MOLECULAR MECHANISMS COMMON TO TYPES I AND II DIABETES

Organizers: Ira Goldfine and Michael Czech

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Molecular Mechanisms Common to Types I and II Diabetes

Keynote Address (Joint)

C 001 HOW G PROTEINS TRANSMIT SIGNALS, Henry R. Bourne, Departments of Pharmacology and Medicine, Cell Biology Program, and Cardiovascular Research Institute, University of California School of Medicine, San Francisco, CA 94143.

Heterotrimeric ($\alpha\beta\gamma$) G proteins transmit signals from cell surface receptors for hormones and sensory stimuli to effector molecules — enzymes and ion channels — that initiate cellular responses. Among recent advances in understanding this signaling system, this lecture focuses on work in many laboratories that has provided a plausible, consistent, and testable model of the relations between structure and function of Gα subunits. Now that 3D crystal structures of Gα subunits have been solved, Gα is arguably the best understood protein signaling machine in biology. We can now identify in Gα the four key functional regions of a generic signaling machine, and begin to describe in detail how they work. Experiments with antibodies, mutations, synthetic peptides, and chimeric Gα subunits all point to the extreme carboxy (C) terminus of Gα as the location of the Gα signal detector region, which interacts with and discriminates among ligand-activated receptors, and then initiates a change in conformation that leads to release of GDP from α·GDP. Work in several labs seeks to pinpoint specific contact points between receptor and Gα. Similar techniques have located the Gα signal generator region, which interacts with and activates effectors such as adenylylcyclase and cGMP-phosphodiesterase (respective targets of the α subunits, α_s and α_t, of G_s and retinal transducin). Evidence from studies of α_s and α_t locates the amino acids that make up the signal generator region in loops situated on the "back" face of Gα, opposite to the face that includes the guanine nucleotide binding site. Mutations, biochemical probes of Gα structure, and analogies with homologous GTPases all point to the Gα region that corresponds to loop 4 and helix α2 of $p21^{ras}$ as the principal switch region, responsible for translating the presence of a γ-phosphate on GTP into a conformational change that enables the signal generator region to activate the appropriate effector. Finally, each Gα subunit possesses a sophisticated built-in timing device that turns off its active conformation by catalyzing hydrolysis of bound GTP, just as GTPase Activating Proteins (GAP's) do for small GTPases like $p21^{ras}$ and Elongation Factor Tu. Recent evidence from this laboratory shows that this timer-turnoff region is composed of an inserted domain of ~120 amino residues, which serves as a built-in GAP and catalyzes GTP hydrolysis via a conserved arginine residue (R201 in α_s).

Genetics of Type II Diabetes Mellitus

C 002 MOLECULAR GENETICS OF NON-INSULIN DEPENDENT (TYPE 2) DIABETES MELLITUS, Graeme I. Bell, Howard Hughes Medical Institute, The University of Chicago, Chicago.

NIDDM is a heterogeneous disorder characterized by defects in insulin secretion and insulin action. Genetic factors play an important role in its development which has prompted a search for diabetes-susceptibility genes. These studies have shown that mutations in candidate genes such as insulin (chromosome 11p15.5), insulin receptor (19p13.3-p13.2) and glucokinase (7p13) can cause diabetes. Association studies have implicated the glycogen synthase gene (19q13.3) and genes in the major histocompatibility complex (6p21.3) in the development of diabetes but no mutations in these genes have been identified. Genetic linkage studies have identified markers for a diabetes-susceptibility gene in the region of the adenosine deaminase gene (20q12) that is responsible for one form of maturity-onset diabetes of the young and studies in the Pima Indians suggest that there is a locus near the fatty acid binding protein 2 gene (4q26) that affects maximal insulin-stimulated glucose uptake. In addition to these nuclear genes, studies of patients with diabetes and deafness syndromes indicate that deletions and mutations in the mitochondrial genome can also lead to the development of diabetes. The present results suggest that none of these genes can account for the high prevalence of NIDDM, ~5%, in most developed countries. However, 0.04% of Americans may be at increased risk of developing NIDDM because they are heterozygous for mutations in the glucokinase gene and glucokinase-deficient diabetes may be one of the most common metabolic disorders described to date. Genetic studies are beginning to provide a better understanding of the causes of NIDDM which will ultimately result in new approaches for treating this disorder and for identifying individuals at increased risk.

Biology of the Beta Cell I - Gene Regulation

C 003 TRANSGENIC MOUSE MODELS FOR STUDYING THE ROLE OF GLUCOSE PHOSPHORYLATION IN ß-CELL FUNCTION, Shimon Efrat, Margarita Leiser, Manju Surana, David Fusco-DeMane, Obaidullah A. Emran, Y-Jian Wu[1], Gordon Weir[1] and Norman Fleischer, Albert Einstein College of Medicine, Bronx, NY 10461, and [1]Joslin Diabetes Center, Boston, MA 02215.

Recent findings have confirmed the key role of glucose phosphorylation in regulation of insulin secretion in ß cells. The high-K_m enzyme glucokinase (GK) accounts for the majority of glucose-phosphorylating activity in normal ß cells. Transformed ß-cell lines derived from tumors arising in transgenic mice that express SV40 T antigen under control of the insulin promoter maintain normal GK expression. Their insulin secretory response to glucose varies according to the activity of the ubiquitous low-K_m hexokinases (HK). Cells that develop abnormally high levels of HK activity become hypersensitive to glucose. The increase in HK activity appears to correlate with enhanced proliferation rates. We are generating conditionally-transformed ß cells to address this possibility.

The findings of heterozygous GK gene mutations in patients with maturity-onset diabetes of the young (MODY) have raised the possibility that a decrease in ß-cell GK activity may impair the insulin secretory response of these cells to glucose and lead to diabetes. To test the effect of a reduced GK activity in ß cells on their ability to maintain glucose homeostasis, we generated transgenic mice expressing an insulin-promoted GK ribozyme. This construct gives rise to a hybrid RNA consisting of two 12-base stretches of GK gene antisense sequence that flank an RNA catalytic domain. This RNA is designed to hybridize specifically to GK mRNA molecules and cleave them, resulting in reduced levels of GK in ß cells. Two mouse lineages which expressed the transgene had about 30% of the normal islet GK activity. In spite of this decrease, the plasma glucose and insulin levels of the mice remained normal. Insulin secretion in response to 5-8 mM glucose was impaired, however higher glucose concentrations elicited normal secretion. These mice may be predisposed to type II diabetes and are likely to provide insights into other genetic and epigenetic diabetogenic factors.

Biology of the Beta Cell II - Biosynthesis and Processing

C 004 ENGINEERING CELLS TO PROCESS AND SECRETE PROTEINS IN THE INSULIN FAMILY OF HORMONES. D. Groskreutz[1], M. Sliwkowski and C. Gorman[2]. Genentech, Inc., 460 Pt. San Bruno Blvd., South San Francisco, CA 94080, current addresses [1] Promega, Madison, Wisconsin; [2] MEGABIOS, San Francisco, CA.

Insulin in normally processed into the mature, active A and B chain complex in the secretory vesicles of pancreatic β-cells which contain the requisite processing enzymes . These specialized vesicles also provide a means of controlling the processing of such proteins as proinsulin and for protecting the mature form of insulin from degradation by intracellular proteases. During processing, the C-peptide, which resides between the B and A peptides in proinsulin, is excised by enzymes that make two separate cleavages: one at the B-C junction (Arg-Arg dibasic site), and one at the C-A junction (Lys-Arg dibasic site). In cells with only the constitutive pathway there are no specialized, regulated secretory vesicles nor are the specialized proinsulin processing enzymes present. Human proinsulin, is not effectively converted to mature insulin in cells which contain only the constitutive pathway of secretion. Our goal in this study is to develop a means by which a wide variety of cell types can produce mature, active human insulin. To create non-endocrine cells with the ability to correctly and efficiently process human proinsulin to mature insulin, we took advantage of the fact that the endogenous, Golgi-anchored, processing enzyme, furin is present in the constitutive pathway of most cells . Using site-directed mutagenesis we engineered proinsulin to be a substrate for furin by introducing new cleavage sites at both the B/C and the C/A junctions of human proinsulin. When the variant human proinsulin cDNA is introduced into human embryonic kidney cells (HEK-293) , it is processed constitutively into active, mature, human insulin. To assess cleavage, protein sequencing was performed on the final cleavage products and the bioactivity of the secreted molecules was determined monitoring autophosphorylation of the insulin receptor. $Lys_{B29}Arg$ insulin appeared to be sensitive to intracellular degradation. To overcome this possibility, we studied a naturally occurring variant of proinsulin that is present in a subset of patients with hyperproinsulinemia. This variant replaces the histidine at position 10 in the B chain with an aspartic acid . When this mutation is introduced into the engineered-processed proinsulin cDNA, the resulting mutant insulin subsequently accumulated at 10 to 100 fold higher levels. Receptor binding and autophosphorylation assays demonstrate that this insulin variant binds and activates the insulin receptor similarly to native insulin. By modifying the amino acid sequence of the insulin precursor, an insulin which can be processed by nearly any cell has been created. This general scheme also offers other interesting possibilities. It should, for example, now be possible to engineer cells so that they secrete their own insulin, eliminating the need to include insulin in the growth medium. One can also imagine that this scheme could be used to engineer cells to express other growth-stimulationg hormones. In addition to gene therapy, this could have important implications for the growth of cells in culture.

C 005 THE UNIQUE REGULATION OF PROINSULIN BIOSYNTHESIS- Christopher J. Rhodes, E.P. Joslin Research Laboratory, Joslin Diabetes Center, Boston.

Proinsulin biosynthesis is controlled by a wide variety of nutrients, most importantly glucose. A rise in external glucose from a basal 2.8mM to stimulatory 16.7mM level specifically stimulates the rate of proinsulin biosynthesis over that of the majority of ß-cell proteins within 20mins reaching up to a 30-fold maximum stimulation by 60mins. Glucose metabolism is necessary to cause the increase in proinsulin biosynthesis, but the actual signal transduction-mechanism which instigates it has yet to be defined. In the short-term (<8 hours) glucose regulated proinsulin biosynthesis is mediated entirely at the level of translation, but in longer-term situations (*e.g.* >8 hours hyperglycemia or starvation/re-feeding *etc.*) there is some additional transcriptional control. However, glucose mediated transcriptional regulation of proinsulin biosynthesis is relatively modest compared that at the translational level. Glucose-mediated translational regulation of proinsulin biosynthesis is not particularly well defined but it is known to involve an increase in transport of preproinsulin mRNA from a cytoplasmic storage pool to active polysomes on the rough endoplasmic reticulum, and a subsequent increase in initiation rates of preproinsulin mRNA translation. Translational regulation of eukaryotic protein synthesis involves the phosphorylation of essential proteins involved in the initiation phase of translation, so-called initiation factors (eIFs). We have studied the glucose-regulated phosphorylation state of three key regulatory initiation factors (eIF-2α, eIF-3 and eIF-4e), but found no change in the phosphorylation of any of the subunits of these proteins. It therefore seems that glucose-regulated proinsulin biosynthesis is not mediated by the phosphorylation of known eIFs. Nevertheless, we have discovered a yet to be identified 65/66kDa protein that binds to the 7-methyl-guanosine 5'-cap of mRNA that increases in phosphorylation at stimulatory glucose concentrations. This phosphoprotein indicates that a glucose-influenced kinase activity might be involved in translational control of proinsulin biosynthesis. The biosynthesis of the endopeptidase that regulates proinsulin conversion (PC-3) is also glucose regulated in parallel to its substrate, proinsulin. In contrast, the biosynthesis of other enzymes involved in proinsulin processing (PC-2 and carboxypeptidase-H (CP-H)) are not glucose regulated. Interestingly, preproinsulin and PC3 mRNAs predict a 'stem-loop' structure in their 5'-untranslated regions, whereas PC-2 and CP-H mRNAs do not. A similar mRNA 'stem-loop' structure has been shown to be key for transitional regulation of ferritin biosynthesis by iron and that of some viral proteins. We are currently investigating glucose regulated proteins which specifically bind to the 'stem-loop' of preproinsulin and PC3 mRNAs.

C 006 ISLET AMYLOID POLYPEPTIDE (IAPP). Per Westermark[1], Gunilla Westermark[1], Arnold Leckström[1], Johan Permert[2], Lars Christmanson[3], and Christer Betsholtz[3], [1]Department of Pathology 1, [2]Department of Surgery, University of Linköping, Linköping, and [3]Department of Pathology, University of Uppsala, Uppsala, Sweden.

The 37-amino acid residue putative polypeptide hormone islet amyloid polypeptide (IAPP) is mainly expressed by islet beta cells. IAPP is stored together with insulin and the two polypeptides are released simultaneously. The basal plasma levels of IAPP are, however, only 1-3% of those of insulin, i.e. around 8 pmol/l. While only slightly higher than normal plasma IAPP levels have been found in some type 2 diabetic individuals, very high levels occur in persons with diabetes associated with pancreatic carcinoma. Many interesting effects have been obtained with IAPP experimentally. Most attention has been drawn to the effects on the glucose metabolism in skeletal muscle and liver in which organs IAPP gives rise to insulin resistance mimicking that seen in Type 2 diabetes. The levels needed are, however, much higher than detected physiologically. The same is probably true of the inhibiting effects of IAPP on the insulin- and glucagon secretions from the islets of Langerhans. Another effect, experimentally obtained with nearly physiological IAPP plasma concentration is suppression of appetite. IAPP was discovered by its property to form amyloid fibrils in the islets of Langerhans and insulinomas. Recent comparative studies have shown that this property is closely dependent on a short segment in the middle of the molecule, that varies strongly between species and that differences in this part explain the species-specific occurrence of islet amyloid in conjunction with diabetes. Like in other forms of amyloid, further factors like over-production of the fibril protein precursor and yet unknown alterations in the intercellular milieu must also be of importance for the amyloid formation.
There are still several unresolved questions: 1. Do specific IAPP receptors exist and, in that case, where? 2. What is the normal function of IAPP? 3. Which part, if any, does IAPP play in the development of Type 2 diabetes? 4. Why is islet amyloid formed in conjunction with Type 2 diabetes? These questions will be dealt with in the presentation.

Molecular Mechanisms Common to Types I and II Diabetes

Biology of the Beta Cell III - Pathways

C 007 MECHANISMS OF ACTION OF INSULINOTROPIC GLUCAGON-LIKE PEPTIDE-1, Joel F. Habener, Colin A. Leech, and George G. Holz, Laboratory of Molecular Endocrinology, Massachusetts General Hospital, Howard Hughes Medical Institute, Harvard Medical School, Boston, MA.

Glucagon-like peptide-1 (GLP-1) is an intestinal peptide hormone released in response to meals. It has potent glucose-dependent insulinotropic activities in stimulating insulin secretion, insulin biosynthesis and transcription of the proinsulin gene. GLP-1 is formed by post-translational processing of proglucagon which generates at least two equivalently potent isopeptides, GLP-1(7-37) and GLP-1(7-36)amide. The β-cell receptor for GLP-1 is a member of a new subclass of seven-membrane-spanning G-protein-coupled receptors that appear capable of coupling to both cAMP-dependent and phospholipid/Ca^{++}-dependent signalling pathways. The GLP-1 receptor in the glucose-responsive β-cell line HIT-TI5 undergoes rapid and reversible homologous desensitization in response to supraphysiological concentrations of GLP-1. In order to characterize the response to GLP-1 at the single cell level, recordings of membrane current (perforated patch, voltage clamp), single channel activity (cell-attached patch), and calcium imaging (dual wave length, fura-2) were obtained from primary rat β-cells and insulinoma cells (HIT). We show signal transduction cross-talk between glucose (glycolysis) and GLP-1 (cAMP) generated signals via closure of ATP-dependent K^+ channels (*sulfonylurea receptors?*) and resultant opening of voltage-dependent Ca^{++} channels (VDCC). The mutually synergistic actions of glucose and GLP-1 on β-cells are abrogated by lowering glucose levels or by prior exposure of the cells to the cAMP antagonist Rp-cAMPs. In addition, GLP-1 (and other glucagon-related hormones such as glucagon and pituitary adenylyl cyclase activating peptide) can elicit an inward current and an elevation in intracellular [Ca] in both primary β-cells and insulinoma cell lines (e.g. HIT-TI5) when VDCCs are prevented from opening by voltage-clamping of the cells at -80mV. Moreover, under voltage clamp at -80mV, inward current and elevations of intracellular [Ca] are inhibited by removal of extracellular Ca^{++}. These findings indicate the existence of GLP-1 mediated mechanisms that are dependent on glucose and extracellular calcium, but independent of VDCCs. Thus, glucose and GLP-1 act synergistically on β-cells through modulation of cAMP levels and the activities of ion channels. GLP-1 renders β-cells competent to respond to glucose and vice versa. It is possible that defective GLP-1-mediated signalling in β-cells of patients with NIDDM may contribute to their altered insulin secretion and glucose homeostasis.

C 008 FUEL SENSING AND METABOLIC COUPLING IN NORMAL AND FUEL ADAPTED PANCREATIC ISLET CELLS, Franz M. Matschinsky[1], Yin Liang[1], Prabakaran Kesavan[1], Guizhu Li[1], Habiba Najafi[1], Donna Berner[1], Peter Ronner[1], Lijun Wang[1], Heather Collins[1], Ian Sweet[1], and Carol Buettger[1], [1]Department of Biochemistry and Biophysics and the Diabetes Research Center, University Pennsylvania School of Medicine, Philadelphia, Pennsylvania.

Three topics will be addressed: Implications of the glucokinase glucose sensor concept for B-cell function in health and disease; The role of free ADP as metabolic coupling factor in fuel stimulated A- and B-cells; and The adaptation of A- and B-cells to chronic over exposure to glucose and fatty acids. Related to topic 1: Kinetic studies with wildtype and mutant recombinant human glucokinase will be discussed (i.e. V_{max}, K_ms for glucose and ATP, n_H, inflection point, hysteretic behavior, ATP binding). Related to topic 2: the role of the creatine kinase/P-creatine system in metabolic coupling in B-cells of normal and transgenic RIP-CKB mice which over express rat brain creatine will be discussed and the involvement of free ADP as central metabolic coupling factor will be emphasized. Related to topic 3: Results of studies with cultured rat islets which have been maintained for prolonged periods (up to 12 days) in high glucose or fatty acids will be discussed including the effect of this treatment on insulin and glucagon release in a dynamic perifusion system, the induction of glucokinase by glucose, glucose metabolism as determined by the use of 5-T-glucose, and protein biosynthesis as assessed by high resolution 2-D PAGE combined with computerized analysis of gel images. The implications of these results for A- and B-cell's role in normal glucose homeostasis and the pathogenesis of diabetes mellitus will be briefly outlined.

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Gene Expression in Type II Diabetes

C 009 ROLE OF SPECIFIC GENES IN TYPE II DIABETES: AN OVERVIEW, Daryl K. Granner, Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN 37232.

Insulin resistance and β cell failure account for the complex clinical presentation of noninsulin-dependent diabetes mellitus (NIDDM). Insulin resistance primarily involves defective regulation of hepatic glucose production and the peripheral utilization of glucose. Considerable progress has been made in understanding the basic molecular biology, biochemistry and physiology of these processes. Similarly, the mechanisms involved in insulin synthesis, processing, storage and secretion are being elucidated. The relative contributions of insulin resistance and β cell failure are difficult to evaluate when the disease is fully established and clinically apparent, but may be more obvious early, such as in persons with impaired glucose tolerance, or in individuals at risk for developing the disease. The latter can be identified because there is a strong genetic determinant for NIDDM; the offspring of two diabetic parents have a markedly increased incidence of the disease. In addition to genetic factors, environmental components contribute to the multifactorial etiology of NIDDM. Efforts to establish the importance of these different factors will be assisted if a metabolic staging of NIDDM can be agreed upon. This staging, which should correlate the pathophysiologic events responsible for the transition from normal glucose tolerance to frank NIDDM with clinical status, would be based on what is known about insulin resistance and β cell function. Staging will also provide for a classification of the number of causes that leads to NIDDM, if indeed, there is more than one cause of the general phenotype. Strategies for defining the gene or genes responsible for NIDDM can be subsequently devised based on the temporal sequence of appearance of pathophysiologic defects, and what is known about the molecular biology of insulin action. Understanding the defective metabolic code that results in NIDDM will require the concerted efforts of investigators from a number of disciplines.

C 010 GLUCOKINASE GENE EXPRESSION IN NEUROENDOCRINE CELLS: A POTENTIAL MARKER FOR EXTRAPANCREATIC GLUCOSE-SENSITIVE CELLS, Mark A. Magnuson, J. Michael Moates, Catherine Postic, Kevin D. Niswender, and Thomas L. Jetton. Vanderbilt University, Nashville, TN 37232.

Previously we have determined that a 294 bp fragment of upstream glucokinase (GK) promoter DNA is transcriptionally active in insulinoma cell lines. We have now examined the cell-specific expression of this fragment of the upstream GK promoter in transgenic mice and in additional neuroendocrine cell lines. Using both immunocytochemistry and RNA-PCR to detect the transgene product in tissues of transgenic mice, we found that this 294 bp promoter fragment was expressed in several neuroendocrine cell types outside the pancreas and pituitary. In brain, cells in the medial hypothalamus were identified that expressed the transgene. In the gut, the transgene product was detected in a subset of enteroendocrine cells of the stomach and duodenal epithelium. Additionally, a low level of transgene expression was observed in C-cells of the thyroid and in rare bronchopulmonary mucosal cells of the lung. To determine whether transgene expression in these sites correctly reflected expression of the endogenous GK gene, we examined these tissues for the expression GK mRNA and either GK immunoreactivity or activity. Using both RNA-PCR and *in situ* hybridization assays, GK mRNA was detected in rare cells in both the brain and gut. GK immunoreactivity was detected in both ependymal cells lining the third ventricle and in periventricular neural cells. Some of the same cells that exhibited GK immunoreactivity were also found to display GLUT2 immunoreactivity. In the gut, a high Km hexokinase activity was detected in jejunal enterocytes. The activity detected had identical chromatographic behavior to hepatic and islet GK and a similar Km. Some enteroendocrine cells that expressed the transgene driven by the upstream GK promoter also contained glucagon-like polypeptide-1 immunoreactivity. To identify the *cis*-regulatory elements involved in the neuroendocrine cell-specific expression of the upstream GK promoter we also tested a series of block transversion mutations of the upstream GK promoter in two pituitary-derived cell lines that express GK mRNA. Mutations that affected Pal-1, one of two conserved palindromic repeat sequences, diminished reporter gene expression in both AtT-20 and GH3 cells. Multiple proteins in both GK expressing and non-expressing cells were identified that bound to both Pal elements in gel mobility shift experiments. While none of these factors appear to be distinctly cell-specific, their relative abundance differed in cells that express GK compared to cells that do not. Together, these studies have provided evidence for upstream GK promoter activity, GK mRNA, and GK immunoreactivity or kinetic activity in certain neuroendocrine cells outside the pancreatic islet. Since these sites of GK expression correlate with physiologic and electrophysiologic studies that have identified glucose sensitive cells in these areas, we propose that GK may function in dispersed neuroendocrine cells as a glucose sensor, much like it does in the pancreatic β cell.

Glucose Transporter Function in Diabetes

C 011 ROLE OF TRANSVERSE TUBULES IN MUSCLE GLUCOSE TRANSPORT, G. Lynis Dohm[1], Cindy M. Wilson[2], Samuel W. Cushman[2] and Ronald W. Dudek[1], [1]Departments of Biochemistry and Anatomy and Cell Biology, School of Medicine, East Carolina University, Greenville, NC, and [2]Diabetes Branch, NIDDK, NIH, Bethesda, MD.

The observation that transverse tubule (T-tubule) membranes contain approximately five times more glucose transporter than sarcolemma raised a question as to where glucose transport occurs in muscle. The T-tubule membrane system is continuous with the surface sarcolemma and is a tubule system in which extracellular fluid is in proximity with the interior of the muscle fiber. Using immunocytochemical techniques we have previously reported that GLUT4 glucose transporter were located on the T-tubule membrane and in vesicles near T-tubules. After combined stimulation by insulin and muscle contraction, we again observed labeling of the T-tubule but in addition found strong GLUT4 immunolabeling of a subsarcolemmal region which was predominately restricted to an area over the I-band and A-I junction where T-tubules are located. This subsarcolemmal region has a complicated ultrastructure containing components of the surface sarcolemma (S), caveolae (C), and T-tubules (T) which we have termed the SCT complex. Quantitation of GLUT4 immunolabeling in the subsarcolemmal region showed that it was 4 times higher at the SCT complex (over the I-band and A-I junction) versus the A-band.

We have reinforced our immunocytochemical findings by using an entirely different technique. Cell surface glucose transporters were photolabeled with the membrane impermeant ATB-[2-3H]BMPA reagent which was localized by autoradiography. Labeling of the glucose transporters by ATB-[2-3H]BMPA was clearly observed within the muscle, presumably on the exterior surface of T-tubules; labeling of the surface sarcolemma was less obvious. The amount of photolabel at the T-tubule was increased in response to insulin and decreased by treatment with cytochalasin B.

Since T-tubules form channels into the interior of the muscle fiber, glucose could diffuse or be moved by a peristaltic-like pumping action into the transverse tubules and then be transported across the membrane deep into the interior of the muscle fiber. This mode of transport directly into the interior of the cell would be advantageous over transport across the sarcolemma and subsequent diffusion around the myofibrils to reach the interior of the muscle. Thus, in addition to the role of the T-tubule in ion fluxes and contraction, this unique membrane system can also provide a pathway for the delivery of substrates into the center of the muscle cell where many glycolytic enzymes and glycogen deposits are located.

C 012 REGULATION OF GLUCOSE TRANSPORT AND METABOLISM BY A HYPOGLYCEMIC AGENT THAT ACTS AS AN INSULIN-SENSITIZER, Cecilia Hofmann[1], Kathryn Lorenz[1], Susan S. Braithwaite[1], Charles W. Edwards III[1], James A. Bonini[1], Barbara J. Palazuk[2], and Jerry R. Colca[2], [1]Loyola Stritch School of Medicine, Maywood IL and Hines VA Hospital Research Service, Hines IL; [2]Metabolic Diseases Research-The Upjohn Company, Kalamazoo MI

A novel structural class of antidiabetic compounds, the thiazolidinediones (*zones*), was discovered empirically by observing their antihyperglycemic effects in animal models of noninsulin-dependent diabetes mellitus (NIDDM). In obese rats and mice wherein insulin resistance is a prominent metabolic effect, analogues of this class lower blood glucose in concordance with decreased insulin levels. Since the compounds are also inactive in the absence of insulin, it appears that the principal effect of such *zones* is an improvement of peripheral tissue responses to insulin. As such changes are accompanied only by minimal increases in insulin receptor binding, metabolic improvements are likely due to enhanced postbinding events, and the agents can thus be considered insulin-sensitizers.

The aim of the present study was to demonstrate effects of pioglitazone on insulin-mediated glucose transport and metabolism in adipose tissue of obese insulin-resistant diabetic mice. To do so, we investigated the expression of the insulin-responsive glucose transporter GLUT4 as well as that of hexokinase-II, the enzyme that catalyzes the first step in cellular utilization of glucose. Such studies used epididymal fat pads of obese insulin-resistant KKAy mice with or without pioglitazone treatment compared to those from nondiabetic control mice. The experimental results in tissues of diabetic compared to nondiabetic C57 mice demonstrated reduction in fat of GLUT4 mRNA abundance by 77% and decrease of hexokinase-II transcripts by 74%. Treatment of the diabetic animals with the insulin-sensitizing drug pioglitazone partially corrected the deficiency of GLUT4 and hexokinase-II transcripts in fat. After treatment of mice with pioglitazone, deficient GLUT4 protein levels (-42%) in KKAy mouse fat were corrected to normal in parallel with improved responsiveness of such adipocytes to insulin for glucose uptake.

Taken together, these findings indicate that GLUT4 and hexokinase-II are underexpressed concordantly in an animal model of NIDDM. Since the diminished expression of these cellular components for uptake and metabolism of glucose can be corrected by treatment of the diabetic condition with an insulin-sensitizing agent, we conclude that such deficiencies may occur secondary to insulin resistance and may exacerbate hyperglycemia. Our results also underscore the potential utility of insulin-sensitizing *zones* in treatment of the insulin-resistant condition.

C 013 MOLECULAR, STRUCTURAL AND FUNCTIONAL DISSECTION OF THE GLUT4 AMINO TERMINUS, David E. James[1], Robert C. Piper[2], Jan W. Slot[3], Dan Studelska[4], [1]Centre for Molecular Biology, University of Queensland, Brisbane, Asutralia, 4072, [2]University of Oregon, Eugene, [3]University of Utrecht, The Netherlands, [4]Washington University, St. Louis, MO.

GLUT-4 is an intracellular membrane protein that can be induced to move to the plasma membrane (PM) in response to insulin in muscle and adipose tissue. GLUT-4 is efficiently sequestered (5-10% at the PM) in tubulo-vesicular elements that are either associated with the trans Golgi Reticulum or clustered in the cytoplasm in non-stimulated adipocytes and when expressed in non-insulin sensitive cells (eg. fiboblasts). We have analysed the targeting of chimeric glucose transporter proteins comprised of portions of GLUT-4 and an homologous isoform, GLUT-1, that is distributed primarily at the PM. Chimeric transporters have been expressed in Chinese Hamster Ovary (CHO) cells using a Sindbis virus expression system. This expression system faithfully recapitulates the differential targeting of GLUT-1 and GLUT-4 in CHO cells: 70% of Sindbis-produced GLUT-1 is targeted to the PM but only 10-15% of Sindbis-produced GLUT-4 is located at the PM. The following methods have been used to analyse targeting of recombinant transporters in CHO cells: two methods of subcellular fractionation; 2-deoxyglucose uptake in whole cells; immunofluorescence microscopy; and, quantitative electron microscopy immunogold labeling. Using each of these techniques we have localized an important targeting domain to the cytoplasmic amino terminus of GLUT-4, that is both necessary and sufficient for intracellular sequestration. By studying the distribution of a series of GLUT-4 constructs containing amino terminal deletion and point mutations we have identified the critical targeting motif to include residues PSGFQQI in the GLUT-4 amino terminus with the phenylalanine playing a dominant role. High resolution immunofluorescence microscopy of PM fragments obtained from CHO cells revealed that GLUT-4 is highly localized to clathrin lattices and pits in the membrane. However, mutating Phe5 to Ala abrogates this localization. Two dimensional solution NMR studies using a GLUT-4 amino terminal synthetic peptide or the mutant peptide (Phe5->Ala) revealed that the GLUT-4 amino terminus contains a Type I beta turn that is not present in the mutant peptide. These studies indicate that GLUT-4 contains a phenylalanine-based internalization motif within its amino terminus that mediates the association of the transporter with clathrin coated pits. This domain is structurally and functionally similar to previously defined internalization motifs that are present within the cytoplasmic tails of recycling receptors. We have also found that the GLUT-4 amino terminus but not the GLUT-1 amino terminus can confer intracellular sequestration in the context of a heterologous protein containing the membrane spanning region and the exofacial domain of the H1 subunit of the asialoglycoprotein receptor. Expression of these heterologous proteins in 3T3-L1 adipocytes using a stable expression system, however, reveals that other domains may also be involved in regulating the targeting of GLUT-4. Both the wild type H1 subunit and the heterologous protein containing the amino terminus of GLUT-4 exhibited insulin-dependent translocation to the PM in adipocytes but not to the same extent as endogenous GLUT-4. We conclude that the amino terminus of GLUT-4 contains an efficient internalization motif that presumably plays an important role in maintaining the intracellular sequestration of GLUT-4 in non-stimulated cells and in the rapid retrieval of the protein from the cell surface upon removal of the insulin stimulus.

C 014 USE OF DIVERSE EXPRESSION SYSTEMS TO DISSECT GLUCOSE TRANSPORTER FUNCTION, Mike Mueckler[1], Bess Adkins Marshall[1], Peter M. Haney[1], Richard C. Hresko[1], Haruhiko Murata[1], Jian-Ming Ren[2], Eric Gulve[2], John Holloszy[2], Walter C. Soeller[3] and E. Michael Gibbs[3], Departments of Cell Biology and Physiology[1] and Medicine[2], Washington University School of Medicine, St. Louis, Missouri 63110, [3]Pfizer Central Research, Groton, CT 06340

We are using Xenopus oocytes, mammalian cell lines, and transgenic mice as expression systems to study the biosynthesis, intracellular trafficking, and physiologic function of the Glut1 and Glut4 glucose transporters. In fat and muscle cells not exposed to insulin, Glut4 is efficiently sequestered in an intracellular membrane compartment from which it redistributes to the plasma membrane in response to insulin. A similar intracellular sequestration occurs when Glut4 is expressed in non-insulin sensitive cell types. The structural basis for the intracellular targeting of Glut4 was investigated by creating chimeric transporters comprised of regions of Glut4 substituted for the corresponding regions of Glut1, a transporter that is targeted constitutively to the plasma membrane. The chimeras were expressed in Xenopus oocytes and L6 myocytes and their subcellular distributions were then compared to the wild-type transporters. Analysis of subcellular fractionation and immunocytochemical data indicated that the carboxyl terminus of Glut4 contains information that is both necessary and sufficient to confer a Glut4-like targeting phenotype on the chimeric molecules. These data disagree with the results of two published studies concerning intracellular targeting domains in Glut4 (1, 2).

To investigate the physiologic role of glucose transport in skeletal muscle, transgenic mice were constructed that overexpress the Glut1 transporter specifically in skeletal muscle. The rat myosin light chain II promoter was used to drive transcription of human Glut1 cDNA. Glut1 protein was overexpressed several-fold in the sarcolemma of all skeletal muscle types examined, and transport activity was increased 3 to 7-fold in muscles isolated from transgenic animals. Muscles isolated from nontransgenic animals exhibited 2-3-fold increases in glucose transport activity after incubation with insulin, but no insulin-stimulated increase in transport was observed in the muscles of transgenic mice. The elevated transport activity in the transgenic mice was associated with a 10-fold elevation of muscle glycogen and a 2-fold elevation in muscle lactate concentrations. These data demonstrate that transport is rate-limiting for glycogen synthesis and glycolysis in the muscle of normal, resting mice. Transgenic animals exhibited decreased plasma glucose, increased lactate, and increased β-OH-butyrate levels relative to their nontransgenic littermates, despite no significant alterations in the level of plasma insulin or glucagon. These observations indicate that glucose transport in muscle plays a key role in whole body glucose homeostasis and that alteration of the level of a glucose transporter in skeletal muscle directly influences the blood glucose set point.

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C 015 TRANSCRIPTIONAL REGULATION OF THE GLUT4/MUSCLE-FAT GLUCOSE TRANSPORTER GENE, Ann Louise Olson, Min-Ling Liu, Jeanne M. Richardson and Jeffrey E. Pessin. The University of Iowa, Iowa City, IA

We have generated transgenic mouse lines carrying several human GLUT4 reporter gene constructs to examine the regulation of GLUT4 expression. A construct which extends 5.3 kb upstream of transcription start and terminates within exon 10 (hGLUT4-11.5) as well as a construct carrying 2.4 kb of the 5' flanking region of the GLUT4 gene fused to the chloramphenicol acetyltransferase reporter gene (hGLUT4[2.4]-CAT) were expressed in an appropriate tissue-specific manner. To investigate the hormonal/metabolic-dependent regulation of GLUT4, the transgenic animals were made insulin-deficient by streptozotocin (STZ) treatment. In these animals, STZ-induced diabetes resulted in a parallel decrease in endogenous mouse GLUT4 mRNA and the transgenic human GLUT4 mRNA in white adipose tissue, brown adipose tissue and cardiac muscle. Similarly, insulin treatment of the STZ-diabetic animals restored both the endogenous mouse and transgenic human GLUT4 mRNA levels. Two additional 5' deletion constructs (hGLUT4[730]-CAT and (hGLUT4[412]-CAT, containing 730 bp and 412 bp of GLUT4 5' flanking DNA respectively, was also expressed in muscle and adipose tissue. Although these reporter genes were not expressed in liver there was substantial expression in the brain. These data suggest that a brain-specific repressor sequence was present in 2.4 kb of the GLUT4 5' flank and which was absent in the 0.73 kb fragment.

As an independent approach to transgenic animals, we examined the transcriptional regulation of the rat GLUT4 gene in the differentiating C2C12 skeletal muscle cell line. Differentiated myotubes displayed a 4 to 5-fold increase in GLUT4 mRNA compared to undifferentiated myoblasts which paralleled the conversion from non-muscle β-actin mRNA to muscle-specific α-actin mRNA expression. Transient transfection of progressive 5' and 3' deletions of the GLUT4 5' flanking DNA identified a 75 bp region located between -522 and -448 relative to the transcription start site which conferred myotube-specific expression. This region contains a consensus TRα1 binding site in addition to a consensus site for the myocyte enhancer factor 2 (MEF2). Mutational analysis of either of these domains prevents myotube-specific expression suggesting that the interaction of both of these sites was required. Thus, these data demonstrate the presence of a 75 bp proximal skeletal muscle-specific activation domain which is necessary for myotube-specific GLUT4 expression.

C 016 HUMPS AND BLIMPS, VAMPS AND SCAMPS-THE COMPOSITION OF GLUT4-CONTAINING VESICLES, Paul F. Pilch, Galini Thoidis, Konstantin Kandror, Jacqueline Stephens, Bassil Kublaoui and Natalio Kotliar, Department of Biochemistry, Boston University School of Medicine, 80 E. Concord St., Boston, MA 02118

Insulin-stimulated glucose transport is characteristic of adult adipose and muscle tissue and therefore requires tissue-specific gene expression, the glucose transporter isoform, GLUT4, being the only gene so far identified. That other genes exist specific for this process is suggested by the fact that transfection of GLUT4 into various cell lines known to already possess insulin receptors is insufficient to recapitulate the response of fat or muscle (1). To identify additional genes involved in insulin responsiveness, we are employing three complementary approaches. The first two lines of attack rely on our ability to purify GLUT4-enriched vesicles from fat cells and use them for 1. biochemical and 2. immunological analysis. In this way we have identified PI-4-kinase (2) and 3-related proteins (GTV3) (3) that partially translocate to the cell surface in response to insulin. Lienhard and co-workers have identified synaptobrevin-related proteins (VAMPS) in GLUT4-enriched vesicles (4). The GTV3 proteins are Heterogeneous, Unglycosylated, Membrane Protiens (HUMPs) of widespread tissue distribution and unknown function. They are antigenically related to Secretory Compartment Associated Membrane Proteins (SCAMPs) identified by Castle and co-workers (5). More recently, we have employed cell surface biotinylation using cell-impermeable analogs of biotin to identify three large glycoproteins that cycle to and from the cell surface through GLUT4-containing vesicles. One of these, p160, has been purified, partially sequenced and characterized (6). We deem this protein BLIMP for Biotinylated, Large Integral Membrane Protein, and we are in the process of determining its membrane distribution in fat cells and its tissue distribution. The third approach to identifying novel genes involved in insulin-activated glucose transport employs the method of differential mRNA display employing reverse transcriptase and PCR technology. We used TNF-treated fat cells as a condition of insulin resistance, and we compared gene expression from these cells to that of insulin-responsive adipocytes. We found over 50 messages that differ in the display gels, and we have obtained novel, partial clones for three of these that are differentially regulated in control *versus* TNF-exposed fat cells. We are obtaining full-length cDNAs for the three isolated clones and determining their species and tissue distributions.

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Early Events in Hormone and Lymphokine Action II (Joint)

C 017 CD45 TYROSINE PHOSPHATASE REGULATION OF TYROSINE KINASES THAT INTERACT WITH THE T CELL ANTIGEN RECEPTOR, Arthur Weiss[1], Andrew Chan, Makio Iwashima, David Strraus, Monica Sieh, Jan Sap[2], Joseph Schlessinger[2], and Dev M. Desai[1], [1]Howard Hughes Medical Institute, The University of California, San Francisco, CA 94143, [2]Department of Pharmacology, New York University Medical Center, New York, NY 10016

Stimulation of the T cell antigen receptor (TCR) induces the protein tyrosine phosphorylation of several proteins, some of which have been implicated in downstream signaling pathways leading to cellular response. The TCR, which does not have intrinsic protein tyrosine kinase (PTK) activity, sequentially interacts with two classes of cytoplasmic PTKs. The PTKs implicated in TCR signal transduction include members of the Src family, Lck and Fyn, as well as members of the Syk/ZAP-70 family. The interaction of ZAP-70 with the TCR in a T cell line requires Lck. Studies in heterologous systems suggest that Lck and ZAP-70 interact synergistically to induce PTK activity. In addition to these PTKs, expression of CD45, a transmembrane protein tyrosine phosphatase (PTPase), is required for the induction of protein tyrosine phosphorylation by TCR stimulation. In CD45 deficient cells, Lck is hyperphosphorylated at a negative regulatory site of tyrosine phosphorylation suggesting a possible physiologic target and control point of CD45 function.

The cytoplasmic portion of CD45 contains 2 invariant PTPase domains, whereas the extracellular domain of CD45 is structurally heterogeneous due to alternative splicing of three consecutive exons (exons 4, 5 and 6). CD45 isoforms are expressed in a cell activation, developmental and tissue specific manner. Studies suggest that CD45 isoforms differentially associate with a number of T cell surface molecules on distinct T cell subsets. To examine the function of the extracellular domain of CD45, we constructed a chimeric molecule, in which the extracellular and transmembrane domains of CD45 were replaced with those of the epidermal growth factor receptor (EGFR). We demonstrate that the EGFR/CD45 chimera restores TCR signal transduction in a CD45-deficient cell line. Thus, expression of the cytoplasmic domain of CD45 is necessary and sufficient for normal TCR signaling. The addition of EGFR ligands before or during TCR signal transduction inhibits TCR-mediated signaling. This modulation of TCR signaling by the EGFR/CD45 chimera is the result of functional inactivation of the chimera, since ligands to the chimera have no effect on TCR signaling in cells which express both the chimera and wild-type CD45. Moreover, ligand-mediated inhibition of EGFR/CD45 chimera function requires dimerization of the chimeric protein. These studies demonstrate that CD45 plays a critical and active role in TCR signal transduction and suggests a very dynamic interaction of proteins involved in the signaling pathway with PTKs and PTPases whose functions can be regulated. These results also suggest that ligand mediated regulation of receptor-PTPases share mechanistic similarities with that of receptor PTKs.

C 018 INTERACTIONS BETWEEN IRS-1, RAS AND RAS-RELATED PROTEINS IN INSULIN ACTION, C. Ronald Kahn[1], Lee-Ming Chuang[1], Christine Reynet[1], and Morris J. Birnbaum[2], [1]Joslin Diabetes Center, and [2]Harvard Medical School, Boston, MA 02215.

Insulin and IGF-1 produce a wide variety of metabolic effects and stimulate cell growth and differentiation. The immediate downstream substrate of the insulin/IGF-1 receptors is the protein IRS-1. Analysis of the sequence of IRS-1 has revealed over 20 potential tyrosine phosphorylation sites, nine of which occur in the sequence motif YMXM or YXXM. Following insulin-stimulated phosphorylation, IRS-1 serves as a docking protein which binds to the 85 kDa subunit (p85) of PI 3-kinase, GRB2 (which is believed to link IRS-1 to ras activation) and other proteins possessing src homology-2 (SH2) domains. To investigate the interaction of IRS-1, SH2-containing signaling molecules and Ras in insulin action, we have microinjected IRS-1 and GST fusion proteins linked to different SH2 domains, as well as ras proteins, into *Xenopus* oocytes to study the pathway(s) by which insulin/IGF-1 mediate their effects. We find that insulin actions are mediated by IRS-1 through two independent but convergent pathways involving p85 of PI 3-kinase and GRB2. Thus, microinjection of GST-fusion proteins of either p85 or GRB2 inhibited IRS-1 dependent activation of MAP and S6 kinases and oocyte maturation, although only the GST-SH2 of p85 reduced insulin-stimulated PI 3-kinase activation. Microinjection of activated [R12, T59] Ras increased basal MAP and S6 kinase activities and also sensitized the oocytes to insulin-stimulated maturation without altering basal or insulin-stimulated PI 3-kinase. The insulin-stimulated Ras enhanced oocyte maturation response, but not the MAP and S6 kinase activation was partially blocked by the SH2-p85, but not SH2-GRB2, whereas all components of this pathway were blocked by injection of a dominant negative Ras (S17N). Our data strongly suggest that ras activation of MAP and S6 kinases alone is not sufficient for the maximal response of insulin on oocyte maturation. Rather, IRS-1 mediated cooperation between the PI 3-kinase pathway and the GRB2/ras pathway is necessary to achieve maximal insulin-stimulation of oocyte maturation.

A second approach which has indicated potential involvement of ras-related proteins in insulin action has come from studies of insulin resistance. Tissue resistance to insulin action is a central feature and the earliest detectable defect in Type II (non-insulin dependent) diabetes mellitus. In an effort to identify gene(s) associated with this insulin resistance, we prepared two subtraction libraries from skeletal muscle of normal and Type II diabetic humans and screened them using subtracted probes. Only one clone out of the 4000 screened was selectively over-expressed in Type II diabetic muscle as compared to muscle of non-diabetic or Type I diabetic individuals. This clone encodes a new 29 kDa member of the *ras*/GTPase superfamily with unique structural features. We have termed this novel *ras*-associated with diabetes *rad*. *Rad* is expressed primarily in skeletal and cardiac muscle, and is increased by an average of 8.6-fold at the mRNA level in the muscle of Type II diabetics. In view of the potential roles of *ras*-related proteins in insulin signaling, *rad* may play a pathogenetic role in the insulin resistance of Type II diabetes or serve as a marker for the disease.

GTP-Binding Proteins in Signal Transmission (Joint)

C 019 RAS IN T LYMPHOCYTE ACTIVATION, Bengt Hallberg, Patricia Warne, Sydonia Rayter and Julian Downward
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In T lymphocytes, Ras proteins are activated in response to stimulation of the antigen receptor, stimulation of protein kinase C by phorbol esters and by cytokines such as interleukin 2. Phorbol ester stimulation of Ras is almost unique to T cells, indicating that regulation of Ras in this system may differ from other cell types. We have reported previously that phorbol ester treatment leads to a reduction in the level of GTPase activating protein (GAP) activity in T cell lysates. This appears to occur through the activation of a competitive inhibitor of GAP. The stimulation of Ras by antigen receptor agonists occurs through both PKC-dependent and PKC-independent, tyrosine kinase mediated pathways. Permeabilisation of T cells using streptolysin O confirms these findings and also shows that the rate of exchange of nucleotide on Ras is constitutively very high.

We have studied guanine nucleotide exchange factors for Ras in this system. Sos 1 and 2 are present and active. There is some binding of Sos to a 36K membrane tyrosine phosphoprotein in response to T cell receptor stimulation. This interaction is mediated by the Grb2 adaptor protein. In addition Shc is phosphorylated and associates with Grb2 on T cell receptor stimulation. In comparison to fibroblasts, only a small proportion of Sos associates with phosphotyrosine containing proteins. The role of the putative Ras exchange factor Vav has also been studied in this system. Vav shares homology with the Rho family exchange factor dbl, but has been reported to act on Ras in activated T cells. Vav fails to interact with Sos. The role of Vav in the Ras signalling pathway will be discussed.

Downstream effectors of Ras include the serine/threonine kinase Raf. This can be shown to be activated in T cells in response to a number of stimuli that activate Ras. Other potential effectors of Ras are being studied in this system. Ras has been shown to be essential, but not sufficient, for activation of IL2 gene transcription in response to phorbol esters and T cell receptor stimulation. Ras therefore clearly occupies a central role in T cell activation mechanisms.

C 020 G-PROTEIN REGULATION OF PHOSPHOLIPASES, John H. Exton, Howard Hughes Medical Institute and Vanderbilt University, Nashville, TN 37232

G-proteins mediate the actions of many agonists to induce the hydrolysis of phosphoinositides (PI) in their target cells. The G-proteins are either sensitive or insensitive to pertussis toxin. The toxin-insensitive G-proteins are now recognized to be members of the G_q family, and the specific targets of their α-subunits are certain β-isozymes ($\beta 1$, $\beta 3$) of PI-specific phospholipase C (PI-PLC). Recently, it has been found that some β-isozymes ($\beta 2$, $\beta 3$) of PI-PLC are activated by G-protein $\beta\gamma$ subunits. There is evidence that this is the mechanism by which the toxin-sensitive G-proteins act. Modification of PI-PLC by proteolysis or mutagenesis indicates that the sites of interaction of α_q and $\beta\gamma$ subunits on the enzyme are distinct. In vitro studies indicate that much higher concentrations of $\beta\gamma$ subunits are required for activation relative to α_q, but this difference can be ascribed in part to the use of GTPγS to activate α_q. A limited comparison of the effects of different combinations of β- and γ- subtypes has not shown striking differences except for a low potency of $\beta_1\gamma_1$.

The findings that $\beta\gamma$-subunits can activate certain PI-PLC isozymes, but can inhibit the stimulatory effect of α-subunits of the G_q family on other isozymes means that there may be considerable cross-talk between G-proteins in the regulation of PI hydrolysis. Although $\beta\gamma$ subunit subtypes do not seem to show in vitro the high degree of effector specificity observed for α-subunit subtypes, studies with intact cells indicate that both β- and γ-subunits are important determinants of receptor specificity in G-protein-mediated cell signalling. Different cells may thus respond differently to the same agonist depending on their complement of receptor subtypes, G-proteins (and their specific α-, β- and γ-subtypes) and specific isoforms of effector proteins.

C 021 *Abstract Withdrawn*

Molecular Mechanisms Common to Types I and II Diabetes

Immunology of Type I Diabetes

C 022 B-CELL AND T-CELL INTERACTIONS IN IMMUNE RESPONSES TO THE AUTOANTIGEN GAD_{65} IN TYPE 1 DIABETES, **Steinunn Bækkeskov, John Kim, Mark Namchuk, Qin Fu, Yuguang Shi, Wiltrud Richter, Departments of Medicine and Microbiology/Immunology, University of California San Francisco, SanFrancisco, CA 94143-0534**

The smaller form of the enzyme glutamic acid decarboxylase, GAD65, is a major autoantigen in two human diseases that affect its sites of expression. Thus both the destruction of pancreatic β-cells in insulin dependent diabetes (IDDM) and the impairment of GABA-ergic neurons in the rare neurological disorder, stiff-man syndrome (SMS), are characterized by circulating IgG autoantibodies to GAD65 in 80-95% of patients. The predominant HLA class II haplotypes associated with those disorders are DR3, DQ2 and/or DR4, DQ8 for IDDM, and DR3, DQ2 for SMS. Furthermore a rare group of individuals with a protective MHC-class II haplotype, DR2, DQ6, identified in families of IDDM patients, can develop a high titer of GAD65 antibodies but rarely progress to overt disease. Each condition, IDDM, SMS and protection, is characterized by a unique humoral GAD65 epitope recognition. We have hypothesized that impairment of GABA-ergic neurons in SMS is mediated by GAD65 antibodies recognizing an SMS specific epitope, whereas β-cell destruction is mediated by GAD65 specific cytotoxic T-cells. B-cells may however play a major role in antigen presentation of the GAD65 molecule to maintain a chronic autoimmune response. The identification of disease associated and protective epitopes suggests i) that the binding of surface IgG to a humoral epitope in the GAD65 molecule, prior to endocytosis and antigen processing may influence the peptide repertoire available for binding to MHC-class II molecules and presentation to T-cells, and ii) that consequent differences in presentation of peptides to T-cells can result in either chronic autoimmunity and disease or protection against disease.

C 023 EARLY DETECTION AND PREVENTION OF TYPE I DIABETES, George S. Eisenbarth, Massimo Pietropaolo, Roberto Gianani, and Liping Yu, Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, Denver CO 80262

With the development of techniques for the identification of relatives of patients with type I diabetes who are themselves at high risk to become diabetic within four years, trials have been initiated for the prevention of diabetes. These trials follow the demonstration in animal models that immunomodulatory therapies such as oral insulin, or parenteral insulin prevent diabetes in NOD mice and BB rats (SQ but not oral insulin). A series of autoantibodies precede the development of type I diabetes including antibodies reacting with insulin, glutamic acid decarboxylase (GAD), ICA69 (the milk bovine albumin related islet antigen (JCI 92:359-371)), Gm2-1 ganglioside, etc. The precise order in which autoantibodies appear has not been defined, though we have frequently observed anti-insulin autoantibodies preceding development of cytoplasmic ICA. With just three biochemical assays for autoantibodies (insulin, GAD and ICA69) essentially 100% of ICA positive prediabetic relatives express at least one autoantibody and approximately 90% express two or more autoantibodies. Thus it is likely that biochemical assays will shortly replace the difficult to standardize ICA assay. Loss of first phase insulin secretion following intravenous glucose allows identification of a subgroup of ICA positive relatives with a more than 90% risk of type I diabetes within four years. In addition, amongst ICA positive relatives with more normal insulin secretion, absence of insulin autoantibodies identifies a subgroup with almost no progression to diabetes within seven years. Combining immunologic and metabolic testing, trials of several therapies for the prevention of diabetes are underway. Nicotinamide appears to be of minimal efficacy while a pilot trial of IV and low dose SQ insulin appears to have prevented type I diabetes for more than three years in 4/5 relatives (versus 7/7 diabetic amongst controls). A large NIH (screening of approximately 40,000 relatives and entry of 400 ICA positive relatives with low first phase insulin secretion) United States trial of IV plus SQ insulin versus placebo has been approved and will likely begin early in 1994. With current understanding of the natural history of type I diabetes, preventive trials are a possibility and it is hoped that clinical prevention with non-immunosuppressive regimens will in the near future become a reality.

C 024 T CELLS IN THE PATHOGENESIS OF NOD IDDM, C. Garrison Fathman, Patricia Rohane, Yang Yang, and Brett Charlton. Stanford University Medical Center, Department of Medicine, Division of Immunology and Rheumatology, Stanford.

The non-obese diabetic (NOD) mouse is an animal model of human insulin-dependent diabetes mellitus (IDDM), a cell-mediated autoimmune disease in which T cells infiltrate pancreatic islets of Langerhans and destroy insulin-producing β cells (1-3). The initiating autoimmune events are not understood. It is unclear whether single or multiple autoantigens are involved and what role T cells play in the early events. To examine the hypothesis that a single initiating autoantigen was recognized early in NOD islets by a monoclonal T cell population leading to inflammatory insulitis, we examined the T cell receptor (TCR) Vβ repertoire of islet-infiltrating T cells in NOD mice 13-15 days of age by PCR analysis. A predominant monoclonal TCR Vβ8.2 gene was expressed by T lymphocytes infiltrating the islets at this age. This finding suggests that autoimmune diabetes may be initiated by a single β cell antigen-specific T cell clone.

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C 025 IMBALANCE HYPOTHESIS OF TYPE I DIABETES MELLITUS Aldo A. Rossini, John P. Mordes, Dale L. Greiner, Peter Gottlieb, and Barbara J. Whalen, University of Massachusetts Medical School, Worcester, MA

Type I diabetes is a T cell mediated autoimmune disorder leading to selective destruction of pancreatic ß cells. We hypothesize that its expression depends on the relative balance between autoimmune effector cells on the one hand, and populations of regulatory cells on the other. We have investigated this hypothesis by inducing the disorder in diabetes-resistant (DR) BB rats. DR rats develop both autoimmune diabetes and thyroiditis after *in vivo* depletion of $RT6.1^+$ T cells. We now report analyses of the interaction between effector and $RT6^+$ regulatory T cells in the DR rat using an adoptive transfer paradigm with histocompatible nude rats as recipients. In the first set of experiments, we found that the DR donor populations capable of the transfer of diabetes and thyroiditis to nude rats are $RT6.1^-$ T cells. Purified T cells from RT6.1-depleted donors transfer diabetes whereas those from intact animals do not. In addition, we found that both $CD4^+$ and $CD8^+$ T cells from RT6.1-depleted DR rats transfer diabetes far more efficiently that either subset alone. Most importantly, the T cell-dependent transfer of diabetes from RT6-depleted diabetic DR rats could be suppressed by co-transfer of equivalent numbers of T cells ($\approx$60% $RT6.1^+$) from intact DR animals. We conclude that the presence of $RT6.1^+$ regulatory cells prevents β-cell destruction and clinical disease. In corollary studies we also investigated the effect of prophylactic subcutaneous insulin treatment on diabetes in the DR rat. Weanling DR rats given insulin before and during anti-RT6.1 antibody treatment (through 60 days of age) do not develop diabetes, whereas induction of thyroiditis remains unaffected. We found that RT6-depleted spleen cells from insulin-treated DR rats do not transfer diabetes to nude recipients (0/7), but efficiently transfer disease after *in vitro* activation with con A (7/7). Since the donor DR cells are $RT6.1^-$ and therefore deficient in $RT6.1^+$ regulatory cells, the mechanism of suppression of disease in this model may involve failure of effector cells either to expand clonally or to activate appropriately in the insulin-treated RT6.1-depleted milieu. Our overall conclusion is that the relative balance between populations of autoreactive $CD4^+$ $RT6^-$ and $CD8^+$ $RT6^-$ effector T cells on the one hand, and regulatory $RT6^+$ T cells determines whether diabetes will occur in the BB rat.

Transgenic Animal Models for Diabetes Research

C 026 EXPRESSION OF NORMAL AND MUTATED HUMAN INSULIN RECEPTORS (IRs) IN THE SKELETAL MUSCLE OF TRANSGENIC MICE, David E. Moller, Pi-Yun Chang and Heike Benecke, Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, MA 02215

In order to create novel animal models with tissue-specific changes in insulin sensitivity, we have expressed normal or putative dominant-negative human IRs in the skeletal muscle of transgenic mice. Use of the myosin light chain (MLC) promoter-enhancer coupled to the normal human IR cDNA resulted in low level (1.5-2-fold) muscle-specific expression of IRs that was associated with modestly increased in vivo insulin sensitivity (Benecke et al, Diabetes 42:206, '93). For the expression of mutated human IRs (Lys^{1030}→Ala or Ala^{1134}→Thr), 3.3 kb of 5' flanking region from the rat muscle creatine kinase (MCK) gene was fused to IR cDNAs with or without a 3.0 kb intron (5' of the IR coding region). MCK/IR constructs without intron failed to express (0/12 founders). In contrast, abundant expression of mutant human IRs was present in muscle from 6/9 founders transgenic for intron-containing contructs. Human IR expression was confined to skeletal and cardiac (50% lower) muscle in both MCK/IR^{1030} and MCK/IR^{1134} mice. The relative amounts of human versus total IRs in skeletal muscle were measured using quantitative immunoassays with human-specific and non-species-specific anti-IR antibodies. The level of total IR expression in skeletal muscle from 5 transgenic MCK/IR lines was increased by 5-20 fold and was accounted for by the marked overexpression of human IRs. Following *in vivo* insulin stimulation (20 and 40 min after intraperitoneal injection), skeletal muscle from MCK/IR^{1134} and control mice was obtained, solubilized, and IR tyrosine kinase activity towards poly-Glu/Tyr was determined. Results of these experiments indicate that maximal insulin-stimulated IR kinase activity (per mg muscle protein) is reduced by 70-80 % in MCK/IR^{1134} mice. Preliminary data reveals that MCK/IR^{1134} mice have a 2-fold increase in fed plasma insulin levels but normal glucose tolerance compared to controls. **Conclusions**: Use of an intron-containing MCK construct results in high level expression of mutant human IRs in muscle and an apparent defect in muscle IR kinase activity. Further studies of these mice will provide new insights into the role of IRs in muscle in *in vivo* glucose homeostasis as well as in the regulation of specific metabolic pathways.

Islet Cell Biology

C 100 CLONING OF A HIGH AFFINITY SULFONYLUREA RECEPTOR FROM RODENT α- AND β-CELLS, Lydia Aguilar-Bryan, Joseph Bryan and Daniel Nelson, Departments of Cell Biology and Medicine, Baylor College of Medicine, Houston, TX 77030.
The antidiabetic sulfonylureas stimulate insulin secretion from β-cells by binding with high affinity to a 140 kDa membrane protein, presumably an ATP-dependent K^+ channel. We synthesized a radioiodinated derivative of the sulfonylurea, glyburide, to photolabel the receptor in hamster insulinoma tumor (HIT) cell membranes. After solubilization with 1% digitonin, the 140 kDa receptor was purified by sequential chromatography on Con A-Sepharose, reactive green 19-agarose and phenylboronate-agarose followed by SDS polyacrylamide gel electrophoresis. Final purification was ~ 2500 fold. The purified receptor increased mobility slightly following digestion with Endoglycosidase F/N-glycosidase F (Endo F) giving an MW_r ~ 137,000. Partial cleavage with V8 protease produced two radiolabeled fragments of 66 kDa and 49 kDa, both increased mobility upon digestion with Endo F. The intact receptor and the two fragments gave the same N-terminal peptide sequence. Residue 9 was an N-linked glycosylation site giving an *asp* residue when Endo F digested polypeptides were sequenced. Twenty-eight residues of sequence were obtained and used to generate two specific antibodies against synthetic peptides, and degenerate oligonucleotides for use as PCR primers. Both antibodies immunoprecipitate radiolabeled hamster, mouse and rat 140 kDa receptors that are displaced by their respective synthetic peptides indicating the sequence is from bona fide receptor. The oligonucleotides were used as PCR primers with random primed mouse alpha cell (αTC-6) cDNA to amplify a 47 bp fragment of the receptor. This fragment was used to screen HIT, αTC-6 and rat insulinoma (RIN m5f) λ libraries. The 5'-end fragments have been recovered from all three species and are highly homologous. Northern analysis shows all three have ~ a 5500 nt message; ~ 35% has been cloned to date. The translated polypeptide has a putative signal peptide preceding the region defined by chemical sequencing. Hydrophobicity plots suggest there are five membrane spanning domains in the first 200 amino acids downstream of the N-terminus defined by chemical sequencing. Database searches give no sequence matches with known proteins. Expression of a full-length clone should resolve whether this receptor is an ATP-dependent K^+ channel.
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C 101 SYNTHESIS OF A BIOLOGICALLY ACTIVE, I^{125} LABELED, DOUBLE MUTANT OF AMYLIN, G. C. Andrews, B. Guarino, R. Suleske, D. Singleton, L. Contillo, A. J. Torchia, J. C. Parker and D. K. Kreutter, Central Research Division, Pfizer Inc., Groton, CT 06340
Amylin suppresses insulin-stimulated glucose uptake and incorporation into glycogen in soleus muscle. The mechanism by which amylin exerts these effects is unknown. It is therefore important to identify amylin specific receptors in target tissues. Iodination of amylin was attempted and the biological activity of the resultant products was examined to determine their suitability as receptor labeling reagents. Human amylin, iodinated at the N-terminus with Bolton-Hunter reagent or at the C-terminus with chloramine T, was devoid of biological activity as measured by the suppression of insulin-stimulated glycogen synthesis in isolated rat soleus muscle. This suggests that both the C- and N-termini of this peptide are important in receptor binding and/or activation. That the loss of activity is a result of the C-terminal iodotyrosyl, rather than the iodination procedure itself was demonstrated by the fact that authentic 3-iodotyrosyl and 3,5-bis-iodotyrosyl amylins produced by solid phase synthesis had no effect on insulin action and that biological activity of chloramine T-iodinated amylin could be restored by catalytic hydrogenolysis. Replacement of the C-terminal tyrosine with phenylalanine (Y37F) yields a biologically acitve peptide. Replacement of phenylalanine 15 with tyrosine in this mutant peptide generates a double mutant (Y37F, F15Y) with a new site for iodination at residue15. Both the Y37F, F15Y mutant and its synthetic Y37F, F15(3,5-bis-iodotyrosyl) derivative were biologically active. A lactoperoxidase catalyzed I125 iodination procedure was developed for the Y37F, F15Y mutant, affording biologically active, I125 labeled Y37F,F15(3,5-bis-iodotyrosyl) amylin which co-migrates on rp-HPLC with authentic cold standard. This labeled mutant will be useful for identification of the amylin receptor in skeletal muscle and other tissues.

C 102 DIVERGENT EFFECTS OF GLUCOKINASE, HEXOKINASE, AND GLUCOKINASE MUTANTS ASSOCIATED WITH MODY ON GLUCOSE-INDUCED INSULIN RELEASE, Thomas C. Becker, Richard Noel, John H. Johnson, and Christopher B. Newgard, Gifford Labs for Diabetes Res., Univ. Texas Southwestern Med. Ctr., Dallas, TX 75235
Glucose-stimulated insulin secretion from β-cells of the islets of Langerhans is controlled by the rate of glucose metabolism, and glucose phosphorylation is thought to be the rate-controlling step for glucose flux. Our laboratory has demonstrated previously that recombinant adenovirus transfers genes to β-cells of the islets of Langherhans with efficiencies of 70% or more. We therefore prepared recombinant adenoviruses containing the cDNAs encoding 1) the islet isoform of glucokinase, 2) hexokinase I, and 3) two mutant glucokinases isolated from families afflicted with Maturity Onset Diabetes of the Young (MODY), one predicted to have sharply reduced enzymatic activity (V203A) and one with near normal enzymatic activity (E279Q) (a gift from G. Bell and J. Takeda). Four days after viral infection of cultured rat islets, western blot analysis indicated that expression of wild-type or mutant glucokinases was increased by approximately 8-fold relative to uninfected islets or islets infected with a virus containing the β-galactosidase gene; similarly, hexokinase was increased by 6-fold relative to its endogenous levels. Glucose-stimulated insulin secretion was studied by sequentially perifusing islets with buffer containing 3, 20, and 3 mM glucose, for 15 minutes under each condition, for 3-4 separate batches of islets for each recombinant virus. Uninfected islets or islets infected with recombinant virus containing the β-galactosidase gene exhibited 198 ± 23 and 193 ± 19 % increases in insulin release in response to the stimulatory concentration of glucose, respectively. Islets expressing the wild type glucokinase or E279Q mutant showed clearly enhanced responses (436 ± 31 and 312 ± 28 % increases, respectively). Islets infected with the V203A glucokinase mutant had a glucose response similar to the control groups (273 ± 50%). Remarkably, islets overexpressing hexokinase I showed a clearly diminished glucose response (123 ± 6%), possibly related to elevated basal insulin release during perifusion with 3 mM glucose. We conclude that overexpression of normal or near-normal alleles of glucokinase can enhance islet glucose responsiveness, but that severely affected alleles of the enzyme or hexokinase I do not provide such a beneficial effect.

C 103 A NOVEL GLUCOSE-DEPENDENT INSULIN SECRETAGOGUE IS A PHOSPHODIESTERASE INHIBITOR. Joel P. Berger, Chhabi Biswas, Edward Brady, Marco Conti*, Catherine Cullinan, Nancy Hayes, Vincent C. Manganiello+, Richard Saperstein, Lu-hua Wang+, Mark D. Leibowitz. Merck Research Laboratories, RY80N-31C, Rahway, NJ 07065; *Stanford University, Palo Alto, CA; +NIH, Bethesda, MD

A novel arylpiperazine (L-686,398) has been shown to be a glucose-dependent insulin secretagogue both *in vivo* and *in vitro*. The characteristics of its activity were strikingly similar to those of the incretin peptide GLP-1. Elevated intracellular concentrations of cAMP are known to result in potentiation of glucose-dependent insulin secretion from pancreatic ß-cells, and the peptide is thought to act in this manner, since it activates adenylate cyclase. We demonstrate that both GLP-1 and L-686,398 increase the accumulation of cAMP in isolated ob/ob mouse ß-cells, but by different mechanisms. The peptide activates adenylate cyclase in membranes from RINm5F rat insulinoma cells with an EC_{50} of ~200 pM, while the arylpiperazine has no effect on the enzyme. However, L-686,398 inhibits the total cAMP phosphodiesterase activity in homogenates of ob/ob mouse pancreatic islets with an EC_{50} of ~50 μM. To determine the mechanism of PDE inhibition by the arylpiperazine, and to examine its specificity, we have studied the kinetics of arylpiperazine inhibition of 2 cloned, expressed phosphodiesterases. A human heart type III PDE and a rat type IVD PDE have been studied using an unlinked, column based phosphodiesterase assay where conversion of substrate is <20%. The arylpiperazine is a competitive inhibitor of both enzymes, although there is evidence for limited drug binding to the enzyme-substrate complex at high drug concentrations. Inhibition of the type III and IV isozymes is characterized by K_i's of 27 and 5 μM, respectively. Therefore, while not extremely potent, the arylpiperazine does exhibit modest selectivity between the type III and IV isozymes. Preliminary data suggest that the arylpiperazine is a particularly weak inhibitor of a Ca/calmodulin activated PDE (type I).

Further exploration of the effects of L-686,398 on ß-cell-specific PDE isoforms will more clearly define the arylpiperazine's mechanism of action. In addition, such studies may reveal novel PDE's as targets for the development of therapeutically useful glucose-dependent insulin secretagogues.

C 104 YEAST PHOSPHOGLUCOMUTASE AND GLUCOSE SENSING, Pam Bounelis, Nupur Dey, David M. Bedwell and Richard B. Marchase, Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294-0005.

The yeast *S. cerevisiae* responds to alterations in extracellular glucose in a variety of ways. In response to glucose deprivation metabolic pathways previously not utilized are activated. This regulation occurs at both the transcriptional and post-translational levels. In addition, glucose-deprived *S. cerevisiae* respond to a reexposure to glucose with rapid and transient increases in both calcium influx and cAMP production. These increases are dependent upon the phosphorylation of glucose to Glc-6-P but not upon further glycolytic metabolism. These responses do not occur in cells that have never been deprived of glucose. Phosphoglucomutase (PGM) is a key enzyme in the interconversion of glucose and glycogen and recently has been shown in *S. cerevisiae* to be a cytoplasmic glycoprotein. PGM is an acceptor for UDP-Glc: glycoprotein Glc-1-phosphotransferase (Glc phosphotransferase) and contains Glc in phosphodiester linkage to O-linked Man. The synthesis and glycosylation of PGM by *S. cerevisiae* in response to glucose deprivation and heat shock have been determined. PGM synthesis in cells that were glucose deprived by growth to an OD_{600} of 4 and also in early log phase cells following a brief heat shock (37°C) was increased as assessed by Western blot analyses, metabolic labeling with [^{35}S]methionine, and PGM enzymatic activity. PGM expressed following heat shock or in glucose-deprived cells was (a) underglucosylated as measured by metabolic labeling with [^{3}H]Glc, (b) a better acceptor in *in vitro* Glc phosphotransferase assays, and (c) less negatively charged by DEAE chromatography compared to PGM in control cells. This underglucosylation occurred despite ample levels of UDP-Glc. Underglucosylated PGM appeared to have a higher enzymatic specific activity than the fully glucosylated form. These responses thus define stimulus-induced changes in the glycosylation of PGM paralleling comparable changes seen in mammalian cells.

C 105 MITOCHONDRIAL GLYCEROL PHOSPHATE DEHYDROGENASE PROTEIN LEVELS IN PANCREATIC ISLETS AND OTHER TISSUES, Laura J. Brown and Michael J. MacDonald, University of Wisconsin Medical School, Madison, WI 53706.

Glucose stimulates insulin release by aerobic glycolysis. Mitochondrial glycerol phosphate dehydrogenase (mGPDH) has been proposed as a component of the glucose sensing mechanism of the islet B cell, as its role in the glycerol phosphate shuttle may help regulate the mitochondrial oxidation of NADH produced during glycolysis. In humans and laboratory animals the assayable enzyme activity of mGPDH in islets is higher in islets than in most other tissues. It is not known whether mGPDH consists of a single protein form, or whether its enzyme activity is regulated by protein quantity or post-translational modifications. We purified mGPDH from rat mitochondria and made a rabbit anti-serum. In Western blots of SDS-polyacrylamide gels, the mGPDH from seven different tissues is seen as a single band of 72 kD apparent Mr. The level of mGPDH protein correlates with mGPDH enzyme activity. Partially purified mGPDH preparations from livers of euthyroid and hyperthyroid rats also demonstrated that the increased mGPDH activity seen in the hyperthyroid rat is due to increased levels of mGPDH protein. We are now using the antiserum to screen expression libraries in order to isolate the mGPDH cDNA. It is hoped that the isolation of this gene will lead to a better understanding of its tissue specific regulation and will allow exploration of its role in insulin secretion. The antiserum will also be useful in studies of animal models of non-insulin dependent diabetes in which low mGPDH activity in islets has been reported.

C 106 INDUCTION OF INTRACELLULAR AMYLOID FIBRIL FORMATION IN COS-1 CELLS TRANSFECTED WITH HUMAN ISLET AMYLOID POLYPEPTIDE, Peter C. Butler, Timothy D. O'Brien, Norman L. Eberhardt, Endocrine Research Unit, Mayo Clinic, Rochester, MN 55905

The pancreatic islets in non-insulin-dependent diabetes mellitus are characterized by the loss of up to 50% of the β-cell mass and concurrent deposition of amyloid (islet amyloid) derived from islet amyloid polypeptide (IAPP). The central region of IAPP shows species heterogeneity with an amyloidogenic hydrophobic sequence in humans, cats, and monkeys (all prone to islet amyloid and NIDDM) but no hydrophobic sequences in rats or mice (which do not develop islet amyloid or NIDDM). Intracellular IAPP-derived amyloid deposits have been observed in β-cells in animal models of non-insulin-dependent diabetes mellitus and in amyloid-producing human insulinomas. We propose that intracellular formation of islet amyloid is cytotoxic and results in necrosis of β-cells in NIDDM, thus linking the two main pathologic features of the pancreatic islets in NIDDM. To test this hypothesis, we transfected COS-1 cells with cDNA clones of either human (amyloidogenic) or rat (non-amyloidogenic) IAPP. At 24 hours after transfection about 75% of cells showed intense cytoplasmic IAPP immunofluorescence and by electron microscopy showed IAPP immuno-gold labeling in discrete cytoplasmic locations. Ninety-six hours after transfection, 90% of cells expressing human IAPP contained amyloid fibrils all of which were dead (employing standard ultrastructural criteria), whereas cells transfected with the human IAPP cDNA in antisense orientation, rat IAPP (i.e., non-amyloidogenic) cDNA in sense orientation, or mock transfected cells lacked intracellular amyloid and were viable. These findings demonstrate that high expression of human but not rat IAPP is associated with the rapid development of intracellular amyloid fibrils and with cellular degeneration and death. Thus, this system mimics some features of the pancreatic pathology of NIDDM and may serve as a useful *in vivo* model.

C 107 EFFECT OF DOWN REGULATING HEXOKINASE II PROTEIN EXPRESSION ON 2-DEOXYGLUCOSE UPATKE IN RAT L6 MYOCYTES, Chu D.T. Blume J.E. and Young D.A. Diabetes Department, Sandoz Research Institute, East Hanover, NJ 07936

Insulin-stimulated glucose clearance in adipose tissue and skeletal muscle requires a coordinated coupling of GLUT4 glucose transporter and hexokinase II (HKII). Theoretically, a defect in this coupling due to a reduction in HKII activity could result in impaired glucose uptake in these tissues. Although no detectable reduction in the amount of muscle GLUT4 mRNA or protein has been found in NIDDM subjects, insulin-stimulated glucose transport is decreased. Recent ^{31}P-NMR data suggested that impairment in glucose transport and glycogen synthesis in NIDDM subjects could be due to a defect in muscle glucose phosphorylation, since basal and insulin-stimulated muscle glucose-6-phosphate levels were reduced (J. Clin. Invest. 89:1069, 1992). The purpose of this study was to evaluate the effect of suppressing HKII protein expression on insulin-stimulated 2-deoxyglucose uptake in differentiated rat L6 myocytes. Antisense oligonucleotides, designed to hybridize to the translation initiation regions of rat HKII mRNA, were introduced to rat L6 myocytes via cationic liposome mediated transfection. Treatment of cells with antisense oligonucleotides resulted in a 20-40% reduction of HKII protein levels as measured by Western blot. In antisense oligonucleotide-treated cells, 2-deoxyglucose uptake was reduced 25% in acute (1 h), and 40% in chronic (18 h) insulin treatments. Liposome- or sense oligonucleotide-treated controls had no effects on HKII protein levels or 2-deoxyglucose uptake. These data indicate that treatment of rat L6 myocytes with antisense oligonucleotides targeted to the translation initiation regions of rat HKII mRNA results in suppression of HKII protein expression and reduction in insulin-stimulated 2-deoxyglucose uptake. This study suggests that a decrease in HKII activity could lead to the decrease in insulin-stimulated glucose uptake seen in NIDDM patients.

C 108 CLONING AND FUNCTIONAL EXPRESSION OF A HUMAN GLUCAGON-LIKE PEPTIDE-1 RECEPTOR.

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A human glucagon- like peptide -1 (GLP-1) receptor has been cloned from the gastric tumor cell line HGT-1. The cDNA clone encodes a protein of 463 amino acids, and is 91% identical to the rat GLP-1 receptor and 48% identical to the rat glucagon receptor. The predicted secondary structure places the human GLP-1 receptor within the superfamily of seven transmembrane domain G protein coupled receptors, in the branch including the receptors for secretin, calcitonin, and parathyroid hormone. Expression of the human GLP-1 receptor in COS-7 cells results in high affinity binding for [^{125}I] GLP-1 (7-36) amide, with an apparent B_{max} of ~2-5 pmol/mg protein. In contrast, mock transfected COS cells show no detectable binding of the radioligand. GLP-1 (7-36) amide inhibits the binding of [^{125}I] GLP-1 (7-36) amide to transfected COS-7 cells with an IC_{50} of 4nM. The binding of [^{125}I] GLP-1 (7-36) amide to the human receptor is inhibited with the rank order of potency: GLP-1 (7-36) amide > glucagon > secretin, characteristic of a GLP-1 receptor. The human GLP-1 receptor expressed in COS-7 cells is functionally coupled to increases in intracellular cAMP. Incubation of cells expressing the human GLP-1 receptor with the GLP-1 (7-36) amide gives rise to a 4-fold increase in cyclic AMP over basal levels, with an EC_{50} of 25 pM. Glucagon is a full agonist at the human GLP-1 receptor, but is 200-fold less potent than GLP-1 (7-36) amide in activating the receptor. The isolation and functional expression of the human GLP-1 receptor cDNA will aid in the discovery of compounds that act via this receptor for potential use in the treatment of Type II diabetes.

C 109 DIFFERENTIAL EXPRESSION OF GLUCOSE TRANSPORTERS IN α- AND β-CELLS OF THE ISLETS OF LANGERHANS

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Glucose exerts inverse effects on α- and β-cells of the endocrine pancreas. The secretory activity of α-cells is suppressed by glucose whereas that of β-cells is stimulated. The molecular basis of this different effect is not yet clarified. The first step in cellular glucose sensing consists in the facilitated transport of the sugar via (a) membrane protein(s) of the GLUT-family. In dispersed rat islet cells, containing 65% β-cells and 20% α-cells, the process of glucose uptake is characterised by a low as well as a high K_m component. Purified β-cell preparations presented only the high K_m component; the low K_m component was recovered in the preparation of islet non β-cells, containing <20% β-cells. The v_{max} of transport in β-cells was 100-fold higher than in non β-cells (8.40 pmol/min./10^3 β-cells compared to 0.08 pmol/min./10^3 non β-cells). Purified β-cells exhibited GLUT2 expression at the mRNA- and protein-level; they also transcribed GLUT1 but the GLUT1 protein was detected only following a 16 h. culture. The β-cell fraction in the non β-cell preparation was further decreased by autofluorescence activated cell sorting. In the resulting preparation, containing <5% β-cells and >80% α-cells, GLUT2 expression levels were below detection limits. Rat GLUT1 cDNA hybridised with α-cell mRNA but its translation product could not be detected by a GLUT1-antibody directed against the GLUT1 C-terminus. Of all fragments amplified from α-cell cDNA via RNA-PCR with degenerated primers targetted to transmembrane domain V and VII, 10% contained the GLUT1 sequence. No other GLUT-like sequences could be identified. Conclusions : a) In normal physiological conditions, β-cells transcribe GLUT1 and GLUT2 mRNA but express only GLUT2 protein. b) The low K_m component of glucose transport found in dispersed islet cells is contributed by the non β-cell fraction. c) The low K_m glucose transporter in α-cells may be GLUT1 expressed at low abundancy or may be a GLUT1-related protein.

# : both authors contributed equally to this study

C 110 IN VITRO RELEASE OF C-PEPTIDE AND INSULIN IS NOT EQUIMOLAR.

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Equimolar quantities of C-peptide and insulin are expected to be produced during proinsulin processing in the B-cell. To determine the proportion of these two peptides released during stimulation and inhibition of B-cell secretion, we cultured neonatal (3-5 days old) rat pancreata in monolayers and then incubated them in 16.7 mM glucose with or without epinephrine 10^{-7} M. C-peptide and insulin immunoreactivity were measured in the medium following static incubations of 15 to 120 minutes. Molar ratios of C-peptide to insulin released were:

Time (min)	Glucose	Glucose + Epinephrine
15	28±3%	44±1% *
30	30±2%	40±4%
60	33±1%	44±3% *
90	33±1%	40±6%
120	29±1%	45±1% *

(x±sem; n=4 per condition; * p<0.05 vs glucose)

Despite expectations, three times as much insulin as C-peptide was released in response to glucose. Thus, although the molar ratio remained constant over time, C-peptide and insulin were not released in equimolar quantities. With epinephrine, both C-peptide and insulin release were inhibited but this inhibition was less for C-peptide (72%) than insulin (80%), so that the molar ratio increased over that observed in glucose alone. Based on these data we conclude that variable proportions of C-peptide and insulin exist in different B-cells or granules.

C 111 INCREASED GLUCOKINASE ACTIVITY IN ISOLATED ISLETS OF EUGLYCEMIC INSULIN RESISTANT RATS, Jack L. Leahy, Chuan Chen, and Hitoshi Hosokawa, Department of Endocrinology, Metabolism, and Molecular Medicine, New England Medical Center, Boston, MA 02215

Euglycemic states of insulin resistance such as obesity cause compensatory hyperinsulinemia. We used an insulin resistant rat model to test the hypothesis that the cause of the increased insulin secretion is a left-shift in the dose response relationship for glucose-induced insulin secretion secondary to increased ß-cell glucose phosphorylation. 12 week old SHR rats were hyperinsulinemic and euglycemic (plasma insulin 2.1±0.2 ng/ml; plasma glucose 134±2 mg/dl) versus Wistar-Kyoto (WK) control rats (insulin 0.5±0.1 ng/ml p<0.001; glucose 140±6 mg/dl p=NS). The dose-response curve for glucose-induced insulin secretion in isolated islets of SHR rats was left shifted with no change in the maximal response (ED_{50} 6.6±1.0 mM glucose SHR vs 9.6±0.5 mM glucose WK, p<0.03). V_{max} (mol glucose/60 min/kg DNA) for glucokinase and hexokinase were measured in islet homogenates.

	Hexokinase V_{max}	Glucokinase V_{max}	DNA (ng/islet)
SHR (5)	6.0±0.8	10.9±0.9	17.4±2.6
WK (5)	5.1±1.2	8.0±0.4	17.7±2.5
P	NS	0.02	NS

Western blot of islet extracts showed no increase in glucokinase protein in SHR rats (95±3% of WK, n=7)

In summary, ß-cells compensate for a higher demand on insulin secretion through increasing the ß-cell sensitivity for glucose-induced insulin secretion. The mechanism appears to be an increase in the catalytic activity of the glucokinase protein resulting in an increase in ß-cell glucose metabolism. Since SHR rats are normoglycemic, the stimulus for the increase in glucokinase catalytic activity must be something other than a sustained change in the plasma glucose level.

C 112 PHOSPHOGLUCOMUTASE: A POTENTIAL LINK BETWEEN GLUCOSE METABOLISM AND CALCIUM REGULATION, Richard B. Marchase, Pam Bounelis, Nancy A. Veyna, †Mary Ann Martone, †Angelica Bayardo and †Mark Ellisman. Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294-0005 and †Department of Neurosciences, University of California, San Diego, La Jolla, CA 92093-0608.

Phosphoglucomutase (PGM), a key enzyme in the interconversion of glucose and glycogen, has been determined to be one of the growing number of cytoplasmic glycoproteins in which carbohydrate modifications serve as a means of modifying enzymatic activity or topography in response to external stimuli. PGM contains an O-linked mannose that can be further modified in a reversible manner by the addition or deletion of phosphodiester-linked glucose. It appears to be the sole protein acceptor for a Glc phosphotransferase that utilizes UDP-Glc as its sugar donor. In rat cortical synaptosomes and PC12 cells the addition of phosphodiester-linked glucose to PGM has been shown to be transient and to be stimulated by depolarization and the subsequent influx of calcium. This modification results in the preferential association of the glycosylated enzyme with membranes and a concomitant loss in its enzymatic activity. In addition to these findings demonstrating that PGM is responsive to calcium, other data suggest that PGM may itself serve as a regulator of cytoplasmic calcium within excitable cells. For instance, in skeletal muscle PGM has been identified as a protein that modulates calcium reuptake by the sarcoplasmic reticulum (SR). Immunofluorescence and immunoelectron microscopic examinations demonstrate a tight association of PGM with the SR and its colocalization with calcium ATPase. Thus, PGM, an enzyme of clear importance to hexose metabolism, is covalently modified in response to stimuli. This modification would appear to play a role in an association of PGM with membranous organelles important to Ca^{++} regulation.

C 113 CHARACTERIZATION OF HOMEOBOX PROTEINS EXPRESSED IN RAT PANCREATIC ISLETS, Christopher P. Miller, Julia C. Lin and Joel F. Habener, Laboratory of Molecular Endocrinology, Massachusetts General Hospital and Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02114

Homeodomain transcription factors are sequence-specific DNA-binding proteins that are known to be important in development and differentiation. We have used polymerase chain reaction (PCR) to amplify homeodomain-containing cDNAs from adult rat pancreatic islets using degenerate amplimers corresponding to two highly conserved amino acid sequence motifs within the DNA recognition helix of the *drosophila* homeoprotein Antennapedia (Antp). Twelve different homeodomain sequences were identified, including those for mRNAs encoding IDX-1/STF-1, Hox1.3, Hox1.4, Hox1.11, Hox2.6, Hox4A, Hox4.3, Cdx-1, Cdx-3, CHox-7, and two previously unidentified homeodomain proteins. The two new sequences encode a Cdx-related homeodomain and a novel homeodomain. The most abundant Antp-like homeodomain sequence corresponds to the mRNA for IDX-1/STF-1, a recently cloned homeoprotein with high sequence similarity to *xenopus* XlHbox8. We have isolated a full-length cDNA encoding IDX-1/STF-1 and have examined the tissue and cell-specific expression of the IDX-1/STF-1 mRNA and protein. IDX-1/STF-1 is expressed in pancreatic islets and ducts, and in the duodenum. IDX-1/STF-1 binds to enhancer sequences in the rat somatostatin, insulin and glucagon gene 5' flanking regions and activates transcription of reporter constructs containing these sequences. Based upon its pattern of expression, relative abundance, and transcriptional regulatory activities, IDX-1/STF-1 is likely to play an important role in gene regulation and cell differentiation in pancreatic islets and the proximal small intestine.

C 114 REGULATION OF GLUCOSE TRANSPORTER GENE EXPRESSION, SYNTHESIS AND TRANSLOCATION IN CULTURED MUSCLE CELLS BY INSULIN AND GLUCOSE, R. J. Pollet, I. El-Kebbi, S. Bradshaw, C.-I. Pao, L. S. Phillips, S. Cushman and C. Wilson, VA/Emory University Medical Centers, Atlanta, GA 30033 and NIDDKD Bethesda, MD 20892.

We investigated changes in glucose transporter gene expression by insulin and glucose in BC3H1 cells, a continuously cultured skeletal muscle cell line lacking the MyoD transcription factor required for cell fusion. Utilizing cDNA probes for GLUT 1 and GLUT 4 mRNAs (G. 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) stimulated a similar increase in GLUT 1 gene expression but pretreatment with PMA for 12 hr, which suppresses several of the protein kinase C isoenzymes, did not inhibit this insulin effect. Physiologically elevated glucose concentrations (5-25 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 xylose and mannose. We used the impermeant photoaffinity label ATB-[2-^{3}H]BMPA (G. Holman) in the presence and absense of digitonin (0.025%) to identify and quantitate the total and cell surface immunoprecipitable GLUT 1 protein, respectively. In starved cells, 85% of the glucose transporters were present at the cell surface in the basal state with little change in response to insulin (200 nM), correlating with lack of change in 2-deoxyglucose uptake in response to insulin. Exposure to glucose (25 mM) for 24 hr resulted in an 80% decrease in the total GLUT1 content relative to starved cells, with only 25% of their glucose transporters present at the cell surface and an 85% reduction in 2-deoxyglucose uptake. Acute stimulation with insulin or PMA led to more than a 2-fold increase in GLUT 1 at the cell surface. In glucose-exposed cells, acute insulin and PMA treatment increased 2-deoxyglucose uptake by approximately 2- and 4-fold respectively, correlating well with the change in cell surface photolabeled GLUT1.

In summary, insulin stimulates a two-fold increase in gene expression and synthesis of GLUT 1, the major glucose transporter in the BC3H1 cultured skeletal myocyte. PMA mimics this action of insulin in a non-additive manner, suggesting a convergence of pathways in this tissue with respect to regulation of specific gene expression, but the lack of effect of chronic pretreatment with high levels of PMA suggests that this effect may be independent of several of the specific isoenzymes of protein kinase C. 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. Glucose feeding decreases the total number of glucose transporters in the BC3H-1 muscle cell and redistributes the major portion to intracellular fractions, where they can be translocated to their surface site of action by physiologically regulated mechanisms such as insulin stimulation. This study demonstates tthe interrelationahip of pretranslational control of gene expression and translocation to the site of action at the cell surface in the overall funcional regulation of glucose transporter action.

C 115 ABNORMAL OSCILLATORY INSULIN SECRETION IN THE INSULIN RESISTANT ZUCKER DIABETIC FATTY (ZDF) RAT:RESPONSE TO TREATMENT WITH PIOGLITAZONE, Kenneth S. Polonsky, Jeppe Sturis and William Pugh, Department of Medicine, University of Chicago, Chicago IL 60637

Insulin secretion in the normal rat pancreas perfused with constant glucose is pulsatile with a period of 5-10 min. Studies to determine if alterations in oscillatory insulin secretion are present in insulin resistant rats with NIDDM were performed using the isolated perfused pancreas in 5 ZDF rats, 5 nondiabetic Zucker controls (ZC) and a lean Wistar control (W) (plasma glucose levels of 33 ± 3 mM (mean $\pm$ SEM), 13 ± 1 mM, and 13 mM, respectively). Oscillations in the perfusate glucose concentration around a mean of 7 mM were produced with two peristaltic pumps programmed to oscillate as sine waves out of phase, keeping the total flow rate constant. Period and amplitude (mean to peak) of the sine wave were set to 10 min and 10%, respectively. Insulin levels were measured at 1-min intervals for 3 h. In ZC and W, the insulin pulses entrained to the oscillating glucose concentration. Entrainment did not occur in the diabetic animals. Normalized spectral power at 10 min was reduced by 90% in ZDF compared to ZC ($p < 0.0001$). In comparison, the spectral power at 10 min for W was within the range of the spectral power of ZC. To define the effects of improvements in insulin sensitivity, ZDF animals were treated with the agent pioglitazone which enhances insulin sensitivity but has no primary effect on the β-cell. Treatment initiated at age 5-6, weeks prior to onset of diabetes, prevented abnormal oscillatory secretion, whereas abnormal oscillations persisted in animals treated 2 weeks after onset of diabetes despite normalization of plasma glucose. In summary 1) pulsatile insulin secretion cannot be entrained by exogenous glucose in insulin resistant ZDF rats; 2) this abnormality is prevented by treatment with pioglitazone prior to onset of diabetes but not corrected if treatment is begun after onset of hyperglycemia despite normalization of glucose levels; 3) these data suggest a causal link between insulin resistance and abnormal β-cell function which may be prevented but not corrected by agents which increase insulin sensitivity.

C 116 ENHANCED TOXIC EFFECT OF STREPTOZOTOCIN ON GLUT-2 EXPRESSING CELL LINES *IN VIVO* AND *IN VITRO*, Wolfgang Schnedl, Sarah Ferber, and Christopher B. Newgard, Gifford Labs for Diabetes Research, Univ. Texas Southwestern Med. Ctr. and BetaGene, Inc, Dallas, TX 75235

Rat insulinoma cell lines engineered for GLUT-2 expression (RIN 36-7) or lacking GLUT-2 (RIN GT1-3) were transplanted into athymic nude rats, obviating the need for encapsulation of the implanted cells. Animals were injected with streptozotocin to induce diabetes 7-10 days after cell implantation. Animals containing tumors of GT1-3 cells became severely hypoglycemic, desptite streptozotocin injection. Removal of the tumor resulted in immediate hyperglycemia, indicating effective streptozotocin-mediated destruction of the islet β-cells. In constrast, injection of streptozotocin into animals implanted with RIN 36-7 cells resulted in immediate diabetes, without the need for tumor removal, despite the fact that RIN 36-7 cells produce slightly more insulin than RIN GT1-3 cells in the *in vivo* setting. The enhanced toxic effect of streptozotocin on GLUT-2 expressing cells *in vivo* was confirmed by *in vitro* analysis, in which 51 ± 9% of GLUT-2 expressing RIN 36-7 cells were found to be killed by 20 mM streptozotocin, while only 22 ± 1 % of non-GLUT-2 expressing RIN cells were affected ($p < 0.01$). In addition, 51 ± 1% of GLUT-2 expressing AtT-20ins cells were killed by 2 mM streptozotocin, compared to 21 ± 4% of GLUT-1 overexpressing and 21 ± 3% of untransfected AtT-20ins cells ($p < 0.01$ for GLUT-2 cells versus either control). These data suggest either that streptozotocin is specifically transported into cells by GLUT-2, but not GLUT-1, or alternatively that streptozotocin binds to GLUT-2 molecules but not GLUT-1 molecules, resulting in inhibition of transport or alteration of other functions of GLUT-2 leading to cytotoxicity. Cell lines expressing different glucose transporter isoforms may provide insight into mechanisms of β-cell cytotoxicity in IDDM, since 1) Immuglobulins from new-onset IDDM patients bind cells that overexpress GLUT-2 but not GLUT-1 (Inman, et al., *Proc. Natl. Acad. Sci.* 90:1281-1284) and 2) Insulin therapy may delay the onset of IDDM in high risk individuals (Keller, et al., *Lancet* 341: 927-928) and also down-regulates β-cell GLUT-2 expression in normal rodents (Chen, et al., *Proc. Natl. Acad. Sci.* 87: 4088-4092).

C 117 INDUCED EXPRESSION OF THE GTP-BINDING PROTEIN Gαs IN TWO MODELS OF ß-CELL PROLIFERATION, Andrea L. Sestak, Susanne Mumby, Michael Appel, and Christopher B. Newgard, Departments of Biochemistry and Pharmacology, Univ. Texas Southwestern Medical Center, Dallas, TX 75235

We have utilized the post-insulinoma rat to attempt to isolate genes involved in ß-cell proliferation. Implantation of insulinoma cells into syngeneic rats results in reduction of ß-cell mass by 70% over 8-10 weeks. ß-cell mass rebounds to normal within 4 days of surgical resection of the tumor through a combination of mitosis and swelling of the ß-cells. RNA was isolated from islets at their peak period of mitotic activity (3 days following tumor removal) and amplified by PCR to create a plasmid library. Differential screening with labeled cDNA was used to eliminate all clones expressed at high levels in AR42J cells, an exocrine pancreatic cell line, and in normal liver. Plasmids containing cDNAs which did not bind either of the control pools were isolated and tested for their expression in an alternate model of ß-cell growth, induced by injection of 5 mg/kg dexamethasone into normal rats for a period of 10 days. Those clones that exhibited enhanced expression in islets of dexamethasone injected compared with control rats were subjected to sequence analysis. Two of the clones isolated by this series of screening steps were identified as the alpha subunit of the stimulatory trimeric GTP binding protein (Gαs). Northern blot analysis demonstrated an increase in Gαs mRNA levels in islets during proliferation induced by dexamethasone relative to islets from untreated controls. This increase was confirmed by *in situ* hybridization, which also showed that the message is present in both α- and ß-cells. Immunocytochemical studies suggested that the increase in islet Gαs protein levels caused by dexamethasone is more pronounced than the increase in mRNA. Indeed, western blot analysis demonstrated a 5-fold induction in the larger 52 kD species of Gαs protein in islets from dexamethasone treated rats to levels roughly equal to those observed in normal brain tissue. We have now prepared recombinant adenoviruses containing cDNAs encoding both the long and short forms of wild-type Gαs, as well as constituitively active point mutants of both forms (Q227L). This system was chosen because our laboratory has shown that the recombinant adenovirus system allows gene delivery to cells of isolated islets with efficiencies of 70% or greater. We are now testing whether overexpression of normal or mutant Gαs proteins in islets will result in altered β-cell growth or insulin secretion.

C 118 **Ca^{2+}-ELEVATING SECRETAGOGUES INHIBIT SERINE/THREONINE PROTEIN PHOSPHATASES IN INSULIN-SECRETING CELLS.**
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Reversible protein phosphorylation is a versatile mechanism by which cells transduce external signals into biological responses. In insulin-secreting cells acute specific inhibition of serine/threonine protein phosphatases (PPases) leads to stimulation of Ca^{2+} entry and insulin release. We have investigated the regulation of PPases in RINm5F insulinoma cells by secretagogues and intracellular second messengers. Stimulation of intact cells with L-arginine (which causes Ca^{2+} influx and polyamine synthesis), L-glutamine (which causes Ca^{2+} influx due to ATP generation), KCl (which causes Ca^{2+} influx through depolarization) or ATP (which elevates cytosolic Ca^{2+} by stimulating ion influx and intracellular mobilization) elicited early (1 min) 10-30 % decreases in type-1 and/or type-2A PPase activity ($P<0.05$) which gradually returned to normal levels. Addition of cAMP, cGMP or prostaglandins to cell homogenates failed to affect PPase activities. Physiological concentrations of adenine nucleotides were found to be potent inhibitors of type-2A PPase, and to a lesser extent PPase-1 (ATP>ADP>AMP>adenosine) when added to cell homogenates. IC_{50} values were for PPase-2A 75 μM and 250 μM for ATP and ADP, respectively. Polyamines dose-dependently suppressed PPase-1 activity when added to homogenates in physiological concentrations. We conclude that physiological stimuli of insulin secretion which elevate cytoplasmic free Ca^{2+}, ATP or polyamines, and certain intracellular second messengers, inhibit RINm5F cell PPase activities which may contribute to the increase in phosphorylation state that occurrs after secretory stimulation. Thus, inhibition of protein dephosphorylation may be a novel regulatory mechamism, equally important as protein kinase activation, which controls the stimulus-secretion coupling in insulin-producing cells.

C 119 HUMAN AMYLIN PROMOTER ANALYSIS REVEALS REQUIREMENT FOR BOTH EXON AND INTRON SEQUENCES DOWNSTREAM FROM THE TRANSCRIPTION INITIATION SITE. Walter Soeller, Maynard Carty, Robin Nelson and Jay Lillquist, Dept. of Molecular Genetics and Protein Chemistry, Pfizer Central Research, Groton, CT.06340

Human Amylin (Islet Associated Polypeptide, IAPP) overexpression in pancreatic ß cells is thought to lead to pancreatic amyloid plaque formation in NIDDM. In order to understand how such overexpression could occur at a transcriptional level we undertook a study of human amylin promoter function by examining transient expression of promoter-CAT reporter constructs in ßTC3 and RIN cell lines. 5' deletion analysis identified a 50 bp sequence 90 bases upstream of the TATA box that is responsible for ß cell-specific transcription and homologous to the FAR/FLAT element in the rat insulin promoter. Deletion of sequences downstream from position +31 resulted in the complete loss of promoter activity. This loss of activity was explored further by selective deletion and/or scanning mutagenesis of sequences within the first exon and intron of the amylin gene. Elimination of intron I resulted in 10 fold lower activity while scanning block mutagenesis of sequences 27 to 53 nucleotides downstream from the initiation site resulted in 3 fold lower activity. Suprisingly, mutagenesis of sequences surrounding the start site itself resulted in a 5 fold increase in activity. DNase I protection analysis using RIN nuclear extracts revealed a footprint in this region. The role of this putative repressor activity in the regulation of amylin transcription will be discussed.

C 120 THE PRESENCE OF PARAFUSIN, A STIMULUS-SENSITIVE PHOSPHOGLUCOPROTEIN, IN RAT PANCREAS AND IN A MURINE ISLET CELL LINE βTC3, Alice Widding and Birgit H. Satir, Department of Anatomy and Structural Biology, Albert Einstein College of Medicine New York, N.Y. 10461

Paramecium Parafusin (pPFUS) is a M_r 63,000 evolutionarily conserved phosphoglucoprotein which has been implicated in regulation of exocytosis in this unicellular eukaryote (Birgit H. Satir et al. 1989 Proc.Natl.Acad.Sci. 86:930). Here we report the presence of parafusin in rat pancreas and a murine pancreatic βcell line (βTC3), which synthesizes, stores and releases insulin as in normal βcells (D'Ambra et al. 1990, Endocrin. 126:2815). The presence of parafusin was tested in two ways 1) by immunoblot analysis using a pPFUS polyclonal synthetic peptide antibody and 2) by incorporation of the UDPGlc analogue (β^{35}S)-UDPGlc. The βTC3 cells were grown in 5% CO_2 - 95% air at 37°C to near confluency, washed in TRIS buffer (10mM) 0.9% NaCL, pH 7.3. The cells were harvested with a rubber policeman and homogenized on ice in TRIS buffer (50mM) containing protease inhibitors in a Dounce homogenizer using 30 strokes. The rat pancreas was homogenized on ice via sonication in the same buffer. Immunoblot analysis showed islet PFUS(iPFUS) present in the homogenates and low (1500xg) and high speed (100,000xg) supernates. Incorporation of (β^{35}S)-UDPGlc using these fractions shows the label to be predominatly in a M_r 63,000 polypeptide, which on Western blot crossreacts with the pPFUS antibody. These results show the presence of iPFUS in rat pancreas and islet βcells and suggests the possibility of a role for iPFUS in insulin secretion.

C 121 THE EXPRESSION OF PROGLUCAGON GENE AND ITS PROTEIN PRODUCTS IN ISLET NEOGENESIS IN THE HAMSTER, Ronit Rafaeloff, Randall Chong, Scott W. Barlow, Lawrence Rosenberg and Aaron I. Vinik, The Diabetes Institutes, Eastern Virginia Medical School, Norfolk VA 23510.

Diabetes mellitus is a disorder of glucose homeostasis characterized by hyperglycemia, insulinopenia, peripheral insulin resistance and increased glucagon release. The proglucagon gene encodes the protein precursor for both glucagon and glucagon like peptide I (GLP-I), which are generated as a result of tissue specific posttranslational processing. Glucagon is hyperglycemic due to gluconeogenesis while GLP-I is a potent insulin secretagogue. The question arises whether in diabetes mellitus there is increased glucagon expression and production or alternately decreased GLP-I release. Thus, understanding the normal developmental regulation of the proglucagon gene and these two peptides is of great importance. We therefore studied the developmental regulation of GLP-I and glucagon by examining the temporal and regional expression of the proglucagon gene and the distribution of glucagon and GLP-I in our wrapped pancreas model of Syrian golden hamster islet neogenesis.

Hamster pancreata were excised 1,2,6,10 and 28 days(d) after cellophane wrapping (CW). *In situ* hybridization analysis showed expression of proglucagon gene in cells associated with the ductular epithelium as early as 6d after CW. Glucagon peptide positive immunocytochemical reactivity was observed in small foci not associated with islets as early as 10 days, while GLP-I was observed in neo-islets at the same time point. In conclusion: the early expression of proglucagon gene in duct epithelium and GLP-I in the developing islets is compatible with evolution of pancreas specific glucagon from primitive ductal elements and suggests an important role of GLP-I in the development of the insulin secretory mechanism.

Insulin Action

C 200 REGULATION OF GLUCOSE TRANSPORT AND GLUT 1 EXPRESSION BY IRON CHELATORS IN MUSCLE CELLS IN CULTURE. Nava Bashan, Smadar Ben-Ezra, Nitzan Kozlovsky and Ruth Potashnik. Department of Biochemistry, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel.

Massive intake of iron, repeated blood transfusion and hemochromatosis resulted in overload of iron in various organs. One of the pathological conditions shown to be associated with iron overload is NIDDM. This led us to investigate the effect of iron and iron chelators on glucose transport, and expression of Glut 1 and Glut 4 transport proteins in L6 muscle cells in culture. Incubation of L6 myotubes in the presence of 20 μg/ml iron for 48 hours resulted in a reduction of 2-deoxyglucose uptake by 40%. Incubation of the myotubes in the presence of the iron chelators deferoxamide (0.5 mM) or bipyridyl (0.1 mM) resulted in a 3-4 fold increase in glucose uptake over a period of 24 h compared to that in cells incubated in normal medium. The effect of iron chelators and insulin were additive suggesting that these stimuli of transport act through independent pathways. In the presence of the chelators, glucose utilization and lactate accumulation in the medium increased by 2.5 folds. Addition of cycloheximide to the medium of cells incubated in the presence of the chelators prevented the induction increase in glucose uptake indicating that *de novo* synthesis of glucose transport proteins was the major means by which cells increased their ability to take up glucose. Western and Northern blot analysis showed that expression of Glut 1 protein and mRNA were significantly elevated in cells incubated in the presence of either deferoxamide or bipyridyl. Glut 4 expression appeared not to be affected. In conclusion, the present results indicate that in L6 myotubes lack of iron induces an adaptive response to increase cellular glucose uptake through elevated expression of Glut 1, whereas overloading of iron inhibit glucose transport. The potential contribution of this response to physiological or pathological conditions remain to be further explored.

C 201 IRS-1 IN RAT BRAIN: CO-LOCALIZATION WITH INSULIN RECEPTORS AND PHOSPHOTYROSINE Denis G. Baskin, Michael W. Schwartz, Barry J. Goldstein, and Morris F. White, Veterans Affairs Medical Center and University of Washington, Seattle, WA 98195; Jefferson Medical College, Philadelphia, PA 19107; Joslin Diabetes Center and Harvard Medical School, Boston, MA 02215

The effects of insulin in the CNS include altered gene expression in discrete populations of neurons. Thus, identification of cells expressing molecules involved in post-receptor signaling events following insulin binding to neurons is of fundamental importance for characterizing the nature of insulin action in the brain. Therefore, we asked whether neuronal populations that have high concentrations of insulin receptors and phosphotyrosine in the olfactory bulb and hippocampus also express insulin receptor substrate-1 (IRS-1), a protein with numerous tyrosines that are phosphorylated after insulin stimulation of peripheral insulin-sensitive cells. In situ hybridization showed that IRS-1 mRNA is co-localized with insulin receptor mRNA in neuron cell bodies of the hippocampus and olfactory bulb. In contrast, IRS-1 immunoreactivity (shown by immunocytochemistry) was found in layers that contain (a) the synapses of these neurons, (b) high insulin binding (as shown by receptor autoradiography) and (c) intense phosphotyrosine immunostaining. The differential anatomical distribution of these mRNAs and proteins suggests that the genes encoding IRS-1 and insulin receptor are transcribed in neuronal perikarya and their cognate proteins are transported to the synaptic regions of the neurons. These results are the first evidence for the presence of IRS-1 mRNA and its co-localization with insulin receptor mRNA in brain neurons. The congruency in the location of brain phosphotyrosine, insulin binding, and IRS-1 suggests that phosphorylation of IRS-1 may play a role in the action of insulin on neurons, and suggests a signal transduction mechanism by which insulin may influence metabolism and gene expression in the brain.

C 202 INHIBITION OF PI-3 KINASE BLOCKS INSULIN-STIMULATED DNA SYNTHESIS, GLUCOSE UPTAKE AND ACTIVATION OF PP70-S6 KINASE

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Insulin stimulates the rapid Tyr-phosphorylation of its endogenous substrate, IRS-1, which in turn binds to SH2 domain containing proteins such as phosphatidylinositol 3-kinase (PI 3-kinase). PI 3-kinase is activated upon binding to IRS-1 leading to phosphorylation on the D-3 position of phosphatidylinositols, and the products are thought to function in regulating cell growth and metabolism. Recently a specific inhibitor of PI 3-kinase (LY294002) was developed and shown to specifically inhibit the activity of the enzyme (*in vivo* and *in vitro*) with an IC_{50}=1.4μM. In the present study we have characterized the effects of this inhibitor on insulin-stimulated growth and metabolic events. Analysis of *in vivo* labelled ^{32}P-phospholipids from 3T3-L1 adipocytes treated with LY294002 showed a specific inhibition of insulin-stimulated PI 3-kinase without affecting insulin receptor or IRS-1 phosphorylation or PI 4-kinase. In *in vitro* reactions, the IC_{50} (1.5 μM) for inhibiting PI 3-kinase associated with anti-IRS-1 immunoprecipitates was identical to that found for PI 3-kinase associated with anti-p85 immunoprecipitates. In CHO cells overexpressing the human insulin receptor, both insulin- and serum-stimulated DNA synthesis were inhibited by LY294002; this effect was reversible. Treatment with inhibitor dramatically reduced both basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes (IC_{50}=1-2 μM). In addition, inhibition of PI 3-kinase prevented insulin-stimulated pp70 S6-kinase activation (IC_{50}= 1-2μM). By contrast, treatment with LY294002 did not affect insulin-stimulated activation of MAPK or pp90^{rsk}. From the current data, it is clear that signals originating at the level of the insulin receptor are both divergent and convergent resulting in a complex signaling network. The findings with LY294002 provide an example of this networking, in that stimulation of DNA synthesis, pp70 S6-kinase, and glucose transport share a common signal(s) that requires PI 3-kinase activity, but separate from the pathways involving activation of MAPK and pp90^{rsk}.

C 203 INSULIN AND IGF-1 RECEPTORS ELICIT DIFFERENT SIGNAL TRANSDUCTION PATHWAYS, Cynthia Corley Mastick, Derek LeRoith*, and Alan R. Saltiel, Department of Signal Transduction, Parke-Davis Pharmaceutical Research, Ann Arbor, MI. 48105, and *Diabetes Branch, NIDDK, NIH, Bethesda, MD. 20892.

Signal transduction pathways were compared in NIH-3T3 cell lines expressing equal numbers (~1x10^6) of insulin (NIR), IGF-1 (WT21), or chimeric IGF-1/insulin receptors (CH1; IGF-1 receptor with the c-terminal tail of the insulin receptor). Lipogenesis is more sensitive and responsive to insulin (in NIR cells) than to IGF-1 (in WT21 cells). Increasing the number of IGF-1 receptors from 9000 (parental cells) to 1x10^6 (WT21 cells) did not change the IGF-1 dose response of lipogenesis. Unlike the IGF-1 receptor, expression of the chimeric receptor enhanced IGF-1 stimulation of lipogenesis. Overexpression of all of the receptors increased the responsiveness of thymidine incorporation to hormonal stimulation to the level observed with 10% serum. However, WT21 and CH1 cells are more sensitive to IGF-1 than NIR cells are to insulin. In contrast to the differences in the cellular effects of these hormones, both the insulin and IGF-1 receptors produced comparable stimulation of tyrosine phosphorylation, including phosphorylation of IRS-1. However, IGF-1 induced the phosphorylation of a unique 220 kD protein in WT21 cells, which was not observed in NIR cells. Although receptor autophosphorylation was comparable in WT21 and CH1 cells, the level of phosphorylation of all endogenous substrates was significantly reduced in CH1 cells. Interestingly, under conditions where there is a dose-dependent stimulation of MAP kinase activity in WT21 cells by IGF-1 (up to 4-fold at 100 nM), there is no activation of MAP kinase in the parental or CH1 cells, or in the NIR cells in response to insulin (from 0.1-100 nM). Therefore MAP kinase activation does not correlate with the ability of receptors to stimulate lipogenesis, or thymidine incorporation. These data indicate that the signal transduction pathways elicited by the insulin and IGF-1 receptors are distinct; insulin stimulates metabolic pathways, while both hormones stimulate mitogenic pathways.

C 204 A NOVEL FORM OF SUPPRESSION OF GLUCOSE TRANSPORT DEFECTS BY DNA REGULATORY ELEMENTS IN THE PROMOTERS OF HeXose Transporter GENES in *S. cerevisiae*. Noel M. Fong, George Theodoris, and Linda F. Bisson, Dept. of Viticulture and Enology, Univ. of California at Davis, Davis, CA 95616

Glucose transport in yeast *S. cerevisiae* is mediated by two kinetically distinct mechanisms of low and high affinity. Members of the HXT (HeXose Transporter) family (*HXT 1-4, SNF3*) are responsible for high affinity transport; a *snf3* null mutation results in a growth defect on low (0.05%) glucose media and loss of high affinity transport. Presence of multiple copies of the promoter region of *HXT4* was sufficient for suppression of the *snf3* growth defect. Deletion analysis localized this suppressing region to 330 bp within an ORF found in the *HXT4* promoter. The suppression was determined to be mediated by the DNA sequence itself, as abolishment of two potential ATGs within the ORF did not diminish the suppression. Our working model of multicopy suppression by this DDSE (DNA-Dependent Suppressor Element) is that DDSE titrates out transcriptional repressors of chromosomal genes which are then expressed to give glucose transport and/or growth on low glucose. The gene(s) turned on by DDSE responsible for restoration of growth on low glucose concentrations might not be directly involved in transport, but merely co-regulated with transporters. The suppressing effects of DDSE requires HXT2 and HXT3 genes, but multicopy DDSE only marginally increases high affinity transport in *snf3* strains. DDSE regions are also present in the promoters of *HXT1, HXT2,* and *HXT3,* but not *SNF3* or other glycolytic genes.

C 205 INDUCTION OF PDH KINASE MAY CONTRIBUTE TO HEPATIC INSULIN RESISTANCE INDUCED BY HIGH-FAT FEEDING, Lee G. Fryer, Karen A. Orfali, Mark J. Holness and Mary C. Sugden Department of Biochemistry Basic Medical Sciences, Queen Mary & Westfield College (London University), London E1 4NS, U.K.

The mitochondrial pyruvate dehydrogenase (PDH) complex of animal tissues is inactivated by phosphorylation by PDH kinase. PDH kinase activity in rat liver mitochondria is increased 2 to 3 fold by 24 to 48 h starvation *in vivo*. In primary hepatocytes, addition of n-octanoate in tissue culture leads to an increase in PDH kinase activity comparable to that induced by starvation *in vivo*, whereas insulin addition reverses the effects of starvation. PDH kinase is thus a direct target enzyme for the interactive effects of fatty acids and insulin. The administration of a high-fat diet has been demonstrated to reduce the suppressibility of hepatic glucose output by insulin *in vivo*. The present study examined the effects of high-fat feeding on the expression of hepatic PDH kinase. Rats were maintained for 4 weeks on either a high fat diet containing (by calories) 47% saturated fat, 20% protein and 33% carbohydrate or a control diet containing (by calories) 9% fat, 20% protein and 71% carbohydrate. Rats were sampled in the absorptive state. PDH kinase was assayed in extracts of hepatic mitochondria. The addition of ATP to mitochondrial extracts resulted in a time-dependent inactivation of the PDH complex. PDH inactivation was complete (<15% of initial value) within 10 min. The rate constant for inactivation of PDH in mitochondrial extracts from control rats was - 0.094 ± 0.011 min^{-1}. The rate of ATP inactivation of PDH complex in extracts of hepatic mitochondria from the fat-fed rats was markedly (2 fold) faster than the control value. Thus, PDH kinase activity is significantly enhanced to an extent comparable to that evoked by prolonged starvation. Previous studies have shown roles for increased PDH kinase activity in restricting PDH activity and pyruvate oxidation in settings of hepatic carbohydrate depletion. The present study demonstrates that a nutritional regime containing fat and carbohydrate in proportions encountered in the Western diet can evoke mechanisms which may lead to inappropriate sparing of carbohydrate by the liver. Since hepatic PDH inactivation is critical for the disposition of pyruvate towards gluconeogenesis, the induction of hepatic PDH kinase by fat-feeding may be an important prelude to the development of hepatic insulin resistance.

C 206 ACTIVATION OF PHOSPHATIDYLINOSITOL 3'-KINASE BY PHOSPHO AND PHOSPHONOPEPTIDES DERIVED FROM IRS-1 CONTAINING TWO YMXM SH2 DOMAIN BINDING MOTIFS, John J. Herbst*, Glenn Andrews*, Leonard Contillo*, Lou Lamphere†, Gustav E. Lienhard† and E.Michael Gibbs*, * Pfizer Central Research, Groton, CT 06340, †Department of Biochemistry, Dartmouth Medical School, Hanover N.H. 03755

The phosphotyrosine form of the major substrate for the insulin receptor tyrosine kinase, the insulin receptor substrate 1 (IRS-1), forms multimeric complexes with effector proteins. IRS-1 has been demonstrated to associate with virtually all the phosphatidylinositol 3'-kinase (PI3K) activity stimulated by insulin. IRS-1 contains nine tyrosine phosphorylation sites within the YMXM or YXXM motifs reported to be involved in activating PI3K through binding to the SH2 domains on the p85 regulatory subunit. We used sequences within IRS-1 as a model for synthesizing both phosphotyrosine and nonhydrolyzable phosphonotyrosine peptides containing these YMXM motifs.We then tested these peptides on their ability to compete with IRS-1 for binding to the SH2 domain of PI3K and for their ability to stimulate PI3K activity. Peptides containing a single phospho or phosphono PYMXM motif and peptides containing a double PYMXM motif connected by a linker [PYMXM-(linker)-PYMXM] were able to compete with IRS-1 for binding to the C-terminal SH2 domain of PI3K conjugated to GST beads at similar micromolar concentrations. Furthermore, at nanomolar concentrations double PYMXM motif peptides stimulated PI3K activity in cellular homogenates to a similar extent as treatment with 100nM insulin. However, those peptides containing only a single PYMXM motif were unable to stimulate PI3K activity even at concentrations as high a 100μM. We conclude from these results that the high affinity activation of PI3K requires the binding of at least two of the phosphorylated YMXM motifs on IRS-1. Additionally, the nonhydrolyzable phosphonopeptide derivatives should prove a useful tool in dissecting the role of PI3K in insulin action.

C 207 ACTIVATION OF MAP/ERK KINASE BY AN INSULIN SENSITIZING AGENT IN 3T3-L1 CELLS, Rolf F. Kletzien and Lisa A. Foellmi-Adams, Metabolic Diseases Research, Upjohn Laboratories, The Upjohn Company, Kalamazoo, MI 49001

Cellular signaling by insulin is initiated by stimulation of its receptor tyrosine kinase resulting in the activation of several cytosolic protein serine/threonine kinases. Recent results from several laboratories have shown that the MAP/ERK family of serine/threonine kinases play a central role in cellular signaling in response to insulin and other growth factors. We have employed the 3T3-L1 cell line as a model for studying hormone activation of the MAP kinases. 3T3-L1 preadipocytes exhibit a requirement for insulin-like growth factor-1 (IGF) for differentiation into adipocytes, which then respond metabolically to insulin. Previous work from our laboratory has established that the insulin sensitizing agent, pioglitazone, enhances the IGF regulated differentiation of 3T3-L1 cells. We evaluated the influence of pioglitazone on MAP kinase both during IGF-regulated differentiation and in the response of mature adipocytes to insulin. When added to preadipocytes, pioglitazone was found to increase basal MAP kinase activity within 30 min to a level equivalent to that observed in IGF treated cells. Longer times (6 hr) of treatment resulted in a decrease in IGF stimulated activity which may reflect a drug-related inhibition or a redistribution of the kinase to the nucleus. Incubation of adipocytes with pioglitazone resulted in activation of MAP kinase within 10 min to a level equivalent to that observed following insulin stimulation of cells. The drug did not further enhance the insulin-regulated activation of MAP kinase nor did it influence the tyrosine phosphorylation of the β-subunit of the insulin receptor. Comparison of the action of okadaic acid versus pioglitazone on MAP kinase activation suggests that the drug is not influencing this family of kinases through an inhibition of protein phosphatases. These results demonstrate that pioglitazone is capable of rapidly activating one of the central protein kinase cascades of insulin action by a post-insulin receptor mechanism.

C 208 INSULIN RESISTANCE MEDIATED BY A PROTEOLYTIC FRAGMENT OF THE INSULIN RECEPTOR, Victoria P. Knutson, Patricia V. Donnelly, Yvonne Balba and Maria Lopez-Reyes, Department of Pharmacology, University of Texas Medical School, Houston, TX 77225

Insulin resistance is a common clinical feature of obesity and non-insulin dependent diabetes mellitus, and is characterized by elevated serum levels of glucose, insulin and lipids. The mechanism by which insulin resistance is acquired is unknown. We have previously demonstrated that upon chronic treatment of fibroblasts with insulin, conditions which mimic the hyper-insulinemia associated with insulin resistance, the membrane-associated insulin receptor ß subunit is proteolytically cleaved, resulting in the generation of a cytosolic fragment of the ß subunit, ß', and that the generation of ß' is inhibited by the thiol protease inhibitor E64 (Knutson, V.P. (1991) *J. Biol. Chem.* 266:15656-15662). In this report, we demonstrate that in 3T3-L1 adipocytes, 1) cytosolic ß' is generated by chronic insulin administration to the cells, and that E64 inhibits the production of ß'; 2) chronic administration of insulin to the adipocytes leads to an insulin resistant state, as measured by lipogenesis, and E64 totally prevents the generation of this insulin-induced cellular insulin resistance; 3) E64 has no effect on the insulin-induced down regulation of insulin receptor substrate-1 (IRS-1), and therefore, insulin resistance is not mediated by the down regulation of IRS-1; 4) under *in vitro* conditions, partially purified ß' stoichiometrically inhibits the insulin-induced auto-phosphorylation of the insulin receptor ß subunit; and 5) administration of E64 to obese Zucker fatty rats improves the insulin resistance of the rats compared to saline-treated animals. These data indicate that ß' is a mediator of insulin resistance, and the mechanism of action of ß' is the inhibition of the insulin-induced autophosphorylation of the ß subunit of the insulin receptor.

C 209 INVOLVEMENT OF GLUCOSAMINE SYNTHETASE (GFAT) IN EGF AND GLUCOSE MODULATED GROWTH FACTOR TRANSCRIPTION, Jeffrey E.Kudlow, Inn-Oc Han, Mark D. Roos and Andrew J. Paterson, University of Alabama at Birmingham, Birmingham, AL 35294

Glucose and EGF stimulate the transcription of the transforming growth factor-α (TGFα) gene. The effect of glucose has been previously shown to require its metabolism to glucosamine through a rate limiting step catalyzed by glutamine:fructose-6-phosphate amidotransferase (GFAT). Mapping of the glucose and EGF response elements in the TGFα gene has shown that both responses can be transduced through the same proximal element in the TGFα gene. We tested the hypothesis that the EGF effect might also require the induction of GFAT. Indeed, EGF stimulated the accumulation of GFAT mRNA but this accumulation occurred 18 hours following EGF stimulation whereas TGFα mRNA accumulation occurred 6 hours after EGF stimulation. Furthermore, an inhibitor of GFAT activity completely blocked the stimulation by glucose of TGFα transcription in MDA468 cells while it only partially attenuated the response to EGF. The effects of maximal doses of EGF and glucose on TGFα transcription were additive, also suggesting different pathways of action of these agents. We have postulated that some of the glucosamine produced by GFAT is involved in *O*-glycosylation of transcription factors or other proteins which, in turn, regulate the transcription of genes typified by the TGFα gene. We examined the effects of glucose, glucosamine and EGF on the pattern of proteins modified by *O*-linked N-acetyl-glucosamine (*O*-GlcNAc). We found that glucose and glucosamine had a marked effect on the content of *O*-GlcNAc in two proteins of about 60 and 68 kDa, whereas EGF did not. The 68 kDa glycoprotein appears to associate with the transcription factor, Sp1, in a sugar-dependent manner. Since Sp1 binds to several sites in the glucose/EGF responsive element of the TGFα promoter, we propose that the 68 kDa glycoprotein may interact with Sp1 to mediate the sugar responsiveness of the TGFα gene. The mechanism for EGF appears to be distinct from that of glucose. These findings suggest a novel mechanism by which glucose modulates gene transcription, perhaps leading to glucotoxicity.

C 210 CLONING AND CHARACTERIZATION OF A NOVEL PTPASE RELATED TO THE DUAL SPECIFICITY PHOSPHATASE VH-1, Seung P. Kwak, Jack E. Dixon, Department of Biological Chemistry, The University of Michigan, Ann Arbor, MI 48109-0606

Phosphorylation of ERK by MEK (MAP-KK) appears to be a common step in the signaling pathway mediated by receptors for insulin, EGF, and other growth factors. *In vitro* studies indicate that mammalian dual specificity PTPases related to the vaccinia H-1 gene product (VH-1) can deactivate ERK that is previously phosphorylated by MEK, suggesting that VH-1-like PTPases are involved in modulating signal transduction of surface receptor kinases at this common step. We isolated a novel member of this PTPase subclass, hVH-3 (human Vaccinia H-1 related clone 3), from human placenta. The hVH-3 gene localizes to the human chromosome at locus 5q25. The mRNA is 2.4Kb in length and is expressed primarily in the placenta and liver. The mRNA encodes a 79Kd protein that possesses a catalytic domain at the carboxyl terminus and an apparent second domain at the amino terminus that is similar to the non-catalytic region of cdc25. Bacterially expressed PTPase hVH-3 exhibits activity towards a phosphotyrosine analog PNPP. A closely related PTPase, CL100, is translocated into the nucleus when expressed in Hela cells, although the localization of hVH-3 is currently under investigation. These findings suggest that this family of PTPases can catalyze substrates such as ERK that are phosphorylated on both Tyr and Ser/Thr residues and reside both in the nucleus and the cytoplasm.

C 211 PHORBOL ESTER STIMULATES PHOSPHORYLATION ON SERINE 1327 OF THE HUMAN INSULIN RECEPTOR

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Phorbol esters stimulate the phosphorylation of the insulin receptor on discrete serine and threonine residues in intact cells. Phosphorylation of the insulin receptor cytoplasmic domain on serine, threonine and tyrosine residues regulates receptor tyrosine kinase activity and signaling. In these studies we demonstrate that phorbol ester treatment of intact COS-1 cells transiently expressing the human insulin receptor stimulates phosphorylation of serine 1327 within the C-terminal tail of the insulin receptor ß subunit. Phosphopeptide maps of wild-type (Ser^{1327}) and mutant (Ala^{1327}) human insulin receptors revealed the absence of a single phosphopeptide in the Ala^{1327} receptors when compared to wild-type receptors from phorbol ester-treated cells. Phosphoamino acid analysis revealed phosphoserine within the phosphopeptide from wild-type receptors that is absent in the Ala^{1327} receptor. The synthetic peptide 1327S (KRSYEEHIPYTHMNGGKK) corresponding to amino acids 1325-1342 of the human insulin receptor is phosphorylated on serine by protein kinase C. After digestion with trypsin, the phosphorylated synthetic peptide co-migrated with the serine-phosphorylated peptide isolated from wild-type insulin receptors that was absent from the Ala^{1327} mutant. Ser^{1327} is proximal to autophosphorylation sites Tyr^{1328} and Tyr^{1334}. The potential effects of serine phosphorylation at position 1327 on subsequent phosphorylation of these tyrosines by the insulin receptor kinase was examined using synthetic peptides. The chemically modified peptide 1327S(P) was synthesized with the stoichiometric addition of phosphate to the side-chain hydroxyl of a serine corresponding to position 1327 of the insulin receptor. Kinetic analysis revealed that the addition of phosphate to the serine improved substrate recognition by the insulin receptor almost two-fold. The average K_m was 1.44 mM for the peptide 1327S(P) vs. 2.64 mM for peptide 1327S. However, in comparison to its unphosphorylated control, the serine phosphorylated peptide 1327S(P) also reduced the V_{max} of the insulin receptor tyrosine kinase 53%. We conclude that the juxtaposition of a serine phosphorylation site adjacent to receptor tyrosine phosphorylation sites provides the potential for regulation of insulin receptor signaling through its carboxyl terminal tail.

C 212 INSULIN RECEPTOR INDUCED RAS-GTP FORMATION: EVIDENCE FOR IRS1 DEPENDENT AND INDEPENDENT SIGNALING, J. Antonie Maassen, D. Margriet Ouwens, Gerard C.M. Vander Zon, Gijsbertus J. Pronk[1], Johannes L. Bos[1], Department of Medical Biochemistry, University of Leiden, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands, [1]Laboratory for Physiological Chemistry, University of Utrecht.

Evidence is emerging that insulin receptors mediate Ras•GTP formation at least in part by Tyr-phosphorylation of IRS-1 and subsequent association to GRB2/SOS, whereas activated EGF-receptors interact directly with GRB2/SOS or indirectly via Shc. In this study, we investigated the contribution of the major Tyr-autophosphorylation sites, Y1158, Y1162 and Y1163, of the insulin receptor to the Tyr- phosphorylation of IRS-1 and Shc, and the formation of Ras•GTP. For that, we constructed CHO cell lines, overexpressing mutant insulin receptors in which the major Tyr-autophosphorylation sites were stepwise replaced by phenylalanines. (Some cell lines and expression vectors kindly provided by C.R Kahn and R.A. Roth respectively). It was found that insulin (5 min., 100nM) stimulates phosphorylation of IRS-1 and Shc on Tyr, and formation of IRS-1/GRB2 and Shc/GRB2 protein complexes, together with an increase in Ras•GTP, in cell lines expressing wild type or mutant F1158Y1162,Y1163 receptors. Cell lines expressing mutant Y1158F1162,F1163 showed insulin-induced Tyr-phosphorylation of Shc, Shc/GRB2 complex formation and Ras•GTP formation, in absence of Tyr-phosphorylation of IRS-1 and formation of IRS-1/GRB2 complexes. The mutant insulin receptor F1158F1162Y1163 and F1158F1162F1163 were inactive in inducing any of these responses. We conclude that phosphorylation of Y1158 and Y1162 of the insulin receptor is linked to distinct post-receptor processes, and that conversion of Ras•GDP to Ras•GTP can occur in absence of Tyr-phosphorylation of IRS-1, suggesting an involvement of Shc/GRB2 complex formation in insulin-induced Ras•GTP formation.

C 213 ANALYSIS OF THE CO-LOCALISATION OF GLUT4 AND TGN38 WITHIN 3T3-L1 ADIPOCYTES, Sally Martin, *Barbara Reaves,* George Banting and Gwyn W. Gould, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, U.K. *Department of Biochemistry, University of Bristol, University Walk, Bristol BS8 1TD, U.K.

The exposure of isolated adipocytes to insulin results in a ~20-fold increase in the rate of glucose transport into the cells. This increase is mediated by the movement of a pool of intracellular vesicles containing the insulin-responsive glucose transporter, GLUT4, to the cell surface. In the resting state, the majority of GLUT4 is sequestered inside the adipocyte in an as yet unidentified intracellular compartment. TGN38 is an integral membrane protein which is predominantly localised to the *trans* Golgi network. We investigated whether GLUT4 and TGN38 are co-localised in the murine 3T3-L1 adipocyte cell line. Immunoadsorption of intracellular vesicles containing GLUT4 using an antibody specific for this isoform of the glucose transporter did not deplete the low density microsomal fraction of TGN38 in these cells. Moreover, no TGN38 was detected in the GLUT4-containing vesicles by immunoblotting with a TGN38 specific antiserum. Immunoadsorption of TGN38-containing vesicles revealed a low but detectable amount of GLUT4 did co-localise with TGN38. The amount of GLUT4 in the TGN38 vesicles did not change in response to insulin. Imunofluorescence analysis of TGN38 and GLUT4 revealed markedly different staining patterns. Reversal of insulin-stimulated glucose transport and subsequent analysis of the vesicles demonstrated that during the recycling of GLUT4 to the intracellular storage site there was no increase in the amount of GLUT4 co-localising with TGN38. These results suggest that the *trans* Golgi network is not the major site of GLUT4 localisation in 3T3-L1 adipocytes and that the recycling of GLUT4 does not involve the *trans* Golgi network.

C 214 DETECTION OF A 60kD TYROSINE PHOSPHORYLATED PROTEIN IN HEPATOMA CELLS.

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Activation of the tyrosine kinase activity of the insulin receptor by autophosphorylation leads to phosphorylation of cellular substrates on tyrosine. Thus far, the best characterized is the insulin receptor substrate (IRS)-1, which has been proposed to serve as a docking protein or binding site for other molecules involved in insulin receptor signal transduction. A number of other proteins that become phosphorylated in response to insulin have been identified, some of these are reported to be tissue-specific. For example, a 60kD phosphoprotein has been detected in adipocytes following insulin stimulation (Lavan, B.E. and G.E. Leinhard, 1993, J. Biol. Chem. 268,5921-5928). We have identified a protein of similar molecular weight in rat hepatoma cells transfected with the human insulin receptor (HTC-IR) which is tyrosine phosphorylated in response to insulin in a dose-dependent manner, with maximal phosphorylation occurring at 50nM insulin. While the dose-response of pp60 phosphorylation mirrors that of the receptor, the timecourse is slightly slower. Maximal phosphorylation of the insulin receptor and IRS-1 can be detected by one minute. Maximal pp60 phosphorylation, however, is not detected until five minutes post-stimulation. Like the adipocyte protein, the 60kD hepatoma protein binds to the N-terminal SH2 domain of the p85 regulatory subunit of PI3 kinase. Studies are underway to further charaterize the chemical and biological properties of this phosphoprotein.

C 215 SEQUENCE OF A NEW PROTEIN WHICH IMMUNOCROSS-REACTS WITH INSULIN RECEPTOR SUBSTRATE-1 HAS A SIMILARITY TO NUCLEAR MITOTIC APPARATUS PROTEIN NuMA, Masaki Nishiyama and Shin-ichi Hayashi, Department of Nutrition, The Jikei University School of Medicine, Tokyo 105, Japan

We studied the molecular structure and physiological function of insulin receptor substrate-1 (IRS-1) (1-3). In the course of the molecular cloning of the IRS-1 associated protein(s) we found a new protein, designated HH-109 which immunologically cross-reacts to anti human IRS-1 monoclonal antibody. The partial cDNA sequence has 1762 nucleotides (n.t.) with a putative polyadenylation signal and a poly A tail in the 681 n.t. of 3' non-coding region. The expression of an expected size of a protein by the estimating the length of the open reading frame (331amino acid) and the immunocross-reactivity to anti human IRS-1 monoclonal antibody by Westernblotting analysis were confirmed. The homology searched by Gene Bank was 52.9% and 58.2 % in the nucleotide sequence for the nuclear mitotic apparatus protein (NuMA) (4) and a rabbit myosin heavy chain , respectively. Interestingly the homologous region, 1-600 n.t./ 1-200 a.a., was restricted to the primary amino acid sequence creating α-helical coiled-coil secondary stucture. Indeed the heptads repeat was observed in the central part and disrupted by the glycine run. And that HH109 and NuMA contain a very basic region in C-terminus. Other similarity to NuMA which has been identified as a phosphorylated protein is that a number of protein kinase recognition sequence motifs are present. Interestingly in the basic region YDIM sequence was found in HH-109 but not in NuMA. YXXM motif has been reported as a preferable substrate for an insulin receptor tyrosine kinase . We speculate that the primary structure of HH109 is fundamentally homologous to NuMA, containing the α-helical domain in the central part and the basic domain in the C-terminus region. It is conceivable that the isolated protein ,HH109, participates in a signal tranduction as some cytoskeletal proteins such as vinculin, foldrin, α-actinin and microtubule-associated protein 2. The physiological function and biochemical properties of HH109 are under investigation.

In this meeting the characterization including the epitope mapping for anti human IRS-1 monoclonal antibodies will be also presented.

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C 216 INTRACELLULAR SIGNALING MOLECULES IN GROWTH FACTOR ACTION, Jerrold M. Olefsky, UCSD, Dept. of Med., La Jolla, CA 92093

Although IRS-1 is clearly a substrate of the insulin and IGF-I receptor, its role as a signaling molecule remains undefined. We utilized a single cell microinjection technique in which inactivating IRS-1 antibodies were introduced into quiescent rat 1 fibroblasts which overexpress human insulin receptors. Our results show that microinjection of anti-IRS-1 antibodies disrupts the signaling properties of IRS-1 such that insulin and IGF-1 are no longer capable of stimulating cells to undergo DNA synthesis. Anti-IRS-1 microinjection had no effect on EGF, FGF, PDGF, or FCS stimulation of DNA synthesis. The effect of IRS-1 to mediate DNA synthesis, may involve grb-2/SOS and ultimate stimulation of p21ras. Shc is another intracellular signaling molecule which can become tyrosine phos-phorylated upon ligand stimulation and bind to grb-2/SOS. Since Shc proteins do not bind to IRS-1, this represents a potential IRS-1 independent pathway for stimulation of ras and mi-togenesis. Microinjection of anti-Shc antibodies into HIRc cells inhibits insulin, IGF-I and EGF stimulated mitogenesis by ~80%. We also find that immunoprecipitation of cell ly-sates with anti-Shc antibody removes ~80% of guanine nucleotide exchange activity. Consistent with this, immunoblotting experiments demonstrated that the majority of intracellular grb2 from insulin or IGF-I stimulated cells associates with Shc, and only a small proportion with IRS-1. Based on these findings, we suggest that: (1) IRS-1 is an intracellular signaling molecule for insulin and IGF-I, but not for the other growth factors and (2) For the insulin and IGF-I signaling cascades, it would appear that the major signaling pathway leading to p21ras stimulation is mediated through Shc and not IRS-1.

C 217 PHOSPHORYLATION AND ACTIVATION OF THE IGF-I RECEPTOR IN *SRC* TRANSFORMED CELLS, **John E. Peterson‡, Michael Kaleko§, Kenneth Siddle[a], and Michael J. Weber‡,** *From the ‡Department of Microbiology, University of Virginia, Health Sciences Center, Charlottesville, VA 22908, §Genetic Therapy, INC., Gaithersburg, Maryland 20878, and the [a]Department of Clinical Biochemistry. University of Cambridge, Cambridge CB2 2QR, United Kingdom*

Our laboratory has previously identified the Insulin-Like Growth Factor receptor (IGF-I receptor) as a protein whose tyrosine phosphorylation correlates with transformation by *src*. To examine the effect of *src* expression on the activity of the IGF-I receptor, the human IGF-I receptor was expressed in fibroblasts co-expressing the temperature sensitive v-*src* mutant, tsLA29. The IGF-I receptor exhibited an elevated level of tyrosine phosphorylation in *src* transformed cells even in the absence of IGF-I treatment. *Src*-induced phosphorylation of the receptor was correlated with an increase in the *in vitro* tyrosine kinase activity of the receptor, both towards itself and exogenous substrates. The *src* induced increase in receptor activity was shown to be dependent on tyrosine phosphorylation, as treatment with a tyrosine-specific phosphatase lowered receptor activity. The tyrosine phosphorylation of IGF-I receptor mutants defective for kinase activity was also examined.

Since $pp60^{v\text{-}src}$ can directly phosphorylate insulin-family receptors and since phosphorylation of the IGF-I receptor correlates with phenotypic transformation in cells transformed by various *src* mutants, we believe our findings suggest a novel pathway by which $pp60^{v\text{-}src}$ (and presumably other *src*-family members) can alter cellular regulation, namely by intracellular, ligand-independent activation of growth-factor receptors.

C 218 RAD: A NOVEL MEMBER OF THE RAS/GTPASE FAMILY OVER-EXPRESSED IN MUSCLE OF TYPE II DIABETIC HUMANS, Christine Reynet and C. Ronald Kahn, Joslin Diabetes Center, Harvard Medical School, Boston, MA 02215

In an effort to identify gene(s) associated with insulin resistance of Type II diabetes, we prepared two subtraction libraries from skeletal muscle of normal and Type II diabetic humans and screened them using subtracted probes. Only one clone out of 4000 clones screened was selectively over-expressed in Type II diabetic muscle as compared to muscle of non-diabetic or Type I diabetic individuals. The sequence of the initial 0.4 kb insert did not match any known sequence in GenBank, and thus we used this clone to isolate several other longer clones from the original diabetic cDNA library, and to obtain the 1.4 kb full-length cDNA. Analysis of the open reading frame of this novel clone revealed 45 to 55 % homology at the nucleotide level with members of the *ras*/GTPase superfamily. We have termed this novel *ras*-related cDNA associated with diabetes *rad*. The deduced amino acid sequence of *rad* contains 269 amino acids with a predicted Mr of 29 KD and presents all five GTPase domains conserved among the *ras*/GTPase-related proteins, but also has some unique structural features. First, the sequence of the putative domain responsible for GTPase-activating protein (GAP) binding is quite divergent from that observed for the p21-*ras* family members. Secondly, there is a glutamic acid in the G3 domain in a position (equivalent to position 60 in N-*ras*) which in other *ras* family members is occupied by a highly conserved glycine. Finally, the C-terminus does not contain the typical CAAX or CCXX prenylation site present in virtually all *ras* family members, although it contains a polybasic domain. We have confirmed that *rad* codes for a GTP-binding protein by testing the GTP binding activity of a glutathione-S-transferase (GST)-*rad* fusion protein. *Rad* is expressed primarily in skeletal and cardiac muscle, and is increased by an average of 8.6-fold at the mRNA level in the muscle of Type II diabetics. Since *ras*-like proteins have suggested to play a role in insulin action, over-expression of *rad* in Type II diabetes could provide an important clue as to the mechanism of insulin resistance and/or genetics of this disease.

C 219 CLONING AND CHARACTERIZATION OF A NOVEL GDP-DISSOCIATION INHIBITOR ISOFORM FROM SKELETAL MUSCLE, Assia C. Shisheva*, Andrew D. Cherniack*, Thomas Sudhof+ and Michael P. Czech*, *Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, MA 01605, +Department of Molecular Genetics, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75235-9050

Insulin-regulated membrane trafficking of glucose transporters appears to involve small GTP-binding proteins such as the Rab proteins. Rab function is regulated by GDP dissociation inhibitors (GDI) that release Rab proteins from membranes and inhibit GDP dissociation. Here we report the isolation of a full length cDNA encoding a novel GDI isoform of 445 amino acids (GDI-2) with deduced molecular weight of 50649, present in insulin-sensitive mouse skeletal muscle. Full length and partial cDNA clones encoding a previously reported GDI protein (GDI-1) were also isolated from cDNA libraries prepared from rat brain and mouse skeletal muscle, respectively. The homologous forms of GDI-1 in mouse muscle and rat brain were almost identical at the protein level (99% similarities). Mouse GDI-2 shows high sequence similarity to mouse and rat GDI-1: 72% at the nucleotide and 86% at the amino acid levels. Northern blot analysis revealed that in human tissues both GDI-1 and GDI-2 transcripts were abundant in brain, skeletal muscle and pancreas, but were weakly expressed in heart and liver. GDI-1 mRNA was expressed in kidney whereas GDI-2 was almost absent, while in lung the relative amounts of these mRNA species were reversed. Both mRNA species were also detectable in cultured 3T3-L1 fibroblasts and L6 myoblasts. Differentiation of these cell lines into highly insulin-responsive adipocytes and myocytes was accompanied by large increases in GDI-1 (7- to 10-fold) and GDI-2 mRNA (3- to 5-fold). These data raise the possibility that GDI-1 and/or GDI-2 may be involved in insulin-regulated membrane trafficking of glucose transporters.

C 220 IDENTIFICATION OF NOVEL GENES POTENTIALLY ASSOCIATED WITH THE INSULIN RESPONSE, Jacqueline M. Stephens and Paul F. Pilch, Department of Biochemistry, Boston University Medical Center, Boston, MA 02118

Chronic treatment of 3T3-L1 adipocytes with tumor necrosis factor (TNF) results in the inhibition of gene expression of the insulin sensitive glucose transporter, GLUT4 (Stephens and Pekala, 1991 and 1992). TNF treatment effects various levels of GLUT4 gene expression including transcription, mRNA accumulation and stability. Chronic TNF treatment results in a total depletion of GLUT4 protein and a state of insulin resistance in the 3T3-L1 adipocytes.

The 3T3-L1 adipocytes (-/+ TNF treatment) are a simple system for comparing insulin sensitive vs. insulin resistant cells. Therefore, this cell culture system is being utilized to identify mRNA's differentially expressed in these particular conditions by using a recently developed technique that relies on the use of a set of oligonucleotide primers and PCR (Liang and Pardee, 1992).

To date, 58 differentially expressed clones have been identified from differential display gels containing insulin sensitive and insulin resistant amplified DNA. Most of these clones have been successfully purified, amplified, and random primed for northern analysis. The majority of these clones correspond to very low copy mRNA's and have been difficult to detect. Northern blots containing polyA+ are now being utilized to detect these clones. However, four differential clones have been detected and are currently being characterized. One of the clones has been identified as GLUT4 and serves as a good internal control for the differential screening . Another clone (#6) hybridizes to a 1.4 kb mRNA and is upregulated about 8-fold in the insulin resistant cells. Sequencing and data bank comparisons of this clone (217 bp which corresponds to the 3'UTR of the mRNA) indicate that it is potentially novel. We are currently screening the cDNA library prepared from the insulin resistant 3T3-L1 adipocytes to obtain the full length clone. Clone 3, is significantly down-regulated in the insulin resistant cells and corresponds to a very low copy 1.0 kb mRNA. Sequence analysis of this clone (120 bp) does not correspond to any other sequences in the databases. We are also examining the expression of these clones in control and diabetic animals.

These experiments provide a strong basis for identifying genes which are potentially involved in the regulation of GLUT4 and/or other aspects of insulin action.

C 221 ROLE OF p85 SUBUNIT OF PI-3-KINASE AS AN ADAPTOR MOLECULE IN INSULIN RECEPTOR SIGNALING. Sung, C.K., Sanchez-Margalet, V, Truitt, K.* and Imboden J.*. Division of Diabetes and Endocrine Research, Mt. Zion Medical Center, University of California, San Francisco; San Francisco, CA 94120; Immunology and Arthritis section *, VA Medical Center, UCSF; San Francisco. CA 94121

Recent studies indicate that after the insulin receptor (IR) is activated, it phosphorylates the docking protein, insulin receptor substrate-1 (IRS-1). IRS-1 then induces the formation of a non-covalent IR signaling complex with proteins containing SH2/3 domains including the p85 regulatory subunit of phosphatidylinositol-3-kinase (PIK). Employing rat HTC hepatoma cells overexpressing human IR and an antiserum to the p85 subunit of PIK (α-p85), we have observed three major tyrosine phosphorylated proteins in α-p85 immunoprecipitates after insulin stimulation: IR; IRS-1; and a new 62 kD protein (p62). To directly study the association of p62 with the subunits of PIK (p110 or p85), we first immunoprecipitated insulin-treated cell lysates with antibodies to the p110 subunit of PIK (α-p110) and subsequently with α-p85. These immunoprecipitates were then analyzed by western blotting with α-PY. In response to insulin, p62 exists complexed to either PIK holoenzyme or p85 alone. However, much more p62 is associated with p85 alone than with the holoenzyme. In order to determine whether p62 directly associates with the p85 subunit of PIK, we performed an in vitro protein association experiment employing maltose binding protein (MBP) fusion proteins containing SH2 domains of the p85. We observed that in addition to IRS-1 (whose direct interaction with the SH2 domains of p85 has already been demonstrated by others), p62 is also directly associated with the SH2 domains of p85. These data suggest that: 1) PIK may serve as an adaptor molecule interacting with and activating other intracellular proteins via their SH2/3 domains.; and 2) p62 may be involved in insulin receptor signaling via PIK.

C 222 *Abstract Withdrawn*

C 223 ACTIVATION AND INACTIVATION OF THE INSULIN RECEPTOR TYROSINE KINASE IN DIFFERENT ENDOSOME SUB-POPULATIONS IN 3T3-L1 ADIPOCYTES, Bo Wang, Patricia V. Donnelly and Victoria P. Knutson, Department of Pharmacology, The University of Texas Medical School, Houston, TX 77225.

Activation of the insulin receptor tyrosine kinase has been shown to be the key element for insulin signal transduction. Insulin-induced insulin receptor endocytosis is involved in insulin degradation, insulin receptor recycling and degradation. However, little is known about the relationship between activation of the insulin receptor tyrosine kinase and insulin receptor endocytosis. In addition, the role of endosomal trafficking in receptor activation and inactivation is not well defined.

Our laboratory has developed a novel technique which uses preparative isoelectric focusing to separate the insulin-induced endosomes into four discrete sub-populations: constitutive (insulin-independent), early, middle and recycling endosomes. After 45 min of treatment with 1 μM insulin, the tyrosine kinase activity of the insulin receptors from each endosome sub-population was quantitated using RCAM-lysozyme as a substrate. We found that insulin receptors from the early as well as the constitutive endosomes contained greater tyrosine kinase activities with dramatically higher affinity for the substrate than the insulin receptor localized at the plasma membrane of the stimulated cells. This suggests that the early phase of insulin receptor endocytosis is related to activation of the insulin receptor tyrosine kinase. On the other hand, insulin receptors from the middle and recycling endosomes had no measurable tyrosine kinase activity, indicating that inactivation of the tyrosine kinase takes place in the middle and late phase of insulin receptor endocytosis. We also found that IGF-1 did not stimulate RCAM-lysozyme phosphorylation, indicating that substrate phosphorylation in the tyrosine kinase assay was mediated by the insulin receptor tyrosine kinase, but not by the IGF-1 receptor tyrosine kinase. These data demonstrate that insulin receptor endocytosis plays an important role in transducing and turning off insulin's biological signal.

Diabetes Mellitus and Transgenic Models

C 300 TRANSGENIC MICE OVEREXPRESSING P-ENOLPYRUVATE CARBOXYKINASE IN THE LIVER DEVELOP NON-INSULIN-DEPENDENT DIABETES MELLITUS, Fatima Bosch, Anna Pujol, Mireia Pelegrin, Carles Hug Ros and Alfons Valera, Department of Biochemistry and Molecular Biology. Autonomous University of Barcelona. 08193-Bellaterra. Spain.

The increase in hepatic gluconeogenesis is believed to be an important factor responsible for the fasting hyperglycemia detected in patients with non-insulin-dependent diabetes mellitus (NIDDM) (type II). P-enolpyruvate carboxykinase (PEPCK) is the main regulatory enzyme of gluconeogenesis. To investigate the role of the expression of PEPCK gene in the development of NIDDM, we have produced lines of transgenic mice carrying a PEPCK minigene regulated by its own promoter. A 7-fold increase in the concentration of PEPCK mRNA was detected in the liver of these animals. The overexpression of the PEPCK gene was related with an increase in glucose production from pyruvate in hepatocytes in primary culture. Transgenic mice had higher serum insulin concentration and lower liver glycogen content than control mice. When intraperitoneal glucose tolerance tests were performed, blood glucose levels were higher than those detected in normal mice. This animal model shows that primary alterations in the rate of liver glucose production are able to induce insulin resistance and NIDDM. Supported by grants from FISss and CICYT.

C 301 LINKAGE ANALYSIS OF GLUT4, FABP2, AND CHROMOSOME 19 MARKERS IN FAMILIAL NIDDM, Steven C. Elbein, Michael Hoffman, and Mark Leppert, Division of Endocrinology, University of Utah, Salt Lake City, UT 84132

Defects of either insulin secretion or insulin action may predispose to familial NIDDM. We examined 3 regions which are strong candidates for defects in insulin action: the insulin responsive glucose transporter region (GLUT4, chromosome 17p;4 markers), the FABP2 locus which has been implicated in insulin resistance in Pima Indians (chromosome 4q), and 7 highly polymorphic microsatellite markers (heterozygosity > 0.7;) spaced at approximately 20 cM intervals on chromosome 19q and 19p bands 12 - 13.2. The latter markers span a region which includes several previously unexamined candidate loci, including ApoC1, ApoC2, ApoE, and glycogen synthase. Each locus was examined in 400 individuals from 16 families selected for ≥ 2 NIDDM siblings. Analysis was performed with the MLINK program (LINKAGE 5.1) under dominant, recessive, and intermediate models assuming a 50% phenocopy rate and an age dependent penetrance factor. Linkage was rejected (LOD < -2) for GLUT4 under all models, and for FABP2 under dominant and recessive models. Although many regions of chromosome 19 could not be excluded (LOD < -2) under all models, the maximum LOD score was positive for only 2 markers. D19S221 (19p13.1) had a LOD score of 1.02 at 5 cM under the intermediate model, and ApoC2 had a maximum LOD of 1.67 at 10 cM under the dominant model. Our data do not suggest a role for GLUT4, FABP2, or most regions of chromosome 19 in NIDDM in this population, but the region near ApoC2 includes many strong candidates genes and merits further evaluation. Typing of additional markers and multipoint analyses of this region are in progress.

C 302 ENHANCED GLYCEMIC CONTROL IN NORMAL AND *db/db* TRANSGENIC MICE OVEREXPRESSING HUMAN GLUT4, E. Michael Gibbs, Jeffrey E. Pessin†, A. J. Milici, Scott C. McCoid, Jeffrey L. Stock, R.W. Stevenson, Judith L. Treadway, and John D. McNeish, Central Research Division, Pfizer Inc., Groton, CT 06340 and †Department of Physiology & Biophysics, University of Iowa, Iowa City, IA 52242

We examined the physiological role of the GLUT4/muscle-fat specific facilitative glucose transporter in regulating glucose homeostasis in transgenic mice expressing high levels of this protein in an appropriate tissue-specific manner. GLUT4 protein overexpression in two independent founder lines on a hybrid background resulted in a substantial reduction of fasting glucose levels (~70 mg/dl) compared to wild type mice (~130 mg/dl). Furthermore, the GLUT4 transgenic mice had a markedly enhanced glucose disposal following an oral glucose challenge showing only a slight elevation in plasma glucose levels (~90 mg/dl) whereas wild type mice displayed a typical 2- to 3-fold increase (~250-300 mg/dl). Parallel to the changes in plasma glucose, insulin levels were approximately 2-fold lower in the transgenic mice compared to the wild type mice. Furthermore, transgenic mice had increased circulating levels of triglycerides, free fatty acids, and ß-hydroxybutyrate indicative of increased lipolysis due to insulinopenia. To test the effects of overexpressing GLUT4 in a diabetic condition we produced GLUT4 transgenic mice on the genetically diabetic inbred strain, C57BL/6 *db/db*. Initial data demonstrate that the transgenic *db/db* mice have lower fasting plasma glucose (~50 mg/dl *vs.* 200 mg/dl) and a markedly enhanced ability to dispose of an oral glucose load compared to nontransgenic *db/db* mice, which corresponded with an increased cell surface GLUT4 content as assessed by *in situ* immunolocalization. Taken together these data demonstrate that increased expression of the human GLUT4 gene *in vivo* results in more efficient metabolic control over fluctuations in plasma glucose concentrations in both normal and genetically-diabetic mice.

C 303 REGULATED EXPRESSION OF HUMAN INSULIN IN THE LIVER OF TRANSGENIC MICE CORRECTS DIABETIC ALTERATIONS, Xavier Gregori, Alfons Valera, Cristina Fillat, Cristina Costa, Jordi Sabater, Joana Visa, Anna Pujol, Tura Ferre and Fatima Bosch, Department of Biochemistry and Molecular Biology. Autonomous University of Barcelona. 08193-Bellaterra. Spain.

Insulin-dependent diabetes mellitus (IDDM) (Type I) results from the autoimmune destruction of the ß cells in the islets of Langerhans in the pancreas, and subsequent metabolic alterations. Although insulin therapy allows most patients to lead active lives, this replacement is imperfect. The high incidence of the IDDM make this illness a good candidate for gene therapy. Diabetes gene therapy would be possible if insulin could be produced by cells other than the pancreatic ß cells. The selection of a suitable promoter to drive the insulin gene is critical in order to achieve a physiologically regulated expression of the hormone. The P-enolpyruvate carboxykinase (PEPCK) gene promoter is highly sensitive to glucagon and insulin, the main regulatory hormones of glucose homeostasis. Glucagon, which increases during diabetes, activates, while insulin inhibits, PEPCK gene expression. Transgenic mice expressing the PEPCK/human insulin chimeric gene have been obtained as a model to study the feasibility of gene therapy for diabetes. These transgenic animals were healthy and normoglycemic and expressed human insulin in a physiologically regulated manner, mainly in the liver. Streptozotocin-treated transgenic mice had high levels of human insulin immunoreactivity in the serum and showed a significant decrease (up to 40%) of glycemia compared to streptozotocin-treated control mice. Expression of endogenous genes and metabolic parameters involved in glucose metabolism were normal in the liver, even when transgenic animals were treated with diabetogenic doses of streptozotocin. These results constitute an indication"in vivo" that diabetes gene therapy is possible, by producing insulin in extrapancreatic tissues.

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C 304 ISLET REGENERATION IN INSULIN-INTERFERON-GAMMA TRANSGENIC MICE. Danling Gu and Nora Sarvetnick, Department of Neuropharmacology, The Scripps Research Institute, La Jolla, CA 92037.

We have previously described lymphocyte-related ß cell destruction in transgenic mice carrying the interferon-gamma (IFN-g) gene linked to the insulin promoter. The lost ß cells are continuously replenished by the regeneration of islet cells differentiated from rapidly dividing duct cells. Within the duct walls, numerous single endocrine cells and bud-like islet structures were revealed by the specific antibody to each of four islet hormones. The regenerating process in adult transgenic mice is similar to that observed in embryonic islet neogenesis. The duct cells express tyrosine hydroxylase (TH) and glutamic acid decarboxylase (GAD), the cell types which are believed to be the forerunner of endocrine cells. The newly differentiated islet cells exhibit characteristics of embryonic endocrine cells. These cells are mitotically active as revealed by the incorporation of BrdU by the immunoreactive hormone-containing cells. The dividing ductal endocrine cells migrated to form islet structures. Taken together, these results suggest that the regeneration of islet cells in IFN-g transgenic mice closely parallels embryonic islet neogenesis and provides a model system for studying factors influencing islet cell differentiation.

C 305 INTERLEUKIN 1 (IL-1) RECEPTORS ARE PRESENT AND FUNCTIONAL IN THE NONOBESE DIABETIC (NOD) MOUSE AT THE PREDIABETIC STAGE, Françoise Homo-Delarche, Abdelaziz Amrani, *Mehrnaz Jafarian Tehrani, Jean-Marie Pléau, ** Pierre Mormède, Mireille Dardenne and *France Haour. CNRS URA 1461, Hôpital Necker and * Pharmacologie Neuro-Immuno-Endocrinienne, Institut Pasteur 75015 Paris, France.** INSERM U259 - Université de Bordeaux II, 33077 Bordeaux Cedex France.

Type 1 diabetes, in particular in the NOD mouse model, has been suggested to be linked to defective IL-1 receptor gene on chromosome 1. However, long-term treatment of NOD mice with IL-1 can affect the development of diabetes. On the basis on these data, we investigated in the NOD mouse strain as compared to control strains, the presence of IL-1 receptors and the effect of the well-known hypoglycemic agent, IL-1, on glycemia. As assessed by autoradiography, IL-1 receptors are expressed in prediabetic, 2-month-old NOD mice in various organs and in particular in the pancreas. These IL-1 receptors are localized to the islets of Langerhans, in both NOD and control mice. The expression of these receptors disappears in diabetic NODs. The effect of mrIL-1α (250 and 500 ng/20 g b.w.) was assessed on NOD mouse glycemia 2 hours after injection, a time at which a maximal effect (around 50% decrease) is observed in C57BL/6 and DBA/2 mice. 2-month-old nondiabetic mice of either sex are insensitive to the two doses of mrIL-1α 2 hrs after injection. Kinetics studies, however, show some effect of mrIL-1α on NOD mouse glycemia, but the effect is delayed, limited and transitory (not exceeding 25% decrease 4 hrs after injection). In contrast, mrIL-1α strongly stimulates corticosterone production in both sexes of NODs. PCR analysis, using specific primers to the 5' region of the IL-1 receptor DNA gene leads to amplification of this region in C57BL/6, but not in DBA/2 or NOD mice. The difference observed in amplication of the IL-1 receptor among the three strains suggests that the IL-1 receptor gene in NOD and DBA/2 strains presents some differences in structure as compared to the C57BL/6 strain (polymorphism or presence of intron). Taken together, these results demonstrate that IL-1 receptors exist and are functional in the NOD mouse. The incomplete response to the hypoglycemic effect of IL-1 may be related, at least in part, to the anti-insulin effect of glucocorticoids.

C 306 THE MITOCHONDRIAL MUTATION AS A CAUSE OF DIABETES IN JAPAN - DEFINITION OF A SUBTYPE OF DIABETES, T. Kadowaki, H. Kadowaki, H. Sakura, S. Otabe, Y. Mori, K. Tobe, R. Hagura, Y. Yazaki, and Y. Akanuma, Third Department of International Medicine, University of Tokyo and Institute for Diabetes Care and Research, Asahi Life Foundation, Tokyo, JAPAN

A genetic defect in mitochondrial (mt) function is a plausible candidate to cause diabetes, since oxidative phosphorylation in the mitochondria has been suggested to play a role in insulin secretion from the pancreatic ß cells. We screened 55 IDDM subjects with a family history (Group I), 100 NIDDM subjects with a family history (Group II), 250 subjects with diabetes randomly taken from a diabetes outpatient clinic (Group III), 7 families having an association of diabetes and deafness (Group IV) for the mitochondrial mutation, an A to G transition at position 3243 of leucine tRNA (3243 bp mutation). We also studied the prevalence of diabetes in 39 subjects with MELAS and their 127 non-MELAS family members (Group V). We identified 26 families (55 subjects) with diabetes associated with the 3243 bp mutation. Three families were from Group I (5.5%), two families from Group II (2.0%), two families from Group III (0.8%), 5 families from Group IV (70%), and 14 families from Group V (36%). In these families, diabetes and the 3243 bp mutation cosegregated in a fashion consistent with material transmission. Sensory hearing disturbance was the symptom most frequently (≅ 60%) associated with diabetes. Diabetes due to the 3243 bp mutation was generally associated with the impaired endogenous insulin secretary capacity of their pancreatic ß cells.

In conclusion, the 3243 bp mutation in the mitochondrial gene represents a significant cause of both IDDM and NIDDM in Japan.

C 307 *Abstract Withdrawn*

C 308 **AUTOIMMUNITY TO VIRAL ANTIGEN BY β-CELL EXPRESSION OF INTERFERON-γ IN VIRAL ANTIGEN TRANSGENIC MICE,** Myung-Shik Lee, Matthias von Herrath, Michael B.A. Oldstone, and Nora Sarvetnick, Department of Neuropharmacology, The Scripps Research Institute, La Jolla, CA 92037

Transgenic mice expressing interferon-γ in pancreatic ß-cells (Ins-IFN-γ mice) develop insulitis, insulin-dependent diabetes mellitus and sensitization to islet tissue. However, the sensitization to specific islet antigens was not demonstrated partly because of lack of defined islet antigens. Transgenic mice expressing a viral antigen in pancreatic β-cells (RIP-LCMV mice) would allow us to study not only specific immunity to a defined antigen but also the role of viruses in the pathogenesis of diabetes. We crossed Ins-IFN-γ mice with RIP-LCMV-NP or -GP mice, and studied the development of diabetes and viral antigen-specific cytotoxicity. All 13 of 13 NP/IFN-γ double transgenic mice became diabetic before 4 month of age, while none of 6 NP/(-) littermates became diabetic. Two of 5 GP/IFN-γ double transgenic mice became diabetic, while none of 5 GP/(-) littermates became diabetic. Lymphocytes from NP/IFN-γ mice showed 20-40% killing of LCMV-infected target cells. They also killed 17-31% of vaccinia-NP-infected targets. Lymphocytes from GP/IFN-γ mice demonstrated 12-22% killing of the LCMV-infected targets. Lymphocytes from GP/(-) or NP/(-) mice did not show significant killing. These results suggest that lymphocytes potentially reactive to self (or integrated viral) antigens are not completely deleted. Instead, they were activated by alteration of the local cytokine environment in the pancreas, which could lead to the development of autoimmune diabetes.

C 309 A 4KB SEGMENT OF DNA SPANNING THE HUMAN INSULIN GENE ENCODES SUSCEPTIBILITY TO INSULIN DEPENDENT DIABETES MELLITUS. A.M. Lucassen[1], C. Julier[2], M. Lathrop[2], J.I. Bell[1]. [1] Molecular Immunology group, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, U.K. [2] Centre d'Etude du Polymorphisme Humain, 27, rue Juliette Dodu, Paris 75010, France.

Several different genes are thought to be important in susceptibility to insulin dependent diabetes mellitus (IDDM) in humans. We have been studying a genomic region on chromosome 11p15.5 spanning the genes for insulin, tyrosine hydroxylase and insulin like growth factor II. This region is associated with IDDM and linked to IDDM in families. To identify the exact mutation(s) encoding susceptibility, a systematic analysis of this region was made by sequence comparisons of eight different haplotypes in diabetics and controls. Multiple polymorphisms were identified throughout a region spanning 20 kb and their association with IDDM studied in 156 random IDDM patients and 96 matched controls. The boundaries of the region of susceptibility could thus be defined as a 4 kb stretch of DNA spanning the insulin gene. This region contains ten candidate disease mutations each significantly associated with IDDM. However, none of these are located in coding or known regulatory sequence of genes, so that the mechanism of susceptibility remains unclear. One of these polymorphisms is a repetitive region known as the 5'INS VNTR. Recently, such repetitive sequences have been implicated in the pathogenesis of several genetic disorders and it may be that the length of the VNTR has an effect on insulin regulation. Two of the polymorphisms lie in the 3' untranslated region of the insulin gene and it is possible that these affect insulin message stability. Only two main haplotypes formed by these polymorphisms are observed in this region and represent the exact complement of each other arguing in favour of strong evolutionary selection pressures. It may be that a combination of polymorphisms rather than individual sites alone is responsible for susceptibility. We have made constructs of the two major haplotypes and are currently investigating their role in insulin regulation through functional assays. We are also studying the distribution of these haplotypes in other ethnic populations.

C 310 IMMUNOLOCALIZATION OF THE HUMAN GLUT4 TRANSPORTER IN MUSCLE AND FAT FROM TRANSGENIC MICE. A.J. Milici, H.A. Stukenbrok, S. C. McCoid, J.L. Treadway, J.E. Pessin* and E. M. Gibbs, Central Research Division, Pfizer Inc., Groton, CT 06340 and * The University of Iowa, Iowa City, IA 52242.

We have generated human GLUT4 transgenic mice which express high levels of this insulin sensitive glucose transporter in fat and striated muscle. Previously, we have demonstrated the functional responsiveness of the human GLUT4 transporter in these mice. To ascertain the cytoplasmic compartments containing the human GLUT4 transporter, we have localized it at the light and electron microscopic level in perfusion fixed animals. Following fixation, small portions of fat, skeletal and cardiac muscle were removed and processed for frozen sectioning. Both thick (~0.5μm) and thin (<0.1μm) sections were labeled with a specific anti-GLUT4 antibody and binding detected with either a fluorescent or gold tagged secondary antibody. At the immunofluorescent level, in the absence of insulin, GLUT4 appears to be distributed in the cytoplasm in discrete punctate bodies. The perinuclear cytoplasm contained the greatest concentration of these fluorescent bodies. A small amount of staining of the plasmalemma was present in the absence of insulin in all tissues in transgenic mice, but not in wild type mice. Thirty minutes after the i.p. administration of insulin (8U/kg) and glucose (1 g/kg), there was a general loss of this punctate staining pattern and an intense plasmalemmal fluorescence was observed. We confirmed these findings at the EM level, in the absence of insulin, where GLUT4 was observed in the tubulo-vesicular compartment in all tissues. In addition, in cardiac and skeletal muscle, GLUT4 appeared to be localized in components of the sarcoplasmic reticulum. Upon treatment with insulin, there was migration of this transporter from the intracellular vesicular compartment to the plasmalemma in all tissues which correlates with a functional increase in glucose transporter metabolism. The GLUT4 localization patterns in the transgenic mice are essentially identical to patterns in wild type mice, moreover, the increased signal in the transgenic mice has facilitated both functional and morphological measurements of GLUT4.

C 311 *Abstract Withdrawn*

C 312 IFN-ALPHA AS THE LINK BETWEEN THE ENVIRONMENT AND THE IMMUNE SYSTEM IN TYPE I DIABETES. T.A. Stewart. X Huang, B Hultgren, L Martin, S Pitts-Meek, J. Hully and J. MacLachlin. Genentech Inc., South San Francisco, CA 94080 USA

Type I diabetes is caused by an auto immune mediated destruction of the insulin producing pancreatic beta cells. Transgenic mice in which the pancreatic beta cells express interferon gamma (IFN-g) develop an autoimmune diabetes. However NOD mice that are genetically deficient in IFN-g still become diabetic and the islets of human diabetics appear to have histologically detectable changes prior to the invasion of the lymphocytes capable to making IFN-g. In contrast, the islets of recent onset diabetics make interferon alpha (IFN-a) and transgenic mice that express IFN-a in the pancreatic beta cells develop a beta cell specific autoimmune diabetes that can be prevented with a neutralizing monoclonal antibody directed against IFN-a. Further, multiple low doses of streptozotocin (MLDS) induce islet expression of interferon alpha, and both MLDS and recombinant interferon alpha will induce islet expression of IL-6, TNF alpha and GMCSF. Both recombinant interferon alpha and poly I/C will exacerbate MLDS induced diabetes. The islets of the BB (DP) (but not the BB (DR)) rats express IFN-a prior to detectable inflammation and prior to expression of Il-6, TNF alpha, IL-1 beta, and interferon gamma. Poly I/C will induce the expression of interferon alpha in the islets of the BB (DR) rats and will lead to diabetes in these animals. These results have lead to the hypothesis that islet expression of IFN-a is causally involved in the development of type 1 diabetes in humans.

C 313 TARGETED DISRUPTION OF THE INSULIN RECEPTOR SUBSTRATE-1 (IRS-1) GENE,

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The IRS-1 is a cytoplasmic protein that is highly phosphorylated on its tyrosine residues within 10 seconds after insulin or IGF-I stimulation of the sensitive tissues. Therefore, IRS-1 is believed to be a major substrate of these receptor tyrosine kinases. There are at least 14 possible tyrosine phosphorylation sites on IRS-1, and 9 of which contain YMXM or YXXM motifs which are binding sites of the src homology 2 (SH2) domain of the phosphatidyl inositol kinase 85kDa subunit. It is highly probable that IRS-1 binds to other SH2 domain-containing proteins, and make large signaling complex. Indeed, we have proved that IRS-1 physically associates with GRB2/ASH, which contains one SH2 domain and two SH3 domains. Lately, GRB2 is shown to bind Sos, a GDP dissociation stimulator for Ras. On the other hand, Shc, an another SH2 domain-containing protein is shown to be phosphorylated on tyrosine by insulin stimulation and also bind GRB2. Shc does not bind IRS-1 and therefore there exist two independent pathways leading to Ras activation in the signaling cascade of insulin action. To clarify the physiological role of IRS-1, we have made mice carrying a mutation on IRS-1 gene locus by homologous recombination in embryonal stem cells. Two homologous recombinant clones have successfully colonized into germ cells. The heterozygous mice were apparently normal, and the phenotypes of homozygous mice will be presented.

C 314 METABOLIC PROFILE AND PERIPHERAL GLUCOSE UTILIZATION IN HUMAN GLUT4 TRANSGENIC MICE

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We have shown (Liu, et al. *PNAS*, in press) that mice expressing the hGLUT4 transgene (TG) in muscle and adipose have decreased fasting plasma glucose and a reduced glucose and insulin response during an OGTT relative to non-transgenic (NTG) controls. We have now examined the metabolic/hormonal profiles of TG and NTG in fed and fasted states, and have begun to assess peripheral glucose utilization in skeletal muscle. Glucose turnover by tracer kinetic technique demonstrated that *in vivo* glucose clearance was 1.5-fold greater in TG relative to NTG (13±2 vs. 8.6±0.3 mg/min/kg; $p<0.05$), but hepatic glucose production (Ra) was unchanged (9±1 mg/min/kg). Fed plasma glucose was lower in TG than NTG (124±6 vs. 157±12 mg%; $p<0.05$), whereas triglycerides, free fatty acids, and ß-hydroxybutyrate were elevated by 32-78% ($p<0.05$). Fasting resulted in further elevation in plasma lipid parameters in TG. Glucagon levels were elevated by 54% ($p<0.001$) in fed TG relative to NTG. To assess peripheral glucose uptake and metabolism, net glycogen content was determined in skeletal and cardiac muscles. Glycogen content was increased 3-fold ($p<0.05$) in diaphram and quadriceps of fed TG relative to NTG, while heart glycogen was even more markedly elevated (27.2±3.4 vs. 5.4±1.1 µmol/g, $p<0.001$). We also observed a 2.6-fold increase in plasma lactate concentration between TG and NTG (13.1±1 vs. 5.0±1.3 mM; $p<0.001$), suggesting increased glycolysis in TG skeletal muscle as well. Liver glycogen did not differ between fed TG and NTG mice and both groups displayed a similar reduction in hepatic glycogen content after an overnight fast. Fasting did not affect glycogen levels in TG heart, but surprisingly, skeletal muscle glycogen content was nearly completely depleted (diaphram, 0.83±0.26 µmol/g; $p<0.01$) or markedly reduced (quadriceps, 4±0.3 µmol/g; $p<0.001$) in fasted TG relative to the fed state. These data suggest that TG undergo accelerated glycogen depletion relative to NTG to maintain substrate availability and prevent hypoglycemia during a fast. We are now measuring glucose transport and metabolism in isolated soleus muscles from TG and NTG *in vitro*, and determining peripheral glucose disposal *in vivo* utilizing an insulin/glucose clamp. In summary, enhanced glycemic control in hGLUT4 transgenic mice appears to be accounted for by increased peripheral glucose clearance and increased glucose uptake and glycogenesis in skeletal and cardiac muscles.

C 315 ß-CELL DEFECTS IN TRANSGENIC MICE EXPRESSING GLUT-2 ANTISENSE RNA INDUCE NON-INSULIN-DEPENDENT DIABETES MELLITUS

Alfons Valera, Gemma Solanes, Josefa Fernández-Alvarez*, Anna Pujol, Jorge Ferrer*, Guillermina Asins, Joana Visa, Ramon Gomis* and Fatima Bosch, Department of Biochemistry and Mol. Biology, Autonomous University of Barcelona and *Department Endocrinology, Hospital Clinic, Barcelona, Spain

An increase in blood glucose is the main signal for insulin secretion by pancreatic ß cells. Glucose must be metabolized to trigger insulin release. Two proteins, the glucose transporter, GLUT-2, and the glucose phosphorylating enzyme, glucokinase, have been implicated in the control of glucose metabolism in ß cells. However, controversy has arisen as to which of these two candidates is the rate-limiting step for the ß-cell glucose-sensing apparatus. To investigate the role of glucose transporter GLUT-2 in the regulation of insulin secretion and in the development of non-insulin dependent diabetes mellitus (NIDDM) (type II), we have obtained transgenic mice expressing high levels of GLUT-2 antisense RNA in ß cells. Western blot and immunohistochemistry analyses showed an 80% reduction in GLUT-2 protein in the ß cells of these animals. Isolated islets from transgenic mice showed impaired glucose-stimulated insulin secretion. In addition, much higher levels of blood glucose were detected in transgenic mice than in controls when intraperitoneal glucose tolerance tests were performed. These results suggest that the reduction of GLUT-2 in the pancreas could be a crucial step in the development of NIDDM.

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C 316 TRANSGENIC MICE OVERPRODUCING HUMAN ISLET AMYLOID POLYPEPTIDE HAVE INCREASED INSULIN STORAGE AND SECRETION

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Islet amyloid polypeptide (IAPP) has been reported to acutely impair both the secretion and peripheral actions of insulin *in vitro* . To determine the effects of chronic elevations of IAPP *in vivo* on islet β-cell function, we measured pancreatic immunoreactive insulin (IRI) content and IRI secretion in transgenic mice expressing a rat insulin promoter-human proIAPP cDNA construct. Transgenic mice expressed human proIAPP mRNA in their pancreas and human IAPP (hIAPP)-immunoreactivity was localized to their islets by immunostaining. Total (mouse plus human) immunoreactive IAPP (IR-IAPP) was 2.5 fold higher in pancreatic extracts of transgenic animals compared to their non-transgenic littermates (6.6±1.3 vs 2.9±0.3 fmol/µg total protein; n=12 pairs; $p<0.05$). Surprisingly, IRI levels in the pancreatic extracts of the transgenic mice were increased, not reduced, compared to controls (415±59 vs 246±29 fmol/µg total protein; n=12 pairs; $p<0.05$). To determine whether insulin secretion was increased in transgenic animals, the IRI and IR-IAPP responses to glucose were assessed using the isolated, perfused pancreas. At basal glucose (4.4 mM), the secretion rate of both IRI (1152±330 vs 416±93 fmol/min; n=5 pairs; $p<0.05$) and IR-IAPP (9.3±1.7 vs 4.8±0.8 fmol/min; n=5 pairs; $p<0.05$) was significantly elevated in transgenic mice. Similarly, when β-cell secretion was stimulated with 16.7 mM glucose, the IRI response (area under curve) was more than 2-fold greater in the transgenic group (613±84 vs 235±64 pmol; n=5 pairs; $p<0.05$). The IR-IAPP response to 16.7 mM glucose was also significantly enhanced in transgenic mice (1234±83 vs 767±215 fmol; n=5 pairs; $p<0.05$). We conclude that pancreatic insulin content and secretion is increased in transgenic mice which overproduce and secrete hIAPP. These changes may arise from a direct effect of hIAPP on the β-cell or as an adaptation to hIAPP-induced insulin resistance.