Application of dual-digitonin-pulse perfusion to the study of hepatic mRNA zonation

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Heterogeneous zonation of hepatic protein expression over the liver lobule has been recognized by using several analytical techniques, including microdissection, selective cell isolation, immunohistochemistry and hybridization of mRNA *in situ*. We previously employed the technique of dual-digitonin-pulse perfusion for the highly selective collection and analysis of periportal and perivenous soluble protein. In the present work we have now documented the feasibility of the application of this technique to the study of zonal distribution of mRNA. By using a split-stream design, both protein and RNA fractions can be simultaneously

collected from hepatic zones. High-quality RNA (average yield ~ 9–33 µg of total RNA per mg of eluted protein) is obtained for analysis. As analysed by immunoblotting and Northern-blot analysis, the zonal distribution of several important cytosolic metabolic enzymes and their mRNAs can be documented. This technique is also applicable to the study of mRNAs for organelle- and membrane-associated proteins that are not recoverable with this digitonin-lysis technique. The application of this experimental technique should allow further molecular insight into the mechanisms underlying zonation of hepatic function.

INTRODUCTION

In the study of hepatic enzymes and other proteins, it has become apparent that many proteins are heterogeneously distributed over the hepatic lobule. In addition, heterogeneous fluxes through several metabolic pathways (e.g. ammonia fixation, gluconeogenesis) has also been observed. This phenomenon, termed metabolic zonation, has been studied by several techniques, including microdissection of hepatic zones, isolation of periportal and perivenous hepatocytes, and immunohistochemistry [1–5]. We have applied the technique of dual-digitonin-pulse perfusion, which allows the selective sampling of digitonin-releasable contents from the cytosolic compartment of the periportal and perivenous regions [2,6–10]. The advantages of this technique include its high selectivity, the rapidity of sampling, and high reproducibility between experiments.

Two general forms of intrahepatic gradients of enzymes and other proteins have been collectively recognized by these techniques, and both types of gradients can be expected to translate into functional heterogeneity as well. First, there is zonal distribution of cellular enzyme/protein content, as judged by enzyme assay, immunohistochemistry, and/or immunoblotting of eluted fractions [1–10]. Second, for at least one interconvertible enzyme, acetyl-CoA carboxylase, there is zonal variation in the actual enzyme specific activity, due to variable enzyme phosphorylation [9,10].

Zonation of the content of any given protein could occur by several mechanisms, including zonal differences in mRNA production/degradation, variable translational efficiency or variable protein degradation. There is a limited amount of information available of relevance to these various possibilities. Zonal variation in mRNA content would suggest heterogeneous gene transcription, mRNA processing or mRNA turnover. To examine such variation, hybridization of mRNA *in situ* with labelled cDNAs has revealed, in some instances, zonation of mRNA paralleling that of the encoded protein. For example, heterogeneous distribution of mRNAs encoding glucose

transporters, glutamine synthetase, carbamoylphosphate synthetase, glucokinase and transcription factor C/EBP have been demonstrated in this fashion [1,11–14]. In general, such studies of hybridization *in situ* have shown that the distribution of protein and the respective mRNAs are co-localized, suggesting that regulation is exerted at the pre-translational level.

The technique of dual-digitonin-pulse perfusion has not previously been applied to the study of hepatic mRNA distribution. Its potential advantages include the very rapid sampling of soluble cell products, the ability to ‘quench’ the eluted samples rapidly in buffer(s) that would minimize RNA degradation, and the ability to detect hybridizable mRNA on Northern blots in order to assess mRNA size(s) and probe specificity. The application of this technique to mRNA assessment would also allow indirect estimation of possible zonal content of membrane- or organelle-associated proteins that are largely not recovered by the digitonin-release technique. In the present study, we have explored the feasibility of the application of this technique to the study of mRNA distribution in the liver and compared it with the simultaneous distribution of the associated protein product, where possible.

EXPERIMENTAL

Animal preparation and dual-digitonin-pulse perfusion sample collection

Male Wistar rats (170–190 g) were used in all experiments. Five groups of 4 rats each constituted the nutritional study. One group was maintained on normal rat chow (Fed); a second group was fasted for 48 h before liver perfusion (Fasted). The remaining animals were also fasted for 48 h and then refed with a high-carbohydrate chow (Bioserv) for 3 h (3 h RF), 24 h (24 h RF) or 48 h (48 h RF). All animals were kept on a 06:00–18:00 h light/dark cycle; all refeeding and fasting periods were begun at 07:30 h. Refeeding of fasted animals employed a high-carbohydrate low-fat chow (Bioserv).

After nutritional manipulation, livers were perfused and

digitonin-lysed eluates from the periportal and perivenous hepatic zones were collected with a split-stream design [2,6,7]. One-half of the eluate fraction was directly collected into an equal volume of ice-cold collection buffer, containing Tris/HCl (40 mM; pH 7.4), NaCl (100 mM), sodium pyrophosphate (60 mM), NaF (100 mM), dithiothreitol (2 mM), sodium vanadate (0.2 mM), ZnCl_2 (10 μM) and sucrose (0.5 M). After mixing and removal of a small portion, the samples were frozen at -80°C for subsequent protein analysis. The other half of the fraction was directly collected into sterile tubes containing ice-cold guanidine thiocyanate (8 M), EDTA (20 mM), Tris/HCl (100 mM; pH 7.5 at room temperature) and β -mercaptoethanol (16%, v/v). These samples were also rapidly frozen and kept at -80°C . All samples diluted the collection buffers about 2-fold, as measured by differential weighing of the individual tubes. Except for analysis of the sample removed for marker enzymes and protein content, all samples were then directly shipped from Copenhagen to Dartmouth on solid CO_2 for analysis. The order of periportal- or perivenous-perfusion sample collection was alternately varied within each set of animals; this order did not affect any of the results obtained (not shown).

Protein analysis of eluted fractions

The initial sample was used in Copenhagen to measure eluate protein and the activities of pyruvate kinase and alanine aminotransferase, markers of zonation, as in [6,7]. On arrival at Dartmouth, samples were rapidly thawed and then centrifuged (160 000 g for 60 min) to remove debris. After confirmation of protein content, samples were prepared for SDS/PAGE. For the purposes of the present paper, protein analysis was largely confined to immunoblotting. Denatured samples were separated on SDS/polyacrylamide gels and electrophoretically transferred to poly(vinylidene difluoride) membranes. After blocking and incubation with primary antibody (see the text), all blots were secondarily probed with horseradish-peroxidase-labelled secondary antibodies. Immunoreactive bands were identified by chemiluminescence (Amersham, ECL reagent) on exposure to Kodak XAR film. Exposure times varied between 2 and 30 s, depending on the analysis performed. The representative data shown in this report were derived by pooling of four periportal or perivenous eluates for each nutritional condition, in order to minimize any intrasample variation. Analysis of individual samples confirmed the reproducibility of the technique of collection, as previously reported [6,7,9,10].

With respect to the enzyme acetyl-CoA carboxylase, eluate concentrations of this protein in non-denatured samples were also determined by avidin/antibody sandwich e.l.i.s.a. [15].

RNA analysis of eluted fractions

After sample receipt at Dartmouth, total RNA was isolated from the guanidinium-quenched samples by the method of Cathala et al. [16], with the following modifications: the concentration of SDS in the solubilization buffer was 1% and the RNAs were precipitated with 3.5 vol. of 4 M LiCl per vol. of homogenate. After determination of RNA content (A_{260}), each individual sample was analysed for integrity by RNA electrophoresis and ethidium bromide staining. Analysis of RNA samples on Northern blots was performed as described in [17], with a single modification. Agarose gels used in the electrophoretic separation of RNA did not contain formaldehyde [18]. cDNA hybridization probes for Northern-blot analysis were either digoxigenin labelled using the Genius Nonradioactive Nucleic Acid Labelling and Detection System from Boehringer Mannheim according to

the manufacturer's instructions (glutamine synthetase, fatty acid synthase, ATP citrate lyase) or ^{32}P labelled with [α - ^{32}P]CTP by using the Random Primers DNA Labeling System (Gibco BRL) [acetyl-CoA carboxylase, phosphoenolpyruvate carboxykinase (PEPCK), glucokinase, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, insulin receptor, actin]. The protocol for Northern blotting (capillary transfer, hybridization, washing, detection) for chemiluminescent probing followed that suggested by the manufacturer; the ^{32}P -cDNA protocol was that detailed in [17].

Miscellaneous methods

Protein concentration was determined by the method of Bradford [19].

Materials

Rats were obtained from the Animal Breeding Facility of the Panum Institute at the University of Copenhagen. [α - ^{32}P]CTP was purchased from DuPont/New England Nuclear. Horseradish-peroxidase-labelled secondary antibodies were obtained from Sigma, as were most standard chemicals. Nitrocellulose and poly(vinylidene difluoride) membranes used for blotting were obtained from Schleicher and Schuell and Millipore, respectively. Guanidinium isothiocyanate was purchased from Fluka. cDNAs for mouse β -actin and guinea-pig HMG-CoA reductase were purchased from American Type Culture Collection.

RESULTS AND DISCUSSION

Characterization of eluate fractions: marker enzymes, protein recovery and RNA integrity

As assessed by marker enzyme and protein recovery, the eluted periportal and perivenous fractions in these experiments are characteristic of those previously reported [6,7]. Alanine aminotransferase displays a periportal preponderance (7–9-fold gradient), whereas pyruvate kinase is nearly equally distributed between periportal and perivenous fractions; protein recovery is reproducibly slightly higher in the periportal eluates (results not shown).

RNA recovery (calculated from A_{260}) ranged from 9 to 33 μg of RNA per mg of protein with no significant differences between periportal and perivenous samples (periportal 12.2 ± 4.3 , perivenous 16.1 ± 9.0 $\mu\text{g}/\text{mg}$ of protein; means $\pm$ S.D. for 4 preparations of each zonal fraction from each nutritional condition). Highest recovery was observed in the 24 h- and 48 h-refed samples, and lowest in fasted samples. As judged by A_{260}/A_{280} , excellent purity is obtained (periportal 1.72 ± 0.04 ; perivenous 1.67 ± 0.07 ; means $\pm$ S.D. for all preparations). When examined by ethidium bromide staining on agarose gels, no RNA degradation was observed and the content of 28 S and 18 S rRNA was constant between all samples, as matched by the A_{260} . When probed on Northern analysis, actin mRNA concentration was constant in all samples, whether analysed individually or after pooling (results not shown).

Specific mRNA and protein content as judged by Northern analysis and immunoblotting

As an index example of a zoned protein where other techniques indicate parallel zonation of mRNA, we chose glutamine synthetase for analysis. Others have reported the nearly exclusive zonation of enzyme activity, protein and mRNA to the

Figure 1 Analysis of glutamine synthetase protein and mRNA in periportal and perivenous eluate fractions

In (a) is shown an immunoblot of glutamine synthetase (42 kDa) in periportal (P) and perivenous (V) eluates from fed (FE), fasted (FA), 3 h-refed (F/R 3), 24 h-refed (F/R 24) and 48 h-refed (F/R 48) animals (pairs left to right, respectively). Each lane represents loading of 20 μ g of eluate protein on the SDS gel. In (b) is shown a representative Northern blot of periportal (P) and perivenous (V) total RNA (20 μ g per lane) from fed animals, probed with digoxigenin-labelled glutamine synthetase cDNA and developed with the Genius Kit. All protein and RNA samples represent the pool of 4 eluates from each zone and in each nutritional condition.

Figure 2 Analysis of PEPCK protein and mRNA and glucokinase mRNA

In (a) is shown immunoblot analysis of PEPCK protein (20 μ g total protein per lane) in pooled eluate samples (see Figure 1) from periportal (P) and perivenous (V) zones of rat livers in each of five nutritional states. The molecular mass of the identified staining band is 69 kDa. In (b) is shown the Northern analysis of PEPCK mRNA in which 20 μ g of total RNA from split-stream-collected pools at each condition is loaded per lane. The blot was probed with 32 P-labelled PEPCK cDNA. The hybridizing RNA is of size 2.6 kb, as determined by reference to stained standards (not shown). In (c) is shown the Northern analysis of glucokinase mRNA in some of the same pools as those probed in (b). This blot was probed with 32 P-labelled glucokinase cDNA. The hybridizing RNA is of size 2.4 kb, as determined by reference to stained standards (not shown).

perivenous hepatic zone [1,12]. As judged by immunoblotting with an anti-(glutamine synthetase) antibody, the enzyme content is expressed nearly exclusively in the perivenous zonal eluates in all five nutritional conditions (Figure 1a). Glutamine synthetase polypeptide content diminishes on fasting and remains lower than the fed state, even after 48 h of refeeding, consistent with known nutritional effects on this enzyme [20]. On Northern

analysis, there is a parallel exclusive perivenous zonation of glutamine synthetase mRNA (Figure 1b; only fed sample pools shown).

For purposes of this semi-quantitative feasibility analysis, protein and RNA samples from each zone and each nutritional state ($n = 4$ preparations from each zone at each nutritional condition) were pooled for analysis. Enzymes of glucose metabolism are known to be zoned within the liver acinus. PEPCK activity is more prominent in the periportal zone, whereas glucokinase is more prominent in the perivenous zone [1,4]. Furthermore, the measured activity of each of these hepatic enzymes varies as a function of the nutritional state; PEPCK activity is highest in fasted rats and declines with high-carbohydrate feeding, whereas glucokinase activity displays the inverse regulation [1,4]. In the analysis of our protein-eluate fractions, PEPCK enzyme protein shows a periportal preponderance in the fasted state and falls to undetectable levels on high-carbohydrate refeeding (Figure 2a). The zonal distribution and change in protein content during refeeding is paralleled by an identical pattern of mRNA distribution/content (Figure 2b). In contrast, glucokinase mRNA shows a relative perivenous zonation and is induced on refeeding (Figure 2c).

The fatty-acid-synthesizing enzymes, acetyl-CoA carboxylase, ATP citrate lyase and fatty acid synthase, are also known to be zoned in the hepatic acinus ([1,2,4,8–10,21]; L. A. Witters, G. Gao, B. E. Kemp and B. Quistorff, unpublished work). Taken together, previous reports indicate that the content of each of these three enzymes may be higher in perivenous hepatocytes, depending on sex and nutritional state, although the activity of acetyl-CoA carboxylase is actually higher in periportal hepatocytes, due to a relative enzyme dephosphorylation [9,10]. All three enzymes are also subject to marked nutritional variation in content, being very low in fasted animals and 'hyperinducing' on refeeding with high-carbohydrate chow [9,10,15]. In a previous study, we reported a small net perivenous/periportal gradient in acetyl-CoA carboxylase content as measured by e.l.i.s.a. that did not reach statistical significance [10]. Employing a larger number of samples from several nutritional states, we have now statistically confirmed this small intrahepatic gradient (L. A. Witters, G. Gao, B. E. Kemp and B. Quistorff, unpublished work). Based on immunoblotting studies, there is also a small perivenous preponderance of fatty acid synthase and ATP citrate lyase protein, most easily observed in the 'induced' state [9,10]. This pattern of nutritional induction, coupled with a slight perivenous predominance of all three mRNAs, is seen on Northern analysis of the eluted fractions (Figure 3).

In analysis of other mRNAs encoding soluble proteins, we also found that pyruvate kinase mRNA was equally distributed between perivenous and periportal fractions in the fed animals, paralleling the lack of zonation of pyruvate kinase activity previously reported (results not shown) [1,2,6,7]. Analysis of 'spot 14', a thyroid-hormone- and carbohydrate-regulated hepatic protein [22], showed parallel marked induction of both protein and mRNA by refeeding, with nearly equal distribution between periportal and perivenous fractions (results not shown).

As judged from the elution pattern of marker enzymes, it is clear that the dual-digitonin-pulse perfusion technique, as used in the present study, does not elute the mitochondrial compartment, since less than 0.5% of the mitochondrial enzymes, glutamate dehydrogenase and citrate synthase, are eluted [23]. However, the degree to which proteins from other organelles such as plasma membrane, endoplasmic reticulum, lysosomes and the nucleus are eluted has not yet been systematically tested. The ability to isolate and analyse mRNA, however, does allow an approach to the study of possible zonation of the latter group

Figure 3 Analysis of lipogenic-enzyme mRNA

The Figure shows three Northern analyses performed after separation of 20 µg of total RNA per lane from the pooled fractions used in Figure 2. Three separate blots were probed with cDNAs for acetyl-CoA carboxylase (ACC) (³²P-labelled; hybridizing mRNA 9.0 kb), ATP citrate lyase (ACL) (digoxigenin-labelled; hybridizing mRNA 4.3 kb) and fatty acid synthase (FAS) (digoxigenin-labelled; hybridizing mRNA 9.5 kb).

Figure 4 Analysis of Insulin-receptor and HMG-CoA reductase mRNA

The Figure shows representative Northern analyses performed after separation of 20 µg of total RNA per lane from some of the pooled fractions used in Figure 2. Blots were probed with either ³²P-labelled insulin-receptor cDNA (upper panel; hybridizing mRNA at 8.5, 7.5 and 5 kb), or HMG-CoA reductase cDNA (bottom panel; hybridizing mRNA 4.5 kb).

of proteins. In these feasibility studies, we have explored three such mRNAs: those for the insulin receptor, HMG-CoA reductase and the GLUT-2 glucose transporter. As shown in Figure 4, there is no marked zonation of HMG-CoA reductase mRNA; an apparent small zonation of the mRNA for the insulin receptor to the periportal side is seen. In addition, insulin-receptor mRNA content appears to be induced in both hepatic zones on refeeding. We are unaware of other studies examining intrahepatic distribution of insulin-receptor mRNA, although HMG-CoA reductase mRNA, measured by hybridization *in situ*, has been reported to have a periportal localization under different nutritional conditions from those employed in the present study [24]. Confirming the results of others, we additionally found no zonation of GLUT-2 mRNA in fed animals (results not shown) [11].

In summary, the results presented clearly indicate the applicability of the dual-digitonin-pulse perfusion technique to the study of mRNA distribution in the liver and provides an

approach to the future study of some of the mechanisms that underlie hepatic zonation. The results that we obtained with respect to a number of mRNAs are generally consonant with those obtained by hybridization *in situ*, although we have not attempted to quantify precisely the degree of zonation in these feasibility experiments. From the observations to date, increased zonal expression of enzyme protein appears to be paralleled by increase in enzyme mRNA, suggesting that zonal differences in gene transcription, mRNA processing or mRNA turnover underlie the protein gradients observed. The ability to collect rapidly both soluble protein and mRNA simultaneously from a well-defined microcirculatory zone within the same liver provides a valuable tool to a molecular dissection of the transcriptional, translational and post-translational events responsible for the heterogeneity of the hepatocytes in the liver lobule.

We are deeply indebted to a number of investigators who supplied either cDNAs or antibodies for the purpose of this study. The following kindly provided these reagents: Dr. Wouter Lamers [University of Amsterdam; anti-glutamine synthetase] antibody and glutamine synthetase cDNA, Dr. Daryl Granner [Vanderbilt; cDNAs for PEPCK and glucokinase and anti-PEPCK antibody], Dr. Ki-Han Kim [Purdue; acetyl-CoA carboxylase cDNA], Dr. Stuart Smith [Oakland Research Institute; fatty acid synthase cDNA], Dr. Nabil Elshourbagy [Smith, Kline and French Laboratories; ATP citrate lyase cDNA], Dr. Joseph Avruch [Massachusetts General Hospital; insulin-receptor cDNA], Dr. Michael Mueckler [Washington University; GLUT-2 cDNA] and Dr. William Kinlaw [Dartmouth; cDNA for 'spot 14']. This study was supported by grants to L.A.W. from the National Institutes of Health (DK35712) and the Juvenile Diabetes Foundation and to B.Q. from the Emil C. Hertz Fund and the Danish Medical Research Council (Grant No. 12-0063). The excellent technical assistance of Lissi Immerdal in liver perfusion is gratefully appreciated.

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