paul Kleine
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SUMMARY

Vanadium, a group Vth transition element, has been shown to mimic most of the biological effects of insulin such as stimulation of glucose transport, glycogen synthesis, lipogenesis and inhibition of lipolysis and gluconeogenesis. In addition, vanadium therapy improves glucose homeostasis in animal models of diabetes mellitus as well as in human subjects. However, the molecular mechanisms by which insulinomimetic effects of vanadium compounds are mediated remains poorly characterized. In particular, it is not known whether vanadium action is mediated through the activation of one or more steps in insulin signaling cascade or by an alternate pathway.

The biological actions of insulin are initiated by binding to α-subunit of its receptor and subsequent β-subunit tyrosine phosphorylation and activation. The activated receptor in turn phosphorylates downstream proteins including insulin receptor substrate-1 (IRS-1) which serves as a docking protein for association and activation of several Src homology 2 (SH2) domain containing signaling molecules such as PI3-k, Syp/SHP-2, Nck and Grb-2. These IRS-1 associated proteins then stimulate two major signaling pathways. The first is the canonical Ras-Raf-MEK-MAP kinase cascade and the second is the PI3-k-PKB-GSK-3 pathway. Recent studies have suggested that the PI3-k pathway might be responsible for mediating most of the metabolic responses of insulin whereas the Ras-MAP kinase cascade may primarily be involved with mitogenic effects. Thus, the objective of the research presented in this dissertation was to examine (a) if vanadium salts activate the two main signaling pathways of insulin action and (b) what is the contribution

of these pathways in mediating the insulinomimetic effects of vanadyl sulfate on glycogen synthesis.

Chinese Hamster Ovary cells overexpressing Human Insulin Receptor (CHO-HIR cells) have been utilized in these studies. Treatment of CHO-HIR cells with different vanadium salts such as sodium orthovanadate (OV), vanadyl sulfate (VS) or sodium metavanadate (MV) resulted in an increase in the tyrosine phosphorylation of two proteins with apparent molecular sizes of 44 kDa (p^{44mapk} or ERK1) and 42 KDa (p^{42mapk} or ERK2). However, under these conditions tyrosine phosphorylation of IR-β subunit was not detected, whereas insulin, as expected, stimulated tyrosine phosphorylation of its 95 kDa β-subunit. Furthermore, similar to insulin, tyrosine phosphorylation of p^{44mapk} and p^{42mapk} by these salts was accompanied by stimulation of MAP kinase activity. In addition, ribosomal protein kinases, p^{90rsk}, which is downstream of MAP kinase in insulin signaling cascade, as well as p^{70s6k}, were also activated by these vanadium salts. Thus, vanadium-induced activation of these signaling molecules may play an important role in mimicking insulin-like effects.

Since MAP kinases were activated by VS, we wished to further explore if signaling components upstream to MAP kinase such as MEK, Raf and Ras are also stimulated by VS. In addition, we also evaluated the role of PI3-k on these signaling molecules by using a PI3-k inhibitor, wortmannin. Incubation of CHO-HIR cells with VS resulted in an increased tyrosine phosphorylation of p^{44mapk} and p^{42mapk} in parallel with an activation of MAP kinase as well as MEK and c-Raf-1 activities. Pretreatment of these cells with wortmannin blocked VS-induced

tyrosine phosphorylation of p^{44mapk} and p^{42mapk} and activation of Raf, MEK, MAP kinase as well as p^{70s6k} activities suggesting that PI3-k may be required for this cascade. Furthermore, pretreatment of these cells with farnesyltransferase inhibitor B581which is known to block Ras function, resulted in an attenuation of VS-induced MAP kinase activation indicating a role of Ras in mediating this effect. Thus, it may be suggested that PI3-k is required for VS-stimulated activation of Ras-MAP kinase and p^{70s6k} pathways.

Subsequent studies were conducted to examine if similar to insulin, VS also stimulated PI3-k activity and whether VS-induced activation of PI3-k, MAP kinase and p^{70s6k} contribute to VS-stimulated glycogen synthesis. Treatment of CHO-HIR cells with VS resulted in an increase in PI3-k activity and glycogen synthesis which were blocked by PI3-k inhibitors, wortmannin and LY294002. By contrast, inhibition of MAP kinase by PD98059 or p^{70s6k} by rapamycin, failed to inhibit glycogen synthesis in response to VS. Moreover, VS-induced activation of PI3-k and glycogen synthesis occured independent of IR-β subunit tyrosine phosphorylation but was associated with an enhanced tyrosine phosphorylation of IRS-1. Furthermore, we detected PI3-k activity in the IRS-1 immunoprecipitate suggesting that PI3-k is recruited to IRS-1 upon VS stimulation. These results therefore suggested that IRS-1 tyrosine phosphorylation and PI3-k activation play a critical role in mediating VS-stimulated glycogen synthesis independent of IR-tyrosine phosphorylation.

Taken together, results from these studies demonstrate that similar to insulin, VS stimulated both PI3-k and Ras-MAP kinase signaling pathways.

However, unlike insulin, VS responses are independent of any detectable increase in IR tyrosine phosphorylation but are mediated with an enhanced tyrosine phosphorylation of IRS-1. These data also provide evidence to suggest that the PI3-k pathway plays a critical role in mediating VS-stimulated glycogen synthesis whereas the Ras-MAP kinase pathway may be important for eliciting some other biological responses such as cell proliferation and differentiation.

RÉSUMÉ

Le vanadium, un élément de transition du groupe V, est reconnu pour mimer la plupart des effets biologiques de l'insuline comme la stimulation du transport du glucose, la synthèse du glycogène, la lipogénèse et l'inhibition de la lipolyse et de la gluconéogénèse. De plus, les thérapies à base de vanadium appliquées à des animaux diabétiques, aussi bien qu'à certains sujets humains, ont démontré une amélioration de l'homéostasie du glucose. Cependant, les mécanismes moléculaires par lesquels les composés de vanadium engendrent leurs effets insulinomimétiques ne sont pas connus. D'ailleurs, on ne sait pas si l'action du vanadium est transmis par une ou plusieurs étapes de la cascade de signalisation de l'insuline ou d'une toute autre façon.

Les actions biologiques de l'insuline sont initiées par sa liaison à la sous-unité α de son récepteur suivie de la phosphorylation des tyrosines et l'activation de la sous-unité β. Le récepteur activé phosphoryle des protéines en aval dont le insulin receptor substrate-1 (IRS-1) qui sert de protéine de liaison pour l'association et l'activation de plusieurs molécules de signalisation possédant des domaines Src homology 2 (SH2) comme PI3-k, Syp/SHP-2, Nck et Grb-2. Ensuite, ces protéines associées à IRS-1 stimulent deux cascades de signalisation. La première est la cascade de Ras-Raf-MEK-MAP kinase et la deuxième est la voie de PI3-k-PKB-GSK-3. Des études récentes suggèrent que la voie impliquant PI3-k serait possiblement responsable de la plupart des réponses métaboliques de l'insuline alors que la cascade de Ras-MAPK serait plutôt impliquée dans les effets mitogéniques. L'objectif de la présente est d'examiner (a) si les sels de vanadium

peuvent activer les deux principales voies de signalisation de l'insuline et (b) quelle est la contribution de ces voies dans l'effet insulinomimétique engendré par le sulfate de vanadyle sur la synthèse du glycogène.

Ces études impliquent des cellules d'ovaires d'hamsters chinois surexprimant le récepteur à insuline humain (CHO-HIR). Le traitement de ces cellules avec différents sels de vanadium comme l'orthovanadate de sodium (OV), le sulfate de vanadyle (VS) ou le métavanadate (MV) résulte en une augmentation de la phosphorylation des tyrosines de deux protéines de poids moléculaire de 44kDa ($p44^{mapk}$ ou ERK1) et 42 kDa ($p42^{mapk}$ ou ERK2). Par contre, dans ces conditions, la phosphorylation des tyrosines des sous-unités β du récepteur à insuline (95 kDa) n'est pas observée, contrairement à une stimulation avec l'insuline. Néanmoins et de façon similaire à l'insuline, la phosphorylation des tyrosines de $p44^{mapk}$ et $p42^{mapk}$ par ces sels est accompagnée d'une stimulation de l'activité MAP kinase. De plus, la protéine kinase ribosomale, $p90^{rsk}$, qui est en aval de la MAP kinase dans la cascade de signalisation de l'insuline est, comme $p70^{s6k}$, activée par ces sels. Ainsi, l'activation de ces molécules par les sels de vanadium joue sûrement un rôle important dans l'effet insulinomimétique.

Comme les MAP kinases sont activées par le VS, nous avons voulu savoir si les composantes de signalisation en amont de la MAP kinase (MEK, Raf et RAS) sont également stimulés par le VS. De plus, nous avons évalué le rôle de la PI3-k envers ces composantes en utilisant un inhibiteur de PI3-k, la wortmannine. L'incubation de CHO-HIR avec du VS résulte en une augmentation de la phosphorylation des tyrosines de $p44^{mapk}$ et $p42^{mapk}$ de même qu'une augmentation

de l'activité de MAP kinase, MEK et c-Raf-1. Un pré-traitement de ces cellules avec de la wortmannine bloque la phosphorylation des tyrosines de $p44^{mapk}$ et $p42^{mapk}$ ainsi que l'activation de Raf, MEK, MAPK et $p70^{s6k}$ induite par le VS, suggérant ainsi que la PI3-K est requise pour l'activation de cette cascade. En outre, le pré-traitement de ces cellules avec un inhibiteur de la farnesyltransférase, B581, bloquant l'action de Ras, résulte en une atténuation de l'activation de MAP kinase induite par le VS. Ceci indique un rôle important pour Ras dans les effets insulinomimétiques du VS. Par ailleurs, il est suggéré que la PI3-k est requise pour l'activation faite par le VS sur la voie de Ras-MAP kinase et $p70^{s6k}$.

Suite à ces études, nous avons voulu savoir si, de façon similaire à l'insuline, le VS peut aussi stimuler l'activité de PI3-k et si l'activation de PI3-k, MAP kinase et $p70^{s6k}$ induite par le VS contribue à la synthèse du glycogène. Le traitement des CHO-HIR avec le VS résulte en une augmentation de l'activité de PI3-k, de même qu'une augmentation de la synthèse du glycogène. Ces derniers étant bloqués par les inhibiteurs de la PI3-k, la wortmannine et le LY294002. Dans un autre ordre d'idée, l'inhibition de MAP kinase par PD98059 ou de $p70^{s6k}$ par la rapamycine n'inhibe pas la synthèse du glycogène en réponse au VS. De plus, l'activation de PI3-k et de la synthèse du glycogène induite par le VS se produit de façon indépendante de la phosphorylation des tyrosines des sous-unités β du récepteur à insuline, mais elle est associée avec une augmentation de la phophorylation des tyrosines du IRS-1. Qui plus est, nous avons détecté l'activité de la PI3-k dans un précipité d'anticorps anti-IRS-1 suggérant que la PI3-k est recrutée au IRS-1 suite à une stimulation de VS. Ces résultats indiquent que la

phosphorylation des tyrosines d'IRS-1 et l'activation de la PI3-k jouent un rôle de premier plan dans la synthèse du glycogène induite par le VS de façon indépendante de la phosphorylation des tyrosines du récepteur à insuline.

Tous ces résultats démontrent, de façon similaire à l'insuline, que le VS stimule la voie de PI3-k et de Ras-MAP kinase. Par contre, contrairement à l'insuline, les réponses induites par le VS sont indépendantes de toute hausse de la phosphorylation des tyrosines du récepteur à insuline, mais sont en association avec une hausse de la phosphorylation des tyrosines du IRS-1. Ces résultats fournissent des évidences suggérant que la PI3-k joue un rôle critique sur la synthèse du glycogène induite par le VS, alors que, la voie de Ras-MAPK serait plutôt importante pour des réponses biologiques comme la prolifération et la différentiation cellulaire.

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
BMOV	Bis Maltalato OxoVanadium
cAMP	Cyclic Adenosine 3′, 5′-Monophosphate
CHO-HIR	Chinese Hamster Ovary cell Overexpressing Human Insulin Receptor
CREB	cAMP Response Element-Binding Protein
EDTA	Ethylene Diamine Tetra-acetic Acid
eEF	eukaryotic Elongation Factor
EGF	Epidermal Growth Factor
ERK	Extracellular signal Regulated Kinase
Gab	Growth factor Adapter Binder
GAP	GTPase Activating Protein
GDP	Guanosine Diphosphate
GI	Gastro Intestinal tract
Grb-2	Growth factor Receptor Binder
GS	Glycogen Synthase
GSK-3	Glycogen Synthase Kinase-3
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
HEPES	(N-(2-HydroxyEthyl)Piperazine-N′-(2-EthaneSulfonic acid)
H_2O_2	Hydogen Peroxide
IDDM	Insulin Dependent Diabetes Mellitus
IGF	Insulin like Growth Factor
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
JAK	Janus Kinase
JNK	Jun N-terminal Kinase
kDa	kiloDalton
LAR	Leukocyte Antigen Related
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen Extracellular regulated Kinase
Mr	relative Molecular weight
mSOS	mammalian Son Of Sevenless
mTOR	mammalian Target Of Rapamycin
MV	MetaVanadate
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide (reduced)
NIDDM	Non-Insulin Dependent Diabetes Mellitus
OV	OrthoVanadate
PDGF	Platelet Derived Growth Factor
PDK-1	Phosphatidyl Inositol (3, 4)P_2 /(PI3,4,5)P_3 Dependent Kinase-1

PH	Pleckestrin Homology
PHAS-I	Phosphorylated Heat and Acid-Stable protein
PI	Phosphatidyl Inositol
PI3P	Phosphatidyl Inositol 3 Phosphate
$PI3,4P_2$	Phosphatidyl Inositol 3, 4 bisphosphate
$PI3,4,5P_3$	Phosphatidyl Inositol 3, 4, 5 trisphoaphate
PI3-k	Phosphatidyl Inositol 3-Kinase
PKB	Protein Kinase B
PKC	Protein Kinase C
PLA_2	PhosphoLipase A_2
PPG-1	Protein Phosphatase Glycogen bound-1
p^{90rsk}	90 k.Da. Ribosomal Kinase
p^{70s6k}	70 k.Da. Ribosomal Kinase
PTB	Protein Tyrosine Binding
PTK	Protein Tyrosine Kinase
PTPase	Protein Tyrosine Phosphatases
SH2	Src Homology-2
Shc	Src Homology Collagen
SHP2	Src Homology Phosphatase-2
STZ	Streptozotocin
TMD	TransMembrane Domain
TNF-α	Tumor Necrosis Factor- α
VS	Vanadyl Sulfate

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DEDICATION

This thesis is dedicated to my beloved mother Mrs. Shanti Pandey who always encouraged and supported me with her special love and kindness. Her teachings have always been in the forefront of my life

CHAPTER I

INTRODUCTION

INTRODUCTION:

1 HISTORICAL PERSPECTIVE OF VANADIUM:

Vanadium, a trace element belonging to the Vth group of transition elements of the chemical periodic table, was discovered in 1830 by a Swedish chemist Nils Sefstrom who named it after the Swedish goddess of beauty, Vanadis (reviewed by Nechay et. al., 1986). Vanadium is found in the biosphere as well as in mammals and constitutes about 0.02% in the earth's crust and the total body pool of vanadium is estimated to be about 100 μg based on a daily intake of 10-60 μg (Nechay et. al., 1986). The first documented use of vanadium as a therapeutic agent was presented in 1899 by Lyonnet, a French researcher who observed a decrease in the urinary output of glucose in two out of three diabetic patients given sodium metavanadate (MV) in drinking water (Lyonnet et.al., 1899). This observation went unnoticed for a long time and interest on the biological importance of vanadium resurfaced in 1977 when Cantley and his associates demonstrated that vanadate can inhibit Na^+-K^+-ATPase activity (Cantley et. al., 1977). Later on Tolman et.al. in 1979, reported *in vitro* insulin-like effects of vanadium on glucose metabolism in rat diaphragm and adipocytes (Tolman et.al., 1979). This observation was confirmed and extended by two different studies demonstrating the insulinomimetic effect of vanadate (Dubyak et.al., 1980; Shechter et.al., 1980). However, a major breakthrough came when Heyliger et. al. in 1985 reported that oral administration of vanadate solution has the capability to normalize blood glucose levels in streptozotocin (STZ) induced-diabetic rats (Heyliger et. al., 1985). Since then several articles have been published reporting various insulinomimetic effects of vanadium. The following section provides a brief overview in which various aspects of vanadium research will be discussed such as its chemistry,

potential uses as an antidiabetic agent, toxicological effects as well as its mechanism of action in relation to the insulin signaling cascade.

2 CHEMISTRY OF VANADIUM COMPOUNDS:

2.1 Vanadium Compounds:

The trace element vanadium can exist in several oxidation states which ranges from -1 to +5. However, it forms mainly three valence states +3, +4 and +5. In aqueous solution it undergoes a series of complex hydrolytic and polymerization reactions (Amos and Sawyer, 1972) and has the capability to react with nitrogen bases and hydroxyl groups (Crans, 1994). Vanadium can form quite stable complexes with various nucleoside such as Nicotinamide Adenine Dinucleotide (NAD), Guanosine Dinucleotide Phosphate (GDP), Adenine Dinucleotide Phosphate, (ADP) as well as thiol groups of several inorganic/organic compounds. For example, it can form a complex with EDTA, (Ethylenediaminetetraacetic acid), which is commonly present in several buffers used during experiments (Amos and Sawyer, 1972). The vanadate-EDTA complex is very stable. Therefore, it appears that EDTA interferes with vanadate as well as other vanadium containing compounds such as diperoxovanadate dependent reactions for example NADH (Nicotinamide Adenine Dinucleotide, reduced form) oxidation (Ravishankar and Ramasarma, 1995a) and oxygen release by catalase (Ravishankar et.al., 1995b). Recent studies have shown that buffering with HEPES, (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] is the best choice among available solutions while using vanadium compounds for any experiment (Huyer et.al., 1997).

In general, tetravalent (IV) and pentavalent (V) valance states of vanadium are ubiquitiously distributed. At physiological pH, trivalent vanadium is unstable and is rapidly

oxidized while tetravalent vanadium is stable at acidic pH as blue vanadyl cation and is less toxic (Macara, 1980). Vanadyl is not easily oxidized and it does not generate superoxide ion upon autooxidation (Kalyani et. al., 1992). Vanadate, the pentavalent vanadium compound (V) oligomerizes at high concentration (V_2 and V_5) (Jaswal and Tracey, 1991). It has been reported that vanadate exist as metavanadate (VO_3^-) in the plasma but reduces to vanadyl state once taken up inside the cell. Intracellularly it is bound to the reduced form of glutathione (GSH) and is less potent as a protein phosphatase inhibitor (Macara, 1980; Elberg et.al., 1994). The possible entry mechanism of vanadium involves an anion transport mechanism (Degani et.al., 1981). As vanadyl it can interact potently with phosphatases and the inhibition is attributed to a five-coordinate vanadate complex which mimics the transition state of the phosphate ester hydrolysis reaction. This is an interesting feature of vanadate species which is exhibited by its similar structure (tetrahedral or trigonal bipyramid) and charge to phosphate. Chemical structures of commonly utilized vanadium compounds are presented in Figure 1.

2.2 Other Vanadium containing complexes:

In an attempt to improve the potential therapeutic efficacy, several vanadium complexes were synthesized by many laboratories. In 1987, Katoda, et. al. observed that when vanadate is mixed with hydrogen peroxide (H_2O_2), a new species named 'pervanadate'/peroxovanadate is generated (Kadota et.al., 1987). Pervanadate is several fold more potent than vanadate in mimicking insulin action (Kadota et.al., 1987; Posner 1994). Some of the insulin actions mimicked by pervanadate include lipogenesis, glucose transport and diminished lipolysis in various tissues and cell lines such as adipose, liver, muscle and CHO-HIR cells (Heffetz and Zick 1989; Fantus et.al., 1989; Shisheva and

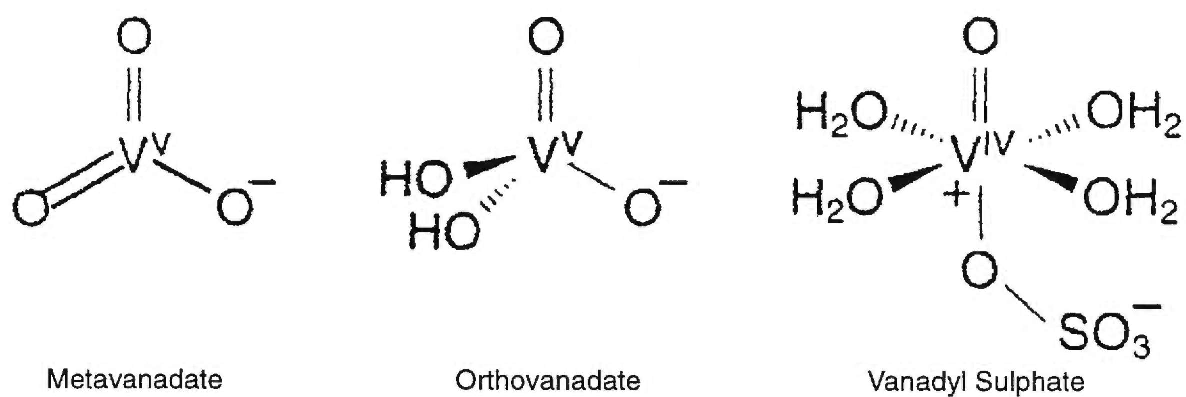


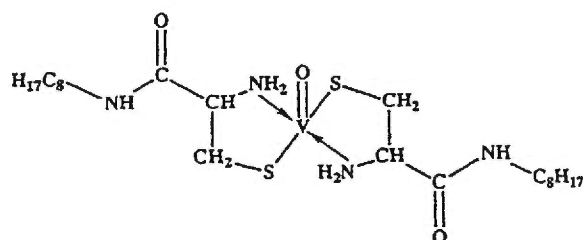
Figure 1: Chemical structures of the most widely used vanadium compounds.

(Adapted from: Brichard and Henquin Trends Pharm. Sci.16, 265-270, 1995)

Shechter 1993a., Posner 1994). This compound, similar to vanadate, also has structural resemblance to the phosphate. Interestingly, the sequential addition of peroxo group(s) 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 present in PTPases (Shaver et.al., 1995).

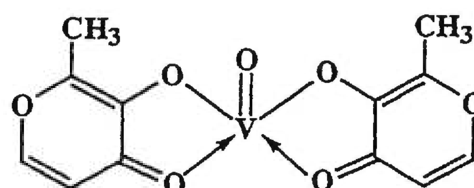
Vanadate possess redox (oxidation-reduction) activities which arise due to the formation of a complex upon reaction with H_2O_2. It has been reported that at acidic, basic and neutral pH ranges, vanadate forms mono, tri and diperoxo-vanadium complexes respectively (Howarth and Hunt, 1979). In this regard 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 slower rate by catalase as compared to free H_2O_2. (Ravishankar et.al., 1995b).

Another class of compounds categorized as "Organovanadium Complexes", exemplified by bis(maltolato)oxovanadium (IV), (BMOV), was used in 1992 by Yuen et.al. to study their effect as an antidiabetic agent (Yuen et.al., 1993). This compound is synthesized by forming a bond between two maltose sugars and a metal with its inner coordination sphere which increases its stability. It was observed that BMOV administered to rodents was absorbed faster with lesser gastrointestinal (GI) toxicity as compared to vanadate. Several other organovanadium compounds were prepared whose properties are described in a later section. Structures of some organovanadium complex are presented in Figure 2.



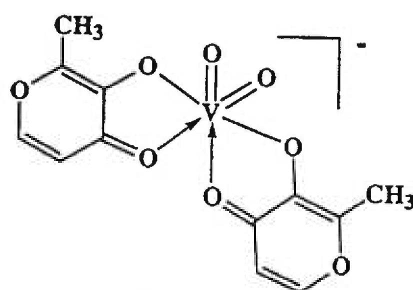
Bis(cysteine, amide N-octyl)oxovanadium(IV)

Bis(N-octylcysteinamide)oxovanadium(IV)-Naglivan.



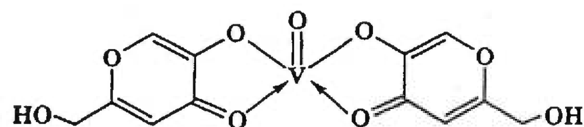
Bis(maltolato)oxovanadium(IV)

Bis(maltolato)oxovanadium(IV)-BMOV.



Bis(maltolato)dioxovanadate(V)

Bis(maltolato)dioxovanadate(V)-BMO2V.



Bis(kojato)oxovanadium(IV)

Bis(kojato)oxovanadium(IV)-BKOV.

Figure 2: Structure of some organovanadium complexes.

(Adapted from: McNeill et. al. Mol. Cell Biochem 153, 175-180, 1995)

2.3 Effect of Vanadium compounds on Various Enzymes:

Cantley and coworkers reported an inhibitory effect of vanadate on purified Na^+ K^+-ATPase which occured within 2-3 minutes (Cantley et. al., 1977). This inhibition was favoured by the presence of Mg^+, K^+ and ATP. Later on vanadate was shown to inhibit PTPases (Swarup et.al., 1982) as well as acid and alkaline phosphatases (Chasteen, 1983). The myosin ATPase and contractile protein dynein are also inhibited by vanadate without any covalent phosphoenzyme intermediate formation (Nechay, 1984). In addition, vanadate also inhibits phosphodiesterase, ribonuclease and Ca^{++}-ATPase (Ramasarma and Crane, 1981). Besides its inhibitory properties, vanadate stimulates some enzymes. For example, it stimulates adenylate cyclase activity which in turn leads to increased cellular concentration of cAMP (Combest and Johnson, 1983; Boyd and Kustin, 1984; Chasteen, 1983). This action of vanadium is mediated by the nucleotide regulatory protein of the adenylate cyclase complex (Erdmann et. al., 1984). Since vanadium stimulates the key enzyme in the cAMP second messenger system, it undoubtedly has important secondary effects on patterns of intracellular protein phosphorylation. In addition it also stimulates glucose-6-phosphate dehydrogenase (Nour-Eldeen et.al., 1985).

3 DIABETES AND VANADATE ACTION:

Diabetes mellitus in humans can be categorized as either type I or type II. Type I is insulin-dependent (IDDM), while type II (in which insulin is present but tissues are resistant to its actions) is classified as non-insulin-dependent (NIDDM). In fact, some diabetic individuals fit into neither category, and treatment regimens may include exogenous insulin administration for both types. Insulin, like many proteins/peptides is not absorbed intact following oral administration, hence in insulin-deficient or resistant state,

such as diabetes, it must be administered parenterally. The potential of vanadium compounds to mimic insulin while being administered orally represents a significant advance over currently available therapies. The following section briefly summarizes studies of vanadium compounds on both types of diabetes.

3.1 Insulinomimetic Effects of Vanadate in Type I diabetes mellitus:

Type I diabetes, as described above, is also characterized by impaired insulin secretory response due to the destruction of β-cells of the pancreas. Rats injected with streptozotocin (STZ), which destroys insulin producing β-cells and therefore makes them diabetic, provides the best animal model to study IDDM. These rats are hyperglycemic besides being deficient in insulin production.

The first report regarding effectiveness of vanadate in producing normoglycemia in STZ-diabetic rats was demonstrated by Heyliger et. al. in 1985. It was observed that when vanadate (0.8 mg/ml) was given in drinking water for a period of 42 days normoglycemia occurred in these rats. The response was observed within a week of treatment and maintained for several months. During this period there was no detectable alteration in the pattern of food intake or body weight although slow growth rate and mild diarrhoea was observed which was corrected by administering 0.5% saline in drinking water. Under these conditions there was no significant change in the level of either plasma insulin or glucose. Similar glucose lowering effect was later on demonstrated by other investigators. Ramanadham et. al. (1989a and 1989b) reported that vanadyl sulfate (VS) was able to lower blood glucose when given to STZ-diabetic rats in drinking water (0.25-1.0 mg/ml) for a 3 week period. This effect was maintained for 13 weeks even after withdrawal of the treatment. This effect could have been due to the fact that vanadyl was retained for a

longer time period in the cells as compared to other forms of vanadium compounds. However, in contrast Meyerovitch et. al. (1987) and Fantus et. al. (1990) observed that discontinuation of therapy could not reverse the effect. In their study, by administering MV at a dose of 0.2 mg/ml in drinking water plasma glucose was decreased but it was above the normal levels. The secondary complications of diabetes such as nephropathy and retinopathy were also improved when therapy was continued for one year (Dai et.al., 1994).

3.1.1 Effects of Vanadium Complexes:

Although inorganic vanadium salts were orally effective, they were not well absorbed by the body system. Therefore, organically chelated vanadium complexes were synthesized with higher lipophilic properties so as to be absorbed easily in the GI. These were more effective at lower doses with less side effects compared to inorganic vanadium salts, which had to be administered at relatively higher doses (McNeil et.al., 1995). For example, administration of BMOV, which was synthesized upon mixing VS with the organic ligand maltol, to STZ-diabetic rats lowered the elevated levels of plasma glucose thereby maintaining euglycemia. In this study an improvement of heart function as well as lowering of lipids were also noticed. It was observed that BMOV was effective in lowering glucose level within 1 day (Yuen et.al., 1993) while approximately 4-7 days was required by inorganic vanadium salts to have the same effect. Similarly some other complexes such as bis(cysteine,amide-N-acetyl) oxovanadium (iv) (Cam et.al., 1993) and bis(picolinato) oxovanadium (Sakurai et.al., 1995) have been shown to produce glucose lowering effects.

Recently a more stable peroxovanadium compound having an oxoligand, one or two peroxoanion(s) with an ancillary ligand was developed by Yale et. al. (1995). These peroxo componds being several fold more active than vanadate were able to lower plasma

glucose in diabetic as well as normal rats (Yale et.al., 1995). It was observed that the blood glucose level was quickly and effectively lowered following peroxovanadium administration, a pattern often observed with insulin. In contrast to inorganic vanadium salts or organovanadium compounds, the peroxovanadium compounds had to be injected intraperitoneally or intravenously to elicit antidiabetic effects.

Vanadium compounds, in addition to normalizing glucose homeostasis, also have an important role in lipid metabolism by restoring the altered plasma lipids and lipoprotein towards normal levels in STZ-diabetic rats (Ramanadham et.al., 1989 a,b; Sekar and Govindasamy, 1991). Brichard et. al. (1994) demonstrated the restoration of mRNA levels of fatty acid synthase, acetyl CoA carboxylase while Pugazhenthi et.al. reported normalisation of malic enzyme and glucose 6-phosphate dehydrogenase (Pugazhenthi et.al., 1991a). Vanadate also suppressed the activity of 3-hydroxy-3-methyl glutaryl-CoA synthase, an important regulatory enzyme of liver ketogenesis (Valera et.al., 1993).

3.2 Insulinomimetic Effects of Vanadium salts in Type II diabetes mellitus:

Glucose intolerance arises due to the resistance of the target tissues to insulin. The characteristic of type II diabetes is a blunted response to insulin which can occur at the receptor and/or postreceptor levels. The antidiabetic effect of vanadium compounds have been examined in several animal models of type II diabetes including the genetically diabetic ob/ob mice which are obese, hyperglycemic and insulin resistant, the fatty (fa/fa) Zucker rats and the diabetic (C57 BL/KsJ) db/db mice.

Brichard et.al. (1990) reported lowering of blood glucose in ob/ob mice upon sodium orthovanadate (OV) feeding in drinking water. Meyerovitch et. al. (1991) showed that OV (0.25 mg/ml) in drinking water given for 7 weeks lowered blood glucose

markedly. In fa/fa Zucker rats oral administration of OV (0.5 mg/ml) for 12 weeks improved elevated glucose levels (Brichard et.al., 1989). The next study reported that vanadate treatment of fa/fa rats during euglycemic hyperinsulinemic clamping, enhanced body glucose disposal mediated by insulin (Jacobs and Cuatrecasas, 1981). Finally, in genetically diabetic db/db mice, OV therapy reduced blood glucose maximally after about 8 weeks (Meyerovitch et. al., 1991) while plasma glucose level was also lowered three months following treatment (Pugazhenthi et.al., 1991b). The glucose lowering effect of peroxovanadium salts or other organic chelated vanadium salts have not been examined in type II diabetes mellitus.

3.3 Effect of Vanadium in Human Diabetes:

As outlined above there are extensive studies documenting the antidiabetic effect of various vanadium compounds in animal models of type I and II diabetes. However, only limited studies on the effect of vanadium in human diabetics have been conducted. It was reported that oral administration of vanadium in capsule form for a period of three weeks (100-125 mg/day/person) improved both peripheral and hepatic insulin sensitivity (Cohen et.al., 1995; Goldfine et.al., 1995). Cohen et. al. studied NIDDM subjects and in their study they gave VS to NIDDM patients. At the end of study period euglycemic-hyperinsulinemic clamps and oral glucose tolerance test was performed. It was observed that sensitivity of target tissues to insulin increased and therefore, resulted in increased glucose uptake, glycogen synthesis and a decrease in hepatic glucose output. By contrast, the investigation of Goldfine et. al. (1995) included both NIDDM and IDDM subjects. Patients were given MV orally (50 mg at each time during breakfast, lunch and supper). It was noticed that glucose utilisation was improved in IDDM patients however, dramatic

effects were seen in NIDDM patients in which sensitivity to insulin was improved in all patients during therapy. In addition to improvement in glucose metabolism, the cholesterol level was lowered markedly in both IDDM and NIDDM patients. Thus, these studies were encouraging, although the long term use of vanadium salts as an antidiabetic agent needs careful evaluation.

4 ROLE OF VANADIUM IN REGULATING MITOGENIC EFFECTS:

Although the major focus of vanadium research has been on its insulinomimetic and antidiabetic properties, its mitogenic actions are also a cause for concern. Vanadium stimulates cell proliferation and differentiation (Klarlund, 1985). This property can be considered as growth factor mimetic effect. Insulin, epidermal growth factor (EGF) and platelet derived growth factor (PDGF), all activate their receptors on tyrosine residues by a process of autophosphorylation (Sefton and Hunter, 1984) and it has been reported that total cell phosphotyrosine content is increased by several fold in transformed cells (Sefton and Hunter, 1984). Vanadium mimics the effect of these growth factors i.e. EGF, insulin, IGF and PDGF in several cell types via the inhibition of PTPase activity (Hori and Oka, 1980; Smith, 1983; English et. al., 1983; Swarup et.al., 1982). Furthermore, it was reported that in cultured embryonic chicken fibroblasts, IGF-1 and OV together stimulated greater (^{3}H)-thymidine incorporation than IGF-1 alone (Lau et.al., 1988). In addition to its growth factor like effects, vanadate causes dose-dependent neoplastic transformation of NRK-1 kidney cells (Klarlund, 1985). Vanadate also induces transformation in hamster embryo cells (Rivedal et.al., 1990), BALB/3T3 cells (Sabbionoi et.al., 1990) and bovine papilloma virus DNA-transfected C3H 10T1/2 cells (Kowlaski et.al., 1992). It was observed that relatively low concentrations of vanadium are mitogenic

depending upon cell type. At concentrations less than 10^{-10} M, vanadate stimulates colony formation of human breast and lung cancer cells while at concentrations of more than 10^{-10} M colony formation is inhibited (Hanauske et.al., 1987). Vanadate at 10 mM inhibits the concanavalin-A-dependent mitogenic response by 50% in thymocyte cultures (Ramanadham and Kern, 1983) while at 50 mM it only slightly inhibits mitogenesis of mouse Leydig tumor cells (Sato et.al., 1987). In another study where an increase in ornithine decarboxylase activity was used as a marker for detecting potential tumorogenic agents, OV and VS enhanced the activity of ornithine decarboxylase in cultured mouse C3H 10T1/2 fibroblasts and minced rat ovary cells respectively (Davison et.al., 1991; Reddy et.al., 1989).

All these biological actions of vanadate appear to be due to the prevention of dephosphorylation of phosphorylated protein tyrosine kinase substrates. The underlying mitogenic mechanism of vanadate is unclear but it involves phosphorylation on tyrosine residues. In summary, vanadate stimulates tyrosine phosphorylation accompanied by cell proliferation and increased mitogenesis in the presence of several growth factors (Klarlund, 1985; Feldman et.al., 1990; Chen Y et.al., 1993; Wang et.al., 1995). In contrast, there are several reports demonstrating inhibition of cell proliferation by vanadate such as in primary chondrocytes, neuronal and glial cells as well as other cell/tissue types (Hanauske et.al., 1987; Cruz et.al., 1995; Faure et.al., 1995; Djordjevic, 1995) but the mechanism remains unidentified.

5 TOXICOLOGICAL STUDIES OF VANADIUM COMPOUNDS:

Vanadium salts have been administered to rodents and humans to produce hypoglycemia and correct other abnormalities associated with diabetes however, the

concern about its toxicity remains an important issue. The reported toxic effects of vanadium compounds include mild to severe diarrhoea (Heyliger et.al., 1985), decrease in body weight gain and death due to dehydration (Brichard et.al., 1988; Domingo et.al., 1991 a,b) as well as an increase serum concentration of urea and creatinine (Domingo et.al., 1991a,b).

Vanadium in most studies is given orally to diabetic rats and mice in drinking water, the optimal toxicological range can vary from 0.2 to 1.1 mg/ml or more (Domingo et.al., 1985; Llobet and Domingo, 1984). Both vanadate and vanadyl are moderately toxic as evidenced by the acute oral toxicity observed in rats and severity of toxic effects increased as the valency increased (Llobet and Domingo, 1984). Vanadyl ions have been shown to be toxic to embryos and also are teratogenic in mice (Paternain et.al., 1990). In some cases chronic treatment with these compounds have been demonstrated to cause renal and bone damage, anemia, low sperm count and some neurological signs such as convulsions and paralysis (Domingo et.al., 1991b). Exposure and inhalation of oxides of vanadium in dust and smoke from burning fuels enriched in vanadium, which are common in industries, can lead to some respiratory problems such as asthma, pulmonary oedema (Vouk, 1979). Excess consumption of MV can cause nausea, vomitting and giddiness and high incidence of coronary insufficiency and bradycardia may occur (Vouk, 1979).

Animals given vanadium salts showed a high mortality rates which was suggested to be due to its high accumulation in tissues (Sakurai et.al., 1990; Domingo et.al., 1991b). By contrast, histopathological examinations of several organs such as heart, lung, liver, ileum and stomach from control, untreated diabetics or OV-treated diabetic animals did not show any differences in vanadium content (Mongold et.al., 1990). Furthermore, Meyerovitch et.al.

(1991) did not observe hepatotoxicity or nephrotoxicity even after 7 weeks of vanadium therapy in a diabetic mouse model. In the light of these toxicological observations, investigators have suggested several means to diminish these phenomenons such as adding sodium chloride and adjusting pH (Heyliger et.al., 1985; Bendayan et.al., 1989). Use of tiron, a chelator, was also shown to reduce the toxic effect of vanadium by preventing its accumulation in tissues (Domingo et.al., 1992). However, recently conducted studies on human subjects have not reported any adverse effects of vanadium salts therapy except occasional salivation and GI discomfort (Goldfine et. al., 1995; Cohen et.al., 1995).

Thus, although vanadium salts possess a great potential to serve as an antidiabetic agent specially in NIDDM, it is important to consider the long term toxic effects that these compounds may exert.

6 VANADATE ACTION AND INSULIN SIGNALING PATHWAYS:

Since the first report by Swarup et. al. (1982) demonstrating that vanadate is a potent inhibitor of PTPases, it was believed that vanadate may act at the cellular level by preventing the dephosphorylation of tyrosine phosphorylated protein substrates. The insulin like effects of vanadium salts therefore, can be considered to be secondary to PTPase inhibition. Thus, it is relevant to describe the signal transduction pathways stimulated by insulin (Figure 3) which will provide us a better understanding of vanadium action at the cellular level.

6.1 Insulin signaling:

Insulin elicits its biological effect by binding to its cell surface receptor. Biochemical characterization has revealed that the insulin receptor (IR) is a heterotetrameric protein consisting of two insulin binding extracellular α-subunits which are linked by

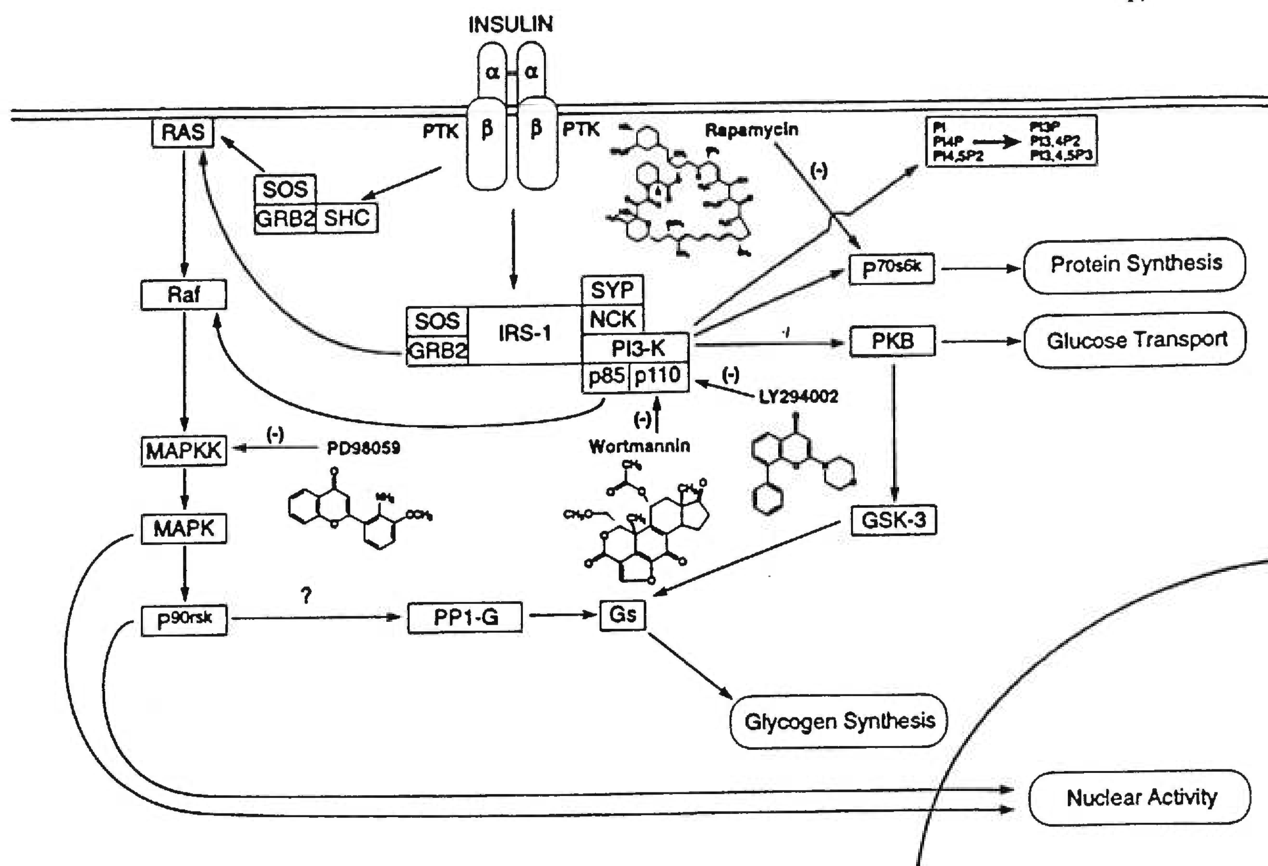


Figure 3: A schematic diagram illustrating insulin signaling pathway. Specific target site of various inhibitors have also been shown.

Insulin binding to the α-subunit leads to the activation of PTK activity of β-subunit. The activated β-subunit in turn phosphorylates its substrates for example, IRS-1 and Shc. Tyrosine phosphorylated IRS-1 in turn recruits several SH2 domain containing proteins, such as SOS, Grb2, Syp, NCK and PI3-k. The IRS-1 associated complex in turn activates the Ras-Raf-MEK-MAP kinase, PI3-k-P^{70s6k} as well as the PI3-k-PKB-GSK-3 cascades. On the other hand the SOS-Grb2-Shc complex may also initiate Ras-Raf-MEK-MAP kinase pathway independent of IRS-1 associated complex. PI3-k is shown to phosphorylate PI, PI4P and PI4,5P to produce PI3P, PI3,4P_2 and PI3,4.5.P_3 respectively. Specific inhibitors and their structures such as the p^{70s6k} inhibitor, rapamycin; the PI3-k inhibitors, wortmannin and LY294002 as well as the MAPKK inhibitor PD98059 have also been indicated. The (-) signs and arrows show the enzymes which are targets of these inhibitors.

disulfide bonds to two signal transducing intracellular β-subunits (White and Kahn, 1994), (Figure 3). A major breakthrough in understanding the cellular pathways of insulin action came when Kasuga et.al. (1982a) demonstrated that the β-subunit of the IR possessed protein tyrosine kinase activity (PTK) which underwent an autophosphorylation reaction upon insulin stimulation. Later on several studies clarified the mechanism involved in activation and regulation of this kinase (Kasuga et.al., 1982b; Petruzzelli, 1982; Avruch, 1982). Identification of the functional domains was provided by the isolation of cDNA of human IR and later on site-directed mutagenesis of specific amino acids helped establish structure-function relationships (Ullrich et.al., 1985; Ebina et.al., 1985a; Ebina et.al., 1985b). Analysis of IR mutants had provided valuable information regarding the role of insulin binding, receptor internalization and PTK in transmitting signals downstream (Kahn and White, 1988; Kahn et.al., 1993; Backer et. al., 1988). A key downstream effector of IR was discovered and named insulin receptor substrate-1 (IRS-1) (White et.al., 1985). IRS-1 acts as a docking protein allowing several other small proteins with Src homology (SH)-2 domain such as growth factor receptor binder, (Grb-2); an adapter of ras-specific guanyl nucleotide exchange factor mammalian Son of Sevenless (mSOS); a non-catalytic adapter protein, Nck, Shc, which can also mediate signaling independent of IRS-1 and Syp/SHP-2, a protein tyrosine phosphatase and p85 subunit of Phosphatidylinositol 3-kinase (PI3-k). As a consequence to recruitment of these proteins, activation of downstream signaling takes place. In the following section some of the salient features of insulin signaling elements are described. A schematic diagram of the insulin signaling pathway has been presented in Figure 3 showing the various signaling components that mediate some of the physiological responses of insulin.

6.1.1 The Insulin Receptor:

The structure of the IR was first provided by biochemical studies. In its native conformation IR consists of two α-subunits and two β-subunits covalently joined together by disulfide (-S-S-) bonds forming an $\alpha_2\beta_2$-heterotetramer (Figure 3).The apparent molecular weight (Mr) of the holoreceptor ($\alpha_2\beta_2$) is approximately 350 kDa under non-reducing conditions while in the reducing state the α–subunit has a Mr. of 135 kDa and β–subunit has a Mr. of 95 kDa (Massague et.al.,1981; Kasuga et.al.,1982a). The insulin binding region resides in the α– subunit whereas the β–subunit possesses an intrinsic PTK activity.

The synthesis of IR takes place first as a proreceptor with a high molecular weight of approximately 180 kDa which is rapidly glycosylated followed by formation of intra and inter subunit disulfide bonds in the rough endoplasmic reticulum. Further post-translational modifications take place in the Golgi apparatus, which includes proteolytic cleavage at the tetrabasic sequence Arg720, Lys721, Arg722 and Arg723, located at the junction of the α and β-subunits. Terminal glycosylation and fatty acid acylation then follows (Hedo, 1983; Hedo and Simpson, 1985).

6.1.1.1 The α– subunit mediates Insulin Binding and Signal Transmission:

The α–subunit of IR is responsible for ligand binding (Ullrich et.al., 1985; Ebina et.al., 1985 a, b; Yip, 1992). The identification of the exact residues in ligand recognition and binding remains difficult to elucidate because mutagenesis experiments may alter conformation and thus the binding domains. Therefore, a chimeric IGF-1 receptor containing the -NH_2-terminal residues (1-68) from IR resulted in a hybrid receptor that can bind insulin with high affinity (Kjeldsen et.al., 1991). It was interpreted from these kinds of experiments that the cystein-rich domain in the IR is a primary determinant in insulin binding (Gustafson and Rutter,

1990). Mutation of Phe89 to Ile, Ser, Trp or His resulted in a dramatic reduction in insulin binding affinity (DeMeyts, 1990). Although it is unclear which residue in the IR interacts directly with insulin, it is clear that -NH_2-terminal domains are required for both high affinity binding and ligand recognition. In one study it was proposed that one Cys per α–subunit located at amino acid position 435, 468 and 524 is involved in this process (Frias and Waugh, 1987), a similar observation was made by Schaffer and Ljungqvist (1992). On the other hand most of the carboxy-terminal region plays an important role in ligand recognition and binding and is also involved in covalent interaction with the β–subunit (Cheatham and Kahn, 1992). This interaction helps to relay the signal from ligand binding to activation of IR-PTK activity.

The α–subunit of IR can also act as a regulatory subunit of the tetrameric holoreceptor because deletion of a certain portion by tryptic digestion or *in vitro* mutagenesis unlocks the inhibitory effect and activates the kinase activity of β–subunit (Shoelson, 1988). Thus, the α–subunit in the absence of insulin maintains a conformational constraint on the β–subunit which otherwise would be constitutively active. The carboxy-terminal of α–subunit has four Cys residues, 647, 682, 683 and 684, which are involved in disulfide linkage with the β–subunit. The Cys 647 to Ser 647 mutation did not cause any change in insulin binding but these mutants were defective in insulin- stimulated PTK activity. They also have strong non-covalent interaction with β–subunits. Therefore, insulin stimulated signal transmission from ligand binding to kinase activation requires proper covalent interaction for normal subunit communication.

6.1.1.2 The β–subunit Possesses an Insulin Stimulated Tyrosine Kinase Activity:

The β–subunit consists of a short extracellular site for glycosylation, a transmembrane domain of 23-amino acids and an intracellular region which has PTK activity required for insulin action. In vitro mutagenesis experiments have provided evidence suggesting that a key lysine residue at 1030 position (Lys1030), which is a ATP binding site, when replaced with other amino acids caused complete loss of kinase activity of β–subunit although insulin binding was unaffected. The metabolic and growth promoting effects of insulin were also blocked in these cells (Chou et.al., 1987; Ebina et.al., 1987; McClain et.al., 1987; Stumpo and Blackshear, 1991).

The transmission of signal from outside to inside of the cell, which results in kinase activation, is not clear. For some growth factors such as PDGF and EGF, ligand induced receptor dimerization or oligomerization precedes receptor activation. It was believed that this oligomerization will produce a conformational change in the receptor resulting in phosphorylation and thus activation of kinase. Some studies have provided evidence suggesting that IR undergoes dimerization/ oligomerization (Heffetz and Zick, 1986; Kahn et.al., 1986). It has also been reported that insulin-stimulated autophosphorylation occurs predominantly within the same holoreceptor where one β–subunit phosphorylates its adjacent β–subunit (Fratalli et.al., 1992; Lee, J. et.al., 1993; Shoelson et.al., 1991; Cobb et.al., 1989), the process termed as transphosphorylation.

The role of transmembrane domain (TMD) in regulation of IR-β–subunit kinase activation has also been studied. With the help of site-directed mutants of TMD, it was suggested that TMD can bear the large changes without losing the signaling function (Fratalli et.al., 1991; Goncalves et.al., 1993; Cheatham et.al., 1993). However,

substituting Gly-Pro to Ala-Ala residues, resulted in about two-fold increase in insulin-stimulated receptor internalization and ligand degradation (Goncalves et.al., 1993). Therefore, wild type receptor internalization will be slower resulting in a decreased rate of receptor degradation and thus increased the half-life of the receptor and prolong the signal.

A complex series of tyrosine phosphorylations in the β–subunit takes place upon insulin binding to the α–subunit. In this process at least six to seven Tyr residues are phosphorylated. (Kahn et.al., 1993; Feener et.al., 1993; Goren et.al., 1987). The major sites of autophosphorylation and kinase regulatory domain are constituted by Tyr 1158, Tyr 1162 and Tyr 1163. Mutation of these residues to Phe results in loss of kinase activation as well as autophosphorylation (Goren et.al., 1987; Ellis et.al., 1986; Wilden et.al., 1992). Two additional sites for autophosphorylation are present near the C-terminal of the β–subunit, Tyr 1328 and Tyr 1334, which may not be essential for kinase activation (Tavare and Siddle, 1993; Tornquist and Avruch, 1994).

The juxtamembrane domain of the IR-β–subunit also plays an important role in IR function. The presence of Tyr 972, which is the site for autophosphorylation, is not needed for kinase regulation and therefore not important for activation of kinase (Feener et.al., 1993). Analysis of point mutation of Tyr972 results in a type of receptor that is impaired in internalization in response to insulin (Backer et.al., 1990; Backer et.al., 1992a). In addition, after a point mutation of Tyr972 to Phe, IR was unable to phosphorylate IRS-1, a crucial substrate for IR, which resulted in a decrease in PI3-k activation as well as a block in glycogen and DNA synthesis (White et.al., 1988; Backer, 1992b). Thus, the juxtamembrane region is a critical domain required for both insulin stimulated signaling via phosphorylation of IRS-1 as well as receptor internalization.

The carboxy-terminal of IR-β–subunit has some Ser/Thr phosphorylation sites. It was observed that phosphorylation of Ser/Thr of receptor occurs under basal conditions. Upon stimulation with insulin there was an increase in both phosphotyrosine as well as phospho Ser/Thr content of the receptor. It has been reported that phosphorylation on Ser/Thr induced by cAMP and phorbol esters resulted in a decrease in IRPTK activity and loss of insulin sensitivity (Takayama, 1988; Stadmauer and Rosen, 1986; Lewis et.al., 1990a; Lewis et.al., 1990b; Chin et.al., 1993). A recent report indicates that the juxtamembrane domain has potential sites for Ser phosphorylation. It may be speculated that phosphorylation on Ser/Thr of IR acts as a switching mechanism to 'turn off' the signal stimulated by insulin, however studies with mutants have not provided adequate information regarding a specific role of these phosphorylation processes in regulating IR-PTK activity (Tavare and Siddle, 1993.). Although the physiological importance of Ser/Thr phosphorylation is not clarified, a report indicates that in the insulin resistant state PKC activity increased (Chin et.al., 1993). It has also been reported that glucose stimulates PKC activity, there is precedence for PKC regulation of insulin signaling in diabetic states (Lee T.S. et.al., 1989).

Thus, the accumulated evidence suggests that the activation of IR is a tightly regulated process. Insulin binding of α–subunit results in a conformational change in the β–subunit via the juxtamembrane domain which causes enhanced tyrosine phosphorylation and activation of β–subunit. This in turns relays the signal to physiological responses which are mediated by several intermediary enzymes/proteins. It appears that autophosphorylation on Tyr causes a positive regulation while Ser/Thr phosphorylation acts as a negative regulator. The hallmark of insulin-stimulated mitogenic and metabolic

responses is the Lysine residue at position 1030 (Lys1030), which is the ATP binding site, because mutation at this site resulted in cessation of biological responses due to failure of IR to undergo autophosphorylation and thus loss of tyrosine kinase activity.

6.1.2 Insulin Receptor Substrate (IRS) Signaling System:

Insulin stimulation results in the phosphorylation of the IR-β–subunit on a tyrosine residues that plays a key role in controling receptor activity directly or enzymes at early steps in the signaling cascade or coordinates the recruitment of multicomponent signaling proteins or docking proteins near the activated receptor. In this way insulin specific docking proteins named Insulin Receptor Substrate (IRS) are also tyrosine phosphorylated and transmit the signal downstream that result in physiological responses such as glycogen synthesis, glucose transport and protein synthesis as well as mitogenic responses.

IRS was originally identified in insulin-stimulated hepatoma cells (White et.al., 1985). Antibodies against phosphotyrosine provided the first evidence for an IR endogenous cellular substrate. Within seconds after stimulation with insulin, a high Mr. tyrosine-phosphorylated protein having a molecular size of about 185 kDa (pp185) was detected either in immunoprecipitates of extracts prepared from (^{32}-P) orthophosphate labelled cells or an immunoblot of whole cell extracts using α–Py antibody (White et.al., 1985). Subsequently this protein was also observed in a variety of different cells and tissues in response to insulin or IGF-1 (Myers et.al., 1994). Since then a few more related proteins were identified which are IRS-2, IRS-3, Growth factor adapter binder, (Gab-1) and p^{62dok} (Sun et.al., 1995; Holagado-Madura et.al., 1996; Yamanashi and Baltimore, 1997). These docking proteins are functionally similar to IRS, although they do not have amino acid sequence similarity to IRS. They share several structural features, for example

(a) they have pleckstrin, a protein substrate for PKC found in platelets, homology (PH) and /or a protein tyrosine binding (PTB) domain at the -NH_2-terminal side that mediates lipid-protein or protein-protein interaction (the PH domain is a conserved region of about 120 a.a. first identified as a major substrate of PKC in platelets which interacts with the lipid environment whereas the PTB domain was shown to interact directly with the autophosphorylation domain of IR using the yeast two hybrid system) (Craparo et.al., 1995), **(b)** multiple carboxy-terminal Tyr residues provide a site for binding of SH2 domain containing proteins, **(c)** the proline rich region engages SH3 and **(d)** the Ser/Thr region that may regulate overall function through other protein-protein interactions.

IRS-2 was identified in skeletal muscle and hepatocytes from IRS-1 knock out mice. IRS-2 migrates with an Mr of about 10 kDa higher than IRS-1 on SDS-PAGE. Both IRS-1 and IRS-2 have conserved PH domains at the NH_2-terminal and a PTB domain that binds to phosphorylated NPXY- motif. (Sun et.al., 1995). Recently it was demonstrated that disruption of IRS-2 in mice impairs insulin signaling and β-cell function that results in the development of type 2 diabetes (Withers et.al., 1998). However, IRS-1 disruption in mice retards growth but does not lead to diabetes because the presence of IRS-2 compensated for the possible impairment that may have arisen due to IRS-1 dysfunction (Araki et.al., 1994).

Among other IRS proteins, Gab-1 was cloned by expression screening with Grb-2. The structure of Gab-1 has similarity to IRS-1 and IRS-2 in that it has a conserved PH domain, multiple COOH-terminal tyrosine phosphorylated sites as well as a motif that can bind to PI3-k but lacks the PTB domain. However, the role of Gab-1 in insulin stimulated responses is not yet known. (Holgado-Madruga et.al., 1996).

The activated receptors for insulin, IGF-1, EGF and various other protein tyrosine kinases such as V-Abl and V-Src were able to associate with a 62-kDa phosphoprotein termed p^{62dok} (Yamanishi and Baltimore, 1997). This protein also contains a PH domain at its NH_2-terminus which was not quite similar to IRS-1, IRS-2 and Gab-1. It was noticed that the PTB domain with arginine residues may bind to a NPEY (Asn-Pro-Glu-Tyr)-motif in IR. There are multiple tyrosine phosphorylated sites at the COOH-terminal which have been observed to bind to several SH2-proteins but not to PI3-k and therefore, it appears that PI3-k is not regulated by p^{62dok} .

Similar to IRS-1 and IRS-2, a 60-kDa protein pp60, termed as IRS-3 ($pp60^{IRS3}$), was identified in rat adipocytes as another member of the IRS family (Lavan et.al., 1997). The presence of PH and PTB domains suggest that it also has an important role as a docking protein. The multiple tyrosine phosphorylated region may help engage with SHP-2, PI3-k and Grb-2 molecules (Lavan et.al., 1997). It was observed that $pp60^{IRS3}$ binds to p85 more rapidly as compared to IRS-1 and IRS-2, which may suggest that it is a better regulator of PI3-k than are IRS-1 or IRS-2. Furthermore, it is the predominant IRS in adipocytes lacking IRS-1 (Smith-Hall et.al., 1997).

6.1.3 Receptor-Substrate Coupling:

To transmit the signal from the receptor, IRS proteins should interact with the activated receptor and amplify the signal to further downstream molecules. The interaction should be transient because a prolonged association will diminish the signal transmission. Therefore, SH2 domain interaction, which is strong, appears to participate less in this process. The PH domain, on the other hand, located at the NH_2-terminus of IRS proteins seems to play an important role in this event. However, the exact mechanism of coupling

in this process is unknown. Present evidence demonstrates a chimeric IRS-1 protein containing a heterologous PH domain derived from different proteins did not engage in coupling whereas the PH domain from IRS-2 or Gab-1 are sensitive to insulin (Burks et.al., 1997).

The PTB domain, on the other hand, binds specifically to phosphorylated NPXY(Asn-Pro-any amino acid-Tyr)- motifs present in the receptors of insulin and IGF-I (Wang et.al., 1993). In some cases the kinase regulatory loop binding (KRLB) domain of IRS-2 or the c-Met binding domain in Gab-1 have been shown to couple receptor (Sawka-Verhelle et.al., 1996; He et.al., 1996). Thus, these interacting domains provide the mechanism by which receptor-substrate coupling takes place.

6.2 The Signaling Pathways Originating From IRS:

The activated IR-PTK phosphorylates IRS-1, which appears to be the major substrate mediating insulin action (Sun et.al., 1991). The tyrosine phosphorylated IRS-1 then binds with several SH2 domain containing proteins to generate downstream signals. These adapter proteins include p85 subunit of PI3-k, Grb-2, SHP2 (or Syp), Fyn, Nck and Crk (Myers et.al., 1994; Skolnik et.al., 1993a; Sun et.al., 1993; Kuhne et.al., 1993; Beitner-Johnson et.al., 1996; Lee C.H. et.al., 1993.). These adapter proteins associated with IRS-1 form a signaling complex which in turn initiates two main cascades: The first is the PI3-k-PKB-GSK-3 pathway and the second is the Ras-Raf-MEK-MAP Kinase-P^{90rsk} pathway as shown in Figure 3. The following section presents a brief description of these two most important signaling pathways stimulated by insulin.

6.2.1 The PI3-Kinase-PKB-GSK-3 pathway:

The PI3-k is a heterodimer consisting of an 85 kDa regulatory subunits ($p^{85\alpha}$ and $p^{85\beta}$) and catalytic subunits of 110 kDa ($p^{110\alpha}$ and $p^{110\beta}$). Interestingly, another isoform of p110 which is not activated by p85 but interacts with G-protein βγ subunit is, p110γ. The p110 catalytic subunit has lipid kinase activity which phosphorylates the inositol ring of phosphoinositides (PI) at the D-3 position of PI, PI4P monophosphate and $PI4,5P_2$ bisphosphate to produce PI3 monophosphate, PI3,4-bisphosphate ($PI-3,4P_2$) and PI-(3,4,$5P_3$) trisphosphate respectively (Carpenter and Cantley, 1996). The p^{85} regulatory subunit has two SH2 and one SH3 domain and through the former it associates with tyrosine phosphorylated IRS-1 (Backer et.al., 1992c; Yonezawa et.al., 1992). Binding of SH2 domain of p^{85} to phosphorylated IRS-1 in YMXM(Tyr-Met-any amino acid-Met)-motifs activates the catalytic domain (Backer et.al., 1992b). There are about 9 YMXM-motifs in IRS-1 and 2 which are key for binding to PI3-k whereas IRS-3 has 4 such motifs to associate with PI3-k.

The roles of PI3-k in insulin stimulated signaling cascade have been investigated by using two approaches: the first is the use of pharmacological agents which are specific inhibitors of PI3-k such as the fungal metabolite wortmannin (having an inhibitory effect in nM range) and Eli Lilly compound LY294002 (inhibits in μM range) and the second is a genetic approach which utilizes the overexpression of a dominant negative form of p^{85}regulatory subunit of PI3-k or overexpressing a constitutively active form of PI3-k.

The two inhibitors described above are structurally and mechanistically different cell permeable agents, inhibit the catalytic activity of p110 and also PI-(3-4,5)P_3 generation in response to insulin (Ui et.al., 1994). It was shown that these inhibitors prevented insulin-induced activation of glycogen synthase, glucose transport and GLUT 4 translocation to the plasma membrane as

well as inhibited lipolysis (Robinson et.al., 1993; Okada et.al., 1994; Clarke et.al., 1994). Several other biological effects of insulin are also inhibited by these agents. For example wortmannin or LY294002 dependent inhibition of insulin effects include glycogen synthase kinase (GSK)-3 β activity (Moule et.al., 1995; Welsh et.al., 1994), and glycogen synthase (Moule et.al., 1995; Welsh et.al., 1994), stimulation of fatty acid synthesis (Moule et.al., 1995), and protein synthesis (Mendez et.al., 1996), activation of the gene that encodes phosphoenol pyruvate carboxy kinase (PEPCK) (Sutherland et.al., 1995), activation of 70 kDa ribosomal protein kinase p^{70s6k} (Von Mateuffel et al., 1996; Chung et.al., 1994; Cheatham et.al., 1994; Hara et.al., 1995) and Ser/Thr kinase, PKB (Kohn et.al., 1995; Alessi et.al., 1996). Some other effects of insulin such as antilipolysis (Okada et.al., 1994), phosphorylation and activation of cAMP phosphosdiesterase (Rhan et.al., 1994), activation of acetyl Co-A carboxylase (Moule et.al., 1995) are also sensitive to these inhibitors. Thus, it appears that PI3-k is a major signaling molecule mediating insulin's metabolic responses.

These pharmacological approaches are excellent tools to investigate the role of PI3-k in insulin stimulated physiological effects, however specificity towards inhibiting PI3-k has always been questioned. Although wortmannin inhibits PI3-k, it can not discriminate between the p^{110} catalytic isoforms and other enzymes sensitive to it. For example both PI3-kinases, one specific to phosphatidyl inositol (Volinia et.al., 1995) and the other is G-protein-activated (also known as $p^{110\gamma}$) (Stoyanov et.al., 1995) , are targets of wortmannin. In addition, a putative target of rapamycin (rapamycin is a p^{70s6k} inhibitor) known as the mammalian target of rapamycin (mTOR), also possesses wortmannin-sensitive protein-kinase activity (Brunn et.al., 1996). In addition, wortmannin has also been shown to inhibit phospholipase A_2 (PLA_2) (Cross M.J. et.al., 1995). It is unknown at this time if these wortmannin sensitive targets are also

important in insulin-induced biological effects or if there exists alternative signaling cascades sensitive to wortmannin.

The second approach to investigate the role of PI3-k is by genetic means. It was observed that overexpression of mutant p^{85} , which is unable to bind to p^{110} owing to the lack of binding sites, inhibited insulin-induced PI3-k activity in IRS-1 immunoprecipitates as well as production of PI-(3,4,5) P_3 (Hara et.al., 1994). Translocation of GLUT4, a major glucose transporter in insulin target tissues/cells and glucose transport were inhibited by either overexpression or microinjection of this mutant p^{85} protein (Quon et.al., 1995; Kotani et.al., 1995). There was marked inhibition of glucose transport as well as GLUT1, a major glucose transporter in fibroblast, translocation (Hara et.al., 1994). In another genetic approach where inter SH2 regions of p85 and p110 of PI3-k are co-expressed which resulted in constitutive expression of PI3-k, It was found that basal MAP kinase or glycogen synthase activation was not attenuated but enhanced DNA synthesis and partially stimulated glucose transport was observed (Frevert and Kahn, 1997).

There are several PI3-k effectors that have been identified such as Ser/Thr kinase PKB/cAkt and some PKCs. The best characterized Ser/Thr protein kinase untill now are the novel PKC isoforms PKCε and PKCη (Toker et. al., 1994) and PKCζ (Toker et.al., 1994; and Nakanishi et.al., 1993) and proto-oncogene product PKB/cAkt (Franke et.al., 1995; Cross D.A.E et.al., 1995).

6.2.2 PKB/cAkt: Downstream Effector of PI3-Kinase:

In 1991 a cellular homolog of the viral Akt oncogene, cAkt encoding a serine/threonine kinase with an SH2-like region was reported by Bellacosa et.al. (1991). In the same year two other groups independently reported a similar protein and was named

PKB (Jones et.al., 1991) and RAC (Coffer and Woodgett, 1991). This enzyme has an Mr. of 55 kDa with a NH_2-terminal catalytic domain similar to the p^{90rsk} and p^{70s6k} kinase catalytic domain. The COOH-terminal domain had similarity to PKA as well as PKC and the amino terminus contains a PH domain possessing a Ser/Thr kinase domain (Kohn et.al., 1995). PKB has been shown to be regulated by PI3-k and the phosphorylated product of PI3-k, PI-3,4P_2 (Cross D.A.E. et.al., 1995; Franke et.al., 1995). It was observed that PKB activation could be blocked by either wortmannin or p^{85} mutant of PI3-k (Khon et.al., 1995; Franke et.al., 1995) suggesting that this Ser/Thr kinase is a downstream effector of PI3-k. The exact mechanism by which PKB is activated by PI3-k is not yet clear. Burgering and Coffer (1995) showed that a receptor mutant of PDGF lacking a tyrosine residue, which is essential for binding to p^{85} regulatory subunit of PI3-k, was unable to activate either PI3-k or PKB, however under these conditions MAP kinase activation occured. Some *in vitro* studies have shown the activation of PKB by D-3 phosphorylated PI (Franke et.al., 1997). By contrast James et.al. (1996) did not observe this effect. Interestingly, it was observed that the NH_2-terminal noncatalytic domain of PKB binds preferably PI3,4P_2 with preference over PI3,4,5 P_3 and mutations at critical amino acid residues within PH domain prevented the binding (Franke et.al., 1997; Klippel et.al., 1997).

A constitutively active PI3-k when overexpressed resulted in marked increase in PKB activation (Klippel et.al., 1996; Marte et.al., 1997; Franke et.al., 1997) There are two sites in PKB, one is serine 308 in the noncatalytic domain and another Thr 473 outside the catalytic domain, whose phosphorylation resulted in activation (Andjelkovic et.al., 1996; Kohn et.al., 1996a; Alessi et.al., 1996). In addition, a protein kinase which phosphorylates Thr 308 in PKB-α has recently been purified (Alessi et.al., 1997). This protein kinase is

activated by both PI-3,4P_2 and PI-(3,4,5) P_3 and has been named phosphatidylinositol (3,4)P_2/PI-(3,4,5)P_3 dependent kinase-1 (PDK-1) (Alessi et.al., 1997) however, the precise role of PDK-1 in insulin-induced activation of PKB and inactivation of GSK-3 needs to be clarified.

Thus, the metabolic effects of insulin mediated by PKB/cAkt is currently the most challenging task and some reports have demonstrated the activation of glycogen synthase, GLUT 4 translocation and glucose uptake by recombinant, constitutively active PKB (Kohn et.al., 1996b; Frevert and Kahn, 1997). Furthermore, the ability of PKB to directly phosphorylate and inactivate GSK-3 in vitro, which in turn inhibits glycogen synthase, represents an important observation (Cross D.A.E. et.al., 1995).

6.2.3 Glycogen Synthase Kinase-3 (GSK-3):

GSK-3 is a 55 kDa polypeptide is expressed as two isoforms α and β (Woodgett, 1990), was first identified as a protein kinase which had the ability to inhibit glycogen synthase by phosphorylation on a Ser residue (Embi et.al., 1980; Parker P.J., 1982). In basal state of the cells, GSK-3 is active, (Ramakrishana and Benjamin, 1988; Hughes et.al., 1992) and subsequently several studies demonstrated that insulin action can result in inhibition of GSK-3 activity (Welsh and Proud, 1993; Stambolic and Woodgett, 1994). This inhibition was attributed to the phosphorylation of a single Ser residue near the COOH-terminus. The phosphorylation of this site can be catalyzed by p^{90rsk}, p^{70s6k} (Sutherland et.al., 1993; Sutherland and Cohen, 1994) as well as by PKB under *in vitro* conditions (Cross D.A.E. et.al., 1995). The insulin stimulated GSK-3 β kinase activity was blocked by wortmannin (Moule et.al., 1995; Welsh et.al., 1994). By contrast, it was recently observed that a specific inhibitor of MEK, PD98059, which inhibits MAP kinase

and p^{90rsk}, as well as rapamycin, a selective inhibitor of p^{70s6k}, were not able to prevent the insulin induced inhibition of GSK-3 in L6 myoblasts (Cross D.A.E. et.al., 1995) and human myotubes (Cross D.A.E. et.al., 1997). Thus, an important action of insulin is inhibition of GSK-3 but the question remains as to how insulin regulates this kinase and what is the contribution of GSK-3 in the regulation of the other downstream effectors such as glycogen synthase or if there exists unidentified GSK-3 isoforms that participate in the regulation of insulin metabolic responses (Lawerence and Roach, 1997).

6.2.4 Ribosomal Kinase, p^{70s6k}:

In 1980, Smith et.al. reported a ribosomal protein S6 from insulin-treated 3T3-L1 adipocytes (Smith et.al., 1980). It was identified as the first insulin-stimulated Ser/Thr protein kinase and was subsequently purified and cloned (Price et.al., 1989; Kozma et.al., 1989; Kozma et.al., 1990; Banerjee et.al., 1990). In 1992, an immunosuppresant drug, rapamycin, was shown to inhibit p^{70s6} kinase activity and phosphorylation of 40 kDa S6 protein (Price et.al., 1992; Calvo et.al., 1992; Chung et.al., 1993). Subsequently it was reported that inhibition occured through functional inhibition of mTOR kinase (Brown et.al., 1995). Thus, the role of p^{70s6} kinase in insulin stimulated biological responses was facilitated by the use of rapamycin. It has been suggested that p^{70s6k} and mTOR mediate insulin-stimulated protein synthesis via an effect on peptide chain elongation factor. Rapamycin treatment inhibits PHAS-1 (phosphorylated heat and acid-stable protein), a protein translational repressor, in response to insulin (Lin and Lawrence, 1994). In the basal state PHAS-1 complexes with the eukaryotic initiation factor-4E (eIF-2) and acted as an inhibitor of translation however, upon insulin stimulation the complex dissociates and initiates translation. However, insulin-stimulated protein synthesis was partially

blocked in rat muscle (Dartvet et.al., 1996) as well as in myeloid progenitor cell line- 32D (Mendez et.al., 1996) by rapamycin suggesting that an alternate pathway may be operating to exert the effect.

The role of p^{70s6k} on insulin-stimulated glucose metabolism was further examined by utilizing rapamycin. Rapamycin had no effect on insulin-stimulated glucose transport, GSK-3 inhibition or antilipolysis (Avruch et.al., 1996). By contrast, a partial inhibition of insulin-induced GS in 3T3-L1 adipocytes (Shephard et.al., 1995), skeletal muscle (Azpiazu et.al., 1996) and human myoblast (Hurel et.al., 1996) was reported. In addition, it has been observed that rapamycin may exert its effect in a cell/tissue specific manner on insulin-stimulated GS/glycogen synthesis because there was no effect observed in rat adipocytes (Lin and Lawrence, 1994; Moule et.al., 1995), skeletal muscle (Moxham et.al., 1996), or CHO-HIR cells (Sakaue et.al., 1995).

In addition to rapamycin, the PI3-k inhibitor wortmannin inhibited p^{70s6} kinase thus placing it downstream to PI3-k (Weng et.al., 1995a; Chetham et.al., 1995). It was also reported that overexpression of PI3-k enhances the phosphorylation of p^{70} sites which could be blocked by wortmannin (Weng et.al.,1995b; Han et.al., 1995; Chung et.al., 1994) suggesting that S6 kinase is regulated by PI3-k. Furthermore activation of p^{70s6} kinase was observed in the cells expressing mutant v-Akt (Burgering and Coffer, 1995) however, it appears that p^{70s6} kinase is not directly phosphorylated by PKB, since a kinase deficient mutant of Akt did not modulate signals for p^{70s6} kinase (Burgering and Coffer, 1995) which suggests that either Akt did not function or is not directly activating p^{70s6} kinase. Taken together these observations suggests that p^{70s6} kinase is downstream to PI3-kinase but whether PKB lies between PI3-k or p^{70s6} kinase remains to be determined.

Although accumulated evidence has indicated a central role of PI3-kinase in insulin action, the precise role of PKB in this process remains to be clarified. Nevertheless, it is quite interesting that PI3-k may play a key role in mediating important metabolic effects of insulin such as glucose transport, glycogen synthesis and GLUT4 translocation.

6.3 The Ras-MAP Kinase Pathway:

The second pathway, which has been extensively studied, consists of Ras-Raf-MEK-MAP kinase-p^{90rsk} molecules. The following section briefly describes the individual components of this cascade as well as other adapter proteins associated with IRS-1 that help initiate this signaling pathway upon insulin stimulation.

6.3.1 Ras: A small GTP binding protein: Ras is a 21 kDa (p^{21ras}) membrane-associated GTP-binding protein that possess an intrinsic GTPase activity and has been shown to play an important role in cell growth, differentiation and tumor formation. It is one of the original members of the superfamily of small GTP-binding proteins which are involved in a variety of cellular processes including protein trafficking, cell growth, vesicular transport such as endocytosis and exocytosis as well as regulation of cytoskeletal structure (Chardin, 1991; Der, 1989; Macara, 1991) and p^{21ras} cycles between an active GTP-bound state and an inactive GDP-bound conformation. In quiescent unstimulated cells, most of the p^{21ras} is present in the inactive GDP-associated form. However, upon stimulation by insulin there is an increase of exchange of GTP for GDP that results in Ras activation (Skolinik et.al., 1993b; Schlessinger, 1993; Boguski, 1993). The regulation of

GTP-GDP exchange on Ras is governed by GTPase activating protein (GAP) and guanine nucleotide exchange factors such as mSOS (Medema et.al., 1993).

Several proteins such as adapter proteins Grb-2, Shc and Ras-specific nucleotide exchanger factor mSOS, have been demonstrated to participate in the regulation of p^{21ras} activation. Grb-2 is a small Mr. cytosolic protein which has one SH2 and two SH3 domains and is devoid of any enzymic activity (Lowenstein et.al., 1992). After insulin stimulation, Grb-2 with its SH3 domains forms a complex with mSOS. The Grb2-mSOS complex then binds with IRS-1 which subsequently links the IR and p^{21ras} signaling pathway resulting in further activation of downstream molecules as shown in figure-3.

Alternatively Shc, another SH2 domain containing protein, can also link IR to the Ras-MAP kinase pathway independent of IRS-1 complex (Pelicci et.al., 1992) (figure 3). In this scheme, insulin stimulation causes Shc phosphorylation on tyrosine residues which provides a binding site for Grb-2 leading to the formation of Shc-Grb-2-mSOS complex (Skolnik et.al., 1993a; Kovacina, 1993; Pronk, 1993). Recently Sasaoka et.al., (1996) have demonstrated Grb-2 binds to phosphorylated Tyr317 of Shc. It has been reported that the microinjection of antibodies against Shc inhibited insulin induced DNA synthesis (Sasaoka et.al., 1994; Sasaoka et.al., 1996). Thus these data suggest Ras-MAP kinase may mediate the mitogenic signal of insulin via Shc.

In the majority of cases p^{21ras} is activated via the Grb2-mSOS-IRS-1 complex which causes the conversion of the GDP-bound form of Ras to a GTP-bound active conformation. The active Ras recruits c-Raf-1, a Ser/Thr protein kinase, to the plasma membrane (Zhang et.al., 1993). This process may take place through the effector domain of Ras which has a high affinity binding site for c-Raf-1. Several studies have provided

evidence from biochemical and genetic approaches indicating cellular differentiation and mitogenesis occured when activated Ras recruited, phosphorylated and thus activated c-Raf-1 (Lowy, 1993; Satoh et.al., 1992.).

6.3.2 Raf: A Serine/Threonine Kinase and Effector of Ras:

Raf kinase was first discovered as a mutant retroviral transforming agent (Rapp et.al., 1988). The cellular homolog, c-Raf-1, is activated by insulin (Kovacina et.al., 1990). The NH_2-terminal of c-Raf-1 is noncatalytic and provides effector sites for incoming signals while the truncation of COOH-terminal kinase domain causes transformation of rodent fibroblasts (Avruch et.al., 1994b). The -NH_2 terminal of Raf-1 binds to active Ras-GTP configuration (Chuang et.al., 1994; Barnard et.al., 1995). This interaction is critical for Raf-1 to be recruited from its cytoplasmic location to the membrane by active Ras. An additional interaction involves c-Raf-1 binding to a different site on Ras through its cysteine rich, zinc finger motif independent of GTP-binding site. Therefore, this dual interaction of c-Raf with Ras results in almost full activation of the kinase. Raf-1 activation requires phosphorylation on Ser/Thr. The exact mechanism by which Raf is activated is still not completely clear. In some studies, a protein Ser/Thr kinase known as KSR (LysSerArg motif) have been identified as ceramide-activated kinase that has the ability to phosphorylate and activate c-Raf-1 (Zhang et.al., 1997) and thus it is suggested that KSR may be involved in the Raf-1 activation by TNF-α and other similar ligands that produce ceramide. Furthermore, PKCα (Kolch et.al., 1993) and non receptor tyrosine kinases of the Src family represents potential candidates for Raf-activation (Marais et.al., 1995).

6.3.3 MEK: A dual Specificity Kinase Downstream to Raf-1:

Mitogen extracellular signal regulated kinase kinase (MEK), also known as MAP kinase kinase or MAPKK, is phosphorylated by activated Raf-1 which leads to its subsequent activation (Kyriakis et.al., 1992; Roberts, 1992). The MEK enzyme has two isoforms MEK1 and MEK2 which are associated with insulin-stimulated MAP kinase signaling . A coherent body of evidence from genetic studies of caenorhabditis elegans and Drosophila indicate that Raf-1 is a potential activator of MEK located downstream of RTK and activated Ras (Avruch, 1994a). It was demonstrated that Ser residues 218 and 222 are phosphorylated in activated MEK (Ahn et.al., 1992). Besides Raf-1 other kinases that activate MEK are c-mos protein kinase and ste11, a mammmalian homolog of yeast, saccharomyces cerevisae (Avruch, 1994b). The activated MEK phosphorylates its further downstream effector, known as MAP kinase. There are two isoforms of MAP kinases p^{44} and p^{42} (ERK1 and ERK2 respectively). MEK phosphorylates these MAP kinases on Thr and Tyr residues with a consensus sequence TEY (ThrGluTyr motif) present in the catalytic domain (Cobb et.al.,1995; Roberts, 1992).

6.3.4 MAP Kinases: Ubiquitously Distributed Kinases:

The stimulation of mitogen activated protein (MAP) kinase by insulin was first identified by Ray and Sturgill in 1987. MAP kinases are Ser/Thr kinases which activate their target proteins by phosphorylating Ser/Thr residues whereas they are activated by Thr/Tyr phosphorylation. Similar to cAMP dependent kinases, they have broad substrate specificity for example transcription factors such as c-fos (Chen et.al., 1993), ribosomal kinase, p^{90rsk} (Sturgill et.al., 1988). Moreover, a binding site for MAP kinase has been identified on

p^{90rsk} protein kinase (Hsiao et.al., 1994). It has been observed that these kinases have an absolute requirement for proline residues adjacent to the Ser/Thr phosphorylation sites on its substrates and therefore sometimes they have been categorized as ''proline directed'' kinases (Mukhopadhyay et.al., 1992). It has also been shown that this sequence site represent a negative determinant for protein kinase A and protein kinase C (Mukhopadhyay et.al., 1992).

The activated MAP kinase and p^{90rsk} have been shown to translocate to the nucleus where they phosphorylate and activate transcription factors such as c-myc, $p62^{tcf}$, ATF-2 (Davis, 1993; Denton and Tavaré, 1995). A recent report shows that MAP kinase activates CREB (cAMP response element-binding protein) kinase which in turn phosphorylates and activates CREB (Xing et.al., 1998). Furthermore, this study revealed that CREB kinase has an amino acid similarity to RSK-2, a member of the p^{90rsk} family (Xing et.al., 1998).

6.3.5 P^{90rsk}: A Physiological Substrate of MAP Kinase:

The immediate downstream molecule to MAP kinase is the 90 kDa ribosomal protein kinase, p^{90rsk}. Despite the great deal of work which has been done on p^{90rsk}, the exact physiological role is still not clarified. Initial studies have demonstrated that insulin-induced p^{90rsk} activates glycogen bound protein phosphatase (PPG-1), which in turn dephosphorylates and activates GS. It was proposed that PPG-1 also dephosphorylates and inactivates both phosphorylase kinase and glycogen synthase (Dent et. al., 1990; Crews and Erikson, 1993). In addition, p^{90rsk} can also phosphorylates and inactivate GSK-3 in vitro (Sutherland et.al., 1993). These earlier observations need to be redefined because recent studies which utilized PD98059, a specific inhibitor of MEK, which blocks MAP kinase and p^{90rsk}, have demonstrated that insulin inhibition of GSK-3 is not reversed

in L6 myoblasts (Cross D.A.E. et.al; 1995). In this study it was suggested that inhibition of GSK-3 by insulin was mediated by the PI3-k-PKB pathway. Furthermore, based on studies, which utilized dominant negative mutants of Ras or SOS or PD98059- it was observed that insulin stimulated glucose uptake and glycogen synthase activation was not affected (Clarke, 1994; Sakaue et.al., 1995a; Sakaue et.al., 1995b; Lazar et.al., 1995) suggesting that this pathway does not participate in this process. However, these dominant negative Ras/SOS mutants have been shown to play a critical role in insulin stimulated DNA synthesis and cell growth (Moller, 1992; Sakaue et.al., 1995b). Thus, the availability of several specific inhibitors as well as recombinants/mutants have aided in dissecting the role of the Ras-MAP kinase pathway in various insulin's actions (Avruch, 1996; Denton, 1995). Moreover, many studies have come to the similar conclusion demonstrating that neither Ras nor linear effectors of Ras such as RAF-MEK-MAPK-RSK contribute significantly to insulin induced glycogen synthase activation and glucose transport. Therefore, with the available information, it may be suggested that the Ras-MAP kinase pathway may have a major role in cell growth and differentiation whereas the PI3-k-PKB cascade may exert metabolic effects in response to insulin.

6.4 Protein Tyrosine Phosphatases and Insulin Signaling:

PTPases catalyze the rapid dephosphorylation and inactivation of receptor kinases and other phosphorylated substrates and thus helps to maintain the steady-state balance between phosphorylated and unphosphorylated proteins. These PTPases are also expressed in insulin-sensitive tissues and have been implicated in the physiological regulation of insulin signaling pathways. They regulate reversible tyrosine phosphorylation of IR and post-receptor substrates in the insulin signaling cascade. This section briefly

describes the involvement of PTPases in the regulation of the insulin signaling cascade which include receptor type PTPases such as LAR (Leukocyte Antigen Related) as well as transmembrane, intracellular and non-receptor enzyme, PTP1B.

The family of PTPases exert positive and negative regulatory actions on various pathways of cellular signal transduction and metabolism (Walton et.al., 1993; Fischer et.al., 1991). It has been observed that the catalytic domain of these enzymes has approximately 230 conserved amino acids with the sequence motif - (I/V)HCXAGXGR(S/T)G, i.e. (Ile/Val) His-Gly-Arg-(Ser/Thr)Gly respectively. The cysteine residue is essential to catalyze the hydrolysis of protein phosphotyrosine residues by forming a cysteinyl-phosphate intermediates (Goldstein, 1995). Two main groups have been defined to include these PTPases. The first is the receptor type, which has an extracellular domain, a single transmembrane segment and one or two tandemly conserved PTPase catalytic domains and the second is a non-receptor type, which have a single PTPase domain and additional protein segments.

Potential PTPases implicated in insulin signaling cascade:

6.4.1 SHP2/SH-PTP2: This class of PTPase is widely expressed (Ahmad and Goldstein, 1995a) and complex with tyrosine phosphorylated IRS-1 as well as autophosphorylated EGF and PDGF receptor (Case et.al., 1994). It was demonstrated that SHP2 can dephosphorylate IR and IRS-1 in an *in vitro* system (Ugi et.al., 1994). However, studies in intact cells were not able to show direct interaction between IR and SHP2 (Yamauchi et.al., 1995). Insulin-stimulated mitogenesis has been shown to be positively regulated by SHP2 (Xiao et.al., 1994). Thus, this PTPase seems to play a

positive role in downstream post-receptor signaling in response to insulin however, IR does not participate in this event.

6.4.2 PTP1B: This enzyme is also widely expressed and was first identified in the cytosolic portion of the placenta (Charbonneau et.al., 1989). The full-length enzyme is 50 kDa with a cleavable C-terminal segment downstream from the PTPase domain which facilitates its attachment to the endoplasmic reticulum (Frangioni et.al., 1992). It was observed that if xenopus oocytes are microinjected with PTP1B, insulin-stimulated S6 kinase activation was blocked and oocyte maturation was retarded (Cicirelli et.al., 1990). In another study, overexpression of PTP1B almost completely dephosphorylated IR-β-subunit (Lammers et.al., 1993). Furthermore, PTP1B antibody loading, by an osmotic shock technique, increased insulin stimulated PI3-k activity and DNA synthesis compared to control cells loaded with pre-immune IgG (Ahmad et.al., 1995b). Under these conditions IR-kinase activity towards an exogenous peptide substrate as well as IRS-1 tyrosine phosphorylation was also increased. A role for PTP1B as a negative regulator of insulin action was also demonstrated by an enhancement of insulin signaling where a catalytically inactive mutant of PTP1B was transfected (Kenner et.al., 1996).

6.4.3 LAR: Insulin-stimulated physiological responses are regulated by the PTPases, LAR. LAR, with a molecular size of 150 kDa, belongs to receptor type PTPases and has extracellular, transmembrane and cytoplasmic domains. It was demonstrated by site directed mutagenesis that Cys-1539 is important for catalytic activity since changing it to serine residue ablates the enzyme activity and thus formation of phosphoenzyme intermediate (Pot et.al., 1991; Streuli et.al., 1990). LAR is widely expressed in insulin sensitive tissues (Goldstein et.al., 1991; Longo et.al., 1993) and it appears that

phosphotyrosine of IR kinase domain is the target of this PTPase (Hashimoto et.al., 1992a; Hashimoto et.al., 1992b). Transfection of hepatoma cells with antisense mRNA to LAR resulted in amplification of insulin-stimulated PI3-k activity. Moreover, decreased LAR expression resulted in augmentation in IRS-1 tyrosine phosphorylation and its complexing with p85 subunit of PI3-k, IRS-1 associated PI3-k activity and activation of both MEK and MAP kinase (Kulas et.al., 1995, 1996). Thus, LAR exerts a negative regulation of the insulin signaling pathway.

6.4.4 RPTPα (LRP) and RPTPε: This receptor-type LRP PTPase or RPTP is expressed in insulin sensitive tissues. It has *in vitro* catalytic activity towards the general dephosphorylation of IR (Hashimoto et.al., 1992a). By using a novel transfection assay for PTPase that negatively regulate insulin action, it was demonstrated that LRP and the closely related transmembrane enzyme RPTP-ε can act as a negative regulator of the IRPTK. The attenuation of GRB-2 mediated signaling has suggested a potential role for LRP in modulating other intermediate signaling cascades (DeHertog et.al., 1994)

7. CELLULAR MECHANISM OF VANADATE ACTION : A postulated mechanism of vanadium's effect is that vanadate behaves as a phosphate analog, stimulating protein-tyrosine phosphorylation by virtue of its inhibitory actions on PTPases (Swarup et. al., 1982; reviewed by Srivastava, 1995). Incubation of cells with vanadate preserves or enhances the phosphotyrosine content of cells (Tracey and Gresser, 1986).

In early studies vanadate was shown to activate tyrosine phosphorylation of solubilized IR but not Ser or Thr residues in a fashion analogous to insulin (Tamura et.al., 1983; Ueno et.al., 1987; Gherzi et.al., 1988). Vanadate also stimulated tyrosine kinase activity of the IR β-subunit (Ueno et.al., 1987; Smith et.al., 1988). Subsequently, it was

observed that vanadate when orally administered failed to change IR kinase activity while exerting an insulin-like glucose-lowering effect in STZ-diabetic rats (Mooney et.al., 1989). In rat adipocytes, vanadate appeared to bypass IR kinase activation, as shown by insulin-independent stimulation of glucose transport and metabolism (Shisheva and Shechter, 1992b). Some other studies have also shown that vanadate acts independent of IR (Strout et. al., 1989; Fantus et.al., 1989). Thus, current evidence suggests a post receptor mechanism of action of vanadium in stimulating glucose utilization, perhaps involving an alternative, cytosolic (i.e. nonreceptor) PTK which is stimulated preferentially by vanadium and is insulin-independent (Srivastava, 1990; Shisheva and Shechter, 1993b). In contrast to vanadium, pervanadate compounds (Posner et.al., 1994) or vanadate plus superoxide radicals (Trudel et.al., 1991) or vanadate with added H_2O_2 (Zick and Sagi-Eisenberg, 1990; Hecht and Zick, 1992) exert insulinomimetic effects by increased autophosphorylation and activation of the IR. Interestingly, it was recently reported that peroxovanadium compounds irreversibly oxidized the thiol (-SH) group(s) of a critical cysteine residues in the catalytic site of PTPase whereas vanadate caused a weaker and reversible inhibition (Huyer et.al., 1997). Thus, it may be suggested that insulin requires the activation of IR to exert its physiological responses, the vanadium salts on the other hand, appear to utilize some of the insulin signaling pathway independent of IR activation to exert their biological effects.

CHAPTER II

Article 1

Vanadium Salts stimulate mitogen-activated protein (MAP) kinase and ribosomal S6 kinases

Sanjay K. Pandey, Jean-Louis Chiasson and Ashok K. Srivastava

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VANADIUM SALTS STIMULATE MITOGEN-ACTIVATED PROTEIN (MAP) KINASES AND RIBOSOMAL S6 KINASES

Sanjay K. Pandey, Jean-Louis Chiasson and Ashok K. Srivastava

Research Group On Diabetes and Metabolic Regulation
Centre de Recherche/Hotel-Dieu de Montreal Hospital and
Department of Medicine, University of Montreal
Montreal (Quebec),H2W 1T8, CANADA

Correspondence should be addressed to:
Dr. Ashok K.Srivastava
Centre de Recherche/Hotel-Dieu de Montreal
3850 Saint-Urbain Street
Montreal (Quebec),
H2W 1T8 ,Canada
Tel: (514) 843-2917
Fax: (514) 843-2911

ABSTRACT

Effect of several vanadium salts, Sodium orthovanadate, vanadyl sulfate and sodium metavanadate on protein tyrosine phosphorylation and serine/threonine kinases in chinese hamster ovary (CHO) cells overexpressing a normal human insulin receptor was examined. All the compounds stimulated protein tyrosine phosphorylation of two major proteins with molecular masses of 42 kDa (p42) and 44 kDa (p44). The phosphorylation of p42 and p44 was associated with an activation of mitogen-activated protein (MAP) kinase as well as increased protein tyrosine phosphorylation of $p42^{mapk}$ and $p44^{mapk}$. Vanadium salts also activated the 90kDa ribosomal s6 kinase ($p90^{rsk}$) and 70 kDa ribosomal s6 kinase ($p70^{s6k}$). Among the three vanadium salts tested, vanadyl sulfate appeared to be slightly more potent than others in stimulating MAP kinases and $p70^{s6k}$ activity. It may be suggested that vanadium-induced activation of MAP kinases and ribosomal s6 kinases may be one of the mechanisms by which insulin like effects of this trace element are mediated.

KEY WORDS: Vanadium salts, MAP kinase, Ribosomal s6 kinases (p^{90rsk} and p^{70s6k}), insulinomimesis, protein tyrosine phosphatase.

INTRODUCTION

Vanadium is a group V transition element and is an endogenous constituent of all or most mammalian tissues (1-3). Vanadium salts such as sodium orthovanadate (Na_3VO_4), sodium metavanadate ($NaVO_3$) and vanadyl sulfate ($VOSO_4$) mimic several of the metabolic and growth promoting effects of insulin (4-24, for a review see reference 25). However, the most remarkable insulinomimetic effect of vanadium salts is their ability to normalise blood glucose in type I (insulin-dependent diabetes mellitus-IDDM) (26,27) and type II (non insulin-dependent diabetes mellitus-NIDDM) animal models of diabetes mellitus (28-30). Vanadium salts also improved the metabolic abnormalities associated with both type I as well as type II models of diabetes mellitus (27,31-43, for a review see reference 44).

It is well established that the biological actions of insulin are initiated by binding of insulin to a specific receptor located on the membrane of target cells (45-46). The insulin receptor is a heterotetrameric glycoprotein composed of two extracellular α-subunits and two transmembrane β-subunits. The α-subunit possesses the insulin binding activity and the β-subunit has an intrinsic protein tyrosine kinase (PTK) activity (44-48). The binding of insulin to the α–subunit of its receptor activates the PTK activity of the β-subunit and results in autophosphorylation of the β-subunit in tyrosine residues as well as the tyrosyl phosphorylation of endogenous substrate (45-48). Insulin receptor β-subunit autophosphorylation and activation of its PTK activity is believed to be a major pathway mediating the insulin action since cells with PTK deficient insulin receptors are unable to elicit several of the biological effects of insulin (49-50).

However, the mechanism by which vanadium salts exert their insulin like effects remain to be clarified.

Vanadium salts are potent inhibitors of protein tyrosine phosphatases (PTPases) (51) and thus were initially thought to activate the insulin receptor PTK activity by preventing the dephosphorylation of insulin receptor β-subunit (11,52-55). However, recent work has suggested that the site of action of vanadium salts might not involve insulin receptor PTK (54-61).

A critical step in insulin signaling cascade appears to be activation of a group of protein serine/threonine kinases called mitogen activated protein (MAP) kinases, 90 kDa ribosomal S6 kinase ($p90^{rsk}$) and 70 kDa ribosomal s6 kinase, $p70^{S6k}$ (48,62-66). We have shown earlier that sodium orthovanadate stimulates the tyrosyl phosphorylation and activation of MAP kinases in an insulin receptor protein tyrosine kinase independent manner (60,61). Therefore, we were interested to examine if $p90^{rsk}$ which is immediately downstream of MAPK in insulin signaling cascade (48) could also be activated under these conditions. In addition, we also questioned whether $p70^{s6k}$ which is also activated by insulin by as yet undefined pathway (48) will be similarly activated by vanadium salts. Furthermore, in the light of the suggestion that vanadyl sulfate may be more potent than sodium orthovanadate in eliciting hyperglycemic effects, (67) we have compared the effects of various salts of vanadium on MAPK, $p90^{rsk}$ and $p70^{s6k}$ activities in chinese hamster ovary cells overexpressing a normal human insulin receptor (CHO-HIRc).

MATERIALS AND METHODS

CHO cells overexpressing a normal human insulin receptor (CHO-HIRc) were a kind gift of Dr. Morris F. White (Joslin Diabetes Centre, Boston, MA,USA).. Insulin was from Eli Lilly Co. Indianapolis,IN,USA). Myelin basic protein (MBP), sodium orthovanadate and sodium metavanadate were purchased from Sigma Chemical Company (St.Louis,MO,USA). Vanadyl sulfate was from Aldrich Chemical Co. (Milwaukee,WI,USA).Antiphosphotyrosine antibody, anti-MAP kinase antibody (raised against a peptide based upon residue 333-367 of the C-terminus of the rat 43 kDa ERK1) that detects several isoforms of MAP kinases including $p44^{mapk}$ and $p42^{mapk}$, antiphosphotyrosine agarose beads and S6 peptide RRRLSSLRA were from Upstate Biotechnology (Lake Placid,NY,USA). Goat anti-mouse or anti-rabbit IgG conjugated to alkaline phosphatase was from Bio-Rad (Mississauga,Ontario,Canada). The $p70^{s6k}$ antiserum (raised against a peptide cerresponding to amino acids 2 to 30 of rat $p70^{s6k}$ (68) and $p90^{rsk}$ antiserum raised against bacterially produced chicken S6 kinase II (69) were generously provided by Drs. Frederic Hall of USC, School of Medicine, Los Angeles and John Blenis of Harvard Medical School,Boston respectively.

Cell Culture:

CHO cells were maintained on HAM's F-12 medium containing 10% fetal bovine serum. Cells were grown to confluence in 100 mm plates and incubated in serum-free F-12 medium for 20 hours prior to the experiment (61).

Detection of Phosphotyrosine-Containing Proteins

Tyrosine phosphorylation of cellular proteins stimulated in the absence or presence of insulin or vanadium compounds was assessed by immunoblotting using antiphosphotyrosine antibodies. Cells were stimulated with insulin or different vanadium compounds. The cells were lysed on ice in 400 µl of buffer A (25 mM Tris-HCl pH 7.5, 25 mM NaCl,1mM Na ortho vanadate,10 mM Na fluoride, 10 mM Na pyrophosphate, 20 nM Okadaic acid, 0.5 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 µg/ml aprotinin, 1% Triton X-100 and 0.1% SDS). The lysates were clarified by centrifugation for 12 minutes at 10, 000 x g. Equal amounts of protein samples were electrophoresed on 10% SDS-polyacrylamide gels and transferred to polyvinilidine difluoride (PVDF) membranes and blotted with antiphosphotyrosine antibody (1:1000) and detected using goat-anti-mouse IgG conjugated to alkaline phophatase (1:3000).

Immunoaffinity Purification of Phosphotyrosyl Proteins and MAP Kinase Immunoblotting

The cell lysates were affinity purified on antiphosphotyrosine-agarose column and immunoblotted using a MAP kinase antibody (61). For affinity purification of phosphotyrosyl proteins, briefly, the clarified lysates from control or stimulated cells were incubated for 1 hour at 4°C with antiphosphotyrosine agarose beads equilibrated in buffer B (20 mM Tris-HCl pH 7.4, 1% Nonidet p-40, 10 mM EDTA, 0.2 mM Na vanadate, 0.01% Na azide and 100 mM NaCl). The beads were washed 3 times with buffer B and phosphotyrosine containing proteins were eluted with 1 mM phosphotyrosine in buffer B. The eluates were boiled in 3x

Laemmli's sample buffer, electrophoresed on 10% SDS-polyacrylamide gels, transferred to PVDF membranes and blotted with anti-MAP kinase antibody (1:500) and detected using goat-anti-rabbit IgG conjugated to alkaline phophatase.

MAP Kinase Assay

For MAP kinase assay cell lysates were prepared in buffer A containing no Triton X-100 or SDS. To 5 µl of cell lysate (approximately 3-4 µg protein) , 40 µl of kinase buffer (25 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothreitol, 40 µM ATP, 1 µM staurosporine, 15 µg MBP, 0.5 mM EGTA and 0.5 µCi (γ^{32}P)ATP) was added. After 12 minutes at 30°C, the reaction was stopped by spotting 20 µl of the reaction mixture onto P-81 filter papers (2 cmx2 cm),washed 4 times in 0.5% phosphoric acid and counted for radioactivity (70).

Assay of $p70^{s6k}$ and $p90^{rsk}$

The clarified lysates prepared in buffer A were normalized to contain equal amounts of protein (100µg) and incubated for 4 hours at 4°C with 3 µl of either $p70^{s6k}$ or $p90^{rsk}$ antibody preadsorbed to protein A sepharose beads (Pharmacia Biotech Inc, Missisauga, Ontario,Canada). The immunecomplex was collected by centrifugation followed by washing 3 times with buffer A and once with buffer B containing 20 mM HEPES pH 7.4, 10 mM $MgCl_2$, 1mM DTT, 10 mM β-glycerophosphate. The beads were resuspended in 20 µl of buffer B containing S6 peptide RRRLSSLRA and phosphotransferase reaction was initiated by adding 5 µl of 100 µM ATP containing 2 µCi (γ^{32}P)ATP,after 15 minutes at 30°C, the reaction was stopped by spotting on P-81 filter paper, washing in 0.5% phosphoric acid and counted for radioactivity.

RESULTS

Effect of various vanadium salts on protein tyrosine phosphorylation

We have shown earlier that sodium orthovanadate (OV) causes the tyrosyl phosphorylation and activation of $p42^{mapk}$ and $p44^{mapk}$ in a concentration and time dependent manner (60,61). Therefore, to examine if a similar response is observed with other salts of vanadium such as sodium metavanadate (MV) and vanadyl sulfate (VS) (Fig. 1), the CHO-HIRc cells were treated with 100 μM of OV, MV or VS and lysates were immunoblotted with antiphosphotyrosine antibodies. As shown in Fig. 2, all the three vanadium compounds stimulated the tyrosyl phosphorylation of two major proteins having molecular masses of 44 kDa (p44) and 42 kDa (p42) respectively. However VS appeared to be about 40% more potent than either OV or MV. Furthermore none of the vanadium compounds stimulated the tyrosyl phosphorylation of either the insulin receptor or any other proteins (Fig. 2).

Effect of Vanadium salts on MAP kinase activity

Since the molecular masses of p44 and p42 corresponded to that of MAP kinases $p44^{mapk}$ and $p42^{mapk}$ which are activated in response to insulin (71) it was of interest to examine if increased tyrosyl phosphorylation of these proteins by MV and VS correlated with an increase in MAP kinase activity. As shown in Fig. 3, stimulation of CHO-HIR cells with all the 3 vanadium compounds resulted in the activation of MAP kinase activity as judged by increased phosphorylation of myelin basic protein (MBP) as an exogenous substrate. The stimulatory effect of VS on MAP kinase activity was slighty more than that of insulin (5.8 fold for VS

versus 4.5 fold for insulin). However, VS was about 2 times more potent than either OV or MV in stimulating the MAP kinase activity (Fig. 3). Vanadium salts-mediated activation of MAP kinase was accompanied by an increase in the phosphotyrosyl content of $p44^{mapk}$ (ERK 1) and $p42^{mapk}$ (ERK 2) as determined by immunoprecipitation using antiphosphotyrosine antibody followed by immunoblotting with a specific antibody to MAP kinase which recognises both ERK 1 and ERK 2 (data not shown, (61)).

Effect of vanadium salts on 90 kDa ribosomal S6 kinase ($p90^{rsk}$)

In insulin stimulated cells MAP kinase activation is associated with the activation of an immediate downstream serine/threonine kinase- a 90 kDa ribosomal S6 kinase ($p90^{rsk}$) (48,63,66). Therefore, to evaluate if vanadium compounds exerted a similar effect, the $p90^{rsk}$ activity was assessed in CHO-HIR cells stimulated with various vanadium compounds. The $p90^{rsk}$ activity was determined by using an immune complex kinase assay in which $p90^{rsk}$ protein from cell lysates was immunoprecipitated using a specific antiserum. This antiserum was prepared against a recombinant chicken rsk S6 protein kinase which specifically immunoprecipitated $p90^{rsk}$ in various cell types (69). The phosphotransferase reaction in the immune complex was performed by using a synthetic peptide substrate corresponding to amino acid 231-239 in human 40 S ribosomal protein S6. As shown in figure 4, incubation of CHO-HIR cells with all the vanadium salts (100 μM) activated the phosphotransferase activity upto 3-fold as compared to untreated control cells. All the salts tested were almost equipotent in activating the $p90^{rsk}$ activity and the response was similar to that observed with insulin.

Effect of vanadium compounds on 70 kDa ribosomal S6 kinase ($p70^{S6k}$)

In addition to $p90^{rsk}$, insulin also stimulates a 70 kDa ribosomal S6 kinase termed as $p70^{S6k}$ (48,64,65). However, in contrast to $p90^{rsk}$, MAPK is not the upstream kinase mediating $p70^{S6k}$ phosphorylation and activation, and the mechanism by which $p70^{S6k}$ is activated remains to be clarified (48). We therefore asked whether similar to insulin, vanadium salts could also activate $p70^{S6k}$ in CHO-HIR cells. For these experiments also an immune complex kinase assay using an antiserum raised against a synthetic peptide corresponding to amino acid 2-30 of rat $p70^{S6k}$ which specifically recognizes $p70^{S6k}$ was performed. The results shown in figure 5 indicate that OV, VS and MV all caused a potent stimulation in the phosphotransferase activity of $p70^{S6k}$. The fold stimulation ranged between 2.5 to 4 fold as compared to untreated control. Among the 3 salts tested, the stimulatory response of VS was higher than that of either OV or MV. VS caused about 4 fold stimulation whereas only 2.5 fold stimulation was observed with OV or MV in comparision to control cells (figure 5). Furthermore the stimulatory response of VS was almost equal to that of insulin (figure 5).

DISCUSSION

In this study we have shown that various vanadium salts activate serine/threonine kinases-MAP kinase, $p90^{rsk}$ and $p70^{S6k}$ in CHO cells overexpressing a normal human insulin receptor. The activation observed by vanadium salts was comparable to that observed with insulin and occured in the absence of detectable tyrosyl phosphorylation of any proteins other than $p44^{mapk}$ and $p42^{mapk}$. Most notably, the tyrosyl phosphorylation of neither insulin receptor β–subunit nor the insulin receptor substrate 1 (IRS-1) could be detected even after 30 minute incubation of cells with any of the vanadium salts. Vanadate-mediated activation of MAPK and $p90^{rsk}$ has previously been shown in other cell types but in these studies no attempts were made to correlate it with tyrosyl phosphorylation of insulin receptor β-subunit (72,73). Our earlier suggestion that tyrosyl phosphorylation and activation of MAP kinases can occur in the absence of IRS-1 phosphorylation (60,61) has recently been confirmed by Tamemoto et. al. who demonstrated that in the livers of mice deficient in IRS-1, MAP kinase activation was not significantly altered as compared to normal mice (74).

Recent studies have implicated MAP kinase signalling pathways in the regulation of glycogen metabolism by insulin (75-78). Dent et. al. demonstrated that an insulin-stimulated protein kinase (ISPK) is able to phosphorylate and thereby activate the regulatory subunit of the glycogen bound form of protein phosphatases-1 (PP1-G) (75). Activated PP1-G dephosphorylates glycogen synthase and phosphorylase kinase and thus stimulates glycogen synthesis (75). ISPK has subsequently been identified as an isoform of $p90^{rsk}$ or rsk II (79,80).

Moreover, both $p90^{rsk}$ and $p70^{s6k}$ catalyze in vitro phosphorylation of glycogen synthase kinase-3 (GSK-3) (81-83). GSK-3, which is able to phosphorylate and inhibit the activity of glycogen synthase is phosphorylated and inactivated in response to insulin, is believed to play an important role in the glycogen metabolism (83). Recent studies with rapamycin , an immunosuppressant and specific inhibitor of $p70^{s6k}$ revealed that $p70^{s6k}$ may not be involved in the phosphorylation and inactivation of GSK-3 (76,77). However, based on experiments using wortmannin, an inhibitor of phosphatidyl -inositol kinase (PI3K), a possible role of MAP kinase/$p90^{rsk}$ signalling pathway in insulin-mediated inactivation of GSK-3 has been suggested (76,77). Furthermore, insulin-stimulated glycogen synthesis and glycogen synthase activation was also blocked by wortmannin and rapamycin in 3T3-L1 adipocytre (78) indicating the involvement of PI3K and $p70^{s6k}$ signalling pathway in this process (78). These results are however in contrast to the studies of Lin and Lawrence who demonstrated that rapamycin did not attenuate the stimulatory effect of insulin on glycogen synthase (84) and suggested that activation of MAP kinase and ribosomal s6 kinases may not be sufficient for the activation of glycogen synthesis in adipocytes (84).

An involvement of PI3K in insulin-mediated glucose transport and antilipolytic effects has also been suggested (85,86). Inhibition of PI3K activity by wortmannin and LY294002,another specific inhibitor of PI3K, resulted in complete inhibition of insulin-stimulated $p70^{s6k}$ as well as glucose uptake in 3T3-L1 adipocytes (85). LY294002 treatment inhibited the translocation of GLUT 4 glucose transporters to the plasma membrane (85). Rapamycin however failed to

exert any effect on insulin-stimulated glucose transport in 3T3-L1 adipocytes (87) suggesting the existence of alternate mechanisms.

Thus, the ability of vanadium salts to stimulate MAP kinase, $p90^{rsk}$ and $p70^{s6k}$ might be one of the mechanisms by which these compounds exert insulinomimetic effects in various systems. This notion is further supported by a recent study in which OV-stimulated glucose transport was partially inhibited by wortmannin treatment (86). In this regard, it is noteworthy that, in circulating mononuclear leucocytes from diabetic subjects, the insulin-mediated activation of ribosomal S-6-kinases (s6k) and MAP kinase was completely diminished, as compared to non-diabetics. $NaVO_3$ therapy of diabetic subjects not only improved the glucose homeostasis but also stimulated the basal s6k and MAPK activity between 1.7 and 3.9-fold (88) further suggesting a role of these serine/threonine kinases in the insulinomimetic effects of vanadium. Involvement of additional mechanisms in insulinomimesis by vanadium salts is also possible. For example, studies of Fantus et.al. have demonstrated a possible role of vanadium (OV) in enhancing insulin sensitivity and prolonging insulin action at the level of insulin receptor PTK via PTPases (89, 90). An involvement of OV-stimulated, staurosporine inhibited cytosolic PTK in vanadium action has been proposed in rat adipocytes (91). It has been suggested that cytosolic PTK activation is secondary to the inhibition of a PTPase (91).

The mechanism by which vanadium salts activate MAP kinase and $p90^{rsk}$ and $p70^{s6k}$ is not clearly understood . A tentative scheme with potential sites where vanadium salts might act on insulin signaling cascade is shown in figure 6.

Vanadium salts are inhibitors of PTPases (51) and by preventing the dephosphorylation are able to increase the phosphotyrosyl content of key protein molecules in insulin signalling cascade (48) (Figure6) and thus result in the activation of the MAP kinases as well as $p70^{S6k}$ activating pathway. The potential target could be insulin receptor β-subunit and/or IRS-1 (Figure 6) however results presented here and elsewhere (58-61) do not support this notion. Shc protein which is tyrosyl phosphorylated and activated in response to insulin (89) may serve as another target. Shc is able to interact with GRB2 in an IRS-1 dependent and independent manner and may activate the $p21^{ras}$ signalling pathway leading to the activation of MAPK and $p90^{rsk}$ (48) (Figure 6). Alternatively vanadium compounds might activate MAP kinase pathway by inhibiting a constitutively active MAP kinase specific PTPase. A vanadium-inhibited MAPK-specific protein tyrosine phosphatase (PTPase) has recently been described in Xenopus-oocytes (9 3) and a similar PTPase might also be expressed in CHO cells. Since several lines of evidence link PI3K with the activation of $p70^{s6k}$ (85,91) and recent studies have shown a modulation of PI3K activity by vanadate (86), it may be suggested that vanadium compounds utilize a similar pathway in activating $p70^{s6k}$ protein.

In conclusion, these results demonstrate that similar to insulin, vanadium salts activate serine/threonine kinases, MAP kinases, $p90^{rsk}$ and $p70^{s6k}$. However, in contrast to insulin vanadium mediated activation is independent of the tyrosine phosphorylation of either the insulin receptor or IRS-1. It may be suggested that

vanadium-mediated activation of MAP kinases, $p90^{rsk}$ and $p70^{s6k}$ plays an important role in mimicking insulin-like effects.

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LEGENDS

FIGURE 1 Structural formula of vanadium salts used.

FIGURE 2 Effect of various salts or insulin on protein tyrosine phosphorylation. CHO-HIR cells were treated in the absence (C) or presence of insulin (INS,100 nM) or 100 μM each of sodium orthovanadate (OV) vanadyl sulfate (VS) or sodium metavanadate for 5 min. Cell lysates were prepared and immunoblotted with anti-phosphotyrosine antibodies as described in materials and methods. The number in the left indicate the position of molecular weight standards. IR denotes the position of the insulin receptor β-subunit. This is a representative result from one of the three experiments.

FIGURE 3 Effect of insulin or vanadium salts on MAP kinase activity from CHO-HIR cells. CHO-HIR cells were treated in absence (CON) or presence of insulin or with different vanadium salts as described in figure 2 and MAP kinase activity was assayed in cell lysates using Myelin Basic Protein (MBP) as exogenous substrate as described in materials and methods. The results are one of three independent experiments performed in triplicate.

FIGURE 4 Effect of insulin or vanadium salts on 90 kDa ribosomal protein s6 kinase activity ($p90^{rsk}$) in CHO-HIR cells. CHO-HIR cells were treated with vanadium salts as described in figure 2 and $p90^{rsk}$ activity was determined in immune complex using S6 peptide, RRRLSSLRA as substrate as described in materials and methods. Results are from one of three independent experiments performed in triplicate.

FIGURE 5 Effect of insulin or vanadium salts on 70 kDa ribosomal protein s6 kinase activity ($p70^{s6k}$) CHO-HIR cells were treated with vanadium salts as described in figure 2

and $p70^{S6K}$ activity was determined in immune complex using S6 peptide, RRRLSSLRA as substrate as described in materials and methods. The results are one of three independent experiments performed in triplicate.

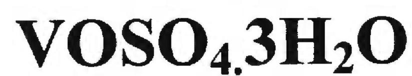
Figure 6 SCHEMATIC MODEL SHOWING POSSIBLE TARGET SITES OF VANADIUM COMPOUNDS IN INSULIN SIGNALLING CASCADE: Insulin initiates its action by binding to α- subunit of its receptor, which is a heterotetrameric protein, and activates the intrinsic protein tyrosine kinase activity of β-subunit of the receptor by autophosphorylation . The activated β-subunit receptor in turn phosphorylates and thereby activates several proteins among which insulin receptor substrate-1 (IRS-1) is most widely characterized. The activated IRS-1 serves as docking protein and forms a complex with several SH2 domains containing proteins e.g. sos (son of sevenless), GRB2, Shc, Nck, Syp (PTPase) and phosphatidyl inositol 3-kinase (PI3K). This complex, in turn ,stimulates Ras, MAPKK , MAPK and $p90^{rsk}$ cascade. Activated MAPK phosphorylates PHAS-1 (a heat stable protein) which is involved in protein synthesis via activation of eIF-4 (elongation initiation factor-4). The stimulated ribosomal protein $p90^{rsk}$ phosphorylates and activates glycogen bound protein phosphatase-1 (PP1-G). The activated PP-1G dephosphorylates glycogen synthase (GS) and phosphorylase kinase (Phk) and stimulates glycogen synthesis. $P90^{rsk}$ mediates phosphorylation of glycogen synthase kinase-3 (Gsk-3). MAPK and $p90^{rsk}$ may regulate nuclear activity by phosphorylating transcription factors. The other form of ribosomal protein s6 kinase , the 70 kDa protein, $p70^{s6k}$ lies on the other side of insulin signalling cascade and among other events is also implicated in modulating glucose transport and protein synthesis. Vanadium compounds are inhibitors of PTPases and by preventing dephosphorylation are able to

increase tyrosine phosphorylation of key proteins involved in insulin signalling cascade. The potential target of this PTPase(s) could be IR-subunit, IRS-1, Shc, MAPK or a cytosolic PTK. Based on the results presented here and elsewhere the IR-β subunit/IRS-1 may not be the sites of vanadium action however, MAPK-specific PTPase and/or Shc could be possible targets of vanadium compounds. With regard to p^{70s6k} activation by vanadium compounds a role of PI3-K may be suggested. The possibility that vanadium may act at other sites can not be excluded.

ABBREVIATIONS

eIF-4: eukaryotic protein synthesis initiation factor-4
GRB-2: growth factor receptor bound protein-2
GSK-3: Glycogen Synthase Kinase-3
IRS-1: insulin receptor substrate-1
ISPK: insulin stimulated protein kinase
MAPK: mitogen activated protein kinase; also known as
ERK: extracellular signal regulated kinase
MAPKK: mitogen activated protein kinase kinase; also known as MEK, MAPK or ERK kinase
PHAS-I: phosphorylated heat and acid stable protein regulated by insulin
PI3K: phosphatidylinositol 3-kinase
PTK: protein tyrosine kinase
PTPase: protein tyrosine phosphatase
rsk: ribosomal s6 kinase
shc: src homology domain containing protein
SOS: son of sevenless

(Sodium Orthovanadate)



(Vanadyl Sulfate)

(Sodium Metavanadate)

Figure 1

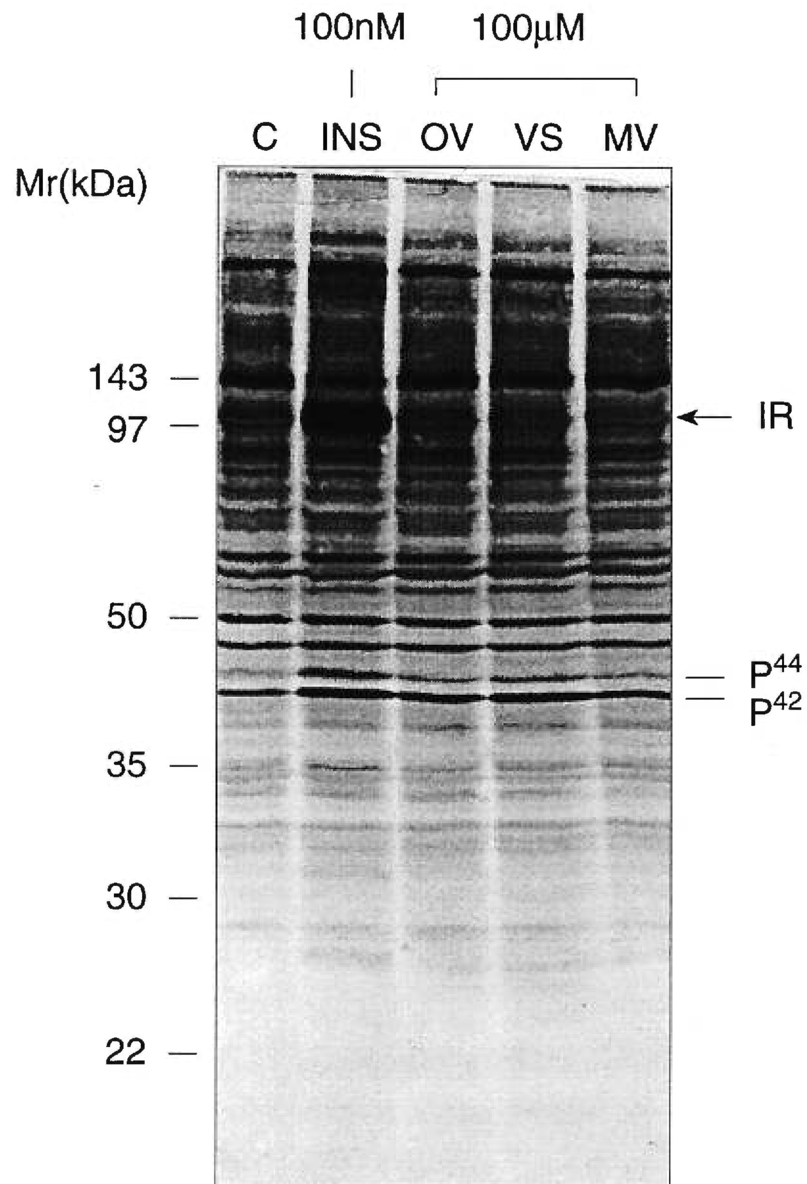


Figure 2

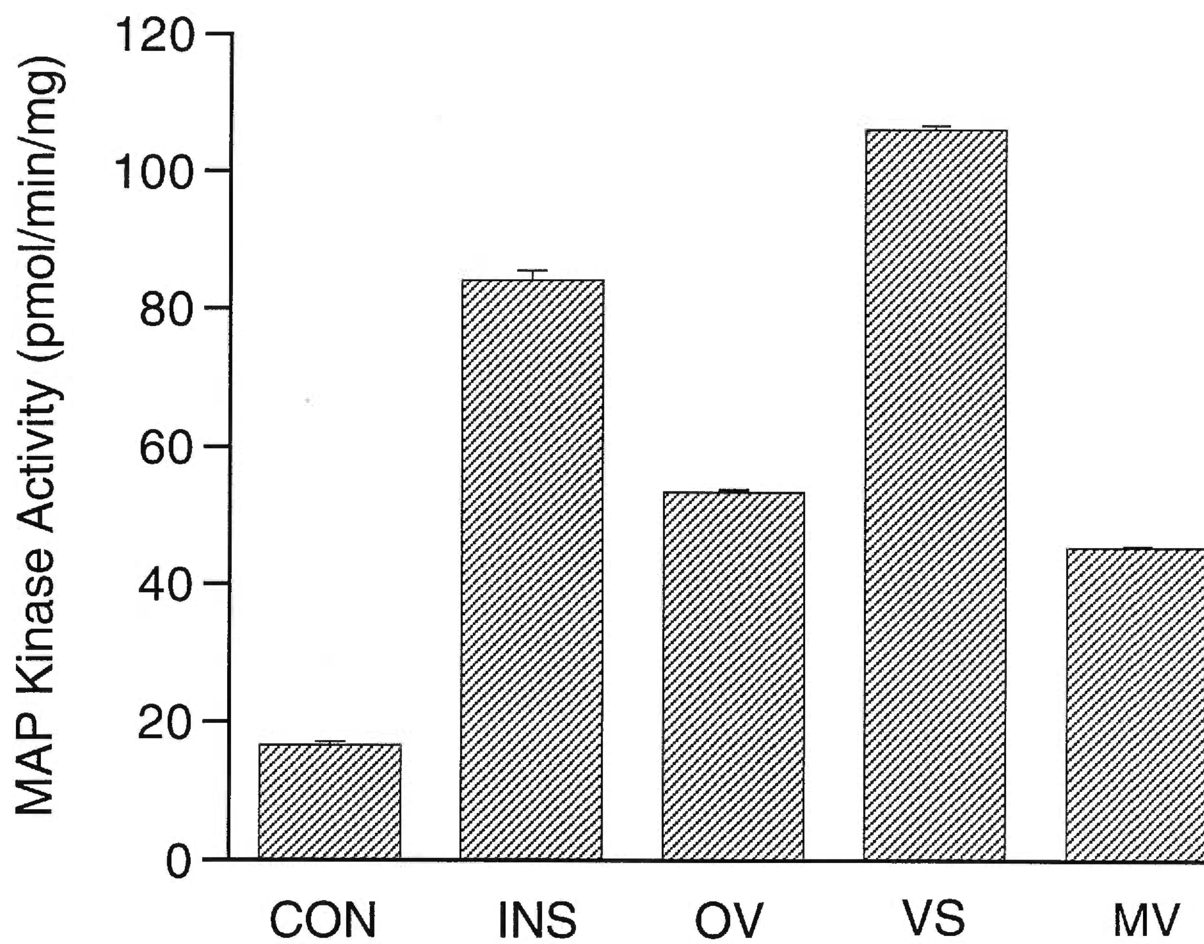


Figure 3

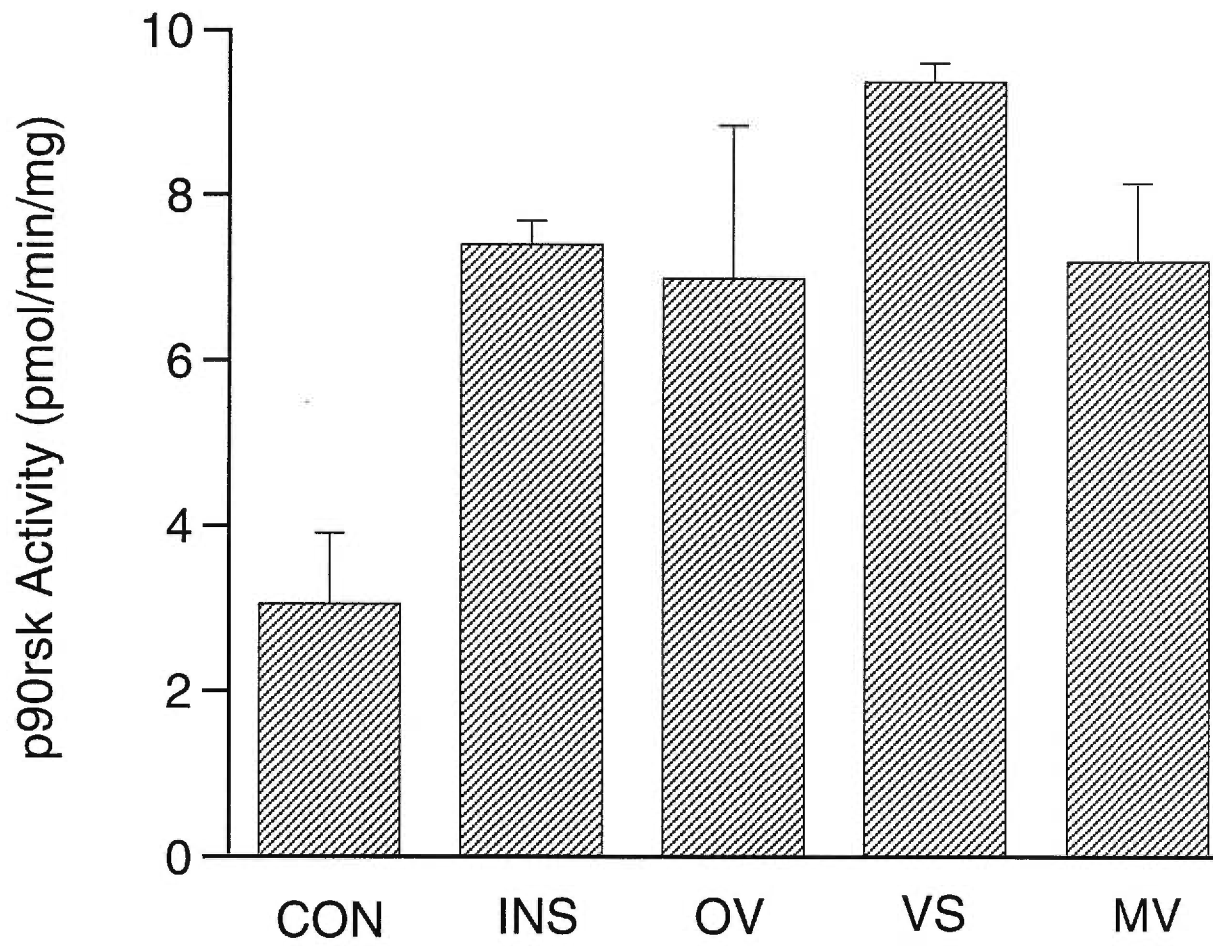


Figure 4

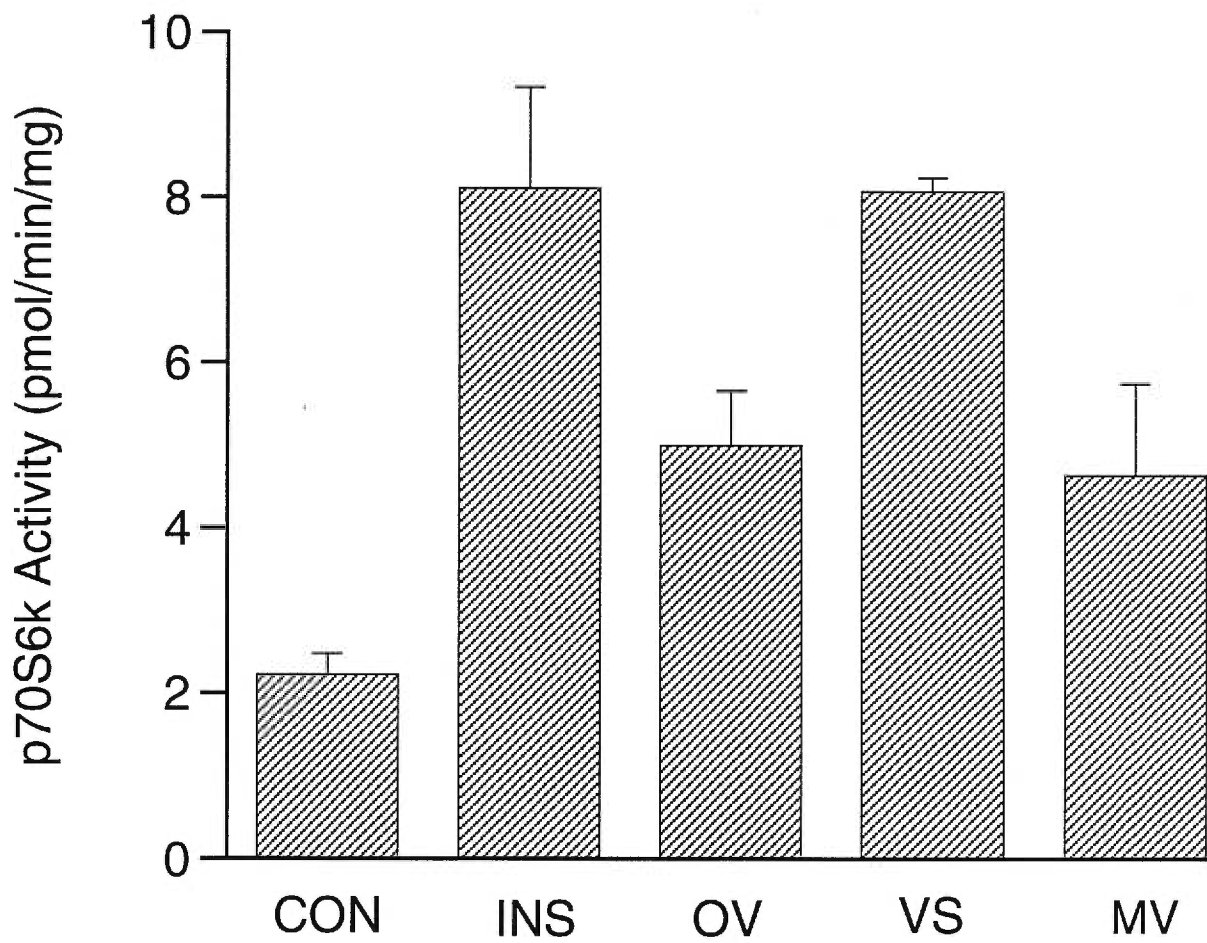


Figure 5

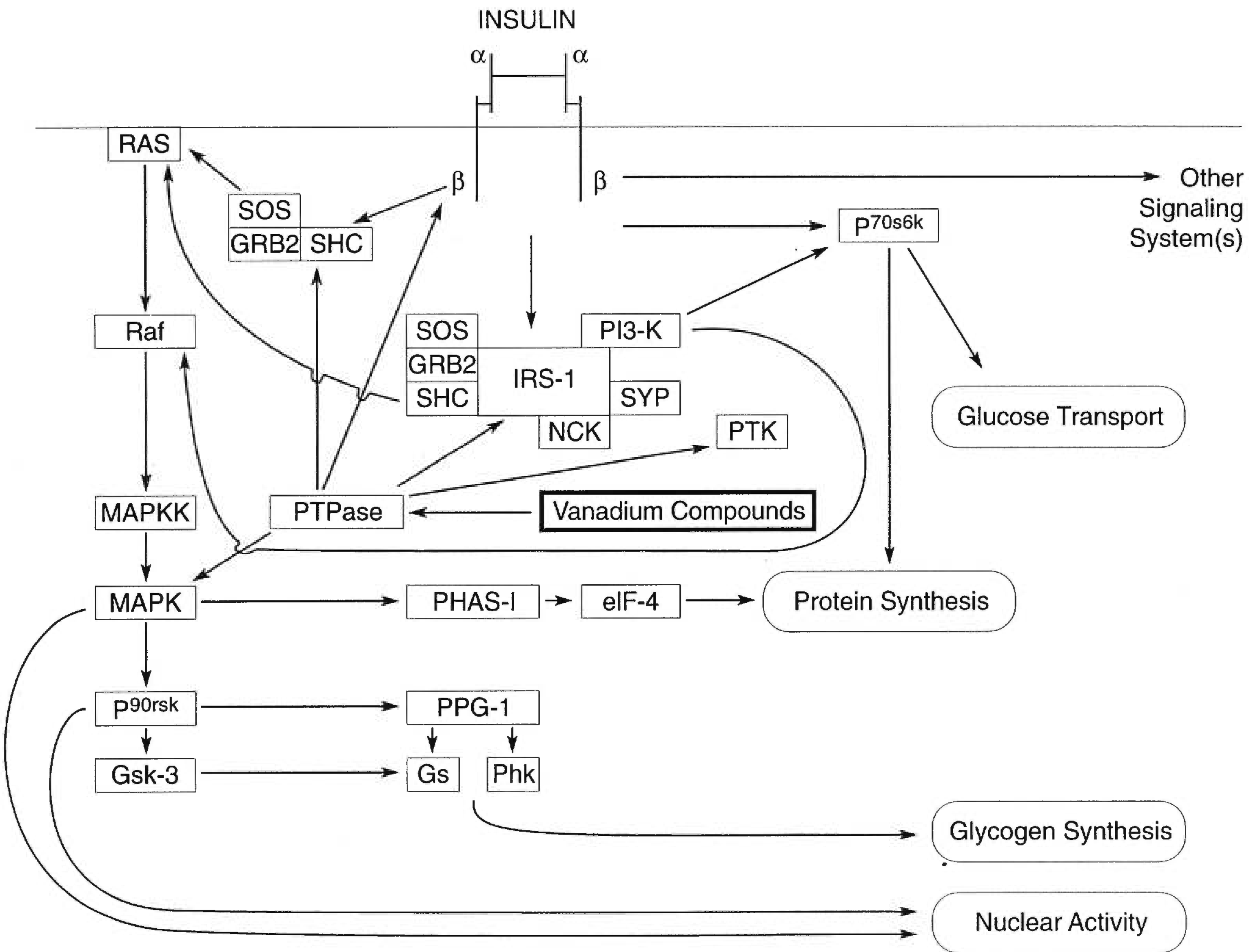


Figure 6

CHAPTER III

Article 2

Vanadyl Sulfate-Stimulated Glycogen Synthesis is Associated with Activation of Phosphatidyl Inositol 3-kinase (PI3-k) and is independent of Insulin receptor tyrosine phosphorylation

Sanjay K. Pandey, Madhu B. Anand-Srivastava and Ashok K. Srivastava

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Vanadyl Sulfate-stimulated Glycogen Synthesis is Associated with Activation of Phosphatidylinositol 3-kinase (PI3-k) and is Independent of Insulin Receptor Tyrosine Phosphorylation †

Sanjay K. Pandey[‡§], Madhu B. Anand-Srivastava[§] and Ashok K. Srivastava[‡*]

Centre de Recherche, Centre Hospitalier Université de Montréal,

Campus Hotel-Dieu[‡] and Departments of Physiology[§] and Medicine[‡]

University of Montréal, Montréal, Québec, H2W 1T8, Canada

Running Title: Role of PI3-kinase in insulinomimesis by vanadyl sulfate

***ADDRESS FOR CORRESPONDENCE**

Ashok K. Srivastava, Ph.D.

Centre Hospitalier Université de Montréal

Centre de Recherche, Campus Hôtel-Dieu

3850, St. Urbain Street , Montréal,

(Québec), H2W 1T8, Canada

Tel: (514) 843-2917

Fax: (514) 843-2911

e-mail: srivasta@ere.umontreal.ca

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Abbreviations: ATP, adenosine 5′-triphosphate; CHO-HIR cells, chinese hamster ovary cells overexpressing human insulin receptor; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; ERK, extracellular signal regulated kinases; Grb, growth factor receptor binder; GSK-3, glycogen synthase kinase-3; Hepes, N-(2-hydroxyethyl) piperazine-N′-2-ethanesulfonic acid; IDDM, insulin-dependent diabetes mellitus; IR, insulin receptor; IRS-1, insulin receptor substrate-1; JNK; c-jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAP kinase kinase; mSOS, mammalian son of sevenless; NIDDM, non insulin-dependent diabetes mellitus; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PEPCK, phosphoenol pyruvate carboxykinase; PI3-k, phosphatidylinositol 3-kinase; PKB, protein kinase B; PMSF, phenylmethylsulphonyl fluoride; PTK, protein tyrosine kinase; PTPases, protein tyrosine phosphatases; rsk, ribosomal S-6 kinases; SDS, sodium dodecyl sulfate; SH2, src homology 2; VS, vanadyl sulfate.

ABSTRACT

Salts of the trace element vanadium, such as sodium orthovanadate and vanadyl sulfate (VS), exhibit a myriad of insulin-like effects, including stimulation of glycogen synthesis and improvement of glucose homeostasis in type I and type II animal models of diabetes mellitus. However, the cellular mechanism by which these effects are mediated remains poorly characterized. We have shown earlier that different vanadium salts stimulate the MAP kinase pathway and ribosomal-S-6-kinase (p^{70s6k}) in chinese hamster ovary cells overexpressing human insulin receptor (CHO-HIR cells) (Pandey, S.K., Chiasson, J.-L. and Srivastava, A.K., Mol. Cell. Biochem. 1995, 153: 69-78). In the present studies, we have investigated if similar to insulin, VS also activates phosphatidylinositol 3-kinase (PI3-k) activity, and whether VS-induced activation of the PI3-k, MAP kinase and p^{70s6k} pathways contributes to glycogen synthesis. Treatment of CHO-HIR cells with VS resulted in increased glycogen synthesis and PI3-k activity which were blocked by pretreatment of the cells with wortmannin and LY294002, two specific inhibitors of PI3-k. On the other hand, PD98059 and rapamycin, specific inhibitors of the MAP kinase pathway and p^{70s6k} respectively, were unable to inhibit VS-stimulated glycogen synthesis. Moreover, VS-stimulated glycogen synthesis and PI3-k were observed without any change in the tyrosine phosphorylation of insulin receptor (IR) β–subunit but were associated with increased tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1). In addition, PI3-k activation was detected in IRS-1 immunoprecipitates from VS-stimulated cells, indicating that tyrosine phosphorylated IRS-1 was able to interact and thereby

activate PI3-k in response to VS. Taken together, these results provide evidence that tyrosine phosphorylation of IRS-1 and activation of PI3-k play a key role in mediating the insulinomimetic effect of VS on glycogen synthesis independent of IR-tyrosine phosphorylation.

INTRODUCTION

Inorganic salts of the trace element vanadium have been demonstrated to mimic most of the biological effects of insulin in several cell types, including stimulation of glucose transport (1-3), glycogen synthase (4), lipogenesis (5) and inhibition of lipolysis (6). They have also been shown to stimulate glycogen synthesis in the liver and diaphragm and inhibit gluconeogenesis in hepatocytes (7). In addition, oral administration of vanadium salts has been reported to improve glucose homeostasis in type I (insulin-dependent diabetes mellitus, IDDM) (8,9) and type II (non-insulin-dependent diabetes mellitus, NIDDM) (10-12) animal models of diabetes mellitus as well as in limited human clinical trials (13,14, reviewed in 15 and 16).

Vanadium salts, potent inhibitors of protein tyrosine phosphatases (PTPases) (17), were initially thought to mimic insulin action by activating insulin receptor (IR) protein tyrosine kinase (PTK) activity by preventing dephosphorylation of the IR β-subunit (18). However, recent studies have suggested that the site of vanadium action may not involve IR-PTK (19-23). Thus, the molecular mechanism(s) of the insulinomimetic effect of vanadium salts remains poorly characterized and requires further elaboration.

Insulin elicits its biological effects by binding to the extracellular IR α-subunit which then stimulates the intrinsic PTK activity of the IR β-subunit (24-27). Activated IR-PTK phosphorylates insulin receptor substrate-1 (IRS-1), which appears to be the major substrate mediating insulin action (28). Tyrosine

phosphorylated IRS-1 interacts with several src homology 2 (SH2) domains containing signaling proteins, such as adapter proteins growth factor receptor binder-2 (Grb-2) (29), Syp/SHP2 (30, 31), Nck (32) as well as the 85-kDa subunit of phosphatidylinositol (PI) 3-kinase (PI3-k) (33, 34) and initiates two main signaling pathways. In one pathway, Grb-2 binding to IRS-1 recruits a nucleotide exchange factor, mammalian son of sevenless (mSOS) which eventually activates p^{21ras}, a low molecular weight GTP-binding protein. p^{21ras} cycles between active GTP-bound conformation and inactive GDP-bound states. p^{21ras} activation leads to the sequential activation of several protein kinases such as Raf, a serine/threonine kinase, which phosphorylates and activates mitogen-activated protein (MAP) kinase kinase or MEK, which in turn phosphorylates and activates two isozymic forms of MAP kinases, p^{44mapk} (extracellular signal regulated kinase 1 or ERK 1) and p^{42mapk} (ERK 2) (35, 36). Activated MAP kinase phosphorylates and activates a downstream ribosomal protein kinase, $p90^{rsk}$ (37). Recently, using a selective inhibitor of MEK, PD98059, it was suggested that MAP kinase activation plays a role in DNA synthesis, cell growth and transcriptional activation of c-fos (38, 39). On the other hand, evidence has been presented to indicate that many other biological effects of insulin, such as glucose transport, glycogen synthesis and lipid synthesis, as well as transcriptional regulation of several genes, such as phosphoenol pyruvate carboxykinase (PEPCK) and hexokinase II, may not require the insulin-induced ras/MAP kinase pathway (39, 41). A 70 kDa ribosomal s6

kinase, p^{70s6k} , is also stimulated in response to insulin by a mechanism as yet uncharacterized (42).

The second pathway stimulated by insulin involves the activation of PI3-k, which is a heterodimer consisting of an 85 kDa (p85) regulatory subunit with two SH2 domains and a 110-kDa (p110) catalytic subunit (27). The p110 catalytic subunit has lipid kinase activity which phosphorylates PI on the D-3 position of the inositol ring (43,44,). Recently, a serine/threonine kinase named protein kinase B (PKB) has been shown to be the target of PI3-k (45). By using two specific inhibitors of PI3-k , wortmannin and LY294002, it has been demonstrated that insulin-induced activation of PI3-k plays a key role in mediating several biological effects of insulin, such as glucose transport (46), glycogen synthesis (47) and transcriptional regulation of several genes (40,41).

Vanadium salts have been reported to mimic the cardinal response of insulin on glycogen synthesis in various in vitro systems (7,18) and to improve the depressed levels of glycogen in animal models of diabetes mellitus (48). However, the possible contribution of insulin-induced signaling pathways in vanadium salt-stimulated glycogen synthesis has not been investigated. We have shown earlier that various vanadium salts activate MAP kinase and p^{70s6k} signaling pathways in an IR-PTK-independent manner (22,49). In the present studies, we have investigated the contribution of the MAP kinase, p^{70s6k} and PI3-k pathway in vanadyl sulfate (VS)-stimulated glycogen synthesis in chinese hamster ovary cells overexpressing human insulin receptor (CHO-HIR cells). The data presented here demonstrate, for the first time, that VS stimulated glycogen synthesis and PI3-k

activity in CHO-HIR cells, and the inhibitors of PI3-k, wortmannin and LY294002, blocked VS-stimulated glycogen synthesis. On the other hand, inhibition of either the MAP kinase or p^{70s6k} pathway failed to inhibit the glycogen synthesis induced by VS. We demonstrate further that VS-induced PI3-k activation occurred in the absence of any detectable tyrosine phosphorylation of IR β-subunit, but was accompanied by enhanced tyrosine phosphorylation of IRS-1.

MATERIALS AND METHODS

Materials

CHO-HIR cells and IRS-1 antibody were kind gifts from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA). Insulin was obtained from Eli Lilly Co. (Indianapolis, IN). Antibody against p85 regulatory subunit of PI3-k was a generous gift from Dr. Louise Larose (McGill University, Montreal) and was also purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Radiolabelled D-(U-^{14}C)-glucose was purchased from Dupont, NEN (Boston, MA). Insulin receptor antibody was a kind gift from Dr. Barry I. Posner (McGill University, Montreal) or purchased from Upstate Biotechnology (Lake Placid, NY). Wortmannin and staurosporine were obtained from Sigma Chemical Co. (St. Louis, MO). MEK inhibitor PD98059 was kindly provided by Dr. Alan Saltiel of Parke-Davis Pharmaceutical Research, (Ann Arbor, MI). Rapamycin was from Calbiochem (La Jolla, CA), LY294002 was from Biomol (Philadelphia, PA) and VS was from Aldrich Chemical Co. (Milwaukee, WI). Anti-phosphotyrosine and anti-MAP kinase antibodies as well as S6 peptide RRRLSSLRA were sourced from Upstate Biotechnology. Goat anti-mouse or anti-rabbit IgG conjugated to alkaline phosphatase was from Bio-Rad (Mississauga, Ontario). Silica gel-60 plates were purchased from Merck & Co., Inc. (Rahway, NJ). Protein A sepharose beads were bought from Pharmacia Biotech Inc. (Mississauga, Ontario). p^{70s6k} antiserum (raised against a peptide corresponding to amino acids 2 to 30 of rat p^{70s6k}) was generously donated by Dr. Frederic Hall of the USC School of Medicine at Los Angeles.

Methods

Cell Culture. CHO-HIR 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 20 hours prior to the experiment, as described earlier (49).

Glycogen Synthesis. Glycogen synthesis was measured by following the incorporation of D-(U-^{14}C)-glucose into glycogen, essentially according to the method described by Lazar et. al. (39). Briefly, near-confluent, serum-deprived CHO-HIR cells in 100- mm dishes were stimulated with VS or insulin with or without pretreatment with inhibitors as described in figure legends. The assay was initiated by adding D-(U-^{14}C)-glucose (1 µCi/dish) and 5 mM glucose (final concentration). It was terminated after 2.5 hour by washing three times with ice-cold phosphate-buffered saline (PBS) and then solubilized in 30% KOH. The solubilized cells were transferred to glass tubes and heated at 100^{0}C for 20 min in a dry bath, then 2.0 mg carrier glycogen was added, and the mixture was boiled for another 30 min. Glycogen was precipitated by the addition of 2 volumes of 95% ethanol, and the precipitate was dissolved in water. The radioactivity incorporated into glycogen was determined by counting the precipitate in a scintillation counter.

PI3-k Assay. The assay was performed essentially as described by Fukui and Hanafusa (50) with minor modifications. Serum-deprived, confluent CHO-HIR cells were stimulated with VS or insulin with or without pretreatment with inhibitors, as described in the figure legends. The cells were washed twice with cold PBS and lysed in buffer A (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM Na

ortho vanadate, 10 mM Na fluoride, 10 mM Na pyrophosphate, 20 nM okadaic acid, 0.5 mM ethylenebis(oxyethylenenitrolo)-tetraacetic acid (EGTA), 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 1% Triton X-100 and 0.1% sodium dodecyl sulfate (SDS)). The cells were scraped and centrifuged at 12,000xg for 12 min. Equal amounts of protein lysates were incubated with 5 μl of p85-antibody for 2 hours at 4°C. Protein A sepharose beads were then added, and the resulting mixture was incubated for an additional 2 hours at 4°C. The beads were centrifuged in a microtube for 15 sec, and the pellet was washed 3 times with HNTG (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% Glycerol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF), once each with PBS, 0.5 M LiCl and 0.1 M Tris, pH 7.5, H_2O and, finally, with 0.1 M NaCl/1 mM EDTA/20 mM Tris-HCl, pH 7.5. The beads were then resuspended in 50 μl of kinase buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.5 mM EGTA), followed by the addition of 1.0 μl of 20 mg/ml PI dissolved in dimethyl sulfoxide (DMSO). The mixture was vortexed, preincubated at room temperature for 10 min, and the kinase reaction initiated by adding (γ-^{32}P)-adenosine 5′-triphosphate (ATP), (10 μCi per assay) and $MgCl_2$ (10 mM, final concentration) at room temperature (30°C). The reaction was stopped after 3 min by the addition of 150 μl of $CHCl_3$:CH_3OH:HCl (100:200:2) and 100 μl of $CHCl_3$ was added to separate the phases. The organic (inferior) phase was taken and washed with 1 volume of CH_3OH:HCl (1:1). Lipid samples were concentrated in vacuo and spotted onto silica gel-60 plates which were later developed in $CHCl_3$:CH_3OH:NH_4OH:H_2O

(43:38:5:7). The phosphorylated lipids were then visualized by autoradiography, and areas corresponding to each spot were quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Insulin Receptor Tyrosine Phosphorylation. Serum-deprived, confluent CHO-HIR cells were stimulated with insulin (100 nM) or VS (100 μM) for 5 min, washed with ice-cold PBS and lysed on ice in 400 μl of buffer A. The lysates were clarified by centrifugation at 10,000 x g for 12 min, pre-cleared with protein A sepharose for 30 min, and incubated with insulin receptor antibody for 4 hours at 4°C. Immunoprecipitates were collected with Protein A sepharose, washed 3 times with 50 mM N-(2-hydroxyethyl) piperazine-N-2-ethanesulfonic acid (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 immunoprecipitates were solubilized by boiling in 2 x sample buffer for 10 min, electrophoresed on 10% SDS-polyacrylamide gels (SDS-PAGE) and immunoblotted with anti-phosphotyrosine antibody (1:1000). The blots were developed by using goat anti-mouse IgG conjugated to alkaline phosphatase (1:3000).

Autophosphorylation of IR β–subunit. CHO-HIR cells were stimulated as described above either with insulin or VS for 5 min and lysates were prepared. Antibodies against phosphotyrosine or IR β–subunit were incubated with protein A sepharose for 2 hours at 4°C. After this, equal amounts of protein from cell lysates were added to the complexes of protein A sepharose and antibody, with incubation continuing for another 4 hours at 4°C. The beads were centrifuged in a microtube for 15 sec, and the pellet was washed 3 times with HNTG and once

with PBS. They were then resuspended in 50 μl of kinase buffer. The mixture was vortexed and preincubated at room temperature with $MgCl_2$ (10 mM) and $MnCl_2$ (5 mM) for 10 min. The reaction was started by adding (γ-^{32}P)-ATP (5 μCi per assay), and incubation was continued for another 10 min at 30°C. It was stopped by the addition of 50 μl of 2 x sample buffer. The contents were boiled, centrifuged, and the solubilized fractions electrophoresed on 10% SDS-PAGE, followed by autoradiography.

IRS-1 Tyrosine Phosphorylation. CHO-HIR cells were stimulated with different concentrations of VS for 5 min. Equal amounts of proteins from cell lysates were added to the anti-IRS-1 antibody-protein A sepharose complex, and incubation was continued overnight at 4°C. The immunoprecipitates were collected by centrifugation in a microtube for 15 sec, and the pellet was washed 3 times with ice-cold PBS. The beads were boiled in 2 x Laemmli's sample buffer and processed further as described above for IR tyrosine phosphorylation.

Assay of MAP Kinase and p^{70s6k} Activity. Carified cell lysates prepared in buffer A were normalized to contain equal amounts of protein (100 μg) and incubated for 4 hours at 4°C either with 3 μl of MAP kinase antibody or p^{70s6k} antibody preadsorbed to protein A sepharose beads. The immunecomplexes were collected by centrifugation, followed by washing 3 times with buffer A and once with buffer B (20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 mM β-glycerophosphate). For MAP kinase assay, the immunecomplexes were suspended in 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 15 μg myelin basic protein (MBP)) and the reaction was initiated by adding 5 μl of 40 μM ATP containing 0.5 μCi (γ-^{32}P)-ATP. For p^{70s6k} assay, the immunecomplexes were suspended in 20 μl of buffer B containing the S6 peptide RRRLSSLRA (3 μg), and the reaction was initiated by adding 5 μl of 100 μM ATP containing 2 μCi (γ–^{32}P)-ATP. After 15 min at 30°C, the reaction was stopped by spotting the aliquots on P-81 filter paper, followed by washing in 0.5% phosphoric acid and counting for radioactivity (49).

Statistical Analysis. For some experiments comparision of the data was performed by Student's paired t-test using SigmaStat 2.0 programme (Jandel Scientific, San Rafael, CA). The P-values <0.05 were considered as significantly different.

RESULTS

Effect of Wortmannin on VS- stimulated Glycogen Synthesis Incubation of CHO-HIR cells with increasing concentrations of the insulinomimetic agent VS stimulated the rate of glycogen synthesis in a concentration-dependent manner, as determined by (^{14}C)-glucose incorporation into glycogen (Figure 1A). Stimulation of glycogen synthesis could be detected with VS doses as low as 1 μM (1.7-fold stimulation) and was maximal at 100 μM where about 3-fold stimulation was observed (Figure 1A).

Since wortmannin, a highly selective inhibitor of PI3-k, has been shown to block insulin-stimulated glycogen synthesis (47,51), we were interested to determine if PI3-k was also implicated in VS-stimulated glycogen synthesis. Figure 1A, illustrates that treatment of CHO-HIR cells with 100 nM wortmannin for 10 min prior to stimulation with different concentrations of VS markedly blocked the stimulatory effect of VS on glycogen synthesis. Under similar conditions, insulin treatment (100 nM for 5 min), as a positive control, also stimulated the glycogen synthesis by about 3-fold over control values which was almost completely blocked by wortmannin pretreatment (Figure 1C).

Effect of LY294002 on VS-stimulated Glycogen Synthesis Since wortmannin has also been shown to inhibit other enzymes, such as phospholipase A_2 (52), we utilized another inhibitor of PI3-k, LY294002, to further confirm a role of PI3-k in VS-induced glycogen synthesis. This inhibitor is structurally different from wortmannin and acts on the ATP-binding site of the enzyme (53). As shown in Figure 1B and 1C, pretreatment of CHO-HIR cells with LY294002

(10 μM) for 30 min markedly inhibited VS- and insulin-induced activation of glycogen synthesis, further supporting a role of PI3-k in VS- as well as insulin-stimulated glycogen synthesis.

Effect of VS on PI3-k Activity To investigate if VS-induced activation of glycogen synthesis was correlated with enhancement of PI3-k activity in CHO-HIR cells, PI3-k activity in lysates of cells treated with different concentrations of VS was assayed in immunecomplexes obtained by using an antibody to the p85 subunit of PI3-k. As shown in Figure 2A and 2B, treatment of CHO-HIR cells with VS for 5 min stimulated PI3-k activity in a concentration-dependent manner. At 100 μM, VS stimulated PI3-k activity by about 3- fold as compared to the controls, and this was markedly inhibited by treatment of cells with wortmannin (100 nM for 10 min) prior to stimulation with VS (Figure 2C and 2D). Similar results were obtained while using insulin as a positive control (Figure 2 C,D).

Effect of PD98059 on VS-stimulated MAP Kinase Activity and Glycogen Synthesis Recently, PD98059, which blocks insulin-induced activation of the MAP kinases ERK 1 and ERK 2, has been used to study the role of the MAP kinase pathway in mediating various biological effects of insulin (39). We, therefore, investigated whether PD98059 modulates VS-induced MAP kinase activation and glycogen synthesis in CHO-HIR cells. As shown in Table 1, both VS and insulin stimulated MAP kinase activity by 4-5-fold, which was markedly inhibited by pretreatment of cells with PD98059 (10 μM for 30 min) . However, PD98059 treatment failed to exert an inhibitory effect on insulin- or VS-stimulated glycogen synthesis (Table 1). These data demonstrate that similarly to insulin, VS-

induced stimulation of glycogen synthesis is independent of MAP kinase activation.

Effect of Rapamycin on VS-induced Activation of P^{70s6k} and Glycogen Synthesis Rapamycin, an immunosuppressant drug which potently inhibits p^{70s6k} activation induced by insulin and other growth factors has been used as a tool to investigate the role of p^{70s6k} in mediating various physiological effects of insulin (54-56). Studies in which rapamycin was employed to examine its effect on insulin-stimulated glycogen synthesis or glycogen synthase activation have produced conflicting results. It has been shown that rapamycin, despite blocking insulin-induced activation of p^{70s6k}, fails to block glycogen synthesis in rat adipocytes (57,58), mouse skeletal muscle (59), CHO (60) and PC12 cells (51). On the other hand, rapamycin treatment has been reported to inhibit insulin-stimulated activation of glycogen synthase and glycogen synthesis in 3T3-L1 adipocytes (47) and rat diaphgram muscle (61). Therefore, it was of interest to investigate if rapamycin could also block VS-stimulated glycogen synthesis in CHO-HIR cells. The results in Table 1 indicate that pretreatment of cells with 50 ng/ml rapamycin for 30 min blocked VS- and insulin-stimulated p^{70s6k} activity without affecting glycogen synthesis. These data suggest that like insulin (60), p^{70s6k} activation is not required for VS-induced glycogen synthesis in CHO-HIR cells.

Effect of VS on IR Tyrosine Phosphorylation and Autophosphorylation. Insulin-induced activation of the PTK activity of IR β-subunit is essential for mediating several biological responses of insulin including glycogen synthesis (62).

We therefore, investigated if conditions which stimulate glycogen synthesis in response to VS also stimulate tyrosine phosphorylation of the IR β-subunit. CHO-HIR cells were incubated with insulin (100 nM) or VS (100 μM) for 5 min. Cell lysates were prepared and subjected to immunoprecipitation by an antibody directed against IR β-subunit followed by immunoblotting with anti-phosphotyrosine antibody. As shown in Figure 3A, VS treatment did not cause any detectable increase in tyrosine phosphorylation of the IR β-subunit, whereas, as expected, insulin markedly stimulated tyrosine phosphorylation of the IR β-subunit as compared to unstimulated cells. Since IR β-subunit tyrosine phosphorylation is associated with stimulation of its autophosphorylation, this observation was further confirmed by performing invitro autophosphorylation reaction in anti-phosphotyrosine and anti-IR immunoprecipitates from control, VS- and insulin-stimulated cells. As shown in Figure 3B and 3C, VS did not cause any increase in autophosphorylation whereas, as expected, heightened autophosphorylation of the 95 kDa IR β-subunit was observed in insulin-treated cells. This suggests that, in contrast to insulin, VS-stimulated glycogen synthesis is not associated with activation of IR-PTK.

Effect of VS on Tyrosine Phosphorylation of IRS-1 and PI3-k Activity Associated with IRS-1. PI3-k activation by insulin requires increased tyrosine phosphorylation of IRS-1. We, therefore, examined whether PI3-k activation by VS was correlated with enhanced tyrosine phosphorylation of IRS-1. CHO-HIR cells were incubated with various concentrations of VS, and cell lysates were subjected to immunoprecipitation with an antibody against IRS-1.

Immunoprecipitates were then analyzed by immunoblotting with anti-phosphotyrosine antibody. As shown in Figure 4A, VS treatment of cells stimulated the tyrosine phosphorylation of IRS-1 in a concentration-dependent manner.

To determine if VS-stimulated tyrosine phosphorylation of IRS-1 results in its association with and activation of PI3-k, we studied the PI3-k activity in IRS-1 immunoprecipitates prepared from CHO-HIR cells stimulated with VS. As shown in Figure 4B and 4C, about a 6 fold rise in PI3-k activity was detected in IRS-1 immunoprecipitates after VS stimulation (100 μM, 5 min) of cells compared to unstimulated control cells. This increase correlated well with an enhanced tyrosine phosphorylation of IRS-1 in response to VS (Figure 4A).

DISCUSSION

In the present study, we have demonstrated that VS, which exerts a variety of insulin-like effects, including the ability to serve as an orally-active antidiabetic agent, stimulates PI3-k activity as well as glycogen synthesis in CHO-HIR cells. Our results also show that two structurally and mechanistically distinct inhibitors of PI3-k, wortmannin and LY294002, attenuate VS-induced PI3-k activation as well as glycogen synthesis. Similar observations have been made in relation to insulin-induced activation of glycogen synthesis or glycogen synthase in several systems, such as 3T3-L1 adipocytes (47), PC-12 cells (51) and rat adipocytes (63). However, the data presented here are the first to show that VS-stimulated activation of glycogen synthesis as well as PI3-k activation are blocked in concert by wortmannin and LY294002 (Figures 1 and 2) and suggest that VS and insulin employ wortmannin/LY294002-inhibitable signaling intermediates to stimulate glycogen synthesis.

A serine/threonine protein kinase, cAKt/PKB, is activated in response to several growth factors including insulin (45,64-65) and vanadate (66). The enzyme is stimulated in vitro by PI3-k-generated phospholipid products (64), and has been implicated in the regulation of glycogen synthesis (65). This contention is based on the fact that glycogen synthase kinase-3 (GSK-3), a potent protein kinase capable of phosphorylating and decreasing the activity of glycogen synthase, is a substrate of cAKt/PKB (65). Interestingly, cAKt/PKB-catalyzed phosphorylation and inactivation of GSK-3 was also inhibited by wortmannin and LY294002 (65).

Recent studies have shown that overexpression of constitutively-active cAKt/PKB did not reproduce the ability of insulin to stimulate glycogen synthesis in 3T3-L1 adipocytes (67). It is not known, however, if cAKt/PKB overexpression was associated with decreased GSK-3 activity in the above studies. Moreover, insulin-induced glycogen synthesis was significantly attenuated in the cells overexpressing the constitutively active form of cAKt/PKB (67).

Studies using cells overexpressing a dominant, negative mutant of the 85-kDa subunit of PI3-k have shown that despite markedly attenuated insulin-dependent activation of PI3-k, glycogen synthase activation was normal (60). This, together with the observation that PI3-k activation in response to other growth factors was not associated with glycogen synthase activation suggests that PI3-k may not be involved in the process (68). Therefore, it is possible that wortmannin and LY294002 exert their inhibitory effect on glycogen synthase activation by suppressing some other form of PI3-k and/or an uncharacterized enzyme involved in glycogen synthase activation. Thus, the contribution of PI3-k or cAKt/PKB in regulating glycogen synthesis appears to be complex and needs further investigation.

Our observation that VS-stimulated glycogen synthesis is not blocked by PD98059, a specific inhibitor of MEK1 and MEK2, the immediate upstream kinase in the ERK1 and ERK 2 signaling cascade (Table 1), demonstrated that ERK activation does not contribute to VS-stimulated glycogen synthesis. Similar conclusions were drawn by Lazar et al. after studying the mechanism of insulin-induced glycogen synthesis in 3T3-L1 adipocytes and L6 myotubes (39). In

addition, experiments using dominant negative ras mutants have revealed that activation of the ras/ERK signaling cascade is not required for insulin-activated glycogen synthesis in PC-12 cells (51) and 3T3-L1 adipocytes (69).

The lack of an effect of rapamycin on VS-stimulated glycogen synthesis (Table 1), which suggests that p^{70s6k} may not have a role in this process is consistent with findings in PC12 cells (51), skeletal muscle (59), rat adipocytes (57,58) and CHO-HIR cells (60) in response to insulin. In some studies, however, rapamycin treatment was shown to inhibit insulin-induced glycogen synthesis (47,61). Thus, the existence of cell-specific pathways for activation of glycogen synthesis may be postulated. Evidence has been presented recently showing that c-jun NH_2-terminal kinase (JNK) and ribosomal kinase III (rsk III) may contribute to insulin-stimulated activation of glycogen synthesis in skeletal muscle *in vivo* (59).

Whereas insulin-induced glycogen synthesis requires functionally-active IR-PTK (62), VS-induced glycogen synthesis occurs in the absence of a detectable IR tyrosine phosphorylation. In fact, using 3 different experimental approaches, we have failed to find any significant increase in tyrosine phosphorylation and PTK activation of IR in VS-stimulated CHO-HIR cells. Similar observations have been made in the rat diaphragm where vanadate-stimulated glycogen synthesis was not associated with enhanced IR tyrosine phosphorylation (19). No effect of vanadate on IR tyrosine phosphorylation in rat adipocytes has also been demonstrated (70). Interestingly, despite having no effect on tyrosine phosphorylation of the IR β-subunit, VS stimulated the tyrosine phosphorylation of IRS-1. Moreover, the fact that PI3-k activation was observed in IRS-1 immunoprecipitates from VS-treated

cells suggests that recruitment of PI3-k by binding to tyrosine phosphorylated IRS-1 may be one of the mechanisms by which VS activates PI3-k. The precise mechanism by which VS stimulates the tyrosine phosphorylation of IRS-1 is not clearly understood at present. Since vanadium salts are potent inhibitors of PTPases (17), it is possible that by suppressing a constitutively-active IRS-1 specific-PTPase, VS causes an increase in the phosphotyrosine content of IRS-1. Alternatively, VS may stimulate a non-receptor PTK which, in turn, may phosphorylate IRS-1. It is noteworthy that some cytokines and other peptide hormones have been shown to stimulate the tyrosine phosphorylation of IRS-1 by activating cytosolic PTKs (71,72). Clearly, additional studies are required to test these possibilities and to identify putative PTKs or PTPases responsible for IRS-1 phosphorylation in response to VS.

In summary, our results demonstrate, for the first time, that VS induces the tyrosine phosphorylation of IRS-1 and activates PI3-k in an IR-PTK-independent manner. Thus it may be suggested that activation of PI3-k and wortmannin/LY294002-inhibitable signaling pathway constitute one of the mechanisms by which inorganic vanadium salts exert insulin-like effects on glycogen synthesis.

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Table 1

Effect of PD98059 and rapamycin on VS- and insulin-stimulated MAP kinase and p^{70s6k} activities as well as glycogen synthesis

Treatment	Kinase activity (pmol/min/mg)		Glycogen synthesis (cpm ± SE)
	MAP kinase	p^{70s6k}	
Control	9.6 ±3.2	2.2±0.5	871±25
Vanadyl Sulfate	52±7.4	8.87±0.4	2738±54
Insulin	50±5.8	8.93±1.0	2646±29
VS+PD98059	19.3±4	---------	2793±73
Ins+PD98059	10.6±5	---------	2522±51
VS+rapamycin	--------	3.2±0.3	2897±88
Ins+rapamycin	--------	2.71±0.4	2650±72

CHO-HIR cells were incubated with or without PD98059 (10 µM) or rapamycin (50 ng/ml) for 30 min. followed by stimulation in the absence (control) or presence of vanadyl sulfate (VS, 100 µM) or insulin (Ins, 100 nM) for 5 min. MAP kinase or P^{70s6k} activity in lysates was determined as described in the Methods section by using MBP or a synthetic peptide RRRLSSLRA as exogenous substrate, respectively. Glycogen synthesis was determined by following the

incorporation of ^{14}C-glucose into glycogen as described in Fig. 1. Values represent the means ± SE from 3 experiments performed in triplicate.

FIGURE LEGENDS

Figure 1. **Effect of wortmannin and LY294002 on VS-stimulated glycogen synthesis** **(A)** Confluent, serum starved CHO-HIR cells were treated with wortmannin (100 nM, filled squares) or vehicle (0.01% DMSO used to dissolve wortmannin, filled circles) for 10 min. prior to stimulation with different concentrations of VS for 5 min. After this period, 5 mM glucose and D-(U-^{14}C)-glucose (1 μCi/sample) was added to the dishes and incubation continued for an additional 2.5 hours. The reaction was terminated by washing with ice-cold PBS and the cells were solubilized in 30% KOH. Glucose incorporation into glycogen was determined by following the incorporation of ^{14}C-glucose into glycogen as described in Methods. Values from 3 separate experiments, each performed in triplicate, are shown as mean ± SE **(B)** Confluent serum-starved CHO-HIR cells were incubated without (control, C) or with VS (100 μM for 5 min) or pretreated with LY294002 (10 μM, 30 min) followed by stimulation with VS (LY294002 +VS). Glycogen synthesis was determined as described in Fig. 1A. Values are from 3 experiments, each performed in triplicate, are shown as means ± SE **(C)** Confluent serum- starved CHO-HIR cells were incubated without (control, C) or with insulin (INS, 100 nM for 5 min) or pretreated with wortmannin (100 nM, 10 min) or LY294002 (10 μM, 30 min), followed by stimulation with INS (W+INS) or (LY294002+INS) respectively. Glycogen synthesis was determined as described under Fig. 1A. Values are from 3 separate experiments, each performed in triplicate, are shown as means ± SE

Figure 2. **Effect of wortmannin on VS-stimulated PI3-k Activity**

(A) Confluent, serum-starved CHO-HIR cells were incubated without (control, C) or with increasing concentrations of VS for 5 min. Cell lysates were prepared and proteins immunoprecipitated with anti-p85 antibody. Immunecomplexes were assayed for PI3-k activity using PI as substrate. Phospholipids were resolved by thin layer chromatography and then exposed to x-ray film. An autoradiogram of a representative experiment is shown. Arrowheads indicate the origin and position of phosphorylated substrate PIP. **(B)** PIP, the phosphorylated product, formed was quantified by PhosphorImager and values from 2 experiments are shown as means ± SE **(C)** Confluent, serum-starved CHO-HIR cells were pretreated with wortmannin (100 nM) or vehicle for 10 min followed by stimulation in the absence, C, or presence of VS, (100 μM) or insulin (INS, 100 nM) for 5 min. Lysates were prepared and PI3-k assay performed as described above. **(D)** The phosphorylated product (PIP), was quantified by PhosphorImager. Values from 4 experiments are shown as means ± SE. *P= 0.01 versus VS-stimulated activity.

Figure 3. **Effect of VS on insulin receptor β-subunit tyrosine phosphorylation and autophosphorylation** **(A)** Confluent, serum-starved CHO-HIR cells were either unstimulated, C or stimulated with 100 nM insulin (INS) or 100 μM VS for 5 min. Cells were lysed and lysates subjected to immunoprecipitation with anti-IR β-subunit antibody, followed by immunoblotting with anti-phosphotyrosine antibody. This immunoblot is the representative of 2 other experiments with similar results. **(B)** and **(C)**. Confluent serum-starved, CHO-HIR cells were either unstimulated, C, or stimulated with 100 nM insulin

(INS) or 100 μM VS for 5 min. Cell lysates were prepared and then immunoprecipitated with either anti-phosphotyrosine antibody **(B)** or anti-IR β-subunit antibody **(C)**. The respective immunoprecipitates were subjected to autophosphorylation reaction followed by autoradiography as described in Methods. The autoradiograms shown are representative of 2 other experiments with similar results.

Figure 4. **Effect of VS on insulin receptor substrate (IRS-1) tyrosine phosphorylation and IRS-1 associated PI3-k activity** **(A)** Confluent, serum-starved CHO-HIR cells were stimulated with the indicated concentrations of VS. Lysates were prepared and subjected to immunoprecipitation with antibody to IRS-1. Immunoprecipitates were seperated on 10% SDS-PAGE followed by immunoblotting with anti-phosphotyrosine antibody as described in Methods. The data are representative of 3 independent experiments with similar results. **(B)** Confluent, serum starved CHO-HIR cells were either unstimulated, C, or stimulated with VS (100 μM) for 5 min. They were lysed and immunoprecipitated with anti-IRS-1 antibody. PI3-k activity was measured in IRS-1 immunecomplexes as described in Fig. 2. This autoradiogram is representative of 2 experiments with similar results **(C)** The phosphorylated product (PIP), was quantified by PhosphorImager. Values from 2 separate experiments are represented as means ± SE.

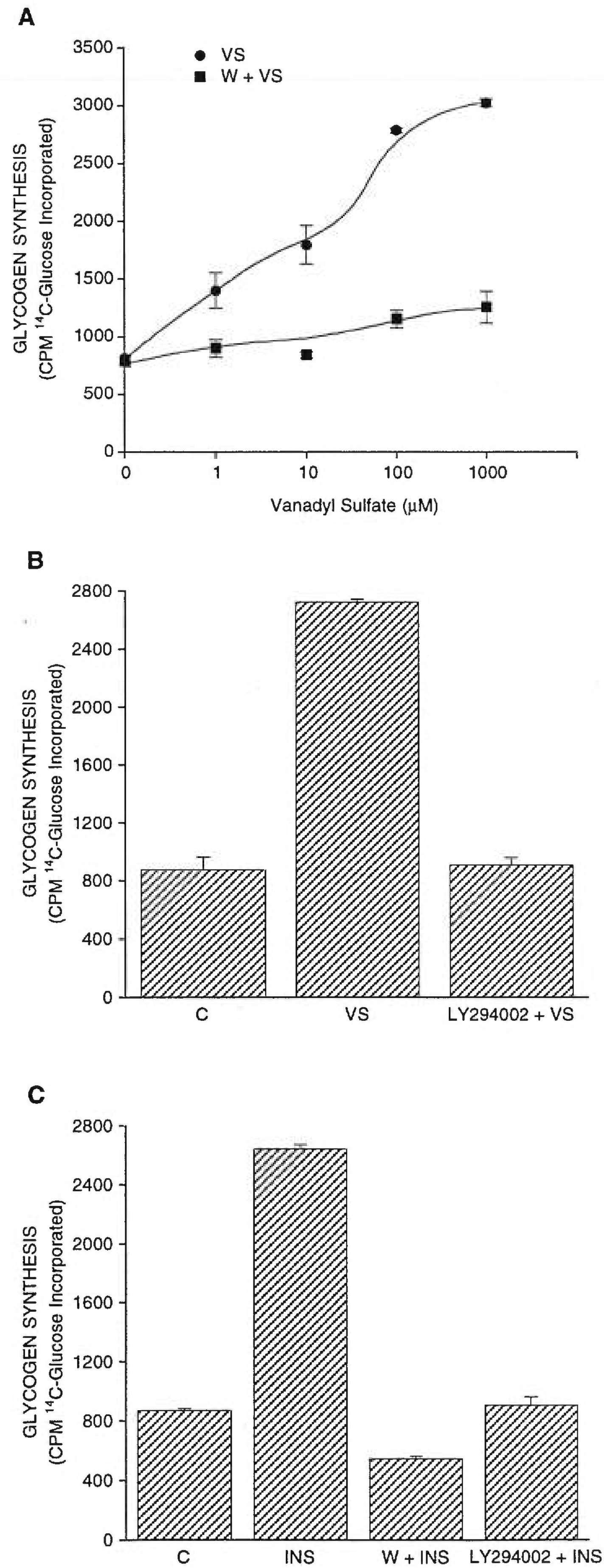


Figure 1

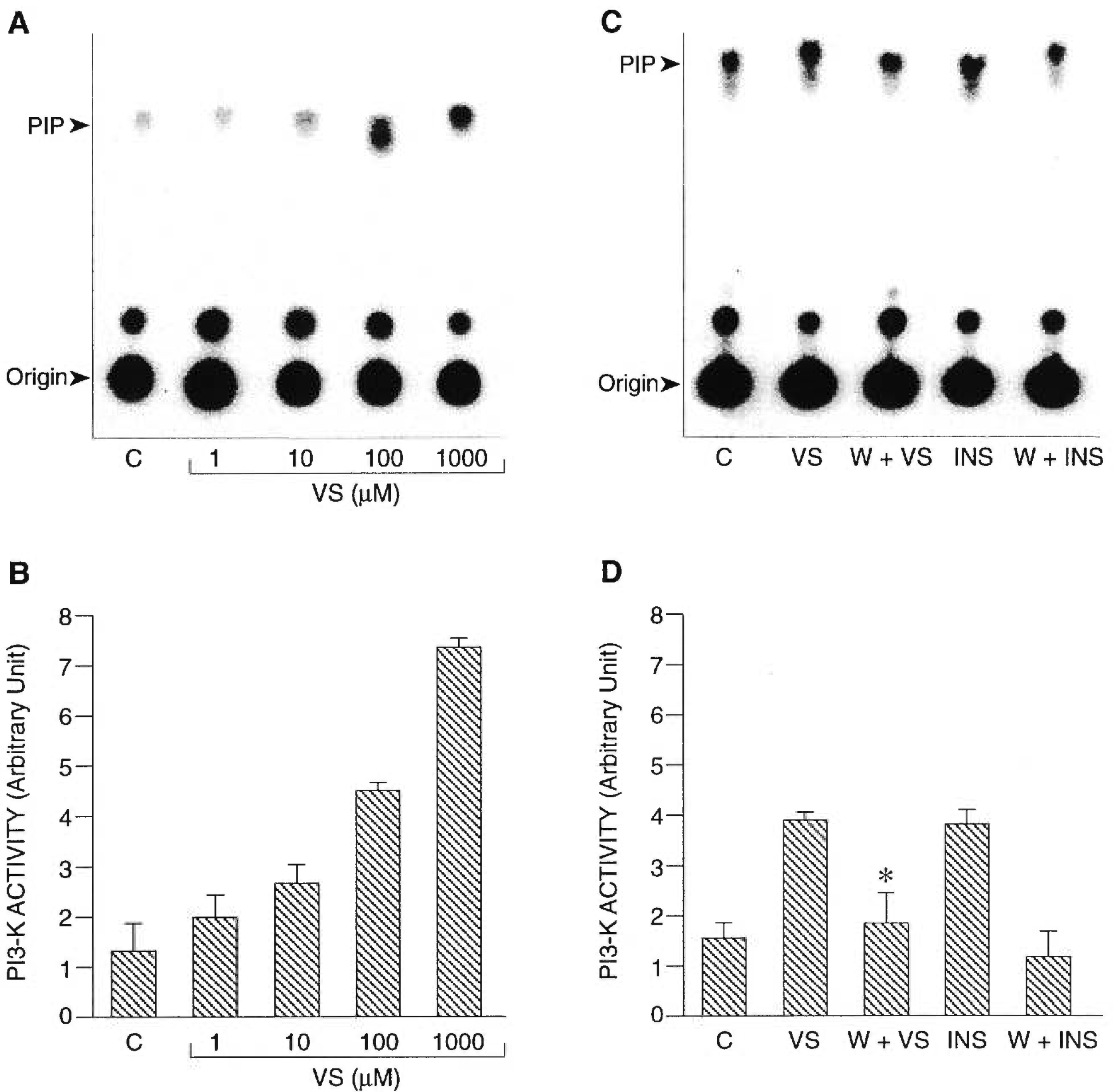


Figure 2

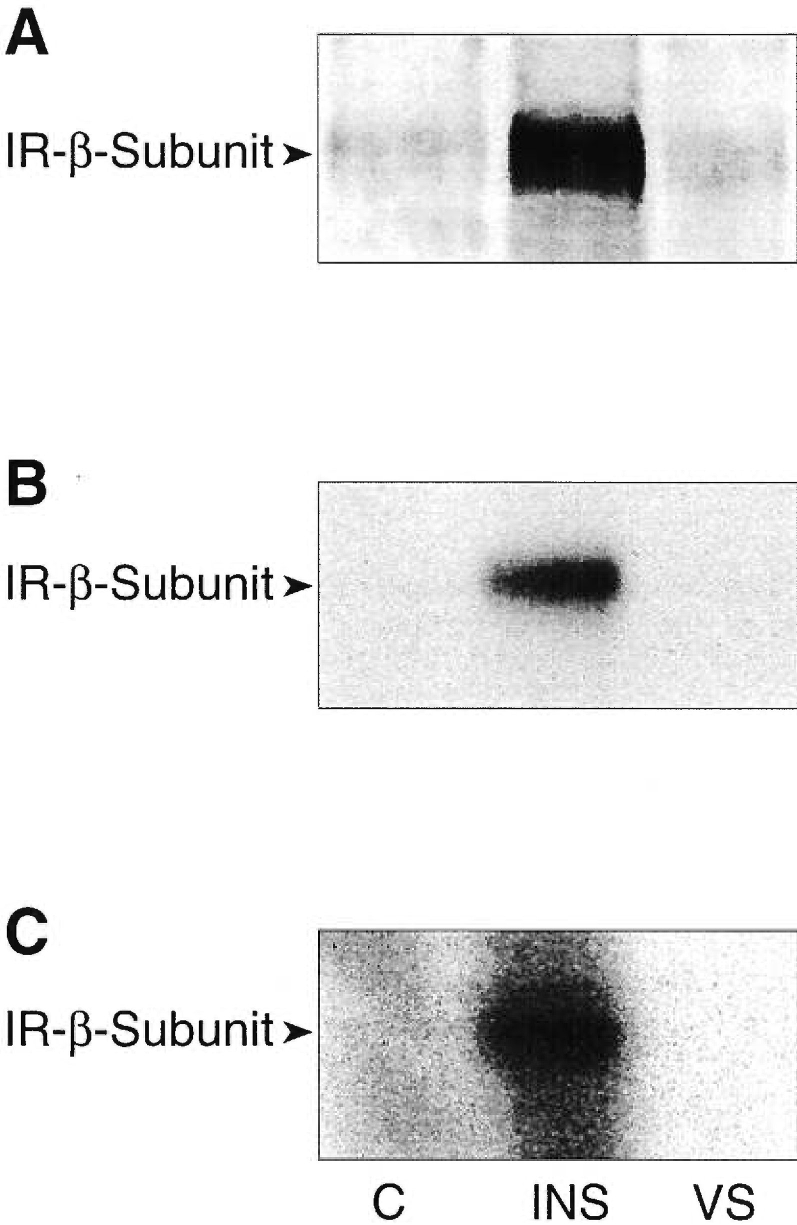
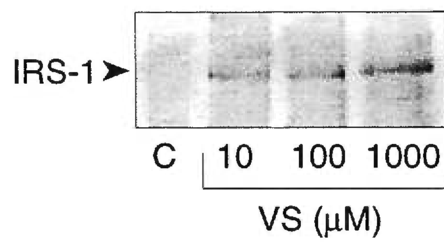
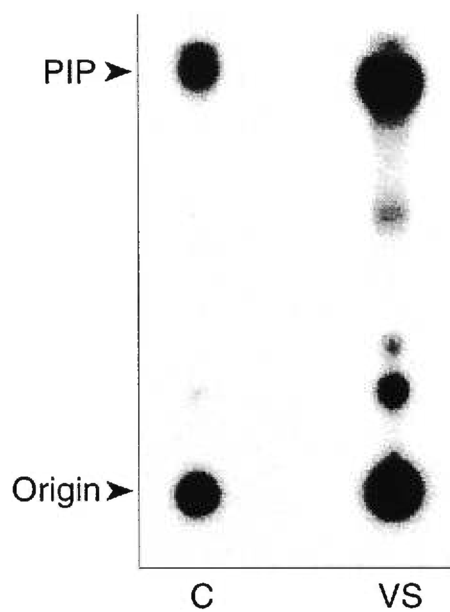


Figure 3





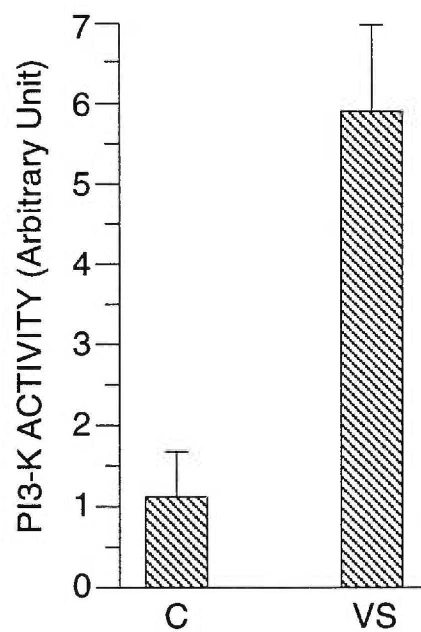


Figure 4

CHAPTER IV

Article 3

Wortmannin, a selective inhibitor of Phosphatidyl Inositol 3-kinase, inhibits vanadyl Sulfate-induced activation of c-Raf-1, MEK, MAP kinases and p^{70s6k} ribosomal kinase

Sanjay K. Pandey and Ashok K. Srivastava

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Wortmannin, a selective inhibitor of Phosphatidyl Inositol 3-kinase, inhibits Vanadyl Sulfate-induced activation of c-Raf-1, MEK, MAP kinases and p^{70s6k} ribosomal kinase †

Sanjay K. Pandey[‡§] and Ashok K. Srivastava[‡*]

Centre Hospitalier Université de Montréal, Centre de Recherche ,
Campus Hotel-Dieu[‡] and Department of Physiology[§] and Medicine[‡],
University of Montréal, Montréal, Québec, H2W 1T8, Canada

Running Title: Regulation of PI3-k dependent MAP kinase pathway by Vanadyl Sulfate

***ADDRESS FOR CORRESPONDENCE**
Ashok K. Srivastava, Ph.D.
Centre Hospitalier université de Montréal,
Centre de Recherche, Campus Hotel-Dieu
3850, St. Urbain Street, Montréal (Québec)
H2W 1T8
Tel: (514) 843-2917.
Fax: (514) 843-2911
e-mail: srivasta@ ere.umontreal.ca.

† Supported by Medical Research Council of Canada

ABSTRACT

Inorganic salts of trace element vanadium, such as sodium orthovanadate and vanadyl sulfate (VS), elicit a wide variety of insulinomimetic effects such as stimulation of glucose uptake, glycogen synthesis and normalization of glucose levels in animal models of diabetes mellitus. However, the mechanism by which these effects are mediated in not clearly understood. We have shown previously that vanadium salts activate mitogen activated protein (MAP) kinase in an insulin receptor (IR) protein tyrosine kinase (PTK)-independent manner in Chinese Hamster Ovary cells overexpressing human Insulin Receptor (CHO-HIRc). In the present study we have examined if signaling components upstream to MAP kinase such as MEK, Raf and Ras are also activated by VS. In addition, we have also evaluated the effect of PI3-k inhibitor, wortmannin, on VS-induced Ras-MAP kinase and p^{70s6k} activation. Treatment of CHO-HIR cells with VS resulted in an increase in tyrosine phosphorylation of p44mapk and p42mapk in parallel with a stimulation in MAP kinase, MEK and c-Raf-1 activities. Preincubation of these cells with wortmannin, a specific inhibitor of PI3-k, blocked VS-stimulated MAP kinase activity as well as tyrosine phosphorylation of p44 and p42 proteins. Furthermore, wortmannin also inhibited VS-stimulated MEK and c-Raf 1 as well as p^{70s6k} activities. Thus, these data suggest that stimulation of MAP kinase in response to VS is accomplished by a coordinated activation of signaling cascade upstream of MAP kinase. In addition, pretreatment of cells with farnesyltransferase inhibitor, B581, resulted in an attenuation of VS-induced MAP activation

suggesting a role of Ras in mediating this effect. Taken together these results also provide evidence suggesting that PI-3k may be required for the stimulation of Ras-MAP kinase and p^{70s6k} pathway by VS.

INTRODUCTION

Salts of the trace element vanadium mimic essentially all of the actions of insulin on glucose and lipid metabolism. These effects include stimulation of glucose transport, glycogen synthesis, glycogen synthase, lipogenesis and inhibition of lipolysis as well as gluconeogenesis (1-7). Furthermore, vanadium salts given orally has been shown to normalize blood glucose levels in animal models of insulin-dependent diabetes mellitus (IDDM or type I) (8,9) and non insulin-dependent diabetes mellitus (NIDDM or type II) (10-12). In addition, recent studies in human subjects have demonstrated that vanadium salts improved many of the abnormalities associated with diabetes mellitus (13, 14, reviewed in 15 and 16).

The diverse metabolic and mitogenic effects of insulin are initiated by binding to cell surface α-subunit of insulin receptor (IR) which results in tyrosine autophosphorylation of their β-subunit and an increase in intrinsic protein tyrosine kinase (PTK) activity (17-20). The activated IR-PTK phosphorylates its major substrate named insulin receptor substrate-1 (IRS-1) (21). The specific tyrosine phosphorylated sequences on IRS-1 then serve as binding sites for Src-homology 2 (SH2) domain containing signaling proteins. These include the Grb-2-SOS complex (22), Syp (SHP2), a protein tyrosine phosphatase (PTPase), Nck (a linker protein whose function in not yet clear) (23), as well as the 85 kDa regulatory subunit of phosphatidylinositol (PI) 3-kinase, (PI3-K), a lipid kinase (24, 25). There are two main signaling cascades that originate from the IRS-1 associated complex. The MAP kinase pathway, which has been studied

extensively, transduces extracellular signals into intracellular responses. Binding of the Grb-2-SoS complex to IRS-1 leads to stimulation of GDP exchange for GTP on p^{21ras} . The activation of p^{21ras} recruits a serine/threonine kinase, Raf, to plasma membrane and contributes to its activation. Raf then phosphorylates and activates MAP kinase kinase or MEK which in turn activates two isozymic forms of MAP kinases, p^{44mapk} (ERK 1) and p^{42mapk} (ERK 2) by phosphorylating at threonine and tyrosine residues (26, 27). The activated MAP kinase phosphorylates and activates a downstream ribosomal protein kinase, $p90^{rsk}$ (28). Insulin stimulates another ribosomal s6 kinase, p^{70s6k}, which lies on different signaling pathway, by a mechanism which is poory characterized (29).

The second emerging pathway that radiates from IRS-1 complex upon insulin stimulation is PI3-k-PKB-GSK-3. PI3-K is composed of an 85 kDa (p85) regulatory subunit and a 110-kDa (p110) catalytic subunit (30) which catalyzes phosphorylation of inositol ring on D-3 position of PI (31,32). A downstream serine/threonine kinase named protein kinase B (PKB) has been shown to be the target of PI3-k (33). PKB in turn phosphorylates GSK-3 and inactivates it (33). The availability of potent inhibitors of PI3-k , wortmannin and LY294002, has aided in investigating the contribution of PI3-k signaling pathways to insulin-stimulated physiological responses. However, in association with PI3-k inhibition, these inhibitors also attenuate other signaling enzymes in MAP kinase cascade suggesting a cross-talk between the Ras/MAP kinase and PI3-k pathways. Wortmannin blocks activation of MAP kinase by insulin in muscle cells (34-35), CHO-T cells (36), and (pre)adipocytes (37,38). Wortmannin also attenuates

vasopressin and pituitary growth hormone induced MAP kinase activation in rat fibroblasts (39) and 3T3-preadipocytes respectively (40), endothelin-1 induced MAPK and Raf-B stimulation in CHO cells (41), and IL-8 and chemoattractant C5a induced activation of Raf-1, C-Raf and MAP kinase in T-cells (42). Furthermore, Karnitz et. al. reported that interlukin-2 stimulated MAP kinase and MEK are inhibited by wortmannin in the T lymphocytes cell line, CTLL2 (43).

The mechanism by which vanadium salts mimic insulin action is believed to be by virtue of their inhibitory effect on protein tyrosine phosphatases (PTPases) (44) which in turn enhance protein tyrosine phosphorylation. In early studies the insulin like action of vanadate was suggested to be due to the activation of IR-PTK activity secondary to an inhibition of dephosphorylation of IR β-subunit (45). Subsequently, it was observed that the site of vanadium action is at the post receptor level (46-50) and does not involve insulin receptor tyrosine phosphorylation (49, 51-52). Thus, the molecular mechanism of insulinomimetic effects of vanadium salts remains poorly characterized and requires further clarification.

We have demonstrated earlier that several vanadium salts activate MAP kinase as well as two ribosomal kinases, p^{90rsk} and $p70^{s6k}$, in an IR-PTK-independent manner (49,53). In the light of results demonstrating that wortmannin blocks agonist-induced Ras/MAP kinase and p^{70s6k} pathways in several cell types, the present study was undertaken to examine whether VS-stimulated MAP kinase is also inhibited by wortmannin and if upstream signaling components to MAP

kinase such as MEK, Raf and Ras are activated by VS in a wortmannin-sensitive manner.

MATERIALS AND METHODS

Materials

CHO cells overexpressing a normal human insulin receptor (CHO-HIRc) were a kind gift from Dr. Morris F. White (Joslin Diabetes Centre, Boston, MA). Insulin was from Eli Lilly Co. (Indianapolis, IN). Antibody as well as substrate to c-Raf 1 was the generous gift of Dr. Rama K. Jaiswal (Case Western University, Ohio). MEK antibody and recombinant p44 were kindly provided by Dr. Sylvain Meloche (Centre de Recherche, Campus Hotel Dieu de Montreal, Montreal). Wortmannin was bought from Sigma Chemical Company (St.Louis, MO). Vanadyl sulfate was from Aldrich Chemical Co. (Milwaukee,WI,). Antiphosphotyrosine antibody and S6 peptide RRRLSSLRA were from Upstate Biotechnology (Lake Placid, NY). Goat anti-mouse or anti-rabbit IgG conjugated to alkaline phosphatase were from Bio-Rad (Mississauga, Ontario). Protein A sepharose beads were bought from Pharmacia Biotech Inc (Mississauga, Ontario). The p^{70s6k} antiserum (raised against a peptide corresponding to amino acids 2 to 30 of rat $p70^{s6k}$) was generously provided by Dr. Frederic Hall of USC, School of Medicine, Los Angeles. Farnesyl transferase inhibitor, B581, was purchased from Bachem Bioscience Inc. (King of Prussia, PA).

Methods

Cell Culture CHO cells were maintained in HAM's F-12 medium containing 10% fetal bovine serum. Cells were grown to confluence in 100 mm plates and incubated in serum-free F-12 medium for 20 hours prior to the experiment (53).

MAP Kinase Assay For MAP kinase assay cell lysates were prepared in buffer A (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1mM Na orthovanadate, 10 mM Na fluoride, 10 mM Na pyrophosphate, 20 nM Okadaic acid, 0.5 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 1% Triton X-100 and 0.1% SDS). The clarified lysates were normalized to contain equal amounts of protein (100μg) and incubated for 4 hours at 4°C with 5 μl of MAP kinase antibody preadsorbed to protein A-Sepharose beads. The immune complex was collected by centrifugation followed by washing 3 times with buffer A and once with buffer containing 20 mM Hepes pH 7.4, 10 mM $MgCl_2$, 1mM DTT, 10 mM β-glycerophosphate). Then 40 μl of kinase buffer (25 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM dithiothretol (DTT), 1 μM staurosporine, 0.5 mM EGTA) and 10 μl myelin basic protein (1mg/ml) were added. The reaction was initiated by adding 5 μl of 1mM ATP containing 0.5 μCi (γ^{32}P) ATP. After 12 minutes at 30°C, the reaction was stopped by spotting 20 μl of the reaction mixture onto P-81 filter papers (2 cm x 2 cm). Papers were washed 4 times in 0.5% phosphoric acid and counted for radioactivity.

Immunoaffinity Purification of Phosphotyrosine Proteins and MAP Kinase Immunoblotting Cell lysates were affinity purified on antiphosphotyrosine-agarose column and immunoblotted using a MAP kinase antibody (49). For affinity purification of phosphotyrosine proteins, the clarified lysates from control or stimulated cells were incubated for 1 hour at 4°C with antiphosphotyrosine agarose beads equilibrated in buffer B (20 mM Tris-HCl pH 7.4, 1% Nonidet p-40, 10 mM EDTA, 0.2 mM Na vanadate , 0.01% Na azide and 100 mM NaCl).

The beads were washed 3 times with buffer B and phosphotyrosine containing proteins were eluted with 1 mM phosphotyrosine in buffer B. The eluates were boiled in 3 X Laemmli's sample buffer, electrophoresed on 10% SDS-polyacrylamide gels (SDS-PAGE), transferred to PVDF membranes, and blotted with anti-MAP kinase antibody, SM1, (1:500) and detected by using goat-anti-rabbit IgG conjugated to alkaline phosphatase.

Detection of Phosphotyrosine-Containing Proteins Tyrosine phosphorylation of cellular proteins was assessed by immunoblotting using antiphosphotyrosine antibodies. Cells either unstimulated or stimulated with insulin or vanadyl sulfate, were lysed on ice in 400 µl of buffer A. The lysates were clarified by centrifugation for 12 minutes at 10, 000 x g . Equal amounts of protein samples were electrophoresed on 10% SDS-polyacrylamide gels, transferred to polyvinilidine difluoride (PVDF) membranes incubated with antiphosphotyrosine antibody (1:1000) and detected using goat-anti-mouse IgG conjugated to alkaline phophatase (1:3000).

Assay of $p70^{s6k}$ Activity The assay was performed as described before (53). Briefly, clarified lysates prepared in buffer A were normalized to contain equal amounts of protein (100µg) and incubated for 4 hours at 4°C with 3 µl of p^{70s6k} antibody preadsorbed 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 containing 20 mM Hepes pH 7.4, 10 mM $MgCl_2$, 1mM DTT, 10 mM β-glycerophosphate. The beads were resuspended in 20 µl of buffer B containing S6 peptide RRRLSSLRA. The phosphotransferase reaction was initiated by adding 5

μl of 100 μM ATP containing 2 μCi (γ^{32}P)-ATP. After 15 minutes at 30°C, the reaction was stopped by spotting on P-81 filter paper. Papers were washed in 0.5% phosphoric acid and counted for radioactivity (53).

MEK Assay Confluent, serum starved CHO-HIR cells were stimulated with or without wortmannin followed by VS. MEK activity was assayed by measuring its ability to increase the MBP kinase activity of recombinant p^{44mapk} . The samples were prepared as described for MAP kinase assay. Equal amount of lysate proteins (100 μg) were incubated for 4 hours with 1 μl of anti-MEK antibody preadsorbed to Protein A-Sepharose beads. The immune complex was washed 3 times with lysis buffer, once with kinase assay buffer (20 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 1mM DTT) and then resuspended in kinase buffer containing 50 μM ATP, 5 μCi of (γ-^{32}P) ATP and 300 ng of recombinant p^{44mapk}. After incubation at 30 °C for 30 min. MBP (0.25mg/ml) was added and the incubation was continued for an additional 10 min. The reaction was stopped by adding 40 μl 3 X Laemmeli's sample buffer. Solubilized proteins were seperated by SDS-PAGE (10% polyacrylamide gel) and were electrophoretically transferred to Immobilin-P (Millipore, Missisauga ,Ontario). Membranes were exposed to phosphorimager (Molecular Dynamics) and radioactivity corresponding to each area was quantified.

Raf Assay For Raf kinase assay, samples were prepared as described for the MEK assay except that immunoprecipitation was performed with 1 μg of anti c-raf-1 antibody. The reaction was carried out using "kinase-inactive MEK" (K52R)

(kindly provided by Dr. Rama Jaiswal of Case Western Reserve University, Cleveland, Ohio) as substrate.

RESULTS

Effect of wortmannin on VS-stimulated MAP Kinase Activity and Protein Tyrosine Phosphorylation Several reports have demonstrated that wortmannin, a selective PI3-k inhibitor, attenuates insulin-stimulated MAP kinase activity in various cell systems (34-43). Therefore, we wished to investigate whether wortmannin could also inhibit VS-stimulated MAP kinase activity. CHO-HIR cells were incubated with or without wortmannin (100 nM for 10 min.) followed by stimulation with either VS (100 μM) or insulin (100 nM, as a positive control) for 5 min. MAP kinase activity was determined using an immune complex assay. Both VS and insulin stimulated MAP kinase activity by about 4 to 5 fold over control. However, wortmannin pretreatment resulted in almost complete inhibition of both VS and insulin-stimulated MAP kinase activity (Fig 1A). To evaluate if wortmannin treatment exerted any effect on the protein tyrosine phosphorylation stimulated by VS or insulin, CHO-HIR cells were incubated as in Fig 1A and cell lysates were subjected to antiphosphotyrosine immunoblotting. As shown in Fig. 1B, VS-treated cells showed increased phosphorylation of two proteins having apparent molecular sizes of 44 (p44) and 42 (p42) kD respectively. Wortmannin pretreatment almost completely blocked VS-stimulated tyrosine phosphorylation of these proteins. On the other hand, insulin treatment resulted in increased tyrosine phosphorylation of 95 kD IR-β subunit as well as p44 and p42. Wortmannin treatment significantly attenuated insulin-stimulated tyrosine phosphorylation of p44 and p42 and not of the IR-β subunit. In contrast, under these conditions VS-stimulated p44 and p42 tyrosine phosphorylation occured in

the absence of any detectable increase in IR-β tyrosine phosphorylation (Fig 1B). Furthermore, neither wortmannin nor its vehicle DMSO alone had any effect on protein tyrosine phosphorylation or MAP kinase activity as compared to unstimulated control (Fig. 1, A and B).

To characterize further the identity of p44 and p42 kDa proteins, lysates from cells stimulated under different conditions were subjected to immunoaffinity purification with agarose-conjugated anti phosphotyrosine antibody. The immunopurified proteins were immunoblotted with anti MAP kinase antibody, SM 1, which detected both p44 (Erk 1) and p42 (Erk 2) isoforms of MAP kinases . As shown in Fig 2, wortmannin pretreatment (100 nM for 10 min.) markedly inhibited VS and insulin-stimulated tyrosine phosphorylation of both p^{44mapk} (Erk 1) and p^{42mapk} (Erk 2) proteins (Fig. 2 lane 3 and 6).

Effect of wortmannin on VS-induced activation of MEK and c-Raf-1

Since MAP kinase activation requires a sequential activation of several upstream signaling molecules such as MEK and c-Raf 1, it was of interest to examine if VS, activates these signaling components similar to insulin. Serum-starved CHO-HIR cells were either unstimulated (C) or stimulated with VS and then MEK catalytic activity was determined in MEK immunoprecipitates. It was observed that VS stimulated MEK activity approximately 3 to 4 fold as compared to control (Fig 3, A and B). Furthermore, based on the results demonstrating that various agonists stimulate MEK by a wortmannin sensitive pathway (43), we were interested to examine whether wortmannin could also block MEK in response to VS. Serum-starved CHO-HIR cells were pretreated with 100 nM wortmannin for 10 min. and

then stimulated with VS (100 μM for 5 min., W+VS). To our surprise, VS stimulated MEK activity was almost completely inhibited by wortmannin (Fig 3A and B) suggesting that PI3-K lies upstream of MEK. Similarly, insulin-stimulated MEK activity was also inhibited by wortmannin (data not shown).

Increased Raf-1 activity is the primary stimulus for MEK activation. Therefore, we examined the effect of VS on Raf-1 activity and its modulation by wortmannin. Serum-starved CHO-HIR cells were incubated with or without 100 nM wortmannin for 10 min. followed by stimulation with VS. Raf kinase activity was determined in immunoprecipitates using "kinase-inactive MEK" (K52R) as substrate. As shown in Fig. 3, C and D that VS enhanced c-Raf 1 activity by about four fold over unstimulated control. Interestingly, wortmannin pretreatment almost completely inhibited c-Raf 1 activity stimulated by VS. Under these conditions insulin stimulated c-Raf activity was also inhibited by wortmannin (data not shown). The results from the above experiments suggest that wortmannin sensitive PI3-k may lie upstream of c-Raf-1 and MEK.

Role of Ras in VS stimulated MAP Kinase Pathway: Although it is generally believed that the Ras-Raf-MEK-MAP kinase cascade follows a linear series however, existence of Ras-independent mechanism(s) for Raf-MEK-MAP kinase activation have also been proposed (54-56). Insulin has been shown to activate p^{21ras} in many cell types (22, 57-58), and activated p^{21ras} is linked to MAP kinase stimulation (59). Since MAP kinases are also activated in Ras transformed NIH 3T3 cells (60-61), their activation provides an excellent marker for the activation of the Ras signal transduction pathway. Therefore, we next examined

the contribution of Ras protein in VS-stimulated responses. For these experiments we studied the effects of B581, a farnesyltransferase inhibitor, on VS and insulin-stimulated MAP kinase activation. Farnesylation of Ras is essential for its localization to plasma membrane and thus its activation. B581 has therefore, been employed to assess the role of Ras protein in MAP kinase activation and cell transformation (61, 73). As shown in Figure 4, pretreatment of CHO-HIR cells with two different concentrations of B581 (50 and 100 μM) for 24 hours almost completly blocked MAP kinase activation in response to VS as well as to insulin (as a positive control). These results demonstrated that pharmacological inhibition of Ras farnesylation blocks VS-induced activation of MAP kinase and suggest that a sequential activation of Ras, Raf and MEK is required in mediating the insulinomimetic effect of VS on MAP kinase activation.

Effect of Wortmannin on VS-stimulated p^{70s6k} Activity: Our previous study demonstrated that 70 kDa ribosomal protein kinase $p70^{s6k}$, which is a downstream effector of PI3-k, was activated by both VS as well as insulin (53). Furthermore, in the light of reports suggesting that wortmannin inhibits $p70^{s6k}$ in response to growth factors (62-63), we were interested in determining whether activities of this enzyme is in response to VS and insulin was also sensitive to wortmannin. CHO-HIR cells were incubated with or without wortmannin (100 nM, 10 min) and then stimulated with insulin (100 nM) or VS (100 μM) for 5 min. To confirm the specificity of the inhibition of $p70^{s6k}$ activity by wortmannin, an immune complex kinase assay was performed. As shown in Fig. 5, both VS and insulin stimulated $p70^{s6k}$ activity by about 4-5 fold over control. Wortmannin

treatment significantly inhibited insulin as well as VS-stimulated $p70^{s6k}$ activity suggesting that stimulation of $p70^{s6k}$ by VS is also mediated by PI3-k.

DISCUSSION

In the present study we have examined the effect of VS, an insulinomimetic agent, on the activation of the Ras/MAP kinase pathway in CHO-HIR cells. We also investigated the role of PI3-k in the activation of this pathway by utilizing a specific inhibitor wortmannin and thus made an attempt to establish a link between these two pathways. Our results show that wortmannin treatment inhibited both VS as well as insulin-stimulated MAP kinase activity and protein tyrosine phosphorylation of p^{44mapk} (ERK 1) and p^{42mapk} (ERK 2) in CHO-HIR cells. Although, similar observations were made previously demonstrating that insulin-stimulated MAP kinase was inhibited by wortmannin in various cell types, such as L6 cells (34,35), CHO-T cells (36) and adipocytes (36,38), the present work provides first evidence to support an involvement of Ras protein and PI3-k activation in mediating the responses of insulinomimetic vanadium salts. LY294002, another structurally and mechanistically different inhibitor of PI3-k, has also been reported to attenuate insulin-induced activation of MAP kinase as well as Raf-1 in 3T3-L1 adipocytes and KB cells, a human epidermoid carcinomal cell line (64-65) while a lack of an effect of LY294002 was observed by Cheatham et. al. in 3T3-L1 adipocytes (66).

The attenuating effect of PI3-k inhibitors is not limited to insulin responsive cells because several other studies have demonstrated that wortmannin blocks MAP kinase activation in response to various growth factors and hormones (39-41, 43, 67). Scheid et.al. have demonstrated that PI3-k inhibitors blocked IL-3-induced activation of MAP kinase in hemopoietic cell, MC-9 (68). Similarly, Van

Willebrand et.al. demonstrated that wortmannin attenuated anti CD3-induced MAP kinase in Jurkat T-cells (69). This conclusion was further supported by studies by Karnitz and Co-workers on IL-2-induced activation of MAP kinase in the T cell line, CTLL-2 (43).

The inhibitory effect of wortmannin does not appear to result from a direct inhibition of the MAP kinase *in vitro* but rather from an effect on a step upstream to the activation of MAP kinase. The activation of MAP kinase requires phosphorylation on both tyrosine and threonine (70) catalyzed by a single dual specificity kinase, MEK (71). Further examination of protein kinase cascade revealed that VS and insulin caused an increase in MEK activity and surprisingly activation of MEK was also sensitive to wortmannin. In this regard Cross et. al. have reported an inhibitory effect of wortmannin on insulin/IGF-induced activation of MEK in rat skeletal muscle cell L6 (34). Similar observation of MEK inhibition by wortmannin was made by Karnitz et. al. while studying the IL-2 stimulated Ras/MAP kinase signaling pathways (43). Thus, the results presented in this study suggest that VS-induced activation of MEK, may require PI3-k activation in a fashion similar to other agonists.

Although considerable evidence now indicates that MEK is activated by Raf, however, Raf does not appear to be major growth factor-activated MEK kinase in some cells (72). Our next search for possible candidates that may relay the signal to MAP kinase in response to VS and insulin lead us to the demonstration that c-Raf 1 is also activated in response to VS. Interestingly, wortmannin treatment also inhibited VS-stimulated Raf-1 kinase. A similar

observation was made by Suga et. al. (65) demonstrating that insulin in CHO-HIR cells stimulated Raf-1 kinase was blocked by wortmannin while EGF stimulated Raf-1 was not. Similarly, Karnitz and Co-workers also reported that IL-2 stimulated Raf-1 kinase was not inhibited by wortmannin (43). Thus, the conflicting observations among various studies may appear to arise either due to the nature of different agonists or cell lines or due to the experimental conditions employed and therefore requires further clarification. Our results however, strongly suggest for the first time that wortmannin sensitive PI3-k is important for VS-stimulated Raf-MEK-MAP kinase cascade.

Activation of Ras is critical to turn on the MAP kinase signaling pathway in response to many agonists. The farnesylation of Ras is essential to localize it to plasma membrane and for its activation. Thus, the participation of Ras in the VS- and insulin-stimulated Raf-MAP kinase cascade was evaluated by utilizing a specific farnesyl transferase inhibitor, B581. It was observed that B581 pretreatment prevented MAP kinase activation in response to VS as well as insulin. This effect was presumambly due to the inhibition of Ras owing to its inability to translocate to the plasma membrane. These data provide strong evidence suggesting that for Ras is an upstream signaling protein that mediates the VS stimulated activation of - Raf, MEK and MAP kinase. Since wortmannin inhibits VS-and insulin-induced activation of Raf, MEK and MAP kinase, it is most likely that Ras would also be inhibited by wortmannin and that PI3-k, may be an upstream regulator of Ras-MAP kinase cascade.

Whereas, the insulin stimulated Ras/MAP kinase pathway requires PI3-k activation dependent on the enhanced tyrosine phosphorylation and activation of IR-β subunit as well as IRS-1 (20), the VS stimulated MAP kinase cascade appeared to be independent of IR protein tyrosine phosphorylation (49). Thus, it seems that PI3-k can transmit the signal to Ras/MAP kinase pathway both in a receptor-independent and dependent fashion. In this regard Hu. and Co-workers have elegantly demonstrated that a constitutively active form of PI3-k can transmit signals in a Ras-dependent manner without requiring receptor stimulation (74). In another study Yamauchi and Co-workers (75) demonstrated that overexpression of a dominant negative p85 subunit of PI3-k can act upstream of Ras and Raf activity.

Ribosomal kinases are important in mediating some of the insulin-induced biological responses such as protein synthesis (76). The observation that similar to insulin, VS-induced activation of p^{70s6k} was also inhibited by wortmannin suggest that both insulin and VS-stimulated p^{70s6k} requires wortmannin sensitive PI3-k. Several other reports have suggested that wortmannin inhibited p^{70s6k} , thus placing it downstream to PI3-k (62-63). It was also reported that co-expression of PI3-k enhances the phosphorylation of p^{70s6k} sites which could be blocked by wortmannin (77-79) suggesting that S6 kinase is regulated by PI3-k. Thus, our results with VS confirm these observations and show for the first time that VS-induced p^{70s6k} is also regulated by PI3-k.

In conclusion, we demonstrate for the first time that stimulation of MAP kinase by the insulinomimetic agent VS requires ras protein and is dependent on

the wortmannin-sensitive PI3-k activation. Thus, it may be suggested that similar to insulin, which utilizes this cascade to exert its mitogenic effects, VS, which promotes similar effects on various cells types (80-81), may require mediation of PI3-k dependent Ras-MAP kinase cascade .

FIGURE LEGENDS

Figure 1.**Effect of Wortmannin on VS and insulin-stimulated MAP Kinase activation and Tyrosine Phosphorylation.** **(A)** Confluent, serum starved CHO-HIR cells were pretreated with wortmannin (100 nM for 10 min) or vehicle (0.01% DMSO, used to dissolve wortmannin) followed by stimulation with either VS (100 μM) or insulin (100nM) for 5 min. Cell lysates were prepared and subjected to: **(A)** MAP kinase assay using Myelin Basic Protein (MBP) as an exogenous substrate described in methods. The values are from three separate experiments each performed in triplicate and are shown as mean ± S.E. **(B)** Confluent, serum starved CHO-HIR cells were stimulated as in **(A)** and cell lysates were subjected to anti-phosphotyrosine immunoblotting as described in methods.

Figure 2. **Identification of p44 and p42 proteins:**

Cells were pretreated without or with wortmannin (100 nM) for 10 min.) followed by stimulation with either VS (100 μM) or insulin (100nM) for 5 min. Lysates from different treatment were subjected to immunopurification with agarose-conjugated anti-phosphotyrosine antibody, analysed with SDS-PAGE and probed with anti-MAPK antibody which detected two isozymic forms of MAP kinase, p^{44mapk} (ERK-1) and p^{42mapk} (ERK-2).

Figure 3. **Effect of wortmannin on VS-stimulated MEK and C-Raf 1 activity**.

(A) and (B) Serum starved confluent CHO-HIR cells were pretreated without or with wortmannin (100 nM) for 10 min followed by stimulation in the absence (C) or presence of vanadyl sulfate (VS, 100 μM) for 5 min and lysates were prepared.

MEK kinase activity was determined as described under methods by following the ability of recombinant ERK to phosphorylate MBP. Kinase reaction products were seperated on SDS-PAGE and transfer to PVDF membrane, exposed to phosphorimager. Area corresponding to each band was quantified by phosphorimager (Molecular Dynamics). The values are representative from three seperate experiments and are presented in A. **(C) and (D)** C-Raf-1 activity was assessed by using kinase dead MEK as substrate. Kinase reaction products were processed as described above and area corresponding to each band was quantified by phosphorimager (Molecular Dynamics). The values are from three seperate experiments and are shown in C.

Figure 4. **Effect of Farnesyltransferase inhibitor B581 on VS-stimulated MAP Kinase activity**. Confluent, serum starved CHO-HIR cells were pretreated with B581 at indicated concentration for 24 hours and then stimulated with VS (100 μM) or insulin (100 nM) for 5 min. Cell lysates were prepared and subjected to MAP kinase assay as described under Figure 1A. The values are from three separate experiments each performed in triplicate and are shown as mean ± S.E

Figure 5. **Effect of Wortmannin on VS-stimulated P^{70s6k} Activity**: Confluent, serum starved CHO-HIR cells were pretreated without or with wortmannin (100 nM) for 10 min followed by stimulation in the absence (C) or presence of vanadyl sulfate (VS,100 μM) or insulin (ins, 100 nM) for 5 min and lysates were prepared. P^{70s6k} kinase assay activity was determined in immunecomplex using S6 peptide RRRLSSLRA as substrate as described in methods. The values are from four seperate experiments each performed in triplicate and are shown as means ± S.E.

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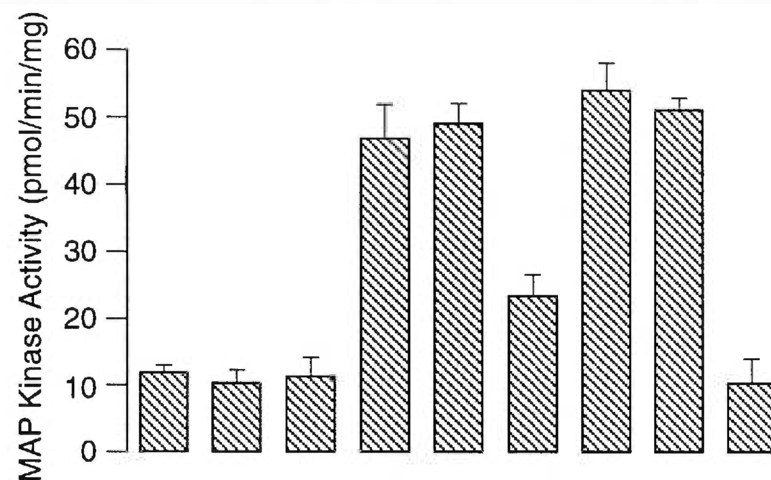
A



B

kDa
143 —
97 —
50 —
35 —
30 —
21 —

← IR β-subunit
← P44
← P42

DMSO	–	+	–	–	+	–	–	+	–
WORTMANNIN	–	–	+	–	–	+	–	–	+
INSULIN	–	–	–	+	+	+	–	–	–
VANADYL SULFATE	–	–	–	–	–	–	+	+	+

Figure 1

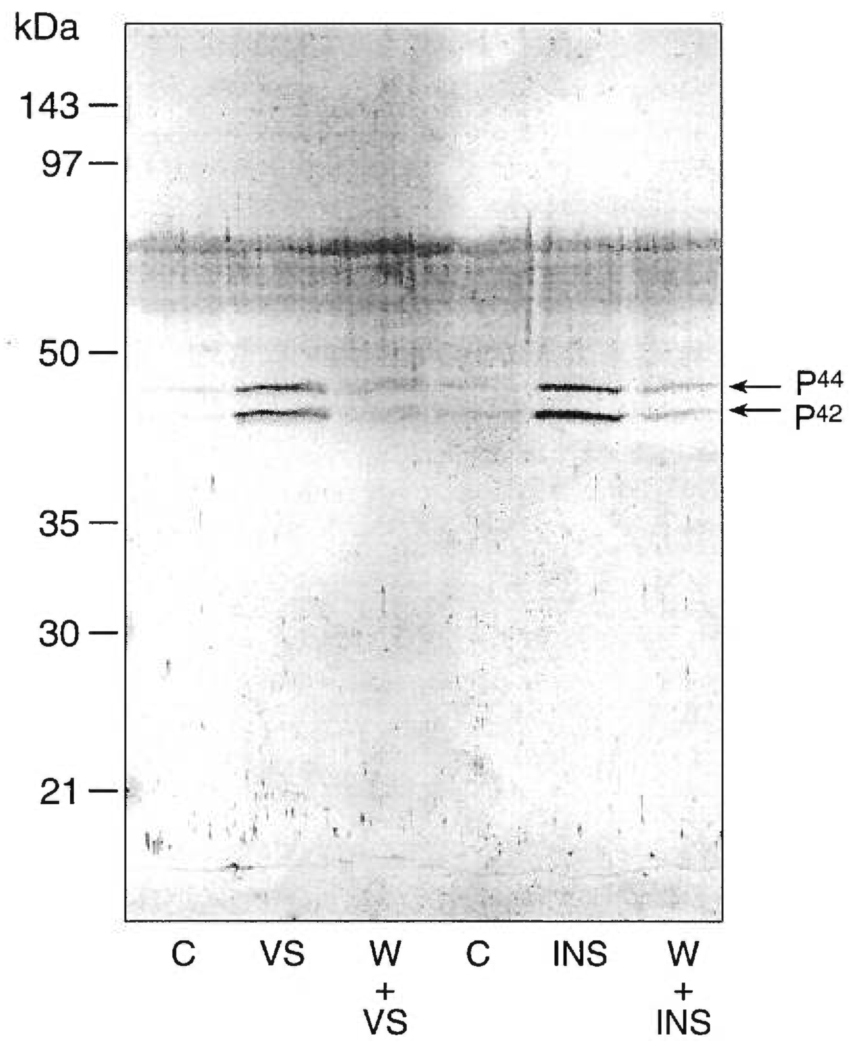


Figure 2

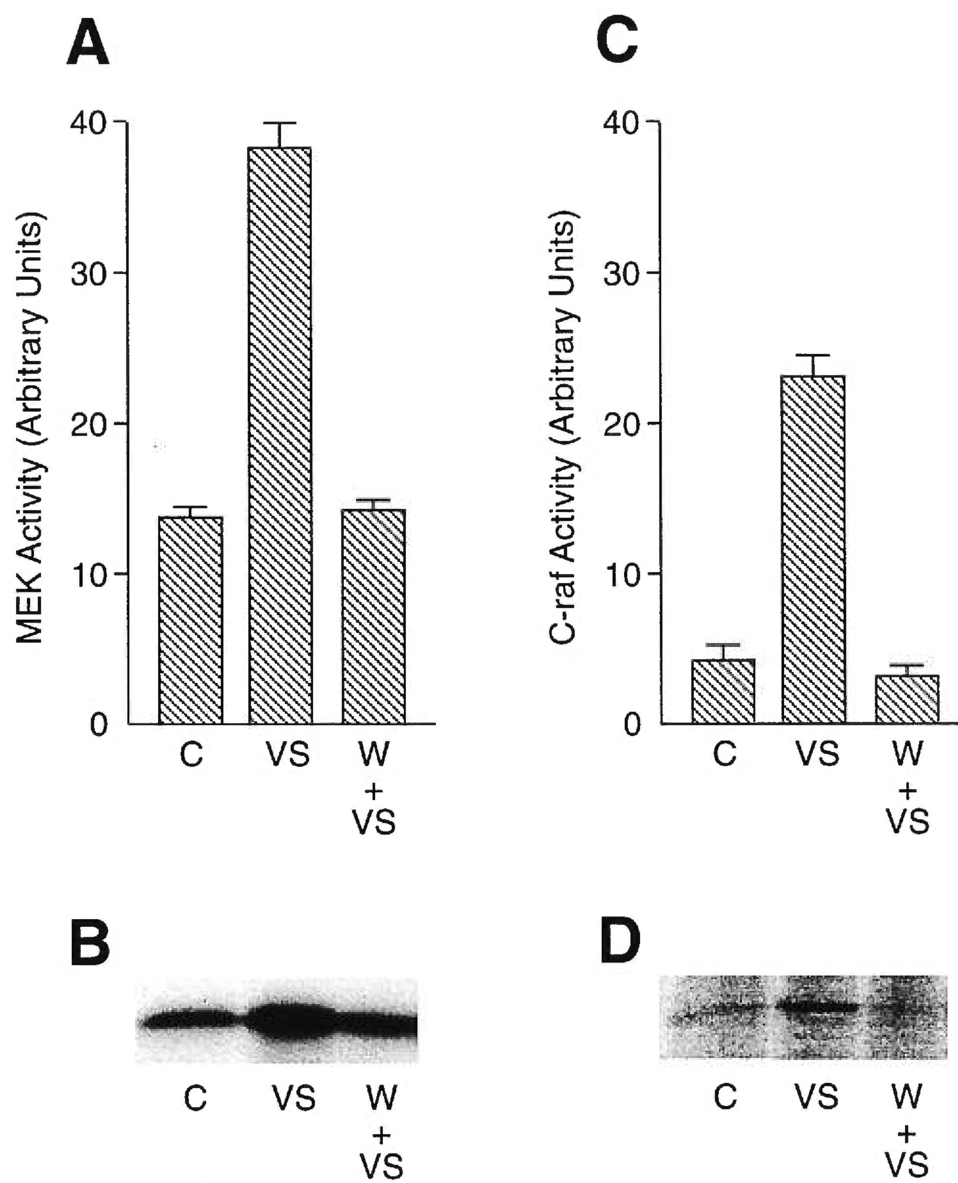


Figure 3

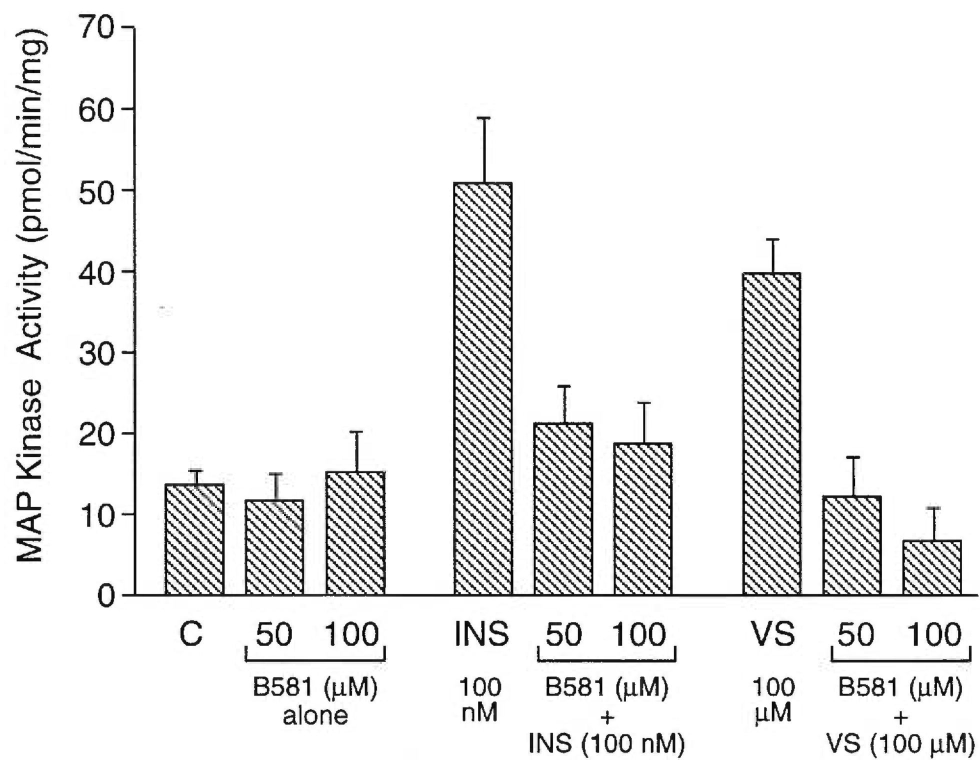


Figure 4

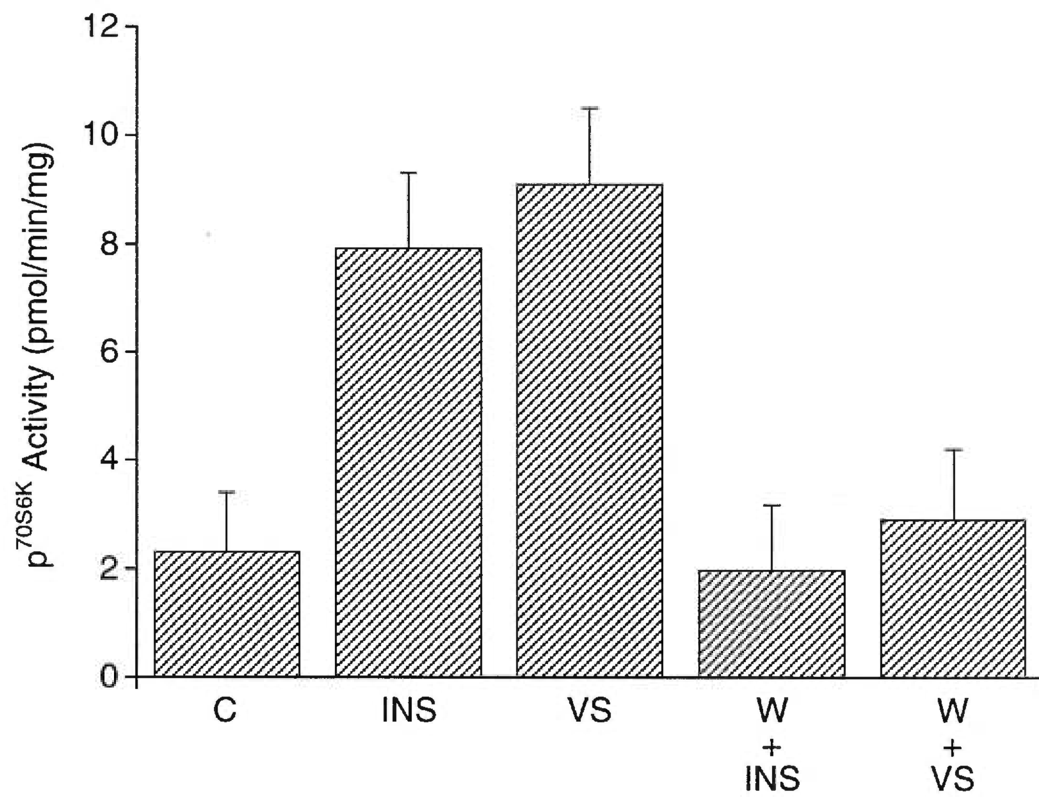


Figure 5

CHAPTER V

General Discussion and Conclusion

DISCUSSION

Over the past two decades considerable evidence has accumulated to show that vanadium salts mimic most of the biological effects of insulin in both *in vitro* and *in vivo* systems. In 1979, vanadium compounds were shown to increase glucose transport and oxidation in adipocytes, to stimulate glycogen synthesis in liver and diaphragm and to inhibit gluconeogenesis (Tolman et. al., 1979). It was not until 1985 when Heyliger et. al. demonstrated that OV administration to STZ-diabetic rats resulted in lowering of blood glucose level, that the interest for elucidating the mechanism by which vanadium salts exert insulin like effects was aroused. Essentially three types of vanadium salts i.e. OV, VS and MV, have been used to study the mechanism of vanadium action as an insulinomimetic agent both at the biochemical and physiological levels. All three salts have proven to be orally effective in lowering blood glucose levels in animal models of diabetes mellitus.

Since the molecular machinery of insulin action at the cellular level is very complex and still poorly characterized, it is understandable that much controversy still surrounds the mechanism underlying the insulin-like effects of vanadium i.e. putative sites of vanadium action are not yet identified. Therefore, with the use of an in vitro model of cultured cell line, CHO-HIR cells, which has been widely used to study insulin action, we set out to examine the insulinomimesis by vanadium salts in relation to the insulin signaling cascade. Several potential sites for vanadium action have been proposed which include both insulin receptor as well as post receptor mechanisms. Most of the insulinomimetic properties of vanadate have been ascribed to an increased tyrosine phosphorylation of proteins involved in

insulin signaling cascades owing to vanadium-induced inhibition of PTPase, the enzyme that catalyzes the dephosphorylation reaction.

The discussion presented here is divided into 4 sections. Section (A) examines the role of receptor PTK in vanadium action. Section (B) discusses the effects of various vanadium salts on MAP kinase and ribosomal kinases. Section (C) examines the contribution of vanadium-stimulated signaling molecules in glycogen synthesis and finally section (D) investigates the possible relationship between MAP kinase and PI3-k pathways.

(A) Vanadium and IR β-Subunit Tyrosine Phosphorylation:

Since phosphorylation of tyrosine residues on the IR β-subunit and IRS-1 constitutes two early steps in the transduction of the insulin signal at the intracellular level, the early studies focused on the possibility that vanadium salts may stimulate tyrosine phosphorylation of IR either directly or through its inhibitory effect on PTPases. Conflicting data was published in the literature demonstrating that vanadate might exert its insulin like effects independent of IR β-subunit tyrosine phosphorylation and PTK activation (Green, 1986; Meyerovitch et.al., 1989; Mooney et.al., 1989; Fantus et.al., 1989; Strout et.al., 1989; Blondel et al., 1990; Shisheva and Shechter, 1992a; D'Onofrio et. al., 1994) or whereas other studies demonstrated that vanadium acted through tyrosine phosphorylation and activation of IR β-subunit (Tamura et. al., 1983; Tamura et. al., 1984; Bernier et. al., 1988; Gherzi et. al., 1988; Pugazhenthi et.al., 1993). Therefore, to further clarify this issue we wished to examine the effect of vanadium salts on IR β-subunit tyrosine phosphorylation and its activation. Treatment of CHO-HIR cells

with three different vanadium salts enhanced the tyrosine phosphorylation of two proteins of molecular sizes of 44 (p^{44mapk}) and 42 (p^{42mapk}) kDa as compared to unstimulated control. However, there was no detectable increase in the tyrosine phosphorylation of IR β-subunit in anti-phosphotyrosine immunoblots from the total cell lysates separated on 10% SDS-PAGE (Figure 2, Chapter 2). By contrast, under similar conditions, a marked increase in tyrosine phosphorylation of IR β-subunit in response to insulin was observed (Figure 2, Chapter 2). These observations were further confirmed by utilizing 3 different approaches where tyrosine phosphorylation and PTK activation of IR β-subunit was performed in the immunoprecipitates obtained by utilizing antibodies against phosphotyrosine and IR β-subunit. In these studies we failed to detect the activation of IR-PTK in response to VS (Figures 3, A, B and C, Chapter 3). Thus, these data clearly demonstrated that, whereas insulin stimulated the tyrosine phosphorylation and autophosphorylation of IR β-subunit, no such effect was observed in response to VS. Therefore, results presented in Figures 3, A, B and C of Chapter 3, indicate that the site of vanadium action is distal to IR-PTK. This notion is further supported by the studies in which OV-stimulated MAPK activation was demonstrated in IR-PTK deficient CHO-1018 cells (D'Onofrio et.al., 1994). It is noteworthy that a possible role of staurosporine (at low concentration)-sensitive cytosolic non-receptor PTK and a membrane associated PTK in mediating the insulinomimetic effect of OV has been suggested in adipocytes (Shisheva and Schechter, 1992a, 1993b; Elberg et. al., 1997). Nonetheless, it is not known if these PTKs play a role in vanadium-induced responses in CHO-HIR cells.

(B) Effect of Vanadium Salts on MAP kinases and Ribosomal kinases:

MAP kinase and S6 kinases have been implicated in the regulation of insulin-induced physiological responses such as glycogen metabolism (Tobe et. al., 1992; Dent et. al., 1990; Welsh et. al., 1994; Shepherd et.al., 1995). It has been demonstrated that p^{90rsk}, a physiological target of MAP kinase, may phosphorylate the glycogen binding subunit of protein phosphatase-1 (PP1-G) resulting in the activation of PP-1 (Dent et. al., 1990). This causes an increase in dephosphorylation and activation of glycogen synthase to enhance glycogen synthesis and dephosphorylation and inactivation of phosphorylase kinase to reduce glycogenolysis (Dent et.al., 1990). The p^{70s6k} and p^{90rsk} isoforms have been shown to phosphorylate and inactivate GSK-3 in vitro (Sutherland et.al., 1993; Sutherland et. al., 1994; Woodget, 1991). Moreover, in the light of suggestions that VS is more potent than OV in eliciting hypoglycemic effects (Pederson et.al., 1989) we investigated this premise at the cellular level by comparing the mechanism of action of different vanadium salts on MAP kinase and ribosomal kinases, p^{90rsk} and p^{70s6k}. Treatment of CHO-HIR cells with these three vanadium salts at 100 μM concentration for 5 min. resulted in an enhanced tyrosine phosphorylation of p^{44} (ERK I) and p^{42} (ERK II) with a parallel activation of MAP kinase activity similar to insulin (Figure 3, chapter 2). Our studies further demonstrated that S6 kinases, p^{90rsk} which is the immediate downstream signaling molecule to MAP kinase, as well as p^{70s6k} , are also activated by vanadium salts and these effects were comparable to that of insulin (Figure, 4 and 5, Chapter 2). Moreover, VS appeared to be slightly more potent than either OV or MV in

stimulating MAP kinase as well as ribosomal kinases. Therefore, results from these experiments suggest that activation of these signaling molecules might constitute one of the mechanisms by which vanadium salts exert insulin like effects. Our observation was further supported by some other reports. In one such study, VS treatment normalized blood glucose concentration which was correlated well with the improved activation of S6 kinase (Hei et. al., 1995). Vanadium salts given intraperitoneally have also been shown to stimulate MAP kinase (Tobe et. al., 1992), p^{70S6k} activity (Kozma et. al., 1989) and S6 peptide activity (Tobe et. al., 1992) in rat liver, but not in rat skeletal muscle (Hei et.al., 1995). In another study, MV therapy of diabetic human subjects, whose S6 kinase and MAP kinase activities were diminished, were increased to the levels similar to those produced by in vitro stimulation with insulin (Goldfine et.al., 1995). Moreover, in these studies MV also improved glucose homeostasis.

As discussed above, several earlier studies suggested that MAP kinase and ribosomal kinases are important for insulin-induced biological effects such as glycogen synthesis, however this notion was challenged in several later studies. A synthetic inhibitor of MEK, PD98059 from the Parke-Davis pharmaceutical company, which specifically inhibits MAP kinase activation and downstream biological responses, was utilized to study the role of MAP kinase and p^{90rsk} in insulin action. It was shown that PD98059 had no effect on insulin-stimulated inhibition of GSK-3 in the myotubes from L6 cell and in cultured human myoblasts (Cross D.A.E, 1995; Hurel et. al., 1996). This inhibitor was also unable to attenuate insulin-induced activation of PP-1, glycogen synthase and glycogen

synthesis (Lazar et. al., 1995; Azpiazu et. al., 1996). In addition, the cells transfected with dominant negative mutants of ras, despite having an attenuated MAP kinase activation in response to insulin, exhibited a normal stimulation of glycogen synthase/glycogen synthesis further supporting the lack of a role of MAP kinase pathway in contributing to these responses (Yamamoto-Honda et. al., 1995). By contrast, MAP kinase signaling has also been implicated in DNA (Dent et.al., 1990) and protein synthesis (Lin et.al., 1994; Pause et.al.,1994). A large number of putative cellular targets such as transcription factors, protein kinases, cytosolic proteins and cytosolic phospholipase A_2 have also the physiological targets of MAP kinase (Johnson et.al., 1994).

Similar to MAP kinase and p^{90rsk}, p^{70s6k} was thought to mediate insulin-stimulated glycogen metabolism. The immunosuppressant drug, rapamycin, which inhibits p^{70s6k}, has been used to explore the role of p^{70s6k} in insulin-stimulated physiological responses. Initially it was shown that rapamycin treatment did not block insulin-induced inactivation of GSK-3 (Cross et. al., 1994; Welsh et. al., 1994; Moule et.al., 1995) suggesting that p^{70s6k} may not regulate glycogen metabolism. Furthermore, lack of inhibition of insulin-stimulated glycogen synthase activation by rapamycin in adipocytes suggested that ribosomal S6 kinase is insufficient to stimulate glycogen synthesis (Lin and Lawerence, 1994). However, a partial inhibition of glycogen synthase activation/glycogen synthesis was observed in 3T3-L1 adipocytes, rat diaphragm and human myoblasts (Shepherd et.al., 1995; Azpiazu et. al., 1996; Hurel et.al., 1996). In addition, ribosomal kinases have been implicated in enhanced S6 phosphorylation which has been

linked to increased rates of protein synthesis in response to insulin and other mitogens (White and Khan, 1994; Sturgil, 1988). By contrast, it has been demonstrated that selective inhibition of p^{70s6k} by rapamycin prevented S6 phosphorylation and inhibited cell proliferation (Kuo et.al., 1992; Chung et.al., 1993). Furthermore, p^{70s6k} has been also shown to play an important role in insulin-stimulated phosphorylation of a translational repressor, PHAS-I which dissociates from its inactive complex, PHAS-I-eIF-4E, upon insulin stimulation and initiates translation (Azpiazu et al., 1996; Lin et.al., 1995). Rapamycin treatment blocks insulin-stimulated PHAS-I phosphorylation and thus protein synthesis. Hence, these studies suggest that insulin, which utilizes S6 kinases and MAP kinase signaling molecules to elicit mitogenic and protein synthesis responses, and vanadium salts which also exert similar effects on many cell types (Klarlund, 1985; Sabbioni et.al., 1993), may utilize these signaling events to promote cell proliferation and differentiation.

(C) Role of PI3-k in VS-Stimulated Glycogen Synthesis:

Since our earlier studies demonstrated that VS was more potent than OV or MV in stimulating MAP kinase and ribosomal kinases, p^{90rsk} and p^{70s6k} (Chapter 2), the subsequent studies utilized VS to study its effect on various other signaling components such as PI3-k as well as examined the contribution of these signaling molecules in VS-stimulated glycogen synthesis. The role of MAP kinase in VS-stimulated glycogen synthesis was evaluated by utilizing a MEK inhibitor-PD98059. Treatment of CHO-HIR cells with PD98059 inhibitor, which almost completely blocked VS-stimulated MAP kinase activity (Table 1, chapter 3), did

not block VS-induced activation of glycogen synthesis, as measured by monitoring (^{14}C)glucose incorporation into glycogen (Table 1, Chapter 3). These data suggested that MEK and downstream signaling molecules may not contribute to VS-stimulated glycogen synthesis. A similar observation was made by Lazar et.al., (1995) demonstrating that insulin-induced glycogen synthesis does not require the MAP kinase pathway. Additional evidence was provided by demonstrating that insulin-stimulated glycogen synthesis was not affected by expression of dominant negative ras mutants that disrupts the Ras/MAP kinase signaling cascade in 3T3-L1 adipocytes (Dorrestijn et.al., 1996) and PC12 cells (Yamamoto-Honda et.al., 1995). However, some of these observations are at variance with other reports described in section B. Thus, further studies are required to establish the precise role of MAPK pathway in VS-stimulated glycogen synthesis.

Since we observed that 70-kDa ribosomal kinase p^{70s6k} was stimulated by VS (Figure 5, Chapter 2), the role of this kinase in VS-stimulated glycogen synthesis was assessed by utilizing an immunosuppressant drug, rapamycin. Treatment of CHO-HIR cells with rapamycin significantly inhibited VS-stimulated p^{70s6k} activity (Table 1, Chapter 3) but failed to inhibit glycogen synthesis in response to VS. Similar observations were made while studying insulin-stimulated glycogen synthesis in various cell types such as PC12 cells (Yamamoto-Honda et.al., 1995), skeletal muscle (Moxham et.al., 1996), rat adipocytes (Lin et.al., 1994; Moule et.al., 1995) and CHO-HIR (Sakaue et.al.,1995a). However, some of these reports showed that insulin-induced glycogen synthesis could be attenuated by rapamycin treatment in 3T3-L1 adipocytes and rat diaphragm (Shepherd et.al.,

1995; Azpiazu et.al., 1996). Thus, the conflict between these studies may be attributed to the existence of alternate pathways that may operate in a cell specific manner. In this regard a recent report demonstrated that JNK and ribosomal kinase III (rsk III) may be required for insulin-stimulated glycogen synthesis in skeletal muscle *in vivo* (Moxham et.al., 1996).

Since PI3-k has been implicated to play a key role in insulin-stimulated metabolic responses, subsequent studies were conducted to assess the contribution of PI3-k in VS-stimulated glycogen synthesis. Two specific inhibitors of PI3-k, wortmannin and LY294002, have been utilized to examine the role of PI3-k in VS-stimulated glycogen synthesis. Both inhibitors are structurally and mechanistically different. Wortmannin, a fungal metabolite, irreversibly inhibits PI3-k in the nanomolar range while LY294002, a synthetic compound from Eli Lilly company, reversibly inhibited PI3-k in the micromolar range. Pretreatment of CHO-HIR cells with these inhibitors significantly blocked VS-stimulated glycogen synthesis presumably by attenuating PI3-k activity and thus suggesting that PI3-k may be mediating this effect. To further confirm this, we have also directly measured the PI3-k activity in p85 regulatory subunit immunoprecipitates. As shown in Figure 2A, (Chapter 3), VS-stimulated PI3-k activity in a concentration dependent manner which was significantly inhibited by wortmannin (Figure 2C, Chapter 3). Our findings are the first to show that PI3-k is activated by inorganic vanadium salts and is required for VS-stimulated glycogen synthesis.

Since PI3-k activation occurs through its association with tyrosine phosphorylated IRS-1, the tyrosine phosphorylation of IRS-1 was assessed by

incubating CHO-HIR cells with increasing concentration of VS followed by immunoprecipitation with anti-IRS-1 antibody and detection by immunoblotting with antiphosphotyrosine antibody. The results revealed that VS enhanced IRS-1 tyrosine phosphorylation in a concentration dependent manner. Subsequent experiments showed that VS stimulated the PI3-k activity in IRS-1 immunoprecipitates suggesting that PI3-k is recruited to IRS-1 in response to VS (Figure 4, Chapter 3). It is noteworthy that PI3-k activity was higher in IRS-1 immunoprecipitates than p85 immunoprecipitates (Figures 2A,B and 4B,C Chapter 3). Although, insulin-stimulated metabolic effects such as glycogen synthesis and glucose transport have been shown to be mediated by PI3-k (Shepherd et. al., 1995; Cheatham et. al., 1994), our observation is the first to show that inorganic vanadium salts-induced activation of glycogen synthesis requires PI3-k. A recent report also demonstrated that vanadate-induced activation of PI3-k and glucose uptake is attenuated by the PI3-k inhibitor, wortmannin, in rat adipocytes (Li et. al., 1997). However, some other insulin-like effects of vanadium such as IGF-BP gene expression, glucose transport and 2-deoxy hexose uptake stimulated by vanadate and pervanadate were not affected by PI3-k inhibitors suggesting that an alternative signaling mechanism might mediate these responses (Band and Posner, 1997; Tsiani et.al., 1997; Ida et.al., 1996). Furthermore, in the light of recent observation that PKB, a Ser/Thr kinase, which lies immediately downstream to PI3-k, is regulated by insulin, vanadate and peroxovanadate in rat adipocytes (Wijkander et. al., 1997) strongly suggest that the IRS-1-PI3-k-PKB pathway may

constitute one of the mechanisms by which insulin-like metabolic responses of vanadium salts are exerted.

Since vanadium salts act independently of IR-PTK to enhance the tyrosine phosphorylation of IRS-1, the mechanism by which IRS-1 was tyrosine phosphorylated remains to be clarified. Vanadium salts are potent PTPase inhibitors (Swarup et. al., 1984) thus suggesting that inhibition of a constitutively-active PTPase may enhance IRS-1 tyrosine phosphorylation. Alternatively Janus Kinases (JAK) might be activated by VS to stimulate phosphorylation of IRS-1 since a recent report implicates JAK in the phosphorylation of IRS-1 and IRS-2 (Waters and Pessin, 1996). In addition, it is also possible that VS can stimulate staurosporine-inhibitable Cyt-PTK which in turn may phosphorylate IRS-1 (Shisheva and Shechter, 1992a., 1993b). However, presently the nature of the kinase(s) or PTPase(s) that cause enhanced tyrosine phosphorylation of IRS-1 is not known and additional studies are required to identify these enzymes.

(D) Requirement of PI3-k by VS-stimulated MAP kinase Pathway:

A role of wortmannin-inhibitable PI3-k for several agonists such as growth factors which include insulin and EGF, cytokines and various hormone-stimulated activation of the MAP kinase pathway has been demonstrated in various cell lines (Cross et.al., 1994; Hurel et.al., 1996; Welsh et.al., 1994; Uehera et.al., 1995; Standaert et.al., 1995; Nishioka et. al., 1995; Kilgour et.al., 1996; Sugawara et.al., 1996). Since we have observed that VS stimulated MAP kinase as well as PI3-k activities, the next questions we asked were: (1) if upstream signaling molecules to MAP kinases are also activated by VS and if so (2) whether these responses could

be sensitive to wortmannin. Our results demonstrated that similar to insulin, VS stimulated MEK and c-Raf-1 activities by about 3-4 fold which were significantly inhibited by wortmannin pretreatment of the CHO-HIR cells (Figure 2, chapter 4). Furthermore, a role of ras was assessed by utilizing a farnesyltransferase inhibitor, B581, that inhibits ras attachment to the plasma membrane as well as its activation and thus downstream signals. Incubation of CHO-HIR cells with B-581 resulted in significant inhibition of both the VS and insulin-induced activation of MAP kinase activity. These data therefore, implicates ras as an upstream regulator of VS-stimulated MEK-Raf-MAP kinase-p^{90rsk} cascade. Thus, the inhibition of VS-induced activation of MEK, Raf, MAP kinase activities by the PI3-k inhibitor wortmannin, suggests that a relationship exists between PI3-k and MAP kinase pathways. However, studies from other laboratories have shown that wortmannin treatment did not result in attenuation of insulin or EGF-stimulated Raf-1 activity (Suga et.al., 1997; Karnitz et.al., 1997). Thus, the conflicting results may seem to be due to either differences in cell lines used or experimental conditions employed.

Thus, our studies demonstrate the VS-induced stimulation of Ras/MAP kinase and PI3-k cascades but only PI3-k pathway contributed to glycogen synthesis, and these effects were independent of tyrosine phosphorylation and activation of IR β-subunit. The MAP kinase pathway, by contrast, may play an important role in other biological responses such as cell differentiation and proliferation. A schematic model of vanadium action in relation to insulin signaling cascade is presented in Figure 1.

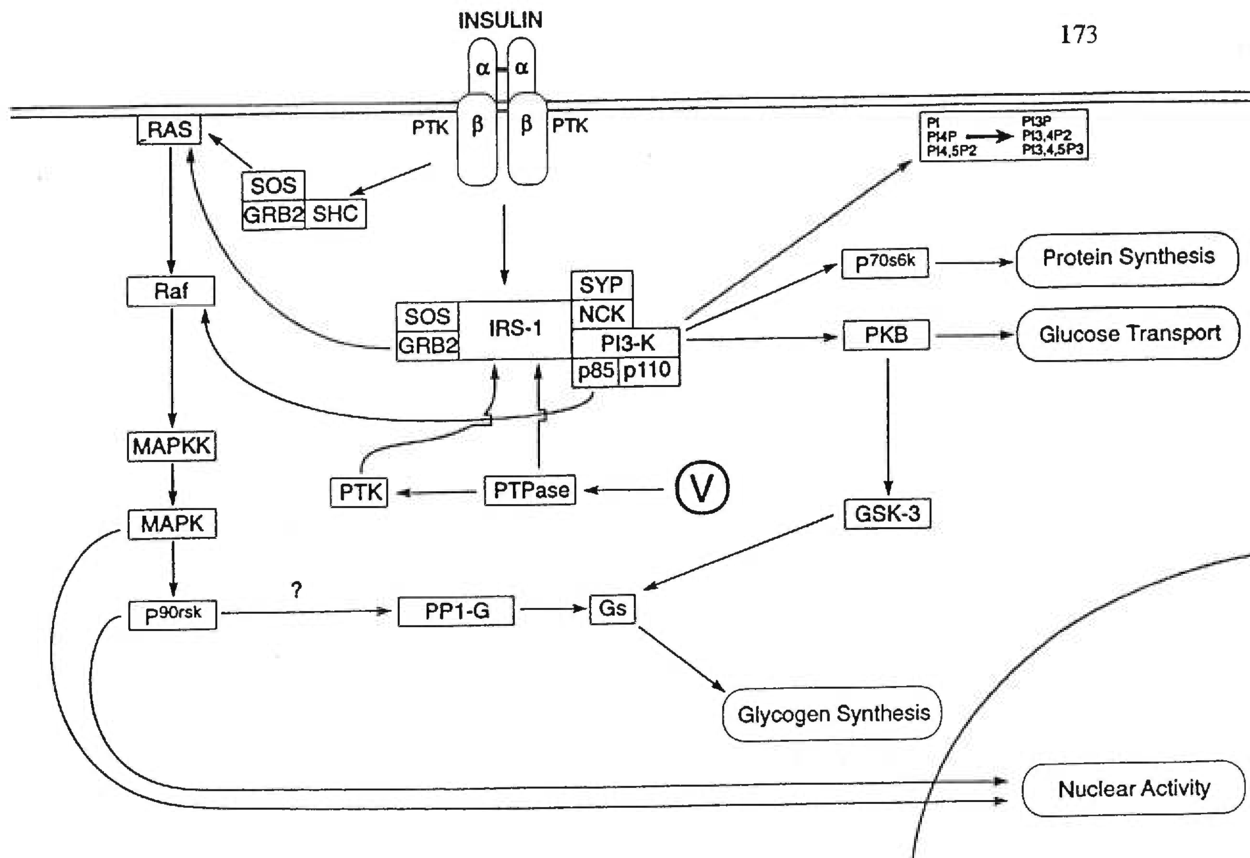


Figure 1: A model representing possible mechanism of vanadium action in relation to insulin signaling pathway.

Vanadium salts (V) mimic insulin-like effects by stimulating Ras-Raf-MAP kinase, PI3-k and p^{70s6k} signaling pathways independent of IR-PTK activation but in association with tyrosine phosphorylation of IRS-1. V-induced tyrosine phosphorylation of IRS-1 may occur either by inhibition of a constitutively active PTPase(s) or activation of cytosolic PTK secondary to PTPase(s) inhibition.

Conclusion and Future direction:

The results presented in this dissertation demonstrate that similar to insulin, VS stimulates both Ras-MAPk-p^{90rsk} as well as PI3-k pathways. Furthermore, similar to insulin, VS-stimulated glycogen synthesis is mediated via the PI3-k dependent pathway. However, in contrast to insulin, which requires functionally active IR-PTK to exert its effects, VS-induced responses occurred in the absence of IR-β subunit tyrosine phosphorylation. In addition, VS interestingly despite having no effect on IR tyrosine phosphorylation, enhanced the tyrosine phosphorylation and association of IRS-1 with PI3-k. Furthermore, we demonstrate for the first time that VS stimulated Ras-MAP kinase cascade requires wortmannin-sensitive PI3-k activation.

The exact mechanism by which IRS-1 is tyrosine phosphorylated in response to VS in not known. Since vanadium salts are inhibitors of PTPases, it may be speculated that VS may inhibit a IRS-1 specific PTPase and results in an enhanced IRS-1 tyrosine phosphorylation. Alternatively a non-receptor cytosolic PTK may be activated by VS which in turn can increase IRS-1 tyrosine phosphorylation. Thus, further studies in identifying the specific PTPase or PTKs would be helpful to explain the mechanism of VS-stimulated tyrosine phosphorylation and activation of IRS-1.

Thus, taken together the present studies suggests that although both arms of the insulin signaling cascade are activated by VS, only the PI3-k dependent cascade mediates VS-stimulated glycogen synthesis whereas the MAP kinase pathway may play a role in some other biological responses such as cell proliferation and differentiation.

Since most of the effects of vanadium salts are due to the inhibition of PTPase, the identification of PTPase(s) may help in understanding the exact mechanism of their action. Some potential targets are PTP1-B and Shp2. Utilization of cells deficient in these PTPases would answer some of the important questions. It is anticipated that knowledge gained will eventually lead to the development of novel therapy for the better management of diabetes mellitus.

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ANNEXE

Contribution of Authors:

In this dissertation all the experiments were performed by myself. Dr. Ashok K. Srivastava provided guidance, insight and very useful ideas throughout this project. He also read my thesis and helped me in editing the articles as well as other parts of thesis. Dr. Madhu B. Anand-Srivastava provided helpful suggestions and also read the articles. Dr. Jean-Louis Chiasson discussed the project and also gave suggestions which were of help.