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DIFFERENCES IN THE EFFECT OF INSULIN ON THE GENERATION BY ADIPOCYTES AND IM-9 LYMPHOCYTES OF A CHEMICAL MEDIATOR WHICH SIMULATES THE ACTION OF INSULIN ON PYRUVATE DEHYDROGENASE[1]

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SUMMARY: Insulin treatment of adipocytes increased the amount or activity of a low molecular weight, acid stable material which, when isolated and partially purified from intact adipocytes, yielded a single active fraction which stimulated pyruvate dehydrogenase. IM-9 lymphocytes contained a similar active material but in contrast to the adipocyte, insulin treatment of IM-9 lymphocytes caused a reduction in the amount or activity of the material either by decreasing production of the material or by increasing production of an inhibitory substance. These findings are consistent with the reported biological effects of insulin on these two cell types and suggest that the active material from these two cell types is the putative insulin chemical mediator.

Recent studies have indicated that the interaction of insulin with its receptor on the plasma membrane results in the generation of a chemical mediator or second messenger from the plasma membrane that is at least partially responsible for the short-term effect of insulin on metabolic processes. The first evidence for the generation of this chemical mediator was the finding that the direct addition of insulin to a subcellular system consisting of plasma membranes and mitochondria from rat adipocytes resulted in a decreased phosphorylation of the alpha sub-unit of mitochondrial pyruvate dehydrogenase with concomitant stimulation of pyruvate dehydrogenase activity in the mitochondria (1,2,3). The stimulation of pyruvate dehydrogenase activity by insulin in this subcellular system was mimicked by the direct addition of concanavalin A or anti-insulin receptor antibody (3). The effect of all three ligands was observed only when the plasma membranes were present, suggesting that a chemical mediator of

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insulin action was being generated by the interaction of insulin with its receptor on the plasma membrane. Larner et al. (4) have reported that insulin increased the amount or activity of a low molecular weight material extracted from rabbit skeletal muscle which inhibited glycogen synthase kinase and stimulated glycogen synthase phosphatase from muscle. In addition, this material stimulated pyruvate dehydrogenase in isolated mitochondria from rat adipocytes (5), thereby simulating the action of insulin on the subcellular system in the presence of plasma membranes. Kiechle et al. (6) have demonstrated that insulin increased the amount or the activity of a similar low molecular weight, acid stable material which, when isolated from intact adipocytes, stimulated pyruvate dehydrogenase activity in adipocyte mitochondria. Furthermore, Kotagal et al. (7) have demonstrated a similar low molecular weight, acid-stable material generated from the plasma membrane of the adipocyte whose activity or quantity is increased by insulin (Kiechle et al., unpublished observations). The stimulation of the pyruvate dehydrogenase activity by insulin and concanavalin A in the adipocyte subcellular system and the stimulation of the enzyme activity by the insulin-sensitive material isolated from rat adipocytes or the adipocyte plasma membrane were all shown to result from activation of the phosphatase activity within the pyruvate dehydrogenase complex and not by alteration of the kinase activity.

Another step necessary in proving that the material from adipocytes and skeletal muscle fulfills the role of the insulin chemical mediator or second messenger is to demonstrate the existence of this material in other cell types, and to show that, where appropriate, insulin can alter the level or the activity of the material. The IM-9 cultured lymphocyte has been extensively studied as a model system for insulin interaction with its receptor (8). Despite extensive studies on the binding of insulin to its receptor on the IM-9 lymphocyte, there are no reports of a biological response of this cell to insulin. It was thus felt that this cell type would be an appropriate model to study in contrast to the adipocyte. The present study demonstrates that a material similar to that isolated from rat adipocytes and rabbit skeletal muscle is present in the IM-9

lymphocyte, but in contrast to the increased amount or activity of this material resulting from insulin treatment of the adipocytes or skeletal muscle, insulin causes a decrease in the amount or activity of the material in the IM-9 lymphocyte.

MATERIALS AND METHODS: Adipocytes were prepared from the epididymal fat pads of 120g Sprague-Dawley rats by collagenase digestion as previously described (1,3). Equal aliquots of adipocytes were incubated in Krebs-Ringer phosphate buffer, pH 7.4, in the presence or absence of 100 μUnits of insulin per ml for 10 min at 37°C. The adipocytes were centrifuged at 1000x g for 1 min and the incubation medium was removed. The insulin-treated and control cells were then heated without buffer for 3 min at 100°C, followed by the addition of 3 volumes of 0.2 M acetate buffer, pH 3.8, containing 0.1 mM EDTA and 0.1 mM cysteine, and reheated at 100°C for 4 min. The mixture was cooled on ice and filtered through glass wool to remove fat and denatured protein. The absorbances at 260 nm were measured for each set of cells and found to be within 5% of each other. The colorless supernatant was treated with acid-washed Norit to remove nucleotides and then lyophilized. The white powder was dissolved in 1.0 ml of 0.05 M formic acid and chromatographed on Sephadex G25 (column, 3x26 cm). Three-ml fractions were collected in the first 9 tubes and 1-ml fractions in the remaining tubes. The eluate was in 0.05 M formic acid. All fractions were collected at a flow rate of 10 drops per min and their absorbance at 230 and 260 nm was determined. Insulin, the β chain of insulin, and uridylyl(3'→5')adenylyl(3'→5')adenosine (UAA; MW 936,7) were used to calibrate the column. Five fractions were collected as previously described (6). Fraction I contained the void volume material, II the material eluting between the void volume and the start of the 230 nm peak, III the front half of that peak, IV the back half of the peak, and V the post-peak fraction. Fractions were diluted to a final volume of 60 ml with 0.05 M formic acid, lyophilized, and redissolved in 1.5 ml of 0.001 M formic acid. For control material, 60 ml of 0.05 M formic acid was lyophilized and redissolved in 1.5 ml of 0.001 M formic acid.

IM-9 lymphocytes were grown in culture as previously described (9). The cells were harvested, and centrifuged to yield a packed cell volume of 0.5-0.75 ml. The cells were resuspended in Krebs-Ringer phosphate buffer, pH 7.4, and washed 2 times. They were then divided into two aliquots with the same buffer and incubated in the presence or absence of 100 μUnits of insulin per ml for 10 min at 37°C. After incubation the cells were handled in a manner identical to the adipocytes for extraction, column fractionation, and preparation of the isolated fractions. In neither cell type did the 260 nm absorbance of the cell extracts prior to Sephadex G25 chromatography differ between the control or insulin-treated cells, indicating equal extraction efficiency.

Each fraction was tested for its ability to alter pyruvate dehydrogenase activity in mitochondria isolated from adipocytes as previously described (3,6). A mixture of 50 μl of each fraction and 150 μl containing 250-500 μg mitochondrial protein, 50 μM Mg^{2+}, 50 μM Ca^{2+}, and 125 μM disodium ATP in 50 mM potassium phosphate buffer, pH 7.4, was preincubated at 37°C for 5 min. The assay was initiated by the addition of 0.25 mM 1-[^{14}C] pyruvate (1mCi/mmol), 0.5 mM NAD, 1 mM coenzyme A, 0.1 mM co-carboxylase, and 0.1 mM dithiothreitol in 50 mM potassium phosphate buffer, pH 7.4. After 2 min, the assay was stopped by the addition of 200 μl of 6 M H_2SO_4. The enzyme activity was determined as the amount of [^{14}C]O_2 produced.

RESULTS

Measurement of the absorbance at 230 or 260 nm of the Sephadex G25 chromatographed extract of IM-9 lymphocytes revealed a peak at a location similar to that

TABLE I

Pyruvate dehydrogenase activity of Sephadex G25 fraction from insulin-treated and control adipocytes[a]

Fraction number	Insulin-treated (nmol/mg/min)	Control (nmol/mg/min)	Δ (nmol/mg/min)
I	3.1±0.1	3.3±0.8	-0.2
II	3.4±0.05	7.5±0.3	-4.1
III	41.2±1.4	13.9±0.7	+27.3
IV	5.4±0.3	3.2±0.2	+2.2
V	2.5±0.05	2.6±0.1	-0.1

[a]The results are from a single representative experiment performed in triplicate. The values represent the mean ± SEM. The Δ column represents insulin-treated values minus control values. ATP was not present in the enzyme assay media. Background activity (no tissue) of 1.9 nmol/mg/min was subtracted from each experimental value.

reported for adipocyte extracts (6) and to that found for the adipocyte extracts used in this study (data not shown). Furthermore, the peak heights observed in the column fraction did not vary between the control or insulin-treated cells for either cell type studied.

Each fraction from the Sephadex G25 column for both control and insulin-treated adipocytes or IM-9 lymphocytes was tested for its ability to stimulate pyruvate dehydrogenase activity in adipocyte mitochondria. The data in Table I demonstrate that for control and insulin-treated adipocytes, fraction III contains the active material that significantly stimulates pyruvate dehydrogenase activity. This fraction had previously been identified as containing the active fraction and was in the molecular weight range of 1,000-1,500 as indicated by the molecular weight markers (6). As in seven other similar experiments with adipocytes, fraction III from insulin-treated cells contained a greater quantity or amount of activity of the active material. In the present experiment, fraction III from insulin-treated cells stimulated pyruvate dehydrogenase activity almost three times that of the control. In other experiments (data not shown), the stimulation by fraction III from insulin-treated cells was two to four times that of controls.

TABLE II

Pyruvate dehydrogenase activity of Sephadex G25 fractions from insulin-treated and control IM-9 lymphocytes[a]

Fraction number	Insulin-treated (nmol/mg/min)	Control (nmol/mg/min)	Δ (nmol/mg/min)
I	1.4±0.3	1.5±0.2	-0.1
II	8.6±0.8	2.1±0.2	+6.5
III	6.6±0.3	21.6±0.9	-15.0
IV	2.9±0.2	4.1±0.1	-1.2
V	1.5±0.2	2.6±0.9	-1.1

[a]The results are from a single representative experiment performed in triplicate. The values represent the mean ± SEM. The Δ column represents insulin-treated values minus control values. ATP was not present in the enzyme assay media. Background activity (no tissue) of 1.8 nmol/mg/min was subtracted from each experimental value.

Table II shows that control IM-9 lymphocytes contain a material in fraction III that stimulates pyruvate dehydrogenase activity much greater than any other fraction. This material is of the same molecular weight range as the active material from adipocytes. In contrast to the adipocytes, insulin treatment of IM-9 lymphocytes resulted in a significant reduction in the ability of fraction III to stimulate the pyruvate dehydrogenase activity. The control fraction III demonstrated 3.4 times more stimulation of pyruvate dehydrogenase activity than the fraction III from insulin-treated cells. In a separate experiment (data not shown), control fraction III stimulated pyruvate dehydrogenase activity 2.2 times greater than the same fraction from insulin-treated cells. In the experiment shown in Table II the pyruvate dehydrogenase activity of fraction II from insulin-treated cells was greater than control cells. This increase in activity has not been reproducible and was not seen in other experiments (data not shown).

DISCUSSION

The present study provides further evidence that the insulin-sensitive material isolated from intact adipocytes (6) and skeletal muscle (4) and generated from adipocyte plasma membranes (7) fulfills the role of the chemical mediator of at least some of the short-term intracellular effects of insulin

on responsive cells. It was confirmed that insulin treatment of adipocytes increased the amount or the activity of a low molecular weight, acid-stable material which, when isolated from intact adipocytes by heat extraction and subsequent Sephadex G25 chromatography, was found in a single active fraction which simulated the ability of insulin to activate pyruvate dehydrogenase. A similar material can be isolated from IM-9 lymphocytes by the same extraction and chromatographic approach. In contrast to the adipocyte, insulin treatment of the IM-9 lymphocyte resulted in a decrease in the amount or the activity of this factor as measured by its ability to activate pyruvate dehydrogenase activity in mitochondria from adipocytes. This finding is consistent with the lack of reports on the ability of insulin to alter metabolic pathways in the IM-9 lymphocyte that are sensitive to the hormone in other tissues. The IM-9 cell was without response to a wide range of insulin concentrations when 3-O-methyl glucose uptake, amino isobutyric acid transport, CO_2 production from glucose or cyclic AMP generation have been measured (J.R. Gavin, III, unpublished observations).

The presence of the chemical mediator substance in IM-9 lymphocytes is the first demonstration of this material in insulin-insensitive cells, and begins to document its ubiquitous nature. The presence of this chemical mediator in IM-9 lymphocytes raises interesting questions concerning the coupling of the insulin receptor to the effector system in the plasma membrane responsible for generating the message from the plasma membrane. It is possible that in the insulin-insensitive cell there is a lack or an inappropriate coupling of the receptor to the effector system. The finding that insulin actually decreased the amount or activity of this mediator substance as measured by its ability to stimulate pyruvate dehydrogenase could occur by at least two mechanisms. Insulin treatment of the cell could result in a decreased production of the material or in the increased production of an inhibitor substance without altering the production of the mediator. Cheng *et al.* (10), in Larner's laboratory, have separated the insulin mediator from skeletal muscle from an active material which had effects

opposite from those of the mediator substance on the cyclic AMP-dependent protein kinase and the glycogen synthase phosphoprotein phosphatase. Similarly, there is preliminary evidence that a low molecular weight substance exists in adipocytes that can inhibit pyruvate dehydrogenase activity (Kiechle et al., unpublished observations). Further purification studies with the active fraction from IM-9 lymphocytes will be required to distinguish between the two suggested mechanisms for the insulin effect on the mediator substance.

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