Carbohydrate-response Element-binding Protein Deletion Alters Substrate Utilization Producing an Energy-deficient Liver*§

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Livers from mice lacking the carbohydrate-responsive element-binding protein (ChREBP) were compared with wild type (WT) mice to determine the effect of this transcription factor on hepatic energy metabolism. The pyruvate dehydrogenase complex was considerably more active in ChREBP (WT) mice to determine the effect of this transcription factor on hepatic energy metabolism. The pyruvate dehydrogenase complex was considerably more active in ChREBP (WT) mice to determine the effect of this transcription factor on hepatic energy metabolism. The pyruvate dehydrogenase complex was considerably more active in ChREBP (WT) mice to determine the effect of this transcription factor on hepatic energy metabolism. The pyruvate dehydrogenase complex was considerably more active in ChREBP (WT) mice to determine the effect of this transcription factor on hepatic energy metabolism.

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ent protein kinase/protein phosphatase 2A. Phosphorylation of ChREBP by protein kinase A and AMP-dependent protein kinase prevents ChREBP entry into the nucleus and inhibits DNA binding, whereas dephosphorylation by the xylulose-5-P-activated protein phosphatase 2A activates ChREBP.

Previous studies (9) of the phenotypic differences between WT and ChREBP−/− mice fed a normal chow diet showed that the ChREBP−/− mice had an increase in plasma glucose from 157 to 190 mg/dl, a 2-fold increase in liver glucose-6-P concentration and a 3-fold increase in liver glycogen compared with control mice. These animals also have a markedly higher liver phosphoenolpyruvate and lower pyruvate concentrations, reflecting their lower LPK activity (10). As a result of the lower levels of most of the enzymes of fatty acid synthesis pathway and also malic enzyme, the ChREBP−/− mice have reduced epididymal fat and brown fat mass and lower serum free fatty acid concentration (10). Hepatic triglyceride content of ChREBP−/− mice were not different from WT mice, reflecting unaltered storage of dietary fat in these animals. Nevertheless, the ChREBP−/− liver appears as though it is deficient in metabolizable substrate, despite a surfeit of energy stores in glycogen and triglyceride, presumably because of reduced rates of lipolysis in adipose tissue and by a decrease in glycolytic flux secondary to a decreased LPK activity.

Remarkably, attempts to feed a high sucrose or high fructose diet to ChREBP−/− mice lead to a further decrease in plasma free fatty acids and death by hypothermia and starvation (10), suggesting that the defect in fatty acid synthesis induced in ChREBP−/− mice leads to a deficit of the important energy and heat-producing substrate, free fatty acids. It further suggests that adequate fatty acid synthesis and free fatty acid release is a prerequisite for homothermic life. We therefore examined more thoroughly the effects of ChREBP deletion on hepatic substrate utilization, cellular redox state, and energy status.

**EXPERIMENTAL PROCEDURES**

**Materials and General Methods**—Measurement of hepatic metabolites were performed using slight modifications of conventional methods of enzymatic analysis (11, 12). 13C-Labeled materials were purchased from Cambridge Isotopes (Andover, MA).

**Animal Treatments**—ChREBP gene-deficient (ChREBP−/−) mice were generated, and the phenotypic characteristics were presented previously (9, 10). Mice were housed in colony cages with a 12-h light/12-h dark cycle and fed a standard chow diet containing 6% fat (Teklad Mouse/Rat Diet 7002 from Harlan Teklad Premier Laboratory Diets) unless otherwise noted. All mice were sacrificed at the end of the dark cycle. For measurement of liver metabolites, mice were sacrificed by cervical dislocation, and the liver was removed and freeze-clamped. For liver perfusion, animals were anesthetized, and the livers were removed for perfusion, with subsequent sacrifice of the animal. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center.

**Substrate Utilization in Perfused Liver**—Three days prior to liver perfusions, mice began eating a high starch diet (60% starch, 20% casein, 15% cellulose, 2.5% vitamins, and 2.5% minerals). On the day of the experiment, livers were isolated from fed mice and perfused in a nonrecirculating preparation at 8 ml/min via the portal vein as described previously (13). The perfusion medium consisted of a standard Krebs-Heinslet buffer containing 1.5 mM [U-13C3]lactate, 0.15 mM [U-13C3]pyruvate, 8 mM [1-13C]glucose, and 0.05 mM free fatty acids (algal mixture of 11.6% palmitoleic, 39.1% palmitic, 14.6% linoleic, and 34.81% oleic acid) and 3% albumin. Livers were perfused for 75 min, and perfusate samples were collected every 15 min to measure oxygen consumption and ketone production. Livers were freeze-clamped, extracted with perchloric acid, and centrifuged. Acid extracts were neutralized with KOH, centrifuged to remove precipitant, and freeze-dried. Extracts were reconstituted in 500 μl of D2O for 13C NMR analysis.

Carbon-13 NMR was performed as described previously (13). Briefly, proton decoupled 13C NMR spectra of liver extracts were acquired in a 5-mm Varian broadband probe using a 45° pulse, 1.5-s acquisition time, and a 1.5-s post-acquisition delay. A WALTZ-16 sequence was used for broadband proton decoupling. 13C NMR multiplets were deconvoluted using NUTS (Acorn NMR, Fremont, CA).

Multiplets from [13C]glutamate resonances were used to determine substrate oxidation, anaplerosis, pyruvate cycling, and gluconeogenesis, all relative to tricarboxylic acid cycle flux. Nonsteady state equations were used to determine relative substrate oxidation of lactate and pyruvate, glucose or FFA, using the C3 and C4 multiplets as described previously (14, 15). The fraction of FFA oxidation in the tricarboxylic acid cycle represents the total fat oxidized from both the perfusate and endogenous triglycerides. Anaplerosis, pyruvate cycling, and gluconeogenesis were determined from a steady state analysis of glutamate multiplets C2, C3, and C4 using the program tcaCALC (16). In this model, program parameters LAC3, LAC123, anaplerosis, PDC, and PK were allowed to change. YPC (anaplerosis) and PK (pyruvate cycling) are reported relative to total tricarboxylic acid cycle turnover. Gluconeogenesis PEP is taken as the difference between YPC and pyruvate cycling (PK + ME). Pyruvate cycling represents a maximal estimation of LPK flux (relative to tricarboxylic acid cycle turnover) because malic enzyme flux cannot be distinguished from LPK flux.

**Immunoblot Analysis**—Tissue powders (50 mg) prepared under liquid nitrogen were homogenized with 0.5 ml of extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5% (v/v) Triton X-100, 2% (v/v) bovine serum, 1 μM leupeptin, 10 μM l-1-tosylamido-2-phenylethyl chloromethyl ketone, and 10 μg/ml trypsin inhibitor). Protein concentrations of tissue extracts were determined with a protein assay kit (Bio-Rad) using bovine serum albumin as standard. Fifty μg of protein were separated on a 12.5% (w/v) SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schüll) by the semidyrid electroblotting method. Western blot analysis was carried out as described previously (17) using an enhanced chemiluminescence immunodetection system (Roche Diagnostics). Immunoblotting was performed with polyclonal rabbit antisera against PDK1, PDK2, PDK3, PDK4, and PDC.

**Measurement of Enzyme Activities and Metabolites**—The activities of PDC and its kinase were measured in Triton X-100
tissue extracts as described previously (18). The metabolites were determined as described (7, 9). ATP was measured spectrophotometrically using hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) by the method described previously (19). All enzymes used were purchased from Sigma.

Calculation of Free Cytosolic Adenine Nucleotides and Nucleotide Ratios in Cytoplasm and Mitochondria—Measurement of total pyridine nucleotides, because of their impermeance to mitochondrial membrane and binding, give no information of the redox state in compartments of interest. Likewise because of intramitochondrial segregation of the bulk of cellular ADP in mitochondria, measurement of total ADP gives no information on the cytosolic free ADP concentration. Therefore, the free nucleotide ratios and numbers were calculated from the measured metabolites of near-equilibrium reactions in the cellular compartment of interest as indicated (20). The free cytosolic [NAD\(^+\)]/[NADH] was calculated from the measured substrates of the lactate dehydrogenase reaction where the \( K_{eq} \) is \( 1.11 \times 10^{-11} \) as shown in Equation 1,

\[
\text{[NAD}^+]_c = \frac{[\text{pyruvate}]}{[\text{lactate}]} = \frac{10^{-7.2}}{K_{LDH}}
\]

(Eq. 1)

The free mitochondrial [NAD\(^+\)]/[NADH] ratio was calculated from the measured substrates of the glutamate dehydrogenase (GIDH) reaction where the \( K_{GIDH} \) is \( 3.87 \times 10^{-13} \) as shown in Equation 2,

\[
\frac{[\text{NAD}^+]_m}{[\text{NADH}]_m} = \frac{[\alpha\text{-ketoglutarate}^2][\text{NH}_3]}{[\text{glutamate}]} = \frac{1}{K_{GIDH}}
\]

(Eq. 2)

The free cytosolic [NADPH]/[NADP\(^+\)] ratio was calculated from the measured substrates of the isocitrate dehydrogenase (IcDH) reaction where the equilibrium constant, \( K_{IcDH} \) is 1.17 M, and the CO\(_2\) concentration at pH 7.2 was assumed to be 1.99 mM given an intracellular [HCO\(_3\)-] of 25 mM as shown in Equation 3,

\[
\frac{[\text{NADPH}]}{[\text{NADP}^+]_c} = \frac{[\text{isocitrate}^2]}{[\alpha\text{-ketoglutarate}^2][\text{NH}_3]} = \frac{1}{K_{IcDH}}
\]

(Eq. 3)

The free cytosolic [ATP]/[ADP] × \([P_i]\) ratio, or phosphorylation potential, was calculated from the substrates of the combined glyceroldehyde-3-phosphate dehydrogenase reaction, the 3-phosphoglycerate kinase reaction, the equilibrium constant of the combined reactions, which is designated as \( K_{G+G'} \), and the triose-phosphate isomerase reaction, and the lactate dehydrogenase reaction (23).

The \( \Delta G' \) of ATP hydrolysis was calculated from the \( \Delta G^0 \) of ATP hydrolysis determined at 38 °C, \( I = 0.25 \), and free [Mg\(^{2+}\)] of 1 mM to be \(-31.8\) kJ/mol (24) and the phosphorylation potential according to Equation 4,

\[
\Delta G'_{\text{ATP}} = \Delta G^0_{\text{ATP}} - RT \ln \left( \frac{[\text{ATP}]}{[\text{ADP}][P_i]} \right)
\]

(Eq. 4)

Liver ATP was measured at a mean of 3.4 mM and liver \( P_i \) found earlier to be about 4.8 mM. From the \( \Delta G' \) of ATP hydrolysis determined from the measure of dihydroxycetone phosphate, 3-phosphoglycerate, lactate, and pyruvate, the free [ADP] was calculated as described previously (20), and see Equation 5.

\[
\text{free cytosolic [ADP]}_M = \frac{[\text{ATP}]}{[P_i]} \times \text{phosphorylation potential}
\]

(Eq. 5)

The free cytosolic [AMP] was calculated from the equilibrium constant for the myokinase reaction, which is 1.05 at pH 7.0, and free [Mg\(^{2+}\)] = 1 mM (25) by substituting measured [ATP] and the free cytosolic [ADP] in Equation 6,

\[
\text{free cytosolic [AMP]} = \frac{[\text{free cytosolic ADP}]^2 K_{MK}}{[\text{measured ATP}]}
\]

(Eq. 6)

The precise adjustment of the apparent equilibrium constants for variations in free [Mg\(^{2+}\)] where the substrates have significantly different Mg\(^{2+}\) binding constants, or of pH, where substrates have acid dissociation constants close to physiological pH, have been described elsewhere (26).

Mitochondria Isolation and Oxidative Metabolism—Liver mitochondria were isolated as described (27). Briefly, 4 fed WT and ChREBP\(^{-/-}\) mice were killed by decapitation between 08:00 and 09:00 a.m. The livers were quickly removed and placed in cold MSN buffer (220 mM mannitol, 70 mM sucrose, 5 mM MOPS, pH 7.4). A 10% suspension (w/v) of the minced liver containing 2 mM EDTA and 0.2% bovine serum albumin saturated by oxygen bubbling was prepared using Potter-Elvehjem homogenizer. Nuclei and cell debris were removed by centrifugation at 700 × g for 10 min, and mitochondria were isolated by centrifugation of the supernatant at 7000 × g for 10 min twice. The resulting mitochondrial pellet was washed twice with MSN buffer and diluted to ~50 mg of mitochondrial protein per ml. Oxygen consumption rate was measured polarographically in a stirred oxygraph vessel. The respiratory medium consisted of 15 mM KCl, 15 mM K\(_2\)HPO\(_4\), 15 mM KH\(_2\)PO\(_4\), 25 mM Tris-HCl, 45 mM sucrose, 12 mM mannitol, 5 mM MgCl\(_2\), 7 mM EDTA, and 0.2% bovine serum albumin. ADP was added to a concentration of 5 mM, and pyruvate and lactate were added to give a concentration of 10 mM (+2.5 mM malate). Ascorbic acid and tetramethylphenylenediamine were used at 7.2 and 1 mM, respectively. Succinate and rotenone were used at 10 mM and 1.25 mM, respectively.

Measurement of β-Oxidation Rate in Hepatocytes from WT and ChREBP\(^{-/-}\) Mice—β-Oxidation of acyl-CoA was measured as the production of tritiated water from [9,10-\(^{3}\)H]palmitic acid (9). Hepatocytes from WT and ChREBP\(^{-/-}\) mice were allowed to attach to culture wells in Dulbecco’s modified Eagle’s culture medium containing 100 mM dexamethasone, 10 mM insulin, and 100 mM 3,3’,5-triiodothyronine and were incubated for 3 h. Nonadherent cells were removed, and the hepatocyte monolayers were incubated an additional 3 h in Dulbecco’s modified Eagle’s medium containing 0.1 mM palmitate plus [9,10-\(^{3}\)H]palmitic acid (Amersham
Gluconeogenesis from PEP (GNGPEP) was estimated from the difference between anaplerosis and pyruvate cycling. Anaplerosis, cataplerosis, and pyruvate cycling spectra of liver glutamate was used to measure relative fluxes incubated at 37 °C for 24 h to allow H2O in the sample to equilibrate with water in the flask (28). The 13H was counted with a scintillation counter. Under these conditions the water exchange ratio was ~60%.

RESULTS

Substrate Selection in Perfused Liver; ChREBP−/− Mice Shift Hepatic Energy Substrate from Fatty Acids to Pyruvate—Isolated livers were perfused with a mixture of [U-13C]lactate, [U-13C]pyruvate, [1-13C]glucose, and long chain free fatty acids to determine whether hepatic substrate oxidation is altered by ChREBP loss of function. NMR isotopomer analysis of the 13C spectra of liver glutamate was used to measure relative fluxes through substrate oxidation and gluconeogenic pathways (Fig. 1A). Nonsteady state NMR isotopomer analysis of the 13C spectra of liver glutamate was used to determine the fractional oxidation of lactate, pyruvate, and FFAs (15) (Fig. 1B). Lactate and pyruvate oxidation in the hepatic tricarboxylic acid cycle was elevated almost 2-fold in the ChREBP−/− compared with WT mice. Glucose oxidation was minor in both cases and not different between ChREBP−/− and WT mice. The contribution of unlabeled substrates, presumably from fatty acids in the perfusate or endogenous liver triglycerides, accounted for 66% of unlabeled substrate oxidation.

Through substrate oxidation and gluconeogenic pathways (Fig. 1A). Nonsteady state NMR isotopomer analysis of the 13C spectra of liver glutamate was used to determine the fractional oxidation of lactate, pyruvate, and FFAs (15) (Fig. 1B). Lactate and pyruvate oxidation in the hepatic tricarboxylic acid cycle was elevated almost 2-fold in the ChREBP−/− compared with WT mice. Glucose oxidation was minor in both cases and not different between ChREBP−/− and WT mice. The contribution of unlabeled substrates, presumably from fatty acids in the perfusate or endogenous liver triglycerides, accounted for 66% of unlabeled substrate oxidation.
lute lactate and pyruvate oxidation is elevated, whereas absolute FFA oxidation is decreased in ChREBP−/− livers compared with WT. Ketone body production by WT livers was small, as expected with livers taken from well-fed animals, but livers from ChREBP−/− mice produced even less acetacetate and β-hydroxybutyrate, consistent with decreased fatty acid oxidation (Fig. 1C).

Steady state isotopomer analysis of liver glutamate 13C NMR spectra (16) was also performed to determine the effect of ChREBP deletion on hepatic anaplerosis, pyruvate cycling, and gluconeogenesis (Fig. 1D). Anaplerosis and cataplerosis relative to tricarboxylic acid cycle flux were taken to represent combined pyruvate carboxylase (PC) and phosphoenolpyruvate carboxykinase (PEPCK) flux, respectively, relative to tricarboxylic acid cycle turnover and was decreased by more than 2-fold in the ChREBP−/− livers (Fig. 1D). This finding is in agreement with impaired fat oxidation that would tend to decrease PC activity by limiting its allosteric activation by fat-derived acetyl-CoA (29). The acetyl-CoA concentrations in WT and ChREBP−/− livers were 51 nmol/g liver and 32 nmol/g, respectively, although it is not clear whether PC activation distinguishes fat-derived or PDC-derived acetyl-CoA. Increased pyruvate oxidation observed by NMR and radiolabeled techniques (see below) also accounts for decreased flux through PC because less pyruvate would be available for carboxylation. Despite impaired flux through PC and PEPCK pathways, gluconeogenesis from PEP (GNGPEP) relative to tricarboxylic acid cycle flux was unchanged in the ChREBP−/− livers compared with WT (Fig. 1D), because pyruvate cycling was also decreased by 3-fold, thus sparing substrate for gluconeogenesis in ChREBP−/− mice. Pyruvate cycling refers to any pathway that converts tricarboxylic acid cycle intermediates derived from pyruvate back to pyruvate. In the present case, decreased pyruvate cycling presumably results from reduced LPK expression, which would spare PEP from conversion back to pyruvate, or reduced malic enzyme (ME) expression, which would spare malate (and thus oxaloacetate) from conversion to pyruvate. Isotope tracer methods employed here do not distinguish these pyruvate cycling pathways, but suppression of their combined action allows normal gluconeogenesis to be sustained despite impaired pyruvate carboxylase and/or PEPCK flux.

Redox and Energy Status of Liver; the Altered Substrate Utilization in ChREBP−/− Mouse Liver Results in Perturbed Redox States of Mitochondria and Cytosol in a Divergent Manner Lowering the Phosphorylation Potential Creating an Energy-deficient Liver—Analysis of intermediary metabolites in freeze-clamped liver of ChREBP−/− mice fed a normal chow diet ad libitum showed no differences in [citrate] or [isocitrate] compared with WT mice. [Dihydroxyacetone phosphate] and [3-phosphoglycerate] were increased 2.8- and 1.7-fold, respectively, with low expression of LPK in ChREBP−/− mice (Table 1). Pyruvate concentration was reduced 2-fold, compatible with inactivation of LPK and ME expression and increased pyruvate oxidation (Fig. 1). Moreover, the concentration of lactate was increased 1.4-fold, indicating a 3-fold reduction in cytosolic NAD+/NADH redox state in the liver of ChREBP−/− mice relative to WT mice. The more reduced cytosolic NAD+/NADH couple in the liver of ChREBP−/− mice could result from decreased mitochondrial reducing power required for mitochondrial ATP synthesis. Interestingly, there was a 2-fold increase in [α-ketoglutarate] in ChREBP−/− mouse liver but unchanged [glutamate] and [NH4+] (Table 1), indicating that the free mitochondrial [NAD+/NADH] ratio was increased 1.7-fold in the liver of ChREBP−/− mice relative to that of the WT mice (Table 2). The mechanism by which ChREBP−/− livers develop a cytosolic redox state that is more reduced (more free NADH) but a mitochondrial redox state that is more reduced (more free NADH) but a mitochondrial redox state that is more oxidized (less free NADH) remains unclear, but it is likely related to the shift in substrate oxidation between the two phenotypes. Impaired mitochondrial fat oxidation is generally concordant with increased mitochondrial [NAD+] /[NADH] ratio, and both of these conditions are features of the ChREBP−/− liver. The total ATP levels in the ChREBP−/− mice were 24% lower than that of WT livers (Table 1). To determine whether impaired production of mitochondrial reducing equivalents affects mitochondrial ATP synthesis, the metabolite concentrations in Table 1 were used to calculate the ΔG′ of ATP hydrolysis and phosphorylation potential. The reduction of the cytosolic NAD(H) couple reflects a decrease in the ΔG′ of ATP hydrolysis by which the two nucleotide couples are linked through the glyceraldehyde-3-P-dehydrogenase/3-phosphoglycerate kinase reactions. The phosphorylation potential in

### Table 1

**Comparison of hepatic content of intermediary metabolites in ChREBP−/− and wild type mice consuming a normal chow diet**

| Metabolite | Wild type | ChREBP−/− |
|------------|-----------|-----------|
| Citrate    | 0.264 ± 0.018 | 0.284 ± 0.017 |
| Isocitrate | 0.0217 ± 0.002 | 0.0200 ± 0.002 |
| Dihydroxyacetone-P | 0.0327 ± 0.002 | 0.0921 ± 0.017 |
| 3-Phosphoglycerate | 0.413 ± 0.030 | 0.693 ± 0.037 |
| Pyruvate   | 0.120 ± 0.010 | 0.058 ± 0.007 |
| l-Lactate  | 1.09 ± 0.09 | 1.49 ± 0.09 |
| l-Glutamate | 1.28 ± 0.08 | 1.51 ± 0.11 |
| α-Ketoglutarate | 0.052 ± 0.009 | 0.105 ± 0.005 |
| NH4+      | 0.606 ± 0.018 | 0.567 ± 0.035 |
| ATP       | 4.1 ± 0.6 | 3.1 ± 0.4 |

*p < 0.01.*

### Table 2

**Comparison of free nucleotide ratios and free nucleotide in livers of ChREBP−/− versus wild type mice as calculated from the measured metabolites given in Table 1**

| Free nucleotide ratio or free nucleotide | Wild type | ChREBP−/− |
|----------------------------------------|-----------|-----------|
| Cytosolic [NAD+/NADH]                  | 658 ± 80  | 222 ± 22* |
| Mitochondrial [NAD+] /[NADH]           | 3.97 ± 0.62 | 6.89 ± 0.89* |
| Cytosolic [NADP+] /[NADPH]             | 0.04438 ± 0.00093 | 0.00914 ± 0.00066* |
| Phosphorylation potential [ATP][ADP][Pi] (m−1) | 8210 ± 1110 | 4530 ± 800* |
| ΔG′ ATP hydrolysis (kJ/mol)             | −55.8 ± 0.4 | −54.2 ± 0.4 |
| Free cytosolic [ADP]                   | 0.101 ± 0.020 | 0.184 ± 0.029 |
| (μmol/g wet weight)                    |            |           |
| Free cytosolic [AMP]                   | 3.92 ± 1.73 | 12.3 ± 4.0 |

*p < 0.01.*

Values are means ± S.E. with (n = 7) in each group. The significance of difference of the means of the two groups as judged by the Mann-Whitney U test is indicated.
the ChREBP<sup>−/−</sup> mouse liver was decreased 1.8-fold (Table 2), and the ΔG′ of ATP hydrolysis decreased from −55.8 to −54.2 kJ/mol. Consequently, the free cytosolic [ADP] concentration is calculated to be increased 1.8-fold, whereas the free cytosolic [AMP] is calculated to be increased more than 3-fold in the ChREBP<sup>−/−</sup> mouse liver. Finally, the free cytosolic [NADP]<sup>+</sup>] /[NADPH] ratio was increased almost 2-fold in the livers of ChREBP<sup>−/−</sup> mice, consistent with diminished lipogenic capacity in the livers of these mice.

Studies in Isolated Mitochondria Show No Defect in Oxidative Phosphorylation or Difference in O<sub>2</sub> Consumption but the Capacity of the Liver for β-Oxidation Is Decreased—Because NMR studies of intact livers indicated altered fat and pyruvate oxidation and metabolite analysis suggested perturbed mitochondrial energetics, we performed experiments to determine whether these results could be observed in mitochondria isolated from hepatocytes of ChREBP<sup>−/−</sup> and WT mice. The oxidative phosphorylation capacity of mitochondria isolated from ChREBP<sup>−/−</sup> liver was normal as measured by respiration rates, respiratory control ratios, and P/O ratios (data not shown). Mitochondrial β-oxidation was measured using tritiated palmitate and was found to be decreased 30% compared with livers from wild type mice (1.63 ± 0.16 versus 2.18 ± 0.16 nmol/h/mg protein), results that are similar to the NMR data from intact isolated livers described above.

PDC and PDK—Because the metabolite analysis indicated that the pyruvate concentration was significantly lower in ChREBP<sup>−/−</sup> livers, but the NMR data indicated that lactate and pyruvate oxidation was higher, the activities of PDC and PDK were determined in the liver. The PDC is a key regulatory enzyme for the supply of pyruvate-derived acetyl-CoA to the citric acid cycle for the generation of ATP (30). The PDC exists in active (dephosphorylated) and inactive (phosphorylated) forms with the interconversion catalyzed by PDKs and pyruvate dehydrogenase phosphatases (31). The actual activity of PDC (PDCa), which refers to the activity of the complex as extracted from the tissue, was much higher in livers from ChREBP<sup>−/−</sup> mice relative to liver from WT mice (Table 3). In contrast, total PDC activity (PDCt), which refers to the activity of the complex after complete dephosphorylation with pyruvate dehydrogenase phosphatase, was not significantly different in the livers of WT and ChREBP<sup>−/−</sup> mice (Table 2). Therefore, 20% of the PDC was active in the livers of ChREBP<sup>−/−</sup> mice but only 7.2% in the livers of WT mice. This difference in PDC activity state and therefore the phosphorylation state can be explained at least in part by 74% less PDK activity in liver extracts of ChREBP<sup>−/−</sup> mice (Table 2). Because induced changes in PDK activity have been found previously (32) to reflect changes in the level of expression of one or more of the four PDKs expressed in tissues (31), immunoblot analysis was conducted on liver extracts prepared from the mice. Surprisingly, PDK3 was found reduced 44% in the liver of ChREBP<sup>−/−</sup> mice relative to that of WT (Fig. 2). PDK1 and PDK2 were detectable by immunoblot analysis but were not reduced in amount (Fig. 2). PDK4 protein was not detectable, as expected for this isomerase in the liver of fed mice (data not shown). Immunoblot analysis of the E1α subunit of PDC showed no change in the amount of this protein between ChREBP<sup>−/−</sup> and WT mice (Fig. 2). Therefore, the lower PDK activity in the liver of ChREBP<sup>−/−</sup> can be explained in part by a reduction in the amount of PDK3 protein. The greater PDC activity in ChREBP<sup>−/−</sup> mice supports the NMR findings that pyruvate and lactate oxidation are elevated in these livers. This increase may indicate a compensatory mechanism to overcome the low pyruvate concentration resulting from the decreased LPK activity. However, this is opposite to what is normally observed. Activation of PDC usually occurs in response to a high pyruvate concentration because pyruvate inhibits PDK activity. It appears therefore that the low pyruvate concentration in the livers of ChREBP<sup>−/−</sup> mice is not sufficient to activate PDC directly.

Table 3: PDC and PDK activities in WT and ChREBP<sup>−/−</sup> liver

|          | WT               | Knock-out       |
|----------|------------------|-----------------|
| PDC<sub>a</sub> (units/g liver) | 0.18 ± 0.01 | 0.57 ± 0.17<sup>*</sup> |
| PDC<sub>t</sub> (units/g liver) | 2.49 ± 0.24 | 2.85 ± 0.10<sup>†</sup> |
| % activity (PDC<sub>a</sub>/PDC<sub>t</sub>) | 7.2 ± 0.6  | 20.2 ± 6.2<sup>‡</sup> |
| PDK activity (ies) | 0.69 ± 0.09 | 0.18 ± 0.03<sup>§</sup> |

<sup>*</sup>p < 0.05.  
<sup>†</sup>p < 0.02.
ChREBP Protein Deletion Produces an Energy-deficient Liver

DISCUSSION

The predominant effects of the ChREBP transcription factor is to increase the amount of the glycolytic enzyme LPK, most of the enzymes necessary for fatty acid synthesis, and the malic enzyme involved in the production of NADPH required for fat synthesis. By impairing lipogenesis, deletion of ChREBP logistically leads to a decrease in the weight of epididymal and brown fat stores and a 50% reduction in circulating fatty acids. ChREBP−/− livers had a near global reduction in hepatic fluxes (Fig. 3). However, impaired hepatic \( \beta \)-oxidation and the resultant change in substrate preference from long chain fatty acids in the WT liver to a preference for lactate and pyruvate oxidation in the ChREBP−/− liver were unanticipated. In fact, one might have predicted a decrease in lactate and pyruvate oxidation because PDH is a common pathway for oxidation of these substrates and for \( de \) novo lipogenesis from carbohydrate. Yet, this remarkable shift in substrate selection is consistent with the 3-fold increase in PDH activity found in the livers of ChREBP−/− mice. The mechanism by which ChREBP deletion stimulates PDH activity involves a 40% reduction in PDK and an oxidized mitochondrial redox state that favors further inactivation of PDK. Inactivation of PDK limits phosphorylation and deactivation of PDH. Ironically, loss of ChREBP function results in a shift away from a lipid economy in the liver by impairing both fat oxidation and fat synthesis. The deleterious effect of this shift on whole body energy homeostasis and hepatic energy metabolism suggests that metabolism of free fatty acids by liver is necessary for the maintenance not only of body temperature but also for a normal redox and phosphorylation state in liver.

The change from fat oxidation to pyruvate and lactate oxidation in the ChREBP null liver results in a more reduced mitochondria but more oxidized cytoplasm as mirrored by the redox pairs, [lactate]/[pyruvate] and [glutamate]/[\( \alpha \)-ketoglutarate]\(^{\ddagger} \), respectively. This observation is unusual because mitochondrial and cytosolic redox states are normally tightly linked and therefore typically change in parallel with one another (20).

The calculated free cytosolic and [NAD\(^{+}\)]/[NADH] ratios were respectively 658 in wild type and 222 in ChREBP−/−, whereas the respective calculated mitochondrial [NAD\(^{+}\)]/[NADH] were 3.97 and 6.89 (Table 2). These differ markedly from the measurement of total NAD and NADH in rat liver reported previously (32) (Table 4).

During starvation, when free fatty acids are elevated and liver is metabolizing lipid, the redox state of all the pyrimidine nucleotide couples becomes reduced. Conversely, when carbohydrate is being metabolized by liver, pyrimidine nucleotide couples become more oxidized. In both cases the redox states of the pyrimidine nucleotide systems reflect the state of reduction of the primary metabolic substrate. Divergent changes in the redox potential of the cytosolic and mitochondrial compartments have been reported when livers are perfused with the uncoupling agent carbonyl cyanide \( p \)-trifluoromethoxyphenylhydrazone (33). In that case the rate of NADH production by the citric acid cycle is insufficient to supply the flux through the uncoupled electron transport chain. More recently in human pyruvate carboxylase deficiency (34), a similar pattern of reduced cytoplasmic free NAD\(^{+}\) and oxidized mitochondrial NADH couple was reported, perhaps resulting from low levels of oxaloacetate and an impaired ability of the tricarboxylic acid to produce adequate reducing power for electron transport. Interestingly, deletion of ChREBP also impairs pyruvate carboxylase flux, although in this case the mechanism by which the mitochondrial and cytosolic redox states diverge from one another remains unclear.

The finding that ChREBP−/− mice have a near 2-fold decrease in plasma free fatty acids from 0.76 to 0.40 \( \mu \)m, and decreased capacity for hepatic \( \beta \)-oxidation, makes this phenotype more dependent upon lactate and pyruvate oxidation as an acetyl-CoA source for the tricarboxylic acid cycle. The latter was documented in perfused livers in which lactate and pyruvate were found to be the major substrates forming acetyl-CoA in the ChREBP−/− mice, whereas fatty acid oxidation was the major substrate for the citric acid cycle in WT mice (Fig. 1). The differences in redox states and \( \Delta G' \) of ATP hydrolysis of the two hepatic phenotypes (supplemental Table 1) can be explained the inherent energy content of the substrates oxidized. For instance, C2 units derived from pyruvate yield −778 kJ/mol C2 unit whereas the C2 unit derived from fatty acids yields −1247 kJ/mol C2 of palmitate (supplemental Table 1). Reliance of the ChREBP−/− liver on the lower energy content of pyruvate compared with long chain fatty acids may account for the energy-deficient state observed in the ChREBP−/− mouse liver. The \( \Delta G' \) of ATP hydrolysis normally varies within a very narrow 10% range (−53 to −60 kJ/mol ATP) among a variety of metabolic states (23, 35). This constancy of the energy of ATP
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TABLE 5
The total measured concentrations of adenine nucleotides in fed rat liver versus the calculated free cytosolic values in μmol/g fresh weight (20)

|         | ATP   | ADP   | AMP   | ATP/ADP × P_i |
|---------|-------|-------|-------|---------------|
| Measured total | 3.38  | 1.32  | 0.294 | 1090          |
| Free cytosolic  | 3.38  | 0.046 | 0.00047 | 27,200       |

hydrolysis occurs even though the oxidation of C2 units responsible for NADH formation in the tricarboxylic acid cycle (and ultimately ATP formation) varies from −778 to −1247 kJ/mol C2 (or 60%). The biochemical consequence of this disparate energy content is that the stoichiometry of total ATP formation is substrate-dependent. Thus, the complete oxidation of the C2 units of palmitate produces 16 ATP (supplemental Table 1), whereas oxidation of pyruvate produces only 10 ATP. These data illustrate that although ATP formation must remain fairly constant to maintain metabolic integrity, cellular redox state can vary widely, dependent on the substrates being oxidized (23, 35), and the efficiency by which the resultant energy can be used to drive ATP synthesis. In the case of the ChREBP]<sup>−/−</sup> liver, the decreased phosphorylation potential leads to a 2-fold increase in the free cytoplasmic [ADP]. Although increased ADP should activate LPK and stimulate glycolysis, this allosteric activation fails to compensate for the 85% reduction in LPK expression as evidenced by decreased pyruvate cycling in the ChREBP]<sup>−/−</sup> liver. As a consequence cytosolic ADP and AMP levels remain relatively high. The latter 4-fold increase in the free cytosolic [AMP] likely activates AMP-dependent protein kinases, resulting in significant and widespread consequences on energy metabolism that have yet to be examined.

Just as measurement of total pyridine nucleotides gives no information on the redox states in cytoplasm and mitochondria, so also measurement of total ADP content gives no information on the free cytoplasmic [ADP] because of sequestration of ADP within the mitochondrial matrix (20). Similarly, the measurement of total AMP gives no accurate estimate of the free cytosolic [AMP], which is in near equilibrium with the free cytosolic [ADP] through the action of myokinase (EC 2.7.4.3).

A comparison of total measured adenine nucleotides versus free cytosolic adenine nucleotides in rat liver has been reported previously (20) (Table 5). The free AMP concentration is calculated from the total ATP concentration, the free cytosolic ADP concentration, using the myokinase equilibrium constant adjusted for the free [Mg<sup>2+</sup>] concentration as described previously (25).

Interestingly, SREBP does not compensate for ChREBP deletion by inducing lipogenesis even though SREBP levels are normal in ChREBP]<sup>−/−</sup> mice. The reason for this is not entirely clear, but it appears that ChREBP provides not only the activation of lipogenic enzyme expression but also provides the metabolic impetus for fat synthesis by triggering the availability of the substrate acetyl-CoA for fat synthesis. Without this substrate SREBP cannot stimulate fatty acid synthesis. Currently there is no direct evidence by which SREBP-mediated insulin signaling activates the glycolytic pathway to generate pyruvate (and ultimately acetyl-CoA). Nevertheless, a confluence of insulin signaling (SREBP) and metabolic (ChREBP) activation may be required to initiate lipogenesis and perhaps explains the reason for the existence of the two major transcription factors SREBP and ChREBP.

CONCLUSIONS

In summary, we conclude that ChREBP deletion causes the liver to become energy-deficient as evidenced by a decrease in phosphorylation potential. This results from low LPK activity and decreased fatty acid oxidation. The liver attempts to compensate for this energy deficit by diminished activity of PDK and activating PDC, but the resulting increase in pyruvate oxidation is incapable of compensating for decreased fat oxidation or maintaining a normal phosphorylation potential.

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REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1997) Cell 89, 331–340
2. Yamashita, H., Takenoshita, M., Sakurai, M., Bruick, R. K., Henzel, W. J., Shillinglaw, W., Arnott, D., and Uyeda, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9116–9121
3. Kuriyama, H., Liang, G., Engelking, L. J., Horton, J. D., Goldstein, J. L., and Brown, M. S. (2005) Cell Metab. 1, 41–51
4. Kabashima, T., Kawaguchi, T., Wadzinski, B. E., and Uyeda, K. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5107–5112
5. Kawaguchi, T., Osatomi, K., Yamashita, H., Kabashima, T., and Uyeda, K. (2002) J. Biol. Chem. 277, 3829–3835
6. Kawaguchi, T., Takenoshita, M., Kabashima, T., and Uyeda, K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13710–13715
7. Liu, Y. Q., and Uyeda, K. (1996) J. Biol. Chem. 271, 8824–8830
8. Nishimura, M., and Uyeda, K. (1995) J. Biol. Chem. 270, 26341–26346
9. Iizuka, K., Miller, B., and Uyeda, K. (2006) Am. J. Physiol. 291, E358–E364
10. Iizuka, K., Bruick, R. K., Liang, G., Horton, J. D., and Uyeda, K. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 7281–7286
11. Bergmeyer, H. U. (1985) Methods of Enzymatic Analysis, 3rd Ed., VCH Verlagsgesellschaft, Weinheim, Germany
12. Lowry, O. H., and Passonneau, J. V. (1972) A Flexible System of Enzymatic Analysis, Academic Press Inc., New York
13. Burgess, S. C., Hausler, N., Merritt, M., Jeffrey, F. M. H., Storey, C., Milde, A., Koshy, S., Lindner, J., Magnuson, M. A., Malloy, C. R., and Sherry, A. D. (2004) J. Biol. Chem. 279, 48941–48949
14. Malloy, C. R., Sherry, A. D., and Jeffrey, F. M. (1990) Am. J. Physiol. 259, 1987–1995
15. Malloy, C. R., Thompson, J. R., Jeffrey, F. M., and Sherry, A. D. (1990) Biochemistry 29, 6756–6761
16. Jeffrey, F. M. H., Storey, C. J., Sherry, A. D., and Malloy, C. R. (1996) Am. J. Physiol. 271, E788–E799
17. Wu, P., Inskeep, K., Bowker-Kinley, M. M., Popov, K. M., and Harris, R. A. (1999) Diabetes 48, 1593–1599
18. Jeoung, N. H., Sanghani, P. C., Zhai, L., and Harris, R. A. (2006) Anal. Biochem. 356, 44–50
19. Lamprecht, W., and Trautschold, I. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed) pp. 2101–2110, Academic Press, New York
20. Krebs, H. A., and Veech, R. L. (1969) in Pyridine Nucleotide Interrelations (Papa, S., Tager, J. M., Quagliarello, E., and Slater, E. C., eds) pp. 329–384, Adriatica Editrice, Bari, Italy
21. Engel, P. C., and Dalziel, K. (1969) Biochem. J. 115, 621–631
ChREBP Protein Deletion Produces an Energy-deficient Liver

22. Londesborough, J. C., and Dalziel, K. (1968) Biochem. J. 110, 217–222
23. Veech, R. L., Lawson, J. W., Cornell, N. W., and Krebs, H. A. (1979) J. Biol. Chem. 254, 6538–6547
24. Guynn, R. W., and Veech, R. L. (1973) J. Biol. Chem. 248, 6966–6972
25. Veech, R. L., Gates, D. N., Crutchfield, C., Gitomer, W. L., Kashiwaya, Y., King, M. T., and Wondergem, R. (1994) Alcohol. Clin. Exp. Res. 18, 1040–1056
26. Kwack, H., and Veech, R. L. (1992) Curr. Top. Cell Regul. 33, 185–207
27. Krahenbuhl, S., Chang, M., Brass, E. P., and Hoppel, C. L. (1991) J. Biol. Chem. 266, 20998–21003
28. Ashcroft, S. J. H., and Randle, P. J. (1968) Biochem. J. 107, 599–600
29. Williamson, J. R., Browning, E. T., and Scholz, R. (1969) J. Biol. Chem. 244, 4607–4616
30. Randle, P. J. (1986) Biochem. Soc. Trans. 14, 799–806
31. Harris, R. A., Bowker-Kinley, M. M., Huang, B., and Wu, P. (2002) Adv. Enzyme Regul. 42, 249–259
32. Wu, P., Blair, P. V., Sato, J., Jaskiewicz, J., Popov, K. M., and Harris, R. A. (2000) Arch. Biochem. Biophys. 381, 1–7
33. Spry, M. (1971) Relationship between the Redox State of the Pyridine Nucleotides, the Phosphorylation State of the Adenine Nucleotides and Substrate Utilization in Animal Tissues. B.Sc. thesis, Oxford University, Oxford, UK
34. Mochel, F., Delonlay, P., Touati, G., Brunengraber, H., Kinman, R., Rabier, D., Roe, C., and Saudubray, J. (2005) Mol. Genet. Metab. 84, 305–312
35. Veech, R. L., Kashiwaya, Y., Gates, D. N., King, M. T., and Clarke, K. (2002) IUBMB Life 54, 241–252