実験的肝障害ラットの hyperinsulinism に関する研究

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実験的肝障害ラットのHyperinsulinism機構に関する研究

—インスリン受容体におけるインスリン結合と分解の相互関係—

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目的） receptor上でのインスリン結合と分解の相互関係および肝障害におけるhyperinsulinism機序を解明したので報告する。方法）CCl_4による実験的肝障害ラット（CCl_4－rats）を作成し，purified liver plasma membrane（PPM）におけるインスリン結合と分解および肝 homogenate の insulin－degrading activity を測定した。結果）CCl_4－rats 肝の 27,000×g 上清の insulinase activity（9.96±0.71）は normal rats（N－rats）（13.40±0.52）のそれよりも有意に低下した。しかしながらGIT activityは不変であった。（CCl_4－rats: 36.01±1.57; N－rats 34.23±1.34）。CCl_4－rats PPMに対する^{125}I－insulin の speacific binding は 5.46±0.71%でN－rats のそれの約½に減少した。また結合インスリン量も35～45%減少し，Scatchard解析より有意な affinity の低下とreceptor数の減少が観察された。またPPMにおける分解も著しく抑制された。インスリン分解の Kinetic analysis において，CCl_4－ratsとN－ratsのKmには有意差がなかったが，Vmaxは⅓に減少し，non competitive inhibitionの存在を示し，receptor の障害を示唆した。結論）receptor へのインスリン結合の低下が分解の低下を惹き起こしたことはインスリンが receptor に specific に結合することがインスリン分解の重要な limiting factor となっていることを示唆した。また肝障害における hyperinsulinism は膵の hypersecretion によるものではなく，肝におけるインスリン分解の低下によるものであることが明らかとなった。

INSULIN BINDING AND DEGRADATION BY LIVER PLASMA MEMBRANE FROM CARBON TETRACHLORIDE - INDUCED LIVER INJURY RAT

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SUMMARY

We measured insulin-degrading activity in both purified liver plasma membranes and liver homogenate of CCl_4-induced liver injury rats (CCl_4-rats) and investigated the interactions of insulin binding with degradation on receptors and the mechanism of hyperinsulinism of liver disease.

Insulinase acyivity per mg protein per min in the 27,000 x g supernate of CCl_4-rats liver (9.96 $\pm$ 0.71) was lower than that of normal rats (13.40 $\pm$ 1.57). The value was about 26 %. However glutathione-insulin transhydrogenase activity was not significantly elevated (36.01 $\pm$ 1.57 compared with 34.23 $\pm$ 1.34 in normal rats).

In the time course experiments, about 6 min lag in the initial appearance of ^{125}I-insulin degradation were observed. The degradation in the preincubation of prebound ^{125}I-insulin to plasma membranes were about the same rates as that in the initial incubation. This indicates that insulin bound to receptors are degraded.

The CCl_4-rats liver plasma membranes have significantly decreased specific binding of ^{125}I-insulin per 100 μg protein (5.46 $\pm$ 0.71 % vs 11.34 $\pm$ 0.25 % ; $p < 0.001$) compared with the normals. In CCl_4-rats the amount of insulin bound was 35-45 % lower than in normals and a significant decrease in the affinity and a significant decrease of concentrations of receptor were ofserved. The amount of insulin degraded in CCl_4-rats markedly also decreased. In the kinetic analysis

of insulin degradation, apparent Km in CCl_4-rats and normal rats was not significantly different, but apparent Vmax in CCl_4-rats was strongly decreased.

These results suggest that the insulin binding to receptors storongly mediate the insulin degradation and, furthermore, that the peripheral hyperinsulinism is due solely to decreased hepatic insulin degradation secondary to parenchymal damage, even though a passing hypersecretion occurred with the progress of the cirrhosis.

INTRODUCTION

Many patients with liver disease have been elevated peripheral immunoreactive insulin (IRI) levels with glucose intolerance.[1,2,3] It is uncertain whether this hyperinsulinism is secondary to increased insulin secretion or decreased degradation by the diseased liver. Recently, it is indicating that this hyperinsulinism resulted in the decreased insulin degradation from the experiments by the simultaneous measurement of insulin and C-peptide levels.[4,5] However, direct mechanism of this hyperinsulinism not been studied in detail.

Insulin is degraded primary by the liver. The initial step of the interaction of insulin with target cell is binding the hormone to a specific receptor.[6] Insulin binding on the membranes may be a more important determining factor in the rate of insulin degradation.[7,8] Alterations of the binding step in the hyperinsulinaemia produced by obase have been

well studied,[9] but alterations of degrading activity and the interaction of insulin binding with degradation are not as well established.

In order to clarify the mechanism of hyperinsulinism of liver disease, we measured insulin-degrading activity in both liver plasma membranes and liver homogenate of CCl_4 induced liver injury rats. We also tried to make sure the interaction of insulin binding with degradation on receptors.

MATERIALS AND METHODS

Experimental animals

Normal male rats of the Wistar strain, weining 220-280 g were used in all experiments. The rats were fed ad libitum Oriental rat chow (Oriental Kobo Kogyo Co. LTD). Male Wistar rats (280 g) with liver disease (CCl_4-rats) were made by the subcutaneous injection of 50 % tetrachloride - 50 % Olive oil (0.5 ml) on Monday and Thursday for 10 weeks. Eight - 10 weeks later the animals were killed by decapitation and the liver rapidly removed for studies. Both fasting plasma IRI and fasting blood glucose levels were significantly ($p < 0.001$) higher in CCl_4-rats as compared with normal rats.

Assay of GIT and insulinase activity

Livers were removed and rinsed with cold 0.154 M KCl. A 1 g of liver was mixed 4 ml of 0.25 M sucrose - 5 mM EDTA (pH 7.5). The pieces were then homogenized with teflon homogenizer.

The homogenates were centrifuged at 27,000 x g for 30 min. Aliquots of supernate were then assayed for determination of glutathione-insulin transhydrogenase (GIT) and insulinase activity. GIT and insulinase activity were measured by the conversion of ^{125}I-insulin to trichloroacetic acid (TCA) soluble products as described Barandani and Tomizawa[10].

Membrane preparation

Liver plasma membranes were prepared by the Ray modification[11] of the method of Neville[12]. In this procedure, partially purified membranes were further purified by centrifugation at 107,000 x g for 2 hours in 16 CN tubes containing 5 ml of 45 % sucrose, 6.2 ml of 41 % sucrose and 2.2 ml of 37 % sucrose. The purified plasma membranes were obtained from the interface between 37 % and 41 % sucrose. The purified plasma membranes were taken out with the helop of a Pasteur pipette, washed free of sucrose and then suspended in an appropriate amount buffer. Plasma membrane protein was assayed by the method of Lowry et al.[13] with bovine serum albumin as a standard. The plasma membrane marker enzyme 5'-Nucleotidase (EC 3.1.3.5) was measured by the method of Auruch and Walloch[14]. There was a 70-fold increase in the activity of 5'-Nucleotidase in the purified plasma membranes compared to the first homogenates.

Insulin binding to liver plasma membranes

Liver membranes (80 - 120 μg protein) were incubated at 30 ℃ in Krebs-Ringer phosphate (KRP) buffer pH 7.5 containing 1.5 % bovine serum albumin, 0.1 nM ^{125}I-insulin (Dainabot RI

Lab. LTD) and varying concentrations of unlabelled porcine insulin (NOBO INDUSTRI). The incubation volume was 500 µl. At the end of the incubation period, tubes were centrifuged immediately at 10,000 x g for 5 min in 4 °C. The supernate were removed and assayed for degradation (see below). The pellet was washed twice with 200 µl of chilled KRP buffer containing 0.1 % bovine serum albumin and the pellet was counted in a gamma counter. Non-specific binding, i.e., binding in the presence of 50 µg native insulin was subtracted from the total binding to determine the specific binding.

Insulin degradation by liver plasma membranes

Degradation of insulin was measured incubating ^{125}I-insulin and unlabelled insulin with plasma membranes as described above. One hundred µl of supernate was added to 1.0 ml chilled KRP buffer pH 7.4 containing 0.2 % bovine serum albumin, and precipitated eith 1.0 ml of 10 % TCA. The mixture was centrifuged at 2,500 rpm for 10 min and 200 µl of the supernate was counted in a gamma counter. The percent of insulin degraded was obtaind by subtracting radioactivity in the supernate of control samples incubated without membranes from that of samples of eigth membrane:

RESULTS

Insulinase and GIT activity ; these enzymes were located in the cell ; of liver homogenate in normal rats and CCl_4-rats is shown in Table 1. A slight but not significant (p 0.1) increase in GIT activity of CCl_4-rats was observed, however

the relatively specific enzyme for insulin degradation, insulinase of CCl_4-rats had significantly decreased degrading activity as compared with normal rats.

Figure 1 shows the time course of binding and degradation of ^{125}I-insulin to normal rats liver plasma membranes. Binding reached a steady state by 30 min at 30°C and degradation increased linearly with time. About 6 min lag in the initial appearance of ^{125}I-insulin degradation products were observed.

Figure 2 shows displacement courve of ^{125}I-insulin at concentrations of unlabelled insulin between 3.5×10^{-11} M and 3.5×10^{-6} M. As shown, the binding of ^{125}I-insulin was consistently reduced by 30 - 60 % in CCl_4-rats at all concentrations of insulin. The CCl_4-rats liver plasma membranes have significantly decreased specific binding of ^{125}I-insulin per 100 μg protein (5.46 ± o.71 % ; vs 11.34 ± 0.25 % ; $p < 0.001$) compared with the normal rats liver plasma membranes.

In CCl_4-rats the amount of insulin bound was 35 - 45 % lower than in normal rats at each insulin concentration (Figure 3). According to Scatchard analysis[15] of insulin binding data from figure 3, dissociation constant (Kd_1 ; high affinity : Kd_2 ; low affinity) in CCl_4-rats and normal rats was 16.80×10^{-9} M : 153.84×10^{-9} M and 4.45×10^{-9} M : 28.85×10^{-9} M, respectively (Figure 4). Maximum binding capacity to liver plasma membranes of CCl_4-rats decreased to about 55 % that of normal rats. These results indicate a significant decrease in the affinity and concentrations of receptor in liver plasma membranes of CCl_4-rats.

Whatever insulin concentration, the amount of insulin degraded in CCl_4-rats markedly decreased (Figure 5). A kinetic analysis of insulin degradation by liver plasma membranes estimated from Lineweaver - Burk plot. Apparent Km in CCl_4-rats (1.13×10^{-7} M) and in normal rats (0.87×10^{-7} M) was not significantly different, whereas apparent Vmax in CCl_4-rats (0.29 ng/min/100 μg protein) was strongly decreased as compared to the normal rats (0.84 ng/min/100 μg protein)(Figure 6).

To clarify whether insulin bound to receptor are degraded or dissociated as intact insulin, ^{125}I-insulin prebinding plasma membranes were reincubated at 30 ℃ for 20 min in ^{125}I-insulin free medium. As shown in Table 2, the prebound ^{125}I-insulin to plasma membranes were degraded. The degradation in the reincubation were about the same rates as that in the initial incubation. Lag time observed in the initial incubation was not seen, because ^{125}I-insulin degradation products appeared immediately after reincubation in the medium.

DISCUSSION

The liver is a major site of insulin degradation and it has been suggested that up to 50 % of secreted insulin is removed in a single rans hepatic passage.[16,17,18,19] Peripheral insulin concentrations therefore reflect a balance between insulin secretion and degradation. It has been indicated that peripheral hyperinsulinaemia in the diseased liver is a result of increased

insulin secretion from pancreatic B cell and decreased degradation by the liver. Recently, Greco et al.[20] have been reporting that inspite of basal peripheral plasma IRI levels were raised in cirrhotic patients, basal portal insulin values was the same as controls. The work of Jonston et al.[4], with simultaneous insulin and C-peptide measurements in patients with liver disease, suggests that the hyperinsulinism is a result of lowered insulin degradation and parenchymal damage may be more important than portal systemic shunting.

In the present study, lowered insulinase activity and decreased insulin binding to and degradation by purified plasma membranes in CCl_4-rats was observed. Specially, in a kinetic analysis of insulin degradation by liver plasma membranes apparent Km in CCl_4-rats was similar to that of normal rats, whereas apparent Vmax in CCl_4-rats was strongly decreased. This finding indicates occurrence of non-competitive inhivition and impediment in insulin degradation by liver plasma membranes in CCl_4-rats. We have also observed that insulin removal rate by the isolated perfused rat liver in CCl_4- rats decreased to 62 % that of normal rats (not shown data). The inhibition of insulin degradation by perfused liver was the same rate as liver plasma membranes. These results suggest that the peripheral hyperinsulinism is due solely to decreased hepatic insulin degradation, especially decreased insulin degradation by plasma membranes.

The relationship between binding and degradation of insulin

by plasma membranes is presently controversial. Freychet et al.[21] have reported that insulin degradation by and binding to receptor in liver plasma membranes are independent processes. On the other hand, Terris and Steiner[7] have suggested that insulin bound to hepatocyte plasma membranes is the substrate for insulin degradation by the liver, and Dial et al.[22] have also reported that insulin binding to receptor was shown to aid in its degradation. Recently, Olefsky et al.[23] found that the low affinity receptors having a rapid dissociation rate do not degrade insulin, and whereas the high affinity receptors having a slow dissociation rate mediate insulin degradation.

In the present study, we have attemted to characterize the interaction of binding and degradation of insulin receptor with the simultaneous measurement of insulin binding to and degradation by purified liver plasma membranes of liver injury rats, persumably possessing the lesion in receptors. In the time course experiment, the lag in the initial appearance of iodinated degradation products were observed. Furthermore, prebound insulin to plasma membranes was degraded insulin more rapidly than non-prebound insulin, but degraded insulin was the same rate (Table 2). This result is in agreement with the observation of Terris and Steiner[7] and Dial et al.[22]. The precise mechanism by which insulin is degraded are unclear, but tentative conclusion are possible for this lag. During the association, the insulin-receptor complexes are altered or translocated in some way as to alow bound insulin to be degraded.

In CCl_4-rats, both the amount of insulin bound and degraded was about 40 % lower than in normal rats. As previously described, in a kinetic analysis of insulin degradation apparent Km in CCl_4-rats was similar to that of normal rats and apparent Vmax was decreased to one-third. This indicates occurrence of non-competitive inhibition, and thereby damage of receptors are considered. A lowering of affinity to insulin binding, as shown from Scatchard analysis data (Figure 4), was resulted in the lesion in receptors. This speculation indicates that decreased insulin degradation is caused by lowered insulin binding.

It is unclear whether in receptor with hormonal biological effect and for degradation, there may be a structural or functional differentiation or not. However insulin binds to binding site on the plasma membranes, some of which are readily compartmentalized. The bound insulin is then either released intact or undergoes in some way processing or internalization and is subsequently degraded without dissociating from receptor. The data presented here suggests that specific binding of insulin to receptor may be a more important limiting factor of insulin degradation.

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Table I

Insulinase and GIT Activities in 27,000 x g Supernatant of liver Homogenate in Normal and CCl_4-Rat

	specific activity (degraded insulin p mol / min / mg protein)	
	Normal rat	CCl_4-rat
Insulinase	13.40 ± 0.52 (n=8)	9.96 ± 0.71 (n=6)*
GIT	34.23 ± 1.34 (n=8)	36.01 ± 1.57 (n=6)

Values are mean ± S.E * $p < 0.01$

GIT : glutathione-insulin transhydrogenase

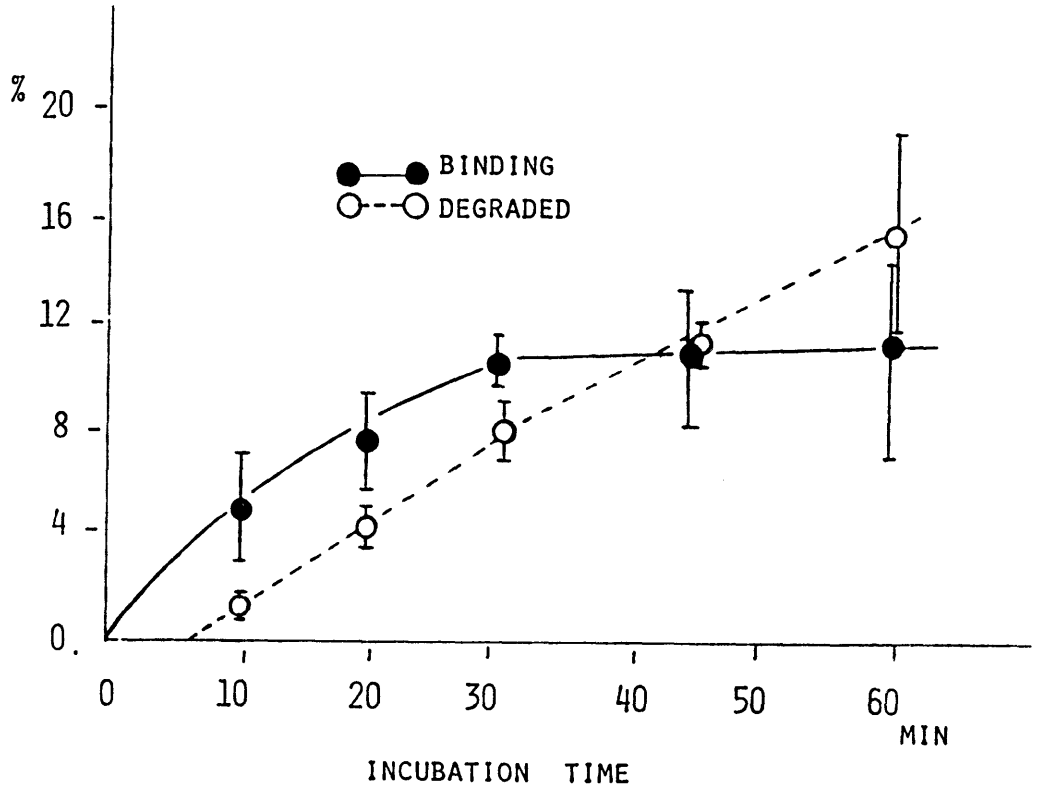

Fig. 1 Time courses of binding and degradation of ^{125}I-insulin by purified rat liver plasma membranes. Plasma membranes (80-120 μg of protein) were incubated at 30 ℃ with ^{125}I-insulin (0.1 nM) in a total volume of 0.5 ml. Data are expressed as the percentage of ^{125}I-insulin bound (● ——●) or degraded (o ---- o) at the appropriate time per 100 μg protein of plasma membranes.

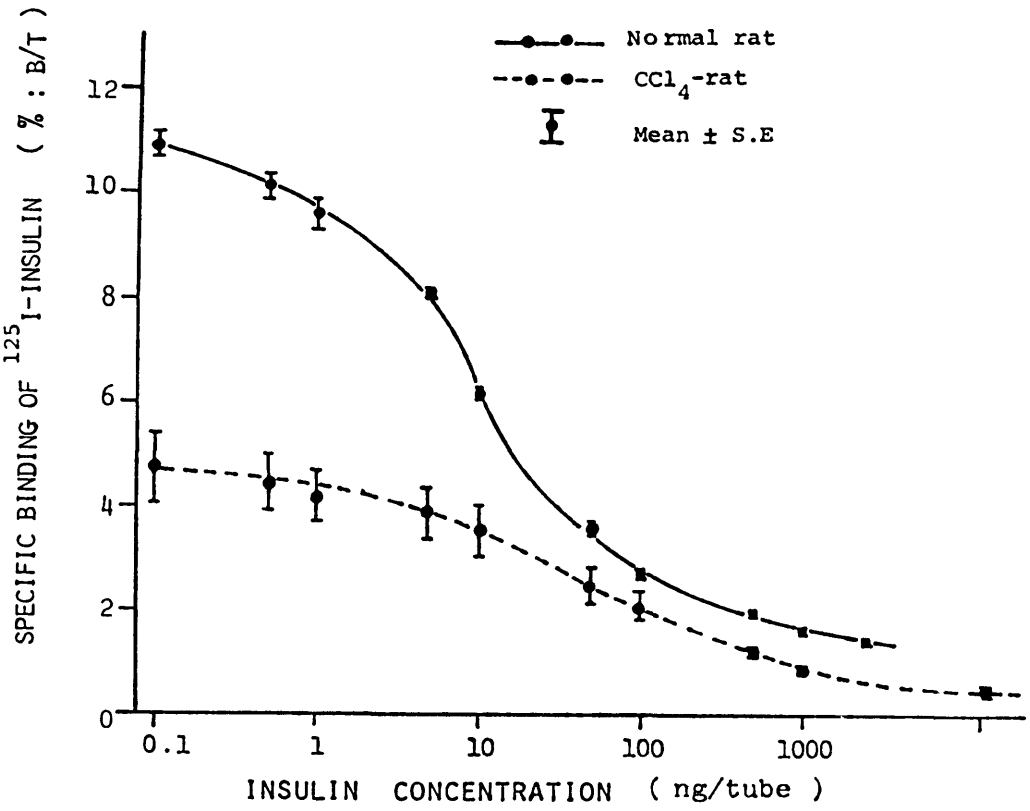

Fig.2 Specific binding of ^{125}I-insulin to liver plasma membranes and the inhibiting effect of unlabeled insulin. The results are the mean ± SE of binding of eight separate membrane preparations.

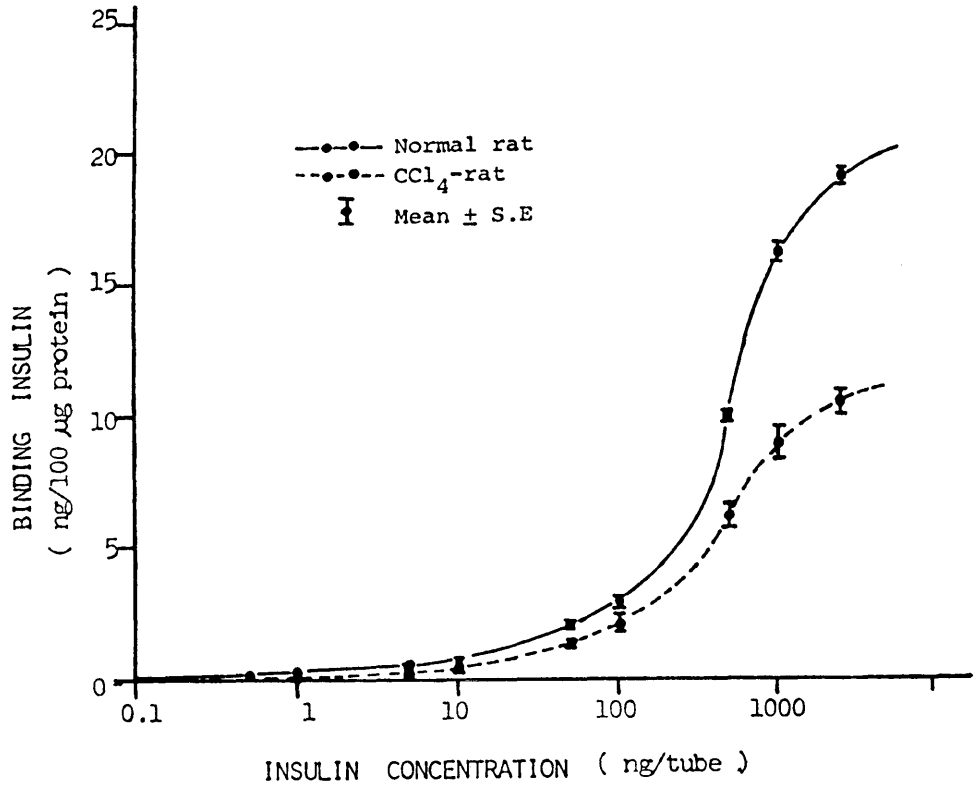

Fig.3 Amount of insulin binding to hepatic purified plasma membranes from CCl_4-rat (n=7) and normal rat (n=8). Purified plasma membranes were incubated at 30 °C for 30 min with 140 pM ^{125}I-insulin and each concentration of native porcine insulin.

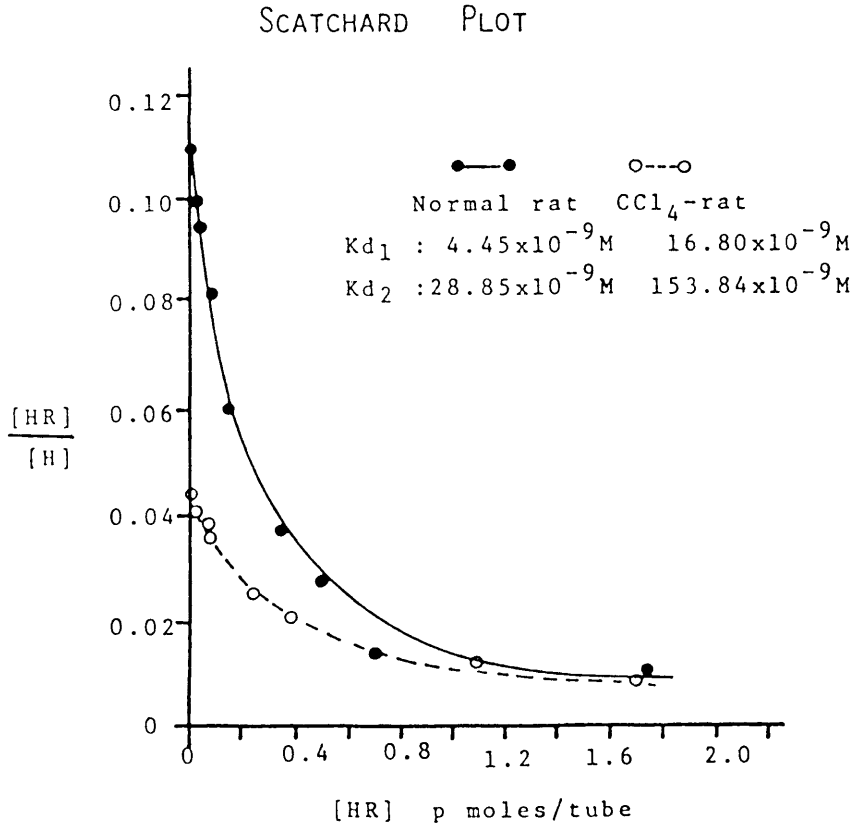

Fig.4 Scatchard plot of the binding results in figure 3.

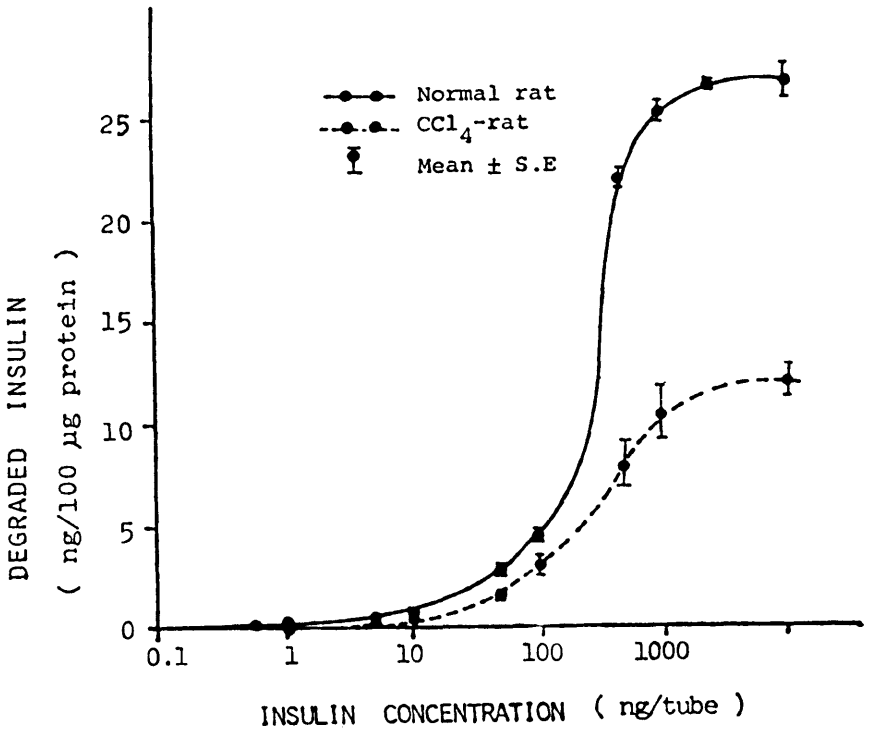

Fig.5 Amount of degraded insulin by hepatic purified plasma membranes from CCl_4-rat (n=7) and (n=8). Purified plasma membranes were incubated at 30 ℃ for 30 min with 140 pM ^{125}I-insulin and each concentration of native porcine insulin.

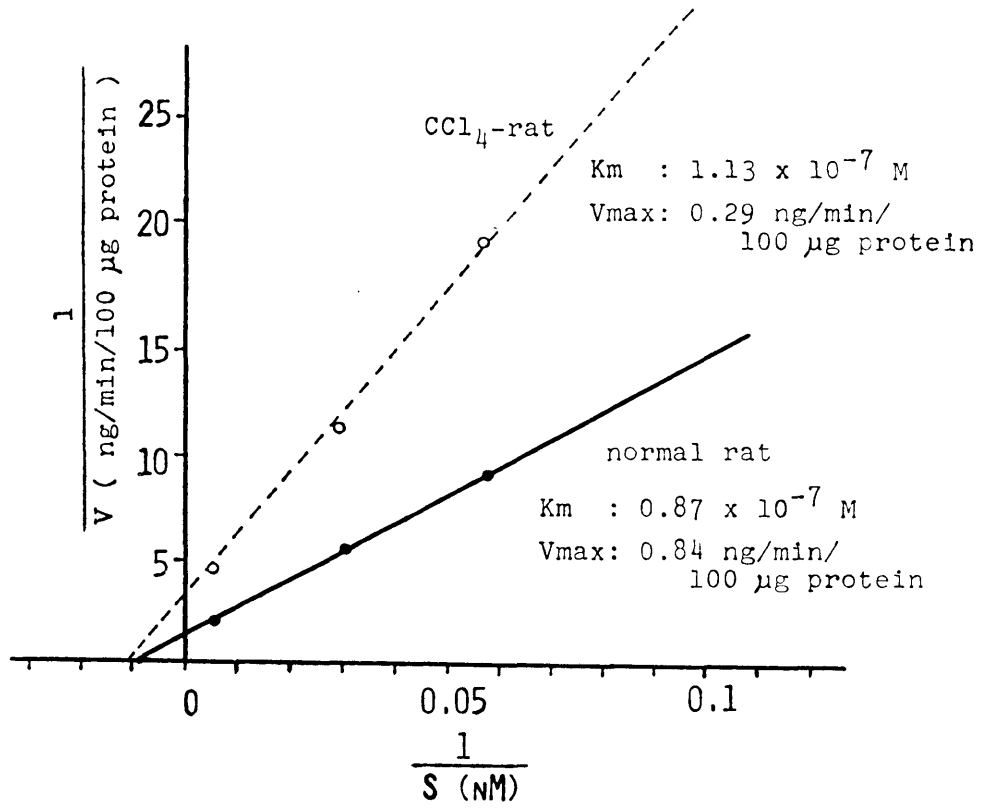

Fig.6. Line weaver - Bruk plot of the data from figure 5. Normal rat (● —— ●) and CCl_4-rat (O ---- O) are exposed to ^{125}I-insulin and unlabeled insulin at 30 °C as described for figure 5. Vmax is determined from the intercept on the vertical axis, and apparent Km is determined from the intercept on the horizontal axis.

Table 2

DEGRADATION OF ^{125}I-INSULIN BOUND TO HEPATIC PLASMA MEMBRANE

INCUBATION		REINCUBATION
	%	
6.00 ± 0.22		6.94 ± 0.25
(N=23)		(N=8)

Values are means ± S.E
^{125}I-Insulin bound per tube was ~1800 cpm.