Insulin Receptors in Virus-Induced Diabetes Mellitus in Mice

KOICHI KAWANISHI¹, MASAYUKI KUBOTA²,
AND FUMITO YAGYU²

¹Department of Internal Medicine, Kagawa Medical School,
Kagawa 761-07, Japan and ²Third Department of
Internal Medicine, Okayama University Medical
School, Okayama 700

Abstract

Quantitative estimations were made of insulin receptors on liver cell membrane of DBA/2 mice infected with M variant of encephalomyocarditis virus. The virus produced an impairment of glucose metabolism on day 3 of infection, which lasted for 5 months. The fasting plasma insulin concentration was markedly decreased on day 14. The specific binding of ¹²⁵I insulin to the membrane receptor was significantly decreased on day 3 of infection. The binding inhibitions were stronger in male mice than in females. The number of insulin receptors began to decrease on day 1, was decreased remarkably by day 3, and returned on day 7 to the level before infection. A decrease of receptor affinity was also observed in infected animals. These results seem to show that changes in insulin receptors are one cause of the impairment of glucose metabolism in the initial phase of virus-induced diabetes.

Viral infection is thought by some to be an etiological factor in diabetes mellitus. This theory is based on evidence from pancreatic pathology (insulinitis), genetics, epidemiology and animal models of virus-induced diabetes. Yoon *et al.* (1979) demonstrated clinical evidence for the etiological role of Coxsackie B₄ virus. They isolated Coxsackie B₄ virus from the pancreas of a boy who died of diabetic ketoacidosis and showed that this virus produced diabetes in mice.

Changes in insulin receptors were recently found after *in vitro* viral infection (Shimizu *et al.*, 1980). There is yet no report of the effect of viral infection on insulin receptors using the target organ of the infected animals. The present study investigated the early changes in the insulin receptors after infection with M variant of

encephalomyocarditis (EMC) virus.

Materials and Methods

Mice.

Seven-week-old DBA-2 mice were used. They were purchased from the Animal Laboratory, Shizuoka, Japan. Seven to nine mice were housed in one cage. Food and water were available *ad libitum*.

Virus.

The M variant of encephalomyocarditis (EMC) virus was kindly supplied by Dr. A. L. Notkins of the National Institutes of Health, USA. Stock pools were grown in cultured fibroblast L-929 (mouse fibrosarcoma line) in Eagle's MEM with 5% calf serum.

Virus titers were expressed as 50% tissue culture infectious doses (TCID₅₀).

Mice were inoculated intraperitoneally with 0.5 ml of virus containing TCID₅₀ at 10⁶/0.1 ml. The same experiment was carried out using Coxsackie B₄ virus that was kindly supplied by Dr. I. Tagaya of the National Institutes of Health of Japan.

Glucose tolerance test (GTT). Mice were fasted

for 18 h and then injected intraperitoneally with a 10% glucose solution at 2 mg/g body weight. Blood was immediately collected after this injection (0 time) and at 60 and 120 min. In males, the GTT was performed on day 3, 7, 14 and 35 and at month 4 and 5. In females, the GTT was performed on day 3. Blood glucose concentrations were measured by a reflectance meter (Kuhl, 1975). Fasting plasma immunoreactive insulin (IRI) concentrations were measured on day 3, 7 and 14 by the two antibody method (Morgan and Lazarow, 1962).

Histology of pancreatic islet cells. Dissected specimens were stained with hematoxylin-eosin and examined under the light microscope.

Insulin receptor assay.

Insulin receptor assays were performed on day 1 and 3 for male mice and day 1, 3 and 7 for female mice. 125-I insulin was prepared by the chloramine-T method (Gavin *et al.*, 1972) at specific activities of 150–200 $\mu\text{Ci}/\mu\text{g}$ for binding to liver cell membranes. Animals were fasted for 18 h before decapitation. The liver was excised and placed in 0.3 M sucrose and homogenized in a polytron PT-10 for 60 seconds. The suspension was centrifuged at $15,000 \times g$ for 25 min at 4°C. The supernatant was centrifuged for 90 min at $100,000 \times g$ at 4°C. This 100,000 g pellet was gently homogenized in a glass homogenizer in Tris HCl buffer at pH 7.4 and pooled at -20°C until use. The protein concentration of the liver cell membrane was determined by the method of Lowry *et al.* (1951).

Binding study. Liver cell membranes (420 and 450 μg) were incubated with 0.1 ng of 125-I insulin at 4°C for 18 h, then precipitated at 4°C for 30 min at 3,000 rpm. The specific binding was calculated by the percentage of 125-I radioactivity bound to

the membrane pellet in the presence of 10^4 ng/ml unlabeled insulin from the percentage bound in the absence of unlabeled insulin.

An average affinity profile was plotted by the method of De Meyts and Roth (1975), $\bar{K}$, or average affinity constant, was calculated as $(B/F)/(Ro-B)$, where Ro is the total number of binding sites or the X intercept on the Scatchard plot and $\bar{K}$ was then plotted as a function of fractional occupancy $Y=(B/Ro)$.

Results

Consumption of food and water in male mice was decreased on day 3 of infection and increased thereafter. In female mice, an insignificant decrease in food consumption was observed on day 3 of infection, but water consumption was increased after infection. There was no significant difference between the sexes as to weight gain after infection (Table 1). At day 3 after infection with M variant of EMC virus, male mice did not show an elevation in fasting blood glucose (FBG), but showed mild glucose intolerance after glucose loading (Table 1). FBG elevation became apparent on day 7. On day 14, the FBG concentration was high, but the blood glucose concentration after glucose loading was

Table 1. Blood glucose concentrations during the glucose tolerance test, the fasting IRI concentrations, food and water consumption and body weight of M variant EMC virus infected mice

Days	Glucose Tolerance Test Blood Glucose (mg/dl)			Fasting IRI ($\mu\text{U}/\text{ml}$)	Water Consumption (ml/day)	Food Consumption (g/day)	Body Weight (g)
	0 min	60 min	120 min				
Males	0	88.7 $\pm$ 6.5	153.7 $\pm$ 5.9	155.3 $\pm$ 16.1	8. 1 $\pm$ 1.01	3.0 $\pm$ 0.1	24.9 $\pm$ 0.1
	3	87.3 $\pm$ 2.4	220.0 $\pm$ 39.1	204.0 $\pm$ 48.3	8. 0 $\pm$ 2.04	2.7 $\pm$ 0.1**	25.2 $\pm$ 0.4
	7	140.2 $\pm$ 14.4**	481.1 $\pm$ 39.4**	426.7 $\pm$ 26.7**	8. 3 $\pm$ 3.26	4.3 $\pm$ 0.4**	24.7 $\pm$ 0.5
	14	179.3 $\pm$ 14.5**	180.5 $\pm$ 33.1	214.5 $\pm$ 35.9	0.57 $\pm$ 0.37**	4.6 $\pm$ 0.3**	24.5 $\pm$ 0.5
	35	79.5 $\pm$ 1.8	187.5 $\pm$ 33.6	202.6 $\pm$ 27.6			
	120	73.6 $\pm$ 5.9	216.3 $\pm$ 27.6	249.8 $\pm$ 33.8*			
	150	55.6 $\pm$ 6.3**	191.8 $\pm$ 17.8*	217.1 $\pm$ 20.2*			
Females	3	89.2 $\pm$ 8.4	201.4 $\pm$ 36.1	189.1 $\pm$ 28.3	#4.5 $\pm$ 0.2**	2.8 $\pm$ 0.3	20.5 $\pm$ 0.1
	21	75.9 $\pm$ 5.2	144.5 $\pm$ 10.9	186.8 $\pm$ 15.8	#4.5 $\pm$ 0.4**	2.8 $\pm$ 0.3	21.6 $\pm$ 0.4

In female mice, water consumption on 0 day was 3.2 ± 0.1 ml/day, food consumption 3.0 ± 0.2 g/day and body weight 20.7 ± 0.2 g

Significant difference versus 0 day (before infection). * $p < 0.05$ ** $p < 0.01$ N=7 M $\pm$ SEM

Fig. 1. Histological findings of the islets of infected mice 7 days after infection. This figure shows moderate damage to islet cells and disrupted architecture, degeneration of insular cells and pyknosis of nuclei are also seen. There were no mononuclear cell infiltrations. Hematoxylin-eosin stain. $\times 400$.

slightly lower than that on day 7. Glucose intolerance with a normal FBG concentration was observed on the fifth week and at month 4 and 5 after infection. There were no significant changes in fasting IRI concentrations on day 3 and 7 after infection. A marked decrease in fasting IRI was observed on day 14. The plasma immunoreactive pancreatic glucagon concentration was significantly decreased to 71.6 pg/ml on day 3 from a normal control value of 101.5 pg/ml. The female mice showed a milder glucose intolerance than male mice. There was no impairment of glucose metabolism in male and female mice infected with Coxsackie B₄ virus.

Table 2. Morphological damage to islet cells of EMC virus infected mice

Days	Number of mice	Mild	Moderate	Severe
3	8	0	2	6
7	9	1	4	4
14	7	0	7	0

Mild: less 20% of cell damage

Moderate: 20% to 80% of cell damage

Severe: over 80% of cell damage

Histological alterations became evident on day 3 in the pancreatic islets of mice infected with EMC virus. Destruction of the overall architecture of the insular tissue, degeneration of insular cells, pyknosis of nuclei and vascular proliferation were observed (Fig. 1). However, infiltration of mononuclear cells was not observed in the islets. Table 2 classifies the pathological changes in the islets after virus infection. These changes were observed until day 7 of infection. There were no pathological changes in mice infected with Coxsackie B₄ virus.

Fig. 2 (males) and 3 (females) show the specific binding of ¹²⁵I insulin to liver cell membranes (A) and an analysis of the binding (B). The specific binding of ¹²⁵I insulin to the liver cell membranes was 24.3% before infection, and 23.2% on day 1, and 8.7% on day 3 after infection in males. In female mice the specific binding of ¹²⁵I insulin to liver cell membranes was 25.2% on day 1, 13.2% on day 3 and 20% on day 7. The specific binding was markedly decreased on day 3 after infection in both males and females. However, in females, the specific binding on day 7 re-

Fig. 2. Displacement curve (A) and quantitative analysis (B) of the specific binding of ^{125}I -insulin to liver cell membrane of infected male mice. The specific binding was 24.3% before infection (●—●), 23.2% on day 1 after infection (○—○), and was decreased to 8.7% on day 3 (□—□). Quantitative analysis indicates a remarkable decrease in the number of insulin receptors on day 3 after infection.

Fig. 3. Displacement curve (A) and quantitative analysis (B) of the specific binding of ^{125}I -insulin to liver cell membrane of infected female mice. The specific binding was 25.2% on day 1 (○—○), and was decreased to 13.2% on day 3 (□—□), but was increased to 20% on day 7 (■—■) after infection. Quantitative analysis indicates a decrease in the number of insulin receptors on day 3 after infection. On day 7, the number of insulin receptors returned to the level before infection.

turned nearly to the level before infection.

Quantitative analysis showed that insulin receptors had a high affinity-low capacity (HA-LC, affinity constant: K_1) and a low affinity-high capacity (LA-HC, affinity constant: K_2). Table 3 shows the affinity constants and insulin bound to liver cell membrane of infected mice. In male mice

the K_2 on day 3 was significantly decreased. The decrease in insulin binding of 420 μg liver cell membrane was slight on day 1 and marked on day 3. These findings indicate that under virus infection there was a decrease in insulin receptor concentration. In female mice, the K_2 was decreased on day 3, similar to K_2 of male mice, and

Table 3. Affinity constants by quantitative analysis of specific binding of 125-I insulin to liver cell membrane of infected mice

		Days			
		0	1	3	7
Males	HA-LC (K_1)	4.747	4.912	4.291	
	($\times 10^8 M^{-1}$)				
	LA-HC (K_2)	0.563	0.598	0.218	
	($\times 10^8 M^{-1}$)				
	Insulin Bound (nM)	4.6	4.1	2.6	
Females	HA-LC (K_1)		4.898	4.582	4.412
	($\times 10^8 M^{-1}$)				
	LA-HC (K_2)		0.596	0.398	0.409
	($\times 10^8 M^{-1}$)				
	Insulin Bound (nM)		4.5	2.7	4.9

Pooled sample. HA-LC: high affinity-low capacity
LA-HC: low affinity-high capacity

then increased on day 7. Insulin binding of 450 μ g liver cell membrane was decreased to 2.7 nM on day 3 after infection. However, the insulin binding on day 7 returned to the level before the experiment.

Fig. 4. shows the average affinity profiles of the data presented in Fig. 2 and 3. The average affinity profiles of male mice (A) demonstrated $\bar{K}_e = 6.9 \times 10^7 M^{-1}$ for preinfection, $\bar{K}_e = 7.1 \times 10^7 M^{-1}$ for postinfection on day 1 and $\bar{K}_e = 4.8 \times 10^7 M^{-1}$ for day 3. In female mice (B), the average affinity profiles show $\bar{K}_e = 7.0 \times 10^7 M^{-1}$ for postinfection on day 1, $\bar{K}_e = 5.3 \times 10^7 M^{-1}$ for day 3 and $\bar{K}_e = 5.1 \times 10^7 M^{-1}$ for day 7. This observation suggests a decrease in the average affinity of insulin receptors in infected animals. The decrease in the average affinity was more prominent in males than in females.

Fig. 4. Average affinity profile for the data presented in Fig. 1 and 2.

(A) shows average affinity profiles in male mice and (B) shows those in female mice. In (A), (●—●) shows preinfection, (○—○) postinfection on day 1 and (□—□) postinfection on day 3. In (B), (○—○) shows postinfection on day 1, (□—□) postinfection on day 3 and (■—■) postinfection on day 7. The average affinity constant ($\bar{K}$) is calculated as $(B/F)/(R_0-B)$, where R_0 is the total concentration of receptors, B is the concentration of bound hormone-receptor complex, and F is the concentration of free hormone. Fractional receptor occupancy ($\bar{Y}$) is calculated as (B/R_0) . $\bar{K}$ is plotted as a function of $\log \bar{Y}$.

Discussion

Infection of DBA/2 mice with M variant of EMC virus produced glucose intolerance. An increase in blood glucose after glucose loading was apparent on day 3 after infection, and a maximal blood glucose rise was observed on day 7. Glucose intolerance continued for 5 months although the severity declined. Glucose intolerance in female mice was milder than in males at the same stage. Changes were not observed in fasting IRI concentrations in infected male mice on day 3 and 7, but a remarkable decrease was observed on day 14.

Histological changes in the pancreatic islet cells became evident on day 3, but infiltrations of mononuclear cells were not observed until the 14th day after infection. In the present study, we compared changes in mice infected with EMC and Cocksackie B₄ virus, but metabolic and pathological changes were not observed in mice infected with Cocksackie B₄ virus. Coleman *et al.*, (1974) reported development of diabetes and acinar cell damage following Cocksackie B₄ virus infection in CD-1 mice. However, Ross *et al.*, (1974) found no evidence of hyperglycemia or damage to beta cells in EMC virus infected DBA/2 mice. Variations in the virus or in susceptibility of the host to infection are important factors in the production of diabetes.

The specific binding of 125-I insulin to liver cell membrane was markedly decreased on day 3 after infection. The binding inhibitions were stronger in males than in females. Quantitative analysis was performed to determine whether the lower binding of 125-I insulin was due to a change in receptor concentrations or receptor affinity. The affinity constant of high affinity-low capacity (K_1) in male mice was not changed on day 1 and 3. However, the affinity constant of low affinity-high capacity (K_2) was slightly decreased on day 1 and signi-

ficantly on day 3. This indicates that the number of insulin receptor began to decrease on day 1, and was significantly decreased on day 3 after infection. In females, the same tendency was observed, and K_2 was decreased on day 3, but increased on day 7. The decrease in insulin receptor was normalized on day 7. $\bar{K}_e$ in male mice was not changed immediately after infection, but was decreased on day 3 of infection. Likewise, a decrease in $\bar{K}_e$ was observed in female mice on day 3 and 7 of infection. Decreased $\bar{K}_e$ was more obvious in males than in females. These findings indicate that decreased insulin binding to liver cell membrane in virus-infected animals is due to a reduction in the number of receptors as well as a decrease in receptor affinity. Shimizu *et al.* (1980) examined the effect of viral infections on insulin receptors using in vitro radioreceptor assay with 125-I insulin on human-amnion (WISH) cells. These investigators showed that herpes simplex virus and vesicular stomatitis virus produced a significant decrease in insulin binding without evidence of insulin degeneration. On quantitative analysis, this decrease in binding was found to be a result of a decrease in receptor concentration without a change in receptor affinity. However in their experiments, EMC virus did not alter the insulin receptors. The discrepancies between their report and ours might be due to differences in experimental conditions.

Craighead and Steinke (1971) showed that infected EMC virus was disseminated in blood after multiplying in the skin and subcutaneous tissues for 24 to 84 h, and viremia usually appeared on or before the third day and persisted for 1 to 3 days. The virus then invaded the tissues. High virus titers were found in tissues by the sixth day. Virus tests in the pancreas were positive for as long as 18 days in some of their experiments. From these findings and our study, it is thought that changes in

insulin receptors develop during viremia and results in changes in insulin receptors and the concomitant disappearance of viremia. The synthesis of new insulin receptors may occur by day 7 after infection and leads to receptor recovery.

It is concluded that in mice infected with the M variant EMC virus, the derangement of receptors by viremia is one cause of the impairment of glucose metabolism in the initial phase, and glucose intolerance in a later phase in due to hypoinsulinemia from disturbed pancreatic beta cells. It is thought that this condition is similar to that found in clinical diabetes.

References

- Coleman, T. J., K. W. Taylor and D. R. Gamble (1974). The development of diabetes following Cocksackie B virus infection in mice. *Diabetologia* 10, 755-759.
- Craighhead, J. E. and J. Steinke (1971). Diabetes mellitus-like syndrome in mice infected with encephalomyocarditis virus. *Am. J. Pathol.* 63, 119-134.
- De Meyts, P. and J. Roth (1975). Cooperativity in ligand binding: a new graphic analysis. *Biochem. Biophys. Res. Commun.* 66, 1118-1126.
- Gavin, J. R., J. Roth and P. Freychet (1972). Insulin receptors in human circulating cells and fibroblasts. *Proc. Natl. Acad. Sci. USA.* 69, 747-751.
- Kuhl, C. (1975). Dipping procedure for blood glucose determination with Dextrostix and the Eyetone reflectance meter. *Acta Med. Scand.* 197, 467-469.
- Lowry, O. W., N. J. Rosenborough, A. L. Farr and R. J. Randall (1951). Protein measurement with folin phenol reagent. *J. Biol. Chem.* 193, 265-275.
- Morgan, C. R. and A. Lazarow (1962). Immunoassay of insulin using a two-antibody system. *Proc. Soc. Exp. Biol. Med.* 110, 29-32.
- Ross, M. E., K. Hayashi and A. L. Notkins (1974). Virus-induced pancreatic disease: Alteration in concentration of glucose and amylase in blood. *J. Infect. Dis.* 129, 669-676.
- Shimizu, F., J. J. Hooks, C. R. Kahn and A. L. Notkins (1980). Virus-induced decrease of insulin receptors in cultured human cells. *J. Clin. Invest.* 66, 1144-1151.
- Yoon, J. W., M. Austin, T. Onodera and A. L. Notkins (1979). Virus-induced diabetes mellitus. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. *N. Engl. J. Med.* 300, 1173-1179.