Quantitative analysis of liver metabolites in three stages of the circannual hibernation cycle in 13-lined ground squirrels by NMR

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Serkova NJ, Rose JC, Epperson LE, Carey HV, Martin SL. Quantitative analysis of liver metabolites in three stages of the circannual hibernation cycle in 13-lined ground squirrels by NMR. Physiol Genomics 31: 15–24, 2007. First published May 29, 2007; doi:10.1152/physiolgenomics.00028.2007.—Thirteen-lined ground squirrels and other circannual hibernators undergo profound physiological changes on an annual basis, transitioning from summer homeothermy [body temperature (Tb) ~37°C] to winter heterothermy (Tb cycling between 0°C and 37°C). We hypothesize that these physiological changes are reflected in biochemical changes that provide mechanistic insights into, and biomarkers for, hibernation states. Here we report the results of an NMR-based metabolomics analysis of liver extracts from ground squirrels in three distinct physiological states of circannual hibernation: summer active (SA), late torpor (LT), and reentering torpor (Ent) after one of the euthermic arousals. Of the 43 identified and quantified metabolites, 36 differed among these three states and fell into two patterns of variation: 1) SA differed from both of the two winter states; or 2) the two winter states differed from each other, but one of the two was not different from SA. Concentrations of hepatic glucose, lactate, alanine, succinate, β-hydroxybutyrate, glutamine, and betaine were identified as robust hepatic biomarkers that together distinguish among animals in these three states of the circannual hibernation rhythm. These data are consistent with a proposed two-switch model of hibernation, in which setting the summer-winter switch to winter enables expression of a distinct torpor-arousal switch. The summer-winter switch is characterized by the metabolites associated with the well-known switch from carbohydrate to lipid fuel utilization during hibernation. The torpor-arousal switch is characterized by the accumulation of metabolites of nitrogen (glutamine) and phospholipid (betaine) catabolism in LT with the capacity to act as protective osmolytes.

metabolomics; nitrogen metabolism; osmolytes; Spermophilus tridecemlineatus; torpor

MAMMALIAN HIBERNATORS are uniquely able to orchestrate and survive extended periods of extremely low body temperature (Tb) and metabolic, respiratory, and heart rates in a state called torpor. The deep, multiday periods of torpor that characterize hibernation alternate with periodic short arousals that reverse the dramatic physiological depressions associated with the torpid state (reviewed in Ref. 5). Thus hibernators, including 13-lined ground squirrels (Spermophilus tridecemlineatus), are homeothermic like most mammals in summer but switch to heterothermy in winter (Fig. 1). The biochemical consequences of periodic arousals from torpor are complex and come with tremendous costs. Although hibernation is a strategy that saves large amounts of energy over the winter compared with remaining euthermic (~90%), most of the energy used in winter (>70%) is used to fuel these interbout arousals (Ref. 22 and references therein). Hence the arousals are an enigma—Why arouse when remaining torpid would save so much more energy? It has been suggested that hibernators must reawarm to restore or remove a metabolic imbalance (Ref. 27, chapter 6).

Metabolomics seeks to identify and quantify the low-molecular-weight endogenous compounds characteristic of a particular physiological state, in this case opposing states of the circannual hibernation cycle. This can be done through collection of global metabolic data with modern spectroscopic techniques and appropriate statistical approaches (29, 36). Nuclear magnetic resonance (NMR) spectroscopy is one of the major techniques for metabolomics analysis because it allows for simultaneous assessment and quantification of small-molecule metabolites in the concentration range above 10 μM. Thus quantitative assessment of various metabolite classes (such as sugars, amino acids, osmolytes, lipids, high-energy phosphates) in tissues and biological fluids can be performed with high-resolution 1H- and 31P-NMR spectroscopy. Biomarker identification is achieved by applying statistical approaches including principal component analysis (PCA) either to these NMR spectra or to the quantitative metabolic data that are derived from them.

The dramatic changes in behavior, physiology, and environment as ground squirrels cycle between summer and winter, taken together with changes in Tb and metabolic rate as winter animals cycle between torpor and arousal, are predicted to lead to metabolic homeostasis characteristic for each stage of this circannual rhythm and hence specific biomarkers that distinguish among them. Here we apply a nonbiased metabolomics approach to identify hepatic metabolites that differ among three stages of the circannual hibernation cycle in ground squirrels: summer active (SA) and two winter stages, late torpor (LT) and entrance (Ent). Animals reentering torpor (Ent) rather than in interbout arousal (IBA) were chosen because aroused hibernators may be in various intermediate stages of reestablishing the components that are permissive for torpor, whereas Ent animals by definition have fully completed this process. The liver was chosen for this work because it is a large and critical component of systemic metabolism in mammals (33). Liver metabolites were extracted and then subjected to proton (1H)- and phosphorus (31P)-NMR. The spectra were analyzed to identify and quantify metabolites. Remarkably, significant differences associated with activity state were found in most of the metabolites identified. PCA analysis was further used to independently assess changes associated with these
Livers were shipped on dry ice and stored at Livers were collected after animals were killed by stunning and with a 12:12-h light-dark cycle for at least 3 wk before experiments. Livers were harvested. The University of Wisconsin Institutional Animal Care and Use Committee approved all animal procedures. Liver acid extraction for NMR. Liver tissues were extracted with 0.1–0.3 g of frozen liver was powdered in a mortar in the presence of liquid nitrogen and then added to 6 ml of ice-cold perchloric acid. After centrifugation for 20 min at 1300 g and 4°C, the supernatants were collected and the pellets were redissolved with 2 ml of perchloric acid, vortexed, and centrifuged. Both supernatants, containing the hydrophilic fraction of the extract, were combined, and the mixture was neutralized (pH 7.0) with KOH before being centrifuged again to remove potassium perchlorate. Supernatants with their water-soluble metabolites (hydrophilic compounds) were then lyophilized overnight to remove water for NMR experiments. The extracted hydrophilic metabolites were dissolved in 0.45 ml of deuterium oxide (D2O) before 1H-NMR. The pellets from the second centrifugation containing the lipid fraction were dissolved in 4 ml of ice-cold water, adjusted to pH 7.0 with KOH, and then lyophilized overnight to remove water for NMR experiments. The lipids were dissolved in 1 ml of deuterated chloroform-methanol mixture (2:1 vol/vol) before 1H-NMR. All deuterated compounds were purchased from Cambridge Isotope (Andover, MA).

Proton quantitative 1H-NMR on liver extracts. For NMR analysis, the dissolved hydrophilic and lipophilic extracts were transferred into 5-mm NMR glass tubes (Wilmad LabGlass, Buena, NJ). First, the extracts were analyzed by high-resolution 1H-NMR spectroscopy with a 500-MHz high-resolution Bruker DRX system equipped with Bruker TopSpin software (Bruker Biospin, Fremont, CA; Refs. 34, 35). An inverse T1X 5-mm probe was used for all experiments. To suppress water residue in the extracts, a standard Bruker water presaturation sequence was used (“zgpr”). An external reference, trimethylsilyl propionic-2,2,3,3-d4 acid (TMSP, 0.5 mmol/l for hydrophilic and 1.2 mmol/l for lipid extracts), was used for metabolite quantification of fully relaxed 1H-NMR spectra and as a 1H chemical shift reference (0 ppm). For metabolite identification in water-soluble and lipid extracts, a two-dimensional (2D) 1H,13C heteronuclear single quantum correlation (HSQC) NMR sequence was used. The 1H-NMR peaks for single metabolites were identified and referred to a metabolite chemical shift library (2). After Fourier transformation and phase and baseline corrections, each 1H peak was integrated with 1D WINNMR (Bruker Biospin). The absolute concentrations of single metabolites were then referred to the TMSP integral and calculated according to the equation (1):

\[ C_i = \frac{I_x N_x C_{\text{M}}}{I_{\text{9}} V M} \]  

where \( C_i \) is metabolite concentration, \( I_x \) is integral of metabolite 1H peak, \( N_x \) is number of protons in metabolite 1H peak (from CH, CH2, CH3, etc.), C is TMSP concentration, I is integral of TMSP 1H peak at 0 ppm (9 since TMSP has 9 protons), V is volume of the extract, and M is weight of liver tissue. The final metabolite concentrations are expressed as micromoles per gram of liver tissue.

Phosphorus quantitative 31P-NMR on liver extracts. The water-soluble (hydrophilic) liver extracts were additionally analyzed by 31P-NMR spectroscopy immediately after 1H-NMR and addition of 100 mmol/l EDTA to chelate divalent ions bound to ATP (30). Phosphorus spectra were obtained on a Bruker 300-MHz Avance spectrometer (31P-NMR frequency: 121.5 MHz) equipped with a 5-mm QNP 31P/13C/19F/1H probe using a composite pulse decoupling (CPD) program. An external standard in a thin capillary, methylene-diphosphonic acid (2.3 mmol/l D2O; Sigma-Aldrich), was placed into the NMR tube to serve as a reference for both chemical shift (18.6 ppm) and phosphorous metabolite quantification (see above).

Statistical analyses. Absolute individual concentrations of each metabolite obtained from each of the three NMR experiments for each sample (1H-NMR on hydrophilic and hydrophobic extracts as well as 31P-NMR on hydrophilic extracts) were analyzed by ANOVA (Excel, Microsoft) followed by Tukey’s post hoc test to identify the groups that differed significantly (Minitab).

PCA was independently applied to the more complex 1H-NMR spectra of the hydrophilic extracts. The PCA prediction and classification (group clustering or “pattern recognition” visualization) and all
mathematical models were built with the AMIX software package (3.5.1, Bruker Biospin) followed by R package (2.00) on spectral segments of hydrophilic 1H-NMR spectra. Briefly, before the segment intensities were loaded into the R package software each 1H-NMR spectral set was divided into 175 segments, i.e., “buckets,” and the spectra were scaled to the total intensity. Fourier transformation and phase and cubic spline base corrections of each 1H-NMR spectrum were performed before reduction to bucket histograms. Each bucket width was 0.05 ppm to provide optimal group separation on the score plots (see RESULTS). The region of 4.65–5.05 ppm was excluded from segmentation and data analysis because of water presaturation and water residue. Bucket values were scaled to the TMS signal region at −0.05 to 0.05 ppm. After bucket histograms were loaded into the R package software, PCA was applied to 1) cluster the samples among SA, LT, and Ent animals (scores $t_i$) and 2) identify markers responsible for this group clustering (plots $p_i$).

RESULTS

Pattern recognition among study groups. The global metabolic profile of liver from SA, LT, and Ent ground squirrels (Fig. 1) was assessed for six animals from each group with 1H- and 31P-NMR. Analysis was performed on both the hydrophilic and lipophilic (1H-NMR only) components of the extracts. A representative 1H-NMR spectrum of one of the hydrophilic extracts from each animal group is presented in Fig. 2. These spectra are unusually distinct, with some regions exhibiting more similarity between the SA and Ent samples and others between LT and Ent. A total of 65 hepatic metabolites were identified in the three spectra obtained from each liver sample (1H- and 31P-NMR on hydrophilic and 1H-NMR on lipophilic extracts). The concentrations of 43 of these metabolites were above the lower limit of quantification (0.01 μmol/g). Of the 43 quantified metabolites all but 7 differed significantly (ANOVA, $P < 0.05$) between at least two groups of ground squirrels (Table 1).

1H-NMR spectra of the hydrophilic compounds were highly complex and contained multiple NMR peaks from various hydrophilic metabolites including sugars, polyols, amino acids, cellular osmolytes, lactate, and glutathione. Thus these spectra were independently analyzed by unsupervised PCA to determine whether their apparent differences were robust enough to distinguish the three sample groups, SA, LT, and Ent. In the first step of this analysis, PCA scores ($t_i$) describe the variation in the sample direction, i.e., similarity or dissimilarity among individual 1H-NMR spectra. Each triangle in Fig. 3A represents the spectral differences in the metabolic profile from an individual ground squirrel sample. The PCA scores revealed a distinct group clustering of the spectra obtained from SA, LT, and Ent animals (Fig. 3A). In the second step of the PCA analysis, the loading plots ($p_i$) describe the variation in the variable direction, i.e., similarity or dissimilarity among variables. This analysis explained the patterns in the previous scores ($t_i$) and identified the specific metabolites that were responsible for the observed group clustering (Fig. 3B). The metabolites condensed at the origin do not contribute to group clustering, but to a lesser extent. The NMR peaks of these metabolites were confirmed based on signal assignment from 2D-H,C-HSQC and 2D-H,H-COSY NMR experiments using the chemical shift database.

Six of these eight metabolites differ between SA and both winter states. Five of these, glucose, lactate, alanine, succinate, and reduced glutathione (GSH), all decreased in the two winter states (both LT and Ent) compared with summer, whereas β-hydroxybutyrate increased (Fig. 4; Table 1). Glucose concentrations were substantially elevated in SA animals (18- and 6.4-fold over LT and Ent hibernators, respectively). Likewise, glycolytic activity in the liver was significantly elevated in SA animals compared with both winter groups; lactate concentrations averaged 18.6-fold higher in SA than in LT and 9.8-fold higher in SA than Ent. Consistent with the direct metabolic dependence of alanine biosynthesis on glycolytic activity and lactate production, alanine was also highest in SA liver, 13.9-fold elevated over Ent and 10.7-fold over LT. A component of the citric acid cycle, succinate, was also elevated 3.5- and 2.7-fold in SA livers compared with LT and Ent livers, respec-
Table 1. Concentrations of liver metabolites in three stages of circannual hibernation

<table>
<thead>
<tr>
<th>SA</th>
<th>LT</th>
<th>Ent</th>
<th>ANOVA</th>
<th>Differences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine</td>
<td>2.45±0.76</td>
<td>8.46±0.32</td>
<td>1.81±0.42</td>
<td>1.31E-12</td>
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<tr>
<td>Glucose</td>
<td>42.53±7.98</td>
<td>2.36±0.82</td>
<td>6.68±3.7</td>
<td>9.57E-10</td>
</tr>
<tr>
<td>Polyols + sugars</td>
<td>87.88±13.71</td>
<td>22.7±2.92</td>
<td>22.17±7.13</td>
<td>1.63E-09</td>
</tr>
<tr>
<td>Lactate</td>
<td>9.28±2.49</td>
<td>0.5±0.07</td>
<td>0.97±0.29</td>
<td>2.56E-08</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.7±0.8</td>
<td>0.26±0.12</td>
<td>0.2±0.09</td>
<td>1.12E-07</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.85±0.55</td>
<td>0.82±0.25</td>
<td>1.04±0.21</td>
<td>1.34E-07</td>
</tr>
<tr>
<td>Cholines</td>
<td>0.13±0.11</td>
<td>1.22±0.42</td>
<td>0.25±0.17</td>
<td>5.90E-06</td>
</tr>
<tr>
<td>Betaine</td>
<td>1.46±1.04</td>
<td>3.09±0.55</td>
<td>0.56±0.2</td>
<td>4.77E-05</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>0.12±0.04</td>
<td>0.68±0.16</td>
<td>0.82±0.31</td>
<td>6.29E-05</td>
</tr>
<tr>
<td>CH3-acetyl</td>
<td>6.95±0.99</td>
<td>8.51±0.99</td>
<td>5.33±0.7</td>
<td>0.0002</td>
</tr>
<tr>
<td>Lys + Arg</td>
<td>0.25±0.11</td>
<td>0.52±0.19</td>
<td>0.16±0.06</td>
<td>0.0007</td>
</tr>
<tr>
<td>GSH</td>
<td>2.09±0.51</td>
<td>1.16±0.17</td>
<td>1.17±0.41</td>
<td>0.0011</td>
</tr>
<tr>
<td>Asparate</td>
<td>0.08±0.04</td>
<td>0.23±0.1</td>
<td>0.06±0.07</td>
<td>0.0028</td>
</tr>
<tr>
<td>GPC</td>
<td>0.31±0.11</td>
<td>1.22±0.42</td>
<td>0.25±0.17</td>
<td>5.90E-06</td>
</tr>
<tr>
<td>Taurine</td>
<td>4.46±1.02</td>
<td>5.11±1.36</td>
<td>7.55±2.14</td>
<td>0.0098</td>
</tr>
<tr>
<td>Nucleotides</td>
<td>1.18±0.65</td>
<td>1.34±0.4</td>
<td>0.5±0.28</td>
<td>0.0173</td>
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<tr>
<td>Other sugars</td>
<td>0.75±0.55</td>
<td>0.12±0.17</td>
<td>1.22±0.92</td>
<td>0.0275</td>
</tr>
<tr>
<td>Total glutathione</td>
<td>4.13±0.95</td>
<td>3.2±0.58</td>
<td>2.75±0.86</td>
<td>0.0293</td>
</tr>
<tr>
<td>Formate</td>
<td>0.47±0.16</td>
<td>0.56±0.09</td>
<td>0.37±0.12</td>
<td>0.0580</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.65±0.43</td>
<td>0.63±0.15</td>
<td>0.43±0.11</td>
<td>0.3342</td>
</tr>
<tr>
<td>Creatine</td>
<td>0.35±0.47</td>
<td>0.52±0.14</td>
<td>0.57±0.4</td>
<td>0.5552</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.28±0.35</td>
<td>0.18±0.05</td>
<td>0.16±0.15</td>
<td>0.6277</td>
</tr>
<tr>
<td>Adenine</td>
<td>2.1±0.37</td>
<td>2.2±0.35</td>
<td>2.01±0.47</td>
<td>0.6857</td>
</tr>
<tr>
<td>Glycogen</td>
<td>0.44±0.33</td>
<td>0.76±0.75</td>
<td>0.68±0.8</td>
<td>0.6917</td>
</tr>
<tr>
<td>Aromatic amino acids</td>
<td>2.2±0.43</td>
<td>2.3±0.34</td>
<td>2.29±0.66</td>
<td>0.9182</td>
</tr>
</tbody>
</table>

Average concentrations (in μmol/g liver tissue) and SD from 6 individuals are reported for 43 metabolites and 3 ratios in hepatic extracts from summer active (SA), late torpor (LT), and torpor-reentering (Ent) (Fig. 1) 13-lined ground squirrels. Hydrophilic compounds were quantified by both 1H- and 31P-NMR and hydrophilic compounds by 1H-NMR. Values for metabolite ratios (which lack units) are given in italics. GSH, reduced glutathione; PME, phosphomonoesters; PC, phosphocholine; PDE, phosphodiester; GPC, glycerophosphocholine; PtdCholine; phosphatidylcholine; TAG, triacylglycerol; PtdEthanolamine, phosphatidylethanolamine; PUFAs, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids. ANOVA value and significant (p < 0.05, Tukey) differences among groups are indicated.

In contrast, betaine and glutamine distinguish between LT and Ent hibernators; these two metabolites differed in the LT animals compared with their concentrations in either SA or Ent animals (Fig. 5; Table 1). Betaine, a hepatic methylyamine oxosomyl and important methyl donor, was 5.6-fold increased in LT over Ent and 2.2-fold increased over SA. Glutamine, an ammonia scavenger and osmolyte, was elevated 4.7-fold over Ent and 3.4-fold over SA in LT animals.
Changes in hepatic energy state and membrane metabolism. The phosphorus-containing hydrophilic metabolites were identified from $^{31}$P-NMR spectra (Fig. 6) of the liver hydrophilic fractions and quantified (Table 1). These include hepatic high-energy phosphates (ATP, ADP, $\text{NAD}^{+}$) as well as phospholipid precursors [phosphocholine (PC) and other phosphomonoesters (PME)] and phospholipid catabolism products [glycerophosphocholine (GPC) and other phosphodiesters (PDE)]. ATP concentration and ratio of concentration of ATP to ADP differed significantly among the three study groups. Surprisingly, livers of torpid hibernators exhibited an increased energy state when compared with SA and Ent winter animals; in LT animals, the ratio of ATP to ADP was four times greater than in SA and 3.2 times greater than in Ent animals. This result should be viewed with caution, however: ATP is labile and may be catabolized to some degree in the tissue between the time of euthanasia and freezing. Because of the low $T_b$ of torpor, this degradation of ATP is likely slowed in LT animals. Nevertheless, these results make it unlikely that ATP is severely depleted during LT and are consistent with an earlier report demonstrating elevated hepatic ATP in LT animals (46) as well as a recent report demonstrating increased brain energy stores in torpid hibernators by in vivo magnetic resonance spectroscopy (23). The phospholipid precursors PC and total PME were significantly decreased in LT hibernators compared with SA (down 3.9- and 5.3-fold, respectively), with no significant differences found between Ent hibernators and SA animals. Conversely, the degradation products of membrane phospholipids, GPC and other PDE, were significantly decreased in extracts of Ent livers compared with the other two groups. GPC, a phospholipid breakdown product and osmolyte, was reduced 4.8-fold in Ent compared with SA and 3.2-fold compared with LT animals. Likewise, PDE was down 4.3- and 3-fold in Ent compared with SA and LT animals, respectively. The low levels of PDE and elevated PME in Ent animals resulted in a large increase in the ratio of PME to PDE in Ent compared with LT animals (16.3-fold). This ratio, which represents a balance between membrane biosynthesis and breakdown, was also increased in Ent compared with SA animals, although to a lesser extent (4.4-fold; Table 1, Fig. 5).

Changes in hepatic lipid profiles. Significant changes were also observed in the lipid metabolic profile as calculated from $^1$H-NMR spectra of the liver hydrophobic fractions. All 10 of the quantifiable lipid metabolites differed significantly among at least two of the three groups (Table 1). Total lipid, triacylglycerol, and fatty acid levels were elevated ~2-fold during LT compared with SA animals and cholesterol was elevated 1.5-fold, providing further evidence of the metabolic fuel switch from carbohydrates in summer to lipids in winter. Phospholipids were the only types of lipids that significantly decreased in LT animals; the decreased concentrations of phosphatidylcholine (PtdCholine, the major membrane phospholipid) in LT compared with both SA and Ent animals correlated well with decreased concentrations of its precursor, PC, in $^{31}$P-NMR spectra in LT animals (Table 1, Fig. 5).

**DISCUSSION**

Liver metabolic profiles of ground squirrels in three stages of their circannual hibernation cycle were compared to identify biomarkers that distinguish among SA animals, hibernators in an extended bout of torpor (LT), and hibernators reentering torpor after a periodic arousal to euthermia (Ent). Significant changes were observed in carbohydrate, amino acid, fatty acid, phospholipid, and energy metabolites as well as in the concentrations of several osmolytes. Remarkably, most (84%) of the 43 liver metabolites that were quantitatively assessed in this unbiased metabolomics screen differed significantly between animals in at least two of these three states of the circannual hibernation cycle. We hypothesize that hibernation in ground squirrels and other circannual hibernators involves two biochemical switches: 1) a summer-winter switch that enables heterothermy and 2) a torpor-arousal switch that enables torpor but can only be activated when the summer-winter switch is in the winter position (Fig. 7). The first step toward testing the predictions of this model is identification of biomarkers that characterize each state; the changing levels of metabolites documented in this study provide such biomarkers. These metabolites also provide insight into the biochemical bases for the proposed summer-winter and torpor-arousal switches. The liver of SA animals is characterized by active carbohydrate
catabolism (high levels of glucose, lactate, alanine, and succinate) compared with winter animals, in which a shift to lipid catabolism is apparent (high lipid, fatty acid, and ketone body levels), particularly in LT animals. The major differences between the two winter groups, Ent and LT, were elevated levels of osmolytes that are related to nitrogen (glutamine) and phospholipid (betaine) metabolism during torpor.

The changing concentrations of hepatic metabolites in hibernators differed both qualitatively and quantitatively from the results of unbiased screens for differential expression of...
mRNAs and proteins, in which only a small proportion of the gene products assessed differed significantly among hibernation groups in a variety of tissues (Ref. 3, reviewed in Ref. 5; see also more recent work in Ref. 44). Specifically in liver, a screen of 3,082 unique cDNA probes on a microarray revealed significant, but typically small (∼2-fold), changes in just 102 mRNAs (3.3%) in golden-mantled ground squirrels. Moreover, only two mRNAs differed significantly between LT animals and aroused hibernators, both by 1.4-fold (43). A relatively low proportion of the proteome was also found to differ with generally smaller fold changes between SA and Ent hibernators in a 2D gel-based proteomics screen of liver; 9% of the ∼900 protein spots analyzed differed between Ent hibernators and SA animals, and the largest fold change was 7.3-fold (11), in contrast to these results, where 37% of the metabolites changed between Ent and SA animals and the largest fold change was 14-fold. Nevertheless, among those protein differences are several enzymes relevant to the metabolites identified here. For example, glucose concentrations are lower in LT than in Ent animals and independent of time since animals last fed in both states (Fig. 8). Thus gluconeogenesis in liver must occur during IBA throughout the long fast of the hibernation season, consistent with an earlier report (18). At the protein level, three enzymes of gluconeogenesis are elevated in Ent compared with SA animals: malate dehydrogenase 1, phosphoenolpyruvate carboxykinase 2, and pyruvate carboxylase (11). 3-Hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial), a key enzyme in ketone body formation, is also elevated almost fivefold in Ent over SA livers, which agrees well with the elevated levels of β-hydroxybutyrate measured in this metabolomics screen.

Elevated levels of glucose, polyols and sugars, lactate, alanine, and succinate in summer compared with LT and Ent, together with the winter elevation of β-hydroxybutyrate and lipid fuels (significant in LT, a trend in Ent; Table 1, Fig. 4), are consistent with the well-documented switch from carbohydrate- to fat-based metabolism between summer and winter in circannual hibernators (reviewed in Refs. 5, 8). Although the efficiency of storing energy as fat rather than carbohydrate offers a clear advantage to hibernators, efficiency may not be the only advantage of switching to a fat-based metabolism in winter. Euthermic, nonfasted animals oxidize glucose and other carbohydrates as their primary energy source. Hypoxic stress followed by reperfusion typically leads to an elevation of glucose consumption and glycolytic activity, resulting in toxic lactate accumulation (e.g., in rat liver; Ref. 30). Anaerobic glycolysis is an ineffective way to produce ATP, and therefore a significant depletion in energy state is seen on ischemia and reperfusion in eutherian animals (30). In the present study, no decrease in the energy state was observed in LT or Ent hibernating squirrels relative to summer animals. Avoidance of lactate accumulation, a switch to lipid oxidation, and retention of energy charge as seen in these hibernators are also features of livers that are preconditioned and therefore resistant to ischemic insults (2, 30, 34, 35).

It is important to question whether hibernators experience ischemia. Entrance into torpor in ground squirrels begins with a dramatic metabolic depression. A drop in Tb follows, further reducing metabolic demand (22). The animals are normoxic when entrance into torpor is initiated, and the combination of their hypometabolic state and thermodynamic effects apparently allows hibernating ground squirrels to maintain normoxia throughout entrance and torpor; it is only during arousal, when...
metabolic rate increases to drive the rewarming process, that the hibernator is known to experience hypoxia (28). Metabolic rates are dramatically elevated before reperfusion is fully completed, leading the arousing hibernator to experience transient hypoxia and oxidative stress in some tissues. For example, during arousal brain levels of hypoxia-inducible factor-1α increase (28), as do plasma levels of urate (39), a marker for reactive oxygen species production; yet little damage occurs (28). In fact, there is a great deal of evidence that a wide variety of tissues including liver are endogenously protected against the types of damage associated with ischemia-reperfusion (I/R) injury in hibernators (6, 9, 10, 14–16, 24, 26). The protected phenotype does not appear to depend on low temperature, but rather is exhibited in tissues from aroused hibernators (9, 25, 26).

GSH is an endogenous antioxidant that is protective against I/R injury (19); hence it would not be surprising to find elevated levels of GSH in winter when the animals are naturally protected against I/R. In this study, however, we found decreased concentrations of hepatic GSH levels in both winter states compared with summer and no difference between Ent and LT. Other measurements of GSH levels in hibernators revealed no state-dependent differences in liver of arctic (39) and intestine of 13-lined (7) ground squirrels. Interestingly, however, compounds related to GSH production (glutamine and betaine) were among the metabolites exhibiting increased concentrations in LT relative to Ent animals in our screen, suggesting the possibility that these precursors to GSH accumulate in torpor before oxidative stress occurs. These precursors could then be consumed to replenish any GSH used in preventing oxidative damage during arousal, thus allowing GSH levels to remain fairly constant throughout the torpor-arousal cycle.

Glutamine has been found to preserve glutathione levels during oxidative stress (20), which may be important for arousal, but its accumulation during LT is likely to be more broadly significant. Glutamine is the major nontoxic ammonia scavenger in the liver and therefore an important intermediate in nitrogen metabolism. In hibernators, protein synthesis virtually ceases during torpor but is restored to summer levels or higher during each IBA (17, 41, 42, 45). Because ground squirrels do not eat for several weeks to months during the hibernation season, they must recycle their amino acid building blocks for protein synthesis from the hydrolysis of cellular proteins. In mammals, however, the nitrogen-containing amino acids cannot be stored to any great extent and the excess amino acids not used immediately for protein synthesis are typically catabolized to ammonia, which is either excreted directly or converted to urea or uric acid in the liver for excretion by the kidney. Hibernating ground squirrels downregulate the steady-state levels of mRNA corresponding to several genes involved in urea synthesis (43) and reduce urine output. Therefore, with protein synthesis greatly decreased, an increase in ammonia production during torpor is inevitable. From the results of this study, it appears that increased glutamine is synthesized from the excess ammonia in the liver, likely to prevent toxic effects of ammonia throughout the body (12). There are reduced levels of glutamine in the brains of torpid ground squirrels, which are restored during IBA (23), providing further evidence that the liver is maintaining nitrogen homeostasis in these animals. During arousal and throughout the IBA states of hibernation, accumulated hepatic glutamine can be utilized (as seen by the reduced concentrations in the Ent animals) to provide additional energy, support protein synthesis and the citric acid cycle, and provide oxidative protection through the γ-glutamyl cycle (4).

Betaine (trimethylglycine) is a primary methyl donor for S-adenosylmethionine (SAM), which is known to defend hepatic GSH concentrations under conditions of oxidative stress (21). Furthermore, betaine is critical for proper liver function, cellular replication, and detoxification reactions (1). Recently, it has been shown that activation of hepatic methylamine synthesis, especially through betaine aldehyde dehydrogenase, results in increased betaine levels and protection against increased urea levels in marine elasmobranchs (40). In the hibernating ground squirrels studied here, the likely source of the increased betaine observed in LT is the breakdown and conversion of membrane phospholipids, which is also consistent with the observed decrease of PtdCholine, the most abundant membrane component in eukaryotes, in LT (32). Thus hibernating ground squirrels apparently catabolize lipid membranes during torpor, using the liberated choline to increase betaine concentrations, which may enhance osmotic protection. During arousal, the accumulated betaine can be used to drive the SAM cycle, providing for oxidative protection and normal liver function.

Significantly, although synthesis of glutamine and betaine occur via distinct biochemical pathways (nitrogen and phospholipid metabolism), both metabolites are important osmolytes in liver. Osmolytes are small molecules that affect protein stability and may be either denaturing or protecting (38). Increased levels of protecting osmolytes, including the glutamine, betaine, and GPC reported here, facilitate protein folding by shielding them from denaturation that occurs as a consequence of solute, cold, and heat stresses in a variety of organisms (31, 37). Interestingly, their levels are not elevated in Ent animals, but rather accumulate in LT animals as a by-product of other important catabolic processes occurring during torpor. Their protective properties can then be exploited when needed most, as the hibernator arouses from torpor.
The hypothesis that ground squirrels arouse to restore or replenish metabolites at euthermic Tb (27) makes predictions about the significance of altered concentrations of critical metabolites as animals cycle between torpor and arousal. If ground squirrels arouse to remove a toxic or inhibitory metabolite that accumulates during torpor, its level is predicted to be high in LT and low in Ent. Alternatively, if they arouse to replenish a metabolite that enables torpor, that metabolite should be found at relatively higher concentrations in Ent than in LT. Both of these patterns are apparent in this data set (Fig. 5). It is unlikely that any one of these metabolites triggers entrance or arousal, because a complex physiological phenotype like hibernation is unlikely to be regulated by the concentration of any single molecule. Nevertheless, the metabolites identified in this study provide insight into the underlying biochemistry of hibernation. Because both betaine and glutamine accumulate in LT and are restored to low levels in Ent, neither can be driving entrance into torpor. As discussed above, these are known protective osmolites and their accumulation in LT could be beneficial during arousal, so it is also unlikely that they are toxic and arousal occurs specifically to remove them. The elevated glutamine, however, is indicative of increasing nitrogen accumulation. A critical level of glutamine may be reached in LT, such that its nitrogen-buffering capacity is no longer able to accommodate catabolic ammonia without harm to the cells. Once this occurs, other metabolites could signal arousal; among the possibilities worth further consideration are the depletion of citric acid cycle intermediates by limiting anaerobic glutamate or accumulation of urea. Interestingly, injection of exogenous urea stimulates arousal in ground squirrels (13).

It is clear from this study of three critical time points of the circannual hibernation cycle that metabolic analyses offer untapped potential to provide significant biomarkers to distinguish the various stages of the circannual hibernation cycle at a biochemical level. These biomarkers in turn provide insight into the molecular mechanisms that underlie the hibernation phenotype. It will be critical in the future to apply these and other high-throughput “omics” methods to additional organisms obtained with a denser sampling regimen across the summer–winter and torpor-arousal cycles, to fully test the predictions of our two-state model of circannual hibernation and identify the crucial components of the two switches. Such data are required to fully understand the mechanisms underlying the tissue protection and metabolic control of natural mammalian hibernation and to inform pharmacological strategies to recapitulate these phenotypes in nonhibernating mammals, including humans.

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REFERENCES