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PRESENCE OF AN ACTIVATOR OF PYRUVATE DEHYDROGENASE IN HUMAN CIRCULATION: ELEVATION FOLLOWING A GLUCOSE LOAD AND POSSIBLE RELATION TO AN INSULIN MEDIATOR

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SUMMARY. Our earlier observation that the chemical mediator of insulin action stimulates lipid synthesis in primary cultures of rat hepatocytes prompted us to examine its presence in human serum and its regulation by changes in insulin levels. Serum samples were obtained from normal subjects following an oral 100 gm glucose tolerance test (GTT; n=10). An acid soluble, heat stable and charcoal non-absorbable substance was extracted from different sera and tested for their ability to stimulate liver mitochondrial pyruvate dehydrogenase (PDH). This substance obtained from GTT samples at 1/1000 final dilution caused significantly higher stimulation of PDH when compared to that obtained from fasting samples. These results demonstrate the presence of an activator of PDH (molecular weight ~ 1000-2000) in human circulation. Since the activator of PDH is modulated by physiological perturbation such as oral glucose ingestion, known to cause changes in circulating insulin levels, it may possibly be related to insulin mediator.

Exposure of various target cells to insulin results in generation of "putative" insulin mediator which enhances the activities of key insulin regulatory enzyme in cell free system(1-3). We have shown that the chemical mediator of insulin action from liver particulate fraction enhanced *de novo* lipid synthesis and down regulated insulin receptors in primary cultures of rat hepatocytes(4). Thus, the mediator is able to mimic insulin action in intact cell system. Similar observations were made by Zhang et al., who demonstrated that the mediator prepared from adipocyte plasma membranes inhibited lipolysis and stimulated lipogenesis in intact isolated adipocytes(5). Recently, Jarett et al found that insulin mediator from rat skeletal muscle stimulated glycogen synthesis in rat adipocytes.(6) It is likely that following insulin action, the mediator is released into the medium in *in vitro* experiments and into the circulation in *in vivo* models in an analogous manner to cyclic AMP(7). The present study demonstrates the presence of the activator of PDH in serum which is modulated by physiological perturbation of circulating insulin levels by oral glucose ingestion, and thus may be related to an insulin mediator.

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MATERIALS AND METHODS

Chemicals. The [1-^{14}C] pyruvic acid (sodium salt, 19.7 mCi/m mol) was purchased from New England Nuclear, Boston, MA. Dithiothreitol (DTT), thiamine pyrophosphate (TPP), Coenzyme A, β-NAD, ATP, sodium pyruvate, hyamine hydroxide, and acid washed charcoal were obtained from Sigma Chemical Co., St. Louis, MO. All other reagents were analytical grade.

Glucose tolerance test. Oral GTT was performed on 10 normal volunteers, age 18-36 years, weight within 10% of their ideal body weight, with no family history of diabetes mellitus, and with no known metabolic disorders. These subjects did not receive any medication for at least one week and they had an isocaloric, well balanced diet for 3 days prior to the test. After an overnight fast, a fasting blood sample was collected and then again at 1/2-, 1-, 2-, 3-, and 4-hours after oral administration of 100 g glucose. Blood glucose was measured by glucose oxidase method using Beckman Glucose Analyzer II(8). Serum insulin levels were determined by double antibody radioimmunoassay(9).

Extraction procedure. The method is similar to that described for rat adipocytes(10) and liver particulate fraction(11) with minor modifications as previously described by us(4). An equal volume of 0.2 M acetate buffer (pH 3.75) containing 0.1 mM EDTA and 0.1 mM DTT was added to the serum and boiled for 5 minutes. The resulting suspension was treated with acid washed charcoal (5 mg/ml) for 10 minutes at 4°C and then centrifuged at 40,000 x g for 30 minutes. The supernatant was lyophilized, suspended in 1 mM formic acid to a final volume which was 1/5th of the original volume of serum, and centrifuged again at 40,000 x g for 30 minutes. The clear supernatant was aliquoted, and stored at -70°C. All mediator samples were assayed for PDH activity in a single batch.

Pyruvate dehydrogenase assay. The activity of pyruvate dehydrogenase was measured by the release of $^{14}CO_2$ from [1-^{14}C] pyruvic acid using liver mitochondria (inner mitochondrial membranes i.e. mitoplasts) by the method of the Saltiel et al(3,11). Liver mitochondria (2 mg/ml) and the serum mediator (1/1000 final dilution) were incubated at 37°C for 15 minutes in 200 µl volume of 50 mM phosphate buffer (pH 7.4) with 50 µM $MgCl_2$, 50 µM $CaCl_2$, and 200 µM ATP. The assay was then initiated by adding a cocktail consisting of 0.25 µM ^{14}C-pyruvate, 0.1 mM TPP, 0.1 mM Coenzyme A, 1 mM DTT and 0.5 mM NAD. Following 10 minutes of incubation at 37°C, the reaction was stopped by adding 300 µl of 3N H_2SO_4. The $^{14}CO_2$ released was absorbed onto 200 µl of 1 M hyamine hydroxide, which was placed in center wells. The $^{14}CO_2$ absorbed onto hyamine hydroxide was counted in a liquid scintillation counter (Packard Tri-Carb 4530). The background $^{14}CO_2$ released in the absence of the mediator and mitochondria, was <8% of total counts and was subtracted from each determination. Both background and control (only 1 mM formic acid) tubes were placed before and after each set of GTT samples. The assay was performed in triplicate. The intra-assay coefficient of variation was <5% for control tubes placed before and after each set of GTT samples.

Statistical analysis. Student's "t" test was used to assess the statistical significance between fasting and different GTT serum samples.

RESULTS

Figure 1 demonstrates the effect of oral glucose administration on blood glucose, serum insulin and stimulation of PDH activity by an acid soluble, heat stable and non-absorbable by charcoal substance when tested at 1/1000 final dilution. The PDH activity in the presence of such substance from 1,2, and 3 hour post glucose samples was significantly greater when compared to that from overnight fasting serum samples ($p < 0.05$). Although the PDH activity at 1/2 and 4 hours was higher than at fasting, it was not significantly different. When the

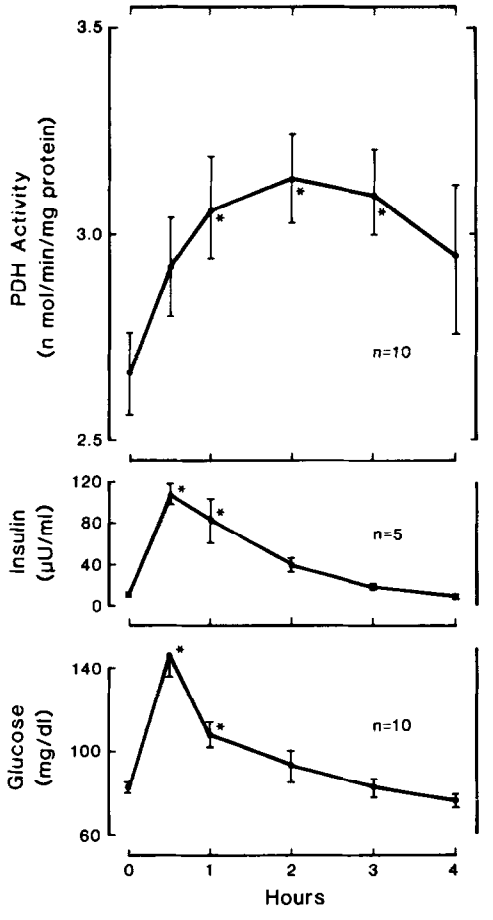

Figure 1: Upper panel: Effect of an acid soluble, heat stable and charcoal non-absorbable substance (1/1000 final dilution) prepared from GTT serum samples of normal subjects on rat liver mitochondrial pyruvate dehydrogenase (PDH) activity. Middle panel: Serum immunoreactive insulin levels following oral GTT in normal subjects. Lower panel: Blood glucose levels following oral GTT in normal subjects. Each point represents mean ± SEM. The level of significance ($p < 0.05$ or less) is shown by an asterisk when the comparison is made against basal i.e. fasting value.

peak (3.36 ± 0.08; nmol/min/mg protein) PDH stimulating activity following oral GTT was compared to that of fasting serum (2.65 ± 0.10), the differences were more pronounced and highly significant ($p<0.001$). The acid soluble, heat stable and nonabsorbable by charcoal substance from fasting serum samples (2.65 ± 0.10) at 1:1000 final dilution significantly stimulated liver mitochondrial PDH activity over buffer control (2.3 ± 0.10; $p<0.05$). We were unable to notice any significant difference in PDH stimulating activity of these preparations of any GTT samples when assayed at higher concentrations (1/10 and 1/100 final dilutions). This could be attributed to a non-specific stimulant introduced during the extraction procedures, presumably calcium from charcoal or present in the post GTT

samples. When the serum activator of PDH was desalted by Amicon filtration (cut off range <500 mol. wt.), the PDH stimulatory activity reduced appreciably but was higher than identically treated buffer control. We were also able to separate the activator of PDH from serum samples which eluted between 1000-2000 molecular weight) and the non-specific stimulant (eluted in the bed volume region) on Sephadex G-25 chromatography. The elution position of the activator of PDH was similar to that of liver particulate insulin mediator preparation.

DISCUSSION

In the present study, we have demonstrated the presence of an acid soluble, heat stable, and charcoal non-absorbable substance in human serum which stimulates the activity of PDH in a cell-free system, an assay most commonly used to demonstrate the generation of mediator from various insulin sensitive tissues. The presence of such a substance in serum could be due to the release of the "putative" mediator or second messenger of insulin action into the circulation following insulin actions. This assumption is based on the following facts: An acid soluble, heat stable, and charcoal non-absorbable mediator extracted from liver and adipose tissue following exposure to insulin, stimulates the PDH activity in various cell-free systems(10,11). The molecular weight of the "putative" mediator isolated from different tissues, e.g., adipocytes(1), liver particulate fraction(3) and muscle(2) is close to that of the activator of PDH in the serum. The changes in circulating levels of insulin by oral glucose administration enhance the ability of this substance to modulate the PDH activity. We and others have demonstrated that the putative mediator(s) from liver, skeletal muscle, and fat cells, is able to mimic some of insulin actions in intact cell systems(4-6). Like cyclic AMP(7), this substance ie. the activator of PDH might be released into the circulation following insulin's biological effects in various tissues. Our results are only suggestive of the existance of insulin mediator in circulation and its possible *in vivo* modulation by insulin. Although not much information is available in the literature on the properties of the mediators the following characteristics should be established to insure that the insulin mediator in serum described here is the authentic mediator: a) alkaline lability; b) retention on anion exchange resins; c) activation or deactivation of other mediator sensitive enzymes, (ie., glycogen synthase, cAMP-dependent protein kinase, cAMP-phosphodiesterase and acetyl CoA carboxylase); d) solubility in organic solvents; e) kinetics of action on enzymes; and f) co-elution of serum-derived material with fat cell, or skeletal muscle material on sizing columns.

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