DIABETES AND INSULIN ACTION

Organizers: Ira Goldfine and C. Ronald Kahn

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Diabetes and Insulin Action

Keynote Address (joint)

CB 001 A MOLECULAR DISSECTION OF VESICULAR TRANSPORT, James E. Rothman, Department of Molecular Biology, Lewis Thomas Laboratory, Princeton University, Princeton, New Jersey 08544-1014.

Protein transport between successive cisternae of the Golgi stack is reconstituted when isolated Golgi membranes are incubated with cytosol and ATP. This transport is due to rounds of budding and fusion of coated vesicles. When transport is blocked by adding the nonhydrolyzable analogue of GTP, GTPγS coated vesicles massively accumulate. The coated vesicles can be purified by density gradient centrifugation following extraction with salt, and contain a number of characteristic polypeptide chains including several GTP-binding proteins, but not clathrin. Following transfer of budded coated vesicles between cisternae, the coats are removed, and fusion proceeds in an ATP dependent process. Fusion requires an NEM-sensitive protein (NSF), a series of soluble NSF attachment proteins (SNAPs), palmityl-CoA as well as other cytosolic factors. The same pathway appears to account for transport from the endoplasmic reticulum to the Golgi, as well as transport within the Golgi, in both animals and in yeast.

Insulin Receptor: Structure, Function and Turnover

CB 002 EVIDENCE THAT INSULIN PLUS ATP CAN INDUCE A CONFORMATIONAL CHANGE IN THE BETA SUBUNIT OF THE INSULIN RECEPTOR WITHOUT INDUCING RECEPTOR AUTOPHOSPHORYLATION, B.A. Maddux and I.D. Goldfine, Division of Diabetes and Endocrine Research, Mount Zion Medical Center of the University of California, San Francisco, CA 94120

The effect of insulin and ATP on insulin receptor beta subunit conformation was studied in vitro with monoclonal antibodies directed at several regions of the receptor beta subunit. These antibodies bound to purified and radioiodinated insulin receptors. Insulin plus ATP inhibited binding. The greatest inhibitory effect of insulin and ATP was seen with antibody 17A3 that recognizes a domain of the beta subunit that is near the major tyrosine autophosphorylation sites at residues 1158, 1162, and 1163. Accordingly, this antibody was employed in additional studies. ATP alone inhibited 17A3 binding with a one-half maximal inhibitory concentration at 186 $\pm$ 7 μM. Insulin potentiated the effect of ATP at concentrations as low as 100 pM; at 100 nM where insulin had a maximal effect, insulin lowered the one-half maximal inhibitory concentration of ATP to 16 $\pm$ 6 μM. Proinsulin was approximately 5% as potent as insulin, and guinea pig insulin was about 2% as potent as insulin. Inhibition by insulin and ATP required the presence of Mn^{+2} but not Mg^{+2}. At 1 mM CTP, GTP, ITP, TTP, and AMP were without effect in either the presence or absence of insulin; ADP in contrast, was inhibitory in the presence of insulin. Of major interest was adenyl-5'-yl imidodiphosphate (AMP-PNP). This nonhydrolyzable analog of ATP inhibited 17A3 binding, and the effect of AMP-PNP (like ATP) was potentiated by insulin. Two insulin receptor beta subunit mutants were then studied. Mutant receptor F3, where the major tyrosine autophosphorylation sites at residues 1158, 1162, and 1163 were changed to phenylalanines, bound to 17A3; antibody binding was inhibited by insulin and ATP in a manner similar to normal receptors. In contrast, mutant receptor M1030, where the lysine in the ATP binding site at residue 1030 was changed methionine, bound 17A3, but unlike either normal receptors or F3 receptors, the binding of 17A3 was not inhibited by insulin and ATP. These in vitro studies demonstrate therefore that insulin and ATP can change the conformation of the insulin receptor beta subunit. This change requires the binding of ATP (or a related analog) but not receptor autophosphorylation. These studies also raise the possibility that, in vivo, ATP binding in the presence of insulin induces a conformation change in the insulin receptor beta subunit which in turn signals some of the biological effects of insulin.

CB 003 **THE RELATION BETWEEN INSULIN RECEPTOR STRUCTURE, FUNCTION, AND SIGNAL TRANSDUCTION**, Morris F. White, C.Ronald Kahn, Jonathan Backer, Peter A. Wilden, Xiao Jian Sun, and Martin G. Myers, Joslin Diabetes Center and Harvard Medical School, Boston MA 02215.
The β-subunit of the insulin receptor is a tyrosine kinase that undergoes autophosphorylation and activation during insulin binding to the α-subunit. We have investigated the relation between IR structure and signal transmission by studying the effect of mutations in the β-subunit on activation of putative signal transduction pathways and on biological activity. At least two regions of the β-subunit of the IR have been shown to be particularly important for normal signal transmission: the regulatory region including the autophosphorylation sites at Tyr_{1146}, Tyr_{1150} and Tyr_{1151}, and the juxtamembrane region; the C-terminus which contains 2 tyrosine autophosphorylation site does not appear to play a significant role. Substitution of each tyrosine residue in the regulatory region with phenylalanine reduces IR autophosphorylation and kinase activity. Moreover, these mutant receptor molecules do not undergo endocytosis through the high affinity mechanism. One mutant in particular in which Tyr_{1146} is replaced by Phe_{1146} (IR_{F1146}) does not mediate insulin-stimulated DNA synthesis; however, it does mediate insulin-stimulated glycogen synthesis normally, suggesting that more than one signal transduction pathway may be involved in insulin action. We have focused our attention on two putative signal transduction pathways: tyrosyl phosphorylation of pp185 and the activation of the phosphatidyl inositol 3-kinase. The IR_{F1146} does not stimulate pp185 phosphorylation, whereas it partially activates PtdIns 3-kinase, suggesting that a single mutation can differentially affect molecular interactions between the IR and cellular proteins. The juxtamembrane membrane region of the IR is a 23 amino acid sequence in the β-subunit located just beyond the transmembrane spanning region; it is encoded entirely by exon 16 of the IR gene. Point mutations or deletion of the C-terminal portion of this region ($IR_{\Delta 960}$) reduces the ability of the receptor to mediate insulin stimulation of glycogen and DNA synthesis, and reduces endocytosis through the high affinity system. The loss of endocytosis may be related to deletion of the consensus amino acid sequence, $NPXY_{960}$, which is required for internalization of some membrane proteins. Moreover, the $IR_{\Delta 960}$ does not stimulate tyrosine phosphorylation of pp185 or activate the PtdIns 3-kinase. In contrast, activation of the kinase by autophosphorylation *in vitro* is normal. Thus, mutations in the juxtamembrane region of the β-subunit have more global effects on IR signal transmission than mutations in the regulatory region, but milder effects on receptor function. In summary, it appears that the juxtamembrane region of the IR β-subunit interacts directly with cellular proteins that are essential for signal transmission and endocytosis, whereas the regulatory region plays an indirect regulatory role.

CB 004 IDENTIFICATION AND CHARACTERIZATION OF THE LIGAND-BINDING DOMAIN OF THE INSULIN RECEPTOR, Cecil C. Yip*, Ronit Rafaeloff[#], Carl Grunfeld[+], Ira D. Goldfine[#] and Dennis M. Hawley[#],*Banting & Best Dept. of Med. Res., Univ. of Toronto, Toronto, Ont. M5G 1L6, Canada [+]Dept. of Medicine, School of Medicine, Univ. of California, & Medical Serv., VA Medical Centre., San Francisco, CA 94121, [#]Div. of Diabetes & Endocrine Res., Dept. of Medicine, Mt. Zion Hosp. & Medical Centre, San Francisco, CA 94120. We previously reported that a 23-kDa receptor proteolytic fragment containing an insulin-binding site was localized within residues 205-316 in the cysteine-rich region of the insulin receptor α subunit, and postulated that the sequence 241-251 is part of the insulin-binding domain (1). In the present study, we have used an antiserum AP-II raised against a synthetic peptide containing the sequence 241-251 to test this postulate, and to study the role of sequence 241-251 in insulin binding. The AP-II immunoprecipitated the 23-kDa fragment, confirming our sequence assignment of this fragment. As expected, it also immunoprecipitated the intact subunit of the insulin receptor that had been denatured by reduction and alkylation. However, the antiserum did not block [^{125}I]-iodoinsulin binding, and did not precipitate either photoaffinity-labeled insulin receptors or insulin receptors labeled with ^{125}I. These findings suggest that sequence 241-251 in the native receptor was inaccessible to the antiserum. However, using a radioactive photoaffinity probe ([^{125}I]-AZAP-insulin) that allows cleavage and removal of insulin after photolabeling, we found that after insulin removal the photolabeled receptor was precipitated by AP-II, suggesting that the sequence 241-251 has thus become accessible after the removal of insulin. We conclude therefore that the sequence 241-251 forms part of the receptor insulin-binding domain and that insulin binding to the receptor induces a conformational change that allows the exposure of this domain after removal of insulin. Such a conformational change may play a role in activation of the receptor and transmembrane signalling. We have previously studied by site-specific mutagenesis the role of this sequence in insulin binding, and found that an alteration of this sequence from PPYYHFQDW to RRYYDFQDW increased the binding affinity of the receptor, as well as the sensitivity to insulin (2). An insulin analog (from Novo Res. Inst.) B10-His Asp was found to bind to the mutant receptor with even higher affinity than insulin. These findings suggest that insulin binding to the receptor involves charge interaction, and that the sequence 241-251 forms part of the insulin binding domain.

1. Yip, C.C., Hsu, H., Patel, R.G., Hawley, D.M., Maddux, B.A., and Goldfine, I.D. (1988) Biochem. Biophys. Res. Commun. 157, 321-329. 2. Rafaeloff, R., Patel, R., Yip, C., Goldfine, I.D., and Hawley, D.M. (1989) J. Biol. Chem. 264, 15900-15904.

Insulin Receptor-Like Receptors

CB 005 TRANSMEMBRANE SIGNALLING OF THE INSULIN/IGF-1 HYBRID RECEPTORS. Jeffrey E. Pessin and Judith L. Treadway, Department of Physiology & Biophysics, The University of Iowa College of Medicine, Iowa City, IA 52242

Both classical insulin and IGF-1 receptors exist as well defined $\alpha_2\beta_2$ heterotetrameric complexes which are assembled from two identical $\alpha\beta$ heterodimeric half-receptor precursors. Recent evidence has suggested that insulin and IGF-1 half-receptor precursors can heterologously assemble to form $\alpha_2\beta_2$ insulin/IGF-1 hybrid receptor complexes *in vivo* and *in vitro*. We have utilized the *in vitro* assembly of wildtype insulin and IGF-1 half-receptors with various mutant insulin half-receptors to examine ligand-stimulated transmembrane signalling. In a concentration dependent manner, incubation of a kinase-defective insulin half-receptor $\alpha\beta_{INS.A/K}$ with the wildtype insulin half-receptor $\alpha\beta_{INS.WT}$ resulted in complete inhibition of ligand-stimulated substrate kinase activity. Similarly, *in vitro* assembly of the $\alpha\beta_{IGF.WT}$ with the $\alpha\beta_{INS.A/K}$ half-receptor also produced an inactive substrate kinase and based upon immunological cross-reactivity was a direct consequence of heterologous receptor formation. The inhibition of substrate kinase activity appeared to result from defective insulin-signalling, since hybrid receptors composed of an autoactivated wildtype half-receptor with the kinase-defective half-receptor displayed normal substrate kinase activity.

To further examine the role of subunit interactions in the intramolecular signalling pathway, insulin-stimulated autophosphorylation of the $\alpha\beta_{INS.WT}$-$\alpha\beta_{INS.A/K}$ hybrid receptor was investigated. In contrast to substrate kinase activity, insulin-stimulated autophosphorylation was completely unaffected in comparison to the wildtype $\alpha\beta_{INS.WT}$-$\alpha\beta_{INS.WT}$ receptor. One possible explanation for an apparently normal β subunit autophosphorylation with impaired substrate kinase activity would be an altered intramolecular autophosphorylation cascade. To address this issue, autophosphorylation was determined in hybrid receptors prepared by the heterologous assembly of the $\alpha\beta_{INS.A/K}$ half-receptor with the β subunit C-terminal truncated $\alpha\beta$ half-receptor $\alpha\beta_{INS.\Delta CT}$. Under these conditions, autophosphorylation exclusively occurred on the $\alpha\beta_{INS.A/K}$ β subunit. These data demonstrate that the ligand-dependent substrate kinase activity of the insulin and IGF-1 holoreceptors requires interactions between two functional β subunits within the $\alpha_2\beta_2$ heterotetrameric complex which occurs via an intramolecular trans-phosphorylation reaction.

CB 006 THE INSULIN RECEPTOR-RELATED RECEPTOR, IRR. Valerie M. Watt and Peter Shier, Department of Physiology, University of Toronto, Toronto, Ontario, Canada, M5S 1A8. IRR is a new member of the mammalian insulin receptor family that we initially identified by nucleotide sequence analysis of human and guinea pig genomic DNA. The predicted primary structure of IRR is as similar to the insulin and insulin-like growth factor-I (IGF-I) receptors as they are to each other. This similarity between the three proteins occurs throughout their entire length with maximum identity (~80%) in the tyrosine kinase domains. To facilitate further studies, we have isolated a portion of rat genomic DNA which by Southern blot analysis is the rat homologue of the human IRR gene. Nucleotide sequence analysis of the region 5' to the initiatior methionine has revealed the presence of elements typical of mammalian promoters. In addition, we have used this rat genomic DNA as probe in Northern blot analysis to determine the tissue-specific expression of the IRR gene. Confirmation of this expression was obtained using polymerase chain reaction techniques as well as by isolation of a cDNA clone. On the basis of its similarity to the insulin and IGF-I receptors in the extracellular binding domain, the IRR is likely to be a novel receptor for a member of the insulin family. The isolation of the cDNA encoding IRR will facilitate determination of the ligand(s) for this receptor.

Substrates and Signals of the Insulin Receptor (joint)

CB 007 INSULIN-STIMULATED ACTIVATION OF THE RAF-1 PROTEIN KINASE, R.-M. Lee and P.J. Blackshear, Howard Hughes Medical Institute Laboratories and Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, NC 27710

One of the most pressing questions in the field of insulin action is how the activation of the insulin receptor protein tyrosine kinase leads to phosphorylation of cellular proteins on serine and threonine residues. Among the serine/threonine protein kinases rapidly activated by insulin in sensitive cells is the Raf-1 proto-oncogene kinase. This kinase is also rapidly phosphorylated on serine residues in response to insulin; however, the question remains as to whether this is the result of activation of an insulin-stimulated Raf-1 kinase kinase or insulin-stimulted Raf-1 kinase autophosphorylation. We have addressed this issue by searching for an insulin-activated Raf-1 protein kinase kinase. Using recombinant Raf-1 protein as a substrate, we have identified a kinase in sensitive cells that can be activated by insulin to phosphorylate the Raf-1 protein within 2-5 min of insulin exposure. Insulin-stimulated phosphorylation of the Raf-1 protein occurs on multiple sites, some of which comigrate with sites phosphorylated in response to insulin in intact cells. Further characterization of this kinase is in progress.

CB 008 INSULIN STIMULATION OF TYROSINE PHOSPHORYLATION AND MEMBRANE ASSOCIATED PHOSPHATIDYLINOSITOL 3-KINASE ACTIVITY IN CELLS EXPRESSING WILD-TYPE AND MUTANT INSULIN RECEPTORS, Richard A. Roth, Kazuyoshi Yonezawa, Bei Zhang, Janice E. Chin and Kristina S. Kovacina, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305-5332.

After binding to its receptor, insulin elicits a diverse array of biological responses. Various mutant receptors have been described which differ in their ability to mediate distinct biological responses. For example, a mutant receptor which lacks two critical autophosphorylation sites has been reported to be capable of mediating insulin's ability to stimulate DNA synthesis but not glucose uptake. This mutant receptor was previously found to exhibit no insulin-stimulated kinase activity in vitro. This mutant was now studied to assess its in vivo tyrosine kinase activity by determining whether it was capable of mediating the tyrosine phosphorylation of a putative endogenous substrate of tyrosine kinases, the type I phosphatidylinositol (PtdIns) 3-kinase. This mutant receptor was found to be ~1/3 as potent as the wild-type receptor to stimulate this response. This level of tyrosine phosphorylation of the PtdIns Kinase was clearly elevated in comparison to the parental cells. Western blotting with anti-phosphotyrosine antibodies also documented that this mutant receptor was capable of insulin-stimulated in vivo tyrosine phosphorylation of various substrates. Moreover, in vitro assays demonstrated that this receptor exhibited approximately 10-times higher basal kinase activity than the wild-type receptor. Finally, insulin was found to stimulate an increase in the membrane associated PtdIns 3-kinase activity in cells expressing both the wild-type receptor as well as this mutant receptor, although the mutant was approximately 1/2 as potent as the wild-type receptor.

These results indicate that: 1) In vitro assessments of the tyrosine kinase activity of various mutant insulin receptors may not accurately reflect their in vivo activity; 2) Tyrosine phosphorylation of PtdIns 3-kinase parallels an increase in membrane associated PtdIns 3-kinase activity; and 3) The ability of the mutant receptor lacking two autophosphorylation sites to mediate insulin-stimulated tyrosine phosphorylation of various endogenous substrates may account for the reported ability of this receptor to mediate some biological responses.

Post Receptor Signals

CB 009 STUDY OF A NATURAL INHIBITOR OF THE INSULIN RECEPTOR TYROSINE KINASE, Alphonse Le Cam, Patrick Auberger, Laurence Falquerho, Gilles Patey, Ginette Le Cam, Jean O. Contrérès[1] and Bernard Rossi[1], Centre CNRS-INSERM de Pharmacologie-Endocrinologie, rue de la Cardonille, 34094 Montpellier, France, and Unité INSERM 260, faculté de médecine, 06034 Nice, France[1].

Tyrosine kinase activity is associated with many growth factor receptors (e.g., EGF, PDGF, IGF-I, insulin). Ligand-induced activation of the kinase activity appears to mediate most if not all of the effects of the growth factors. Among other effectors, natural inhibitors may participate to the physiological regulation of tyrosine kinase activity. Two examples of such inhibitors have so far been described: 1) the Müllerian Inhibiting Substance (MIS), reported to inhibit the EGF receptor kinase (1); 2) a 63 kDa phosphoprotein (PP^{63}) secreted by rat hepatocytes that inhibits the insulin receptor kinase, which we have characterized (2). PP^{63} is an acidic (pI=4.8-5.2), fucose-rich glycoprotein phosphorylated at a serine residue. A single 1.8 kb mRNA species was detected in the liver. The affinity purified protein inhibits both hormone-stimulated autophosphorylation and tyrosine kinase activity towards exogenous substrates of the solubilized insulin receptors, but has no effect on the EGF receptor kinase. Dephosphorylation of PP^{63} virtually abolishes these effects. Hormonal stimulation of receptor autophosphorylation is also inhibited in intact hepatoma FaO cells. Concomitantly, PP^{63} blocks the stimulation of ^{3}H-thymidine incorporation by insulin but has no effect on so-called metabolic actions of the hormone, in the same cells. PP^{63} sequence, deduced from cDNA cloning, was found to share more than 70% identity with human α2-HS glycoprotein and bovine fetuin, proteins which belong to the cystatin superfamily. The molecular mechanism of kinase receptor inhibition involves neither inhibition of hormone binding, nor proteolysis or rapid dephosphorylation of the receptor by PP^{63}.

The gene encoding PP^{63}, which appears to be unique, was cloned and sequenced. It comprises 7 exons separated by 6 introns of variable sizes. A major transcription initiation site was mapped 73 bp ahead of the translational initiator ATG. Proximal promoter elements were identified and functionally characterized.

1) Coughlin, J.P., Donahoe, P., Budzik, G.P. and MacLaughlin, D.T. (1987) Mol. Cell. Endo. 49, 75-86.
2) Auberger, P., Falquerho, L., Contrérès, J.O., Pagès, G., Le Cam, G., Rossi, B. and Le Cam, A. (1989) Cell, 58, 631-640.

CB 010 INSULIN RECEPTOR REGULATION BY SERINE AND THREONINE PHOSPHORYLATION, Robert E. Lewis and Michael P. Czech, Department of Biochemistry and the Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605

As a means toward understanding insulin receptor mediated signal transduction we are attempting to discern the functional role of insulin receptor phosphorylation on serine and threonine residues and to identify the kinase(s) responsible for these phosphorylations. Based on peptide mapping studies and the comigration of receptor-derived and synthetic phosphopeptides we previously proposed that serine 1293/1294 and threonine 1336 of the human insulin receptor were phosphorylated following insulin and phorbol ester addition to intact cells. We now show the discrete loss of these phosphorylation sites following site-directed mutatgenesis of the insulin receptor cDNA which confirms their identity. In an attempt to identify the kinase responsible for phosphorylating the insulin receptor in intact cells we tested the possibility that protein kinase C (PKC) mediates insulin stimulated receptor phosphorylation on serine and threonine residues. COS-1 cells transiently expressing the human insulin receptor were treated chronically with 500 nM PMA or DMSO (control) for 41 h to down regulate PKC. Transfectants were then labeled with ^{32}P and left untreated, or treated with 100 nM insulin or 100 nM PMA. Phosphoamino acid analysis of in vivo labeled insulin receptor revealed that PKC down regulation blocked the effect of PMA, but had no effect on insulin stimulated phosphorylation of the insulin receptor β subunit. HPLC phosphopeptide mapping demonstrated that specific phosphorylation of threonine 1336 in response to insulin was unaffected by PKC down regulation, while PMA stimulated phosphorylation of threonine 1336 was blocked by PKC down regulation. We conclude that insulin stimulated serine/threonine phosphorylation of the insulin receptor is not mediated by PKC. Treatment of ^{32}P labeled COS-1 transfectants with 0.5 μM okadaic acid has no effect on insulin receptor phosphorylation in response to insulin or PMA. These results suggest that phosphatases 1 and 2A may not play a major role in the regulation of these serine/threonine phoshorylation sites on the β subunit. Furthermore, the data suggest that the kinase which phosphorylates the insulin receptor in intact cells may be distinct from other recently identified kinases whose activity is regulated by serine/threonine phosphorylation. A candidate for the insulin receptor directed serine kinase has been identified as a copurifying serine kinase (IRSK) in affinity purified preparations of insulin receptor from human placenta. IRSK phosphorylates synthetic peptides identical to insulin receptor serine/threonine phosphorylation sites in an insulin dependent manner, suggesting that IRSK is activated by the insulin receptor. Identification and purification of IRSK is facilitated by antibodies which, upon immunoblotting, recognize a putative serine kinase in insulin receptor preparations distinct from the insulin receptor β subunit. Identification of IRSK should lead to an improved understanding of the mechanisms underlying its activation, and the initial events in insulin receptor signal transduction.

CB 011 REGULATION OF MAP (MITOGEN-ACTIVATED PROTEIN) KINASE BY TYROSINE AND THREONINE PHOSPHORYLATION, D. Michael Payne[√], Anthony J. Rossomando[√], Paul A. Martino[**], Alan K. Erickson[*], Donald F. Hunt[**], Michael J. Weber[√], and Thomas W. Sturgill[**], Departments of [√]Microbiology, [**]Chemistry, and [*]Pharmacology and Internal Medicine, University of Virginia, Charlottesville, Virginia 22908.
Insulin, as well as a diverse array of other potential mitogens, rapidly stimulate the activity of a 42 kDa serine/threonine kinase upon addition to responsive quiescent cells. We have termed this enzyme MAP kinase, a name derived originally from the in vitro substrate Microtubule-Associated Protein 2, and now standing for Mitogen-Activated Protein kinase, to reflect its promiscuous stimulation by mitogens. Active p42 MAP kinase contains phosphotyrosine and phosphothreonine, and both phosphorylations are required for activity (1). We have now purified murine p42 MAP kinase and isolated and sequenced the regulatory tryptic peptide containing these phosphorylation sites by mass spectrometry. The sequence obtained is similar to a sequence encoded by a rat cDNA (ERK1) recently isolated by Cobb and co-workers for an insulin-stimulated MAP2 kinase, which in turn is closely related to KSS1, a kinase involved in cell cycle control in yeast. The immediate sequence surrounding the phosphorylation sites is conserved in yeast and mammals.

1. Anderson, N.G., Maller, J.L., Tonks, N.K., and Sturgill, T.W. (1990) Nature 343, 651-653.
2. Boulton, T.G., Yancopoulos, G.D., Gregory, J.S., Slaughter, C., Moomaw, C., Hsu, J., and Cobb, M.H. (1990) Science 249, 64-67.

The Beta Cell-Mechanisms of Signalling, Desensitization and Cell Growth

CB 012 ION CHANNELS AND THE SULFONYLUREA RECEPTOR, Boyd, A.E. III, Department of Medicine, Baylor College of Medicine, Houston, TX 77030
In the pancreatic beta cells the proximal step in sulfonylurea signal transduction is the binding of these clinically important drugs to high affinity receptors in the beta cell membrane. Using HIT cells as a model system, we have established an extremely close correlation between the affinity of binding of glyburide and its analog, iodo"glyburide", and the activation of various steps in stimulus-secretion coupling - inhibition of 86Rb+ efflux, increase in [Ca2+]i resulting from gating of voltage-gated calcium channels by cell depolarization and the exocytosis of insulin. Two different L-type channel cDNAs have been identified in a HIT cell library, one "neuroendocrine" in type and one more "cardiac"-like.
A HIT cell membrane protein of Mr = 140,000, which we believe to be the high affinity sulfonylurea receptor, can be covalently linked to 5125iodo-2-hydroxyglyburide by ultraviolet irradiation. The receptor has been solubilized and retains binding activity and the same rank order of displacement of the 5125iodo-2-hydroxy-glyburide as observed with the native receptor. The 140,000 molecular weight protein has been partially purified and the amino acid sequence of three proteolytic fragments have been used to design oligonucleotides to screen HIT cell cDNA libraries. Since the binding constant of glyburide or iodo"glyburide" is closely correlated with the ability of these compounds to inhibit the ATP-sensitive K+ channel, increase [Ca2+]i and elicit insulin secretion, we have identified the 140,000 molecular weight protein as the sulfonylurea receptor. Expression of the cloned cDNA should allow us to test this hypothesis directly.

CB 013 THE ROLE OF DIACYLGLYCEROL AND PROTEIN KINASE C IN INSULIN SECRETION, Michael L. McDaniel, Richard A. Easom, Bryan A. Wolf, and John Turk, Department of Pathology, Washington University School of Medicine, St. Louis MO 63110

Agents which activate protein kinase C, such as phorbol esters (TPA) and synthetic diacylglycerols (OAG) are known to stimulate insulin secretion from pancreatic islets. Whether protein kinase C is directly involved in the release of insulin in response to a variety of different types of physiological insulin secretagogues remains controversial. In the present studies, the insulin secretagogues carbachol, glucose, and the combination of carbachol plus glucose were examined for their effects on protein kinase C activity by determination of their ability to elevate endogenous diacylglycerol levels, to translocate protein kinase C from cytosol to membranes, and to induce the phosphorylation of an endogenous 80 kDa protein kinase C substrate present in islets.

Insulin secretion induced by the muscarinic receptor agonist carbachol was found to be associated with an increase in diacylglycerol accumulation in islets which contained both arachidonate and sterate and which appeared to arise from the hydrolysis of glycerolipids including phosphoinositides. Carbachol-induced insulin secretion was also associated with the activation of protein kinase C in islets as reflected by the translocation of protein kinase C from the cytosol to a membrane-associated state, and by the enhanced phosphorylation of the 80 kDa endogenous protein kinase C substrate. The combination of carbachol and a submaximally stimulating concentration of glucose (10 mM) also exerted a marked synergistic effect on the phosphorylation of the 80 kDa substrate. In contrast, glucose alone at insulin stimulatory concentrations induced little change in the total islet content of diacylglycerol, although conversion of ^{14}C-glucose conversion to ^{14}C-diacylglycerol was demonstrable.This de novo synthesis of diacylglycerol from glucose in islets occured too slowly to participate in acute signal transduction, but the process continued to occur in a time and glucose-dependent manner upon prolonged incubation. Furthermore, glucose-induced insulin secretion did not appear to be associated with the translocation of protein kinase C or enhanced phosphorylation of the endogeous 80 kDa substrate of protein kinase C.

These data indicate that the activation of protein kinase C may play an important role in carbachol-induced insulin secretion and also in the potentiation by carbachol of insulin secretion induced by glucose. However, the activation of protein kinase C does not appear to be a primary determinant of insulin secretion induced by glucose alone.

CB 014 OVERVIEW OF INSULIN SECRETION IN DIABETES, R. Paul Robertson, Department of Medicine and Cell Biology, University of Minnesota Medical School, Minneapolis, MN 55455

This overview will focus on mechanisms of defective insulin secretion in patients with diabetes mellitus. Since type I diabetes mellitus is characterized by death of the pancreatic islet beta cell, this autoimmune disease is characterized by total lack of insulin secretion in the basal and stimulated state. In contrast, patients with type II diabetes mellitus have functioning beta cells that continue to secrete insulin in the basal state and remain responsive to all non-glucose stimuli. Consequently, the defective insulin response in type II diabetes mellitus can be characterized as glucose-specific wherein the beta cell appears to have selectively lost the ability to recognize glucose signals.

The only manner in which patients with type I diabetes mellitus can recover the ability to secrete insulin is through successful pancreas transplantation. Such patients have normal fasting glucose levels and normal phasic glucose-induced insulin secretion. In contrast, patients with type II diabetes mellitus can be treated with several pharmacologic agents that are capable of partially restoring defective first and second phase glucose-induced insulin secretion. In addition, defective first phase glucose-induced insulin secretion can be partially restored in the absence of pharmacologic therapy if the patient's circulating glucose level can be kept in the normal range for at least 20 hours preceding glucose challenge. The latter phenomenon gives support to the hypothesis that glucose desensitization secondary to prolonged hyperglycemia represents a pathogenic force in abnormal insulin secretion in patients with type II diabetes mellitus.

Diabetes and Insulin Action

Mechanisms and Control of Insulin Secretion

CB 015 The Molecular Biology and Actions of Amylin, Luskey, Kenneth L., Ogawa, A., Alam, T., Chen, L., Leffert, J., Lee, Y. and Stein, D., Departments of Internal Medicine and Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Tx. 75235.

Amylin (or islet amyloid polypeptide) is a 37 amino acid peptide, homologous to calcitonin gene-related peptide, that is cosecreted with insulin from the pancreatic β-cell. It was initially purified from extracellular amyloid deposits in the islets of patients with noninsulin dependent diabetes mellitus (NIDDM). Both amylin secretion and mRNA levels are regulated in parallel with insulin in the islet. A 12 day insulin infusion to induce prolonged hypoglycemia (blood sugar $\approx$50 mg/dl) resulted in a >95% suppression of both insulin and amylin RNA. In contrast, an infusion of 50% glucose for 5 days induced both amylin and insulin RNAs. Under a wide variety of conditions parallel changes in the secretion of insulin and amylin are also observed. In normal rats, a 72 hour fast results in approximately a 70% suppression in both insulin and amylin secretion from the isolated perfused pancreas . In states of acquired or genetic insulin resistance parallel increases in insulin and amylin secretion are seen. However, the molar ratio of amylin secreted relative to insulin remains at about 5%, unchanged from the ratio in normal rats. In obese Zucker rats that develop diabetes, both insulin and amylin expression are reduced from levels observed in nondiabetic, insulin-resistant animals. Thus it appears that although amylin hypersecretion is present in an animal model of NIDDM, it parallels changes in insulin secretion. Potential actions of amylin are currently being explored. Studies by other laboratories have shown that amylin inhibits glycogen synthesis in skeletal muscle and stimulates gluconeogenesis in hepatocytes. Infusion of amylin at high concentrations (nM plasma levels compared to control levels of <20pM) resulted in hyperglycemia and increased plasma lactate concentrations. These effects at pharmacologic levels are consistent with the *in vitro* actions; however, the physiologic relevance or potential role in the insulin resistance of NIDDM is still unclear.

CB 016 PROCESSING AND SORTING OF ISLET PROHORMONE PRECURSORS, S.P. Smeekens, C. Albiges-Rizo, L.A. Phillips, S.J. Chan, R. Carroll, W. Chutkow, A.G. Montag, H. Swift, M. Schwen, R. Hammer, and D.F. Steiner, Howard Hughes Medical Institute, and the Departments of Molecular Genetics and Cell Biology and Pathology, the University of Chicago, Chicago, IL 60637 and Howard Hughes Medical Institute, the University of Texas Southwestern Medical Center, Dallas, TX 75235

The biosynthesis of neuroendocrine peptide precursor proteins and their subsequent intracellular transport and packaging into secretory vesicles is a multistage process which presumably requires both an appropriate structure in the native precursors as well as the expression of sets of genes encoding proteins involved in the processing, storage and regulated release of hormones and/or neuropeptides. Studies in our laboratory of a mutant human proinsulin (His B10 Asp) have shown that this single a.a. replacement does not impair the folding or processing of this proinsulin into insulin but nevertheless causes a significantly greater proportion of it to be released via an unregulated, or constitutive, pathway than is normally the case. Studies with transgenic mice expressing various mutated versions of the human insulin gene in combination with the Asp^{10} replacement are in progress to determine whether the elevated insulin receptor binding affinity of the mutant prohormone is related to its altered secretory behavior.

In other studies we have identified and are characterizing two yeast kex2-related proteases (PC2, PC3) in islet and neuroendocrine cells which we believe are involved in the proteolytic processing of hormone precursors, most likely in regulated pathway secretory vesicles. Like CP'ase H these enzymes lack hydrophobic transmembrane anchors but may be bound to secretory granule membranes via amphipathic helical segments near their C-termini. Antibodies to PC2 demonstrate the presence of this protein within secretory granules in the β cells of the islets of Langerhans. Further aspects of the expression, properties and cellular localization of PC2 and PC3 will be discussed. (Work from these laboratories is supported by the Howard Hughes Medical Institute and by NIH grant DK 13914.)

CB 017 TRANSLATIONAL REGULATION OF PROINSULIN BIOSYNTHESIS.

Sandra L. Wolin and Peter Walter, Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143.

In pancreatic β cells, the translation of proinsulin is regulated in response to the glucose concentration of the extracellular medium. When primary cultures of rat pancreatic islets are incubated in high concentrations of glucose, the synthesis of proinsulin increases five-fold within minutes, and is due to increased translation of pre-existing preproinsulin mRNA (1,2). As a first step towards understanding the molecular mechanism by which the translation of proinsulin is regulated, we devised a sensitive assay to determine the distribution of translating ribosomes on an mRNA with single nucleotide precision (3). Because the assay uses unlabeled RNA fragments that can be part of a complex mixture, it can be used to probe ribosome transit in vivo as well as in vitro. Using this assay to monitor ribosome movement along two model secretory proteins, bovine preprolactin and rat preproinsulin, during translation in wheat germ and reticulocyte extracts, we found that ribosomes pause during initiation and termination of translation. Signal recognition particle, a small ribonucleoprotein required for the targeting of secretory proteins to the endoplasmic reticulum, halts translation at a natural pause site after the signal peptide has emerged from the ribosome (3,4). We have now used this assay to probe the mechanism by which the translation of preproinsulin mRNA is regulated in pancreatic islets. Our results indicate that preproinsulin translation is regulated at both the initiation and elongation levels.

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Beta Cell Destruction and Growth

CB 018 THE ROLE OF GROWTH HORMONE AND PROLACTIN IN THE GROWTH OF THE BETA CELL, Jens Høiriis Nielsen, Annette Møldrup, Nils Billestrup and Elisabeth D. Petersen, Hagedorn Research Laboratory, DK-2820 Gentofte, Denmark

Growth hormone (GH) prolactin (PRL) and placental lactogen (PL) were previously shown to stimulate both insulin production and proliferation of pancreatic islet cells from new-born rats (1,2). Both somatogenic and lactogenic receptors have been demonstrated on the rat insulinoma cell line RIN 5AH (3). The mechanism of action of these hormones on the insulin producing cells is, however, not known. By transfection of RIN 5AH cells with cDNA coding for the cloned liver GH-receptor we have demonstrated that the insulinotropic effect is mediated via this receptor. By using the incorporation of 5-bromo-deoxyuridine as a measure of the mitogenic effect in new-born rat beta cells, GH, PRL and PL were found to exert the same maximal effect. Treatment of rats with GH was also found to increase the mitotic activity of the beta cells in vivo. In the contrast to other cells, the effect of GH on the beta cell seems not to be mediated by insulin-like growth factor I (IGF-I), as shown by the absence of IGF-I mRNA in GH-stimulated islet cells as well as the lack of effect of antibodies to IGF-I on the mitogenic effect of GH. Furthermore, IGF-1 did not influence nor mimick the effect of GH. The glucose induced insulin release exhibited a monophasic response during GH-stimulation, whereas a biphasic response was recovered one week after removal of GH from the cultured islet cells. These results suggest that GH, PRL and PL have a direct mitogenic effect on the pancreatic beta cell which is not mediated via IGF-I, and that these hormones in vivo play a crucial role in the growth of the beta cell mass during periods with increased demand for insulin, e.g. adolescence and pregnancy. Lack of such compensatory growth may lead to development of impaired glucose tolerance, insulin resistance and/or diabetes.

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CB 019 ISLET CELL DESTRUCTION AND REGENERATION IN IFN-γ TRANSGENIC MICE. Nora Sarvetnick, Department of Neuropharmacology BCR-1, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, California, 92037

IFN-γ is produced in response to infection and has immune-stimulatory and proliferative activities. To investigate the potential role of IFN-γ in inflammatory autoimmune diseases, transgenic mice expressing IFN-γ in pancreatic beta cells were created[1]. These mice became diabetic following the appearance of increasing numbers of lymphocytes within and surrounding the islets. Backcross experiments with SCID immunodeficient mice have demonstrated that these lymphocytes are causative agents in the observed beta cell loss[2]. Grafting experiments and *in vitro* CTL assays indicate that these transgenic mice have become immunologically sensitized to normal islets[2,3]. Interestingly, a secondary proliferative/regenerative response opposes the lymphocyte destruction. This is initiated by duct cell proliferation and with the appearance of more primitive neuroendocrine progenitor cells along the apical regions of the ducts. The regenerative process in the ins-IFN-γ transgenic mice appears similar to the events that occur during embryonic islet cell development. However some abnormal structures and and unusual cell types accumulate as these "lesions" progress. The potential stimulus for this proliferative/regenerative response will be discussed. These studies underscore the lymphokine's ability to initiate a complex "transdifferentiation" pathway within a terminally differentiated structure.

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The Glucose Transporter: Structure and Function (joint)

CB 020 THE GLUCOSE TRANSPORTER FAMILY. Graeme Bell, Howard Hughes Medical Institute, University of Chicago, Chicago, IL 60637 The oxidation of glucose represents a major source of metabolic energy for mammalian cells. However, because the plasma membrane is impermeable to polar molecules such as glucose, the cellular uptake of this important nutrient is accomplished by plasma membrane-associated carrier proteins that bind and transfer it across the lipid bilayer. Two classes of glucose carriers have been described in mammalian cells (Table 1): Na^+-dependent glucose transporters and facilitative glucose transporters. Sodium- dependent glucose transporters are expressed in the small intestine and kidney where they are responsible for the dietary absorption of glucose and its reabsorption from forming urine, respectively. By contrast, facilitative glucose transporters are widely distributed in mammalian tissues and present on the surface of most if not all cells. A family of five structurally-related and functional facilitative glucose transporters have been described (Table 1) as well as a glucose transporter pseudogene. Each facilitative glucose transporter isoform has a distinct pattern of expression. The amino acid sequences of the facilitative and Na^+-dependent glucose transporters are structurally unrelated. The diversity of glucose transporters expressed in mammalian tissues contributes to the precise regulation of glucose uptake under varying physiological conditions.

Table 1. Human glucose transporters

Isoform	Major sites of expression
A. Facilitative glucose transporters	
1. GLUT1/Erythrocyte-HepG2	Ubiquitous; abundant in fetal tissues, placenta and adult brain, kidney and colon
2. GLUT2/Liver	Liver, pancreatic ß cell, kidney and small intestine
3. GLUT3/Brain	Ubiquitous; most abundant in brain
4. GLUT4/Muscle–fat	Muscle (skeletal and cardiac) and adipose (brown and white) tissue
5. GLUT5/Small intestine	Small intestine (jejunum)
6. GLUT3P1 (formerly GLUT6)	Pseudogene-like sequence related to GLUT3
B. Sodium-dependent glucose transporters	
1. SGLT1 (Na^+/glucose cotransporter)	Absorptive epithelial cells of small intestine and kidney

CB 021 OVERVIEW OF ALTERATIONS IN INSULIN ACTION IN HUMAN DIABETES. José F. Caro, Madhur K. Sinha, Walter J. Pories, Hisham A. Barakat, Prabhaker G. Khazanie, G. Lynis Dohm, Departments of Medicine, Surgery, Clinical Pathology, and Biochemistry, School of Medicine, East Carolina University, Greenville, NC 27858.

Insulin resistance and diminished insulin secretion are present in all patients with type II diabetes. One of the metabolic consequences of these alterations is hyperglycemia due to increased glucose production by the liver and decreased glucose utilization by skeletal muscle and adipose tissue.

In order to understand the mechanism(s) of insulin resistance at the cellular level, we have developed methods to isolate human hepatocytes, adipocytes and muscle fibers from morbidly obese patients with and without type II diabetes undergoing gastric bypass surgery.

It is clear that an abnormality in the coupling between insulin and insulin receptor or in the abundance of insulin receptor protein are not responsible for insulin resistance in type II diabetes. However, the ability of insulin to stimulate insulin receptor tyrosine kinase is markedly impaired in the liver, muscle and adipose tissue from these patients resulting in decreased tyrosine phosphorylated endogenous substrates. Furthermore, a novel pathway of "cross-talk" between the insulin receptor and Gi proteins, which seems to be independent of tyrosine kinase activation, is impaired in type II diabetes albeit $Gi_{1\text{-}2\alpha}$ immunodetection is normal and $Gi_{3\alpha}$ is only slightly decreased. Therefore, these two separate pathways of insulin signalling are abnormal in type II diabetes and likely responsible for insulin resistance.

Additionally, abnormalities in the expression of the facilitative glucose transporters family are present in type II diabetes. In adipose tissue GLUT-4 protein is decreased by ~ 20% in obesity and by over 50% in diabetes qualitatively consistent with the alterations in insulin stimulated glucose transport. In skeletal muscle the decrease in GLUT-4 is ~ 20% and identical in obese patients with or without type II diabetes suggesting that additional defects in translocation and/or activation must be present in this tissue.

Liver GLUT-2 protein and mRNA are similar in obese patients with or without diabetes. However, obese patients with impaired glucose tolerance who presumably represent a pre-diabetic state have dramatic decrease in GLUT-2 protein and mRNA. If in the liver GLUT-2 serves as a glucose sensor, a decrease in GLUT-2 may result in an erroneous homeostatic increase in glucose production initiating a cascade of events that lead to the end stage hyperglycemic syndrome recognized today as type II diabetes. Once the full-blown diabetic syndrome is established, it is impossible to determine which of the target tissues and beta cell defects, if any, are primary. Since a genetic marker for type II diabetes is not available yet, detailed clinical investigation of subjects with impaired glucose tolerance may allow understanding type II diabetes before the complex end stage hyperglycemic syndrome becomes established.

CB 022 MECHANISMS OF GLUCOSE TRANSPORT REGULATION: ROLE OF PROTEIN KINASE C I.A. Simpson, S.W. Cushman, J.J. Egan, W. Heath, C. Londos, H. Nishimura, J. Saltis. Eli Lilly Co., Indianapolis, In., and NIDDK, NIH, Bethesda, Md.

Activation of protein kinase C is associated with an increase in glucose transport activity in a variety of different cell types. Insulin, vasopressin, epinephrine and bombesin elicit a comparable stimulation of protein kinase C activity in rat adipose cells although these C-kinase activities may be associated with different isoenzymes. Glucose transport activity is stimulated 3-4-fold by phorbol 12-myristate 13-acetate (PMA) as well as all of these hormones, except for insulin which induces a 40-fold stimulation. In addition to magnitude, several other distinctions can be made between the transport responses to insulin and the other hormones. The time course for activation by insulin is much more rapid than for the other hormones ($t_{1/2} \approx 2$ min. cf $t_{1/2} \approx 8$ min.). Glucose transporter concentration in plasma membranes, as measured by cytochalasin B binding, is increased 6-fold by insulin as compared to a 3-fold increase in response to PMA and other hormones. In addition, the relative levels of GLUT1 and GLUT4 isoforms that are translocated to the plasma membrane are markedly different. Whereas, the translocation of GLUT1 is similar in both cases insulin induces a 2.5-fold greater increase in GLUT4 translocation. These observations confirm that GLUT4 is the primary transporter in rat adipose cells and also suggest that GLUT4 is innately more active than GLUT1. The likelihood that different mechanisms are responsible for these transport responses is supported by the distinct effects induced by catecholamines (R_s) and adenosine (R_i) on the responses. Whereas these agents modulate the intrinsic activity of the insulin mediated response they alter the translocation response to the other hormones. Although the mechanisms are clearly distinct they are also interactive, as preincubation with PMA both accelerates and augments the actions of insulin. Whether these distinctions reflect the involvement of different isoenzymes of protein kinase C or are due to completely unrelated mechanisms remains to be determined.

Glucose Transporter Translocation and Activation

CB 023 GLUCOSE TRANSPORTERS AND INSULIN RESISTANCE IN TYPE II DIABETES MELLITUS, W. Timothy Garvey, Department of Medicine, Indiana University, VA Hospital 111-E, 1481 West 10th Street, Indianapolis, IN 46202. Insulin resistance contributes to hyperglycemia in NIDDM, and has been shown to be due, at least in part, to impaired stimulation of the glucose transport system in isolated adipocytes and muscle explants. To better understand the biochemical basis of insulin resistance, we have examined the role of facilitative glucose transporter proteins (GLUT) in both adipose and muscle tissues. In isolated adipocytes, we employed the cytochalasin B binding assay, a chemical affinity method which measures all GLUT isoforms, and demonstrated cellular depletion of transporters in NIDDM and obesity. Since the GLUT4 isoform appears to mediate the major portion of insulin-stimulated glucose uptake, we then specifically assessed GLUT4 gene expression. The number of GLUT4 in adipocytes was measured in total post-nuclear and subcellular membrane fractions on Western blots, and levels of encoding mRNA were quantitated on Northern blots. Relative to lean controls, the cellular content of GLUT4 was decreased 40% in obesity and 85% in NIDDM in total cellular membranes, and corresponding decrements were observed in cellular levels of GLUT4 mRNA. In obesity, cellular depletion of GLUT4 primarily involved low-density microsomes (LDM) leaving fewer transporters available for insulin-mediated recruitment to the plasma membrane (PM). In NIDDM, GLUT4 loss was profound in all membrane subfractions (PM, LDM, and HDM). The relative decrements in GLUT4 could totally explain decreased glucose transport rates and cytochalasin B binding sites in these cells. Patients with Gestational Diabetes were also studied; omental adipocytes were obtained at term Caesarian section and found to be markedly depleted in GLUT4 protein and mRNA compared to that observed in term non-diabetic pregnancies.

Studies were also conducted in skeletal muscle, the most important tissue for insulin-mediated glucose uptake. In post-nuclear membrane fractions from *vastus lateralis* biopsies, GLUT4 content (relative units per mg protein) was similar among lean (1.0± .2), obese (1.5 ± .3), NIDDM (1.2 ± .2), and IGT (1.4 ± .2) subgroups (p=NS). GLUT4 mRNA levels were also similar. Because muscle fibers (types I & II) exhibit different capacities for insulin-mediated glucose uptake, we tested whether a change in fiber composition could cause insulin resistance without altering overall GLUT4. We found that quantities of fiber-specific isoenzymes (phospholamban, and types I & II Ca^{++}ATPase and calsequestrin) were similar in all subject groups, as were levels of GLUT4 normalized for amounts of these isoenzymes. In further studies, a period of intensive insulin therapy in NIDDM was found to ameliorate insulin resistance, as evidence by a 78% increase in maximal insulin-stimulated glucose uptake during euglycemic glucose clamps, without altering muscle GLUT4 content.

To the extent that GLUT4 regulation in *vastus lateralis* is representative, insulin resistance in muscle may involve impaired GLUT4 function and/or translocation, and not transporter depletion (as is observed in adipocytes). However, we have obtained data in streptozotocin-induced diabetic rats which suggests that GLUT4 could be regulated differentially in muscle groups which differ in fiber composition. Diabetes-induced suppression of GLUT4 was most marked in *soleus* (type I fibers), intermediate in *gastrocnemius* (mixed, type IIa>IIb), and did not occur in *vastus lateralis* (IIb>IIa).

In conclusion: 1) In adipocytes, pretranslational suppression of GLUT4 is an important mechanism of insulin resistance in NIDDM, Gestational Diabetes, and obesity; 2) Overall GLUT4 expression in *vastus lateralis* is normal in NIDDM and obesity; 3) Further studies are needed in muscle to test the hypotheses that GLUT4 expression in diabetes is muscle- or fiber-specific, or that abnormalities in GLUT4 function and/or translocation impair glucose transport.

CB 024 REGULATION OF GLUCOSE TRANSPORTERS IN MUSCLE CELLS: BIOSYNTHESIS AND SUBCELLULAR DISTRIBUTION. Amira Klip, Philip Bilan, Dimitrios Dimitrakoudis, Toolsie Ramlal, Elena Burdett, Ulla-Maija Koivisto and Mladen Vranic. The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8.

Skeletal muscle is the main tissue responsible for glucose utilization in the fed state. Under most conditions, glucose transport across the cell membrane is the rate limiting step in glucose utilization by this tissue, and is regulated both acutely and chronically by insulin and glucose. The precise molecular events underlying such regulation begin to be unravelled, based on new approaches to fractionate skeletal muscle and to quantitate glucose transporters (GTs). Acute (30 min) exposure to insulin, whether in vivo or in the perfused hindquarter in the rat, causes translocation of GTs from an intracellular membrane (IM) organelle to the plasma membrane (PM). This organelle is neither the sarcoplasmic reticulum (cisternae or longitudinal tubes) nor the transverse tubules. The translocation is specific for the GLUT-4 GT, while the GLUT-1 GT resides almost exclusively in the PM in the basal and insulin-stimulated states. Mild diabetes, with normal fasting insulinemia but elevated glycemia, lowers the amount of GLUT-4 GT and increases GLUT-1 GT in the PM, while decreasing glucose uptake. In these diabetic rats, GLUT-4 GTs still translocate from the IM to the PM. Although the total number of GTs present in the IM is lower, the amount of GTs leaving this compartment in response to insulin is the same for diabetic than for control rat muscles. Correction of glycemia with phlorizin injections results in recovery of GLUT-4 GT levels in the PM but not in the IM, suggesting that the number of GTs in PM is under specific control of ambient glucose concentrations.

In order to study the regulation of GTs by glucose or insulin independently, cells of the L6 skeletal muscle line were exposed to each agent, and 2-deoxyglucose uptake was determined. (1) These cells express both the GLUT-1 and GLUT-4 GTs, but the ratio of GLUT-1/GLUT-4 is higher than in adult rat skeletal muscle. Acute (<1h) exposure of L6 cells to insulin or IGF-1 caused and increase in hexose uptake accompanied by translocation of GTs, both GLUT-1 and GLUT-4, from IM to PM. (2) Chronic (24 h) exposure to insulin elevated hexose transport, and this increase was totally protein synthesis-*dependent*. This was accompanied by an increase in the amount of both GLUT-4 and GLUT-1 GTs in the PM, an increase in GLUT-1 and a decrease in GLUT-4 in the IM, and a corresponding increase in the cellular levels of GLUT-1 mRNA and small decrease in GLUT-4 mRNA. This suggests that over 24 h insulin stimulates hexose transport primarily through an increased synthesis of GLUT-1 GTs, and perhaps also through a protein synthesis-dependent translocation of GLUT-4 GTs. (3) The effect of high glucose (25 mM) was compared to that of glucose deprivation (in presence of 25 mM xylose) over a 24 h period. Glucose deprivation caused an increase in hexose transport that was protein synthesis-*independent*, accompanied by increased amounts of GLUT-1 and GLUT-4 GTs in the PM, and increased GLUT-1 mRNA but not GLUT-4 mRNA levels. This suggests that the elevated GLUT-1 mRNA levels do not contribute to the stimulation of hexose transport by 24 h of glucose deprivation, but rather that the translocation of GTs to the PM plays a major role.

In conclusion, the subcellular distribution of GTs appears to be under the control of glucose and insulin, independently of the regulation of GT gene expression.

Insulin, Diabetes and Gene Regulation

CB 025 CLONING AND CHARACTERIZATION OF AN INSULIN SENSITIVE DNA BINDING PROTEIN THAT REGUALTES GAPDH GENE EXPRESSION X.F. Kong, Nargis Nasrin, Isabelle Dugast , M. Denaro, P. Banerjee, B. Kahn and M. Alexander-Bridges, Mass. General Hospital, HHMI Boston, MA 02114

We have previously shown that the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA is markedly induced by insulin in cultured 3T3-L1 adipocytes and in the fat and liver of rats fasted and refed a high-carbohydrate, low-fat diet. The human GAPDH gene contains two independent elements, IRE-A and IRE-B, which can confer an inductive effect of insulin on CAT gene expression in transiently transfected H35 hepatoma cell lines. Binding of a nuclear protein to the IRE A is increased 3- to 4-fold in insulin-stimulated 3T3 adipocytes and in nuclei isolated from the liver of fasted- refed rats(1).

During the induction of diabetes GAPDH mRNA levels are regulated in the fat, but not the muscle, of diabetic rats treated with insulin. Nuclear extracts prepared from control, diabetic and insulin-treated diabetic rats show a marked increase in the activity of the IRE-A DNA binding protein. The the protein is not detected in the nuclear extracts of muscle.

To define the molecular mechanisms involved in these events we have used the Singh-Sharp Southwestern technique to screen a rat adipocyte library and detect a fusion protein that specifically binds the IRE-A motif. This cDNA interacts with a mRNA 7 kb in size that is regulated when 3T3 adipocytes are exposed to insulin overnight and during the process of fasting and refeeding. This clone is expressed in fat and liver, not in muscle. This finding accounts in part for the tissue specificity of the hormone response of GAPDH to insulin. We have isolated and partially sequenced a human gene that shares a highly conserved domain with the rat cDNA. This region encodes a novel DNA binding motif.

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CB 026 TISSUE-SPECIFIC EXPRESSION AND REGULATION OF THE GLUCOKINASE GENE, Mark A. Magnuson, Departments of Molecular Physiology and Biophysics and of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232. Glucokinase (GK) is expressed in both the hepatocyte and the pancreatic β cell and plays a key role in the metabolism of glucose by both cell types. The expression of GK is differentially regulated: hepatic GK is stimulated by insulin and inhibited by cAMP whereas islet GK is stimulated by glucose. Our studies have been directed towards understanding the molecular basis for the tissue-specific expression and differential regulation of this gene. Characterization of the single copy GK gene has shown that it contains two different transcription control regions; the upstream control region is active in the β cell while the downstream control region is active in liver. In this presentation I will discuss our most recent findings regarding the cell type-specific expression of this gene in the pancreatic β cell. To identify the *cis*-acting elements and *trans*-acting factors which regulate expression of the gene in the β cell, a detailed fusion gene analysis of the upstream transcription control region was performed. We found that a relatively small DNA fragment (-280 to +14 bp relative to +1 of transcription) contained all the necessary sequence information for efficient expression in permanent insulinoma cell lines. Within this 294 bp DNA fragment are two copies of the sequence motif TGGTCACCA and three other sequences that are similar at greater than 5 of 9 bp. Mutagenesis of these sequence motifs is detrimental to transcriptional activity in β-TC-1 cells. The dyad symmetry of this element suggests it is a binding site for a factor (presumably a dimer) which stimulates GK gene expression in the β cell. Gel mobility shift experiments support this notion since we have detected proteins in insulinoma cells lines that bind to the TGGTCACCA motif. Studies to characterize this binding activity are currently underway. Expression of the GK gene is also affected at a post-transcriptional level by alternate RNA splicing of the GK pre-RNA. Hepatic and islet GK differ by 15 amino acids at the N-terminus due to the alternative splicing of tissue-specific first exon sequences. In addition, the use of an alternate splice acceptor site in the 4th exon of the gene has been detected in insulinoma and cultured islets. The effect of this alternate splicing event is to delete 51 bases from the mRNA and remove 17 amino acids from the protein generated. Measurement of the ability of the different islet GK isoforms to phosphorylate glucose indicates that the protein missing 17 amino acids is enzymatically inactive. The role of alternate RNA splicing in regulating expression of GK activity in the islet remains uncertain but could be a mechanism for modulating gene product activity.

Late Abstract

STRUCTURE AND FUNCTION OF THE GLUCOSE TRANSPORTER, Mike Mueckler, Juan Garcia, Marilyn Strube, and Konrad Keller*, Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110 and *Institute of Pharmacology, Free University of Berlin, D-1000 Berlin 33, Germany

Facilitative glucose transport in the human is mediated by at least five homologous transporter isoforms. All five are predicted to possess nearly identical 2-dimensional membrane topologies and are likely to have very similar tertiary structures. The transporters are predicted to span the membrane in the form of 12 α-helical segments. Five of the twelve transmembrane segments are capable of forming amphipathic helixes. We have proposed a simple model in which amphipathic helixes cluster to form a thermodynamically stable aqueous pore through the lipid bilayer. According to the model, the walls of the hydrated channel are lined with hydroxyl and amide-containing side chains that form the substrate-binding sites by contributing hydrogen bond donors and acceptors to the hydroxyl groups of the sugar.

We are investigating the structure/function relationships of the GLUT 1 transporter by using site-directed mutagenesis in conjunction with expression in Xenopus laevis oocytes. The human GLUT 1 transporter exhibits a Km of 21 mM and a turnover of 2,000/sec for 3-O-methylglucose under equilibrium exchange conditions when expressed in Xenopus oocytes. The Km decreases to ~10 mM under zero trans influx conditions, suggesting that the GLUT 1 transporter exhibits accelerated exchange in oocytes and thus behaves in a similar fashion as the human erythrocyte transporter. Several amino acid positions have been identified that appear to be involved in transport function. Amino acid substitutions at Trp-388 and Trp-412 inhibit transport activity. These two residues represent possible sites of covalent photolabeling of the transporter by cytochalasin B, which is believed to inhibit transport by binding to the inward-facing substrate-binding site of the transporter. Mutations at these sites do not appear to affect equilibrium binding of cytochalasin B to the transporter. Substitutions at Asn-411 and Gly-332 also disrupt transport function. Asn-411 may line the proposed aqueous tunnel and participate in hydrogen-bonding with glucose. Gly-332 is a highly conserved residue lying in the cytoplasmic linker domain connecting transmembrane segments 8 and 9. It forms part of a pentameric amino acid motif that is conserved in many members of the Transporter Superfamily, and may play an important role in the formation of a basic transporter-type folding pattern.

Insulin Receptors

CB 100 LOCALIZATION AND ALTERATION OF LIGAND-SPECIFICITY OF THE INSULIN AND IGF-I RECEPTORS, Asser S. Andersen, Thomas Kjeldsen, Finn C. Wiberg, Jesper Skou Rasmussen, Karin Bach Møller and Niels Peter H. Møller, Molecular Genetics, Bioscience, Corporate Research, Novo Nordisk A/S, DK-2880 Bagsværd, Denmark.

The insulin and IGF-I receptors, like their respective ligands, are highly homologous proteins. Each receptor binds, but only weakly, the ligand for the other receptor. In an attempt to define what regions of the receptors are involved in determining the ligand specificity, expression vectors encoding soluble chimeric insulin/IGF-I receptors were prepared. The chimeras were expressed in Baby Hamster Kidney cells and partially purified before characterization. We have previously shown, that a chimera with the N-terminal 283 amino acids of the IGF-I receptor replacing the corresponding sequence of the insulin receptor (encoded by exon 1,2 and 3) bound ligand with the characteristics of a native IGF-I receptor (i.e. high affinity for IGF-I and low affinity for insulin). Additional chimeras within this region, show that the 68 N-terminal amino acids of the insulin receptor are involved in high affinity binding of insulin, whereas the cysteine rich domain of the IGF-I receptor seems to confer high affinity for IGF-I. We propose that these to regions are part of a common ligand binding site and involved in determining the ligand specificity of this receptor family.

CB 101 OVEREXPRESSION OF THE HUMAN INSULIN RECEPTOR IN A BACULOVIRUS SYSTEM K. Baltensperger, C.W. Woon, P. Vissavaijhala*, A.H. Ross* and M.P. Czech, Dept. of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, MA 01605, and *Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545

The baculovirus-directed expression of the entire human insulin receptor (bHIR) protein allows us to produce milligram quantities of recombinant bHIR. In contrast to a recent report we used a novel transfer vector (pVL1393), which leads to nonfused protein expression. In spinner cultures, maximum expression of bHIR occurs at about 72 hrs post infection and yields approximately 10^6 cell-surface insulin binding sites per cell. Our data indicate that the bHIR is only partially cleaved to the heterotetrameric form. When ^{125}I-insulin was crosslinked to cell surface receptors of infected cells or to total cell lysates, the heterotetramer but not the uncleaved proreceptor could be detected in immunoprecipitates obtained with the anti-receptor monoclonal antibody CT-1. The antibody precipitates both the proreceptor and the heterotetramer as shown by precipitation of [^{35}S]-methionine metabolically labelled receptors. Despite insulin binding to the cleaved receptor, no insulin-stimulation of isolated bHIR autophosphorylation could be demonstrated. However, bHIR phosphorylation in ^{32}P-labelled cells was sligthly stimulated by insulin. Interestingly, phosphoamino acid analysis of the *in vivo* phosphorylated bHIR reveals a high phosphoserine-content (40-50% of total phosphoamino acid content) as well as phosphotyrosine in both receptor forms. The data show that both forms of bHIR are substrates for serine kinases in Sf9 cells. Identification of the involved kinases and the phosphorylation sites on the bHIR could add new information about regulatory mechanisms affecting the insulin receptor tyrosine kinase.

CB 102 INCREASED MITOGENIC POTENCY OF HIGH AFFINITY INSULIN ANALOGUES IN MOUSE NIH 3T3 FIBROBLASTS, Steen Gammeltoft and Kirsten Drejer, Department of Clinical Chemistry, Bispebjerg Hospital, Copenhagen and NOVO-Nordisk, Bagsværd, Denmark.

The receptor binding and mitogenic effect of 3 monomeric insulin analogues were determined in mouse NIH 3T3 fibroblasts. These cells express IGF-I receptors whereas insulin receptors are not detectable, and IGF-I bound with 700 times higher affinity than insulin (Ki 0.6 nM vs. 0.4 μM). The insulin analogues [B10Asp]insulin and [A8His, B4His, B10Glu, B27His]insulin bound to the IGF-I receptor with 3 and 7 fold greater affinity relative to insulin and [B9Asp, B27His]insulin was 0.2 fold as potent. These relative affinities correlate with the relative potencies in the free fat cell lipogenesis and hepatoma HepG2 cell binding assays. IGF-I stimulated DNA synthesis with 400 times higher potency than insulin (ED_{50} 0.25 nM vs 0.1 μM). [B10Asp]insulin and [A8His, B4His, B10Glu, B27His]insulin were 20 and 400 fold more potent than insulin as stimulants of thymidine incorporation, whereas [B9Asp, B27His]insulin was 0.5 fold as potent. The rates of degradation of ^{125}I-labelled insulin and analogues during the assay of thymidine incorporation (24h at 37°C) were identical. It is concluded that amino substitutions in insulin which results in increased receptor binding affinity lead to a disproportionately enhanced mitogenicity compared with metabolic potency. This may result from a slower dissociation rate of the analogues from the IGF-I receptor compared with insulin leading to an increased mitogenic signal.

CB 103 INDUCED MEMBRANE EXPRESSION OF INSULIN RECEPTORS ON LYMPHOCYTES IS REGULATED BY A NOVEL POST TRANSLATIONAL MECHANISM David W. Goodman and Peter C. Isakson, Department of Pharmacology, University of Virginia, Charlottesville, Va 22908

Activation of resting lymphocytes results in the appearance of insulin receptors on the cell surface. To establish the molecular basis for inducible receptor expression on lymphocytes, we assessed insulin receptor mRNA levels in resting and Con A activated rodent splenocytes. Quantitative analysis using a ribonuclease protection assay indicated that equivalent amounts of insulin receptor mRNA were expressed in resting and activated T lymphocytes, and Northern blots of RNA from both cell types showed equivalent expression of the two major insulin receptor mRNA species. This suggests that the increased membrane expression of insulin receptors on lymphoblasts is not due to enhanced accumulation of insulin receptor mRNA. To determine whether resting T cells contained insulin receptors that are not expressed on the cell surface, ^{125}I insulin binding assays were performed on Triton X-100 solubilized cells. In the presence of detergent, equivalent numbers of insulin binding sites were found in both resting and Con A activated T cells. In contrast, intact resting T cells expressed less than 10% of the insulin binding sites found on activated T cells. Detergent solubilized receptors from resting lymphocytes showed both high and low affinity binding sites by Scatchard analysis, and approximately 1/3 of the receptors bound to lectin affinity columns (Con A and wheat germ agglutinin). These data suggest that resting lymphocytes synthesize mature insulin receptors but fail to transport them to the cell surface. Lymphocyte activation may allow membrane expression of insulin receptors by facilitating transport via a novel undefined mechanism.

CB 104 THE CYSTEINE-RICH DOMAINS OF THE INSULIN AND INSULIN-LIKE GROWTH FACTOR I (IGFI) RECEPTORS ARE PRIMARY DETERMINANTS OF HORMONE BINDING SPECIFICITY: EVIDENCE FROM CHIMERIC RECEPTORS, Thomas A. Gustafson* and William J. Rutter, Hormone Research Institute, University of California, San Francisco, CA 94143-0534 and * Current address: Department of Physiology, University of Maryland School of Medcine, 655 West Baltimore Street, Baltimore, MD 21201.

To delineate the the structural determinants of the insulin receptor (IR) and insulin like growth factor I receptor (IGFIR) which affect hormone binding specificity we have constructed seven chimeric receptor cDNAs and stably expressed them in CHO cells. Clonal cell lines expressing high levels of each receptor chimera were analyzed for insulin and IGFI binding activity. Measurements of tracer hormone binding and immunoprecipitation of metabolically labelled receptors showed that all chimeras were properly processed and expressed at the cell surface. The binding data indicate that 56 amino acids of the IR and 52 amino acids of the IGFIR located in corresponding regions of the cysteine-rich domains are the primary determinants of hormone binding specificity. These regions are located between amino acids Asn 230 and Ile 285 on the IR and between His 223 and Met 274 on the IGFIR. In addition, the αIR-3 antibody, which competes for IGFI binding, was found to interact with the same 52 amino acids of the IGFIR which determines hormone specificity. Other antibodies which interfere with insulin binding (5D9, MC51, and MA20) interact with epitopes in the C-terminal 288 amino acids of the α subunit. We conclude that 56 and 52 amino acids of the cysteine-rich domains of the IR and IGFIR contain the major determinants of hormone binding specificity although other, more C-terminal regions of both receptors contribute to hormone binding. Lastly, we have examined the chimeric receptors' ability to phosphorylate their β subunits and cellular substrates *in vivo* and present data suggesting that although the efficiency of signal transduction varies with each chimera in most cases the receptors retain the ability to transduce a signal.

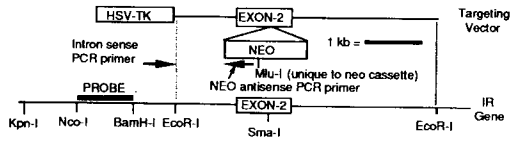
CB 105 PURIFICATION OF THE Mr 160,000 PHOSPHOTYROSYL PROTEIN, A PUTATIVE MEDIATOR OF INSULIN ACTION. Susanne R.Keller, Kouichiro Kitagawa, Ruedi Aebersold*, Gustav E.Lienhard, and Charles W.Garner#. Dept.of Biochemistry, Dartmouth Med. Sch., Hanover, NH 03756, *Biomedical Research Center, University of British Columbia, Vancouver, Canada V6T 1W5, # Dept. of Biochemistry and Molecular Biology, Sch. of Med., Texas Tech University, Lubbock TX 79430.

Signaling from the insulin receptor is thought to proceed by the phosphorylation of target proteins on tyrosine. In many different cell types a protein of MW 160,000 to 185,000 (pp160) is the most abundant phosphotyrosyl protein, along with the insulin receptor itself, detected after exposure of cells to insulin. We developed a procedure for the isolation of pp160 from insulin-treated 3T3-L1 adipocytes: pp160 was adsorbed from the soluble proteins of the cell extract with a monoclonal antibody against phosphotyrosine (1G2) and eluted with phenyl phosphate; SDS PAGE of the eluate yielded pure pp160. 30 μg of pp160 was purified from 1000 10-cm plates (equivalent to 10^{10}cells) and the aminoacid sequences of several peptides were obtained. An antipeptide antibody was used for immunoblotting of 3T3-L1 adipocyte cell lysates, with purified pp160 as standard. These results showed that the amount of pp160 is about 250,000 copies per cell and is thus in a ratio of 1 to 1 with the insulin receptor, and that 80% of cellular pp160 is phosphorylated on tyrosine in response to insulin. The aminoacid sequences are now being used as the basis for cloning the cDNA encoding pp160.

CB 106 INSULIN AND IGFs STIMULATE RECEPTOR AUTOPHOSPHORYLATION AND TYROSINE PHOSPHORYLATION OF A 70 kDa SUBSTRATE IN INTACT FETAL CHICK NEURONS, Kenner, K.A. and Heidenreich, K.A. Dept of Medicine, University of California, San Diego, La Jolla, CA 92093
In this study we examined the effects of insulin, IGF-I, and IGF-II on tyrosine phosphorylation in intact cultured fetal chick neurons. Ligand binding curves demonstrated the presence of both insulin receptors (6,000 high affinity sites/cell) and IGF-I receptors (45,000 sites/cell). After 5 days in culture, brief exposure to insulin, IGF-I, or IGF-II increased the tyrosine phosphorylation of an 87 kDa membrane-associated protein in a rapid, dose-dependent manner. Comparison of the dose-response curves for insulin and IGF-I suggested that each peptide stimulated autophosphorylation of its own receptor β-subunit: the maximal response evoked by IGF-I was approximately 10-fold higher than by insulin (3-5 fold), consistent with the larger number of IGF-I receptors in these cells. The dose response data for IGF-II suggested that it could act through either receptor type. Changes in the tyrosine phosphorylation of other proteins were not detected in neurons cultured for 5 days, however neurons cultured only 3-5 hours contained a predominant 70 kDa protein that was also phosphorylated on tyrosine in response to all three hormones (pp70). Brief hormone exposure had maximal effects on the tyrosine phosphorylation of pp70 after 2-5 hours in culture, and was not detected in neurons cultured longer than 24 hours. The time course and dose dependence of pp70 phosphorylation paralleled that of insulin and IGF-I receptor autophosphorylation. These data suggest that both insulin and IGF-I receptors are active tyrosine kinases in fetal neurons, and that pp70 represents an endogenous substrate for the insulin and IGF-I receptor kinases in neurons.

CB 107 PROTEOLYTIC PROCESSING OF THE BETA SUBUNIT OF THE MATURE INSULIN RECEPTOR. Victoria P. Knutson, Department of Pharmacology, The University of Texas Medical School at Houston, Houston, TX 77225.
The addition of insulin to tissues and intact cells results in the time-dependent loss of insulin binding activity, in a process referred to as down regulation. We have previously found this process to involve at least two discreet steps: The rapid ($t_{½} \approx 10$ min) translocation of insulin receptor from the plasma membrane to an intracellular site, followed by the slower ($t_{½} \approx 2$ h) intracellular inactivation of the receptor. Utilizing a monospecific polyclonal antibody generated against the carboxy terminus of the cytosolic domain (ß subunit) of the insulin receptor, we find that upon addition of insulin to the intact cells, the cytosolic domain of the receptor is lost from the cellular membranes with a time course that parallels the loss of insulin binding activity from the cells. The loss of the cytosolic receptor domain from the cellular membranes is accompanied by a corresponding increase in the production of antibody immunoreactivity in the cellular cytosol. Immunoblot analysis reveals that the cytosolic component, ß', has a molecular mass of approximately 61,000 Da. The insulin-induced generation of ß' can be inhibited by cycloheximide, leupeptin and the calpain inhibitor E-64, implicating protease activity in the production of the fragment. Preliminary evidence indicates that upon generation of ß', the insulin receptor α subunit can be found in the lumen of endosomal vesicles, indicating that the protease activity may be localized in the endosomes. With a mass of 61,000 Da, the intact tyrosine kinase domain of the insulin receptor may be present in the cytosolic fragment. Experiments are currently underway to investigate this possibility.

CB 108 INSULIN RECEPTOR GENE TARGETING WITH HOMOLOGOUS RECOMBINATION. Bradford B. Lowell, David E. Moller, Vedrana S-Susulic and Jeffrey S. Flier. Departments of Endocrinology and Medicine, Beth Israel Hospital, Boston, MA 02215.
Transfecting cDNA's encoding mutants of the insulin receptor (IR) into cultured cells has led to insight into IR structure-function relationships. A major limitation of these studies has been the lack of cells that have insulin sensitive processes but few or no endogenous IRs, the expression of which makes the analysis of mutant receptors difficult. We are attempting to generate a 3T3 adipocyte cell line without functional IR genes by homologous recombination. We have cloned 18 kb of the mouse IR gene surrounding exon 2 and have constructed the targeting vector shown below. The second exon is interrupted at a unique Sma-I site via insertion of a neomycin (NEO) resistance cassette (pMC1NEOpolyA) which disrupts the IR gene and permits positive selection for cells with an integrated construct. The HSV thymidine kinase expression vector (HSV-TK, provided by Cappechi et al), which sensitizes cells to the lethal effects of ganciclovir (GAN), is at the 5' end for negative selection. In random integrations, the entire construct will insert into the genome making such cells NEO-R, GAN-S. In specific recombinations, the HSV-TK will be lost and the cells will be NEO-R, GAN-R. Successful recombinants will be identified with the PCR primers shown below with successful recombination resulting in an amplified product of 1.6 kb. PCR positive clones will be confirmed via Southern blotting using Kpn-I and Mlu-I digested genomic DNA probed with the 1.0 kb Nco-I - BamH1 fragment shown below. Following a single allele knockout, a second allele will be targeted using the same vector in which hygromycin resistance is inserted into exon 2. Due to the heteroploid nature of this cell line, we expect most cells with 2 disrupted alleles to retain additional functional IR genes. These cells will be purged using an insulin-diphtheria toxin chimera which will kill all cells still bearing IRs but be nontoxic to cells without receptors. We will report on the results of our current positive-negative selection experiments.



CB 109 **EXPRESSION VECTOR FOR HEPATOCYTE PLASMA MEMBRANE ECTO-ATPase: A POTENTIAL PHYSIOLOGICAL SUBSTRATE FOR PHOSPHORYLATION BY THE INSULIN RECEPTOR TYROSINE KINASE,** Sonia M. Najjar, Domenico Accili, and Simeon I. Taylor, Diabetes Branch, NIDDK, NIH, Bethesda, Maryland 20892

The insulin receptor possesses tyrosine-specific protein kinase activity that has been postulated to be crucial in mediating the intracellular actions of insulin. In addition to autophosphorylation activity, the insulin receptor phosphorylates many artificial and endogenous cellular proteins such as a 120 kDa rat liver plasma membrane glycoprotein (pp120). The amino acid sequence of immunoaffinity purified pp120 matched the deduced amino acid sequence of a hepatocyte plasma membrane ecto-ATPase. cDNA from rat liver mRNA was synthesized by reverse transcriptase and amplified by *Taq* DNA polymerase using 20-mer primers deduced from the ecto-ATPase nucleotide sequence. The obtained cDNA was then cloned into a pGEM-4Z plasmid, and the nucleotide sequence of the 1.6 kb insert was found to be identical to that of the coding region of the rat plasma liver ecto-ATPase.

In order to study the role of the phosphorylation of pp120 by insulin receptor tyrosine kinase in mediating insulin action, we constructed ecto-ATPase expression vectors for transient and stable transfections. The availability of these expression vectors for ecto-ATPase will enable us to directly test our hypothesis that ecto-ATPase is a substrate for phosphorylation by the insulin receptor.

CB 110 **PURIFICATION AND SEQUENCING OF PP185** Paul Rothenberg[1], William Lane[2], Xiao-Jian Sun[3], Morris White[3], and C.R. Kahn[3], [1]Dept. of Pathology, University of Pennsylvania School of Medicine, Philadelphia, PA, [2]Dept. of Biology, Harvard University, and [3]Joslin Diabetes Center, Harvard Medical School, Boston, MA.

Insulin causes tyrosine phosphorylation of an M_r=185 kDa protein (**pp185**) in diverse cell types. PP185 is a putative cytosolic substrate of the insulin receptor tyrosine kinase and pp185 is hypothesized to be an important intracellular transducer of the mitogenic and/or metabolic regulatory effects of insulin. However, the nature and function of pp185 is unknown. To identify pp185 directly, we purified pp185 from insulin-stimulated rat liver using a new, denaturation-based extraction procedure which blocks endogenous tissue phosphatases and thus allows a high-yield and facile, single-step isolation of phosphotyrosyl proteins (PYPs) by absorption to an immobilized anti-phosphotyrosine antibody matrix. Absorbed PYPs were eluted with pNPP and resolved by 1-D SDS PAGE. From 50 rat livers we recovered 100 picomoles of pp185 and also 340 picomoles of the 95 kDa insulin receptor β-subunit. Following transfer to nitrocellulose, pp185 was cleaved in situ with trypsin, and the resulting tryptic peptides separated on a narrow-bore reverse phase HPLC. Direct Edman degradation of 10 such internal pp185 peptides yielded novel amino acid sequences which are not identical to any reported protein or gene sequence. Thus, pp185 is a new, previously unknown protein. Based on pp185 sequence data, polyclonal anti-peptide antibodies were raised which specifically recognize a single 185 kDa, insulin-stimulated phosphotyrosyl protein in liver, skeletal muscle and adipose tissue, and also several cultured cell lines. Immunocytochemical localization of pp185 reveals a diffuse, predominantly cytoplasmic distribution in hepatocytes. Synthetic oligonucleotide probes based on the pp185 amino acid sequences have been used to successfully screen a rat liver cDNA library to obtain near full-length pp185 cDNA clones. The purification and sequencing of pp185 opens new avenues for investigating the structure and function of this receptor substrate and for further evaluating the role of tyrosine phosphorylation in the insulin signal transduction pathway.

CB 111 *Abstract Withdrawn*

CB 112 IMMUNOASSAYS FOR DETERMINING THE CONCENTRATION AND ACTIVATION STATE OF INSULIN-, IGF-I- AND HYBRID RECEPTORS IN HUMAN TISSUES.

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The aim of this work was to develop specific and sensitive assays for receptors in small samples of human tissues, in order to study the concentration and activation of receptors *in vivo* . A two-site assay format was used, in which samples were incubated with a pair of monoclonal antibodies recognizing distinct antigenic sites. One antibody was adsorbed to the wells of plastic microtitre trays (solid phase Ab) and the second antibody conjugated to alkaline phosphatase (labelled Ab). The labelled Ab bound to the well was quantified by a sensitive enzymatic cycling system, and the measured signal was proportional to antigen concentration. To assay total insulin receptors we used solid phase Ab CT-1 (directed against the carboxyterminus of the ß-subunit) and labelled Ab 83-14 (directed against the α-subunit). The detection limit of the assay was ~10^9 receptors (extract from ~10µg liver, ~100µg muscle or 10^3 NIH 3T3HIR transfected fibroblasts). The assay response was not influenced by insulin, added either to cells or within the assay . To assay activated insulin receptors we used solid phase Ab PY-22 (anti-phosphotyrosine) and labelled Ab 83-14. In this case the assay response showed the expected dependence on prior exposure of cells to insulin. We have also generated a number of antibodies to human IGF-I receptors, which will permit development of similar assays for these receptors and for insulin receptor/IGF-I receptor hybrids.

CB 113 THE ISOLATION OF A cDNA MOLECULE ENCODING THE INSULIN RECEPTOR SUBSTRATE "pp185", Sun, X.J., Rothenberg, P.A., Kahn, C.R., Goldstein,B.J.,Backer, J.M., Wilden, P.A., Aroki, E., Cahill, D.A.,Schroeder, G., and White, M.F., Joslin Diabetes Center, Boston MA 02215

The insulin receptor is a tyrosine kinase that undergoes autophosphorylation and activation during insulin binding. Insulin signal transmission may occur, at least partially, through tyrosine phosphorylation of cellular proteins; however, the structures and functions of cellular substrates have not been revealed. A phosphotyrosine-containing protein called "pp185" has been immunoprecipitated from many insulin-stimulated cell lines with anti-phosphotyrosine antibodies. The apparent molecular weight of this protein by SDS-PAGE ranges from 160 kDa in 3T3-L1 cells to 185 kDa in Fao hepatoma cells. Recently, a 175 kDa phosphoprotein with the characteristics of pp185 was partially purified from rat liver on immobilized antiphosphotyrosine antibodies. Partial amino acid sequence was determined from 6 tryptic peptides, and degenerate oligonucleotide probes constructed. One of these probes hybridized with a single clone in a lambdaZAP rat liver cDNA library containing a 3200 bp insert. Additional overlapping clones were isolated which determine a 5356 bp cDNA sequence that contains a 3705 bp open reading frame encoding a 131 kDa protein; the deduced amino acid sequence contains all six tryptic peptides obtained from the "pp185". Northern analysis with probes prepared from this cDNA hybridize with a 9.5 Kb mRNA isolated from rat liver, muscle, kidney, spleen and brain. In addition, polyclonal antipeptide antibodies prepared in rabbits with a 15 amino acid sequence of the cloned protein immunoprecipitated a 170 kDa phosphoserine-containing protein from [^{32}P]phosphate-labeled Chinese hamster ovary cells. Insulin immediately stimulates tyrosine phosphorylation of this protein in CHO cells which overexpress the wild-type human insulin receptor, but not in CHO cells expressing mutant insulin receptors. The deduced amino acid sequence is hydrophilic (average hydrophobicity = -0.7) and contains several putative tyrosine phosphorylation sites, which is consistent with previous biochemical results. In summary, we have cloned a phosphoprotein from rat liver with the expected characteristics of the insulin receptor substrate "pp185."

CB 114 AUGMENTED MITOGENIC SIGNALLING BY A MUTANT INSULIN RECEPTOR LACKING THE TWO TYROSINES OF THE CARBOXY-TERMINUS, Y. Takata, N.J.G.Webster and J.M.Olefsky, Department of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, CA 92093

A mutant human insulin receptor(HIR) with a carboxy-terminal(CT) 43 amino acid deletion exhibits normal I mediated kinase activity, but impaired metabolic signalling and augmented mitogenesis(JBC 263:8904, 8912, 1988; 264:12820,1989). To determine the role of the two CT tyrosines(Y 1316, Y1322) in these biological effects, the two Ys were changed to phenylalanine(F) by site directed mutagenesis(Y/F2). The mutant cDNA was transfected into rat 1 fibroblasts and compared to cells expressing wild type receptors(HIRc). HIRY/F2 is synthesized, processed, transported to the cell surface, binds I with normal affinity and internalizes normally. HIRY/F2 autophosphorylated in response to I and had normal kinase activity toward Glu:Tyr. Both cell lines displayed enhanced sensitivity to I for stimulation of glucose transport. Additionally, however, HIRY/F2 cells showed more than a 4 fold enhanced sensitivity for I stimulated DNA sythesis compared to HIRc cells. In conclusion:1) the apparently normal kinase activity of HIRY/F2 indicates that these CT Ys do not modulate kinase functions, 2) the CT Ys do not appear to play a role in IR metabolic signalling, but do appear to normally inhibit mitogenic signalling and their removal converts the IR into a very active growth signaling receptor.

CB 115 STUDIES ON THE AUTOPHOSPHORYLATION OF TYROSINES 1158, 1162 AND 1163 OF THE HUMAN INSULIN RECEPTOR

Jeremy M. Tavaré*#, Martin Dickens*, Bei Zhang+, Richard A. Roth+ and Leland Ellis#. *Department of Biochemistry, University of Bristol, U.K., #Howard Hughes Medical Institute, Dallas, TX and +Department of Pharmacology, Stanford University, CA.

Insulin-stimulated autophosphorylation of the human insulin receptor β-subunit occurs on at least seven tyrosine residues within three distinct regions of the receptor primary amino acid sequence. Three tyrosines (1158, 1162 and 1163) of the kinase domain are very rapidly phosphorylated, and their phosphorylation closely correlates with kinase activation. Two tyrosines (1328 and 1334) near the C-terminus of the β-subunit are also rapidly phosphorylated, but their functional role is unclear. Additional tyrosines (965, 972 and 984) at the juxta-membrane region may also be phosphorylated, but only significantly in purified receptor preparations.

We have employed a series of insulin receptor mutants, in which tyrosines 1158, 1162 and 1163 have been substituted with phenylalanine(s), to investigate the role of tyrosine phosphorylation at these sites in transmembrane signalling. Studies with these mutants have provided insight into the potential order of insulin-stimulated phosphorylation of tyrosines 1158, 1162 and 1163 in the intact receptor. Furthermore, tyrosine 1162 plays a crucial role in mediating insulin-stimulated phosphorylation of threonine, but not serine, residues on the β-subunit in intact cells.

CB 116 THE HUMAN INSULIN RECEPTOR GENE PROMOTER IS INDUCIBLE BY DEXAMETHASONE IN RAT 1 FIBROBLASTS. Nicholas J.G. Webster. Department of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, CA 92093.

In 1987 MacDonald and Goldfine showed that transcription of the insulin receptor gene (hIR) is induced by dexamethasone 3-4 fold in IM-9 cells. The cloned 1.8Kb promoter region contains two weak homologies to a glucocorticoid response element (GRE) at -306 and -1354 relative to the translational initiation codon. It has been shown that the partially purified glucocorticoid receptor can bind to these elements in-vitro. To test whether these elements are functional, we transfected a reporter gene consisting of the 1.8Kb hIR promoter upstream of the coding sequence for the chloramphenicol acetyl transferase (CAT) gene into HepG2 hepatoma and Rat 1 fibroblast cells. Dexamethasone had no effect on the level of CAT activity above controls in both cells. However, if an expresion vector containing the cDNA for the human glucorticoid receptor is cotransfected with the reporter plasmid then the CAT activity is increased 2.5 fold upon treatment with dexamethasone in Rat 1 fibroblasts but not in HepG2 hepatoma cells. The lack of induction in HepG2 cells may be related to the fact that the promoter is more active in these cells, the binding of the GR in the presence of dexamethasone may interfere with another transcription factor leading to no net induction. Indeed, one can imagine that the repressive effects of glucocorticoids in adipocytes may be mediated by such a mechanism.

CB 117 IDENTIFICATION OF THE LIGAND BINDING DOMAIN OF THE INSULIN RECEPTOR: INSIGHTS FROM CHIMERIC INSULIN /IGF-1 RECEPTORS. Jonathan Whittaker and Sujata Pawagi, Department of Medicine, SUNY at Stony Brook, Stony Brook, NY11794

The insulin (IR) and IGF-1 (IGFR) receptors are structurally homologous proteins. Thus it should be possible to identify their functional domains by creating chimeric receptors in which the corresponding domains of the two proteins have been exchanged and examining the resulting changes in function. To identify the ligand binding domains of the insulin receptor, we have substituted coding regions for predicted domains of the IR a subunit for the corresponding regions of the IGFR cDNA, using a predicted model of their tertiary structure (BBA 916:220, 1987). Ligand binding studies of the expressed proteins suggested that aas.1-119 and 311-428 were responsible for the ligand specificity of IR. In contrast, the cysteine rich domain, aas. 155-310, made little contribution to ligand specificity. Alanine scanning mutagenesis of these regions is being performed to confirm this and to further localize this binding domain.

Glucose Transporters

CB 118 PHOTOLABELING OF THE ERYTHROCYTE GLUCOSE TRANSPORTER WITH A HYDROPHOBIC PHOTOACTIVABLE REAGENT-2[^{3}]H-DIAZOFLUORENE.

Sujata Bhat*, Anil K. Lala* and Stephan A. Baldwin+ , *Department of Chemistry, Indian Institute of Technology, Bombay, Powai, Bombay 400 076, India and +Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K.

A hydrophobic photoactivable reagent, 2-[^{3}H]diazofluorene (2-[^{3}H]DAF) was used to photolabel the human erythrocyte glucose transporter. This photolabel partitions into the membrane hydrophobic core and on photolysis, inserts into the transmembrane segments of membrane spanning proteins providing an insight into the topography of such proteins. The glucose transporter was purified from human erythrocytes and then photolysed in the presence of 2-[^{3}H]DAF. Analysis of the photolysate on SDS-PAGE indicated a peak in the 55 Kda region of the gel showing that DAF labels the transporter. This was further confirmed by immunoprecipitation of the transporter with an anti-C terminal peptide antibody. The photolabeled transporter was subjected to mild tryptic digestion to obtain the glycosylated 35 Kda fragment and the 18 Kda fragment. Electrophoretic analysis of this showed the labeling of both the 35 and 18 Kda fragments. The 18 Kda fragment was further immunoprecipitated with anti 450-467 peptide antibody. Analysis of the immunoprecipitated sample indicated a labeled 18 Kda peptide. However, attempts to immunoprecipitate the 35 Kda fragment with an anti 148-156 peptide antibody which recognizes the denatured transporter did not give any peak of radioactivity in the 35 Kda region.

CB 119 THE INSULIN-REGULATABLE GLUCOSE TRANSPORTER (GLUT4) IS EXPRESSED IN RENAL MICROVESSELS. F. C. Brosius III, J. Briggs, K. Shogren, P. Goel, M. Barac-Nieto, M. J. Charron, Dept. of Internal Medicine, University of Michigan School of Medicine, Ann Arbor, MI 48109; Depts. of Pediatrics and Biochemistry, Albert Einstein College of Medicine, Bronx, NY; and Whitehead Institute for Biomedical Research, Cambridge, MA

Previously, we showed that GLUT4 is expressed in renal glomerulus and in cultured glomerular mesangial cells. In order to determine whether GLUT4 is expressed in renal microvessels, we performed immunocytochemical studies on rat kidney sections using polyclonal antipeptide antisera to both the carboxy- and amino-termini of the rat GLUT4 protein. Both antisera detected GLUT4 in what appear to be the smooth muscle cells of the renal afferent arterioles and interlobular arteries. This localization to microvessels was confirmed by the use of specific GLUT4 cDNA primers and the polymerase chain reaction (PCR) to amplify GLUT4 cDNA from cDNA prepared from mRNA of dissected rat and rabbit afferent arterioles and interlobular arteries. We have studied the expression of GLUT4 in the kidneys of one pair of fasted and refed rats. Immunocytochemical results show a marked decrease in renal GLUT4 protein in glomeruli and microvessels from rats who were fasted for 3 days when compared to rats who were similarly fasted and then refed with intraperitoneal glucose. Thus, the insulin-regulatable glucose transporter is expressed in renal glomerulus and in preglomerular vascular tissues. Furthermore, GLUT4 expression and presumably glucose uptake in these tissues is decreased during fasting a hypoinsulinemic state. These findings suggest that glucose transporter expression may be involved in the renal hemodynamic response to conditions of altered insulin secretion.

CB 120 PROTEIN SYNTHESIS INHIBITORS ACTIVATE GLUCOSE TRANSPORT WITHOUT INCREASING PLASMA MEMBRANE GLUCOSE TRANSPORTERS IN 3T3-L1 ADIPOCYTES, Brian M. Clancy, Scott A. Harrison, Joanne M. Buxton and Michael P. Czech, Program in Molecular Medicine, Univ. of Mass. Med. Center, Worcester, MA 01605

Exposure of 3T3-L1 adipocytes to 300 μM anisomycin (A) or 500 μM cycloheximide (C) caused a marked stimulation of hexose transport. A maximal increase in hexose uptake of about 7-fold was achieved within 4-8 h after cells were treated with either A or C. In contrast, 100 nM insulin (I) elicited about a 10-fold increase in glucose transport within 30 min. Analysis of transport as a function of 3-O-methyl-[^{14}C] glucose concentration revealed that the effects due to either 100 nM I (0.5 h) or 300 μM A (5h) could be attributed to 2.9-fold and 2.5-fold increases, respectively, in Vmax, coupled with 2.1-fold and 2.2-fold decreases, respectively, in the apparent K_m of the transport process. Total cellular amounts of GLUT1 and GLUT4 remained constant after treating cells with either A or C for 5 h. Although I, A and C elicited robust increases in hexose transport, a striking discrepancy between the fold-increase in transport and the relative amount of glucose transporters in the plasma membrane fraction was observed. I elicited a 2.8-fold and a 1.6-fold increase in the relative amounts of GLUT4 and GLUT1, respectively, in the plasma membrane fraction. In contrast, the amounts of GLUT4 and GLUT1 in the plasma membrane fraction were not significantly affectedby either A or C. In addition, glucose transport in the plasma membrane fraction prepared from A-treated cells (5h) was 2-3-fold greater than transport measured in plasma membranes derived from control cells. These observations suggest that the effect of A or C on glucose transport in differentiated 3T3-L1 cells is due to an increase in the intrinsic activity of one or both transporter isoforms.

CB 121 MEMBRANE DISTRIBUTION AND TRANSLOCATION OF GLUCOSE TRANSPORTERS IN SKELETAL MUSCLE, G. Lynis Dohm, Jacob E. Friedman, Wilhelm R. Frisell, and Ronald W. Dudek, Department of Biochemistry, East Carolina University School of Medicine, Greenville, NC 27858

To investigate the cellular and subcellular distribution of glucose transporters in skeletal muscle we have utilized the techniques of immunocytochemical localization in human muscle and isolation of muscle membranes from rat muscles. GLUT1 and GLUT4 isoforms of the glucose transporter were localized by electron microscopy using immunogold labelling. Glucose transporters in isolated membranes were quantitated by Western blot. Specific labelling for GLUT4 was observed in human muscle between the myofibrils within the triad (on both terminal cisternae and transverse tubules) and sarcoplasmic tubules. GLUT4 was not observed in endothelial cells or on the surface plasma membranes of the muscle cells. The GLUT1 transporter isoform was found along the sarcolemma (plasma membrane) and within endothelial cells. Our data give strong evidence that the GLUT1 transporter isoform is located on the surface membrane of muscle in both the basal and insulin stimulated state and may play an important role in basal glucose transport in skeletal muscle. The GLUT4 transporter resides in intracellular membranes under basal conditions and appears to be translocated to triad membranes in response to insulin. This suggests the novel concept that glucose may diffuse down the transverse tubule where it is then transported inside the muscle cell in response to insulin.

CB 122 REGULATION OF GLUCOSE TRANSPORTER GENE EXPRESSION IN BC3H-1 CULTURED MUSCLE CELLS BY INSULIN, GLUCOSE, AND GLYCEROLIPIDS. Imad M. El-Kebbi, C.-I. Pao, John C. Coleman, Lawrence S. Phillips, and Robert J. Pollet, Emory Univ. and VA Medical Centers, Atlanta, GA.

We and others have previously investigated the contribution of insulin-induced changes in membrane glycerolipids toward mediating insulin-stimulated glucose transport and intracellular metabolism in muscle and adipose tissue. We now examine the regulation of glucose transporter gene expression by insulin, glucose, and glycerolipids in BC3H-1 cells, a continuously cultured skeletal muscle cell line lacking the transcription factor required for cell fusion. Utilizing cDNA probes for GLUT 1 and GLUT 4 mRNAs (Dr. Graeme Bell), the major transporter synthesized in this tissue is GLUT 1. Insulin in the nM concentration range stimulated a specific two-fold increase in GLUT 1 mRNA which was maximal at 12 hr. Phorbol myristate acetate (PMA) and other diacylglycerol analogs also stimulated a similar increase in GLUT 1 gene expression and glucose transporter synthesis. Pretreatment of the BC3H-1 myocytes with PMA for 12 hr., which suppresses several of the protein kinase C isoenzymes, did not inhibit insulin stimulation of glucose transporter gene expression. Physiologically elevated glucose concentrations ($\geq$ 25 mM) suppressed basal, insulin- and PMA-stimulated glucose transporter gene expression by more than 75%. This suppression was specific for glucose as shown by the lack of effects of identical concentrations of xylose.

In summary, insulin stimulates a two-fold increase in gene expression and synthesis of GLUT 1, the major glucose transporter in the BC3H-1 cultured skeletal myocyte. PMA and other diacylglycerol analogs mimic this action of insulin in a non-additive manner, suggesting that the common pathway for several insulin actions in this tissue also extends to the level of regulation of specific gene expression. As for other insulin responses in this and other tissues, the lack of effect of chronic pretreatment with high levels of PMA suggests that these effects may be independent of several of the specific isoenzymes of protein kinase C. Finally, suppression of GLUT 1 gene expression by physiologically high levels of glucose in this muscle tissue may serve as a model of glucose toxicity and insulin resistance, and appears to be independent of the above stimulatory pathways.

CB 123 THE C-TERMINAL CYTOPLASMIC DOMAIN OF THE RAT GLUT4 GLUCOSE TRANSPORTER RESTORES CATALYTIC ACTIVITY TO AN INACTIVE TRUNCATE OF THE HUMAN GLUT1 PROTEIN, S. A. Harrison, C.-W. Woon, J. M. Buxton and M.P. Czech, Dep. of Biochem. and Mol. Biol., Univ. of Massachusetts Med. Center, Worcester, MA 01605. A family of at least five hydrophobic proteins are responsible for the facilitated transport of sugar through the plasma membranes of mammalian cells. While overall conservation among the transporter primary amino acid sequences is very high, each transporter isotype has a unique C-terminal domain, consisting of approximately 30 amino acids, which is located in the cell cytoplasm. In order to examine the structure/function relationships of these unique transporter domains, we constructed a cDNA encoding a chimeric protein, GLUT1-4c, which consisted of a human HepG2 glucose transporter protein (GLUT1) with a rat skeletal muscle glucose transporter (GLUT4) C-terminus. In the chimeric cDNA construct, rat GLUT4 nucleic acid sequences encoding the C-terminal domain of that transporter, were substituted for the homologous human GLUT1 nucleic acid sequences. Additionally, a GLUT1 transporter cDNA was constructed that encodes a 29 amino acid C-terminal truncation of the GLUT1 protein. This truncate, GLUT1t, terminates at isoleucine$_{463}$, 13 amino acids C-terminal to the twelfth putative membrane spanning domain. These 13 amino acids are highly conserved among mammalian and bacterial sugar transport proteins. These mutant transporter cDNAs, GLUT1-4c and GLUT1t, and the cDNA encoding the intact human HepG2 GLUT1 protein, were then subcloned into a mammalian expression vector and transfected separately into Chinese hamster ovary fibroblasts. The resultant cell lines were examined for expression of the native and mutant transporter proteins and for hexose transport activity associated with the expressed protein. We now report that cells expressing this truncated GLUT1 protein at the cell surface do not exhibit increased rates of hexose transport, whereas cells expressing either the intact human GLUT1 or the GLUT1-4c chimeric transporter exhibit elevated cell surface protein levels and corresponding increases in sugar transport rates. Michaelis-Menton kinetic parameters measured for 0-trans 3-O-methyl glucose influx demonstrate the similarity between the catalytic activities of the GLUT1 and the GLUT1-4c proteins. Thus, removal of 29 C-terminal amino acids from the GLUT1 protein results in loss of sugar transport activity and addition of the homologous GLUT4 C-terminus to the truncate restores that catalytic activity.

CB 124 HYDRODYNAMIC STUDIES AND GLUTERALDEHYDE CROSS-LINKING OF THE HUMAN ERYTHROCYTE GLUCOSE TRANSPORTER, Daniel N. Hebert and Anthony Carruthers, Program in Molecular Medicine, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, Ma. 01605.

It has been suggested that the oligomeric structure of the glucose transporter may be a determinant of carrier activity. Hydrodynamic and cross-linking techniques are employed here to elucidate the oligomeric structure of the human erythrocyte glucose transporter (Glut 1) in intact and solubilized membranes. Glut1 was purified in the presence and absence of 25 mM dithiothreitol (DTT). Reducing SDS-PAGE, western blot analysis of the two preparations shows that the +DTT preparation consists of monomeric glucose transporter while the -DTT preparation contains both monomeric and multimeric components. Sucrose gradient ultracentrifugation in 20 mM cholate resolves a 3.9S and a 7.5S component in both transport preparations. Carrier purified in the presence of DTT is largely resolved as a 3.9S fraction with a minor 7.5S fraction. The major and minor components of the -DTT preparation are 7.5S and 3.9S, respectively. Size-exclusion chromatography (SEC) in 20 mM cholate resolves a 48.5Å and 62.5Å fraction in +DTT and -DTT preparations, respectively. Gluteraldehyde cross-linking of the -DTT purified transporter in intact membranes produces a classical multimeric profile with increasing cross-linker concentration. Cross-linking of the +DTT purified transporter results in a monomeric profile. Transporter solubilized from erythrocyte ghosts is resolved by SEC as a 63Å fraction irrespective of the presence or absence of DTT. These studies suggest that Glut1 may exist as a multimeric complex in both detergent/micelles and in the membrane lipid bilayer.

CB 125 DISSOCIATION BETWEEN IN VIVO INSULIN ACTION ON GLUCOSE UPTAKE AND THE EXPRESSION OF GLUT1 AND GLUT4 IN SKELETAL MUSCLE OF DIABETIC RATS, Barbara B. Kahn, Luciano Rossetti, Harvey F. Lodish and Maureen J. Charron, Beth Israel Hospital, Boston, MA 02215, Whitehead Institute, Cambridge, MA 02139 and University of Texas Health Science Center, San Antonio, TX 78284.

Diabetes is characterized by resistance to the stimulatory action of insulin on glucose uptake in muscle. We investigated the mechanisms for this insulin resistance in streptozocin diabetic rats. Immunofluorescence using antipeptide antibodies in soleus muscle co-localizes Glut4 with dystrophin, intrinsic to muscle fibers, while Glut1 appears to be in perineurial sheath. Immunoblotting total membranes from gastrocnemius/soleus muscle shows that Glut1 and Glut4 protein levels are unaltered from control levels at 7d of diabetes but decrease to ~20% of control at 14d of diabetes. This decrease is prevented by insulin treatment. In 7d diabetic rats, the lack of decrease in Glut4 protein in muscle markedly contrasts with ~90% depressed levels of Glut4 in adipose cells. Thus, Glut4 undergoes tissue specific regulation in response to diabetes. Northern blotting reveals that Glut1 and Glut4 mRNA levels in muscle are decreased 63-70% at both 7d and 14d of diabetes and are restored by insulin treatment. At 7d of diabetes, in vivo insulin stimulated glucose uptake measured by euglycemic clamp is severely impaired (54% of control). Defects are present in both glycogen synthesis (6.8 ± 1.4 diabetic vs. 13.4 ± 0.9 control, mg/kg/min) and glycolysis (11.3 ± 0.8 vs. 19.9 ± 0.9). The contribution to overall glucose disposal of glycogen synthesis (34 vs. 36%) and glycolysis (56 vs. 53%) is similar in 7d diabetic and control rats. The concentration of muscle glucose-6-phosphate, the substrate common to these two pathways, is decreased ~30% in diabetic rats. These findings suggest a probable defect in glucose transport in muscle of diabetic rats which occurs before reduction in Glut1 or Glut4 levels and may result from impaired glucose transporter translocation and/or diminished intrinsic activity. Subsequently, suppression of Glut1 and Glut4 gene expression may contribute further to the insulin resistant glucose uptake characteristic of diabetes.

CB 126 EXPRESSION AND REGULATION OF GLUCOSE TRANSPORT AND TRANSPORTERS DURING RAT AND HUMAN MUSCLE CELL DIFFERENTIATION. Amira Klip, Yasuhide Mitsumoto, Andrew Grant, Elena Burdett, Philip Bilan, Toolsie Ramlal, Lawrence Leiter, Vivian Sarabia, Loretta Lam, Dimitrios Dimitrakoudis and Nava Bashan. The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8.

In skeletal muscle, glucose transport is mediated by the GLUT-1 and GLUT-4 glucose transporters. L6 muscle cellsof rat skeletal muscle origin differentiate in culture from myoblasts into myotubes through cell alignment and fusion (myogenesis). Their basal rate of transport decreased during myogenesis and the insulin response of glucose transport increased. In myoblasts, GLUT-1 predominated whereas only trace GLUT-4 was detected. GLUT-1 was present as a doublet; the lower molecular size form increased and the higher molecular size almost disappeared during differentiation. In contrast, the GLUT-4 ontent increased during muscle cell differentiation. Both insulin and the oral hypoglycemic drug metformin stimulated glucose uptake in myotubes but not in myoblasts. Metformin recruited GLUT-1 and GLUT-4 from internal membranes to plasma membranesto whereas insulin and IGF-Ipreferentially recruited the GLUT-4. These results indicate that maturation of myoblasts into myotubes is required to express full regulation of glucose transport by insulin, IGF-I or metformin.

Human muscle cells were grown from satellite cells present in adult muscle biopsies, and clonally selected for fusion potential into myotubes. These cells showed saturable hexose uptake inhibitable by cytochalasin B and stimulatable by nM insulin and IGF-I. High glucose (25 mM) down-regulated glucose uptake activity, and metformin stimulated glucose uptake at concentrations similar to those circulating in humans undergoing metformin therapy. These human cells expressed easily detectable levels of GLUT-1 but barely detectable levels of GLUT-4 in Western blots of cell extracts. The limited amount of material yielded by clonal primary cultures precluded the isolation of subcellular fractions, but polymerase chain reaction and in situ hybridization allowed to probe for the presence of GLUT-4 during human muscle cell differentiation.

CB 127 THE *HXT1* GENE OF *SACCHAROMYCES CEREVISIAE* IS A NEW MEMBER OF THE FAMILY OF HEXOSE TRANSPORTERS. Deborah A. Lewis and Linda F. Bisson, Department of Viticulture and Enology, University of California, Davis, CA. 95616.
Two novel genes involved in hexose transport in the yeast *Saccharomyces cerevisiae* have been identified. The gene *HXT1* (hexose transporter) was isolated from pSC7, sequenced and found to encode a hydrophobic protein, highly homologous to the sugar transporter proteins from eucaryotes and procaryotes. Multicopy expression of *HXT1* restored high affinity glucose transport to the *snf3* mutant, which is significantly deficient in high affinity glucose transport. The proteins HXT1, HXT2 (66% homologous to HXT1) AND GAL2 (69% homologous to HXT1) were found to have a putative leucine zipper motif at a concensus location in membrane spanning domain 2 and SNF3 (27% homologous to HXT1) has a degenerate zipper motif at this position. Loss of HXT1 resulted in a decrease in high affinity glucose and mannose, but not fructose transport. Deletion analysis of pSC7 revealed another gene (called ORF2) which restored both high and low affinity glucose transport to the snf3 mutant and restored good growth on galactose to a *gal 2* mutant. The functional 1.5 kb ORF2 region is too small to encode a 60 kD sugar transporter. *Lac Z* promoter fusion analysis of *HXT1* has revealed that *HXT1* is maximally expressed in the absence of *SNF3* and under repressing glucose concentrations, although expression of *HXT1* high affinity glucose transport is only at derepressing glucose concentrations. This suggests a model of post-translational regulation of the expression of glucose-repressible hexose transport in yeast.

CB 128 STIMULATION OF PROTEIN SYNTHESIS BY INTERNALIZED INSULIN, David S. Miller, Laboratory of Cell & Molec Pharmacol, NIEHS-NIH, Res Triangle Pk, NC 27709.
Previous studies showed that microinjected insulin stimulates transcription and translation in Stage IV Xenopus oocytes by acting at nuclear and cytoplasmic sites [Science 240:506(1988); JBC 264:10438(1989)]. Here I consider the question of whether hormone, internalized from an external medium, acts on those sites to alter protein synthesis. Both intracellular accumulation of undegraded ^{125}I-insulin and incorporation of ^{35}S-methionine into oocyte protein were measured. Anti-insulin antibody was microinjected into the cytoplasm of insulin-exposed cells to block hormone action through internal sites. Cells exposed for 2 h to 7 or 70 nM external insulin exhibited increased methionine incorporation, but minimal intracellular (nucleus & cytoplasm) hormone accumulation and no effect of microinjected antibody. With 24 h exposure, methionine incorporation again increased, but now accumulation of hormone was substantial (3 and 25 fmol with 7 and 70 nM, respectively), and microinjected antibody significantly reduced the insulin-stimulated component of incorporation; basal incorporation was not affected. Maximal inhibition by antibody was 40% of the insulin-stimulated component. Together, the data show that inhibition of insulin-dependent translation by cytoplasmic anti-insulin antibody was associated with intracellular accumulation of hormone. Thus, in cells exposed to external insulin translational control arises from hormone action at both surface and internal sites.

CB 129 SINGLE AMINO ACID SUBSTITUTION AT RESIDUE 412 MARKEDLY DECREASES THE INTRINSIC ACTIVITY OF GLUT1 GLUCOSE TRANSPORTER, Yoshitomo Oka, Tomoichiro Asano and Hideki Katagiri, Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Tokyo, Japan
GLUT1 glucose transporter cDNA was modified such that tryptophan 412, putative cytochalasin B photoaffinity labeling site, was mutated to leucine. Although the mutated transporter was expressed into plasma membranes of Chinese hamster ovary cells, an increase in the uptake of 2-deoxy-D-glucose (0.1 mM) remained 15-30 % of the increase induced by expressing a similar amount of normal glucose transporter. A similar decrease in 2-deoxy-D-glucose uptake was also observed at 10 mM, which is above the Km of this glucose transporter isoform. Importantly, cytochalasin B labeling was not abolished but decreased 50 % and the results with side-specific glucose analogs suggested that the outer glucose binding site of the mutant was intact but the inner binding site was modulated. These results indicate 1) cytochalasin B labeling site is not tryptophan 412, although tryptophan 412 is located in the inner glucose binding site of the GLUT1 glucose transporter, 2) substitution of leucine for tryptophan 412 decreases the intrinsic activity of the glucose transporter.

CB 130 EVIDENCE THAT FUNCTIONAL ERYTHROCYTE-TYPE GLUCOSE TRANSPORTERS (GLUT 1) ARE OLIGOMERS.-Anna Pessino, Chee W. Woon, Brian M. Clancy, Scott A. Harrison, Joanne M. Buxton, Daniel N. Hebert, Anthony Carruthers and Michael P. Czech, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical School, Worcester, MA 01605
Previous studies on the native size of the glucose carrier, estimated by irradiation-inactivation experiments, suggested the presence of dimeric or tetrameric forms of glucose transporters in human erythrocytes (Jung et al., 1980; Jarvis et al., 1986). We explored the hypothesis that the erythrocyte-type glucose transporter (GLUT 1) exists as a multimer by using a CHO cell line expressing a glucose transporter chimeric protein. The chimeric transporter was composed of the first 463 amino acids of human GLUT 1 and the 30 C-terminal amino acids of rat skeletal muscle glucose transporter (GLUT 4). Transfection of CHO fibroblasts with this corresponding hybrid cDNA (GLUT 1-4 c) resulted in overexpression of functional chimeric glucose transporters (approximately 10-fold higher than endogenous GLUT 1, as assessed by [^{125}I]IAPS-forskolin labeling of total membranes and 2-deoxyglucose uptake in parental and transfected CHO fibroblasts). Total membranes from transfected cells were solubilized in 2% Nonidet P-40 and subjected to immunoprecipitation using anti-GLUT 1 and anti-GLUT 4 C-terminal peptides antisera (αGT1 and αGT4, respectively). Immunoprecipitation of chimeric transporters, performed with αGT4, allowed a nearly complete recovery of endogenous GLUT 1 molecules in the immunoprecipitated material, detected with αGT1 in Western blot analysis. In Nonidet P-40 lysates from both total and low density microsomal membranes from 3T3-L1 adipocytes, no co-immunoprecipitation of native GLUT 1 and native GLUT 4 molecules was detected. Our results support the following conclusions: 1) the specific co-immunoprecipitation of native GLUT 1 and GLUT 1-4 c from transfected CHO cells indicates that functional GLUT 1 exists in an oligomeric state; 2) no evidence exists for GLUT 1/GLUT 4 heteromultimeric complexes in 3T3-L1 adipocytes.

CB 131 SOMATOTROPIN (GH) INDUCES TRANSLOCATION OF AN INSULIN-REGULATABLE GLUCOSE TRANSPORTER (IRGT) IN CULTURED RAT PRIMARY ADIPOCYTES, J.W. Tanner, K.A. Leingang*, M.M. Mueckler* and K.C. Glenn, Biological Sciences, Monsanto Co., St. Louis, MO 63198 and *Department of Cell Biology and Physiology, Washington University Medical School, St. Louis, MO 63110.
The cellular mechanism whereby acute GH exposure increases adipocyte glucose uptake was investigated using cultured, in vitro-differentiated rat adipocytes. Preadipocytes, isolated from collagenase digested inguinal fat pads of 75 g rats, were differentiated in a manner similar to 3T3-L1 preadipocytes (i.e., isobutylmethylxanthine, insulin, dexamethasone). On day six following the induction of differentiation, cells were incubated for 18-20 h in serum-free DMEM containing 1% BSA. Subsequently, the acute effect (20 min) of recombinant bovine GH (bGH; 4.5 nM) or insulin (100 nM) on glucose uptake was determined by assessing cellular incorporation of [^{14}C]-2-deoxyglucose in the absence or presence of cytochalasin B. bGH and insulin increased glucose uptake 3- and 3.4-fold, respectively, over glucose uptake for unstimulated cells. Western analysis of subcellular fractions demonstrated a 4.5- and 6.5-fold enrichment for IRGT protein in plasma membranes and a concomitant decrease in low density microsome IRGT from cells treated with bGH or insulin, respectively, compared to control cells. These data demonstrate that the insulin-like effect of GH on adipocyte glucose uptake, like insulin, involves translocation of the IRGT molecule to the plasma membrane. Furthermore, the stimulation of Glut4 translocation by both GH and insulin may indicate a common cell signalling element between the adipocyte GH and insulin receptors or, alternatively, the existence of multiple cellular mechanisms for stimulating Glut4 translocation.

CB 132 THE NH_2-TERMINAL DOMAIN OF THE HUMAN GLUT1 CONFERS A HIGH LEVEL EXPRESSION OF GLUCOSE TRANSPORTERS IN CHINESE HAMSTER OVARY FIBROBLASTS, Chee-Wai Woon, S.A. Harrison, J.M. Buxton, S. Perregaux and M.P. Czech, Dept. of Biochem. and Mol. Biol., University of Massachusetts Med. Center, Worcester, MA01655. We have constructed chimeric cDNAs in which different lengths of the rat Glut4 is replaced by the corresponding regions of the human Glut1. One set of chimeras referred to as Glut1(199)/Glut4 and Glut4(215)/Glut1, replaces the NH2-terminal approximately 200 amino acids of one transporter with the corresponding sequence of the other. The second set of chimeras referred to as Glut1/Glut4(30) and Glut4/Glut1(29), replaces the COOH terminal approximately 30 amino acids with the corresponding residues of the other. Stable expression of the native rat Glut4, Human Glut1 and the chimeric transporters in Chinese hamster ovary(CHO) cells resulted in the isolation of clonal lines exhibiting two distinct phenotype. High levels of expression (10 fold or higher over endogenous Glut1 as estimated by immunoblotting and [^{125}I]IAPS-forskolin labelling of total membranes) were obtained for the native Glut1 and the chimeras Glut1(199)/Glut4 and Glut1/Glut4(30). In each case the gene products contain the NH2-terminal polypeptide of Glut1. These transporters also catalyzed 2-deoxyglucose transport. Detectable expression was obtained for the native Glut4 and the chimeras Glut4(215)/Glut1 and Glut4/Glut1(29) by Western blotting. [^{125}I]IAPS-forskolin labelling in this second group did not show a significant level of expression of the transfected cDNAs over that in the control CHO cells. This pattern of overexpression suggests that the NH2-terminus of the glucose transporter polypeptide plays a crucial role in regulating the level expression of these transporters in CHO fibroblasts. In addition the results suggest that the expressed chimeric transporters Glut1(199)/Glut4 and Glut1/Glut4(30) are both functional. A similar pattern of expression has been observed in the 3T3-L1 fibroblasts. These results provide the first evidence for a major role of the NH_2-terminus of the glucose transporters in determining tissue specific expression.

Islet Cell Physiology

CB 200 GROWTH HORMONE RECEPTORS IN INSULIN PRODUCING CELLS: LOCALIZATION OF THE DOMAIN RESPONSIBLE FOR THE INTERNALIZATION OF GH. Giovanna Allevato, Nils Billestrup, Annette Møldrup, and Jens Hoiriis Nielsen, Hagedorn Research Laboratory, DK-2820, Gentofte, Denmark.

Growth hormone (GH) stimulates both β-cell proliferation and insulin biosynthesis in normal rat islets and in the rat insulinoma cell line RIN-5AH. Specific receptors for GH (GH-R) have been demonstrated in RIN-5AH cells, similar to the recently cloned rat liver GH-R. The signal transduction mechanism of the GH-R is not yet known. The aim of the present study was to characterize the structural requirement for internalization of GH in RIN-5AH cells transfected with cDNA coding for the full length wild type (wt) GH-R_{1-638}, mutated GH-R_{1-294} retaining 5 amino acid residues of the cytoplasmic domain (mut.1), and mutated GH-R_{1-455} retaining 214 amino acid residues of the cytoplasmic domain(mut.2). The mutants were unable to transmit the insulinotropic effect. The clones expressing wt, mut.1 and mut.2 GH-R's showed an increased binding of GH up to 12 fold with the same affinity as the untransfected cell line. The internalization was measured by incubation of $5x10^5$ cells with 1 μCi ^{125}I-hGH in monolayer culture for different time period at 37 C followed by washing four times with HBSS at 4 C. The bound ^{125}I-hGH was removed by washing for 3 minutes at 4 C with 0.15 M NaCl/5 mM glycine pH 2.5. The parent cell line showed a maximal internalization of 50% of the specifically bound hormone after 90 minutes at 37 C, the transfected clones showed 59% (wt) and 50% (mut 2), whereas mut.1 showed no internalization. In conclusion, these results suggest that the domain of the GH-R required for internalization of the ligand is located in the cytoplasmic part between 5 and 294 amino acid residues proximal to the transmembrane domain.

CB 201 ELEVATION OF AMYLIN GENE-EXPRESSION IS CONSISTENT WITH A ROLE FOR THIS HORMONE IN THE CAUSATION OF INSULIN RESISTANCE IN THE RAT, Garth. J.S. Cooper, Andrew A. Young, Bronislava Gedulin, M. Jacqueline Johnson & Huei-Jen S. Huang. Amylin Corporation, Towne Centre Drive, La Jolla, CA 92121

Amylin is a peptide hormone secreted from the β-cells of the pancreatic islets of Langerhans in response to nutrient stimuli. Administration of amylin produces insulin resistance in skeletal muscle and liver. However, aberrant amylin gene-expression has not previously been demonstrated in insulin-resistant disease states. The LA/N-cp rat, when homozygous for the cp gene, is obese, insulin resistant and hyperinsulinemic, and exhibits impaired glucose tolerance and type IV hyperlipoproteinemia. We studied pancreatic expression of the amylin and insulin genes in LA/N-obese rats (n = 5), compared with LA/N-lean (n = 5) and Wistar (n = 5) control rats, by determination of pancreatic mRNA levels and circulating blood hormone concentrations. LA/N-obese rats had similar fasting blood glucose levels, but impaired glucose tolerance in comparison with control rats. RNase-protection analysis was used to demonstrate that LA/N-obese rats had comparable 7-fold increases in pancreatic levels of amylin and insulin mRNA. Blood levels of both hormones were markedly elevated in LA/N-obese rats compared with controls, consistent with observed elevations in fasting mRNA levels. To examine the effect of increasing amylin levels in the presence of elevated insulin concentrations, we also studied the effect of rat amylin on insulin action in rat skeletal muscle *in vitro*. Amylin induced insulin resistance in a dose-dependent fashion, independent of insulin concentration, and in the presence of maximally-stimulating insulin. Elevated blood levels of amylin measured in LA/N-obese rats in the present study were similar to those previously observed to induce insulin resistance in normal rats *in vivo*. These results are consistent with a role for amylin in the induction of insulin resistance and associated metabolic abnormalities, such as obesity, in rats.

CB 202 CYTOSOLIC FREE Ca^{2+}, O_2 CONSUMPTION AND INSULIN SECRETION OSCILLATE IN GLUCOSE-STIMULATED RAT PANCREATIC ISLETS. B. E. Corkey, E. A. Longo, K. Tornheim, J. T. Deeney, B. A. Varnum, D. Tillotson, & M. Prentki, Boston U. Med. Ctr. Boston, MA.

Insulin secretion in the intact organism and by the perfused pancreas and groups of isolated perifused islets is pulsatile. We have proposed a metabolic model of glucose-induced insulin secretion in which oscillations in the ATP/ADP ratio drive alterations in metabolic and electrical events that lead to insulin release. A key prediction of our model is that metabolically driven Ca^{2+} oscillations will also occur. Using the fluorescent Ca^{2+} probe, fura 2 and sensitive O_2 electrodes, we investigated cytosolic free Ca^{2+} responses and O_2 consumption in perifused rat islets maintained in culture for 1 to 4 days. We found that elevated glucose increased the average cytosolic free Ca^{2+} level, the ATP/ADP ratio and O_2 consumption. Oscillatory patterns were obtained for Ca^{2+}, O_2 and insulin secretion with 10 and 20 mM glucose. Evaluation of the Ca^{2+} responses of a large series of individual islets monitored by digital image analysis and perifused at both 3 and 10 mM glucose indicated that the rise in glucose concentration caused a doubling of the average cytosolic free Ca^{2+} value and a 5-fold increase in the amplitude of the oscillations, with little change in period. The pattern of Ca^{2+} change within the islets suggested that these increases resulted from recruitment of responding cells. The coexistence of oscillations with similar periods in insulin secretion, O_2 consumption and cytosolic free Ca^{2+} is consistent with the model of metabolically driven pulsatile insulin secretion.

CB 203 SEXUAL DIMORPHISM OF ß-CELL DEGENERATION IN TRANSGENIC MICE OVER-EXPRESSING RAS PROTEINS IN PANCREATIC ß CELLS, Shimon Efrat, Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

The human *H-ras* oncogene induces cell degeneration and diabetes when expressed in pancreatic ß cells in multiple lineages of transgenic mice (Efrat *et al.*, 1990, *Mol. Cell. Biol.* **10**, 1779-1783). The disease develops predominantly in male mice between 5-8 months of age and is characterized by the appearance of large cavities in the islets of Langerhans and by the development of hyperglycemia, without an obvious autoimmune response. A similar phenotype is induced by the wild type human H-ras gene, suggesting that overexpression, rather than the presence of mutant Ras proteins, is responsible for the induction of ß-cell degeneration. Most transgenic female mice do not manifest this phenotype, even at much greater ages. However, ovariectomy induces female ß-cell degeneration similar to that of the males. In contrast, castration or the presence of the testicular feminization mutation do not alter the course of the disease in males. Treatment of males and ovariectomized females with estrogen prevents the development of diabetes. These results suggest that testicular androgens and a functional testosterone receptor are not required for the increased susceptibility of male ß cells to the effects of Ras overexpression, and that the relative resistance of female ß cells is mediated by estrogen. In addition, a genetic component of female ß-cell resistance to Ras is revealed by breeding the transgenic mice into the C3HeB/FeJ genetic background, which results in a pronounced increase in the incidence of female diabetes.

CB 204 EVIDENCE THAT LOSS OF PREPROINSULIN mRNA IS A SECONDARY EVENT IN CYPROHEPTADINE-INDUCED INHIBITION OF INSULIN SYNTHESIS, L.J. Fischer, C.P. Miller and S.J. Giddings, Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI 48824 and Washington University Medical Service, Veterans Administration Medical Center, St. Louis, MO 63106

Cyproheptadine (CPH) administration to rats has been reported to reversibly deplete preproinsulin mRNA (PPImRNA), inhibit proinsulin synthesis and deplete pancreatic insulin. Current studies investigated whether an initial drug-induced loss of PPImRNA was the cause of subsequent changes in insulin cell function. Analysis of rat pancreatic PPImRNA and proinsulin after a single dose of CPH (45 mg/kg) showed that a 50% loss of proinsulin, present at 1.5 hr, preceded a reduction in PPImRNA observed 3 to 6 hours after the dose. Results from isolated rat islets indicated that direct exposure to CPH (10 µM) inhibited proinsulin synthesis (30 min incorporation of ^{3}H-leu) without altering PPImRNA in islet tissue. Culture of RINm5F and HIT-T15 cells with 10 µM CPH produced depletion of cellular insulin but no alteration in PPImRNA. These data from in vivo and in vitro experiments indicate that a loss of PPImRNA may be associated with, but not the initial cause of, CPH-induced inhibition of insulin synthesis and cellular depletion of the hormone.

CB 205 CO-ORDINATE REGULATION OF INSULIN AND AMYLIN SECRETION FROM RAT ISLETS OF LANGERHANS AND HIT-T15 ß-CELLS, Peter Hammonds, Lisa Palmieri, Jennifer Troge and Robert Mertz, Division of Endocrinology, Glaxo, Inc., Research Triangle Park, NC 27709

The contribution of amylin to impaired carbohydrate metabolism and non-insulin dependent diabetes (NIDDM) remains equivocal. In the present study, we have investigated insulin and amylin secretion induced by nutrient or pharmacological secretagogues in rat islets and clonal HIT-T15 ß-cells. Insulin was measured by ELISA, amylin by radioimmunoassay. In islets incubated under basal conditions (3 mM glucose, 2h), insulin release was 148.3 ± 11.1µU/islet (n=3); amylin was 86.0 ± 8.0. pg/islet (n=3). Raising glucose from 3 to 20mM evoked a 2-fold stimulation of both insulin and amylin secretion. In addition, 10mM mannose induced a 1.6 fold stimulation of both hormones. In HIT-T15 cells (n=4), as in islets, co-ordinate stimulatory effects on insulin and amylin secretion were also induced in response to nutrient secretagogues. Moreover, in the presence of 11mM glucose, HIT-T15 insulin (I) and amylin (A) secretory responses were potentiated co-ordinately in response to 40 µM glibenclamide (I = 1.6 fold; A = 1.3 fold), 10µM forskolin (I = 2.3 fold; A = 1.5 fold) or 5 µM bombesin (I = 2.0 fold; A = 1.4 fold). In conclusion, ß-cell insulin and amylin secretory responses appear to be regulated co-ordinately *in vitro*.

CB 206 DETECTION OF LIVER-TYPE (GLUT2) AND ERYTHROCYTE / BRAIN-TYPE (GLUT1) GLUCOSE TRANSPORTER cDNA IN PANCREATIC ß-CELLS, Harry Heimberg, Erik Quartier and Frans Schuit, Department of Biochemistry, Vrije Universiteit Brussel, 1090 Brussels, Belgium

A family of glucose transporter (GT) genes, each encoding transmembrane proteins which facilitate the cellular uptake of glucose, has recently been described. It is of physiological interest to study their role in ß-cells from the endocrine pancreas, which are considered as one of the primordial sites regulating glucose homeostasis. Previous studies showed the presence of liver-type GT mRNA in isolated islets of Langerhans (Thorens et al., 1988, Permutt et al., 1989), but this tissue is still composed of several endocrine and non-endocrine cell types. We have now studied GT gene expression in pure ß-cells, obtained after autofluorescense-activated cell sorting of dispersed islet cells. cDNA fractions derived from up to 10^5 ß-cells as well as from muscle, brain and liver tissue were amplified with two degenerate oligonucleotide primers directed against stretches of conserved sequences encoding the 5th and 7th homologous transmembrane segments of the currently known GT's. Amplified DNA was dot-blot hybridized with oligonucleotide probes or mapped after restriction enzyme digestion. Confirmation of the already reported expression patterns of GLUT1, GLUT2 and GLUT4, in brain, liver and muscle tissue was obtained with these techniques. Brain-type and liver-type GT cDNA were detected with comparable intensity in isolated ß-cells but no muscle-type was found in these conditions. The pattern of GT gene expression and its detection in small numbers of isolated ß-cells make it possible to investigate if GT's play any role in the heterogeneous glucose sensitivity and glucose responsiveness of individual ß-cells that we recently described (Schuit et al., 1988).

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CB 207 DEFICIENCY OF AMYLIN EXPRESSION IN THE PANCREAS OF AUTO-IMMUNE BB/WOR DIABETIC RATS, H.-J. Su Huang, Garth J.S. Cooper, Andrew A. Young and M. Jacqueline Johnson, Amylin Corporation, 9373 Towne Centre Drive, Suite 250, San Diego, CA 92121

Amylin is a peptide hormone co-secreted with insulin from pancreatic B-cells in response to nutrient secretagogues. Amylin exerts modulatory effects on processes of carbohydrate metabolism in liver and skeletal muscle. We used RNase protection analysis to measure the tissue specific expression of amylin and insulin in both normal (Wistar) and auto-immune diabetic (BB/Wor) rats. Of 20 different tissues examined in normal Wistar rats, amylin mRNA was expressed primarily in the pancreas and to a lesser extent (1-5% of pancreatic levels) in the stomach, duodenum, jejunum, ileum, colon and lung. Insulin expression was detected only in the pancreas, indicating that amylin can be expressed independently of insulin. Amylin mRNA levels in the pancreas were approximately 5% those of insulin. Diabetes in the BB/Wor rat model resembles the human type I disease in that both are the consequence of autoimmune-mediated pancreatic B-cell destruction. Our results reveal that amylin, but not insulin, was expressed in the pancreases of a subset of animals having an absolute requirement for insulin therapy to prevent ketoacidosis, suggesting that amylin can be produced from insulin-free pancreatic cells; the concentration of amylin mRNA in the pancreas was approximately 10-15% of that in normal controls. Amylin mRNA was not detected in the pancreases of animals with similar clinical symptoms after an overnight fast, indicating that amylin transcription in insulin-free pancreatic cells is subject to dietary regulation. In a subset of BB/Wor rats characterized by late onset of diabetes, insulin and amylin mRNA were detected in the pancreas although the levels of both were markedly decreased. This suggests that the B-cells in this subset of animals were not completely destroyed. In this group, amylin mRNA was present at 20-30% and insulin mRNA was present at 2-10% of the level seen in normals. This study indicates that the ratio of pancreatic amylin to insulin mRNA in BB/Wor rat may be elevated during development of the disease as the result of lesser autoimmune attack upon insulin-free amylin producing cells. Animals with diabetes showed no detectable compensatory increase in levels of amylin mRNA in extra-pancreatic tissues.

CB 208 CARBOXYPEPTIDASE H IS CO-SECRETED IN RAT INSULINOMA-DERIVED CELL LINES WITH OR WITHOUT PEPTIDE HORMONE SUBSTRATE, David Parkinson and Fred T. Fiedorek, Departments of Cell Biology & Physiology and Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110.

Carboxypeptidase H (CP-H, EC 3.4.17.10) removes C-terminal basic amino acids from a wide range of peptide hormone intermediates (including insulin, glucagon, and somatostatin) in endocrine and neural tissues. We have characterized CP-H in two insulinoma-derived (RIN) cell lines. The RIN 1046-38 cell line produces 31.5 ± 7.8 (mean ± SEM) nanograms of insulin per 10^6 cells per day as its sole peptide hormone and 1.17 ± .24 mU of CP-H per 10^6 cells per day. RIN 1046-44 cells produce neither insulin nor a variety of other pancreatic and extrapancreatic peptide hormones, but they continue to secrete 61% of the amount of CP-H released by 1046-38 cells. CP-H from RIN cells is stimulated 8-fold by 2 mM cobalt, is potently inhibited by GEMSA, and shows maximal enzyme activity at pH 5.5. Approximately 20% of CP-H is soluble at pH 5.5 and the remaining 80% exists in a membrane-associated form which is released by treatment with 0.5% Triton X-100. Immunoblots of both acid soluble and Triton-releasable extracts from RIN cells containing equivalent enzyme activity reveal a single band of 54 kD that was stained to equal intensity in both fractions. Exposure of RIN 1046-38 cells to 40 mM KCl stimulated the release of insulin from 0.40 ± .05 to 0.83 ± .04 nanograms and CP-H from 74 ± 1 to 184 ± 7 µU per 10^6 cells per 20 minutes. The amount of insulin secreted was approximately 15-fold greater on an estimated molar basis than the total CP-H produced during both basal and KCl stimulated periods of release in RIN 1046-38 cells. Release of CP-H from RIN 1046-44 cells was stimulated to a similar extent by KCl. The inclusion of 10 mM $MgCl_2$ with 40 mM KCl blocked the release of insulin in 1046-38 cells and attentuated CP-H release in both RIN cell lines. Thus, CP-H is produced and secreted from both RIN cell lines in a regulated manner even though known peptide hormone substrate (proinsulin) is present only in RIN 1046-38 cells.

CB 209 ISLET CELL ANTIGENS AND AUTOANTIGENS: FURTHER CHARACTERISATION, R.Raju, S.Srikanta and N.Kochupillai, Department of Endocrinology, All India Institute of Medical Sciences, New Delhi 110 029, INDIA.

To enable characterisation of molecular nature of pathogenetically relevant islet autoantigen(s) we have recently generated a series of murine monoclonal islet cell antibodies (n=18; immunogen=human insulinoma homogenates; screening=immunohistochemistry). Differentiation antigens (ICAgs) recognised by these MAbs displayed varied intracellular and 'surface-like' cytological distribution; MAbs I-39 and -45 reacted with all islet endocrine cells, I-51 and -52 reacted with peripheral islet alpha cells only. Exposure to 100 degree C for 1 hr (dry heating of pancreatic sections) did not significantly alter immunoreactivity of islet antigens recognised by human ICAb and MAbs I-39 and -52; those recognised by I-45 was less heat stable. The extraordinary heat stability of the islet antigen has potential for exploitation in its isolation and may be consonant with its proposed carbohydrate nature (glycolipid/glycoprotein). Islet cells share I-45 differentiation antigen with other neuroendocrine cells viz. anterior pituitary, adrenal medulla and gut endocrine cells. Distribution of I-51 and I-52 antigens were more restricted.

CB 210 THE GENERATION AND SCREENING OF NOVEL GLUCAGON ANTAGONISTS BY SATURATION MUTAGENESIS, Robert A. Smith., Rob Sisk, and Peter Lockhart, Zymogenetics, Inc., 4225 Roosevelt Way NE, Seattle, WA 98115.

Glucagon clearly has an important role in the regulation of glucose hemostasis, and it has long been speculated *that glucagon antagonists may be effective therapeutic agents in the control of type II diabetes.* We have attempted to isolate such antagonists by creating a library of mutant glucagon coding sequences, expressing them in a yeast secretion system, and screening for clones that produce analogs which inhibit the glucagon stimulation of rat hepatocyte membrane adenylate cyclase. The library was constructed by allowing random misincorporation during oligonucleotide synthesis of the glucagon coding sequence at a rate that resulted in almost all clones producing a glucagon species with at least one amino acid substitution. We have developed a simplified cyclase assay that allows us to directly test culture broths from these clones for their ability to stimulate or inhibit the glucagon dependent activity, and plan to use this method to screen a large enough portion of the library to be sure of having tested a number of different amino acid substitutions in each of the 29 residues of glucagon.

CB 211 AMYLIN AND INSULIN EXERT COMPLEMENTARY CONTROL OVER CORI CYCLE ACTIVITY, Andrew A. Young, Ming-Wei Wang, Garth J.S. Cooper and David M. Mott*, Amylin Corporation, San Diego, CA 92121 and *CDNS, NIH-NIADDK, Phoenix, AZ 85016

The protein hormone amylin co-secreted with insulin, stimulates hepatic glycogenolysis, gluconeogenesis, skeletal muscle glycogenolysis and inhibition of insulin-mediated glycogen synthesis. It is the principal component of the islet amyloid observed in the majority of type 2 diabetics. Relative to 6 control rats, bolus intravenous injections of 25 nmol into 6 somatostatin-infused rats (light halothane anesthesia) resulted in rapid hyperglycemia, hyperlactemia and increased endogenous glucose production (measured by non-steady-state 3-^{3}[H]-glucose dilution). The hyperlactemia, hyperglycemia and increased glucose production persisted for 2,3 and 5 hours respectively. We propose that the dual β-cell signal (insulin + amylin) exerts coordinated control over the lactate component of Cori cycle (glucose → 3-carbon molecule → glucose) activity, with insulin controlling carbon flux into peripheral stores and amylin controlling its retrieval.

Gene Regulation

CB 212 DECREASED PROTEIN KINASE C α and β ACTIVITIES IN INSULIN RESISTANT TISSUES OF ZUCKER OBESE (fa/fa) RATS. Denise R. Cooper and James E. Watson, Research Service, J. A. Haley Veterans Hospital and Dept. of Medicine, University of South Florida College of Medicine, Tampa, FL 33612

The downregulation of protein kinase C (PKC) by activators such as 12-0-tetradecanoyl phorbol 13-acetate (TPA) in various cell systems is accompanied by diminished PKC-dependent responses. The stimulation of glucose transport (GT) activity and inactivation of glycogen synthase are PKC-dependent responses. In the perfused heart of obese rats, GT is poorly stimulated by TPA or insulin when compared to lean controls; PKC activity is altered and poorly activated by TPA, and TPA-induced glycogen synthase inactivation is also absent in hepatocytes from obese rats (Van de Werve *et al, Diabetes* 36:310, 1987). In this study, we compared the hydroxyapatite profiles of PKC isozymes in insulin-resistant tissues of the obese rat with its lean counterpart. Total PKC activity was decreased 38 ± 4% ($p<0.001$, n=8) in liver, heart, diaphragm and soleus muscle (but not brain extracts) of obese rats when compared to their lean controls. All four tissues expressed PKC α and β (or PKC-III and II). Liver and soleus expressed predominately PKC-III, but PKC-II and III activities were equally expressed in diaphragm and hearts from lean rats, and both forms were diminished in tissues from obese rats. Activity of a previously unidentified PKC species was also observed in Zucker tissues, and was decreased in obese animals. Additionally, decreased immunoreactive levels of PKC were detected by Western blot with peptide-specific antisera in obese tissues. Thus, diminished PKC levels in insulin resistant tissues may be a result of increased activation and turnover of PKC due to hyperinsulinemia and hyperglycemia and/or an alteration in PKC expression. In either case, decreased PKC levels may result in decreased GT and insulin resistance.

CB 213 INSULIN INDUCES INITIAL ACCUMULATION OF GLUCOKINASE mRNA IN PRIMARY CULTURES OF NEONATAL RAT HEPATOCYTES, Jean Girard, Michael Narkewicz, Jean Francois Decaux, Pascale Bossard, Patrick Iynedjian*, Pascal Ferré. Centre de recherches sur la Nutrition, Meudon, France, Institut de Biochimie Clinique*, Genève, Suisse.

We have investigated the effect of insulin on the regulation of glucokinase (GK) mRNA in primary cultures of hepatocytes from 10-12-day-old suckling rats. GK mRNA was undetectable in such cells after 48 h of culture in serum-free medium devoid of hormones. Addition of insulin to the medium resulted in induction of GK mRNA. The effects of insulin were dose-dependent. Dexamethasone alone did not induce GK mRNA, but enhance the response to insulin. Induction of GK mRNA by insulin was not affected when the medium glucose concentration was varied between 5 and 15 mM, nor when culture was conducted in glucose-free medium supplemented with lactate and pyruvate or galactose. The time course of initial accumulation of GK mRNA in response to insulin was characterized by a lag of 12 h and an induction plateau reached after 36 h. If hepatocytes were then withdrawn from insulin for 24 h and subsequently subjected to a secondary stimulation by insulin, GK mRNA re-accumulated with much faster kinetics and reached the fully induced level within 8 h. Both primary and secondary responses to insulin were abolished by actinomycin D. Insulin did not stimulate GK transcription in isolated nuclei during the initial exposure but markedly stimulated it during re-exposure. These results provide insight into the role of insulin in the ontogenic development of hepatic glucokinase.

CB 214 EXPRESSION OF BOTH α AND β SUBUNITS OF CASEIN KINASE II PROTEIN ARE REQUIRED FOR EXPRESSION OF CATALYTIC ACTIVITY IN COS-1 CELLS, R.A. Heller-Harrison and M.P. Czech, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, MA 01655.

Casein kinase II (CK-II), a nucleotide independent serine threonine protein kinase exists as an $\alpha_2\beta_2$ or $\alpha'_2\beta_2$ subunit structure. We have cloned the cDNAs for the α and β subunits of human CK-II into the expression vector pCMV1 and have co-transfected or individually transfected these constructs into COS-1 cells to test the hypothesis that both subunits of CK-II are required for CK-II activity to occur. Immunoblot analysis utilizing anti-CK-II antiserum of 22 k x g supernatant and pellet fractions from transient transfected COS-1 cells showed 1.5-2.0 fold increases in immunoreactive α subunit in both the α and (α + β) transfected cells in both supernatants and pellets relative to control cells. Immunoreactive β subunit in supernatants was increased 12.5 fold and 7.7 fold in the β and (α + β) transfected cells respectively, while the β subunit in pellets was increased 14.3 fold and 16.4 fold in the β and (α + β) transfected cells respectively. Supernatant and pellet fractions assayed for CK-II activitywere found to be increased by an average 3.5 and 4.4 fold respectively in the (α + β) transfected cells relative to control cells while cells transfected with the α or β subunit alone demonstrated no increases in CK-II activity. Immunoprecipitation of CK-II from ^{32}P-orthophosphate labeled COS-1 cells that were co-transfected with the α and β subunits showed the expressed β subunit to be phosphorylated in vivo. These results demonstrate 1) that human CK-II protein can be expressed in COS-1 cells and that the expressed protein has biological activity; and 2) that this activity is regulated in a manner similar to the endogenous protein in that increases in both subunits are required for an increase in CK-II activity.

CB 215 INSULIN STIMULATES MACROMOLECULAR UPTAKE INTO AND EFFLUX FROM ISOLATED H35 HEPATOMA CELL NUCLEI. L. Jarett, A. Peralta Soler, and R.M. Smith, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

The effect of insulin and other agents on the uptake of bovine serum albumin labeled colloidal gold particles (Au-BSA) into H35 hepatoma cell nuclei was determined. Au-BSA was prepared using 10, 15 or 24 nm diameter particles. Isolated intact nuclei were incubated for 60 min at 24° C with 15 nM Au-BSA in the presence or absence of 0.5 to 100 ng/ml of insulin and prepared for electron microscopy. Au-BSA was excluded from the nucleus in the absence of insulin irrespective of the size of the gold particle. Insulin caused a dose dependent nuclear uptake of 10 nm diameter, but not 15 or 24 nm, Au-BSA through the nuclear pores. By contrast, glucagon, EGF, PDGF and IGF-I did not stimulate uptake of Au-BSA. The insulin-stimulated uptake of Au-BSA was blocked by concanavalin A but wheat germ agglutinin mimicked the insulin effect. Efflux of Au-BSA from the nuclei was studied in nuclei previously loaded with Au-BSA in the presence of insulin. Nuclei were washed and reincubated with or without insulin in the presence or absence of 3 mM ATP for 60 min at 24°C. Efflux of Au-BSA was negligible in the absence of ATP. ATP alone resulted in the efflux of ~ 40% of the Au-BSA. Insulin stimulated the ATP-dependent efflux by 60%. These data indicate that insulin stimulates the uptake of non-karyophilic macromolecules into the nucleus via an energy independent pathway that can be blocked by concanavalin A. The insulin-stimulated uptake of non-karyophilic macromolecules was dependent on the size of the macromolecules, indicating that insulin has a limited effect on the functional diameter of the nuclear pores. In contrast to the energy independent nuclear uptake, the insulin-stimulated efflux of the macromolecules from the nucleus required the presence of ATP. These results suggest there are two insulin-sensitive macromolecular exchange routes between the nucleus and cytoplasm with different energy requirements.

CB 216 REGULATION OF KEMPTIDE INSULIN-STIMULATED KINASE (KIK) BY REVERSIBLE PHOSPHORYLATION,, Jes K. Klarlund, Andrew P. Bradford, Nazer Khalaf and Michael P. Czech, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester MA 01655.

We recently purified a novel insulin sensitive serine kinase (KIK) from livers of control and insulin treated rats [1]. In the presence of phosphatase inhibitors, activation of KIK by insulin is stable and the purified kinase exhibits up to 10-fold increases in specific activity (V_{max}) relative to KIK isolated from control rats. Treatment of purified insulin-stimulated KIK with protein phosphatase 2A (PP2A) results in a significant decrease in kinase activity (up to 17 fold) in a time and dose dependent manner. In contrast much smaller decreases (2-5 fold) are seen in activity of KIK from control rats . Inactivation of KIK by PP2A was not observed when phosphatase was added after the preincubation period and was completely blocked by the presence of nitrophenyl phosphate. Preincubation of purified KIK with protein phosphatase 1 had little effect on the activity of the kinase. In other experiments, treatment of isolated rat adipocytes with okadaic acid, a potent inhibitor of PP2A, results in a significant increase (2-4 fold) in cytosolic Kemptide kinase activity similar to that previously reported in response to insulin [2]. This insulin/ okadaic acid sensitive kinase activity comigrates with KIK on Mono Q FPLC chromatography. The effects of insulin and okadaic acid are not additive. These results are consistent with our hypothesis that the insulin stimulation of KIK is mediated by reversible phosphorylation catalysed by a yet unidentified kinase kinase.

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CB 217 INDUCTION OF aP2 BY AN INSULIN SENSITIZING AGENT, Rolf F. Kletzien, Peter K.W. Harris, Lisa A. Foellmi, Beatrice M. Wyse, Jerry R. Colca and Steven D. Clarke, Metabolic Diseases Research, Laboratory of Diabetes, Upjohn Company, Kalamazoo, MI, 49001

Pioglitazone is anti-diabetic agent that increases sensitivity of target tissues to insulin through an undefined mechanism. aP2 is a fatty acid binding protein which may mediate some actions of insulin in adipcoytes. The aP2 gene is transcriptionally regulated during differentiation of preadipocytes to mature adipocytes. Pioglitazone accelerates the insulin or IGF induced differentiation of 3T3-L1 cells. Analysis of the kinetics of drug-related changes in the cellular phenotype indicate that pioglitazone is inducing changes in gene expression during the first few hours of treatment. We have studied the effect of the drug on specific mRNA species and have found that the aP2 mRNA is elevated 10-fold within four hours of treatment of appropriately staged 3T3-L1 cells. Several other insulin-regulated mRNA species (c-fos, Glut-4, LPL) were not induced by treatment with pioglitazone alone. Thus, the aP2 gene is candidate for direct action of the drug. Induction of aP2 by pioglitazone was also observed in animal models. Treatment of ob/ob mice with various levels of the drug for six weeks caused a dose-dependent increase in aP2 mRNA in epididymal fat. The increase in epididymal fat pad aP2 mRNA correlated positively with the changes in the physiological parameters effected by the drug. Pioglitazone treatment of KKA^y mice resulted in a 2 to 3-fold increase in aP2 mRNA. A possible direct effect of pioglitazone on the aP2 gene is being evaluated by analysis of aP2 promoter/CAT plasmids transfected into 3T3-L1 cells.

CB 218 AN INSULIN RECEPTOR, AN INSULIN GENE, AND INSULIN-INDUCED PROTEIN PHOSPHORYLATION IN NEUROSPORA CRASSA, John Lenard, Ganapathy Muthukumar and Hemanta Kole, Department of Physiology and Biophysics, UMDNJ-Robert Wood Johnson Med School, Piscataway, N.J. 08854-5635.

N. crassa secretes insulin as detected by RIA. The wall-less ("slime") strain responds to mammalian insulin in several ways, including increased glucose utilization and increased glycogen production arising from activation of glycogen synthase. These observations suggest the existence of an insulin-mediated signal transduction pathway in N. crassa. Three recent findings support this hypothesis: (i) A gene encoding an insulin-like protein has been identified and sequenced. (ii) A membrane-bound insulin-binding protein, a putative receptor, has been purified to homogeneity. It has an apparent m.w. of 66 kDa, pI=7.4. Several mammalian insulins bind equally well (Kd=ca. 20 nM) but proinsulin, IGF-I and IGF-II are not bound. (iii) Mammalian insulin induces a ca. 25% enhancement of incorporation of ^{32}Pi into TCA precipitable material in 30 min. Two-dimensional gels showed differences in phosphorylation of at least 8 specific proteins. Most were phosphorylated on ser/thr, but several were alkali stable, indicating tyr phosphorylation. Insulin also stimulated phosphorylation by ATP of specific proteins in isolated membranes, at both ser/thr and tyr. One or more of these proteins may be involved in mediating insulin-induced metabolic effects in N. crassa.

CB 219 TRANSLATIONALLY REGULATED INSULIN-SENSITIVE GENE PRODUCTS, Richard M. Levenson and Perry J. Blackshear, Howard Hughes Medical Inst. (PJB) and Dept.of Pathology, Duke University Medical Center and GRECC, Durham VA Medical Center (RML), Durham, NC 27705.

Cells exposed to insulin and other growth promoting substances can respond by increasing their overall rate of protein synthesis; the synthesis of individual proteins may also be preferentially affected (Levenson et al., JBC, 264:11904-11911). We used ultra-high resolution ("giant") two-dimensional gel electrophoresis to define early (0-5 h) protein synthetic responses to insulin and to various growth factors and PMA. The cell model employed is the HIR 3.5 cell line (Whittaker et al., PNAS, 84: 5237), NIH 3T3 cells transfected with a cDNA encoding the human insulin receptor. After treatment and metabolic labeling, cell lysates are separated on giant gels and the resulting autoradiographs are digitized and analyzed using Bio Image 2-D gel software. More than 20 of the 2,500 proteins visualized are preferentially synthesized following insulin treatment. This set includes the protein, elongation factor 2, which itself plays a role in protein synthesis. Kinetics for these inductions vary, with some attaining maximum synthetic rate within 1 h of treatment. We find that the synthesis of the majority of the early insulin-responsive proteins, including EF-2, is regulated at the translational, not the transcriptional, level, since actinomycin D, an inhibitor of RNA transcription, does not block their synthesis and for some proteins actually acts synergistically with insulin. While all the insulin-induced proteins so far detected are also induced by the PKC-activator, PMA, we find that insulin markedly inhibits the synthesis of at least one protein unaffected by PMA, and conversely, PMA induces the synthesis of at least 3 additional proteins which are not sensitive to insulin. These PMA-inductions are also translationally regulated, thus providing a model of agent-specific, probably PKC-dependent, translational regulation. PDGF, among other growth factors tested, also induces the translation of apparently PDGF-specific proteins. The sensitivity of these inductions to transcriptional inhibitors is under investigation.

CB 220 INHIBITION AND STIMULATION OF C-MYC GENE TRANSCRIPTION BY INSULIN IN RAT HEPATOMA CELLS: INSULIN ALTERS THE INTRAGENIC PAUSING OF C-MYC TRANSCRIPTION, Joseph L. Messina, Department of Physiology, SUNY Health Science Center at Syracuse, Syracuse, New York 13210.

One of insulin's least studied actions is its ability to induce DNA synthesis and cell division. In rat H4IIE hepatoma cells insulin, acting through its own receptor, stimulates cell division. However, little is known about the molecular mechanisms involved in this effect. The proto-oncogene c-myc is a cellular gene which when expressed at abnormal levels is often associated with the process of tumorigenesis. Expression of the normal cellular myc gene may be necessary for growth factor-induced cell cycling. In the present work, insulin was shown to regulate cellular accumulation and transcription of the c-myc gene in rat hepatoma cells. The control of c-myc by insulin was complex, with an initial insulin-induced decrease in c-myc transcription to 50% of control values at 15 and 30 min. This was followed by an increase in transcription of about 3-fold by 60-120 min. Similar to the initial inhibitory effect of insulin, the protein synthesis inhibitors cycloheximide or anisomycin decreased c-myc transcription. However, there was no secondary induction of c-myc transcription by protein synthesis inhibitors. The effects of both insulin and protein synthesis inhibitors were shown to be through alterations in intragenic pausing of transcription of the sense mRNA, not through changes in initiation of transcription.

CB 221 EXPRESSION AND BIOCHEMICAL ASSAY OF NORMAL AND NOVEL GLUCOKINASE cDNAS, **Christopher B. Newgard, Christian Quaade, and Steven D. Hughes**, Center for Diabetes Research and Department of Biochemistry, UT Southwestern Medical Center, Dallas, TX 75235

Glucokinase has been ascribed a central role in the regulation of glucose-stimulated insulin secretion from islet β-cells. A number of variant transcripts of the single rat glucokinase gene have recently been described that are expressed in islets, liver, and anterior pituitary. In order to begin to address the physiological significance of the multiple glucokinase gene products, four of these naturally occuring transcripts were prepared as cDNA and were expressed in bacteria. The constructs used were as follows: 1) pGKB1, the normal glucokinase cDNA expressed in islets and anterior pituitary; 2) pGKB3, a variant glucokinase cDNA of islets and anterior pituitary with a 52 nucleotide deletion in exon 2; 3) pGKL1, normal liver glucokinase cDNA, and 4) pGKL2, normal liver glucokinase cDNA with a 151 bp insert between exons 1 and 2 plus the 52 nucleotide deltion in exon 2. Expression of constructs pGKB1 and pGKL1 resulted in a glucose dependent, glucokinase-like activity, 10-fold and 100-fold, respectively, above the background bacterial glucose phosphorylation activity when assayed at 50 mM glucose. Expression of pGKB3 and pGKL2, in contrast, did not result in any glucokinase-like activity. Expression of protein products from all four DNA constructs was verified using two anti-glucokinase antibodies, one directed against the N-terminal peptide specific for islet glucokinase (U343) and the other against a peptide from a region of common sequence in the liver and islet protein (V980). The glucokinase activity in crude extracts of bacteria expressing pGKB1 or pGKL1 was unaffected by mixing with either of the enzymatically inactive gene products, suggesting that these proteins do not directly regulate glucokinase activity. Immunofluorescence analysis with antibody U343 reveals that glucokinase is not randomly distributed in the cytoplasm of cultured cell lines derived from islet and anterior pituitary, but rather is found as aggregates. We are currently investigating whether alternate glucokinase transcripts might play a role in determining the intracellular distribution of the active glucokinase enzyme.

CB 222 STIMULATION OF mRNA TRANSPORT FROM ISOLATED NORMAL AND DIABETIC NUCLEI BY CALMODULIN AND THE INSULIN RECEPTOR, Dorothy Schumm and Saroj Larroya-Runge, Department of Physiological Chemistry and the Comprehensive Cancer Center, The Ohio State University College of Medicine, Columbus, OH 43210

A number of possible modulators of the insulin effects on nuclei were tested in a cell-free system containing only defined components. This system has been shown to release mature mRNA in a time and energy-dependent manner. Only slight (<15%) stimulation was observed with protein kinase C, trisinositol phosphate and insulin (10^{-6} to 10^{-12} M). Calmodulin at a concentration of 1.0 μM increased RNA release from normal rat liver nuclei by 36% and at 5.0 μM, 100% in a 30 min. period. Increases were slightly higher for diabetic nuclei (125% at 5 μM calmodulin). Similar stimulation was observed for poly A (+) RNA. This stimulation was eliminated in the absence of calcium or the presence of specific calmodulin inhibitors. Calmodulin binding to nuclei and calmodulin stimulation of RNA transport showed similar pCa curves. Purified activated insulin receptor also markedly stimulated release of both total and poly A (+) RNA from both types of nuclei. It also decreased the calmodulin concentration required for maximal stimulation of RNA release from 5.0 μM to approximately 2 μM without altering the pCa curve. Studies are continuing on the processing and release of specific mRNAs which are known to be stimulated (albumin), inhibited (PEPCK) or unaffected (β-actin) by insulin in vivo.

CB 223 CHARACTERIZATION OF A NOVEL CYTOSOLIC, INSULIN- STIMULATED SERINE KINASE FROM RAT LIVER L. Wolfe and M.P. Czech, Dept. of Biochemistry and Molecular Biology and Program in Molecular Medicine, Univ. of Mass. Medical Center, Worcester, MA 01605

A cytosolic insulin-sensitive serine kinase (ISSK) has been purified to apparent homogeneity in parallel from livers of control or insulin-treated rats. ISSK is very labile and requires rapid purification for stability. ISSK migrates with an apparent MW of 90,000 on denaturing gels and elutes as a monomer on Superose 12 gel filtration FPLC. Purified ISSK from insulin-treated rats shows a 2-3 fold higher specific activity compared to enzyme prepared from control rats, suggesting a covalent modification as the mechanism of activation. Substrates of ISSK include kemptide, microtubule-associated protein 2 (MAP2), and myelin basic protein (MBP), with MBP being a 10-50-fold better substrate than MAP2. ISSK also phosphorylates histones H1, H2B, H3, and H4, with histone H2B being phosphorylated to a stoichiometry of 0.6 to 0.7 pmol Pi per pmol H2B. ISSK does not autophosphorylate to a significant stoichiometry. The activity of ISSK is inhibited by fluoride, glycerophosphate, heparin, and potassium phosphate using MBP as substrate, but is unaffected by calcium, cAMP or vanadate. Interestingly, the activity of the enzyme is inhibited by polylysines using MBP as substrate, but is stimulated by polylysines using MAP2 as substrate. ISSK will utilize magnesium (10 mM) as well as manganese (1 mM). The inability to autophosphorylate, the ability to phosphorylate histones, and the lack of effect of vanadate on the activity distinguish this enzyme from the 90 kDa S6 kinase from Xenopus. These and other properties distinguish this kinase from those previously described, indicating that ISSK is a novel insulin-stimulated serine kinase.

Diabetes

CB 224 INSULIN-MIMETIC EFFECTS OF ORAL VANADYL SULFATE IN THE STREPTOZOTOCIN-DIABETIC RAT, Margaret C. Cam, Raymond A. Pederson, Roger W. Brownsey, Julie Faun and John H. McNeill, Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, B.C., V6T 1W5 Canada.

The effects of oral vanadyl sulfate were studied in vivo at several time points after onset of diabetes. Wistar rats made diabetic with streptozotocin and age-matched controls were administered vanadyl sulfate trihydrate 0.75 mg/ml ad libitum in the drinking water. Treatment was initiated at 3 (DT3), 10 (DT10), and 17 (DT17) days following induction of diabetes. Food and fluid intake was dramatically reduced in the diabetic-treated (DT) rats to control levels following treatment, although average body weight did not differ from untreated diabetics. At the concentration given, euglycemia was achieved in 44% of total DT rats (responders: 33% in DT3, 64% in DT10, and 38% in DT17) and maintained without apparent toxicity for 5 months. Plasma cholesterol and triglycerides at termination in the DT rats did not differ from control. Plasma insulin was reduced in the control-treated (CT) group compared to C but was not significantly increased in the various DT groups. Oral glucose tolerance tests performed at 5 months of treatment showed an improved glucose tolerance in the DT groups without substantial insulin release. Thus, oral vanadyl sulfate increases tissue sensitivity to insulin in response to an oral glucose load and corrects various aberrations in plasma parameters even after the occurrence of a two-week long diabetic state. (Supported by CDA).

CB 225 A MUTATION IN THE TYROSINE KINASE DOMAIN OF THE INSULIN RECEPTOR THAT IMPAIRS INSULIN ACTION AND INCREASES BINDING AFFINITY FOR INSULIN, Alessandro Cama, Maria de la Luz Sierra, Laura Ottini, Simeon I. Taylor, Diabetes Branch NIH, Bethesda, MD 20892.

Insulin resistance is an important factor predisposing to the development of noninsulin-dependent diabetes mellitus. In addition, obesity appears to play an important role in causing insulin resistance. Nevertheless, even among obese people, there is considerable variability in insulin sensitivity. To evaluate the interaction between obesity and genetic risk factors, we have sequenced the insulin receptor gene in an obese patient with insulin resistance (weight, 82 kg; height, 157 cm). The patient, a 30 yr old woman, had a history of acanthosis nigricans and hirsutism as an adolescent. However, the acanthosis nigricans had disappeared approximately 10 years earlier, and the hirsutism had been treated by medical therapy and electrolysis.

The patient was heterozygous for a mutation substituting isoleucine for methionine at position 1153. Met-1153 is located in the intracellular domain of the receptor near the cluster of tyrosine phosphorylation sites at positions 1158, 1162, and 1163. Studies of the Ile-1153 mutant receptor expressed in NIH-3T3 demonstrated that the Ile-1153 mutation impairs the ability of insulin to activate receptor tyrosine kinase activity. Interestingly, this mutation in the intracellular domain increased the affinity with which insulin bound to the extracellular domain of the receptor. In addition the mutation appears to impair the ligand induced receptor internalization.

In conclusion, the Ile-1153 mutation inhibits the ability of insulin to activate receptor autophosphorylation, and impairs the ability of the receptor to mediate insulin action in vivo. In addition, the observation that the mutation increases the affinity of insulin binding provides evidence of the allosteric interactions between the intracellular and extracellular domains of the insulin receptor. Furthermore, this patient provides a paradigm in which genetic factors act in concert with other risk factors such as obesity to cause clinically important insulin resistance.

CB 226 MOLECULAR ANALYSIS OF THE INSULIN RECEPTOR GENE IN FAMILIAL TYPE 2 DIABETES, Steven C. Elbein, Lise Sorensen, & Michelle Taylor, Division of Endocrinology, VAMC and University of Utah School of Medicine, Salt Lake City, UT 84105.

Insulin resistance and decreased insulin receptor (IR) tyrosine kinase activity have been described in type 2 diabetes. To test the hypothesis that these phenomena result from IR β-chain mutations, we examined members of 19 white pedigrees ascertained for $\geq$2 diabetic siblings by linkage analysis with IR RFLP, northern blot analysis of RNA from lymphoblastoid cell lines, RNA mismatch analysis of the β-chain, and PCR based screening by single strand conformation analysis and sequencing of exons 13, 14, and 17. No single allele could account for affected status in 8/13 pedigrees analyzed by linkage. Northern blot analysis of RNA from 29 individuals with diabetes or hyperinsulinemia showed patterns and IR RNA quantities similar to control samples. RNA mismatch analysis detected no β-chain mutations with probes covering exons 14 - 22. In PCR conformation analysis of >110 individuals 2 exon 17 mutations were found, representing 9% and 35% of individuals, but both appear to be silent. Two rare mutations remain to be evaluated. Mutational patterns were also found with exons 13 (14%) and 14 (12%), but the latter was silent in analysis of one individual. IR mutations, particularly of the β-chain, do not appear to be common in familial diabetic pedigrees when examined by several methods, but additional analyses are in progress.

CB 227 GLUCOSE TRANSPORT DEFICIENCY IN DIABETIC ANIMALS IS CORRECTED BY TREATMENT WITH AN ORAL HYPOGLYCEMIC AGENT, Cecilia Hofmann, Kathryn Lorenz, and Jerry R. Colca, Loyola Stritch School of Medicine, Maywood, IL 60153, Hines VA Research Service, Hines, IL 60141, The Upjohn Company, Kalamazoo, MI 49001
Pioglitazone is a thiazolidinedione hypoglycemic agent which may be useful for management of patients with diabetes. Genetically obese, *insulin-resistant* (IR) KKAy mice and streptozotocin-injected *insulin-deficient* (ID) rats were utilized to examine the effects of diabetes and hypoglycemic drug~ and/or insulin treatment on glucose transport activity and expression of GLUT4 glucose transporter mRNA in fat and muscle. Blood glucose compared to nondiabetic control animals was significantly elevated in IR (162 vs 499 mg/dl) and ID (165 vs 502 mg/dl) animals. In contrast, insulin levels compared to respective controls were significantly higher in the IR mice (14 vs 496 µU/ml), but lower than controls in the ID rats (22 vs 3 µU/ml). GLUT4 mRNA abundance was decreased significantly compared to nondiabetic control levels in fat (-49%) and muscle (-42%) of IR diabetics as well as in fat (-52%) and muscle (-28%) of ID diabetics. Following pioglitazone therapy (20mg/kg/day for 4 days), elevated blood glucose levels were lowered significantly (-70% for IR and -42% for ID) and deficient GLUT4 mRNA levels were restored to control levels in both IR and ID animals, although the ID animals required partial insulin replacement (6U Ultralente/day) for this action. In ID rats, neither drug alone nor insulin at this low dose resulted in significant correction. Since the presence of insulin was requisite for correction of glucose transport deficiencies by pioglitazone, it appeared that the agent acted to amplify cellular responses to insulin.

CB 228 INHIBITION OF GLUCOSE OXIDATION IN DIABETES, STARVATION AND OBESITY: A MOLECULAR BASIS. Alan L. Kerbey, Philip J. Randle & Ian D. Caterson*, Dept of Clinical Biochemistry, John Radcliffe Hospital, Oxford, U.K. and *Dept of Endocrinology, Royal Prince Alfred Hospital, Sydney, Australia.
In animals including man, glucose oxidation is decreased by diabetes, starvation, obesity, high-fat feeding and oxidation of lipid fuels, and increased by exercise, carbohydrate intake and inhibitors of lipid oxidation. The major determinant of rates of glucose oxidation under these conditions is the activity of mitochondrial pyruvate dehydrogenase complex (PDHC) which is regulated by reversible phosphorylation (phosphorylation is inactivating). We have investigated the molecular basis of enhanced phosphorylation of PDHC in IDDM and starvation (rat heart, liver) and in NIDDM with obesity (mouse heart) and give evidence that longer-term mechanisms involve a stable increase in activity of PDH kinase (PDHK) brought about by increased specific activity of a mitochondrial protein activator of PDHK (referred to as PDHK activator protein, KAP)(Mr: native protein, 75kDa; subunit, 36kDa). On the basis of experiments with primary rat hepatocytes and cardiac myocytes in tissue culture, we give evidence that interconversion of KAP between more-active and less-active forms is subject to regulation by hormones (insulin inactivates; glucagon activates) and by oxidation of lipid fuels (activate); and that KAP activity is inversely correlated with PDH complex activity in vivo. KAP has been identified in mitochondrial extracts of rat heart, hind-limb muscle, liver, kidney and white and brown adipose tissue (IDDM or starvation increase KAP activity approx. 3-fold in these tissues). KAP is present in an active form in mitochondrial extracts of rat brain but it is not increased in activity when mitochondria are prepared from starved animals. We suggest that modulation of KAP between more-active and less-active forms may be of importance in tissues where operation of the glucose-fatty acid cycle is fundamental to fuel selection; and that KAP is likely to be of substantial importance in mediating effects of IDDM and NIDDM on whole body glucose homeostasis.

CB 229 INSULIN RESISTANCE AND DIABETES DUE TO DIFFERENT MUTATIONS IN THE TYROSINE KINASE DOMAIN OF BOTH INSULIN RECEPTOR GENE ALLELES, J. Kusari, Y. Takata, E. Hatada, G. Freidenberg, O. Kolterman, and J.M. Olefsky, UCSD, Dept. of Med., La Jolla, CA 92093 and the VAMC, San Diego, CA 92161.
Mutations in the insulin receptor gene can lead to in vivo and in vitro insulin resistance and also diabetes in selected patients. We have studied a diabetic woman (22 yr) with Type A insulin resistance and acanthosis nigricans. Insulin binding to the patient's erythrocytes, monocytes, adipocytes, fibroblasts and transformed lymphocytes was decreased. Receptor autophosphorylation and tyrosine kinase activity were reduced in partially purified insulin receptors from the proband's transformed lymphocytes. Determination of the nucleotide sequence of the patient's insulin receptor cDNA revealed that the subject was a compound heterozygote who inherited two different mutant insulin receptor gene alleles. The paternal allele contains a missense mutation encoding the substitution of glutamine for arginine at position 981 in the tyrosine kinase domain of the receptor. The maternal allele contains a nonsense mutation causing premature termination after amino acid 988 in the ß-subunit, thereby deleting most of the kinase domain. In summary: (1) we have identified a patient and her family with a genetic form of insulin resistance and diabetes due to a defect at the level of the insulin receptor, (2) the proband is a compound heterozygote displaying a missense mutation (position 981) in one allele and a nonsense mutation (position 988) in the other insulin receptor gene allele, (3) The missense mutation is in the kinase domain and encodes a receptor with impaired in vitro kinase activity, and (4) based on the in vitro and in vivo phenotype the kinase domain mutation at position 981 is biologically significant leading to insulin resistance.

CB 230 ALTERATIONS OF PHOSPHORYLASE IN DIABETIC RATS AND THE EFFECTS OF INSULIN-MIMETIC AGENT VANADYL SULPHATE TREATMENT. Heyi Liu and John H. McNeill, Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, B.C., V6T 1W5 Canada.

A supersensitivity to isoproterenol (ISO)-induced phosphorylase activation in hearts from diabetic rats and a decrease in the total hepatic phosphorylase activity in diabetic rats have been previously reported. The nature of the supersensitivity and the effect of vanadyl sulphate (VS) treatment on hepatic phosphorylase are investigated in this study. It was observed that both basal and ISO-stimulated phosphorylase kinase activities were significantly increased in diabetic heart. Verapamil (50 nM) was shown to abolish the supersensitivity of cardiac phosphorylase activation by ISO (5 nM), suggesting that calcium levels may be partially responsible for this alteration. Treatment with VS in drinking water for five months completely restored the decrease in total and phosphorylase a activities in the liver from diabetic rat. The effects of verapamil on cardiac phosphorylase kinase and the effects of VS on the supersensitivity of cardiac phosphorylase in diabetic rats are currently under investigation. [Supported by MRC (Canada) and CDA.]

CB 231 CHARACTERIZATION OF NATURALLY OCCURRING KINASE-DEFICIENT INSULIN RECEPTOR MUTATIONS David E. Moller, Heike Benecke, Atsushi Yokota, and Jeffrey S. Flier, Beth Israel Hospital Boston, MA 02215.

Mutations in the insulin receptor (IR) gene have been reported in patients with severe insulin resistance, however insulin signalling via these mutant IRs has not been described. We have recently identified 2 mutations in the IR tyrosine kinase domain in subjects with the Type A syndrome: $Trp^{1200} \rightarrow Ser^{1200}$ and $Ala^{1134} \rightarrow Thr^{1134}$. Both mutant IR cDNAs were expressed in CHO cells; IR processing and insulin binding affinity were normal. IR autophosphorylation (in intact cells and with solubilized IRs) was severely impaired (< 5% of control IRs). Kinase activity towards an endogenous substrate (pp185) was undetectable with both IR/Ser^{1200} and IR/Thr^{1134} cells. Both mutant IRs failed to phosphorylate an exogenous substrate *in vitro*. Several biologic actions of insulin were studied in transfected cells. Insulin stimulation of [^{3}H]thymidine incorporation and specific gene expression (GLUT-1) was impaired with both mutants. Surprisingly, IR/Ser^{1200} cells retained insulin sensitivity for glucose uptake and [^{14}C]glucose incorporation into glycogen while the equally kinase-defective IR/Thr^{1134} cells failed to signal these actions. Conclusions: 1) Transfected kinase-deficient IR mutations derived from insulin resistant subjects have distinct defects in the ability to mediate insulin action. 2) Ala^{1134} and Trp^{1200} are highly conserved residues which are likely to be important for the function of tyrosine kinases. 3) These studies demonstrate divergence of insulin signalling pathways at the IR and suggest that IR kinase activity is not always required for certain important IR mediated biologic effects.

CB 232 GLUCOSE TRANSPORTER LEVELS IN TISSUES OF THE SPONTANEOUSLY DIABETIC ZUCKER (fa/fa) RAT, Lawrence J. Slieker, Karen L. Sundell, William F. Heath, H. Edward Osborne and Julie Bue. Diabetes Research Division, Lilly Research Laboratories, Indianapolis, IN 46285

We have used antibodies to the fat/muscle glucose transporter (GLUT4) and the liver glucose transporter (GLUT2) to measure levels of these proteins in various tissues of obese nondiabetic and obese spontaneously diabetic male Zucker (fa/fa) rats. This strain develops overt diabetes associated with decreased plasma insulin levels. Depending on the diet used and the age of the rats, age matched lean, obese and obese spontaneously diabetic rats can be generated as a model of human NIDDM.

Group (30 wks)	Weight	Fed plasma glucose	Fed plasma insulin
Lean (L), n=5	476 ± 7 g	130 ± 3 mg/dl	1.2 ± 0.28 ng/ml
Obese (O), n=8	693 ± 13	175 ± 6	14.7 ± 1.8
Diabetic (D), n=6	562 ± 24	392 ± 33	2.9 ± 0.48

In fat, GLUT4 levels in O did not differ from L, but levels in D were reduced 50% when normalized to either protein ($p<0.002$) or DNA ($p<0.02$). In liver, GLUT2 levels were increased 30-40% in D vs O or L on both a protein and DNA basis ($p<0.01$). In heart, O GLUT4 levels did not differ from L on a DNA basis, but were reduced 25% on a protein basis ($p<0.02$). In both cases, D were reduced 35% compared to L ($p<0.01$) and 15-30% compared to O. In soleus, GLUT4 was only reduced in D (25%, $p<0.02$), and only on a protein, not a per cell, basis. These data suggest that in this model of NIDDM, glucose transporter levels in muscle, adipose tissue and liver are regulated differently in response to changes in insulin and glucose, and that alterations in GLUT2/4 protein levels are not associated with obesity *per se*, but are secondary to the diabetic state.

CB 233 **Elevated FFA Levels Inhibit the Insulin Receptor Binding and Degradation in Isolated Rat Hepatocytes without Influencing the Tyrosine Kinase Activity in Solubilized Receptors.** Jan Svedberg, Per Björntorp, Ulf Smith and Peter Lönnroth, The Wallenberg Laboratory and the Department of Medicine, Sahlgren's Hospital, University of Gothenburg, Sweden.

Abdominal obesity is associated with insulin resistance both in the liver and peripheral tissues. This effect could be due to elevated FFA levels which may directly influence insulin action.
In the present study, we investigated the effect of different FFA concentrations on insulin binding and action in isolated rat hepatocytes. High physiological FFA levels (0.4 mM) decreased specific insulin binding ≈ 40 % without changing apparent receptor affinity. There was a significant linear correlation between the decrease in insulin binding and the decrease in hormone degradation, measured by TCA-precipitation, by different FFA concentrations. This inhibitory effect was energy-dependant and associated with FFA oxidation. Etomoxir, a specific inhibitor of carnitine palmitoyl transferase (CPT 1), shifted the dose-response curve for inhibitory effect of FFA 2-3 fold to the right. Preincubating the cells with FFA did not influence insulin binding to partially purified receptors from subsequently solubilized cells. Receptor tyrosine kinase activity, both basal and insulin-stimulated, was unchanged by pretreating the cells with FFA. **Conclusion:** Elevated FFA levels decrease insulin binding and degradation in rat hepatocytes through an energy-dependant mechanism which can be overcome by Etomoxir, a CPT 1 inhibitor. However, binding to partially purified receptors, as well as the basal and insulin-stimulated tyrosine activity are not changed. These data are compatible with an effect of FFA on the receptor recycling in intact cells.

CB 234 DIFFERENTIAL FOOD INTAKE IN DIABETIC RATS WITH DIFFERENT ROUTES OF INSULIN DELIVERY. A. E. Willing, E. K. Walls, & H. S. Koopmans.
Department of Medical Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4.

Two major theories of the control of food intake state that increasing systemic plasma insulin levels should reduce daily intake. Infusing insulin in doses of 2.0 and 3.0 U/day i.v. into either the vena cava or the portal vein of diabetic rats should lead to differences in feeding since the liver removes half of the insulin that passes through it. Food intake in the vena cava group increased from 71.6 ± 1.5 kcal/day during prediabetic baseline to 93.9 ± 1.3 and 112.0 ± 0.9 kcal/day at 2.0 and 3.0 U/day, respectively ($p < .001$). In the portal group, there were significant increases in intake from 74.2 ± 2.4 to 91.4 ± 1.4 at 2.0 U/day and only 93.9 ± 1.3 kcal/day at 3.0 U/day. While there was no difference between daily intakes during the 2.0 U/day infusion, at 3.0 U/day ,intake was significantly less in the portal group ($p < .001$). Weight gain in the vena cava animals increased from 1.2 ± 0.05 to 3.6 ± 1.9 g/day ($p < .001$); the portally infused group gained 1.5 g/day throughout the study. Therefore, the higher systemic insulin levels that occur with vena cava rather than portal insulin infusion induce overeating and rapid weight gain. These results contradict the two major theories.

This research was supported by grants from MRC, NIH, NSERC, and AHFMR.

CB 235 EFFECT OF NIDDM ON THE GLUCOSE TRANSPORT SYSTEM IN SKELETAL MUSCLE.. J.F. Youngren, R.J.Barnard and S.H. Sheck.
Department of Kinesiology, UCLA, Los Angeles, CA 90024-1527

Insulin resistance in skeletal muscle is known to be a principal aspect of NIDDM. The purpose of this study was to investigate the glucose transport system in muscle samples obtained from NIDDM patients and lean controls. NIDDM patients showed significantly elevated plasma glucose levels (182.4 ± 23.2 vs 97.7 ± 6.7 mg/dl). Basal glucose transport was not different between the control and NIDDM groups (32.6 ± 9.5 vs 30.9 ± 4.4 pmol/mg protein). The number of insulin receptors was reduced in NIDDM (0.82 ± 0.03 vs 1.63 ± 0.18 pmol/mg protein) and the affinity for insulin binding increased (kd 93.3 ± 3.1 vs 138 ± 11.9 nM). Tyrosine kinase activity, as assessed from the incorporation of ^{32}P-ATP into Glu 4:Tyr 1, was reduced in NIDDM at all insulin concentrations from 1 to 100nM. Maximum kinase activity was reduced (1.88 ± 0.04 vs 2.97 ± 0.07 fmol ^{32}P/fmol insulin binding). The number of microsomal glucose transporters was not significantly different between the groups (7.65 ± 0.9 vs 7.01 ± 1.4 pmol/mg protein). These results show that alterations in both insulin receptor number and function may play a substantial role in skeletal muscle insulin resistance in NIDDM.

Late Abstracts

INSULIN-STIMULATED PHOSPHATIDYLINOSITOL 3-KINASE ACTIVITY IS ASSOCIATED WITH pp185 IN CHO CELLS EXPRESSING THE HUMAN INSULIN RECEPTOR, JM Backer, GG Schroeder, X-T Sun, P Rothenberg, CR Kahn, and MF White, Joslin Diabetes Center, Boston, MA 02215

Insulin induces a 10-fold stimulation of phosphatidylinositol 3-kinase (PIK) activity in CHO cells expressing human insulin receptors (CHO/IR). The PIK can be detected in vitro in antiphosphotyrosine (αPY) immunoprecipitates from insulin-stimulated cells and may be a substrate of the IR. However, the stimulated PIK is weakly associated with the IR itself, as only 20% of insulin-stimulated PIK activity is detectable in anti-receptor immunoprecipitates. To determine whether other substrates might mediate the interaction between the IR and the PIK, insulin stimulated CHO/IR cells were immunoprecipiated with an antipeptide antibody to the endogenous substrate pp185 (αpp185) and assayed for PIK. The αpp185 immunoprecipitates contained levels of PIK activity similar to that seen in αPY precipitates. The activity was stimulated 10-20-fold by insulin and its immunoprecipitation was blocked by excess pp185-derived peptide. Moreover, during HPLC analysis the deacylated lipid products obtained from αPY or αpp185 immunoprecipitates were identical and eluted as gPI-3-P. These data show that pp185 associates with the PIK in insulin stimulated cells. We propose that the tyrosyl-phosphorylated pp185 may serve as a coupling protein between the IR and the PIK, analogous to the coupling of PIK to $pp60^{c\text{-}src}$ by the polyoma middle T.

CHARACTERIZATION AND PROCESSING OF TWO HUMAN INSULIN RECEPTORS WITH CYS TO SER MUTATONS IN THE C-TERMINAL PORTION OF THE α SUBUNIT, Bentley Cheatham, Chris J. Rhodes, and C. Ronald Kahn, Joslin Diabetes Center, Boston, MA 02215.

The Cys residues in the C-terminal portion of the α-subunit have been suggested as likely candidates to be involved in the α-β interaction. In order to determine the structural and functional significance of these C-terminal α-subunit Cys residues, two site-directed mutants were constructed which modify all four Cys residues in this region: C647S contains a point mutation converting Cys^{647} to a Ser; C682,3,5S is a triple mutant in which $Cys^{682,683,685}$ have been converted to Ser residues. Since these mutants are defecient in Cys residues that may play a critical role in the initial folding of the proreceptor, these mutants may not be properly processed. Recently an *in vitro* processing assay has been established based on a previously uncharacterized proteolytic activity which has been found in secretory granules from insulinoma tissue and in purified rat liver golgi vesicles. This processing activity has been shown to convert the 190 kDa IR precursor to its α and β subunits. Using *in vitro* translated wild-type human IR, C647S, and C682,3,5S in the *in vitro* processing assay we find that wild-type IR and both Cys mutants are converted from 152 kDa unglycosylated proreceptor forms to an 82.5 kDa and 69.7 kDa unglycosylated α and β-subunits respectively. These results show that an *in vitro* synthesized unglycosylated insulin proreceptor is also a substrate for the proprotein processing endopeptidase. Furthermore both Cys mutants appear to be recognized by the proteolytic processing enzyme(s). Preliminary characterization of these receptors expressed in COS cells has shown that cells transfected with wild-type IR, C647S, or C682,3,5S result in a net 2-4.5 fold increase in insulin binding as compared to mock transfected cells. Both mutant IR display binding affinities similar to the wild-type receptor. Expression of these mutant receptors and their apparent normal ligand binding suggest that the α and β subunits are probably still intact. The establishment of stably transfected CHO cell lines and structural analysis of these mutant IR are underway and should provide a more detailed analysis with respect to their subunit structure, binding kinetics, and signaling capabilities.

REGULATION OF INSULIN, EPIDERMAL GROWTH FACTOR AND TRANSFORMING GROWTH FACTOR ALPHA LEVELS BY GROWTH FACTOR DEGRADING ENZYMES, Barry D. Gehm and Marsha Rich Rosner, Ben May Institute and Dept. Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637.

The mechanisms by which growth factors are degraded and the role this process plays in the regulation of cell growth are not well understood. Insulin degradation is believed to be mediated by a specific metalloprotease, insulin degrading enzyme (IDE). We have previously shown that IDE can also degrade transforming growth factor alpha (TGFα) but not epidermal growth factor (EGF) in vitro. This selectivity was surprising since TGFα and EGF are structurally similar and bind to the same receptor with comparable affinities. Using a spectrum of protease inhibitors, we have now analyzed the in vivo degradation of TGFα, EGF, and insulin by human hepatoma HepG2 cells. The results suggest that bacitracin-sensitive metalloproteases are involved in the degradation of TGFα and EGF as well as insulin, and that the degradation of TGFα, but not EGF, is mediated in part by the IDE. Inhibiting the activity of these metalloproteases decreased growth factor depletion, suggesting that these enzymes play an important role in the control of extracellular growth factor levels. The existence of separate degradative pathways for EGF and TGFα may explain how the two factors exert differential effects in some systems, and degradation of TGFα by IDE would provide a possible mechanism for crosstalk between the insulin and TGFα/EGF signalling systems. Supported by the American Diabetes Association, the Juvenile Diabetes Foundation, and the University of Chicago Diabetes Research Center.

PHORBOL ESTER ONLY PARTIALLY MIMICS INSULIN'S EFFECTS ON GLUCOSE TRANSPORT AND GLUCOSE TRANSPORTER DISTRIBUTION, E. Michael Gibbs, David M. Calderhead, Geoffrey D. Holman and Gwyn W. Gould, Department of Biochemistry, University of Glasgow, Glasgow, G12 7AY, Scotland

12-O-Tetradecanoyl phorbol-13-acetate (PMA; 1 μM, 60 min) increased hexose transport 3-fold in 3T3-L1 adipocytes. This effect was mediated by translocation of two isotypes of glucose transporters (GLUT 1 and 4) to the plasma membrane, as determined by 1) labeling cell surface transporters with galactose oxidase and [3H]borohydride, 2) photoaffinity labeling with a membrane impermeant glucose analogue, and by 3) immunoblotting subcellular fractions. The PMA effect on both transport and transporter translocation was substantially less than that of insulin. Whereas insulin stimulated transport 15- to 20-fold and increased plasma membrane GLUT 1 and GLUT 4 transporters 4- and 17-fold, respectively, the PMA-induced increase in plasma membrane GLUT 1 and GLUT 4 amounted to only about 40% and 10% of that induced by insulin. The PMA-induced decrease in intracellular transporters of both types was about half of that caused by insulin. Finally, protein kinase C down-regulation prevented acute stimulation of hexose transport by PMA, but not by insulin. These findings suggest that transport stimulation by insulin and PMA occurs via different mechanisms, which is manifested by insulin's ability to induce a much greater increase in the plasma membrane GLUT 4 content than the phorbol ester.

THE INSULIN DEGRADING ENZYME REGULATES BOTH INTRACELLULAR AND EXTRACELLULAR INSULIN LEVELS VIA AN INSULIN SHUNT, Wen Liang Kuo, Barry D. Gehm and Marsha Rich Rosner, Ben May Institute and Dept. Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637.

Several lines of evidence have suggested that the insulin degrading enzyme (IDE), a nonlysosomal evolutionarily conserved enzyme, can initiate cellular insulin degradation in mammalian cells. To test this hypothesis and to determine whether the IDE can regulate insulin degradation, we examined the effects of overexpression of IDE activity on both intracellular and extracellular insulin levels. We have recently cloned a Drosophila homologue of the IDE (dIDE) that has 48% amino acid identity with the human IDE, degrades insulin with comparable efficiency, and is readily expressed in mammalian cells. Transient expression of the dIDE in monkey kidney cells (COS) leads to approximately 3-fold increase in the rate of degradation of extracellular insulin. To determine where this degradation was occurring, we monitored the fate of prebound insulin upon internalization. Transfection with dIDE increased the rate of degradation of internalized insulin. The results indicate that 1) the IDE can mediate cellular degradation of insulin; 2) the IDE can regulate extracellar insulin levels; 3) the primary site of insulin degradation by the IDE is intracellular. In these cells a significant fraction of the internalized insulin is recycled to the outside through an insulin shunt, and the IDE serves to regulate the extent to which the recycled insulin remains intact. Supported by grants from American Diabetes Association, Juvenile Diabetes Foundation, and University of Chicago Diabetes Research Training Center.

MODULATION OF GLUCOSE TRANSPORTER EXPRESSION (GLUT 4) IN ANIMAL MODELS OF IMPAIRED INSULIN ACTION, Paul F.Pilch, Peter Brecher, Kim Chen, Stuart Chipkin, Lise Coderre, Natalio Kotliar, Wilfred Mamuya, Merce Monfar, Neil Ruderman and Galini Thoidis, Boston, MA

In experimental models of diabetes in rats, the expression of GLUT 4 mRNA and protein can be dramatically reduced in both muscle and fat tissues. Administration of insulin restores GLUT 4 to normal levels indicating that GLUT 4 expression may be under transcriptional control, and that alterations of that expression may be found in other pathological states of altered insulin action. Thus, we have begun to look at the expression of GLUT 4 in a model of cardiac hypertrophy induced by DOC/salt treatment and in denervated rat hind limb. In both these rodent models of human pathology, GLUT 4 mRNA and protein are reduced. After one week of the DOC/salt regimen, GLUT 4 protein and mRNA are depressed in cardiac tissue to about 40% of normal levels, and they remain so for up to three weeks. In denervated hind limb muscle, a decrease in GLUT 4 is evident as soon as a day after the sciatic nerve is severed, and protein levels drop to about 10-20% of controls 7 days after denervation. We are in the process of determining the fiber specificity of the denervation response and whether the response to experimental hypertension can be extended both to skeletal muscle and to other animal models of this pathological state.

A TRANSIENT EXPRESSION SYSTEM FOR FUNCTIONAL EXPRESSION OF GLUCOSE TRANSPORTER PROTEINS IN A VARIETY OF CELL TYPES, Robert Piper[¶]; Charles Rice[§]; Henry Huang[§]; and David James[¶]. Departments of Cell Biology and Physiology[¶] and Microbiology [§], Washington University, St. Louis, MO 63110.

Five isoforms of the family of facilitative glucose transporter family have now been cloned. Characterization of these isoforms with respect to their kinetic properties and their intracellular sorting behavior has been hampered by the inability to express these transporters in appropriate cell types. To address these problems, we have developed a transient expression system by using Sindbis virus, an enveloped single stranded RNA virus. Both GLUT 1 and GLUT 4 were inserted into a cDNA of the Sindbis virus genome immediately downstream of a subgenomic mRNA promoter. Full length genomic RNA was synthesized *in vitro* and transfected into baby hamster kidney (BHK) cells to produce a viral stock which was subsequently used to infect various cell lines. Infection of chicken embryonic fibroblasts with GLUT-1-producing virus resulted in a 2 fold increase in 2-deoxyglucose (2DG) uptake after 4 hours and a 7 fold increase in uptake after 8 hours. No increase in 2DG uptake was observed with insulin. The production of GLUT-1 protein was confirmed by immunoblot analysis and was proportional to the increase in transport. Infection with GLUT-4 producing virus produced similar amounts of transporter as the GLUT-1 virus but resulted in only a 2 fold increase in 2DG uptake after 8 hours. These data suggest that GLUT-4 is targeted to an intracellular compartment. This was confirmed by immunofluorescence localization of transporters in infected cells using specific anti-C-terminus antibodies. GLUT-1 containing virus also was able to infect a wide variety of cells including BHK fibroblasts, 3T3-L1 fibroblasts, 3T3-L1 adipocytes, HepG2 cells; and EL4 lymphocytes. Thus, this transient Sindbis expression system provides the opportunity to examine the functional and targetting characteristics of the glucose transporters .

ACTIVATION OF PHOSPHATIDYLINOSITOL 3-KINASE (PI3K) BY INSULIN, N. Ruderman, R. Kapeller, K. Chen, M. Yoakim, B. Schaffhausen, J. Backer, M. White and L. Cantley, Diabetes and Metabolism Division, Evans Department of Medicine, Boston University School of Medicine, Boston, MA 02118

Insulin causes a 10-20 fold increase in anti-phosphotyrosine (APY) immunoprecipitable PI3K activity in CHO cells transfected with human insulin receptor (CHO/IR). This effect of insulin occurs at physiological concentrations, it is evident within one minute and it is associated with tyrosine phosphorylation of the 85KD subunit of the PI3K. In the intact cell activation of PI3K is paralleled by an increase in phosphate incorporation into its reaction products PI-3,4-P_2 and PIP_3. CHO cells expressing the insulin receptor mutant (CHO/IR_{F960}) are defective in transmitting the insulin signal, but undergo autophosphorylation normally. The increase in anti-P-Y immunoprecipitable PI3K caused by insulin is 20-40% of that in CHO/IR and phosphorylation of the 85KD subunit is markedly depressed. In addition, insulin fails to stimulate phosphate incorporation into PI-3,4-P_2 in intact CHO/IR_{F960}, although it stimulates phosphate incorporation into PIP_3 to the same extent as in CHO/IR. The results suggest that PI3K is a target protein of the insulin receptor and that the juxtaglomerular region of the insulin receptor is important for its activation. They also suggest that PI-3,4-P_2 and PIP_3 formation are differentially regulated in the CHO cell and that production of the former more closely parallels insulin action.

INSULIN STIMULATION OF GUANYLATE CYCLASE ACTIVITY IN RAT CARDIOMYOCYTES, Michael F. Shanahan, Edward W. Lam, Suganthi Chinnaswamy and Barbara A. Guebert, Department of Physiology, Southern Illinois University School of Medicine, Carbondale, IL 62901

We have previously shown that agents which increase cellular cGMP levels exhibit an insulinomimetic effect on 3-O-methylglucose transport in isolated rat cardiomyocytes(Endocrinology **125**,1074-1081,1989). We have extended our studies on the mechanisms involved in insulin-induced elevation of cGMP levels in these cells by measuring guanylate cyclase activity in cardiomyocytes permeablilized with digitonin. Permeabilization of ventricular myocytes with digitonin allows study of the hormone sensitive guanylate cyclase with accesability to exogenous GTP in the presence of a substrate regenerating sytem. These conditions overcome the difficulties associated with broken cell or subcellular fractionation in which regulation of guanylate cylase by both insulin and isoproterenol is lost. Guanylate cyclase activity measured in the presence of phosphodiesterase inhibitors (isobutylmethylxanthine and/or zaprinast) indicate that both insulin and isoproterenol increase cGMP levels by stimulating guanylate cyclase. Maximal levels of stimulation appear to require the presence of ATP as well as Mn^{2+} in the presence of Mg^{2+}. Experiments using inhibitors of guanylate cyclase, methylene Blue and Ly 83583, strongly suggest that insulin elevates cell cGMP by stimulating the soluble form of the cyclase. Inhibition of insulin-stimulated guanylate cyclase activity by both these agents also blocks insulin stimulation of 3-O-methylglucose transport in a concentration-dependent manner. Adenosine also stimulates guanylate cyclase in these cells and the effect is additive to insulin. These and previously reported observations suggest that elevation of cGMP may be at least one of several potential mediator pathways by which insulin stimulates glucose transport in heart cells.

COMPARISON OF INSULIN RECEPTOR ENZYMATIC FUNCTION IN MUTANT RECEPTORS REPLACING TYROSINES 1146, 1150 & 1151 WITH PHENYLALANINE OR SERINE, Peter A. Wilden, C. Ronald Kahn, and Morris F. White, Joslin Diabetes Center, Boston, MA 02215
The tyrosine autophosphorylation sites (1146, 1150, 1151) in the regulatory region of the insulin receptor β-subunit were replaced by either phenylalanine or serine to examine their role in insulin receptor enzymatic function. Wild-type and mutant insulin receptor cDNAs were expressed in Chinese hamster ovary (CHO) cells at equal levels. In cells labeled with (^{32}P)-ortho-phosphate, insulin stimulated phosphorylation of the wild-type insulin receptor and the triple serine mutant (IR_{3S}) insulin receptor 8-fold. In contrast, the triple phenylalanine mutant (IR_{3F}) insulin receptor phosphorylation was stimulated 3-fold by insulin. However, when the insulin receptors were immunoprecipitated with an anti-phosphotyrosine antibody the mutant IR_{3S} receptor showed only 30% of the wild-type insulin receptor phosphorylation; whereas, the mutant IR_{3F} receptor was not precipitated by this antibody. When these three insulin receptor molecules were partially purified and *in vitro* autophosphorylated, insulin stimulated wild-type insulin receptor phosphorylation 6-fold, the mutant IR_{3S} receptor phosphorylation was stimulated 3-fold by insulin, and the mutant IR_{3F} receptor phosphorylation was not stimulated by insulin. Insulin stimulated the kinase activity of the wild-type insulin receptor 10-fold. Autophosphorylation, further increased the receptor kinase activity to 20-fold. In contrast, the mutant IR_{3S} receptor showed a 3-fold activation of its kinase activity by insulin and an additional 2-fold increase upon autophosphorylation. The mutant IR_{3F} receptor kinase was activated 2-fold by insulin, but showed no further activation by autophosphorylation. Thus, replacement of these tyrosine residues with phenylalanine greatly reduces or eliminates insulin-stimulated autophosphorylation while replacement with serine shows normal or only slightly reduced phosphorylation which only partially activates the insulin receptor kinase.