DISSERTATION

TRANSLOCATION OF INSULIN RECEPTORS INTO PLASMA MEMBRANE

MICRODOMAINS IN RESPONSE TO INSULIN AND TO INSULIN-ENHANCING

VANADIUM AND CHROMIUM COMPOUNDS

Submitted by

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We HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY ABEER S. AL-QATATI ENTITLED TRANSLOCATION OF INSULIN RECEPTORS INTO PLASMA MEMBRANE MICRODOMAINS IN RESPONSE TO INSULIN AND TO INSULIN-ENHANCING VANADIUM AND CHROMIUM COMPOUNDS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

TRANSLOCATION OF INSULIN RECEPTORS INTO PLASMA MEMBRANE
MICRODOMAINS IN RESPONSE TO INSULIN AND TO INSULIN-ENHANCING
VANADIUM AND CHROMIUM COMPOUNDS

We have examined the translocation of insulin receptors into specialized, cholesterol-enriched membrane microdomains called lipid rafts following treatment of RBL-2H3 cells with insulin, bis-maltolatooxovanadium (BMOV) and tris(pyridinecarbxylato) chromium(III) ($Cr(pic)_3$). Isopycnic sucrose gradient ultracentrifugation was used to subfractionate membrane fragments and insulin receptors were identified within low or high buoyant density membrane fractions using insulin receptor-specific antibodies and western blotting. Single particle tracking methods were used to confirm the confinement of individual insulin receptors within small membrane compartments on intact, viable RBL-2H3 cells. We demonstrated that insulin receptors translocate into lipid rafts upon binding insulin or following exposure to BMOV or $Cr(pic)_3$. Phosphorylated insulin receptors also appeared in membrane raft fragments in response to insulin and/or insulin-mimicking compounds.

Extraction of cholesterol from lipid rafts disrupted these microdomains and caused a decrease in the number of unphosphorylated and phosphorylated insulin receptors within these compartments. In addition to their ability to induce translocation of insulin

receptors into lipid rafts, BMOV and Cr(pic)$_3$ caused an increase in the number of phosphorylated IRS-1 molecules within these membrane fragments. To determine why Cr(pic)$_3$ and BMOV might affect the distribution of insulin receptors in non-raft and raft compartments, membrane fluidity was evaluated in Cr(pic)$_3$ and BMOV treated cells.

Fluidity, as suggested by a decrease in lipid packing, was increased following treating 2H3 cells with either BMOV or Cr(pic)$_3$. These results suggest that changes in lipid packing resulting from exposure of cells to either Cr(pic)3 and BMOV may affect the distribution of receptors in non-raft and raft compartments. Increased receptor localization in rafts or small membrane compartments evaluated by single particle tracking studies, would result in increased likelihood of insulin receptor phosphorylation within these signaling platforms. Thus rafts may be an important membrane structures involved in cell signaling events mediated by insulin receptors.

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CHAPTER 1

I. Introduction to Diabetes Mellitus

Diabetes mellitus is a syndrome characterized by disordered metabolism and abnormally high blood sugar. It is a common disorder, persons of all age groups world-wide are affected. An estimated 20.8 million people in the United States have diabetes and, of those, only 14.6 million have been diagnosed (1). Diabetes is associated with long-term complications that affect almost every part of the body. The disease often leads to blindness, heart and blood vessel disease, stroke, kidney failure, amputations and nerve damage. At least 65% of those with diabetes die from heart disease (2) (Figure 1).

There are two major types of diabetes, called type 1 and type 2 (3). Type 1 diabetes has also been called insulin dependent diabetes mellitus (IDDM), or juvenile onset diabetes mellitus. It is generally an autoimmune disease in which the immune system attacks and destroys the insulin-producing beta cells in the pancreas resulting in little or no insulin production (4). A person who has type 1 diabetes must take insulin daily to live. It is believed that the ability to develop abnormal antibodies in type 1 diabetes is, in part, genetically inherited (5). Certain viral infections such as mumps and coxsackie viruses or some other environmental toxins might generate abnormal antibody responses that cause damage to the pancreatic islet cells where insulin is made (6). Type 1 diabetes tends to occur in young, lean individuals, usually before 30 years of age (7). However, occasionally, older patients do present with this form of diabetes. This case is referred to as latent autoimmune diabetes in adults. Type 1 diabetes accounts for about 5

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to 10% of diagnosed diabetes in the United States (8). It occurs equally among males and females but is more common in whites than in non whites. This disease is rare in most African, American Indian, and Asian populations. However, some northern European countries, like Finland (9) and Sweden have high rates of type 1 diabetes. The symptoms of this disease usually occur over a short period, although beta cell destruction can begin years earlier. Symptoms include increased thirst and urination, constant hunger, weight loss, blurred vision, and extreme fatigue. If not treated with insulin, the patient can suffer from life-threatening diabetic coma, known as diabetic ketoacidosis.

Type 2 diabetes has also been referred to as non-insulin dependent diabetes mellitus (NIDDM) or adult onset diabetes mellitus. It is the most common form of diabetes; about 90-95% of people with diabetes have type 2. A major distinguishing characteristic of type 2 diabetes is a lack of sensitivity to insulin by the cells of the body, particularly fat and muscle cells (10). The defective responsiveness to insulin may involve the insulin receptor in cell membranes. In addition to the genetic factors which are strongly associated with this form of diabetes (11), there are other predisposing risk factors including central obesity (12), a previous history of gestational diabetes, physical inactivity, and certain ethnicities. This disease occurs more often in African Americans, American Indians, some Asian Americans, native Hawaiians and other Pacific Islander Americans, and Hispanics/Latinos. Type 2 diabetes usually occurs in individuals over 30 years old and the incidence increases with age. The symptoms of type 2 diabetes develop gradually and their onset does not occur rapidly as in type 1 diabetes. These symptoms include fatigue, frequent urination, increased thirst and hunger, weight loss, blurred vision, and slow healing of wounds or sores.

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Traditional management of the symptoms of type 1 and advanced type 2 diabetes is by insulin injections. Many problems involving physical and mental pain, and some side effects can be caused by insulin injections. Many researchers in the field of diabetes have therefore tried to find orally active therapeutic compounds to use in place of insulin injections and other synthetic pharmaceuticals. Several metals, such as chromium, cobalt, molybdenum, zinc and vanadium have been found to produce insulin-like effects.

II. Insulin structure

Insulin is the most potent anabolic hormone known because of its ability to promote the synthesis and storage of carbohydrates, lipids and proteins and to inhibit their degradation and release back into the circulation (13). It is produced in the islets of Langerhans in the pancreas whose name comes from the latin *insula* for "island". Insulin is a peptide hormone composed of 51 amino acid residues and has a molecular weight of 5808 Da and is constructed of two peptide chains, A chain of 21 amino acid residues and B chain of 30 residues (Figure 2). The A chain contains 2 sections of alpha helix (A2 Ile- A8 Thr and A13 Leu- A19 Tyr) (14). In between these sections is a flat ribbon that enables the helices to lie alongside one another. The B chain appears to wrap around the A chain. It consists of a larger section of alpha helix (B9 Ser- B19 Cys) and the glycine residues at 20 and 23 allow it to fold into V shape (14).

The three dimensional structure of insulin is maintained by disulfide bridges (15). These form between thiol groups on cysteine residues. Three disulfide bridges are formed: two between the A and B chains (between A7, B7, A20 and B19) and one within the A chain (A6 and A11). In addition to that, there are many salt bridges as well

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as van der Waals forces and a polar exterior that stabilize the molecule tertiary structure. Insulin can form granules consisting of hexamers in which 6 insulin molecules are grouped around 2 zinc ions (16). This doughnut-shaped form is the one in which insulin is stored in the pancreatic islets and secreted into the circulation. Insulin can form dimers but the active form is usually the monomeric one (14).

After the insulin mRNA leaves the nucleus, the translation process of preproinsulin occurs on the rough endoplasmic reticulum (RER). This is followed by rapid cleavage to proinsulin that is released into the intracisternal spaces of RER in which it folds and forms the native disulfide bridges of insulin. Proinsulin is then transported to the Golgi apparatus. The granules budding from the trans-Golgi cisternae are rich in proinsulin and contain proteolytic enzymes like prohormone convertases (PC1 and PC2) and exoprotease carboxypeptidase E (17). Because of the action of these enzymes, the central portion of proinsulin (C-peptide) is cleaved from the C- and N- terminal ends of the molecule giving the mature insulin.

III. Insulin release from pancreatic islet cells

Insulin is released from beta cells of pancreatic islets in response to elevated blood glucose levels. Glucose enters the beta cells through the glucose transporter GLUT2 in which glucose goes into glycolysis and the respiratory cycle where multiple molecules of ATP are produced by oxidation. Dependent on ATP levels, the ATP-controlled potassium channels close and the cell membrane depolarizes. Because of depolarization voltage gated calcium channels open which allows calcium ions to flow into beta cells. An increased calcium levels causes activation of phospholipase C, this enzyme will cause the cleavage of phosphatidyl inositol 4,5-bisphosphate into inositol

1,4,5-triphosphate (IP3) and diacylglycerol. IP3 binds to receptor proteins in the endoplasmic reticulum (ER) which leads to the release of calcium from ER via IP3 gated channels. Significantly increased amounts of calcium in beta cells causes the release of previously synthesized insulin that is stored in secretory vesicles (14). For 1-2 hrs following a meal insulin is released from the pancreas is hormone pulses with oscillation over a period of 3-6 minutes. Thus insulin concentration changes from more than 800 pmol/l to less than 100 pmol/l (18). This oscillation is important to avoid down regulation of insulin receptors in target cells and to assist the liver in extracting insulin from the blood (18).

In addition to glucose, there are other substances that are known to stimulate insulin release including acetylcholine from vagus nerve endings, cholecystokinin from enteroendocrine cells of intestinal mucosa, glucose-dependent insulinotropic peptide (GIP), and amino acids from ingested proteins which include alanine, glycine, and arginine. These three amino acids act similarly to glucose by altering the beta cell membrane potential.

When glucose levels return to basal values after a meal, insulin release from the beta cells slows or stops. Release of insulin is strongly inhibited by the stress hormone norepinephrine which leads to increased blood glucose levels during stress.

When insulin molecule has docked onto its receptor, it may be released back into the extracellular space or it may be degraded by the cell. Insulin is degraded by endocytosis of the insulin-receptor complex followed by the action of insulin degrading enzyme. Most insulin molecules are degraded by liver cells. A typical insulin molecule

that is produced endogenously by the beta cells is degraded about 71 minutes after its initial release into the blood stream.

IV. Insulin functions

Insulin-mediated signaling via the insulin receptor leads to storage of glucose in liver and muscle cells in the form of glycogen, increased esterification of fatty acids and synthesis of triglycerides from fatty acid esters in adipose cells, increased amino acid and potassium uptake, increased DNA replication and protein synthesis, and relaxation of arterial wall muscle which increases blood flow in microarteries. Insulin also decreases proteolysis, lipolysis, and gluconeogenesis.

V. Insulin receptor structure

Insulin action is mediated through an insulin-specific receptor. The insulin receptor is a member of the tyrosine kinase family of transmembrane signaling proteins that are important regulators of cell differentiation, growth and metabolism (19). The insulin receptor is found in approximately all vertebrate tissues but its concentration differs from as few as 40 receptors on circulating red blood cells to more than 200,000 receptors on adipocytes and hepatocytes. The receptor gene is located on the short arm of human chromosome 19, is more than 150 kilobases in length and contains 22 exons which encode a 4.2-kb cDNA(19). The physiologically relevant insulin receptor is composed of 2 α and 2 β subunits linked by disulfide bonds (19) (Figure 3). Both subunits are derived from a single proreceptor by proteolytic processing at a cleavage site consisting of 4 basic amino acids. The existence of one site of alternative splicing surrounding exon 11 creates 2 receptor isoforms differing by 12 amino acids near the C-terminal end of the α subunit (20). The fully mature receptor contain N-linked carbohydrate side chains

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capped by terminal sialic acid residues. Two structurally-related molecules belong to insulin receptor family and include the insulin-like growth factor (IGF-1) receptor and an orphan receptor called insulin receptor-related receptor. These two receptors share more than 80% amino acid sequence identity in the kinase domain but has low amino acid sequence identity in the extracellular domain.

The α subunit of the insulin receptor is composed of 723 amino acid residues, has a molecular weight of 135,000, is located entirely extracellularly and is responsible for ligand binding (21). *In vitro* mutagenesis and biochemical studies suggested that the N-terminal domain of the insulin receptor is required for high affinity insulin binding (21). The α subunit is considered to be the regulatory part of the enzymatic portion of insulin receptor. The unoccupied α subunit inhibits the tyrosine kinase activity of the β subunit. If the α subunit is removed by proteolytic cleavage, deletion mutagenesis or if there are certain point mutation within this subunit ($Arg^{86} \rightarrow Pro$), then the inhibition will be relieved. The β subunit of the receptor has 620 amino acids and composed of a short extracellular domain that contains sites for N- and O-linked glycosylation, a 23- amino acid transmembrane domain, and an intracellular domain that contains a Tyr-specific protein kinase (22).

Many functional regions have been defined in the β subunit including the ATP binding domain and autophosphorylation sites in the intracellular juxtamembrane region (Tyr^{960} and possibly Tyr^{953} and Tyr^{972}), a regulatory region (Tyr^{1146}, Tyr^{1150}, and Tyr^{1151}) and the COOH terminus (Tyr^{1316} and Tyr^{1322}) (23). Autophosphorylation of all three tyrosine residues of the regulatory region stimulates kinase activity 10-20 fold (23). If 1, 2, or 3 tyrosine residues in the regulatory region are mutated, then insulin-stimulated

kinase activity will be reduced with a loss in the biological function (24). The juxtamembrane region of β subuit is encoded by exon 16 of the receptor gene and it is a highly functional domain required for both insulin-stimulated receptor endocytosis and for signaling via phosphorylation of IRS-1 protein, one of the molecules involved in transducing insulin signal (25). The C-terminus of the β subunit does not appear to be important for activation of the kinase. Many studies using a mutant insulin receptor lacking the last 43 amino acids showed that the autophosphorylation and kinase function normally, the complete C-terminal domain is required for normal insulin-stimulated activation of glycogen synthesis.

Besides its role in signal transduction, the insulin receptor mediates internalization of insulin. Through endocytosis of the insulin-receptor complex, insulin will be degraded and most of the unoccupied receptors recycle to the plasma membrane. After prolonged insulin stimulation, the receptor is degraded causing attenuation of the insulin signal (26). Internalized insulin receptors are functional as kinases which means that insulin-stimulated internalization is important for signal transduction. The two major pathways for endocytosis that exist to different degrees in all cells are the coated pit-mediated pathway which requires a functional insulin receptor kinase, tri-phosphorylation in the receptor's regulatory region, and two tyrosines containing β turns in the juxtamembrane region (27). The other pathway is a constitutive and nonsaturable internalization route that does not require receptor autophosphorylation or an intact juxtamembrane region. This route is important for insulin receptor internalization in Chinese hamster ovary cells.

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Upon dissociation of insulin, both the receptor and its substrates undergo a rapid dephosphorylation and thus signal termination. Protein tyrosine phosphatases (PTPases), especially the cytoplasmic phosphatase PTP1B have been implicated in this process (28). Insulin action can also be temporally controlled by lipid phosphatases that dephosphorylate phosphatidylinositol 3,4,5-triphosphate. Microinjection of the phosphoinositide phosphatase SHIP2 blocks insulin action. The insulin receptor is phosphorylated on serine and threonine residues in the basal state and in response to cell stimulation by phorbol esters, cAMP analogues, and insulin. Numerous *in vitro* studies have shown that the tyrosine kinase activity of the receptor decreases as a consequence of serine/threonine phosphorylation. An example of this is the phosphorylation of the receptor or IRS proteins on serine residues by GSK3 or mTOR, both of which are activated downstream targets of IRS phosphorylation (29). The exact serine phosphorylation sites which are inhibitory are unknown, but might include Ser[1293], Ser[1294], and Ser[1336] and the enzymes involved in receptor Ser/Thr phosphorylation *in vivo* remain uncertain.

VI. Signal transduction by insulin receptor

The biological effects of insulin are mediated through a multi-component signaling system that is conserved across a wide range of species. When insulin binds to its receptor, it will stimulate a cascade of signaling events that leads to modification in a number of physiological processes. Binding of insulin to the α subunit of insulin receptor will induce the transphosphorylation of one β subunit by another on specific tyrosine residues in an activation loop causing an increase in the catalytic action of the kinase

enzyme. This in turn leads to phosphorylation of intracellular protein substrates like the insulin receptor substrate (IRS) proteins (30), Shc (31), APS (32) and c-Cbl (33).

IRS proteins are important because they act as an interface between insulin receptor and the downstream effector molecules. Four IRS protein molecules have been identified to date. IRS-1 is mainly involved in somatic cell growth and insulin action in muscle and adipose tissue (30). IRS-2 is important in β cell survival and growth, insulin action in the liver, brain growth, reproduction and food intake (34). IRS-3 and IRS-4 are predominantly expressed in adipose and neuroendocrine tissues, respectively, although their exact roles are not clear (30). Gene knockout experiments showed that mice deficient in IRS-1 are viable (35), they exhibit extreme defects in both embryonic and postnatal growth. These mice are insulin resistant with impaired glucose tolerance associated with hypertriglyceridaemia and hypertension (35). Diabetes never develops in these mice because of the compensatory hypersecretion of insulin (35). IRS-2 mice also exhibit insulin resistance in both peripheral tissues and liver, have defective growth in some tissues including certain regions of the brain, islets and retina (30). Insulin resistance combined with decreased β cell mass causes type 2 diabetes in these mice (30). IRS-3 and IRS-4 knockout mice have normal or near normal growth and metabolism (30).

During insulin stimulation, tyrosine phosphorylation sites in the IRS proteins bind to the Src-homology-2 (SH2) domain in a number of downstream signaling molecules including phosphatidylinositol 3-kinase (PI-3K), growth factor receptor binding protein 2 and SH2-containing protein-tyrosine phosphatase 2. Both metabolic and mitogenic effects of insulin are mediated by PI-3K (36). In skeletal muscle and adipose tissues,

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stimulation of PI-3K pathway will enhance glucose utilization by modulating the expression or subcellular localization of glucose transporters (GLUT4) and the storage of glucose as glycogen or fat (37). In addition, activation of this pathway by insulin stimulates protein synthesis in most cell types. When the regulatory subunit of PI-3K (P85) interacts with IRS protein, it becomes activated. The activation of PI-3K catalyses the phosphorylation of phosphoinositides on the 3-position to produce phosphatidylinositol-3-phosphates, particularly PtdIns(3,4,5)P3, that bind to the PH domains of a number of signaling molecules to change their activity or subcellular localization. Phosphatidylinositol-3-phosphates regulate the AGC family of serine/threonine protein kinases. PDK1 is one of the well known AGC kinases that phosphorylates and activates the serine/threonine kinase Akt/PKB (38). Akt along with the atypical protein kinases C (PKCζ and PKCλ) seem to be required for insulin-stimulated glucose transport. In addition to that, Akt phosphorylates and inactivates glycogen synthase kinase 3 (GSK-3), decreasing the rate of phosphorylation of glycogen synthase, thus increasing its activity state leading to glycogen accumulation (39).

Target of rapamycin (mTOR) is a tool utilized by insulin to increase the synthesis and blocks the degradation of proteins. mTOR is a member of the PI-3K family of proteins but it acts primarily as a serine rather than lipid kinase. The stimulation of this protein kinase involves PI-3K activation. mTOR regulates the mammalian translation machinery by direct phosphorylation and activation of p70 ribosomal S6 kinase (p70[rsk]).

Insulin stimulates cellular growth by activation of the mitogen-activated protein (MAP) kinase extracellular signal-regulated kinase (ERK). This route involves the tyrosine phosphorylation of IRS proteins and/or Shc, which in turn interact with the

adapter protein Grb-2 recruiting the son-of-sevenless (SOS) exchange protein to the plasma membrane for activation of Ras. Ras is also activated by stimulation of the tyrosine phosphatase SHP2, through its interaction with receptor substrates such as Gab-1 or IRS1/2. When activated, Ras stimulates a serine kinase cascade through activation of Raf, MEK, and ERK (40). Once ERK is activated it will translocate to the nucleus to catalyze the phosphorylation of transcription factors such as $p62^{TCF}$. This transcriptional event will lead to cellular proliferation or differentiation. Inhibition of this pathway by dominant negative mutants or pharmacological inhibitors prevents cell growth by insulin, but without any effect on the metabolic actions of the hormone (41) (Figure 4).

Along with PI-3K pathway of insulin, other signals are required for insulin-stimulated glucose uptake. The second pathway involves tyrosine phosphorylation of the Cbl protooncogene (33). The phosphorylation of Cbl requires its recruitment to the adapter protein APS which contains SH2 and PH domains (32). APS is found as a preformed homodimer and interacts with the 3 phosphotyrosines in the activation loop of the insulin receptor upon its activation via its SH2 domain (32). Each part of the APS homodimer binds to a β subunit of the receptor. When APS binds to insulin receptor it becomes phosphorylated on a C-terminal tyrosine resulting in the recruitment of Cbl via its SH2 domain. The Cbl associated protein (CAP) is recruited with Cbl to the insulin receptor:APS complex. CAP belongs to a family of adapter proteins with a common organization containing 3 SH3 domains in its COOH terminus and an NH2-terminal region of similarity to the gut peptide sorbin called the sorbin homology (SoHo) domain (42). CAP is expressed in insulin-sensitive tissues, is mainly induced during adipocyte differentiation and its expression is increased by insulin-sensitizing peroxiosome

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proliferators-activated receptor-γ agonists (43). The COOH-terminal SH3 domain of CAP associates with a PXXP motif in Cbl so that these two proteins are constitutively associated. Upon phosphorylation, the Cbl-CAP complex translocates to lipid raft domains in the plasma membrane through the interaction of SoHo domain of CAP with flotillin, one of lipid raft proteins. Overexpression of dominant-interfering CAP mutants that do not bind to Cbl or flotillin blocked translocation of phosphorylated Cbl to lipid rafts and inhibited insulin-stimulated glucose uptake and GLUT4 translocation (44). Upon tyrosine phosphorylation, Cbl can interact with the protein CrkII, an SH2/SH3-containing adapter protein (45). CrkII binds to specific phosphorylation sites on Cbl via its SH2 domain and is associated with the nucleotide exchange factor C3G via its SH3 domain. As a result, insulin binding initiates the translocation of both CrkII and C3G to lipid rafts. When C3G is translocated to lipid rafts it causes the activation of the small G proteins TC10α and TC10β (46). Overexpression of a dominant-interfering TC10 mutant inhibits insulin-stimulated glucose uptake and GLUT4 translocation (45). The overexpression of the wild-type TC10 can also inhibit insulin action because, in this case, a large fraction of TC10 may saturate the endogenous exchange factor without producing the down-stream signal in the target cellular compartment (45). TC10 interacts with a number of effector molecules when it is activated. One of these is a splice variant of the adapter protein CIP4. CIP4 contains 1 FCH domain, 2 coiled coil domains, and 1 SH3 domain. The FCH domain interacts with microtubules, and the 2nd coiled-coil domain interacts with TC10 in a GTP-dependent manner. CIP4 translocates to the plasma membrane from an intracellular compartment upon insulin stimulation (47). The expression of mutant forms of CIP4 with diminished TC10 binding inhibits insulin-

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stimulated GLUT4 translocation which indicates that CIP4 is required for insulin-stimulated glucose transport as a downstream effector of TC10 (47). TC10 also interacts with Exo70, a component of the exocyst complex which is essential for the tethering or docking of secretory vesicles. Inhibition of insulin-stimulated glucose uptake occurs as a result of the expression of a mutant form of Exo70 by preventing the fusion of the GLUT4 vesicle with the plasma membrane. In addition to that overexpression of sec8 and sec6, which are components of the exocyst complex, increased glucose transport following insulin stimulation in 3T3-L1 adipocytes. All of these studies suggest the important role of the exocyst system in insulin-regulated GLUT4 exocytosis.

The PI-3K and CAP/Cbl/Crk/C3G/TC10 pathways of insulin signaling can intersect. A typical PKCs can be phosphorylated by PDK1 downstream of PI-3K. PKCζ/λ is then recruited to lipid rafts in a TC10-dependent manner, via Par3 and Par6 proteins. The a typical PKC act as a meeting point for the PI-3K and TC10 signaling pathways. This shows the extent of complexity of the signaling pathways of insulin in regulating GLUT4 recycling.

VII. Plasma membrane structure

The cellular plasma membrane acts as an impermeable barrier to the passage of water-soluble molecules. Being an active border is not the membrane's only function. It also acts as a gatekeeper, as a receiver and an amplifier and as a regulator and a static stabilizer. The lipid bilayer constitutes the basic structure for the membrane. In 1972, Singer and Nicholson proposed a new view of the membrane, the Fluid-Mosaic model (48). In this model, the proteins are embedded in a fluid bilayer, either partly integrated or partly peripherally attached, with sugar moieties anchored on many proteins on the

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outer surface (48). According to their model, both lipids and proteins diffuse laterally in the plane of the membrane, at 2 μm and 1μm per second, respectively (48). This picture may be valid in the space scale of 10 nm. However, the plasma membranes contain different structures or domains that can be classified as protein-protein complexes, lipid rafts, pickets and fences formed by actin-based cytoskeleton and large stable structures such as synapses or desmosomes.

The lipid composition of the plasma membrane is very different from that of the other membranes of the cell. All cellular membranes contain glycerophospholipids, comprising one of several head groups attached via glycerol to two acyl chains, one of which is usually unsaturated. In addition to glycerophospholipids that are sufficient to form bilayers, most eukaryotic cells contain two additional classes of lipids, sterols and sphingolipids. Sphingolipids are based on ceramide. They differ from the glycerophospholipids in that both acyl chains are often saturated. The sterols are based on a rigid four-ring structure, in which cholesterol is the principle form found in vertebrates.

The ternary mixtures of sphingomyelin, unsaturated phosphatidylcholine and cholesterol with a wide range of compositions and temperatures proved the existence of a cholesterol-poor, liquid disordered (Ld) phase along with a liquid-ordered (Lo) phase enriched in sphingolipids and cholesterol (49). Cholesterol is crucial for the formation of the Lo phase which is characterized by a high degree of acyl chain ordering but with translational mobility similar to that of the Ld phase (49). The more tightly packed acyl chains are found in the Lo phase and consequently there is a reduction in cross-sectional area per lipid. Fluorescently labeled GPI-anchored proteins such as Thy-1,

glycosphingolipids such as ganglioside GM1, and saturated phospholipid probes were found to partition preferentially into an Lo phase in macroscopically phase-separated model membranes, whereas lipids with short or unsaturated acyl chains, and most transmembrane proteins are preferentially excluded from the Lo phase.

VIII. Plasma membrane microdomains

Phase separation may explain the observation that cellular membrane lipids are not completely solubilized by nonionic detergents such as Triton X-100 (50). Low-density detergent-resistant membranes (DRMs) that are enriched in sphingolipids and cholesterol and have a clear bilayer appearance were isolated from mammalian cell lysates (50). Several lines of evidence suggest that DRMs are in the Lo phase. First, model membranes that are very close to DRMs in terms of composition, i.e., rich in sphingolipids and cholesterol, are in the Lo phase (51). Second, a good correlation was made between the presence of Lo phase and detergent insolubility in model membranes; increasing the concentration of sphingolipids and cholesterol in model membranes can promote both Lo phase formation and detergent insolubility and detergent insolubility is not observed unless the Lo phase is present (51). Third, detergent insolubility was reduced by deletion of sphingolipid and cholesterol in cells (52). Finally, the fluorescence polarization of diphenylhexatriene showed that the acyl chain fluidity of DRM is similar to that in Lo phase bilayers (53). However it should be noted that the existence of Lo phase domains in cell membranes has not been directly demonstrated. Furthermore, if these domains are present, they may be similar but not identical to the Lo phase described in model membranes.

Lipid rafts are small, heterogeneous, highly dynamic, sterol and sphingolipid-enriched domains that compartmentalize cellular processes (54) (Figure 5). The lipid raft model proposes that interactions between the carbohydrate heads of the glycosphingolipids causes sphingolipids to associate laterally with one another. Any spaces between associating sphingolipids are filled by cholesterol molecules which act as functional spacers. The closely associated sphingolipid-cholesterol clusters behave as assemblies within the exoplasmic leaflet, where the intervening fluid regions are occupied by unsaturated phosphatidylcholine molecules.

Studies on epithelial cell polarity were the basis for lipid raft hypothesis. In epithelial cells, plasma membranes are polarized into apical and basolateral domains (55), with the former being enriched in sphingolipids and the latter in phosphatidylcholine. The tight junctions between the apical and basolateral cell-surface domains prevent the mixing of these lipid molecules. Studies on the delivery of newly synthesized sphingolipids in epithelial MDCK cells showed that a simple glycosphingolipid, glucosylceramide, was preferentially transported to the apical membrane (56). Delivery to the apical domain was explained by the proposal that glycosphingolipid clusters formed within the exoplasmic leaflet of the Golgi membrane, these microdomains were considered to be sorting centres for proteins destined for delivery to the apical plasma membrane (56). In support of this model, GPI-anchored proteins use glycolipid anchors as apical sorting determinants.

Resistance to cold non-ionic detergents, especially Triton X-100 and the sensitivity to cholesterol depletion were considered to be the two major criteria for the definition of lipid rafts. Many cellular processes that are mediated by lipid rafts have

been shown to be sensitive to cholesterol depletion. Several methods can be used for cholesterol depletion including cholesterol binding, use of chelating compounds such as filipin, inhibiting cholesterol biosynthesis with statins, or removing cholesterol from the plasma membrane with cyclodextrin (57, 58). Extraction with cold Triton X-100 and the recovery of detergent resistant microdomains (DRM) as low-density fractions during sucrose gradiant centrifugation has become a widely used method for identifying lipid raft components. Non-ionic detergent extraction method has some pitfalls regardless of its usefulness. A raft protein can be connected to the cytoskeleton, so it will not float after detergent extraction, or its association with rafts can be so weak that it is solubilized by the detergent. In addition to that, changes in detergents and extraction conditions can produce different results.

Different proteins vary in their affinities for lipid rafts. Proteins with raft affinity include glycosylphosphatidylinositol (GPI)-anchored proteins (59), doubly acylated proteins, such as Src-family kinases or the α-subunits of heterotrimeric G proteins (60), cholesterol-linked and palmitoylated proteins such as Hedgehog (61) and transmembrane proteins such as influenza virus hemagglutinin and β-secretase (62). Some transmembrane proteins may reside in lipid rafts for short time but when they become crosslinked or oligomerized, their affinities for lipid rafts increase.

Western blotting strategies were the basis for the identification of GPI-anchored proteins as selectively partitioning into detergent-resistant membrane domains (63). This observation has been confirmed in numerous proteomics analyses in which proteins such as 5′-nucleotidase (64), Thy-1, DAF (65), and CD59 (66) have been identified. In addition to the identification of the above proteins, both caveolin and flotillin, initially

reported to be in detergent-resistant membranes, were also identified in proteomics analyses. The consistent identification of caveolin, flotillin and GPI-anchored proteins in proteomics analyses from lipid rafts prepared by a wide variety of methods suggests that these are true resident raft proteins and hence valid markers for these domains.

Cytoskeletal and adhesion proteins including actin, myosin, vinculin, cofilin, cadherin, filamin, and erzin are also routinely identified in lipid raft preparation (66-68). The presence of cytoskeletal proteins in rafts is not an indication that these are integral raft proteins but rather that rafts interact with the cytoskeleton and therefore, when isolated, the rafts retain some of their associated cytoskeletal proteins.

In order to visualize rafts in the plasma membrane, researches have attempted to observe the concentration of putative raft molecules in specific regions. It was very difficult to observe rafts in resting cells. Imaging of rafts in steady-state cells using light and electron microscopy has revealed that, in almost all of the cases, the sample preparation protocol involved induction of the clustering of raft molecules, using crosslinkers like antibodies and fixatives, use of probes that might crosslink raft molecules (69), such as cholera toxin and latex beads conjugated with antibodies, or employment of low temperatures. Therefore, rafts can only be seen after crosslinking or ligating raft molecules or at lower temperatures (70). This suggests that the raft size in the resting cell is smaller than the optical diffraction limit ($\approx$ 300nm). Stabilization of raft molecules by crosslinking or lowering the temperature might increase raft size. Employing single particle tracking and photonic force microscopy techniques for the indirect estimates of the sizes of lipid raft domains in living cells suggest even smaller sizes ranging between 50 and 200nm in diameter (71). FRET and pulse EPR spin-

labeling experiments suggested that the rafts are even smaller and/or short-lived, and/or the raft molecules rapidly diffuse in and out on a time scale of 100µs or less (72).

Lipid rafts have important roles in many cellular processes, including membrane sorting and trafficking, cell polarization and signal transduction. Different receptors that are present in raft regions play a role in cell targeting by some pathogen. Both CD4 (73) and CCR5 (74) were found to associate with the raft region. CD4 is the binding site for HIV-1 virus, whereas CCR5 strongly enhances the virus entry. For Coxackievirus A9, the MHC class I is required for virus internalization. MHC Class I and integrin $\alpha v \beta 3$, which is a coreceptor reside in DRMs (75). In addition, disruption of lipid raft by methyl-β-cyclodextrin, a powerful cholesterol binding molecule both on erythrocyte membrane and parasite vacuolar membrane inhibits *Plasmodium falciparum* infection of the erythrocytes (76).

The distribution of proteins inside and outside of rafts has implications for cell function. Raft proteins are estimated to constitute about 4% of the total membrane proteins. The close proximity of proteins residing in rafts allows their interactions. Simons and colleagues proposed that in order to organize the signal complex in space and time, rafts have to cluster together forming a larger platform in which the adaptors, scaffolds and anchoring proteins would interact (77). The formation of clustered rafts would lead to signal amplification through the concentration of signaling molecules, as well as to exclusion of unwanted modulators (77). Abundant evidence exists for the formation of larger, stable rafts formed by the clustering of raft-philic molecules. An example is the crosslinking of Thy1, a GPI-anchored protein (78). Crosslinking of this protein causes cholesterol and glycosphingolipids to become concentrated, leading to the

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formation of larger stabilized rafts with lifetimes longer than several minutes. Also, in neutrophils, after chemotactic stimulation, large ordered membrane microdomains were formed (79).

Receptors could behave in three different ways in rafts (77). First, receptors that are associated with lipid rafts could be activated through ligand binding. Second, receptors which have weak affinity for lipid rafts could oligomerize on ligand binding and this would increase residency times in rafts. Third, the membrane may contain multiple types of rafts which differ in their protein composition, some activated receptors might recruit crosslinking proteins that bind other proteins in adjacent rafts. Clustering would coalesce rafts which would then contain a new mixture of molecules.

An important example on the involvement of lipid rafts in signal transduction is in T cells (80). In these cells, T-cell receptor (TCR) and negative regulators of signal transduction like CD43 and CD45 reside outside lipid microdomains. LAT, Fyn, Lck and CD48 which are the positive regulators of signal transduction reside in rafts (80). Such localization of proteins is normally observed in resting cells and prevents unnecessary signaling. Upon stimulation, the small dynamic rafts combined to form bigger platforms supporting signal transduction. As soon as the small, dynamic microdomains join together forming bigger ones, immunological synapse (IS) will be formed by recruitment of signaling proteins into raft regions. The redistribution of several receptors into IS is interrelated to their gained resistance to solubility in Triton solutions. As a result of these changes, a signaling center will be formed to inform the cell about the contact it has been occupied with. The cell response depends on which

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costimulators have been cross-linked, e.g. CD28/TCR or CD48/TCR costimulation leads to raft coalescence, while CD45/TCR cross-linking downregulates the signaling.

IX. Detection of lipid rafts in the exoplasmic leaflet

Liquid-ordered lipid microdomains have distinguishing parameters in terms of size (5 nm-1μM), stability (0.1s-10 min) and abundance (13-80% of the membrane surface) depending on studies on lipid-raft marker molecules. Multiple techniques, such as single particle tracking (SPT) (81), laser trapping (71), single-molecule microscopy (82), and fluorescence resonance energy transfer (FRET) have been utilized to obtain a consistent measurement of the size of lipid rafts and their stability *in vivo* . The experiments yielded a broad range of raft diameters from ≈ 50 up to ≈ 700 nm. The smallest size of lipid raft was proposed using a laser trap to confine the motion of a bead bound to a raft protein to a small area. In this way, the local diffusion was measured by high resolution particle tracking on the membrane of live BHK fibroblasts and PtK_2 cells (71). The experiments showed that the diffusion constant of raft-associated proteins was significantly reduced compared to the diffusion constant of non-raft proteins. Raft associated GPI-anchored proteins reside in lipid rafts for long periods reaching up to 10 minutes (71). The diffusion of raft associated proteins was increased to values equal to or higher than that of non-raft proteins by cholesterol deletion. In addition to that, the average radius of lipid raft was measured to be 26±13 nm (71).

A single molecule study using fluorescent lipid probes revealed much larger sizes of lipid domains (83). To sense liquid-ordered domains in live human coronary artery smooth muscle cells, a saturated acyl-chain fluorescent lipid probe, DMPE-Cy5 was used (83). This saturated lipid probe showed a high partitioning into domains with an average

size of 0.7 µm. Within these domains DMPE-Cy5 had a long residence time of ≈13 s. These domains covered about 13% of the membrane area (83). In contrast, an unsaturated lipid probe, DOPE-Cy5 showed free diffusion outside the domains proving the specificity of the saturated lipid probe. Experiments with a higher concentration of the saturated lipid probe indicated that the observed domains were stable for minutes.

GPI-anchored proteins have been widely used in particle tracking studies to observe lipid rafts by detecting confined diffusion. A study in C3H 10T1/2 murine fibroblasts revealed that 35-37% of the GPI-anchored protein Thy-1 as well as the ganglioside GM1 are transiently confined (7-9 s) in a lipid domain with a diameter of 260-330 nm (84). Reduction of cell glycosphingolipid synthesis by 40% caused a decrease in the percentage of trajectories of Thy-1 exhibiting confined diffusion. The size of confining domain was also reduced by ≈ 1.5 fold, indicating that confinement was resulted from diffusion inside lipid rafts. It is worthy to note that the trajectories exhibiting confinement and the size of the domains were not affected by Triton X-100 treatment of the cells indicating that the observed domains were detergent-resistant (84).

In another study of Thy-1 and GM1 in the same cell type using single particle tracking technique, it was shown that the availability of lipid microdomains and their size was reduced by cholesterol deletion (85). It was also noted that these lipid domains can be visited again within tens of seconds after the original escape of a particle and the diffusion within the zones was reduced by a factor of 2 compared to the diffusion outside the zones (85). The same study proved that fluorescent lipid probe with unsaturated acyl chains spend much less time in these lipid domains compared to Thy-1, GM1 or lipid probe with saturated acyl chain. Single particle tracking studies showed that 200-300 nm

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cholesterol-sensitive domains are present in the plasma membrane of many cell types. These domains can trap GPI-anchored proteins and gangliosides, they are stable for at least tens of seconds.

In addition to the single particle tracking methods, fluorescence resonance energy transfer (FRET) microscopy has been used to study lipid rafts. Extremely small rafts could be detected using this technique because FRET is a phenomenon that occurs when fluorophores are in very close proximity (0.1-10 nm). In order to study the occurrence of lipid rafts in the apical membrane of MDCK cells, fluorescently-labeled donor and acceptor antibodies were used to investigate the distribution of the GPI-anchored protein 5′ nucleotidase (5′ NT) (86). The efficiency of energy transfer correlated with the surface density of the acceptor-labeled antibody. This revealed that the GPI-anchored proteins were randomly distributed in contrast with the expected clustering in lipid rafts. In another study, the level of energy transfer between fluorescent analogues bound to a GPI-anchored folate receptor was measured using fluorescence depolarization method (87). In contrast to what was obtained for 5′ NT, the extent of energy transfer for this GPI-anchored folate receptor was independent of receptor density and it was sensitive to cholesterol removal (87). From this study, it was concluded that the GPI-anchored folate receptor is clustered in cholesterol-dependent microdomains of < 70 nm in diameter in CHO and CaCo cells. From these two different data one can conclude that there is no single lipid raft type.

It was suggested that rafts could exist as transiently stabilized structures and if rafts would be stable, they will form small structures that cover insignificant fraction of the cell surface and contain a limited amount of GPI-anchored proteins and gangliosides.

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This hypothesis is supported by the work of Sharma et al. (88). They used homo-FRET to analyze the organization of different GPI-anchored proteins expressed in various cell lines (88). These homo-FRET studies were supported by hetero-FRET experiments. The study showed that 20-40% of the GPI-anchored proteins in the cell membrane are organized as clusters that consist of, at most, four GPI-anchored proteins, whereas the remaining population of GPI-anchored proteins is present as monomers outside the clusters. Subsequent experiments indicated that the clusters are sensitive to cholesterol removal and they disappear when a transmembrane anchor is used instead of a GPI-anchor, supporting the hypothesis that the clusters are lipid rafts. From this FRET-based study, it seems that lipid rafts are very small (<5 nm) cholesterol-dependent clusters that contain a slight fraction of the GPI-anchored proteins in the cell membrane.

Inter-leaflet coupling of membrane domains is an important issue because it facilitates signal transduction which involves the linkage of transmembrane proteins with the molecules anchored in the cytoplasmic leaflet. In a study to investigate whether lipid rafts are involved in the interactions between the cytoplasmic leaflet-anchored tyrosine kinase Lyn and the IgE receptor, IgE-FcεRI, Pyenta and colleagues used cross-correlation analysis to study inter-leaflet coupling (89). For this purpose, a GFP linked to the membrane anchoring sequence of Lyn was constructed and its behavior compared to a GFP fused to the membrane-anchoring sequence of K-Ras, a protein with low affinity for lipid rafts (89). The ganglioside GD_{1b} and the GPI-anchored protein Thy-1 were used as markers for exoplasmic leaflet rafts (89). When IgE-FcεRI, GD_{1b} and Thy-1 were cross-linked to form large patches in RBL-2H3 cells, cross-correlation analysis was used to quantify the co-redistribution of the cytoplasmic leaflet anchored GFPs. It was found that

GFP with the Lyn membrane anchor co-redistributed significantly more with exoplasmic leaflet lipid raft markers and IgE receptor than the GFP with the K-Ras membrane anchor. These results showed that the important communication between Lyn and cross-linked IgE receptor could be enhanced by their co-association with lipid rafts and provides the first evidence for inter-leaflet coupling.

X. Caveolae structure and functions in cell signaling

Caveolae are small, flask-shaped invaginations observed at the plasma membrane of many cell types but are especially plentiful in adipocytes, smooth muscle cells, endothelial cells and fibroblasts. They were first described over 40 years ago in electron micrographs of endothelial cells as 50-100 nm structures. Caveolae are dynamic structures having a characteristic coat that consists largely of oligomerized caveolin proteins. Three distinct caveolin genes were identified by molecular cloning, they are caveolin-1, caveolin-2, and caveolin-3 (90,91). Two isoforms of caveolin-1(Cav-1α and Cav-1β) are derived from alternate initiation during translation. Adipocytes, endothelial cells, and fibroblasts express Caveolin-1 and -2 proteins , whereas the expression of caveolin-3 is muscle specific. Co-immunoprecipitation and dual labeling experiments have shown that caveolins-1 and -2 form stable hetero-oligomeric complex and they are co-localized . Caveolae were missing in caveolin-1 knockout mice but these mice were not lethal (92). In these mice the lungs showed hypercellularity with thickened alveolar septa and an increase in the number of vascular endothelial cells, the vascular abnormality was found in endothelium-dependent relaxation, contractility, and myogenic tone. Such mice developed pulmonary hypertension and dilated cardiomyopathy (93). Caveolin-2 knockout mice were similar to caveolin-1 knockout mice. The lung

parenchyma in caveolin-2 knockout mice showed hypercellularity, with thickened alveolar septa and an increase in the number of endothelial cells (94). Mutation of caveolin-3 gene is involved in the pathogenesis of limb-girdle muscular dystrophy in humans (95). In mice with caveolin-3 knockout, there was an elimination of the dystrophin-glycoprotein complex from lipid rafts and abnormalities in the organization of the T-tubule system (96). Caveolae are enriched in cholesterol that is essential for their appropriate morphology and function.

Various molecules involved in mediating cellular signaling have been found in caveolae (97). These molecules include G protein-coupled receptors, Ras, various growth hormone receptors, NOS, adenylyl cyclase or protein kinase C. Detection of these molecules in the same fraction as caveolin after sucrose density gradient based cellular protein fractionation or detergent-based cell solubilization proved that these proteins were localized in caveolae.

In addition to its role in scaffolding, caveolin can regulate the activity of various enzymes including kinases. It was shown that the basal activity of G proteins was suppressed by inhibiting GDP/GTP exchange. This inhibition was caused by a region of caveolin-1 cytoplasmic N-terminal domain. Various molecules, such as growth factor receptors, NOS, adenylyl cyclase, or PKC have been shown to be inhibited by the same peptide (97). This caveolin-derived protein domain has been named the caveolin-scaffolding domain that is also involved in the formation of multivalent homo-oligomers of caveolin. Not all caveolin subtypes have the same inhibitory effect on the above molecules. A similar inhibitory effect on the enzymatic activity was caused by caveolin-1 and -3, while cavolin-2 shows no effect. Also within the same enzyme family, not all

the subtypes are regulated to the same degree by caveolin. The scaffolding domain peptide from caveolin-1 as well as caveolin-3 inhibited type 3 and type 5 adenylyl cyclase isoforms while the same peptide had no effect on type 2 isoform (98).

Caveolin-binding signaling molecules contain a common sequence motif that is recognized by the caveolin scaffolding domain. Two related caveolin-binding motifs were elucidated (99). $\Phi XXXX\Phi XX\Phi$ and $\Phi X\Phi XXXX\Phi$ (where Φ is an aromatic amino acid) exist within most caveolae-associated proteins like most Gα subunits and the kinase domains of many distinct families of tyrosine and serine/threonine protein kinases including; MAP kinase, EGF, PDGF and insulin receptors. Thus the caveolin scaffolding domain act similarly as SH2, SH3 and PH protein domains to form membrane-bound oligomeric complexes that contain signaling molecules and cytoskeletal elements.

Different models were proposed to explain how caveolae participate in cell signaling (100); 1- Hormonal transduction. Caveolae close and open a membrane channel for the passage of molecules or ions (second messengers). This is a way by which caveolae can respond to the signal induced by binding of a hormone to its receptor. 2- Receptor transduction. Some GPI-anchored proteins in caveolae are hormone receptors. Caveolae have Src kinases on their cytoplasmic surface. A phosphorylation cascade is stimulated when ligand binding causes the GPI receptor to associate with the kinase. 3- Mechanical transduction. Caveolae interact with elements of the cytoskeleton through actin filaments. Stretching or compressing the cell will cause changes in the organization of the cytoskeleton that can be transmitted to caveolae by a change in filament tension. The force applied by the actin filaments changes the caveolae, which

opens membrane channels/carriers that allow messenger molecules or ions to enter the cytoplasm.

XI. Lipid and protein compartmentalization in membranes and the role of the cytoskeleton

Singer-Nicolson model or the two-dimensional continuum fluid model can not explain two basic problems of the membrane molecular dynamics . First, the diffusion coefficients for both proteins and lipids in the plasma membrane are smaller than those found in artificially reconstituted membranes or liposomes by factors of 5 to 50 (101). Second, when membrane receptors and other signaling molecules associate to form oligomers or molecular complexes, their diffusion rates severely decreased or they may be temporarily immobilized (102). Understanding the mechanism for these two issues was cleared by single molecule observation; high-speed single particle tracking of an unsaturated phospholipid, L-α-dioleoylphosphatidylethanolamine (DOPE) showed that the plasma membrane is partitioned into submicron-sized compartments with regard to the translational diffusion of lipid molecules (103). Statistical and quantitative analyses indicated that the average compartment size was 230 nm and the average residency time within each 230 nm compartment was 11 ms in the case of NRK cells (103). It was concluded that lipid diffusion in the cell membrane is slow because the plasma membrane is compartmentalized with regard to the translational diffusion of phospholipids. Because lipid molecules undergo hop diffusion over these compartments and it takes time to hop from a compartment to an adjacent compartment, this explains the slow diffusion of membrane molecules in the plasma membrane compared to that in liposomes or reconstituted membranes. In order to examine whether plasma membrane

compartmentalization was universal, Murase and coworkers used DOPE to examine plasma membrane compartmentalization in nine mammalian cell types (104). They found that the compartment size varied greatly from 30 nm up to 230 nm and also the residency time of DOPE ranges between 1 and 17 ms (104).

To explain the temporal corralling of transmembrane proteins, the "membrane-skeleton fence" or "membrane-skeleton corralling" models were proposed (105). According to these models, transmembrane proteins extend into the cytoplasm and their cytoplasmic domains become closely associated with the membrane skeleton. This induces temporal confinement or corralling of transmembrane proteins within the membrane-cytoskeleton network (Figure 6) which is followed by a hop to an adjacent compartment.

Fujiwara et al. (103) and Murase et al. (104) examined the involvement of the membrane skeleton, as well as the effects of the extracellular matrices, the extracellular domains of membrane proteins, and the cholesterol-rich raft domains on phospholipid hop diffusion by modulating these membrane-associated molecules and structures. They found that the phospholipid movement was affected only when they modulated the membrane skeleton (103,104). Fujiwara and colleagues observed DOPE diffusion in membrane blebs as well as in liposomes, they also reduced the actin-based membrane skeleton by treating cells with latrunculin. They found that DOPE molecules undergo rapid simple Brownian diffusion with a diffusion coefficient of $\approx 9\ \mu m^2/s$ in these membranes (103). These findings indicate that the membrane skeleton is involved in both the temporal corralling and hop diffusion of phospholipids.

It is important to note that DOPE molecules are located in the extracellular leaflet of the membrane, whereas the membrane skeleton is located on the cytoplasmic surface of the membrane. Thus, it is difficult for DOPE and the membrane skeleton to interact directly with each other. To explain this challenge, the "anchored transmembrane protein-picket model" was proposed. In this model, various transmembrane proteins that are tightly associated with the membrane skeleton (fence) effectively act as rows of pickets to inhibit the free diffusion of phospholipids due to steric hindrance as well as the hydrodynamic-friction like effects (106) of immobilized anchored membrane protein pickets. A series of Monte Carlo simulation by Fujiwara and coworkers showed that 20-30% coverage of the intercompartmental boundary by these anchored transmembrane pickets is sufficient to make the residency time of 11 ms in a 230 nm compartment in NRK cells (103) . These rows of pickets on the membrane-skeleton fences act as a diffusion barrier that confine the movement of phospholipid molecules for some time. There is no need for these transmembrane picket proteins to be stably attached to the membrane skeleton for a long time. If a transmembrane protein is bound to the membrane skeleton for at least 10 μs, then it will be sufficient for this protein to act as an effective picket to form the diffusion barrier. The anchored transmembrane protein pickets would work for any molecule incorporated in the membrane including transmembrane proteins. So, the diffusion of the transmembrane proteins in the membrane will be suppressed by both the fence and picket.

Suppression of diffusion of membrane molecules upon oligomerization or molecular complex formation is caused by membrane compartmentalization induced by membrane skeleton "fences" and the anchored-protein "pickets". Such confinement

induced by oligomerization or molecular complex formation is called "oligomerization-induced trapping" (107). The macroscopic diffusion coefficient of the transmembrane protein E-cadherin was decreased by a factor of 40 upon oligomerization which reflects the size of the clusters ranging in size from dimers to large protein oligomers (107). The macroscopic diffusion rate of DOPE was slowed by a factor of 5 upon artificial crosslinking (108). When the monomers of membrane molecules are associated together to form molecular complexes, these complexes as a whole have to hop all at once and because of that, their rate of hopping between the compartments will be slow.

Temporary confinement of a cytoplasmic signal at the very early stages of signal transduction may require oligomerization-induced trapping . When an extracellular signal is received by a receptor, the receptor will form oligomers and signaling complexes. Because of the "oligomerization-induced trapping", these complexes will be found in the same membrane-skeleton compartment as that where the extracellular signal was received. Therefore, the cytoplasmic signal can be confined to a place where the extracellular signal was received. This can be made possible by the membrane-skeleton fence and the anchored transmembrane-protein pickets.

In addition to what was mentioned previously, the ''membrane-skeleton fence'' and the ''anchored-transmembrane protein pickets'' may provide large diffusion barriers in the cell membrane. Nakada et al. found that neuronal cells develop diffusion barriers in the plasma membrane using the fence and picket mechanisms (109). The neuron has two distinct domains: somatodendritic domain and axonal domain, each domain has its characteristic membrane proteins. In order to prevent the intermixing of the membrane molecules located in each of these two domains, the cell must have a diffusion barrier in

the boundary region between them. The region that separates these two domains is called the initial segment (IS), an elongated domain in the mature neurons located at the foot of the axon. The proteins and lipids in the cell membrane will mix with each other if the barrier at the IS is missing . Nakada and coworkers observed the unsaturated phospholipid DOPE using single molecule techniques including single fluorescent molecule video imaging (SFVI), single particle tracking (SPT), and single-molecule dragging by laser tweezers. At 1 day *in vitro*, DOPE diffuses rapidly everywhere in the cell membrane including the IS membrane. In contrast, the diffusion of DOPE in the IS membrane was severely restricted in 10 days, whereas it was still free elsewhere on the cell membrane (109). Furthermore, the optical tweezers could not drag the colloidal-gold probes attached to DOPE in the mature IS membrane whereas those attached to the younger neurons could be dragged freely. These results suggest that a diffusion barrier to even phospholipids exists and is formed during neuronal development. Nakada et al. (39) examined the mechanism by which the diffusion barrier formed following the development of a neuron and found that the formation of the diffusion barrier occur concurrently with the concentration of actin, ankyrin-G and Na^+ channels in the IS area. Partial depolymerization of the actin filaments made DOPE and the Na^+ channels mobile again whereas suppression of DOPE diffusion in the IS membrane appeared after stabilization of actin filaments. Ankyrin-G is needed to anchor various transmembrane proteins on the membrane skeleton (110). All of these findings suggested that the confined concentration of various transmembrane proteins and membrane-skeletal proteins and their interactions with one another can create the diffusion barrier in the IS

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membrane. The presence of very dense rows of anchored-protein pickets in the IS membrane can block the macroscopic diffusion of phospholipids in the IS domain.

Transient confinement zones (TCZs) are membrane domains in which diffusion of a membrane molecule is slowed. They are defined as domains where the stay of an observed molecule, normally tagged with a colloidal gold particle, will be much longer than expected based on the average diffusion coefficient of the molecule (111). DRM-philic molecules exhibit TCZs much more often than DRM-phobic molecules and TCZ depend on cholesterol for its formation. The membrane compartments and TCZ have been confused, but they are two completely different structures. The membrane compartments occupy the entire cell membrane except for other membrane structures like clathrin-coated pits, caveolae and cell-to-cell adhesion structures. Continuity of these compartments is one of the important characteristics of the compartmentalized structure. These observations agree with what was obtained from the fence and picket models because these models showed that the compartments are separated by narrow fences or by the pickets that keep these fences near the bilayer and so there is no significant diffusion-distance between adjacent compartments. In contrast to the membrane compartments, contiguity of TCZs with each other is not seen; TCZ are generally separated from each other by a micron or more. The residency time within the membrane compartment is 1-25 ms on average, whereas that within a TCZ is on average on the order of a second. While the diffusion coefficient within the compartment is comparable to those found in artificial membranes (5-10 $\mu m^2/s$), the accurate measurements of the diffusion rates of the molecules in TCZs have not been made. Most of the molecules examined including phospholipids, transmembrane proteins, gangliosides, and GPI-

anchored proteins exhibited hop diffusion (112). Even after partial deletion of cholesterol, these molecules undergo hop diffusion, whereas TCZ behavior is severly decreased because raft-philic molecules appear to reside in TCZ for statistically significant time fractions.

It is important to distinguish between confinement of a molecule in corrals and the effects from lipid rafts. Wawrezinieck and coworkers have proposed a new type of fluorescence correlation spectroscopy (FCS) experiment for classifying membrane domains known as the FCS Diffusion Law (113). Two classes of microdomains are considered. The first consists of a mesh-like series of diffusion barriers that mimics the actin cytoskeleton model. The diffusion of the molecules is free within each grid but between adjacent grids it becomes restricted by the probability of the diffusing species crossing the diffusion barrier. The second class, the lipid raft, consists of isolated circular domains in which the partition coefficient of the diffusing species for the domains control the movement in and out of a given domain, and molecules can also have different diffusion coefficients within domains and outside of domains. Experimentally, the FCS measurements at different waist sizes of the laser was used to test the presence of these two classes of microdomains. By graphing the diffusion time versus the radius squared of the waist, a regression line can then be fit to the data, and the theoretical diffusion time in a waist of zero nm (t_o) can be calculated (113). Using this approach, a positive (t_o) is indicative of diffusing molecules that interact with isolated domains (lipid rafts), a negative (t_o) denotes mesh-like actin cytoskeletal constraints on diffusion and a zero t_o indicates free diffusion (113,114). The slope of this graph is related to the diffusion coefficient (D). Combining this method with treatments such as cholesterol deletion or

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cytoskeletal disruption will test for shifts from confined to free diffusion to confirm the possible mechanisms of diffusional trapping. By this method, it is found that the association of a fluorescent sphingolipid analog and several GPI-anchored proteins was mainly with lipid rafts whereas the transferrin receptor was confined by cytoskeletal corrals (114). In addition to that, fluorescent glycerophospholipid analogs were not confined by either mechanism.

Most of the studies have investigated the mobility of a single type of protein or lipid at a time, comparing them against other molecules and across treatments. It would be useful to be able to determine the relationship between the movements of multiple molecules at the same time within the same cell. To take advantage of multiple labeling, single molecule tracking techniques were used to investigate signaling by crosslinked GPI-anchored proteins (115). In this two-part study, colloidal gold molecules were used to both induce crosslinking and serve as markers for the clusters of cross-linked proteins (115). The researchers showed that GPI-anchored receptor clusters become transiently immobilized and undergo simple diffusion. Then, by visualizing the clustered GPI-anchored proteins and GFP-tagged versions of intracellular signaling proteins at the same time they were able to investigate the recruitment of signaling proteins to these sites. This enabled them to know the types of intracellular proteins that were recruited to the GPI-anchored receptor clusters, in addition to the relationship between the recruitment of signaling proteins and the temporary immobilization of the GPI-anchored proteins. They also tested for a role for lipid rafts in mediating protein recruitment to these sites by comparing the behavior of Lyn-GFP and LynN20-GFP, a construct consisting of the N-terminal 20 amino acid sequence of Lyn known to target the protein to lipid rafts (115).

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LynN20-GFP was recruited to the GPI-anchored proteins clusters more often than controls but less frequently than Lyn-GFP itself suggesting that lipid rafts are important in targeting it to the cluster but protein-protein interactions are also essential (115).

XII. The role of lipid rafts and caveolae in insulin receptor function

A number of proteins involved in signal transduction are localized to caveolae and lipid rafts which suggests that these membrane domains are involved in cellular signaling. A possible role for caveolae and caveolins in insulin signaling was proposed when Saltiel and coworkers showed that addition of insulin to 3T3-L1 adipocytes was associated with tyrosine phosphorylation of caveolin-1, with maximal phosphorylation being detected at 5 minutes after the insulin pulse (116). Tyrosine phosphorylation of caveolin did not occur in adipocytes after stimulation with either platelet derived growth factor (PDGF) or epidermal growth factor (EGF), this is in contrast to what was obtained after insulin stimulation. The same group also showed that tyrosine phosphorylation of caveolin by insulin did not occur in undifferentiated preadipocytes, although both insulin receptor and caveolin were expressed in these cells (116). Using a monoclonal antibody specific for caveolin-1 phosphorylated on tyrosine-14, Lee and colleagues also found a rapid increase in caveolin-1 tyrosine phosphorylation after insulin stimulation of 3T3-L1 adipocytes (117). No effect was seen with PDGF, EGF, bovine fibroblast growth factor, tumor necrosis factor α or interleukin-6. Lee et al. (117) showed that in 3T3-L1 fibroblasts, insulin was able to induce caveolin tyrosine phosphorylation, in contrast to the results obtained by the first group. This inconsistency may result from the different methods and reagents used by the researchers.

By using double immunogold transmission electron microscopy and detergent-free isolation of caveolin-enriched membrane domains (CMD), Gustavsson et al. (45) proposed that caveolin and both subunits of the insulin receptor were colocalized in approximately 50 nm round structures that represent the individual caveolae or clusters of these structures (118). This colocalization was confirmed by immunofluorescence microscopy in paraformaldehyde-fixed 3T3-L1 adipocyte plasma membrane sheets (118). The immunofluorescence labeling pattern appeared as doughnut-like spots of 0.3-0.5 μm in diameter which corresponds to the size of the caveolae clusters seen by electron microscopy. Biochemically, the insulin receptor cofractionated with caveolin in detergent-free isolation of CMD from rat adipocyte plasma membranes (118). The researchers found that addition of insulin was not associated with a significant change in localization of the insulin receptor as determined biochemically or by immunofluorescence (118).

Direct protein-protein interactions between the insulin receptor and caveolae make these reseptors segregate preferentially into these membrane structures. These interactions involve the caveolin scaffolding domain and hydrophobic motifs in the caveolin-interacting proteins. The insulin receptor contains this motif. After preparing CMD from CHO cells that overexpressed the insulin receptor and IRS-1 by a detergent-free method, Yamamoto et al. found that the insulin receptor, but not IRS-1, cofractionated with caveolin-1 (119). These researchers demonstrated a direct effect of caveolin scaffolding domains on insulin receptor tyrosine kinase activity using *in vitro* assays (119). The cytoplasmic tyrosine kinase domain of the insulin receptor and full-length IRS-1 were expressed in insect cells and subsequently purified. These

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polypeptides were then incubated with the scaffolding domain peptides of caveolin-1, -2, and -3. The increase in tyrosine phosphorylation of IRS-1 by the kinase domain of insulin receptor was noticed in the presence of caveolin-1 and -3 peptides whereas caveolin-2 peptide showed no effect (119). In another study supporting the importance of caveolin scaffolding domain in insulin signaling, wild type caveolin-1 was overexpressed in COS-7 cells. The overexpression of caveolin-1 enhanced insulin-induced Elk-1 phosphorylation and inhibited ERK2 phosphorylation (120). The mutant form of caveolin-1 with disrupted scaffolding domain had no effect on insulin signaling (120).

To analyze whether insulin receptor associates with lipid rafts in hepatoma cells, Vainio and colleagues evaluated Triton X-100 insolubility at 4°C (121). In their study, insulin-stimulated and basal cells were lysed in 0.1% Triton X-100 on ice and the lysates were subjected to Optiprep gradient fractionation in the presence of the detergent. Western blot analysis of the proteins precipitated from the fractions indicated that in hepatoma cells, ligand binding is needed for the partitioning of insulin receptors into detergent-resistant domains (121).

Lipid rafts and caveolae also provide a necessary organizing structures for insulin-stimulated glucose transport. Karlsson and colleagues found that GLUT4 translocates to caveolae in response to insulin (122). The biochemical isolation of the caveolar fraction from purified plasma membranes of freshly isolated rat adipocytes was used to confirm the morphological localization of GLUT4 to caveolae. In addition to the biochemical technique, the electron microscopic examination of the caveolae fractions showed that 50-70% of the membrane fragments were labeled with antibodies against

caveolin (122). GLUT4 labeling was largely found in the same membranes as those labeled for caveolin (122). In addition to that, Baumann et al. reported that flotillin-1, a raft protein, recruits a complex of tyrosine-phosphorylated Cbl and Cbl-associated protein (CAP) to rafts (44). Localization of the CAP-Cbl complex to lipid raft subdomains generates a pathway that activates TC10, a Rho GTPase protein. TC10 can directly interact with many effectors that are known to modulate cytoskeletal function leading to translocation of GLUT4 and thus glucose entry into the target cells.

Immunoelectron microscopy data have not agreed with insulin-stimulated movement of GLUT4 to caveolae. Malide and colleagues labeled ultra-thin cryosections of embedded white and brown rat adipocytes with anti-GLUT4 immunogold particles (123). The increase in the gold particles seen after insulin stimulation was found at the plasma membranes but not in caveolae specifically. The researchers estimated that only 2% of the GLUT4 label was in caveolae (123).

If lipid rafts and caveolae play an important role in insulin signaling, then disruption of these membrane microdomains would inhibit insulin receptor function. To demonstrate this, Gustvsson et al. (124) incubated 3T3-L1 adipocytes for 50 minutes with β-cyclodextrin which binds cholesterol and extracts it from the plasma membrane. This treatment led to flattening of caveolar invaginations and caused an inhibition of insulin-stimulated glucose uptake, downstream tyrosine phosphorylation of IRS-1 and protein kinase B, and insulin-dependent binding of IRS-1 to the insulin receptor (124). The same treatment had no significant effect on insulin binding to intact adipocytes or on the levels of insulin receptors and caveolin within the plasma membrane (124). Both insulin receptor autophosphorylation and activation of the MAP kinase pathway were not

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affected by β-cyclodextrin treatment (124). The researchers noted the similarities between these cell culture results and what was found in many cases of clinical insulin resistance and suggested a connection between caveolae and the pathology of this disorder.

In another study, human hepatoma cells were incubated with methyl-β-cyclodextrin for 15 minutes at 37°C (121). This resulted in 50% depletion of cellular cholesterol (121). Cholesterol extraction resulted in an approximately 60% decrease in insulin receptor phosphorylation (121). Insulin-dependent tyrosine phosphorylation of IRS-1 was also impaired by approximately 50% (121).

Tumor necrosis factor α (TNF-α) induces insulin resistance in type 2 diabetes via a mechanism that is not clear. Inokuchi and colleagues found a discriminating increase in the acidic glycosphingolipid ganglioside GM3 in 3T3-L1 adipocytes treated with TNF-α, suggesting a specific function for GM3 (125). They were also used living animals including obese Zucker *fa/fa* rats and *ob/ob* mice where GM3 synthase mRNA levels in the white adipose tissues are higher than in the lean controls (125). In DRM from TNF-α treated 3T3-L1 adipocytes, GM3 levels were doubled compared to results in normal adipocytes. It was also noted that insulin receptor accumulation in DRMs was decreased while caveolin and flotillin levels were unchanged (125). The authors believed that elimination of insulin receptors from the DRMs by TNF-α treatment is due to an excessive accumulation of GM3 in these microdomains, especially since preventing GM3 synthesis using D-PDMP attenuated the elimination of insulin receptors from the DRMs (125). These findings provide an evidence that, in insulin resistance defect associated

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with type 2 diabetes, the insulin metabolic signaling abnormality may result from the loss of insulin receptors from the microdomains as a result of the accumulation of GM3.

XIII. Pharmacological properties of vanadium salts and vanadium-containing compounds

Vanadium is the chemical element with the symbol **V** and atomic number 23 (126). Vanadium is a trace element that is essential for mammalian growth and has demonstrated biological effects which include reducing cholesterol levels and cholinesterase activities. Combustion of fossil fuels released large quantities of vanadium to the atmosphere which has been implicated in air pollution induced diseases.

Vanadium is characterized by wide distribution in which it constitutes about 0.02% of the earth's crust. It was first discovered in 1801 by the mineralogist Del Rio who gave it the name panchromium because of its color changes as a function of its oxidation state. Unfortunately, a French chemist incorrectly stated that Del Rio's new element was only impure chromium (127). This transitional element was then rediscovered in 1831 by the Swedish chemist Nils Gabriel Sefstrom who named the element in honor of the Scandinavian-goddess Vanadis because of its beautiful multicolored compounds. Vanadium of 99.3 to 99.8% purity was not produced until 1927 (128). Vanadium is found in about 65 different minerals among which are camotite and vanadinite. It is also found in phosphate rock and certain iron ores and is present in some crude oils in the form of organic complexes (129). Commercial production from petroleum ash is considered to be an important source of the element. Pure vanadium is a bright white metal that is soft and ductile. It has good corrosion resistance to alkalis and salt water but oxidizes readily above 660 C°. Vanadium is a mixture of two isotopes,

50V and 51V. 50V is highly radioactive with long half-life. 17 other unstable isotopes are recognized. The principal use of vanadium is as an alloying addition to iron and steel (129), particularly in high strength steel and to a lesser extent in tool steels and castings. Vanadium foil is used as a bonding agent in cladding titanium to steels. Vanadium pentoxide is used in ceramics and as a catalyst.

Occupational exposure to vanadium containing dusts is encountered in the mining of vanadium bearing ores. Background vanadium concentrations in unpolluted air range from 0.02-2 ng/cu m (129). Rural sites may have vanadium levels as high as 65 ng/cu m while industrialized urban centers with a high consumption of residual fuel oil may have maximum levels up to 10000 ng/cu m (129).

Most foods are found to be a poor source of vanadium with low nanogram levels or less (130). Only a few items, such as spinach, parsley, mushrooms and oysters have higher values. Cereals, liver and fish tend to have intermediate levels and drinking water does not generally considered to be an important source of vanadium (130). Vanadium is poorly absorbed from the gastrointestinal tract, most ingested vanadium is transformed in the stomach to VO_2^+ and remains in this form as it passes through the duodenum (130). Vanadium has been shown to be stored in the bone (main storage depot), kidney and liver following i.p.injection. Feces are the major route of excretion when vanadium is taken orally but urine is the major route in the case of intravenous injection of vanadium (130).

It can be estimated that daily dietary intake is in the order of a few tens of micrograms, though it may vary over wide limits. Vanadium from some foods, mainly vegetables may be greatly increased by exogenous vanadium from soil and dust. In humans, the total body pool of vanadium is estimated to be 100-200µg and its

intracellular concentration is approximately 20nM. Vanadium requirements for growth are minimal as indicated by the very low levels of vanadium found in various milks and eggs.

Vanadium exists in several valence states (-3, -1, 0, +1 to +5) and the expression of a given form is highly pH dependent. In biological systems vanadium is found predominantly as the vanadate (+5) and vanadyl (+4) forms. In plasma, vanadium exists in both oxidation states.

Vanadium plays a very limited role in biology. A vanadium-containing nitrogenase is used by some nitrogen-fixing microorganisms. 10% of the blood cell pigment of the sea cucumber is vanadium, vanadium in the vanabin pigment causes the blood color of sea cucumber to be yellow. Several species of macrofungi accumulate vanadium up to 500 mg/kg in dry weight.

XIV. The use of vanadium in the management of diabetes

Daily insulin administration and/or diet help millions of diabetic patients in controlling their diabetes. Although insulin replacement is the easiest method of controlling chronic diabetes, insulin can be degraded orally and must be taken by injection. Thus, there is a great interest in using orally active insulin mimics instead of insulin injections, particularly vanadium compounds. The earliest documented evidence of the insulin-like effects of the inorganic vanadium salt, sodium orthovanadate (Na_3VO_4) was published by Lyonnet and colleagues in 1899 (131). They observed that oral Na_3VO_4 administration decreased glucosuria in 2 out of 3 diabetic patients (131). Their study was unnoticed for a long time but when Tolman et al. demonstrated an *in vitro* insulino-mimetic effect of vanadium salts in 1979, the interest in vanadium came back

(132). They showed that several inorganic vanadium compounds stimulated glucose transport and oxidation in adipocytes, increased glycogen synthesis in rat diaphragm and hepatocytes and inhibited gluconeogenesis in liver cells (132). Since then, numerous studies have revealed various insulino-mimetic effects of vanadium compounds *in vitro* including the stimulation of lipogenesis (133) as well as the inhibition of lipolysis (134).

An important accomplishement occurred in 1985 when Heyliger and coworkers demonstrated that oral administration of vanadate to streptozocin-treated diabetic rats (STZ rats) lowered their high levels of blood glucose to normal values (135). STZ-induced diabetic rats are considered to be a model of type 1 diabetes because STZ treatment destroys insulin-producing cells of the pancreas . Unlike insulin, vanadate can permeate plasma membranes and the intestinal wall easily because it is a low-molecular-weight substance and a phosphate analog. Sodium metavanadate ($NaVO_3$) in drinking water was found to work best at a concentration of 0.2 mg/ml (136). Under these conditions, blood glucose levels of STZ-diabetic rats dropped to near normal values within 4 days of treatment and stable normoglycemia was obtained for 2-3 weeks as long as $NaVO_3$ was supplied in a continous fashion (136). Several laboratories then showed that vanadate therapy decreased tissue abnormalities resulting from chronic hyperglycemia. For example, insulin binding capacity in the liver was restored. Key enzymes involved in carbohydrate metabolism became functional and the level of glycogen, which was greatly diminished in these diabetic rats, was normalized following a 2 week of oral treatment (137). Glucokinase, which was essentially lacking, was restored to 65% of normal value and glycogen was restored from 40 to 109% (137). The activity of 6-phosphofructo-2-kinase, an essential enzyme for maintaining normal

glycolytic flux, and nonelevated gluconeogenesis was shifted from 20 to 122% in vanadate-treated STZ-rats (137). Vanadium induced a correction of the decreased amounts of glucose transporters as well as of the low activity of glycogen synthase, because of that the ability of insulin to stimulate glucose disposal by muscles was enhanced.

The effects of vanadium on adipose tissue were less clear. A reduction of the high rate of lipolysis has been observed but the decreased expression and activity of lipogenic enzymes were not modified. Despite these improvements, vanadium therapy is not expected to exert the same effects of insulin in insulin-deficient diabetic models. As an example, STZ-rats are largely catabolic (138), insulin therapy completely restored the daily weight-gain in these animals that is only partially corrected (20-30% of insulin effect) by vanadium therapy. The catabolic nature of the untreated STZ-rat model is likely due to an increased rate in muscle protein degradation, a process that can be stopped by insulin, but not by vanadium. Thus, it appears that vanadium therapy might be more beneficial for the treatment of type 2 diabetes.

The most common genetic rodent models for type 2 diabetes are *ob/ob* mice, *db/db* mice, *BB* rats and *fa/fa* rats. These hyperglycemic and hyperinsulinemic rodents demonstrate a reduced response to insulin at the receptor and post-receptor levels. Reducing blood glucose levels or diminishing any of the defects associated with utilization of glucose can not be achieved by exogenously administered insulin . Glucose homeostasis as well as oral glucose tolerance were improved after the administration of sodium orthovanadate (NaOV) to *fa/fa* Zucker rats, *db/db* mice and *ob/ob* mice either in drinking water or food (136). A nutritionally induced model of diabetes and insulin

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resistance, *Psammomys obesus* (sand rat) (137) was treated with vanadium, this treatment resulted in prolonged restoration of normoglycemia and normoinsulinemia. Vanadyl sulfate (VS) treatment in this model was associated with a normal glucose tolerance test and a decreased level of the hepatic gluconeogenic enzyme phosphoenol pyruvate carboxykinase. Low-level insulin is required for vanadium to work in these animals as indicated by the inability of VS to produce any effect in sand rats that had lost their insulin secretory capacity. Thus, vanadium appears to act as an insulin potentiator instead of being a mimicker in improving insulin resistance in sand rats (137).

Hypertension is frequently associated with hyperinsulinemia and insulin resistance. It would be expected that if vanadium corrected these defects, it would also decrease blood pressure. Hypertensive rats (SHR) (138) and fructose hypertensive rats (139), models of genetic and acquired hypertension, respectively were given VS. VS reduced plasma insulin levels and blood pressure in both types of animals (140). These results reveal the antihypertensive potential of vanadium *in vivo*.

In addition to their action on glucose metabolism, vanadium compounds can adapt lipid metabolism *in vivo*. NaOV treatment of insulin-resistant, sucrose-fed diabetic rats and *fa/fa* Zucker rats significantly lowered plasma triglycerols (141). VS decreased plasma cholesterol levels in humans without a significant effect on either plasma free fatty acid or triglyceride fractions (142). Vanadate has also been shown to reduce total and free cholesterol levels in normal subjects (143) which may be due to inhibition of the steps involved in cholesterol biosynthesis.

The demonstration of a beneficial effects of vanadium compounds in both type 1 and type 2 animal models of diabetes mellitus has resulted in the initiation of many

clinical trials to determine whether vanadium therapy is useful in human subjects with diabetes. In type 1 diabetes, sodium metavanadate (NaMV) (125mg/day) for 2 weeks had no effect on fasting plasma glucose levels, 2 out of 5 patients respond well to such treatment in which there was a decrease in daily insulin requirements and an increase in glucose utilization (144). In type 2 diabetes, NaMV resulted in increased insulin sensitivity due to enhanced non-oxidative glucose disposal (144). In addition to that, VS improved insulin resistance in type 2 diabetes, this improvement was associated with a slight decrease in fasting plasma glucose and glycosylated haemoglobin (HbA$_{1c}$), increase in insulin-mediated glucose uptake, and suppression of hepatic glucose production (145). Because of the treatment of small number of subjects with low doses of vanadium for a short period of time in those early studies, 2 groups investigated the effect of longer treatment with higher VS doses in a relatively larger number of subjects with type 2 diabetes. Goldfine and colleagues (146) treated 16 patients with VS doses ranging from 75 to 300 mg/day for 6 weeks and noticed that fasting glucose declined significantly only in the 300 mg VS group whereas HbA$_{1c}$ decreased in both the 150 and 300 mg groups (146). These treatments had no effect on suppression of hepatic glucose production in response to insulin. Similar study conducted by Cusi and coworkers (142), they have treated 11 patients of type 2 diabetes with VS at a dose of 150 mg/day for 6 weeks. They found that both HbA$_{1c}$ and fasting plasma glucose were significantly reduced. In this study, VS decreased endogenous glucose production and increased insulin-mediated glucose disposal (142). It is important to note that, in contrast to animal studies, none of the clinical research done so far has resulted in complete elimination of

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hyperglycaemia. The difference may be due to the duration of therapy and also due to the much lower blood vanadium level reached in patients than in animals.

Vanadium salts have been seriously considered as a possible treatment for diabetes. Since inorganic vanadium compounds are poorly absorbed from the gastrointestinal tract and show multiple signs of toxicity, many attempts have been made to elevate the insulino-mimetic or insulin-enhancing activity of vanadium without increasing its toxicity. Several organically-chelated vanadium compounds such as bis (maltolato)oxovanadium (BMOV) have been synthesized (143). BMOV accumulates primarily in bone, liver, and kidney tissue (144). The more beneficial pharmacological effect of BMOV compared to that of inorganic vanadium has been attributed to increased absorption from the gastrointestinal tract due to its low molecular weight, acceptable lipophilic/hydrophilic balance, and the neutral charge of the vanadyl complex (145). Interactions of BMOV with the serum proteins, apo-transferrin and albumin were studied using electron paramagnetic resonance (EPR). BMOV binding to human serum apo-transferrin resulted in EPR spectra that were similar to those for vanadyl sulfate binding to apo-transferrin. By contrast, the interaction of BMOV with human serum albumin produced an EPR spectrum noticeably different from that of vanadyl sulfate. Adduct formation with albumin could both protect BMOV from the effects of oxidation (147) and increase relative efficacy by slowing transit time through the bloodstream (148).

Passive diffusion is the route by which BMOV can be orally absorbed (149). It was administered to STZ-diabetic rats in the drinking water at a maximal concentration of 0.75 mg/ml for 6 months, this administration normalized plasma glucose levels in 8 out of 12 animals and restored elevated plasma lipid parameters and heart function in all

diabetic treated rats (150). There was a strong relation between improved heart function and long term glucose control. However, BMOV treatment did not result in a sustained euglycemic response following withdrawal of treatment (150). A number of acute experiments were conducted to compare the glucose-lowering properties of BMOV and vanadyl sulfate. BMOV and vanadyl sulfate were administered by oral gavage at a concentration of 175 mg/kg or by intraperitoneal injection at a concentration of 20 mg/kg (151). The efficiency of BMOV was found to be 2-3 times higher than vanadyl sulfate by either route of administration. Following a single administration, the lower plasma glucose levels were maintained with BMOV for up to 14 weeks in 15-20% of animals (62). Intravenous administration of vanadium produced a plasma glucose lowering effect only when given by infusion over an extended period of time. BMOV and inorganic vanadium had similar effects on elevated blood pressure and hyperinsulinemia in SHR animals (152).

In order to examine the effectiveness of organic vanadium in type II diabetes, BMOV has been used in *fa/fa* (fatty) Zucker rats. BMOV at a maximal concentration of 0.5 mg/ml for 14 weeks of treatment reduced plasma insulin levels from 180 to normal (50 μU/ml) by week 4. At these concentrations BMOV did not affect body weight gain in lean controls but did significantly reduce body weight in the fatty treated group (153). BMOV administration at a maximal concentration of 0.2 mg/ml did not affect food and fluid intake, body weight gain or plasma cholesterol levels in fatty treated animals. At the lower concentration, both plasma glucose and triglyceride levels were reduced in response to BMOV. An oral glucose tolerance test showed an improved glucose tolerance in fatty treated animals regardless of the concentration of BMOV.

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Treatment of hyperglycemia in diabetes mellitus and the prevention of the secondary complications associated with this disorder can be made by the use of organic vanadium compounds. Organically chelated vanadium complexes appear to be as effective insulin-mimetic agents at significantly lower doses. These compounds reduced the gastrointestinal side effects of vanadium treatment and did not effect body weight gain and food and fluid intake in control treated animals.

XV. Cellular effects of vanadium salts and vanadium-containing compounds

The mechanism of action of vanadium in producing its anti-diabetic effects *in vivo* is unclear. *In vitro* and *in vivo* data demonstrate that vanadium does affect various aspects of the insulin signaling pathway. It has been postulated that the ability of vanadium to mimic insulin effects result from vanadium acting as a phosphate analog. It inhibits protein tyrosine phosphatases (PTPases) (154) and because of that protein tyrosine phosphorylation can be stimulated. Support for this notion comes from studies in which *in vitro* treatment of hepatocytes (155) and hepatoma cells (156) and *in vivo* therapy of diabetic rodents with various vanadium compounds (157) inhibit the activities of several PTPase forms including SH-2 domain-containing PTPase (SHP-2) (155), PTP-1B (158) and total PTPase (159). In contrast to the effects on isolated cells and diabetic animal models, there was no significant effect on total PTPase of muscle homogenates from VS-treated human subjects. The cause for this difference is not clear but it is possible that only specific types of PTPasas are inhibited by vanadium which may be missing in crude tissue extracts containing many different forms of PTPases. The ability of vanadium to inhibit PTPase activity may be one of the mechanisms by which it exerts insulino-mimetic and anti-diabetic actions, in which the expression levels of PTPases,

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including PTP-1B are reduced in rodent models of diabetes (160) and the reduction of PTP-1B by antisense oligonucleotides (ASOs) has been shown to improve insulin sensitivity in *ob/ob* and *db/db* mice (161). Another target of vanadium could be lipid phosphatases like tensin homolog (PTEN) which is capable of down regulating phosphatidyl inositol 3-kinase signaling. This is supported by the notion that specific inhibition of PTEN expression by ASO resulted in normalization of blood glucose in *db/db* and *ob/ob* mice (162).

Vanadium was shown to activate a staurosporine-sensitive cytosolic protein tyrosine kinase (Cyt PTK) distinct from insulin receptor tyrosine kinase (163), this was revealed by a study conducted in intact rat adipocytes . This activation was linked to glucose oxidation and lipid synthesis but dissociated from glucose uptake and inhibition of lipolysis. Cyt PTK would then be implicated only in specific cellular response. Neither insulin nor phorbol ester had any affect on Cyt PTK activity which means that Cyt PTK would be highly selective for vanadium.

Because vanadium salts are potent inhibitors of protein tyrosine phosphatases and the activation of the insulin receptor requires increased tyrosine phosphorylation of the insulin receptor β subunit (IR-β), vanadium may activate insulin receptor protein tyrosine kinase (IR-PTK) by preventing dephosphorylation of IR-β subunit. A previous study showed that vanadate stimulated tyrosine phosphorylation of the IR-β subunit in rat adipocytes (164) and it was also demonstrated that sodium orthovanadate (NaOV) treatment partially reversed the decreased tyrosine phosphorylation of the IR-β subunit in STZ diabetic rats without significantly affecting IR-PTK in the liver (165). In subsequent experiments, NaOV or VS did not cause any change in IR-β subunit tyrosine

phosphorylation in adipocytes (133) and IR-overexpressing Chinese hamster ovary cells (CHO-HIR) (166,167). Rat diaphragm showed similar observations in which NaOV did not stimulate tyrosine phosphorylation of the IR-β subunit (168). These and other data indicated that tyrosine phosphorylation of the IR-β subunit and IR-PTK activation may not be the early events of the insulin-like effects of inorganic vanadium salts. Unlike the inorganic salts of vanadium, BMOV was found to increase the tyrosine phosphorylation of IR-β subunit (169). Although there is no effect of vanadium salts on tyrosine phosphorylation of IR-β subunit, several studies have reported an increase in the tyrosine phosphorylation of IRS-1 in response to VS and NaOV (167). VS treatment increased IRS-1 phosphorylation which was associated with its increased binding to the p85 subunit of PI3-K. The putative kinase responsible for the action of VS remains unidentified till now. However, some have reported that vanadium compounds could activate epidermal growth factor receptor (170) and non-receptor PTKs like focal adhesion kinase (171) which may participate in IRS-1 phosphorylation.

Vanadium induced PI3-K activation which is important for the stimulation of glycogen synthesis, this effect has been demonstrated in both adipocytes (172) and CHO-HIR cells (167). In these studies, pharmacological inhibition of PI3-K by wortmannin attenuated NaOV or VS-stimulated increases in glycogen synthesis (172). Wortmannin also blocked vanadate-stimulated lipogenesis in adipocytes (173). These experiments support an important role of wortmannin-sensitive PI3-K activity in a number of insulino-mimetic effects of vanadium compounds including glucose uptake, glycogen and lipid synthesis. PKB, a downstream effector of PI3-K, also was shown to be activated in response to VS in CHO-HIR cells and to NaOV in adipocytes (173), L-6 myotubes (174)

and adult cardiomyocytes (175). Because PKB has been implicated in facilitating the physiological effects of insulin on glucose uptake and glycogen synthesis, it is possible that one of the mechanisms by which vanadium compounds produce insulin-like effects is via the activation of the PI3-K/PKB signaling system. On the other hand, some studies have indicated the involvement of a PI3-K-independent pathway in vanadium-induced glucose transport (176). The nature of this pathway remains unidentified at present but perhaps may involve a CAP/APS/Cbl/TC10 dependent route.

PKB-catalysed phosphorylation of its downstream substrates, forkhead transcription factor (FKHR) and glycogen synthase kinase-3 (GSK-3) results in their inactivation. Since the inactivation of these proteins reduces the gene expression of 2 gluconeogenic enzymes, PEPCK and G6Pase, it is possible that vanadium inhibits gluconeogenesis by this pathway.

NaOV and VS have also been shown to stimulate the phosphorylation and activation of MAPK family member ERK1/2 in several cell types, including CHO-HIR and adipocytes. VS-induced ERK1/2 activation was associated with sequential stimulation of upstream components of the ERK1/2 signaling system like MEK-1, c-raf and c-ras (177).

It was postulated that vanadium improved glucose uptake by an increase in the intrinsic activity or enhanced targeting of GLUT-4 to the cell surface. In isolated rat adipocytes (178) and in cultured L-6 myotubes (176), NaOV can stimulate GLUT-4 translocation to the cell surface. Vanadium exposure of STZ diabetic rats can restore the expression and/or cell surface translocation of GLUT-4 in skeletal muscle (179) and heart (180). Also, treatment of a nutritionally induced, insulin-resistant model (*p.obesus*) with

VS restored membrane-associated as well as total protein and mRNA content of GLUT-4 in the gastrocnemius muscle (137).

Under *in vivo* conditions, trials have been made to examine the association between the glucoregulatory response of vanadium and its actions on various elements of the insulin signaling cascade. VS therapy of type 2 diabetic patients was found to modify several components involved in insulin signaling like the increase in basal IR and IRS phosphorylation and PI3-K activation in muscle homogenates (181). Treatment of diabetic subjects with sodium metavanadate causes stimulation of basal $p70^{s6k}$ and MAPK activity in circulating mononuclear monocytes (182). In contrast to these clinical data, treatment of STZ diabetic or *fa/fa* Zucker rats with BMOV was not associated with any significant change in the activation of PI3-K (183), PKB (184) or GSK-3 (185) in skeletal muscle.

Another proposed mechanism of vanadium action is by changing membrane fluidity. The definition of a fluid membrane can vary greatly. According to the cell biologist, the membrane is fluid when it contains protein and lipid molecules capable of rapid and extensive diffusion motions in the plane of the membrane bilayer. Several hydrophobic fluorescent molecules have been used as probes of membrane fluidity. The ANEP (aminonaphthylethenylpyridinium) dyes are the most sensitive probes for detection of submillisecond membrane potential changes. The electronic structure and the fluorescence spectra of these dyes will be changed in response to changes in the surrounding electrical field. This optical response is sufficiently fast to detect transient potential changes in excitable cells. These dyes are nonfluorescent in water and upon

binding to membranes they become strongly fluorescent. Fluorescence is not affected by pH and photostability is generally high.

Vanadium compounds must cross cell membranes to act as anti-diabetic agents, evidence for the interaction of vanadium compounds with membranes and lipid interfaces has been presented by Dr. Crans and coworkers who used NMR spectroscopy to examine interaction of [VO_2dipic]⁻ with reverse micelles. These studies demonstrated that vanadium compounds interact with the lipid interface. Such a result suggested that vanadium could change the distribution of membrane lipids and induce their packing as a result of its ability to be inserted in the bilayer.

XVI. Vanadium toxicity

Despite the beneficial effects of vanadium as an anti-diabetic agent, vanadium compounds have been associated with several toxic effects. The most common are diarrhea, decreased fluid and food uptake, dehydration and reduced body weight gain (186). These symptoms can be prevented by adding NaCl to the drinking water, adjusting the pH of the solution to neutrality and gradually increasing the dose of vanadium (187). Organic vanadium compounds are safer as anti-diabetic agents than inorganic vanadium salts. Diabetic rats receiving organic vanadium compounds did not show any gastrointestinal side-effects and did not develop diarrhea (188). Tiron as a chelater can be used to decrease vanadate toxicity without changing its anti-diabetic effects (189).

Other toxic effects of vanadium salts include hepatotoxicity, nephrotoxicity and teratogenicity as well as developmental/reproductive toxicity. They stimulate mitogenesis and cell proliferation in cultured cells (190) and have the potential to exert

tumorigenesis/carcinogenic activity. In contrast to these data, it was demonstrated that vanadium compounds inhibited serum and growth factor-stimulated mitogenesis and possess anti-tumor activity (191).

Many other studies have indicated that vanadium compounds did not cause any change in the levels of urea, creatinine, glutamic oxaloacetic transaminase and glutamic pyruvic transaminase indices of kidney and liver functions (192). In addition to that, no significant changes in the histopathology of several tissues including the liver, spleen, stomach, heart and lung have been observed among control and VS-treated animals (193). Some reports have revealed that behavioural changes were seen in rats treated with NaMV (194).

Gastrointestinal discomfort was the most common toxic effect in the patients treated with vanadium salts (144). The clinical studies utilized lower doses than those given to animals and have been of short duration. The long term toxicity of vanadium and the efficacy of organo vanadium compounds in humans need further exploration.

Although animal studies have been sufficient to prove the important role of vanadium in diabetes, a similar role in controlling human diabetes is yet to be recognized. Early trials with vanadium in diabetic human volunteers have shown hopeful results and within the coming few years the possible pharmacological effects of vanadium compounds should be more clearly known.

XVII. Pharmacological properties of chromium-containing compounds

Chromium is a chemical element which has the symbol **Cr** and atomic number 24. It is a member of the transition metals in group VI. Chromium is a steely-gray hard metal that has a high melting point. It is odorless and tasteless. The name of the element

is derived from the Greek word ''chroma'' meaning color because many of its compounds are powerfully colored (195). The most common oxidation states of chromium are +2, +3, and +6 with +3 being the most stable. +1, +4 and +5 are rare. Hexavalent chromium is corrosive and causes chronic ulceration of skin surfaces. Chromium compounds of oxidation state +6 are powerful oxidants. Exposure to these compounds can cause corrosive burns by denaturation of tissue proteins. They are characterized by being soluble and readily absorbed from the GIT, skin and lungs. They have also been responsible for asthma and discoloration of teeth.

Chromium is the 21st most abundant element in Earth's crust with an average concentration of 100 ppm (196). Erosion of chromium-containing rocks release chromium to the environment. Chromium can be distributed by volcanic eruptions. The concentrations range in soil is between 1 and 3000 mg/kg. Chromium is mined as chromite ore (197). About two-fifths of the chromite ores in the world are produced in South Africa, while India, Russia and Turkey are also substantial producers. Naturally occurring chromium is composed of three stable isotopes; ^{52}Cr, ^{53}Cr and ^{54}Cr with ^{52}Cr is the most abundant.

Westerners noticed the existence of chromium in the 18th century. In 1761, Johann Lehmann found an orange-red mineral in the mines of Ural mountains which he named *Siberian red lead* (198). At first, it was misidentified as a lead compound with selenium and iron components, but later the mineral was recognized as Crocoite with a formula of $PbCrO_4$ (198). In 1770, Peter Simon visited the same site as Lehmann and found a red lead mineral, he proposed that this mineral had useful properties as a pigment in paints. In 1797, Louis Nicolas Vauquelin received samples of crocoite ore. He

produced chromium oxide by mixing crocoite with HCL. Vauquelin discovered that metallic chromium could be isolated by heating the oxide in a charcoal oven (199).

Chromium was regarded with great interest because of its high corrosion resistance and hardness. It was discovered that adding chromium and nickel to form stainless steel will make this steel highly resistant to corrosion and discoloration. Chromium is known for its luster when polished. It is used as a protective and decorative coating on many items like car parts, plumbing fixtures, furniture parts, usually applied by electroplating. Chromium is also used in leather tanning. Metal alloys now account for 85% of the use of chromium.

People should get all their chromium requirements from food as recommended by many nutritionists . A daily intake of 50-200 µg chromium is regarded to be safe and adequate for optimal health (200). Chromium in food is mainly in the trivalent form. Dietary sources of chromium include brewer's yeast, lean meats, cheese, pork kidney, whole-grain breads and cereals, molasses, spices and some bran cereals. Low amounts of chromium are found in Vegetables, fruits and most refined and processed foods. Individuals who consume excessive amounts of sugary foods, old people and pregnant women are most likely to be deficient in chromium. Some of the important deficiency symptoms of chromium are anxiety, attention deficit disorder, aortic cholesterol plaque, impaired growth, infertility, obesity, depression, peripheral neuropathy and high blood cholesterol.

Exogenous and endogenous factors significantly change the absorption and the bioavailability of chromium. One study of men over age 60 showed that the absorption of trivalent chromium from dietary utilization was approximately 1.8 % (201). Other

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sources indicated that chromium absorption ranges between 0.5 and 2 % (202). Differences in the absorption of trivalent chromium can be due to multiple factors including the type of chromium ingested, competing minerals and the effect of vitamins, proteins, drugs and other nutritional factors used in combination. Chromium absorption is influenced by dietary factors such as starch, ascorbic acid, minerals, oxalate and amino acid intake. Carbohydrate intake has been shown to influence chromium urinary excretion and tissue concentration. Higher concentrations of chromium in blood and tissue were found in mice fed ^{51}Cr-labelled chromium III chloride together with starch compared to those fed with chromium III chloride mixed with sucrose, fructose or glucose (203). Diets high in simple sugars have also been shown to increase urinary excretion of chromium by 10-300% (204). Animals fed ascorbic acid with chromium supplementation demonstrated increased absorption (205). Some amino acids have been found to increase absorption of chromium from the intestine. Amino acids like histidine and glutamic acid that can easily form complexes with chromium were shown to increase absorption (206). A number of minerals influence absorption. In studies utilizing rats, zinc supplementation reduced chromium absorption (207). Iron, manganese and calcium have all been shown to lower intestinal transport of chromium.

A number of drugs have different impact on chromium absorption . Aspirin and indomethacin increased the blood level of chromium (208). In contrast to the effects of aspirin and indomethacin, antacids significantly decrease blood and tissue levels of chromium (208), this decrease has been attributed to a competitive inhibition by the minerals in the antacids.

The multiple forms of trivalent chromium differ in the extent of absorption in which the inorganic complexes have lower levels compared to organic complexes. Chromite ores, chromic oxide and chromium III chloride have shown to have the lowest levels of absorption (209).

The degree of chromium retention in various tissues is of a major concern. A large quantity of Cr was found concentrated in the kidneys as compared to liver, spleen, heart, lung and gastronemius muscle of rats fed a high-chromium diet over a three-week period (209). The specific chromium compound had different effect on being concentrated in the kidney, with chromium picolinate have a greater effect than chromium nicotinate or chromium chloride.

XVIII. Role of chromium in diabetes and lipid metabolism

In 1950s Schwarz and Mertz demonstrated the existence of a new dietary factor which was absent in the diet of rats fed Torula yeast as the only protein source (210). Rats consuming the diet were unable to remove glucose efficiently from the bloodstream, this effect was reversed by adding foods rich in chromium or by adding synthetic inorganic chromium III complexes to the diet (210). Confirmation of the important action of chromium in humans comes from patients receiving total parenteral nutrition (TPN) (211). A patient receiving TPN developed severe signs of diabetes, including weight loss and hyperglycemia that does not respond to high doses of insulin. Supplemental chromium reduced the signs and symptoms of diabetes, in which there was an improvement in the glycemic status and a reduction in insulin requirements. Other studies of the beneficial effects of chromium in patients receiving TPN have also been

documented. Chromium is now routinely added to TPN solutions. The results of these studies strongly supported chromium as a significant cofactor in the action of insulin.

There have been several studies involving Cr supplementation of subjects with diabetes. Most of these reports included type 2 diabetic patients but there are smaller studies involving Cr and type 1 diabetes. The absorption of chromium in type 1 diabetic patients is approximately double that of control or subjects with type 2 diabetes (212). Urinary excretion of subjects with type 1 diabetes is also more than twice that of control subjects (213). It seems that people with type 1 diabetes need additional Cr for their metabolic control mechanisms which is reflected by higher absorption but this increased absorption does not appear to be utilized and is ultimately excreted in the urine.

In a study involved 48 type 1 diabetic patients, Ravina and coworkers showed that these patients were able to reduce their insulin dosage by 30% and their blood sugar variations were much smaller following 10 days of supplemental Cr (200 μg per day of Cr as Cr picolinate) (214). Following three months of Cr supplementation in a 28-year-old female with an eighteen year history of type 1 diabetes, Chromium (200 μg three times daily as Cr picolinate) decreased glycosylated hemoglobin from 11.3% to 7.9% (215). Other researchers have also reported beneficial effects of supplemental Cr on people with type 1 diabetes.

Chinese patients with type 2 diabetes receiving Cr picolinate practiced significant improvements in HbA1c, fasting plasma glucose, 2-h glucose and fasting and 2-h insulin (216). Other investigators studied the effects of brewer's yeast (23.3 μg chromium/day) and chromium chloride (200 μg chromium/day) on glucose tolerance, serum lipids and antidiabetic drug dosage in a 16-week randomized, double-blind, crossover trial that

included 78 patients with type 2 diabetes (217). Both forms of chromium supplementation decreased mean fasting plasma glucose, 2-h glucose and fructosamine (217). Chromium treatment also slightly reduced required doses of antidiabetic drugs mainly glibenclamide, a sulfonylurea (217). Treatment of 833 patients with type 2 diabetes with Cr picolinate (500 µg/day) for 10 months improved both fasting plasma glucose and postprandial plasma glucose versus baseline and reduced the occurrence of diabetic symptoms including fatigue, thirst and frequent urination (218).

Not all studies have demonstrated significant effects of chromium supplementation in patients with diabetes. Chromium supplementation of 250 µg/day for 7-16 months does not have any significant effect on serum glucose levels in 76 patients aged 42-83 years (25 of whom had type 2 diabetes) with atherosclerotic disease (219). These results agreed with another small-scale trial that indicated no significant actions of chromium supplementation (200 µg/day for 2 months) versus placebo on either blood glucose or HbA1c in 30 patients with type 2 diabetes.

There are a number of reasons for the variations between the studies dealing with the effects of chromium supplementation on glucose metabolism. First of all, the subjects included in the human studies were originated from diverse genetic and nutritional backgrounds and they lived in environments of varying degrees of stress, all of which may affect Cr metabolism. Other factors including the diet, selection of subjects, the duration of the study and the amount and type of supplemental Cr will cause varying results of supplemental Cr. 200 µg/day of supplemental Cr as Cr chloride did not account for any beneficial Cr effects in diabetic patients (220), this is in contrast to the use of 400 µg or more of Cr as Cr chloride that reported positive effects (221). The more

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bioavailable Cr picolinate compound have reported greater effects at 1000 µg/day than at 200 µg/day (222). In addition, response to Cr is related to the degree of glucose intolerance. Subjects with good glucose tolerance who do not need additional Cr do not respond to supplemental Cr (223). Subjects using sufficient Cr and well balanced diets also do not respond to additional Cr (224).

In addition to improvements in blood glucose and insulin due to supplemental Cr, at least 8 studies have showed the effectiveness of Cr supplementation in improving blood lipids. Such improvements are usually greatest in subjects with the highest blood lipids, but significant changes may take several months to appear. In the study of Abraham and colleagues, 6 to 16 months were needed to increase HDL cholesterol and decrease triglycerides after the use of 250 µg Cr as Cr chloride (219).

Chromium supplementation has also shown to be effective in improving glucose and insulin metabolism in women with gestational diabetes. Thirty women with gestational diabetes (20-24 gestational week) were divided into three groups and given 0, 4 or 8 µg of Cr per kg body weight as Cr picolinate for eight weeks (225). Chromium supplementation of women with gestational diabetes improved glucose intolerance and lowered hyperglycemia by decreasing fasting levels of glucose, insulin and C-peptide (225). The pregnant women who received 8 µg of Cr/kg body weight showed greater positive effects than those who received 4 µg of Cr/kg body weight (225). So, chromium picolinate supplementation may be a useful choice when the diet is not sufficient to achieve normoglycemia in women with gestational diabetes.

Glucocorticoid administration leads to insulin resistance in experimental animals (226) and humans (227). Steroid-induced diabetes is more evident in subjects who have

impaired glucose tolerance or diabetes prior to the glucocorticoid treatment. Since chromium is important in improving insulin sensitivity, it may be involved in the prevention and regulation of steroid-induced diabetes. Chromium supplementation of three patients with steroid-induced diabetes led to a reversal of the signs and symptoms of diabetes. To confirm these results, 50 patients with uncontrolled steroid-induced diabetes were supplemented with Cr (228). All of the patients had fasting blood glucose values greater than 13.9 mmol/L that did not improved following the use of hypoglycemic drugs and/or insulin therapy. The steroid-induced diabetes of 47 of the 50 patients was controlled by supplemental Cr, 200 μg of Cr as Cr picolinate (228). 5 patients were able to stop taking all forms of hypoglycemic medications and blood glucose remained normal by taking 200 μg of Cr daily (228).

XIX. Biological mechanisms of chromium action

In the 1980s, Wada, Yamamoto and co-workers reported the isolation and characterization of a unique chromium-binding oligopeptide named low-molecular weight chromium-binding substance (LMWCr) or chromodulin (229). The oligopeptide possesses a MWT of 1500 Da and is comprised of only 4 types of amino acid residues: glycine, cysteine, glutamate and aspartate (230). Chromodulin binds 4 equivalents of chromic ions. The oligopeptide appears widely distributed in mammals. It has been isolated and purified from rabbit liver (230), porcine kidney (231), bovine liver (232) and colostrums (233), dog liver (234), mouse and rat liver (235).

In the presence of chromium, chromodulin can enhance the ability of insulin to stimulate the metabolism of glucose by isolated rat adipocytes (230). In the absence of insulin, chromodulin does not affect the protein kinase activity of rat adipocytes but the

kinase activity was stimulated by 8-fold in the presence of insulin (236). Chromodulin is stored in its apo-form in the cytosol and nucleus of insulin-sensitive cells. Increases in plasma insulin concentrations have been found to result in a movement of chromium from the blood to insulin-dependent cells (237). This transfer is likely mediated by the metal transport protein transferring (238). Apochromodulin can sequester chromium in response to chromic ion flux. Binding of the newly generated holochromodulin to the insulin-stimulated insulin receptor will maintain its active conformation and amplify insulin signaling. As soon as the blood concentrations of insulin decrease and receptor signaling must be terminated, chromodulin needs to be eliminated from cells (237). The loss of chromodulin from cells is associated with an increase in urinary chromium concentrations after carbohydrate intake. The way by which apochromodulin is replaced is unidentified, but the oligopeptide may be synthesized as a proprotein which is modified post-translationally to give the oligopeptide.

Chromium also inhibits phosphotyrosine phosphatase (PTP-1), a rat homolog of a tyrosine phosphatase (PTP-1B) that inactivates the insulin receptor. The specific inhibition of insulin receptor phosphotyrosine phosphatase activity needs to be studied carefully since chromodulin has also been shown to activate a membrane phosphotyrosine phosphatase (239). Stimulation of insulin receptor kinase activity and the inhibition of insulin receptor tyrosine phosphatase by chromium would cause increased phosphorylation of the insulin receptor which is associated with increased insulin sensitivity.

Theories for explaining the mechanisms by which chromium participates in the function of insulin range from direct interaction of Cr with insulin to a role of Cr in

increasing insulin receptor number as well as increasing membrane fluidity and the rate of insulin internalization. Evans and colleagues demonstrated that chromium picolinate is the most lipophilic among a group of chromium-containing compounds. It has the greatest effect on membrane fluidity (240). They proposed that chromium complexes most likely increased membrane fluidity by becoming inserted within the hydrophobic core of the liposomal lipid bilayer, thus decreasing hydrogen bonding between adjacent fatty acid residues on the membrane phospholipids (240). Because of the lipophilicity of chromium picolinate, this compound may cause insulin internalization by increasing membrane fluidity for efficient removal of the insulin-receptor complex from the surface of the plasma membrane (240).

In an attempt to elucidate the role of chromium in enhancing insulin action, Chen and co-workers showed that chromium picolinate treatment recruits intracellular localized GLUT4 to a region juxtaposed to the cytoplasmic side of the plasma membrane (241). In the presence of insulin treatment, these subplasma membrane-localized transporters fuse to the plasma membrane and enhance insulin-stimulated glucose transport (241). Their data reveal that the beneficial action of chromium picolinate on GLUT4 redistribution is cholesterol dependent (241). The mechanism by which chromium picolinate decreases cholesterol is rough but may occur as a result of stimulation of 5′-AMP-activated kinase (AMPK). Activation of this enzyme has been shown to suppress the expression of a sterol regulatory element binding protein (SREBP-1). This protein is a member of a family of lipogenic transcription factors directly implicated in the expression of more than 30 genes required for the synthesis and uptake of cholesterol and other lipids.

It is possible that Cr action may involve the actin cytoskeleton. A role for actin in insulin-stimulated GLUT4 translocation has been implicated by several studies. Further studies are required to show if there is a relation between chromium action on GLUT4 trafficking and actin cytoskeleton dynamics.

XX. Chromium toxicity

Some studies of several cell cultures using supraphysiological doses proposed that chromium especially in the form of chromium picolinate may increase the probability of DNA damage. There is no evidence that chromium increases DNA damage *in vivo*. There have also been isolated reports on the ability of chromium picolinate treatment to induce kidney failure but the relationship of chromium to these events is not clear. The Institute of Medicine have concluded that chromium picolinate is safe. Results from clinical trials have revealed that chromium supplementation at doses up to 1000 µg/day and for periods as long as 64 months does not have any toxic effects (200).

Goals of this study

In this study, we will examine the effects of vanadium and chromium-containing compounds on membrane fluidity and their ability to cause translocation of the insulin receptor into lipid rafts. Biophysical techniques will be the primary methods used in this project. Some transition metals appear to have some activity that mimics the action of insulin on certain cells. Their effects may be mediated through their actions on the plasma membrane and the organization of proteins and lipids within the lipid bilayer. These interactions could perturb the membrane lipid organization and facilitate the translocation and confinement of insulin receptor into membrane microdomains that serve as signaling platforms and, because of that, they may enhance insulin-mediated

effects and reduce insulin resistance. Introducing such compounds that normalize blood glucose and lipid levels and reduce the risk of developing cardiovascular problems associated with diabetes would be of great pharmacologic interest.

CHAPTER 2

Insulin receptors translocate to plasma membrane microdomains following binding of insulin or exposure to insulin-mimicking compounds

ABSTRACT

Signal transduction of insulin receptors can be mediated by translocation of these receptors into membrane structures that are characterized by insolubility in Triton X-100 and low density in sucrose gradients. In our study, we describe the translocation of insulin receptors from the bulk membrane into membrane rafts following treatment of 2H3 cells expressing insulin receptors with insulin and/or insulin-enhancing compounds. Receptor translocation to lipid rafts was reduced when cells were pretreated with 1% MβCD which reduces membrane cholesterol and disrupts rafts. The fold increase in the number of phosphorylated insulin receptors within raft fragments was high following exposure of 2H3 cells to insulin and/or insulin-mimicking compounds, this increase was compared to untreated cells. Disruption of rafts by MβCD reduces the appearance of phosphorylated insulin receptors in these membrane compartments in response to insulin. There was a significant increase in the number of phosphorylated IRS-1 molecules within lipid rafts under the effects of insulin, BMOV or Cr(pic)$_3$, which may indicate the importance of these structures in harbouring some of the signaling molecules involved in insulin action. Single particle tracking of individual insulin receptors showed that these receptors become confined in small compartments when exposed to insulin and/or

BMOV or Cr(pic)$_3$, significantly smaller than the regions accessed by the untreated receptors. Finally, BMOV or Cr(pic)$_3$ cause an increase in membrane fluidity, one way by which these compounds can elicit translocation of insulin receptors into rafts where signaling molecules are concentrated.

INTRODUCTION

One level of spatial organization proposed for the plasma membrane is that of the liquid –ordered phase. This phase is enriched in cholesterol and lipids with saturated acyl chains, such as sphingolipids and glyco-sphingolipids but is relatively depleted of other phospholipids. Simons and Ikonen proposed a model to describe lipids in the liquid-ordered phase (242). According to this model, lipids pack together to form dynamic "rafts" in the plasma membrane and certain molecules, including signaling proteins, are recruited to these domains (242). The segregation of signaling proteins into lipid rafts is essential for signal transduction in a variety of cell types because of the accessibility of those proteins to regulatory or effector molecules. Several lines of evidence suggest the importance of lipid rafts in compartmentalization of insulin signaling in adipocytes (116). Direct interaction between resident raft proteins (caveolins and flotillin-1) and insulin-signaling molecules may cause the organization of these molecules in proper space and time to ensure transduction of the insulin signal. Two questions raised by these observations are whether insulin function is dependent on receptor translocation into rafts and whether these structures are sites for insulin receptor phosphorylation.

In recent years, several metals like vanadium and chromium have been found to exert insulin-like effects. Vanadium compounds have been demonstrated to improve insulin sensitivity and glucose homeostasis in animal models of type 1 (135) and 2 diabetes mellitus (136) as well as in small number of diabetic human subjects (144).

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Several potential theories have been suggested to explain the molecular mechanism responsible for the insulin-like effects of vanadium compounds. One proposed mechanism is that vanadium induces the activation of several components of the insulin-signaling pathways such as ERK1/2 (177), P38MAPK, and PI3-K/PKB (173-175). Because of the importance of these pathways as mediators of the insulin response, it may be suggested that vanadium-induced activation of these signaling molecules contributes to its insulin-like action. In view of these facts, an important question here is whether translocation of insulin receptors into lipid rafts induced by vanadium represents another mechanism by which vanadium compounds elicit their insulin-like effects.

Chromium has been demonstrated to inhibit phosphotyrosine phosphatase and activate insulin receptor kinase leading to increased phosphorylation of the insulin receptor (236) and increased insulin sensitivity. In addition, it has been suggested that chromium enhances insulin binding, insulin receptor number (243), insulin internalization (241), and β-cell sensitivity. As with vanadium compounds, it is of interest to know whether chromium compounds affect translocation of insulin receptors into lipid rafts.

To address these questions, we have isolated membrane fragments from insulin and/or BMOV or Cr(pic)$_3$ 2H3-treated cells expressing insulin receptors. To further demonstrate the localization of functional insulin receptors within rafts, we have treated cells with MβCD which efficiently removes cholesterol from the plasma membrane of live cells (85,86) and thus disrupts raft structure. To assess the importance of raft fragments in mediating the phosphorylation of insulin receptors in response to insulin and/or insulin-enhancing compounds, we evaluated the relative number of

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phosphorylated insulin receptors within these fragments using western blotting techniques and anti-phospho IR antibody. A discontinuous sucrose gradient and ultracentrifugation methods were used to separate raft fragments from the bulk membrane. Then we examined whether disruption of membrane rafts is accompanied by altered phosphorylation of insulin receptors within these compartments in response to insulin. In an attempt to elucidate the biological significance of lipid rafts in mediating insulin receptor signaling cascade, we studied the effects of insulin, BMOV, or $Cr(pic)_3$ on phosphorylation of IRS-1 within these membrane fragments.

To independently examine the effects of insulin and/or insulin-mimicking compounds on insulin receptor motion within the plasma membrane, we have used single-particle tracking methods to evaluate the size of membrane compartments accessed by individual receptors as visualized microscopically on viable cells (85).

Finally, the effects of BMOV or $Cr(pic)_3$ on membrane fluidity was examined. Changing lipid packing and organization within the membrane may be a possible explanation of how these compounds can enhance the shift of insulin receptors to low-density, high-buoyancy membrane fragments that represent lipid rafts. Such translocation of insulin receptors could be necessary to recruit downstream signaling molecules needed to enhance insulin action.

MATERIALS AND METHODS

Materials

Minimum Essential Medium (MEM) with Earle's Balanced Salts was purchased from Thermo Scientific (Logan, Utah). Fetal bovine serum (FBS) was purchased from Gemini BioProducts (Woodland, CA). Insulin from bovine pancreas and anti-phospho-insulin receptor (PTyr^{972}) antibody were purchased from Sigma-Aldrich (St. Louis, MO) as well as methyl-β-cyclodextrin. Insulin Rβ (C-19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit IgG, horseradish peroxidase conjugated was purchased from Pierce Biotechnology (Rockford, IL). Dr. Debbie Crans from Colorado State University kindly provided us with bis-maltolatooxovanadium (BMOV) and tris(2-pyridinecarbxylato)chromium(III) (Cr(pic)$_3$) compounds. Colloidal gold(40 nm) was purchased from TedPella,Inc (Redding, CA). Aminonaphthylethenylpyridinum (di-4-ANEPPDHQ) dye as well as Qdot605-streptavidin were purchased from Invitrogen (Carlsbad, CA). Insulin receptor β-biotin IgG and p-IRS-1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines

To test whether the rat wild type insulin receptor becomes associated with membrane rafts following binding of ligand, rat basophilic leukemia-2H3 cells (RBL-2H3) were maintained in cell medium that included MEM supplemented with Earle's Balanced Salts and containing FBS, 200 mM L-glutamine, 10,000 U/ml penicillin G, 10

mg/ml streptomycin and 25 μg/ml fungizone (Gemini BioProducts (Woodland, CA). In some experiments, cells were serum-starved for at least 16 hours to remove a source of insulin from the cell media. In these experiments, the MEM was supplemented as described with the exception of FBS.

Isolation of plasma membrane rafts

2H3 cells were incubated with 200nM insulin for 1 hour at 37°C and/or with 10 μM BMOV or 10 μM Cr(pic)$_3$ for overnight or 1 hour at 37°C. To isolate membrane rafts from 2H3 cells, $5x10^7$ cells were washed two times with phosphate-buffered saline, pH 7.2 (PBS) and lysed for 5-10 minutes on ice in 1 ml of a buffer containing 25 mM MES, 150 mM NaCl, 2mM EDTA, 0.25% Triton-X100, and protease inhibitors including aprotinin, leupeptin, EDTA, and PMSF (Roche). A low speed 300x g spin was used to remove cell nuclei and large cell debris. 1 ml of the supernatant from this spin, which contained plasma membrane fragments, was then combined with 1 ml of 80% sucrose containing 0.25% Triton-X100 and protease inhibitors to produce a 40% sucrose solution. A discontinuous sucrose gradient from 10-80% was created with the sample in 40% sucrose layered within this gradient. The gradient was loaded into a Beckman SW-41 swinging bucket rotor and spun at 175,000x g for 20 hours at 4°C. After the spin, eighteen 650 μl fractions were carefully collected from the top of the gradient downward. A 50 μl aliquot from each fraction was diluted 1:1 with 95% SDS and 5% β-mercaptoethanol. After separation of proteins from each fraction using SDS-PAGE and transfer of proteins to nitrocellulose, the insulin receptor was identified using 150 μl of 200 μg/ml insulin Rβ polyclonal IgG antibody (Santa Cruz Biotechnology, Santa Cruz,

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CA). In experiments evaluating raft distributions of phosphorylated IR and phosphorylated IRS-1 under the effects of insulin and/or insulin-mimicking compounds, 50 µl aliquots from fractions 7-12 were diluted 1:1 with 95% SDS and 5% β-mercaptoethanol and, after separation of proteins from each fraction using SDS-PAGE and transfer of proteins to nitrocellulose, the phosphorylated insulin receptor was identified using 10 µl of anti-phospho-insulin-receptor (Sigma-Aldrich, St. Louis, MO) and the phosphorylated IRS-1 was determined using 75 µl of anti-p-IRS-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The amount of receptor in each fraction was determined using a Bio-Rad GS-800 calibrated densitometer. The sucrose concentration in each fraction was determined using a Bausch and Lomb refractometer.

In some experiments, cells were pretreated for 1 hour at 37°C with 10 mM methyl-β-cyclodextrin (MβCD) in serum-free MEM prior to incubation with insulin or with 200 µg/ml insulin Rβ polyclonal IgG antibody for 45 min at room temperature followed by a second 45 min incubation with 0.8 mg/ml anti-rabbit IgG (Pierce Biotechnology). MβCD neither binds nor inserts in the plasma membrane of cells but rather extracts cholesterol by including it in a central non-polar cavity of cyclic oligomers of glucopyranoside in α-1,4-glycosidic linkages (244). At this concentration, it was non-toxic to cells and did not compromise cell integrity (111).

Single-particle tracking of insulin receptors on individual cells using gold particles

Lateral dynamics and the size of domains accessed by individual insulin receptors were evaluated using single-particle tracking methods as described by Kusumi and coworkers (85). For studies using 40 nm nanogold particles, the particles were

conjugated with the lowest possible concentration of insulin Rα antibody (Santa Cruz Biotechnology, Santa Cruz, CA) needed to stabilize the gold solution and were then incubated with 2H3 cells expressing insulin receptors. The antibody-gold concentration, typically 32μg/ml, was then further reduced by addition of 1% BSA in PBS until there were approximately 1-4 gold particles per cell. This binding was specific for insulin receptors; when cells were preincubated with a 10-fold excess of insulin Rα antibody, no antibody-gold particles were detected on cells. In some experiments, cells were treated with 200nM insulin for 30 minutes after labeling of insulin receptor with insulin Rα antibody or with 10 μM Cr(pic)$_3$ for overnight or for 1 hour before labeling of insulin receptor with insulin Rα antibody. In new set of experiments, 2H3 cells were pretreated with 1% MβCD for 1 hour prior to labeling with anti insulin receptor antibody.

Individual nanoparticles were imaged by differential interference contrast with a 1.4 N.A. 63x objective in a Zeiss Axiovert 135 microscope. Images were acquired using a Dage IFG-300 camera and were recorded for two minutes (3600 frames) at approximately 30 nm/pixel under the control of Metamorph software from Universal Imaging. The trajectories for individual gold particles were segmented into domains by calculation of statistical variance in particle position over times using a procedure similar to that previously reported (104,245). The variance of a particle's position can be calculated within windows of varying duration. These windows are translated along the particle trajectory, producing a variance plot that exhibits peaks that indicate inter-domain boundaries. These results can be analyzed to yield the domain size and residence time for each particle. Effective macroscopic diffusion constants were calculated as the

square of the domain diagonal divided by four times the residence time in the domain as previously described (246).

In experiments using quantum dots to track insulin receptors, cells were labeled first with insulin receptor β-biotin IgG and then with quantum dot Qdot605 that was already attached to streptavidin. Wide-field fluorescence images were collected from the apical cell surface on a Zeiss Axiovert 200M using a 63x W 1.2 NA objective and Cy3 filter set. Images were collected every 33 or 100ms for up to 5 minutes at a final magnification of 315x onto an Andor Ixon EM camera resulting in a final pixel size of 49.6nm. Macroscopic diffusion rate and compartment sizes were determined using custom analysis programs.

Effects of vanadium and chromium-containing compounds on membrane fluidity

To study the effects of vanadium and chromium-containing compounds on membrane fluidity, we used di-4-ANEPPDHQ dye. This dye was dissolved in 10% DMSO in distilled water to produce a stock solution of 300μM in concentration. 2H3 cells were seeded onto sterile culture dishes to produce 50% confluence. The cells are either untreated, treated with 200nM insulin for 30 minutes, or with 10μM BMOV for at least 16 hours, or treated with different concentrations of Cr(pic)$_3$ for either 1 hour or at least 16 hours. 2H3 cells were then labeled with 3μM di-4-ANEPPDHQ in PBS buffer for 1 hour at room temperature. Cell images were collected simultaneously in two channels (long-pass 660nm channel 1 and long-pass 510nm channel 2) using an Olympus microscope 1x-70 equipped with a Fluoview confocal scanning unit.

Background correction, fluorescence intensity ratio calculation and red/green overlay creation were performed using the Image J program (rsbweb.nih.gov/ij).

RESULTS

Insulin receptors appear in membrane rafts following insulin binding

After isopycnic centrifugation of plasma membrane fractions from 2H3 cells expressing insulin receptors, unoccupied insulin receptors were found in sucrose fractions with relatively high densities (Figure 7). Immunoblots of insulin receptors identified by insulin-receptor specific antibody were developed as previously described (247) and the relative amount of insulin receptors in each fraction was quantified using densitometry. Over 80% of insulin receptors were localized in sucrose fractions 11-18 where sucrose concentration ranged from approximately 43-80%. Following treatment of cells with 200nM insulin for 1 hour, there was a change in the distribution of receptors to lower density sucrose fractions. Over 40% of insulin treated insulin receptors consistently appeared in fractions 1-10 which contained 10-39% sucrose.

Insulin receptors appear in membrane rafts following BMOV treatment

Following overnight treatment of cells with 10μM BMOV, there was a marked change in the distribution of receptors to lower density sucrose fractions. Approximately 60% of insulin receptors appeared in fractions 1-10 which contained 10-39% sucrose (Figure 7). A similar change in the distribution of insulin receptors between non-raft and raft membrane fragments occurred when cells were treated with 10 μM BMOV for 1 hour (Figure 8). When 2H3 cells were treated with both 200nM insulin for 1 hour and 10 μM

BMOV for at least 16 hours, over 50% of the receptors were found in low-density, high buoyancy membrane fractions (Figure7).

Insulin receptors appear in membrane rafts following Cr(pic)₃ treatment

Following overnight treatment of cells with 10μM Cr(pic)$_3$, there was a marked redistribution of receptors and an increase in insulin receptors in lower density sucrose fractions. Over 60% of insulin receptors appeared in fractions 1-10 which contained 10-39% sucrose (Figure 9). A similar change in insulin receptor distribution occurred when cells were treated with 10 μM Cr(pic)$_3$ for 1 hour (Figure 10) and when 2H3 cells were treated sequentially with 200nM insulin for 1 hour and 10 μM Cr(pic)$_3$ for at least 16 hours (Figure 9).

Deleting membrane cholesterol disrupts membrane rafts and decreases the number of insulin receptors in lipid rafts

Preincubation of cells for 1 hour with 1% MβCD, a cholesterol sequestering agent, reduced the number of insulin receptors appearing in high buoyancy membrane fractions. Over 75% of untreated insulin receptors were found in fractions 10-12 following exposure of cells to 1% MβCD (Figure 11) and more than 60% of insulin receptors were found in fractions 10-12 after treatment of 2H3 cells with 250nM insulin for 30 minutes and with 1% MβCD for 1 hour (Figure 11).

Phosphorylation of insulin receptors in lipid rafts following binding of insulin and/or insulin mimicking compounds

After isopycnic centrifugation of plasma membrane fractions from 2H3 cells expressing insulin receptors, the overall distribution of phosphorylated insulin receptors in raft fragments (lanes 7-9) is nearly the same as the relative number of insulin receptors in non raft fragments (lanes 10-12) following treatment of the cells with 200nM insulin for 1 hour and/or 10μM BMOV for at least 16 hours (Figure 12), with 200nM insulin for 1 hour and/or 10μM Cr(pic)$_3$ for at least 16 hours (Figure 13). Representative western blots obtained from cell samples separated on sucrose density gradients demonstrate the same results (Figure 14). However, the fold increase in the number of phosphorylated insulin receptors was high in raft fragments compared to untreated cells following treatment of 2H3 cells with insulin and/or BMOV or Cr(pic)$_3$. These results are summarized in Table 1. Preincubation of cells for 1 hour with 1% MβCD disrupted membrane rafts containing insulin receptors and, as a result, over 75% of phosphorylated insulin receptors treated with 50nM insulin were found in fractions 10-12 following exposure of cells to 1% MβCD. More than 50% of those receptors treated with 250nM insulin were found in fractions 10-12 following exposure of cells to 1% MβCD (Figure 15).

IRS-1 phosphorylation occur within lipid rafts following insulin treatment

Treating 2H3 cells with 200nM insulin for 30 minutes was accompanied by a marked increase in IRS-1 phosphorylation within low-density, high buoyancy membrane fractions. More than 60% of phosphorylated IRS-1 molecules were found in these

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membrane fragments following treatment of 2H3 cells with the hormone (Figure 16). MβCD treatment will disrupt membrane rafts and because of that fewer phosphorylated IRS-1 molecules will appear in rafts following such treatment. Figure 16 indicated that only 9% of phosphorylated IRS-1 molecules were in raft fragments when 2H3 cells were treated with 1% MβCD for 1 hour.

Phosphorylation of IRS-1 in lipid rafts following exposure of 2H3 cells to insulin mimicking compounds

BMOV and Cr(pic)$_3$ caused a significant increase in IRS-1 phosphorylation in raft fragments. Our data showed that over 85% of phosphorylated IRS-1 molecules were found in lipid rafts when 2H3 cells were exposed to 10μM BMOV for 1 hour (Figure 17). Cr(pic)$_3$ treatment of 2H3 cells was also shown to cause an increase in the number of phosphorylated IRS-1 molecules in rafts in which more than 55% of these molecules were found in rafts following Cr(pic)$_3$ treatment (Figure 17).

Single particle tracking of insulin-occupied insulin receptors demonstrates " trapping" of insulin receptors in small membrane compartments

To independently asses the localization of insulin-treated insulin receptors in membrane compartments, single particle tracking methods were used. This technique identifies individual insulin receptors on the surface of viable cells and tracks their motions over approximately two minutes. The centroid for a 40nm gold particle attached to an individual receptor can be identified visually on video obtained from each experiment and its motions can be quantitatively described. Figure 18 shows a

representative particle tracks for an untreated insulin receptor and for an insulin receptor on cells treated with insulin and demonstrates that these receptors accessed different membrane areas in the presence and absence of insulin. Insulin treatment reduced the size of compartments containing insulin receptors from 216 ± 23 to 113 ± 16 nm (Table 2) while the average residence time for receptors within microdomains and the number of domains, 6 ± 2 and 8 ± 2, did not differ for untreated and insulin-treated cells, respectively. Nonetheless, the rate of receptor diffusion (D) within each domain was reduced following insulin treatment. Figure 19 shows these results for individual receptors on untreated or insulin-treated cells; as receptors access larger membrane microdomains, their rate of diffusion within these microdomains increases. Single particle tracking of insulin receptors also demonstrates confinement of these receptors in small membrane compartments after treating 2H3 cells with $10\mu M$ $Cr(pic)_3$ for 1 hour (Figure 18) or at least 16 hours (Table 2). Under both conditions more than 80% of receptors appear in compartments with diameters of less than 75nm (Figure 20).

Because MβCD pre-treatment largely reversed the effects of insulin on compartment size and receptor lateral diffusion, it seems plausible that compartments retaining insulin-treated receptors are rafts. As shown in table 2, MβCD pre-treatment results in significant increase in the size of membrane compartments containing insulin receptors. The size of these compartments does not differ significantly from those accessed by untreated insulin receptors and, together with results from sucrose density gradient centrifugation experiments, suggests that these unbound receptors are residing in the bulk membrane rather than rafts.

When quantum dots were used to track the movement of individual insulin receptors on 2H3 cells, the domain size was reduced from 472 ± 9 to 419 ± 6 nm after treating cells with 10μM BMOV for 2 or 12 hours as shown in Figures 21 and 22. An individual receptor's rate of diffusion (D) within each domain was also reduced (Figure 23). When cells were treated with BMOV at various concentrations for at least 16 hours, insulin receptor diffusion was generally reduced as was the size of compartments accessed by the receptor although to different extents as shown in Table 3.

Cr(pic)₃ and BMOV treatments decrease lipid order in the plasma membrane

The relative extent of lipid ordering was evaluated in cells treated with either BMOV, Cr(pic)₃ or with metal-containing compounds and insulin. Insulin treatment alone had no effect on the ratio of fluorescence emission compared to untreated cells and suggests that insulin does not alter lipid ordering. In contrast, Cr(pic)₃ treatment increased the fluorescence emission ratio from 0.7 for untreated cells to 1.1 (Figure 24). This is shown visually in Figure 25 as an overlay of red and green confocal fluorescence images. These results indicate that Cr(pic)₃ alone increases plasma lipid disorder and thus increases membrane fluidity. The effect of Cr(pic)₃ treatment was compared to that of MβCD which sequesters membrane cholesterol, as a result, increases lipid disorder. . Figure 24 indicates that preincubation of 2H3 cells for 1 hour with MβCD, like Cr(pic)₃ treatment, increases the ratio of fluorescence emission from 0.7 for the basal line to 1.3.

To determine whether lipid effects of Cr(pic)₃ are dependent on concentration, 2H3 cells were treated with Cr(pic)₃ at concentrations ranging from 10μM to10nM. As shown

in Figure 26, 100nM Cr(pic)$_3$ had no effect on the ratio of red/green fluorescence emission while higher concentrations increased this ratio.

Similar measurements using 2H3 cells treated with 10μM BMOV were obtained. The ratio of fluorescence emission was increased from 0.7 for untreated cells to 0.94 with BMOV treatment and the magnitude of this increase was similar to the effect of 1% MβCD for 30 minutes (Figure 27). The overlay of red/green fluorescence of confocal images was shown in Figure 28. Treating cells with cholesterol for 1-2 hours decreases the ratio of fluorescence emission to 0.6 (Figure 27). By reloading cells with cholesterol, lipid order increases and, in these experiments is not statistically different from lipid order in untreated membranes.

DISCUSSION AND FUTURE DIRECTIONS

Our results demonstrate that insulin receptors, either occupied by insulin or exposed to insulin-enhancing compounds, translocate from the bulk membrane into small nm-diameter plasma membrane compartments with characteristics of so-called rafts. Moreover, the regions in which hormone-treated insulin receptors are confined are small. These results are similar to those of Vainio and coworkers (121) that studied Triton X-100 insolubility of insulin receptor at 4°C. In their study, insulin-stimulated and basal cells were lysed in 0.1% Triton X-100 on ice and the lysates were subjected to Optiprep gradient fractionation in the presence of the detergent. By western blot analysis, this group found that in hepatoma cells insulin receptors can partition into detergent-resistant domains and this association is dependent on ligand binding.

It is likely that insulin receptors preferentially segregate into caveolae, a subset of lipid rafts, due to receptor interactions with caveolin, a protein marker for caveolea. These interactions involve the caveolin scaffolding domain and hydrophobic motifs in the caveolin-interacting proteins. 2 related caveolin-binding motifs have been elucidated (248). $\Phi XXXX\Phi XX\Phi$ and $\Phi X\Phi XXXX\Phi$ (where Φ is an aromatic amino acid) exist within most caveolae-associated proteins like most Gα subunits and the kinase domains of many distinct families of tyrosine and serine/threonine protein kinases including; MAP kinase, EGF, PDGF and insulin receptors (248). In insulin receptor, this motif exists in the kinase domain of the beta-subunit (WSFGVVLW, amino acid residues 1193-1200).

It appears that membrane rafts may serve as a signaling platforms for insulin receptors although the specific signaling event(s) are uncertain. Spatial coordination of key signaling proteins in lipid rafts may provide a rapid, efficient and specific mechanism for promoting signal transduction from extracellular to intracellular mechanisms while also preventing cross-talk between pathways (249, 250). Lipid rafts and caveolae also play an essential role in insulin stimulated glucose transport. GLUT4 is induced to translocate to caveolae in response to insulin (122). GLUT4 labeling was largely found in the same membranes as those labeled for caveolin. They biochemically isolated caveolar fraction from purified plasma membranes of freshly isolated rat adipocytes. Electron microscopic examination of the caveolae fractions confirmed that 50-70% of the membrane fragments were labeled with antibodies against caveolin. In addition to that, translocation of GLUT4 to the membrane, and thus glucose entry into target cells, can be mediated by localization of CAP-Cbl complex to lipid raft (44). This complex is recruited to lipid rafts by flotillin-1 which is a resident lipid raft protein. Appearance of CAP-Cbl complex in lipid raft will create a pathway that stimulates TC10 that is known to regulate actin filaments in many cell types (251). Modulation of cytoskeletal function can enhance translocation of GLUT4 from inside stores to the surface of cell.

If rafts play a major role in concentrating some proteins needed for signal transduction, then cholesterol deletion and raft disruption may disperse raft-associated proteins and reduce the likelihood of protein-protein interactions necessary for cell signaling. Indeed, MβCD treatment reduced FRET between GPI-anchored proteins (87) and signal transduction by these proteins (252). Other studies have shown that cholesterol is important for lipid raft formation and that its depletion decreases signal

transduction efficiency (111, 253). In addition, insulin-stimulated glucose uptake, down stream tyrosine phosphorylation of IRS-1 and protein kinase B, and insulin-dependent binding of IRS-1 to insulin receptor were inhibited when 3T3-L1 adipocytes were incubated with MβCD for 50 minutes (124).

Our data show that the fold increase in the number of phosphorylated insulin receptors within raft fragments was high when these receptors were treated with insulin or exposed to BMOV or Cr(pic)₃ as compared to untreated receptors. Because vanadium compounds are potent inhibitors of protein tyrosine phosphatases, these compounds can activate insulin receptor protein tyrosine kinase by preventing dephosphorylation of IR-β subunit. It was shown that vanadate stimulated tyrosine phosphorylation of the IR-β subunit in rat adipocytes (164) and sodium orthovanadate was able to reverse the decreased tyrosine phosphorylation of the IR-β subunit in STZ diabetic rats (165). Similarly, insulino-mimetic organo-vanadium compound BMOV was also found to increase the tyrosine phosphorylation of IR-β subunit (169). However, exposure of adipocytes (254) or IR-overexpressing Chinese hamster ovary cells (255,256) to NaOV or vanadyl sulfate did not cause any effect on IR-β subunit phosphorylation.

IRS-1 is one of the most important molecules involved in insulin receptor signaling pathway. We have investigated the possibility of the presence of phosphorylated IRS-1 within lipid raft fragments under the effects of insulin and/or insulin mimicking compounds. We found that high numbers of phosphorylated IRS-1 molecules were present in raft fragments following treating 2H3 cells with insulin, BMOV or Cr(pic)₃. These data may strengthen the idea of the biological importance of lipid rafts in mediating insulin signaling.

In agreement with what we get, several reports have indicated the potential importance of some vanadium salts in inducing IRS-1 phosphorylation. Pandey and coworkers (167) showed that VS and NaOV have a stimulatory action on IRS-1 phosphorylation.

Stimulation of insulin receptor kinase activity and the inhibition of insulin receptor tyrosine phosphatase by chromium would cause increased phosphorylation of insulin receptor. Wang and coworkers (257) found that insulin-stimulated tyrosine phosphorylation of the human insulin receptor in CHO-IR cells was enhanced following Cr(His)$_3$ treatment. Using immuno-blotting, they also reported that cellular chromium increased the insulin-stimulated tyrosine phosphorylation of IRS-1 at submaximal doses of insulin (257). This is in agreement with reports indicating that membrane-associated GLUT4, PI3K activity and Akt phosphorylation were increased following exposure of JCR-LA rats (a model for insulin resistance) (258, 259) or human subjects with type 2 diabetes (260) to chromium.

Disruption of membrane rafts reduced translocation of phosphorylated insulin receptors into these membrane compartments. This result is reasonable if hormone-mediated signaling is most efficient within rafts where higher concentrations of downstream signaling molecules exist. Nonetheless, some signaling proteins remain available within the bulk membrane, albeit at reduced concentrations, where they remain capable of relaying a productive signal in response to insulin binding. Treating human hepatoma cells with MβCD for 15 minutes at 37°C resulted in 50% depletion of cellular cholesterol (121). Extraction of cholesterol caused a reduction in insulin receptor

phosphorylation by $\approx 60\%$ (121). Insulin-dependent tyrosine phosphorylation of IRS-1 was also impaired by $\approx 50\%$.

Single particle tracking studies provide some insight into the nature of insulin receptor-containing structures. Upon binding ligand or exposed to insulin-enhancing compounds, the insulin receptors become largely confined within small compartments. For the most part, the receptor remains within these regions for comparatively long times and appear to diffuse pseudo-randomly before being captured within another compartment of similar size. Similar behavior has been described and analyzed by Kusumi and coworkers (105) for selected phospholipids and for transferrin receptor (261) and by Daumas et al.(245) for the μ opioid receptor, a G protein-coupled receptor involved in pain responses. Daumas suggest that confinement of μ opioid receptor within a specific domain results from interaction of the receptor with the confining molecules. Alternatively, Ritchie and colleagues (262) proposed that these interactions may be with proteins forming a continuous barrier (fences) or discontinuous protein barrier (pickets). Fences or pickets allow confinement of receptor diffusion within small membrane compartments and, at the same time, permit intermittent escape from a compartment followed by faster diffusion in the bulk membrane. By using fluorescence photobleaching recovery methods, Roess et al. (263) suggested that actin filament dynamics can provide fences or organizing structures for pickets that confine the lateral diffusion of LH receptors. One should interpret these results carefully, however, because the compartments identified in signal particle tracking studies may be different from structures identified in biochemical studies such as plasma membrane rafts. The

relationship of membrane compartments visualized via single-particle tracking to biochemically-identified rafts remains a complicated issue.

One of the possible ways by which insulin-mimicking compounds can induce translocation of insulin receptors into raft fragments is through changes in membrane fluidity. Our data indicate that treating 2H3 cells with Cr(pic)$_3$ increases membrane fluidity. Chromium effects may be mediated through its effects on the plasma membrane which, in turn, causes redistributions of both proteins and lipids within the lipid bilayer. These interactions could perturb the membrane lipid organization and favor the translocation of insulin receptor into membrane microdomains and retention in those compartments. This agrees with what was obtained by Evans and coworkers (241). They demonstrated that anisotropy (r), the inverse of membrane fluidity, was decreased when either chromium picolinate or chromium nicotinate was added to synthetic liposomal membranes. This group (241) suggested that chromium complexes increased membrane fluidity by becoming inserted within the hydrophobic core of the liposomal lipid bilayer. As a result, the hydrogen bonding between adjacent fatty acid residues on the membrane phospholipids will be diminished leading to a decrease in membrane rigidity.

Changes in lipid packing in plasma membranes in the presence of vanadium-containing compounds are consistent with the possibility that membrane fluidity is influenced by these compounds. Our data show that lipid fluidity of 2H3 cells is increased following treatment with BMOV. Moreoever, disrupting the integrity of lipid rafts by extracting cholesterol from membranes using MβCD causes an increase in lipid disorder and thus lipid fluidity as indicated by our data. Reloading 2H3 cells with cholesterol restores normal membrane fluidity value. Our results differ from those of

Yang et al.(264). They evaluated lipid fluidity using erythrocyte membranes treated with various vanadium-containing compounds and demonstrated that, to varying degrees, several compounds including $NaVO_3$, $VO(acac)_2$, BMOV, and $[VO_2dipic]^-$, decrease membrane fluidity and increase lipid order in erythrocyte membranes.

There are, however, a number of questions that should be addressed in the near future. First, we would like to establish whether small membrane compartments accessed by insulin-occupied receptors are the same structures isolated in low density sucrose fractions and whether rafts are essential for insulin receptor function. These questions seem likely to be resolved only if it can be demonstrated that forcing insulin receptor, either with or without ligand, into the raft environment, can produce a downstream signal and if some of the factors participating in insulin receptor mediated signaling can be localized with insulin receptors in the same membrane compartments.

Second, we have not examined the effects of re-loading cholesterol into membranes following cholesterol depletion in single particle tracking experiments. Presumably, the addition of cholesterol to membrane would cause re-formation of membrane compartments. Re-formation of these compartments can reconstitute effective signaling in response to hormone. These studies would support the notion that membrane compartments are essential for insulin receptor-mediated signaling.

Third, an evaluation of fluorescence resonance energy transfer between insulin receptors and GM1, a membrane raft component, would further strengthen the hypothesis that insulin receptors are found in membrane rafts during signaling. We would anticipate that energy transfer will not occur between insulin receptor and GM1 before binding of insulin receptor to its ligand. Only upon binding insulin, or exposure to insulin-

mimicking compounds would energy transfer be seen. This FRET would be eliminated by MβCD treatment and raft disruption.

Finally, it has not been shown conclusively that lipid rafts exist on viable cells. Although few researchers believe that they are artifacts, these structures may in fact be very short lived and structurally different from the biochemically isolated structures examined in this project. Along these same lines, it has not been conclusively shown that lipid rafts serve as organizing structures of proteins and lipids involved in signal transduction. Although it is attractive to think that gathering proteins involved in cellular signal transduction facilitates and organizes signal transduction, the role of lipid rafts in this process remains a topic of active debate.

Table 1: Fold increase in phosphorylated insulin receptors/untreated cells

Treatment	7	8	9	10	11	12
Insulin(200nM)	3	21	73	>100	>100	>100
BMOV (10μM)	1.1	2.2	3	3	>100	>100
BMOV/I	2	4	1	34	>100	>100
Cr(pic)$_3$(10μM)	37	1	1	4	>100	>100
Cr(pic)$_3$/I	16	>100	>100	>100	>100	>100

Table 2: Tracking of individual insulin receptors before and after treating 2H3 cells with insulin and/or Cr(pic)$_3$

Treatment(s)	# of particles analyzed	Number of domains/ 2 min trajectory	Domain diameter (nm)	D (macroscopic diffusion coefficient) (10^{-11} cm2/sec)	Residence time (seconds)	D (microscopic diffusion coefficient) (10^{-11} cm2/sec)
None	20	6±2	216±23	1±0.4	21±4	2.4±0.4
200 nM insulin (30 minutes)	20	8±2	113±16	0.5 ±0.2	15±3	4±0.7
10 μM Cr(pic)3 (overnight)	20	8±2	119±19	0.6 ±0.3	15±3	3±0.5
10μM Cr(pic)3 (1h)	20	8±2	81±16	0.4 ±0.3	16±4	1±0.1
10μM Cr(pic)3 /200nMinsulin (30 minutes)	20	8±2	98±16	0.4 ±0.2	15±3	2±0.2
1% MBCD (1 hour)	10	7±2	253±35	2±0.6	16±3	2±0.1

Table 3: Both compartment size and diffusion rate within these compartments for insulin receptors were reduced in response to insulin or BMOV with different concentrations. These values were comparable to those obtained for unoccupied receptors.

Treatment	Diffusion Coefficient	Compartment size (nm)
No treatment	$10 \pm 0.5 \times 10^{-11}$ cm2sec-1	471 ± 9
50 nM insulin	$3 \pm 0.7 \times 10^{-11}$ cm2sec-1	239 ± 22
250 nM insulin	$2.7 \pm 0.2 \times 10^{-11}$ cm2sec-1	266 ± 11
0.1 µM BMOV	$7 \pm 0.4 \times 10^{-11}$ cm2sec-1	383 ± 9
1.0 µM BMOV	$6 \pm 0.3 \times 10^{-11}$ cm2sec-1	404 ± 8
10 µM BMOV	$6 \pm 0.2 \times 10^{-11}$ cm2sec-1	419 ± 6
100 µM BMOV	$6 \pm 0.2 \times 10^{-11}$ cm2sec-1	418 ± 7

Figure 1: Estimated number of new cases of diagnosed diabetes in people aged 20 years or older, 2007. From National Diabetes Statestics. www.diabetes.niddk.nih.gov/dm/pubs/statestics.

Figure 2: Insulin structure. From: www.chemistryexplained.com/Hy-Kr/insulin.html.

Figure 3: Insulin receptor structure. From: Cheatham B and Kahn R 1995 Insulin action and the insulin signaling network. Endocrine Reviews 16: 117-142.

Figure 4: Insulin signaling pathway. From: Rhodes CJ and White MF 2002 Molecular insights into insulin action and secretion. European Journal of Clinical Investigation 32: 3-13.

Figure 5: Lipid rafts. From: Publications.nigms.nih.gov/insidethecell/ch2.

Figure 6: Membrane skeleton fence model. From: Lommerse PHM, Spaink HP and Schmidt T 2004 *In vivo* plasma membrane organization: results of biophysical approaches.Biochimica et Biophysica Acta 1664: 119-131

Figure 7: Before exposure to insulin, insulin receptors appear in high density fractions of the sucrose gradient. After treatment of 2H3 cells with 200nM insulin and/or 10μM BMOV, receptors appear in lower density fractions. Membrane fractions were isolated and the relative insulin receptor content was assessed as described in Materials and Methods. Results shown are the mean and standard error of the mean (S.E.M.) for at least 3 individual experiments. The sucrose concentration in each fraction was evaluatein five separate experiments using a Bausch and Lomb refractometer together with standard curve. Because, for any given fraction, the sucrose concentration did not vary appreciably from experiment to experiment, the average sucrose concentration for five representative experiments is shown.

Figure 8: Before exposure to BMOV (1 hour), insulin receptors appear in high density membrane fractions. After treatment of 2H3 cells with 10μM BMOV for 1 hour, receptors appear in lower density fractions. Results shown are the mean and standard error of the mean (S.E.M.) for at least 3 individual experiments. The sucrose concentration in each fraction was evaluated as described in Figure 7.

Figure 9: In the absence of hormone or Cr(pic)$_3$ treatment, insulin receptors appeared in high density sucrose fractions. After overnight treatment of 2H3 cells with 10μM Cr(pic)$_3$ in the absence of insulin or together with 1 hr treatment with 200nM insulin, receptors appeared in lower density fractions. Results shown are the mean and standard error of the mean (S.E.M.) for at least 3 individual experiments.

Figure 10: Untreated insulin receptors appear in high density fractions of the sucrose gradient. After treatment of 2H3 cells with 10 μMCr(pic)$_3$ for 1 hour, most receptors appear in lower density fractions. Results shown are the mean and standard error of the mean (S.E.M.) for at least 3 individual experiments.

Figure 11: Disruption of plasma membrane rafts by treatment of cells with 1% MβCD resulted in most receptors remaining in high density sucrose fractions despite the presence of insulin. Results shown are the mean and S.E.M for 5 separate experiments.

Figure 12: The overall distribution of phosphorylated insulin receptors in raft fragments is nearly the same as in non raft fragments following treatment of 2H3 cells with insulin and/or BMOV. Membrane fractions were separated as described in Materials and Methods, and densitometry was used to estimate the relative amount of phosphorylated insulin receptors contained in each fraction. Results shown are the mean and SEM for at least 5 individual experiments.

Figure 13: The overall distribution of phosphorylated insulin receptors in raft fragments is nearly the same as in non raft fragment following treatment of 2H3 cells with insulin and/or Cr(pic)₃. Membrane fractions were separated as described in Materials and Methods, and densitometry was used to estimate the relative amount of phosphorylated insulin receptors contained in each fraction. Results shown are the mean and SEM for at least 5 individual experiments.

Figure 14: Representative western blots obtained from cell samples separated on sucrose density gradients demonstrate that phosphorylated insulin receptors distribute equally in raft fragments (lanes 7-9) and non raft fragments (lanes 10-12) after treating 2H3 cells with insulin and/or BMOV or Cr(pic)₃.

Figure 15: Disruption of plasma membrane rafts by extraction of cholesterol from the plasma membrane caused a shift of large number of phosphorylated insulin receptors into high density sucrose fractions. Result shown are the mean and SEM for 2 separate experiments.

Figure 16: Insulin increases the number of phosphorylated IRS-1 molecules within low density sucrose fractions. Disruption of plasma membrane rafts by treating 2H3 cells with 1% MβCD resulted in most phosphorylated IR-1 molecules remaining in high density sucrose fractions. Results shown are the mean and S.E.M for at least 2 separate experiments.

Figure 17: In the absence of insulin, phosphorylated IRS-1 molecules appeared in high density sucrose fractions. After treatment of 2H3 cells with 10µM BMOV or 10µM Cr(pic)$_3$ for 1 hour, a significant fraction of phosphorylated IRS-1 molecules was found within lipid rafts. Results shown are the mean and S.E.M for at least 2 separate experiments.

Figure 18: Representative trajectories for a single insulin receptor on an untreated cell, a cell treated with insulin or treated with $Cr(pic)_3$ for 1 hour. Each track represents data obtained during a single experiment of approximately two minutes. Individual compartments within a given particle trajectory were identified as described in Materials and Methods.

Figure 19: Single particle tracking of individual insulin receptors labeled with gold-conjugated anti-insulin receptor antibody. The compartment size and diffusion coefficient for insulin receptor within that compartment were calculated as described in Materials and Methods. Results shown are from individual cells that were untreated, treated with insulin after labeling of receptors with gold-conjugated anti-insulin receptor antibody, or treated with Cr(pic)₃ for 1 hour or at least 16 hours. Data presented in this figure for each condition are from cells examined in separate experiments on 3 different days.

Figure 20: Compartment sizes obtained from single particle tracking of insulin receptors. Individual values for compartment sizes were calculated from particle tracks of insulin receptors before and after treating them with insulin and/or Cr(pic)₃. These treatments shift over 75% of insulin receptors into smaller 1-100nm diameter compartments.

Figure 21: Representative trajectories for a single insulin receptor using quantum dots on an untreated cell, cell treated with 50nm insulin, or a cell treated with 10μM BMOV. The representative tracks for the hormone treated or BMOV treated cells show receptor confinement within small compartments compared to untreated cells. Individual compartments within a given particle trajectory were identified as described in Materials and Methods.

Figure 22: Values for compartment size from single particle tracking of insulin receptors using quantum dots. Individual values for compartment sizes were calculated from particle tracks of insulin receptors before and after treating cells with insulin or BMOV. These treatments cause a reduction in size of compartments accessed by insulin receptors compared to untreated cells. At least, 30 nanogold particles were tracked for each treatment.

Figure 23: Values for diffusion rate from single particle tracking of insulin receptors using quantum dots. Individual values for diffusion rate were calculated from particle tracks of insulin receptors before and after treating cells with insulin or BMOV. These treatments cause a reduction in diffusion rate of insulin receptors compared to untreated cells. At least, 30 nanogold particles were tracked for each treatment.

Figure 24: Measurement of fluorescence emission values as indicative for membrane fluidity. The ratio does not change after treating 2H3 cells with insulin compared to the basal value, whereas the ratio was increased following exposure of cells to $10\mu M$ $Cr(pic)_3$ alone or with 200nM insulin. Incubation of cells with MβCD extracts cholesterol from the membrane and increases the ratio of fluorescence emission.

Panel A

Panel B

Panel C

Figure 25: Red/Green overlay confocal fluorescence images of 2H3 cells stained with 3μM di-4-ANEPPDHQ. (*A*) Untreated cells; (*B*) cells treated with 10μM Cr(pic)₃ for 1 hour; (*C*) cells treated with 1% MβCD for 1 hour.

Figure 26: Effect of different doses of Cr(pic)₃ on membrane fluidity. Both 10μM and 1 μM concentrations of Cr(pic)₃ cause an increase in membrane fluidity as indicated by the increase in fluorescence emission ratio. 100nM concentration of Cr(pic)₃ have returned the ratio to the basal line value.

Figure 27: Measurement of fluorescence emission values as indicative for membrane fluidity. The ratio does not change after treating 2H3 cells with insulin compared to the basal value, whereas the ratio was increased following exposure of cells to 10μM BMOV. Incubation of cells with MβCD extracts cholesterol from the membrane and increases the ratio of fluorescence emission. Reloading cells with cholesterol decreases the ratio to 0.6. 25 cells were analyzed for each treatment.

A

B

C

Figure 28: Red/Green overlay confocal fluorescence images of 2H3 cells stained with 3μM di-4-ANEPPDHQ. (*A*) Untreated cells; (*B*) cells treated with 10μM BMOV; (*C*) cells treated with 1% MβCD for 30 minutes.

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LIST OF ABBREVIATION

IDDM: Insulin dependent diabetes mellitus

NIDDM: Non-insulin dependent diabetes mellitus

Da: Dalton

Ile: Isoleucine

Thr: Threonine

Leu: Leucine

Tyr: Tyrosine

Ser: Serine

Cys: Cysteine

RER: Rough endoplamic reticulum

PC1, PC2: Prohormone convertases

GLUT2: Glucose transporter 2

ATP: Adenosine triphosphate

IP3: Inositol 1,4,5-triphosphate

GIP: Glucose-dependent insulinotropic peptide

cDNA: Complementary DNA

IGF-1: Insulin-like growth factor

Arg: Arginine

Pro: Proline

IRS-1: Insulin receptor substrate-1

PTPase: Protein tyrosine phosphatase

SHIP2: Phosphoinositide phosphatase

cAMP: Cyclic adenosine monophosphate

GSK3: Glycogen synthase kinase

mTOR: Mammalian target of rapamycin

SH2: Src-homology-2

PI-3K: Phosphatidylinositol 3-kinase

GLUT4: Glucose transporter 4

PtdIns(3,4,5)P3: Phosphatidylinositol-3-phosphate

PH: Pleckstrin homology domain

PDK1: Phosphoinositide-dependent kinase 1

PKB: Protein kinase B

PKC: Protein kinase C

GSK-3: Glycogen synthase kinase-3

P70^{rsk}: P70 ribosomal S6 kinase

eIF-4E: Initiation factor 4E for eukaryotic translation

MAP: Mitogen-activated protein

ERK: Extracellular signal-regulated kinase

Grb-2: Growth factor receptor-bound protein 2

SOS: Son of sevenless

Gab-1: GRB2-associated-binding protein 1

CAP: Cbl associated protein

SoHo: Sorbin homology

SH3: SRC homology 3

GTP: Guanosine triphosphate

PDK1: Phosphoinositide-dependent kinase-1

Ld: liquid disordered

Lo: Liquid ordered

GPI: Glycosylphosphatidyl inositol

DRM: Detergent-resistant membranes

Thy-1: Thymus cell antigen

MDCK: Madin-Darby canine kidney

DAF: Decay accelerating factor

CD59: Cluster of differentiation 59

CCR5: Chemokine receptor type 5

HIV-1: Human immunodeficiency virus-1

MHC: Major histocompatibility complex

TCR: T-cell receptor

Lck: Leukocyte-specific protein tyrosine kinase

IS: Immunological synapse

SPT: Single particle tracking

FRET: Fluorescence resonance energy transfer

BHK: Baby hamster kidney

5′NT: 5′nucleotidase

CHO: Chinese hamster ovary

CaCo: Colorectal-adenocarcinoma

IgE: Immunoglobulin E

FcεRI: Fc epsilon receptor I

RBL-2H3: Rat basophilic leukemia-2H3

GFP: Green Fluorescent protein

Cav-1: Caveolin-1

NOS: Nitric oxide synthase

GDP: Guanosine diphosphate

EGF: Epidermal growth factor

PDGF: Platelet derived growth factor

DOPE: Dioleoylphosphatidylethanolamine

SFVI: Single fluorescent molecule video imaging

TCZ: Transient confinement zones

FCS: Fluorescence correlation spectroscopy

D: Diffusion coefficient

CMD: Caveolin-enriched membrane domains

TNF-α : Tumor necrosis factor

DRM: Detergent resistant membrane microdomains

Na_3VO_4: Sodium orthovanadate

STZ: Streptozocin-treated diabetic rats

$NaVO_3$: Sodium metavanadate

VS: Vanadyl sulfate

SHR: Spontaneously hypertensive rats

HbA_{1c}: Glycosylated haemoglobin

BMOV: Bis(maltolato)oxovanadium

EPR: Electron paramagnetic resonance

SHP-2: SH2 domain-containing PTPase

ASO: Antisense oligonucleotides

PTEN: Lipid phosphatase like tensin homolog

Cyt PTK: Staurosporine-sensitive cytosolic protein tyrosine kinase

IR-β: Insulin receptor-β

IR-PTK: Insulin receptor protein tyrosine kinase

CHO-HIR: Insulin receptor-overexpressing Chinese hamster ovary cells

FKHR: Forkhead transcription factor

G6Pase: Glucose-6-phosphatase

ANEP: Aminonaphthylethenylpyridinium

ppm: part per million

mg/kg: milligram per kilogram

Cr: Chromium

HCL: Hydrochloric acid

μg: microgram

TPN: Total parental nutrition

HDL: High density lipoprotein

LMWCr: Low-molecular weight chromium-binding substance

AMPK: 5′-AMP-activated kinase

SREBP-1: Sterol regulatory element binding protein

DNA: Deoxy ribonucleic acid

MEM: Minimal essential medium

FBS: Fetal bovine serum

Insulin Rβ: Insulin receptor β

Cr(pic)$_3$: Tris(2-pyridinecarboxylato)chromium(III)

Di-4-ANEPPDHQ: Aminonaphthylethenylpyridinum

Qdot: Quantum dot

PBS: Phosphate-buffered saline

EDTA: Ethylene diamine tetraacetic acid

MβCD: Methyl-β-cyclodextrin

BSA: Bovine serum albumin

SEM: Standard error of the mean