Glucagon stimulates phosphorylation of different peptides in isolated periportal and perivenous hepatocytes

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Abstract The perivenous and periportal zones of the liver acinus differ in enzyme complements and capacities for gluconeogenesis, glycolysis and other metabolic processes. The biochemical factors governing this metabolic zonation are still poorly understood. Glucagon-mediated protein phosphorylation is an important factor in the regulation of hepatic metabolism. Here we show, by comparing the ^{32}P-labelling pattern of isolated periportal and perivenous hepatocytes, that glucagon promotes the phosphorylation of zone-specific peptides as well as three common peptides (glycogen phosphorylase, glycogen synthase and pyruvate kinase) in the two cell types. We propose that the zone-specific phosphorylation of peptides is an important factor governing the short-term zonation of metabolic processes in the liver.

Key words: Glucagon; Protein phosphorylation; Liver; Hepatic zonation; Glucose metabolism

1. Introduction

Hepatocytes from the perivenous and periportal zones of the microcirculatory unit of the liver, the acinus, differ in their enzyme complements and subcellular structures [1]. The periportal hepatocytes, which are exposed to more oxygenated blood rich in absorbed nutrients, possess a greater mitochondrial volume (20% by volume) and have higher activities of mitochondrial enzymes than perivenous hepatocytes. In functional terms, the periportal hepatocytes have higher activities of gluconeogenic enzymes (pyruvate carboxylase, phosphoenolpyruvate carboxykinase, fructose 1,6-bisphosphatase and glucose 6-phosphatase) and oxidative and tricarboxylic acid (TCA) cycle enzymes, whereas perivenous hepatocytes have higher activities of glycolytic enzymes (glucokinase, fructose 6-phosphate 1-kinase and pyruvate kinase), lipogenic enzymes (ATP-citrate lyase, acetyl CoA carboxylase and fatty acid synthase) and ketogenic enzymes. All hepatocytes within the acinus possess the machineries for glycogen synthesis and degradation (glycogen phosphorylase and glycogen synthase) and also the capacity for the synthesis and degradation of the regulatory metabolite fructose 2,6-bisphosphate via the bifunctional enzyme 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (hereafter referred as fructose 6-phosphate 2-kinase) [2]. Periportal hepatocytes have a greater capacity for glycogen synthesis from gluconeogenic precursors than perivenous hepatocytes [3], raising the possibility that zonation is a major factor determining the relative contributions of the direct and indirect pathways to hepatic glycogen synthesis.

Many biochemical processes in the liver are regulated by reversible protein phosphorylation. Glucagon promotes hepatic glycogenolysis and gluconeogenesis via phosphorylation of glycogen phosphorylase, glycogen synthase, fructose 6-phosphate 2-kinase and pyruvate kinase. Cytosolic peptides in the liver that are phosphorylated in response to acute challenge with glucagon have been identified by one- and two-dimensional electrophoresis [4–10]. A number of these proteins, including pyruvate kinase and fructose 1,6-bisphosphatase, are expressed at different levels in periportal and perivenous hepatocytes. Although periportal and perivenous hepatocytes both possess receptors and the signalling pathway for glucagon [3,11,12], they may differ in their sensitivity to glucagon either as a result of a periportal-perivenous glucagon gradient across the acinus or from other factors. In this context, glycogen degradation in periportal hepatocytes is more sensitive to stimulation by glucagon compared to perivenous hepatocytes [13]. Histochemical studies [14,15] suggest that this greater sensitivity may be the consequence of higher glycogen phosphorylase activities in the periportal zone, although studies on freshly isolated periportal and perivenous hepatocytes show no differences in glycogen phosphorylase activities [11]. The question as to what extent differences in glucagon sensitivity might be exerted at the level of protein phosphorylation has not been addressed before. In this paper we have compared isolated periportal and perivenous heptocytes with respect to their sensitivity to glucagon-stimulated phosphorylation of cytosolic proteins identified by one-dimensional SDS-PAGE.

2. Materials and methods

2.1. Chemicals

Reagents were obtained from the sources specified in [10]. The Micro BCA protein assay reagent kit was purchased from Pierce (Rockford, Illinios, USA), whereas digitonin and RQ1 RNase-free DNase (deoxyribonuclease) were obtained from the Sigma Chemical Company (St. Louis, Mo, USA). Alanine aminotransferase activity was measured using the ALT (ALAT/GPT) kit from Boehringer Mannheim GmbH (Mannheim, Germany).

2.2. Glucagon challenge of ^{32}P-labelled periportal and perivenous hepatocytes and the isolation of the cytosolic protein fraction

Periportal and perivenous hepatocytes were isolated from fed male Wistar rats (150–200 g) by site-specific digitonin cell lysis followed by collagenase perfusion as described by Lindros and Penttilä [16]. The crude cell suspension was briefly treated with DNase in bicarbonate/Hepes buffer at pH 7.4 followed by centrifugation twice at 400 × *g* for 20 s and filtration of the cell suspension using nylon mesh (100 μm). Cell viability, assessed by exclusion of Trypan blue, ranged between 85–95%. Isolated periportal hepatocytes were confirmed to have alanine aminotransferase activities at least twice those of perivenous cells.

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The final pellet ($2–4 \times 10^6$ cells/ml) was suspended in a low-phosphate (0.1 mM) Krebs–Ringer bicarbonate buffer containing 16 mM L-lactate and 4 mM pyruvate as described by Garrison and Wagner [6].

The hepatocyte preparations were first labelled with $^{32}PO_4^{3-}$ (0.5 mCi) in a shaking waterbath (37°C) for 45 min to incorporate ^{32}P-label into the intracellular ATP pool [10]. The cells were challenged with different concentrations of glucagon (10^{-12} to 10^{-7} M) for 5 min. Thereafter, the cells suspension was diluted 2-fold in digitonin fractionation buffer (4 mg/ml digitonin in 10 mM TES at pH 7.4 containing 50 mM NaF, 10 mM EDTA, 5 mM EGTA and 200 mM sucrose) and centrifuged at $13,000 \times g$ for 6–8 s to terminate the reaction and release cytosolic proteins [10]. The cytosolic fractions were prepared by boiling the cytosolic proteins immediately for 5 min in SDS buffer (0.0625 M Tris-HCl, pH 6.8 containing 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS and 0.001% (w/v) Bromophenol blue).

2.3. One-dimensional gel electrophoresis and densitometric analysis

The cytosolic protein fraction was resolved by one-dimensional SDS-PAGE by a modification of the method of Laemmli [17] using a 5 to 15% (w/v) linear gradient acrylamide resolving gel and a 4% (w/v) stacking gel. The use of linear gradients of acrylamide was found to increase resolution of low and high molecular mass peptides. Samples of the cytosol fraction (20 μg protein) were loaded onto polyacrylamide gels and electrophoresed [10]. Gels were then stained, destained and vacuum dried in a gel drier and the dried gels exposed on a Fujix Phosphor Imaging casette for approx. 24 h. The resultant images were scanned on a Fujix BAS 1000 Phosphor Imager using the MacBas V1.01 programme and the ^{32}P-labelled phosphopeptides were analysed densitometrically using the NIH Image V1.57 programme. The protein content of cytosolic fractions was determined by the Micro BCA protein assay reagent kit. The molecular masses of individual peptides were determined as described in [10].

2.4. Calculation and expression of results

The extent of phosphorylation of the different peptides in response to glucagon challenge in isolated periportal and perivenous hepatocytes was determined by comparison to controls (no glucagon) for six separate experiments, the results being expressed as means ± S.E.M. The statistical significance of differences between groups of data was determined by ANOVA.

3. Results

3.1. Identity of the peptides phosphorylated in response to acute glucagon challenge in periportal and perivenous hepatocytes

Previous research based on mixed rat hepatocytes isolated by conventional techniques [10] has established that glucagon consistently stimulates the phosphorylation of a number of cytosolic peptides, including the α- and β-subunits of glycogen phosphorylase kinase, glycogen phosphorylase, glycogen synthase, pyruvate kinase, fructose 6-phosphate 2-kinase, phenylalanine hydroxylase, fructose 1,6-bisphosphatase and ribosomal protein S6 as well as two as yet unidentified peptides with molecular masses of 48 kDa and 46 kDa. In the present study, the phosphorylation of these peptides was compared in isolated periportal and perivenous hepatocytes after challenge with dif-

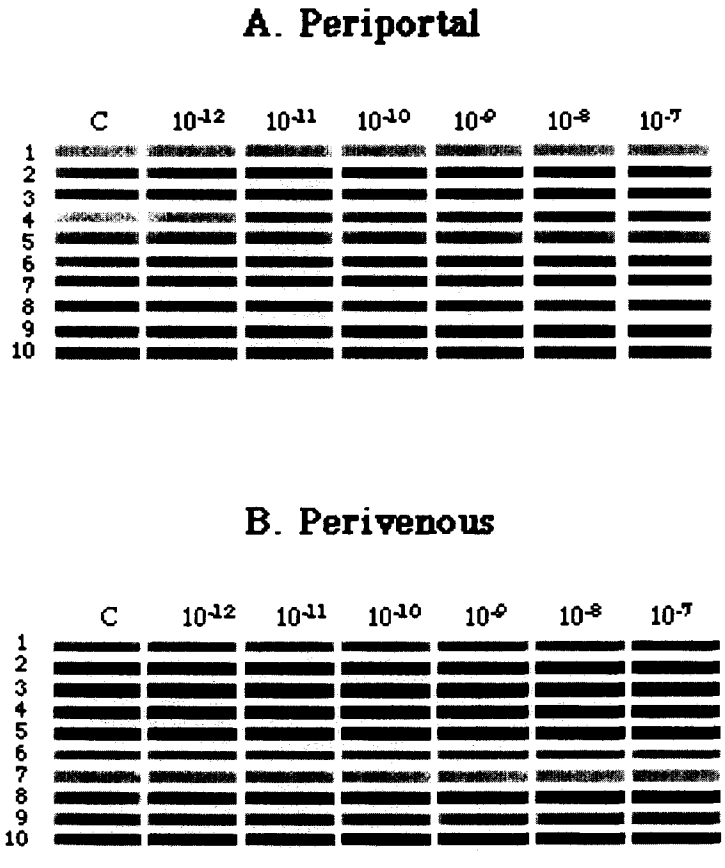

Fig. 1. Typical phosphor images of the cytosolic peptides phosphorylated in response to different concentrations of glucagon (10^{-12}–10^{-7} M) in isolated (A) periportal and (B) perivenous hepatocytes. The phosphor images of the one-dimensional gels show approximately thirty cytosolic ^{32}P-labelled peptides making it difficult to see changes in the ^{32}P-labelling of individual peptides, particularly those present as minor components. To overcome this problem, the figure shows only the images of those bands that correspond to peptides with molecular masses of (1) 123, (2) 93, (3) 85, (4) 61, (5) 55, (6) 52, (7) 48, (8) 46, (9) 42 and (10) 34 kDa (see text for details). C refers to control (no glucagon).

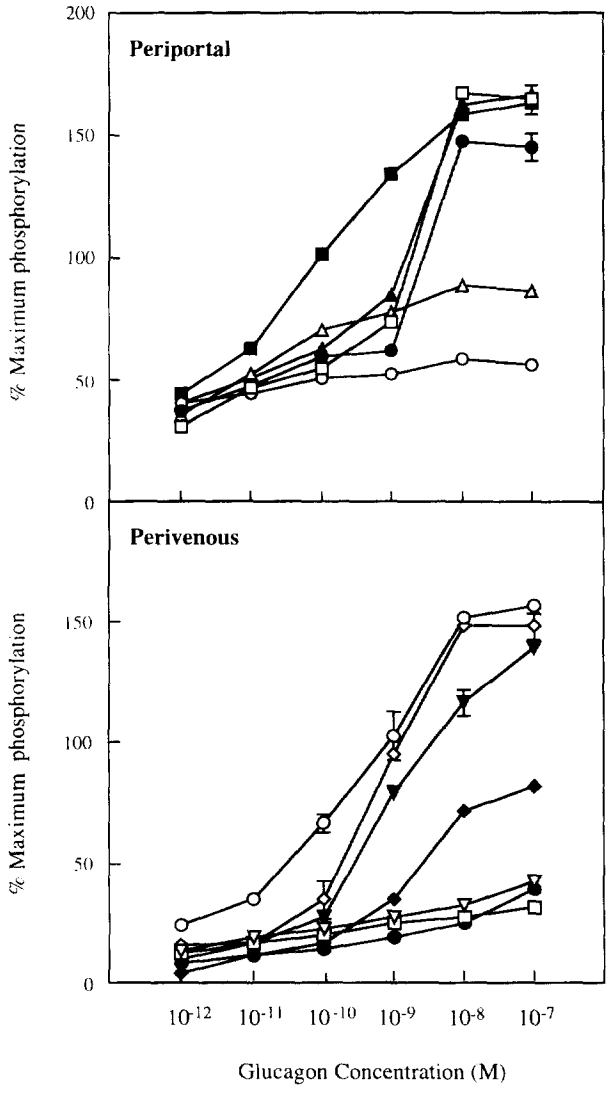

Fig. 2. The phosphorylation of cytosolic peptides in response to different concentrations of glucagon (10^{-12}–10^{-7} M) in isolated periportal and perivenous hepatocytes. The results shown are means ± S.E.M. for six separate experiments, the extent of ^{32}P-labelling of the individual peptides being expressed as increases (%) above basal (no glucagon) values. The phosphopeptides shown are glycogen phosphorylase (□), glycogen synthase (●), pyruvate kinase (○), phenylalanine hydroxylase (△), fructose 1,6-bisphosphatase (■), the β-subunit of glycogen phosphorylase kinase (▽), fructose 6-phosphate 2-kinase (▼), ribosomal protein S6 (♦) and two as yet unidentified peptides of molecular masses of 48 kDa (▲) and 46 kDa (◇).

ferent concentrations of glucagon (10^{-12}–10^{-7} M) that encompass the physiological range (10^{-10}–10^{-8} M).

When cytosolic protein fractions from both periportal and perivenous hepatocytes were subjected to one-dimensional SDS-PAGE, at least forty-five peptide bands were consistently detected after Coomassie blue-staining. Approximately thirty of these peptides incorporated ^{32}P during pre-equilibration with $^{32}PO_4^{3-}$. Many, but not all, of the peptides showed higher basal (no glucagon) phosphorylation in perivenous compared to periportal hepatocytes (Fig. 1). This higher basal phosphorylation may reflect differences in endogenous protein kinase and protein phosphatase activities between the two cell types. Glucagon was found to increase the phosphorylation of different subsets of peptides in periportal and perivenous hepatocytes (Figs. 1 and 2). Glucagon stimulated the phosphorylation of six cytosolic peptides with molecular masses of 93, 85, 61, 52, 42 and 48 kDa in periportal hepatocytes. The molecular masses of these ^{32}P-labelled phosphopeptides were highly reproducible and agree closely with our previously published studies [10], allowing identification of five of these phosphopeptides as glycogen phosphorylase (93 kDa), glycogen synthase (85 kDa), pyruvate kinase (61 kDa), phenylalanine hydroxylase (52 kDa) and fructose 1,6-bisphosphatase (42 kDa). The 48 kDa phosphopeptide remains unidentified. By contrast, glucagon stimulated the phosphorylation of seven peptides of molecular masses of 123, 93, 85, 61, 55, 34 and 46 kDa in perivenous hepatocytes (Figs. 1 and 2). Only three of peptide substrates (glycogen phosphorylase; 93 kDa, glycogen synthase; 85 kDa and pyruvate kinase; 61 kDa) are the same as those phosphorylated in periportal hepatocytes. Our previous studies [10] indicate that of the other four three correspond to the β-subunit of glycogen phosphorylase kinase (123 kDa), fructose 6-phosphate 2-kinase (55 kDa) and ribosomal protein S6 (34 kDa), respectively, whereas the 46 kDa phosphopeptide remains unidentified.

3.2. Extent and concentration-dependency of glucagon-stimulated peptide phosphorylation in periportal and perivenous hepatocytes

There were differences between periportal and perivenous hepatocytes not only in the subsets of cytosolic peptides phosphorylated in response to glucagon challenge but also in the extent of the stimulation of this phosphorylation (Table 1), including that of the common peptide substrates. In periportal hepatocytes glucagon at 10^{-7} M (compared to 10^{-12} M) increased the phosphorylation of glycogen phosphorylase, glycogen synthase and pyruvate kinase by 107%, 82% and 11%, respectively, whereas in perivenous hepatocytes it increased the phosphorylation of these peptides by 18%, 29% and 107%, respectively. Thus, although glucagon stimulates the phosphorylation of common substrates in periportal and perivenous hepatocytes, the extent of phosphorylation of these substrates is significantly different ($P < 0.001$) between the two cell types. Peptides also differed in their responsiveness to glucagon at different concentrations. Half-maximal phosphorylation (EC_{50}) of the peptides occurred at 1×10^{-10} to 4×10^{-9} M glucagon, except that the glucagon response curves of phenylalanine hydroxylase in periportal hepatocytes and those of glycogen phosphorylase and glycogen synthase in perivenous hepatocytes were flattened (Table 1).

4. Discussion

This paper represents the first systematic attempt to quantitate the impact of hepatic zonation on the responsiveness of cytosolic peptides to glucagon-stimulated protein phosphorylation. One-dimensional SDS-PAGE of ^{32}P-labelled cytosolic peptides from periportal and perivenous rat hepatocytes pre-equilibrated with $^{32}PO_4^{3-}$ indicates that glucagon stimulates the phosphorylation of different subsets of peptides in the two cell populations. Whereas glucagon promotes the phosphorylation of phenylalanine hydroxylase, fructose 1,6-bisphosphatase and an as yet unidentified 48 kDa phosphopeptide in periportal

Table 1
Identity of the cytosolic peptides phosphorylated in response to glucagon in periportal and perivenous hepatocytes and the extent and EC_{50} of their phosphorylation

Identified proteins	Molecular mass (kDa)	Increase in phosphorylation (%)		EC_{50} ($\times 10^{-10}$ M)	
		PP	PV	PP	PV
Phosphorylase kinase β-subunit	123	nd	25 ± 1	–	fr
Glycogen phosphorylase	93	107 ± 2[b]	18 ± 1[a,c]	20	fr
Glycogen synthase	85	82 ± 2[b]	29 ± 1[b,c]	40	fr
Pyruvate kinase	61	11 ± 1	107 ± 2[b]	fr	9
Fructose 6-phosphate 2-kinase	55	nd	118 ± 2[b]	–	1
Phenylalanine hydroxylase	52	37 ± 1[a]	nd	2	–
Unidentified protein	48	90 ± 2[b]	nd	30	–
Unidentified protein	46	nd	117 ± 2[b]	–	8
Fructose 1,6-bisphosphatase	42	83 ± 2[b]	nd	4	–
Ribosomal protein S6	34	nd	75 ± 2	–	20

Statistically significant increases in phosphorylation at 10^{-12} M glucagon (expressed as means ± S.E.M. relative to 10^{-12} M glucagon) are denoted as [a]$P < 0.01$ and [b]$P < 0.001$, whereas differences in the extent of phosphorylation of the two common phosphopeptide substrates (glycogen phosphorylase and glycogen synthase) between periportal and perivenous hepatocytes are denoted as [c]$P < 0.001$. nd denotes not detected in respective hepatocytes. EC_{50} refers to the glucagon concentrations giving half-maximal phosphorylation, whereas fr denotes that the glucagon response curve was flattened.

hepatocytes, it stimulates the phosphorylation of the β-subunit of glycogen phosphorylase kinase, fructose 6-phosphate 2-kinase, ribosomal protein S6 and an as yet unidentified 46 kDa phosphopeptide in perivenous hepatocytes. In addition to these zone-specific peptides, glucagon stimulates the phosphorylation of three common substrates (glycogen phosphorylase, glycogen synthase and pyruvate kinase) in both cell types, albeit to significantly different extents. The identities of the glucagon-stimulated phosphopeptides have been previously confirmed by two-dimensional SDS-PAGE [10].

The major question raised by this study is why the zonal patterns of phosphorylation of at least certain enzymes (fructose 1,6-bisphosphatase, fructose 6-phosphate 2-kinase, pyruvate kinase and glycogen phosphorylase) do not coincide with the reported zonal distributions of their activities. Fructose 1,6-bisphosphatase and pyruvate kinase, for example, are reported, respectively, to be largely, but not exclusively, periportal (60–78%) and perivenous (55%) in location [2,12,18–21], whereas our results indicate that their phosphorylation is exclusively periportal or perivenous, respectively (Table 1). There is also a discrepancy between the reported equal capacities of periportal and perivenous hepatocytes for the synthesis and degradation of fructose 2,6-bisphosphate [2] and our finding that the phosphorylation of fructose 6-phosphate 2-kinase is exclusively perivenous in location. Similarly, we show that the glucagon-stimulated phosphorylation of glycogen phosphorylase is substantially greater in isolated periportal than in perivenous hepatocytes, whereas studies on isolated hepatocytes [11] indicate that glycogen phosphorylase activity is evenly distributed between the two cell populations. These findings raise the intriguing question of whether zonal differences in phosphorylation may constitute a mechanism to explain the short-term dynamic zonation of glycolysis, gluconeogenesis and other metabolic processes. This mechanism proposes that the zonation of these biochemical processes is determined by zonal differences in sensitivity to phosphorylation rather than zonal differences in the distribution of the target enzymes per se. This hypothesis raises the question of the identity of the factor(s) that determine zonal differences in sensitivity to glucagon. Both periportal and perivenous hepatocytes possess glucagon receptors and the machinery for glucagon signalling via cyclic AMP and cyclic AMP-dependent protein kinase, although there may be a periportal-perivenous gradient in glucagon concentrations and in glucagon receptor density across the acinus. The inference is that differences in glucagon signalling via cyclic AMP are not the primary factors governing zonal differences in phosphorylation. One factor that may account for the apparent zonal differences in sensitivity to glucagon may relate to differences in the activities of protein phosphatases, particularly protein phosphatases 1 and 2A. Another factor may be the levels of regulatory metabolites that modulate glucagon-stimulated phosphorylation. The cyclic AMP-dependent inactivation of L-type pyruvate kinase is inhibited by phosphoenolpyruvate and fructose 1,6-bisphosphate, effects that are counteracted by Mg-ATP and certain amino acids [22–24]. The implication is that effectors that alter the conformation of regulatory proteins may alter their susceptibility to phosphorylation. Thus, if the levels of regulatory metabolites were to differ between periportal and perivenous hepatocytes, the possibility exists that these levels determine phosphorylation in the different cell populations. One other factor that may influence the ^{32}P-labelling of peptides in response to glucagon may be the level of occupancy of phosphorylation sites on peptides by unlabelled phosphate groups prior to glucagon challenge. One approach to address these issues will be to relate changes in the phosphorylation of specific peptides in response to glucagon to changes in enzyme activities and metabolite levels.

A final question relates the functional importance of the zonal differences observed in glucagon-stimulated phosphorylation. Our knowledge of certain of the peptides (phenylalanine hydroxylase, ribosomal protein S6 and the 48- and 46 kDa peptides) is so limited that it is difficult to determine the functional important of zonal differences in their phosphorylation. For the other peptides, our results imply that periportal hepatocytes are the primary site of phosphorylation of the enzymes of glycogen metabolism, whereas the phosphorylation of pyruvate kinase and fructose 6-phosphate 2-kinase, both key enzymes in glycolysis, is primarily perivenous in location.

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