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Insulino-mimetic and Anti-diabetic Effects of Zinc

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Résumé

Il est connu depuis longtemps que le zinc (Zn) est primordial pour la croissance et maintien des systèmes biologiques. De plus, le Zn a aussi démontré des propriétés insulino-mimetiques et anti-diabétiques, comparables à l'insuline dans des cellules isolées, des tissus, ainsi que chez différents types d'animaux ayant un diabète de type 1 et de type 2. Le traitement au Zn améliore les anomalies du métabolisme des glucides et des lipides. Dans les cellules isolées, il rehausse le transport du glucose, la synthèse du glycogène et des lipides, et empêche la gluconéogenèse et la lipolyse.

Il a été démontré que les mécanismes moléculaires responsables des effets Zn, comparables à ceux induits par l'insuline, impliquent l'activation de plusieurs composantes clés de la voie de signalisation de l'insuline. Celles-ci incluent les extracellular signal-regulated kinase1/2 (ERK1/2), le phosphatidylinositol 3-kinase (PI3-K), et la protéine kinase B (PKB). Cependant, les mécanismes moléculaires par lesquelles le Zn active ces voies de signalisation ne sont pas encore très clairs. Bien que les signaux induits par le Zn sont associé à la phosphorylation des tyrosines du récepteur de l'insuline (IR), ainsi que l'activation de l'Insulin-like Growth Factor-1 Receptor (IGF-1R) et de l'Epidermal Growth Factor Receptor (EGFR) dans des différents systèmes cellulaires, la contribution spécifique de chacun de ces récepteurs protéine tyrosine kinase (R-PTK) dans la médiation de la signalisation de ERK 1/2 et PKB induite par le Zn n'est pas encore établis. De plus, le besoin de l'activité PTK du IR dans les effets induits par le Zn reste controversé, et aucune preuve pour le rôle de IGF-1R dans ce processus n'a été donnée. Puisque les voies ERK

1/2 et PI3-K/PKB sont responsables pour les effets mitogènes et métaboliques de l'insuline, respectivement, l'objectif principal de cette étude est d'étudier le rôle des R-PTK dans l'activation de ERK 1/2 et PKB par le Zn.

En utilisant des inhibiteurs pharmacologiques, ainsi que des cellules surexprimant des formes normales et mutantes de IR et IGF1R, nous avons étudié le rôle de plusieurs R-PTKs dans la phosphorylation de ERK1/2 et PKB induite par le Zn. Nos résultats démontrent que le Zn stimule la phosphorylation de PKB et ERK1/2 d'une manière dose et temps dépendante dans les cellules CHO-IR. Le prétraitement avec AG 1024, inhibiteur de IR et IGF1R, bloque la phosphorylation de PKB et ERK 1/2 en réponse au Zn, mais l'AG 1478, inhibiteur du EGFR, était sans effet. Dans les cellules CHO surexprimant une forme inactive du IR (CHO-1018), le Zn induit toujours la phosphorylation de PKB et ERK1/2, tandis que la phosphorylation de PKB et ERK1/2 induite par l'insuline est aboli dans cette lignée cellulaire. Aussi, la phosphorylation de PKB et ERK 1/2 induite par le Zn est atténué dans les cellules IGF1R KO. Ensemble, ces résultats suggèrent que la phosphorylation de PKB et ERK 1/2 induite par le Zn est dépendante de IGF1R-PTK, mais indépendante de IR- et EGFR-PTK.

Mots-clés : Zinc, Agents insulino-mimétique, Récepteur d'insuline, Insulin-like Growth Factor-1 Receptor, Protein Kinase B, Extracellular signal-regulated kinase 1/2, Diabètes

Abstract

It has long been known that Zn is crucial for the proper growth and maintenance of normal biological functions. Zn also has been shown to exert insulin-mimetic and anti-diabetic effects. These insulin-like properties have been demonstrated in isolated cells, tissues, and different animal models of type 1 and type 2 diabetes. Zn treatment has been found to improve carbohydrate and lipid metabolism in rodent models of diabetes. In isolated cells, it enhances glucose transport, glycogen and lipid synthesis, and inhibits gluconeogenesis and lipolysis.

The molecular mechanisms responsible for the insulin-like effects of Zn compounds have been shown to involve the activation of several key components of the insulin signalling pathways, which include the extracellular signal-regulated kinase1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI3-K) / protein kinase B/Akt (PKB/Akt). However, the precise molecular mechanisms by which Zn triggers the activation of these pathways remain to be clarified. Although Zn-induced signalling has been linked to an increase in the tyrosine-phosphorylation of insulin receptor (IR), as well as activation of insulin-like growth factor-1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR) in different cell systems, the specific contribution of each of these receptor protein tyrosine kinases (R-PTKs) in mediating Zn-induced ERK1/2 and PKB signalling remains to be established. In addition, the requirement for insulin receptor (IR) PTK activity in Zn-mediated effects is still controversial, and no clear evidence for a role of IGF-1R in this process has been provided. Since the ERK 1/2 and PI3-K/PKB pathways are implicated in mediating the mitogenic and metabolic effects of insulin respectively, the main objective of

this study was to investigate the role of R-PTKs in Zn-induced ERK 1/2 and PKB/Akt activation. By using pharmacological inhibitors, as well as cells that overexpress normal and mutant/inactive forms of IR and IGF-1R, we have investigated the role of various R-PTKs in Zn-induced ERK1/2 and PKB phosphorylation. Zn stimulated ERK1/2 and PKB phosphorylation in a dose and time-dependent manner in Chinese hamster ovary cells overexpressing IR (CHO-IR). Pretreatment with AG1024, an inhibitor for IR and IGF-1R, blocked Zn-induced ERK1/2 and PKB phosphorylation, but AG1478, an inhibitor for EGFR was without effect. In CHO cells overexpressing tyrosine kinase deficient IR (CHO-1018), Zn was still able to induce the phosphorylation ERK1/2 and PKB/Akt, whereas insulin-induced ERK1/2 and PKB/Akt phosphorylation was abolished in these cells. Moreover, Zn had no effect on the tyrosine phosphorylation of IR-β-subunit and IRS-1 in CHO-IR cells. Furthermore, in IGF-1R knockout cells, both IGF-1 and Zn were unable to stimulate the phosphorylation of ERK1/2 and PKB. Taken together, these data suggest that Zn-induced ERK1/2 and PKB/Akt phosphorylation is independent of IR- or EGFR-PTK, but requires IGF-1R-PTK.

Keywords : Zinc, Insulin signal mimicry, Insulin Receptor, Insulin-like Growth Factor-1 Receptor, Protein Kinase B, Extracellular signal-regulated kinase 1/2, Diabetes

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Chapter 1 – Introduction

1.1 – Metals and Diabetes: An Odd Pairing

Diabetes Mellitus is a major global health problem characterised by hyperglycaemia, polyurea, polydipsia, and polyphagia (*1*). Two major forms of the disease exist: Type 1 diabetes, or insulin-dependent diabetes mellitus, formerly also known as juvenile diabetes, and type 2 diabetes, or non-insulin-dependent diabetes mellitus.

In type 1 diabetes, there is an uncontrolled autoimmune response against insulin secreting pancreatic β-cells, leading to the destruction of the latter, and leaving the patient with a lack of insulin (*2;3*). Up to 10% of diabetics suffer from this form of the disease, and it generally occurs in young and lean patients (*4*). In type 2 diabetes, pancreatic β-cells still produce insulin, however, due to peripheral insulin resistance, there is a decrease in the uptake and transport of blood glucose by muscle and liver tissue (*5;6*). Under these conditions, to compensate for the excess glucose in the blood stream, the β-cells increase their production of insulin. This overproduction of insulin results in an increase in the pancreatic β-cells mass, which can cause hyperinsulinemia, further exacerbating the insulin resistance (*7*), leading to full fledged type 2 diabetes (*8-10*). Over 90% of the diabetic population suffers from type 2 diabetes.

According to the World Health Organization, it is estimated that around 171 million people world-wide suffered from diabetes mellitus in 2000, and that number may double by

the year 2030 (*11*). Current treatments, such as daily insulin injections, in conjunction with or independently of other oral anti-diabetic medications, such as biguanides, sulfonylureas, and thiazolidinediones (TZDs), work to either reduce hepatic glucose production, to increase pancreatic β-cell insulin production and secretion, and to increase insulin sensitivity in peripheral tissues (*12*). These medications help patients reach euglycaemia, yet are not without their own side effects, namely weight gain, increased LDL cholesterol, edema, hypoglycemia and increase of baseline waist circumference (*13*). Therefore, newer therapeutic approaches are needed to treat diabetes more efficiently, and more cost effectively.

It has long been known, even before the discovery of insulin, that many metals, as well as their salt derivatives, possess insulin mimetic and anti-diabetic effects. For example, in 1899, 22 years before the discovery of insulin, sodium orthovanadate (Na_3VO_4), a vanadium salt, was shown to decrease glucosuria in two out of three diabetic patients (*14*). However, it took almost one century for interest in this area of research to peak, when it was shown that multiple vanadium salts were able to stimulate glucose transport and oxidation (*15-18*), as well as lipogenesis in adipocytes (*15-20*). These salts also enhanced glycogen synthesis in the rat diaphragm and hepatocytes (*15;21;22*), and inhibited gluconeogenesis in the liver (*15*) . The demonstrations that Na_3VO_4 was capable of normalizing hyperglycaemia in animal models of diabetes mellitus (*23;24*) sparked the interest of clinicians in the metal and it was found that Na_3VO_4 and vanadyl sulphate (VS) were able to improve hyperglycaemia in several limited human studies (*25-30*).

Molybdenum (Mo) is also a transition metal, in the group VI B, and like vanadium, it can exist in multiple oxidation states (five to be exact: 2, 3, 4, 5, and 6), and exerts anti-diabetic properties (*31;32*). Molybdate was found to increase glucose transport and oxidation in adipocytes and decrease blood glucose in Streptozotocin-induced diabetic rats (*33;34*), and in a genetically obese, insulin-resistant ob/ob model of mouse (*35*). Molybdate also increased insulin receptor (IR) autophosphorylation, as well as the phosphorylation of IR substrate pp160 (*36*).

Chromium (Cr) is yet another transition metal with insulin-mimetic and insulin-enhancing effects. Also a group VI trace metal, it is considered an essential trace mineral (*37*). Cr can exist in four different oxidation states, and due to this, its ions and complexes, similar to those of vanadium and molybdenum, take on a variety of beautiful colours. In its biologically stable trivalent form, Cr has been shown to improve insulin response to glucose in rats fed a high sucrose-low Cr diet, hypothetically by preserving normal peripheral tissue insulin sensitivity, and by playing a role in the maintenance of normal β-cell glucose sensitivity (*38*). It has also been shown that insulin-stimulated glucose transport was improved by Cr, by increasing the amount of the glucose transporter 4, GLUT4, being mobilised to the plasma membrane of adipocytes, as well as increasing membrane fluidity by decreasing plasma membrane cholesterol (*37*). Similar findings were shown in vivo, when Cr enhanced insulin stimulated GLUT4 translocation, as well as insulin sensitivity and glucose disappearance, and improved the lipid profile in obese and insulin resistant rats (*39*).

Zinc (Zn) is yet another metal with potent insulin-sensitizing and insulin-mimicking effects. This work will focus on the insulin-mimetic and anti-diabetic effects of Zn, and discuss in detail its chemistry, its role in type 1, as well as type 2 diabetes mellitus, and its mechanism of action at the molecular level.

1.2 – Historical Aspects of Zinc

Zinc (Zn) is a group 12 trace element, and is the 23rd most abundant element in the earths crust (*40*). It is the second most common trace metal found in the body, after iron, and is the fourth most used metal in the world, after iron, aluminium and copper (*41*). Zn has been used since the time of the ancient Egyptians, in the form of zinc oxide (ZnO), to aide in the healing of wounds and burns (*42*). It was also used in later centuries by the ancient Greeks and Romans, taken from calamine ores (zinc ores discovered in the Belgian city Calamine) in a copper-zinc alloy to produce brass. By the late 1300's, Zn was recognised as a new metal in India, and was mined extensively in Zawar, India, for both brass production and medicinal purposes. In the western world, the discovery of pure Zn is most often credited to the German chemist Andreas Marggraf, when in 1746, he extracted Zn by heating calamine ores and carbon together (*43*).

In nature, Zn occurs as a lustrous bluish-white metal. It is interesting to note that in nature, this trace metal is only present in its divalent state, Zn(II).

In humans, Zn is found in all tissues and tissue fluids. In an average 70 kg male, the total quantity of Zn is estimated to be approximately 2.3 grams, making it the most prevalent trace metal found in tissue (only iron is found in higher concentrations in the human body at about 4 grams, but is located primarily in blood (*44*)) Over 75% of this Zn is located in skeletal muscle and bone tissue (*40*). As well, apart from iron, zinc is the only other element for which nutritional requirements have been established (*45*).

Important sources of Zn are found in most unprocessed food sources, yet Zn concentrations may vary greatly from one type of tissue to another, in the same food source; for example, lean beef contains 43mg of Zn /kg raw meat, whereas a cut of fat beef contains only 10mg/kg raw meat (*46*). Other important sources of Zn are cheddar cheese (40mg/Kg), lentils (31mg/kg), wholemeal wheat (30mg/kg), although it is also found in chicken, rice, pork and sweet corn in moderate quantities (7-20mg/kg), and fish, milk, potatoes and butter in small quantities (1.5-10mg/kg) (*46*).

It has been shown that zinc is essential in growth and development (*47*) and a zinc deficiency has been shown to play a role in multiple diseases, such as malabsorption syndrome, sickle cell disease, chronic liver disease and diabetes (*48*). Zinc deficiency is common in developing nations, yet is also observed in a great number of men and women, from varying economic classes and cultures, suffering from varying clinical conditions, in the United States (*47;49*). The recommended dietary allowance for zinc is 8mg/day for women and 11mg/day for men (*50*).

1.3 – Chemistry of Zinc

1.3.1 – Inorganic Zinc Compounds

As stated earlier, Zn is only present in its divalent state, Zn(II), in nature. It is of low to intermediate hardness (malleable at 100-150 °C), yet is considered a very dense metal, at 7.13g/cm^3, and is therefore called a heavy metal. Being amphoteric, Zn dissolves in both strong alkaline and acidic solutions, and can thus react easily with the latter or other inorganic compounds to form a variety of salts, all of which are nonconducting and nonmagnetic and for the most part, white in colour, with very few exceptions, such as chromate (*51*), which changes colours, from yellow to blue-green, depending on its oxidation state. In industry, this metal is mainly used as a protective coating over iron and other metals against corrosion, in a process called "galvanizing". This protective effect comes from the elements ease of oxidation, due to the fact that it only has two electrons on its outer most shell. It is also extensively used in the construction industry to form metal alloys, and also as an electrode in dry cell batteries, due to its ability of reducing other metal states (*51*).

Zinc oxide (ZnO) is one of the oldest known inorganic zinc compounds. It is an odourless, white/greyish coloured powder. ZnO is soluble in acids and bases, yet not soluble in water or alcohols. It is widely used as a pigment in paints, as an absorber of ultraviolet light (*52*), and as a vulcanizing agent in rubber products (*53*). Other common Zn

compounds, such as zinc chloride ($ZnCl_2$), zinc sulphate ($ZnSO_4$), and zinc nitrate ($Zn(NO_3)_2$) are, on the other hand, very soluble in water and alcohols (*54*).

1.3.2 – Organic Zinc Compounds

In contrast to inorganic Zn compounds, organo-metallic Zn compounds do not exist naturally in the environment (*41*). The latter are synthesized, and are frequently used as topical antibiotics and fungicidal lotions (*55*). Organic Zn compounds can be separated into three main groups (*56*) :

- Organic Zn halides (R-Zn-X), X being any halogen.
- Di-organic Zn molecules (R-Zn-R), R being any alkyl or aryl group.
- Lithium or magnesium Zn compounds ($M^+R_3Zn^-$), M being either lithium or magnesium.

The first organo-Zn compound ever synthesized was Diethyl zinc $(C_2H_5)_2Zn$. It was also the first ever compound to display a sigma bond between a metal and a carbon molecule (*57*). It reacts violently with water and catches fire with ease when in contact with air, and therefore must be handled under nitrogen. It is for this reason that $(C_2H_5)_2Zn$ is known as a hypergolic fuel, because it ignites on contact with air. These types of compounds are often used to propel rockets. Some common reactions used to obtain organo-Zn compounds are oxidative reactions, like the one used by Frankland and Duppa to produce diethyl zinc (*57*), halogen zinc exchange reactions, and transmetalation reactions.

Many organic zinc compounds are used for their medicinal or biological functions. For example, Zn-etheylene-bis(dithiocarbamate)), also known as Zineb, and Zn-dimethyl-dithiocarbamate, or Ziram, are often used as fungicides to protect crop harvests from disease and deterioration while still in the field, in storage, and during transport (*58;59*). Zinc carbonate, which is a mineral ore of zinc, is often used in to treat skin problems, and zinc caprylate is most often used in ointments as a topical antifungal cream (*54;60*).

1.3.3 – Zinc in Biological Systems

It has been know for more than a century that Zn is crucial to the proper growth and maintenance of biological systems, when Raulin showed for the first time in 1869 that Zn was indispensable for the growth of the fungus *Aspergillus niger* , also known as the black mold (*61*). It took almost sixty years after that discovery to prove that Zn was also essential for the growth of higher forms of plants (*62-64*). In 1934, it was proven for the first time that zinc was not only essential in plant life, but in mammalian life as well, when it was shown that Zn deficiency caused growth retardation and loss of hair in the rat (*65*), as well as in the mouse (*66*). Later studies showed that multiple other species of animals, including birds, pigs, sheep, cows and dogs, can also suffer from Zn deficiency, which causes retardation in growth, loss of hair, deformed nails, testicular atrophy, hyperkeratosis, conjunctivitis, and skin lesions (*67-71*).

It was not until 1961 though, that Zn^{2+} was assigned a biological role in humans (*72*). It was discovered by Prasad et al. that a group of adult males from Iran, who were

anemic, yet who also suffered from dwarfism, hepatosplenomegaly and hypogonadism, were not only iron deficient, but were also Zn deficient. When given Zn containing iron supplements, the anemia was corrected, the hepatosplenomegaly improved, there was growth of pubic hair, and an increase in genitalia size (*73*). Sandstead later confirmed that the cause of all the symptoms seen by Prasad in Iran were due to Zn deficiency, and not just iron deficiency (*74*). He showed that Zn supplementation accelerated growth, more than would an iron and protein supplementation alone, in a group of Egyptian patients. As well, Zn supplementation initiated puberty within 7 to 12 weeks of Zn supplementation in these patients, who were already adults, a phenomenon which would come to completion, with full development of secondary sexual characteristics between 12 and 20 weeks (*74*) (Figure 1).

As stated earlier, Zn is found in most tissues of the human body. It is located predominantly in the pancreas, which contains 20 to 30 μg/ g of tissue, in the liver, which contains 40 to 60 μg/ g of tissue, and in the bone, containing as well 40 to 60 μg/ g of tissue (*42*). Zn is found in relatively small quantities in the blood, as compared to intracellular stores. Zn blood plasma concentrations are on average 12.5 μM (*75*), yet can vary from 10.7μM, the lower limit of normal (after a morning fast) (*76*), to 21.1μM (*77*) in healthy individuals.

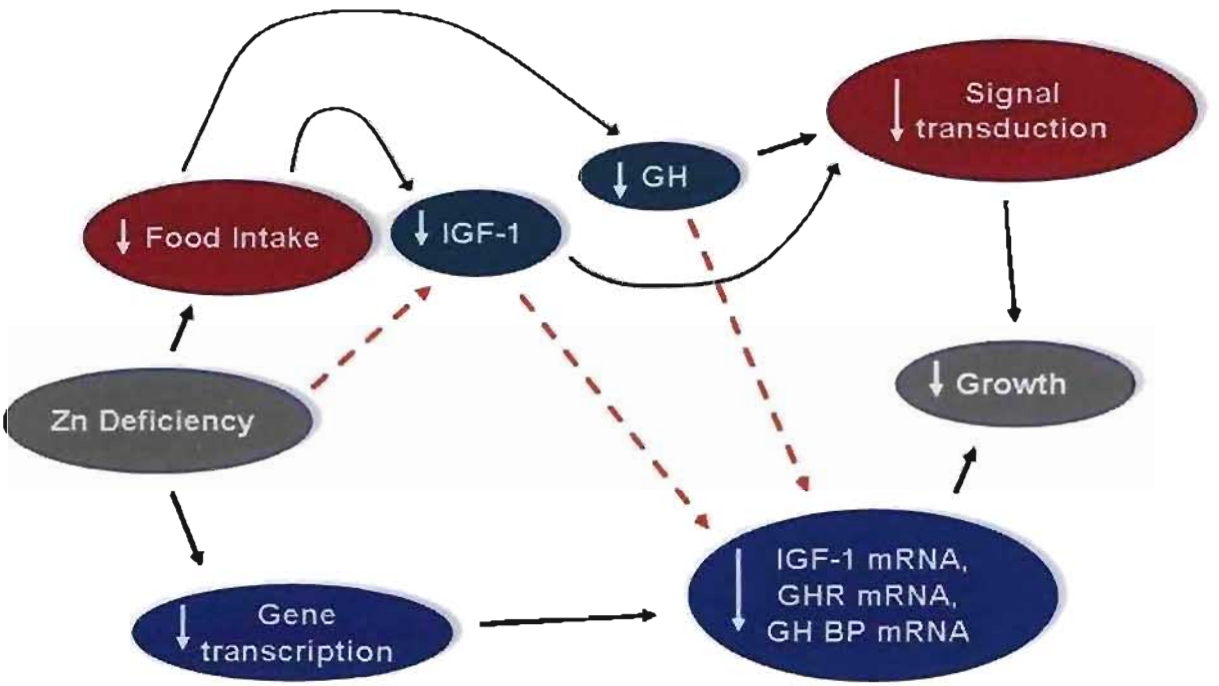

Figure 1: Effects of Zinc deficiency on metabolic processes associated with growth.

Reduced food intake may be a protective mechanism to allow survival during Zn deficiency. Also, Zn is an integral part of many cellular processes, like growth. Growth requires cellular division, as well as DNA, RNA, and protein synthesis, implicating Zn as a cofactor for many enzymes, as well as influencing gene expression through transcription factors. Furthermore, growth hormone (GH) and insulin-like growth factor-I (IGF-1), through reduced food intake and/or Zn deficiency not associated with reduced food intake (dashed orange arrow) may negatively affect IGF-1, growth hormone binding protein (GHBP) and growth hormone receptor (GHR) mRNA levels in an direct fashion (orange dashed arrows). This diagram illustrates the relationship between Zn deficiency and decreased growth, through several different, but related mechanisms. (inspired from (*78*)).

At the molecular level, Zn is an exceptionally important micronutrient, required for over 300 enzymatic reactions that take place in the body (*79*), and is part of more than 2000 Zn dependent transcription factors and other proteins (*80*). Zinc is present in the cell nucleus, nucleolus, and the chromosomes. It plays a vital role in the synthesis of DNA and RNA, through zinc metalloenzymes such as RNA polymerase (*81*), reverse transcriptase and transcription factor IIIA (*82*), and has a stabilizing role in the structure of DNA, RNA and ribosomes (*82*). Zn helps to form certain structures in these metalloenzymes, which allow the proper functioning of the latter. The most common structure is the zinc finger domain, in which the zinc ion forms a loop in the polypeptide chain by creating a bridge between cysteine and histidine residues (*78*). The zinc finger domain in these enzymes was found to be essential for the binding of eukaryotic regulatory proteins to specific DNA sequences (*83*). More recently, Zn fingers have also been shown to bind RNA, as well as being involved in protein-protein interactions, which may help explain why there are Zn fingers that do not bind either DNA or RNA (*84;85*).

Zn homeostasis is at least, in part, controlled by metallothionein. Metallothioneins (MT) are small proteins (approx. 6200Da) rich in cystein, with a very high affinity for divalent heavy metal ions, such as cadmium, mercury, platinum, silver and Zn (*86;87*). MT are primarily found in the liver, pancreas, kidneys and intestinal mucosa. In this sense, they help protect cells and tissues against heavy metal toxicity, facilitate the exchange of metals functioning as anti-oxidants in tissues, as well as protecting the cell against hydroxyl free radicals, which can cause an oxidative stress induced apoptosis (*87;88*). It has been suggested that MT play an important role in Zn-induced cell proliferation and

differentiation (*89*), since they are over expressed in proliferating tissues, such as regenerating and developing rat livers, and certain types of tumors (*90-93*). MT also play a role in metal exchange with Zn dependant enzymes, such as carbonic anhydrase (CA), a zinc metalloenzyme that catalyzes the reversible conversion between carbon dioxide and the bicarbonate ion, through the hydration of carbon dioxide (CO_2) (*94-96*). CA, and by association, Zn, is therefore involved in vital physiological processes liked with respiration, transport of CO_2 / bicarbonate between lungs and tissues, pH and CO_2 equilibrium, ureagenesis, gluconeogenesis, and lipogenesis, (*97-99*) the latter two linking Zn to diabetes and its insulino-mimetic effects, which will be discussed later in this text.

1.4 – Zinc and Diabetes

In 1980, it was shown for the first time that Zn, in the form of zinc chloride ($ZnCl_2$) can mimic insulin in its ability to stimulate lipogenesis in rat adipocytes (*100*), even though a connection between Zn and diabetes had been made 14 years earlier, when Quarterman et al. demonstrated that Zn deficient animals were less sensitive to insulin, causing them to conclude that Zn was in some way involved with insulin action (*101*). The fact that it was already known that Zn is stored in and secreted from the pancreas along with insulin (*102*), as well as Zn being included in insulin preparations, prompted investigators to test the anti-diabetic potential of Zn in animal models of diabetes.

1.4.1 – Zinc and Animal Models of Type 1 Diabetes

In animal studies, subcutaneous Zn injections were shown to partially prevent hyperglycaemia and development of diabetes in streptozotocin (STZ) treated rats, through the increased activation of MT acting as an oxygen free radical scavenger, as well as a reductant of lipid peroxidation in the pancreas and liver (*103*). It should be noted that animals treated with STZ, a compound that specifically destroys insulin producing β cells of the pancreas, are considered a good model of type 1 diabetes mellitus (*104*). Later studies proved that these effects were not entirely due to the increased activation of MT, as Zn pre-treatment of STZ-induced diabetic MT-null mice improved their diabetic state (*105*).

The aforementioned studies were confirmed by Ohly et al. The latter gave C57BL/6 mice multiple low doses of STZ (MLD-STZ) to induce diabetes. Mice were then given Zn (in the form of zinc sulphate)-enriched drinking water, and it was shown that the Zn-enriched drinking water up-regulated MT, as well as prevented MLD-STZ diabetes and loss of pancreatic β-cell function (*106*). The MLD-STZ mice treated with Zn-enriched drinking water had a significant decrease of their blood glucose concentrations, and maintained a blood glucose concentration under the euglycaemic threshold of 13.9 mmol/l, while the non-Zn treated MLD-STZ mice had blood glucose concentrations much higher than the euglycaemic threshold. Furthermore, following the results of an oral glucose tolerance test, it was shown that Zn-enriched drinking water significantly countered MLD-STZ loss of glucose tolerance, an indicator that Zn treatment prevented essential β-cell dysfunction. The group went on to conclude that these anti-diabetic effects were due to the activation of Zn-induced MT, which provides a defense against OH^- groups generated in β-cell by the inflammatory reactions, most probably due to the MLD-STZ treatment (*106*).

In further support of the anti-diabetic effects of Zn, Tobia et al. showed that high Zn supplementation (HZn) (1000 ppm) dramatically delayed the onset and reduced the severity of diabetes in BioBreed (BB) Wistar rats, an inbred rat strain that spontaneously develops diabetes, as compared to BB rats fed a normal Zn diet (NZn) (50 ppm), and low Zn (LZn) (1ppm) (*107*). The study demonstrated that the NZn and LZN groups became diabetic almost two weeks before the first HZn became diabetic, at 85 days of age. At 100 days of age, only 19% of the HZn became diabetic, as compared to 53% for the NZn group and

44% for the LZn group, which equates to a 60% drop in the onset of diabetes. Also, the HZn group had improved glucose tolerance, which can be justified by the higher serum and pancreatic insulin content, as well as increased insulin release, compared to the animals in the other two groups (*107*).

1.4.2 – Zinc and Animal Models of Type 2 Diabetes

The anti-diabetic potential of Zn compounds has also been examined in animal models of type 2 diabetes mellitus. In 1985, Begin-Heick et al. showed that a high Zn supplementation (1000mg Zn/Kg diet) for 4 weeks attenuated fasting hyperglycaemia and hyperinsulinemia in *ob/ob* mice (*108*), a well established model of type 2 diabetes mellitus. In these studies, high Zn supplementation elevated insulin in pancreatic islets, and attenuated of the abnormally high insulin secretory response to glucose in isolated pancreatic islets (*108*).

It was later shown that even a lower dose of Zn (300 ppm) for 6 weeks was sufficient to decrease fasting hyperinsulinemia and hyperglycaemia, as well as in reducing the amount of body weight in *db/db* mice, another mouse model of type 2 diabetes (*109*). Furthermore, in the same 6 week time span, a Zn deficient diet (3 ppm) aggravated fasting hyperglycaemia in *db/db* mice, compared to those fed a diet adequate in Zn content (30 ppm). Zn supplementation also increased pancreatic Zn content of *ob/ob* mice to normal levels (*109*). It was therefore concluded that a high pancreatic Zn content and low circulating insulin levels due to Zn supplementation in *db/db* mice enhanced peripheral

insulin sensitivity and pancreatic β-cell function, and as a result, less circulating insulin was necessary to invoke glucose uptake (*109*).

In yet another recent study, a complex formed between Zn and Cyclo (His-Pro) (CHP), a metabolite of thyrotrophin-releasing hormone or product of peptide or amino acid sources, was studied in the Goto-Kakizaki (GK) rat, a non-obese type 2 diabetes animal model, exhibiting a mild fasting hyperglycaemia (*110*). CHP metabolism is thought to be directly related to glucose metabolism (*110*), since STZ-diabetic rats exhibited an increase of CHP levels which decreased after insulin treatment (*111*), suggesting that blood glucose metabolism is related to gut CHP concentrations (*112*). The Zn-CHP compound was shown to improve short term and long term oral glucose tolerance, as well as decreased insulin resistance, in aged GK rats (*110*). This decreased insulin resistance is thought to be due to the supply of new Zn brought into the system through the Zn-CHP complex. Zn then becomes part of new enzymes and proteins that remove partially digested protein fragments from the cell, and goes on to increase synthesis and translocation of GLUT4 (*110*). The integration of Zn into the system happens through its chelation by CHP, causing its increased intestinal absorption and uptake in muscle tissue (*113*), hence increasing Zn metabolism.

1.4.3 – Organic Zinc Complexes and Diabetes

In most of the studies using Zn as an anti-diabetic molecule, either a high dose of Zn (1000ppm) (*107*) or a long treatment period (up to 8 weeks) (*114*) was used , both of which may be toxic to the organism being treated. At the time, it was discovered that Bis-

maltolato-oxo-vanadium (BMOV), an organo-vanadium compound, was more potent than inorganic vanadium salts in improving hyperglycaemia in STZ-diabetic rats (*115*). This discovery prompted Sakurais' group to synthesize Bis-maltolato-Zinc (II) ($Zn(Mal)_2$), which was modeled after BMOV (*116*). This compound, at 500 μM, was found to inhibit free fatty acid release from epinephrine-stimulated rat adipocytes to a greater extent than $VOSO_4$ and $ZnSO_4$ (*116*). It was also shown that $Zn(Mal)_2$ inhibited free fatty acid release from epinephrine-stimulated rat adipocytes to a greater extent than insulin or $Zn(Mal)_2$ alone, suggesting that this compound was not only an insulinomimetic compound, but also an insulin enhancer (*117*).

In animal experiments, using insulin-resistant KK-A^y type 2 diabetic mice, it was observed that intraperitoneal (i.p.) injection of 68.8 μM $Zn(Mal)_2$ /Kg body weight for the first two days decreased blood glucose from above 20mM to just under 11mM, which was maintained at that level for two weeks (*117*). After two weeks of treatment, serum insulin and triglyceride levels dramatically improved in treated mice, as compared to a non-treated KK-A^y control group. Also, blood urea nitrogen, an indicator of renal disturbance, glutamic oxaloacetic transaminase, and glutamic pyruvic transaminase, indicators of liver disturbance, were unchanged in treated mice, as compared to non-treated KK-A^y control group (*117*), showing that $Zn(Mal)_2$ did not disturb renal or hepatic functioning. Furthermore, oral glucose tolerance tests after the two week treatment showed greatly improved glucose tolerance in KK-A^y- $Zn(Mal)_2$ treated group (*117*).

Subsequently, several new organic Zn compounds were synthesized and were tested for their anti-diabetic potential. Two such compounds, Bis(picolinato)zinc(II), $Zn(pic)_2$, and Bis(methylpicolinato)zinc(II), $Zn(mpa)_2$ were synthesized by Yoshikawa et al. (*118*). Results of tests done in isolated rat adipocytes and in KK-A^y type 2 diabetic mice were similar to tests done using $Zn(Mal)_2$ (*117*), with the difference that $Zn(mpa)_2$ was found to be more potent than $Zn(Mal)_2$ and $Zn(pic)_2$. $Zn(mpa)_2$ was shown to improve cholesterol metabolism and decrease HbA_{1C}, or glycosylated hemoglobin, an indicator of glycaemic control (*119;120*), to a greater extent than $Zn(Mal)_2$, $Zn(pic)_2$, $VOSO_4$ and $ZnSO_4$, while exhibiting no alteration in hepatic and renal functions (*118*).

Since then, many other organic Zn compounds have been synthesized, such as bis (allixinato)Zn(II), $ZN(alx)_2$ (*121*), bis(2-aminomethyl-pyridinato)Zn(II) (*122*), bis (dimethyldithiocarbamate)Zn(II) and bis (diethyldithiocarbamate)Zn(II) (*123*), all of which have exhibited potent insulinomimetic and insulin enhancing properties in *in vitro* as well as *in vivo* experiments (figure 2). Not only are these compounds more potent than their predecessors, but also improve complications of diabetes, such as improving obesity-linked hypertension in mice, hyperleptinemia, as well as adiponectine serum levels (*123*), which were shown to play a protective role against insulin resistance (*124*). Also, in early 2007, it was shown for the first time that bis(pyrrolidine-N-dithiocarbamate)Zn(II), $Zn(pdc)_2$, improved glucose tolerance and had a hypoglycaemic effect in KK-A^y mice through oral administration, and not just through i.p. injections (*123*).

The precise mechanism by which Zn and its compounds improve hyperglycaemia and glucose homeostasis in diabetes remains unclear. However, the ability of Zn compound to increase glucose transport, glycogen synthesis, and lipogenesis, and to inhibit gluconeogenesis and lipolysis, and to modulate key elements of the insulin signalling pathway(*125-132*) have been suggested to contribute to this response. Since activation of insulin-induced signalling cascade is critical to exert on insulin-like effect, it is important to discuss this pathway in detail.

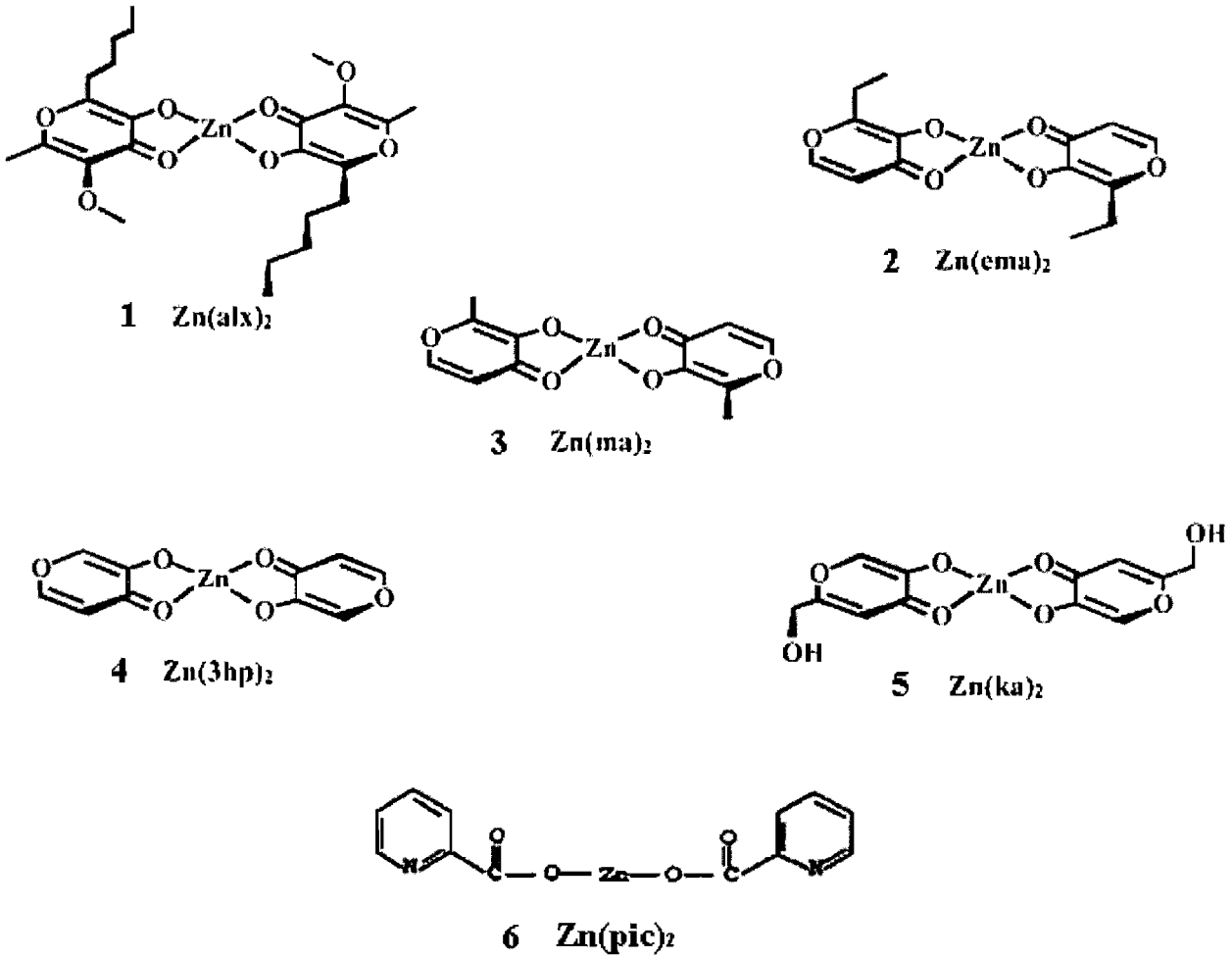

Figure 2: Structure of several organo-Zn compounds exhibiting anti-diabetic effects.

1: bis(allixinato)Zn(II), 2: bis(ethylmaltolato)Zn(II), 3: bis(maltolato)Zn(II), 4: bis(3-hydroxypyronato)Zn(II), 5: bis(kojato)Zn(II), 6: bis(picolinato)Zn(II). (Inspired from (*121;133*))

1.5 – The Insulin Signalling Cascade

Insulin is the primary hormone involved in blood glucose control. In response to increasing blood glucose levels, pancreatic β-cells secrete insulin, which stimulates glucose uptake in muscle and fat tissues (*134*) and triggers inhibition of gluconeogenesis and glucose release by the liver.

The first event in insulin action involves the binding of insulin to its receptor. The insulin receptor (IR) is composed of two extracellular α-subunits and two transmembrane β-subunits linked to each other by disulfide bonds (*135*) (Figure 3). Both the α- and β- subunits are derived from a single proreceptor by proteolytic processing in the Golgi apparatus. The α-subunit is located entirely outside of the cell and contains the insulin-binding site, while the intrinsic insulin-regulated tyrosine kinase activity possessing β-subunit is the intracellular component of the receptor. The IR-β subunits consists of five main regions. The first region consists of short extracellular sites for glycosylation. The transmembrane region, composed of 23 amino acids, anchors the IR to the plasma membrane. The intracellular region, which is separated into 3 distinct functional regions, has protein tyrosine kinase activity required for insulin action. It is separated into the juxtamembrane region, containing two tyrosine residues, which are autophosphorylated in response to insulin binding. Of these, tyrosine 972 is instrumental in binding the phosphotyrosine binding domain (PTB) domains of Insulin Receptor Substrate-1 (IRS-1) and Shc (*135*). The regulatory domain (Tyr 1146, Tyr 1150 and Tyr 1151), contains adenosine triphosphate (ATP)-binding domains, as well as the enzymatic

active site of the receptor, and the C terminus domain(Tyr 1316 and Tyr 1322), not critical for receptor activation, yet is thought to bind Insulin Receptor Substrate-2 (IRS-2) and to play a regulatory role that is essential for signalling (*135*).

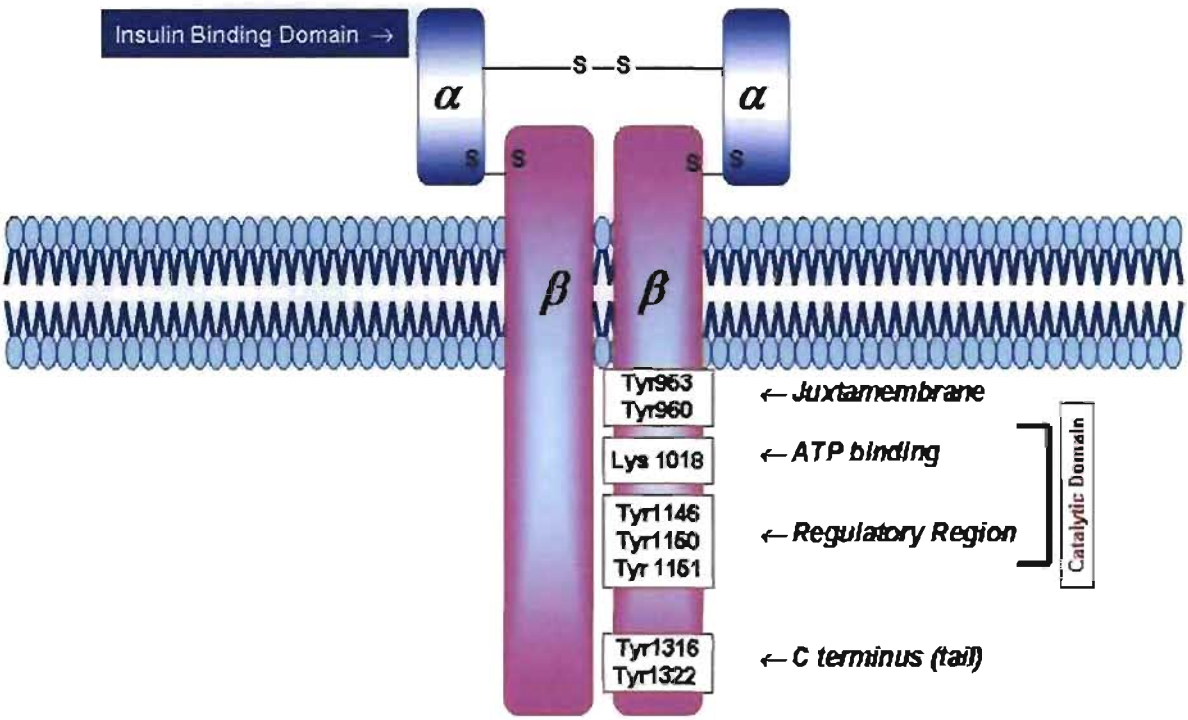

Figure 3: Schematic representation of the structure of the insulin receptor

The insulin receptor, composed of two α-subunits and two β-subunits, linked by disulfide bonds. The extracellular α-subunits contain the insulin binding domains. The transmembrane β-subunit has several tyrosine autophosphorylation sites, ATP binding domain and regulatory region, necessary for the activation of its intrinsic protein tyrosine kinase activity. (Inspired from (*136*)).

Binding of insulin to the α-subunit of IR causes a conformational change, leading to an enhanced protein tyrosine kinase (PTK) activity of the β-subunit by multi-site tyrosine phosphorylation (*137*). IR can then phosphorylate several scaffolding proteins, including insulin receptor subtrates (IRSs), Src homology collagen (Shc) and adaptor protein with pleckstrin homology (PH) and Src homology 2 (SH2) domains (APS) (*138*). These phosphorylated scaffolding proteins then bind and activate other signalling kinases, initiating several different pathways which mediate the multiple effects of insulin. Zn has been shown to phosphorylate IR-β subunit in 3T3-L1 fibroblasts, as well as in rat adipocytes (*139*). This may be one of the possible mechanisms through which Zn exerts its insulin-mimetic effects, since activation of IR leads to activation of the insulin signalling cascade (Figure 4).

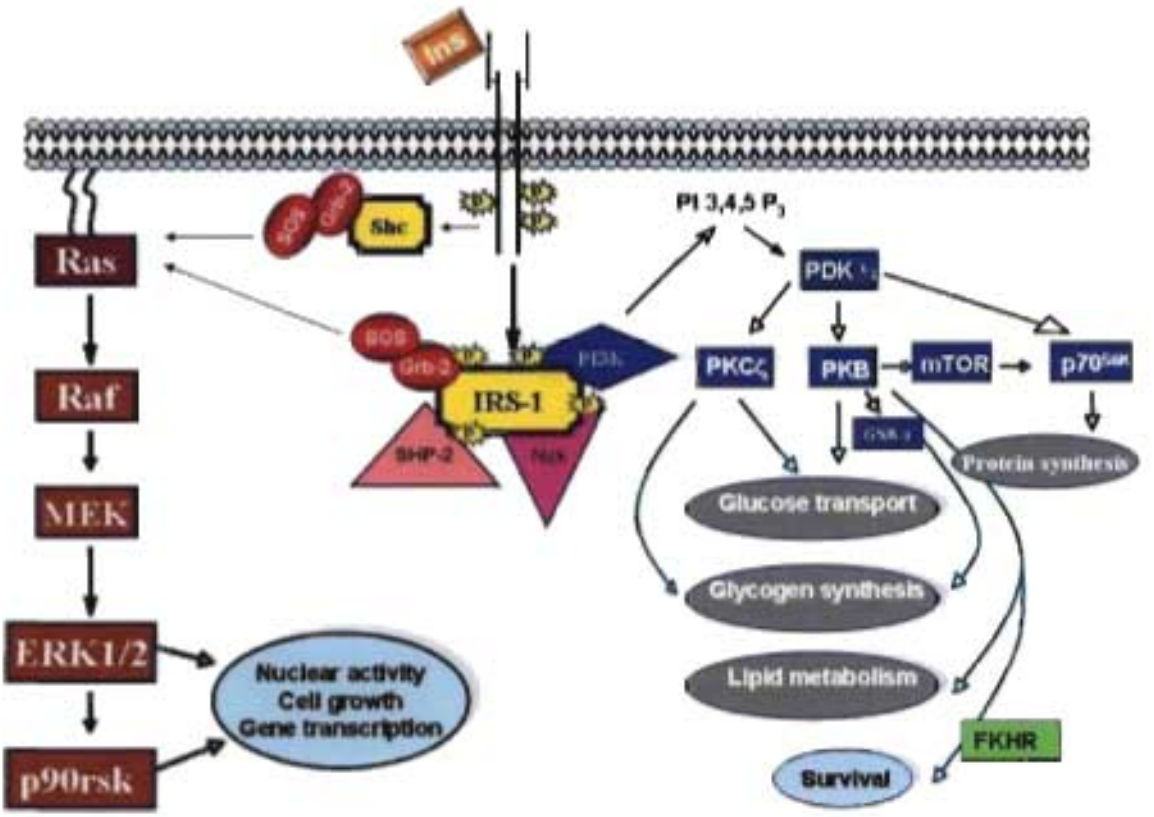

Figure 4: Schematic model showing key elements of the insulin-signaling cascade

Insulin-induced tyrosine phosphorylation and activation of IR/IRS leads to recruitment of SH-2 domain-containing signalling proteins, such as Grb-2-SOS and the p85 regulatory subunit of PI3-K, initiating 2 signalling pathways. One pathway, known as the MAPK pathway, consists of Ras/Raf/MEK/ERK1/2 and p90rsk. Another pathway involves PI3-K activation, which results in the generation of PIP3. PIP3 activates a variety of downstream signalling components involved in a glucose transport system, protein and glycogen synthesis (inspired from (*140;141*)).

In one pathway, IRS-1 with growth factor receptor binder-2 (Grb-2) complexed with mammalian son of sevenless (mSOS) results in the activation of Ras and Raf, serine/threonine kinases. Activated Raf phosphorylates MEK (MAPK kinase), which in turn phosphorylates extracellular signal-regulated kinase (ERK1/2) on Thr and Tyr residues located in the activation loop of the kinase. ERK1/2 then phosphorylates and activates a downstream ribosomal protein kinase, $p90^{rsk}$. Both ERK1/2 and $p90^{rsk}$ can be translocated to the nucleus, where they phosphorylate transcription factors, such as c-Jun, CHOP, CREB and MEF-2 (reviewed in(*142*)), and contribute to the mitogenic and growth-promoting effects of insulin (reviewed in (*143*)) (Figure 5).

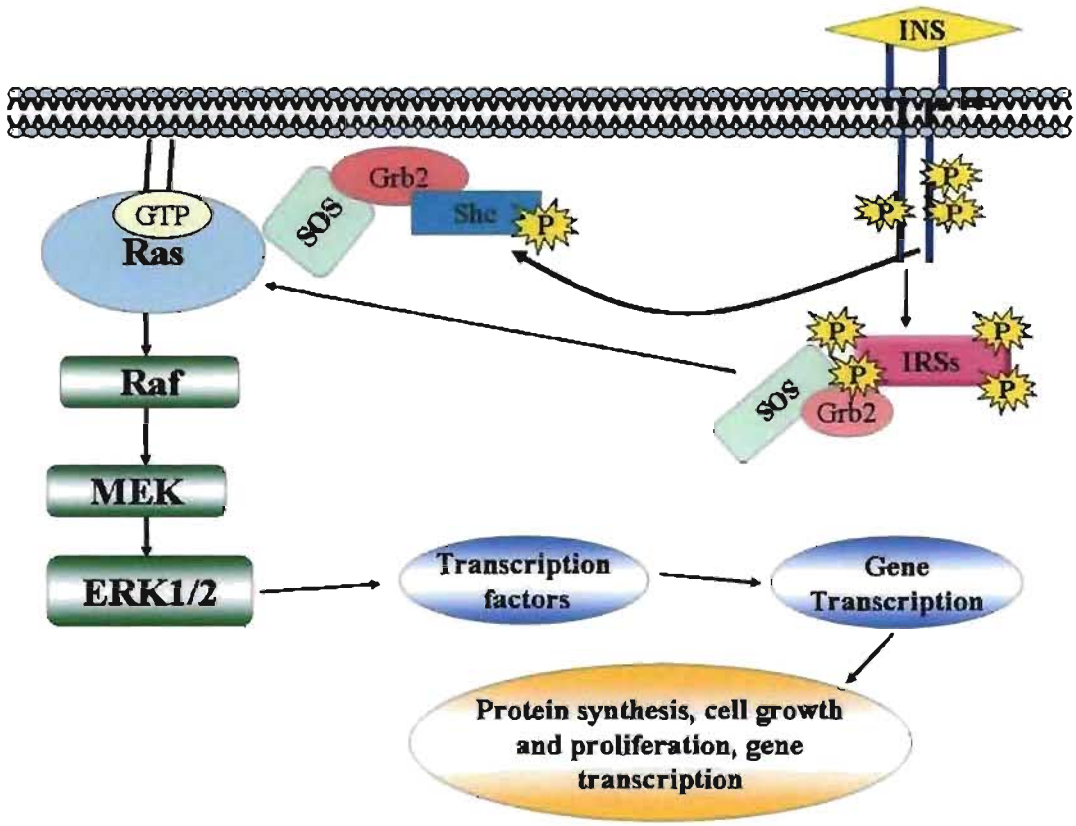

Figure 5: Schematic diagram showing key steps involved in insulin-induced activation of ERK1/2

In response to insulin, Shc or IRS-1 become phosphorylated and bind the Grb2-SOS complex, leading to p21-Ras stimulation by GTP loading, with subsequent activation of Raf, MEK, and two isozymic forms of MAPK, ERK-1 and ERK 2. Both ERK1/2 and and other transcription factors can be translocated to the nucleus, activating transcription and other cellular processes. (Inspired from (*142;144;145*)).

In the other main pathway mediated by insulin action, the p85 subunit of Phosphatidylinositol 3- Kinase (PI3-K) activates the p110 catalytic subunit of PI3-K catalyses phosphorylation of phosphatidylinositol (PI) lipids at position 3 of the inositol ring, and generates 3-phosphorylated forms of PI, such as phosphatidylinositol 3, 4, 5 triphosphate (PIP_3) and phosphatidylinositol 3, 4 diphosphate (PIP_2) (*146*). The formation of PIP_2 and PIP_3 generates recognition sites for Plecstrin Homology (PH) domain containing proteins, principally 3'-phosphoinositide-dependent kinase 1 (PDK1), which is translocated to the plasma membrane along with Protein Kinase B (PKB) (*146;147*), and other and related serine/threonine protein kinases, which are responsible for the phosphorylating and activating several downstream signalling protein kinases, such as PKB, protein kinase C-zeta (PKC-ζ), and p70 ribosomal S6 kinase ($p70^{s6k}$) (*148;149*) (Figure 6).

The following sections will briefly describe some of the key components of the insulin signalling cascade, of which the insulin receptor has already been described earlier.

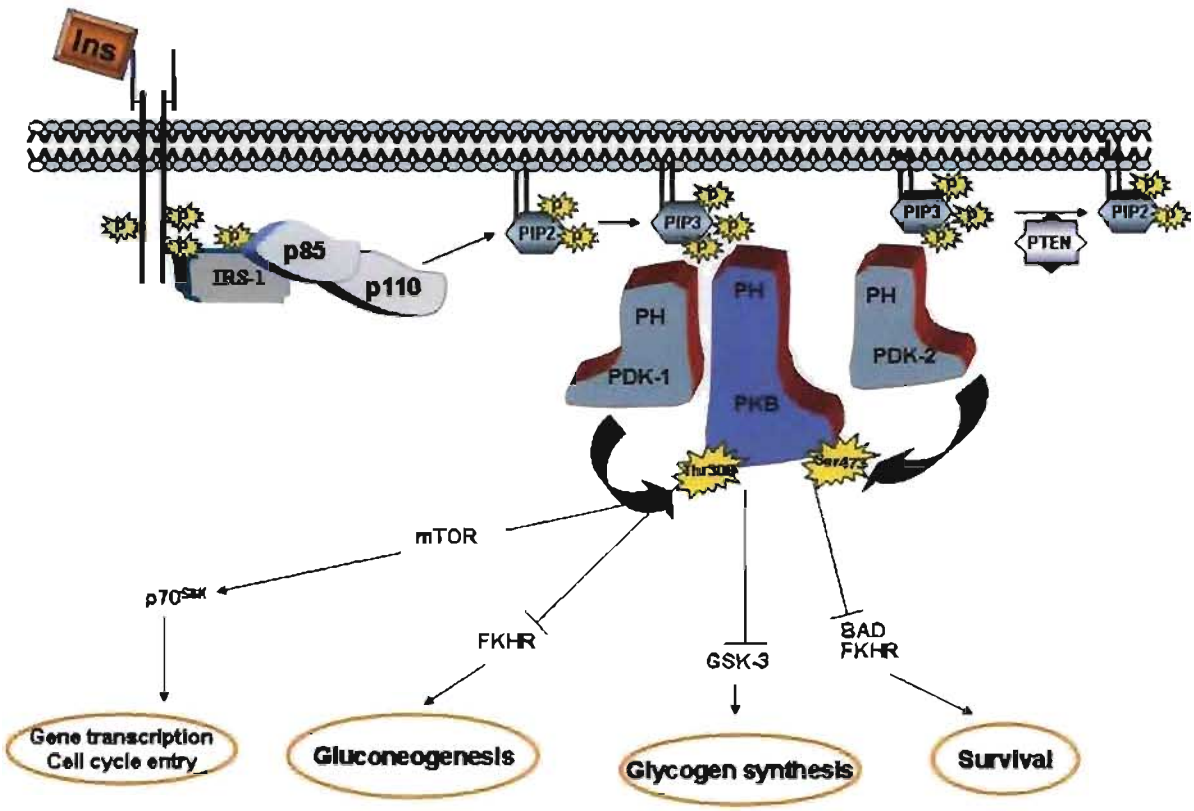

Figure 6: Schematic representation of PKB activation and its physiological role

Phosphorylated IRS-1 recruits PI3-K, which catalyzes the phosphorylation of PIP2 leading to the formation of PIP3. PIP3 recruits PH domain containing proteins to the plasma membrane including PDK-1/2 and PKB, where PKB becomes phosphorylated and activated. PTEN, a lipid phosphatase, dephosphorylates PIP3 to PIP2 and thus inhibits activation of PKB. (Inspired from (*150-152*)).

1.5.1 – Insulin Receptor Substrates

IRS proteins serve as docking sites for effector molecules responsible for transmitting the insulin signal (*153*). At least four main IRS proteins have been identified in mammals. IRS-1 and IRS-2 are widely expressed in all tissues. IRS-3 is present in rodents and is highly restricted to adipose tissues. IRS-4 is only expressed in the brain, thymus, kidney and β-cells (*154;155*). IRS proteins are composed of a NH2-terminal PH and PTB domain, followed by a COOH-terminal tail composed of multiple tyrosine and serine/threonine phosphorylation site residues (*140;153*).

PTB domain mediates interactions of IRSs with the IR, insulin-like growth factor type 1 receptor (IGF-1R) and interleukin-4 (IL-4) receptors through phosphorylated-NPXY motifs located in these receptors (*156*). The tyrosine phosphorylated residues in the COOH-terminal serve as docking sites for SH2 domain-containing signal transducers, such as the p85 subunit of PI3-K, the SH2 domain-containing tyrosine phosphatase (SHP2) and Src-like kinase Fyn (*157*). Adaptor molecules, such as Grb-2, nck, Crk, SHB and others that mediate downstream signals can also dock in the COOH-terminal (reviewed in (*153*)). The function of serine/threonine (ser/thr) phosphorylation sites in IRSs has not yet been fully understood, but several studies have indicated that insulin resistance inducing factors, such as free fatty acids, lead to increased ser/thr phosphorylation of IRS-1 and consequently to impairment of insulin signal transduction (*158*).

Although IRSs are similar in composition, it has been shown that each type has a specific role. For example, mice in which IRS-1 has been knocked out have a decreased

growth, and suffer from insulin resistance, yet do not develop diabetes (*159*). On the other hand, mice in which IRS-2 has been knocked out develop type 2 diabetes, have a decreased β-cell function, and are insulin resistant (*160*). In experiments using 3T3-L1 fibroblasts and rat adipocytes, Zn treatment was not able to increase tyrosine phosphorylation of either IRS-1 or IRS-2 (*139*). As mentioned earlier, activation of PI 3-kinase caused by insulin is via the association of tyrosine-phosphorylated IRS proteins with the p85 subunit of PI 3-kinase, yet IRS-1 and IRS-2 may not play an important role in Zn induced PI3K/PKB activation.

1.5.2 – Phosphatidylinositol 3-Kinase (PI3-K)

PI3-Ks are a family of lipid kinases that phosphorylate the D3 hydroxyl group (3'-OH) of the inositol ring in phosphatidyl inositol (PI) (*143*). Products of the PI3-K reaction include phosphatidylinositol-3-monophosphate (PIP), phosphatidylinositol-3,4-bisphosphate (PIP2) and phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) (*161*).

Based on sequence homology and substrate preference, PI3-Ks have been divided into classes I, II and III. Class I PI3-Ks are heterodimeric proteins, each of which consists of a 110 kDa catalytic subunit and an associated regulatory subunit. This class is further divided into classes IA and IB, of which class IA has three isoforms (α, β and δ) of the catalytic p110 subunit and several forms of regulatory subunits (p85α, p55α, p50α, p85β and p55γ). Class IB, on the other hand, has only one member of the catalytic subunit called p110γ and one form of the regulatory subunit p101. Class IA is activated by receptor PTK (RPTK), while class IB is activated by heterotrimeric G protein-coupled receptor (GPCR) (*162*). In vitro, PI, PI 4 P, and PI 4,5 P are phosphorylated by class I PI3-K enzymes to form PIP, PIP2 and PIP3, yet phosphorylation of PI 4,5 P is favoured in vivo, which is responsible for the formation of PIP3 (*162;163*).

Class II PI3-Ks, consisting of two major mammalian subclasses, α and β, contain a carboxy-terminal C2 domain, a protein module originally observed in PKC molecules, with phospholipid binding sites. There are no known regulatory subunits in this class, which may not even be necessary.

Class III PI3-Ks are thought to represent the primordial PI3-K that gave existence to the other classes, due to the fact that it is the only class of PI3-K enzymes present in yeast. PI is the only substrate recognized by this class and is phosphorylated to generate PI3P (*164;165*). Class III PI3-Ks induce local increases in PI3P, which are thought to be required for agonist-independent membrane trafficking processes (*165*).

PI3K derived PIP_2 and PIP_3 bind to the PH domains of downstream targets, such as PDK1/PDK2, which are translocated to the plasma membrane, and participate in the activation of PKB. Direct stimulation of PI3K by Zn was shown in HNN8 cells (*128*) and Swiss 3T3 cells (*129*).

1.5.3 – Protein Kinase B/Akt (PKB/Akt)

PKB was discovered as an oncogene within the mouse leukemia virus AKT8 (*166*). Sequence analysis of the viral oncogene and its cellular homologue revealed that it encodes a Ser/Thr kinase (*167*). PKB is a 57 kDa protein, and was finally given the name PKB due to its high homology with protein kinase A (PKA) and PKC. It is known to exist as three isoforms, PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (*168*). All PKB isoforms have an amino-terminal PH domain, a central catalytic Ser/Thr kinase domain and a carboxy-terminal regulatory domain that contains the hydrophobic motif (HM) (*150;169*). The PH domain of PKB interacts with membrane lipid products, such as PIP2 and PIP3, produced by PI3-K (*170*). The kinase domain of PKB is very similar to other cAMP-dependent/ cGMP-dependent/PKC (AGC) kinases, such as PKA, PKC, $p70^{S6K}$ and $p90^{rsk}$, containing the conserved Thr residue whose phosphorylation is required for enzymatic activation (*150*). The carboxy-terminal regulatory domain of PKB is composed of approximately 40 amino acids. Phosphorylation of the Ser or Thr residue in this HM is necessary for full activation. This motif is identical in all mammalian PKB isoforms, and is of great importance, as it has been shown that a deletion mutant motif completely abolishes PKB enzymatic activity.

Phosphorylation site mapping of PKB from quiescent cells with no stimulation, or serum starved cells stimulated with IGF-1 revealed that while Ser 124 and Thr 450 are constitutively phosphorylated and seem to contribute to stabilization of the proteins, phosphorylation of Th308 in the activation loop and Ser 473 in the carboxy-terminal site is

detected in stimulated cells (*169;171*). Also, the constitutive localization of PDK-1 at the plasma membrane is thought to be because C terminal PH domain in PDK-1 binds phospholipids with 10-fold higher affinity than the PKB PH domain. The following scenario for PKB activation has therefore been proposed (*150;151;172*): The activation of PI3-K leads to the production of PIP_2 and PIP_3. These phospholipids interact and cause the translocation of PKB to the inner membrane, where PDK-1 is located. Additionally, it is thought that binding of 3'-phosphoinositides to PKB changes its conformational status, making the regulatory residues more accessible to phosphorylation. PDK-1, which is thought to be constitutively active, phosphorylates Thr308 in PKB (*173*), stabilizing the activation loop in an active form. This phosphorylation is a prerequisite for kinase activation, but phosphorylation of Ser 473 is necessary for full PKB activation. The nature of putative Ser 473-PKB kinase called PDK-2 is still controversial (*174*), although several candidates have been suggested (*175*)

PKB contributes to a variety of cellular responses, including cell growth, cell survival and metabolism. It also plays a primordial role in the mediation of glucose transport, glycogen synthesis, gluconeogenesis, lipogenesis, and protein synthesis, through the following mechanisms: Increasing the number of GLUT4 transporters available for glucose uptake (*172;176;177*), by activation of Glycogen Synthase (GS) via phosphorylation of GSK-3 by PKB on Ser 9 resulting in the inhibition of its catalytic activity, increasing glycogen synthesis (*178*), by suppressing glucocorticoid and cAMP signaling-stimulated glucose production by downregulating the transcription of PEPCK and G6Pase, as well as phosphorylation of FOX01, to downregulate gluconeogenesis

(*179;180*), and by activation of mTOR (mammalian target of Rapamycin), a 4E-BP1 kinase, which deactivates 4E-BP1, releasing eIF-4E, promoting translation initiation (*151*).

Zn has been shown to activate PKB in BEAS-2B human bronchial cells(*181*), HepG2 cells (*131*), as well as in 3T3-L1 fibroblasts and rat adipocytes (*139*). The activated PKB contributes to the regulation of glucose transport, glycogen synthesis, gluconeogenesis, lipogenesis, and protein synthesis, through the activation or inhibition of downstream effectors, as mentioned earlier. Zn –induced GSK-3 and FOX01 phosphorylation has also been shown (*131*), which may mediate the response of Zn on increased glycogen synthesis by upregulation of GS, and inhibition of gluconeogenesis (*182*)

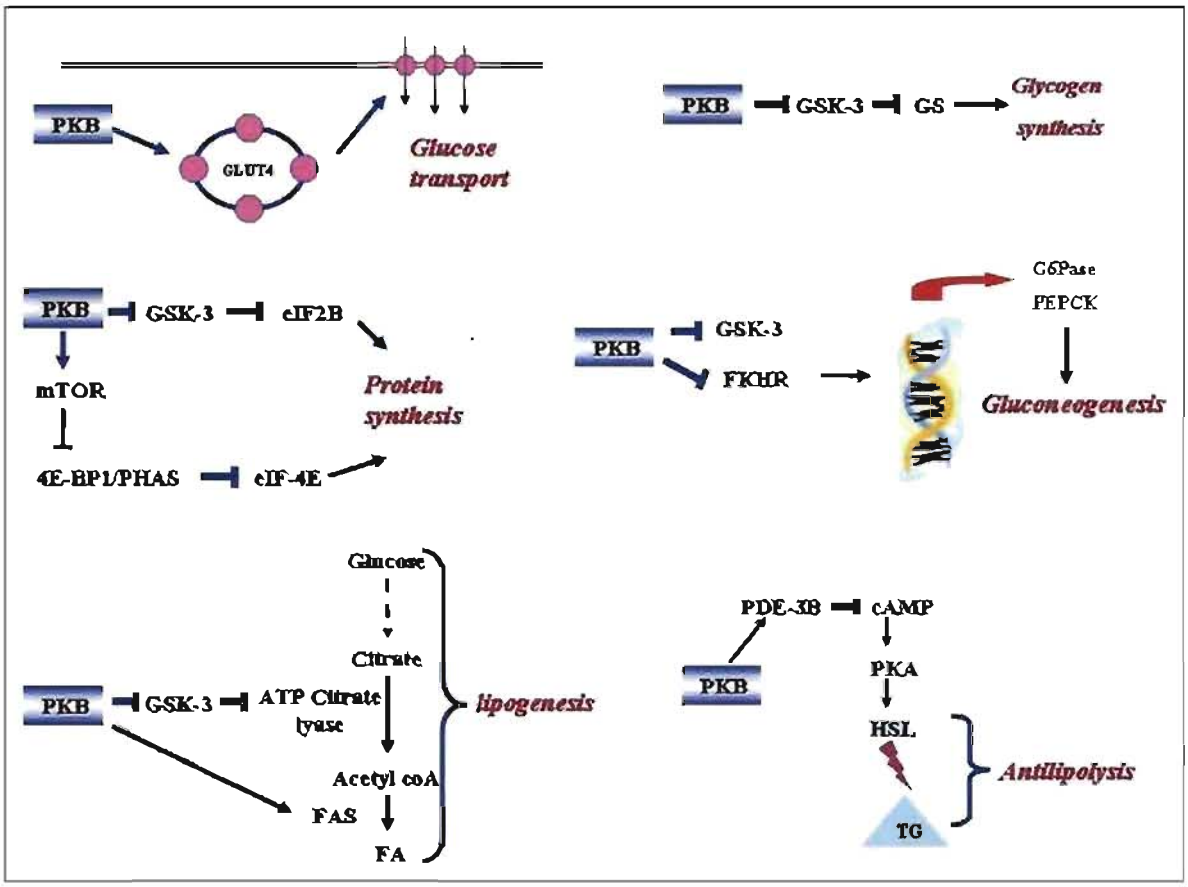

Figure 7: Graphic representation showing a role of PKB in the regulation of carbohydrate metabolism

Inspired from (*151;172*)

1.5.4 – Mitogen Activated Protein Kinase (MAPK/ERK1/2) Pathway

MAPKs are a family of Ser/Thr protein kinases, which have been widely conserved among eukaryotes. They are involved in cell proliferation, cell differentiation, cell movement and cell death, amongst other cellular responses (*142;144;183;184*).

In mammalian cells, five MAPK families have been identified, including ERK1, 2, 5, 7, Jun N-terminal kinase 1, 2 and 3 (JNK1/2/3), and p38α, β, γ, and δ. These pathways all follow a similar system of activation, in which a stimulus activates a MAPKKK, which will then activate a MAPKK, which is an upstream activator of MAPK, which leads to a cellular response. The MAPK is the final effector of the cellular response. MAPKKKs are Ser/Thr kinases, and are activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli. Activation of MAPKKKs phosphorylate and activate MAPKK, which then phosphorylates Thr and Tyr residues in the activation loop of the kinase, stimulating MAPK activity. Once activated, MAPKs phosphorylate target substrates on Ser or Thr residues followed by a praline (figure 5).

ERK1/2 is the principal MAPK pathway activated by insulin. This pathway consists of the MAPKKKs (A-Raf, B-Raf and Raf-1), the MAPKKs (mitogen and extracellular signal regulated kinase 1 and 2 (MEK1/2)), and the MAPKs (ERK-1 and ERK-2). Signals from activated receptor tyrosine kinase (RTK) or GPCR to Raf/MEK/ERK are transmitted through different isoforms of the small GTP-binding protein such as Ras. As mentioned earlier, following activation of the IR and its substrates, Grb-2 associates with SOS, and this causes

the release of GDP, as well as binding of GTP to Ras (forming Ras-GTP), which is accompanied by a conformation change in Ras, allowing it to bind to a wide range of downstream effector proteins, including isoforms of the Ser/Thr kinase Raf. Raf then binds to, and phosphorylates, the dual-specificity protein kinase MEK-1 and -2, the latter of which phosphorylate ERK1/2 within a conserved Thr-Glu-Tyr (TEY) motif in their activation loop. ERK1/2 can then be translocated to the nucleus where it can phosphorylate and activate a number of transcription factors involved in gene activation. It can also activate a number of cytosolic proteins, such as $p90^{rsk}$ through its proline directed Ser/Thr kinase activity.

Zn was shown to activate ERK1/2 in HT-29 colorectal cancer cells (*125;127*), as well as in human epidermal A431 cells, which leads to activation of the mitogenic effects of the insulin signalling cascade, namely cell growth and gene transcription. This activation by Zn has been suggested to function through both a epidermal growth factor receptor –dependent and –independent pathway (*126*).

1.5.5 – Protein Tyrosine Phosphatases (PTPases) Implicated in the Insulin Signalling Cascade

PTPases are important regulators of the insulin signalling pathway. They catalyze the rapid dephosphorylation and inactivation of IR-β subunit and IR substrates (*185*). PTPases can be divided into four classes: 1) classical receptor PTPases (R-PTPases), which include leucocyte antigen receptor (LAR), PTPσ, PTPδ (type Iia); PTPμ, PTPκ, PTPλ/ψ/o (type Iib); PTPφ; PTPα, PTPε (type IV); PTPζ/β, PTPγ (type V); 2) classical non-receptor PTPases (NR-PTPases) including PTP1B, SHP-1, SHP2, PTP-PEST; 3) dual specificity PTPases (DS-PTPases); and 4) low molecular weight PTPases (*186;187*). R-PTPases and NR-PTPases are specific only to phosphotyrosine residues, whereas DS-PTPases recognize phosphotyrosine, phosphopthreonine and phosphoserine residues.

PTP1B was the first PTPase isolated and characterized in detail from the human placenta (*188*). Because a decrease in its expression level resulted in increased insulin signalling and action, PTP1B has been considered as a negative regulator of insulin action (*189*). For example, glucose transport and GLUT-4 translocation are decreased in PTP1B-overexpressing cells (*189*), and osmotic loading of neutralizing PTP1B antibodies in rat hepatoma cells increased insulin-induced IRS-1 phosphorylation, PI3-K activity and DNA synthesis (*190*). Also, a role of PTP1B, and other PTPases has been suggested in the pathogenesis of diabetes, because PTPase expression has been shown to be impaired in rodent models of type 1 and type 2 diabetes mellitus, as well as in human subjects (*191-194*).

LAR, a type of R-PTPases, is widely expressed in insulin-sensitive tissues (*195*). Treatment of cells with insulin results in increasing association between LAR and IR. In the plasma membrane, LAR physically interacts with the IR and promotes its dephosphorylation (*196*) Furthermore, fat tissue of obese patients, as well as 3T3-L1 adipocytes pre-incubated with high glucose, show high LAR expression levels (*192*).

SHP2 (also called PTP1D, SH-PTP2, SH-PTP3, Syp and PTP2C) is a ubiquitously-expressed cytosolic PTPase (*197*), composed of a central phosphatase domain, two amino-terminal SH2 domains and a carboxy-terminal tail containing two tyrosyl phosphorylation sites (*186*). Overexpression of dominant negative SHP2 in rat adipocytes impairs insulin-induced GLUT-4 translocation. It also blocks insulin-stimulated mitosis in 3T3-L1 adipocytes and insulin-induced Ras activation. In contrast, disrupting binding between IRS-1 and SHP2 results in increased the Tyr phosphorylation of IRS-1 and heightened insulin-dependent activation of PI3-K as well as protein synthesis but this mutation fails to alter MAPK activity and cell proliferation (reviewed in (*186*)).

It has been shown that the enzymatic activity of PTP1B is inhibited by Zn in C6 rat glioma cells (*198*), as well as in airway epithelial cells (*199*), which can potentially increase the phosphorylation of IR, or other R-PTKs, through inhibition of its dephosphorylation.

1.5.6 – PTKs Other than the IR as Potential Targets of Zinc

As mentioned earlier, Zn has been shown to potently inhibit PTPs (*198-200*), induce tyrosine phosphorylation of insulin receptor-β subunit (IR-β) (*139*), and activate the PI3K/PKB pathway (*128;129;131;139*), all integral parts of the insulin signalling cascade. In addition, Zn has also been shown to induce the tyrosine phosphorylation of epidermal growth factor receptor in A431 human epidermal carcinoma cells, B82 mouse lung fibroblasts, and primary HAE cells (*126;201;202*), as well as cause IGF-1R-β subunit phosphorylation in C6 glioma cells (*198*). Therefore, it is possible that Zn-induced signalling responses may be mediated through the activation of growth factor receptor-PTKs, which we will examine here.

1.5.6.1 – Epidermal Growth Factor Receptor (EGFR)

The EGFR is a receptor tyrosine kinase that is ubiquitously expressed in a variety of cell types, and is most abundant in epithelial cells and many cancer cells (*203-205*). It belongs to a family containing three other members (ErbB2, ErbB3, and ErbB4) that undergo homodimerization or heterodimerization to induce autophosphorylation and receptor tyrosine kinase activation in response to ligand binding (*204;206*). The EGFR contains an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase autophosphorylation and regulatory domain. Dimerization of the receptor activates the intrinsic tyrosine kinase activity of the intracellular domain at different residues, resulting in the recruitment of the SH-containing domain proteins, which trigger downstream

events. The phosphorylation of EGFR on tyrosine 1068 is followed by recruitment of the adaptor protein Grb2, leading to the activation of Ras/ERK1/2 pathway.

1.5.6.2 – Insulin-like Growth Factor type 1 - Receptor (IGF1-R)

IGF-1R is also a receptor tyrosine kinase that is very similar to the IR in structural and functional homology. In fact, in cells that express both receptors, hybrid receptors appear to form readily, yet the biological consequence of these hybrids is not clear (*207*). The receptor is a tetramer consisting of 2 extracellular α-chains and 2 intracellular β-chains. An intracellular tyrosine kinase domain is found in the the β-chain which is believed to be essential for most of the receptors' biological effects (*208*). In response to IGF-1, IGF-1R activation mediates tumor cell proliferation, motility, and protection from apoptosis. Binding of IGF-1 or insulin, at very high, unphysiological concentrations, induces the activation of PTK domain of IGF-1Rβ subunit, activating the autophosphorylation of the receptor (reviewed in (*207*)). Phosphorylation of adaptor/docking proteins, such as insulin receptor substrate (IRS-1 or IRS-2), Shc and Grb2 then takes place (*153;209*). IRS-1, as mentioned earlier, contains multiple tyrosine phosphorylation sites that recognize and bind SH2-containing signalling molecules, such as Grb2, Nck, the p85 subunit of PI3-K and the SHP2. Binding of Grb2/Sos to tyrosine-phosphorylated IRS-1 activates Ras, which then stimulates the Raf-1/MAPK cascade (*210*). The activated IGF-1R also triggers PI3K, and its downstream targets, PKB/Akt and p70s6k (*211;212*).

1.6 – Anti-Diabetic Effects of Zinc

1.6.1 – Effect on Glucose Transport

The stimulatory effect of Zn on glucose transport has been observed in rat adipocytes (*213;214*), as well as in 3T3-L1 fibroblasts and 3T3-L1adipocytes (*139*). In most of these studies, high Zn concentrations were required to enhance glucose transport. For example, May and Contoreggi used up to 1000 μM Zn (in the form of zinc chloride) to stimulate glucose transport (*213*). Ezaki later confirmed these experiments, using 1000 μM Zn to stimulate glucose transport in isolated rat adipocytes by a post receptor mechanism, which was 67% the value achieved with 1 nM insulin (*214*). Further investigations into the functioning of the molecular mechanisms of the anti-diabetic effects of Zn showed that Zn activated glucose transport in adipocytes and 3T3-L1 fibroblasts through activation of PI3K and PKB/Akt (*139*), and in contrast to earlier studies, it was shown that 20 to 50 μM was sufficient to significantly increase glucose transport in 3T3-L1 adipocytes. Considering that normal Zn serum concentration range at approximately 15 μM, concentrations varying from 20 to 50 μM may be sufficient to evoke a physiologically-relevant increase in glucose transport (*139*). Noteworthy is the fact that in the study, Zn had a greater effect on glucose transport in 3T3-L1 adipocytes thank 3T3-L1 fibroblasts. This is explained by the fact that 3T3-L1 adipocytes predominantly express insulin-sensitive glucose transporter protein type 4 (GLUT4), and 3T3-L1 fibroblasts express insulin-insensitive GLUT1, showing that Zn acts here as an insulin-mimetic, since insulin-stimulated glucose transport is mediated by GLUT-4

(*215;216*). Ilouz et al. also showed that physiological concentrations of Zn (10 μM), in the form of $ZnCl_2$, stimulated glucose uptake in GLUT4 expressing isolated mouse adipocytes, to a much greater extent than in C6 rat glioma cells and GP8 rat brain endothelial cells, which both express GLUT1, and not GLUT4 (*217*). They go on to show that physiological concentrations of Zn inhibited glycogen synthase kinase 3β (GSK-3β), and suggest that inhibition of GSK-3β by phosphorylation is essential for glucose uptake (*217*).

1.6.2 – Effect on Glycogen Metabolism and Gluconeogenesis

Another physiological response modulated by Zn is its action on glycogen synthesis. As early as 1985, it was shown that rat livers exposed to Zn salts for 30 days contained twice as much glycogen as those of control rats (*218*). Not only was there more glycogen, but the storage of glycogen in the liver of the rats was also accelerated by feeding them Zn, in the form of zinc acetate (*218*). More recent studies have shown that Zn, in the form of $ZnCl_2$, phosphorylates GSK-3β at physiological concentrations (15 μM) in HEK-293 cells (*217*). GSK-3β and Forkhead box protein 01 (FOX01) were also shown to be phosphorylated by $ZnSO_4$ in HepG2 cells. Phosphorylation of GSK-3β inactivates it, causing increased glycogen synthesis by upregulating glycogen synthase (*182*). Phosphorylation of FOX01, a transcription factor largely involved in glucose metabolism, causes it to become inactive. FOXO1 is therefore excluded from the nucleus causing an inhibition of hepatic glucose production, through the inhibition of gene expression of gluconeogenic enzymes (*179*). An inhibitory effect of Zn on gluconeogenesis in rat renal cortex slices, as well as rat liver parenchymal cells, has also been demonstrated (*219;220*). It may be suggested that Zn –

induced activation of PI3K/PKB signalling through GSK-3 and FOX01 contributes to enhanced glycogen synthesis, as well as decreased gluconeogenesis.

1.6.3 – Effect on Lipogenesis and Lipolysis

In addition to their action on glucose metabolism, Zn has been reported to alter lipid metabolism both *in vivo* and *in vitro*. As mentioned earlier, Coulston and Dandona showed that Zn, in the form of zinc chloride ($ZnCl_2$) can mimic insulin in its ability to stimulate lipogenesis in rat adipocytes (*100*). Later studies by May and Contoreggi showed that Zn was able to inhibit lypolysis in isolated rat adipocytes stimulated with the β-adrenergic agent ritodrine (*213*), albeit at a high concentration (500μM). This effect was thought to be due to Zn-induced H_2O_2 generation, since exogenous catalase reversed the inhibited lypolysis (*213*), and since H_2O_2 had already been shown to mimic a variety of insulins' actions, including lipogenesis and inhibition of lipolysis (*221*). In 1992 though, Shisheva et al. showed that $ZnCl_2$ increased the uptake of glucose to lipids in rat adipocytes, with a maximum stimulation of lipogenesis 55-80% of maximum insulin response (*222*). They also showed that this event was not due to H_2O_2 production by $ZnCl_2$ as catalase treatment did not inhibit $ZnCl_2$ induced glucose oxidation and its incorporation into lipids (*222*). More recently, insulin mimicking suppression of lipolytic activity was exhibited when both $ZnSO_4$ and $Zn(Mal)_2$ (*116*), as well as $Zn(alx)_2$ (*121*) showed a potent inhibition of free fatty acid release from epinephrine-stimulated rat adipocytes.

1.6.4 – Goal of this Study

As discussed above, Zn compounds have been found to have anti-diabetic, insulin-mimetic, and insulin-potentiating in a plethora of cellular studies, as well as in rodent models of type 1 and type 11 diabetes mellitus, and in a limited number of human studies. These compounds have been shown to enhance and/or mimic insulin action, which includes increasing glucose transport, glycogen synthesis and lipogenesis as well as inhibition of gluconeogensis and lipolysis. In *in vitro* systems, the molecular mechanism of Zn compounds correlates with the activation of different components of the insulin signalling pathway, such as PI3-K and ERK1/2. However, the precise mechanism by which Zn compounds enhance or mimic insulin-like effects is still poorly characterized. There are conflicting data and the precise upstream elements responsible for mediating the Zn-induced activation of ERK1/2 and PKB signalling have not yet been identified. Also, a precise role of IR in mediating the insulin-like effects of Zn *in vivo* and *in vitro* systems remains to be established.

Therefore, the studies presented in this work were undertaken to elucidate a possible role for IR, IGF-1R and EGFR receptor tyrosine kinases on PKB and ERK1/2 phosphorylation by Zn in CHO cells. These studies have used standard protocols of cellular and molecular biology such as cell culture, immunoprecipitation and western blotting, and have also used cells that express wild type or tyrosine kinase deficient forms of IR and IGF-1R.

Chapter 2

Extracellular Zn^{2+}-induced activation of ERK1/2 and PKB phosphorylation requires insulin like growth factor-1 receptor (IGF-1R) and is independent of insulin receptor (IR) protein tyrosine kinase (PTK).

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Extracellular Zn^{2+}-induced activation of ERK1/2 and PKB phosphorylation requires insulin like growth factor-1 receptor (IGF-1R) and is independent of insulin receptor (IR) protein tyrosine kinase (PTK).

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Running title: Zn^{2+}-induced ERK1/2 and PKB activation required IFG-1R

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ABBREVIATIONS

CHO, Chinese hamster ovary; CHO-HIR, CHO cells overexpressed with human insulin receptor; CHO-1018, CHO cells over-expressing a mutant human insulin-receptor in which Lysine 1018 has been replaced by alanine causing a complete loss of the PTK activity of insulin receptor; ERK1/2, extracellular signal-regulated kinase 1 and 2; EGF, epidermal growth factor; EGFR, EGF receptor; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor; IR, insulin receptor; IRS-1, insulin receptor substrate-1; MAPK, mitogen-activated protein kinase; MCF-7, human breast carcinoma MCF-7 cells; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PMSF, phenylmethylsulphonyl fluoride; PVDF, polyvinilidene difluoride.

Abstract

Recent studies have demonstrated that Zn^{2+} exerts insulin-mimetic and anti-diabetic effects in rodent models of insulin-resistance. Zn^{2+} was also shown to activate ERK1/2 and PI3K/PKB, two key components of the insulin signaling pathway. Zn^{2+}-induced signaling was associated with an increase in the tyrosine-phosphorylation of insulin receptor (IR), as well as phosphorylation of insulin-like growth factor-1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR), however, the specific contribution of each of these receptor protein tyrosine kinases (R-PTKs) in Zn^{2+}-induced responses remains to be established. Therefore, in the present studies, by using a series of pharmacological inhibitors and cells that overexpress normal and mutant/inactive forms of IR and IGF-1R, we have investigated the role of R-PTKs in Zn^{2+}-induced ERK1/2 and PKB phosphorylation. Zn^{2+} stimulated ERK1/2 and PKB phosphorylation in a dose and time-dependent manner in Chinese hamster ovary cells overexpressing IR (CHO-HIR). Pretreatment with AG1024, an inhibitor for IR- and IGF-1R-PTK, blocked Zn^{2+}-induced ERK1/2 and PKB phosphorylation, but AG1478, an inhibitor for EGFR was without effect. In CHO cells overexpressing tyrosine kinase deficient IR (CHO-1018), Zn^{2+} was still able to induce the phosphorylation of these two signaling molecules, whereas insulin effect was significantly attenuated. Furthermore, both Zn^{2+} and IGF-1 failed to stimulate ERK1/2 and PKB phosphorylation in IGF-1R KO cells. In addition, Zn^{2+} had no effect on the tyrosine phosphorylation of IR-β-subunit and IRS-1 in CHO-HIR cells. Taken together, these data suggest that Zn^{2+}-induced ERK1/2 and PKB phosphorylation is independent of IR- or EGFR-PTK, yet nonetheless requires IGF-1R-PTK.

Zinc (Zn^{2+}), one of the most abundant trace metals, is fundamental for structural and regulatory cellular functions. It plays critical roles in insulin biosynthesis, storage and insulin secretion from pancreatic β cells (*1-4*). Zn^{2+} has also been shown to exert insulin-mimetic (*5-9*) and anti-diabetic (*10-14*) effects in various rodent models of insulin resistance, and has also been shown to activate lipogenesis and glucose transport in adipocytes (*9;15;16*). Zn^{2+}-deficient rats have abnormal glucose tolerance, which could be reversed by Zn^{2+} repletion (*5*). Additionally, dietary Zn^{2+} supplementation has been reported to attenuate hyperglycemia in leptin receptor deficient db/db mice (*10*). Furthermore, type 2 diabetic subjects have been reported to exhibit lower serum levels of Zn^{2+} as compared to healthy individuals (*17;18*), indicating that Zn^{2+} deficiency might be linked with insulin resistance.

In a plethora of studies, Zn^{2+} was demonstrated to activate several key components of the insulin signaling pathways, such as mitogen-activated protein kinases (MAPKs), extracellular-regulated protein kinase 1 and 2 (ERK1/2) (*19-23*), phosphatidylinositol 3-kinase (PI3K) (*22;24*), protein kinase B (PKB/Akt) (*8;21;22;24-27*), mammalian target of rapamycin (mTOR), as well as p70S6K in several cell types (*24;28*). Early studies did not detect any increase in insulin receptor protein tyrosine kinase (IR-PTK) in rat adipocytes stimulated with Zn^{2+} (*15*). Also, certain in vitro studies suggested an inhibitory effect by Zn^{2+} on insulin-induced IR autophosphorylation (*29*). In contrast, later studies have shown an increase in the Zn^{2+} -induced tyrosine phosphorylation of IR-PTK(*26;30-32*). However, divergent effects of Zn^{2+} on upstream tyrosine kinases responsible for activating these signaling pathways have been suggested. For example, Zn^{2+} treatment of 3T3-L1

fibroblasts and adipocytes showed an increased phosphorylation of the β-subunit of insulin receptor (IR) (*26*), whereas it had no effect on IR-PTK in rat adipocytes (*15*). Moreover, in vitro studies demonstrated that Zn^{2+} exerted an inhibitory effect on insulin-induced autophosphorylation of IR (*29*). Additionally, Zn^{2+} was found to increase tyrosine phosphorylation of insulin-like growth factor type I receptor (IGF-1R) in C6 glioma cells (*33*), and an important role of epidermal growth factor receptor (EGFR) in Zn^{2+}-induced signal transduction has also been suggested in human epidermal A431 cells (*34;35*). Thus, the precise upstream elements responsible for provoking the Zn^{2+}-induced activation of ERK1/2 and PKB signaling remain to be elucidated. Furthermore, in view of the emerging insulin-like effects mediating the *in vivo* and *in vitro* systems, a potential role of IR in this process remains to be established. Therefore, in the present studies, by using a series of pharmacological inhibitors of EGFR, IR and IGF-1R protein tyrosine kinases, as well as cells that overexpress normal and mutant/inactive form of IR and IGF-1R, we explored the molecular mechanism of extracellular Zn^{2+}-induced phosphorylation of ERK1/2 and PKB.

Material and Methods

Antibodies and reagents:

Cell culture reagents were procured from Invitrogen Corp. (Grand Island, NY). Monoclonal phospho-specific ERK1/2 antibody and polyclonal ERK1/2 antibody; horseradish peroxidase-conjugated goat anti-mouse immunoglobulin were from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-Ser^{473}-specific-PKB antibody, the PKB antibody and anti-rabbit antibody were procured from Cell Signaling (Beverly, MA). PTKs inhibitors, AG1024 and AG1478; and PI3K inhibitor, Wortamannin, were procured from Calbiochem (San Diego, CA). Insulin was purchased from Eli-Lily Co. (Indianapolis, IN). $ZnSO_4$ was obtained from Fischer Chemical (Pittsburg, PA). The enhanced chemiluminescence (ECL) detection system kit was from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada).

Cells and Cell culture: Parental Chinese hamster ovary (CHO) cells, CHO cells over-expressing a normal human insulin receptor (HIR), CHO-HIR cells and CHO cells over-expressing a mutant human insulin-receptor in which Lysine 1018 has been replaced by Alanine, causing a complete loss of the PTK activity of insulin receptor (CHO-1018), were a kind gift from Dr. Morris F. White (Joslin Diabetes Center, Boston, MA, USA) (*36*). CHO-HIR and CHO-1018 cells were maintained on F-12 medium (Invitrogen, Burlington, ON, Canada) with 10% fetal bovine serum (FBS).

MCF-7 breast cancer cells, MCF-7 wild type (MCF-7 WT) and MCF-7 stably transfected with an antisense IGF-1R cDNA (MCF-7 SX13) cells were a gift from D. LeRoith (NIH, Bethesda, Maryland) (*37*). MCF-7 normal cells were maintained on DMEM high glucose

medium with 10% FBS, while MCF-7 SX13 cells were grown on DMEM high glucose medium with G418 0.5g/L and 10% FBS (*37*). All cells were grown to 80% confluence in 60 mm plates and incubated in serum-free corresponding medium containing for 20 h and refresh with serum free corresponding medium 1h prior to the experiment.

Immunoblotting : Cells incubated in the absence or presence of stimulating agents were washed twice with ice-cold PBS and lysed in 200 μl of buffer A (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM Na orthovanadate, 10 mM Na fluoride, 10 mM Na pyrophosphate, 2 mM benzamidine, 2 mM ethylenebis (oxyethylenenitrolo)-tetraacetic acid (EGTA), 2mM ethylenediamine-tetraacetic acid (EDTA), 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 1% Triton X-100 and 0.1% sodium dodecyl sulfate (SDS), 0.5 μg/ml leupeptin on ice. The cells were scraped, collected and centrifuged at 12,000 g for 10 min. Protein concentrations were determined by using Bradford assay. Equal amounts of protein were subjected to 10% SDS-PAGE, transferred to polyvinilidene difluoride (PVDF) membranes and incubated with monoclonal phospho-specific-ERK1/2 antibody (1:4000), with polyclonal phospho-Ser^{473}-specific-PKB antibody (1:2000), detected by a horseradish peroxidase conjugated second antibody (1:4000), and visualized with an ECL detection kit, as described earlier (*38*). The same blots were subsequently reprobed with ant-ERk1/2 or anti-PKB antibodies to detect the total amount of these proteins.

Immunoprecipitation: Cleared cell lysates after stimulation (as prepared for immunoblotting) were incubated either with the insulin receptor (IR) or insulin receptor substrate-1 (IRS-1) antibodies at 4°C overnight. The antigen-antibody complexes were immunoprecipitated with protein A sepharose beads for 2h at 4°C. The immunoprecipitated

protein was then washed once with PBS 1X containing inhibitors (1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml aprotinin, 0.5 μg/ml leupeptin and 1 mM Na orthovanadate) and twice with cold lysate buffer, before solublization in Laemmli's sample buffer.

Data analysis: Results from immunoblots were quantified by densitometric analysis of bands using NIH-image J, as well as the Syngene GeneTools software programs. Values presented here are means ± S.D. of at least three independent experiments.

Results

Characterization of Zn^{2+}-induced ERK1/2 and PKB phosphorylation

As shown in Fig 1, treatment of CHO-HIR cells with Zn^{2+} caused a dose-dependent (Fig.1A) and time-dependent (Fig.1B) increase in the phosphorylation of ERK1/2 and PKB. Increased phosphorylation of both ERK1/2 and PKB peaked at around 100 μM of Zn^{2+}. A time course analysis using 50 μM Zn^{2+} revealed that Zn^{2+} treatment resulted in a time-dependent increase in the phosphorylation of both ERK1/2 and PKB in CHO-HIR cells. The phosphorylation was rapid and could be detected as early at 2 min of the treatment and showed a peak at 5 minutes in case of ERK1/2 and 15 min in case of PKB phosphorylation. These effects were sustained up to 60 minute time points.

Effect of Zn^{2+}-induced ERK1/2 and PKB phosphorylation is blocked by AG1024

Zn^{2+} has been shown to increase the tyrosine phosphorylation of EGFR (*34;35;39*), IGF-1R and IR (*26;30-32*), implying a role of these R-PTKs in Zn^{2+}-induced signaling events, therefore, we examined a role of these R-PTKs in Zn^{2+} -induced ERK1/2 and PKB phosphorylation in CHO-HIR cells. For these experiments, we used AG1478, an inhibitor of EGFR-PTK (*40*) and AG1024, an inhibitor for IR-PTK and IGF-1R-PTK (*41;42*). As shown in Fig.2A, pretreatment of CHO-HIR cells with AG1024 significantly blocked Zn^{2+}-induced ERK1/2 and PKB phosphorylation, whereas AG1478 exerted no significant inhibition (Fig.2B).

Effect of Zn^{2+}on tyrosine phosphorylation of IR-β subunit and IRS-1

In addition to inhibiting IGF-1R-PTK, AG1024 also blocks the IR-PTK activity (*41*). Moreover, Zn^{2+} has been shown to enhance tyrosine phosphorylation of IR-β subunit in multiple cell types (*26*). Therefore, we directly assessed the effect of Zn^{2+} on tyrosine phosphorylation of IR-β subunit. As shown in Fig.3A, Zn^{2+} treatment failed to enhance the tyrosine phosphorylation of IR-β subunit, while as expected, insulin treatment resulted in a robust increase in IR-β subunit tyrosine phosphorylation. Since IRS-1 phosphorylation is critical to propagate insulin/IGF-1-induced signaling, we next investigated if stimulation of PKB in response to Zn^{2+} was associated with IRS-1 tyrosine phosphorylation. As shown in Fig.3B, similar to its effect on IR-β subunit phosphorylation, Zn^{2+} had no effect on IRS-1 phosphorylation, whereas insulin and IGF-1, when used as a positive control, had a stimulatory effect.

Effect of Zn^{2+}on ERK1/2 and PKB phosphorylation in IR-PTK deficient CHO-1018 cells

To further analyze the involvement of IR-PTK in Zn^{2+}-induced responses, we utilized CHO cells overexpressing a mutant form of human IR, where the lysine 1018 in the ATP-binding domain was mutated to alanine. This mutant form is unable to bind ATP and lacks the phospho-transferase activity of IR-PTK (*43*). As shown in Fig.4, both Zn^{2+} and insulin enhanced the ERK1/2 and PKB phosphorylation in CHO cells overexpressing wild type IR (active IR) CHO-HIR. However, in CHO-1018, the insulin-induced ERK1/2 and PKB phosphorylation was significantly attenuated, whereas Zn^{2+}-induced effect was unaffected.

Zn^{2+}-induced ERK1/2 and PKB phosphorylation is mediated by IGF-1R

The data presented above suggested that Zn^{2+}-induced effects on these signaling components were independent of IR-PTK activity. Since AG1024, which is also an inhibitor of IGF-1R-PTK, blocked the stimulatory effect of Zn^{2+} on ERK1/2 and PKB phosphorylation in CHO-HIR cells, we next examined the involvement of this PTK in mediating the response of Zn. For these experiments we used MCF-7 cells which express normal IGF-1R (IGF-1R, WT cells) and MCF-7 cells which are stably transfected with a mutant negative IGF-1R (IGF-1R KO SX-13 cells cells) (*37*). As depicted in Fig.6, ERK1/2 stimulation of IGF-1R WT cells with either 10 nM IGF-1 or 100 uM of Zn^{2+} for 5 minutes resulted in a robust phosphoryation of ERK1/2 and PKB. However, in IGF-1R KO cells, both IGF-1 and Zn^{2+}-induced effects were significantly attenuated.

Discussion

In the present studies, we have demonstrated that extracellular Zn^{2+} induces the phosphorylation of ERK1/2 and PKB, two well established mediators of insulin action. By using pharmacological and molecular approaches, we have also shown that Zn^{2+}-induced responses on these signaling components primarily require activation of IGF-1R PTK.

Earlier studies have also documented stimulatory effects of Zn^{2+} on MAPKinases, ERK1/2, p38mapk, c-JNK, p70s6k and PI3-K signaling in many different cell types (*19-24;28*). In addition, Zn^{2+} treatment was shown to induce transactivation of EGF-R in B 82L fibroblasts, as well as in A431 cells (*35;39*). Moreover, Zn^{2+}-induced effects were linked with an increase in tyrosine phosphorylation of cellular proteins (*31*), and were suggested to be associated with the activation of IR (*26;30;32*). In contrast, other investigators have shown that Zn^{2+}-induced actions were independent of IR (*8;15;29*) and EGF-R phosphorylation (*35*).

Our studies are the first to investigate an involvement of EGF-R/IR/IGF-1R in Zn^{2+}-induced ERK1/2 and PKB phosphorylation. Our data showing that AG1478, a highly specific inhibitor of EGF-R-PTK, failed to attenuate Zn^{2+}-induced increase in ERK1/2 and PKB phosphorylation suggested that these events are independent of EGF-R PTK. In contrast to our current study, by using PP-153035, another inhibitor of EGF-R-PTK, a role of EGF-R in Zn^{2+}-induced ERK1/2 phosphorylation in A431 cells has been suggested (*35*). Existance of an EGF-R-independent mechanism for Zn^{2+}-induced effects has also been suggested in studies demonstrating that PP2, a Src-PTK inhibitor, despite blocking Zn^{2+}-induced EGF-R tyrosine phosphorylation had no effect on Zn^{2+}-induced ERK1/2

phosphorylation (*35*). Thus, it may be possible that both EGFR-PTK –dependent and -independent pathways contribute to Zn^{2+}-induced signaling.

Zn^{2+} has been suggested to exert various insulin-like effects (*5-9*). Insulin signaling is triggered when insulin binds to the α-subunit of the insulin receptor (IR) on the cell membrane resulting in conformational changes that activate intrinsic protein tyrosine kinase (PTK) activity of the β-subunit by its autophosphorylation in multiple tyrosine residues (*44-46*). In line with this, Zn^{2+} was shown to increase tyrosine phosphorylation of insulin receptor ß-subunit in 3T3-L1 fibroblasts, as well as adipocytes (*26*). However, under these conditions, Zn^{2+} failed to enhance IRS-1 or IRS-2 tyrosine phosphorylation in these cells (*26*). Contrary to these observations, Zn^{2+} did not increase tyrosine phosphorylation of IR in rat adipocytes (*15*) suggesting that Zn^{2+} exerts insulin-like effects through an IR-independent pathway. Further studies have supported the later view and suggested that Zn^{2+} mimics several actions of insulin, both *in vitro* and *in vivo* by a mechanism unrelated to insulin signaling (*8*). The inability of Zn^{2+} to increase tyrosine phosphorylation of IR-b and IRS-1 in our studies support these later studies, and suggests that Zn^{2+}-induced effects are not associated with IR-PTK functions. Our results showing that among various pharmacological inhibitors tested, only AG1024, which is an inhibitor of IR/IGF-1R-PTK, attenuated Zn^{2+}-induced responses pointed towards an intermediary role of IR/IGF-1R in this process. However, the fact that phosphorylation of ERK1/2 and PKB in response to Zn^{2+} was identical in CHO-HIR and IR-PTK-deficient CHO-1018 cells clearly demonstrated that Zn^{2+} signals in an IR-PTK-independent fashion. Since AG1024 also targets IGF-1R-PTK, we further tested the involvement of IGF-1R receptor in Zn^{2+}-induced

ERK1/2 and PKB phosphorylation. We found that Zn^{2+}, as well as IGF-1 induced ERK1/2 and PKB phosphorylation, was almost completely suppressed in IGF-1R KO cells, but not in the cells with normal IGF-1R expression. Normally, IGF-1-induced signaling requires IRS-1 to propagate downstream signaling events to activate MAPK and PI3K/PKB pathways; exceptionally in this study, we observed that Zn^{2+} was unable to induce IRS-1 phosphorylation in CHO-HIR cells. Similar findings have also been reported by others (*26;33*), and additional work in transgenic IRS-1 knockout mice has suggested the existence of IRS-1-independent signaling pathways (*47;48*). It has also been suggested that the p85 subunit of PI-3Kinase directly interacts with IR (*49*) and that a direct interaction of p85, Syp and GAP, and the IGF-1R is possible (*50*). Thus Zn^{2+}-induced responses of ERK1/2 and PKB phosphorylation might be mediated through direct interaction of P85/PI-3Kinase with IGF-1R, as suggested earlier (*50*).

In summary, our studies have provided first experimental evidence for a role of IGF-1R-PTK in Zn^{2+}-induced activation of ERK1/2 and PKB signaling pathway, independent to the action of EGF-R and IR-PTK. We have also demonstrated that IR/IRS-1 tyrosine phosphorylation may not be necessary to transduce Zn^{2+}-induced responses in Zn^{2+}-induced responses. These data provide novel insight into the cellular mechanism of Zn^{2+} action.

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Figure Legends

Figure 1. Zn^{2+}-induced dose response and time course of ERK1/2 and PKB phosphorylation: Serum-starved quiescent CHO-HIR cells were treated with or with out Zn^{2+} at indicated concentrations for 5 min, and at 50 μM Zn^{2+} at the indicated time points (Fig.1B). Cell lysates were prepared and equal amounts of protein was separated on 10% SDS-PAGE. ERK1/2 and PKB phosphorylation was detected by immunoblot analysis with phospho-specific ERK1/2 and PKB antibodies. Blots were also analyzed for total ERK1/2 and PKB. The blots represent at least three independent experiments.

Figure 2. Effect of EGF-R, IR and IGF-1R tyrosine kinase inhibitors on Zn^{2+}-induced ERK1/2 and PKB phosphorylation: Serum-starved quiescent CHO-HIR cells were treated with or with out insulin (100 nM), EGF (100 nM) or Zn^{2+} (100 μM) in presence or absence of 10 μM of AG1024 (IR/IGF-1R PTK inhibitor) or AG1478 (EGF-R PTK inhibitor) pre-incubation for 30 minutes, as indicated. Cell lysates were prepared and equal amounts of protein was separated on 10% SDS-PAGE. ERK1/2 and PKB phosphorylation was detected by immunoblot analysis with phospho-specific ERK1/2 and PKB antibodies. Blots were also analyzed for total ERK1/2 and PKB. The blots represent at least three independent experiments.

Figure 3. Zn^{2+} dose not stimulate IR-β or IRS-1 in CHO-HIR cells: Serum-starved quiescent CHO-HIR cells were treated with or with out insulin (100 nM), IGF-1(10ng/ml) or Zn^{2+} (100 μM) for 5 minutes, (Fig.3A,B) for 5 minutes. For IR- phosphorylation cleared cell lysates were immunoprecipitated with IR and immunoblotted with p-Tyr. To see the

status of IRS-1 phosphorylation, cleared cell lysates were immunoprecipitated with IRS-1 and immunoblotted with p-Tyr. Results shown here represent at least three independent experiments.

Figure 4. Zn^{2+} induces ERK1/2 and PKB phosphorylation in IR-deficient CHO-1018 cells. Serum-starved quiescent CHO-HIR and CHO-1018 cells were treated with or with out insulin or Zn2+ for various concentrations as indicated for 5 minutes. Cell lysates were prepared and equal amounts of protein was separated on 10% SDS-PAGE. ERK1/2 and PKB phosphorylation was detected by immunoblot analysis with phospho-specific ERK1/2 and PKB antibodies. Blots were also analyzed for total ERK1/2 and PKB. A. Insulin-stimulated ERK1/2 and PKB phosphorylation in CHO-HIR and CHO-1018 cells, B. Zn2+-stimulated ERK1/2 and PKB phosphorylation in CHO-HIR and CHO-1018 cells. Values are the means ± SE of at least three independent experiments.

Figure 5. Zn^{2+}-induced ERK1/2 and PKB phosphorylation is attenuated in IGF-1R-defecient SX-13 cell: Serum-starved quiescent MCF-7 and MCF-7 SX13 cells were treated with or with out IGF-1 (10 nM) or Zn2+ (100 μM) for 5 minutes. Cell lysates were prepared and equal amounts of protein was separated on 10% SDS-PAGE. ERK1/2 and PKB phosphorylation was detected by immunoblot analysis with phospho-specific ERK1/2 and PKB antibodies. Blots were also analyzed for total ERK1/2 and PKB. Values are the means ± SE of at least three independent experiments.

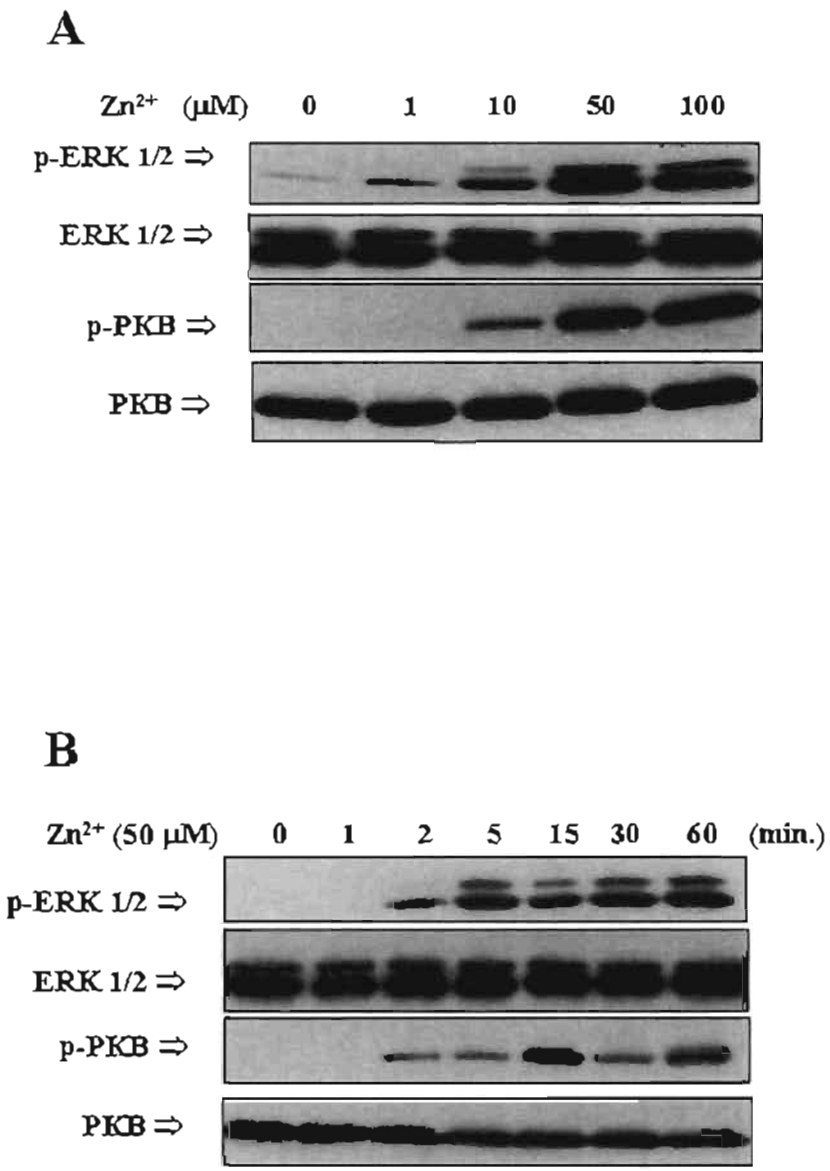
A
Zn2+ (μM) 0 1 10 50 100
p-ERK 1/2 ⇒
ERK 1/2 ⇒
p-PKB ⇒
PKB ⇒
B
Zn2+ (50 μM) 0 1 2 5 15 30 60 (min.)
p-ERK 1/2 ⇒
ERK 1/2 ⇒
p-PKB ⇒
PKB ⇒

Figure 1

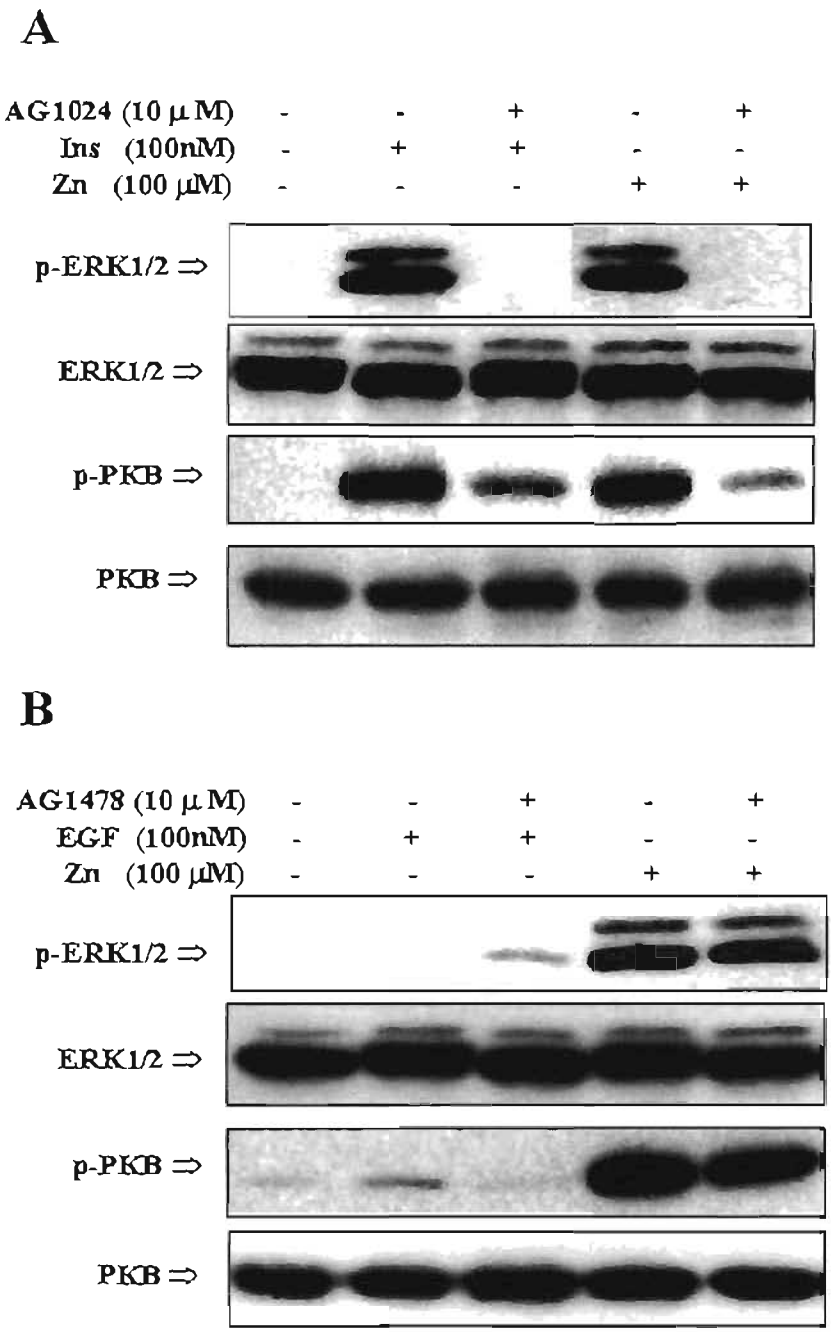
A
AG1024 (10 μM) - - + - +
Ins (100nM) - + + - -
Zn (100 μM) - - - + +
p-ERK1/2 ⇒
ERK1/2 ⇒
p-PKB ⇒
PKB ⇒
B
AG1478 (10 μM) - - + - +
EGF (100nM) - + + - -
Zn (100 μM) - - - + +
p-ERK1/2 ⇒
ERK1/2 ⇒
p-PKB ⇒
PKB ⇒

Figure 2

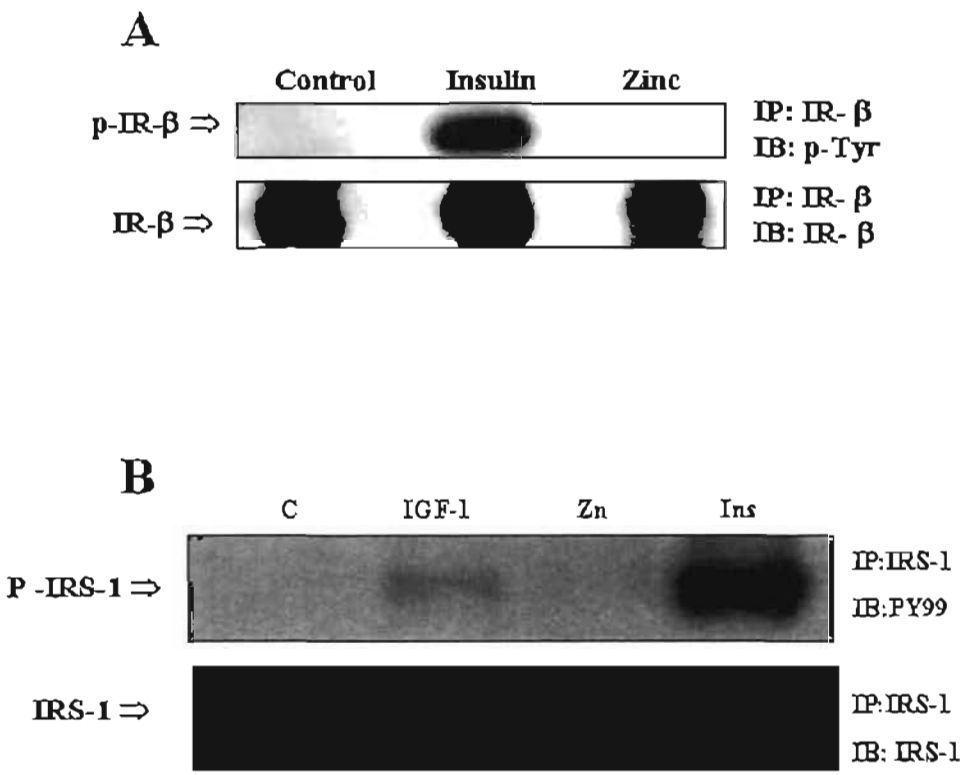
A
Control
Insulin
Zinc
p-IR-β ⇒
IP: IR- β
IB: p-Tyr
IR-β ⇒
IP: IR- β
IB: IR- β
B
C
IGF-1
Zn
Ins
P -IRS-1 ⇒
IP:IRS-1
IB:PY99
IRS-1 ⇒
IP:IRS-1
IB: IRS-1

Figure 3

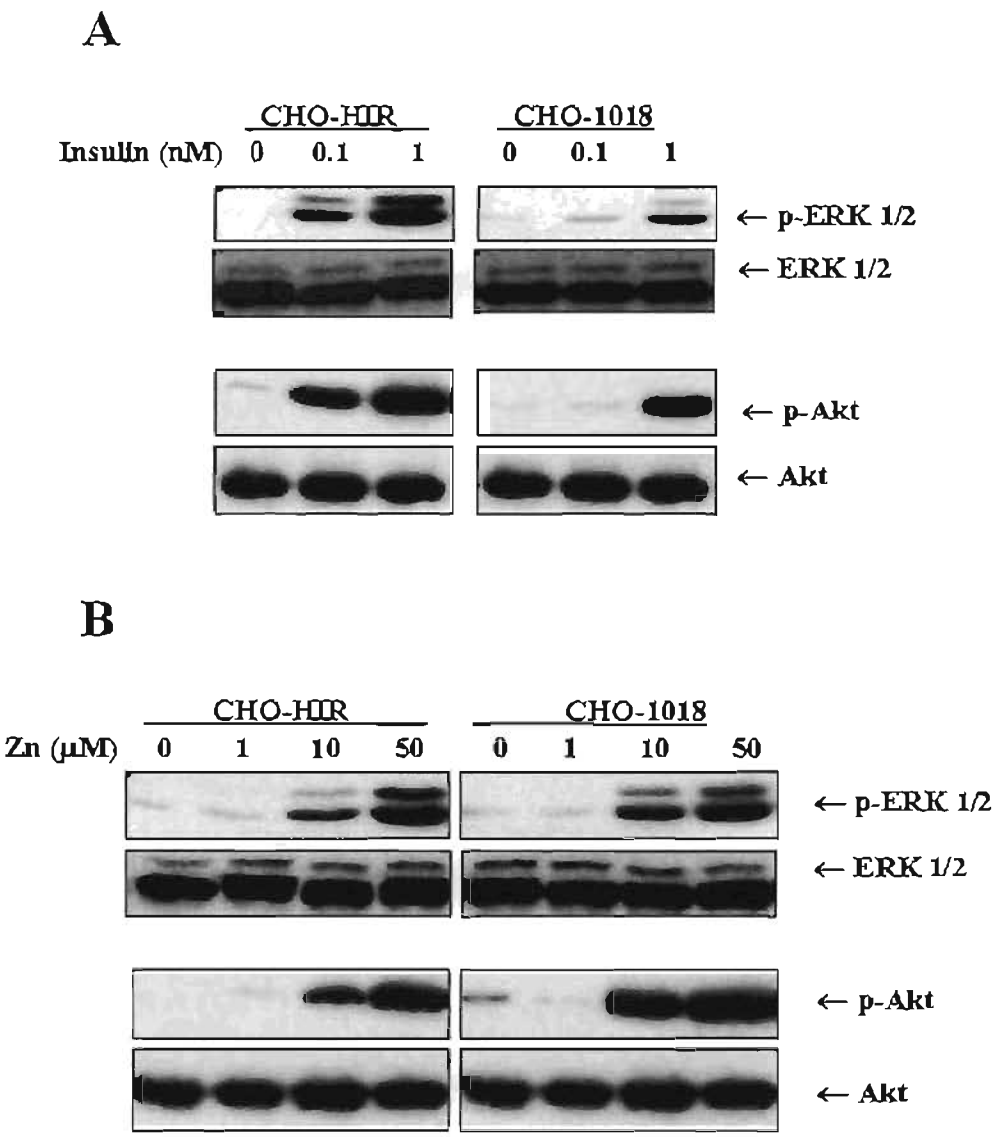
A
CHO-HIR
CHO-1018
Insulin (nM) 0 0.1 1 0 0.1 1
← p-ERK 1/2
← ERK 1/2
← p-Akt
← Akt
B
CHO-HIR
CHO-1018
Zn (μM) 0 1 10 50 0 1 10 50
← p-ERK 1/2
← ERK 1/2
← p-Akt
← Akt

Figure 4

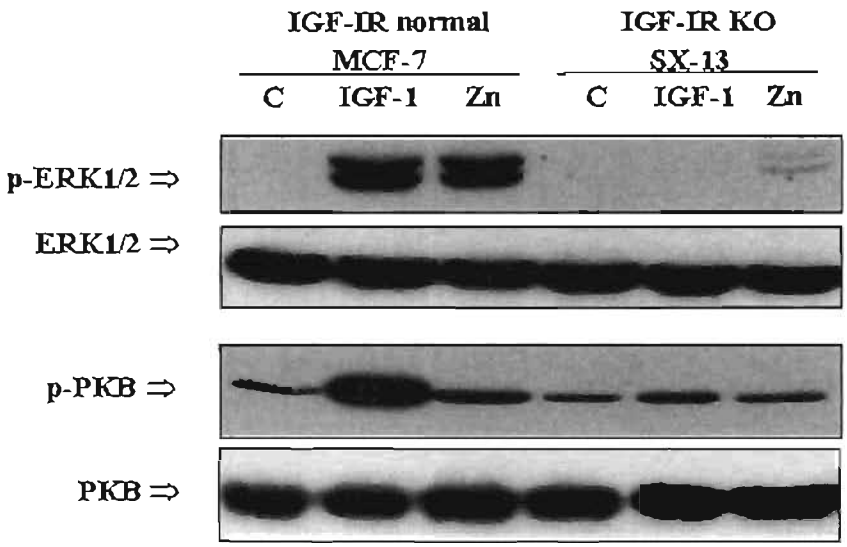
IGF-IR normal
MCF-7
IGF-IR KO
SX-13
C
IGF-1
Zn
C
IGF-1
Zn
p-ERK1/2 ⇒
ERK1/2 ⇒
p-PKB ⇒
PKB ⇒

Figure 5

Chapter 3 – General Discussion

A potential role of Zn in insulin action was suggested as early as 1966, when it was found that Zn deficient animals were less sensitive to insulin (*101*). The demonstrations a decade later that Zn could stimulate lipogenesis in rat adipocytes further strengthened the insulin-mimetic role of Zn (*100*). Later studies examined the effect of Zn on glucose transport in rat adipocytes (*213;214*), 3T3-L1 fibroblasts and 3T3-L1adipocytes (*139*), and showed that treatment of these cells with $ZnCl_2/ZnSO_4$ induced glucose uptake by about 2 fold. Since then, these findings were extended to include the effects of Zn treatment in animal models of type 1 and type 2 diabetes mellitus. These studies revealed that Zn treatment partially prevented hyperglycaemia and development of diabetes in STZ rat model of type 1 diabetes (*103*). Similarly, Zn supplementation attenuated fasting hyperglycaemia and hyperinsulinemia in *ob/ob* mouse model of type 2 diabetes mellitus (*108*).

Multiple studies were also performed at the cellular level, showing that Zn can mimic insulin and activate several key elements of the insulin signal transduction pathway. For example, activation of PI3K (*129*), as well as PKB/Akt, in multiple cell types, such as in airway epithelial cells, HT-29 colorectal cancer cells, 3t3-L1 fibroblasts and rat adipocytes (*127;139;181;222;237*) was shown. Similarly, Zn was found to activate ERK1/2 in HT-29 cells (*125*) and bronchial epithelial cells (*236*).

Zn treatment also enhanced IR phosphorylation in rat adipocytes, digestive gland cells, and 3T3-L1 fibroblasts (*36;139;240;241*), although earlier studies had not detected

any increase in IR-PTK or IR-phosphorylation in rat adipocytes stimulated with Zn. In fact, some studies even suggested an inhibitory effect of Zn on insulin-induced IR autophosphorylation (*239*). A role of IGF-1R and EGFR in Zn-induced ERK 1/2 and PKB signal initiation was also suggested in C6 glioma cells (*198*) and human epidermal A431 cells (*126;242*), respectively.

In view of these conflicting data on the role of various receptor PTKs in initiating Zn-induced signalling events, the present studies were undertaken to investigate a role for receptor PTKs in Zn-induced ERK1/2 and PKB/Akt phosphorylation. For these experiments, we have used AG1478, an inhibitor of EGFR-PTK and AG1024, an inhibitor for IR-PTK and IGF-1R-PTK. Our data suggests that EGFR does not participate in either PKB or ERK1/2 activation by Zn in CHO-IR cells, since AG1478 had no inhibitory effect on PKB or ERK1/2 phosphorylation in response to Zn. Thus, our results are in conflict with earlier studies in A431 cells where a role of EGFR activation in Zn-induced ERK1/2 and PKB activation was demonstrated (*126;242*). However, our work is supported by the studies where PP2, a Src-PTK inhibitor, despite blocking Zn-induced EGFR tyrosine phosphorylation, failed to inhibit Zn-induced ERK1/2 phosphorylation (*126*), and suggests the existence of an EGFR-independent mechanism of Zn-induced signalling.

As for AG1024, it significantly blocked Zn-induced ERK1/2 and PKB phosphorylation, implying that either IR or IGF-1R plays a role in the signal transduction.

To further probe the role of IR/IGF-1R in this process, we assessed the effect of Zn on tyrosine phosphorylation of IR-β subunit. Zn^{2+} treatment failed to enhance the tyrosine

phosphorylation of IR-β subunit, while as expected, insulin treatment resulted in an increase in IR-β subunit tyrosine phosphorylation. Our results differ from earlier studies showing that Zn enhances tyrosine phosphorylation of IR-β subunit in multiple cell types (*36;139;240;241*).

To further analyze the involvement of IR-PTK in Zn-induced responses, we utilized CHO cells overexpressing a mutant form of human IR, in which the lysine 1018 in the ATP-binding domain was mutated to alanine (*249*). Both Zn and insulin enhanced the ERK1/2 and PKB phosphorylation in CHO-IR cells. However, in CHO-1018 cells, insulin-induced ERK1/2 and PKB phosphorylation was significantly attenuated, whereas Zn-induced effect was unaffected, providing further evidence that IR is not necessary for Zn-induced ERK1/2 and PKB activation in CHO cells.

Since AG1024 also blocks IGR-1R, we examined the involvement of this PTK in mediating the response of Zn. For these experiments we used MCF-7 cells which express normal IGF-1R (IGF-1R, WT cells) and MCF-7 cells which are stably transfected with a mutant IGF-1R negative SX-13 cells (IGF-1R KO cells). We found that stimulation of IGF-1R WT cells with Zn resulted in a robust phosphorylation of ERK1/2 and PKB. However, in IGF-1R KO cells, both IGF-1 and Zn-induced effects were completely attenuated, providing the first evidence for a role of IGF-1R in Zn-induced ERK1/2 and PKB activation, independent to EGFR activation and IR/IRS-1 tyrosine phosphorylation.

Taken together, we have demonstrated for the first time that IGF-1R is required for Zn-induced activation of ERK1/2 and PKB in CHO-IR cells, and that this activation is

independent of IR/IRS-1 and EGFR-PTK activation, providing original insight into the cellular mechanism of Zn action.

Chapter 4 – Conclusion

The results presented here demonstrate for the first time the requirement of the IGF-1R in mediating Zn-induced phosphorylation of ERK1/2 and PKB/Akt in CHO-IR cells. We also show that the activation of ERK1/2 and PKB/Akt by Zn occurs independently of the action of IR and IRS-1. These results were obtained by using AG1024, a specific pharmacological inhibitor of IR/IGF-1R-PTK, as well as mutant/inactive IR overexpressing cells, and IGF-1R KO cells. In contrast to other studies, our studies show that AG1478, a highly specific inhibitor of EGFR-PTK, failed to attenuate Zn-induced increase in ERK1/2 and PKB phosphorylation, suggesting that these events are independent of EGFR-PTK.

The precise mechanism by which Zn activates IGF-1R in CHO cells, however, remains unclear. Nonetheless, since Zn is known to have a potent PTPase inhibitory effect, it may be suggested that Zn treatment can shift the equilibrium of the phosphorylation-dephosphorylation cycle, resulting in a net increase of the tyrosine phosphorylation of IGF-1R and/or other proteins, which may trigger the IGF-1R-PTK signalling cascade. Even though our work provides novel insight into the cellular mechanism of Zn action, further studies are required to elucidate the precise mechanism through which IGF-1R is activated, and transmits its signal to induce phosphorylation of ERK1/2 and PKB/Akt pathways, which, in the long run, may help to develop new therapies to manage / treat diabetes and associated complications.

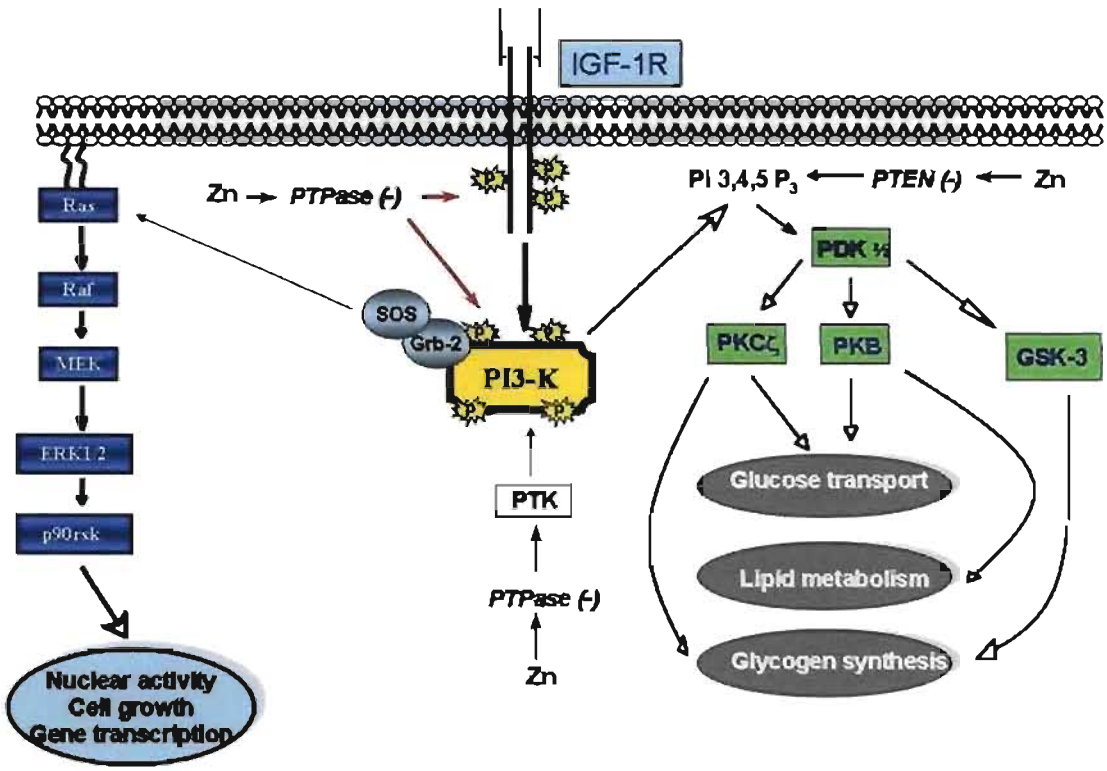

Figure 8: A model summarizing the mechanisms of Zn-induced responses in IGF-1R signalling.

IGF-1R seems to be required for Zn-induced phosphorylation of ERK1/2 and PKB, since these events were blocked by AG 1024. The mechanism by which Zn stimulates ERK1/2 and PKB phosphorylation is still unknown, but the ability of Zn to inhibit PTPases, as well as to stimulate IGF-1R and PI3-K might contribute to this effect.

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