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# INSULIN RESISTANCE IN THE LIVER IN FASTING AND DIABETES MELLITUS: THE FAILURE OF INSULIN TO STIMULATE THE RELEASE OF A CHEMICAL MODULATOR OF PYRUVATE DEHYDROGENASE

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SUMMARY. To further define the mechanism(s) of insulin resistance in the liver associated with diabetes and fasting, we evaluated the ability of insulin to release an activator of pyruvate dehydrogenase activity from a liver particulate fraction. Insulin reproduceably and significantly enhanced the release of mediator from the liver particulate fraction of control animals. The particulate fractions from fasted and diabetic animals were resistant to this effect of insulin. Refeeding and insulin treatment, respectively, restored responsiveness to insulin. These data support the concept that alterations at or near the plasma membrane can be responsible for or accompany the insulin resistance observed in the liver in fasting and diabetes mellitus.

INTRODUCTION. Previous studies from our laboratory have shown that insulin stimulates fatty acid synthesis in both freshly isolated and primary cultures of heptocytes and that this response to insulin is markedly blunted in hepatocytes from fasted (1,2) and nonketotic streptozotocin-diabetic rats (2,3). We demonstrated that this insulin resistance occurs in the presence of normal to increased insulin binding suggesting a defect in insulin action at a site distinct from the binding of insulin to its receptor (4). The exact site of this resistance to insulin action is, however, unknown.

Several recent studies have presented evidence for the production and release of one or more chemical substances from membranes of skeletal muscle (5,6), adipose tissue (7-12) liver (13,14) and hepatoma cells (15) following their incubation with insulin. These putative mediators of insulin action are of small molecular weight and are capable of modulating several insulin sensitive enzymes including pyruvate dehydrogenase (6-16),

Abbreviations. PDH, pyruvate dehydrogenase; TPP, Thiamin pyrophosphate; DTT, dithiothreitol.

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glycogen synthase (5), low km cAMP phosphodiesterase (15), $Ca^{++}$, $Mg^{++}$ATPase (16) and adenylate cyclase (14).

With regard to altered metabolic states, Begum et al (12) have demonstrated a decrease in the ability of insulin to stimulate pyruvate dehydrogenase activity in a cell free preparation containing adipocyte plasma membranes from animals fed a high fat diet. These studies suggest that a portion of the insulin resistance observed in adipocytes from rats fed high fat diets may be due to a decreased ability of insulin to generate the putative chemical mediator of insulin action. To further define the mechanism(s) underlying insulin resistance in the liver of diabetic and fasted rats, we have evaluated the ability of insulin to release an activator of pyruvate dehydrogenase activity from a crude liver particulate fraction from these animals.

## METHODS

Male Sprague-Dawley rats (125-250g) were used for all studies. Animals had free access to water and , except when indicated, were fed ad libitum. For studies using fasted rats, food was withheld for 60 h. Nonketotic diabetes mellitus was induced in fed rats by the injection of streptozotocin (85 mg/kg) via a femoral vein under ether anesthesia as previously reported (2-4). Diabetic animals were used between 5 and 20 days after the induction of diabetes. Diabetic rats treated with insulin received 0.025 U/g body weight of PZI insulin for 7 d. Fasted-refed rats were refed for 3 to 4 d prior to sacrifice.

Streptozotocin ws a gift from Dr. D. Kaiser of the Upjohn Co. and crystalline porcine insulin from Dr. R. Chance of Eli Lilly and Co. (1-$^{14}$C) Pyruvic acid was obtained from Amersham, PZI insulin from Eli-Lilly and Co. and all other chemicals from Sigma. The liver particulate fraction and putative insulin mediator were prepared as described by Saltiel et al (13,14). The particulate fraction was resuspended in 10mM potassium phosphate buffer, pH 7.4, to achieve a final protein concentration of 15-20 mg/ml and incubated for 15 min at 37°C in the absence and presence of 2nM insulin. Following this incubation an equal volume of 0.2M acetate buffer, pH 3.75, containing 0.1 mM EDTA and 0.1 mM DTT was added and centrifuged and treated as described by Saltiel et al (12,13). The supernatant was lyophilized, the powder dissolved in 1 mM formic acid and centrifuged at 10,000 xg for 10 min. This final supernatant is referred to as the extract or mediator fraction and its concentration as the equivalence of the original protein content of the liver particulate fraction (0.16 and 1.6 mg/ml).

Rat liver mitoplasts enriched in PDH activity were prepared by the method of Parsons et al (17) as modified by Saltiel et al (14) and used to assay the mediator activity. Mitoplasts wre suspended in 10 mM potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose at a protein concentration of 8-10 mg/ml and stored for up to 2 weeks at -70°C with no loss of activity. Mitoplasts (0.1mg) were preincubted in 10 mM potassium phosphate buffer, pH 7.4, containing 0.13 mM ATP for 15 min at 37°C in the presence of 50 uM $CaCl_2$, 50 uM $MgCl_2$ and the mediator fraction in a final volume of 0.1 ml. PDH activity was assayed by monitoring the release of $^{14}CO_2$ from (1-$^{14}$C) pyruvate (0.22 uCi/ml) for 10 min at 37°C in a final reaction volume of 0.125 ml containing 1 mM DTT, 0.1 mM TPP, 0.1mM CoA, 0.5 mM NAD and 0.25 mM unlabeled pyruvate in 50 mM potassium phosphate buffer, pH 8.0. The reaction was stopped by adding 0.2 ml 6N $H_2SO_4$ and the $^{14}CO_2$ released trapped in 0.2 ml ethanolamine: ethylene glycol monomethyl ether (1:2 v/v). All assays were performed in triplicate.

Protein was determined by the method of Lowry et al (18). Statistical analyses were performed by the Student's t test.

RESULTS. Pyruvate dehydrogenase activity in mitoplasts prepared from the livers of normal control rats was 8.52 ± 0.32 nmol $^{14}CO_2$ released/mg protein per min. In the presence of 0.13 mM ATP this activity was reduced to 0.94 ± 0.13 nmol $^{14}CO_2$ released/mg protein per min which represents 89% suppression of PDH activity. Upon the addition of material released from the liver particulate fraction prepared from normal control animals (weight = 190 ± 3 g, serum glucose = 184 ± 10 mg/dl) PDH activity increased (Table I). Over the range of extract concentrations used in these studies, no inhibition of PDH activity was observed. The supernatant from liver particulate fractions treated with insulin led to further enhancement of PDH activity (Table 1). In preliminary studies using insulin concentrations from 0.1 to 20 nM it was found that the concentration of insulin giving maximal generation or release of the stimulator of PDH activity was 2 nM (data not shown). As a result, we used this concentration in all subsequent experiments. Using a liver particulate fraction from control animals, insulin led to approximately a 32% increase in PDH activity at both 1:10 and 1:1 dilutions of the released material. The absolute amount of insulin induced stimulation of PDH activity was, however, substantially greater at lower extract dilutions (Table 1). The stimulation of PDH activity ( $\Delta$ ) was significantly greater by extracts from insulin treated membranes at both the 1:10 ($p < 0.005$) and 1:1 ($p < 0.001$) dilutions.

As previously observed for total lipid and fatty acid synthesis in both freshly isolated and primary cultures of hepatocytes from nonketotic streptozotocin-diabetic animals (2,3), the liver particulate fraction from these animals was resistant to the ability of insulin to generate the activator of PDH activity (Table I). Extracts from non-insulin treated liver particulate fractions from diabetic animals (animal weight = 201 ± 14g, serum glucose = 495 ± 10 mg/dl) stimulated PDH ativity to the same degree as extracts from the non-insulin treated particulate fractions from normal animals. In contrast, however, insulin had no significant effect on generation of the activator of PDH activity and in fact led to a small decrease in PDH activity which was significant at an extract dilution of 1:1 (Table I, $p < 0.025$). At both the 1:10 and 1:1 dilutions of extract the ability of insulin to generate the activator of PDH measured as the absolute change ( $\Delta$ )

TABLE I

THE ACTIVATION OF MITOCHONDRIAL PYRUVATE DEHYDROGENASE BY A FACTOR RELEASED FROM LIVER MEMBRANES DERIVED FROM ANIMALS WITH DIFFERENT METABOLIC STATES

| | Pyruvate Dehydrogenase Activity (nmol $^{14}CO_2$ released/mg protein per min) | | | |
|---|---|---|---|---|
| Extract Dilution | -Insulin | +Insulin | Absolute Change ($\Delta$) | % stimulation |
| | | Control N=10 | | |
| 1:10 | 1.22±0.28 | 1.61±0.26 | 0.40±0.07 | 32.8% |
| 1:1 | 2.57±0.31 | 3.38±0.44 | 0.82±.0.18 | 31.9% |
| | | Diabetic N=6 | | |
| 1:10 | 1.15±0.17 | 1.05±0.16 | -0.10±0.05 | -8.7% |
| 1:1 | 2.88±0.40 | 2.35±0.33 | -0.52±0.17 | -18.1% |
| | | Diabetic-treated N=3 | | |
| 1:10 | 2.16±0.19 | 2.49±0.17 | 0.33±0.02 | 15.3% |
| 1:1 | 3.50±0.30 | 4.49±0.34 | 0.99±0.27 | 28.3% |
| | | Fasted N=4 | | |
| 1:10 | 1.47±0.24 | 1.24±0.15 | -0.23±0.11 | -15.6% |
| 1.1 | 2.65±0.38 | 2.71±0.29 | 0.05±0.15 | 1.9% |
| | | Fasted-Refed N=4 | | |
| 1:10 | 1.82±0.30 | 1.98±0.28 | 0.16±0.05 | 8.8% |
| 1.1 | 2.24±0.54 | 2.58±0.63 | 0.34±0.12 | 15.2% |

Rat liver particulate fractions from control, diabetic, fasted and fasted-refed animals were incubated in the absence and presence of 2nM insulin for 15 min at 37° C and the supernantant treated as described in the Methods Section. Extract dilutions of 1:10 and 1:1 are equivalent to the supernatant from an initial membrane concentration of 0.16 and 1.6 mg protein/ml. Mitoplasts were prepared from normal control rats. Pyruvate dehydrogenase activity was assayed as described in the Methods Section.

was significantly less in liver particulate fractions from diabetic as compared to normal control animals ($p < 0.001$). Following the in vivo treatment of diabetic animals with insulin for 7 d (weight = 214 ± 2 g, serum glucose = 30 ± 2 mg/dl), PDH activity is increased by extracts of both insulin and non-insulin treated particulate fractions. The response to insulin ( $\Delta$ ) is restored (Table I) ($p < 0.001$ and $p < 0.005$ at the 1:10 and 1:1 dilutions, respectively, vs particulate fractions from untreated diabetic animals) and is not significantly different from control animals. In four animals treated for only 2 d (serum glucose = 110 ± 12 mg/dl) insulin responsiveness was restored to a lesser ($\Delta$ =0.29 ± 0.13 nmol $^{14}CO_2$/mg protein per min at a 1:1 dilution) but significant ($p < 0.02$) degree.

This restoration of insulin responsiveness with regard to generation of the activator of PDH activity is similar to the restoration of insulin stimulated lipogenesis in hepatocytes from diabetic animals treated with insulin in vivo (2).

The particulate fractions isolated from the livers of fasted animals (weight = 142 ± 20 g, serum glucose = 111 ± 7 mg/dl) are similar to those from diabetic animals in that they are resistant to the ability of insulin to stimulate the activity of PDH (Table I). The stimulation of PDH activity ( Δ ) by insulin in the particulate fraction from fasted animals is significantly less than that from control animals ($p < 0.001$ at both extract dilutions). This data is again similar to that which we have reported using hepatocytes from fasted animals (1,2). These hepatocytes are resistant to the ability of insulin to stimulate total lipogenesis and fatty acid synthesis. As observed in the particulate fractions from diabetic animals, there is a slight but insignificant inhibition of PDH activity following insulin treatment at an extract dilution of 1:10 (Table I). With refeeding for 3 to 4 days (animal weight = 191 ± 7 g, serum glucose = 208 ± 7 mg/dl) the ability of insulin to stimulate PDH activity ( Δ ) is restored at the 1:10 dilution ($p < 0.025$ and $p < 0.1$ at 1:10 and 1:1 dilutions, respectively, vs fasted animals). While the ability of insulin to stimulate PDH activity in fasted-refed rats was not significantly diffrent from control animals ($p < 0.1$ at 1:10 dilution), the restoration of responsiveness is not as impressive as that in insulin treated diabetic animals. The reason for this is presently unknown.

DISCUSSION. Hepatic lipogenesis is resistant to stimulation by insulin in fasting and diabetes mellitus (1-3). In hepatocytes from fasted animals , insulin responsiveness can be restored in primary cultures by in vitro treatment with insulin (1, 2). The insulin responsiveness in the liver of diabetic animals can be restored by in vivo treatment of animals with insulin but not by in vitro treatment of primary cultures of hepatocytes (2). This suggests that in vivo factors other than insulin may be important in reversing the hepatic insulin resistance of diabetes. Since insulin binding is normal to increased in hepatocytes from fasted and diabetic animals (4), we have suggested that the observed insulin resistance is due to postbinding mechanisms.

The studies of others have defined a putative chemical mediator of insulin action common to several biological responses and several tissues (5-16). This putative mediator is produced and released when membrane fractions are incubated with insulin. Since the

insulin resistance in diabetes mellitus and fasting appears not to be related to alterations in insulin receptor number or affinity (4), it is of interest to evaluate possible post-binding sites which may be responsible for or accompany the decreased ability of the liver to respond to insulin. The present studies were undertaken to further evaluate the site of this insulin resistance. The data indicate that the generation of the putative mediator of insulin action, as measured by the release of a stimulator of PDH activity from a liver particulate fraction treated with insulin, is markedly and significantly depressed in fasting and nonketotic diabetes mellitus. Furthermore, refeeding and insulin treatment, respectively, restore the ability of insulin to generate this chemical mediator. These data are in agreement with previous data from our laboratory evaluating insulin responsive lipogenesis in freshly isolated and primary cultures of hepatocytes from fasted and diabetic animals (1-3) and suggest that the inability of insulin to generate either the production or release of this and possibly other mediators may be an important site of insulin resistance in the liver in these two metabolic states. Whether this represents the primary site of insulin resistance and whether the inability of insulin to generate this putative mediator is responsible for or related to other metabolic pathways which are resistant to insulin is presently unknown. The fact that amino acid uptake is not resistant to insulin in hepatocytes from diabetic animals but is resistant to insulin in hepatocytes from fasted animals (4) suggests that other mediators may be involved in this biological response to insulin. Also, we have not evaluated the possible role of an inhibitory chemical substance which may be produced in excess following the exposure of particulate fractions from livers of fasted and diabetic animals to insulin (14). It is possible that the generation of an inhibitor and not the lack of generation of a stimulator is responsible for the results observed in this study. Further studies will be necessary to answer these questions. However, the present data support the concept that alterations at or near the plasma membrane level can be responsible for or accompany the insulin resistance observed in the liver in fasting and diabetes mellitus.

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