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DECREASED INSULIN-GENERATION OF PYRUVATE DEHYDROGENASE INHIBITOR IN INSULIN RESISTANT STATES*

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SUMMARY. Insulin resistance produced in rats by feeding a high fat diet or by dexamethasone administration (50 ug/day, sc for 4 days) resulted in 50-70% decrease in the generation of pyruvate dehydrogenase inhibitor by insulin exposed liver particulate fractions. The inhibition was dose dependent. Treatment of insulin mediator preparations with neuraminidase and B-D-galactosidase resulted in inactivation of the pyruvate dehydrogenase inhibitor. Presence of exogenous enzyme substrates during enzyme digestion partially protected the inhibitor from inactivation. Protease treatment did not affect the inhibitor while the stimulatory activity of the insulin mediator was abolished by trypsin treatment. These results together with the previous report suggest that insulin resistance results in a decrease in the generation of both of the mediators of insulin action. This may result from a decrease in insulin binding, shown earlier, or from a decrease in precursor availability.

Recent reports from many laboratories have shown that the exposure of plasma membranes from rat adipocytes, skeletal muscle and liver to insulin results in the release of one or more low molecular weight chemical substances which in turn can affect certain enzymes in broken cell preparations (1-6). Thus, activation of glycogen synthetase, pyruvate dehydrogenase (PDH), cyclic AMP phosphodiesterase and acetyl CoA carboxylase by insulin mediator(s) have been demonstrated in vitro. In many cases, the effects of insulin on the generation of this chemical substance appear to be biphasic with respect to the concentrations of hormone (1,2,4-7). Low concentrations of insulin produce a stimulator of PDH, glycogen synthetase and acetyl CoA carboxylase, whereas the degree of stimulation is decreased at higher insulin concentrations. It has been suggested that these opposite effects of insulin might be attributable to the release of two antagonistic substances. Cheng

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The abbreviation used was pyruvate dehydrogenase (PDH).

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et al (8) described the separation of stimulatory and inhibitory activities of glycogen synthetase by high voltage electrophoresis. Seals and Czech (7) reported that both an activator and an inhibitor of PDH were released from adipocyte plasma membranes and these two activities could be distinguished by the kinetics of their generation by insulin. Saltiel et al (5) reported the separation from liver particulate fractions of these two antagonistic activities of insulin mediator by alcohol extraction. The liver plasma membrane derived ethanol extracts inhibited adenylate cyclase, PDH and acetyl CoA carboxylase whereas the ethanol insoluble fractions stimulated the activity of these enzymes (5,6).

Recent reports from our laboratories have shown that the insulin resistance induced in rats by fat feeding or dexamethasone administration results in decreased generation of PDH activator by insulin exposed liver and adipocyte plasma membranes (9,10). This decreased activation of PDH could involve a decrease in the stimulatory activity of PDH activator or an increase in the inhibitory activity. The present studies were initiated to discover whether insulin resistance is accompanied by abnormal production of PDH inhibitor by liver plasma membranes of fat fed and dexamethasone injected rats.

MATERIALS AND METHODS

Porcine insulin was a kind gift from Dr. Mary Root (Eli Lilly). [1-^{14}C] pyruvate and hyamine hydroxide were obtained from Amersham Searle. Clostridium perfringes neuraminidase (SA 0.5 U/mg solid) was from Worthington Biochemical Corporation. All the other reagents were from Sigma Chemicals.

Animals and Treatment. Young male Sprague Dawley rats (70-80 g initial weight) were fed one of the two synthetic diets for 5-7 days. A third group of rats was fed Purina Laboratory Chow. Both the synthetic diets contained 33% of the calories as casein with 67% lard or glucose (11). The diets were supplemented with salt and vitamin mixtures.

In studies on the effects of dexamethasone treatment, rats (90-100 g) were injected with dexamethasone (50 g/day, sc, divided in 2 doses) for 4 days. Control rats were injected with an equal volume of physiological saline. All rats had free access to chow and water adlibitum.

Generation of Insulin Mediators. These were generated from rat liver particulate fractions by the method of Saltiel et al (5). Livers (4-5 g) were homogenized in 10 mM potassium phosphate buffer, pH 7.4 and centrifuged at 30,000 x g for 25 min at 4^{o}C. The pellets were resuspended in 15-20 ml of the above buffer to a final protein concentration of 25-40 mg/ml. The particulate fractions (with a total protein content of 450 mg) were divided into 2 portions. One portion was incubated with insulin (100 μU/ml) for 15 min at 37. The other portion was incubated with buffer. Mediators released into the supernatant were extracted as described (5), and the lyophilized powders were resolved into alcohol soluble and insoluble fractions (5).

Aliquots of these fractions were tested on liver mitochondrial PDH for their inhibitory and stimulatory activity.

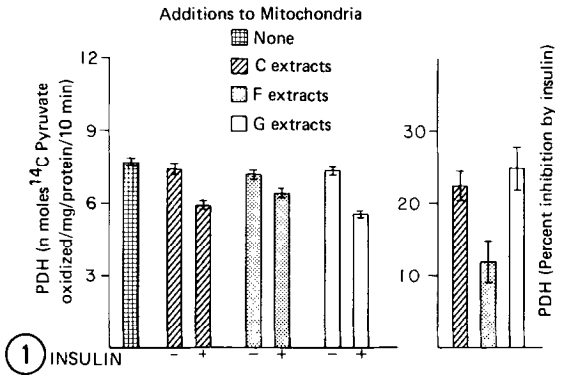
In experiments in which the effects of glycosidases and proteases were studied, liver particulate fractions were exposed to insulin and centrifuged. Separate portions of the supernatants were treated with neuraminidase (100 µg/ml) or galactosidase (100-200 mU/mg protein) or proteases (100 µg/ml) for 10 min at 37°C. After digestion, the supernatants were acidified, treated with charcoal, lyophilized and extracted with alcohol (5).

Preparation of liver mitochondria and assay of PDH activity

Liver mitochondria were prepared by the method of Parsons et al (12) as described earlier (9). PDH was assayed by monitoring the conversion of [1-^{14}C] pyruvate to $^{14}CO_2$ (1) by rat liver mitochondria. Liver mitochondria (2 mg protein/ml) were incubated for 15 min with 10-20 µl of formic acid extracts in the presence of 50 mM potassium phosphate buffer, PH 8.0, containing 50 mM $CaCl_2$ and 50 mM $MgCl_2$ (final volume 0.1 ml). The assay was initiated by addition of 25 µl of assay buffer (1) and was monitored for 10 min.

RESULTS AND DISCUSSION

In confirmation of the earlier observations of Saltiel et al (4,5), we found that the exposure of liver plasma membranes to insulin results in the generation of two antagonistic materials. The alcohol soluble fraction inhibits liver mitochondrial PDH and the alcohol insoluble residue stimulates PDH. When liver particulate fractions were incubated without insulin, alcohol extracts of supernatants produced inhibition of mitochondrial PDH to some extent. The enzyme was further inhibited by extracts from insulin exposed liver particulate fractions. Extracts prepared from fat fed rats showed a significant decrease in the inhibitory activity when compared to preparations from high glucose diet or stock diet fed rats (Fig. 1). Control extracts (not exposed to insulin) prepared from the 3 groups of rats inhibited PDH to the same extent. The inhibition was dose-dependent (Fig. 2). Addition of 20 ul of the extracts from control rats or glucose diet fed rats inhibited PDH by 50-50%, while similar preparations from fat fed rats showed only 26% inhibition. This decrease in the inhibitory activity in preparations from fat fed rats is significant at both low (10 ul) and high concentrations (20 ul) of the extract. The magnitude of decrease observed in the inhibitory activity of PDH inhibitor in fat fed rat preparations is comparable to the previously reported decrease in the stimulatory activity of PDH stimulator (9).



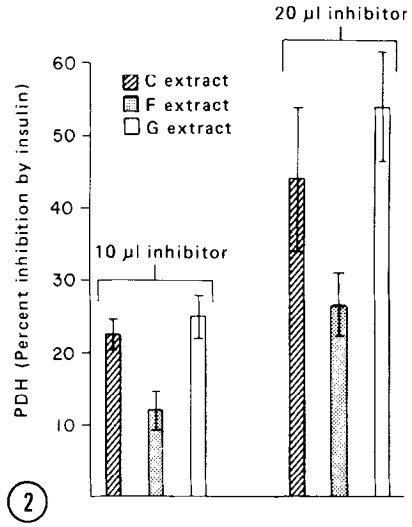


Figure 1. Effects of high fat and high carbohydrate diets on insulin-generaton of PDH inhibitor from liver particulate fractions. PDH inhibitors were prepared as described in the text. Aliquots (10 µl) were tested for their ability to inhibit liver mitochondrial PDH. Results are the means ± SE of 6 individual experiments performed in triplicate. P values comparing insulin induced inhibition by F preparations were < 0.05 compared to G or C (Duncan's multiple range test).

Figure 2. Dose response relationship of the inhibitors extracted from insulin exposed liver praticulate fractions from rats on chow, high fat and high carbohydrate diets. Results are the means ± SE of 6 individual experiments performed in triplicate. P values comparing F groups with C or G by Duncan's multiple range test were < 0.05.

Dexamethasone induced insulin resistance also resulted in a significant decrease in the generation of PDH inhibitor by insulin exposed liver particulate fractions (Fig. 3) when compared to control preparations. These results suggest that the insulin resistance induced by fat feeding or by dexamethasone administration results in inhibition of generation of both the mediators of insulin action. This inhibition could be due to a decrease in insulin binding to liver plasma membranes (13,14) or it could be due to an alteration in the composition of plasma membrane assembly which is involved in the generation of insulin mediators,coupled with post receptor defects.

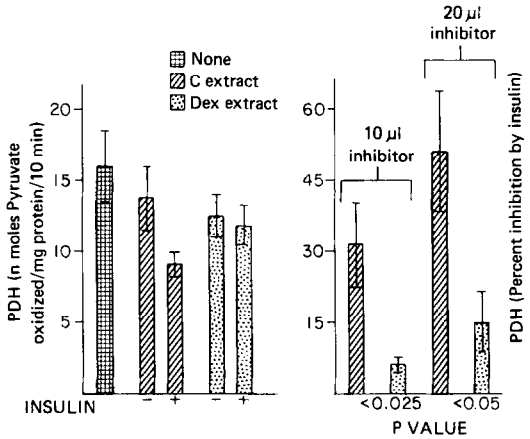


Figure 3. Effect of dexamethasone on insulin-generation of PDH inhibitor from liver particulate fractions. PDH inhibitor was prepared as described in the text. Aliquots (10-20 µl) were tested for their ability to inhibit liver mitochondrial PDH. Results are the means ± SE of 6 individual experiments performed in triplicate.

We recently found that the inhibition of insulin generation of PDH activator by plasma membranes of adipocytes treated in vitro with dexamethasone is partially prevented by the addition of arachidonate or prostaglandin E_2 (unpublished). This suggests that the insulin resistance induced by dexamethasone administration is in part related to a decreased availability of arachidonic acid derivatives which may be involved in the insulin generation of PDH modifiers.

Preliminary attempts to characterize the PDH inhibitor show that the inhibitory material released on exposure to insulin is sensitive to glycosidases (Tables 1 and 2). This suggests that sialyl and galactosyl residues on some component of the supernatant fraction from membrane incubations are necessary for the insulin mediator activity. A previous report from our laboratory showed that neuraminidase treatment caused inactivation of PDH stimulator derived from rat liver plasma membranes (9). It is interesting to note that the sensitivity of PDH inhibitor released spontaneously from liver plasma membranes not exposed to insulin is different from that of the inhibitor produced in response to insulin (Table 1).

Table 1. Effect of neuraminidase and galactosidase on the activity of PDH inhibitor isolated from liver particulate fractions of rats maintained on stock diet.

Expt. #	Treatment	PDH (nmoles ^{14}C pyruvate oxidized/mg protein/10 min) Basal activity	+control extract	+insulin extract	Δ	% inhibition
1	None	11.41±1.60	6.94±1.24	5.42±1.05	1.52±0.20	24±2.2
	Neuraminidase		7.19±1.34	6.67±1.20	0.50±0.20	5±4.5
	P value			a	< 0.01	< 0.005
2	None	15.66±2.10	5.30±1.13	1.79±0.23	3.52±1.1	55±11.5
	Galactosidase		4.93±1.00	4.71±1.32	0.33±1.01	1.0±16.20
	P-value			a	< 0.01	< 0.005
3	None	22.54±5.02	9.5 ±2.50	6.23±2.50	3.3 ±0.06	44±12.2
	Neuraminidase and Galactosidase		9.4 ±2.90	8.12±2.50	1.3 ±0.5	13.4± 4.90
	P value				< 0.05	< 0.005

Insulin mediators were exposed to glycosidases and were extracted into alcohol soluble and insoluble fractions. Aliquots (10 µl in Expt #1 and 20 µl in Expt #2 and 3) of the alcohol soluble fractions were tested for their ability to inhibit mitochondrial PDH. Results are the means ± SE of 6 individual experiments performed in triplicate. "a" denotes paired differences $P < 0.005$.

Whether these two materials are chemically different is not known. A recent report by Cherqui et al (17) also showed that N-glycosidic linkages are important in some of the actions of insulin and lectins on lipogenesis and protein synthesis. These effects of insulin are blocked by treatment of cells with neuraminidase.

Table II Effect of exogenous enzyme substrates on the inactivation of PDH inhibitor by neuraminidase.

Treatment	PDH (nmoles ^{14}C pyruvate oxidized/mg protein/10 min Basal activity	+Control extract	+Insulin extract	Δ	% Inhibition
1) None	17.4±3.1	10.4±1.60	7.7±1.0	2.7±0.50	25.3±2.1
2) Neuraminidase		10.0±1.82	9.6±1.6	0.4±0.60	2.9±4.1
3) Neuraminidase + Substrates		9.2±1.40	7.5±1.0	1.7±0.60	16.9±3.3
4) Substrates		9.9±1.61	7.2±1.3	2.6±0.73	26.7±6.5
P Value					
1 vs 2		NS	< 0.05	< 0.05	< 0.05
2 vs 3		NS	< 0.05	< 0.05	< 0.05
1 vs 3		NS	NS	NS	NS
2 vs 4		NS	< 0.05	< 0.05	< 0.05
3 vs 4		NS	NS	NS	NS

Insulin mediators were exposed to neuraminidase in the presence and absence of exogenous enzyme substrates (0.6 mg mucin + 0.6 mg human glycoprotein fraction v1/ml). The mediators were extracted with alcohol and the soluble fractions (20 µl) were tested for their ability to inhibit liver mitochondrial PDH. Results are the means ± SE of 5 individual experiments performed in triplicate.

Table III Effect of proteases on the inactivation of PDH inhibitor

Expt. #	Treatment	PDH (nmoles ^{14}C pyruvate oxidized/mg protein/10 min) Basal activity	+control extract	+insulin extract	Δ	% inhibition
1	None	14.6±1.4	9.4±2.0	6.9±1.4[a]	2.6±0.8	26±4.0
	Trypsin		9.8±1.7	6.9±1.5[a]	2.0±0.4	21±4.8
2	None	20.2±3.7	10.0±1.0	6.4±0.66[a]	3.6±0.8	35±5.2
	Chymotrypsin		9.5±0.9	5.7±0.73[a]	3.8±0.7	36±1.0
	P value				NS	NS

Insulin mediators were exposed to proteases and were extracted with alcohol and were tested on liver mitochondria as described in the text. Results are the means ± SE of 5 individual experiments performed in triplicate. "a" denotes paired differences $P < 0.005$.

Tables 3 and 4 show the effects of proteases on the inactivation of PDH inhibitor and stimulator. Trypsin at a concentration of 0.1 mg/ml totally inactivated PDH stimulator (Table 4) while the PDH inhibitor did not appear to be affected by trypsin and chymotrypsin (Table 3). These results suggest that the two antagonistic materials released upon incubation of liver plasma membranes with insulin are similar in their sensitivity to glycosidases while they appear to be different in their sensitivity to proteases. Our experiments do not rule out the possibility that glycosidases might be interfering with the mitochondrial response to the mediators. However, the basal enzyme activity (mitochondria alone) was not affected by proteases and glycosidases at the concentrations used in the experiment. Seals and Czech (2) reported the inactivation of adipocyte plasma membrane derived PDH activator by trypsin and chymotrypsin. Larner et al (18) reported that the

Table IV Effect of trypsin on the inactivation of PDH stimulator prepared from insulin exposed liver particulate fractions.

Treatment	Basal activity	+Control extract	+Insulin extract	Δ	% Stimulation
None	2.61±0.55	4.98±1.15	7.94±1.79	2.96±0.78	68±13.2
Trypsin		4.82±1.00	4.84±1.40	0.02±0.56	0.4±10.4
P Value		NS	< 0.025	< 0.05	< 0.025

Insulin mediators exposed to trypsin were extracted with alcohol and alcohol insoluble fractions (20 µl) were tested on liver mitochondria for their ability to stimulate liver mitochondrial PDH (The PDH system contained 200 µM ATP in addition to the ions described in the text). Results are the means ± SE of 6 individual experiments performed in triplicate.

stimulatory mediators released from insulinized muscle extracts were partially inactivated by subtilisin and were totally destroyed by hydrolysis in 6 N HCl and were found to contain amino acids in the hydrolysate. The peptidic nature of these regulators has not been fully characterized. We are not aware of similar studies of the inhibitory factor. Nevertheless, the PDH inhibitory material is of interest in terms of its regulatory potential in the control of insulin induced alterations in the phosphorylation of proteins.

In summary, we have shown that two insulin resistant states are accompanied by decreased generation of both inhibitory and stimulatory PDH regulators by liver plasma membranes.

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