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CHEMICAL MEDIATOR OF INSULIN ACTION STIMULATES LIPID SYNTHESIS AND DOWN REGULATES THE INSULIN RECEPTORS IN PRIMARY CULTURES OF RAT HEPATOCYTES

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SUMMARY. Incubation of rat liver particulate fraction with insulin causes the release of a substance that stimulates lipid synthesis and down regulates the insulin receptor in primary cultures of isolated rat hepatocytes. This substance may be similar to putative mediator(s) of insulin action which has been shown to modulate the activity of key enzymes of lipid and carbohydrate metabolism in various cell free systems. Our data demonstrate that the mediator of insulin is also biologically active in an intact cell system. Down regulation of the insulin receptor by the mediator supports the concept that this phenomenon is a post binding event of insulin action.

INTRODUCTION. There is evidence to suggest that insulin binds to its plasma membrane receptor with subsequent generation of a peptide-like material which mediates some of the intracellular effects of insulin. These include cAMP-dependent protein kinase (1), glycogen synthetase (1), pyruvate dehydrogenase (2-5), adenylate cyclase (6), acetyl CoA carboxylase (7), low km cAMP phosphodiesterase (8-9) and Ca, Mg ATPase (10). The biological action of this putative mediator(s) has been studied in various cell-free systems. In this study, we have evaluated the effects of the mediator of insulin action on lipid synthesis and down regulation of the insulin receptor in primary cultures of rat hepatocytes. The cultured hepatocytes for this study were chosen because the liver is an important target organ of insulin action, and cultured hepatocytes bind, process, and respond to insulin (11, 12). The present work demonstrates that the mediator of insulin action can also mimic biological activities of insulin in an intact cell system.

MATERIAL AND METHODS

Chemicals. Carrier-free (^{125}I) Na, (^{14}C) leucine, (^{125}I) glucagon, (^{125}I) growth hormone, (methoxyl-^{3}H) inulin, 3-O-(^{14}C) methyl-D-glucose and (^{14}C) acetate were obtained from New England Nuclear, Boston, MA. Crude collagenase (4117 CLSII 41K22) was purchased from Worthington Biochemical Corporation, Freehold, N.J. Crystaline procine insulin and glucagon were kindly provided by Dr. Ronald Chance of Eli Lilly and Company, Indianapolis, IN. All other chemicals were reagent grade.

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Primary cultures of rat hepatocytes. Liver perfusion, isolation and culture of hepatocytes from male-Sprague Dawley rats (200-250 g) were performed as previously described (11, 12).

Generation of Insulin Mediator. Insulin mediator was generated from rat liver particulate fraction as reported by Saltiel et al (6). The final material, referred to as "extract generated in the absence or presence of insulin," contains material from 10 g wet weight of initial liver/ml of 1 mM formic acid. This material stimulates the activity of pyruvate dehydrogenase in liver mitoplasts (6) by monitoring the conversion of $(1\text{-}^{14}C)$ pyruvate to $(^{14}CO_2)$ (Sinha and Caro; manuscript in preparation).

Binding of (^{125}I) labeled hormones. Insulin was iodinated (1 Ci/μmol) using chloramine-T according to the method of Cuatrecasas (13). Specific (^{125}I) labeled insulin, glucagon and growth hormone bindings, at apparent steady state, were performed as previously reported (11-15).

Lipid Synthesis. Cultured hepatocytes were incubated at 37°C either for 2 hours or 16 hours with (^{14}C) acetate (0.5 μCi/ml, 5 mM) in the absence and presence of insulin ($1x10^{-9}$ and 1×10^{-7}M) or different dilutions of the extract generated in the absence or presence of insulin (1×10^{-9} and 1×10^{-7}M). Following incubation for either 2 or 16 hours, total lipids were extracted into a mixture of chloroform, methanol and water as previously described (16).

Indicators of cell viability and yield. (^{14}C) leucine incorporation into proteins was determined by the filter paper disc method (17), and the incorporation was linear for at least 4 hours. The intracellular water space was estimated, utilizing 3-O(^{14}C) methyl-D-glucose and adjusting for the trapping of extracellular water by the $(\text{methoxyl-}^{3}H)$ inulin space measurement (18). The total amount of cellular protein and DNA per plate was also determined (19).

RESULTS AND DISCUSSION

Figure 1 demonstrates the effect of different dilutions of the extract generated from liver particulate fraction in the absence and presence of insulin (1×10^{-7}M) on lipid synthesis. Extract generated in the presence of insulin, at 1/1000 dilution, stimulates the incorporation of (^{14}C) acetate into total lipids by a value similar to that stimulated by 1×10^{-7}M insulin itself. Dilutions of the extract higher than 1/10,000 were totally ineffective in regard to lipid synthesis, and therefore the data are not represented in the figure. Figure 1 also shows the importance to establish appropriate controls in these experiments. Different dilutions of the extract generated by incubating the liver particulate fraction with insulin are compared with the same dilutions of the extract generated in the absence of insulin. This is particularly relevant since this latter extract, i.e., generated with buffer alone at dilutions of 1/100 and 1/10,000 respectively, inhibits and stimulates lipid synthesis when compared with that of untreated cells. Thus, it appears that material from liver particulate fraction generated in the absence of insulin has the capacity to modulate lipid synthesis. Insulin only enhances the formation of this material.

Stimulation of lipid synthesis by the mediator of insulin action could be due to contamination with insulin in the preparations of mediator tested in

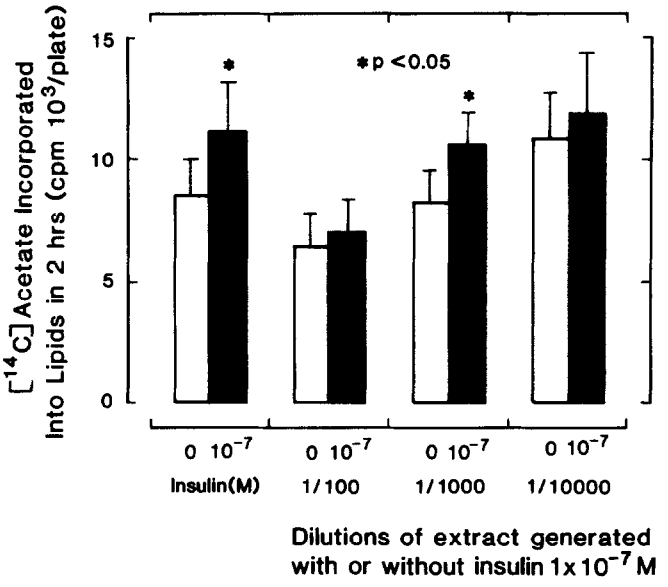

Figure 1. Effect of insulin and different dilutions of extract generated from liver particulate fraction with or without insulin (1 x 10^{-7}M) on lipid synthesis in primary cultures of hepatocytes. Primary cultures of isolated rat hepatocytes were incubated in serum-free medium for 16 hours. The incorporation of (1-^{14}C) acetic acid (sodium salt, 57 mCi/mmole) into lipids was assessed in Hanks-Hepes buffer containing 8 mM glucose, pH 7.4, after washing the plates free of incubation medium.

Plates were incubated with (1-^{14}C) acetate (0.5 µCi/ml, 5 mM) in the presence and absence of insulin (1 x 10^{-7}M) or different dilutions of extract generated in the presence or absence of insulin (1 x 10^{-7}M). The method of generation of extracts is detailed in the text. Two hours later, the medium was poured off, the plates were washed with cold phosphate buffer, and cellular lipids were extracted into a mixture of chloroform, methanol and water. The (^{14}C) radioactivity present in the total lipid fraction was counted in a Beckman liquid scintillation counter. The data represents the means (± standard error) of triplicate plates from eight separate experiments.

present studies. However, the contamination of the extract with insulin was excluded by demonstrating that there was no detectable insulin in the extract generated with or without insulin (1 x 10^{-7}M) as assessed by radioimmunoassay (20). In addition, the specific (^{125}I) insulin binding to normal cultured hepatocytes in the presence of 1/100 dilution of the extract generated with or without insulin (1 x 10^{-7}M) at 4°C (n=4) was not significantly different from each other. These experiments demonstrate that there is no insulin in the extract to compete with tracer concentrations of (^{125}I) insulin (1 x 10^{-10}M) for the insulin binding sites. Furthermore, it also suggests that the putative chemical mediator of insulin action does not occupy the specific insulin binding sites. Finally, an identical procedure to generate the insulin mediator was used as described before; but instead of native insulin alone, (^{125}I) insulin (1 x 10^{-10}M) with or without 1 x 10^{-7}M native insulin was utilized to generate

the mediator. Only 2.4% of the initial radioactivity added as (^{125}I) insulin to the particulate fraction was recovered in the final extract. The radioactive material present in the extract was chromatographed on a Sephadex G-50 column (11). Less than 1% of the radioactivity coeluted with insulin. From these experiments, it can be calculated that when a 1/1,000 dilution of the crude extract generated with insulin (1 x 10^{-7}M) is used, less than 1 x 10^{-13}M insulin, if any, may be present in the cultured hepatocytes. Clearly, this concentration of insulin is ineffective in stimulating any of its bilogical functions in cultured hepatocytes.

We have previously suggested that "down regulation" of the insulin receptors may be one of the many post binding effects of insulin action (21). Incubation with insulin for 16 hours is needed to significantly "down regulate" the insulin receptors. Liver cells were incubated with mediator and (^{14}C) acetate to determine if the mediator sustains biological effect over that time period. Figure 2

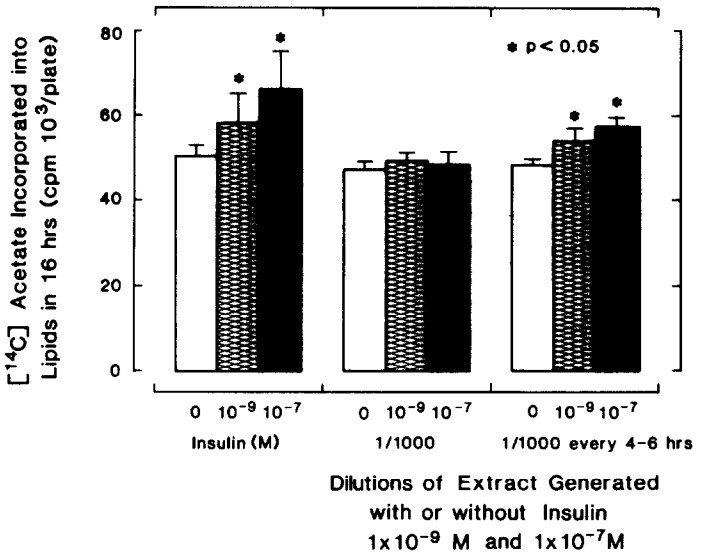

Figure 2. Effects of insulin and different dilutions of extract generated from liver particulate fraction with or without insulin on lipid synthesis in primary cultures of hepatocytes. At the time of the first medium change 4 hours after plating, (1-^{14}C) acetate (0.5 μCi/ml, 5 mM) was added to each plate in the presence or absence of insulin (1 x 10^{-9}M or 1 x 10^{-7}M) or different dilutions of extract generated in the presence or absence of insulin (1 x 10^{-9}M or 1 x 10^{-7}M) added either initially only, or also re-added every 4-6 hours. Sixteen hours after the addition of (1-^{14}C) acetate, the medium was poured off, the plates were washed with cold phosphate buffer, and cellular lipids were extracted into a mixture of chloroform, methanol and water. The (^{14}C) radioactivity present in the total lipid fraction was counted in a Beckman liquid scintillation counter. The data represent the means (± standard error) of triplicate plates from six different experiments.

demonstrates that if the insulin mediator is re-added every 4-6 hours over a 16 hour period a small, but significant, stimulation of lipid synthesis is observed.

Figure 3 shows that insulin mediator at 1/100 dilution, re-added to the culture medium every 4-6 hours over a period of 16 hours, significantly decreases specific (^{125}I) insulin binding when examined at tracer concentrations of (^{125}I)-insulin (1 x 10^{-10}M). Separate binding experiments (n=2) performed with (^{125}I)-insulin and increasing concentrations of native insulin (11) (data not shown) suggested that the observed decrease in insulin binding in the presence of the extract was due to approximately 30% reduction in the number of binding sites and not due to change in the affinity. The decrease in (^{125}I) insulin binding by the insulin mediator was not due to competition with insulin for the specific insulin binding sites as discussed earlier. Also, the (^{125}I) insulin binding

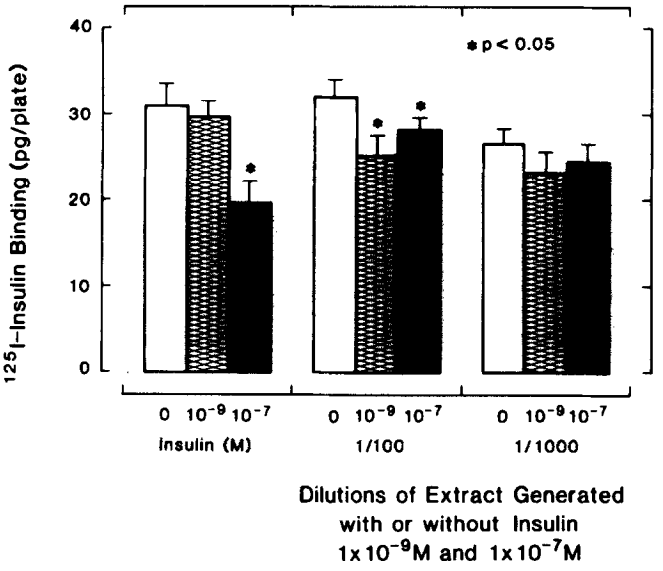

Figure 3. Effects of insulin and different dilutions of extract generated from liver particulate fraction with or without insulin on (^{125}I) insulin binding to primary cultures of hepatocytes. At the time of the first medium change 4 hours after plating, primary cultures of isolated rat hepatocytes were incubated in serum-free medium with or without insulin (1 x 10^{-9}M - 1 x 10^{-7}M) or different dilutions of extract (1/100 - 1/1000) generated with or without insulin (1 x 10^{-9}M - 1 x 10^{-7}M). Both insulin and extracts were re-added to the cultures every 4-6 hours. After 16 hours of incubation, the plates were washed with Hanks-Hepes buffer to remove prebound insulin or extract material. Then, (^{125}I) insulin (1 x 10^{-10}M, 1 Ci/μmole), prepared by the chloramine-T method, was added to the cultures incubated in Hanks-Hepes buffer with and without unlabeled insulin (1 x 10^{-6}M) for 6 hours at 4°C. The cells were then washed with cold phosphate saline buffer, pH 7.4, to remove free insulin and the specific (^{125}I) insulin binding was calculated. The data represent the means (± standard error) of triplicate plates from four different experiments.

experiments were performed at 4°C; therefore, the differences in (^{125}I) insulin binding observed cannot be explained by differences in insulin degradation or internalization since these two processes are nearly absent at this low temperature (11). Furthermore, the viability of cells was not affected by the different experimental conditions as assessed by light microscopy, intracellular water space, total protein and DNA concentration per plate, and by the incorporation of (^{14}C) leucine into proteins (Table I). Finally, the decrease in (^{125}I) insulin binding induced by the insulin mediator appeared to be specific since it did not

Table I.

Cell Treatment	Specific binding of ^{125}I-labeled Glucagon (pg /plate)	Specific binding of ^{125}I-labeled Growth Hormone (pg/plate)	(^{14}C) Leucine Incorporated into proteins (cpm10^2/plate)	Intracellular Water Space (µl/plate)	Protein (mg/plate)	DNA (µg/plate)
Extract 1/100 generated without Insulin	13.2 ± 0.9	22.5 ± 0.7	82 ± 6	1.5 ± 0.1	1.0 ± 0.1	20 ± 1
Extract 1/100 generated with Insulin 1 x 10^{-9}M	13.4 ± 0.9	27.8 ± 2.4	78 ± 5	1.6 ± 0.1	1.0 ± 0.1	18 ± 2
Extract 1/100 generated with Insulin 1 x 10^{-7}M	13.8 ± 0.8	25.6 ± 1.9	78 ± 12	1.6 ± 0.1	0.9 ± 0.1	17 ± 1

Table I. The effects of the 1/100 dilution of the extract generated with or without insulin (1 x 10^{-9}M or 1 x 10^{-7}M) on hormone binding and cell viability. Hepatocytes were incubated for 16 hours in serum free medium in the absence and presence of extract generated from liver particulate fraction in the presence or absence of insulin (1 x 10^{-9}M or 1 x 10^{-7}M) which was readded every 4-6 hours.

At the end of the incubation period, cells were washed with Hank-Hepes buffer and the following experiments were done in the same buffer. Specific (^{125}I) labeled glucagon (0.6 nmol/L) and human growth hormone (0.1 nmol/L) binding was determined at 4°C after 6 hours of incubation in the absence and presence of a large excess of same unlabeled hormone. ^{14}C-leucine incorporation into proteins by the filter paper disk method was determined at 37°C and was linear for at least 4 hours. The intracellular water space was estimated utilizing 3-O (^{14}C) methyl-D-glucose and adjusting for the trapping of extracellular water by the (methoxyl-^{3}H) inulin space measurement. The total amount of cellular protein and DNA per plate was also determined after the cells were washed with cold phosphate-saline buffer containing 2 mM EDTA. The numbers are means (± standard error) of triplicate samples from three different experiments.

affect either (^{125}I) glucagon or (^{125}I) growth hormone binding (Table I).

The present studies have demonstrated that a water soluble material which is heat stable, acid stable, and non absorbable by charcoal is generated by insulin from liver particulate fraction and mediates some of the intracellular effects of insulin in intact cultured hepatocytes. These include stimulation of (^{14}C)-acetate into lipids and down regulation of (^{125}I) insulin binding. It has been recently shown that a similar material stimulates the activity of acetyl CoA carboxylase in liver cytosol (7). In our cell system high concentrations of mediator preparations are required to observe a significant biological effect. In contrast, in cell-free systems highly concentrated mediator preparations are less effective than more dilute samples (1-9). However, we do not know how much of the mediator is internalized or degraded by the hepatocytes. Also, the mechanism of insulin mediator penetration into the intact hepatocytes to stimulate lipid synthesis is not known and is being investigated.

In view of the fact that this mediator mimics some of the insulin actions in intact cells from target organs, it may be postulated that cells resistant to insulin due to defect(s) in the generation of the insulin mediator (22,23) would respond to the mediator obtained from normal cells.

After the completion of this study, Zhang et al., (24) have reported that a similar mediator of insulin action mimics the cAMP-lowering action as well as lipogenic and antilipolytic effects of insulin in isolated adipocytes.

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