

Functional Consequences of Metabolic Zonation in Murine Livers: Insights for an Old Story

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BACKGROUND AND AIMS: Zone-dependent differences in expression of metabolic enzymes along the portocentral axis of the acinus are a long-known feature of liver metabolism. A prominent example is the preferential localization of the enzyme, glutamine synthetase, in pericentral hepatocytes, where it converts potentially toxic ammonia to the valuable amino acid, glutamine. However, with the exception of a few key regulatory enzymes, a comprehensive and quantitative assessment of zonal differences in the abundance of metabolic enzymes and, much more important, an estimation of the associated functional differences between portal and central hepatocytes is missing thus far.

APPROACH AND RESULTS: We addressed this problem by establishing a method for the separation of periportal and pericentral hepatocytes that yields sufficiently pure fractions of both cell populations. Quantitative shotgun proteomics identified hundreds of differentially expressed enzymes in the two cell populations. We used zone-specific proteomics data for scaling of the maximal activities to generate portal and central instantiations of a comprehensive kinetic model of central hepatic metabolism (Hepatokin1).

CONCLUSIONS: The model simulations revealed significant portal-to-central differences in almost all metabolic pathways involving carbohydrates, fatty acids, amino acids, and detoxification. (HEPATOLOGY 2021;73:795–810).

The liver is the central metabolic organ of higher vertebrates and continuously adapts its metabolic capacities to the actual physiological status of the individual. After a carbohydrate-rich meal, a substantial portion of glucose is taken up by the liver from the plasma and is transiently converted into glycogen and triacylglycerol. Conversely, under fasting conditions, the liver produces glucose by phosphorylation of glycogen and *de novo* synthesis from amino acids, lactate, and glycerol to prevent a potentially life-threatening drop in plasma glucose below 55 mg/dL.⁽¹⁾ Intriguingly, the capacity of hepatocytes to take up or to produce glucose varies with their location within the liver acinus. Hepatocytes close to the

Abbreviations: Acta2, alpha smooth muscle actin 2; ADH, alcohol dehydrogenase; Arg1, arginase 1; CPS1, carbamoyl-phosphate synthase 1; CYP1A2, cytochrome P450 1A2; CYP2E1, cytochrome P450 2E1; DNL, de novo lipogenesis; E-Cad, E-cadherin; Emr1, EGF-like module containing, mucin-like, hormone receptor-like sequence 1; FFAs, free fatty acids; Gck, glucokinase; Gfap, glial fibrillary acidic protein; Gl2, glutaminase 2; GLUL, glutamine synthetase; GLUT2, glucose transporter 2; Krt8, keratin 8; Krt18, keratin 18; Krt19, keratin 19; Ldha, lactate dehydrogenase A chain; LFQ, label-free quantification; PC, pericentral; PCHs, pericentral hepatocytes; PP, periportal; PPHs, periportal hepatocytes; TAG, triacylglycerol; VLDL, very-low-density lipoprotein.

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portal vein (periportal hepatocytes; PPHs) possess a higher gluconeogenic capacity than hepatocytes close to the central vein (pericentral hepatocytes; PCHs). Conversely, PCHs have a higher glycolytic capacity than PPHs. Gradual spatial separation of anabolic (ATP-consuming) gluconeogenesis from catabolic (ATP-generating) glycolysis may represent a principle in tissue metabolism to limit the extent of ATP-wasting futile cycles. Another prominent example of zone-dependent metabolic differences is ammonia detoxification. Ammonia fixation in urea is predominantly located in the periportal (PP) zone, whereas glutamine synthesis is predominantly located in the pericentral (PC) zone.^(2,3)

Zonal differences in the proteome of hepatocytes are driven by portocentral gradients of oxygen, metabolites (e.g., glucose), hormones and morphogens such as Wnt, Hedgehog, and hepatocyte nuclear factor alpha.^(4,5) The opposing effects of the pancreatic hormones, insulin and glucagon, on the zonation of glycolytic and glucogenic enzymes have been demonstrated in the earlier pioneering *in vitro* studies of Jungermann.⁽⁶⁾ Subsequently, flow bioreactors were used to study the impact of selected regulators of zonation by exposing hepatocytes to controlled concentrations gradients of oxygen, metabolites, or xenobiotics.^(7,8) Using a tissue model of the liver acinus and taking into account known relationships between the protein translation rate and oxygen, glucose, insulin, and glucagon

concentrations, we were able to recapitulate the zonation of hepatic glucose metabolism under fasting and feeding conditions.⁽⁹⁾

Over several decades, the investigation of differential gene expression along the portocentral axis has been restricted to a few key regulatory enzymes. More recently, the group of Shalev Itzkovitz was successful in quantifying the entire transcriptome of thousands of mouse liver cells together with their lobule coordinates.⁽¹⁰⁾ They found that ~50% of liver genes are significantly zoned, but a comprehensive and quantitative analysis of the functional consequences of this zone-specific enrichment was not provided in this pioneering work. Therefore, we have strived to map the zone-dependent expression data for metabolic enzymes and membrane transporters onto a mechanistic model of the central hepatic metabolism. Therefore, we isolated hepatocytes from mouse liver by collagenase perfusion and separated PPHs and PCHs by immunostaining and subsequent cell sorting. We then used shotgun proteomics to explore the proteome of the purified hepatocytes. Specifically, we used relative protein abundances to scale the maximal activities of enzymes and membrane transporters included in a recently published kinetic model of hepatocyte metabolism⁽¹¹⁾ to assess the implications of zone-dependent gene expression for a multitude of different metabolic functions (Fig. 1). A complete list of all enzymes and transporters considered in the model is given in Supporting Table S1.

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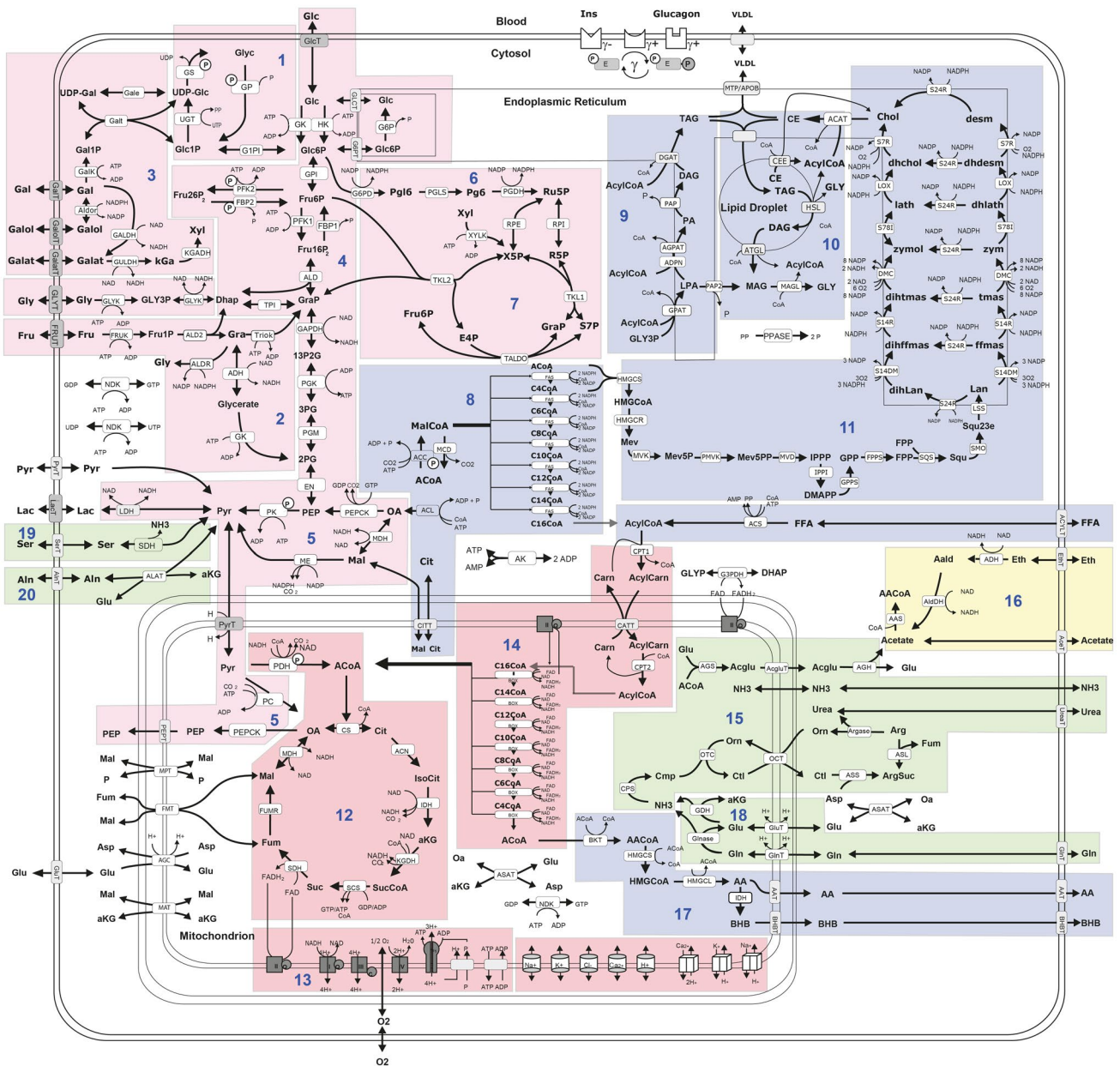


FIG. 1. Schematic model representation. Reactions and transport processes between compartments are symbolized by arrows. Single pathways as defined in biochemical text books are numbered and highlighted by different coloring: (1) glycogen metabolism, (2) fructose metabolism, (3) galactose metabolism, (4) glycolysis, (5) gluconeogenesis, (6) oxidative pentose phosphate pathway, (7) nonoxidative pentose phosphate pathway, (8) fatty acid synthesis, (9) triglyceride synthesis, (10) synthesis and degradation of lipid droplets and synthesis of VLDL lipoprotein, (11) cholesterol synthesis, (12) tricarbalic acid (TCA) cycle, (13) respiratory chain and oxidative phosphorylation, (14) β -oxidation of fatty acids, (15) urea cycle, (16) ethanol metabolism, (17) ketone body synthesis, (18) ammonia formation, (19) serine utilization, and (20) alanine utilization. Small cylinders and cubes symbolize ion channels and ion transporters. Double arrows indicate reversible reactions, which may proceed in both directions according to the value of the thermodynamic equilibrium constant and cellular concentrations of their reactants. Reactions are labeled by the short names of the catalyzing enzyme or membrane transporter given in the small boxes attached to the reactions arrow. Red boxes indicate enzymes that are regulated by hormone-dependent reversible phosphorylation. Metabolites are denoted by their short names. Full names of metabolites and kinetic rate laws of reaction rates are outlined in Berndt et al.⁽¹³⁾

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Materials and Methods

ISOLATION OF PRIMARY MOUSE HEPATOCYTES

Primary hepatocytes were isolated by collagenase perfusion of mouse liver as described in an earlier work.⁽¹²⁾ Isolated hepatocytes were cleared of nonparenchymal cells by differential centrifugation.⁽¹³⁾ We have demonstrated the purity of our primary hepatocyte isolation by this method elsewhere, where we looked for the expression of typical markers of mature hepatocytes such as *Arg1* (arginase 1), *Gck* (glucokinase), *Gls2* (glutaminase 2 [liver, mitochondrial]), *Ldha* (lactate dehydrogenase A chain), *Krt18* (keratin 18), and *Krt8* (keratin 8). For the nonparenchymal cell population (e.g., hepatic stellate cells, vascular smooth muscle cells, Kupffer cells, and cholangiocytes), we analyzed *Gfap* (glial fibrillary acidic protein), *Acta2* (actin, alpha 2, smooth muscle, aorta), *Emr1* (EGF-like module containing, mucin-like, hormone receptor-like sequence 1), and *Krt19* (keratin 19).⁽¹³⁾

MAINTENANCE OF THE MICE AND FEEDING

Male C57Bl/6N mice were maintained in a pathogen-free facility. The animals were kept on a 12:12 h LD cycle, according to the German guidelines, the World Medical Association and the Declaration of Helsinki for the care and safe use of experimental animals. The mice had free access to regular chow (ssniff M-Z V1124-0, composed of 22.0% protein, 50.1% carbohydrate, 4.5% fat; usable energy: 13.7 kJ/g; ssniff Spezialdiäten GmbH, Germany) and tap water throughout the experiment. The C57Bl/6N mice were sacrificed at 3 months of age, between 9:00 and 11:00 am, after administration of an anesthetic mixture of ketamine, xylazine and atropine.

Other detailed methods are available in the Supporting Information.

Results

ISOLATION OF PP AND PERICENTRAL HEPATOCYTES BY FLOW CYTOMETRY

To sort the isolated primary hepatocytes into populations of PPHs and PCHs, specific marker proteins

with no overlap in zonal expression (Fig. 2A) were chosen for intracellular staining. After excluding cell doublets in the forward scatter-area versus forward scatter-height plot and cell debris in the forward scatter versus side scatter plot, positive cell populations were gated against matching isotype controls (Supporting Figs. S1 and S2). The relative proportion of positively labeled cells in both stainings reflects the extended PP pattern of E-cadherin (E-cad). in contrast to the small, one-cell-layer PC localization of glutamine synthetase (GLUL), as indicated by immunohistochemistry staining (Supporting Fig. S3). E-Cad labeling (Supporting Fig. S3, left panel) resulted in large populations of ~30%-50%, whereas GLUL labeling (Supporting Fig. S3, right panel) resulted in small populations of ~1.5%-3.0%. To give a global overview about the purity of the sorting method, the data from proteome analysis were analyzed with regard to all proteins of the individual cell fractions. The results of this analysis show that 995 proteins (Supporting Table S2, sheet 3 and sheet 4) of a total of 3,736 (Supporting Table S3) detected proteins have a significant zoned pattern. A selection of the 40 most potent zoned proteins shows that well-known zoned proteins can be found in both the PC and the PP fraction (Fig. 2B; Table 1). Also, western blotting analysis of PPH and PCH marker proteins demonstrated the purity of the isolation method. Therefore, prominent PC-expressed proteins, such as GLUL, cytochrome P450 1A2 (CYP1A2), and cytochrome P450 2E1 (CYP2E1), could not, or could only weakly, be found in PPHs. The dominant PP-expressed protein, E-Cad, could not be detected in the PC fraction. Expression of carbamoyl-phosphate synthase 1 (CPS1) showed stronger expression in the PP zone; however, this protein is also found in PCHs (Fig. 2C,D). This corresponds to the rather homogeneous distribution of this protein, in which there is a tendency toward expression only in the PP region.⁽¹⁴⁾ In addition, Gaasbeek et al. showed that in the adult rat liver, some PC hepatocytes harbor also carbamoylphosphate synthetase, albeit the number of glutamine-synthetase-positive cells diminishes.⁽¹⁵⁾

DIFFERENCES IN THE PROTEIN ABUNDANCES OF PPHs AND PCHs

Shotgun proteome profiling yielded protein intensities for 3,736 proteins that could be identified in PPHs, PCHs, and the reference hepatocytes. Statistical

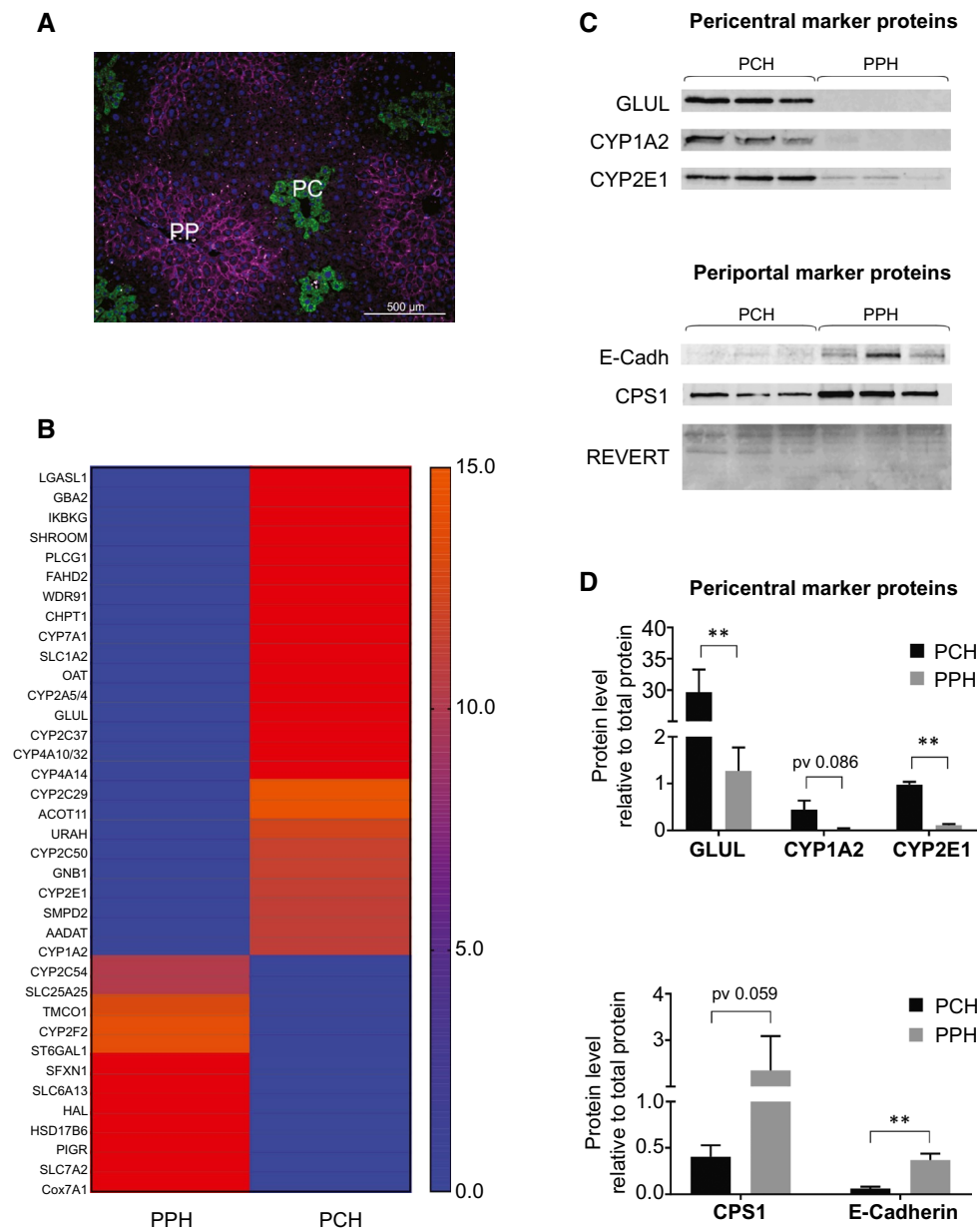


FIG. 2. Sorting of PC and PP hepatocytes by flow cytometry. (A) Immunofluorescence double staining shows distinct PP (E-Cad, magenta) or PC (GLUL, green) location along the portocentral axis. (B) Fold-change heatmap of the 39 strongest zoned proteins. Red boxes indicate out-of-range values (>15-fold expression). The fold change of PPH and PCH cell populations to each other was calculated with the normalized LFQ intensities from the proteomic analysis. (C) Western blotting analysis of zone-specific markers in lysates from sorted GLUL-positive (PCHs; $n = 3$) and E-cad-positive (PPHs; $n = 3$) hepatocytes. (D) Western blotting protein quantification normalized to total protein. Unpaired t test, $*P \leq 0.05$. Abbreviations: AADAT, aminoacidate aminotransferase; ACOT11, acyl-coenzyme A thioesterase 11; CHPT1, choline phosphotransferase 1; CYP2A5/4, cytochrome P450, family 2, subfamily a, polypeptides 5 and 4; CYP2F2, cytochrome P450, family 2, subfamily f, polypeptide 2; CYP4A10/32, cytochrome P450, family 4, subfamily a, polypeptides 10 and 32; CYP4A14, cytochrome P450, family 4, subfamily a, polypeptide 14; CYP2C37, cytochrome P450, family 2, subfamily c, polypeptide 37; CYP2C50, cytochrome P450, family 2, subfamily c, polypeptide 50; CYP2C54, cytochrome P450, family 2, subfamily c, polypeptide 54; FAHD2, fumarylacetoacetate hydrolase domain-containing protein 2; GBA2, glucosylceramidase beta 2; GNB1, G protein subunit beta 1; IKBKG, inhibitor of nuclear factor kappa B kinase regulatory subunit gamma; PLCG1, phospholipase C gamma 1; pv, P value; SLC1A2, solute carrier family 1 member 2; SLC25A25, solute carrier family 25 member 25; SLC6A13, solute carrier family 6 member 13; SLC7A2, solute carrier family 7 member 2; SMPD2, sphingomyelin phosphodiesterase 2; ST6GAL1, ST6 beta-galactoside alpha-2,6-sialyltransferase 1; TMCO1, transmembrane and coiled-coil domains protein 1; URAH, 5-hydroxyisourate hydrolase; WDR91, WD repeat domain 91.

TABLE 1. Mean LFQ of Most Potent Zonated Proteins

Protein IDs	Protein Names	Mean LFQ E-Cad Mouse 1-6	Mean LFQ GLUL Mouse 1-6
P16045	Galectin-1	0.00E+00	1.40E+07
Q69ZF3;B1AWI3	Nonlysosomal glucosylceramidase	0.00E+00	3.01E+06
A3KG44;Q8VC91;E9Q2Y3;Q7TSS3;O88522; A3KG40;A3KG41;A3KG38	NF-kappa-B essential modulator	0.00E+00	5.28E+06
A7TU71;A2ALU4	Protein Shroom2	0.00E+00	4.17E+06
A2A4A6;Q62077;G3UXP4;F6SLLO	Phosphoinositide phospholipase C;1- phosphatidylinositol 4,5-bisphosphate phosphodies- terase gamma-1	0.00E+00	6.03E+06
A0A0R4J094;Q3TC72	Fumarylacetoacetate hydrolase domain-containing protein 2A	0.00E+00	1.37E+07
S4R1X1;Q7TMQ7;D3Z101;D3Z0V8	WD repeat-containing protein 91	0.00E+00	1.49E+07
Q8C025;D6RCW3;D3Z6W4;D3YU39	Cholinephosphotransferase 1	0.00E+00	2.41E+07
Q64505	Cholesterol 7-alpha-monooxygenase	0.00E+00	1.69E+07
P43006;A2APL5;F6ZRK3;F7CAM6;A2AQI7	Excitatory amino acid transporter 2;Amino acid transporter	0.00E+00	9.49E+07
P29758	Ornithine aminotransferase, mitochondrial	2.53E+07	6.29E+09
Q91X75;P20852;P15392;F7B9W9;F6SHL3; E9Q593	Cytochrome P450 2A5;Cytochrome P450 2A4	9.41E+06	1.33E+09
P15105;D3YVK1;D3Z121	Glutamine synthetase	3.60E+08	3.13E+10
P56654	Cytochrome P450 2C37	2.33E+07	9.19E+08
A2A977;O88833;A2A8T1;F8WGU9;A2A8S9; Q91WU1;A2A8S8	Cytochrome P450 4A10	8.75E+06	2.34E+08
O35728;A0A087WS15	Cytochrome P450 4A14	1.03E+07	1.97E+08
Q64458;Q3UT49;H3BLM0	Cytochrome P450 2C29	2.04E+08	3.28E+09
A2AVR6;Q8VHQ9;F6SS52;E0CYS0; E0CYA0;E0CXP8	Acyl-coenzyme A thioesterase 11	5.23E+05	7.76E+06
A0A1BOGSD0;Q9CRB3;A0A1BOGR99; A0A0A0MQC3	5-hydroxyisourate hydrolase	1.29E+07	1.78E+08
Q91X77	Cytochrome P450 2C50	3.73E+08	4.58E+09
P62874;H3BKR2;H3BLF7;Q61011	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	1.16E+06	1.34E+07
Q05421;A0A1BOGSV7;Q0PGA1	Cytochrome P450 2E1	2.63E+08	3.03E+09
O70572;D6RGM4	Sphingomyelin phosphodiesterase 2	9.86E+05	1.12E+07
Q9WWM8;Q3UNSS	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial	7.50E+06	8.36E+07
P00186	Cytochrome P450 1A2	3.82E+08	4.25E+09
Q6XVG2	Cytochrome P450 2C54	7.58E+07	8.35E+08
A2ASZ8;Z4YLR9;A0A0A6YWC1;F6RC04	Calcium-binding mitochondrial carrier protein ScaMC-2	1.28E+07	1.26E+06
A0A0A6VVS2;Q921L3	Transmembrane and coiled-coil domain-containing protein 1	3.94E+07	3.85E+06
P33267;A0A0U1RPL9	Cytochrome P450 2F2	3.10E+09	2.39E+08
Q64685	Beta-galactoside alpha-2,6-sialyltransferase 1	7.43E+06	5.39E+05
Q99JR1	Sideroflexin-1	7.85E+08	5.69E+07
P31649;D3YVW5	Sodium- and chloride-dependent GABA transporter 2	1.95E+07	1.04E+06
P35492;F8WH73	Histidine ammonia-lyase	1.13E+09	4.20E+07
Q9R092	17-beta-hydroxysteroid dehydrogenase type 6	1.04E+08	2.28E+06
O70570;D3YVM4;D3Z2D3	Polymeric immunoglobulin receptor;Secretory component	6.31E+07	6.15E+05
E9QJY0;P18581	Cationic amino acid transporter 2	6.82E+06	0.00E+00
A0A140LIU4;P56392	Cytochrome c oxidase subunit 7A1, mitochondrial	1.31E+07	0.00E+00
A0A0R4IZW5;P09803	Cadherin-1;E-Cad/CTF1;E-Cad/CTF2;E-Cad/CTF3	1.78E+07	0.00E+00

analysis of differences between the protein intensities in PPHs and PCHs by means of paired-sample *t* test at a significance level of $P = 0.05$ revealed that 21.9% of the detected proteome was zoned. There were only marginal differences in the percentage of proteins with higher expression levels in either the PPHs (13.4%) or the PCHs (13.2%).

From the whole detected proteome, we selected 316 proteins that are associated with enzymes and membrane transporters of the kinetic model (i.e., “model proteins”). Within this subgroup, 58.2% of proteins exhibited significant portal-to-central expression differences, which was more than twice as high as in the total proteome. Moreover, a larger fraction of metabolic enzymes had higher protein abundances in PPHs (42.7%), whereas the fraction of metabolic enzymes with higher expression in the PCHs was 22.5%. A complete list of differentially expressed proteins can be found in Supporting Table S2.

PARAMETERIZATION OF ZONE-SPECIFIC KINETIC MODELS

To obtain zone-specific instantiations of the metabolic model for each laboratory animal, the maximal activities of enzymes and transporters in the PCHs and PPHs were computed according to Equation 1 (see Materials and Methods). The average percentage of enzymes and transporters for which a protein intensity value was available from the proteomics analysis was 80.0%, with the lowest coverage of 64.4% and the highest coverage of 88.5%. To fill the gaps, we applied a statistical imputation method that estimates missing values of a given target protein based on the expression profiles of other predictor proteins with sufficiently similar intensity profiles taken from the same proteome. This method increased the average coverage of measured protein intensity values to 84.1%, with 77.9% the lowest coverage and 88.5% the highest coverage.

First, we compiled a list of potential predictor proteins for which measured protein intensities for the three cell fractions (PP, PC, or whole liver) were available for all 6 mice. This resulted in three sets of 1,482, 1,571, and 1,064 predictor proteins. Second, for a given target protein with missing protein intensity values in some mice, we determined a group of significantly Pearson-correlated ($P < 0.05$) predictor proteins also exhibiting a Spearman correlation larger than a critical threshold value $\rho > 0.95$. Linear regression analysis

with each of these predictor proteins yielded estimates of the missing values. Finally, the missing intensity values were filled by the mean of the estimates obtained for all predictor proteins. For the 6 mice included in this study, the measured and imputed label-free quantification (LFQ) intensities for the proteins of the kinetic model are available for the reference cells, PPHs and PCHs in Supporting Table S4.

MODEL-BASED FUNCTIONAL CHARACTERIZATION OF PPHs AND PCHs

For the assessment of functional differences between PPHs and PCHs, we used zone-specific kinetic models to compute steady-state load characteristics describing the change of a metabolic function in response to changes in the concentration of a distinct plasma metabolite. Steady-state model computations assume that the characteristic time for the change of the plasma metabolites is much longer than the characteristic time required for establishing a stationary state of the metabolic network where the time derivatives of all metabolites are practically zero.

Given that the cellular metabolic response depends on the extracellular concentrations of insulin, glucagon and all exchangeable external metabolites, we were confronted with a quasi-continuum of external conditions at which load characteristics can be constructed. Therefore, we decided to choose two different types of external conditions. For load characteristics at varying plasma levels of glucose, we used the phenomenological transfer functions, glucose-hormone transfer and hormone-phosphorylation transfer function (see Materials and Methods) to compute the plasma concentrations of insulin and glucagon from the plasma glucose level and the phosphorylation state of hormone-sensitive interconvertible enzymes. For all other load characteristics, we fixed the external conditions in strict correspondence to the assay conditions applied in the experiments that were used to calibrate the generic kinetic model, Hepatokin1. The external conditions for each load characteristic are given in Supporting Table S5.

Zonation of Carbohydrate Metabolism

The maximal gluconeogenic capacity of PPHs and PCHs was analyzed by computing the exchange flux through the glucose transporter at varying concentrations

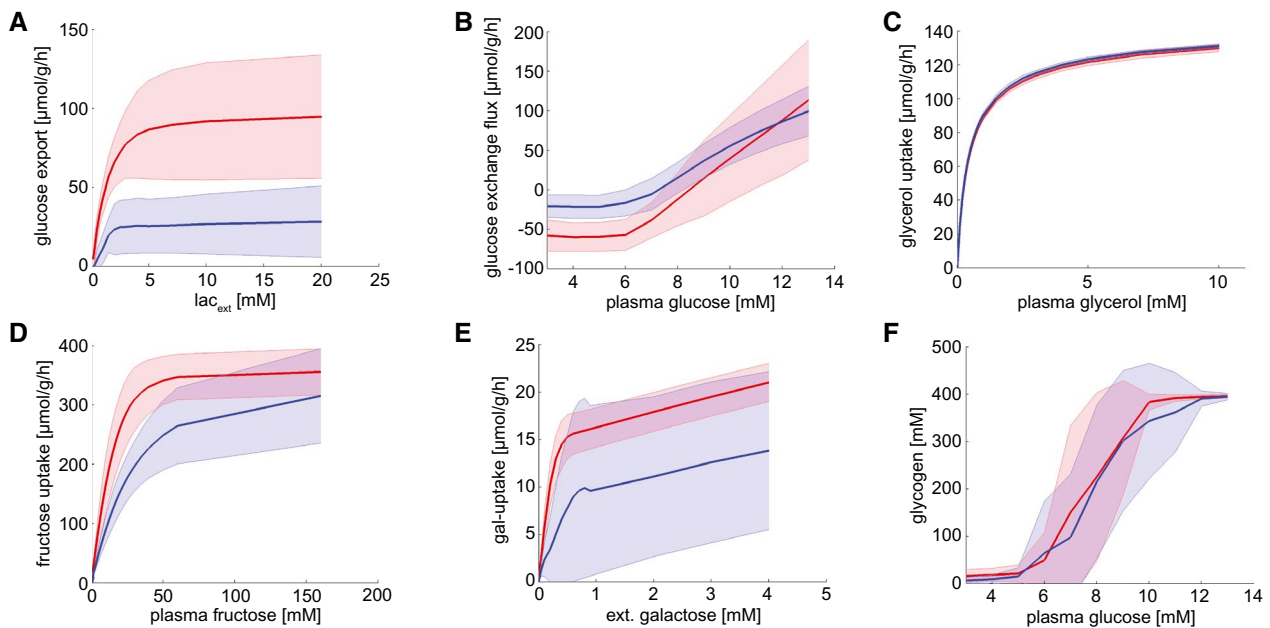


FIG. 3. Differences in hepatic carbohydrate metabolism of PP and PC hepatocytes. Red, PPH; blue, PCH. Solid lines represent the mean of 6 animals, and shaded areas indicate the standard deviations. The external conditions for each load characteristics are given in Supporting Information Supplement S6. (A) Rate of gluconeogenesis in dependence of plasma lactate concentration. (B) Glucose exchange flux in dependence of plasma glucose concentration. (C) Glycerol uptake flux in dependence of plasma glycerol concentrations. (D) Fructose uptake flux in dependence of plasma fructose concentration. (E) Galactose uptake flux in dependence of plasma galactose concentration. (F) Glycogen content in dependence of plasma glucose concentration.

of the precursor metabolite, lactate (Fig. 3A). Given that lactate is converted to pyruvate, this calculation provides a realistic assessment of the gluconeogenic capacity even under conditions where amino acids serve as the major gluconeogenic substrate. PPHs are predicted to possess a significantly larger gluconeogenic capacity than PCH, and the maximal capacities of the two cell variants differ by more than a factor of 2. For both cell types, the half-maximal rate of gluconeogenesis is reached at lactate concentrations of ~ 1 mM, representing the normal physiological plasma value.

Differential capacities of gluconeogenesis and glycolysis in PPHs and PCHs have a large impact on glucose exchange with the blood. Figure 3B depicts the glucose exchange flux as a function of the external glucose concentration in a range between 3 and 12 mM, spanning physiological conditions from fasting (low insulin, high glucagon) to feeding (high insulin, low glucagon).⁽⁸⁾ PPHs are more responsive to changes in plasma glucose levels; that is, PPHs are more gluconeogenic at low glucose concentrations and become more glycolytic at high glucose concentrations. At physiological plasma concentrations of glucose in the range

of 6–8 mM, PCHs are more glycolytic than PPHs. Importantly, the set point (i.e., the external glucose concentration at which the hepatocyte switches from glucose production to glucose consumption) shifts from 6.5 mM for PCHs to 8 mM for PPHs.

The capacity of PPH and PCH to metabolize glycerol and the two important food-derived monosaccharides, fructose and galactose, is depicted in Fig. 3C–E. Although there was no zonal difference in the glycerol uptake rate, PPHs had a higher capacity to metabolize fructose and galactose than PCHs. Figure 3F shows that the ability to store glycogen is equal in the two zones.

Zonation of Fatty Acid Metabolism

Zone-dependent differences in the metabolism of fatty acids were examined by computing the cellular uptake rate of free fatty acids (FFAs) and the rate of central pathways of lipid metabolism in dependence of external FFA concentrations (Fig. 4). Up to external concentrations of FFAs of ~ 0.8 mM, the uptake rate of FFAs is independent of the zonal localization

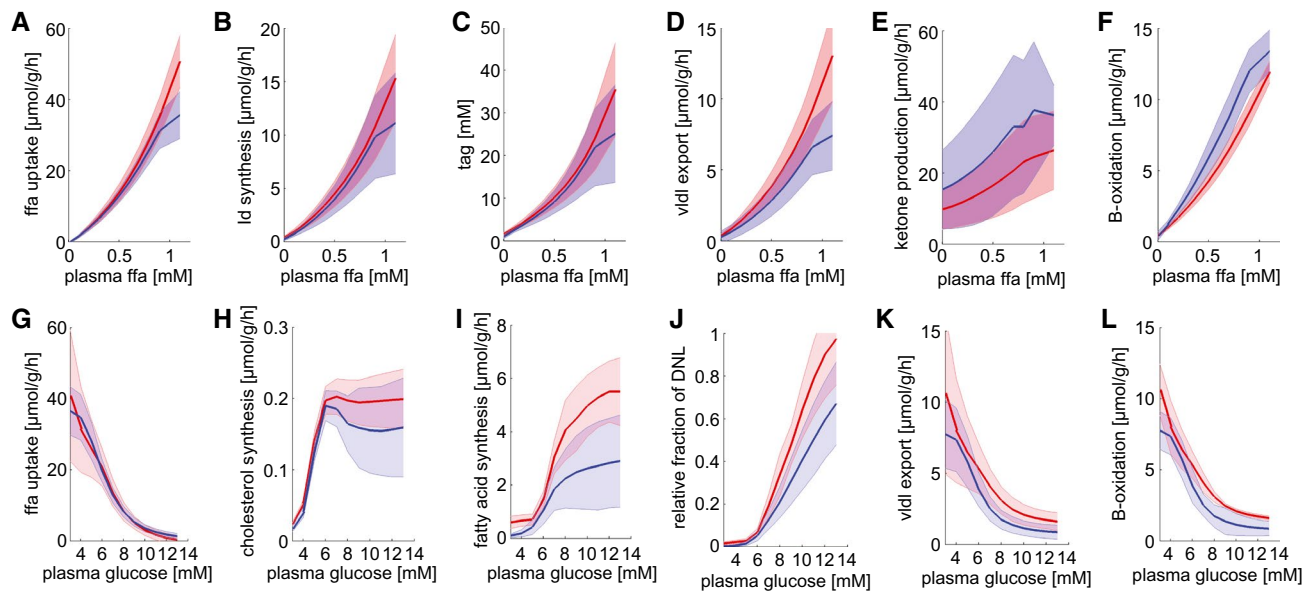


FIG. 4. Functional differences in hepatic fatty acid metabolism of PP and PC hepatocytes. Red, PPH; blue, PCH. Solid lines represent the mean of 6 animals, and shaded areas depict standard deviations. See Supporting Information Supplement S6 for the external concentrations of hormones and exchangeable metabolites used for the computation of the load characteristics. (A) Fatty acid uptake rate in dependence of plasma fatty acid concentrations. (B) Rate of TAG synthesis in dependence of fatty acid uptake rate. (C) Rate of TAG content in dependence of fatty acid uptake rate. (D) Rate of VLDL export in dependence of fatty acid uptake rate. (E) Rate of ketone body production in dependence of fatty acid uptake rate. (F) Rate of β -oxidation in dependence of fatty acid uptake rate. (G) Rate of fatty acid uptake in dependence of plasma glucose concentrations. (H) Rate of cholesterol synthesis in dependence of plasma glucose concentration. (I) Rate of fatty acid synthesis in dependence of plasma glucose concentration. (J) Relative share of *de novo* synthesized TAG (DNL) on total fatty acid utilization. (K) Rate of VLDL secretion in dependence of plasma glucose concentrations. (L) Rate of β -oxidation in dependence of plasma glucose concentrations. Abbreviation: Id, lipid droplet.

of the hepatocyte. At higher plasma concentrations, the uptake rate of PCH reaches saturation, whereas the uptake capacity of PPHs does not. The same saturation kinetics in the load characteristics for PCHs were observed in the synthesis rate of triacylglycerol (TAG), the size of the TAG pool, and the synthesis rate of very-low-density lipoprotein (VLDL), which, however, differed only marginally in PPHs and PCHs at lower concentrations of FFAs. A stronger response of PCH to variations of external concentrations of FFAs was predicted for the β -oxidation of FFAs and the synthesis of ketone bodies.

Hepatic uptake of glucose and FFAs is counter-regulated. At low plasma glucose levels, the liver operates as a glucose producer, and lipolysis in adipose tissue increases the plasma level of FFAs and promotes their uptake into hepatocytes. At high plasma glucose levels, the liver operates as a glucose consumer, and the high insulin level promotes the uptake and esterification of FFAs in adipose tissue. This inverse relationship between the plasma glucose level and FFA

uptake is reflected in the load characteristics shown in Fig. 4G. The load characteristics indicate that for glucose concentrations above 4 mM, PPHs and PCHs do not differ in the glucose-dependent uptake of FFAs. However, clear differences exist in the glucose dependency of FFA-utilizing pathways, such as the synthesis of cholesterol, FFAs, and VLDL. Generally, PPHs appear to possess a higher lipid-synthesizing capacity than PCHs. This is also reflected in a higher share of *de novo* synthesized TAG (*de novo* lipogenesis; DNL) in the total cellular TAG pool.

Zonal Differences in Detoxification Capacities

Clearance of potentially harmful metabolites from the blood is one of the most important liver functions. We analyzed the capability of PPHs and PCHs to extract ammonia and ethanol from the plasma. In the liver, ammonia can be fixed in the form of either urea or glutamine. The urea cycle catalyzes

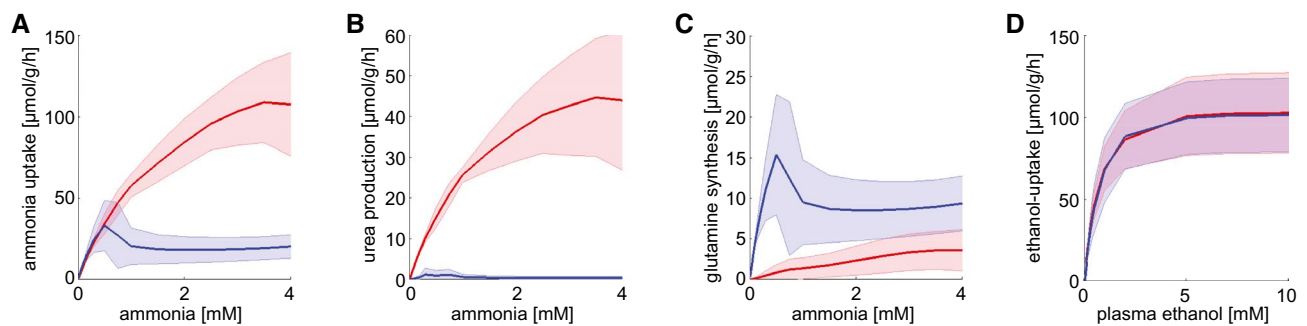


FIG. 5. Comparison of ammonia and ethanol detoxification in PP and PC hepatocytes. Red, PPH; blue, PCH. Solid line represents mean of 6 animals, and shaded area depicts standard deviation. (A) Rate of ammonia uptake in dependence of plasma ammonia concentrations. (B) Rate of urea production in dependence of plasma ammonia concentrations. (C) Rate of glutamine synthesis in dependence of plasma ammonia concentrations. (D) Rate of ethanol uptake in dependence of plasma ethanol concentrations.

irreversible ammonia fixation, whereas ammonia fixation by glutamine synthesis is reversible, given that ammonia can be liberated from glutamine by glutaminases. Figure 5A–C depicts the rates of ammonia uptake, urea production, and glutamine synthesis in PPHs and PCHs at varying external ammonia concentrations. Averaged across the 6 animals, the ammonia uptake was ~4 times higher in PPHs and the metabolic fate of ammonia was completely different in the two cell fractions. Whereas PPHs fix the ammonia almost exclusively in the form of urea, PCHs have a very high rate of glutamine synthesis. No zone-dependent differences were obtained for ethanol detoxification.

Zonal Differences in Energy Metabolism

Given that PPHs and PCHs exhibit differences in central energy delivery and energy consumption pathways of carbohydrate, fatty acid, and ammonia metabolism, it seems obvious that there are also zonal differences in the energetic state of the two cell fractions. We thus analyzed the energetic state (measured by the adenylate charge and the ATP/ADP ratio), rate of gluconeogenesis, rate of ammonia detoxification, and rate of TAG synthesis in response to varying oxygen concentrations (Fig. 6A–H). Our computations predicted a significantly lower ATP/ADP ratio and adenylate charge of PCHs than of PPHs. Gluconeogenesis of PCHs was also more sensitive to falling oxygen pressure. Generally, all metabolic

functions become independent of oxygen pressure at $pO_2 > 25$ mm Hg because of oxygen saturation of complex IV of the respiratory chain.

Discussion

METABOLIC ZONATION: THE QUANTITATIVE VIEW

The aim of our work was to unravel the functional implications of the heterogeneous allocation of protein mass to the central metabolism of hepatocytes residing in the portal and central regions of the acinus. Numerous articles have already reported on the zonation of metabolic enzymes revealed at the transcriptional and translational levels of gene expression or by directly assaying enzyme activities. However, inferring from such data the flux changes of the related metabolic pathways is problematic in view of the enzyme-regulatory mechanisms operating after gene expression.⁽¹⁶⁾ Therefore, we have chosen an approach that combines experimentally determined protein abundances in zone-specific cell fractions with a kinetic model of hepatocyte metabolism that has been extensively validated based on metabolic experiments with hepatocytes, liver tissue, and perfused livers. This approach enabled us to overcome the conventional, ontology-based classification of zone-dependent pathway capacities into “higher or lower or equal” by a quantitative assessment of cell functions in response to typical changes of the blood plasma.

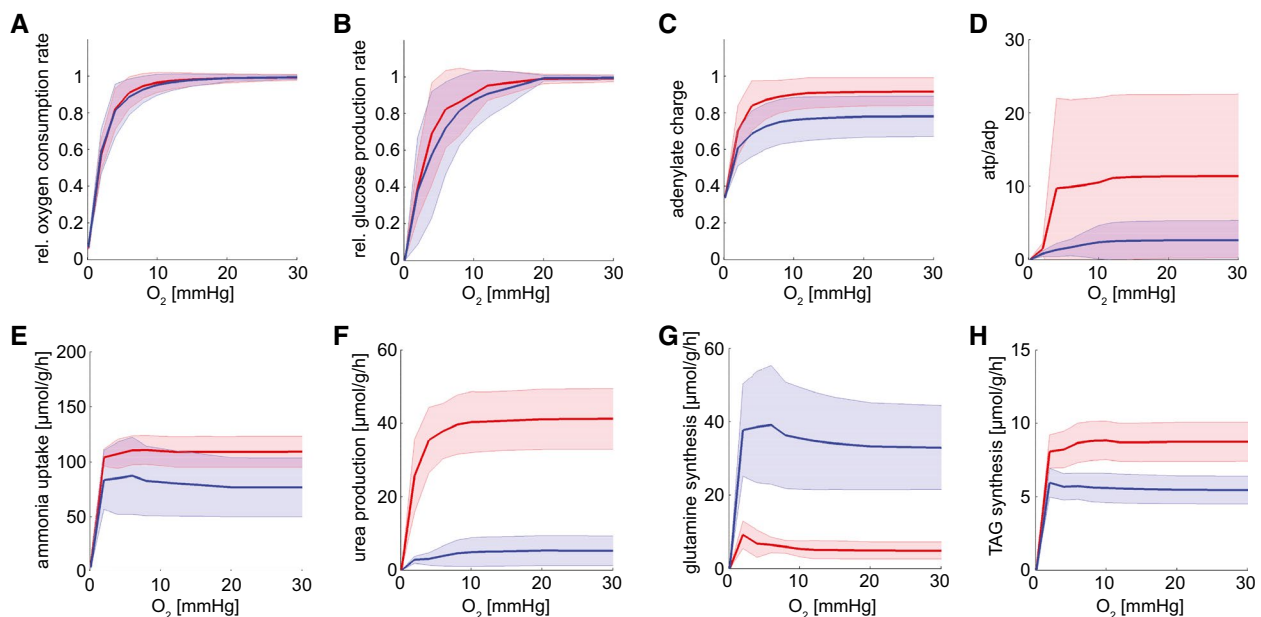


FIG. 6. Metabolic functions of PPH and PCH in dependence of oxygen concentration. Red, PPH; blue, PCH. Solid line represents mean of 6 animals, and shaded area depicts standard deviation. (A) Relative rate of oxygen consumption. (B) Relative rate of glucose production. (C) Adenylate charge $AC = \frac{ATP + 0.5 ATP}{AMP + ADP + ATP}$. (D) ATP/ADP ratio. (E) Uptake rate of ammonia. (F) Rate of urea production. (G) Rate of glutamine synthesis. (H) Rate of TAG synthesis. Abbreviation: rel., relative.

ISOLATION OF PP AND PC HEPATOCYTES BY FLOW CYTOMETRY

During the past two decades, successful attempts to isolate pure hepatocyte populations from whole liver by flow cytometry have been made,⁽¹⁷⁻²⁰⁾ overcoming hurdles such as the vast hepatocyte size and intense autofluorescence. However, up to now, flow cytometry has been used predominantly for the isolation and study of hepatocyte populations derived from the whole liver, and early attempts at the acinar zone-specific isolation of hepatocytes by cell sorting did not prevail.⁽²¹⁾ More recently, imaging techniques, such as immunohistochemistry and *in situ* hybridization, have been developed, enabling the isolation of cells based on the presence of specific marker proteins.⁽²²⁾ For the separation of hepatocytes from the PP and PC regions by flow cytometry, we exploited the fact that surface protein E-Cad and the metabolic enzyme, GLUL, are strictly zoned in the mouse acinus.^(23,24) The analysis of the proteomic data of our sorted cells confirms the classical pericentral zoned proteins such as GLUL, ornithine aminotransferase (OAT), cytochrome P450

family 7 subfamily A member 1 (Cyp7A1) as well as many proteins of drug metabolism (e.g., CYP2E1, CYP1A2, and cytochrome P450, family 2, subfamily c, polypeptide 29 [CYP2C29]) for which heterogeneous distribution has been known for many years.^(25,26) The PP fraction also contains the classical representatives, such as histidine ammonia lyase (HAL), mitochondria proteins like sideroflexin 1 (SFXN1), cytochrome C oxidase subunit 7A1 (COX7A1),^(5,27) as well as hydroxysteroid 17-beta dehydrogenase 6 (HSD17B6)⁽²⁸⁾ and polymeric immunoglobulin receptor (PIGR).⁽²⁹⁾ In addition, the analyses also show a clear zonation of proteins, that the heterogeneous distribution of which along the liver acinus has not been known, such as galectin 1 (LGALS1) as well as shroom family member (SHROOM), in the PCH fraction. However, if looking at the functions of these proteins, it does not seem so unlikely that they are subject to a clear zonation. For example, the strongly zoned expression of galectin could also be confirmed by its function as an immune/inflammatory regulator which acts both extra- and intracellularly, modulating innate and adaptive immune responses.⁽³⁰⁾ In the past decade, evidence shows that many processes of innate

immunity are also not homogeneously distributed in the liver. In this regard, MacParland et al. could show that the PC localized endothelial cells probably have the largest proportion of the innate immune response in the healthy liver.⁽³¹⁾ Taken together, this separation technique improved the specificity of isolated hepatocytes from the different zones compared to the commonly used digitonin-collagenase perfusion.⁽³²⁾ Proteomics-based determination of enzyme abundances in the separate cell fractions and usage of protein abundances in an advanced kinetic model of hepatocyte metabolism enabled us to make *in silico* predictions of the metabolic performance of PPHs and PCHs. The results obtained by our systems biology approach confirmed some already-known features and revealed additional features underlying the “division of labor”⁽¹⁰⁾ in liver metabolism.

CARBOHYDRATE METABOLISM

Earlier studies on the zonation of carbohydrate metabolism in the liver have provided evidence that PPHs and PCHs differ in their gluconeogenic and glycolytic capacities.^(6,33) However, the extent of these zone-dependent differences is now known to be highly dynamic, depending, for example, on nutritional state and circadian rhythms.⁽³⁴⁾ In concordance with results in an earlier work,⁽³⁵⁾ our data suggest that both populations of hepatocytes act as a “glucostat,”⁽³⁶⁾ producing glucose when blood glucose levels are below a critical set point (fasting) and utilizing glucose when blood glucose levels are above the set point (e.g., after a meal; see Fig. 3B). In agreement with literature data, our model computations suggest that PCHs are more glycolytic than PPHs. In PPHs, the protein abundances of the lactate transporter, glucose transporter 2 (GLUT2), and PCJP, as well as key regulatory enzymes of gluconeogenesis, such as phosphoenolpyruvate carboxylase, fructose biphosphatase 2/phosphofructokinase 2, and glucokinase, are higher than in PCHs. In line with this, Ogawa et al.⁽³⁷⁾ found a stronger expression of the insulin-independent, but concentration-dependent, glucose transporter, GLUT2, in PPHs.

Gluconeogenesis from lactate is reported to be more prominent in PPHs.⁽³⁸⁾ This is confirmed by our computations, suggesting a remarkably diminished gluconeogenic capacity of PCHs (Fig. 3A). Our computations also suggest zone-dependent differences

in the utilization of the two important food-derived monosaccharides, galactose and fructose (see Fig. 3D,E). Several enzymes involved in the metabolism of these monosaccharides, such as aldolase B (portal/central abundance ratio, $R = 2.48$), triose kinase ($R = 1.88$), GLUT2 ($R = 1.9$), fructokinase ($R = 1.28$), and the galactose transporter ($R = 1.9$), have a higher abundance in cells within the PP region.

LIPID METABOLISM

Fatty acids formed *de novo* (DNL), by lipolysis of TAG stores in lipid droplets or taken up from the plasma, can be used to yield acetyl-CoA in the process of β -oxidation or can be esterified with glycerol to form TAG stored in lipid droplets or lipoproteins (VLDL). Our analysis revealed almost identical uptake rates of FFAs up to plasma concentrations of ~ 0.8 mM, which is higher than the values observed in C57Bl/6N mice under an oral fat load.⁽³⁹⁾ Thus, the observation that the accumulation of fat (steatosis) in adults starts preferentially in the PC region cannot be explained simply by differences in the capacity of PPHs and PCHs. It should be noted, however, that we have assessed the uptake rate of FFAs under conditions of very low external glucose concentrations also applied in related *in vitro* experiments.

Another source for hepatic fatty acids is DNL from cytosolic acetyl-CoA derived from citrate by the ATP-dependent citrate, lyase. Our analysis suggests that PPHs synthesize more FFAs and possess a higher fraction of synthesized TAG attributable to the higher abundance of fatty acid synthase (PP/PC, $R = 1.4$), acetyl-CoA carboxylase ($R = 1.2$), and ATP citrate lyase ($R = 2.8$) compared with PCHs. The literature findings on the zonation of DNL are contradictory. Our results are in line with those of Evans et al.,⁽⁴⁰⁾ who showed a preponderance of all three mentioned enzymes in the PPHs. However, the majority of publications conclude that DNL is more pronounced in PCHs.⁽⁴¹⁾ It is important to note that the relative contribution of DNL to the total pool of cellular fatty acids depends strongly upon the external glucose concentration. A steep increase in DNL occurs at high external glucose concentrations when plasma concentrations of FFAs are low and the uptake rate of fatty acids is low in both PPHs and PCHs (Fig. 4J).

Regarding the synthesis and export of VLDL, our analysis predicts a strong increase with increasing

concentrations of FFAs in concordance with previous findings.⁽⁴²⁻⁴⁴⁾ The export capacity for VLDL of PPH exceeds that of PCH regardless of the fatty acid uptake rate (Fig. 4D). In particular, at low glucose, the VLDL release rate in PPHs is significantly higher than in PCHs. This model-based prediction is supported by the fact that some marker proteins involved in the assembly and secretion of VLDL particles are enriched in PPHs, including microsomal triglyceride transfer protein (PP:PC, R = 1.24), protein disulfide-isomerase A (R = 2.1), and vesicle-trafficking protein SEC22 homolog B (R = 1.6).

Likewise, we found higher rates of cholesterol synthesis in PPHs than in PCHs (Fig. 4H). In agreement with this finding, Cheng et al.⁽⁴³⁾ also found a predominance of cholesterol synthesis in the PP zone. Moreover, Li et al.⁽⁴⁵⁾ and Singer et al.⁽⁴⁶⁾ reported that hydroxymethylglutaryl coenzyme A reductase, the main regulatory enzyme of cholesterol synthesis,⁽¹¹⁾ is almost exclusively found in the first hepatocytes in the PP area. A higher capacity for cholesterol synthesis in PPHs than in PCHs fits well with the higher VLDL secretion rates, given that cholesterol and cholesterol esters are major constituents of VLDL particles. The higher capacity of PPHs than PCHs to export TAG in terms of VLDL could also explain the disease pattern of early hepatic steatosis in adults, which is predominantly PC centered.^(41,47) Understanding differences in the lipid metabolism of PPHs and PCHs *in vivo* also requires taking into account portocentral differences in the sinusoidal blood flow and plasma concentration of metabolites (in particular oxygen, glucose, and FFAs) and hormones.

AMMONIA METABOLISM

In addition to the zonal effects of carbohydrate metabolism, the ammonia detoxification is the best examined zoned liver function. The liver possesses two alternative pathways for ammonia detoxification, which are strictly separated along the portocentral axis: conversion to urea or glutamine. This view is confirmed by our analysis. Interestingly, the total capacity for ammonia detoxification in the physiological range (plasma levels of ammonia <0.5 mM) is almost the same in the two zones (Fig. 5A). That is, despite large zonal differences in the capacity of the urea cycle and glutamine synthesis, significant portocentral differences in the total ammonia-detoxifying

capacity are expected for external ammonia concentrations that lie far above the physiological value of ~80 μ M in mice.⁽⁴⁸⁾ In the PP zone, ammonia detoxification is achieved almost exclusively by urea formation, whereas in the PC zone, glutamine synthesis accounts for almost all ammonia removal. The strict division of ammonia detoxification between the urea cycle and GLUL fits in with the work of Braeuning et al.,⁽²⁹⁾ who found a complete lack of GLUL in the PP zone and a preferential location (PP/PC abundance ratios of 1.5-1.77:1) of mRNAs of urea cycle enzymes (ornithine transcarbamylase, argininosuccinic acid synthetase, argininosuccinic acid lyase, and arginase) in the PP area. We found protein abundance ratios of 0.84, 1.64, and 4.72 for the three enzymes. More importantly, CPS1, the main regulatory enzyme of the urea cycle, occurs with 2.76-fold higher abundance in the PP zone than in the PC zone.

ENERGY METABOLISM

Hitherto, little was known about the zonal distribution of adenine nucleotides and energetic state between the PP and PC zones. The proteomics data point to an increased capacity of PPHs, compared to PCHs, to generate ATP by oxidative phosphorylation because of the increased abundance of some proteins of the respiratory chain, including complexes I-IV (R = 1.2-2.2), F₀F₁-ATPase (R = 1.76), adenine nucleotide translocator (R = 1.73), and mitochondrial phosphate exchanger (R = 1.53). The better endowment of PPHs with proteins of oxidative phosphorylation does not necessarily imply a higher ATP level, given that PPHs also have a higher capacity for excessively ATP-demanding processes, such as gluconeogenesis and urea synthesis, than PCHs. Balancing the opposing facts against each other, the model computations suggest a higher adenylate charge as well as a higher ATP/ADP ratio in PPHs than in PCHs. This finding is in agreement with the portal-to-central declining ATP level measured in a millifluidic media perfusion system that enables cells to be cultured in a slow-flow environment. Closer inspection of the regulatory circuits entailing this energetic difference between PPHs and PCH yields the following explanation: mutual regulation of fatty acid oxidation and aerobic glycolysis as the main source of ATP production proceeds by reduced nicotinamide adenine dinucleotide- and

acetyl-CoA–dependent inhibition of the pyruvate dehydrogenase (PDHC) reaction, the entry point of glucose-derived carbons into the citric acid cycle. At reduced rates of β -oxidation, rising nicotinamide adenine dinucleotide and CoA levels activate PDHC, rendering glycolytic pyruvate the prevailing energy-delivering substrate. The switch from fatty acids to glucose goes along with a falling mitochondrial redox state and a transient and moderate down-regulation of the respiratory chain and ATP production rate that is subsequently compensated for by a kinetic up-regulation of the respiratory chain as a consequence of the reduced ATP level. Therefore, the computed zonal differences in ATP levels reflect zonal differences in substrate preference rather than in the local energy demand of hepatocytes.

ETHANOL METABOLIZATION

In the healthy liver, ethanol is predominantly degraded by alcohol dehydrogenase (ADH), converting ethanol to acetaldehyde and the acetaldehyde dehydrogenase (ALDH) converting acetaldehyde to acetate. Zone-dependent expression differences with a PP:PC value of $R = 1.6$ for ADH has been reported by Salsano et al.,⁽⁴⁹⁾ whereas our data yield $R = 1.2$ for ADH and $R = 0.45$ – 4.50 for mitochondrial ALDHs. Based on these zone-dependent expression differences, our model yielded only marginal differences in the ethanol detoxification capacities of PPHs and PCHs (Fig. 5D).

METABOLIC CAPACITIES OF THE INTERMEDIATE ZONE

While the focus of our study was clearly on the elucidation of possible metabolic differences between the periportal and pericentral zone, we also analyzed the metabolic capacities of E-Cad-negative and chondroitin sulfate-negative cells stemming from the intermediate zone (preliminary data given in Supporting Table S6). These preliminary results suggest that the intermediate zone has comparable capacities for the uptake and release of glucose as the PC zone whereas the uptake capacities for fructose and galactose correspond to those of the PP zone. The capacities for TAG synthesis and VLDL export were higher and the capacity for β -oxidation was slightly lower than in the PP and PC zones. Ammonia uptake

and metabolization were comparable to the PP zone, whereas ethanol detoxification was constant throughout all three zones. The capacities for the synthesis of fatty acid and cholesterol synthesis were just between those of the PP and PC zone.

Our analysis shows that PPHs and PCHs exhibit significant differences in the protein abundance of approximately two-thirds of metabolic enzymes. These differences result from the fact that extracellular conditions, (e.g., nutrients and hormones), have a strong impact on gene expression, which vary along the sinusoidal bloodstream.⁽⁹⁾ Our model-based analysis suggests that the functional differences implied by unequal enzyme abundances along the portocentral axis depend essentially on the actual plasma concentrations of metabolites and hormones. For example, despite large zonal differences in the capacity of the urea cycle and glutamine synthesis, significant portocentral differences in the total ammonia-detoxifying capacity are only expected for external ammonia concentrations that are far above the physiological value. Similarly, clear discrepancies in TAG synthesis and VLDL export are predicted to occur at FFA concentrations above the physiological value of ~ 1 mM.⁽⁵⁰⁾ Generally, it appears that portal-to-central differences in liver metabolism become more pronounced if the plasma concentrations of glucose and FFAs start to deviate from their physiological values. It must be emphasized that this conclusion holds only for short-time deviations where the liver's proteome does not adapt to a nutritional status that is different from the well-fed nutritional status of the mice in our study.

How variable gene expression will change the metabolic performance of PPHs and PCHs under diabetes, hyperammonia, or dyslipidemia still remains to be addressed to gain a comprehensive mechanistic view of liver metabolism.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.31274/supinfo.