The Journal of Biochemistry, Vol. 63, No. 5, 1968 Dedicated to K. Kaziro on His 70th Birthday.

Studies on the Xanthurenic Acid-Insulin Complex

II. Physiological Activities*

By Yahito Kotake, Toshiko Sotokawa, Edahiko Murakami,† Ayako Hisatake,†† Masakazu Abe††† and Yoshio Ikeda†††

(From the Department of Biochemistry, School of Medicine, Nagoya University, Nagoya, †the Department of Chemistry and ††the Department of Domestic Science, Aichi University of Education, Nagoya Branch, Nagoya and †††the Jikei University, School of Medicine, Tokyo)

(Received for publication, June 1, 1967)

1. The activity of the xanthurenic acid-insulin complex on the blood sugar levels of rabbits is about 50% of that of native insulin.

2. Formation of the complex also decreased the activities of insulin on the uptake of glucose by epididymal fat pads and by isolated diaphragms of rats.

A method for preparation of the xanthurenic acid (XA)-insulin complex and some of its characteristics were described in a previous paper (*1*). In the present study the physiological activity of this complex is compared with that of native insulin.

MATERIALS AND METHODS

XA-insulin Complex—The XA-insulin complex was prepared as described previously (*1*).

Determination of Blood Sugar—Rabbits were injected subcutaneously and dogs were injected intravenously. The blood glucose levels of rabbits were determined by the Hagedron-Jensen method (*2*) and those of dogs by the glucose oxidase [EC 1.1.3.4] method (*3*). The glucose oxidase method (*4*) was used for crossing-over experiments on rabbits.

Glucose Uptake by Epididymal Fat Pads of Rats—The method of modification by Ikeda (*5*) was used for the assay. Male Donryu rats, reared for five to six weeks on CE-2 laboratory chow at room temperature, were divided into groups. Non-fasted rats were sacrificed by a blow on the head or by decapitation, and the testicular epididymal fat pad was quickly cut out with scissors and soaked in the Krebs-Ringer bicarbonate buffer (20°C). After 30 min of soaking, 5 to 6 slices of about 40 mg wet weight were prepared from the distal end and put into a buffer containing 1 mg of glucose per 1 ml of buffer.

Determination of glucose uptake was performed as follows: the reaction mixture was composed of (a) about 40 mg of epididymal fat pad, (b) 1 mg of glucose in 1 ml of the Krebs-Ringer phosphate (pH 7.4) with 0.2 mg of dihydrostreptomycin, and (c) the sample of insulin or the XA-insulin complex. The reaction was carried out in the Baeyer bottle with a rubber stopper. The gas phase was replaced by 100% O_2. The bottle was shaken in an incubator at 37°C. After 4 hr of shaking, 10 μl of the reaction mixture was taken out and mixed with the reagents for the assay of glucose by the glucose oxidase method, and the tissues were weighed on a torsion balance. The uptake of glucose is expressed by mg of glucose/100 mg of wet tissue/4 hr. The uptake was calculated from those obtained with and without the sample.

Glucagon-free crystalline insulin was purchased from NOVO Co, Ltd. and was used as a standard. The insulin was stored in N/30 hydrochloric acid as a 1 unit/ml solution at −20°C and used within 30 days.

Glucose Uptake by Isolated Rat Diaphragm—Thirty day old Donryu rats were fed for a week on CE-2 laboratory chow. Rats weighing 80—100 g were used for this experiment. After 18 hr of starvation, rats

* Dedicated to Dr. Koozoo Kaziro, Professor Emeritus of the Nippon Medical School, on the occasion of his 70th birthday.

were quickly sacrified by a blow on the head or by decapitation, and the whole diaphragm was carefully removed. The diaphragm was immediately cooled on ice and put into the Krebs-Ringer bicarbonate buffer (pH 7.4). The diaphragm was cut into halves with scissors and soaked in buffer for 30 min. During this period, the buffer was changed two or three times. The diaphragm was then transferred to a buffer containing the same concentration of glucose as the reaction medium. The diaphragm was removed, blotted lightly with a piece of filter paper, and transferred to the reaction bottle (10 ml).

The reaction medium was composed of 1 ml of the Krebs-Ringer bicarbonate buffer containing 1 mg of glucose and 0.2 mg of dihydrostreptomycine. The incubation conditions and the method of determination of the glucose content were as described in the previous section, except that the incubation period was 90 min. The glucose uptake was calculated as the mean of at least four measurements, each on a different hemidiaphragm. The uptake of glucose is expressed by mg of glucose/g (wet weight) of tissue/90 min of incubation.

An aliquot of 10 μl of the reaction mixture was taken from the reaction medium and directly added to the reagents for the assay by the glucose oxidase method without previous deproteinization.

RESULTS

Determination of the Blood Glucose Level—Fig. 1 shows the blood glucose levels observed with native insulin and with the XA-insulin complex. The results show that the sugar level decreasing activity of the complex is much less than that of insulin. The relative activity was calculated by the following equation (*6*);

$$\text{Relative activity} = \frac{\text{Decrease of blood sugar by sample}}{\text{Decrease of blood sugar by control}} \times 100$$

$$\text{Decrease of blood sugar} = \left(1 - \frac{\text{Blood sugar after injection}}{\text{Blood sugar before injection}}\right) \times 100$$

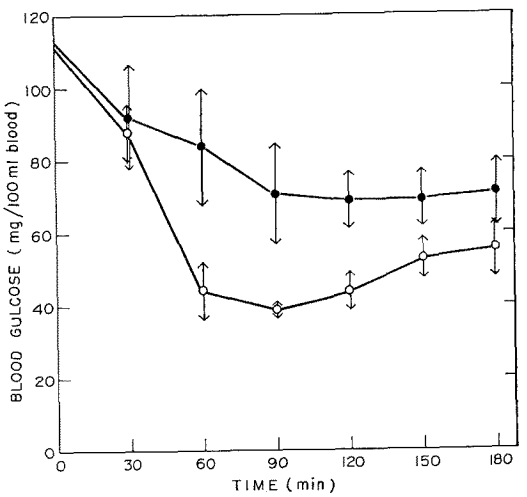

FIG. 1. Effects of native insulin and the complex on the blood sugar levels of rabbits.

Six rabbits weighing about 2 kg were used in each case. 18—21 μg of sample per kg of rabbit was injected. Blood glucose is expressed as mg of glucose per 100 ml of blood.

—○— Native insulin.

—●— XA-insulin complex.

TABLE I

Crossing-over experiment on insulin activity.

Blood glucose was determined by the glucose oxidase method. Values in parenthesis indicate per cent decrease of blood glucose. 15 μg of sample per kg was used in each case.

	Rabbit	Sample	Before injection	After injection			
				1 hr	2 hr	3 hr	5 hr
I	a	Insulin	149	63 (58%)	60 (60%)	68 (54%)	92 (38%)
	b	XA-insulin	120	71 (41%)	70 (42%)	80 (33%)	179 (149%)
II	a	XA-insulin	93	58 (38%)	59 (37%)	74 (20%)	96 (3%)
	b	Insulin	92	43 (53%)	52 (43%)	61 (34%)	76 (19%)

where the blood sugar after injection is the mean value of the blood sugar levels measured 60 min, 90 min, or 120 min after injection of the sample. When the activity of the control insulin was taken as 100, the activity of the complex was 49.

Stochastic analysis of the change with time in the data shown in Fig. 1 (*7*) showed that there is a difference between the activities of native insulin and the complex.

Crossing-over Experiment—The results of a crossing-over experiment using two rabbits reared under the same conditins are summarized in Table I. The blood glucose level was measured by the glucose oxidase method (*4*). The results also show that the activity of the complex is less than that of native insulin.

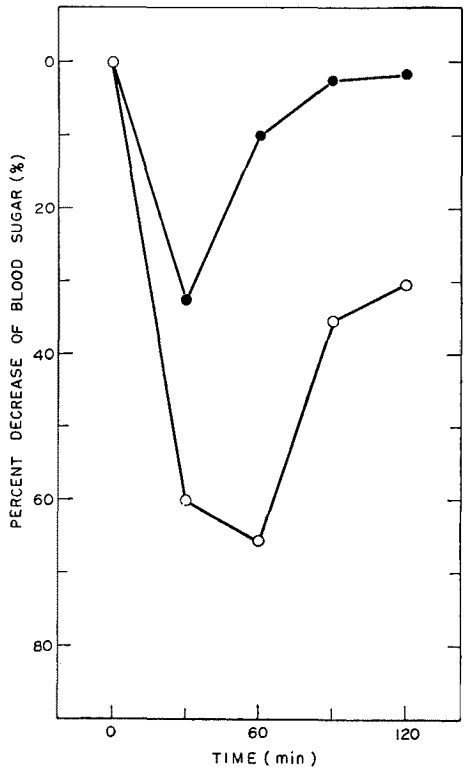

FIG. 2. Effect of native insulin and the complex on the blood sugar levels of dogs.

The values for the complex were the mean values for two dogs.

—○— Native insulin.

—●— XA-insulin complex.

Determination of Blood Glucose Levels in Dogs—Fig. 2 shows the results on the blood sugar levels of dogs. In this case 12.5μg of sample per kg was injected intravenously. The blood sugar level was determined by the glucose oxidase method (*3*). The data again show that the activity of the complex is less than that of native insulin.

Uptake of Glucose by Epididymal Fat Pads of Rats—Table II shows the effect of XA on the uptake of glucose by epididymal fat pads of rats. These results show that XA does not influence the uptake of glucose by epididymal fat pads of rats. Table III shows the effect of the complex on the uptake of glucose under the same conditions as for Table II.

TABLE II

Effect of XA on the uptake of glucose by epididymal fat pads of rats.

Experimental details are given in "MATERIALS AND METHODS". Glucose uptake; glucose in mg/100 mg of wet tissue/4 hr. Values in parenthesis indicate the numbers of experiment.

Concentration in the medium		Glucose uptake mg/100 mg tissue wt/4 hr
Basal (Glucose 100 mg/dl)		0.25±0.07 (6)
XA	1.0/dl	0.29±0.11 (5)
	10.0/dl	0.23±0.06 (6)
	100.0/dl	0.31±0.07 (7)

TABLE III

Effect of native insulin and the XA-insulin complex on the uptake of glucose by epididymal fat pads of rats.

Conditions are as for Table II.

Sample	Glucose uptake mg/100 mg tissue wt/4 hr.
Basal (Glucose 100 mg/dl)	0.25±0.07 (6) 0.36±0.09 (12)
Insulin 4 mμg/ml	0.60±0.14 (6) 0.59±0.12 (16)
XA-insulin 4 mμg/ml	0.38±0.12 (7) 0.31±0.05 (10)

TABLE IV

Effect of the XA-insulin complex on the uptake of glucose by isolated rat diaphragm.

Details are given in "MATERIALS AND METHODS." The uptake of glucose is expressed by mg glucose/g (wet weight) of tissue/90 min. Values in parenthesis are the number of experiment.

Sample	Uptake of glucose
Glucose (1 mg/ml)	1.77±0.53 (13)
Insulin (4 μg/ml)	2.09±0.38 (20)
XA-insulin (4 μg/ml)	1.41±0.41 (16)
XA(100 μg/ml)	1.31±0.42 (16)

The results show clearly that the complex has a less activity of accelerating the uptake of glucose than native insulin.

Glucose Uptake by Isolated Rat Diaphragm—Table IV shows the effects of native insulin, the XA-insulin complex and XA on the uptake of glucose by isolated rat diaphragm. The data indicate that the complex and XA decrease the uptake of glucose, in contrast to the case with epididymal fat pads of rats. The amount of XA in the complex is so small (0.14μg in this case) that the effect of XA liberated from the complex may be neglected.

DISCUSSION

The present results show that the hormonal activity of the XA-insulin complex is much less than that of native insulin. We may suggest from these results that experimental diabetes in the presence of XA may be induced by the following mechanism:

Tryptophan

$\xrightarrow{\text{Vitamin } B_6 \text{ deficiency or excess fat}}$ High production of XA in the body

$\xrightarrow{\text{Binding with insulin}}$ XA-insulin complex

$\xrightarrow{\text{Reduced insulin activity}}$ Diabetes

$\xrightarrow{\text{Overload on } \beta\text{-cells}}$ β-cells damaged slowly.

This mechanism is based on the fact that XA is a metabolite of tryptophan and the excretion of XA increases on administration of a high fat and high protein diet. The present study may throw light on the problem of human diabetes.

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