Interplay between glucose, fatty acid and bile salt metabolism in mouse models of fatty liver

Herrema, Hillechien Jeltje

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Zonation of hepatic metabolism

The fed to fasting transition differentially affects PEPCK protein expression in pericentral and periportal murine hepatocytes

Hilde Herrema, Julius F.W. Baller, Michael Müller, Folkert Kuipers, Albert K. Groen, and Aldo Greffhorst

In preparation
ABSTRACT

Hepatic glucose metabolism is zonated. Glycolysis is mainly localized in hepatocytes surrounding the hepatic vein (pericentral zone) whereas gluconeogenesis is mainly carried out by hepatocytes surrounding the portal vein (periportal zone). Expression of the gene encoding the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK, PCK1) is reduced upon feeding. PEPCK is considered a periportal enzyme but the effects of feeding-fasting transition on PEPCK zonation are unknown. In the present study, laserdissection microscopy, immunohistochemistry and primary periportal and pericentral hepatocytes were used to assess the effects of the feeding-fasting transition on zonation of PEPCK gene and protein. PEPCK protein expression was reduced in pericentral but not in periportal hepatocytes in fed versus fasted mice. In alloxan-induced type 1 diabetic mice, which lack insulin, Pepck mRNA expression and protein levels did not increase upon fasting. Nevertheless, PEPCK was still zonated along the liver acinus suggesting. In conclusion, our data show that the effects of the feeding to fasting response on PEPCK protein expression are likely mediated by insulin and mostly affect pericentral hepatocytes. Nevertheless, additional, yet to be determined factors regulate zonation of PEPCK.
INTRODUCTION

Hepatocytes are functionally distinct depending on their localization in the hepatic acinus, the functional unit of the liver. The metabolic activity of hepatocytes localized around the portal vein differs from the activity of hepatocytes lining the central vein. Metabolic processes in the liver are, therefore, functionally separated which is referred to by the model of metabolic zonation. The liver expresses ~15,000 genes: the location within the acinus is known of only a few. Even less is known about posttranscriptional regulation of genes along the liver acinus, e.g., zonation of posttranscriptional regulation of metabolically enzymes, nuclear receptors and transcription factors.

Carbohydrate metabolism is a well-studied example of hepatic zonation. Glycolysis is pericentrally located while gluconeogenesis is mainly carried out by portal hepatocytes. In light of this well-known zonation of carbohydrate metabolism, information about zonation of gene expression and proteins is of key relevance to study and understand hepatic carbohydrate metabolism and perturbations herein such as in diabetes. Remarkably, however, metabolic zonation is still largely disregarded. Expression levels of several glycolytic and gluconeogenic genes and proteins, and hence enzyme activity, depend on the nutritional status of an organism. Periportal and pericentral hepatocytes are exposed to significantly different concentrations of oxygen, metabolites and hormones which might determine zone-specific hepatocyte functions in response to variations in energy demand. Additionally, it has been shown that hormones can differentially impact on periportal and pericentral gluconeogenesis suggesting that, next to differences in hormone concentrations, there are hormone-specific actions on periportal and pericentral hepatocytes.

The feeding-to-fasting transition has significant effects on plasma concentrations of the regulatory hormones insulin and glucagon. Despite the sparse studies on zonation of insulin signaling that suggest that the insulin receptor is mainly localized in the pericentral zone, the effects of insulin on zonation of metabolic processes are largely unknown. Studies investigating the role of insulin in zonation of hepatic metabolism will provide important insight in insulin-related derangements in hepatic metabolism such as insulin resistance and diabetes. Therefore, the present study addressed the effects of the feeding-fasting transition, insulin infusion and type 1 diabetes on the zonation of protein and mRNA of phosphoenolpyruvate carboxykinase (PEPCK), an enzyme that catalyzes the first step in hepatic gluconeogenesis. Pepck gene expression is decreased by glucose-induced insulin secretion and therefore Pepck expression increases upon fasting when insulin levels are low. Although a few publications on zonation of PEPCK exist, there is no consensus on how this zonation is regulated and how it relates to regulation of glucose metabolism in health and disease.
Confirming previous findings, QPCR analysis of laser-dissected periportal and pericentral hepatocytes from fed mice revealed that Pepck mRNA was increased in periportal hepatocytes compared to pericentral hepatocytes. Compared to hepatocytes from fed mice, hepatocytes from fasted mice had an increased Pepck expression in both zones with the largest induction in pericentral hepatocytes, resulting in an absent Pepck mRNA zonation. Immunohistochemical PEPCK staining and PEPCK immunoblotting on primary periportal and pericentral hepatocytes showed that PEPCK protein was zonated only in livers of fed mice with highest expression levels in periportal hepatocytes. Fasting increased total PEPCK expression and blunted its zonation. Because insulin infusion reduced total liver Pepck mRNA expression, we assessed whether insulin mediates PEPCK protein zonation in alloxan-induced type 1 diabetic mice that lack insulin. Interestingly, PEPCK protein was still zonated in fed alloxan-treated mice which suggests that other factors than insulin mediate PEPCK zonation. Altogether, our results indicate that fasting perturbs the well-known PEPCK zonation. Although insulin might play a role herein, it is probably not the only factor involved.

MATERIALS AND METHODS

Animals

Male C57Bl6/J were purchased from Charles River Laboratories (L’Arbresle, France). Mice were used for experimental procedures at 12 weeks of age. Mice were housed in a temperature- and light-controlled facility were fed commercially available laboratory chow (RMH-B; Arie Blok, Woerden, The Netherlands). All experiments were approved by the Ethical Committee for Animal Experiments of the University of Groningen.

Fasting-feeding-refeeding protocol and insulin deficient mice

Mice were terminated by cardiac puncture under isoflurane anesthesia in an ad libitum fed condition at 8 AM, after an overnight (11PM - 8AM) fast or in a refed state (6h refeeding after a 24h fast). Insulin deficiency was induced by intravenous administration of alloxan (70 mg/kg in sterile PBS, Alloxan Monohydrate, Sigma Aldrich). Ten days after alloxan injections, mice were terminated in an ad libitum fed condition at 8 am or in the fasted condition (3 am – 8 am). Plasma was obtained by centrifugation (3000 rpm, 10 min at 4°C) and stored at -20°C until analysis. The liver was removed and weighed. A small section of the large liver lobe was excised, embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek, Sakura Finetek U.S.A., Inc., Torrance, California), frozen in isopentane and stored at -80°C. The remaining part of the liver was snap-frozen in liquid nitrogen and stored at -80°C until further analysis.
Liver perfusion

Primary periportal or pericentral hepatocytes were isolated from fed and overnight fasted mice using liver perfusion. All buffers were adjusted to pH 7.4 (at room temperature) and oxygenated with carbogen (95% CO2 and 5% O2). Livers remained *in situ* during all perfusion procedures. Mice were anesthetized by an intraperitoneal (IP) injection of Hypnorm (1 ml/kg) and Diazepam (10 mg/kg) in sterile PBS. The portal vein and superior vena cava were canulated. The inferior vena cava was ligated rostral to the kidney. The liver was pre-perfused for 10 min at 37°C with Hanks 1 buffer (112 mmol/l NaCl, 5.4 mmol/l KCl, 0.9 mmol/l KH2PO4, 0.7 mmol/l Na2HPO4, 25.0 mmol/l NaHCO3, 10.0 mmol/l D-glucose and 0.5 mmol/l EGTA). The perfusion rate was kept constant at 3 ml/min using a peristaltic pump (GE Healthcare, peristaltic pump P-1).

To obtain a hepatocyte isolate enriched in periportal or pericentral cells, a down-scaled approach of selective destruction with digitonin as described in rats was applied. After pre-perfusion, digitonin solution (Sigma-Aldrich, 4 mg/ml prepared at 100°C in Hanks 1 buffer) was infused at 37°C via either canula at a rate of 1 ml/min until the characteristic selective destruction pattern on the liver surface was optimal, usually after 40-60 sec (Supplemental Figure 1). Subsequently, the liver was perfused through the canula opposite the digitonin pulse with collagenase-containing Hanks 2 buffer (112 mmol/l NaCl, 5.4 mmol/l KCl, 0.9 mmol/l KH2PO4, 0.7 mmol/l Na2HPO4, 25.0 mmol/l NaHCO3, 10.0 mmol/l D-glucose, CaCl2 5.0 mmol/l) containing 15 mg/250 ml collagenase type 4 (Sigma-Aldrich, 523 units/mg solid, 2.8 units/mg FALGPA) at 37°C. The perfusion rate was 3 ml/min and was ended after collagenase-digestion of the liver, usually after ~30 min.

After collagenase digestion, the liver was excised and hepatocytes were released from the liver capsule in Krebs buffer (NaCl 118 mmol/l, KCl 5.0 mmol/l, KH2PO4 1.2 mmol/l, MgSO4 1.1 mmol/l, CaCl2 2.5 mmol/l, NaHCO3 25.0 mmol/l, D-glucose 10.0 mmol/l, HEPES 10.0 mmol/l, BSA 1% w/v) at 4°C and filtered. The cells were purified twice by centrifugation (2 min, 500 rpm, 4°C) in 50 ml Krebs buffer in which the obtained pellet was gently resuspended in fresh Krebs after discarding the supernatant. Viability was determined by dye exclusion using Trypan-blue (0.4% in PBS). Cells were stored at -80°C in PBS or TRI Reagent (Sigma, St. Louis, MO) until further analysis.

Hyperinsulinemic euglycemic clamp

The hyperinsulinemic euglycemic clamp was performed on 9h fasted mice as described before. Mice were terminated by cardiac puncture under isoflurane anesthesia after the 6h infusion period. The liver was removed and a small section of the large liver lobe was excised, embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek, Sakura Finetek U.S.A., Inc., Torrance, California), frozen in
isopentane and stored at -80°C. The remaining part of the liver was snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

**Laser dissection microscopy (LDM)**

Frozen sections (6 µm thick) were mounted onto uncoated PEN membrane slides (Zeiss membrane slide 1.0 PEN) and stored at -80 °C until further processing. Slides were acclimatized to RT for 30 min and stained with RNase-free hematoxylin for 25s. Slides were rinsed with DEPC tap water and dehydrated with subsequently 75% and 100% RNase-free ethanol. Slides were placed on the laser microscope stage (Leica LMD6000, Germany). The areas to be micro dissected were identified, cut by UV laser and catapulted into a collection tube (Greiner, PCR-tubes 0.2ml) containing 30 µl RLT lysis buffer (RNeasy microkit Qiagen) and 1% (v/v) β-mercaptoethanol. From each liver 60 portal and 60 central areas from 2 cell layers thick were isolated. Endothelial cells lining the portal veins were excluded. Material was stored in RLT lysis buffer at -80 °C until further analysis.

**RNA isolation and amplification from laser dissected hepatocytes**

RNA was isolated using RNeasy microkit (Qiagen) according to manufacturer’s protocol. RNA was amplified using Ambion WT Expression Kit according to manufacturers' protocol (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Amplified RNA was isolated and purified using cRNA binding magnetic beads. RNA quality was verified by lab on a chip analysis (Bioanalyzer 2100; Agilent, Amstelveen, The Netherlands). cDNA was synthesized using random primers and enzyme supplied by the manufacturer. Real-time PCR was performed on a 7900HT FAST real-time PCR system using FAST PCR master mix and MicroAmp FAST optical 96 well reaction plates (Applied Biosystems Europe, Nieuwekerk ad IJssel, The Netherlands). Primer and probe sequences have been published before (www.labpediatricsrug.nl). PCR results were normalized to 36b4.

**RNA isolation from liver and hepatocytes**

Total RNA was isolated from liver using TRI-reagent (Sigma, St. Louis, MO) according to the manufacturers’ protocol. cDNA was produced as described. Real-time PCR was performed on a 7900HT FAST real-time PCR system using FAST PCR master mix and MicroAmp FAST optical 96 well reaction plates (Applied Biosystems Europe, Nieuwekerk ad IJssel, The Netherlands). Primer and probe sequences have been published before (www.labpediatricsrug.nl). PCR results were normalized to 36b4.
Immunoblotting procedures

PEPCK, GLUL and Actin levels were measured in homogenates made in homogenization buffer (30 mM Tris-Cl, pH 7.4; 2.5 mM EDTA, pH 8.0; 150 mM NaCl; 0.5 mM Na3VO4; 5 mM NaF; 5 mM MgCl2; 1.3 M glycerol and protease inhibitors (Complete; Roche Diagnostics)) and cleared by centrifugation. The protein concentrations were measured using the BCA Kit (Pierce). The samples were mixed with 4x SDS loading buffer (0.15 M Tris-chloride, pH 6.8; 12% SDS; 0.02% bromophenol blue; 30% glycerol; 6% β-mercaptoethanol) and equal amounts of protein per sample were subjected to SDS-PAGE and immunoblot analysis. PEPCK, GLUL and the loading control actin were determined using antibodies against PEPCK raised in rabbit (Cayman Chemical, Ann Arbor, MI), against GLUL raised in mouse (BD Biosciences, Franklin Lakes, NJ) and against actin raised in rabbit (Sigma). Finally, horseradish peroxidase-conjugated anti-rabbit or anti-mouse from donkey (Amer sham Pharmacia Bioscience) and SuperSignal West Pico Chemiluminescent Substrate System (Pierce) were used.

Immunohistochemical staining

Frozen sections (4 um thick) were mounted on a glass slide, dried and fixated in acetone. After inhibition of the endogenous peroxidase activity, the slides were incubated with antibodies against PEPCK raised in rabbit (Cayman Chemical) or against GLUL raised in mouse (BD Biosciences). The second incubation was with biotin-labeled anti-rabbit or anti-mouse from goat (DAKO, Glastrup, Denmark) subsequently followed by an incubation with horseradish peroxidase-conjugated streptavidin (DAKO) and 3-Amino-9-Ethylcarbazole (AEC).

Statistics

All values are represented as mean ± standard deviation. Statistical analysis was assessed using the Mann-Whitney-U-test (SPSS 12.0.1 for Windows). P-values were corrected for multiple comparison errors.

RESULTS

Gene expression levels in periportal and pericentral hepatocytes

Periportal and pericentral hepatocytes were isolated from livers of fed and 10h-fasted C57Bl/6j mice using laser dissection microscopy (LDM). Gene expression levels were determined using QPCR. Expression of glutamine synthetase (Glu1), which is exclusively expressed in pericentral hepatocytes 12, and cholesterol 7a-hydroxylase (Cyp7a1), which is mainly expressed in pericentral hepatocytes 17, were indeed nearly absent in periportal hepatocytes of fed and fasted mice (Figure 1). Pepck expression was increased in periportal hepatocytes of fed mice compared to pericentral hepato-
cytes. Upon fasting, *Pepck* expression was higher in both periportal and pericentral hepatocytes and this resulted in similar expression levels in both zones. Expression levels of Forkhead box O1 (*Foxo1*), an important regulator of *Pepck* transcription, were increased upon fasting in both periportal and pericentral hepatocytes and no zonation was detectable. The consequences of feeding and fasting for gene expression patterns in periportal and pericentral hepatocytes will be studied using a comparative Affymetrix microarray approach.

Differential zonation of PEPCK upon feeding and fasting

Zonation of PEPCK protein in livers of fed and fasted mice was assessed immunohistochemically. The strongly zonated protein GLUL was used as marker for pericentral hepatocytes. PEPCK clearly showed zonation in fed mice as it was mainly localized in periportal hepatocytes. Fasting increased total PEPCK protein levels but blunted its zonation (Figure 2).

LDM yields insufficient amounts of protein to study protein expression. Therefore, to study the effects of fasting on PEPCK protein in more detail, periportal and pericentral enriched hepatocyte isolates were obtained from fed and fasted mice using the digitonin-collagenase infusion technique. Digitonin permeabilizes cell membranes by precipitating cholesterol from cell membranes. Administration of

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**Figure 1.** Relative gene expression levels of periportal and pericentral hepatocytes. Hepatocytes were isolated from liver slides of fed and overnight fasted mice using laser dissection microscopy. * p<0.05 periportal vs pericentral, # p<0.05 fed vs fasted, N=5 mice per group. Gene expression levels were normalized to 36B4.
Zonation of hepatic metabolism

Digitonin via the portal vein will dissolve membranes of portal hepatocytes thereby releasing their content and vice versa (see Materials and Methods for details).

Digitonin infusion gave rise to the typical decolourisation (Supplemental Figure 1) as published by Lindros and coworkers who used a similar approach to isolate periportal and pericentral hepatocytes from rats. Expression of Glul and Cyp7a1 were increased in pericentral compared to periportal hepatocyte isolates (Figure 3), confirming that isolated hepatocytes were enriched in periportal and pericentral hepatocytes. Fasting did not affect Glul and Cyp7a1 zonation. As expected, Pepck

![Supplemental Figure 1. Typical decolourisation of liver after infusion of digitonin via the central or portal vein.](image)

Figure 2. Localization of PEPCK in liver slides of fed and overnight fasted mice. PEPCK protein is mainly expressed in periportal hepatocytes of livers from fed mice. Upon fasting, zonation of PEPCK is blunted and the protein is expressed in with more involvement of periportal hepatocytes. Glutamine synthetase (GLUL), which is exclusively expressed in pericentral hepatocytes, was used as marker for pericentral areas. C; pericentral, P; periportal.
Chapter 7

Figure 3. Relative gene expression levels of primary hepatocyte isolates enriched in periportal or pericentral hepatocytes. Hepatocytes were isolated from fed and overnight fasted mice using digitonin-collagenase perfusion (see Materials and Methods for details). * p<0.05 periportal vs pericentral, # p<0.05 fed vs fasted, N=5 mice per group. Gene expression levels were normalized to 36B4.

Figure 4. Western blot analysis of PEPCK expression in primary periportal and pericentral hepatocyte isolates. PEPCK is mainly expressed in periportal hepatocytes upon feeding. Upon fasting, total PEPCK expression was increased. Additionally, zonation of PEPCK was blunted in this condition. GLUL was used as marker for pericentral areas.

and Mcad expressions increased upon fasting but were not zonated (Figure 3). In contrast to Pepck expression in hepatocytes isolated using LDM (Figure 1), Pepck expression did not differ between the primary periportal and pericentral hepatocytes. PEPCK protein was assessed by immunoblotting of periportal and pericentral
Zonation of hepatic metabolism isolates (Figure 4). GLUL could not be detected in isolates enriched in periportal hepatocytes confirming proper enrichment of the isolates in periportal or pericentral hepatocytes. PEPCK expression was reduced in pericentral hepatocytes from livers of fed mice. This zonation pattern was blunted in periportal and pericentral isolates from fasted mice. The results obtained with the immunoblotting analyses confirmed the immunohistochemical findings (Figure 2).

**Insulin regulates the adaptive responsive of Pepck expression levels during feeding and fasting, but does not regulate zonation of PEPCK protein**

Fasting reduces insulin levels. Since zonation pattern of PEPCK also changes upon fasting, the role of insulin on hepatic Pepck gene expression and PEPCK expression were determined in mice infused with insulin, i.e., mice subjected to a hyperinsulinemic euglycemic clamp. Insulin infusion resulted in a ~60% reduction of hepatic Pepck expression (Figure 5). In addition, PEPCK protein was expressed mainly in pericentral hepatocytes of insulin-infused mice. Altogether, these data suggest that insulin is indeed a regulator of PEPCK zonation.

![Figure 5](image.png)

**Figure 5.** Relative gene expression of Pepck in livers of mice that were subjected to hyperinsulinemic euglycemic clamp. Pepck expression is reduced upon insulin infusion (A). PEPCK protein shows strong zonation upon insulin infusion (B). * p<0.05, N=6 mice per group, C; pericentral, P; periportal. Gene expression levels were normalized to 36B4.

To assess whether insulin is a determinant of PEPCK zonation in the adaptive response to feeding and fasting, hepatic Pepck expression levels were determined in alloxan-treated and thus insulin-deficient fed and fasted mice. Alloxan-treated mice were hyperglycemic, hypoinsulinemic and had reduced body weight compared to healthy counterparts in both fed and fasted state (Table 1). Fasting increased plasma NEFA concentrations in both control and alloxan-treated mice. Control mice accumulated triglycerides in the liver upon fasting, however, fasting did not affect hepatic triglyceride contents of alloxan-treated mice. As expected, fasting increased
Table 1. Plasma and liver parameters of control mice (fed, overnight fasted or refed) and alloxan-induced type 1 diabetic mice (fed or fasted). * p<0.05 vs control fed, N=5 till 7 mice per group.

<table>
<thead>
<tr>
<th></th>
<th>Control fed</th>
<th>Control fasted</th>
<th>Control Refed</th>
<th>Alloxan fed</th>
<th>Alloxan fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>9.54 ± 1.84</td>
<td>4.16 ± 0.3 *</td>
<td>10.6 ± 1.6 *</td>
<td>31.4 ± 2.8 *</td>
<td>22.6 ± 1.9 *</td>
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<tr>
<td>BW (g)</td>
<td>25.6 ± 1.7</td>
<td>23.26 ± 1.0</td>
<td>25.2 ± 2.3</td>
<td>21.4 ± 2.1</td>
<td>21.8 ± 1.3</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>0.25 ± 0.17</td>
<td>0.11 ± 0.00</td>
<td>1.86 ± 1.00 *</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>NEFA (μmol/l)</td>
<td>225 ± 55</td>
<td>798 ± 176 *</td>
<td>150 ± 31</td>
<td>118 ± 99</td>
<td>487 ± 131</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.13 ± 0.3</td>
<td>0.86 ± 0.12 *</td>
<td>1.20 ± 0.49</td>
<td>0.79 ± 0.22</td>
<td>0.97 ± 0.36</td>
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<tr>
<td>CH (mmol/l)</td>
<td>1.55 ± 0.11</td>
<td>1.56 ± 0.16</td>
<td>2.02 ± 0.16</td>
<td>2.32 ± 0.42</td>
<td>2.07 ± 0.19</td>
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<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Liver (% BW)</td>
<td>5.07 ± 0.62</td>
<td>4.21 ± 0.27</td>
<td>5.1 ± 0.2</td>
<td>6.3 ± 0.3</td>
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</tr>
<tr>
<td>TG (μmol/g)</td>
<td>4.0 ± 1.4</td>
<td>61.7 ± 16.0 *</td>
<td>27.2 ± 10.6 *</td>
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<tr>
<td>CH (μmol/g)</td>
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</tr>
<tr>
<td>PL (μmol/mg)</td>
<td>35.4 ± 9.7</td>
<td>32.5 ± 2.1</td>
<td>26.9 ± 5.9</td>
<td>28.9 ± 2.9</td>
<td>36.0 ± 9.2</td>
</tr>
</tbody>
</table>

Figure 6. Relative gene expression levels in livers of control and alloxan-induced type 1 diabetic mice. Fasting increased gene expression levels of Pepck and Mcad in control mice. Expression levels of Pepck and Mcad were increased in fed type 1 diabetic mice compared to controls and remained unaffected upon fasting. * p<0.05 vs control fed, N=5 till 7 mice per group. Gene expression levels were normalized to 36B4.
Pepck expression levels in control mice (Figure 6): expression levels were restored upon refeeding. In alloxan-treated mice, in contrast, fasting did not increase hepatic Pepck expression. PEPCK immunoblotting revealed that total PEPCK protein levels remained unaffected in alloxan-treated mice. In control mice, however, fasting induced total hepatic PEPCK protein concentrations (Figure 7). Interestingly, immunohistochemical analysis revealed that PEPCK was still zonated in both fed and fasted alloxan-treated mice (Figure 8).

**DISCUSSION**

The concept of metabolic zonation has been known for long. Although it is complicated to assess ‘zone specific’ metabolism, it is of importance to consider the concept when studying hepatic metabolism. In this work, we used laser dissection microscopy, immunohistochemical staining and periportal and pericentral enriched hepatocyte isolates to investigate zone-specific PEPCK gene and protein expression.
patterns. Gene expression profiling was analyzed using QPCR and will be followed by an Affymetrix microarray approach in the near future.

Q-PCR analysis of laser dissected hepatocytes showed that *Pepck* gene expression appeared to be zonated only in fed mice. Immunohistochemistry and immunoblotting revealed that PEPCK protein was also zonated in fed but not in fasted mice. PEPCK gene and protein expression levels were highest in the periportal area. Upon fasting, total PEPCK protein expression increased, zonation, however, was blunted. Similar to results found in livers from fed mice, insulin infusion reduced total hepatic *Pepck* gene expression. Additionally, hepatic PEPCK protein was strongly zonated in insulin-infused mice. This led us to hypothesize that insulin is a strong determinant of PEPCK protein zonation. In fed alloxan-treated type 1 diabetic and thus insulin-deficient mice, however, PEPCK protein was still zonated. Altogether, these data suggest that insulin plays a role in PEPCK zonation in the feeding to fasting response, but additional mechanisms regulate this zonation in livers of type 1 diabetic mice.

Using *in situ* hybridization, Krones *et al.* found that *Pepck* mRNA is preferentially peripor tally localized in livers of fed rats. Another study showed that the area in which *Pepck* mRNA is expressed increases upon fasting with more involvement of pericentrally localized hepatocytes. Thus, the difference in *Pepck* mRNA concentrations is the highest between hepatocytes directly lining a portal or central vein. The intra-acinar distribution of PEPCK activity has also been shown to be gradually distributed. In our studies, *Pepck* mRNA zonation was only detectable in periportal and pericentral hepatocytes obtained by LDM. This zonation was absent in periportal and pericentral enriched hepatocytes isolated using digitonin-collagenase perfusion technique. Isolates enriched in periportal and pericentral hepatocytes by digitonin-collagenase likely have a higher variability in hepatocyte population, masking zonation of *Pepck* mRNA. *Cyp7a1* and *Glul* mRNA have a stronger zonation compared to *Pepck*, i.e., these mRNAs are expressed in hepatocytes directly lining a central vein. Therefore, zonation of *Cyp7a1* and *Glul* was still detectable in hepatocytes isolated using digitonin-collagenase perfusion. Nevertheless, in contrast to our observations, *Pepck* mRNA has recently been shown to be localized mainly in periportal hepatocytes in isolates of periportal and pericentral hepatocytes from mice using digitonin-collagenase infusion. Because of discrepancies between studies but also variation of isolation within studies, we suggest that periportal and pericentral hepatocytes obtained by LDM should be used as “map” in the study of gene expression patterns in these cells. The major drawback of hepatocytes isolated with LDM is that this method does not yield sufficient material for protein analysis. In the near future, novel GC/MS techniques might be able to study proteomics of hepatocytes isolated with LDM. Currently, primary hepatocyte isolates obtained with the digitonin-collagenase perfusion technique are suitable to assess protein expression, as shown in Figure 4. In addition, this isolation method might be suitable to determine activity of enzymes involved in gluconeogenesis and glycolysis.
in portal and central zones. Predominance of pathways in specific zones of the liver cannot be solely subscribed to the presence of enzymes. Reversal of substrate flux in the liver, e.g., by means of retrograde perfusion, changes the rate of gluconeogenesis and glycolysis in portal and central zones. Thus, substrate concentration and supply determine to a great extent the activity of metabolic pathways in hepatocytes. Additionally, the predominance of gluconeogenesis in periportal hepatocytes is in accordance with thermodynamics. Gluconeogenesis should be coupled to oxidative energy metabolism and is favored in aerobic zones, i.e., the periportal area.

Several hormones have been shown to differentially impact periportal or pericentral gluconeogenesis. Glucagon enhances periportal gluconeogenesis whereas adrenaline stimulates gluconeogenesis mainly in pericentral hepatocytes. The oxygen concentration gradient across the hepatic acinus has been hypothesized to partly underlie these differential effects. Our data from fed and fasted mice suggest that the feeding-to-fasting response influences mainly pericentral PEPCK gene and protein expression, possibly via regulatory actions of insulin on Pepck gene transcription in this zone. Corresponding with this presumably pericentral localized action of insulin is the presence of insulin receptor protein (IR) mainly in the pericentral area. In previous and the current experiments, however, gene expression of the insulin receptor was not zonated. Thus, posttranscriptional modifications might be involved in zonation of the insulin receptor.

The transcription factor FOXO1 is an important mediator of insulin’s effects on gene expression. Insulin induces phosphorylation of FOXO1 thereby reducing its transcriptional control. FOXO1 response elements are present in promoter sites of gluconeogenic genes such as Pepck and G6Pase. We show that Foxo1 mRNA is not zonated in livers of mice. To gain more insight in the zonation of insulin-mediated gene transcription, analysis of FOXO1-DNA binding patterns in specific hepatocytes are currently being performed.

From the experiments using alloxan-treated mice it is clear that insulin is not the only factor regulating PEPCK zonation. PEPCK was zonated in both fasted and fed alloxan-treated mice. Because alloxan-treated mice still produce glucagon, this hormone might be involved in zonation of PEPCK. Glucagon has been shown to act on periportal gluconeogenesis by increasing oxygen uptake in these cells, especially during fasting. In addition, posttranslational modifications of the PEPCK protein, e.g., acetylation and O-GlcNAcylation, might all influence hepatic PEPCK zonation. These modifications also require further research. If insulin differentially affects carbohydrate and lipid metabolism in periportal and pericentral areas, this would partly explain the “triad” of hyperinsulinemia, hyperglycemia and hyperlipidemia typically seen in type 2 diabetes. The present studies are the first to address the role of insulin in differential regulation of key players in metabolism in periportal and pericentral hepatocytes. More studies are required to provide insight in the role of zonation of metabolism in healthy and diabetic livers.
Zonation of hepatic metabolism


