Insulin secretion defects in liver cirrhosis can be reversed by glucagon-like peptide-1

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Abstract

Liver cirrhosis is often accompanied by a disturbed carbohydrate metabolism similar to type 2 diabetes. To investigate the severity of the defect in insulin secretion in this form of diabetes, we measured insulin release from isolated pancreatic islets of rats with CCl₄-phenobarbital-induced liver cirrhosis. Cirrhosis was confirmed by clinical signs, elevated liver enzymes and histology. Fasting venous plasma glucose concentrations were equal in rats with liver cirrhosis and in controls. Plasma insulin and glucagon concentrations were significantly greater ($P < 0.01$) in cirrhotic rats than in control animals. Glucose (16.7 mM)-induced stimulation of insulin release from pancreatic islets revealed a twofold increase in control and cirrhotic rats. Basal and stimulated insulin secretion, however, were significantly lower in cirrhotic animals. The incretin

hormone, glucagon-like peptide-1 (GLP-1), has therapeutic potential for the treatment of type 2 diabetes. Therefore, islets from control and cirrhotic animals were incubated with GLP-1 in concentrations from 10^{-11} to 10^{-6} M. GLP-1 stimulated insulin release in a concentration-dependent manner. In islets from cirrhotic rats, basal and stimulated insulin secretion was blunted compared with controls. These data show that the hyperinsulinemia observed in liver cirrhosis is not due to an increase of insulin secretion from islets, but could be explained by decreased hepatic clearance of insulin. GLP-1 may ameliorate diabetes in patients with liver cirrhosis.

Journal of Endocrinology (2000) **164**, 13–19

Introduction

The association of liver disease with abnormalities of carbohydrate metabolism was recognized by Naunyn (1898) 100 years ago, and the metabolic disturbances of glucose and insulin homeostasis have been intensively investigated in both humans and animal models (Conn *et al.* 1971, Felig & Sherwin 1976, Petrides & DeFronzo 1989a, b). Most patients with liver cirrhosis are intolerant to oral glucose, even when their fasting blood glucose concentration is normal. Impaired regulation of carbohydrate metabolism includes glucose intolerance, hyperinsulinemia and hyperglucagonemia (Holdsworth *et al.* 1972, Silva *et al.* 1988). On the basis of World Health Organization Criteria, more than 70% of patients with cirrhosis have oral glucose intolerance, and diabetes is more prevalent in these patients than in the general population (Kruszynska & McIntyre 1991). The impaired glucose tolerance is due mainly to insulin resistance of peripheral tissues (Kruszynska *et al.* 1991, 1993). Both insulin resistance and consecutive relative impairment of

insulin secretion (Taylor *et al.* 1985) have been considered important. The exact cause of these abnormalities remains unclear. Petrides & DeFronzo (1989a) suggested that hyperinsulinemia is caused by increased insulin secretion and diminished insulin degradation by the liver in cirrhosis. However, the picture is complicated by divergent data. Sirinek *et al.* (1991) demonstrated that extrahepatic porto-systemic shunting plays little or no role in the hyperinsulinemia of cirrhosis. Nakamura *et al.* (1988) showed that insulin release from the isolated perfused pancreas of rats with cirrhosis was decreased. Moreover, these results are in contrast to clinical investigations using the minimal model technique or euglycemic and hyperglycemic clamps (Kruszynska *et al.* 1991). Insulin secretion was greater in patients with cirrhosis, both in the fasting state and during the hyperglycemic clamp. After an oral glucose load, however, the increase in serum C-peptide concentration was relatively delayed and the insulin secretion index was not increased. Hepatic insulin extraction was reduced in the fasting state and during the hyperglycemic clamp. Thus, hypersecretion and decreased

insulin clearance were postulated to result in increased insulin concentrations in patients with cirrhosis.

Numerous studies have shown that glucagon-like peptide-1 (GLP-1) a 30-residue gastrointestinal hormone released from the enteroglucagon cells (L-cells) in the small intestine (Orskov *et al.* 1987) stimulates insulin secretion *in vitro* and *in vivo* (Schmidt *et al.* 1985, Kreymann *et al.* 1987, Orskov 1992, Ritzel *et al.* 1995), and potently influences postprandial glycemic concentrations. Moreover, it is also effective in patients with diabetes (Gutniak *et al.* 1992, Nathan *et al.* 1992), normalizing blood glucose concentrations in patients with type 2 diabetes (Nauck *et al.* 1993) and improving glycemic control in type 1 patients (Creutzfeldt *et al.* 1996), raising the possibility of its use as a therapeutic agent.

GLP-1 has, however, a plasma half-life of only 1–2 min *in vivo* (Mentlein *et al.* 1993, Pridal *et al.* 1996). The short insulinotropic action results from the degradation of the peptide by dipeptidyl peptidase IV that degrades GLP-1 at the N-terminus by cleaving off the first two amino acids, generating biologically inactive fragments (Deacon *et al.* 1995). Another enzymatic cleavage site of GLP-1 is located between amino acids 8 and 9. This metabolic instability, together with the formation of a metabolite [GLP-1(9–36)amide] that can antagonize the effects of GLP-1 (Knudsen & Pridal 1996) may limit the therapeutic potential of native GLP-1. In previous experiments, we demonstrated that GLP-1 analogs with alterations in the positions 2 or 2 and 8 significantly increased insulin release in the presence of 16.7 mM glucose, and were resistant to degradation by dipeptidyl peptidase IV (Siegel *et al.* 1999).

In the present study, we characterized the dynamics of insulin secretion in response to D-glucose to determine whether pancreatic endocrine function is altered in rats with cirrhosis, augmenting the type 2 diabetes-like metabolic situation that is associated with liver cirrhosis.

Materials and Methods

Peptide synthesis

GLP-1 was synthesized by solid-phase methodology on the polyamine resin Ultrosyn A (Pharmacia LKB, Freiburg, Germany) using N-fmoc-protected amino acid pentafluorophenylesters in an automatic peptide synthesizer (Novasyn Crystal; Calbiochem, Heidelberg, Germany) (Dryland & Sheppard 1986). GLP-1 was cleaved from the resin by treatment with 5% (v/v) thioanisole in trifluoroacetic acid for 2 h. The peptide was purified by gel filtration on Sephadex G 10 and preparative HPLC on a C18 Nucleosil 300–7 wide-pore MN column (30 nm, 1 × 25 cm) (Machery and Nagel, Düren, Germany). The quality of the synthetic peptides was assessed by mass spectrometry and automatic amino acid analysis (Schmidt *et al.* 1988).

Oral glucose tolerance tests

Animals were fasted for 12 h before oral glucose tolerance tests. After pentobarbital anesthesia, one polyethylene catheter was inserted into the left carotid vein for blood sampling. D-Glucose (3 g/kg body weight) was administered through an orogastric tube into the distal esophagus. Blood samples (125 µl) were taken 10 min before and 10, 30, 60, 120 and 180 min after glucose administration, for the measurement of plasma glucose concentrations (Glucose Analyzer II, Beckman) and serum insulin concentrations (radioimmunoassay).

Induction of cirrhosis

Cirrhosis was induced with CCl₄ and phenobarbital as described by Jimenez *et al.* (1985). For CCl₄ application, animals were placed in a plastic box (80 × 40 × 40 cm). Flowmeter-controlled compressed air was passed through a flask containing CCl₄ and introduced into the box. Animals inhaled CCl₄ twice a week for 0.5 min at a flow of 2.5 l/min initially, followed by stepwise increases in exposure time up to 8 min at the 10th week of treatment. Drinking water was supplemented with phenobarbital in a concentration of 0.03%. Control animals were kept under identical conditions except for the exposure to either agent. Cirrhosis was verified at the end of individual experiments by gross inspection of the liver, by the presence of ascites, and by histopathologic examination of the liver using standard techniques for fixation and staining (Batts & Ludwig 1995). Rats not showing cirrhosis by microscopic examination (<5% of treated animals) were excluded from further analysis. By the time of the experimental studies, both cirrhotic and control rats had a body weights of 280–300 g.

Stimulation of isolated rat islets with GLP-1 and determination of insulin release

Pancreatic islets were isolated by collagenase digestion (Siegel & Creutzfeldt 1985) from male Lewis rats weighing 250–300 g after an overnight fast; collagenase was from Biochrom, Berlin, Germany. Batches of 200–400 islets with a diameter of greater than 150 µm were maintained in tissue culture medium (RPMI-1640, Biochrom) for 20–24 h at an ambient glucose concentration of 11 mM. The medium was supplemented with 10% fetal calf serum (Biochrom), 100 U/ml penicillin G and streptomycin 100 µg/ml. The islets were maintained at 37 °C, pH 7.4 in an atmosphere saturated with water and gassed with an air–5% CO₂ mixture.

After the maintenance period, the islets were washed and incubated in a modified Krebs–Ringer bicarbonate buffer (KRB-HEPES) as described previously (Siegel & Creutzfeldt 1985). Ten islets per vial were incubated for 30 min in 2 ml KRB-HEPES at 37 °C, pH 7.4 containing

Table 1 Liver function tests in normal and cirrhotic rats. Values are mean $\pm$ S.E.M. for $n=15$ rats per group

Group	ASAT (U/l)	ALAT (U/l)	AP (U/l)	Bilirubin (mg/dl)	Total protein (g/l)	GGT (U/l)
Control	42.57 $\pm$ 5.35	21.27 $\pm$ 1.7	145.7 $\pm$ 50.1	0.08 $\pm$ 0.01	5.57 $\pm$ 0.06	1.14 $\pm$ 0.14
Cirrhosis	266.1 $\pm$ 46.7	164.7 $\pm$ 32.9	316.6 $\pm$ 45.8	0.57 $\pm$ 0.16	5.00 $\pm$ 0.25	4.30 $\pm$ 0.96
$P^\dagger$	0.003	0.005	0.030	0.041	0.11	0.029

† For comparison between control and cirrhosis groups.

the respective stimulating agent. Insulin release was measured by radioimmunoassay (Siegel & Creutzfeldt 1985).

Liver function tests

To assess liver function in control and cirrhotic rats after 10 weeks of treatment, serum was collected from $n=15$ rats per group to determine activities of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (AP), and gamma glutamyl transferase (GGT) using the Automatic Analyzer 717 from Boehringer-Mannheim, Mannheim, Germany. Further serum analyses included total protein and total bilirubin concentration (Table 1).

Determination of serum glucagon concentration

For the quantitative determination of pancreas-specific glucagon in rat serum, a glucagon radioimmunoassay kit from DRG Diagnostics (Marburg, Germany) was utilized. The glucagon antibody showed no crossreactivity greater than 0.1% with oxyntomodulin. Assay sensitivity was 20 pg/ml. The interassay coefficient of variation was less than 10%, and the intra-assay variation was below 6%.

Statistical analysis and calculations

Variance of individual group means was calculated with a one-way ANOVA test, and a Fisher's *post-hoc* test was used for group comparisons.

Results

The rats in the CCl₄-treated group showed an unhealthy appearance and transparent ascites was seen in most rats with cirrhosis when the abdomen was opened. The increase in body weight was not significantly different between the groups. The size and weight of the livers in the CCl₄-treated rats were increased and the surface demonstrated typical appearances of micronodular cirrhosis. The mean liver weight ($\pm$ S.E.M) was 7.5 ± 0.7 g in the control group and 12.3 ± 0.9 g in the cirrhosis

group. The ratio of liver weight to body weight was $2.54 \pm 0.13\%$ in the control group and $4.24 \pm 0.08\%$ in the cirrhosis group. The liver weight and the ratios of liver weight to body weight were significantly greater in cirrhotic animals ($P<0.01$).

The results of the liver function tests are shown in Table 1. Blood glucose concentrations were not different in the two groups of animals studied (controls: 84 ± 6.3 mg/dl; cirrhotic rats: 86.7 ± 5.8 mg/dl, not significant). Cirrhotic rats had significantly greater insulin and glucagon plasma concentrations than control rats, thus demonstrating hyperinsulinemia and hyperglucagonemia (Table 2).

Oral glucose tolerance tests

As depicted in Fig. 1, basal plasma glucose concentrations were not different in control and cirrhotic rats (5.61 ± 0.23 mM compared with 5.23 ± 0.19 mM). Basal insulin concentrations, however, were significantly greater in cirrhotic rats than in controls (1.63 ± 0.1 ng/ml compared with 0.63 ± 0.06 ng/ml). After an oral glucose load, blood glucose concentrations in untreated and cirrhotic animals were within normal limits throughout the experiment.

Insulin concentrations declined over time, but remained increased in the CCl₄-treated group, even after decrease of blood glucose concentration to normal fasting values after 180 min. In cirrhotic rats, insulin concentrations increased 1.7-fold upon stimulation with orally administered glucose, whereas healthy rats had a 3.3-fold increase in insulin concentration.

Table 2 Plasma insulin and glucagon concentrations in control rats and rats with liver cirrhosis. Results are expressed as mean $\pm$ S.D. ($n=15$ rats per group)

	Controls	Cirrhotic
Insulin (μ U/ml)	10.3 $\pm$ 2.8	39.9 $\pm$ 5.6**
Glucagon (pg/ml)	54.5 $\pm$ 5.3	157.6 $\pm$ 22.4**

** $P<0.01$ compared with controls.

Figure 1 Oral glucose tolerance test. Plasma concentrations of glucose (lower panel) and insulin (upper panel) in control (◆) and cirrhotic (●) rats. Values represent means $\pm$ S.E.M., $n=15$ rats per group. Oral glucose was given at $t=0$ min. *Significant difference ($P<0.01$) compared with the control group at corresponding time points.

Stimulation of insulin secretion

Glucose (16.7 mM) induced a twofold increase in insulin release from isolated pancreatic islets of both normal rats (basal 3.0 ± 0.2 ng/10 islets per 30 min; stimulated 6.0 ± 0.5 ng/10 islets per 30 min; $P<0.05$) and cirrhotic rats (basal 1.1 ± 0.1 ng/10 islets per 30 min; stimulated 1.9 ± 0.2 ng/10 islets per 30 min; $P<0.05$) (Fig. 2). GLP-1 exerted insulinotropic effects in islets from normal and cirrhotic rats in a dose-dependent manner. Addition

Figure 2 Stimulation of insulin release by glucose. Pancreatic islets from control and cirrhotic rats were isolated by collagenase digestion as described in Material and Methods. Islets were incubated after 24-h maintenance in tissue culture in KRB-HEPES buffer for 30 min. Insulin release was measured in the presence of 2.8 and 16.7 mM glucose. Values are means $\pm$ S.E.M., $n=15$ rats per group, ** $P<0.01$.

of 10^{-9} M GLP-1 resulted in a significant ($P<0.05$) enhancement of insulin release, to 3.10 ± 0.17 ng per 10 islets from cirrhotic rats. A concentration of 10^{-7} M of GLP-1 caused a further increase (4.05 ± 0.44 ng/10 islets) that was almost 200% compared with glucose alone. When additional concentrations of GLP-1 were tested in separate series, no effect was seen with 10^{-10} M and no further increase at 10^{-6} M compared with the effect achieved with 10^{-7} M (Fig. 3).

Discussion

In this study, an experimental model of liver cirrhosis in rats was used to characterize insulin secretion in this condition, which often is accompanied by a form of diabetes similar to type 2 diabetes. Of the several ways to induce cirrhosis by CCl_4 alone or combined with other substances (Tamayo 1983, Qazi & Alam Mahmood 1988), we utilized a combined method with CCl_4 and pento-barbital. The mortality of rats in the present study was $<5\%$. Most rats in the cirrhosis group showed ascites. The macroscopic appearance, the increased liver weight and the ratio of liver weight to body weight, the histologic features and the abnormal liver function test all indicate that hepatocyte degeneration and necrosis persisted throughout the entire course of induction of cirrhosis. This model showed fasting hyperinsulinemia and a degree of insulin resistance during euglycemic hyperinsulinemic

Figure 3 Effect of GLP-1 on insulin release by pancreatic islets from control and cirrhotic rats. Rat islets were isolated by collagenase digestion and preincubated for 24 h. Static incubations of 10 islets per vial were performed for 30 min in the presence of 16.7 mM glucose and with the indicated concentrations of GLP-1. Insulin release was measured by radioimmunoassay. Values are means $\pm$ S.E.M. for 15 separate experiments. * $P < 0.05$, ** $P < 0.01$, compared with corresponding controls at 16.7 mM glucose in the absence of GLP-1.

clamp studies comparable to those reported in humans (Meyer-Alber *et al.* 1992). Furthermore, compared with values in healthy rats, after i.v. infusion of insulin, increased serum insulin concentrations were observed. This phenomenon has also been described in patients with cirrhosis, and has been explained at least partially by decreased hepatic function and porto-systemic shunting (Petrides & DeFronzo 1989b). The pancreatic tissue of the CCl₄-treated rats showed normal histology of the exocrine and endocrine portions of the pancreas consistent with the observations of Nakamura *et al.* (1988) and Wu *et al.* (1994). Therefore, the abnormal glucose metabolism and insulin secretion do not seem to have been a consequence of direct toxic action by CCl₄ on the pancreatic islets.

Hyperinsulinemia in cirrhosis may be caused by several factors. In our study, peripheral hyperinsulinemia did not seem to be caused by increased insulin secretion, as insulin release by isolated islets from the CCl₄-treated rats showed both reduced glucose-induced insulin secretion and reduced basal release. In clinical studies, increased insulin secretion in patients with liver cirrhosis has been considered to be a compensatory mechanism for the development of peripheral insulin resistance in order to maintain normal blood glucose concentrations (Petrides & DeFronzo 1989a). In the present study, the finding of

normal fasting glucose concentrations does not support the idea of compensatory secretion. It seems more reasonable that the hyperinsulinemia can be attributed to reduced liver insulin extraction, due to impaired hepatocyte function. A large number of investigators (Nygren *et al.* 1985, Petrides & DeFronzo 1989a, b, Kruszynska *et al.* 1991) have, directly or indirectly, shown that reduced insulin clearance by the liver cirrhosis, contributes to hyperinsulinemia, and this may be the main explanation for the hyperinsulinemia encountered in this study.

Little is known about the *in vitro* function of pancreatic islets in cirrhosis. Gomis *et al.* (1994) demonstrated, *in vitro* in isolated pancreatic islets, that the defect in the β -cell response to glucose stimulation contrasts with the unimpaired response to arginine. This may reflect a specific defect in glucose metabolism in the pancreatic β cells. The insulinotropic action of arginine is attributed to the depolarization of the β -cell membrane (Malaisse *et al.* 1989); thus a normal response to arginine stimulation suggests that the cytoplasmic membrane and ionic channels are preserved in β cells from cirrhotic rats. Kruszynska *et al.* (1998) demonstrated that insulin hypersecretion in non-diabetic alcoholic patients might result from enhanced islet β -cell sensitivity to glucose or an increased maximal secretory capacity. Conversely, impaired insulin secretion in diabetic alcoholic patients

with cirrhosis could be due to decreased β -cell sensitivity or diminished secretory capacity. Previous findings by Kruszynska *et al.* (1993) of a prompt output of insulin in response to i.v. tolbutamide in diabetic patients with cirrhosis who lacked a first-phase insulin response to glucose suggest that the insulin secretory defect may be relatively specific for glucose.

The finding that the acute insulin secretory response to arginine in diabetic patients with cirrhosis was not significantly different from that of controls or non-diabetic patients with cirrhosis supports this idea. Specific alterations in glucose metabolism may impair insulin secretion by interfering at the intracellular level, suggesting that the abnormal insulin response to glucose stimulation exhibited by pancreatic islets from rats with cirrhosis and diabetic patients with cirrhosis could reflect a defect at the mitochondrial level. In addition, there is a possibility that hyperammonemia could impair the pancreatic response to a glucose stimulus by decreasing NADPH (Sener *et al.* 1978) and increasing intracellular pH (Smith & Pace 1983). This possibility should be considered, because hyperammonemia is a component of the portal hypertension syndrome.

Our study shows that isolated islets from rats with CCl₄-induced liver cirrhosis exhibit an impaired insulin secretion in response to stimulation with glucose *in vitro*. The endocrine dysfunction in cirrhosis is further supported by our observation *in vivo* after an oral glucose tolerance test. Cirrhotic and healthy rats had identical serum glucose concentrations after an oral glucose load, whereas insulin concentrations were significantly different at all timepoints of the experiment. In cirrhotic rats, serum insulin concentrations were greater at baseline and increased 1.7-fold at maximum, 40 min after glucose instillation. In the control animals, serum insulin concentrations increased 3.3-fold at maximum.

We conclude that GLP-1 exerts insulinotropic effects on islets from normal and cirrhotic rats in a dose-dependent manner. Our study also shows that isolated pancreatic islets from rats with cirrhosis exhibit an abnormal endocrine pancreatic secretion, characterized by impaired insulin secretion in response to glucose stimulation. Studies of insulin secretion in the islets from CCl₄-treated rats revealed that basal and glucose-stimulated insulin release was reduced. Cirrhotic rats had a normal glucose tolerance test in the presence of marked hyperinsulinemia. This raises the question whether hypersecretion of insulin or reduced degradation by impaired liver function account for the increased basal and postprandial insulin concentrations in cirrhosis. As the magnitude of the response of insulin secretion was decreased both *in vitro* and *in vivo*, our findings are in favor of a reduced insulin degradation.

The gastrointestinal hormone, GLP-1, is the most important incretin stimulating insulin secretion after a carbohydrate-rich meal (Schmidt *et al.* 1985, Kreyman

et al. 1987). As it also stimulates insulin secretion and inhibits glucagon secretion in patients with type 2 diabetes, the use of GLP-1 might be also a potential therapeutic option for the treatment of diabetes associated with liver cirrhosis.

Acknowledgements

The authors express their gratitude to M Witt and E Jürgen for excellent technical assistance. This study was supported by a grant from the Christian-Albrechts-University (Hensel foundation).

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Received 19 January 1999

Revised manuscript received 4 August 1999

Accepted 18 August 1999