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INSULIN STIMULATES PHOSPHORYLATION OF A HEAT-STABLE PROTEIN IN RAT ADIPOSE TISSUE

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Insulin in rat adipose tissue acts to increase the phophorylation about 2.5-fold of a low molecular weight protein in the cytosol designated phosphoprotein m. Isoproterenol had no effect on the phosphorylation of phosphoprotein m. Some of the properties of phosphoprotein m are : soluble in 1% trichloro acetic acid, heat-stable and has a molecular weight of 23,000 on polyacrylamide gels in the presence of sodium dodecyl sulfate. Phosphoserine and phosphothreonine are the phosphorylated amino acid residues of phosphoprotein m. The physical and chemical properties of phosphoprotein m are similar to those of previously described inhibitor and modulator proteins.

Insulin acts to increase the phosphorylation of ATP-citrate lyase in rat adipose tissue (1-3). Benjamin and Singer (2) suggested that an early step whereby insulin produces this effect is for it to increase the activity of a protein kinase with specficity for ATP-citrate lyase and other proteins. The finding that insulin acts to increase the phosphorylation of acetyl CoA carboxylase (4), S6 ribosomal protein (5,6) and other proteins (7,8), prompted Denton et al (9) also to suggest that insulin acts to increase the activity of a cyclic AMP-independent protein kinase. Paradoxically it is known that a major effect of insulin in vivo is to increase the dephosphorylation of enzymes of glucose and glycogen metabolism (10-12).

The notion that insulin acts to increase protein kinase activity is supported by the observation that insulin's binding to its receptor induces the phosphorylation of one of the receptor's subunit proteins and increases intrinsic receptor-bound protein kinase activity (13,14). These new observations, coupled with those that define ATP-citrate lyase phosphorylations on three amino acid

The abbreviations used are : SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloro acetic acid.

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residues on two separate peptide sequences (15-17), suggest a novel pathway for insulin action. In this scheme, a soluble phosphoprotein controls protein kinase and/or phosphatase activities by its state of phosphorylation. This phosphoprotein connects and amplifies the hormone-produced signal at the cell surface. The Mg.ATP-dependent protein phosphatase system is known to be regulated by cyclic AMP-dependent and independent protein kinase by modulator and inhibitor proteins (18,19). Therefore, our formulation was that the activity of such a protein phosphatase would be regulated by insulin-controlled phosphorylation of a soluble protein that regulates phosphoprotein phosphtase activity. Because inhibitor-1 is known to be regulated by cyclic AMP-dependent protein kinase (20), we designed experiments to determine whether the phophorylation of a protein with physical and chemical properties of a modulator protein (21) is regulated by insulin action.

MATERIALS AND METHODS

Materials : Insulin, crystalline porcine, 28.5 units/mg (lot 615-075-256 A) was a gift from Eli Lilly. Isoproterenol bitartarate, SDS and other biochemicals were of the highest grade available from Sigma. ^{32}Pi was purchased from New England Nuclear.

Incubation of Adipose Tissue: Young (125-150 g), fed male Sprague-Dawley rats were killed in the morning by cervical dislocation. Epididymal fat pads were rinsed in phosphate-free Krebs-Ringerbicarbonate buffer containing 2.5 mM calcium ion (2). The distal portion of each fat pad was cut into 2 pieces and placed in fresh bicarbonate buffer. During incubations, the buffer was gassed with 95% O_2-5% CO_2 and maintained at 37°C. Four fat pad pieces were transferred to 3 ml of phosphate-free modified Krebs-Ringer bicarbonate buffer containing 2% bovine serum albumin, 20 mM HEPES and 2.5 mM glucose (2,17). After 15 min of incubation ^{32}Pi(0.5 mCi/ml) was added and the adipose tissue incubations were continued for 120 min. Insulin (7 ng/ml) or isoproterenol (0.3 μM) was added and incubations were continued for an additional 10 min.

Preparation of cell-free extracts : At the conclusion of the incubations, fat pad pieces were rinsed in bicarbonate buffer containing phosphate and immediately frozen on an aluminum block at -80°C. Adipose tissue from 6 or 9 animals was pooled according to the experimental protocol and homogenized in a Potter-Elvehjem homogenizer in 4 ml per animal of cold 25 mM potassium phosphate (pH 7) containing 2 mM dithiothreitol, 10% glycerol, 1 mM $MgSO_4$, 1 mM EDTA, 50 mM NaF, 5 μM TLCK and 1 mg/L each leupeptin and pepstatin A (buffer A). The homogenates were centrifuged at 5,000 x g for 10 min and the aqueous extracts saved. The floating fat layer and pellet were rehomogenized in an additional 1 ml of buffer per animal, centrifuged and the aqueous phase pooled with that of the first extraction. Pooled extracts were centrifuged for 60 min at 125,000 x g. The supernatant fluid below the opalescent miniscus was carefully removed and filtered through glass wool to remove traces of floating lipid.

^{32}P-ATP-citrate lyase : Hormone-induced ATP-citrate lyase phosphorylation was measured by determining the ratio of the specific radioactivity of fat pad ATP-citrate lyase from hormone treated compared to control samples. Rabbit

serum containing antibodies to ATP-citrate lyase was added to an aliquot of the clarified homogenate. Immunoprecipitated ATP-citrate lyase was washed and dissolved in sample buffer (17). ATP-citrate lyase specific radioactivity was determined by electrophoresing the immunoprecipitates on 7% SDS-polyacrylamide gels and measuring the amount of protein and radioactivity by densitometry and liquid scintillation counting respectively (17).

Purification of protein m : To isolate proteins with the physical-chemical properties of modulator protein (20) ammonium sulfate was added to the clarified fat pad supernatant solution to 60% saturation. After stirring for 2 h at 4°C the precipitate was collected by centrifugation (20,000 x g for 30 min) dissolved in 2 ml of buffer A, discarding the undissolved precipitate. The solution was added drop wise to 1 ml of boiling buffer B (25 mM Tris-HCl pH 7 containing 15 mM 2-mercaptoethanol) and boiled for an additional 10 min. The cloudy solution was cooled on ice for 30 min and the precipitate was removed by centrifugation (10 min at 20,000 xg). The precipitate was extracted with 1 ml of buffer B, centrifuged and the clear supernatant sulutions combined and dialyzed overnight against buffer B. The dialyzed extract was adsorbed onto DEAE-Sephadex A-50 (0.5 x 10 cm) equilibrated in buffer B. Unadsorbed proteins were removed from the column with buffer B and additional proteins were eluted with 0.1 M NaCl in buffer B. Proteins still adsorbed to the column were eluted with either a step gradient or a linear gradient of 0.1 M to 0.30 M NaCl in buffer B. Proteins in each fraction were resolved by SDS-PAGE and the ^{32}P-proteins were analyzed by autoradiography. Fractions containing radiolabeled proteins of interest were pooled, dialyzed against buffer B and concentrated by dialysis against buffer B containing 10% polyethylene glycol (6000 MW). The concentrate was passed through a column (0.5 x 5 cm) of blue Sepharose equilibrated in buffer B and washed with an additional three column volumes of buffer B. Proteins still adsorbed to the column were eluted in a step wise fashion with 0.1, 0.2 and 0.6 M NaCl in buffer B. The eluates were concentrated by dialysis against 10% polyethylene glycol in buffer B. Aliquots were assayed for radiolabeled proteins by SDS-PAGE as described (2).

RESULTS AND DISCUSSION

In experiments to look for insulin-induced changes in protein phosphorylation amongst proteins of low molecular weight we confirmed the expected increase in the specific radioactivity of ATP-citrate lyase (Table 1). Note that in the low molecular weight region of the electrophoretogram of the fat pad supernatant fractions there was a slight increase in radioactivity associated with a radioactive band designated m in the insulin treated sample compared to control (Fig. 1). After ammonium sulfate fractionation there was a significant increase in radioactivity associated with band m. This increased protein phosphorylation was even more obvious in the boiled, soluble-protein fraction. In six experiments insulin action increased protein m phosphorylation 2.4-fold. Interestingly, though in these experiments both insulin and isoproterenol increased ATP-citrate lyase phosphorylation only insulin increased phosphoprotein m phosphorylation. The heat soluble fraction was further purified by DEAE-Sephadex and blue Sepharose chromatography. As shown in groups B and

TABLE I

Effect of insulin and isoproterenol on the phosphorylation of ATP-citrate lyase and phosphoprotein m

Treatment	ATP-citrate lyase		Phosphoprotein m	
	cpm/μg	%	content	%
1. Control (6)	1150±277[b]	100	4.8±1.3	100
2. Insulin (6)	2572±675[b]	230±69[b]	16.2±5.2[a]	336±83[a]
3. Isoproterenol (3)	2460±841[c]	196±12[b]	6.7±4.4	114±48

The ^{32}P-content of ATP-citrate lyase was determined by immunoprecipitation of the enzyme from cell-free extracts followed by SDS-PAGE and is represented as counts/min/μg protein. The relative ^{32}P-content in phosphoprotein m was measured in the boiled fractions as per cent of total radioactivity in the boiled fraction by SDS-PAGE and densitometric scanning of autoradiograms. The values for control, insulin and isoproterenol are means±S.D. from number of experiments given in parenthesis. The percentage values are presented relative to the control (100%). Statistical significance was calculated by using paired Students t test : a, $P<0.001$; b, $P<0.002$; c, $P<0.02$.

C (Fig. 2) only the fractions from insulin treated samples demonstrated an increase in radioactivity associated with peptide m. Using standard protein markers it was found that peptide m has an apparent molecular weight of 23,000 by SDS-PAGE. Phosphoprotein m was soluble in 1% TCA and was precipitated by

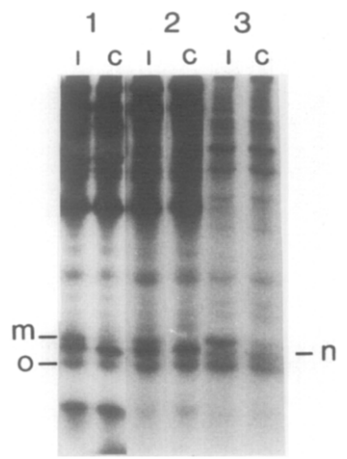

Fig. 1 : Effect of insulin on the phosphorylation of phosphoprotein m in rat adipose tissue. After incubation with ^{32}Pi and hormone, the adipose tissue was homogenized and the crude extract (1), ammonium sulfate fraction (2) and boiled supernatant (3) were prepared as described in the text. Autoradiography was for 7 days for the crude extract samples (lane 1 and lane 2) and for 1 day for other samples. I, designates insulin and C, control.

20% TCA. To determine the identity of the phosphorylated amino acids of protein m the fraction after blue Sepharose chromatography that contained highly radiolabeled pure band m (Fig. 2, lane C-3) was hydrolyzed in 6 N HCl. The radiolabeled amino acids were determined by high voltage electrophoresis (15). In two separate determinations from two different experiments radiolabeled phosphoserine and phosphotheronine were found in equal amounts whereas radiolabeled phosphotyrosine was not detected (Fig. 3). To determine whether protein m could be phosphorylated *in vitro* either by the catalytic subunit of cyclic AMP-dependent protein kinase (22) ATP-citrate lyase kinase (23) or a phosphotyrosine kinase partially purified from rat spleen (24) protein fractions containing partially purified protein m were incubated with these protein kinases and [γ-^{32}P]ATP as described (15). Protein m was not phosphorylated by any of these protein kinases under conditions in which ATP-citrate lyase is phosphorylated or the phosphotyrosine kinase is autophosphorylated (15,24). These observations suggest that yet another cyclic AMP-independent protein

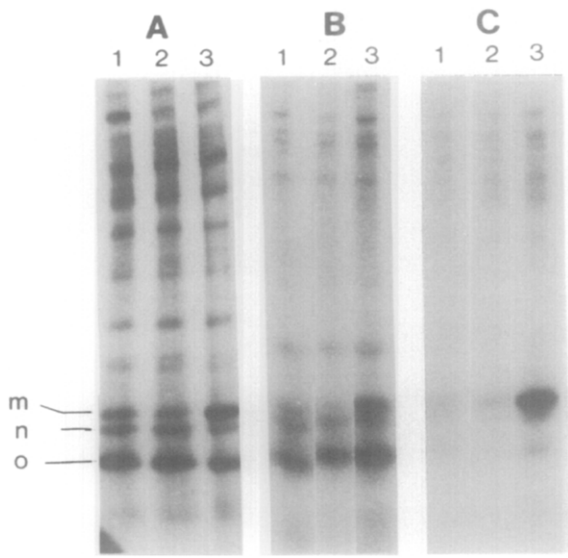

Fig. 2 : Effect of insulin and isoproterenol on the phosphorylation of fat cell phosphoprotein m. Lane 1, control; lane 2, isoproterenol; and lane 3, insulin. The samples are: boiled supernatants (A), first DEAE-Sephadex fractions (B) and second DEAE-Sephadex fractions (C). The molecular weight of phosphoprotein m was 23,000 compared to standard proteins run on the same gel.

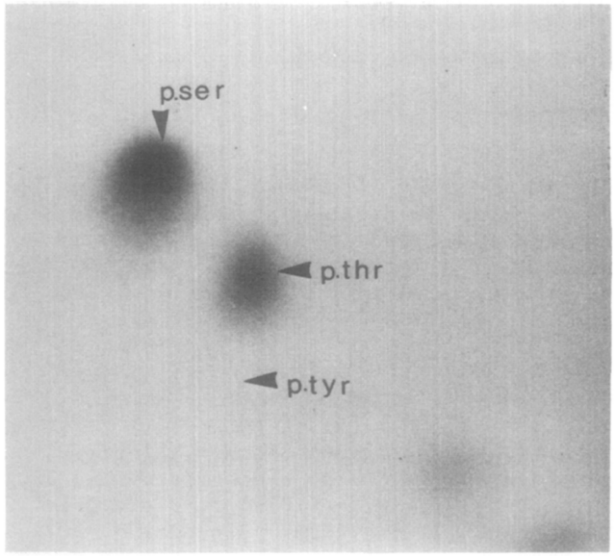

Fig. 3 : Phosphoamino acids from phosphoprotein m : Blue Sepharose fraction containing phosphoprotein m as the major radioactive band was hydrolyzed with 6 N HCl for 3 h. Elctrophoresis in the first dimension was from right to left at pH 1.9 and in the second dimension was from bottom to top at pH 3.5 (27). Even though in this Fig. phosphoserine content was more than phosphothreonine, in two additional experiments in which phosphoprotein m from blue Sepharose fraction was resolved by SDS-PAGE, eluted from the gel, hydrolyzed and phosphoamino acids separated by 2-dimensional thin layer electrophoresis demonstrated equal amounts of phosphoserine and phosphothreonine.

kinase is present in adipose tissue and that it could be under specific hormonal control.

In preliminary experiments, purified blue Sepharose fractions containing protein m enhanced Mg.ATP-dependent phosphatase activity 2-3-fold using radiolabeled phosphorylase as substrate (25). These unpublished observations suggest that fractions containing protein m have phosphatase modulator activity. However, when samples after blue Sepharose chromatography that contained only radiolabeled protein m were resolved by SDS-PAGE and stained by the silver staining method (26) it was found that these fractions contained a significant amount of low molecular weight peptides of less than 20,000 daltons. This result indicates that further purification is necessary before we can identify and determine whether phosphorylated protein m has phosphatase modulator activity. After the completion of these studies we noted that Belsham and Denton (7) described a TCA-soluble fraction that contained a protein the phosphorylation of

which was increased by insulin action. Interestingly, however, they found that epinephrine action increased its phosphorylation. The relationship of their findings to ours is unknown.

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