Vol. 100, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1981 Pages 872-878

INSULIN STIMULATES THE PHOSPHORYLATION OF ACETYL-CoA CARBOXYLASE

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Received April 17, 1981

SUMMARY: The activity of acetyl-CoA carboxylase is subject to regulation by both allosteric modulation and changes in covalent enzyme phosphorylation. The present study was carried out to investigate changes in the acetyl-CoA carboxylase phosphorylation state following insulin or glucagon exposure in rat hepatocytes. Glucagon leads to an increase in enzyme phosphorylation. Unexpectedly, insulin also causes a small but significant increase in enzyme phosphorylation; the effects of insulin plus glucagon on phosphorylation are roughly additive. Thus, acetyl-CoA carboxylase joins a growing list of intercellular proteins whose phosphorylation is increased by insulin and is the first example of an enzyme substrate whose activity is known to be acutely modulated by insulin.

Acetyl-CoA carboxylase (E.C.6.4.1.2) is a major rate-limiting enzyme of fatty acid synthesis. Previous studies in liver and adipose tissue have indicated that the enzyme is subject to both acute and long-term hormonal regulation. While the latter appears to be due to changes in enzyme content, acute intercellular regulation of enzymatic activity may be a consequence of both allosteric modulation and changes in covalent enzyme phosphorylation (1-5). Phosphorylation of the enzymes by the cAMP-dependent protein kinase is accompanied by a decrease in enzyme activity and an increase in the Ka for citrate (6). Dephosphorylation of the enzyme by various phosphoprotein phosphatase preparations in turn activates the enzyme (7,8).

We have been particularly interested in the short-term regulation of acetyl-CoA carboxylase activity by hormones in intact cells. Previous work from our laboratory and work by others have indicated that enzymatic activity is acutely modulated by insulin and glucagon in the liver (9,10) and insulin and epinephrine in adipose tissue (11,12); insulin exposure leads to enzyme inactivation, while glucagon and epinephrine treatment lead to inactivation. We have previously established that the effects of glucagon in the liver and

0006-291X/81/100872-07$01.00/0

epinephrine in adipose tissue are accompanied by increases in enzyme phosphorylation, presumably due to hormone-stimulated activation of the cAMP-dependent protein kinase (3). The observations have been confirmed in other laboratories (4,5). The effects of insulin on enzyme phosphorylation have not been reported. The present study was undertaken to document the effects of insulin on acetyl-CoA carboxylase phosphorylation.

MATERIALS AND METHODS

Male CD rats (130-150 g) were obtained from Charles River Breeding Laboratories. $Na^{32}P$-phosphate was obtained from New England Nuclear.

Hepatocytes were prepared by collagenase digestion of the isolated perfused rat liver by a previously published technique (13). All rats employed in the present experiment were fasted for 48 hours and refed for 48 hours with a low fat, high carbohydrate diet(ICN Pharmaceuticals; Fat-Free Test Diet). Hepatocyte incubation and ^{32}P-labeling were carried out as previously described (3).

The ^{32}P content of acetyl-CoA carboxylase in control and hormonally-stimulated cells was determined by two techniques. After hormone exposure, ^{32}P-labeled cells were pelleted at 1,000 x g x 30 sec, and the pellet was resuspended and homogenized in KP_i (100 mM, pH 7.5), EDTA (2 mM), NaF (50 mM), β-mercaptoethanol (10 mM) and sucrose (0.25 M). Homogenization was carried out at 4°C employing an Ultraturrax homogenizer at full speed for 60 sec. Following homogenization, a high-speed supernatant (105,000 x g x 60 min) was prepared. In the first analytic technique, supernatants from control and hormonally-stimulated cells were matched for total protein concentration and subjected to polyacrylamide gel electrophoresis, employing 7.2% slab gels at pH 9.81 as designed by Neville (14). Gels were stained and destained as previously described (15), dried with a slab gel dryer (BioRad) and the dried gels subjected to radioautography with XR-5 film (Eastman Kodak). ^{32}P content of acetyl-CoA carboxylase was determined by quantitative scanning densitometry of these autoradiographs (15).

The second analytic technique employed immunoprecipitation and cylindrical polyacrylamide gel electrophoresis. High-speed supernatants from control and hormonally-stimulated cell homogenates were subjected to immunoprecipitation with anti-acetyl-CoA carboxylase antiserum, as previously described (3). The immunoprecipitates were resuspended in a buffer containing Tris·HCl (50 mM, pH 7.4), DTT (5 mM), EDTA (1 mM), SDS (1% w/v), pyronin-Y (.012 mg/ml) and sucrose 4% (w/v) and subjected to polyacrylamide gel electrophoresis, using cylindrical gels (5.0%, pH 9.18) by the method of Neville (14). Following staining and destaining, the amount of Coomassie-stained acetyl-CoA carboxylase was determined by scanning densitometry of the stained gel; quantitation of the peak area on the scan was determined by weighing the area of 240,000 dalton peak on the tracing. Linearity of the peak response to varying loads of immunoprecipitate was established in preliminary experiments, and all analyzed samples were included in this linear range. An arbitrary unitage of Coomassie-stained protein was set at 1 unit = 1 mg paper. Following scanning, the gel was sliced into 2 mm segments, and the slices counted as Cerenkov radiation in 10 ml of H_2O with a Packard Tri-Carb Liquid Scintillation Counter. Final results were expressed as cpm ^{32}P per Coomassie-stained unit.

Protein determination was by the method of Lowry, et al. (27). Statistical analyses were performed by the Student's *t* test for paired data.

RESULTS

We have previously established that glucagon treatment of ^{32}P-labeled hepatocytes from fasted/refed rats lead to an increase in the phosphorylation of acetyl-CoA carboxylase coincident with enzyme inhibition (3).

Insulin, as the sole hormone, also leads to a small but significant increase in enzyme phosphorylation, as judged by scanning densitometry of radioautographs prepared from polyacrylamide gel electrophoresis of cytosolic fractions (Table I). When both insulin and glucagon are added simultaneously at their maximally effective concentrations (1 mU/ml and 10^{-8} M, respectively; 15)), the increase in phosphorylation is greater than that observed with either hormone alone. A similar pattern of the phosphorylation of a 216,000 dalton phosphopeptide in adipose tissue by insulin and epinephrine was previously noted in our laboratory (16). This phosphopeptide has since been identified as ^{32}P-acetyl-CoA carboxylase (3).

The pattern of insulin-stimulated phosphorylation of acetyl-CoA carboxylase was confirmed by immunoprecipitation analysis. As shown in Table II, in five experiments, insulin leads to a 39.0 ± 9.0% increase in ^{32}P content over that observed in control preparations. A variability in absolute ^{32}P content between experiments for both control and insulin-stimulated ^{32}P-enzyme is noted; this is likely due to a two-fold variability in the specific activity of the $^{32}P_i$ used in the incubation (500-100 mCi/mmol) coupled with a two-fold variability in measured ATP^{32} specific activity in these cell preparations (unpublished observations). Fig. 1 is an autoradiograph of immunoprecipitates obtained from a representative experiment in which labeled cells were exposed to either no hormone (C), insulin (C), glucagon (G) or glucagon plus insulin (GI). As noted, both insulin and glucagon, as sole hormones, lead to an increase in ^{32}P content of acetyl-CoA carboxylase and exposure of cells to both hormones

TABLE I

^{32}P Content of Acetyl-CoA Carboxylase as Determined by Scanning Densitometry of Radioautographs

Control	Insulin	Glucagon	Glucagon Plus Insulin
1.000	1.202^{*} ±0.070	1.267^{**} ±0.088	$1.462^{+,++}$ ±0.093

The results are expressed as ^{32}P content relative to control = 1.00, as judged by scanning densitometry of radioautographs of dried polyacrylamide slab gels prepared from cytosolic fractions. All hormone exposures were for 10 min: insulin 1 mU/ml (n = 13 hepatocyte preparations), glucagon 10^{-8} M (n = 13), and glucagon plus insulin at the preceding concentrations (n = 12). $^{*}p < 0.01$ control vs insulin; $^{**}p < 0.01$ control vs glucagon; $^{+}p < 0.001$ control vs glucagon plus insulin; $^{++}p < 0.02$ glucagon vs glucagon plus insulin.

simultaneously results in a greater ^{32}P incorporation than that seen with either hormone alone.

DISCUSSION

The results of the present studies indicate that the phosphorylation of acetyl-CoA carboxylase is increased by insulin. Insulin-stimulated protein phosphorylation appears to be a ubiquitous phenomenon in several tissues, including rat adipocytes (16-19), rat hepatocytes (15,20), 3T3-L1 cells (21) and cultured myoblasts (22). While all of the substrates of insulin-stimulated

TABLE II

^{32}P Content of Acetyl-CoA Carboxylase as Determined by Immunoprecipitation

Exp.	Control	Insulin	Insulin/Control
1	10.47	18.18	1.74
2	37.36	44.80	1.20
3	18.98	25.80	1.36
4	19.54	25.84	1.32
5	60.74	81.20	1.34
			Mean 1.39 ± 0.09*

^{32}P content of acetyl-CoA carboxylase was determined by immunoprecipitation analysis in five separate hepatocyte preparations, as described in Methods. The results are expressed as cpm ^{32}P/unit area of Coomassie-stained enzyme. Each data point represents the mean of three analyses on each immunoprecipitate. *indicates $p < 0.01$ by the Student's t test for paired data.

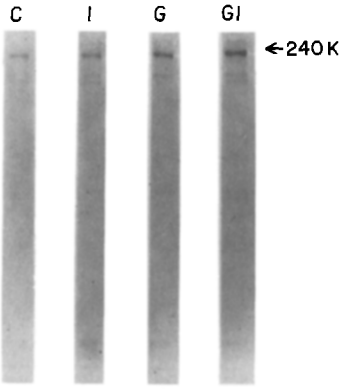

Figure 1. ^{32}P-Acetyl-CoA Carboxylase from Control and Hormonally-Stimulated Cells.

Shown is a radioautograph of polyacrylamide slab gel electrophoresis of immunoprecipitates obtained from 105,000 x g supernatant fractions from control (C), insulin-stimulated (I), glucagon-stimulated (G) and glucagon plus insulin-stimulated (GI) hepatocytes. The time of hormone exposure was 5 min. Cell incubations and immunoprecipitation were performed as described in Methods. The amount of Coomassie-stained 240,000 dalton peptide was identical in each lane, as judged by scanning densitometry of the stained gel (not shown).

phosphorylation have not been identified, ATP-citrate lyase (liver, adipose tissue) and the S6 ribosomal protein (3T3-L1 cells) represents two of these substrates. Acetyl-CoA carboxylase represents an important addition of this growing list, in that it is the first example of insulin stimulation of phosphorylation of an enzyme substrate, whose activity is known to be acutely modulated by insulin.

The activity of acetyl-CoA carboxylase in vitro is increased by dephosphorylation, employing different phosphoprotein phosphatases (7,8). Since insulin increases enzyme activity in adipose tissue (11,12) and liver (10), one might have predicted that any effects of insulin as the sole hormone on the phosphorylation state of the enzyme would be in the direction of dephosphorylation. We have found no evidence for this. It should be recognized that acetyl-CoA carboxylase, like many of the other interconvertible enzymes, possesses multiple, not single, sites of phosphorylation. The isolated rat liver enzyme, in our hands, contains 5-6 moles of alkali-labile phosphate/

mole of 240,000 dalton subunit (23). While the regulatory role(s) of multi-site phosphorylation has not been fully elucidated, there are examples of so-called "second site" phosphorylation in which occupancy of the "second site" regulates the rate of dephosphorylation of other sites (24-26). Whether such a model might explain the seeming paradox of insulin-stimulated phosphorylation of acetyl-CoA carboxylase awaits further study. It is already clear that a detailed analysis of the effects of hormone on enzyme phosphorylation will require analysis of site-specific phosphorylation changes.

Insulin stimulation of the phosphorylation of acetyl-CoA carboxylase appears to be mediated via a cAMP-independent pathway. Several lines of evidence support this view. First, we have previously established that, under the present incubation conditions, insulin, as the sole hormone, has no effect on cAMP accumulation or the activity of the cAMP-dependent protein kinase (15). Secondly, the ability of insulin to stimulate enzyme phosphorylation is evident at both basal and stimulated (glucagon) levels of cAMP accumulation and cAMP-dependent protein kinase activation. Furthermore, the effects of insulin and glucagon together, both at their maximally active concentrations, is greater than with either hormone alone and are roughly additive. There are several possible explanations for the ability of insulin to stimulate enzyme phosphorylation, including stimulation of a cAMP-independent protein kinase, inhibition of a phosphoprotein phosphatase or ligand-mediated alterations in the enzyme substrate that results in a change in its relativity with kinase or phosphatase. Each of these possibilities will have to be tested.

ACKNOWLEDGMENTS

The author wishes to express thanks to Dr. Joseph Avruch for helpful suggestions and to Ms. Martha Chambers for manuscript preparation.

This work was supported by NIH grant AM19270 and a grant from the Juvenile Diabetes Foundation. Dr. Witters is the recipient of a Research Career and Development Award AM00520.

This work was presented in part at the American Society of Biological Chemists Meeting in New Orleans, Louisana in June 1980 (Fed. Proc. 39, 1156, 1980).

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