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Hepatic Insulin Removal Following Oral Glucose Loading in Nonobese Subjects with Mild Type II Diabetes Mellitus

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ABSTRACT

Non Insulin Dependent Diabetes Mellitus (NIDDM) is a heterogeneous disorder characterized by multiple defects leading ultimately to the derangements in carbohydrate metabolism. Studies of the possible factors implicated in the pathogenesis of NIDDM are frequently conflicting, however there seems to be agreement on some of the features common to the majority of affected patients. The most commonly feature observed is insulin resistance, present in both peripheral and hepatic cells. Insulin secretion is inappropriate, varying from low to high. There seems to be an inverse relationship between fasting glucose values and the secretory response. Fasting insulin values are usually normal or increased. Decreased binding of insulin to its receptors is associated with a deficit in their number and, possibly, with the efficacy of the transduction of the hormonal signal.

Although glucose intolerance is a common feature among these patients, the question of the primary factor involved in the development of the multiple derangements in glucose metabolism remains open. One of the early features in this disease is hyperinsulinemia. This can arise both from an increased secretion and a decreased removal. Since removal of insulin takes place primarily in the liver, this study has concentrated on evaluating the possible contributions of changes in liver uptake to glucose intolerance. Insulin secretion was simultanecusly monitored.

The level of hepatic uptake of insulin during basal conditions and after glucose loading was assessed in non-obese subjects with mild diabetes and compared to that of a healthy control group. This was done using a noninvasive

approach based on the insulin and C-peptide responses to an oral glucose tolerance test combined with the assessment of the basic kinetic parameters following the bolus injection of C-peptide and tracer insulin. The differences in hepatic handling of insulin and C-peptide were utilized in the experimental design.

The study was performed in two stages:

Stage 1; The population studied consisted of 32 adult volunteers, divided into three groups based on their level of glucose tolerance (controls, subjects with mild diabetes and subjects with advanced diabetes). This was done based on the results of an oral glucose tolerance test (OGTT) according to the diagnostic guidelines of the National Diabetes Data Group. The data based on the OGTT were also analyzed in the context of overall secretory response to the glucose stimulation. The areas under the glucose, insulin and C-peptide curves were calculated. The ratios of total C-peptide response to that of insulin were examined. The correlation of these ratios to the degree of glucose intolerance expressed as the area under the glucose concentration curves was then investigated.

Stage 2; Five control subjects and six subjects with mild diabetes (age, weight and sex matched) were administered a bolus injection of C-peptide and labeled insulin. The responses obtained from the decay curves combined with the C-peptide and insulin data from the OGTT were used to calculate the total secretion of both peptides during this test. The assessment of insulin secretion was made based on the C-peptide data. Insulin data was used to calculate posthepatic insulin appearance. The fractional extraction by the liver was then evaluated based on the ratio of the posthepatic to the prehepatic insulin appearance.

In order to perform calculations, it was necessary to make the following assumptions:

- the insulin system is piecewise linear in the range considered,

- the hepatic uptake of C-peptide is negligible,

- the metabolic clearance of insulin is different during basal conditions and after glucose loading, but stable during both periods and is directly proportional to its hepatic uptake.

A set of equations based on those assumptions was employed to calculate the desired parameters.

In addition, free fatty acid (FFA) concentrations were measured during the basal state and at hourly intervals thereafter.

It was demonstrated that metabolic clearance was identical under basal conditions (13.1±1.5 ml $kg^{-1}ml^{-1}$ - controls and 13.6±2.0 ml $kg^{-1}ml^{-1}$ - subjects with mild diabetes). Both the metabolic clearance rate and hepatic extraction of insulin fell by 20% in the control group following the glucose load (10.5±1.5 ml $kg^{-1}ml^{-1}$ 39±9%, n.s). However, the decrease in the group of subjects with mild diabetes was found to be approximately 50% ($p<0.05$). The ratios of the integrated insulin to C-peptide concentrations (in arbitrary units) showed a close relationship to the decline in the fractional hepatic extraction after glucose loading. It was found to be 0.21±0.03 for the control group and 0.38±0.05 for the subjects with mild diabetes($p<0.05$). These ratios also demonstrated a correlation with a degree of glucose intolerance expressed as the integrated glucose concentrations for each subject ($r=0.6$). This implies that the ratios of areas under the concentration curves can be used as a rough estimate of changes in the

hepatic extraction of insulin. The increase in the levels of circulating insulin resulted primarily from the fall in the hepatic insulin extraction, because based on the C-peptide data, there was no significant increase in the insulin secretion. The integrated insulin concentrations were 100% greater in the glucose intolerant group when compared with controls (137±24 vs. 73±12), while there was no significant difference in the integrated C-peptide concentrations (369±51 vs. 340±28). It is suggested, therefore that the decrease in insulin removal by the liver, and not hypersecretion can explain the hyperinsulinemia seen in the patients with mild diabetes which were studied here and therefore could be one of the early defects in this disease.

Although the levels of free fatty acids were higher in the group of subjects with mild diabetes, basal levels were not significantly different and decreased to the same extent in both cases, suggesting that this is not the major determinant of the changes in hepatic insulin uptake.

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1. INTRODUCTION

Diabetes mellitus is the most common metabolic disease. This disorder resulting from a variable interaction of hereditary and environmental factors is characterized by abnormal insulin secretion, elevated blood glucose levels and a number of end organ complications like nephropathy, retinopathy, neuropathy and accelerated atherosclerosis (Atkinson et al, 1990). The prevalence of diabetes has increased dramatically over the past 20 years and depending on ethnicity and geography varies from 0.8% to 30.3% (Davidson et al, 1991). In North America, the prevalence in the general population is estimated to be close to 3 %, but again the precise figure depends very much on ethnic origin of the studied population. Over 75% of those afflicted with the disease suffer from a form called Non Insulin Dependent Diabetes Mellitus or NIDDM (Krolewski et al, 1987). The remaining group would be classified as Insulin Dependent Diabetes Mellitus or IDDM (Davidson et al, 1991).

It has been demonstrated that the mechanisms leading to the development of IDDM are of an autoimmune nature. Genetic makeup is thought to play an important role (Todd, 1990). The exact cause of the destruction of insulin secreting beta cells of the pancreas has not yet been discovered, but a cross-reaction of beta cell antigen with viral antigens or other proteins such as cow's milk protein is thought to be involved (Krolewski, 1987). It seems that in the susceptible individual an external "insult" causes a cascade of immune responses resulting in slow but progressive damage (Atkinson et al, 1990). The classical symptoms appear after the silent process has done damage to approximately 80% of the islets of Langerhans. Lack of insulin affects the complicated mechanism by which the body regulates the metabolism of carbohydrates, lipids and proteins. Increased plasma concentrations of counter-regulatory hormones (epinephrine,

cortisol, growth hormone, and glucagon) exaggerate the rate and magnitude of metabolic decompensation induced by insulin lack, by promoting gluconeogenesis, glycogenolysis, proteolysis, lipolysis, and ketogenesis while decreasing glucose utilisation and glucose clearance. Excessive glucose production and defective utilization result in hyperglycemia with glucosuria when the renal threshold of approximately 180 mg/dl is exceeded. The resultant osmotic diuresis produces polyuria, urinary loss of electrolytes, dehydration, and compensatory polydipsia. If this process is allowed to continue, dehydration and acidosis is followed by coma and, if untreated, death occurs (Figure 1). Weight loss, hunger, thirst and frequent urination are the major symptoms of newly diagnosed IDDM. The only available treatment is replacement therapy with daily injections of insulin (Foster, 1987).

The primacy of impaired insulin secretion in the development of NIDDM remains questionable (Luft, et al, 1981, Garvey,1989). Usually at the time of diagnosis the patient has been suffering from diabetes for some time and shows some degree of insulin resistance as well as inappropriate insulin secretion (DeFronzo et al, 1983). The question as to which lesion is primary in the chain of pathological events leading to glucose intolerance remains open. A large amount of data collected over the years indicates that NIDDM is a very heterogeneous disorder, characterized by multiple defects. These defects do not occur only at the level of pancreatic beta cells, but also in the target tissue, namely liver, muscle and adipose cells (Luft et al, 1981). The results of recent studies are very often conflicting and may depend to a great extent on the population studied. However, there are certain features which are common to NIDDM patients (DeFronzo et al, 1983; Olefsky et al, 1981; Ciaraldi et al, 1982):

- Insulin plasma levels are normal or increased in the fasting state

- Insulin response to the glucose load varies from low to increased, the magnitude of the response being related to the fasting glucose values
- Insulin resistance is almost universally found, both in peripheral and hepatic tissues
- Decreased binding of insulin to its receptor is most commonly associated with a deficit in the number and/or performance of the receptors

Based on the results of an oral glucose tolerance test (OGTT) it has been shown that insulin secretion following a glucose load varies among the diabetic subjects examined (Feaven et al 1983; DeFronzo, 1988). The plasma insulin response appears to be deficient in most but not all patients with fasting hyperglycemia. The severity of the secretory impairment tends to be related to the level of the fasting hyperglycemia (Bogardus et al, 1984). Most of the NIDDM patients with fasting glucose levels exceeding 160-180 mg/dl would display severe impairment in the plasma insulin response. There is however a significant number of patients with fasting glucose levels of 140-160 mg/dl who show an increased insulin response. The group of patients with normal fasting glucose values and hyperglycemia following meals, classified as suffering from Impaired Glucose Tolerance (IGT) exhibit in most cases elevated insulin levels, both fasting and after the stimulation with an oral glucose load. The increased insulin is still insufficient, however, to promote effective glucose uptake by the cells which suggests the presence of tissue insensitivity to insulin (DeFronzo et al, 1982).

The presence of insulin resistance was demonstrated conclusively for the first time by Himmsworth in 1942 (Himmsworth et al, 1942). By comparing the effect of exogenous insulin on glucose disposal during the OGTT in a group of NIDDM

patients and a healthy control group, he found that the majority of diabetic subjects displayed an attenuated response. This observation were subsequently confirmed by numerous investigators using a variety of research techniques (Jackson et al, 1973; Savage et al, 1975; Reaven et al, 1976; DeFronzo et al, 1982). In the 1970's the quadruple infusion technique developed by Reaven was used to quantitate the insulin mediated glucose disposal (Reaven et al, 1977). Beta cell secretion was blocked by the infusion of epinephrine. The stimulatory effect of epinephrine on hepatic glucose production was assumed to be blocked by the combination of infused propranolol and insulin. Under these conditions the steady state plasma glucose level was used to assess the tissue sensitivity. His results although questioned at the time, because the epinephrine acts as a potent insulin antagonist, demonstrated the presence of insulin resistance. These observations were confirmed later by Harano et al (1977) using the more physiologic insulin clamp technique. The combined action of propranolol and epinephrine was substituted by an infusion of somatostatin. Insulin was infused at an elevated level and basal glucose maintained by frequent monitoring and a variable glucose infusion. In these studies again higher steady state plasma glucose levels were found among diabetic subjects as compared with the control group. According to Luft et al (1981) insulin resistance is uniformly present among patients with any degree of clinically detectable glucose intolerance, regardless of their secretory response. This resistance is present both in the liver and peripheral tissues, principally in the muscle. The resistance in the liver is expressed as impaired glucose uptake following a meal and failure to suppress glucose production in the fasting state. Peripheral resistance manifests itself as a diminished uptake of glucose by the muscle in response to insulin stimulation.

Insulin exerts its action by binding to the receptors present widely on a variety of cells (Eaton et al, 1984). The receptor's molecular structure has been identified as a tetrameric glucoprotein composed of two alpha and beta subunits linked by disulfide bonds. The alpha subunit includes the site for insulin binding. It seems to be well established that insulin binding induces activation of tyrosine kinase with subsequent phosphorylation of tyrosine residues and possibly some other substrates within the cell (Exton et al, 1991). Binding of the insulin to its receptor provokes a cascade of events stimulating glucose transport and activating a number of the enzymes participating in glucose metabolism (Kahn et al, 1985; Shia et al, 1983; Taira et al, 1989). The precise nature of signal transduction within the cell is still not completely known. This is partially due to problems with the identification of the second messengers for the insulin action. A number of solutions have been proposed. For example, it has been suggested that the signal may be transmitted by small peptides generated by means of proteolysis from the receptor or some other substrate (Van Obberghen et al, 1981) such as insulin receptor substrate (Exton, 1991). The resulting transport of glucose is facilitated by the transporter system translocated to the cell membrane. The transporter system is described as a glycoprotein with a molecular weight of about 50,000 (Grunberger et al, 1983).

The binding of insulin to its receptor can result in a number of possible events: it may dissociate intact from its receptor, it may undergo some structural changes affecting its activity or it may undergo internalization as a receptor-insulin complex through the invagination of the section of the membrane occupied by clustered complexes (Kahn et al, 1985). The majority of the internalized receptors are recycled, possibly after reprocessing in the Golgi

apparatus, to the cell surface. Most of the internalized insulin however undergoes irreversible degradation in the lysosomes (Fehlman et al, 1982).

It has been demonstrated that both insulin action and degradation are the results of binding to the same receptor (Eaton et al, 1980). The extent of the binding depends on the number of receptors and their affinity for insulin. Studies on insulin binding to circulating monocytes from patients with NIDDM and IGT have demonstrated a decrease in binding capacity without the loss of binding affinity (Olefsky et al, 1981, Comi et al, 1987). Although the monocyte is not the typical target tissue, its binding mimics binding to classical target tissues such as liver and muscle cells as well as adipocytes (Olefsky et al, 1985). The decreased number of insulin receptors can be then, responsible for the insulin resistance. The question arises as to the possible mechanism of the receptor deficit. Numerous studies have shown that the number of insulin receptors is regulated by the insulin concentration in the surrounding tissues (Proietto et al, 1984; Marangou et al, 1986; Rizza et al, 1985). It follows then, that increased levels of insulin can be responsible for the downregulation of its own receptor (DeFronzo et al, 1983). As mentioned above, the insulin levels among NIDDM and IGT patients are frequently elevated. The elevated levels of circulating insulin can, by the mechanism of negative feedback, result in downregulation of the insulin receptors. This inverse relationship between insulin levels and insulin binding was shown during studies on diabetic and healthy volunteers (Lonnroth et al, 1983; Caro et al, 1987).

Based on the information gathered so far, the following hypothesis was proposed by DeFronzo. (1983). The primacy of the initial defect - beta cell or target cell derangement has not been established. Any of the initial defects, however would lead to hyperglycemia. Basal hyperinsulinemia would result in

a decreased number of insulin receptors and insulin resistance. In advanced cases of diabetes, investigators such as Kolterman and his colleagues (1981) proposed the presence of a postinsulin-binding defect such as diminished protein kinase activity. In the long run, exhaustion of beta cells would ensue, due to prolonged stimulation by hyperglycemia (DeFronzo et al, 1988).

Hyperglycemia is a very important factor in DeFronzo's proposal. According to studies from his and other laboratories a sustained increase in the glucose level leads to "toxicity" expressed in multiple ways. The effect on the pancreas was already mentioned. Hyperglycemia also affects the glucose transporter system which was shown to decrease in efficacy in the presence of high glucose concentrations. In experiments performed in vitro on epithelial cells isolated from diabetic rats, Matthaei et al (1986) have shown that chronic hyperglycemia causes a 40 -50 % decrease in the number of glucose transport units. Secondly, sustained high levels of glucose may cause desensitization of beta cells to a persistent stimulus (Rossetti et al, 1987) . It was first shown by Bonner-Weir et al (1983) and Kergoat et al (1987) in experiments on partially pancreatectomized rats that sustained hyperglycemia in the presence of reduced beta cell mass leads to a decreased insulin response. Secretion in response to other secretagogues e.g. arginine and leucine was preserved. However, this beta cell desensitization seemed to operate only after the initial defect in the secretory capacity had already taken place.

An additional factor may be implicated in the pathogenesis of NIDDM. It has been observed (Svedberg et al, 1990) that increased levels of free fatty acids (FFA) inhibit insulin binding and degradation by isolated rat hepatocytes. The same authors in their recent study (Svedberg et al, 1991) examined the influence of FFA on hepatic insulin clearance in obese rats. The results suggest that hepatic

insulin clearance is inversely related to the degree of obesity and concentration of the FFA, especially in the portal circulation and thus may be implicated in the development of the observed hyperinsulinemia.

FFA also play other regulatory roles in glucose metabolism. An early exploration of this subject was made 30 years ago by Randle et al (1963). They showed that increased oxidation of FFA, by altering the redox potential of the cell, inhibits several enzymatic steps in the intracellular metabolism of glucose. Oxidation of FFA leads to accumulation of acetyl-CoA which was shown to inhibit pyruvate dehydrogenase. In the process, increased amounts of hydrogen atoms are released which are transferred to nicotinamide adenine dinucleotide (NAD) producing its reduced form - NADH. This results in the slowing down of the Krebs cycle and accumulation of citrate, leading to the inhibition of phosphofructokinase. This defect in the early step of intracellular glucose metabolism leads to the accumulation of glucose-6 phosphate resulting in decreased glucose transport into the cell (Felber et al, 1977; Golay et al, 1984). Later it was established that increased FFA oxidation causes dissociation of glycogen synthase subunits. This explains why both glucose storage and oxidation are affected (Taskinen et al, 1985). Further work of Felber et al (1987) demonstrated that basal plasma levels of FFA and lipid oxidation are elevated in human obesity and NIDDM and fail to suppress after glucose ingestion. These observations were substantiated recently by DeFronzo et al (1985) and Chen et al (1987). The elevation of both fasting FFA levels and the basal rate of lipid oxidation measured by indirect calorimetry was shown to be a common feature among obese diabetic and obese nondiabetic subjects when compared with a nonobese, nondiabetic control group. Further studies performed on these subjects demonstrated failure to suppress those elevated levels during the

euglycemic insulin clamp. In this studies the rate of glucose oxidation was inversely related to the rate of FFA oxidation. Increased lipolysis also affects hepatic glucose metabolism. Evidence from the studies on obese insulin resistant diabetic patients shows that an elevated rate of FFA oxidation stimulates basal hepatic glucose production and gluconeogenesis (Ferrannini et al, 1983; Groop et al, 1986).

In contrast to these observations, the basal rate of lipid oxidation among nonobese NIDDM patients was shown to be normal and insulin mediated suppression was not impaired (Taskinen, et al, 1985). Thus increased levels of FFA cannot always be implicated in the etiology of insulin resistance and hyperinsulinemia in NIDDM.

The liver is a major organ in carbohydrate, protein and lipid metabolism and as such the primary site of action and degradation of many regulatory hormones (Foster, 1987). Its potential role in modulating insulin levels based on changing extraction therefore needs to be examined. The liver receives about a quarter of the cardiac output, but because of its anatomical position, it is exposed to considerably greater concentration of insulin than any other tissue. Insulin reaches the liver via the portal vein before any dilution in the systemic circulation. On each recirculation the liver is exposed to the systemic insulin in addition to that synthesized de novo, greatly increasing the total amount of the hormone that is extracted. According to various data between 40 and 60 % of insulin secreted into the portal vein undergoes first-pass hepatic extraction (Harding et al, 1975; Field et al, 1980; Waldhäusl et al, 1982; Pacini et al, 1990; Chap et al, 1987). In studies that combined hepatic vein catheterization with C-peptide measurements and the euglycemic clamp technique it has been suggested that hepatic uptake stays at the same level or increases slightly going

from basal to moderately elevated concentrations of insulin (Tranberg et al, 1978; Ferrannini et al, 1987). It appears to saturate at insulin levels reaching values above 400-500 μU/ml.

The results of recent studies performed on animals using the euglycemic clamp technique with constant insulin infusion seem to disagree with these data. A number of different insulin concentrations was achieved by varying the insulin infusion rate in several experiments. It was found that the metabolic clearance rate (mcr) of insulin falls as the insulin concentration increases (Morishima et al, 1992). The decrease in mcr over the range studied, 20 to 1000 μU/ ml, was calculated to be 60 %, which is in agreement with the 66 % shown by Sonksen et al,(1973). The saturability of hepatic insulin extraction under physiological or even pathological circumstances thus remains somewhat controversial (Ferrannini et al, 1987; Morishima et al, 1992; Sonksen et al, 1973).

There are numerous pathological circumstances however, where hepatic clearance of insulin is indisputably compromised. It has been observed that in liver cirrhosis diminished hepatic removal of insulin can be attributed to either cellular damage, porto-systemic shunting, or both (Johnston et al, 1977; Iwasaki et al, 1978; Rossel et al, 1983; Marchesini et al, 1990). The precise nature of the derangement has yet to be clarified, but of great importance is the fact that due to this abnormality hepatic handling of insulin is altered resulting in diminished uptake (Johnston et al, 1978; Marchesini et al, 1982). A number of studies have demonstrated that cirrhotic patients are characterized by both fasting and postprandial hyperinsulinemia (Taylor et al; 1985, Proietto et al, 1984; Sirinek et al, 1991). Measurements of insulin secretion based on the levels of C-peptide suggest that the beta cell response is enhanced in cirrhosis (Marchesini et al, 1990). Concomitantly with the hyperinsulinemia many cirrhotic patients display

impaired glucose tolerance (Riggio et al, 1982; Petrides et al, 1989; Cavallo-Perin et al, 1985).

The derangements in carbohydrate metabolism present in liver disease strikingly resemble those observed in NIDDM. It is then possible that similar derangements in the handling of insulin by the liver may play a role in the onset of pathological events leading ultimately to the glucose intolerance of NIDDM. The proposed sequence of events operating here would be as follows (Figure 2):

- Due to the proposed diminished insulin extraction by the liver, there is an increase in the level of circulating hormone.
- High levels of insulin present in the circulation result by the mechanism of negative feedback in the downregulation of the insulin receptors. (decreased number and/or effectiveness).
- Insulin resistance follows - more insulin needed to get the same degree of glucose removal.
- Hyperglycemia subsequently ensues.
- Hyperglycemia in turn stimulates the pancreas in the direction of further insulin secretion, worsening the vicious cycle already operating.

The relative inaccessibility of the liver together with its affluent and effluent blood vessels makes the investigation of the hepatic uptake of insulin a difficult task. The most accurate data can be obtained under steady state conditions by the direct measurements of insulin entering and leaving the liver (Field, 1973; Horwitz et al, 1975). Surgical implantation of catheters in the vessels that supply and drain the hepatic bed makes these measurements technically easy. However

the results obtained during these studies vary widely indicating the possibility of serious sampling error, due most likely, to uneven distribution of insulin in the portal blood (Field, 1973). The need for noninvasive approaches in human studies prompted development of other methods based on insulin and C-peptide kinetics using tracer techniques. Both peptides secreted by the pancreas are the products of proinsulin conversion taking place in the beta cells. They are secreted at equimolar rates but their metabolism is different (Eaton et al, 1980; Faber et al, 1978; Madsbad et al, 1983). C-peptide, in contrast to insulin, is not extracted by the liver to an appreciable degree (Polonsky et al; 1983, Morishima et al, 1986). The primary site of its removal is the kidney (Kuhl et al, 1978).

The study presented here makes an attempt to improve our understanding of mechanisms contributing to glucose intolerance among NIDDM subjects. The aim of this work is the assessment of the hepatic insulin uptake among non-obese NIDDM subjects with fasting glucose values ranging from 90 to 115 mg/dl as compared with that of a healthy control group. The preliminary assumption is made that in this experimental setting, kinetics of both C-peptide and insulin can be described in a linear fashion. The linearity of the C-peptide system has been well established in recent years (Radziuk et al, 1986; Morishima et al, 1986). Insulin removal was shown to be inversely related to the plasma concentration, in that it appears to saturate only at levels which are physiologically high. What is even more important, over the range considered in these studies the removal is near linear under non- steady conditions (Morishima et al, 1992).

The experimental design is based on metabolic and insulin responses to the oral glucose tolerance test combined with the assessment of the basic kinetic parameters following the bolus injection of C-peptide and tracer insulin (Radziuk et al, 1986). The injection response data obtained from the decay curves is used

in an integral equation to calculate the total secretion of both peptides. Based on the C-peptide data an assessment of total insulin secretion is made (Polonsky et al, 1984). Insulin data yields the posthepatic insulin appearance. The ratios of posthepatic to prehepatic insulin appearances are then examined. A decrease in the hepatic uptake would increase this ratio because of the slower disappearance of the insulin from the system. This approach enables us to gain valuable information concerning hepatic uptake of insulin in humans using a noninvasive technique. It will be applied here to compare the insulin uptake by the liver in normal controls and patients with impaired glucose tolerance presumed to represent a relatively early stage in the development of NIDDM.

2. MATERIALS AND METHODS

2. 1 SUBJECTS

2.1.1 General information

The studied population consisted of 32 adult volunteers. The detailed characteristics (age, sex, body weight and body mass index) of each subject can be found in Table 1. All subjects were ambulatory for several weeks prior to the study and continued their normal daily activities. None of them habitually undertook excessive physical activity or was a trained athlete. They were in good general health as assessed by their medical history and present status. None of the patients suffered from liver or endocrine disorders other than diabetes. Routine laboratory examination, with emphasis on liver, kidney and hematological indices, confirmed their health status. None of the control subjects was taking medication known to affect glucose metabolism. Healthy volunteers stayed on the diet containing a minimum of 200 g of carbohydrate a day for three days prior to the OGTT. All others were instructed to follow the diet prescribed by their physician and were asked to withdraw medication (if any) for at least three days prior to the test. Each volunteer was informed about the nature and purpose of the study. All possible risks were explained in detail. The experimental protocol was approved by the Human Ethics Committee of the Ottawa Civic Hospital.

2.1.2 Selection criteria

The study was specifically aimed at a nonobese population to eliminate, as much as possible, the influence of obesity on insulin metabolism (DeFronzo,

1983). Obesity, defined as an excess of body adipose tissue can be measured and characterized accurately using sophisticated methods not available in clinical practice. Where precise measurement is not critical, several techniques are used to obtain reasonable estimates. Two techniques are commonly used to assess the degree of obesity. Relative weight is calculated by dividing the subject's weight by a "desirable" weight, defined as a midpoint value recommended for the "medium frame" person by the 1983 Metropolitan Life Insurance Tables. This method does not differentiate between the excess of muscle or fat in the body composition (Schroeder et al, 1989). The body mass index (BMI) is increasingly used since it is a better reflection of the presence of the excess of adipose tissue. It was therefore employed in this study. BMI was calculated by dividing the subject's weight measured in kilograms by squared height given in meters. According to the National Institutes for Health ideal BMI should fall in the 20-25 kg/m^2 range. Mild obesity is diagnosed when the index exceeds 27.5 kg/m^2. Moderate obesity is diagnosed when BMI ranges between 30 and 40 kg/m^2. It has been suggested that age is another factor that should be taken into consideration when diagnosing obesity. Generally the older population has a slightly increased BMI without ill consequences. Because the subjects participating in this study belonged predominantly to this age group, only those whose BMI exceeded 30 kg/m^2 were excluded from participation.

Based on the results of an OGTT, subjects were tentatively divided into three groups according to the guidelines of the National Diabetes Data Group (National Institutes of Health Diabetes Data Group, 1979). According to these guidelines, glucose concentrations in the plasma at two time points and one interval during the first two hours of the test were taken into consideration.

- Non-diabetic status was determined when fasting glucose was lower than 115 mg/dl, the 2 hour value was lower than 140 mg/dl, and at no time point between the start of the test and 2 hours did the glucose concentration exceed 200 mg/dl.

- Impaired Glucose Tolerance was diagnosed when a subject with normal fasting glucose concentration (< 140 mg/dl) showed a 2 hour value in the 140 - 200 mg/dl range and the glucose concentration equaled or exceeded 200 mg/dl at any time point during the test.

- Diabetes was identified when fasting glucose levels exceeded 140 mg/dl and/or the 2 hour value was equal to or greater than 200 mg/dl and glucose concentration measured at any other time point during first two hours of the test was equal to or exceeded 200 mg/dl.

In order to further divide the patients into experimental groups the following criteria were used. The control group consisted of subjects with normal glucose tolerance. The patients with diabetic glucose tolerance and fasting glucose levels exceeding 115 mg/dl were assigned to the group of subjects with advanced diabetes. Two patients with impaired glucose tolerance and five subjects with diabetic glucose tolerance, but fasting glucose concentrations not exceeding 115 mg/gl were assigned to the group of patients with mild diabetes, as representing a relatively early stage in the development of this disease.

2. 2 EXPERIMENTAL PROTOCOL

The study was performed in two stages. Informed consent was obtained prior to each step.

2.2.1 Experimental protocol - Stage 1

The first stage was utilized to establish the basic parameters pertaining to glucose metabolism such as basal concentrations of glucose, insulin, C-peptide and FFA and those following the glucose ingestion. The results then were used to determine the total areas under the concentration curves and ratios of C-peptide to insulin concentrations. This part of the study combined with the medical history determined the experimental group into which the subjects would fall and was therefore effectively a screening procedure.

The oral glucose tolerance test was administered after an overnight fast (12 - 14 hours). An intravenous catheter (20 GA, Becton Dickinson, Sandy, Utah) was inserted into one of the forearm veins and a saline infusion was started to maintain the patency. The subject's forearm and hand were warmed using a heating pad in order to arterialize the blood (Jackson et al, 1973). A three-way stopcock was put in line to allow for the intermittent sampling of the arterialized blood. Using this method the peripheral uptake of the glucose, insulin and C-peptide was minimized. After the baseline samples were drawn a 75g glucose solution was taken orally at time 0. Then 5 ml samples were taken at 10, 20,30, 45, 60, 80, 100, 120, 150, 180, 210 and 240 minutes into heparinized tubes (Becton Dickinson, Mississauga, Ont) and divided into separate tubes for the further analysis of glucose, insulin, C-peptide and free fatty acids concentrations. Trasylol (1000 KIU),an anti-protease was added to the plasma drawn for the measurement of C-peptide (0.1 ml for each 1 ml of blood), in order to inhibit proteolysis. The samples were kept on ice during the experiment, then centrifuged and the plasma was separated and stored at -20°C until further analysis.

2.2.2 Experimental protocol - Stage 2

The second stage of the study was performed in order to obtain decay curves for labeled insulin and for C-peptide following their injection. The experimental data (fitted in the form of the double exponential curves) allowed for the further assessment of the metabolic clearance rates and the kinetic parameters. This allowed more precision in the estimates of the rates of appearance.

Patients with advanced diabetes were excluded from participation in this part. Five control subjects (4 male and 1 female) and six patients with mild diabetes (5 male and 1 female) were chosen to participate. The subjects were admitted to the hospital ward after an overnight fast and remained recumbent during the course of study. They were not permitted to eat or smoke. Water was allowed in small amounts. The indwelling catheter was inserted into one of the dorsal hand veins and saline infusion started as described previously. The contralateral arm was reserved for injections. Baseline samples for the measurements of plasma insulin and C-peptide concentrations were drawn at time -10 and -5 minutes. Purified C-peptide (IAF International, Laval, Canada) was injected in the amount of approximately 35 pmol. Approximately 10 μCi of insulin in the form of the labeled analog (^{3}H-insulin) was given immediately afterwards. At the midpoint of the injection the clock was started and samples were taken at 1, 2, 4, 7, 10,15, 20, 30, 45, 60, 75 minutes. The blood samples were collected into heparinized tubes and treated as previously described.

2. 3 PREPARATION OF THE INFUSATES

Tritiated insulin was prepared semisynthetically as outlined by Halban et al (1975). It was purified further using reverse-phase liquid chromatography (C Hamilton PRP-3 column, 3.9 x150 mm) with a solvent delivery system from Shimadzu (Kyoto, Japan). The elution was carried out using a linear gradient of 25-35% acetonitryle in 0.1 % trifluoroacetic acid (TFA) over 20 min. The purified insulin was then lyophilized in human serum albumin and reconstituted in 0.9 % NaCl which was sterile and pyrogen - free. The infusate was then passed through a sterile filter (MSI, Westboro MA) into a sterile vial (Omega, Montreal, PQ) and tested for pyrogenicity (LAL -test, Biowhittaker, Walkersville, MD) and for aerobic sterility (Bactrec NR 6A, Becton - Dickinson, Townson, MD). The final specific activity ranged from 205 to 477 dpm/μU.

Unlabelled C-peptide was obtained from IAF International (Laval P.Q.). Purification was carried out on a PRP-1 column (Hamilton, Reno, NE) using an acetonitrile in 0.1 % TFA gradient (20 -30 % over 30 min). The eluate was monitored by a UV Spectrophotometer (Shimadzu, Kyoto, Japan) at 214 nm. The appropriate peak was collected in human serum albumin (0.4 ml, 25 %) and lyophilized. The final preparation was carried out as described for tritiated insulin.

2.4 ANALYTICAL METHODS

2.4.1 Measurements of tritiated insulin in plasma

Two ml of plasma were applied to a polypore RP cartridge (4.6 x 3 cm, Applied Biosystems Inc., San Jose, CA). After washing out the plasma proteins

using 0.1 % TFA in high pressure liquid chromatography (HPLC) grade water, the cartridge was placed in line with a PRP-3 analytical column. The insulin was eluted from the column using an acetonitrile (0.1 % TFA) gradient from 25 to 35% over 20 minutes. The position of the ^{3}H insulin was verified each morning by using a marking standard which underwent the same preparation as the sample except that the eluate was collected in 1 minute fractions. Each fraction was then mixed with scintillation fluid (Formula 989, Biotechnology systems, Boston , MA) and counted using a scintillation counter (Canberra-Packard, Downer's Grove, IL). The insulin-containing fractions were lyophilized and reconstituted with distilled water .

The recovery of labeled phenylalanine from the original labeled insulin was tested. Standard samples were diluted in plasma and treated in the same manner as analyzed samples. The recoveries determined were 89±2.9 %.

2.4.2. Measurements of plasma C-peptide and immunoreactive insulin

Plasma C-peptide and immunoreactive insulin (IRI) levels were determined using the Linco double antibody radioimmunoassay (Linco Research Inc., St Louis, MO). The crossreactivity for the C-peptide antiserum was less than 4% with proinsulin. The insulin antiserum shows less than 0.1% crossreactivity with complete proinsulin and its split fractions, des-31 and des-32 proinsulin (R. Gingerich unpublished data, Linco Research Inc, St. Louis MO).

Plasma glucose concentrations were measured using the glucose oxidase method (Beckman glucose analyzer). The determination is based on the enzymatic reaction of ß-D glucose with oxygen. Oxygen depletion proportional to the glucose concentrations is monitored using an oxygen electrode. The

relationship between oxygen consumption and glucose concentration is determined quantitatively by calibration with a standard solution of glucose.

The concentrations of FFA were determined using an enzymatic assay from Boehringer-Mannheim (Dorval, Canada). Free fatty acids are, in the presence of the enzyme acyl-CoA synthetase, converted by ATP and coenzyme A into acyl-coenzyme A,. Simultaneously AMP and pyrophosphate are produced. Acyl CoA reacts with oxygen in the presence of acyl-CoA oxidase to form 2,3-enoyl-coenzyme A. The resulting hydrogen peroxide converts 2,4,6-tribromo-hydroxy-benzoic acid and 4-aminoanipyrine to a red dye in the presence of peroxidase. The dye is measured in the visible range at 546 nm (Shimizu, 1980).

2.5 CALCULATIONS

The estimation of the hepatic insulin extraction in subjects with mild diabetes and healthy subjects is the main objective in this research project.

The difference in insulin and C-peptide responses to the glucose load, examined by comparing the ratios of the integrated insulin concentration to the integrated C-peptide concentration, provides some information on general patterns of secretion and removal of insulin among the subjects studied. Since the two peptides exhibit different kinetics as well as inter - individual variations these factors should be taken into consideration as much as possible in obtaining estimates of insulin uptake by the liver. The preliminary information can be derived from the OGTT alone, but the latter, more precise, figure is assessed more reliably using the response to the single injection boluses of the C-peptide and labeled insulin.

The following assumptions have been made:

- The kinetics of C-peptide are linear - the metabolic clearance rate is constant (Faber et al, 1978; Morishima et al, 1986). The uptake by the liver is negligible (Kuhl et al, 1978; Polonsky et al, 1983).

- The metabolic clearance rate of insulin is different in the basal state and following glucose loading and is assumed constant through these periods. Thus the changes are assumed to be stepwise although the actual changes would be more gradual. Only the overall change in the clearance from the basal to the postprandial period is therefore examined.

- Hepatic extraction influences the metabolic clearance rate in a directly proportional fashion (Pye et al, 1993).

To calculate the hepatic extraction of insulin during the basal state and following glucose loading the following set of equations was used.

The estimation of basal metabolic clearance for insulin can be obtained from (Faber et al, 1978):

$$mcr_{ib} = M^* / \int_0^\infty h_{ib}(t)dt \qquad [1]$$

where M* is the total amount of ^{3}H-insulin injected as a bolus, and $h_{ib}(t)$ is the plasma insulin response to this injection.

By the same token basal metabolic clearance of C-peptide can be assessed using the equation:

$$mcr_{cpb} = M / \int_0^\infty h_{cpb}(t)dt \qquad [2]$$

where M denotes the total amount of injected C-peptide and $h_{cpb}(t)$ is the plasma C-peptide response to the bolus injection.

Functional forms for the plasma responses to the bolus injections, $h_i(t)$ and $h_{cpb}(t)$, are obtained by fitting the experimental data to a set of two exponentials. The fitting was done using nonlinear least squares methodology (Delta Graph Professional, Macintosh). The experimental function has the general form $y=a_1\exp(-s_1t) + a_2\exp(-s_2t)$ for the labeled insulin, and $y=a_1\exp(-s_1t) + a_2\exp(-s_2t) + b$ for the C-peptide (b = basal values for C-peptide concentrations), where a_1, a_2, s_1, s_2 are constant. The area under the decay curve (integral) is then determined from the formula:

$$\int_0^\infty h(t)dt = \frac{a_1}{s_1} + \frac{a_2}{s_2} \qquad [3]$$

Having the estimate of metabolic clearance rates of insulin and C-peptide, it is possible to calculate the rates of appearance for both peptides.

These are defined as follow:

$$R_{ib} = mcr_{ib}C_{ib} \qquad [4]$$

and

$$R_{cpb} = mcr_{cpb}C_{cpb} \qquad [5]$$

where C_{ib} and C_{cpb} are the basal concentrations of insulin and C-peptide respectively.

The calculated C-peptide rate of appearance corresponds to its prehepatic secretion rate as it does not undergo hepatic extraction. It therefore appears

peripherally at the same rate as it does in the portal vein. This value therefore equals the prehepatic rate of insulin appearance as these two peptides are secreted at equimolar rates (Waldhausl et al, 1979). The calculated rate of insulin appearance is posthepatic as it is based on the peripherally obtained plasma samples. It follows then, that the fraction of insulin that escapes the first-pass hepatic uptake during the basal state can be estimated from:

$$a_b = \frac{R_{ib}}{R_{cpb}} \qquad [6]$$

Thus the fraction undergoing extraction can be obtained as:

$$h_b = 1 - a_b \qquad [7]$$

In order to calculate the metabolic clearance rates and the amount of insulin and C-peptide removed from the circulation over the time of the experiment the following equations were used:

$$\int R_i(t)dt = mcr_i \int C_i(t)dt \qquad [8]$$

and

$$\int R_{cp}(t)dt = mcr_{cp} \int C_{cp}(t)dt \qquad [9]$$

where $\int C_i(t)dt$ and $\int C_{cp}(t)dt$ are obtained from the experimental data for insulin and C-peptide during the OGTT. The integration takes place over the period where concentrations of hormones differ from their basal levels. These equations use the convolution integral relating the hormone concentration with its rate of appearance. The assumption of constant metabolic clearance rate for both peptides during the loading period determines the final form of the equation originally described in an earlier paper (Radziuk et ai, 1986).

The fraction of insulin that escapes hepatic extraction is now estimated as:

$$a = \frac{\int R_i(t)dt}{\int R_{cp}(t)dt} \qquad [10]$$

As noted above the rates of hepatic extraction and metabolic clearance rates of insulin are assumed to be proportional to each other, being different but constant during basal and absorptive periods. Thus, it can be written

$$\frac{mcr_{ib}}{1-a_b} = \frac{mcr_i}{1-a} \qquad [11]$$

where $1\text{-}a_b$ and 1-a are the basal and postprandial hepatic extractions of insulin.

From equations [10] and [8] a is calculated as:

$$a = mcr_i \frac{\int C_i(t)dt}{\int R_{cp}(t)dt} \qquad [12]$$

By transformation of equation [12]we can then obtain the formula for the calculation of mcr_i:

$$mcr_i = \frac{a}{\frac{\int C_i(t)dt}{\int R_{cp}(t)dt}} \qquad [13]$$

which can be used to substitute mcr_i in the equation [11]. The resulting formula;

$$\frac{mcr_{ib}}{1-a_b} = \frac{a}{1-a} \frac{1}{\frac{\int C_i(t)dt}{\int R_{cp}(t)dt}} \qquad [14]$$

then can be transformed into:

$$\frac{a}{1-a} = \frac{mcr_{ib}}{1-a_b} \frac{\int C_i(t)dt}{\int R_{cp}(t)dt} \qquad [15]$$

Defining α as :

$$\alpha = \frac{mcr_{ib}}{1-a_b} \frac{\int C_i(t)dt}{\int R_{cp}(t)dt} \qquad [16]$$

yields the final formula:

$$\frac{a}{1-a} = \alpha \qquad [17]$$

which can be transformed into:

$$a = \frac{\alpha}{1+\alpha} \qquad [18]$$

Since $mcr_{cpb} = mcr_{cp}$, $\int R_{cp}(t)dt$ can be calculated from equation [9]. This allows for the estimation of insulin fraction escaping hepatic uptake (a) and hepatic extraction following glucose load. Metabolic clearance of insulin following the glucose loading can be then assessed from equation [11] and $\int R_i(t)dt$ can be derived from equation [8].

2.6 Statistical Analysis

The glucose, insulin, C-peptide and FFA curves were compared using paired one way analysis of variance (ANOVA) where time was used as a repeated measure (Dowdy at al, 1983). The dispersion among the sample means between the groups, measured by their variance, was compared with the dispersion within the experimental groups. The hypothesis of the equality of the curves considered was rejected when the first variance was greater than the second.

The differences in the mean values between two groups were tested for significance using the unpaired two-tailed Student's t-test (Dowdy at al, 1983). In order to test whether two populations have different means the following statistic was used:

$$t = \frac{(\bar{x} - \bar{y}) - m_{\bar{x}-\bar{y}}}{\sqrt{\frac{s_p^2}{n_1} + \frac{s_p^2}{n_2}}} \qquad [19]$$

where $\bar{x}$ and $\bar{y}$ are the mean values of the experimental results, n_1 and n_2 are the degrees of freedom and m_{x-y} is hypothesized to be 0. Pooled sample variance (s_p^2) represents the average common variance weighted by the degrees of freedom and is calculated from:

$$S_p^2 = \frac{\sum(x-\bar{x})^2 + \sum(y-\bar{y})^2}{n_1 + n_2 - 2} \qquad [20]$$

where x and y are the values of experimental results.

In order to find out whether following glucose loading the fall in the insulin metabolic clearance and insulin hepatic uptake was significant within both examined groups, the matched-pair Student t-test was used (Dowdy at al, 1983) based on the following statistic:

$$t = \frac{\bar{y} - \mu_o}{s/\sqrt{n}} \qquad [21]$$

where $\bar{y}$ is the sum of differences between paired results, s is sample standard deviation, n is the number of pairs (degrees of freedom) and μ_o is hypothesized to be 0.

In addition, the relationship between the ratios of integrated insulin concentrations to C-peptide concentrations and integrated glucose concentrations was tested using Spearman's rank correlation analysis (Anderson et al, 1986). This procedure was used to test the hypothesis that two variables in the examined population are independent. This was computed using the following formula:

$$r_s = 1 - \frac{6\sum_{i=1}^{n} d_i^2}{n(n^2-1)}$$

where n =11, and d^2 is squared difference in ranking of the two variables.

In order to establish, whether the relationship between the ratios of integrated insulin concentrations to C-peptide concentrations and integrated glucose concentrations was linear and how well one variable can be predicted from the other, simple linear regression and correlation analyses was used.

3. RESULTS

The subject characteristics are presented in Table 1. Based on the results of the OGTT using the diagnostic guidelines (as described above), subjects were divided into three groups according to their level of glucose tolerance (Table 2). The group of healthy volunteers consisted of seven subjects (5 men , 2 women). The age varied from 42 to 55 years (mean 50.6±1.7). The second examined group consisted of nine patients (5 men, 4 women) aged between 41 and 65 years (mean 50.7±3.3). This group demonstrated a relatively mild degree of glucose intolerance as judged by normal fasting glucose values (referred to later as patients with mild diabetes). Finally the largest group of patients with established diabetes consisted of fifteen subjects (11 men , 4 women) aged from 37 to 68 (mean 55. 5±2.5). BMI was very similar among the examined groups (mean for controls 25.1±1.0 kg/m^2, subjects with mild diabetes 25.5±1.1 kg/m^2 and subjects with advanced diabetes 24.8±0.6 kg/m^2). The basic experimental parameters are summarized in Tables 3, 4, and 5 for the control group, subjects with mild diabetes and patients with advanced diabetes, respectively. The data based on the OGTT alone was analyzed only in the context of overall secretory response to glucose stimulation. The areas under the glucose, insulin and C-peptide secretion curves were calculated using the trapezoidal rule. The ratios of the total insulin response to that of C-peptide were then examined.

Those subjects who subsequently underwent insulin and C-peptide bolus injections are separately characterized in Table 6. The control group consisted of five participants (4 men, 1 woman) age 48 to 55 (mean 51.6±1.3). BMI varied from 24.4 to 29.3 kg/m^2 (mean 26.2±0.9). Six subjects with mild diabetes (5 men, 1 woman) were between 48 to 65 years old (mean 55.0±3.5). BMI was in the 22.0 - 27.4 kg/m^2 range (mean 25.1±1.0). Subjects with established diabetes were

excluded from participation in the second stage of the study. The basic experimental data is included in Table 7.

Quantitative estimates of the metabolic clearance and hepatic extraction of insulin were obtained in these subjects as outlined in the Methods. Data based on the results of OGTT among the participants of both experimental parts shows that physiological indices were very similar in the fasting state in the groups. Glucose concentration was 91.8 ±2.7 mg/dl for the controls and 101.5±8.2 mg/dl for the subjects with mild diabetes ($p>0.05$). Insulin concentration also did not differ significantly between the examined groups. It was 7.7±0.8 μU/ml (0.05±0.006 pmol/ml) for the controls and 9.5±2.8 μU/ml (0.06±0.02pmol/ml) for the patients with mild diabetes ($p>0.05$). The C-peptide concentration was found to be 0.39± 0.03 pmol/ml in the control group and 0.48±0.05 in the group of the glucose intolerant patients ($p>0.05$). Following glucose loading, measured parameters showed large differences between the examined groups. It can be seen (Figure 3) that postprandial glucose peaked at t=100 min reaching level of 250±13.3 mg/dl among the subjects with mild diabetes while the control group reached maximal values of 173.4±8.1 mg/dl at 45 minutes ($p<0.05$). It is apparent that glucose intolerant subjects display prolonged hyperglycemia after the first hour of the OGTT. The glucose concentration values are still elevated at the end of the experiment as opposed to the control group where values returned to basal levels at 180 minutes. The insulin concentrations follow a very similar pattern (Figure 4). Insulin concentrations are identical up to 60 minutes in both groups, but continue to rise after that time among subjects with mild diabetes reaching the value of 131.4±35.1 μU/ml (0.91±0.24 pmol/ml), while decreasing steadily in the control group. The integrated insulin values calculated for the subjects with mild diabetes are 100% greater then those for the control group (Table 3 and 4).

In contrast to these observations, the C-peptide concentrations (Figure 5) do not show any significant differences ($p>0.05$), although the calculated area was 15% greater in the subjects with mild diabetes.

The ratio of the integrated insulin concentrations to integrated C-peptide concentrations was examined in patients participating in the full experiment and those undergoing OGTT only. As can be seen (Figure 6) the average ratio for the control group was found to be 0.21 ± 0.03 as opposed to 0.38 ± 0.05 for the subjects with mild diabetes ($p<0.05$). It is almost identical when the group of the subjects participating in the OGTT only (Figure 7) is examined. In the subjects with advanced diabetes, the average ratio was calculated to be 0.34 ± 0.1, however, the actual ratios vary greatly as the patients represent a very non-homogenous population characterized by various degrees of hyperinsulinemia and deficient insulin secretion.

The rank correlation coefficient was calculated in order to determine any association between the insulin to C-peptide concentration ratio and the degree of glucose intolerance expressed as the area under the glucose concentration curve. This was done jointly for controls and patients with mild diabetes and was found to be significant (coefficient=0.72).

A linear correlation was found between these two factors, especially on examination of the group of subjects selected for the participation in the full experiment. The correlation between the area under the glucose concentration curve and the $\int C_i / \int C_{cp}$ is more pronounced when considering the group of controls and subjects with mild diabetes only (Figure 6), than when all OGTT participants are examined (Figure 8). The regression coefficient in the group of subjects participating in the combined study was $b= 0.065$ and the correlation

coefficient r=0.6. When looking at the group of controls and subjects with mild diabetes that underwent OGTT only, the results of statistical analysis are similar (b=0.07, r=0.6). It is less apparent when data obtained from all the experimental subjects is analyzed (Figure 8). The calculated regression coefficient is b=0.028 and the correlation coefficient r= 0.3. Once again, this is mainly due to the presence of the subjects with more advanced diabetes and highly variable secretory responses to the glucose ingestion in this case.

Because the ratio of integrated insulin concentrations to the integrated C-peptide concentrations can be regarded as an index of hepatic extraction, it follows that a correlation exists between the degree of glucose intolerance and the hepatic insulin uptake.

Under basal conditions the metabolic clearance rate for insulin is identical, 13.1±1.5 ml $kg^{-1}min^{-1}$ for the control group and 13.6±2.0 ml $kg^{-1}min^{-1}$ for the group of subjects with mild diabetes. Calculated basal metabolic clearance rate for C-peptide is also similar (3.53±0.6 ml $kg^{-1}min^{-1}$ for controls versus 3.00±0.2 ml $kg^{-1}min^{-1}$ for subjects with mild diabetes). The basal rate of appearance for insulin and C-peptide was found to be 0.67±0.08 pmol $kg^{-1}min^{-1}$ and 1.34±0.19 pmol $kg^{-1}min^{-1}$ respectively for healthy volunteers and 0.74±0.38 pmol $kg^{-1}min^{-1}$ and 1.44±0.21 pmol $kg^{-1}min^{-1}$ respectively for the subjects with mild diabetes. Hepatic extraction during basal conditions was estimated to be 49±7% for controls and 50±7% for the group of patients with mild diabetes (Table 8).

The estimation of the total appearance of C-peptide was based on the area under the concentration curve following the glucose load and with the metabolic clearance rate assumed to be the same as during the basal state. The rates do not differ significantly between examined groups. There is, however a large

difference in the calculated metabolic clearance rate of insulin (Figure 9, Panel B). The clearance fell from 13.1±1.5 to 10.5± 1.6 ml $kg^{-1}min^{-1}$ among controls and from 13.6±2.0 to 6.7±1.0 ml $kg^{-1}min^{-1}$ among the subjects with mild diabetes. This implies a 19.8% decrease among controls ($p>0.05$ n.s) and a significant 50.7% decrease among the patients ($p<0.05$). The decline in the metabolic clearance rate was therefore more than double that in the control group. This difference can be attributed to the changes in the hepatic extraction (Figure 9, Panel A), as it was assumed that these two variables are interdependent in a directly proportional fashion. The estimated hepatic uptake fell by 20% in controls and by 52% in the patients, which represents a difference of 61.5% when the two groups are compared.

Finally there is a close relationship between the ratios of integrated insulin to C-peptide concentrations and fractional extraction by the liver after the ingestion of 75 g of glucose (Figure 10). The decline in the fractional hepatic extraction seems to coincide with the increase in these ratios. This implies that the rise in the peripheral appearance of newly secreted insulin is responsible for the increase in the concentration ratios, since the peripheral appearance of C-peptide is similar in both groups. This observation carries the practical implication of using the ratios of the areas under the concentration curves as a rough estimate of hepatic insulin uptake.

The free fatty acids concentrations measured during basal conditions do not show a significant difference between examined groups ($p>0.05$). However, the concentration curves based on the measurements obtained in hourly intervals following the glucose load (Figure 11) differ significantly ($p<0.05$). The mean basal value for the controls was determined to be 0.47±0.1 mmol/l and for the glucose intolerant 0.68±0.1 mmol/l ($p>0.05$). During the course of the study

measured concentrations fell to 0.06±0.03 mmol/l among control subjects and to 0.19±0.05 mmol/l among examined patients ($p<0.05$).

4. DISCUSSION

4.1 GENERAL CONSIDERATIONS

Non-insulin dependent diabetes mellitus is a very heterogeneous disorder. Although the origin of the multiple derangements in glucose metabolism is largely unknown, there are common features known to contribute to the pathogenesis of this disease (DeFronzo et al, 1983). The most important finding among the diabetic subjects is the nearly universal presence of insulin resistance (Caro et al, 1986; Olefsky et al 1982). The source of this phenomenon has not been clearly elucidated. It has been proposed that a primary cellular defect within the insulin receptor leads to the compensatory hypersecretion of insulin (DeFronzo; 1988; Grunberger et al, 1991; Kolterman et al, 1981). Because of the insulin resistance, glucose uptake is affected resulting in hyperglycemia. This in turn leads to further hypersecretion of insulin by the pancreas. The chronically elevated levels of circulating insulin, in turn, impair the receptors' performance by the mechanism of downregulation. In the long run this compensatory mechanism fails, resulting in secretory failure. In view of this hypothesis, the hyperinsulinemia and subsequent insulin resistance are the consequences of the abnormalities within the receptor (DeFronzo, 1988; Olefsky et al, 1985).

On the other hand one can hypothesize that persistent hyperinsulinemia per se can be implicated as a primary factor in the development of insulin resistance. It has been observed that a persistent increase in the amount of circulating hormone can decrease the pool of the available receptors or reduce their effectiveness resulting in the insulin resistance (DeFronzo et al, 1983; Sheehan et al, 1986; Faber et al, 1981; Rizza et al, 1985). In fact it has been found that hyperinsulinemia is frequently observed among apparently healthy subjects who

develop overt diabetes later in life (Davidson et al, 1991). It has been documented that secretory deficiency is usually found among patients with advanced diabetes (Pacini et al, 1990) However, subjects with normal or slightly elevated fasting glucose levels most commonly display hyperinsulinemia following meals or glucose loads (Johnston et al, 1982, DeFronzo et al 1988). Furthermore it has been found that hyperinsulinemia accompanying liver cirrhosis leads to the development of insulin resistance and glucose intolerance (Proietto et al, 1980). The elevated insulin levels in this case result from the variable combination of decreased hepatic uptake due to the cellular defect and portal-systemic shunting (Petrides et al, 1989). Furthermore in studies with a surgically-induced diversion of the pancreatic venous drainage to the peripheral circulation it was shown that resulting hyperinsulinemia is accompanied by a decrease in the insulin sensitivity of approximately 50% (Radziuk et al , 1993).

The question arises as to the source of the observed hyperinsulinemia among diabetic subjects. It has been suggested that increased plasma insulin concentration is the result of hypersecretion (DeFronzo et al, 1983). However the results of this study indicate that alternate pathways exist which may contribute to a systemic hyperinsulinemia. Although the subjects with mild diabetes demonstrated a significant degree of hyperinsulinemia in the response to the glucose loading, the increase in insulin secretion was at best minor. This observation is based on the equivalent C-peptide concentrations among the examined groups. The metabolic clearance of the C-peptide in the subjects with mild diabetes was also identical to that of the controls. The source of the apparent hyperinsulinemia during observed perturbations seems to lie in the lower metabolic clearance rate of this peptide. Since the liver is the major site of the insulin removal (Faber et al, 1981; Cobelli et al, 1988; Eaton et al, 1983;

Ferrannini et al, 1987) it can be assumed that the changes in the hepatic uptake will be reflected in the metabolic clearance rate . It was assumed, therefore, that in the same subject the proportionality factor between the metabolic clearance rate and hepatic insulin extraction would remain the same. Using the calculation based on this assumption it was demonstrated that there was a significant decrease in the insulin removal among the glucose intolerant subjects following glucose loading. The basal metabolic clearance rate in both experimental groups was identical. Hepatic extraction was shown to be approximately 50% for all subjects. Following glucose loading however the decrease in hepatic uptake was small for the controls (20%, n.s.), but fell by approximately 50% among patients with mild diabetes. This change is also reflected in the decreased metabolic clearance rate. The increase in the peripherally measured insulin concentrations can be thus attributed primarily to the decrease in hepatic insulin extraction.

It was demonstrated that insulin clearance is nonlinear presumably based on saturation of hepatic uptake, especially when insulin concentrations reach high nonphysiological levels (Morishima et al, 1992; Pye at al, 1993). In the case of this study this is very unlikely since, based on the C-peptide data, there was little evidence of hypersecretion. The levels of circulating insulin were not high enough to affect the metabolic clearance of insulin under these non-steady state conditions.

It has also been proposed that the increased levels of free fatty acids interfere with insulin uptake. In fact, it was demonstrated in obese diabetic subjects that the increase in the oxidation of FFA is associated with a fall in the hepatic insulin removal (Golay et al, 1984; Groop et al, 1986). Abdominal (especially visceral) obesity which is associated with the greatest degree of insulin resistance could result in increased levels of mobilized FFA particularly in the portal circulation

(DeFronzo et al, 1985; Ferrannini et al, 1983). The data derived from this study indicate that other mechanisms may also be responsible for a decreased hepatic uptake of insulin. Although the difference in the concentrations measured during the perturbations can account for some of the observed changes, the levels of FFA measured prior to glucose ingestion were similar and within the physiological limits in the groups examined (< 0.7mmol/l). It is possible then, that while in the case of obesity, particularly of the android type, increased levels of FFA in the portal vein may contribute to the decrease in the hepatic insulin uptake, in the case of non-obese patients who have impaired glucose tolerance other mechanisms, not related to lipid metabolism, may predominate. On the other hand, the decrease in the uptake of insulin in hepatic cirrhosis seems to originate in a primary cellular defect.

4.2 METHODS

The oral glucose tolerance test served a dual purpose in this study. First of all it allowed to establish the precise level of glucose intolerance among the experimental subjects. Concentrations of glucose, insulin and C-peptide were measured in the basal state and following the glucose load. The degree of glucose intolerance was assessed based on the glucose levels using the guidelines of the National Diabetes Data Group. The areas under the insulin and C-peptide concentration curves were also determined. The large increase in the insulin concentration area among the subjects with mild diabetes was not accompanied by a similar rise in the C-peptide concentration. In fact the integrated C-peptide concentrations were similar among the studied groups, while the area calculated under the insulin concentration curve in the group of subjects with mild diabetes was approximately twice as large as in the control group. This observation per se suggested that a decrease in the insulin clearance was responsible for the

hyperinsulinemia which was present. It can be further supported by the fact that, the calculated average ratio $\int C_i / C_{cp}$ in the subjects with mild diabetes was found to be twice as high as in the control group. In addition, there seems to be a correlation between these ratios and the degree of glucose intolerance. Based on the results of the OGTT participants and subjects who underwent both stages of the study, the correlation appears to be stronger when subjects with advanced diabetes are excluded from the considerations. This can be explained by the fact that subjects with advanced diabetes differ greatly in their secretory responses to the glucose ingestion.

In order to gain more quantitative information a number of assumptions based on the different kinetics of the examined peptides were made (see Calculations). The most important of these is that insulin clearance is directly related to its hepatic extraction. As mentioned above the liver takes up approximately 50% of the insulin on each pass and because of that can be regarded as the major insulin removal site. In fact it was demonstrated in animal studies using a double tracer technique that the metabolic clearance of insulin and its hepatic removal follow a nearly parallel course (Pye et al, 1993). It is most likely then, that metabolic clearance reflects the degree of hepatic extraction.

Another assumption is that the metabolic clearance rate is constant but different during the basal conditions and following the glucose load. The change was assumed to be stepwise. The assumption of piecewise linearity of the insulin system allows the calculation of a mean metabolic clearance rate during the period that follows glucose loading. The small error likely made in the estimates would be similar for both experimental groups. If the metabolic clearance rate of insulin was assumed to be constant throughout the experiments, the peripheral

appearance of insulin calculated would have been overestimated. The calculation based on the inappropriately high clearance rate during the absorption period would result in the underestimation of hepatic extraction. By assuming the changing metabolic clearance in this study it was possible to avoid this inconsistency.

It has been reported that among patients with advanced diabetes and with severe hyperglycemia the increased levels of proinsulin and its split products may contribute to the overall levels of insulin measured using polyclonal antibodies. This frequently leads to the artificially overestimated concentrations of insulin (Horwitz et al, 1975). This is very unlikely to happen in this study. The particular antibody used in the insulin assays has a very low affinity to proinsulin. It is been assessed to be below 0.2%. The proinsulin split products such as des 31, 32 proinsulin were shown to have a cross-reactivity close to zero. It is safe to assume that measured insulin concentrations represent primarily this hormone. Moreover the relatively mild diabetes observed among the participants in this study makes it highly unlikely that there was an increased level of proinsulin and its split products present in the plasma. As pointed out (see Subjects) most of them demonstrated normal fasting glucose levels and the hyperglycemia following the glucose load was relatively mild.

4.3 SUMMARY

This study examined the decrease in the hepatic uptake of insulin as a possible factor responsible for the hyperinsulinemia frequently observed among patients in the early stages of non-insulin dependent diabetes mellitus. This was done by comparing healthy volunteers with subjects who presented with mild

abnormalities in glucose tolerance. The insulin concentrations following a glucose load were elevated among patients when compared with controls. C-peptide levels remained very similar in the two compared groups. This suggests a decrease in the removal of insulin during the observed perturbations. Because the liver is the major organ contributing to insulin clearance it is a reasonable assumption that a decrease in hepatic insulin extraction is primarily responsible for the decreased removal. A set of calculations based on legitimate assumptions was used to estimate the basal and postprandial metabolic clearance rate. This was shown to be equal under basal conditions in the groups examined, but decreased considerably following glucose loading among the subjects with mild diabetes. The postprandial hyperinsulinemia seen may therefore result from a fall in the metabolic clearance and not only from hypersecretion. In the group of glucose intolerant patients studied here, this is even more likely since insulin secretion was shown to be similar to controls. The results of this study suggest that decreased insulin removal by the liver is a possible early defect contributing to the development of non-insulin dependent diabetes mellitus among non obese subjects.

Figure 1. The derangements in carbohydrate, lipid and protein metabolism resulting from insulin deficiency.

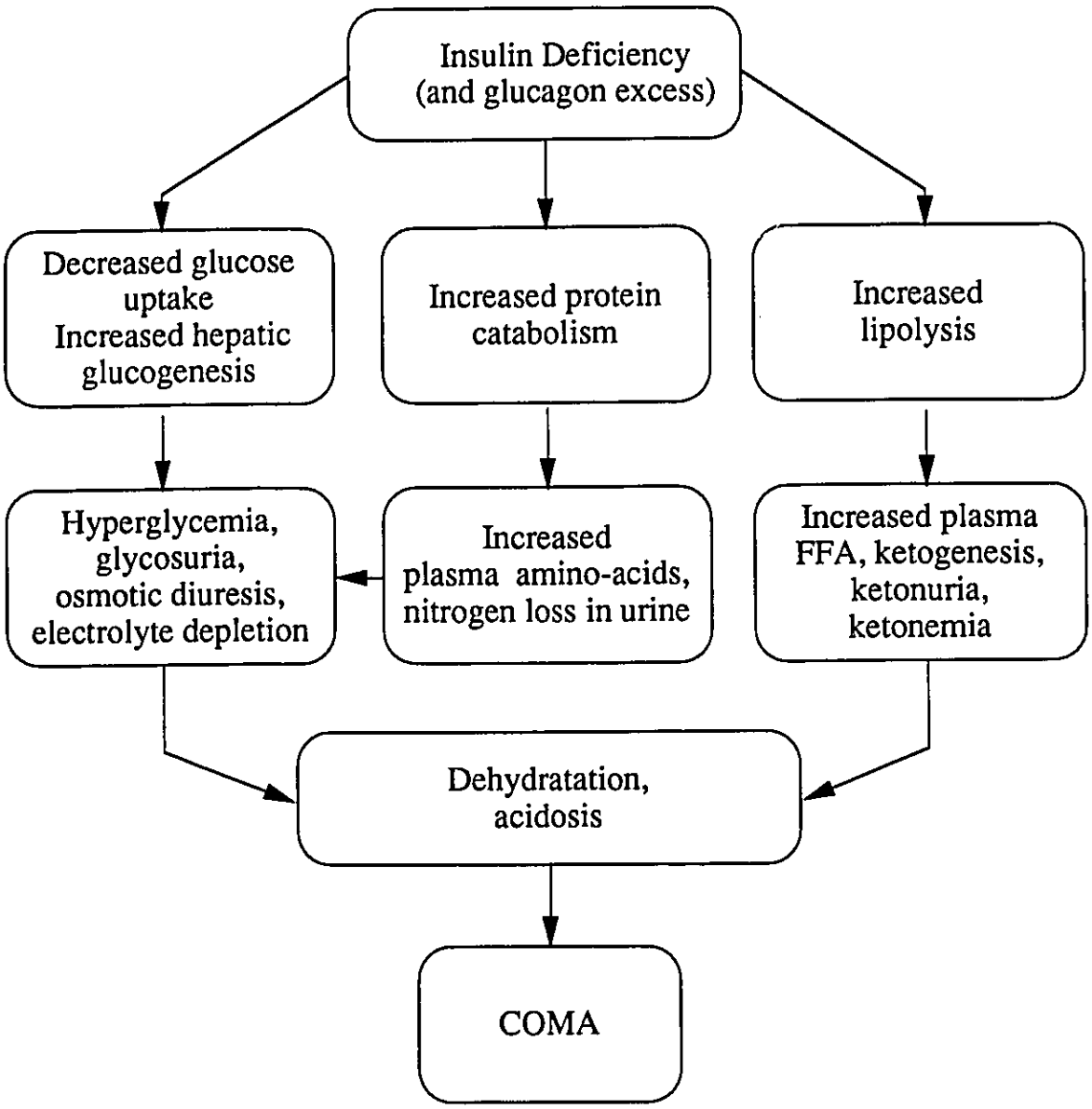

Insulin Deficiency
(and glucagon excess)
Decreased glucose uptake
Increased hepatic glucogenesis
Increased protein catabolism
Increased lipolysis
Hyperglycemia, glycosuria, osmotic diuresis, electrolyte depletion
Increased plasma amino-acids, nitrogen loss in urine
Increased plasma FFA, ketogenesis, ketonuria, ketonemia
Dehydratation, acidosis
COMA

Figure 2. The sequence of events operating in the pathogenesis of NIDDM. The initial defect has not been established, however, any of the implicated factors can cause hyperglycemia as an end result. The proposed decrease in hepatic insulin extraction would lead to the hyperinsulinemia resulting in the further downregulation of the insulin receptors and insulin resistance.

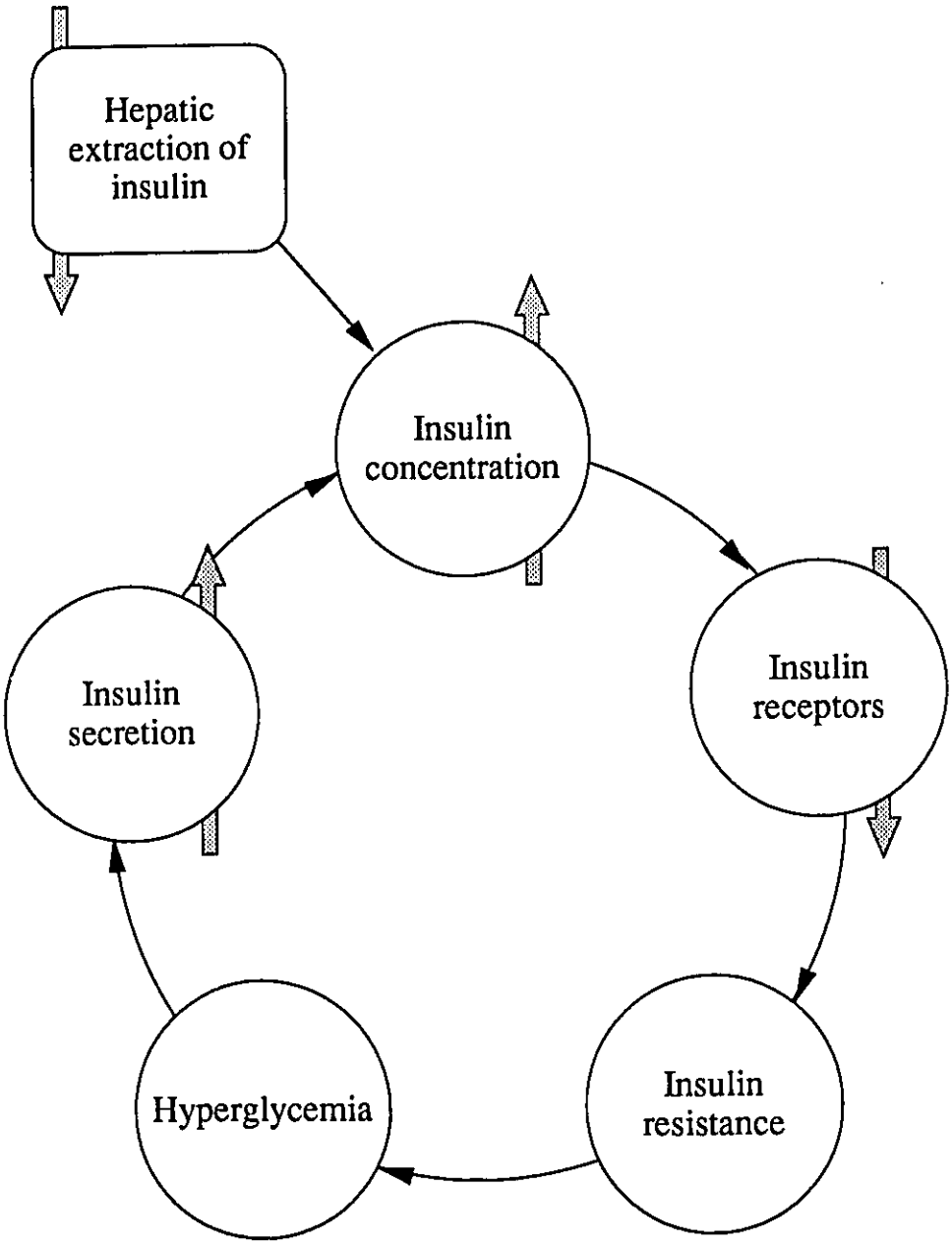
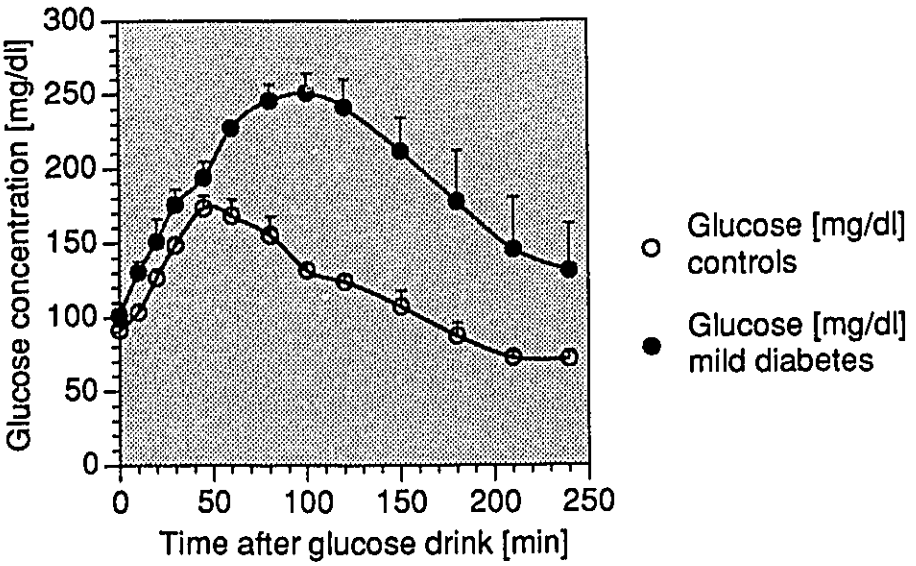
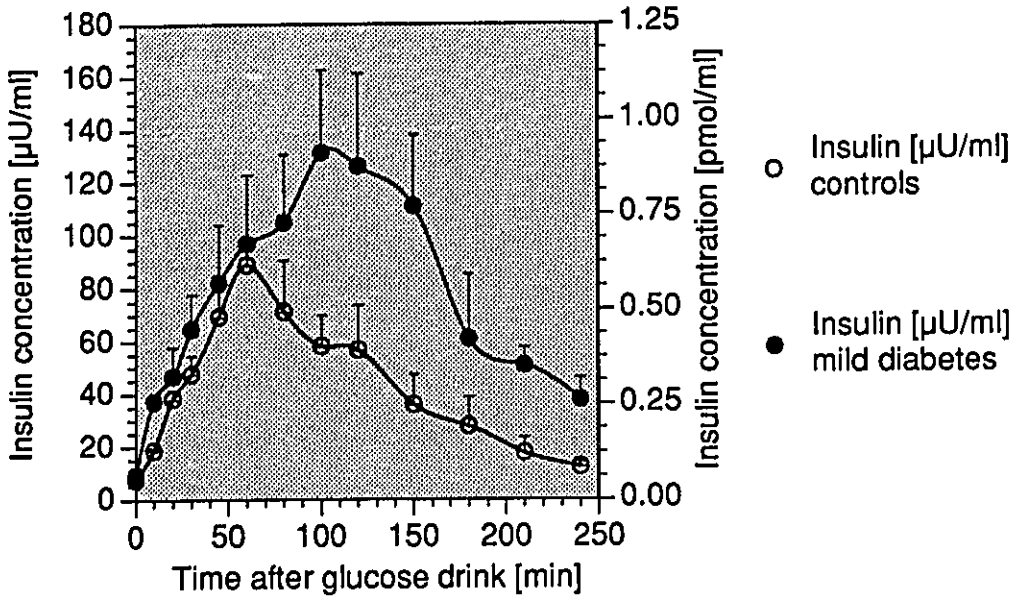

Hepatic extraction of insulin
Insulin concentration
Insulin receptors
Insulin resistance
Hyperglycemia
Insulin secretion

Figure 3. A 75 g glucose drink was administered to normal healthy controls and patients with mild diabetes (normal fasting glucose levels). This figure shows the average glucose concentrations in controls and subjects with mild diabetes.



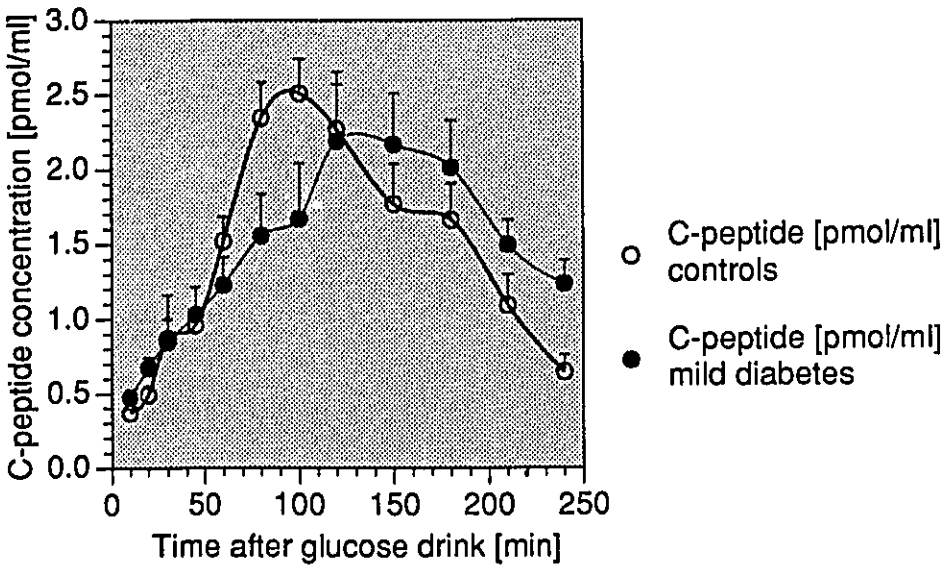
Time [min]	Glucose [mg/dl] controls	Std error	Glucose [mg/dl] mild diabetes	Std error
0	91.8	2.7	101.50	8.18
10	103.6	5.2	130.33	7.11
20	126.8	5.9	151.00	14.96
30	148.8	4.5	175.67	10.28
45	173.4	8.1	194.00	10.51
60	168.4	10.8	227.17	4.56
80	155.2	12.5	245.83	11.09
100	131.6	4.7	250.67	13.32
120	124.0	4.3	241.50	18.54
150	107.2	10.8	211.67	22.66
180	87.4	8.7	177.67	34.55
210	72.6	3.5	145.83	35.06
240	71.2	5.7	131.17	31.43

Figure 4. A 75 g glucose drink was administered to normal healthy controls and patients with mild diabetes (normal fasting glucose levels). This figure shows the average insulin concentrations in controls and subjects with mild diabetes. Insulin concentrations are given both in standard and SI units - μU/ml and pmol/ml.



Time [min]	Insulin [µU/ml] controls	Std error	Insulin [pmol/ml] controls	Std error	Insulin [µU/ml] mild diabetes	Std error	Insulin [pmol/ml] mild diabetes	Std error
0	7.66	0.8	0.05	0.01	9.3	2.8	0.06	0.02
10	18.72	3.4	0.13	0.02	37.2	11.3	0.26	0.08
20	38.24	7.0	0.27	0.05	46.6	13.1	0.32	0.09
30	47.56	9.9	0.33	0.07	64.7	21.9	0.45	0.15
45	69.26	7.6	0.48	0.05	82.2	26.0	0.57	0.18
60	88.88	19.3	0.62	0.13	96.9	26.0	0.67	0.18
80	71.32	11.8	0.50	0.08	104.8	31.3	0.73	0.22
100	58.16	16.9	0.40	0.12	131.4	35.1	0.91	0.24
120	56.78	11.6	0.39	0.08	126.1	27.3	0.88	0.19
150	35.88	11.1	0.25	0.08	111.0	24.4	0.77	0.17
180	27.68	5.9	0.19	0.04	61.0	6.8	0.42	0.05
210	17.48	2.2	0.12	0.01	50.7	8.3	0.35	0.06
240	12.28	1.3	0.09	0.01	37.7	6.3	0.26	0.04

Figure 5. A 75 g glucose drink was administered to normal healthy controls and patients with mild diabetes (normal fasting glucose levels). This figure shows the average C-peptide concentrations in controls and subjects with mild diabetes.

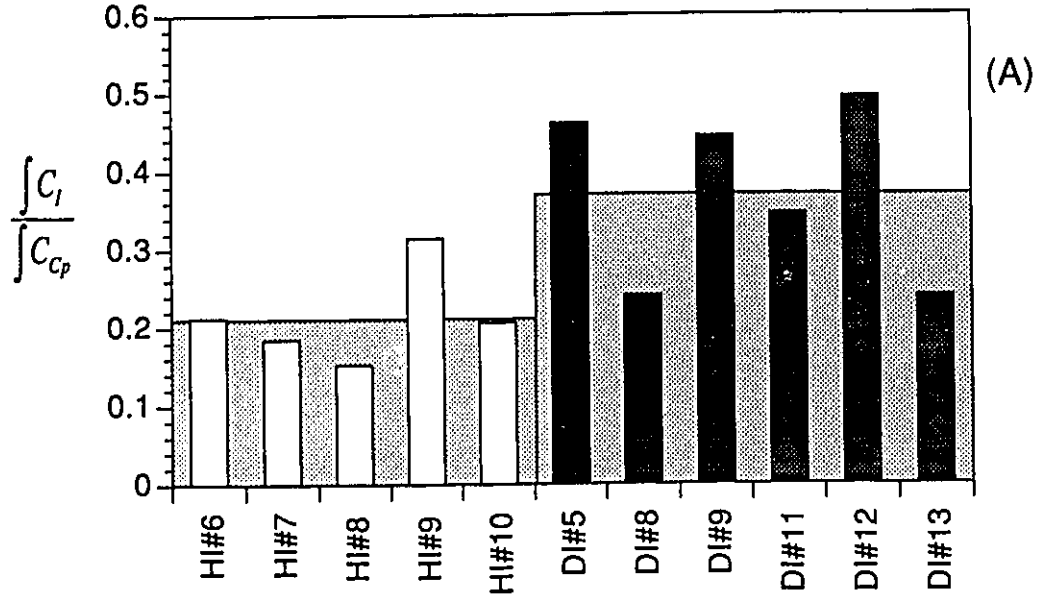


Time [min]	C-peptide [pmol/ml] controls	Std error	C-peptide [pmol/ml] mild diabetes	std error
0	0.37	0.03	0.47	0.05
10	0.49	0.06	0.67	0.08
20	0.87	0.29	0.85	0.15
30	0.96	0.09	1.03	0.19
45	1.52	0.16	1.23	0.18
60	2.34	0.24	1.55	0.28
80	2.51	0.23	1.67	0.37
100	2.27	0.39	2.19	0.38
120	1.76	0.27	2.17	0.34
150	1.66	0.25	2.01	0.31
180	1.09	0.21	1.49	0.17
210	0.64	0.11	1.24	0.16
240	0.53	0.05	1.02	0.18

Figure 6. The insulin and C-peptide responses following a 75 g oral glucose load were integrated over the observation period (4 hours after the drink).

Panel A. The ratios of the integrated insulin to C-peptide concentrations for controls and subjects with mild diabetes (in arbitrary units), are shown. The average ratio in the control group was 0.21±0.03, and in the group of patients with mild diabetes was 0.38±0.05.

Panel B. The ratios of integrated insulin to C-peptide concentrations were plotted against the corresponding area under the glucose concentration curve for controls and patients with mild diabetes. The regression line was plotted. The correlation coefficient was calculated to be 0.6.


(A)
$\frac{\int C_I}{\int C_{Cp}}$
0.6
0.5
0.4
0.3
0.2
0.1
0
HI#6
HI#7
HI#8
HI#9
HI#10
DI#5
DI#8
DI#9
DI#11
DI#12
DI#13

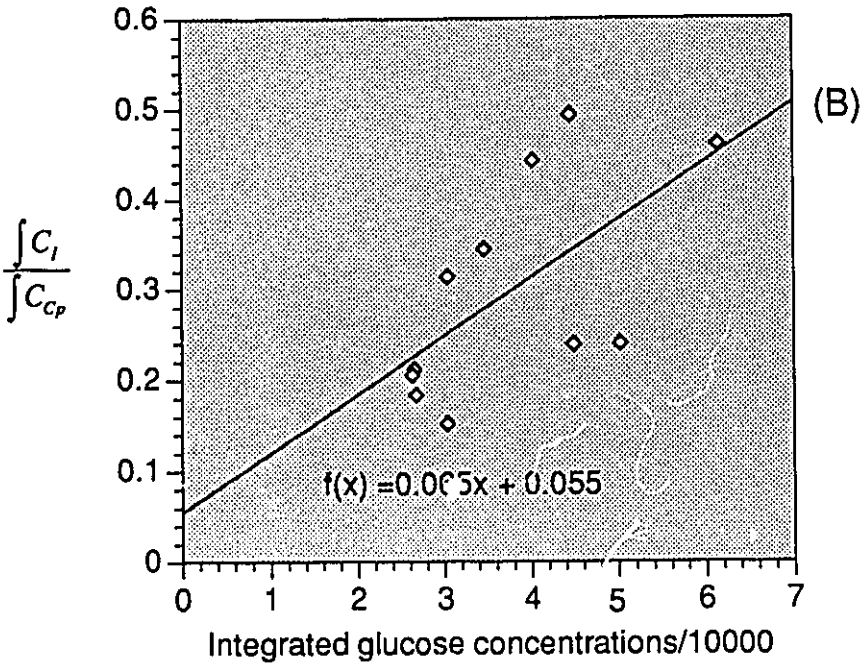

(B)
$\frac{\int C_I}{\int C_{Cp}}$
0.6
0.5
0.4
0.3
0.2
0.1
0
f(x) =0.0
5x + 0.055
0
1
2
3
4
5
6
7
Integrated glucose concentrations/10000

Figure 7. The insulin and C-peptide responses following a 75 g oral glucose load were integrated over the observation period (4 hours after the drink).

Ratios of integrated insulin to C-peptide concentrations are shown for all subjects participating in the OGTT. The average ratio was: 0.21±0.02 for controls, 0.37±0.04 for subjects with mild diabetes, and 0.34±0.04 for subjects with advanced diabetes. The last group demonstrated the most variation in the ratios (as well as in the insulin secretory responses).

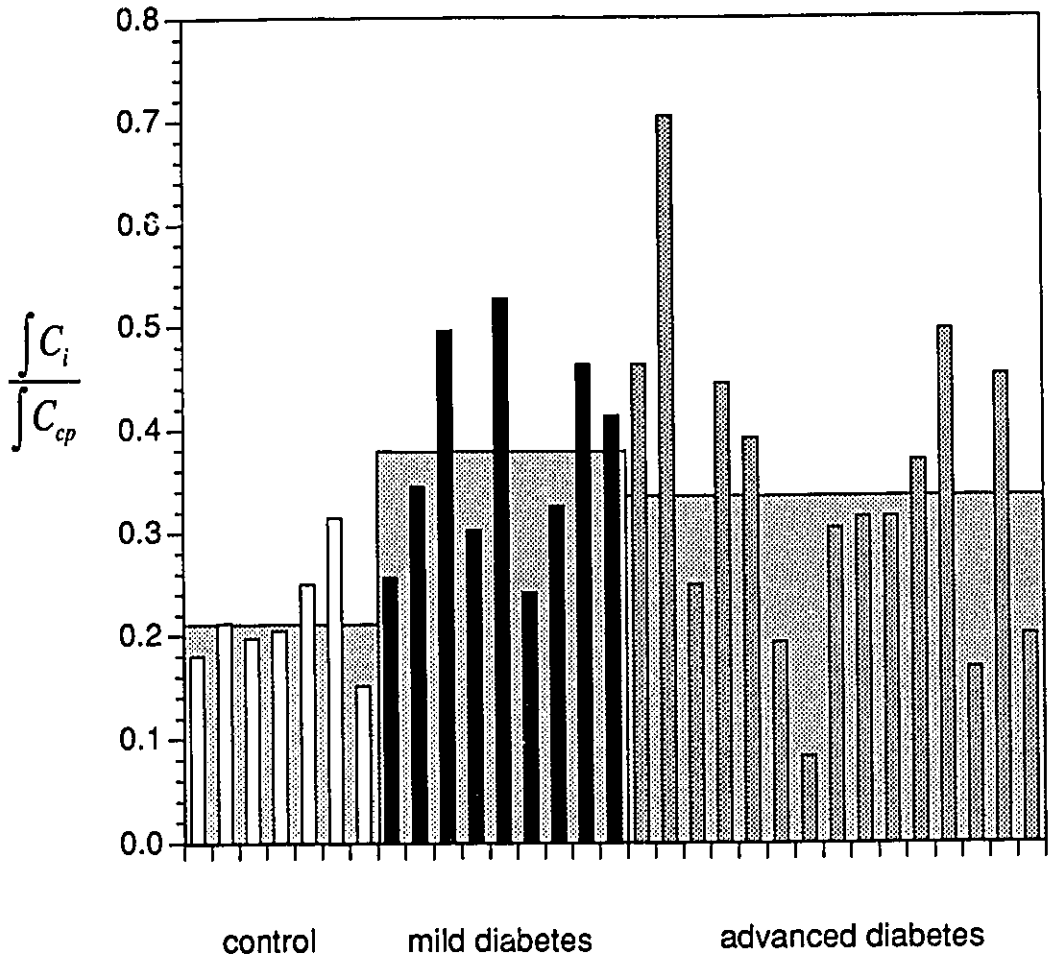

$\frac{\int C_i}{\int C_{cp}}$
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
control
mild diabetes
advanced diabetes

Figure 8. The insulin and C-peptide responses following a 75 g oral glucose load were integrated over the observation period (4 hours after the drink).

The ratios of the integrated insulin to C-peptide concentrations were plotted against the corresponding area under the glucose concentration curve for all participants in the OGTT. The regression lines are plotted for all subjects (broken line) and for controls and subjects with mild diabetes (unbroken line). Since the subjects representing advanced diabetes were characterized by a more variable insulin secretion after stimulation with an oral glucose load, the correlation was less pronounced when this group was included in the analysis.

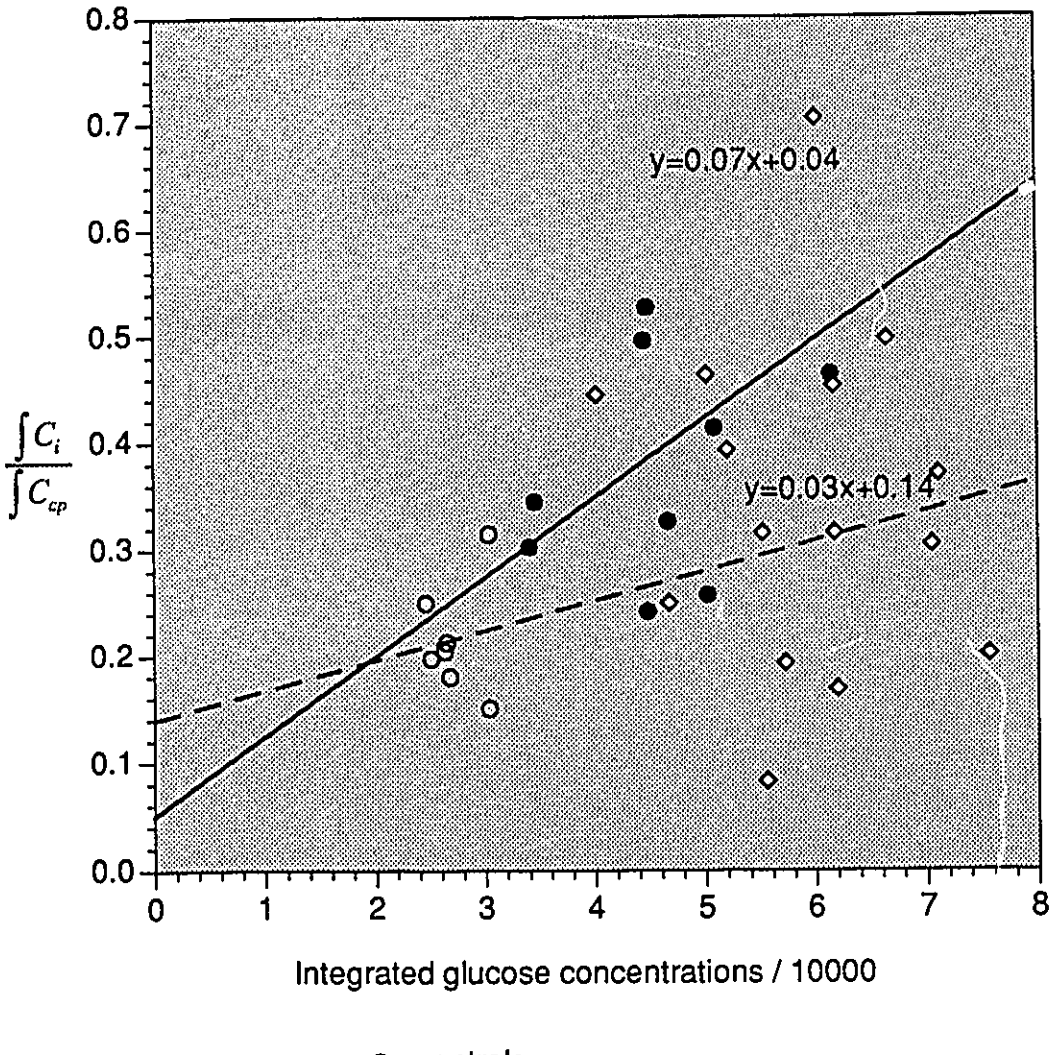
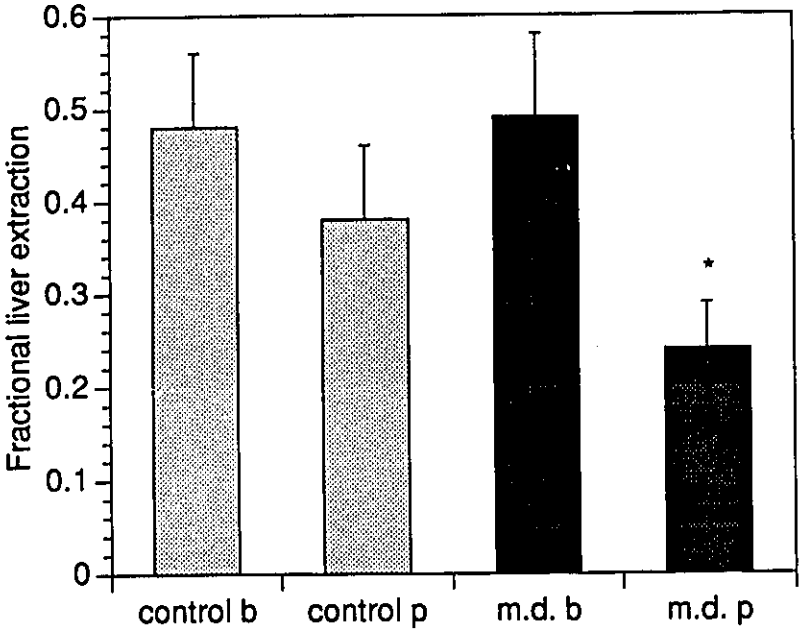

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
8
y=0.07x+0.04
y=0.03x+0.14
$\frac{\int C_i}{\int C_{cp}}$
Integrated glucose concentrations / 10000
controls
mild diabetes
advanced diabetes

Figure 9.

Panel A. The mean fractional hepatic extraction of insulin was calculated before (basal) and after a 75 g oral glucose load. The data are shown in both control subjects and patients with mild diabetes (m.d.) both under basal conditions (b) and postprandially (p). There was a small decrease after glucose loading in the control subjects (not significant) and a significant (*, $p<0.05$) decrease following the glucose drink in patients with mild diabetes.

Panel B. Similar results were seen for the metabolic clearance rate of the insulin.


(A)
Fractional liver extraction
0.6
0.5
0.4
0.3
0.2
0.1
0
*
control b
control p
m.d. b
m.d. p

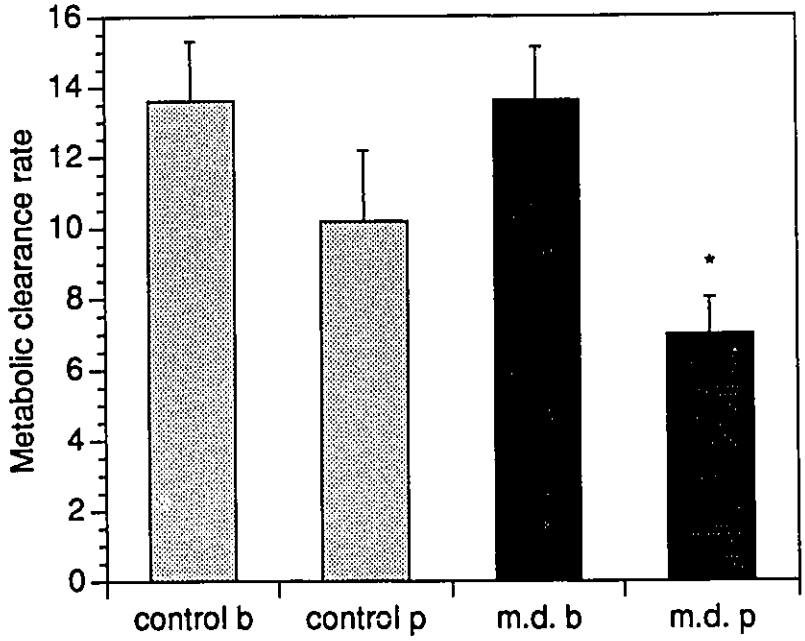
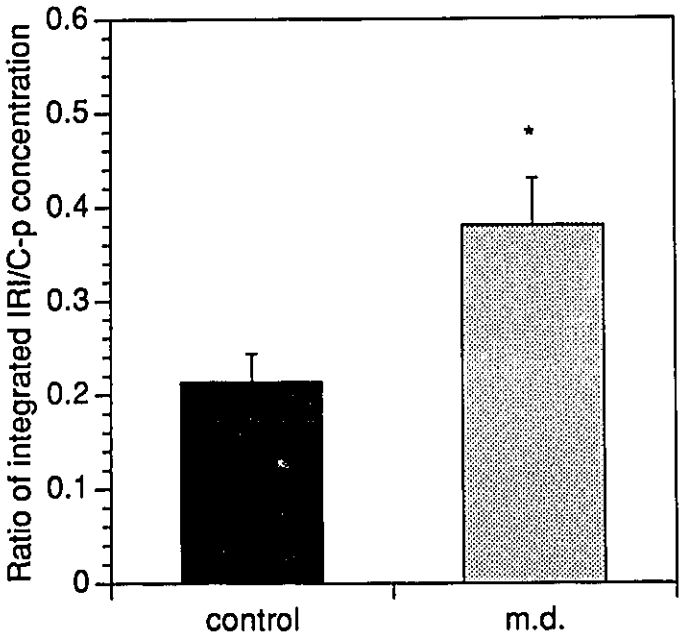
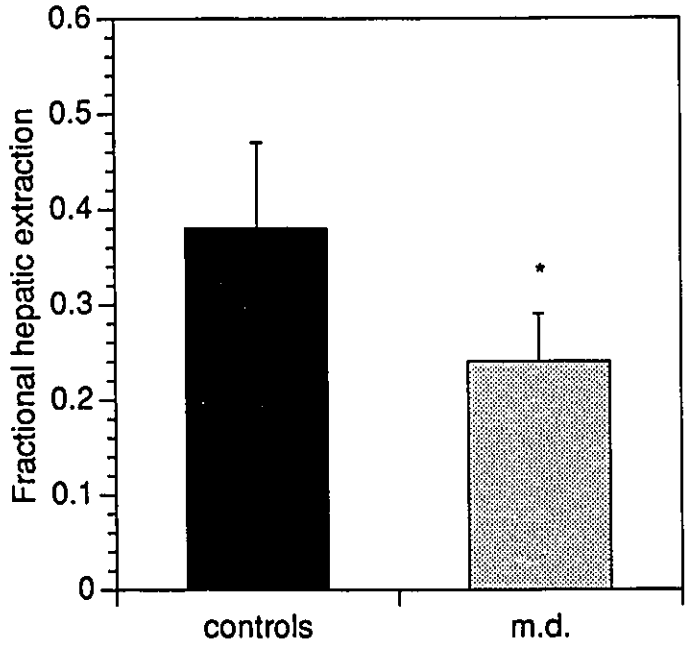

(B)
Metabolic clearance rate
16
14
12
10
8
6
4
2
0
*
control b
control p
m.d. b
m.d. p

Figure 10. The insulin and C-peptide responses following a 75 g oral glucose load were integrated over the observation period (4 hours after the drink). Based on the ratios of the integrated insulin to C-peptide concentrations and the decay curves for the insulin and C-peptide, the fractional hepatic extraction of insulin was calculated. Panel (A) shows an increase in the mean ratio ($p<0.05$) and panel (B) a corresponding decrease in the fractional hepatic extraction of insulin.

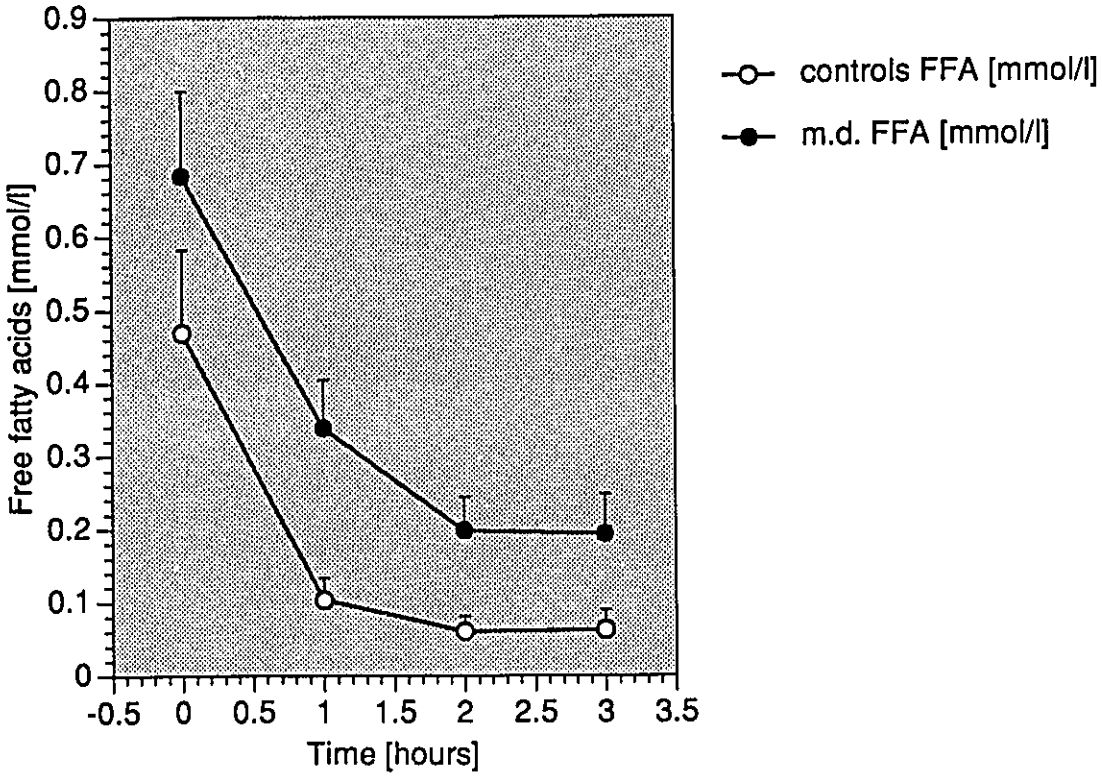

Ratio of integrated IRI/C-p concentration
0.6
0.5
0.4
0.3
0.2
0.1
0
*
control
m.d.

(A)


Fractional hepatic extraction
0.6
0.5
0.4
0.3
0.2
0.1
0
*
controls
m.d.

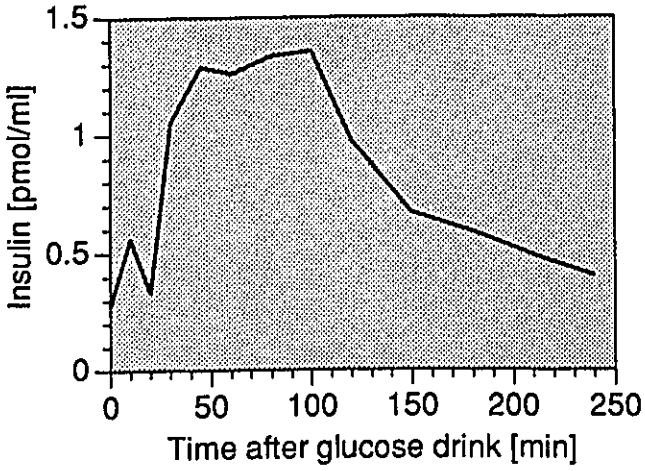
(B)

Figure 11. A 75 g glucose drink was administered to normal healthy controls and patients with mild diabetes (normal fasting glucose levels). This figure shows the average free fatty acids concentrations in controls and subjects with mild diabetes (m.d.)

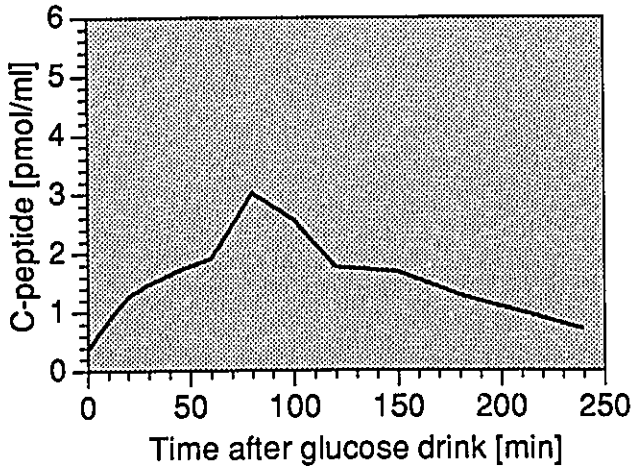


Time [hours]	controls FFA [mmol/l]	Std err	m.d. FFA [mmol/l]	Std err
0	0.469	0.113	0.683	0.116
1	0.103	0.030	0.338	0.067
2	0.061	0.020	0.198	0.046
3	0.062	0.027	0.193	0.054

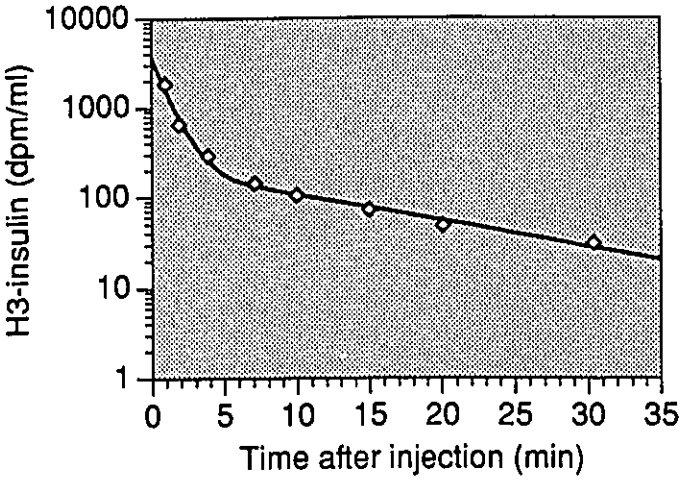
Figure 12. Insulin and C-peptide concentrations during OGTT and the results of a double exponential fitting of the preliminary decay curves following bolus injections of 3H-insulin and C-peptide. These results are from a single study in a patient with mild diabetes (DI#12).



$$\int C_I(t)dt = 201$$

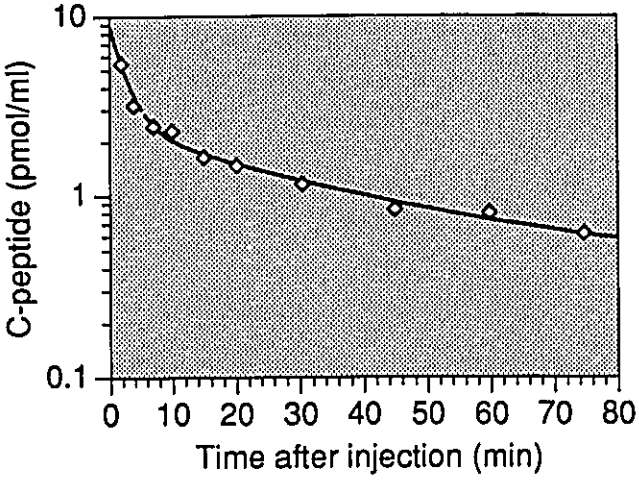


$$\int C_{C-p}(t)dt = 407$$



$$h_I(t) = 3338.1e^{-0.915t} 206.4e^{-0.066t}$$

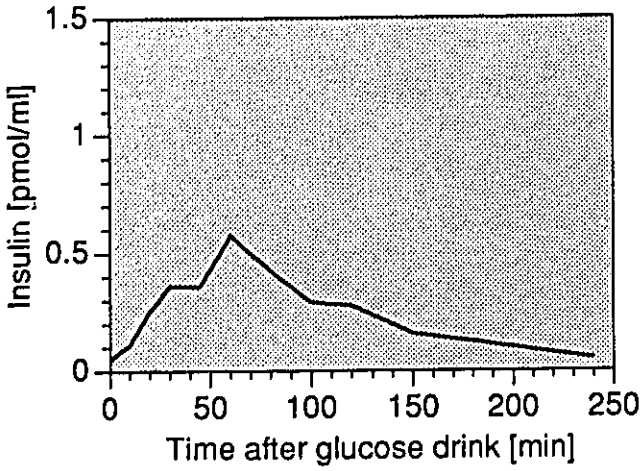
$$\int_0^\infty h_I(t)dt = 6775.5$$



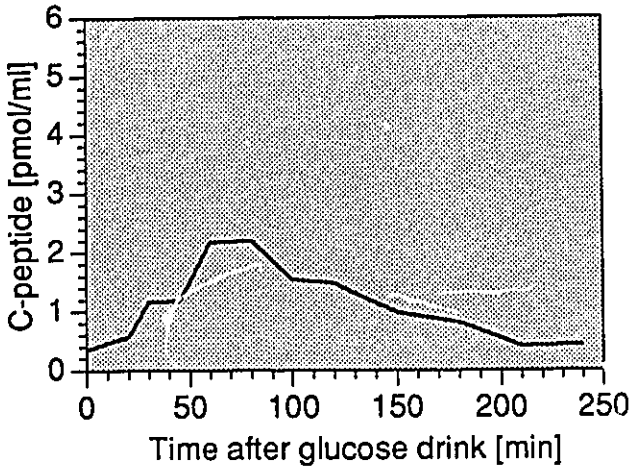
$$h_{C-p}(t) = 6.42e^{-0.38t} + 2.01e^{-0.03t} + 0.4$$

$$\int_0^\infty h_{C-p}(t)dt = 84.1$$

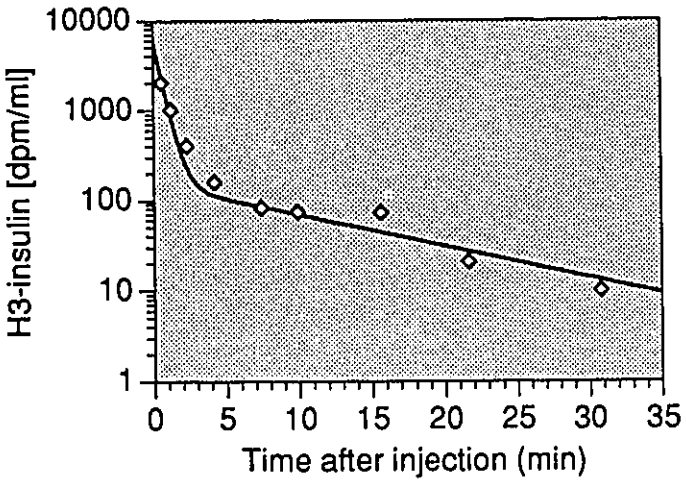
Figure 13. Insulin and C-peptide concentrations during OGTT and the results of a double exponential fitting of the preliminary decay curves following bolus injections of 3H-insulin and C-peptide. These results are from a single study in a healthy control subject (HI#10).



$$\int C_I(t)dt = 57$$

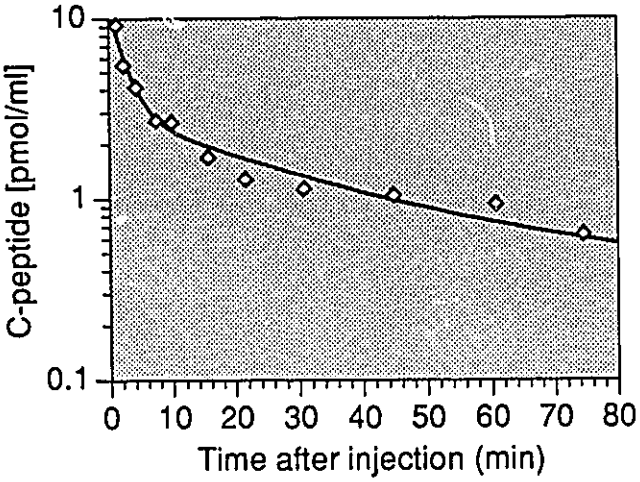


$$\int C_{C-p}(t)dt = 277$$



$$h_I(t) = 5517.4e^{-1.751t} 154e^{-0.08t}$$

$$\int_0^\infty h_I(t)dt = 5076.0$$



$$h_{C-p}(t) = 8.72e^{-0.41t} + 2.52e^{-0.03t} + 0.37$$

$$\int_0^\infty h_{C-p}(t)dt = 105.3$$

Table 1. Personal data for all subjects studied (from the top: controls, patients with mild diabetes and patients with advanced diabetes).

	HEIGHT [cm]	WEIGHT [kg]	BMI [kg/m^2]	AGE [years]	SEX
BI	182	83.0	25.1	55	M
LN	165	66.4	24.4	54	F
LR	180	88.0	27.2	48	M
ND	173	74.5	24.9	51	M
MM	180	95.0	29.3	50	M
SI	182	80.0	24.2	54	F
HR	173	61.0	20.4	42	M
AVG	176.4±2.4	78.3±4.5	25.1±1.0	50.6±1.7	
MJ	166	75.0	27.2	53	M
JPM	184	78.0	22.2	65	M
CM	160	57.0	22.0	48	F
RP	181	89.0	27.2	61	M
MCB	176	85.0	27.4	43	M
AB	178	90.0	28.4	41	M
BF	162	56.0	21.5	34	F
LE	173	90.0	30.0	56	F
MJP	158	61.8	24.0	55	F
AVG	170.9±3.2	75.8±4.7	25.5±1.1	50.7±3.3	
JLT	184	72.5	21.4	50	M
HT	162	72.0	27.4	37	M
BB	180	73.3	22.6	59	M
BS	175	75.2	24.4	60	M
JS	176	72.7	23.5	48	M
CF	176	72.7	23.5	68	M
KD	179	73.0	22.7	62	M
NL	176	86.0	27.7	42	F
MII	179	72.7	22.0	45	M
GP	176	87.7	27.5	63	M
MB	160	65.4	25.6	59	F
BR	181	81.0	24.4	63	M
ESL	161	64.5	24.9	60	F
VL	164	74.4	27.7	68	F
CK	173	79.8	26.7	48	M
AVG	173.9±1.9	82.1±7.7	24.8±0.6	55.5±2.5	

Table 2. Glucose concentrations after a 75 g glucose loading at time points: t = 0, 0.5, 1.0, and 2.0 hours, for all the experimental subjects (from the top: controls, patients with mild diabetes and patients with advanced diabetes). The control group consisted of subjects with a normal glucose tolerance curve. The subjects with fasting glucose levels below 115 mg/dl and an abnormal glucose tolerance curve were assigned to the group of patients with mild diabetes. The remaining subjects with a pronounced glucose intolerance (both fasting and after glucose loading) were excluded from the participation in the second stage of the study since they represented a group with more advanced diabetes.

	Glucose [mg/dl] t=0	Glucose [mg/dl] t=0.5	Glucose [mg/dl] t=1.0	Glucose [mg/dl] t=2.0
LN	86	134	156	122
BI	86	160	142	116
HR	88	136	160	122
MM	92	148	156	116
SI	92	138	124	112
ND	95	146	198	127
LR	100	156	190	139
JPM	71	151	239	276
CM	92	210	217	176
RP	93	174	236	236
LE	92	180	252	139
AB	112	199	244	233
MCB	112	146	235	234
BF	114	166	236	223
MJ	114	198	222	305
MJP	114	144	240	240
JLT	118	232	290	273
HT	118	172	281	320
BB	129	234	300	270
BS	127	175	212	220
JS	131	206	274	273
CF	137	206	268	295
KD	141	207	255	292
NL	145	250	344	326
MII	145	271	275	273
GP	147	190	247	305
BR	149	232	278	316
MB	147	235	314	363
ESL	155	205	283	322
VL	162	231	280	302
CK	190	260	335	366

Table 3. Baseline experimental data in the control subjects who participated in an oral glucose tolerance test (75 g) after an overnight fast. Basal glucose, insulin and C-peptide values are represented in the top panel. The areas under the glucose, insulin and C-peptide concentration curves seen after the administration of the glucose load are summarized in the lower panel. The ratios of the integrated insulin to C-peptide concentrations are also included.

	Glucose baseline [mg/dl]	IRI baseline [µU/ml]	IRI baseline [pmol/ml]	C-p baseline [pmol/ml]
BI	86	8.3	0.06	0.50
LN	86	4.9	0.03	0.34
LR	100	8.0	0.06	0.32
ND	95	9.8	0.07	0.40
MM	92	7.3	0.05	0.37
SI	92	9.2	0.06	0.25
HR	88	9.2	0.06	0.24
AVG	91.3±1.9	8.1±0.6	0.06±0.004	0.35±0.03

	C glucose	C insulin	C C-p	C ins/ C c-p
BI	26565	57	269	0.21
LN	26780	74	402	0.18
LR	30364	57	375	0.15
ND	30415	119	379	0.31
MM	26330	57	277	0.21
SI	24635	58	231	0.25
HR	25075	52	264	0.20
AVG	27116±881.9	67.7±8.9	313.9±26.0	0.21±0.02

Table 4. Baseline experimental data in the subjects with mild diabetes who participated in an oral glucose tolerance test (75 g) after an overnight fast. Basal glucose, insulin and C-peptide values are represented in the top panel. The areas under the glucose, insulin and C-peptide concentration curves seen after the administration of the glucose load are summarized in the lower panel. The ratios of the integrated insulin to C-peptide concentrations are also included.

	Glucose baseline [mg/dl]	IRI baseline [µU/ml]	IRI baseline [pmol/ml]	C-p baseline [pmol/ml]
MJ	114	20.0	0.14	0.43
JPM	71	14.5	0.10	0.74
CM	92	5.5	0.04	0.40
RP	93	3.8	0.03	0.39
MCB	112	2.7	0.02	0.40
AB	112	9.2	0.06	0.50
BF	114	8.6	0.06	0.24
LE	99	11.4	0.08	0.47
MJP	114	15.5	0.11	0.39
AVG	102.3±4.9	10.1±1.9	0.07+0.013	0.44±0.04

	C glucose	C insulin	C C-p	C ins/ C c-p
MJ	61450	100	217	0.46
JPM	50275	59	246	0.24
CM	34658	194	562	0.35
RP	44585	201	407	0.49
MCB	44903	97	406	0.24
AB	44878	223	424	0.53
BF	46690	71	219	0.32
LE	34135	116	384	0.30
MJP	50910	126	305	0.41
AVG	45831±2776.0	131.8±19.8	352.2±117.4	0.37±0.04

Table 5. Baseline experimental data in the subjects with advanced diabetes who participated in an oral glucose tolerance test (75 g) after an overnight fast. Basal glucose, insulin and C-peptide values are represented in the top panel. The areas under the glucose, insulin and C-peptide concentration curves seen after the administration of the glucose load are summarized in the lower panel. The ratios of the integrated insulin to C-peptide concentrations are also included.

	Glucose baseline [mg/dl]	IRI baseline [µU/ml]	IRI baseline [pmol/ml]	C-p baseline [pmol/ml]
JLT	118	18.5	0.13	0.35
HT	118	16.7	0.12	0.30
BB	129	6.9	0.05	0.32
BS	127	10.4	0.07	0.42
JS	131	13.6	0.09	0.42
CF	137	7.0	0.05	0.39
KD	141	6.8	0.05	0.39
NL	145	6.3	0.04	0.71
MII	145	53.8	0.37	0.55
GP	147	17.3	0.12	0.60
MB	147	7.0	0.05	0.15
BR	149	22.0	0.15	0.54
ESL	155	16.4	0.11	0.65
VL	162	15.0	0.10	0.17
CK	190	24.0	0.17	0.84
AVG	142.7±4.8	16.1±3.1	0.11±0.02	0.45±0.05

	C glucose	C insulin	C C-p	C ins/ C c-p
JLT	50185	159	344	0.46
HT	60168	142	201	0.70
BB	46775	66	263	0.25
BS	40273	168	379	0.44
JS	52030	124	316	0.39
CF	57210	69	360	0.19
KD	55525	52	626	0.08
NL	70460	137	451	0.30
MII	55200	118	374	0.31
GP	61788	100	319	0.32
MB	71108	49	132	0.37
BR	66505	93	187	0.50
ESL	62005	59	349	0.17
VL	61713	118	261	0.45
CK	75593	51	256	0.20
AVG	59102±960	100.3±10.6	321.1±83.3	0.34±0.04

Table 6. Selection of personal data for subjects undergoing the extended and glucose tolerance test - both healthy controls (HI) and subjects with mild diabetes (DI). Based on the results of OGTT subjects were divided into three categories: controls, subjects with mild diabetes and subjects with advanced diabetes. The latter group was excluded from the participation in the extended study, since they represent a nonhomogenous population characterized by more variability in their insulin secretory response to glucose stimulation.

	HEIGHT [cm]	WEIGHT [kg]	BMI [kg/m^2]	AGE [years]	SEX
HI#6	182	83.0	25.1	55	M
HI#7	165	66.4	24.4	54	F
HI#8	180	88.0	27.2	48	M
HI#9	173	74.5	24.9	51	M
HI#10	180	95.0	29.3	50	M
AVG	176.0±3.14	81.0±5.0	26.2±0.9	51.6±1.3	
DI#5	166	75.0	27.2	53	M
DI#8	184	78.0	22.2	65	M
DI#9	175	75.2	24.4	60	M
DI#11	160	57.0	22.0	48	F
DI#12	181	89.0	27.2	61	M
DI#13	176	85.0	27.4	43	M
AVG	173.0±3.71	76.5±4.5	25.1±1.0	55.0±3.5	

Table 7. Baseline experimental data in the control subjects who participated in both, an oral glucose tolerance test (75 g) and an extended study. Basal glucose, insulin and C-peptide values are represented in the top panel. The areas under the glucose, insulin and C-peptide concentration curves seen after the administration of the glucose load are summarized in the lower panel. The ratios of the integrated insulin to C-peptide concentrations are also included.

	Glucose baseline [mg/dl]	IRI baseline [µU/ml]	IRI baseline [pmol/ml]	C-p baseline [pmol/ml]
HI#6	86	8.3	0.06	0.5
HI#7	86	4.9	0.03	0.34
HI#8	100	8.0	0.06	0.32
HI#9	95	9.8	0.07	0.4
HI#10	92	7.3	0.05	0.37
AVG	91.8±2.7	7.7±0.8	0.05±0.00	0.39±0.03
DI#5	114	20.0	0.14	0.43
DI#8	71	14.5	0.10	0.74
DI#9	127	10.4	0.07	0.42
DI#11	92	5.5	0.04	0.4
DI#12	93	3.8	0.03	0.4
DI#13	112	2.7	0.02	0.5
AVG	101.5±8.2	9.5±2.8	0.07±0.02	0.48±0.05

	C glucose	C insulin	C c-p	Ratio IRI/C-p
HI#6	26565	57	269	0.21
HI#7	26780	74	402	0.18
HI#8	30364	57	375	0.15
HI#9	30415	119	379	0.31
HI#10	26330	57	277	0.21
AVG	28090±940	73±12	340±28	0.21±0.03
DI#5	61450	100	217	0.46
DI#8	50275	59	246	0.24
DI#9	34658	194	562	0.35
DI#11	44585	201	407	0.49
DI#12	44903	97	406	0.24
DI#13	44878	223	424	0.53
AVG	46792±3588	136.5±24	369±51	0.38±0.05

Table 8. Summary of the experimental parameters which were determined in a group of subjects (normal controls and patients with mild diabetes). At time=0 hr, a 75 g oral glucose load was administered and glucose, insulin and C-peptide concentrations were determined for 4 hours thereafter. A preliminary determination of the metabolic clearance of both insulin and C-peptide was made under basal conditions using plasma responses to the bolus injections of C-peptide and ^{3}H-insulin.

Parameters		Control	Mild diabetes
Basal glucose concentration (mg/dl)		91.8±2.7	101.5±8.2
Basal insulin concentration (μU/ml)		7.7±0.8	9.5±2.8
	(pmol/ml)	0.05±0.006	0.07±0.020
Basal C-peptide concentration (pmol/ml)		0.39±0.03	0.48±0.05
Basal metabolic clearance of insulin (ml/kg/min)		13.1±1.5	13.6±2.0
Metabolic clearance of C-peptide (ml/kg-min)		3.53±0.6	3.00±0.2
Basal appearance rate of insulin (pmol/kg-min)		0.67±0.06	0.74±0.38
Basal appearance rate of C-peptide (pmol/kg-min)		1.34±0.19	1.44±0.21
Basal fractional extraction by liver (%)		49±7	50±7
Metabolic clearance of insulin during OGTT (ml/kg-min)		10.5±1.6	6.7±1.0
Appearance of insulin during OGTT (pmol)		693±91	834±138
Appearance of C-peptide during OGTT (pmol)		1202±205	1148±192
Fractional extraction by liver (%)		39±9	25±4
Integrated insulin concentration		73±12	137±24
Integrated C-peptide concentration		340±28	369±51
Ratio of integrated insulin /C-p concentrations		0.21±0.03	0.38±0.05
FFA (mmol/l)	Basal	0.469±0.11	0.68±0.12
	1 hr	0.10±0.03	0.34±0.07
	2 hr	0.06±0.02	0.20±0.05
	3 hr	0.06±0.03	0.19±0.05

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APPENDIX: Sample Calculations

Figures 12 and 13 represent examples of C-peptide and ^{3}H-insulin decay curves after bolus injections were given, together with the results of the double exponential fit and parameters used during fitting. The insulin and C-peptide response to the glucose load together with calculated areas under the concentration curves are included.

The experimental data from the combined experiments on two subjects (healthy - HI#10 and subject with mild diabetes - DI#12) from both examined groups is used here to demonstrate the calculations in detail.

Under basal conditions, the metabolic clearance rate can be calculated when the response to the bolus injection is known. The procedure of numerical fitting of the experimental data to a double exponential $y=a_1*\exp(-s_1*t) +a_2\exp(-s_2*t)$ for insulin, and $y=a_1*\exp(-s_1*t) +a_2\exp(-s_2*t) + b$ for the C-peptide (b = basal values for C-peptide concentrations) was performed using the least square technique on the Macintosh computer using Excell software.

The results were as follow:

HI# 10	^{3}H insulin	C-peptide
	a_1=983.7	a_1=27.78
	a_2=136.1	a_2=2.67
	s_1= 0.466	s_1=1.051
	s_2=0.06	s_2=0.023

DI#12	^{3}H insulin	C-peptide
	a_1=3338.1	a_1=6.742
	a_2=206.4	a_2=2,020
	s_1=0.915	s_1=0.383
	s_2=0.066	s_2=0.03

The area under the decay curve calculated from the formula:

$$\int_0^\infty h(t)dt = \frac{a_1}{s_1} + \frac{a_2}{s_2}$$

was

$$\int_0^\infty h_i(t)dt = 5076.0 \text{ (HI\#10) and } \int_0^\infty h_i(t)dt = 6775.5 \text{ (DI\#12)}$$

and

$$\int_0^\infty h_{cp}(t)dt = 105.3 \text{ (HI\#10) and } \int_0^\infty h_{cp}(t)dt = 89.0 \quad \text{(DI\#12)}$$

The total ^{3}H-insulin injected was calculated to be:

M*= 7x703582=4925074 dpm (HI#10)

and

M*=7x817610=5723370 (DI#12).

The total C-peptide injected was:

$$M=4.6x7027=32324 \quad \text{(HI\#10)}$$

and

$$M=4x4140=16560 \quad \text{(DI\#12).}$$

The metabolic clearance rate was calculated from equations [1] and [2] (see Calculations). The result was subsequently divided by the subjects' weight, 95 kg and 89 kg for the control and glucose intolerant patient respectively and are expressed in ml $kg^{-1}min^{-1}$:

HI#10

$$mcr_{ib}=6701527/7576.8/95.0=10.2$$

$$mcr_{cpb}=32324/105.3/95.0=3.23$$

DI#12

$$mcr_{ib=}4925074/6775.5/89=9.5$$

$$mcr_{cpb=}16560/89.0=2.22$$

The basal appearance rate can be obtained from equations [4] and [5]. Fasting values of insulin and C-peptide are used for this purpose, multiplied by the appropriate mcr. The final value for the appearance rate of insulin is divided by 144 in order to express both results in pmol/ml. The factor is calculated based on the fact that 1 mg of insulin equals 24 U, and the molar weight of the insulin molecule is 6000. For HI#10, basal insulin and C-peptide concentrations were: C_i= 7.3 μU/ml and C_{cp}=0.37 pmol/ml and for DI#12, C_i=2.7 μU/ml and C_{cp}=0.4 pmol/ml. Therefore we have for HI#10

$$R_{ib}=(10.2 \times 7.3)/144=0.518$$

$$R_{cpb}=3.23 \times 0.37=1.196$$

and for DI#12

$$R_{ib}=(9.5 \times 2.7)/144=0.178$$

$$R_{cpb}=2.22 \times 0.4=0.888$$

From the equation [6] the fraction that escapes the hepatic extraction was calculated for HI#10 as:

$$a_b=0.518/1.196=0.43$$

and for the DI#12 as:

$$a_b=0.178/0.888=0.20$$

Hepatic extraction under basal conditions was therefore estimated to be:

$h_{ib}=1-0.43=0.57$ for HI#10

and

$h_{ib}=1-0.20=0.80$ for DI#12.

In order to proceed with the assessment of the parameters during the glucose loading, first the integrals of the insulin and C-peptide concentrations were calculated using the trapezoidal rule. For the HI#10 these were estimated to be:

$$\int C_i(t)dt = 57$$

$$\int C_{cp}(t)dt = 277$$

For the DI#12 corresponding numbers were:

$$\int C_i(t)dt = 201$$

$$\int C_{cp}(t)dt = 407$$

As the metabolic clearance rate of the C-peptide is the same as during the basal conditions the rate of appearance during the glucose loading can be calculated based on equation [9]. It is

R_{cp}=3.23 x 277=895 pmol/kg-min for HI#10

and

R_{cp}=2.22 x 407=903 pmol/kg-min for DI#12.

In order to asses the hepatic uptake during glucose loading the equation [16] first was solved:

For HI#10 it was:

$$\alpha = \frac{10.2}{0.57} \times \frac{57}{895} = 1.14$$

and for DI#12:

$$\alpha = \frac{9.5}{0.80} \times \frac{201}{903} = 2.64$$

Based on the equation [18] fraction of the insulin escaping the hepatic extraction during the glucose loading is calculated as:

$$a = \frac{1.14}{1+1.14} = 0.53 \text{ for HI\#10}$$

and

$$a = \frac{2.64}{1+2.64} = 0.73 \quad \text{for DI\#12}$$

It follows then, that fraction of insulin undergoing hepatic extraction during the glucose loading for HI#10 is:

$$1\text{-}a=1\text{-}0.53=0.47$$

and for DI#12

$$1\text{-}a=1\text{-}0.73=0.27$$

This means it fell from 57% to 47% in the healthy HI#10 subject and from 80% to 27% in the glucose intolerant DI#12 patient.

It is possible now, to calculate the postprandial metabolic clearance rate of insulin from equation [11]:

$$mcr_i = \frac{10.2 \times 0.53}{0.57} = 8.39 \text{ ml/kg-min} \qquad \text{for HI\#10}$$

and

$$mcr_i = \frac{9.5 \times 0.27}{0.80} = 3.26 \text{ ml/kg-min} \qquad \text{for DI\#12}$$

Finally, the rate of insulin appearance was calculated (based on eq. [8]) to be:

$$\int R_i = 8.39 \times 57 = 478 \text{ pmol kg}^{-1}\text{min}^{-1} \qquad \text{for HI\#10}$$

and

$$\int R_i = 3.26 \times 201 = 660 \text{ pmol kg}^{-1}\text{min}^{-1} \qquad \text{for DI\#12}$$