Defective carbohydrate metabolism in mice homozygous for the tubby mutation

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Defective carbohydrate metabolism in mice homozygous for the tubby mutation. Physiol Genomics 27: 131–140, 2006. First published July 18, 2006; doi:10.1152/physiolgenomics.00239.2005.—Tubby is a member of a small gene family, the tubby-like proteins (TULPs), with predominant expression in neurons. Mice carrying a mutant in Tubb cause retinal and cochlear degeneration as well as late-onset obesity with insulin resistance. During behavioral and metabolic testing, we found that homozygous C57BL/6J-tub/tub mice have a lower respiratory quotient than C57BL/6J controls before the onset of obesity, indicating that tubby homozygotes fail to activate carbohydrate metabolism and instead rely on fat metabolism for energy needs. In concordance with this, tubby mice show higher excretion of ketone bodies and accumulation of glycogen in the liver. Quantitation of liver mRNA levels shows that, during the transition from light to dark period, tubby mice fail to induce glucose-6-phosphate dehydrogenase (G6pdh), the rate-limiting enzyme in the pentose phosphate pathway that normally supplies NADPH for de novo fatty acid synthesis and glutathione reduction. Reduced G6PDH protein levels and enzymatic activity in tubby mice lead accordingly to reduced levels of NADPH and glutathione (GSH), respectively. mRNA levels for the lipolytic enzymes acetyl-CoA synthetase and carnitine palmitoyltransferase are increased during the dark cycle and decreased during the light period, and several citric acid cycle genes are dysregulated in tubby mice. Examination of hypothalamic gene expression showed high levels of preproorexin mRNA leading to accumulation of orexin peptide in the lateral hypothalamus. We hypothesize that abnormal hypothalamic orexin expression leads to changes in liver carbohydrate metabolism and may contribute to the moderate obesity observed in tubby mice.

The tubby mutation was identified as a G-to-T transversion in the splice donor site of the last intron of a novel gene, leading to the loss of the COOH-terminal amino acid sequence encoded by the last exon (16, 31) and consequently to a loss of tubby function (41). The tubby gene is a member of a small gene family that also includes three tubby-like protein (TULP) genes (Tulp1, Tulp2, and Tulp3) (30, 32). The biochemical function of the TULPs is currently not fully understood. Roles as transcription factors (37), as intermediates in insulin signaling (14), or in intracellular transport (3, 7, 8) have been proposed.

Although the mutation was first described more than a decade ago, relatively little is known about the physiological abnormalities underlying the obesity in this model. Here we report that tubby mice have a low respiratory exchange ratio (respiratory quotient; RQ) that does not increase after feeding. This RQ abnormality is accompanied by changes in gene expression and altered metabolism in the liver that may contribute to the observed phenotype of slowly progressive moderate obesity.

MATERIALS AND METHODS

Animals. All animal studies were performed with the approval of The Jackson Laboratory Animal Care and Use Committee (protocol no. 99089). Seven-week-old C57BL/6J.Cg-Tub+/+Jkn (B6-tub/tub) mice, as well as their heterozygote (B6-tub/+ ) and wildtype (B6-/+ ) littermates, were obtained from the Research Animal Facility at The Jackson Laboratory. For collection of the Comprehensive Laboratory Animal Monitoring System (CLAMS) data, homozygous B6.Cg-HbbTub+/+J mice and control littermates were obtained from Jackson Research Systems. The mice were maintained on an NIH-31 mouse/rat diet with 4% fat (11% calories from fat, no. 5K54; PMI Feeds, St. Louis, MO), fed ad libitum, with free access to water (HCl acidified, pH 2.8–3.2) under controlled temperature and humidity with a 12:12-h light-dark cycle (lights on from 0600 to 1800). Mice were killed between 1600 and 1700 (light cycle sample) and between 2300 and 2400 (dark cycle sample), and liver and brain were harvested for RNA isolation and protein and enzymatic assays.

CLAMS. CLAMS (Columbus Instruments, Columbus, OH) is a set of five-in-one cages for automated, noninvasive, and simultaneous monitoring of horizontal and vertical activity, feeding and drinking, O2 consumption (V̇O2), and CO2 production (V̇CO2). Seven- to eight-week-old tubby mice and controls were placed in individual CLAMS cages between 1130 and 1300 on the first day of a 3-day test period. A 3-day test period allows examination of response to a novel environment from data collected on day 1 and leaves two additional days for diurnal patterns to be established. Raw data from each mouse were converted from the archive format to Excel and Statistica files for examination and analysis, respectively. Raw data for each of the measures were plotted as a function of time over each of the three consecutive 24-h periods. Mean values from each animal were examined for outliers in each measure relative to the control B6 mice at several different epochs. The epochs examined included exploratory,
daily, light, and dark periods and the onset of the 12-h dark period relative to the final portion of the light period.

Data are collected in three files for each animal, including an activity file, bout file, and total file. The activity file displays all activity data recorded every 10 s over the 72-h test period, and the bout file contains number and duration of bout activity during the test. The total file displays all measurements for each parameter (V\textsubscript{O\textsubscript{2}}, V\textsubscript{CO\textsubscript{2}}, respiratory exchange ratio, heat, accumulated feed, accumulated drink, XY total activity, XY ambulatory activity, and Z activity). The parameters are recorded from a single cage during a 30-s period every hour, beginning with the first cage of the system and ending with the last cage in the circuit. An analysis software program was created to compare each parameter during the explore period (first 3 measurements after placement in the cage), postexplore period (next 3 measurements after placement in the cage), daily means, three measurements before lights are turned off (BF), three measurements after lights are turned off (AF), three measurements before lights turn on (BN), and three measurements after lights are on (12).

Real-time quantitative RT-PCR. Brain (hypothalamus), liver, muscle, and adipose tissue were collected and immediately placed in liquid nitrogen. RNA was isolated using TRIzol reagent (Life Technologies) according to the manufacturer’s recommendation. Total RNA was further treated with RNase-free DNase I (Ambion). RNA quality and quantity were evaluated by UV spectrophotometry and a total RNA Nano Assay (Agilent Technologies 2100 Bioanalyzer-Bio Sizing, version A.02.01 S1232), respectively.

Oligonucleotide sequences used for real-time PCR assays, length of amplified fragment, and GenBank accession no. of the cDNA from which the primer sequence was derived are in Table 1.

Real-time RT-PCR assays were performed as previously described (6, 34). Real-time PCR primers for the quantitative detection of target mRNAs were designed using the Primer Express computer software (Applied Biosystems). The real-time measurements were carried out on an ABI PRISM 7700 SDS instrument. Samples were analyzed in triplicate in three independent runs. Table 1 lists the forward and reverse primers used for the real-time RT-PCR analyses. The size of the PCR products varied from 201 to 215 bp.

To quantify the gene expression profiles, we used the comparative threshold cycle (Ct) method. The Ct value is defined as the cycle number in which the detected fluorescence exceeds the threshold value. Each Ct value was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18s rRNA. The normalized Ct values for each selected gene were analyzed using the two-way ANOVA method including genotype (B6-+/+, B6-tub/tub), condition (dark/light), and their interactions in the model. In all experiments, the threshold value used to determine Ct during analysis was kept constant. Student’s t-tests were used to compare tubby to B6 mice during light and dark periods separately. Ct values were converted to fold differences in expression according to the equation 2\[\text{Ct1(18s rRNA)} – \text{Ct2(18s rRNA)}\] / [2\[\text{Ct1(tub/tub)} – \text{Ct2(tub/tub)}\]], where Ct1(18s rRNA) and Ct2(18s rRNA) represent the Ct values for the 18s rRNA gene in the tubby and normal samples, respectively.

### Table 1. Gene-specific primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>NCBI</th>
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<tbody>
<tr>
<td>Glucokinase</td>
<td>TGTTGCGACATGGTGAATGAC</td>
<td>ACAATCATGCCGACCTCAGAT</td>
<td>L38990</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase</td>
<td>GATCTGCGACACCCCTACTCT</td>
<td>TGAAGGCGGCCGAGCATTAG</td>
<td>BC019512</td>
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<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>TCAAGAGACCTGGATGACTGAG</td>
<td>TGGCGCCACCTGCACGAT</td>
<td>Z19111</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>TTTGAGGGAACCTGAGCCGATCTC</td>
<td>AGGCGGCTTCTGTGCTGATCAAAG</td>
<td>NM021514</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>GCAGGATGCGAGAAGCAGTCT</td>
<td>GCCCGGAGCCGACGTTACG</td>
<td>D67676</td>
</tr>
<tr>
<td>Acetyl-CoA synthetase</td>
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<td>CCGTGAGGAGGAGGAGGAG</td>
<td>AF310087</td>
</tr>
<tr>
<td>Carnitine palmitoyltransferase1</td>
<td>TGGCCGACAAGGAGAAGAAG</td>
<td>CCTGACATGGTTGCTGCTG</td>
<td>AF017175</td>
</tr>
<tr>
<td>Fatty acid synthase</td>
<td>TTTACACAGGGCATGGTTTT</td>
<td>GGGGCAAGGCGCTGTTAGTT</td>
<td>AF213073</td>
</tr>
<tr>
<td>Phosphoenergypyruvate carboxykinase</td>
<td>GATGATGGCGATGGCATGATG</td>
<td>CATATCGGCTGTAACAGAGGAT</td>
<td>NM011044</td>
</tr>
<tr>
<td>Citrate synthase</td>
<td>GCCCGCTTCTTACCTCATCATCTCAT</td>
<td>CGACACCTGCCGAGATGGA</td>
<td>NT032416</td>
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<tr>
<td>Ioscitrate dehydrogenase</td>
<td>TGAGGCCGAGCTGAGCTGACG</td>
<td>CGAGCAGGCAAATCCCTGTGAC</td>
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<tr>
<td>Ketoglutarate dehydrogenase</td>
<td>TTCTCGGCGGATGACTGAG</td>
<td>CTCACGCTTCTGACGACGAAAG</td>
<td>XM004889</td>
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<tr>
<td>Succinyl-CoA dehydrogenase</td>
<td>TGTTGGGACACCCGAGAGAAGAAG</td>
<td>CCTGAGGCGGAGGAGAAGAAG</td>
<td>AF299839</td>
</tr>
<tr>
<td>Succinyl-CoA synthetase</td>
<td>GATTCCTTCTTGTGCTGCTGCTGCTG</td>
<td>CGCGGCTGAGGCGATACCC</td>
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<tr>
<td>Preproorexin</td>
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<td>AGGCGACGGAAGAGAAGAAGAAG</td>
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<td>Agouti-related protein</td>
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<td>AGGAGCTTGGATGACGTGAC</td>
<td>NM007427</td>
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<tr>
<td>Neuropeptide Y</td>
<td>TGTTGGGACACCCGAGAGAAGAAG</td>
<td>CGCGGCTGAGGCGATACCC</td>
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</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>AGACCGCTGAGGCGAGTGAGAAGAAG</td>
<td>AGGAGCTTGGATGACGTGAC</td>
<td>AF299839</td>
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</tbody>
</table>

NCBI, National Institute for Biotechnology Information.
After the transfer, the membranes were incubated for 1 h in 25 mM Tris/HCl, pH 8.3, and 250 V for 30 min. After the transfer, the membranes were incubated for 1 h in Blotto A (1× TBS containing 5% nonfat milk, 0.05% Tween 20) at room temperature. Subsequently, the membranes were incubated overnight at 4°C in Blotto A in the presence of 1:5,000 diluted rabbit anti-human glucose-6-phosphate dehydrogenase (G6PDH) polyclonal antibodies (cross-reactive between human and mouse, no. ab993, Abcam). Antibodies were detected using Amersham’s enhanced chemiluminescence (peroxidase-conjugated secondary antibodies, 1:1,500 dilution) and detected with the use of chemiluminescent reagents (Amersham, Uppsala, Sweden).

Metabolites. Blood was collected from the orbital sinus via heparinized capillary tubes, and plasma was obtained by centrifugation at 4,000 g for 10 min (4°C). Sampling times were between 1000 and 1100 for nonfed samples, between 1600 and 1700 for light period samples, and between 2000 and 2100 for dark period samples.

Glycogen was extracted from liver using hot potassium hydroxide and ethanol precipitation (10). After acid hydrolysis, glucose levels were determined in the hydrolysate as below.

Plasma glucose and free fatty acid (FFA) levels were determined spectrophotometrically using a colorimetric assay (no. 1383175, Roche Diagnostics) or in a Beckman Coulter Synchrom CX5 Delta chemistry analyzer (Beckman Coulter).

β-Hydroxybutyrate levels were determined in duplicate using a spectrophotometric assay (no. 310-A, Sigma).

Pyruvate was extracted from liver using the methanol-chloroform-water (M/C) method (21). The tissue was kept under liquid nitrogen and ground to a fine powder with a mortar and pestle. Ice-cold solvent (reagent-grade methanol and chloroform in a ratio of 2:1 (vol/vol)) was added to the frozen sample (0.3 ml/100 mg tissue) and mixed to form a slurry. After thawing and transfer to centrifuge tubes, the tissue was kept under liquid nitrogen and ground to a fine powder with a mortar and pestle. Ice-cold solvent (methanol and water) was separated from the lower phase (organic) using a glass syringe, and both fractions were dried at room temperature.

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Protein determination. The concentration of protein in homogenates was determined by the method of Lowry et al. (24) using BSA as a standard.

Statistical analysis. All of the statistical analyses were conducted in JMP (v.6.0; SAS Institute, Cary, NC) and StatView (v.4.5; Abacus Concepts, Berkeley, CA). The analyses of differences between two means of different genotypes in the physiological and expression data were assessed by Student’s t-tests. For all the factorial experiments, two-way ANOVA analyses were performed with genotype (B6–+/+, B6–tub/tub) and condition (dark/light) as factors, to access the significance of the main effects of each factor and of their interactions. Whereas a main effect indicates a difference in means due to a factor averaged over the levels of the other factor in the design, interaction addresses whether the effects of each factor are simply additive.

RESULTS

Tubby mice have an abnormally low respiratory exchange ratio in both fed and nonfed states. CLAMS is a mouse caging system with integrated instrumentation that allows for 24-h automated data collection on individual mice. Monitored are feeding, drinking, and exploratory, ambulatory, and rearing activity as well as V\(\text{O}_2\) and V\(\text{CO}_2\). To test the performance of the system in detecting mutant mice, 7–8-wk-old C57BL/6J (B6–+/+) and condition (dark/light) as factors, to access the significance of the main effects of each factor and of their interactions. Whereas a main effect indicates a difference in means due to a factor averaged over the levels of the other factor in the design, interaction addresses whether the effects of each factor are simply additive.

Figure 1 shows that amounts and patterns of activity in a tubby mouse are generally similar to those of the normal B6 mouse (Fig. 1, A and B). V\(\text{O}_2\) and V\(\text{CO}_2\) of the B6 mouse parallel activity levels (Fig. 1, A and C) over the same 24-h period. Analysis of V\(\text{O}_2\) in B6 mice demonstrated that CO\(\text{O}_2\) release is slightly lower than V\(\text{O}_2\) during periods of relative inactivity (light, 0600–1800) and almost the same as V\(\text{O}_2\) during periods of higher activity (dark, 1800–0600). This reflects a normal metabolic shift from fat utilization (lower CO\(\text{O}_2\)-to-O\(\text{O}_2\) ratio) as the primary fuel source to increased carbohydrate use (higher CO\(\text{O}_2\)-to-O\(\text{O}_2\) ratio).

In tubby mice (Fig. 1, B and D), average V\(\text{O}_2\) is lower than in B6 mice, most notably during high activity (in the dark period). Shortly after entering the dark period (~1900), V\(\text{O}_2\) is similar to that in B6. On the other hand, V\(\text{CO}_2\) is always lower in tubby mice compared with B6 regardless of activity levels (light and dark). Consequently, the RQ in tubby mice is lower than in B6 mice and does not increase after transition to the dark cycle. Figure 2 shows the values for V\(\text{O}_2\) (Fig. 2A), V\(\text{CO}_2\) (Fig. 2B), RQ (Fig. 2D), and ambulation (Fig. 2C) for all animals averaged over distinct time periods (such as light or dark period, etc.). Increased activity typically increases RQ values, as is evident in the values plotted for normal mice (e.g., light vs. dark; Fig. 2, C and D). The activity-related shift in RQ values is almost entirely absent in the mutant mice [Fig. 2D; 0.67 ± 0.22 (B6–tub/tub) vs. 0.76 ± 0.02 (B6), P < 0.0001 light period; 0.67 ± 0.02 (B6–tub/tub) vs. 0.83 ± 0.04 (B6), P < 0.0001 dark period].

The body weights of the tested tub/tub mutant mice were not significantly different from that of their littermate controls (males 26.1 ± 0.0 vs. 25.4 ± 1.1 g, P = 0.48; females 21.4 ± 0.6 vs. 21.4 ± 2.3 g, P = 0.92). Averaged over the entire light and dark periods, tubby mice had a tendency toward lower food intake than their littermate controls in the 4% standard diet (Fig. 2E), particularly during the light period, although this difference reached significance only for females during the light period (P < 0.0001). Tubby mice also showed a trend toward lower activity levels (Fig. 2F; again, this reached significance only for females in the light period (P < 0.0003).
Gene expression of key enzymes analyzed by real-time quantitative RT-PCR. To gain initial insights into the causes for the low RQ in tubby homozygous mice, we examined mRNA levels for key metabolic genes across the light-dark transition. We focused on liver because of its role in carbohydrate metabolism. Real-time kinetic quantitative PCR was used to determine mRNA levels for selected genes in glycolytic, gluconeogenic, lipolytic, and lipogenic pathways that had been reported in the literature to show diurnal rhythms in expression (13, 27, 44), with maximal expression levels between 2200 and 0600 and minimal expression levels between 1400 and 1800. On the basis of these published periods and the activity and feeding patterns obtained from our CLAMS data, we selected 1600–1700 to collect the light-period samples and 2300–2400 for the dark-period samples. The relative expression levels comparing tubby with B6 mice during light and dark periods are presented in Fig. 3.

The majority of the genes studied showed aberrant expression in tubby mice. While mRNA expression levels for representative enzymes that are active in glucose uptake (glucokinase; GK), glycolysis (PFK), gluconeogenesis (phosphoenolpyruvate carboxykinase; PEPCK), and lipogenesis (fatty acid synthase; FAS) were comparable to B6, genes in the lipolytic pathway showed regulation consistent with continued activity through the dark period (Fig. 3, E–K). At the same time, these genes are downregulated in tubby mice during the light period compared with B6. Examples for this mRNA pattern are isocitrate dehydrogenase (IDH; Fig. 3H), acetyl-CoA synthetase (ACS; Fig. 3E), and carnitine palmitoyltransferase-1 (CPT1; Fig. 3F). The genes in the tricarboxylic acid (TCA) cycle were downregulated during the light period in tubby mice compared with B6 controls, when food intake was slightly lower in tubby mice. During the dark period, where food intake was comparable, the TCA cycle genes showed higher expression in tubby mice than in B6 controls (Fig. 3P).

We analyzed the normalized Ct values for each selected gene using the two-way ANOVA method, including genotype (B6+/−, B6-tub/tub), condition (light/dark), and their interactions in the model (see Supplemental Materials; the online version of this article contains supplemental data). ANOVA analyses indicated a significant main effect of condition on gene expression level for the genes encoding ACS, agouti-related protein (Agrp), FAS, G6PDH, and PEPCK (P < 0.0001) and for genes GK and IDH (P < 0.0005). ANOVA analyses also indicated a significant main effect of genotype on expression level for the genes encoding Agrp, G6PDH, and orexin (P < 0.0001) as well as for ACS and neuropeptide Y (NPY; P < 0.0005). The interactions between genotype and condition were significant for ACS, CPT1, and G6PDH (P < 0.0001) and for succinyl-CoA synthetase and succinate dehydrogenase (P < 0.001); thus, in those cases, specific comparisons between tubby and B6 mice under each condition were most informative. Student’s t-tests comparing tubby with B6 mice during light and dark periods separately were carried out for all the selected genes. The t-test results are presented in Fig. 3, where Ct values were converted to fold differences in expression, to illustrate the true changes.

The most dramatic dysregulation in tubby mice was seen for G6PDH. Although G6PDH mRNA showed comparably very low expression in the light period in both mouse strains, it is highly induced in B6 mice after the onset of feeding, whereas it fails to be induced in tubby mice (P < 0.00001; Fig. 3L). This failure to induce G6PDH mRNA leads to a 46% reduction in protein levels, as ascertained by Western analysis, and a concomitant 34% reduction of liver enzyme activity compared with B6 mice (Table 2).

Liver enzyme activities and metabolites. In the analysis of liver enzyme activities and metabolite concentrations, two-way ANOVA was performed to access the significance of the main effect factors, genotype (B6+/−, B6-tub/tub) and condition
The greater reliance of tubby mice on fat metabolism for energy needs would be expected to be reflected in higher ketone body production. Plasma levels of β-HBA as a measure for ketone body formation were significantly higher in tubby mice compared with B6 controls (Table 2) during both the light (46 ± 6 vs. 32 ± 2 mg/dl, \( P < 0.02 \)) and the dark periods (61 ± 4 vs. 33 ± 4 mg/dl, \( P < 0.008 \)).

Conversely, reduced use of carbohydrate as an energy source would be expected to lead to increased liver glycogen stores in the nonfed state. Tubby mice were found to have higher liver glycogen levels than B6 mice 1 h before the end of the light cycle (25.8 ± 7.0 mg/dl, \( n = 4 \), vs. 11.9 ± 0.2 mg/dl, \( n = 4 \); \( P < 0.007 \)).

**DISCUSSION**

The rate of energy expenditure and fuel utilization can be noninvasively assessed by measuring \( V_\text{O}_2 \) and \( V_\text{CO}_2 \). The ratio of the two measurements, the RQ, gives an indication of the energy source utilized in an organism. Metabolism of triglycerides is normally associated with an RQ of ~0.70, and an RQ of 1.0 would indicate total reliance on carbohydrates as an energy source, which in normal individuals is only approached under investigation, Student’s \( t \)-tests were conducted to compare B6 with tubby mice during light and dark periods separately; \( P \) values obtained from \( t \)-tests are summarized in Table 2 along with means ± SE for each group.

**Tubby mice show abnormal expression of hypothalamic neuropeptides.** Because Tub is not expressed in liver (32) but has prominent expression in the central nervous system, we investigated the expression of key hypothalamic neuropeptides and proteins as possible mediators of the abnormal regulation of liver metabolism in tubby mice. Quantitative real-time PCR analysis showed that NPY (\( \text{Npy} \)), Agrp (\( \text{Agrp} \)), and prepro-orexin (\( \text{Hcrt} \)) were upregulated between 3- and 64-fold in both the light and the dark periods compared with B6 controls (Fig. 3, \( M–O \)). The higher expression levels of \( \text{Hcrt} \) in tubby mice lead to higher levels of orexin protein in the cell bodies of orexin neurons in the lateral hypothalamus, as determined by immunohistochemistry (Fig. 4). Quantitation by Western analysis showed that orexin protein levels in brain extracts from tubby mice at 1600 were significantly higher than from B6 mice (means ± SE: 178.3 ± 26 ng/g, \( n = 3 \), vs. 51.9 ± 1.7 ng/g, \( n = 3 \); \( P < 0.0086 \); Fig. 4).

**Fig. 3.** Relative changes in gene expression levels between B6-tub/tub mice and littermate controls during light and dark periods. Expression levels were determined by quantitative real-time PCR assays. mRNA from 3 animals per genotype and time point was assayed. PCR assays were performed in triplicate. \( A–O \): relative changes (fold change) in gene expression levels between B6-tub/tub and littermate controls during light and dark periods. The lowest expression level for each gene was set to equal 1. \( P \): gene expression levels for citric acid cycle enzymes in B6-tub/tub mice relative to B6 controls before and after the onset of the dark phase. \( \text{GK} \), glucokinase; \( \text{PFK} \), phosphofructokinase; \( \text{ACS} \), acetyl-CoA synthetase; \( \text{CPT1} \), carnitine palmitoyltransferase-1; \( \text{FAS} \), fatty acid synthase; \( \text{PEPCK} \), phosphoenolpyruvate carboxykinase; \( \text{CS} \), citrate synthase; \( \text{IDH} \), isocitrate dehydrogenase; \( \text{SDH} \), succinate dehydrogenase; \( \text{OGDH} \), 2-oxoglutarate dehydrogenase; \( \text{HBA} \), 3-hydroxybutyrate; \( \text{G6PDH} \), glucose-6-phosphate dehydrogenase; \( \text{Agrp} \), agouti-related protein; \( \text{Npy} \), neuropeptide Y. • \( P < 0.01 \); ** \( P < 0.001 \). (dark/light), and of their interactions. The ANOVA analyses revealed a significant main effect of condition to increased activities during the dark period. For the factor genotype, its effect averaged over light and dark periods is significant for the plasma levels of FFA (\( P < 0.01 \)) and β-hydroxybutyric acid (β-HBA) and the enzyme activities of PFK (\( P < 0.001 \)) and G6PDH (\( P < 0.0001 \)). The interactions between genotype and condition were significant for G6PDH, PFK, and PDH actual activity (\( P < 0.01 \)). For all of the metabolite and liver enzymes under investigation, Student’s \( t \)-tests were conducted to compare B6 with tubby mice during light and dark periods separately; \( P \) values obtained from \( t \)-tests are summarized in Table 2 along with means ± SE for each group.
Although tubby mice gradually increase their food intake as they age (Nishina PM and Naggert JK, unpublished observation), their food intake surpasses that of control littermates only after the tubby mice weigh significantly more. The increase in food intake in older tubby mice may, therefore, reflect higher substrate utilization in other tissues as well. If glucose was used as energy source to a significant extent in other tissues, such as in adipose tissue or muscle, then an increase in RQ should have been observed during the light-dark transition.

Table 2. Metabolite levels and activities of liver enzymes in B6-tub/tub and B6-+/+ mice during light and dark periods

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Light</th>
<th>Dark</th>
<th>Main Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B6-+/+</td>
<td>B6-tub/tub</td>
<td>B6-+/+</td>
</tr>
<tr>
<td>PFK mU/mg</td>
<td>6.1±1.3</td>
<td>6.1±1.3</td>
<td>11.2±0.9</td>
</tr>
<tr>
<td>G6pase U/min⁻¹·g⁻¹</td>
<td>44.2±1.2</td>
<td>44.2±1.2</td>
<td>41.1±3.6</td>
</tr>
<tr>
<td>Total activity mU/g</td>
<td>1,273.4±96.3</td>
<td>1,273.4±96.3</td>
<td>1,008.4±155.7</td>
</tr>
<tr>
<td>Actual activity mU/g</td>
<td>76.6±13.2</td>
<td>76.6±13.2</td>
<td>71.1±4.2</td>
</tr>
<tr>
<td>% Total activity</td>
<td>6.4±1.7</td>
<td>6.4±1.7</td>
<td>7.1±1.0</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE; n (in parentheses) = no. of animals represented. *Based on units (U)/g liver wet wt and U/mg protein. †P value of the main effect of condition (light vs. dark) was obtained using 2-way ANOVA. ‡Model includes a significant interaction term. ND, not determined; NS, not significant; PFK, phosphofructokinase; G6pase, glucose-6-phosphatase; PDH, pyruvate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; β-HBA, β-hydroxybutyric acid; FFA, free fatty acid.

Fig. 4. Orexin A-immunoreactive neurons in the lateral hypothalamic area of B6-+/+ and B6-tub/tub mice (A) and quantitation by enzyme immune assay (B). Orexin A peptide was determined in three 7-wk-old male B6-tub/tub and B6-+/+ mice, respectively. Values are expressed as means ± SE.
A large fraction (77%) of the genes whose mRNA levels were measured showed abnormalities in diurnal pattern and/or levels of expression. These abnormalities are already present before the onset of obesity in the tubby mice. Fig. 5 summarizes our findings. Genes in the fatty oxidation pathway are downregulated (relative to B6) during the light cycle, perhaps reflecting the reduced food intake during this period. However, during the dark period, when food intake in tubby mice is similar to that in control mice, CPT1, which controls mitochondrial uptake of fatty acids for β-oxidation, and ACS, which activates the end product of β-oxidation, are upregulated in tubby mice. Genes in the TCA cycle through which the acetyl groups are oxidized show a similar pattern of downregulation during the day and upregulation at night. These changes may be expected if, as hypothesized, fatty acid oxidation is the main source of energy during the dark period in tubby mice. It is interesting to note that in Caenorhabditis elegans worm carrying a mutation in the tub-1 gene, β-oxidation also serves to limit lipid accumulation, since a mutation in the β-oxidation pathway enzyme 3-ketoacyl-CoA thiolase interacts with the tub-1 mutation to impair β-oxidation and increase fat storage (26).

Genes in the glycolytic pathway either show the same pattern of regulation as in B6 mice or, like pyruvate kinase (PK) as well as the rate-limiting enzyme for glycolysis, PDH, are upregulated in the dark period compared with B6. Because the RQ in tubby mice does not rise in the dark period, it appears that glycolysis has to be inhibited posttranslationally in tubby mice because glycogen content is markedly increased. Indeed, the activities of key regulatory enzymes in the glycolytic pathway such as PFK and PDH are lower in tubby mice, during both the light and dark cycles (Table 2).

In addition, it is possible that a higher level of β-oxidation could lead to increased levels of acetyl-CoA, which would feedback inhibit PDH and thus reduce glycolysis. Increased acetyl-CoA could then be available for de novo synthesis of fatty acids or ketone bodies. As expected, we do find increased levels of plasma ketone bodies in tubby mice, even in the light period. A reduction in glycolysis would also be expected to leave increased levels of glucose for storage. Again we find higher levels of liver glycogen in tubby mice at the end of the light period, when these stores should be largely depleted.

An unexpected finding was the downregulation of G6PDH and the complete lack of G6PDH induction in the dark period. G6PDH is the key regulating enzyme in the pentose phosphate pathway, the main functions of which are to supply reducing equivalents in the form of nicotinamide adenine dinucleotide phosphate (NADPH) for the de novo synthesis of fatty acids and for the maintenance of intracellular reduced glutathione (GSH) concentrations. Perhaps as a consequence of the lack of G6PDH induction in tubby mice, GSH and NADPH levels are lower than those in B6 mice during the dark period. However, the ratio of NADPH to NADP⁺ is similar in B6 and B6-tub/tub mice. Additional NADPH can be generated by the pentose phosphate pathway itself through conversion of the five-carbon sugar via transaldolase/transketolase reactions to fructose-6-phosphate, which can then reenter the pathway. NADPH can also be generated by alternate routes, for example in the malic enzyme-catalyzed reaction converting malate to pyruvate concomitant with a reduction of NADP⁺ to NADPH. Lower production of NADPH via the pentose phosphate pathway and lower levels of pyruvate in tubby mice would favor this reaction to provide reducing equivalents for fatty acid synthesis. Another source for NADPH is the cytoplasmic conversion of citrate to ketoglutarate by isocitrate dehydrogenase, which is fed by shuttling citrate out of the mitochondrion in exchange for malate and ketoglutarate into the mitochondrion by transporting malate out again. Isocitrate dehydrogenase as a source for NADPH has previously been shown to be important for fatty acid synthesis (18).

Because up to 30% of liver glucose oxidation may occur via the pentose phosphate pathway (23, 43), the failure to induce...
G6PDH in tubby mice contributes to their lower glucose utilization and RQ. Whether the lack of G6PDH induction is a direct effect of the tub mutation, a property of the genetic background, or secondary to the altered metabolic and hormonal environment in tubby mice is currently not known. Direct involvement of the TUB protein is, however, unlikely because of the very low expression of tub in liver. G6PDH is primarily regulated posttranscriptionally; i.e., after a stimulus, mature mRNA is recruited to the cytoplasm after processing of a nuclear pre-mRNA pool (15, 36). Insulin, glucose, and thyroid hormone induce mature G6PDH mRNA (36), but the components of the signal transduction pathway are not known. It is thought that the stimulatory effect of glucose (and fructose) is mediated by a glycolytic intermediate, and insulin may act through stimulation of glycolysis (5). Reduced flux through the glycolytic pathway in tubby mice could, therefore, contribute to lower G6PDH levels.

The profound changes in liver metabolism in tubby mice are presumably the result of defects in central regulation, since tub is expressed primarily in the central and peripheral nervous system and is only at very low levels in the liver (32). However, the liver is innervated by both the sympathetic and parasympathetic nervous system. Transneuronal tracings show that neuronal populations in the lateral hypothalamic area, the ventromedial hypothalamic nucleus, and the suprachiasmatic nucleus are retrogradely labeled (19). It is also well known that sympathetic activity is correlated with fatty acid oxidation in peripheral tissues (38, 39). In the search for potential central causes of the observed abnormalities in liver metabolism, we examined the expression of key neuroptides and their receptors in the hypothalamus. The most notable difference between B6-tub/tub and B6 mice was an ~60-fold upregulation of preproorexin mRNA. This upregulation was also reflected in increased orexin A protein levels.

It has been shown previously that intracerebroventricular injection of orexin A can lead to an increase in metabolic rate and a decrease in RQ, depending on the metabolic state of the animal (25). Similarly, electrical stimulation of the ventromedial hypothalamic nucleus (VMH) leads to increased basal metabolic rate and reduced RQ (35). It may be important in this context that the orexin neurons of the lateral area of the hypothalamus project to the VMH, which in turn projects to the autonomic regions of the paraventricular nucleus, thus providing a path for autonomic signals to the liver (19). This suggests the hypothesis that the increase in orexin A in tubby mice is causative of the abnormal liver metabolism via the autonomic nervous system. It also appears from this work that the mutation in the tub gene primarily affects pathways leading to increased fat deposition while leaving mechanisms resisting weight gain, such as increased lipid oxidation, intact. Tubby mice may, therefore, be a useful model system to further study the central regulation of energy metabolism.

REFERENCES


GRANTS

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