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STIMULATION OF PYRUVATE DEHYDROGENASE ACTIVITY BY INSULIN-DEXTRAN COMPLEX IN MOUSE ADIPOSE TISSUE

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SUMMARY

Soluble and stable insulin-dextran complex was prepared. Pyruvate dehydrogenase activity, as assayed by $^{14}CO_2$ formation from [1-^{14}C]-pyruvate in crude mitochondria of mouse adipose tissue, was increased after incubation of fat pads with native insulin or insulin-dextran. The direct addition of insulin or insulin-dextran to mitochondria was without effect. At submaximal stimulation, insulin-dextran was 10 times less effective than native insulin but the degree of maximal stimulation and the time course of activation by insulin and insulin-dextran were similar. The results favor the concept that the activation of pyruvate dehydrogenase in fat cells does not need the entry of insulin into cells.

Insulin activates pyruvate dehydrogenase (EC.1.2.4.1.) in rat epididymal adipose tissue. In addition to the stimulation of glucose uptake, the activation of pyruvate dehydrogenase is considered to be responsible for the stimulatory effect of insulin on lipogenesis (1-6). The prevailing concept of insulin action is that insulin binds to the cell surface receptor to exert its effect. As pyruvate dehydrogenase is an enzyme complex existing in mitochondria (7,8), the mechanism of signal transfer from plasma membrane receptors to mitochondria for the activation of pyruvate dehydrogenase has been a matter of recent investigation (6). Recently, however, Goldfine and Smith found specific binding sites for insulin in nuclei from rat liver cells, and suggested an intracellular action of insulin (9). Previously, we reported on a preparation of biologically active insulin-dextran complex, which did not penetrate the plasma membrane and did not liberate free insulin during incubation with adipose tissue (10). The present study aims to test whether this insulin-dextran is capable of activating pyruvate dehydrogenase in mouse adipose tissue like native insulin.

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MATERIALS AND METHODS

Animals Eight to ten week old male DDY mice (a non-inbred, closed colony in Japan), fed ad libitum with standard laboratory chow (Animal Research, Tokyo) were used throughout the study.

Chemicals Monocomponent pork insulin was obtained from Novo Laboratory (Copenhagen). Dextran T-70 was purchased from Pharmacia Fine Chemicals A.B. (Uppsala), [1-^{14}C]-pyruvate (11.7 mCi/mmol) from New England Nuclear Corp. (Boston), dithiothreitol, CoA-SH, cocarboxylase and bovine plasma albumin (Fraction V) from Sigma (St Louis, Mo). Other reagents were of analytical grade

Preparation of insulin-dextran complex Dextran T-70 was activated by cyanogen bromide according to a method previously reported for binding of protein to polysaccharides (11), and coupled to monocomponent pork insulin, using the method which was described in detail elsewhere (10). Insulin-dextran preparation was used for the experiments after two successive gel filtrations on a Sephadex G 75 column. Its molecular weight was about 450,000. The amount of insulin bound to dextran was 37.2 units per gram of the complex (10). The same lot was used throughout these studies. The immunoreactivity of the complex with anti-insulin serum was about 50% lower than that of native insulin. In order to test the stability of the complex, insulin-dextran (10 mU) was incubated for 60 minutes in 10 ml of Krebs-Ringer bicarbonate buffer containing about 100 mg adipose tissue. At the end of incubation, 5 ml of medium was lyophilized, dissolved in 3 M acetic acid and chromatographed on a Sephadex G 75 column (0.9 x 45 cm), and the eluates were assayed by insulin radioimmunoassay. No detectable free insulin was released from the complex (Fig. 1).

Adipose tissue incubations Epididymal fat pads from fed mice were each cut into two pieces (about 120 mg each) and were incubated individually in closed plastic vials containing 10 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) under 95% O_2-5% CO_2, with 11 mM glucose and 0.2% bovine serum albumin for 30 minutes at 37°C. After the preincubation, the fat pads were incubated for 30 minutes at 37°C in fresh buffer of the same constitution in the presence or absence of native insulin or insulin-dextran complex. After the incubation, the fat pads were immediately frozen at -80°C on dry ice. The frozen fat pads were homogenized in ice cold 30 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM dithiothreitol and 50 mM NaCl in a volume of 1 ml per 100 mg of tissue for 90 seconds by the use of a homogenizer (Omega Electric, Type SH-3, Tokyo) at a setting of 3.0. The homogenate was centrifuged for 10 minutes at 700 G. The intermediate fraction between pellet and floating fat (crude mitochondria fraction) was used for the pyruvate dehydrogenase assay.

Pyruvate dehydrogenase assay Pyruvate dehydrogenase activity was assayed by measuring the rate of formation of $^{14}CO_2$ from [1-^{14}C]-pyruvate (1,3,4). 0.4 ml of crude mitochondria fraction was added through a rubber by a syringe to a 15-ml plastic vial containing 0.5 ml incubation mixture, which consisted of 30 mM potassium phosphate (pH 7.0), 0.5 mM dithiothreitol, 50 mM NaCl, 1 mM NAD, 0.27 mM CoA, 1 mM cocarboxylase and 0.25 μM [1-^{14}C]-pyruvate. The vials were incubated for 5 minutes at 37°C. The oxidation of [1-^{14}C]-pyruvate was measured by collecting $^{14}CO_2$ in a small plastic cup suspended in the vial and containing Whatman glassfiber paper and 200 μl of β-phenethylamine. The reaction was stopped by cooling the vials in an ice cold bath and injection of 0.6 ml 2.5 M H_2SO_4 through the rubber stopper. The vials were gently shaken at 24°C for 30 minutes. The glassfiber papers were transferred to the plastic counting vials containing 10 ml toluene-ethanol-POPOP counting fluid. The radioactivity was measured by a Packard liquid scintillation spectrometer. Pyruvate dehydrogenase activity was expressed in μmol CO_2/10 minutes/g wet weight.

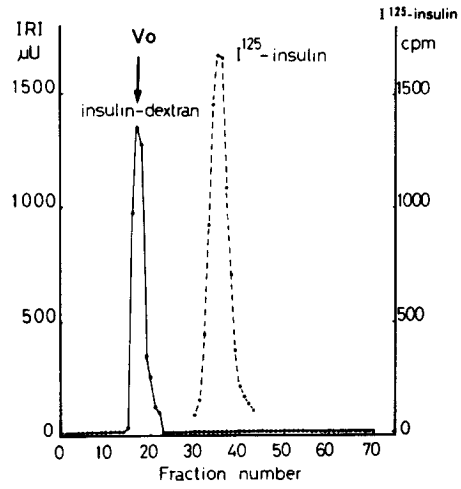

Fig. 1. Gel filtration profile of insulin-dextran complex after incubation with mouse adipose tissue. Adipose tissue (about 100 mg) was incubated for 30 minutes with 10 mU insulin-dextran complex. Following incubation, the medium was chromatographed on Sephadex G 75. Immunoreactive insulin (IRI) was detected only ahead of the region where insulin (dotted line) was eluted.

RESULTS

In mouse epididymal adipose tissue preparation, 1 mU/ml of native insulin stimulated pyruvate dehydrogenase activity slightly in the absence of substrate (Table 1). Additions of glucose or fructose enhanced the insulin activation of pyruvate dehydrogenase significantly. The augmentation of insulin effect reached a plateau at 5 mM of glucose. Subsequent experiments were performed in the presence of 11 mM glucose. In the absence of insulin, glucose alone appeared to increase pyruvate dehydrogenase activity slightly, but the difference was not significant (Table 1).

As shown in Fig. 2A, pyruvate dehydrogenase activity was increased in a dose-dependent manner by the addition of increasing concentrations of both native insulin and insulin-dextran. About 10 times greater concentration of insulin-dextran than that of insulin on the insulin molar basis, however, was necessary to activate pyruvate dehydrogenase to the same extent as did native insulin. At maximal stimulation, native insulin and insulin-dextran enhanced the pyruvate dehydrogenase activity to the similar magnitude. The time course of activation

Table 1. Effects of insulin on pyruvate dehydrogenase (PDH) activity in mouse adipose tissue.

Experiment	Additions		PDH activity (μmoles/g/10 minutes)	
	Substrate	Insulin		
1.	a. None	-	0.45 ± 0.05	
	b. Glucose (5 mM)	-	0.51 ± 0.05	
	c. Glucose (10 mM	-	0.62 ± 0.13	c.vs a. ns.
	d. None	+	0.69 ± 0.05	d.vs a. $p < 0.01$
	e. Glucose (1.25 mM)	+	0.73 ± 0.03	
	f. Glucose (2.5 mM)	+	0.80 ± 0.06	f.vs d. ns.
	g. Glucose (5 mM)	+	1.06 ± 0.07	g.vs d. $p < 0.01$
	h. Glucose (10 mM)	+	0.99 ± 0.10	h.vs d. $p < 0.02$
2.	a. Fructose (11 mM)	-	0.47 ± 0.05	
	b. Fructose (11 mM)	+	0.96 ± 0.17	b.vs a. $p < 0.01$
	c. Glucose (11 mM)	-	0.45 ± 0.04	
	d. Glucose (11 mM)	+	1.17 ± 0.31	d.vs c. $p < 0.01$

3.	Additions to fat pad	Additions to crude mitochondria fraction	PDH activity (μmoles/g/10 minutes)	
	a. None	None	0.21 ± 0.01	
	b. Insulin	None	0.56 ± 0.01	b. vs a. $p < 0.01$
	c. None	Insulin	0.20 ± 0.03	c. vs a. ns.

In experiments 1 and 2, epididymal fat pads were incubated in medium containing various concentration of glucose or fructose and in the presence or absence of 1mU/ml insulin. In experiment 3, adipose tissue pieces were incubated in the presence or absence of 1 mU/ml insulin in Krebs-Ringer bicarbonate buffer (11 mM glucose) for 30 minutes, and then crude mitochondria fraction was prepared and incubated in the presence or absence of 1 mU/ml insulin for 15 minutes. In all experiments, PDH activity was determined as described in the text. Each value represents the mean ± SD of 6 determinations.

was shown in Fig. 2B. A 10-times greater concentration of insulin-dextran than of native insulin was used in this experiment. The pyruvate dehydrogenase activity continued to increase for 20 minutes, and then reached a plateau. This time course was similar whether native insulin or insulin-dextran was used.

Table 1 shows the effect of addition of native insulin directly to crude mitochondria fraction. By this procedure, the pyruvate dehydrogenase activity was not affected at all. This is in contrast to the activation when insulin was added to fat pads before the homogenization.

DISCUSSION

The present study demonstrated that insulin activates pyruvate dehydrogenase in mouse adipose tissue, and the activation is dependent on hexose as was already known for rat adipose tissue (1-6). The particular interest of this

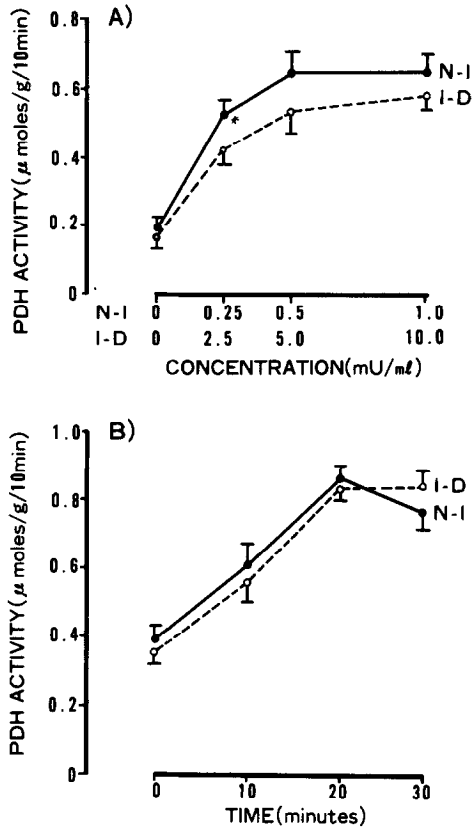

Fig. 2. A) Dose response curve of pyruvate dehydrogenase (PDH) activation in mouse adipose tissue by native insulin and insulin-dextran complex. Pieces of adipose tissue were incubated in 10 ml Krebs-Ringer bicarbonate buffer containing various concentrations of native insulin (N-I) or insulin-dextran complex (I-D) for 30 minutes. Ten times higher concentration of I-D than that of N-I was used. The asterisk (*) represents the significance of difference between PDH activities stimulated by N-I and by I-D ($p<0.05$). B) Time courses of PDH activation by native insulin and insulin-dextran complex. Adipose tissue pieces were incubated in the presence of 1 mU/ml native insulin (solid line) or 10 mU/ml insulin-dextran complex (dotted line) for 30 minutes at 37°C. Each value is the mean $\pm$ SD of 6 determinations.

action of insulin lies in that pyruvate dehydrogenase is an enzyme complex exclusively located in mitochondria (7,8), apart from the plasma membrane receptors. Not only native insulin but also insulin-dextran activated pyruvate dehydrogenase in this system. At maximal stimulation, pyruvate dehydrogenase activity was increased about 3-fold both by insulin and insulin-dextran, but at submaximal stimulation, insulin-dextran was less effective than native insulin. We have previously reported that insulin-dextran was 10-times less

effective on mouse adipose tissue when glucose oxidation, lipogenesis and anti-lipolysis were taken as indexes of insulin action (10). This difference in biological activities was again demonstrated in the activation of pyruvate dehydrogenase. The steric hindrance of insulin-receptor interaction by the huge size of the molecule seems to be the most plausible explanation for the smaller activation by insulin-dextran.

Pyruvate dehydrogenase is known to be inactivated by phosphorylation involving ATP and a kinase reaction and activated by a phosphatase reaction (12-15). For the mechanism of insulin activation, Weiss et al (6) proposed that insulin lowers the mitochondrial ATP/ADP ratio possibly via the decrease in acyl CoA concentration, thereby inhibiting pyruvate dehydrogenase kinase reaction, and increases mitochondrial Mg^{++} and Ca^{++} concentrations which favor the phosphatase reaction (2,4,12, 16-19). On the other hand, a concept was recently proposed that insulin or its fragments might enter the cell and play some role in the action of insulin (9,20). Release of free insulin from insulin-Sepharose complex was reported (21), but there was no release of free insulin from the insulin-dextran complex after incubation with fat pads for 60 minutes. Time course of activation by insulin-dextran was not delayed as compared to that of native insulin. The addition of insulin directly to crude mitochondria fraction was without effect on pyruvate dehydrogenase activity, in accordance with the data of Martin et al (2). These results seem to favor the concept that the action of insulin on fat cells is primarily mediated by its interaction with plasma membrane receptors without the need of its entrance into the cells. They do not exclude the possibility, however, that some non-immunoreactive insulin fragments are liberated during the incubation, enter the cell and are involved in the activation of pyruvate dehydrogenase.

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