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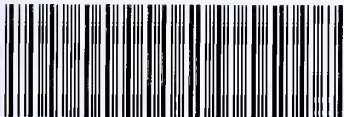
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STUDIES ON THE MOLECULAR MECHANICS OF
INSULIN RESISTANCE IN
PANCREATOGENIC DIABETES

by
Ajoy V. Mishra

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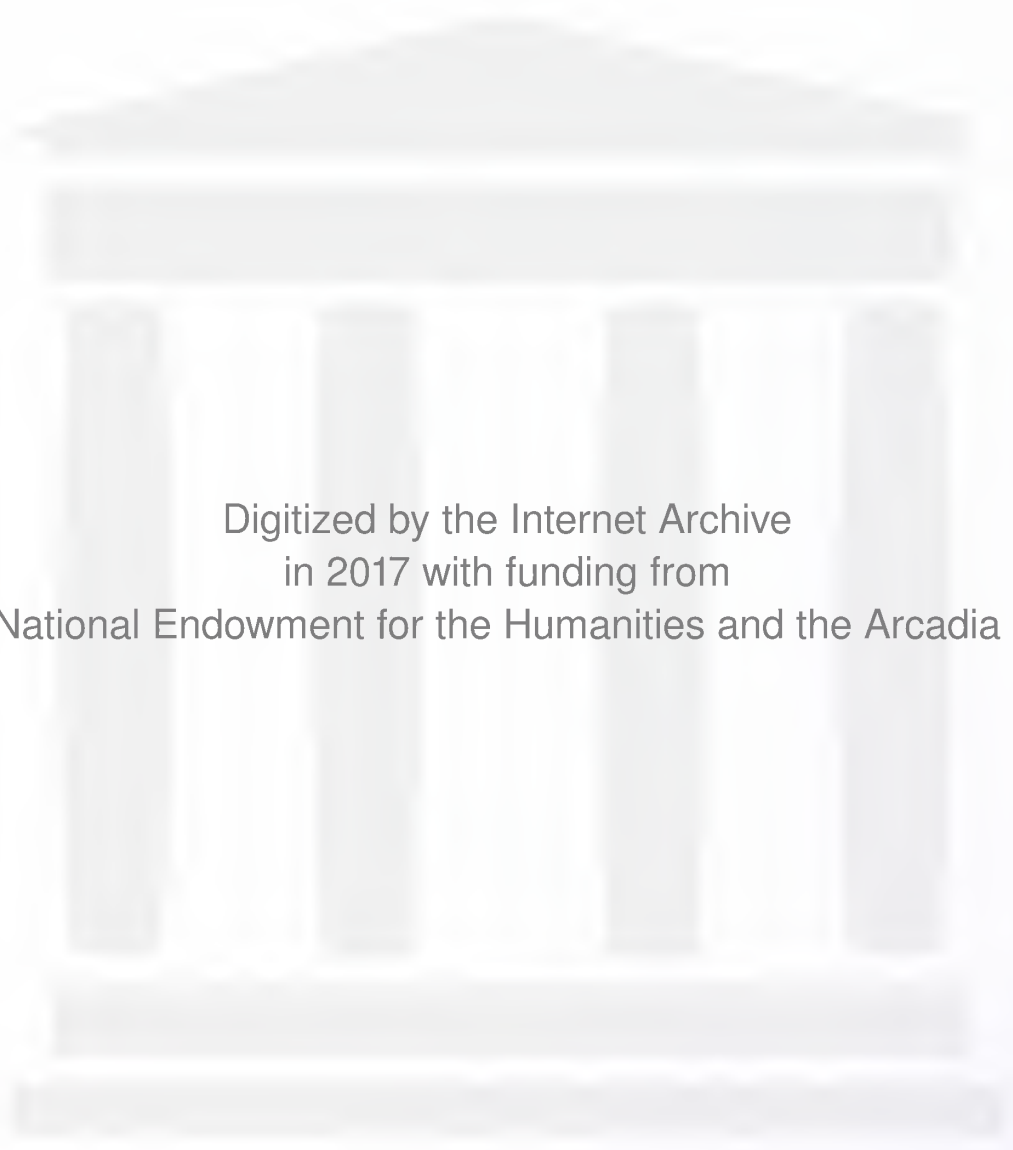
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An anatomical drawing of a human torso, showing the muscles and internal organs. The drawing is detailed and appears to be a study or a reproduction of a historical anatomical work. The title is centered over the upper chest area.

**STUDIES ON THE MOLECULAR MECHANICS OF INSULIN RESISTANCE IN
PANCREATOGENIC DIABETES**

**A THESIS SUBMITTED TO THE
YALE UNIVERSITY SCHOOL OF MEDICINE
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF MEDICINE**

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STUDIES ON THE MOLECULAR MECHANICS OF INSULIN RESISTANCE IN PANCREATOGENIC DIABETES. Ajay V. Maker, Michael Eisenberg, Kumudesh C. Sritharan, John P. Geibel, Bhanu P. Jena, and Dana K. Andersen. Departments of Surgery and Physiology, Yale University School of Medicine, New Haven, CT.

Chronic Pancreatitis (CP) and surgical alteration of the pancreas results in altered glucose metabolism (pancreatogenic diabetes). ~70% of patients with CP develop oral glucose intolerance and ~20% require insulin therapy. Previous experiments have shown altered expression and availability of hepatic insulin receptor (IR) and glucose transporter 2 (GLUT2) in CP, however, the cellular mechanism of resistance has not been established. To assess whether changes in IR and GLUT2 phosphorylation mediate gluco-regulatory dysfunction, rats with CP (n=12), induced by 99% oleic acid injection into the pancreatic ducts, and sham-operated rats (n=12) were studied. Animals were fasted 24 hours, with half of each group fed intraduodenally prior to liver harvest. IR and GLUT2 were isolated from the liver homogenates by immunoprecipitation with anti-IR or anti-GLUT2 antibodies and probed for phosphorylated tyrosine (P-Tyr) or phosphoserine (P-Ser) residues respectively. Total tissue phosphotyrosine phosphatase (PTPase) activity was measured by the paranitrophenyl phosphate assay. To determine if IR and GLUT2 organized spatially in a receptor/transporter complex, the proteins were analyzed with immunofluorescence confocal microscopy and co-immunoprecipitation. IR-P-Tyr increased 24-fold after feeding in sham animals, but increased only 13-fold in CP. Similarly, GLUT2-P-Ser increased ~5-fold in sham tissues, but demonstrated minimal phosphorylation in CP animals. This corresponded to ~12-fold less IR-P-Tyr ($p < 0.05$) and almost 5-fold less IR-P-Ser ($p < 0.05$) in pancreatic animals vs. sham animals in response to feeding. Total PTPase activities were not different. Confocal microscopy demonstrated colocalization of IR and GLUT2 on the hepatocyte membrane and the proteins coimmunoprecipitate significantly over a ubiquitous hepatocyte control protein ($p < 0.05$). These data confirm that hepatic IR and GLUT2 are normally phosphorylated *in vivo* on tyr and ser residues, respectively, upon feeding. This phosphorylation is impaired in livers from rats with CP, and the absence of altered PTPase activity suggests that impaired IR-Tyr kinase activity may contribute to the inappropriate hepatic glucose production seen in pancreatogenic diabetes secondary to CP. This parallel defect in phosphorylation implies a spatial and chemical relationship, and coimmunofluorescence and coimmunoprecipitation confirm their colocalization on the hepatocyte membrane, providing evidence for a receptor/transporter complex.

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TABLE OF CONTENTS

INTRODUCTION	1
CHRONIC PANCREATITIS	2
<i>Classification and Pathology</i>	2
<i>Prevalence and Causes.....</i>	3
<i>Pathogenesis.....</i>	4
PANCREATOGENIC DIABETES	6
<i>Hepatic Insulin Resistance in Pancreatogenic Diabetes.....</i>	8
INSULIN ACTION	9
<i>The Insulin Receptor.....</i>	10
<i>Functional domains of the insulin receptor.....</i>	11
<i>Insulin resistance.....</i>	11
<i>Proximal insulin receptor signaling events</i>	13
<i>Downstream signaling events.....</i>	14
<i>The role of protein tyrosine phosphatases.....</i>	15
GLUCOSE TRANSPORT PROTEIN 2 (GLUT2).....	16
SUMMARY, STATEMENT OF PURPOSE, AND HYPOTHESIS	19
SUMMARY	19
STATEMENT OF PURPOSE AND HYPOTHESIS.....	20
METHODS.....	22
PHOSPHORYLATION STUDIES.....	22
<i>Animals.....</i>	22
<i>Liver Harvest</i>	22
<i>Immunoprecipitation</i>	24
<i>Electrophoresis and Immunoblotting</i>	25
<i>Phosphatase activity.....</i>	26
CO-LOCALIZATION STUDIES	27
<i>Liver harvest and tissue preparation for confocal studies</i>	27
<i>Immunostaining</i>	27
<i>Hepatocyte Tissue Culture.....</i>	28
<i>Co-Immunoprecipitation</i>	29
<i>Electrophoresis and Immunoblotting</i>	30
RESULTS.....	32
PHOSPHORYLATION STUDIES	32
COLOCALIZATION STUDIES	34
<i>Hepatic Histology.....</i>	34
<i>Immunolocalization of GLUT2 with goat anti-GLUT2 and anti-goat FITC</i>	35
<i>Immunolocalization of IR with rabbit anti-IR and anti-rabbit Texas Red.....</i>	35

<i>Colocalization of GLUT2 and IR and Fluorescent Resonance Energy Transfer (FRET) in the hepatocyte</i>	36
<i>Confocal Microscopy Controls</i>	38
<i>Co-immunoprecipitation</i>	38
DISCUSSION	40
REFERENCES	47
APPENDIX	56
PUBLICATIONS	56
INVITED TALKS.....	56
RESEARCH AWARDS	57

LIST OF FIGURES

FIGURE 1: PHOTOMICROGRAPHS OF THE HUMAN PANCREAS.....	3
FIGURE 2: ABDOMINAL CT SCAN IN A PATIENT WITH CP.....	5
FIGURE 3: ERCP IN A PATIENT WITH CP.....	5
FIGURE 4: ABDOMINAL X-RAY IN A PATIENT WITH CP.....	6
TABLE 1: COMPARISON OF TYPES OF DIABETES MELLITUS.....	7
FIGURE 5: HEPATIC GLUCOSE PRODUCTION.....	9
FIGURE 6: THE INSULIN RECEPTOR.....	12
FIGURE 7: A SCHEMATIC OF THE IR PHOSPHORYLATION CASCADE.....	16
FIGURE 8: GLUTs RESPONSE TO INSULIN.....	18
FIGURE 9: PATHOLOGICAL AND HISTOLOGICAL VERIFICATION OF CHRONIC PANCREATITIS IN THE RAT MODEL.....	23
FIGURE 10: IMMUNOPRECIPITATION OF THE INSULIN RECEPTOR.....	26
FIGURE 11: FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET).....	28
FIGURE 12: IR TYROSINE PHOSPHORYLATION.....	33
FIGURE 13: GLUT2 SERINE PHOSPHORYLATION.....	33
FIGURE 14: FEEDING INDUCED PHOSPHORYLATION.....	34
FIGURE 15: PHOSPHOTYROSINE PHOSPHATASE (PTPASE) ASSAY.....	35
FIGURE 16: DOUBLE-LABELED IMMUNOFLUORESCENCE COLOCALIZATION OF GLUT2 AND IR.....	37
FIGURE 17: COIMMUNOPRECIPITATION OF INSULIN RECEPTOR AND GLUCOSE TRANSPORTER 2.....	39

INTRODUCTION

In 1788, Thomas Cawley observed glycosuria in a “free living young man” who died of emaciation and diabetes (1, 2). The post mortem exam revealed multiple pancreatic calculi and atrophy of the pancreas. These initial observations were confirmed by other physicians, and the critical role of the pancreas in diabetes was definitively confirmed in 1889 when Minkowski and Von Mering demonstrated that pancreatic extirpation in dogs led to acute diabetes (3). In 1909 the name “insulin” was proposed for a hypothetical substance secreted by the islets named by Langerhans, and the hormone was isolated and characterized in 1922 by Banting and Best, who administered their crude preparation to pancreatectomized dogs (4). It has been two centuries since Cawley’s early description of diabetes secondary to chronic pancreatitis, and thousands of reports on the disease have been published in the literature, however, glucose intolerance in “...chronic pancreatitis remains an enigmatic process of uncertain pathogenesis, unpredictable clinical course, and unclear treatment.” (5)

Chronic Pancreatitis

Classification and Pathology

Chronic pancreatitis (CP) is a progressive and irreversible inflammatory disease resulting in permanent damage to the exocrine and endocrine function of the pancreas (6). It is distinguished from acute pancreatitis by structural and functional parameters. In acute pancreatitis, the gland reverts to normal despite an attack, whereas, in CP, the gland is abnormal before or after the attack, or both (7).

Morphologically, CP is characterized by acute inflammation, edema, and necrosis superimposed upon chronic fibrosis, inflammation, and loss of exocrine tissue (see figure 1). The ductal system may be dilated and obstructed with calcified protein plugs (see figures 2 and 3) (5, 7). Severe atrophy of the acini and fibrous replacement of the islets is often seen in the advanced CP patient. Beta cell mass is usually reduced, and it has been shown that a 20% reduction of beta cell mass is seen in CP. In severe forms of CP, a marked loss of endocrine as well as exocrine tissue can occur, and usually results in a deficiency of all islet cell hormones, in addition to insulin (8-13).

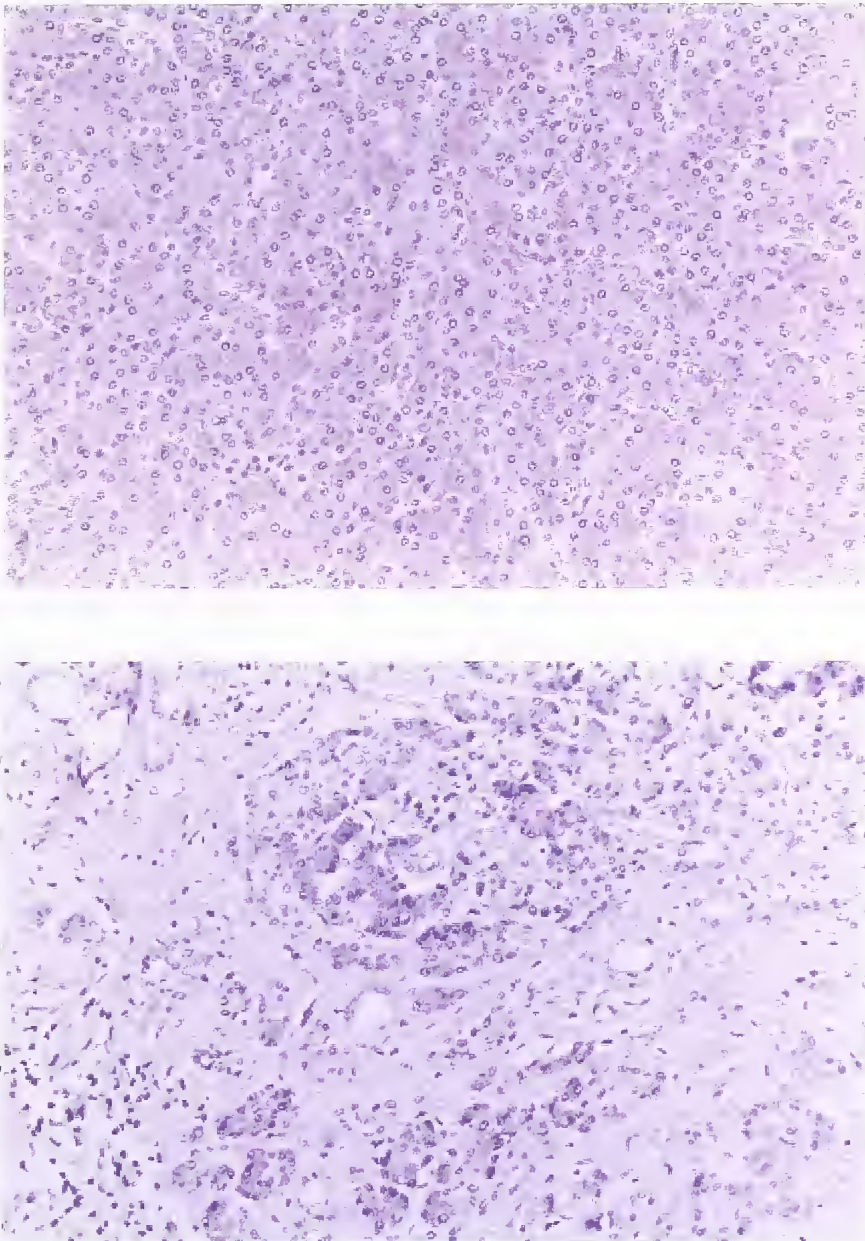


Figure 1: Photomicrographs of the Human Pancreas

The top panel shows normal pancreatic tissue, and the lower panel shows changes in chronic pancreatitis, characterized by a marked increase in interlobular fibrous tissue, atrophy of the acini, and a chronic inflammatory infiltrate (H&E x95) (5).

Prevalence and Causes

CP is clinically silent in a number of individuals and many other patients with abdominal pain elude diagnosis. Still, mean estimates of prevalence worldwide are up to

5% of the population in one study (14). Variation in the level of alcohol consumption worldwide and non-uniform diagnostic criteria have made the true epidemiology of the disease unclear, however, one large scale study based on autopsies in Denmark estimate a mean prevalence of 13% with a range from .2%-66% in that country (15).

The causes of CP are many. In some developed countries, 60-70% of patients present with a 6-12 year history of heavy (150-175 grams per day) alcohol consumption (5), inspiring Freidrich to coin the term “drunkard’s pancreas” in 1878. Furthermore, the rise in the prevalence of CP in Denmark correlates with a parallel rise in the amount of alcohol consumption in the country (16). CP also occurs secondary to obstruction of the pancreatic duct. The many etiologies include recurrent gallstone pancreatitis, pancreas divisum, post-traumatic strictures, pseudocysts, morphological changes in the sphincter, and tumors. Furthermore, CP may occur in patients with cystic fibrosis or CFTR gene mutations, tropical ingestion of cassava fruit cyanogens, hyperparathyroidism, and various hereditary diseases of autosomal dominance with incomplete penetrance (14). Some of these gene mutations involve the cationic trypsinogen gene, which inhibits enzymatic inactivation, and permits intrapancreatic autodigestion (6). A significant minority of all CP cases, however, remain idiopathic in nature (5).

Pathogenesis

Secretions of the diseased gland contain a disproportionately high level of protein from acinar cells compared to concentrations of fluid and bicarbonate secretion from ductal cells (17). As a result, this protein precipitates within the ductal network. The

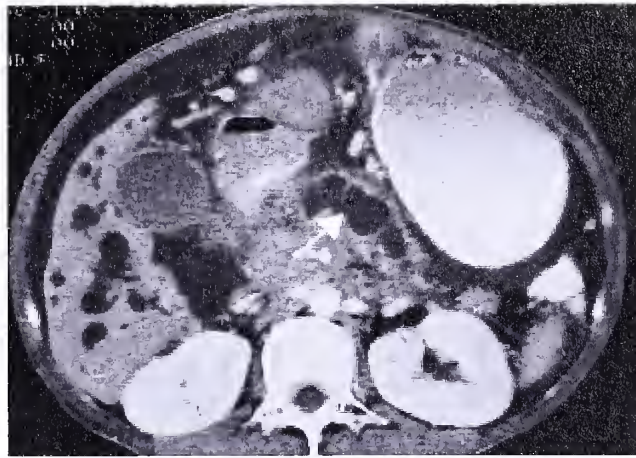


Figure 2: Abdominal CT scan in a patient with CP

CT scan of the abdomen shows gross enlargement of the pancreatic duct (arrow). This patient has CP with a stricture due to a carcinomatous mass in the head of the pancreas (6)

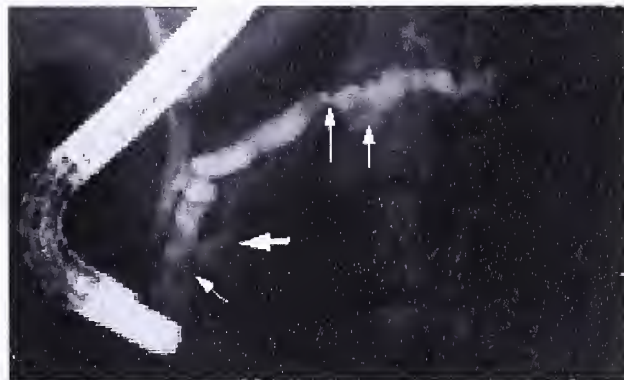


Figure 3: ERCP in a patient with CP

An endoscopic retrograde pancreatogram in a patient with CP, revealing a dilated, tortuous main duct that contains stones or protein plugs (lucencies marked by short arrows). A stricture is visible (long arrow) as is a dilatation of the uncinus process branch (thick arrow) (5).

precipitate eventually progresses into a laminar plug composed of necrotic cells (see figure 3). Calcium carbonate may precipitate in the amorphous plug, forming the characteristic intraductal stones (see figure 4).



Figure 4: Abdominal X-Ray in a patient with CP

Plain radiograph of the abdomen showing diffuse calcifications throughout the pancreas (arrows) in an alcoholic patient with CP (5).

Pancreatogenic Diabetes

The pancreas is critical for the control of glucose metabolism, and pancreatectomy always produces diabetes mellitus (8). The loss of functional pancreatic tissue in CP correlates linearly with the presence of diabetes, so that the greater the destruction of endocrine tissue, the greater the glucose intolerance (18). Thus, 70% of CP patients with calcifications demonstrate overt diabetes, another 20% have abnormal glucose tolerance tests, and another 8% have impaired insulin secretion (9, 16, 19, 20). In the absence of calcifications, the total number affected drops from 98% to ~75% (20). This glucose intolerance secondary to pancreatic resection or destruction is referred to as pancreatogenic diabetes.

Pancreatogenic diabetes occurs due to a decrease in beta cell reserve, causing a reduction in the maximum capacity for insulin release, and is not secondary to an impaired threshold for insulin secretion, as is seen in Type II (non-insulin dependent diabetes mellitus, NIDDM) (20). Although there is a loss of beta cell responsiveness to glucose, the pancreatogenic diabetes seen in CP differs from the glucose intolerance seen in type I (insulin dependent diabetes mellitus, IDDM) and type II NIDDM in that it is brittle, rarely induces ketoacidosis, is prone to severe insulin-induced hypoglycemia due to impaired glucagon secretion, and is characterized by a deficiency of all islet cell hormones (18, 21-23). Pancreatogenic diabetes also demonstrates little or no insulin response to feeding, as opposed to adult onset diabetes patients, whose response to a carbohydrate load is exaggerated and whose plasma insulin levels are normal or elevated (see table 1) (8). Additionally, the classic diabetic complications of retinopathy, nephropathy, and microangiopathy are less common in these patients (18, 24, 25).

	Type I IDDM Juvenile onset	Type II NIDDM Adult onset	Type III Pancreatogenic Postoperative onset
KETOACIDOSIS	Common	Rare	Rare
HYPERGLYCEMIA	Severe	Usually Mild	Mild
HYPOGLYCEMIA	Common	Rare	Common
PERIPHERAL INSULIN SENSITIVITY	Normal or Increased	Decreased	Increased
HEPATIC INSULIN SENSITIVITY	Normal	Normal or Decreased	Decreased
INSULIN LEVELS	Low	High	Low
GLUCAGON LEVELS	Normal or High	Normal or High	Low
PP LEVELS	High	High	Low
TYPICAL AGE OF ONSET	Childhood or Adolescence	Adulthood	Any

Table 1: Comparison of types of diabetes mellitus

Hepatic Insulin Resistance in Pancreatogenic Diabetes

The liver is essential for regulation of metabolic homeostasis. Its central role in control of glucose production and transport in response to stress, fasting, and feeding is fundamental for proper glucose metabolism. This role of the liver is dependent on pancreatic glucoregulatory hormones. Surgical alteration or destruction of the pancreas alters the balance of the hormones, insulin, glucagon, and pancreatic polypeptide, in the body, resulting in a disruption of net hepatic glucose production and utilization by tissues (8).

High rates of hepatic glucose production have been shown to contribute to the abnormal glucose tolerance seen in pancreatogenic diabetes. In fact, there remains a high rate of hepatic glucose production in patients with CP despite physiologic levels of insulin (26, 27). Clinical and laboratory models of pancreatic resection and CP have consistently demonstrated a loss of hepatic sensitivity to insulin, resulting in unsuppressed hepatic glucose production (28). As seen in figure 5, normal animals have an exquisitely sensitive response to insulin, decreasing hepatic glucose production by up to 95%, while subjects with pancreatogenic diabetes demonstrate only a 30% suppression in response to the hormone (29). In addition, basal glucose production is seen to be elevated in patients with pancreatogenic diabetes despite normal peripheral glucose uptake, suggesting that the insulin resistance is specific for the liver (30, 31). Ex-vivo isolated, single pass liver perfusion studies have examined the hepatic sensitivity to insulin in the absence of extrahepatic confounders, demonstrating a loss of insulin induced inhibition of hepatic glucose production in pancreatogenic diabetic animals (28, 32). In these euglycemic glucose-clamp studies, sham operated rats responded to an

insulin infusion with a dose dependent suppression of hepatic glucose production, while CP rats demonstrated uninhibited hepatic glucose production (28). Together, these results confirm that pancreaticogenic diabetes is associated with a primary hepatic resistance to insulin.

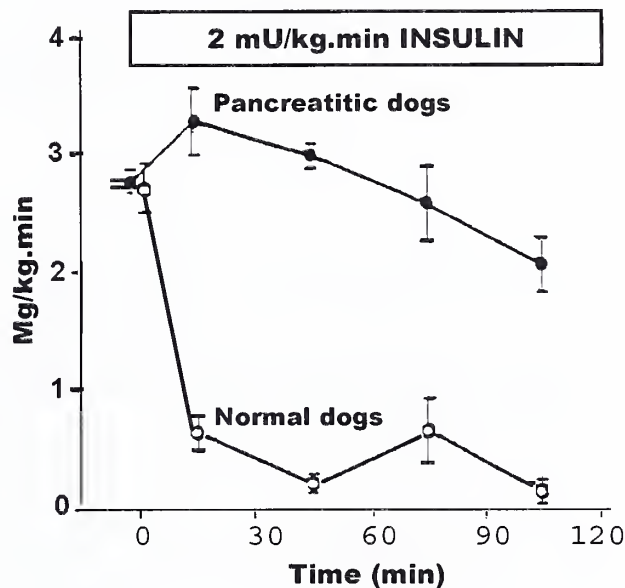


Figure 5: Hepatic Glucose Production

Both groups of animals begin at the same basal rates of hepatic glucose production. In response to an insulin infusion there is an exquisitely sensitive response and almost complete suppression of hepatic glucose production (y-axis) in normal dogs, whereas, pancreatic animals show an impairment in the insulin mediated suppression of hepatic glucose production (29, 31).

Insulin Action

Insulin action initiates a large number of specific effects on a broad spectrum of target cells. These effects can be divided into four pathways: 1) Stimulation of membrane transport processes (ions, glucose, amino acids); 2) Anabolic effects via glycogen synthesis, lipogenesis, and protein synthesis; 3) Anti-catabolic effects inhibiting glycogenolysis, lipolysis, and proteolysis; and 4) Growth promoting effects via

stimulation of nucleic acid synthesis (33). All of these various effects are initiated by the binding of insulin to a specific insulin receptor (IR), potentiating a wave of signal transduction at a post-binding level. The finding that insulin was internalized upon binding to the IR opened a new field of research in glucose metabolism; that of the implication of insulin binding and endocytosis in the physiopathology of diabetes and insulin resistant states (34-37).

The Insulin Receptor

The insulin receptor is comprised of two extracellular alpha ($M_r = 135$ kDa) and two transmembrane beta ($M_r = 95$ kDa) subunits, linked together by sulfide bonds to create a heterotetrameric complex (36, 38, 39). Binding of insulin to the alpha subunit extracellular domain of the insulin receptor induces a conformational change and transmits a signal across the plasma membrane, activating a receptor kinase located on the intracellular beta subunit (see figure 6) (40-42). The receptor then autoactivates and initiates a series of transphosphorylation reactions where beta subunits phosphorylate adjacent tyrosine residues. It has been suggested that specific tyrosine residues have distinct functions upon phosphorylation. For instance, carboxy tail residues have been shown to be critical to the mitogenic effects of insulin.

The autophosphorylation of IR on tyrosine residues results in the phosphorylation and dephosphorylation of many downstream cellular protein substrates (38, 43-47). This tyrosine kinase activity of the IR beta subunit may mediate many or all of the effects of insulin via a phosphorylation cascade, and/or through direct covalent and noncovalent protein interactions (42, 48).

Functional domains of the insulin receptor

The alpha subunit of the IR exists in two isoforms, with the binding domain located in amino acids a.a.83-103. Insulin interaction with the receptor is further modulated by cysteine rich regions, lysine a.a.460, and key amino acid sequences at the C-terminal end (33).

The beta subunit of the IR contains 620 amino acids, 403 of which are a well preserved intracytoplasmic sequence containing the tyrosine kinase domain. This sequence contains thirteen tyrosine (tyr) residues, six of which become phosphorylated upon insulin binding (33, 49). Tyrosines at 1158, 1162, and 1163 in figure 6 contain 50-60% of the phosphate after IR ligand binding, and are crucial for autoactivation of the tyrosine kinase (33). Of note, it is also postulated that serine phosphorylation at amino acids 1293 and 1294 and threonine phosphorylation at 1336 may induce a conformational change, thereby inhibiting IR kinase activity (33).

Insulin resistance

Insulin resistance occurs when normal physiologic concentrations of the beta cell secreted hormone are insufficient to effect appropriate systemic responses (13, 50). By definition, then, insulin resistance is dysfunctional signal transduction by the IR, due to abnormalities in the ligand (insulin), the IR itself, or post-IR events (51).

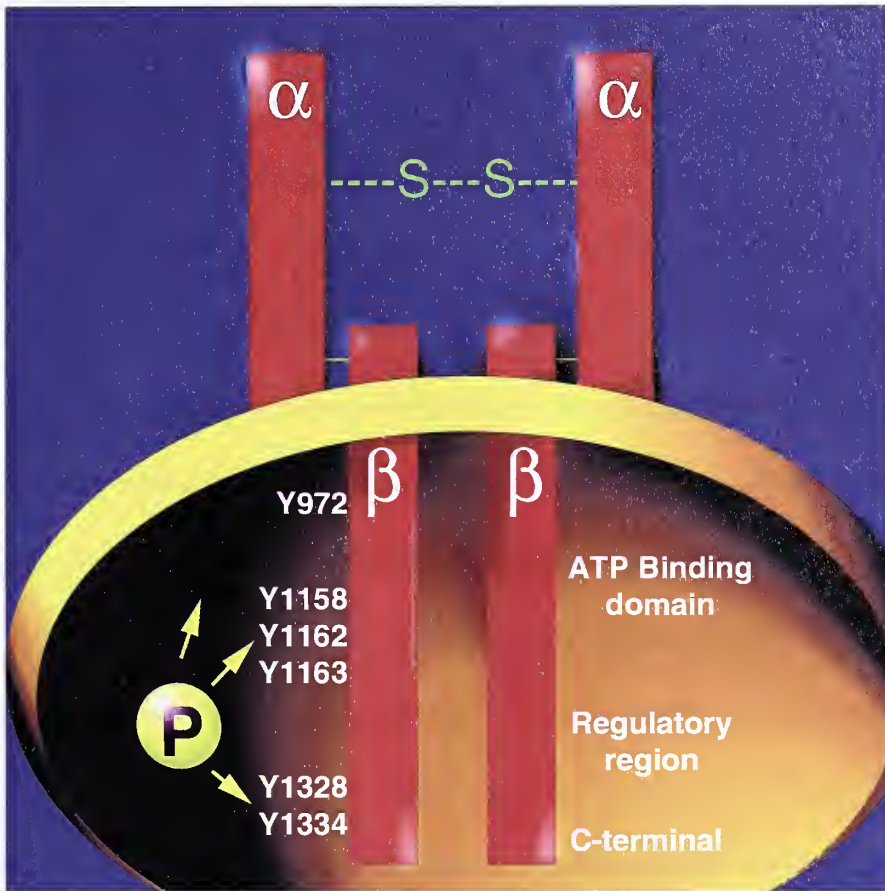


Figure 6: The Insulin Receptor

Insulin binds to the extracellular alpha subunit and induces a conformational change, activating a receptor kinase located on the intracellular beta subunit. Six of the thirteen tyrosine residues on the beta subunit, labeled here according to their location in the amino acid chain, are autophosphorylated upon insulin binding. Dephosphorylation of the same residues by tyrosine phosphatases may modulate the insulin response. *A. Maker*

Various models of cellular insulin resistance, including melanoma cell lines, obese animal models, animal models of insulinopenic diabetes, and cells from patients with severe insulin resistance type A; demonstrate defects in the insulin receptor kinase (33). In the case of obesity, studies demonstrate diminished insulin binding to plasma membrane receptors (52-58), a decrease in the number of IR's, and normal ligand affinity (59, 60), while in non-obese diabetic animals and in gestational diabetes, the degree of insulin resistance correlates with decreased numbers of cell surface IR's (61-64). However, in patients with glucose intolerance secondary to long term glucocorticoid administration, the observed decrease in insulin binding is secondary to diminished IR affinity for its ligand (65). Furthermore, changes in IR expression, binding, phosphorylation state and/or kinase function may be responsible for these and other insulin resistant phenotypes (51).

In addition, it is possible that inhibition of specific tyrosine residues occurs. This is most evident in mutations in the tyrosine kinase domains of the insulin receptor that result in dominantly inherited forms of insulin resistance including the type A syndrome, leprechaunism, Rabson-Mendenhall syndrome, and lipotrophic diabetes (66).

Proximal insulin receptor signaling events

The activated IR functions as a tyrosine kinase on a number of proximal substrate proteins. These include members of the insulin receptor substrate protein family (IRS1/2/3/4), the Shc adaptor protein isoforms, the SIRP family members, Gab1, Cbl, and APS. This downstream signaling induces mitogenic effects via IRS dependent or Shc (src homology and collagen) dependent pathways, i.e., via Grb2, SOS, ras, raf1,

MEK, and Erk (see figure 7). Metabolic effects are also transduced by IRS signals stimulating glucose transport and glycogen synthesis. Much work on IRS1 and IRS2 has been performed and some of their unique metabolic functions have been identified. In IRS-1 knockout mice, for example, mild states of insulin resistance are observed (67), while IRS-2 knockout mice demonstrate frank diabetes with impaired insulin secretion and peripheral insulin resistance (68). It is possible this discrepancy is due to a prominence of IRS1 in skeletal muscle mediation of insulin action, while IRS2 demonstrates prominence in the liver (69).

Phosphorylated IRS proteins contain recognition sites for various cell signaling proteins containing Src homology 2 domains, including Grb2, Nck, SHP2 protein tyrosine phosphatase, and phosphatidylinositol 3 kinase (PI3 kinase). These multiple proteins have been studied extensively to evaluate how they couple growth factor stimulation to intracellular signaling pathways, and to characterize their location and role in the insulin induced cascade. However, much remains to be elucidated.

Downstream signaling events

The function of type 1A PI3 kinase has been extensively investigated using pharmacological inhibitors, blocking antibodies, and expression of dominant interfering and constitutively active mutants (51). These studies conclude that PI3 kinase is necessary for glucose uptake in skeletal muscle and adipocytes via stimulation of GLUT4 translocation from an intracellular pool to the cell surface (70). Moreover, impaired PI3 kinase activity in GLUT1 containing vesicles in fibroblasts from a patient with Werner's syndrome is associated with defective translocation/docking of GLUT1 with the cell

membrane, possibly causing the molecular defect responsible for insulin resistance in the disease. Nawano and his colleagues examined the critical role of the liver in glucose homeostasis in the Zucker diabetic fatty rat. Their group concluded that chronic hyperglycemia reduces the efficiency of PI3 kinase activation of the serine/threonine kinase Akt / Protein Kinase B (Akt/PKB), and that this mechanism accounts for hyperglycemia induced insulin resistance in the liver (71).

Furthermore, current research has shown a 40-55% decrease in insulin stimulated tyrosine phosphorylation of IRS1 and PI3 kinase activity in type 2 diabetic patients, which implicates decreased glucose transport with decreased phosphorylation of molecules downstream to the phosphorylated IR and upstream to the glucose transport proteins.

Although the downstream targets of PI3 kinase are controversial, the serine/threonine kinase Akt (PKB) has been identified as one such protein substrate. Normal expression of Akt in 3T3L1 adipocytes allows GLUT4 localization to the plasma membrane, whereas the dominant mutant Akt disrupts glucose fluxes in the adipose tissue by inhibiting GLUT4 translocation (51, 72). PI3 kinase activation of protein kinase C is also thought to play a role in peripheral glucose metabolism, however, the exact mechanisms are uncertain (see figure 7).

The role of protein tyrosine phosphatases

Protein tyrosine phosphatases (PTPase's) reset pharmacologically important tyrosine kinase receptors to their original dephosphorylated state. Active research is investigating the tyrosine phosphatases specific to dephosphorylate the insulin receptor

(73). Working in conjunction with serine/threonine kinases, these PTPase's may be intimately involved in modulating the IR's proximal signal cascade.

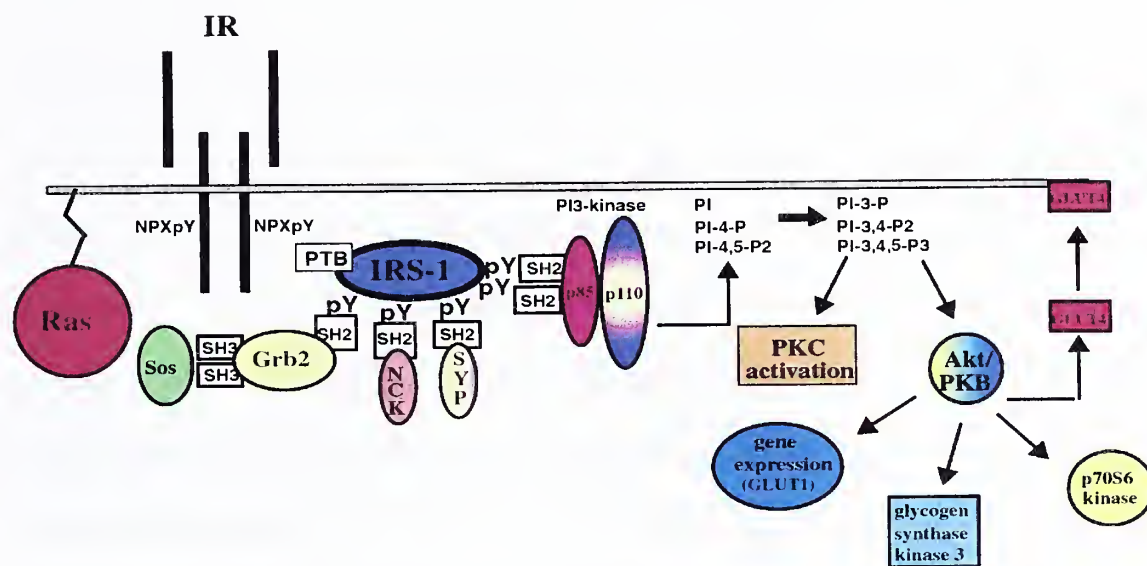


Figure 7: A Schematic of the IR Phosphorylation Cascade

Insulin binds to the IR, inducing autophosphorylation of the intracellular beta subunit. The tyrosine kinase activity of the activated IR phosphorylates insulin receptor substrates, initiating a cascade of events including stimulation of PI3 kinase and Akt/PKB. The effects of IR kinase action effect glucose transport, protein regulation, translocation, glucose metabolism, gene expression, and mitogenesis.

Glucose Transport Protein 2 (GLUT2)

At least eight functional mammalian facilitated hexose carriers (GLUTs) have been identified by molecular cloning, and their specificity and affinity have been characterized. GLUT2, a 52 kDa protein encoded on chromosome 3, is found in tissues carrying large glucose fluxes, such as intestine, kidney, liver, and beta-cells, and facilitates membrane transport of the polar glucose molecule over a wide range of glucose concentrations via an energy-independent mechanism (74-78). The rate of

glucose uptake into the cell increases in proportion to the rise in blood glucose above the physiologic range due to the transporter's low affinity ($K_m=17$) (79).

In the normal animal, it is believed that insulin binding to its receptor propagates a cascade of signals, resulting in translocation of glucose transporters from an intracellular pool to the cell membrane where they dock, fuse, and are activated, as previously described in the case of GLUT 4 (80, 81). Models of streptozotocin induced diabetes have not resulted in dysfunction of hepatic GLUT2, however, trafficking of the transporter is affected in CP, with decreased internalization of the protein seen in the diseased state versus normal animals (82). The failure of insulin mediated removal of GLUT2 off the hepatocyte membrane in CP has been shown to directly correlate with the unsuppressed hepatic glucose production seen in the disease state (32, 82), possibly implying that GLUT2 is normally removed from the cell membrane in response to insulin.

Molecular models studying glucose transport proteins have correlated translocation and changes in glucose transport with changes in the phosphorylation state of the GLUT protein, though no such mechanism has been demonstrated for hepatic GLUT2. In vitro, beta cell GLUT2 has been shown to be activated through the cAMP cascade, and glucose transport into the cell may be modulated by protein kinase A dependent phosphorylation of serine and threonine residues on GLUT2, however, much remains to be elucidated (see figure 8) (83).

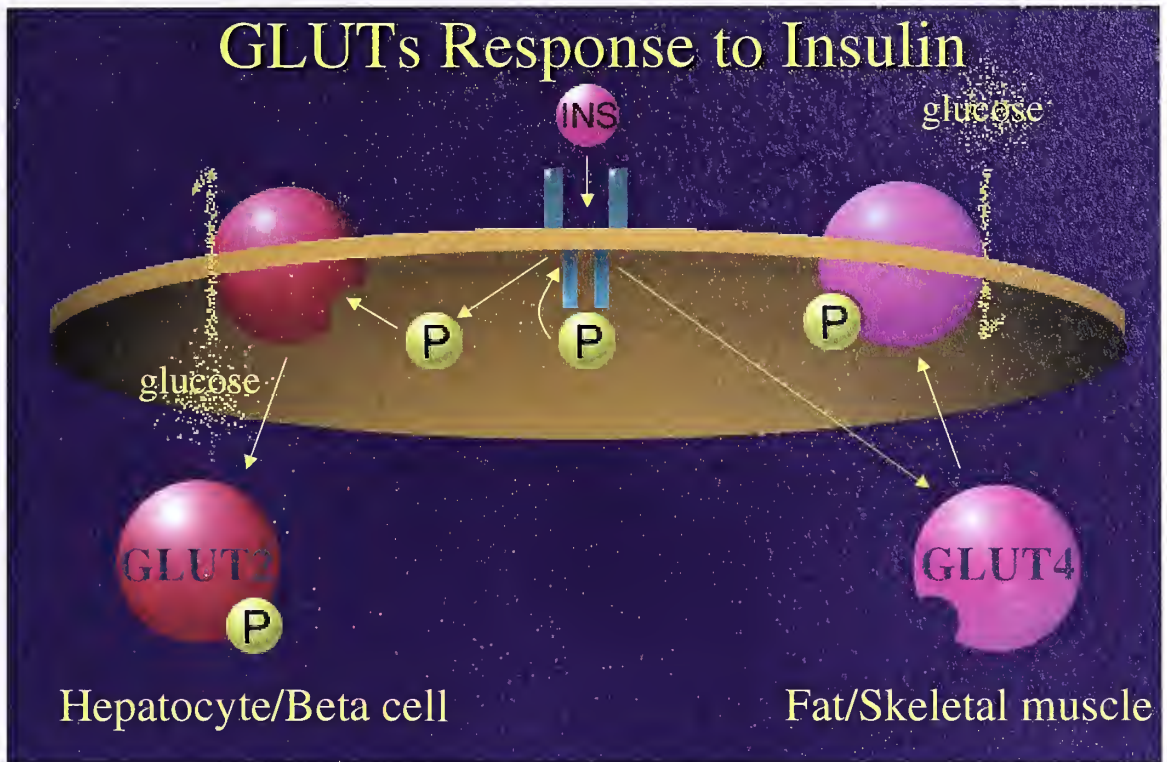


Figure 8: GLUTs response to insulin

Insulin binding to the insulin receptor stimulates the peripheral uptake of glucose and inhibits hepatic glucose production, glycogenolysis, and gluconeogenesis. In the adipocyte and skeletal muscle cell, insulin binding stimulates translocation of GLUT4 to the cell membrane via a series of phosphorylation reactions including PI3 kinase and Akt/PKB, thereby allowing glucose influx into the cell. On the other hand, insulin binding to the hepatocyte IR may stimulate endocytosis of GLUT2, inhibiting glucose efflux. Past studies have correlated serine phosphorylation of GLUT2 with decreased glucose transport in beta cells, and we implicate GLUT2 phosphorylation with decreased glucose transport in hepatocytes. *A. Maker*

SUMMARY, STATEMENT OF PURPOSE, AND HYPOTHESIS

Summary

Impaired function of the tyrosine kinase domains of the insulin receptor frequently results in insulin resistance. Clinically, acquired dysfunction of the insulin receptor's response to insulin stimulation results in glucose intolerance and a diabetic state. Previous experiments in our laboratory have suggested that this impairment of glycemic control may in fact be due to dysfunction of the stimulated insulin receptor and membrane bound GLUT2.

Aberrant protein expression is not solely responsible for the observed insulin-signaling defects in glucose transport seen in CP or diabetes. In fact, various models of insulin resistance and diabetes mellitus have demonstrated that the expression of insulin receptor, IRS-1, IRS-2, MAP kinase, and glycogen synthase are similar between type 2 diabetic and control subjects. Although there is evidence to support that peripheral insulin resistance is secondary to impaired recruitment of intracellular pools of GLUT 4 in skeletal muscle and adipose tissue in diabetes mellitus models, the defect may lie in signaling of the translocation.

Multiple studies in various models of insulin resistance have demonstrated phosphorylation defects at various points in the IR kinase cascade. In particular, it has been shown that the PI3 kinase activation of protein kinase B/Akt is critical to the translocation of glucose transport protein 4 (GLUT4) in response to insulin, and that impaired phosphorylation of PKB/Akt creates an insulin resistant state. The exact mechanisms of cell signaling have yet to be elucidated, however, similar models have

been implied in GLUT 1 and 3. It is clear that GLUT2 is intimately responsible for sensing glucose levels and effecting appropriate beta cell secretion of insulin, and it has been shown that hepatic GLUT2 is critical for controlling hepatic glucose production, however, the control of GLUT2 expression and function is unclear. Though studies in glucose transport activity have been correlated with GLUT2 phosphorylation state, there has been no evidence to support a direct link between IR and GLUT2.

Statement of purpose and hypothesis

We propose that the endpoint of one IR kinase phosphorylation cascade may be the hepatic glucose transporter 2 (GLUT2). Our lab has observed impaired endocytosis of GLUT2 in response to feeding without changes in GLUT2 expression in diabetic models, further strengthening the argument for a defect in cell signaling upstream of the glucose transporter (84). We hypothesized that autophosphorylation of the IR in response to ligand binding sets up a phosphorylation cascade, similar to that implicated for GLUT4, with GLUT2 being intimately connected, both chemically and spatially, with the hepatic IR. We therefore suspected that the pancreatogenic diabetes characteristic of CP is caused by defects in both IR-kinase stimulation and GLUT2 phosphorylation.

Therefore, we hypothesize that:

1. Feeding activates IR and GLUT2 phosphorylation in vivo.
2. The impaired response to insulin and glucoregulatory dysfunction seen in CP may have a basis in insulin receptor dysfunction at the level of tyrosine autophosphorylation.

3. The uninhibited hepatic glucose production seen in CP has a basis in dysfunctional cell signaling, resulting in impaired GLUT2 serine phosphorylation.
4. IR and GLUT2 interact as a functional unit in a receptor/transporter complex, and, as such, are intimately associated spatially as a functional unit in a receptor/transporter complex.

Our first objective was to establish that the hepatic insulin resistance and unsuppressed glucose production characteristic of pancreatogenic diabetes is associated with dysfunction in hepatic IR tyrosine autophosphorylation (IR-P-Tyr) and/or GLUT2 serine phosphorylation (GLUT2-P-Ser) *in vivo*. In the current study, a rodent model of CP that mimics the physiological state of human CP was used to compare phosphorylation states of IR-Tyr and GLUT2-Ser residues in control and CP rats in response to feeding. We also intended to demonstrate an intimate spatial relationship between GLUT2 and IR in the normal rat hepatocyte using double-labeled fluorescent dye confocal imaging and co-immunoprecipitation techniques.

METHODS

Phosphorylation Studies

Animals

Chronic pancreatitis was induced in 150-200 gram male Sprague Dawley rats (n=12) (Charles River, Raleigh, NC). A 2.5 cm midline abdominal incision was made, the loop of the duodenum was mobilized, and the sphincter of Oddi identified. Fifty μ L of 99% oleic acid (Sigma Chemical Company, St. Louis, MO) was infused at 12.5 μ L/minute for 4 minutes into the cannulated pancreatic duct at laparotomy, according to the method of Mundlos et al (85). An atraumatic clamp was applied to the common hepatic duct at its bifurcation to prevent reflux of oleic acid into the liver. Age and weight matched sham-operated animals (n=12), that underwent laparotomy and manipulation of the pancreas only, served as controls. Animals were explored for gross confirmation of CP prior to sacrifice. Histologic verification of chronic pancreatitis has been confirmed previously in this model (see figure 9). Intraperitoneal anesthesia was sodium pentobarbital (0.07mg/g body weight).

Liver Harvest

Fifty-nine to sixty-six days post-induction, rats were fasted 24 hours in metabolic cages, weighed, and anesthetized. All animals underwent a ventral midline incision, a 2mm gastrotomy incision, and transgastric duodenal intubation. Fed animals received a bolus meal (0.5cc/100g) of nutritional supplement (Osmolite HN, Ross Products

Division, Columbus, OH) injected intraduodenally over a 1-2 minute period with circumferential pyloric pressure to prevent reflux. Fasted animals underwent duodenal intubation alone. Thirty-five minutes after intubation, a 2 gram section of the distal aspect of the left main lobe of the liver was excised, rinsed in Ringers

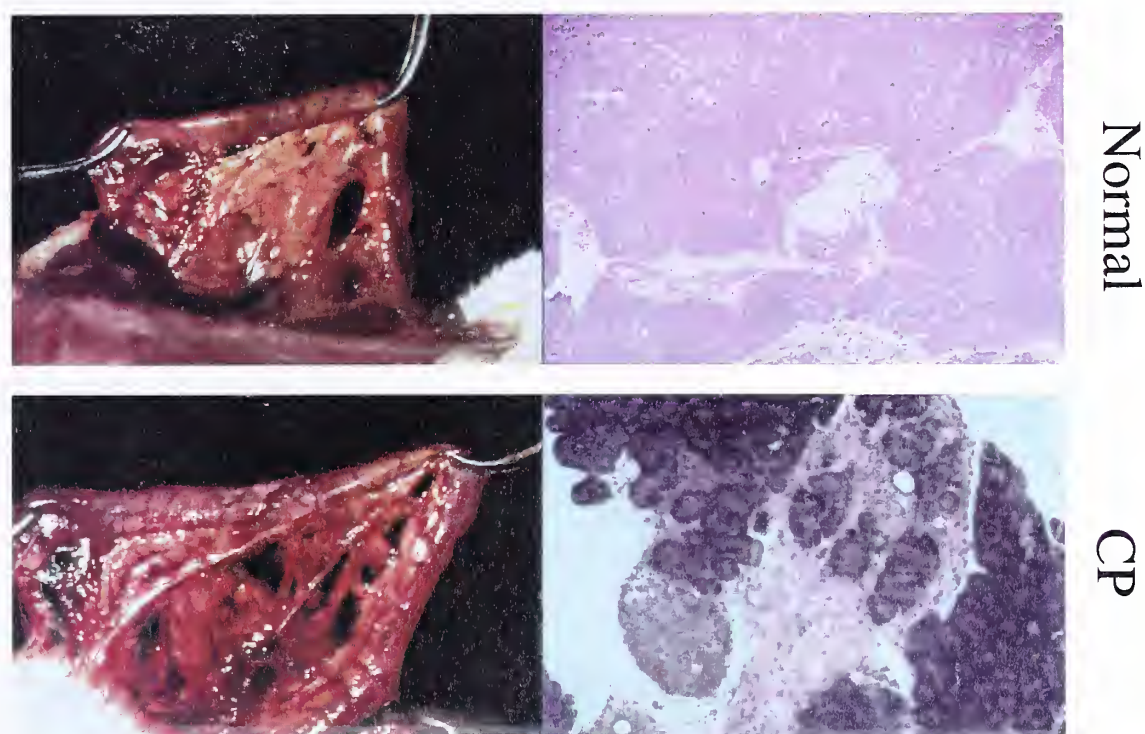


Figure 9: Pathological and histological verification of chronic pancreatitis in the rat model

In the normal animal (upper panel), there is healthy pancreatic tissue in the loop of the duodenum obscuring the common bile duct. Normal islets and acini are verified histologically. However, in the CP animal (lower panel), there is loss of parenchyma in the loop of duodenum, demonstrated here by the clear visibility of the common bile duct through the scant areas of remaining tissue. There is a loss of pancreatic mass, blunting of lobules, and fibrous replacement. Histology confirms fibrosis, fat replacement of exocrine tissue, and inflammation of the gland.

lactate solution, and promptly minced, homogenized, and strained at 4 °C in 2.5 mL of fresh homogenization buffer (10 mM triethanolamine, 10 mM acetic acid pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM PMSF, 1 mM DTT, 1 mM Benzamidine, 10 mM sodium orthovanadate, and 1 micromolar Okadaic acid). PMSF and benzamidine were

included as protease inhibitors, and DTT as a reducing agent. Vanadate was added to inhibit active ATPases and phosphate transferring enzymes (86, 87), and okadaic acid was titrated to inhibit active protein phosphatases completely and specifically once the tissue was procured (88). Protein content was assayed according to the Bradford reaction (89) and 1 mg of total protein homogenate was immediately solubilized in 1 mL of 0.1% Triton X-100 (BioRad, Hercules, CA) at 4 °C.

Animals were paired in sham and CP groups to insure liver harvest at comparable days post-CP induction and handling under similar conditions. Livers from paired animals were harvested concomitantly and samples were run on the same electrophoresis gels to minimize intra-assay variation.

Immunoprecipitation

Five mg of Protein A immobilized on sepharose CL-4B beads (Sigma Chemical Company, St. Louis, MO) were suspended in a 20 µL slurry of PBS and incubated at room temperature with 10 µL of antibody to the IR beta chain (InsulinR Beta C-19, Santa Cruz Biotechnology, Santa Cruz, CA) or to GLUT2 (Biogenesis, Sandown, NH) for 30 minutes. Solubilized samples were centrifuged at 14,000 rpm at 4 °C for 5 minutes, after which 0.5 mg of homogenate was added to the beads. Samples were incubated for two hours on a rotating platform at 4 °C. Samples were then washed twice at room temperature with 0.9 mL of buffer, containing 400 mM NaCl, 50 mM Tris/HCl, and 0.02% sodium azide as a preservative (pH 7.5), and followed by two more washes of 0.9 mL of buffer containing 500 mM NaCl, 10 mM Tris/HCl, and 0.02% sodium azide (pH 7.5). Samples were centrifuged for 3.5 minutes at 10,000 rpm at 4 °C between

resuspensions. The series of four washes was followed by resuspension in SDS reducing buffer for 30 minutes at room temperature. A four minute 12,000 rpm centrifugation separated the sample from the bead pellet (see figure 10). Negative control proteins were precipitated concomitantly.

Electrophoresis and Immunoblotting

Immunoprecipitated samples for IR were incubated in a 100 °C water bath for five minutes. All samples and negative antibody controls were run on a 4-15% stacking gel (BioRad, Hercules, CA) for 33-40 minutes at 200 volts (25-45 mA). Samples to be probed for IR and phosphotyrosine were run on the same gel as were samples to be probed for GLUT2 and phosphoserine. Electrophoresed samples were transferred to a nitrocellulose support at 100V for one hour, then blocked with 3% high purity bovine serum albumin (Sigma) and probed with 1:1000 (200 µg/mL) anti-phosphotyrosine antibody (Santa Cruz Biotechnology) or 1:6000 (37mg/mL stock concentration) anti-phosphoserine antibody (Sigma). Concomitantly, blots were blocked with 3% milk and probed with 1:1000 anti-IR antibody (Santa Cruz Biotechnology) or 1:50000 anti-GLUT2 antibody (Biogenesis). HRP linked secondary antibody was analyzed with the ECL immunodetection system (Amersham Life Science Ltd., Buckinghamshires, England). Protein bands were analyzed with scanning densitometry (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA) and ImageQuaNT™ software (Molecular Dynamics).

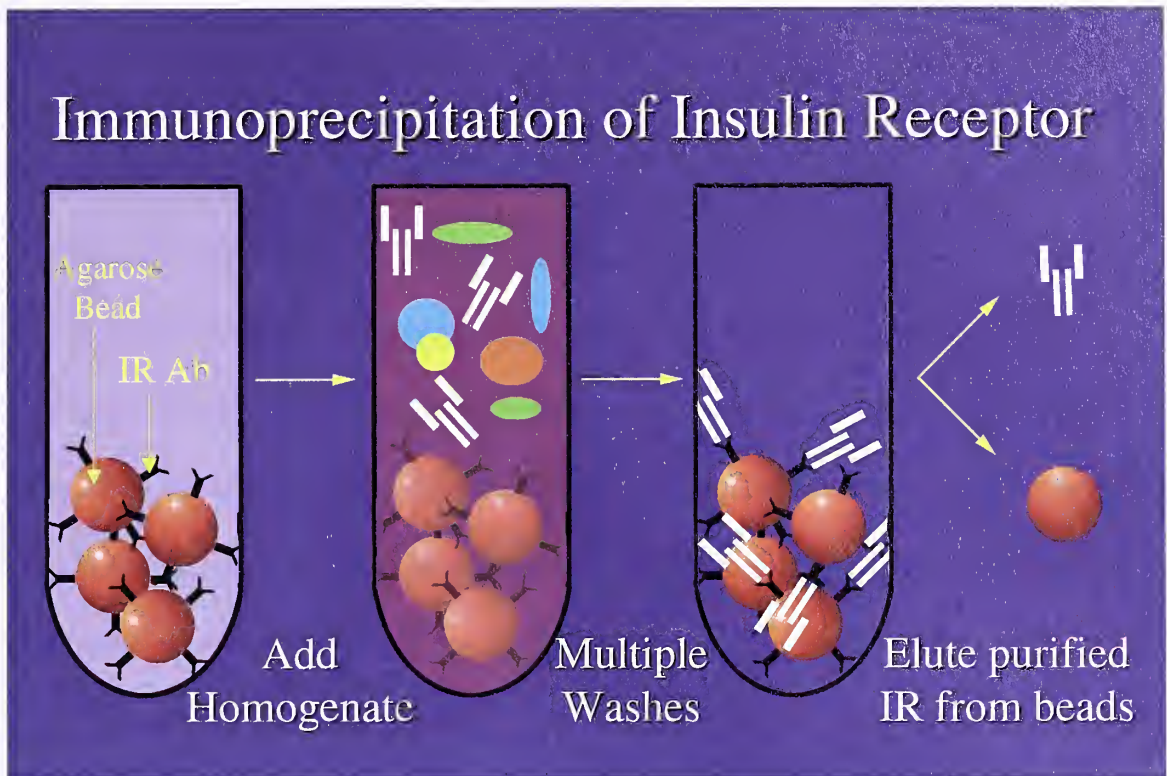


Figure 10: Immunoprecipitation of the insulin receptor

Fresh homogenized liver tissue is added to a slurry of beads coated with antibody to the insulin receptor. A series of washes with graded salt concentrations specifies the antibody-IR interaction. The purified IR can then be eluted from the beads, run on a SDS-PAGE gel, and probed with two antibodies; one to quantify the amount of phosphorylated tyrosine, and the other to quantify the total amount of IR purified from the homogenate. *A Maker*

Phosphatase activity

Total non-IR specific tissue phosphotyrosine phosphatase (PTPase) activity was determined in liver homogenates by the para-nitro phenylphosphate (p-NPP) method. p-NPP is a colorless compound that forms a yellow color reaction when dephosphorylated. In our assay, 1 mg/mL p-NPP in 50mM imidazole buffer, pH 7.4, was used. Three different concentrations of total homogenates were assayed by mixing with 100 μ L of the assay mixture in 1.5 mL Eppendorf tubes at 30 $^{\circ}$ C for 30 minutes. The reaction was terminated by the addition of 100 μ L of 0.2N NaOH. The optical density of the resultant mixture is measured at 410nm, to determine relative PTPase activity in each sample.

Colocalization Studies

Liver harvest and tissue preparation for confocal studies

300-400 gram male Sprague Dawley rats were anesthetized intraperitoneally with sodium pentobarbital (0.07mg/g body weight). At laparotomy, the distal aspect of the right main lobe of the liver was excised, rinsed in Ringers lactate solution, and promptly minced at 4 °C. Samples were processed in 72% sucrose solution and re-equilibrated in phosphate buffered saline at the time of sectioning.

Tissue was snap frozen in blocks for sectioning on a sliding cryostat and cut in 5 micron sections. Sections were fixed in cold acetone and rehydrated in 120 mM phosphate solution for 40 minutes. Samples were then blocked with 8% bovine serum albumin in 120 mM phosphate solution and 0.7% Triton-X for one hour.

Immunostaining

Primary antibodies to GLUT2 (goat anti-GLUT2, Santa Cruz Biotechnology, Santa Cruz, CA) and IR (rabbit anti-IR, Santa Cruz Biotechnology, Santa Cruz, CA) were prepared in a 1:20 dilution in 1% BSA-120mM phosphate, and incubated overnight at 4 °C. Slides were then washed and incubated with donkey anti-goat IgG fluorescein (FITC) conjugated antibody (1:100, Santa Cruz) and donkey anti-rabbit Texas Red conjugated antibody (1:100, Santa Cruz) in the dark for three hours at room temperature. Slides were then washed, mounted with glycerol-PBS, and placed at 4 °C until imaged. Specimens were viewed on a Zeiss LSM Axiovert 100 confocal microscope (Zeiss,

Germany) with a 60x water immersion lens. Images were displayed at 512x512 pixels and processed using Adobe Photoshop v. 5.5 software. FITC was stimulated at 488 nm and emission collected with a 515-565 nm filter system in accordance with peak excitation and emission spectra for the fluorochrome. Similarly, Texas Red was stimulated at 568nm and emission collected in the 575-640 nm range. Fluorescent resonance energy transfer (FRET) was stimulated at 488 nm and collected at 670-810 nm to avoid any possible overlap with FITC emission wavelengths (see figure 11). All background staining was adjusted for and standardized when viewing experimental sections at the same time on the same day.

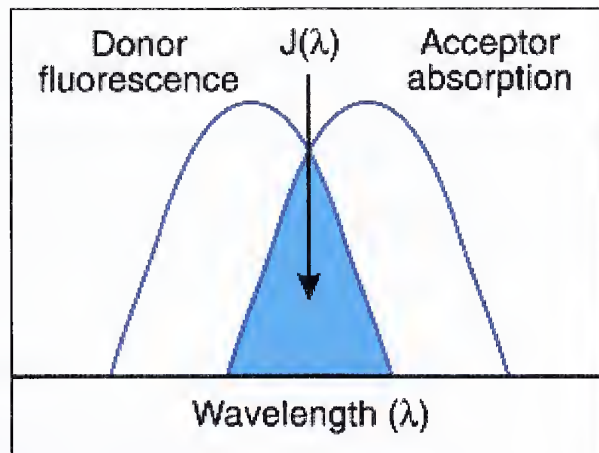


Figure 11: Fluorescence Resonance Energy Transfer (FRET)

FRET is a distance-dependent interaction between the excited states of two dye molecules. Excitation of a donor molecule is transferred to an acceptor molecule without photon emission, making it an important technique for analyzing molecular proximity. $J(\lambda)$ is the spectral overlap integral (90).

Hepatocyte Tissue Culture

(at the Yale Tissue Culture Facility)

150-200g Sprague-Dawley rats were anesthetized with phenobarbital. At laparotomy, the portal vein was then identified and ligated. Heparin (500units/100g

body weight) was injected into inferior vena cava and allowed to circulate for 1 minute. The portal vein was cannulated and the IVC was incised to permit a free flow of perfusate. The liver was then perfused with Hanks A (120 mM NaCl, 5mM KCl, 0.4 mM KH₂PO₄, 0.2 mM Na₂PO₄, 25 mM NaHCO₃, 0.5 mM EGTA, 0.1% D-Glucose) for 3-4 minutes. Next, the liver was perfused with Hanks B (120 mM NaCl, 5mM KCl, 0.4 mM KH₂PO₄, 0.2 mM Na₂PO₄, 25 mM NaHCO₃, 0.4 mM MgSO₄, 0.5 mM MgCl₂, 3 mM CaCl₂, 0.1% D-Glucose) with collagenase (0.05%/400ml Hanks B) until liver digestion became visible. The liver was then excised and rinsed in Leibovitz L-15 media (Life Technologies, Rockville, MD). Glisson's capsule was removed and the digested parenchyma was manually harvested. The tissue suspension was filtered sequentially through an 80 µm mesh followed by a 45 µm mesh. The suspension was then centrifuged at 4 °C for 2 minutes at 400 rpm and the pellet was resuspended in 14 mL of saline buffer. This step was repeated, resuspending the final pellet in 7mL.

Co-Immunoprecipitation

(performed with Michael Eisenberg)

4.5x10⁵ adult male rat hepatic cells from culture were vortexed in 1150 µL of lysis buffer (140 mM NaCl, 10 mM KCl, 8.2 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 1 mM DTT, 1 mM PMSF, 1 mM Benzamidine, 1% Triton, pH=7.5). Lysates were cleared of insoluble material by centrifugation at 14,000 rpm for 10 minutes at 4 °C. The supernatant was precleared with 20 µL of a 1:1 slurry of Protein G-Agarose (Sigma Chemical Company, St. Louis, MO) and PBS for 1 hour at 4 °C. The supernatant was separated by centrifugation at 1,500 rpm for 1 minute and precipitated with 20 µL of

Protein G beads (incubated overnight at 4 °C with 1 µg of either anti-IR or anti-GLUT2 antibody). The beads were collected by centrifugation for 6 minutes at 4 °C and washed twice with Solution I (400mM NaCl, 50mM Tris/HCl, 0.02% sodium azide) and with Solution II (500mM NaCl, 10mM Tris/HCl, 0.02% sodium azide). The beads were then isolated by centrifugation at 6,000 rpm for 4 minutes at 4 °C. GLUT2 and IR were eluted with 60 µL of reducing buffer at 100 °C for 3 minutes. The same protocol was used to immunoprecipitate GLUT2 and IR with antibodies to the hepatocyte membrane sodium potassium ATPase (donated by Dr. Michael Kashgarian, Yale University), to be used as a control protein for comparison.

Electrophoresis and Immunoblotting

Samples were separated by SDS-PAGE on a 7.5% acrylamide gel (Bio-Rad, Hercules, CA) and electrotransferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked with 3% skim milk for 1 hour, washed with PBS-1% Tween 20, and probed with rabbit anti-IR antibody (1:250, Santa Cruz), or blocked with 5% BSA for 1 hour, washed, and probed with donkey anti-GLUT2 antibody (1:500, Santa Cruz) for 1 hour. Membranes were then washed, incubated in anti-rabbit IgG (1:1000, Amersham Life Science Ltd, Buckinghamshires, England) or anti-donkey IgG (1:10000, Pierce Chemical Co.) for 1 hour. The HRP linked secondary antibody was analyzed with the ECL immunodetection system (Amersham Life Science Ltd.). Protein bands were analyzed with scanning densitometry (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA) and ImageQuaNTTM software (Molecular Dynamics). The expected

molecular weights for IR were taken at 95kDa and for GLUT2 at 59 kDa. The data was normalized to an internal control of total cell lysate run on each gel.

All data are represented as a mean \pm the standard error of the mean, unless otherwise stated. Statistical analysis were performed by the student's t-test. Values of p less than 0.05 were regarded as significant.

All experiments heretofore were conducted by Ajay V. Maker unless otherwise stated. The induction of chronic pancreatitis and liver harvests were performed with the assistance of Jinping Wang, M.D., and Lori Slezak, M.D.

RESULTS

Phosphorylation studies

Duodenal feeding resulted in an increase in phosphorylation of tyrosine residues on IR (IR-P-Tyr) in sham animals. IR protein recovery revealed no significant change between sham and CP samples, nor between fasted and fed animals. All comparisons were made on samples run on the same gel and analyzed concurrently. Corrected for IR quantity, IR-P-Tyr increased 395 ± 112 optical density units (ODU) ($p < 0.005$) over the fasted state in sham rats. This corresponded to over a 20-fold increase ($p < 0.05$) in P-Tyr in the sham fed state over the fasted state. IR-P-Tyr also increased in CP animals by 326 ± 87.7 ODU ($p < 0.005$) in the fed state, however, this corresponded to only a 9-fold increase in tyrosine phosphorylation over the fasted state ($p < 0.005$). Thus, CP animals demonstrate about 12-fold ($p < 0.05$) less Tyr phosphorylation compared to sham animals upon feeding.

In a similar manner, phosphorylation of serine residues on GLUT2 (GLUT2-P-Ser) increased in 5/5 sham fed animals, while decreasing in CP fed animals. Densitometric quantification of phosphorylation was corrected for GLUT2 protein volume for each sample. Sham animals increased 4.83 (fold) ± 1.2 upon feeding while CP animals increased only 0.13 (fold) $\pm .77$. This corresponded to almost 5-fold ± 1.19 ($p < 0.05$) less Ser phosphorylation in CP vs. sham animals (see figures 12-14).

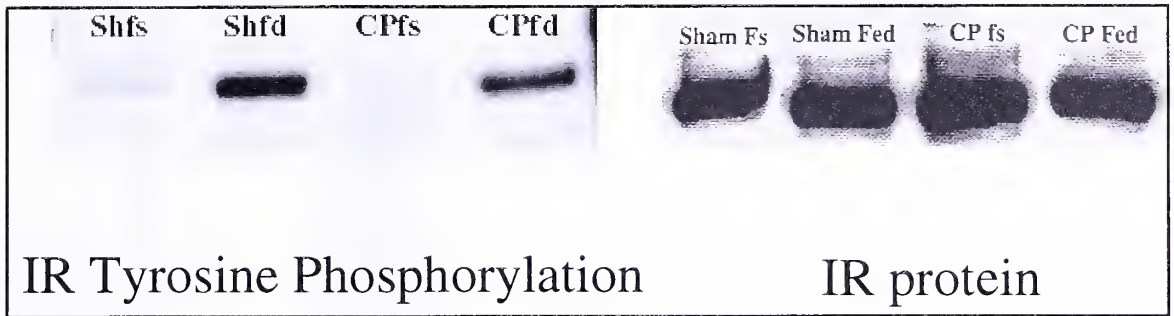


Figure 12: IR tyrosine phosphorylation

In this representative Western blot, IR probed with an anti-phosphotyrosine antibody demonstrates significant increases in total tyrosine phosphorylation in the fed state over the fasted state. Note the gross increase in phosphorylation in the sham animals in response to feeding and the relatively diminished increase in phosphorylation in animals with CP. There is no significant difference in the total amount of IR protein in the different experimental groups, either sham or CP, fasted (fs) or fed (fd).

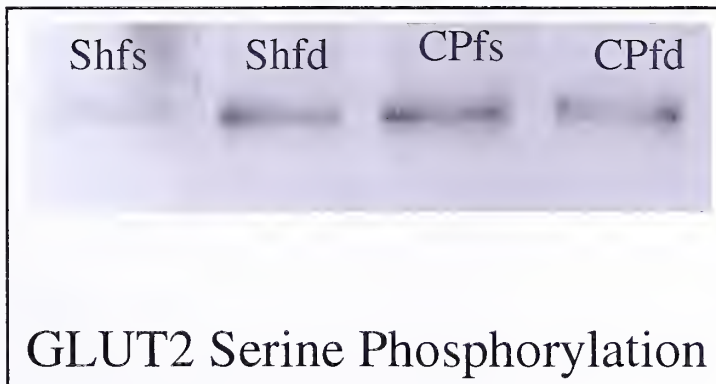


Figure 13: GLUT2 Serine Phosphorylation

In this representative Western blot, there are significant increases in total GLUT2 serine phosphorylation in the sham fed (Shfd) state over the sham fasted (Shfs) state versus a relatively diminished change in phosphorylation in response to feeding in animals with CP.

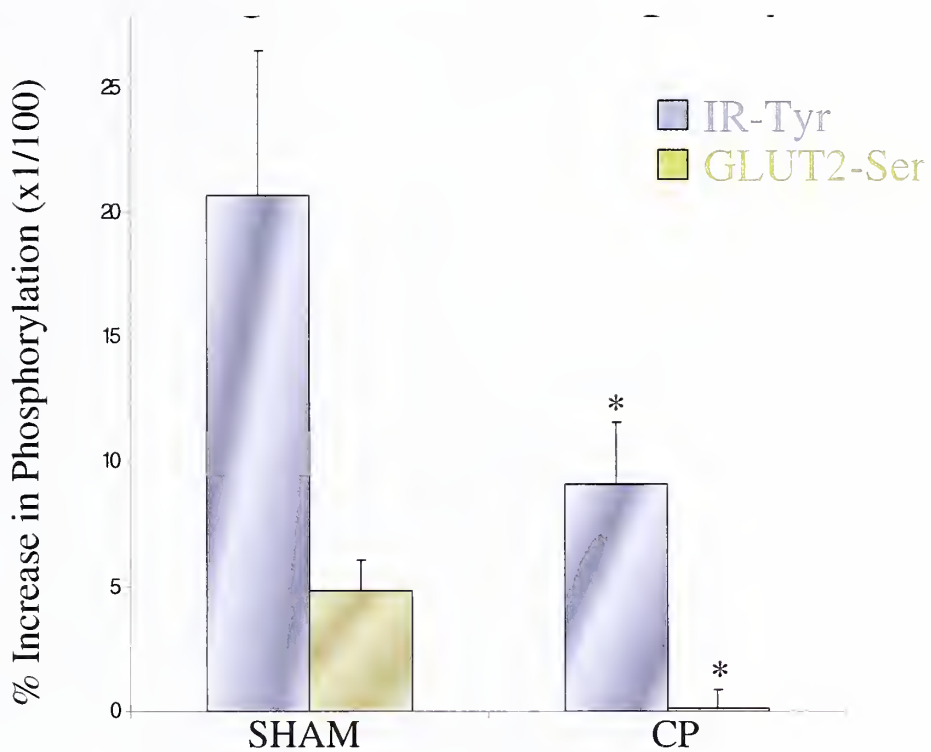


Figure 14: Feeding induced phosphorylation

Sham animals demonstrate ~20-fold increase in insulin receptor tyrosine phosphorylation (IR-P-Tyr) and ~5-fold increase in GLUT2 serine phosphorylation (GLUT2-P-Ser) in response to feeding. CP animals reveal ~12-fold less increase in IR-P-Tyr and ~5-fold less GLUT2-P-Ser in response to feeding.

Total PTPase relative activity of sham fed animals was $1.10 \pm .14$ vs. $.913 \pm .13$ in CP fed animals (n=3,3), revealing no significant relative differences in sham vs. CP fed livers (see figure 15).

Colocalization Studies

Hepatic Histology

Snap frozen sections of rat hepatic tissue stained by H and E were reviewed after rehydration. The tissue appeared healthy and displayed normal sinusoidal architecture (data not shown).

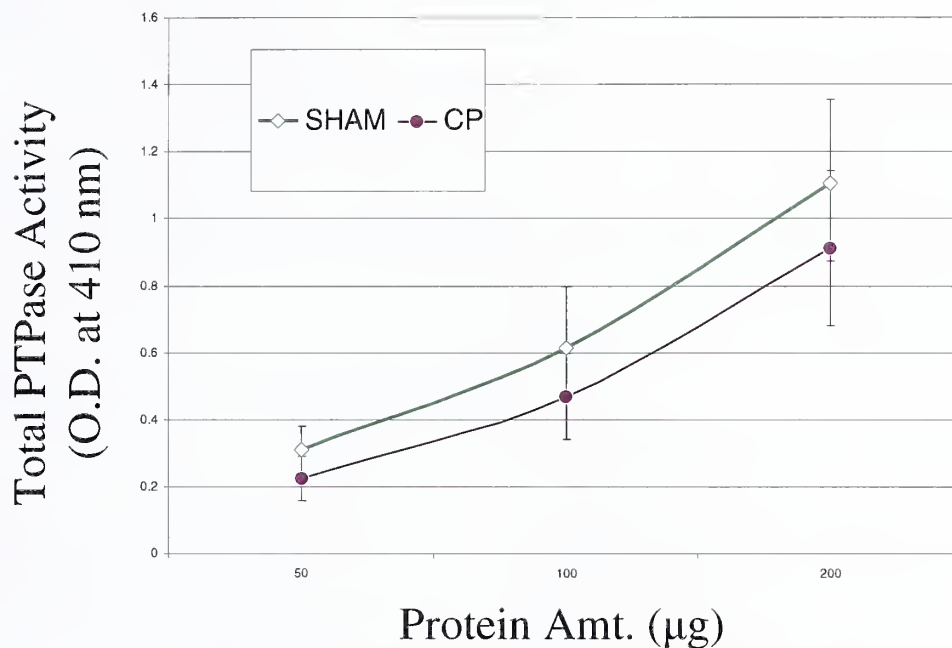


Figure 15: Phosphotyrosine Phosphatase (PTPase) Assay

One possible reason for decreased phosphorylation could be an increase in tyrosine phosphatase activity. No significant changes in total non-IR specific PTPase activity were noted between the sham and CP groups.

Immunolocalization of GLUT2 with goat anti-GLUT2 and anti-goat FITC

The cellular localization of GLUT2 in the adult rat liver is shown in figure 16. Numerous hepatocytes show positive cell membrane staining with punctate low signal cytoplasmic staining. The nucleus is clearly visualized.

Immunolocalization of IR with rabbit anti-IR and anti-rabbit Texas Red

The cellular localization of IR in the adult rat liver is shown in figure 16. Numerous hepatocytes show positive cell membrane staining with punctate low signal cytoplasmic staining. The nucleus is clearly visualized.

Colocalization of GLUT2 and IR and Fluorescent Resonance Energy Transfer (FRET) in the hepatocyte

The same sections of hepatic tissue used to localize GLUT2 and IR independently are superimposed upon each other and viewed in RGB mode. The strongest signals present are on the hepatocyte cell membrane. The majority of cells are seen to display a yellow halo effect in areas where pixels of FITC labeled GLUT2 and Texas Red labeled IR colocalize on the periphery of the cells.

Assuming the absorption spectrum of the acceptor (525-610 nm for Texas Red) overlaps with the fluorescence emission spectrum of the donor (520-600 nm) for FITC, FRET depends on the inverse of the sixth power of intermolecular separation. That distance at which energy transfer occurs is between 10-100 Angstroms. To be more exact in the case of GLUT2 and IR, we approximated the Forster radius (R_0) based on the spectral properties of the donor and acceptor dyes, that is, the distance at which energy transfer is 50% efficient and 50% of excited donors are deactivated. $R_0 = (8.8 \times 10^{23} \times K^2 \times n^{-4} \times QY_D \times J(\lambda))^{1/6}$ Angstroms, where K^2 = the dipole orientation factor, QY_D = the fluorescence quantum yield of the donor in the absence of the receptor, n = the refractive index, and $J(\lambda)$ = the spectral overlap integral. Using the donor/acceptor pair of FITC and tetramethylrhodamine (a dye with similar spectral properties to Texas Red), the Forster radius is approximated to ~55 Angstroms (90).

Samples stimulated in the FITC frequency and collected in the Texas Red emission spectra reveal immunofluorescence on the hepatocyte membrane and diffusely throughout the cytoplasm.

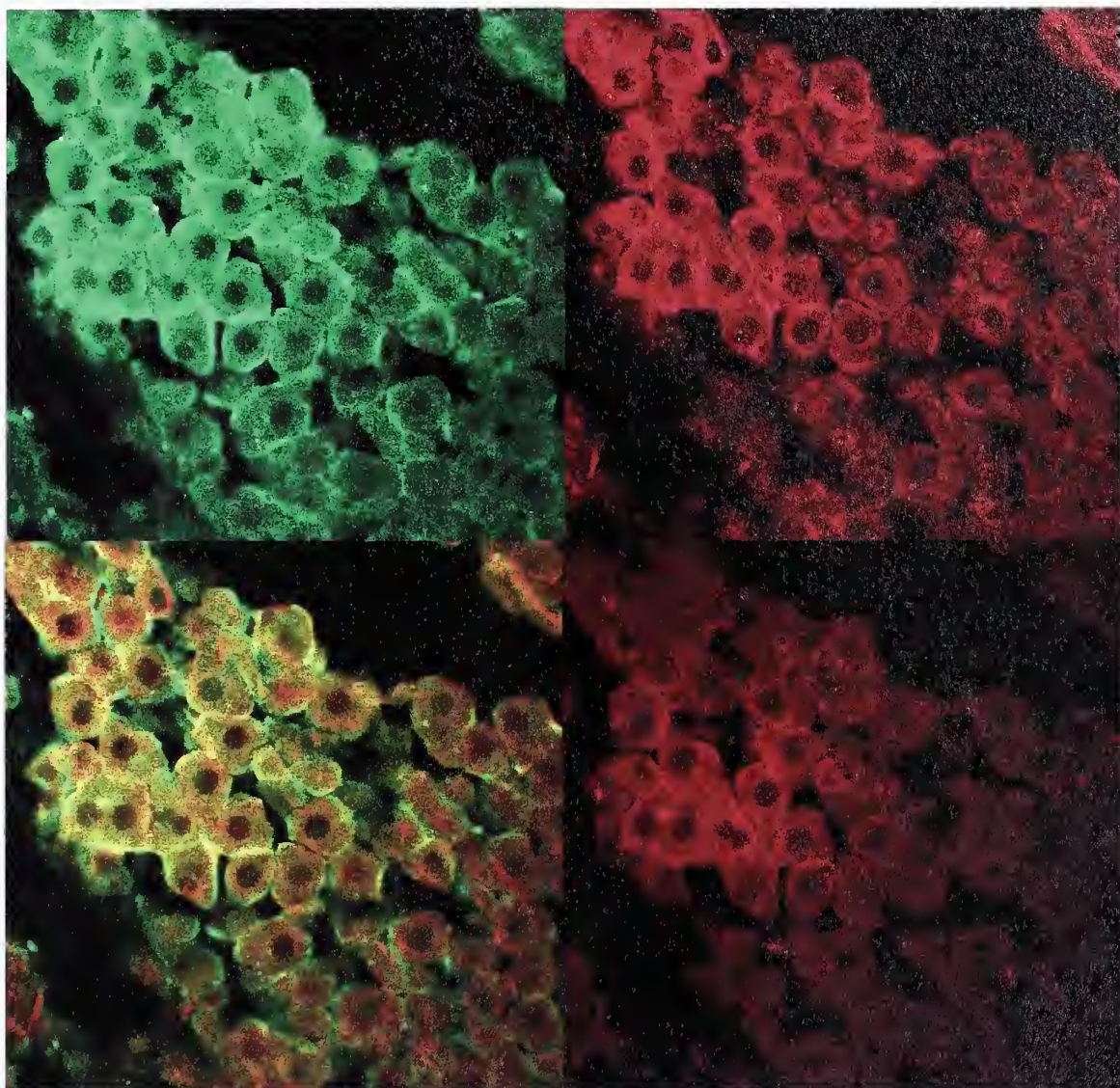


Figure 16: Double-labeled immunofluorescence colocalization of GLUT2 and IR

In these representative images, IR is localized to the cell membrane with FITC (green) conjugated secondary antibody (upper left panel). GLUT2 is localized to the cell membrane with Texas Red (red) conjugated secondary antibody (upper right panel). When both images are viewed together (lower left), colocalization is evident by yellow pixels in areas where GLUT2 and IR are in close proximity. Fluorescent resonance energy transfer (lower right) demonstrates immunofluorescence in the Texas Red emission spectrum after excitation in the FITC spectrum, implying colocalization within 100 Angstroms.

Confocal Microscopy Controls

There is an absence of signal when samples were stimulated for either FITC or Texas Red and emission is collected in both spectra for: blocking solution only, blocking solution only and both secondary antibodies independently and together, only primary antibodies independently and together, rabbit anti-IR primary and anti-goat FITC, and goat anti-GLUT2 primary and anti-rabbit Texas Red.

Co-immunoprecipitation

IR and GLUT2 amounts are expressed as a ratio of total cell lysate signal. When probed for the presence of IR, lysates precipitated with anti-GLUT2 recovered 40.2% of the IR compared to precipitations done with anti-IR antibody (0.43 +/- 0.06 au vs. 1.07 +/- 0.11 au). When probed for the presence of GLUT2, lysates precipitated with anti-IR recovered 25.6% of the GLUT2 compared to precipitations done with anti-GLUT2 antibody (0.31 +/- 0.03 au vs. 1.21 +/- 0.31 au). These results were a significant deviation ($p < 0.05$) from an irrelevant control antibody to the hepatocyte membrane sodium potassium ATPase, which recovered 1.2% of IR and 1.8% of GLUT2 (see figure 17).

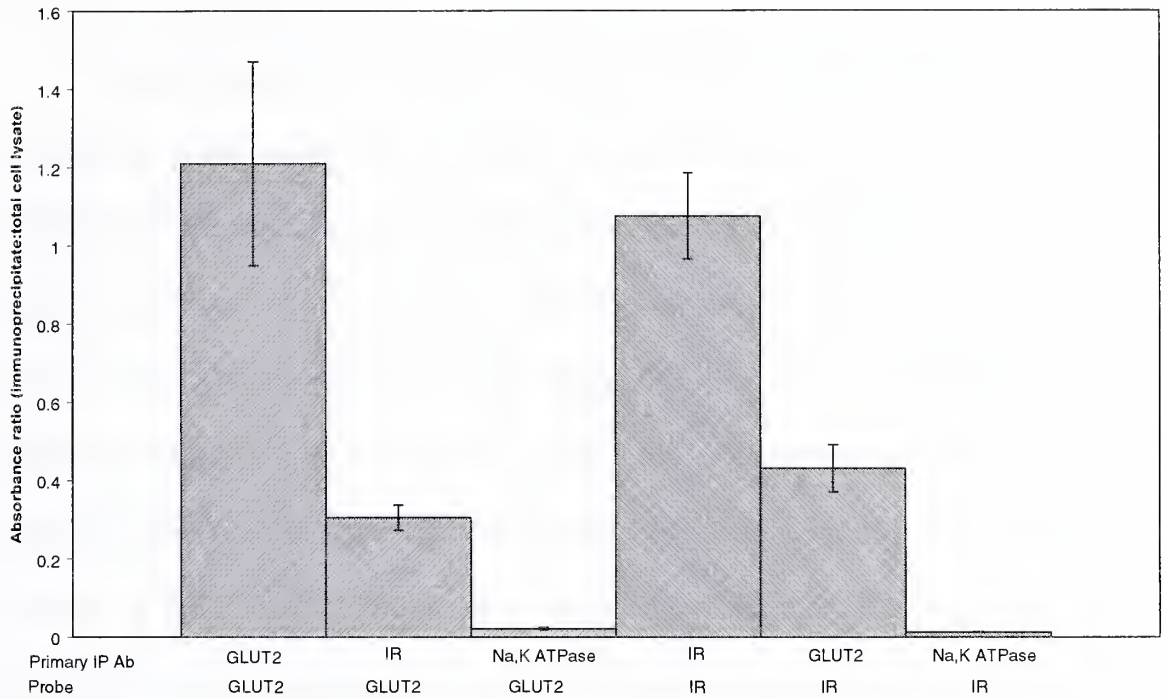


Figure 17: Co-immunoprecipitation of insulin receptor and glucose transporter 2

When probed for the presence of IR, lysates precipitated with antibodies against GLUT2 recovered 40.2% of the IR compared to precipitations done with anti-IR antibody. When probed for the presence of GLUT2, lysates precipitated with antibodies against IR recovered 24.2% of the GLUT2 compared to precipitations done with anti-GLUT2 antibody. Hepatocyte transmembrane antibody against Na, K ATPase recovered only 1.2% of IR and 1.8% of GLUT2.

DISCUSSION

Autophosphorylation of tyrosine residues on IR upon insulin binding may be the first response to the hormone and mediates intracellular protein kinase activities, including glucose regulation (91). Our data demonstrate a 20-fold increase in IR-Tyr phosphorylation and ~5-fold increase in GLUT2-Ser phosphorylation after feeding in sham animals. Although previous studies have shown similar trends in IR-kinase autophosphorylation, these effects have been induced by exogenous or *in vitro* administration of insulin (92). The response seen in sham rats after feeding, therefore, confirms an *in vivo* physiologic model of insulin action that is clinically applicable. In the present study, we have also examined IR and GLUT2 phosphorylation in hepatic cells of both normal and CP rats, and have shown that feeding-stimulated autophosphorylation of IR-Tyr is inhibited ~12 fold and that GLUT2-Ser phosphorylation is decreased ~5 fold in animals with pancreatogenic diabetes.

Activation of a receptor-kinase by IR-Tyr autophosphorylation could be a key mechanism in activating intracellular phosphorylation-dephosphorylation cascades. Furthermore, autophosphorylation of IR alone may be a sufficient catalyst, independent of protein kinase and phosphatase activity, in stimulating the physiologic response of insulin. Therefore, the diminished IR-Tyr phosphorylation can account, at least in part, for the insulin resistance exhibited by rats with pancreatogenic diabetes (93, 94). Additional studies to address the possibility of receptor downregulation, IR trafficking, and ligand binding affinity may further elucidate the molecular mechanisms involved. Alternatively, decreased IR-Tyr phosphorylation may be the first step, with or without

decreased activation of a second messenger, ultimately affecting GLUT2 phosphorylation and glucose transport out of the hepatocyte. That this mechanism is dysfunctional in CP may account for uninhibited hepatic glucose production and subsequent hyperglycemia.

Previous studies *in vitro* have demonstrated changes in glucose transport rates in beta-cells directly accountable to changes in serine phosphorylation, and our results imply a similar *in vivo* mechanism with liver GLUT2 (83). The hepatocyte, however, is unique in that glucose efflux is associated with the fasted state, while the fed state is associated with a decreased rate of transport, or inhibited glucose efflux, *i.e.* in response to an insulin surge. Thus, our data support that the translocation of GLUT2 on and off the hepatocyte cell membrane may be directly linked to the phosphorylation state of the protein, accounting for the dysfunction of hepatic GLUT2 translocation in response to feeding seen in CP (32). Dominant negative or kinase dead insulin receptor studies that compare IR and GLUT2 phosphorylation states may address this mechanism. Accordingly, this characteristic of the hepatocyte implies the opposite direction of net glucose transport, and GLUT translocation, compared to every other cell in the body under the same conditions. It also appears that hepatic GLUT2 functions inversely to GLUT4, with phosphorylation of the transport protein causing a decrease in GLUT2 mediated glucose transport simultaneously with an increase in GLUT4 mediated glucose transport.

The cascade initiated by ligand-receptor binding is regulated by a reciprocal quenching of transduction by the receptor. The mechanisms of quenching are involved in inhibiting the IR response, and include receptor internalization via endocytosis,

receptor desensitization by feedback phosphorylation, receptor ubiquitination and degradation or decreased expression, and dephosphorylation via PTPases. In the CP model studied in our laboratory, IR internalization is inhibited, as measured by an endosome to plasma membrane ratio (84), IR expression has been shown to be reduced (67), and membrane bound IR is diminished (93), though we did not see a significant difference in the total hepatocyte IR purified between sham and CP animals. We have controlled for this possible decrease in plasma membrane IR by expressing and comparing changes in IR-P per amount of IR purified from the cell. It is possible, however, that receptor desensitization by feedback phosphorylation of serine and threonine residues on the IR modulates the insulin response, and this needs to be investigated further in the CP model (33). Previous studies on aged rats have demonstrated insulin resistance to be associated with decreased hepatic IR autophosphorylation, due to elevated IR PTPase activity (95). In addition, studies have shown that the impaired translocation of GLUT4 resulting from overexpression of PTP 1B can be reversed when cells are treated with a PTPase inhibitor (96). Therefore, we chose to investigate if the decrease in IR phosphorylation in pancreatogenic diabetes was secondary to increased tyrosine PTPase activity. Our total PTPase assay revealed that total tissue PTPase activity is not significantly altered in CP. However, it is possible that IR tyrosine-specific PTPase activity may account for the results observed (73). Further studies to assess this possibility are currently in progress.

Insulin binding to the insulin receptor induces a conformational change and autophosphorylation of the intracellular beta subunit, propagating activation of multiple

downstream targets, including PI3-kinase. Recently, multiple new components of the insulin receptor signaling cascade have been discovered and their cDNA clones isolated. Particularly exciting has been the current implications of PI3 kinase and Akt/PKB activation as a required component in glucose transport regulation by insulin in skeletal muscle cells, adipocytes, and in the liver (51, 71, 72). To date, however, there has been no study to imply a direct connection between the IR and a glucose transport protein, either spatially or chemically.

The importance of hepatic glucose production and regulation, however, cannot be over emphasized, and the role of the low affinity, high Km hepatic glucose transport protein GLUT2 in response to insulin is as yet unclear. Previous experiments in our laboratory have confirmed a parallel increase in endosome to plasma membrane ratios upon feeding in both IR and GLUT2, suggesting physical or signal coupling between the proteins (84). Moreover, studies have correlated glucose transport with the in vitro phosphorylation state of GLUT2 (83), and our current study shows decreased phosphorylation of both GLUT2 and IR in CP, implying an intimate connection between GLUT2 and a kinase, or a series of kinase proteins in the hepatocyte. These data, in conjunction with insulin resistant and frankly diabetic syndromes with genetic defects in GLUT2, i.e., Fanconi-Bickel syndrome and homozygous GLUT2 knockout mice, support a cell-signaling relationship between insulin, via the hepatic IR, and GLUT2.

We show, for the first time, that GLUT2 is colocalized spatially and chemically to the hepatic IR, supporting their function as a receptor/transporter complex. GLUT2 and IR can be co-immunoprecipitated significantly relative to a ubiquitous non-tyrosine kinase hepatocyte protein, displaying ~33-fold recovery of IR by an anti-GLUT2

antibody over the control, and ~14 fold recovery of GLUT2 by an anti-IR antibody over the control. Further experiments to specifically address differences in phosphorylated amino acid residues between co-immunoprecipitated and non-co-immunoprecipitated proteins may strengthen the argument for a receptor-transporter communication complex.

That each protein co-precipitates a fraction of the other implies that they are not complexed together in all parts of the cell. A significant portion of each must exist independent of the other, though some of these pools may be subunits of whole protein, inactive molecules, or compartmentalized intracellularly. That ~25% of GLUT2 is bound to all of the hepatocyte IR suggests alternate regulation of the protein 3/4 of the time. However, it is important to note that significantly more (40%) IR co-precipitates when immunoprecipitated with all of the hepatocyte GLUT2, demonstrating that GLUT2 more often colocalizes with IR than vice-versa. It is known that the majority of GLUT2, over 90%, sits on the hepatocyte membrane (97), therefore, it is feasible to conclude from the data that colocalization is more quantifiable on the cell membrane than intracellularly, and this is supported by confocal microscopy imaging.

Double label immunofluorescence has been established as a method of colocalization in a number of studies (98-101). Although IR has been colocalized with IGF/IR hybrid receptors, GLUT4 has been localized to the cell surface, and GLUT2 has been localized to the beta cell membrane, IR has not been studied in relation to GLUT2 or 4 (82, 102-104). When viewed with confocal microscopy in our experiments, both GLUT2 and IR clearly colocalize on the hepatocyte membrane. FRET analysis of the liver tissue confirms energy transfer between the proteins of interest. That distance at which energy transfer occurs is calculated to be between 10-100 Angstroms. To be more

exact in the case of GLUT2 and IR, we calculated the Forster radius (R_o) based on the spectral properties of similar donor and acceptor dyes, at approximately 55 Angstroms. Combined, these data support a unique and specific physical interaction between the proteins. It is implied from these results that GLUT2 and IR maintain a fixed spatial relationship as a complex on the cell membrane and through endocytosis.

In conclusion, it has been shown that phosphorylation defects in the hepatic IR are concomitant with decreased phosphorylation in GLUT2 in pancreatogenic diabetes. Our data imply that this parallel deficiency may not be coincidental, but secondary to a direct cellular signaling cascade from the IR to GLUT2. It is possible that defects in this cross-talk between hepatic GLUT2 and IR are responsible for insulin resistance and unimpaired hepatic glucose production seen in pancreatogenic diabetes. The dysfunction may be at the level of the IR, in intermediate signaling proteins, in docking and fusion proteins, or in spatial relationships between GLUT2 and IR, opening further directions for study into the phosphorylation states of PI3 kinase, Akt/PKB, and IRS proteins; the functional roles of vesicle associated membrane proteins (VAMPs), soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), and other vesicle proteins; and real time atomic force microscopy of IR-GLUT2 interactions.

Furthermore, it appears that hepatic GLUT2 and IR are arranged on the hepatocyte membrane in a specific spatial relationship. That they co-immunoprecipitate significantly relative to a ubiquitous control protein implies an alternate, functional cytoarchitectural arrangement. We therefore propose that an IR-GLUT2 complex exists in hepatocytes, and that this receptor-transporter unit serves as the principal regulatory mechanism of hepatic glucose production. Together, these novel experiments introduce

an elegant intracellular relationship between a receptor and a transporter, explaining how the insulin response is propagated, how involved proteins are signaled and translocate, and how dysfunction causes insulin resistance; stimulating exciting directions for further research in cell signaling of the IR and the molecular mechanisms of insulin resistance. Pharmacologic and gene targeting of these defects holds promise for the future of patients with pancreatogenic diabetes and diabetes mellitus.

REFERENCES

LIVER, n. A large red organ thoughtfully provided by nature to be bilious with. The sentiments and emotions which every literary anatomist now knows to haunt the heart were anciently believed to infest the liver; and even Gascoygne, speaking of the emotional side of human nature, calls it "our hepaticall parte." It was at one time considered the seat of life; hence its name -- liver, the thing we live with. The liver is heaven's best gift to the goose; without it that bird would be unable to supply us with the Strasbourg pate_.

-Ambrose Bierce (1842 - 1914), *The Devil's Dictionary*

*I'm tired of all this nonsense about beauty being only skin-deep. That's deep enough.
What do you want, an adorable pancreas?*

- Jean Kerr

1. Cawley T. 1788. A singular case of diabetes consisting entirely in the quality of the urine with an inquiry into the different theories of that disease. *London Medical Journal*. 9:286-308.
2. Marks IN, Bank S. Etiology, clinical aspects, and medical management. In: *Bockus Gastroenterology*. Philadelphia: W.B. Saunders; 1985. p 4020-4040.
3. Von Mering J, Minkowski O. 1889. Diabetes mellitus nach pankreas extirpation. *Arch Exp Pathol Pahrmacol*. 26:371-381.
4. Bliss M. *The Discovery of Insulin*. Chicago: University of Chicago Press; 1982.
5. Steer ML, Waxman I, Freedman S. 1995. Chronic Pancreatitis. *The New England Journal of Medicine*. 332(22):1482-1489.
6. Mergener K, Baillie J. 1997. Chronic Pancreatitis. *Lancet*. 350:1379-85.
7. Samer M, Cotton P. 1984. Classification of Pancreatitis. *Gut*. 25:756-9.
8. Slezak LA, Andersen D. 2000. Pancreatic resection: effects on glucose metabolism. *World Journal of Surgery*. (in print).
9. Nealon WH, Beauchamp RD, Townsend CM Jr, Boyd G, Shabot M, Thompsom J. 1986. Diagnostic Role of Gastrointestinal Hormones in Patients with Chronic Pancreatitis. *Ann Surg*. 204:430-437.

10. Valenzuela JE, Taylor IL, Walsh J. 1979. Pancreatic polypeptide response in patients with chronic pancreatitis. *Dig Dis Sci.* 24:862-864.
11. Glaser B, Vinik AI, Sive AA, Jr JF. 1980. Plasma human pancreatic polypeptide responses to administered secretin: effects of surgical vagotomy, cholinergic blockade, and chronic pancreatitis. *J Clin Endocrinol Metab.* 50:1094-1099.
12. Donowitz M, Hendler R, Spiro HM, Binder HJ, Felig P. 1975. Glucagon secretion in acute and chronic pancreatitis. *Ann Int Med.* 83:778-781.
13. Kalk WJ, Vinik AI, Bank S, Buchanan KD, Keller P, Jackson W. 1974. Glucagon responses to arginine in chronic pancreatitis: possible pathogenic significance in diabetes. *Diabetes.* 23:257-263.
14. Owyang C, Levitt M. Chronic Pancreatitis. In: Yamada T, Alpers DH, Owyang C, Powell DW, Silverstein SE, editors. *Textbook of Gastroenterology.* New York: Lippincott; 1991. p. 1874-93.
15. Olsen T. 1978. The incidence and clinical relevance of chronic inflammation in the pancreas in autopsy material. *Acta Pathol Microbiol Scand.* 86(sect. A):361-365.
16. Larsen S. 1993. Diabetes mellitus secondary to chronic pancreatitis. *Danish Medical Bulletin.* 40(2):153-160.
17. Sachel J, Sarles H. 1979. Modifications of pure human pancreatic juice induced by chronic alcohol consumption. *Dig Dis Sci.* 24:897-905.
18. Bank S, Marks IN, Vinik A. 1975. Clinical and hormonal aspects of pancreatic diabetes. *Am J Gastroenterol.* 64:13-22.
19. Larsen S, Hilsted J, Tronier B, Worning H. 1987. Metabolic control and beta cell function in patients with insulin dependent diabetes mellitus secondary to chronic pancreatitis. *Metabolism.* 36:964-967.
20. Vinik A. Insulin secretion in chronic pancreatitis. In: Tiengo A, Alberti K, Del Prato S, Vranic M, editors. *Diabetes Secondary to Pancreatopathy.* New York: Elsevier Science Publishers; 1988. p. 35-50.
21. Linde J, Nilsson LH, Barany FR. 1977. Diabetes and hypoglycemia in chronic pancreatitis. *Scand J Gastroenterol.* 12:369-373.
22. Alberti K. Diabetes secondary to pancreatopathy: an example of brittle diabetes. In: *Diabetes secondary to pancreatopathy.* Amsterdam: Elsevier Science Publishers; 1988. p. 211-214.

23. Raue G, Keim V. 1999. Secondary diabetes in chronic pancreatitis. *Zeitschrift für Gastroenterologie*. Suppl 1:4-9.
24. Larsen S, Hilsted J, Philipsen EK, Lund-Andersen H, Parving HH, Worning H. 1990. A comparative study of microvascular complications in patients with secondary and type I diabetes. *Diabet Med*. 7:815-818.
25. Dresler CM, Fortner JG, McDermott K, Bajorunas D. 1991. Metabolic consequences of total pancreatectomy. *Ann Surg*. 214:131-140.
26. Terrettaz J, Jeanrenaud B. 1983. In vivo hepatic and peripheral insulin resistance in genetically obese (fa/fa) rats. *Endocrinology*. 112:1346-1351.
27. DeFronzo RA, Simonson D, Ferranini E. 1982. Hepatic and Peripheral Insulin Resistance: A Common Feature of type-2 and type-1 Diabetes Mellitus. *Diabetologia*. 23:313-319.
28. Seymour NE, Turk JB, Laster MK, Tanaka Y, Rosenberg HE, Rademaker EA, et al. 1988. In vitro hepatic insulin resistance in chronic pancreatitis in the rat. *J Surg Res*. 46:450-456.
29. Tanaka Y, Druck P, Brunicardi FC, Chance RE, Gingerich RL, Elahi D, et al. 1987. Reversal of abnormal glucose production in chronic pancreatitis by administration of gastric inhibitory polypeptide and pancreatic polypeptide. *Surg Forum*. 38:149-151.
30. Yki-Jarvinen H, Kiviluoto T, Taskinen M. 1986. Insulin resistance is a prominent feature of patients with pancreatogenic diabetes. *Metabolism: Clinical and Experimental*. 35(8):718-27.
31. Andersen DK. The role of pancreatic polypeptide in glucose production. In: JC Thompson, editor. *Gastrointestinal Endocrinology Receptor and Post-receptor Mechanisms*. San Diego: Academic Press; 1990. p. 333-357.
32. Andersen DK, Ruiz CL, Burant C. 1994. Insulin regulation of hepatic glucose transporter protein is impaired in chronic pancreatitis. *Ann Surg*. 219(6):679-687.
33. Haring H, Obermaier-Kusser B. The Insulin Receptor: Its role in insulin action and in the pathogenesis of insulin resistance. In: Alberti K and Krall LP, editors. *The Diabetes Annual/5*. New York: Elsevier Science Publishers; 1990. p. 537-555.
34. Carpentier J. 1989. The cell biology of the insulin receptor. *Diabetologia*. 32:627-635.

35. Goldfine ID, Purello F, Vigneri R, Clawson G. Direct regulation of nuclear functions by insulin. In: Czech M, editor. *Molecular Basis of Insulin Action*. New York: Plenum Press; 1985. p. 329.
36. Goldfine I. 1987. The Insulin Receptor: molecular biology and transmembrane signaling. *Endocrine Reviews*. 8(3):235-255.
37. Podlecki DA, Smith RM, Kao M, Tsai P, Huecksteadt T, Brandenburg D, et al. 1987. Nuclear translocation of the insulin receptor: a possible mediator of insulin's long term effect. *J Biol Chem*. 262:3362-3368.
38. White MF, Kahn C. 1994. The insulin signaling system. *J Biol Chem*. 269:1-4.
39. Rosen O. 1989. Structure and function of the insulin receptor. *Diabetes*. 38:1508-1511.
40. Treadway JL, Fratelli AL, Pessin J. 1992. Intramolecular subunit interactions between insulin and IGF-1 alpha beta half receptors induced by ligand and Mn/Mg ATP binding. *Biochemistry*. 31:11801-11805.
41. Kasuga M, Karlsson FA, Kahn C. 1982. Insulin stimulates the phosphorylation of the beta subunit of its own receptor. *Science*. 217:185-187.
42. Kasuga M, Zick M, Blithe DL, Karlsson FA, Haring HU, Kahn C. 1982. Insulin stimulation of phosphorylation of the beta subunit of the insulin receptor. *J Biol Chem*. 257:9891-9894.
43. Cobb MH, Rosen O. 1984. The insulin receptor and tyrosine protein kinase activity. *Biochem Biophys Acta*. 738:1-8.
44. Kahn C. 1985. The molecular mechanism of insulin action. *Annu Rev Med*. 36:429-451.
45. Pillion DJ, Kim S, Kim H, Meezan E. 1992. Insulin Signal Transduction: The role of protein phosphorylation. *The American Journal of the Medical Sciences*. 303(1):40-51.
46. Sun XJ, Wang LM, Zhang Y, Yenush L, Myers MG Jr, Glasheen E, et al. 1995. Role of IRS-2 in insulin and cytokine signaling. *Nature*. 377:173-177.
47. Saltiel A. 1996. Diverse signaling pathways in the cellular actions of insulin. *Am J Physiol*. 270:E375-E385.
48. Kasuga M, Zick Y, Blithe DL, Crettaz M, Kahn C. 1982. Insulin stimulates tyrosine phosphorylation of the insulin receptor in a cell-free system. *Nature*. 298:667.

49. White MF, Shoelson SE, Keutmann H, Kahn C. 1988. A cascade of tyrosine autophosphorylation in the beta subunit activates the insulin receptor. *J Biol Chem.* 263:2969-80.
50. Beck-Nielsen H, Hother-Nielsen O. Insulin resistance. In: Alberti KG, Krall L, editors. *The Diabetes Annual*. New York: Elsevier Science Publishers; 1988. p. 565-591.
51. Pessin JE, Saltiel A. 2000. Signaling pathways in insulin action: molecular targets of insulin resistance. *J Clin Invest.* 106(2):165-170.
52. Kahn CR, Neville DM Jr, Roth J. 1973. Insulin receptor interaction in the obese hyperglycemic mouse: a model of insulin resistance. *J Biol Chem.* 248:244-250.
53. Soll AH, Kahn CR, Neville DM Jr, Roth J. 1975. Insulin receptor deficiency in genetic and acquired obesity. *J Clin Invest.* 56:769-780.
54. Kern P, Picard J, Caron M, Veissiere D. 1975. Decreased binding of insulin to liver plasma membrane receptors in hereditary diabetic mice. *Biochem Biophys Acta.* 389:281-289.
55. Archer JA, Gordon P, Roth J. 1975. Defect in insulin binding to receptors in obese man: amelioration with caloric restriction. *J Clin Invest.* 55:166-174.
56. Harrison LC, Martin FI, Melick R. 1976. Correlation between insulin receptor binding in isolated fat cells and insulin sensitivity in obese human subjects. *J Clin Invest.* 58:1435-1441.
57. Olefsky J. 1976. The insulin receptor: its role in insulin resistance in obesity and diabetes. *Diabetes.* 27:1175-1181.
58. Beck-Nielsen H. 1978. The pathogenic role of an insulin receptor defect in diabetes mellitus of the obese. *Diabetes.* 27:1175-1181.
59. Soll AH, Kahn CR, Neville DM. 1975. Insulin binding to liver plasma membranes in the obese hyperglycemic mouse: demonstration of a decreased number of functionally normal receptors. *J Biol Chem.* 250:4702-4707.
60. Caro JF, Sinha MK, Raju SM, Ittoop O, Pories WJ, Flickinger EG, et al. 1987. Insulin receptor kinase in human skeletal muscle from obese subjects with and without noninsulin dependent diabetes. *J Clin Invest.* 79:1330-1337.
61. Olefsky JM, Reaven G. 1974. Decreased insulin binding to lymphocytes from diabetic patients. *J Clin Invest.* 54:1323-1328.

62. Olefsky JM, Reaven G. 1977. Insulin binding in diabetes: relationships with plasma insulin levels and insulin sensitivity. *Diabetes*. 26:680-688.
63. Maegawa H, Shigeta Y, Egawa K, Kobayashi M. 1991. Impaired autophosphorylation of insulin receptors from abdominal skeletal muscles in nonobese subjects with NIDDM. *Diabetes*. 40:815-819.
64. Damm P, Handberg A, Kuhl C, Beck-Nielsen H, Molsted-Pedersen L. 1993. Insulin receptor binding and tyrosine kinase activity in skeletal muscle from normal pregnant women with gestational diabetes. *Obstet Gynecol*. 82:251-259.
65. Olefsky JM, Johnson J, Liu P, Jen P, Reaven G. 1975. The effect of acute and chronic dexamethasone administration on insulin binding to isolated rat adipocytes. *Metabolism*. 24:517-524.
66. Krook A, O'Rahilly S. 1996. Mutant insulin receptors in syndromes of insulin resistance. *Baillieres Clin Endocrinol Metab*. 10:97-122.
67. Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, et al. 1994. Insulin resistance and growth retardation in mice lacking IRS1. *Nature*. 372:182-186.
68. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, et al. 1998. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature*. 391:900-904.
69. Kido Y, Burks DJ, Withers D, Bruning JC, Kahn CR, White MF, et al. 2000. Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS1, and IRS2. *J Clin Invest*. 105(2):199-205.
70. Czech MP, Corvera S. 1999. Signaling mechanisms that regulate glucose transport. *J Biol Chem*. 274:1865-1868.
71. Nowano M, Ueta K, Oku A, Arakawa K, Saito A, Funaki M, et al. 1999. Hyperglycemia impairs the insulin signaling step between PI3 kinase and Akt/PKB activations in ZDF rat liver. *Biochem Biophys Res Commun*. 266(1):252-6.
72. Kohn AD, Summers SA, Birnbaum MJ, Roth R. 1996. Expression of constitutively active Akt ser/thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem*. 271:31372-31378.
73. Walchli S, Curchod ML, Gobert RP, Arkininstall S, Hooft van Huijsduijnen R. 2000. Identification of tyrosine phosphatases that dephosphorylate the insulin receptor. *J Biol Chem*. 275(13):9792-6.

74. Thorens B, Sarkar HK, Kaback HR, Lodish H. 1988. Cloning and functional expressions in bacteria of a novel glucose transporter present in liver, intestine, kidney, and pancreatic islet. *Cell*. 55:281-290.
75. Thorens B, Cheng ZQ, Brown D, Lodish H. 1990. Liver glucose transporter. *Am J Physiol*. 259:C279.
76. Mueckler M. 1990. Family of glucose transporter genes: implications for glucose homeostasis and diabetes. *Diabetes*. 39:6-11.
77. Bell GI, Kayano T, Buse JB, Burant CF, Takeda J, Lin D, et al. 1990. Molecular biology of mammalian glucose transporters. *Diabetes Care*. 13:198-208.
78. Thorens B, Charron BJ, Lodish H. 1990. Molecular physiology of glucose transporters. *Diabetes Care*. 13:209-218.
79. Thorens B. 1996. Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes. *Am J Physiol*. 270:541-53.
80. Suzuki K, Kono T. 1980. Evidence that insulin causes translocation of glucose transport activity to the plasma membrane from an intracellular storage site. *Proc Natl Acad Sci USA*. 77:2542-2545.
81. Cushman SW, Wardzala L. 1980. Potential mechanism of insulin action on glucose transport in the isolated rat adipose cell: apparent translocation of intracellular transport systems to the plasma membrane. *J Biol Chem*. 255:4758-4762.
82. Nathan JD, Zdankiewicz, Wang JP, Andersen D. 1997. Internalization of hepatocyte glucose transport protein after feeding is impaired in chronic pancreatitis. *Pancreas*. 15:448.
83. Thorens B, Deriaz N, Bosco D, DeVos A, Pipeleers D, Schuit F, et al. 1996. Protein Kinase A-dependent phosphorylation of GLUT2 in pancreatic beta cells. *J Biol Chem*. 271(14):8075-8081.
84. Slezak L, Nathan JD, Zdankiewicz PD, Spector SA, Wang JP, Jena BP, et al. 1998. Impaired Hepatocyte Insulin Receptor Internalization in Chronic Pancreatitis. *Surg Forum*. 49:152-3.
85. Mundlos S., Adler G, Scharr M, Koop I, Arnold R. 1986. Exocrine pancreatic function in oleic acid-induced pancreatic insufficiency. *Pancreas*. 1(1):29-36.
86. Beynon RJ, Bond J, editors. *Proteolytic Enzymes: A Practical Approach*. IRL Press; 1989.

87. Seargeant LE, Stinson R. 1979. Inhibition of human alkaline phosphatases by vanadate. *Biochem J.* 181:247-250.
88. Cohen P, Klumpp S, Schelling D. 1989. An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. *FEBS Lett.* 250(2):596-600.
89. Bradford M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72:248-254.
90. Molecular Probes Inc. *Handbook of Fluorescent Probes and Research Chemicals.* Eugene, Oregon; 7th ed. 2001.
91. Rizzo MA, Romero G. 1998. The role of G proteins in insulin signalling. *J Basic Clin Physiol Pharmacol.* 9(2-4):167-195.
92. Ahmad F, Goldstein B. 1995. Alterations in specific protein-tyrosine phosphatases accompany insulin resistance of streptozocin diabetes. *The American Physiological Society.* 0193-1849:E932-E940.
93. Seymour NE, Volpert AR, Lee EL, Andersen DK, Hernandez C. 1995. Alterations in hepatocyte insulin binding in chronic pancreatitis: effects of pancreatic polypeptide. *Am J Surg.* 169:105-109.
94. Andersen DK, Ruiz CL, Dong X, Hull EC, Seymour NE, Durant C. 1994. Rapid hormone induced changes in hepatic insulin binding are impaired in chronic pancreatitis. *Dig Dis Sci.* 39:1741.
95. Nadiv O, Shinitzky M, Manu H, Hecht H, Roberts CT, LeRoith D, et al. 1994. Elevated protein tyrosine phosphatase activity and increased membrane viscosity are associated with impaired activation of the insulin receptor kinase in old rats. *Biochem J.* 298:743-50.
96. Chen H, Cong LN, Li Y, Yao ZJ, Wu L, Zhang ZY, et al. 1999. A phosphotyrosyl mimetic peptide reverses impairment of insulin-stimulated translocation of GLUT4 caused by overexpression of PTP 1B in rat adipose cells. *Biochemistry.* 38(1):384-9.
97. Ciaraldi TP, Horuk R, Matthaei S. 1986. Biochemical and functional characterization of the rat liver glucose transport system. *Biochem J.* 240:115-123.
98. Reddy S, Young M, Poole CA, Ross J. 1998. Loss of GLUT2 precedes insulin loss in the nonobese diabetic and the low dose streptozotocin mouse models: a comparative immunohistochemical study by light and confocal microscopy. *Gen Comp Endocrinol.* 111(1):9-19.

99. Reddy S, Elliott RB, Poole CA, Ross J. 1997. Double-label immunofluorescence study of glutamic acid decarboxylase in the fetal and ovine pancreas by light and confocal microscopy: evidence for predominant beta cell coexpression. *Gen Comp Endocrinol.* 106:301-309.
100. Wouterlood FG, VanDenderen JCM, Blijleven N, Minnen JV, Hartig W. 1998. Two-laser dual immunofluorescence confocal laser scanning microscopy using cy-2 and cy-5 conjugated secondary antibodies: unequivocal detection of co-localization of neuronal markers. *Brain Research Protocols.* 2:149-159.
101. Silver MA, Stryker M. 2000. A method for measuring colocalization of presynaptic markers with anatomically labeled axons using double label immunofluorescence and confocal microscopy. *J Neurosci Methods.* 94:205-215.
102. Karagiannis SN, King RH, Thomas P. 1997. Colocalization of insulin and IGF-1 receptors in cultured rat sensory and sympathetic ganglion cells. *J Anat.* 191(3):431-40.
103. Noma Y, Bonner-Weir S, Latimer JB, Davalli AM, Weir G. 1996. Translocation of glucokinase in pancreatic beta cells during acute and chronic hyperglycemia. *Endocrinology.* 137(4):1485-91.
104. Malide D, Dwyer NK, Blanchette-Mackie EJ, Cushman S. 1997. Immunocytochemical evidence that GLUT4 resides in a specialized translocation post-endosomal VAMP2-positive compartment in rat adipose cells in the absence of insulin. *J Histochem Cytochem.* 45(8):1083-96.

APPENDIX

Publications

1. **Maker AV**, Jena BP, Aspelund G, Slezak L, Sritharan K, Ranganath S, Andersen DK. In vivo phosphorylation of the hepatic insulin receptor is inhibited in chronic pancreatitis. *NSRF Syllabus*, 67, 1999.
2. **Maker AV**, Slezak L, Aspelund G, Sritharan K, Jena BP, Andersen DK. Impaired phosphorylation of hepatic insulin receptor and GLUT2 in chronic pancreatitis. *Surgical Forum*, Vol L, 14. 1999.
3. Sritharan K, Aspelund G, Slezak L, **Maker AV**, Jena BP, Andersen DK. Impaired recruitment of hepatocyte vesicle associated membrane proteins (VAMP) by feeding in chronic pancreatitis, 2000. (in preparation)
4. **Maker AV**, Slezak L, Sritharan K, Jena BP, Andersen DK. Insulin receptor and GLUT2 phosphorylation dysfunction in chronic pancreatitis, 2000. (in preparation)
5. **Maker AV**, Eisenberg M, Sritharan K, Geibel J, Andersen DK. Colocalization of glucose transport protein 2 and the hepatic insulin receptor, 2000. (in preparation)
6. Eisenberg M, **Maker AV**, Sritharan K, Geibel J, Andersen DK. Colocalization of insulin receptor and glucose transporter 2 proteins in rat hepatocytes. *Gastroenterology*. 2001. (abstract in press)

Invited presentations

1. "In Vivo Phosphorylation of the Hepatic Insulin Receptor is Inhibited in Chronic Pancreatitis," oral presentation, National Student Research Forum, Galveston, TX, 1999.
2. "Impaired Phosphorylation of Hepatic Insulin Receptor and GLUT2 in Chronic Pancreatitis," oral presentation, The American College of Surgeons 85th Annual Clinical Congress, San Francisco, CA, 1999.
3. "Mechanisms of Insulin Resistance in Pancreatogenic Diabetes," oral presentation, Surgical Grand Rounds, Yale School of Medicine Department of Surgery, 2000.
4. "Colocalization of insulin receptor and glucose transporter 2 proteins in rat hepatocytes," poster presentation, American Gastroenterological Association Annual Meeting, Atlanta, GA, 2001.

Research awards

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