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PUTATIVE MEDIATORS OF INSULIN ACTION REGULATE HEPATIC ACETYL CoA CARBOXYLASE ACTIVITY

Alan R. Saltiel, Adam Doble, Steven Jacobs and Pedro Cuatrecasas

Department of Molecular Biology, The Wellcome Research Laboratories
3030 Cornwallis Road, Research Triangle Park, North Carolina 27709, U.S.A.

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SUMMARY. Incubation of rat liver plasma membranes with insulin enhances the production of small molecular weight substances which regulate the activity of liver acetyl CoA carboxylase. While low concentrations of insulin cause the release of a carboxylase stimulator from membranes, concentrations greater than 10^{-9} M generate less stimulating activity. This biphasic concentration curve for insulin can be resolved by differential alcohol extraction into two fractions which have antagonistic activity. The production of both substances is enhanced by insulin. Chemical and chromatographic evidence suggest that these substances are identical to the previously described "mediators" which regulate both pyruvate dehydrogenase and adenylate cyclase activities.

The mechanism by which insulin controls target cell metabolism is poorly understood. Recent evidence suggests that subsequent to the binding of insulin to its cell surface receptor, one or more small molecular weight chemical mediators are generated from plasma membranes which alter certain cellular enzymes, including cAMP-dependent protein kinase (1), glycogen synthetase (1), pyruvate dehydrogenase[1] (2-5), low K_m cAMP phosphodiesterase (6,7), adenylate cyclase (8) and Ca, Mg ATPase (9). Preliminary experiments suggest that these mediators may act by controlling the state of phosphorylation of these enzymes.

Acetyl CoA carboxylase is a prominent rate limiting enzyme in fatty acid biosynthesis. The enzyme is activated acutely by insulin in both liver and adipose tissue, and it is inactivated by glucagon and epinephrine (10-15). Moreover, enzyme activity is modulated by a phosphorylation-dephosphorylation process (13,16-18). Like other insulin-sensitive enzymes, the mechanism by which the binding of insulin to its receptor is coupled to regulation of acetyl CoA carboxylase remains unexplained. In this report, we demonstrate

1. The abbreviation used was pyruvate dehydrogenase (PDH).

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that two distinct small molecular weight substances, released from liver plasma membranes by insulin, can modulate the activity of acetyl CoA carboxylase measured in liver cytosol. These compounds appear to be identical to the previously described insulin mediators that regulate pyruvate dehydrogenase and adenylate cyclase (5,8).

MATERIALS AND METHODS

Materials. Male rats (100-125 g) were from Sprague-Dawley. All reagents were from Sigma, with the exception of CoEnzyme A (P-L Biochemicals), [1-^{14}C]pyruvic acid and [^{14}C]potassium bicarbonate (Amersham), and hydroxide of hyamine (Packard). Highly purified monocomponent bovine insulin was obtained from The Wellcome Research Laboratories, Beckenham, England. HPLC columns were from Water Associates.

Generation of Insulin Mediators. These were generated from a rat liver particulate fraction by a modification of the method described previously (8). Perfused rat livers were homogenized in 0.25 M sucrose, 10 mM potassium phosphate buffer, pH 7.4, and centrifuged at 1,100 x g for 10 min. The supernatant was centrifuged at 10,000 x g for 10 min. The resulting supernatant was diluted 1:1 with 10 mM phosphate buffer and centrifuged at 45,000 x g for 25 min. The pellet was resuspended in 10 mM potassium phosphate, pH 7.4, and incubated with insulin at 37°C. Mediators released into the supernatant were extracted as described, and the lyophilized powders were resolved into ethanol-soluble and insoluble fractions (8). These fractions were chromatographed on HPLC sizing columns (5).

Assay of Pyruvate Dehydrogenase (PDH) Activity. This was assayed in liver mitoplasts (8) by monitoring the conversion of [1-^{14}C]pyruvate to $^{14}CO_2$ (5). 0.5 mM pyruvate was used.

Assay of Acetyl CoA Carboxylase Activity. This was measured in whole liver cytosol. One rat liver was homogenized in 10 volumes of 20 mM Tris-HCl, pH 7.4, containing 0.2 mM phenylmethylsulfonyl fluoride, 1.0 mM dithiothreitol and 0.25 M sucrose. The homogenate was centrifuged at 9,000 x g for 10 min. Cytosol was prepared by centrifuging the supernatant at 45,000 rpm for 1 hr in a Beckman Ti-50 rotor.

Acetyl CoA carboxylase activity was measured by following the incorporation of KH[^{14}C]O_3 into malonyl CoA (19). Cytosol (5-10 mg protein/ml) was preincubated with 5 mM sodium citrate for 10 min at 30°C in the presence of 1 mM dithiothreitol, 1 mM EDTA and 10 mM $MgCl_2$ in 50 mM Tris, pH 7.4, in a total volume of 0.15 ml. To this mixture was added 0.05 ml of the agent to be tested. The reaction was started by the addition of 0.05 ml of assay medium containing 5 mM ATP, 0.5 mM acetyl CoA and 50 mM KH[^{14}C]O_3 in 50 mM Tris, pH 7.4. The reaction was stopped after 3 min by the addition of 0.05 ml 10% perchloric acid. Insoluble material was removed by centrifugation, and 0.1 ml aliquots were dried under a hair dryer. Radioactivity incorporated into malonyl CoA was measured as described elsewhere (19). The reaction was linear throughout 3 min and it was linearly dependent upon protein concentration. All results are the means of triplicate determinations.

RESULTS AND DISCUSSION

Among the numerous effects of insulin on cellular metabolism is the rapid activation of fatty acid biosynthesis. This action appears to depend on

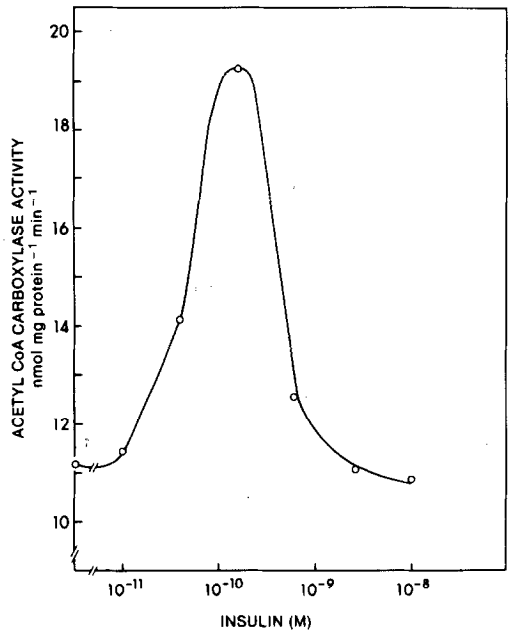

Fig. 1 - Regulation of Acetyl CoA Carboxylase Activity by Insulin. Liver particulate fraction (9 mg protein/ml) was incubated with the indicated concentrations of insulin for 5 min at 37°C in 0.2 ml of 10 mM potassium phosphate, pH 7.4. Following the incubation, solutions were acidified by the addition of 0.20 ml of 10 mM formic acid and membranes were centrifuged at 45,000 x g for 15 min. 50 µl aliquots of the supernatant were added to cytosol preactivated with citrate, and carboxylase activity was monitored as described in "Methods." The final potassium bicarbonate concentration was 20 mM. Carboxylase activity measured in the absence of added supernatant was 5.24 ± 0.11 nmole mg protein^{-1} min^{-1}.

stimulation of the activities of PDH and acetyl CoA carboxylase, key rate limiting enzymes. Although experiments have suggested that insulin activates both enzymes by controlling their state of phosphorylation (20-22), the mechanism by which hormone binding to cell surface receptors is linked to these intracellular actions remains unexplained. Recent studies demonstrating the existence of chemically undefined, small molecular weight substances which mediate the actions of insulin on PDH and other enzymes (1-9) suggested the experiments described here on regulation of acetyl CoA carboxylase activity.

It was of interest to determine whether exposure of liver plasma membranes to insulin results in the generation of acetyl CoA carboxylase regulators. Addition of insulin to a liver particulate fraction enhanced the release of a modulator of carboxylase (Fig. 1). Without incubation with insulin, acidified supernatants from liver membranes produced a twofold stimulation of cytosolic

carboxylase activity. The enzyme was further stimulated by supernatants from membranes incubated with low concentrations of insulin. The effect was maximal at 0.15 nM insulin. At higher insulin concentrations, the degree of stimulation declined. The spontaneous generation of a carboxylase stimulator, as well as the bimodal effect of insulin on generation of this activity, is similar to the effects observed for the PDH regulator (5).

Since the supernatants released from liver membranes are known to contain two antagonistic regulators of PDH (8), techniques used to resolve these antagonists were employed for separation of the putative carboxylase regulators (Table I). Liver membranes were incubated in the presence or absence of 10 nM insulin and the supernatants were extracted as detailed. Lyophilized powders were extracted with 100% ethanol, centrifuged and both the ethanol extract and the residue were dissolved in dilute formic acid. Their effects on acetyl CoA carboxylase were assayed. As was demonstrated for PDH modulators (8), ethanol-soluble material inhibited carboxylase activity. Although inhibitory activity was released spontaneously, exposure of membranes to insulin enhanced the

Table 1. Effect of insulin on the generation of acetyl CoA carboxylase regulators.

Preparation	Dilution	Acetyl CoA Carboxylase Activity* (% Control)		P
		- Insulin	+ Insulin	
Ethanol Extract	1:80	1.24 ± 0.01 (108)	1.11 ± 0.06 (96)	0.05
	1:20	1.23 ± 0.12 (107)	1.04 ± 0.09 (90)	NS
	1:5	0.62 ± 0.07 (54)	0.44 ± 0.03 (38)	0.05
Ethanol Residue	1:80	1.13 ± 0.08 (98)	1.06 ± 0.04 (92)	NS
	1:20	0.91 ± 0.01 (79)	1.33 ± 0.07 (116)	0.005
	1:5	1.97 ± 0.011 (171)	2.78 ± 0.06 (242)	0.0005

*Expressed as nmoles malonyl-CoA mg protein^{-1} min^{-1}. Control value = 1.15 ± 0.06.

Liver particulate fraction was incubated for 5 min at 37°C in the presence or absence of 10 nM insulin. Material released into the supernatant was extracted as described and lyophilized. Powders were then extracted with 100% ethanol and centrifuged to separate ethanol extract from residue. Both fractions were dissolved in 1 mM formic acid and added to the acetyl CoA carboxylase assay at the indicated concentrations. $KHCO_3$ was added at a 10 mM final concentration. Paired samples were compared using the Student "t" test.

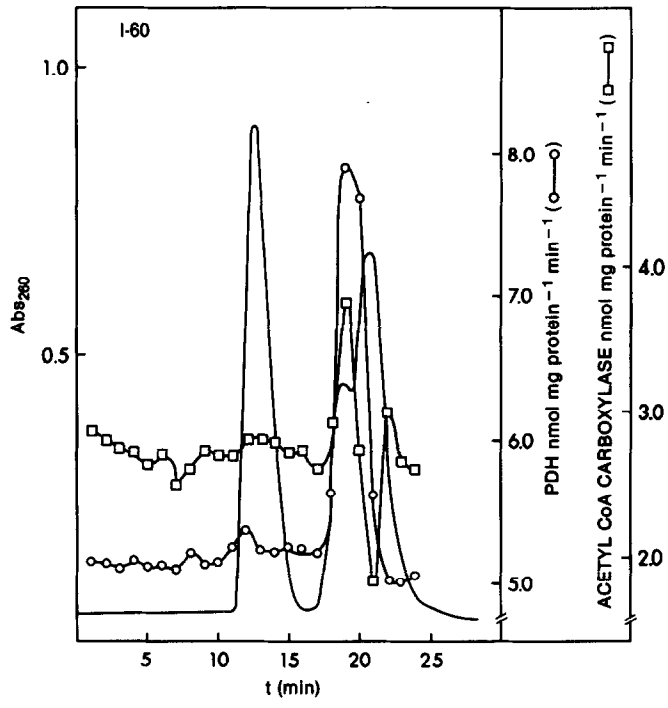

Fig. 2 - High Performance Liquid Chromatography of Ethanol Insoluble Mediator. Ethanol residue was dissolved in 0.2 M ammonium formate, pH 4.0, and chromatographed on two Waters I-60 columns in series, eluting with the same buffer at 1 ml/min. Fractions were lyophilized, dissolved in 1 ml of 1 mM formic acid and assayed for effects on both PDH and carboxylase.

production of this material. Similarly, the ethanol residue which contains the PDH stimulator also stimulated carboxylase activity. Although this substance was also produced spontaneously, it was nevertheless enhanced by insulin.

Interestingly, low concentrations of the ethanol residue could elicit an inhibitory effect on acetyl CoA carboxylase (data not shown). To further resolve these activities, the ethanol-insoluble material was chromatographed on an HPLC molecular sieve column (Fig. 2). Fractions were assayed for effects on both PDH and carboxylase. Stimulatory activity for both enzymes eluted at 19 to 20 min. Some carboxylase inhibitory activity eluted in the two fractions following the stimulator, although no effect on PDH was seen in these fractions. These data suggest that perhaps these two components are not completely resolved by ethanol extraction. Although this is not evident by assay of PDH (8), the carboxylase assay may be more sensitive to the inhibitory activity.

To further compare the regulators of acetyl CoA carboxylase with those of PDH and adenylate cyclase, some of their chemical properties were examined. As has been observed for both the stimulator of PDH and the inhibitor of PDH and adenylate cyclase, both carboxylase regulators were insensitive to boiling, acid stable, alkaline labile and not adsorbed to charcoal. Furthermore, these substances appear to copurify on HPLC columns with the PDH and cyclase modulators (data not shown).

The kinetic behavior of the carboxylase regulators was also similar to that observed on PDH and adenylate cyclase. The stimulator and inhibitor both apparently alter the V_{max} of the enzyme. Like the action of insulin on carboxylase in isolated hepatocytes, they had no significant effect on the EC_{50} for citrate, nor was the biphasic nature of the citrate concentration curve altered (14). Although the inhibitor was effective even in the absence of citrate, stimulation of the enzyme was most pronounced after preactivation with 2 to 5 mM citrate. Furthermore, the enzyme which was partially purified on a Sephadex G-50 column was equally responsive to both mediators (data not shown).

These results suggest that the actions of insulin on acetyl CoA carboxylase activity in liver may be mediated by the same chemical messengers which regulate other insulin-sensitive enzymes. Thus far, chemical and chromatographic studies have shown that the two antagonistic modulators of carboxylase released from liver plasma membranes are identical to those which regulate PDH and adenylate cyclase activities. Data presented here indicate that the production of these substances is regulated by insulin in a manner similar to that described for regulation of other insulin-sensitive enzymes. Since the mechanism by which hormones modulate the activity of acetyl CoA carboxylase remains in question, the generation of these mediators may explain how insulin binding is coupled to enzyme activation. Recent reports (21,22) have indicated that insulin activates acetyl CoA carboxylase via phosphorylation of a site distinct from that phosphorylated by glucagon or epinephrine. Future studies will

evaluate the influence of these mediators on the state of phosphorylation of the enzyme.

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