ORIGINAL

Increasing early insulin secretion compensate adequately for hepatic insulin resistance in CCl_4-induced cirrhosis rats

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Abstract : A number of recent publications have reported an increased frequency prevalence of glucose intolerance with hyperinsulinemia in liver cirrhosis. The aim of this work was to detect, in CCl_4-induced liver cirrhosis rat, the presence and starting point of muscle and liver insulin resistance. Eighteen rats received intraperitoneal injection of 2 ml of soybean oil containing of CCl_4 twice a week for 20 weeks. We executed standard oral glucose tolerance and clamp study to evaluate systemic insulin resistance. Hepatic glucose uptake was much lower in CCl_4 group than that in control group, but peripheral glucose uptake was not decreased in this study. In contrast, early-phase insulin secretion was enhanced in CCl_4 rat using oral glucose load during clamp methods. These data suggested that increased early insulin secretion compensate adequately for hepatic insulin resistance in rats. However there was a report that peripheral glucose uptake was decreased in the case of human liver cirrhosis, which was formed in the course of time. In a chronic condition, this may be associated with reduced insulin content and developed systemic insulin resistance in liver cirrhosis. Then a long term observation study will be required to examine the presence of muscle insulin resistance in liver cirrhosis. J. Med. Invest. 57 : 54-61, February, 2010

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INTRODUCTION

In the presence of hepatic disease, the metabolic homeostasis of glucose is impaired as a result of metabolic disorders such as diabetes mellitus (1-4). A number of recent publications have reported an increased frequency of glucose intolerance with hyperinsulinemia in liver cirrhosis (5, 6). The incidence of a diabetic response to a standard oral glucose tolerance test was 4.4% after a 2-year and 21.2% after a 4-year follow-up in stable cirrhosis (5). The patient with cirrhosis and diabetes mellitus suffers

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from more frequent complications, which can cause death (7-9). The high prevalence of diabetes is due to the insulin resistance of liver cirrhosis (10). In general, insulin resistance is considered to be composed of the following three metabolic defects ; 1) reduced glucose uptake by the peripheral (muscle) tissues ; 2) decreased splanchnic (hepatic) glucose uptake ; 3) overproduction of glucose by the liver (11). Imano, *et al.* showed that splanchnic (hepatic) glucose uptake in patients with liver cirrhosis was markedly decreased compared with normal, and peripheral (muscle) glucose uptake was also decreased in liver cirrhosis (12). The mechanisms by which liver cirrhosis produces insulin resistance and diabetes mellitus have not been clearly established. In case of animal model, liver cirrhosis was induced in male Sprague-Dawley rats by intraperitoneal injection of CCl_4 (13). Then, the aim of this work was to examine the relationship to insulin resistance between liver and muscle in CCl_4-induced liver cirrhosis.

MATERIALS AND METHODS

Animals

All rats were cared for in accordance with the NIH Guidelines for care and use of Laboratory animals. The protocol was approved by the University of Tokushima animal Care and Use Committee. Thirty six male Sprague-Dawley (SD) rats, 5 weeks of age (weight 130-150 g) were purchased from Japan SLC (Hamamatsu, Japan) and were housed in individual cages kept at $23 \pm 1^{\circ}C$ under 12-hour dark/light cycles, with free access to standard laboratory chow and tap water. The animals were divided into 2 groups. Eighteen rats received intraperitoneal injection of 2 ml of soybean oil containing of 0.5 ml of CCl_4 twice a week for 20 weeks (CCl_4 group). The remaining 18 rats received intraperitoneal injections 2 ml of soybean oil twice a week for 20 weeks (control group). The amount of CCl_4 was adjusted according to the change in body weight (BW). The initial dose in 5 and 6 week old rats was 0.5 ml/kgBW, increasing to 1.0 ml/kgBW in 7 to 24 week-old rats. Moreover, glucose metabolism was evaluated by using oral glucose tolerance test, hepatic glucose uptake and early-insulin secretion under a hyperinsulinemic euglycemic clamp with oral glucose load, and insulin-stimulated glucose utilization methods.

Standard oral glucose tolerance test (OGTT) and capability of glucose storage in liver

Control (n=6) and CCl_4-induced (n=6) rats at 24 weeks old were subjected to an oral glucose load of 2 g/kgBW. Catheterization was performed as described previously (14). In the glucose loading study, under sodium pentobarbital anesthesia (50 mg/kgBW), a silicon rubber catheter was inserted into the left femoral vein, and the line of the catheter was led out through subcutaneous tissue to an intravenous hyperalimentation (IVH) kit (Bio-Cannula ; Bio-Medica, Osaka, Japan). On the fifth day after catheterization, tests were conducted after an overnight fast and while the animals were conscious. Blood sample for glucose and immunoreactive insulin determination were collected from the femoral vein in microtubes containing EDTA at 0, 15, 30, 60, 120, and 180 min. Moreover, on the fifth day after the study, blood samples were collected under nonanesthesia from the tail vein for the measurement of plasma glucose, insulin and glucagon levels, and under diethyl ether anesthesia from the left jugular vein for the measurement of clinical parameter. Rats were subsequently sacrificed for harvest and weight measurement liver, visceral fat and pancreatic samples.

Within 2 wk of sampling, the liver tissues were analyzed by microscopic examination after hematoxylin-eosin staining and AZAN staining to evaluate the degree of hepatic fibrosis (with tissue necrosis, regeneration nodules, extensive bridging fibrosis, and no signs of acute inflammation). All samples from kidney, esophagus, and small and large intestines were normal.

Glycogen was measured as previously reported (15). Samples (50 mg) of the liver were incubated at $85^{\circ}C$ in 0.5 ml of 0.5M-NaOH for 20 min. After cooling, 0.15 ml was mixed with the same amount of acetate buffer to make a final pH of 4.7. The samples were incubated at $37^{\circ}C$ for 60 min with amyloglucosidase (0.2 mg/ml). The precipitated proteins were eliminated by centrifugation at 8,000 g for 10 min. The glucose produced was measured by the glucose oxidase method (16).

Plasma glucose levels were determined by the glucose oxidase method (Advantage II, Roche Diagnostics, Germany), and insulin and glucagon levels were determined by a commercially available ELISA kit (Morinaga, Yokohama, Japan) and (WAKO Osaka, Japan), respectively. Serum alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase,

total bilirubin, creatinine, blood urea nitrogen, total protein, albumin, triacylglycerol, total cholesterol, free fatty acid were measured using the several kit (WAKO, Osaka, Japan).

Hyperinsulinemic euglycemic clamp study and measurement of early insulin secretion

In the clamp study, silicon rubber catheters (FT-025, Bio-Medica) were inserted into the left femoral vein and into the left jugular vein at 24 weeks old rat, and the clamp study was conducted on the 5th day after catheterization. Catheterized rats were kept in a special IVH cage (BG-781, Bio-Medica) and were continuously infused with physiologic saline until the test. Insulin-mediated whole-body glucose uptake was measured in conscious rats using the hyperinsulinemic euglycemic clamp technique as reported previously (17). After an overnight fast, blood samples were drawn at baseline to determine fasting plasma glucose and C-peptide levels. Blood samples for determination of plasma glucose level were obtained from the catheter in the femoral vein at 3-minute intervals throughout the study. Data on total body glucose uptake were represented by the mean values of last 20 minutes for the glucose infusion rate (GIR) at 120 min. After determination of baseline GIR during the clamp, glucose was orally administrated at a dose of 0.2 g/kgBW (18). Thereafter, the clamp was continued and the extent of decrease of GIR was monitored for 1.5 hours in order to evaluate hepatic glucose uptake (HGU), which was employed as an indicator of insulin sensitivity in the liver. Total HGU was evaluated in this clamp study, as previously described in detail (19). Furthermore, to evaluate the ability of endogeneous early-phase insulin secretion, serum C-peptide levels were measured at 0 and 10 min during a clamp with oral glucose load study (20). Serum C-peptide levels were measured by a C-peptide radioimmunoassay (RIA) kit (Linco Research, St. Louis, MO).

Determination of insulin-stimulated glucose utilization during clamp study

The glucose utilization index for peripheral tissues (gastrocnemius muscle, diaphragm, epididymal fat pads, musculus quadratus lumborum) was measured in the same group of rats during another hyperinsulinemic-euglycemic clamp test using the 2-deoxy-D-[1-^{3}H] glucose ([^{3}H]2DG) technique as described by Ferre, *et al.* (21) and James, *et al.* (22). At the end of the clamp test, peripheral tissues and blood samples were rapidly treated, and tissue glucose uptake (defined as the glucose metabolic index, Rg') was calculated, as previously described in detail (17). Tissue glucose uptake was calculated using the following equation described by

Rg' (μmol/100 g/min)

$= Cp \times Cm^* (60) / \int_{(0\text{-}60)} Cp^*(t)\, dt$

where Cp is the steady state plasma glucose concentration over a 60 min period of observation (mmol/L) ; Cm* is tissue accumulation of [^{3}H]2DG 6-phosphate per unit mass at 60 min (dpm/mg wet weight) ; Cp*(t) is the plasma [^{3}H]2DG concentration (dpm/mL) ; and t equals 0 when the tracer is administered as a bolus.

Statistical analysis

All results were expressed as mean ± SEM. The statistical significance of the differences in mean values between the control and CCl_4 groups were evaluated using Student *t*-test by StatView software (windows, version 5.0).

RESULTS

1, Body and organ weight and clinical chemistry

Body weight in CCl_4 group was significantly lower than that in control group although the liver and pancreas weights significantly increased in CCl_4 group (Table 1). The irregularity of hepatic surface, the obtuse angle edge and the atrophy of the volume were recognized. Hepatic lobule structures in CCl_4 group were damaged with obvious collagen fibrils and pseudolobule formation, which were characteristic of liver cirrhosis (Fig. 1). Total bilirubin (T-Bil), aspartate aminotransferase (AST), alanine

Table 1 Body and organ weight, and hepatic glycogen content in 24 weeks old CCl_4 and control rat

	Control	CCl_4	
BW (g)	576 ± 10	486 ± 2	**
Liver (g/kgBW)	22.8 ± 0.2	29.3 ± 1.3	*
Pancreas (g/kgBW)	2.0 ± 0.2	2.5 ± 0.5	**
Soleus muscle (g/kgBW)	0.9 ± 0.1	0.9 ± 0.1	
Mesenteric fat (g/kgBW)	16.3 ± 2.1	16.0 ± 1.3	
Epididymal fat (g/kgBW)	17.6 ± 2.1	13.2 ± 1.7	
Retroperitoneal fat (g/kgBW)	24.4 ± 2.0	16.7 ± 2.9	
Glycogen contents in liver (nmol/g tissue)	345.1 ± 121.4	34.6 ± 6.1	*

Results are mean ± SE. n=6 per group. *p< 0.05, ** p< 0.001 versus control.

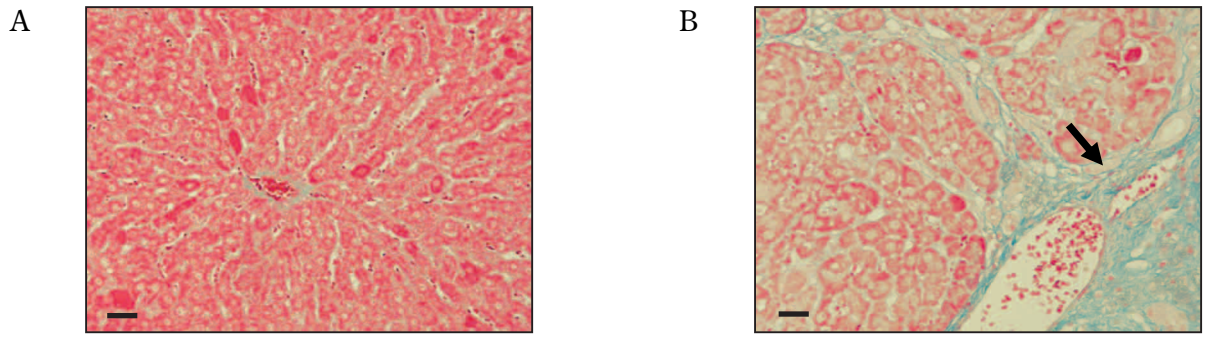

Fig. 1 Histopathological evaluation of the livers in 24 weeks old CCl_4-induced rat
Azan staining was performed for histopathological characteristics of liver in control rat (A) and CCl_4 injection rat (B). Note the presence of hepatic fibrosis (arrow) in (B). Scale size is 100 μm. Original magnification×100.

aminotransferase (ALT), alkaline phosphatase (ALP), total cholesterol (T-cho) and glucagons were significantly increased, and triglyceride (TG) was significantly decreased in CCl_4 group (Table 2). Furthermore, the amount of liver glycogen in the CCl_4 group was lower than that in control ($p<0.05$) (Table 2).

Table 2 Plasma Parameters in 24 weeks old CCl_4 and control rat

	Control	CCl_4	
T-Bil (mg/dl)	0.03 ± 0.004	0.26 ± 0.07	*
AST (IU/L)	111 ± 13	882 ± 117	**
ALT (IU/L)	69 ± 3	502 ± 60	**
ALP (IU/L)	587 ± 78	1410 ± 108	**
TP (g/dl)	6.7 ± 0.1	6.6 ± 0.1	
Alb (g/dl)	3.8 ± 0.03	3.8 ± 0.02	
BUN (mg/dl)	22.2 ± 0.8	9.1 ± 1.2	
Cre (mg/dl)	0.34 ± 0.01	0.33 ± 0.02	
T-cho (mg/dl)	75 ± 5	125 ± 7	**
TG (mg/dl)	192 ± 18	47 ± 4	**
Insulin (ng/ml)	1.80 ± 0.33	2.07 ± 0.25	
Glucagon (pg/dl)	60.7 ± 8.1	112.9 ± 6.7	*
FFA (mEq/l)	0.76 ± 0.18	0.97 ± 0.09	

ALP, alkaline phosphatase ; AST, aspartate aminotransferase ; ALT, alanine aminotransferase ; T-Bil, total bilirubin ; Cre, creatinine ; BUN, blood urea nitrogen ; TP, total protein ; Alb, albumin ; TG, triacylglycerol ; T-Cho ; total cholesterol ; FFA, free fatty acid.
Results are mean ± SE, n=6 per group. *$p<0.05$, ** $p<0.001$ versus control.

2, Standard oral glucose tolerance test (OGTT)

There was no significant difference between two groups (CCl_4 and control) in regard to plasma glucose levels during the OGTT (Fig. 2A). Plasma insulin levels at 15 min during the OGTT were significantly increased in CCl_4 group (5.60 ± 0.62 ng/ml) compared with those obtained in control groups (2.87 ± 0.73 ng/ml, $p<0.01$) (Fig. 2B, 2C). Area under the curve of plasma insulin levels for 180 min was significantly higher in CCl_4 group than control group during OGTT.

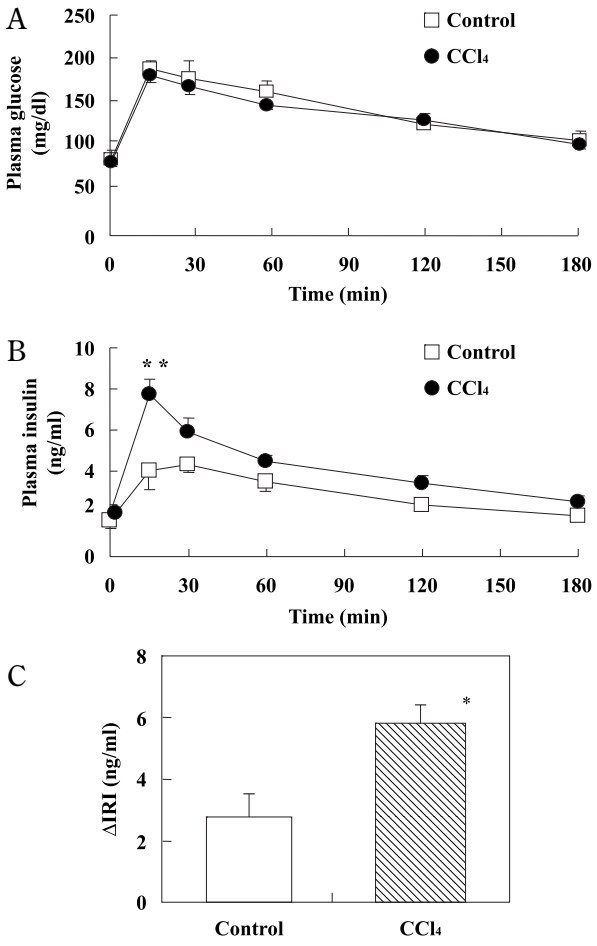

Fig. 2 Changes in plasma glucose and insulin levels during oral glucose tolerance test in 24 weeks old CCl_4-induced rat
(A) Plasma glucose and (B) plasma insulin levels in CCl_4-induced rat and control rat. (C) Incremental ratio of insulin (ΔIRI) was calculated using the formula : IRI (15 minutes)-IRI (0 minute). Results are mean ± SE, n=6 per group. *$p<0.05$, **$p<0.01$ versus control.

3, Glucose utilization and early-phase insulin secretion during clamp study

Insulin sensitivity was evaluated by hyperinsulinemic euglycemic clamp test with oral glucose load, as described in Materials and Method. The glucose infusion rate (GIR), which reflected the insulin sensitivity in peripheral tissues, was identical in both cirrhotic and control rats (Fig. 3A). In contrast, the rate of hepatic glucose uptake (HGU), which might reflect insulin sensitivity in the liver, was significantly lower in CCl_4 group than in control group (Fig. 3B). Furthermore, changes in C-peptide levels, to evaluate early-phase insulin secretion, during the clamp with oral glucose load are shown in Fig. 3C-3E. The increase in C-peptide response (CPR) at 10 minutes was enhanced in CCl_4 groups than those in control groups.

4, Glucose metabolic index

The glucose metabolic index (Rg) was considered as representative of insulin-stimulated glucose uptake in different types of tissue. The Rg in gastrocnemius muscle and epididymal fat pads (Fig. 4),

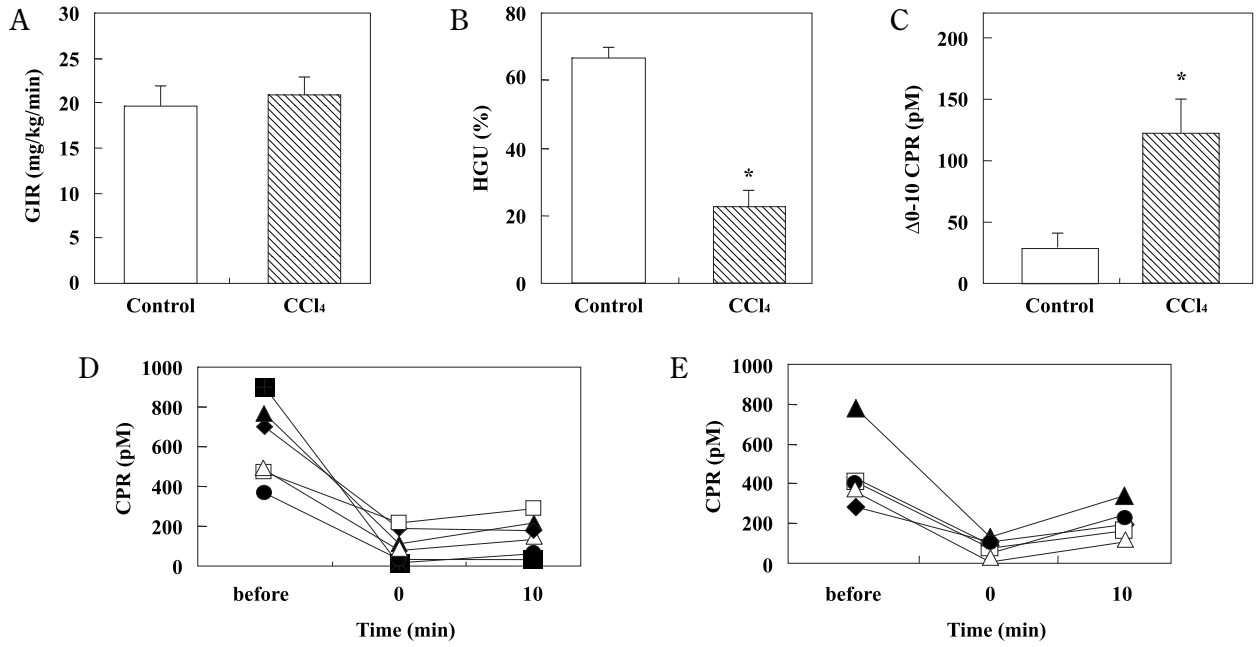

Fig. 3 Measurement of insulin resistance and early-phase insulin secretion during hyperinsulinemic-euglycemic clamp in 24 weeks old CCl_4-induced rat.
(A) Glucose infusion rate (GIR). (B) Hepatic glucose uptake (HGU). (C) Incremental ratio of C-peptide response (CPR) was calculated using the formula : CPR (10 minutes)-CPR (0 minute). Changes in serum CPR levels during clamp at baseline (before clamp), steady state (0 minute), and 10 minutes in control rat (D) and rat with CCl_4 injection (E). Results are mean ± SE, n=6 per group. * $p < 0.01$ versus control.

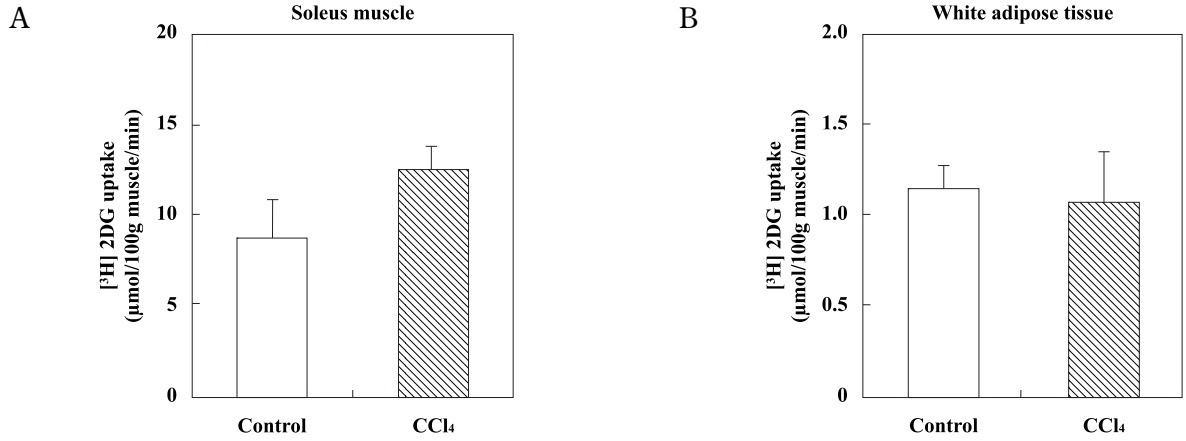

Fig. 4 Insulin-stimulated glucose utilization during clamp
2-deoxyglucose (2-DG) uptake in skeletal muscle (A) and white adipose tissue (B). Results are mean ± SE, n=6 per group.

diaphragm and musculus quadratus lumborum (data not shown) did not show any differences in both groups.

DISCUSSION

Insulin resistance in target organ such as muscle or adipose tissue and hyperinsulinemia seem to be the pathophysiologic bases of diabetes in liver disease. Moreover, an impaired response of the islet beta cells of the pancreas and hepatic insulin resistance are also contributory factors. In this study, cirrhosis was induced in rats by intraperitoneal injection of CCl_4 for 19 weeks. The model of cirrhosis induced experimentally by chronic intoxication with CCl_4 has been widely used for studies of morphology, pathophysiology and pharmacology of liver disease. Laboratory data and pathological findings were similar to the previous reports (23, 24).

We simultaneously measured hepatic glucose uptake (HGU) by using the euglycemic hyperinsulinemic clamp with oral glucose load. HGU in cirrhosis was much lower than that in control. Therefore, the amounts of hepatic glycogen in liver cirrhosis were lower than those in normal. There were no differences in both glucose infusion rate (GIR) and glucose uptake of peripheral tissue (muscle and adipose tissue) between cirrhosis and control groups. Accordingly there was not insulin resistance in skeletal muscle in this experimental study. Holland-Fischer, *et al.* reported the muscle insulin-dependent glucose transporter GLUT4 protein content in the cirrhosis group was not different from that the controls (25), which might explain our results. However, Imano, *et al.* showed that splanchnic (hepatic) glucose uptake in patients with liver cirrhosis was markedly decreased compared with normal, and peripheral (muscle) glucose uptake was also decreased in liver cirrhosis (12). The difference of two reports concerning muscle glucose uptake might be dependent on the duration of liver cirrhosis. The duration of liver cirrhosis in human was longer than that in these experimental rats. Then hepatic insulin resistance might be recognized in the early stage of liver cirrhosis and successively peripheral (muscle) insulin resistance might be appended in the late stage of liver cirrhosis.

The plasma C-peptide level of the early-phase insulin secretion at 10 minutes after glucose load during clamp was significantly higher in CCl_4 than in control. Similarly, plasma insulin levels increased at 15 min during OGTT in CCl_4 group, and subsequently maintained high concentrations compared with control group. It is reported that importance role of rapid insulin secretion suppressed hepatic glucose production (26, 27). Consequently, this response induces the suppression of postprandial hyperglycemia. In the present study, it was not observed in OGTT and peripheral insulin-induced glucose uptake was normal. These results raise the possibility that increased insulin levels might compensate for hepatic insulin resistance.

Hyperinsulinemia in cirrhosis may be caused by two factors. One is decrease of hepatic clearance of insulin. A large number of investigators have, directory or indirectory, showed that reduced insulin clearance by the liver cirrhosis, contributes to hyperinsulinemia (28-31). It has been proposed that insulin extraction by damaged liver and portosystemic shunts result in hyperinsulinemia which is potentiated by raised levels of contra-insulin hormones (glucagon, insulin-like growth factor, free fatty acids and cytokines) (3, 7, 32, 33). The other is increase of insulin secretion from islet. A recently study reports that in patients with Child B grade liver cirrhosis the hyperinsulinemia may be produced by an increase of the pancreatic beta-cell sensitivity to glucose, whereas disturbance of hepatic insulin extraction does not seem to have a significant role (34). Moreover, serum human hepatocyte growth factor (HGF) levels in patients with acute hepatitis, chronic hepatitis and cirrhosis were found to be slightly higher than those in normal subjects (35). Garcia-Ocaña A., *et al.* showed that HGF overexpression in the beta-cell specifically upregulates insulin secretion in response to glucose, independent of islet size (36), although there were no significant differences in the fasting insulin levels between HGF transgenic mice and control. Similar results were obtained in the present study (37). Therefore, CCl_4-induced rat may be chronic compensatory hyperinsulinemia until the islet beta cells are exhausted. However we could not examine the islet size and serum HGF levels. Further examination was needed in these points.

In conclusion, hepatic glucose uptake was much lower in CCl_4-induced rat than that in control although peripheral (muscle) glucose uptake was not decreased in this study. These data suggested that increased early insulin secretion might compensate adequately for hepatic insulin resistance. In a chronically condition, this compensation may be associated with reduced insulin content and developed

systemic insulin resistance in liver cirrhosis. Therefore, a long term observation is required to examine the presence of muscle insulin resistance in liver cirrhosis.

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