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Hepatic zonation of insulin-stimulated tyrosine phosphorylation

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Zonal distribution of insulin stimulation of hepatic protein tyrosine phosphorylation, detected by immunoblotting with an anti-phosphotyrosine antibody, has been studied in the in situ perfused rat liver by dual-digitonin-pulse perfusion. Insulin promotes the rapid and sustained tyrosine phosphorylation of two proteins (pp150 and pp69) that are present only in the perivenous hepatocytes, while three others (pp46, pp48 and pp96) are stimulated identically in the periportal and perivenous cells. The ability of insulin to rapidly activate acetyl-CoA carboxylase is indistinguishable between the hepatic zones. Hepatic zonation of insulin-stimulated tyrosine phosphorylation could underly differential hepatic insulin responses and might provide clues to the identification of tyrosine phosphorylated proteins linked to insulin regulation of intracellular events.

Tyrosine phosphorylation; Insulin receptor; Hepatic zonation; Acetyl-CoA carboxylase

1. INTRODUCTION

Several lines of experimental evidence have firmly established that the tyrosine kinase activity of the β-subunit of the insulin receptor is important for the insulin signalling mechanism. The strongest evidence for its importance has been derived from cell transfection studies with cDNA receptor constructs of normal or mutated β-subunit structure. These experiments indicate that the tyrosine kinase activity is essential for the insulin stimulation of glucose transport, glycogen synthesis, ribosomal S6 kinase activation and mitogenesis [1-2]. However, the nature and identity of the important intracellular substrates for this tyrosine kinase activity, other than the receptor itself which activates on autophosphorylation [3-5], remain unknown. Several candidate substrates, identified by ^{32}P labeling of intact cells or immunoblotting of cell/tissue extracts with anti-phosphotyrosine antibodies, have emerged from studies of the effects of insulin in several cultured cell lines, freshly isolated rat cells, cell lines transfected with high copy numbers of functional insulin receptors and intact liver [6-13].

In order to better characterize insulin's effects on intact tissues, we have investigated, by immunoblotting, hepatic zonal distribution of insulin-stimulated tyrosine phosphorylation, employing perfusion of the rat liver with sampling of the perivenous and periportal zones by dual-digitonin pulse perfusion.

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2. MATERIALS AND METHODS

2.1. *Liver perfusion and sample collection*

Overnight fasted male Wistar rats (150-180 g) were anesthetized with an intraperitoneal injection of pentobarbital (7 mg). Prior to liver perfusion [14], heparin (500 IU) and an α-adrenergic antagonist (Tolazolin; 0.25 mg) was given intravenously in the inferior vena cava. Livers were perfused in situ with Krebs-Henseleit buffer for 5-15 min, followed by an interval where insulin (10^{-7} M) was included in the perfusate. Four groups of animals were employed: control perfusion, 2 min, 5 min and 10 min of insulin perfusion; three or more individual experiments in separate animals were performed in each of these groups. Total perfusion time including the insulin interval was approximately 15 min in all experiments. All perfusates were equilibrated with O_2/CO_2 (95%/5%) at 37°C, giving an input oxygen tension to the liver of 500-600 Torr. The output oxygen tension from the liver was 100-150 Torr and the oxygen consumption, measured as described previously [13], was 2.12 ± 0.17 μmol per g of liver wet weight (n = 12 perfusions). Oxygenation of the medium was discontinued before the addition of insulin.

Immediately following the insulin perfusion interval, the dual-digitonin-pulse perfusion [15] was initiated: 10 s of Krebs-Henseleit buffer without calcium, but with digitonin (5 mg/ml) at a flow rate of 10 ml/min, applying the antegrade (portal vein to hepatic vein) perfusion direction. This was followed by a reversal of flow and washout for 40 s at 20 ml/min. The 5-30 s eluate fractions (periportal) were collected into 5 ml of an ice-cold collection buffer (see below), which was rapidly mixed and transferred to an ice bath. The same sequence was repeated with inverted flow direction (hepatic vein to portal vein), providing eluate from the perivenous zone [15].

The total eluate volume from both zones (~8 ml) was collected directly into 5 ml of an ice-cold buffer containing sodium phosphate (0.25 M; pH 7.4), EDTA (10 mM), EGTA (10 mM), sodium fluoride (250 mM), sodium orthovanadate (10 mM), sodium pyrophosphate (50 mM), dithiothreitol (5 mM), sucrose (1.25 M), PMSF (2.5 mM), benzamidine (5 mM) and aprotinin (400 kallikrein inactivating units/ml). The collected samples of periportal and perivenous eluates were divided into several aliquots for processing: 1 ml was frozen at −80° for later estimate of protein concentration and activity of marker enzymes [15], an SDS gel sample was immediately prepared, as in [16] and frozen at −80°, and the remainder of the fractions were

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frozen in aliquots and stored at −80° until shipment to Dartmouth on dry ice together with the gel samples. Marker enzymes for zonation, alanine aminotransferase and pyruvate kinase, were assayed in Copenhagen [15].

2.2. *Sample processing and analysis*

After shipment to Dartmouth, aliquots of the original eluates were thawed on ice and a second set of SDS gel samples prepared, after matching for protein content. The results with these samples did not differ from those prepared immediately after collection, indicating no alterations had occurred on storage or shipment (not shown). Additional aliquots of the original eluates were centrifuged at 183 000 × *g* for 60 min. The high speed supernatants (cytosol) were matched for protein and SDS gel samples prepared. The high speed pellets were resolubilized in the original collection buffer containing 2% SDS and triturated by repeated passage through a 25-G needle. About 12% of total eluate protein was recovered in this pellet fraction. After matching of these samples for protein, SDS gel samples were also prepared. Lastly, for analysis of acetyl-CoA carboxylase, aliquots (periportal and perivenous separately) from 3 control perfusions and 3 insulin perfusions (10 min) were pooled and subjected to high speed centrifugation. This supernatant was fractionated by 40% NH_4SO_4 precipitation; these pellets were then resuspended and dialyzed for analysis of acetyl-CoA carboxylase activity and content, as in [17,19].

All gel samples were analyzed by immunoblotting after separation of proteins on both 7.2% and 12% SDS polyacrylamide gels. Immunoblotting with a polyclonal rabbit anti-phosphotyrosine antibody was performed as previously [18]; bound antibody was detected with ^{125}I-goal anti-rabbit Ig (0.1 μCi/ml). Acetyl-CoA carboxylase activity was assayed at variable citrate concentrations (0–10 mM) as in [19]; enzyme mass in assayed fractions was determined by an avidin-based ELISA assay with ACC isozyme-specific antibodies, as in [20]. Enzyme kinetics were analyzed by Eadie-Hofstee plots and citrate K_a determined from these plots by linear regression analysis (Cricket Graph 1.2) on a MacIntosh IIcx computer.

2.3. *Miscellaneous methods*

Protein concentrations in eluates and cytosolic fractions were assayed by the method of Bradford [21]. In high speed pellet SDS extracts, protein was determined by the bichinchonic acid method (Pierce); in these assays the standard curve (bovine serum albumin) contained an amount of SDS equal to that added with the samples.

3. RESULTS AND DISCUSSION

On perfusion of the in situ liver of overnight fasted rats, insulin (100 nM) rapidly stimulates the tyrosine phosphorylation of several hepatic proteins (Figs 1 and 2). These responses are maximal after two minutes of insulin stimulation and persist to the same extent for at least ten minutes (Fig. 1). Selected, representative analyses are shown in this figure for each of the time points; each sample represents a pool of material from a minimum of 3 control perfusions and 3 insulin perfusions with sample collection after 2, 5 and 10 min of stimulation. All detectable insulin-stimulated proteins are evident by 2 min of insulin stimulation; no additional stimulated proteins are revealed in the 5 and 10 min samples. Immunostaining of many non-insulin stimulated proteins remains constant as compared to

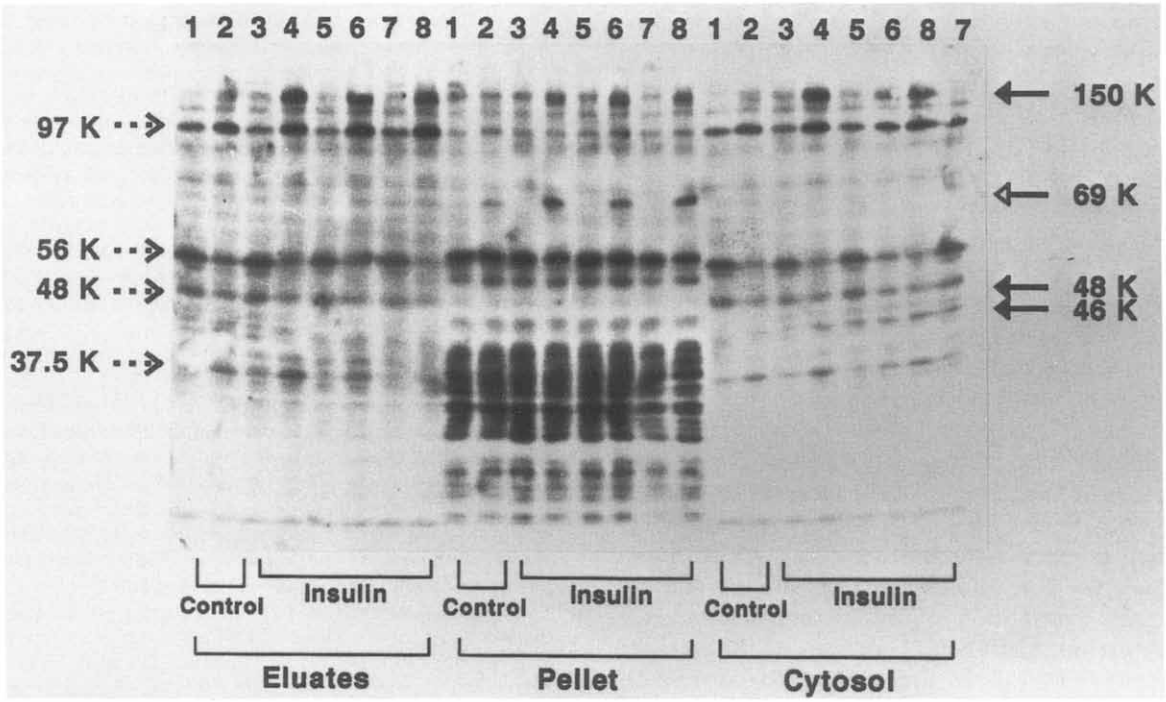

Fig. 1. Immunoblot analysis of periportal and perivenous eluates, cytosol and pellets from control and insulin-stimulated livers. Shown is a representative immunoblot of pooled samples obtained from three control perfusions (lanes 1, 2) and three (for each time point) separate insulin perfusions (2 min: lanes 3 and 4; 5 min; 5 and 6; 10 min; 7 and 8). Initial eluate, pellet and cytosolic proteins from perivenous (odd numbered lanes) and periportal (even numbered lanes) fractions were separated on a 12% acrylamide SDS gel (50 μg protein per lane) prior to blotting. Note that lanes 7 and 8 are reversed in the cytosol panel. Based on migration relative to pre-stained molecular weight standards, the apparent molecular weights of cytosolic insulin-stimulated proteins (closed arrows), membrane/pellet insulin-stimulated proteins (open arrows) and non-stimulated, but zonated, immunostained proteins (arrows with dashed lines) are shown.

the control perfusion shown over the 10 min experimental interval, attesting both to the absence of a changing baseline and to the selectivity of insulin's effects.

The stimulation of tyrosine phosphorylation by insulin, though discernable in the initial eluates, is best visualized in the analysis of cytosolic and pellet fractions (Figs 1 and 2). The dual-digitonin pulse perfusion technique, which allows rapid sampling of zonal hepatic proteins, is principally a technique for the isolation of the soluble proteins liberated on cellular exposure to digitonin [14,15]. However, insoluble components, here termed pellet, are also routinely recovered in these eluates. The exact origin of this material is not certain in that marker enzymes for plasma membrane are not present and electron microscopy does not show significant membrane fragments [Quistorff, Rømert and Tranum-Jensen, unpublished observation]. The term 'pellet' used here is therefore an operational definition, pending further clarification of the nature of this fraction; in part, it may represent proteins rendered insoluble in the digitonin and/or collection buffer.

The most striking aspect of these data is the nearly complete zonation of insulin-stimulated tyrosine phosphorylation of two substrates to the perivenous zone of the liver acinus. One insulin-stimulated protein of molecular mass 150 000 Da (pp150), evident even in the unfractionated eluates, is nearly exclusively perivenous in distribution (Fig. 1). While present predominantly in the cytosolic fraction, a stimulated protein(s) of identical, though somewhat broader, migration is also noted in the perivenous pellet fractions. A second exclusively perivenous insulin-stimulated protein of molecular mass 69 000 Da (pp69) is evident only in the pellet fractions (Fig. 2). In contrast, three other insulin-stimulated proteins have been identified that do not appear to be zonated to either the periportal or perivenous cells. In the cytosolic fractions from both hepatic zones, insulin stimulates the tyrosine phosphorylation of two proteins of molecular mass 48 000 (pp48) and 46 000 (pp46) Da (Fig. 1), while in the pellet fractions a non-zonated stimulation of a protein of molecular mass 96 000 Da (pp96) is evident (Fig. 2). The latter has a molecular weight very similar to that of the insulin receptor β subunit; however, we have been unable to precipitate this protein from Triton X-100 extracts of these pellets with a polyclonal antibody to the insulin receptor, even though traces of insulin binding activity are present in these pellet fractions (not shown).

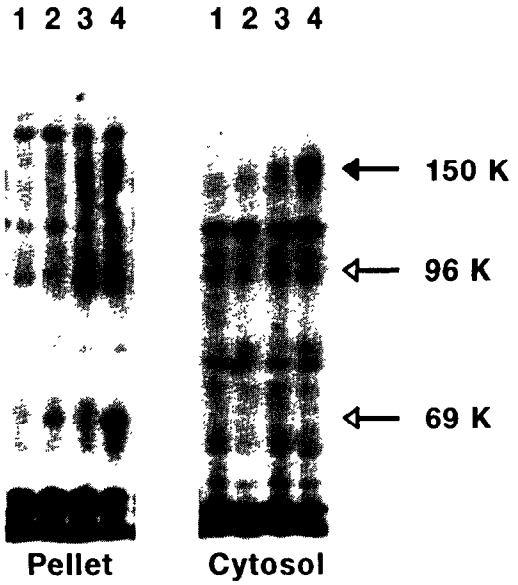

Fig. 2. Immunoblot analysis on 5% SDS gels of periportal and perivenous cytosol and pellet protein from control and insulin-stimulated livers. Representative cytosolic and pellet proteins from the 5 min time point shown in Fig. 1 were additionally blotted after electrophoresis on a 5% polyacrylamide gel to better resolve higher molecular weight insulin-stimulated proteins. Shown are periportal samples (lanes 1 and 3) and perivenous samples (lanes 2 and 4) from control (lanes 1 and 2) and insulin perfusions (lanes 3 and 4). The apparent molecular weights of cytosolic insulin-stimulated proteins (closed arrows) and pellet insulin-stimulated proteins (open arrows) are indicated to the right.

Several other cytosolic immunostaining proteins that are not stimulated by insulin appear to be zonated to either the periportal or perivenous zones as seen in eluates from control livers (perivenous: molecular masses 97 000 and 37 500 Da; periportal 56 000 and 48 000 Da) (Fig. 1). However, caution is advised in assigning phosphotyrosine content to these proteins, since the antibody employed in this study can react with proteins that do not contain phosphotyrosine. An increase in staining on immunoblotting does, in our view, constitute strong evidence for an increase in phosphotyrosine content (as above).

In order to correlate the observed insulin-stimulated tyrosine phosphorylations with another effect of insulin in this system, we have measured the activity of acetyl-CoA carboxylase (ACC), an insulin-sensitive enzyme [19], in periportal and perivenous eluates of control and insulin-perfused livers. As analyzed by citrate kinetics, ACC activation by insulin is quantitatively the same in eluates from both zones (Fig. 3), although, as previously reported [16], there is a basal specific activity gradient to the periportal zone of ACC specific activity. In addition to activation of ACC activity at V_{max}, insulin also diminishes the K_a for citrate in both zones (periportal: control, 2.9 mM, insulin 1.9 mM; perivenous: control, 3.9 mM; insulin 2.9 mM). These responses to insulin are identical to those that have previously been observed in hepatoma cells [19]; the higher K_a values seen in the present experiments likely reflect the fasting state of the animal prior to perfusion [22,23]. Since these changes in citrate kinetics accompany ACC dephosphorylation [19,22,23], it is quite likely that this insulin-stimulated response reflects the same alterations in the serine phosphorylation state of ACC in response

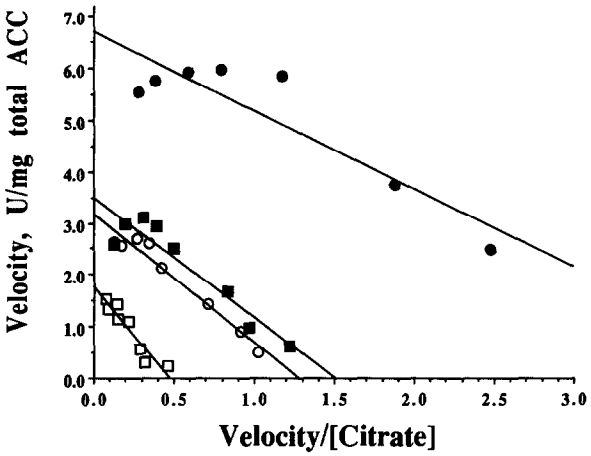

Fig. 3. Kinetic analysis of control and insulin-stimulated acetyl-CoA carboxylase (ACC) in periportal and perivenous eluates. Cytosolic fractions from three control perfusions and three insulin perfusions (10 min) were pooled and processed as in section 2. ACC activity was then assayed in the dialysed NH_4SO_4 pellets at variable citrate concentrations (0–10 mM) in replicate. The data are presented as Eadie-Hofstee plots of enzyme velocity (corrected for citrate-independent activity) per mg total ACC protein (determined by ELISA) vs velocity/[citrate] for control periportal (■), control perivenous (□), insulin periportal (•) and insulin perivenous (○) isolates.

to insulin, as has been directly shown in the hepatoma cell lines [19].

The mechanisms underlying the zonation of two of the stimulated phosphorylations (pp150 and pp69) to the perivenous zone are of some interest. These response could represent zonation of insulin receptor mass or stimulatable insulin receptor tyrosine kinase to the perivenous plasma membrane or apparent zonation due to an increased mass of these kinase substrates within the perivenous cells. The pp150 protein has a mass similar to that of a soluble insulin-stimulated protein(s) of variable molecular mass (160–185 000 Da) reported in several cell systems and after insulin injection into the portal vein [6,11,13]. The pellet-associated pp69 is similar in molecular mass to that of a hepatocyte protein whose tyrosine phosphorylation is increased by insulin and which binds to GDP-agarose [24]. However, we have been unable to fractionate this protein by this technique in Triton X-100 extracts of these pellets. Proteins of masses similar to that of the three non-zonated insulin-stimulated proteins (pp48, pp46 and pp96) have also been observed in some cell systems and intact liver [7,11,13]. Since acetyl-CoA carboxylase is equally stimulated by insulin in both acinar zones, the most unitary explanation is that its activation is linked to that of one or more of these non-zonated stimulated proteins, rather than either of the perivenous-zonated stimulated proteins (pp150 and pp69).

It should be stressed that the zonal selective sampling of the dual-digitonin-perfusion technique, as applied, does not allow analysis of more centrilobular zones of the liver [25]. Therefore, because of selective zonal sampling and because of potential limitations of immunoblotting analysis (e.g. reactivity of the antibody with all phosphotyrosine-containing proteins, potential sensitivity to low protein mass), these data should not be interpreted as revealing of all potential insulin-stimulated tyrosine kinase substrates in the intact liver. Nonetheless, with respect to coupling of putative receptor kinase substrates to other cellular responses, future studies of other rapid actions of insulin in this perfusion system could reveal responses that are localized to the perivenous or periportal zones or occur without hepatic zonation. Such studies, with parallel analysis of patterns of insulin-stimulated tyrosine protein phosphorylation, could provide important information concerning the protein substrates that link the activity of the insulin receptor tyrosine kinase to insulin-stimulated cellular regulation.

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