Transcriptome profiling of brown adipose tissue during cold exposure reveals extensive regulation of glucose metabolism

Qin Hao,1 Rachita Yadav,1,2 Astrid L. Basse,1 Sidsel Petersen,1 Si B. Sonne,1 Simon Rasmussen,2 Qianhua Zhu,3 Zhike Lu,3 Jun Wang,1,3,4,5,6 Karine Audouze,2,7 Ramneek Gupta,2 Lise Madsen,1,8 Karsten Kristiansen,1,3 and Jacob B. Hansen1

1Department of Biology, University of Copenhagen, Copenhagen, Denmark; 2Department of Systems Biology, Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark; 3BGI-Shenzhen, Beishan Industrial Zone, Yantian District, Shenzhen, China; 4Princess Al Jawhara Center of Excellence in the Research of Hereditary Disorders, King Abdulaziz University, Jeddah, Saudi Arabia; 5Macau University of Science and Technology, Avenida Wai Long, Taipa, Macau, China; 6Department of Medicine, University of Hong Kong, Hong Kong; 7Université Paris Diderot, Inserm UMR-S973, Paris, France; and 8National Institute of Nutrition and Seafood Research, Nordnes, Bergen, Norway

Submitted 18 June 2014; accepted in final form 10 December 2014

Hao Q, Yadav R, Basse AL, Petersen S, Sonne SB, Rasmussen S, Zhu Q, Lu Z, Wang J, Audouze K, Gupta R, Madsen L, Kristiansen K, Hansen JB. Transcriptome profiling of brown adipose tissue during cold exposure reveals extensive regulation of glucose metabolism. Am J Physiol Endocrinol Metab 308: E380–E392, 2015. First published December 17, 2014; doi:10.1152/ajpendo.00277.2014.—We applied digital gene expression profiling to determine the transcriptome of brown and white adipose tissues (BAT and WAT, respectively) during cold exposure. Male C57BL/6J mice were exposed to cold for 2 or 4 days. A notable induction of genes related to glucose uptake, glycolysis, glycogen metabolism, and the pentose phosphate pathway was observed in BAT from cold-exposed animals. In addition, glyceral-3-phosphate dehydrogenase 1 expression was induced in BAT from cold-challenged mice, suggesting increased synthesis of glycerol from glucose. Similarly, expression of lactate dehydrogenases was induced by cold in BAT. Pyruvate dehydrogenase kinase 2 (Pdk2) and Pdk4 were expressed at significantly higher levels in BAT than in WAT, and Pdk2 was induced in BAT by cold. Of notice, only a subset of the changes detected in BAT was observed in WAT. Based on changes in gene expression during cold exposure, we propose a model for the intermediary glucose metabolism in activated BAT: 1) fluxes through glycolysis and the pentose phosphate pathway are induced, the latter providing reducing equivalents for de novo fatty acid synthesis; 2) glycerol synthesis from glucose is increased, facilitating triacylglycerol synthesis/fatty acid re-esterification; 3) glycogen turnover and lactate production are increased; and 4) entry of glucose carbon into the tricarboxylic acid cycle is restricted by Pdk2 and Pdk4. In summary, our results demonstrate extensive and diverse gene expression changes related to glucose handling in activated BAT.

thermogenesis; glycolysis; pyruvate dehydrogenase

EXCESSIVE ACCUMULATION of adipose tissue causes obesity that predisposes to hypertension, diabetes, and cardiovascular diseases. Adipose tissue functions not only as an energy storage depot but also as an endocrine organ secreting numerous adipokines acting on the brain, liver, muscle, and other tissues (3, 23, 54). There are two main types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). A white adipocyte is specialized in storage of lipids during feeding and release of lipids during starvation. In contrast, brown fat is specialized in energy expenditure and performs a major role in nonshivering thermogenesis during cold acclimation. Metabolically active BAT is present in adult humans, leaving BAT as an appealing therapeutic target to combat obesity and metabolic disease (8, 38, 51, 61, 62).

In addition to classical brown and white adipocytes, inducible uncoupling protein 1 (UCP1)-expressing adipocytes have been identified in WAT and are designated brite (brown-in-white) or beige adipocytes (5, 7, 41, 52, 70, 74). The abundance of brite/beige adipocytes in mice is highly dependent on mouse strain and location of the adipose depot and is increased in response to cold exposure or treatment with β-adrenergic agonists (11, 71). The subcutaneous inguinal WAT (iWAT) depot has a very high capacity for recruiting brite/beige adipocytes, whereas the visceral gonadal WAT depot in mice is more refractory to brite/beige adipocyte recruitment (11, 50, 63).

BAT is the principal site of nonshivering thermogenesis (3, 17, 18, 39). During thermogenesis, brown adipocytes take up large amounts of lipids and glucose from the circulation (2). Fatty acids are major fuel substrates for brown adipocyte thermogenesis through β-oxidation (32). Cold exposure or β-adrenergic activation of BAT causes lipolysis in brown adipocytes, thereby increasing the supply of fatty acids for oxidation. It has been suggested that glucose taken up by brown adipocytes is used for oxidation and fatty acid synthesis, but there is evidence pointing to a substantial fraction of glucose carbon ending up in lactate and glycerol. Furthermore, lactate and glycerol release are increased following norepinephrine infusion (19, 24, 28). Gene expression profiling has been applied in a number of studies to characterize cold-induced gene expression changes in adipose tissue from different species (12, 49, 53, 64, 65, 67, 72, 75). These studies have addressed a number of interesting topics related to the effect of cold exposure, but none have provided detailed information on gene regulation influencing intermediary glucose metabolism.

For three reasons, we have applied digital gene expression profiling (DGEP) to obtain a comprehensive understanding of gene expression changes related to glucose metabolism during cold-activated adipose thermogenesis and browning: 1) glucose uptake is remarkably increased in BAT in response to cold; 2) activated BAT and brite/beige adipose tissue improve systemic glucose homeostasis; and 3) knowledge of the gene regulatory circuitry controlling glucose handling in adipose...
tissue from cold-exposed mice is sparse (16, 22, 58). We sequenced the transcriptome of interscapular BAT (iBAT) and two WAT depots [browning-prone iWAT and browning-resis-
tant epididymal WAT (eWAT)]. We analyzed three time points: day 0 (control, thermoneutrality) and 2 and 4 days of exposure to 4°C. Time points were selected based on previous reports (14, 36, 66) and the aim of focusing on a phase of high ther- mometric output from BAT and ongoing browning of subcu-
taneous WAT. Overall, we found that relatively few cold-
regulated genes were shared between iBAT and iWAT, whereas a larger proportion of biological processes was in common. Gene expression changes during cold exposure were consistent with profound alterations in glucose metabolism, including apparent increases in glucose uptake, glycogen turn-
over, glycolytic flux, pentose phosphate pathway (PPP) flux, and synthesis of glycerol 3-phosphate and lactate.

MATERIALS AND METHODS

Experimental mice and tissue sampling. In experiment 1, 8-wk-old male C57BL/J6 mice were kept at thermoneutrality (28–30°C) on a 12:12-h light-dark cycle. Mice were fed a standard chow diet ad libitum at all times. After 8 days of acclimation, mice were divided into three groups (n = 8). Two groups were transferred to 4°C for 2 or 4 days. The third group was killed without cold exposure (control mice). In experiment 2, 10-wk-old male C57BL/J6 mice were kept for 8 days at thermoneutrality (28–30°C) (n = 4) or 4°C (n = 4). This experiment was used for Fig. 5. Mice were killed by cervical dislo-
cation. iBAT, iWAT, and eWAT were dissected as previously de-
scribed (15). All mouse experiments were approved by the Norwegian or Danish Animal Research Authority.

RNA isolation from tissue. Adipose tissues were snap-frozen in liquid nitrogen and stored at −80°C. Tissues (40–150 mg) were homogenized in 1 ml TRIzol (Life Technologies), and total RNA was extracted. RNA concentrations were determined by NanoDrop 2000 (Thermo Scientific). The integrity of RNA was confirmed by electro-
phoresis and/or a 2100 Bioanalyzer (Agilent Technologies).

DGEP protocol. Tag library preparation was performed using the Illumina NlaIII Tag Profiling Sample Prep Kit according to the instructions of the manufacturer: 1 µg of total RNA was used for mRNA capture by magnetic oligo(dT) beads. First- and second-strand cDNA were synthesized, and bead-bound cDNA was digested with NlaIII, which cuts at CATG sites. Twenty-one base pair tags were ligated to the free
end of the cDNA fragment. The GEX NlaIII adapter contains a restriction site for MmeI, which cuts 17 bp downstream from the NlaIII recognition site. Twenty-one base pair tags starting with the NlaIII recognition sequence were released after MmeI digestion and ligated with a second adapter (GEX adapter 2) at the site of MmeI cleavage. The adapter-ligated cDNA tags were enriched using PCR primers annealing to the adapter ends. Finally, the resulting fragments were purified and checked by a 2100 Bioana-
lyzer. Each sample was sequenced in one lane of an Illumina flowcell using the Illumina Genome Analyzer II system (BGI-Shenzhen) according to the instructions of the manufacturer. Image analysis, base calling, and extraction of tags were performed using the Illumina pipeline. The data set has been deposited in NCBI’s Gene Expression Omnibus and are accessible through the series accession number GSE65301.

Evaluation and mapping of tags. The sequenced tags from individual samples were checked using FastQC, and counts were summarized by custom shell scripts. Before the tags were mapped to transcript databases, we used cutadapt (31) to eliminate the contamination from sequencing primer and adapters. Mouse mRNA reference (refMmra, 2012_08) was downloaded from the UCSC database. The informative 17 bp of the read were mapped to mRNA reference using bwa (27), and only perfect and unique matches were kept. Information of the NlaIII recognition position in mRNA sequences was extracted by custom perl scripts. The matched tags were filtered by comparing the matched position with the NlaIII recognition CATG sites. Tags with a >1-bp difference were excluded. Approximately 40–50% of total tags passed the screening, and tag counts for each gene were summarized into a count matrix for individual samples. The number of total tags in each sample was normalized to one million tags, and genes with a tag count below five in more than one-third of the samples were removed from the count matrix.

Statistical analysis and gene functional classification for sequenc-
ing data. Principal component analysis (PCA) was performed and visualized using the R package FactoMineR (26). Bioconductor package edgeR in R was used to perform statistical analysis for finding differentially expressed genes (45–47). Gene ontology (GO) enrich-
ment analysis was performed using R package GObi (9). REVIGO was used to summarize and visualize the enriched GO terms (57).

Cell culture. WT-1 cells (59) were propagated and differentiated in Dulbecco’s modified Eagle’s medium (MEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Life Technolo-
gies). Two-day postconfluent cells (day 0) were induced to differen-
tiate with MEM containing 10% FBS, 1 µM dexamethasone (Sigma-
Aldrich), 0.5 mM methylisobutylxanthine (Sigma-Aldrich), 5 µg/ml insulin (Roche), and 0.5 µM rosiglitazone (Cayman Chemical). At day 2, the cells were refed with MEM containing 10% FBS supple-
mented with 5 µg/ml insulin and 0.5 µM rosiglitazone. At days 4 and 6, the cells were refed with MEM containing 10% FBS. Adipocytes were considered mature at day 8. At day 8 the cells were stimulated with 0.1 µM isoproterenol (Sigma-Aldrich) or vehicle for 6 h. Harvesting of cells and RNA isolation were performed as described (37).

Protein lysates and immunoblotting. Homogenization was per-
formed in a lysis buffer described previously (13) with a Preccellys 24 homogenizer (Bertin Technologies). Protein concentrations were determined, and equal amounts of BAT protein (10 µg) from each animal were loaded on 4–12% Bis-Tris gradient gels (NuPAGE; Life Tech-
ologies) and blotted onto Hybond-P membranes (GE Health-
care). Membranes were stained with Amido Black 10B (Sigma-
Aldrich) and probed with primary antibodies against p44/p42 MAPK (mitogen-activated protein kinase, no. 9102) (Cell Signal-
ing Technology), gyceraldehyde-3-phosphate dehydrogenase (GAPDH, ab28245) (Abcam), glycogen synthase (GYS) 1/2 (no. 3886) (Cell Signaling Technology), lactate dehydrogenase (LDH) A (sc-27230) (Santa Cruz Biotech), pyruvate carboxylase (PCX, sc-67021) (Santa Cruz Biotechnology), pyruvate kinase, muscle (PKM) (SAB4200095) (Sigma-Aldrich), glycogen phosphorylase, liver (PYGL, HPA004119) (Sigma-Aldrich), and UCPI (ab19083) (Abcam). Secondary antibod-
ies were horseradish peroxidase-conjugated (Dako). Enhanced chemi-
luminescence (Biological Industries) was used for detection. The FusionCap Advance FX7 software (Velber Lourmat) was used for quantification.

Real-time quantitative PCR. cDNA synthesis and real-time quanti-
tative PCR (qPCR) were performed as described (30, 37). Primers for qPCR (Supplemental Table S1) were designed using NCBI/Primer-
BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK-
LOC=BladHome). Target gene expression was normalized to TATA-
binding protein (Tbp) expression. All data are presented as means ± SE. The significance of differences in gene expression levels was tested by one-way ANOVA and a Tukey post hoc test. A P value below 0.05 was considered as significantly different. Statistics were performed separately in individual tissues. The GraphPad Prism 4 (GraphPad Software) was used for statistical tests.

RESULTS

Overview of the data set. Total body weight and the weight of iBAT, eWAT, and liver were not significantly affected by the cold exposure (Fig. 1A). The weight of the iWAT depot
was reduced by ~50% after both 2 and 4 days of cold exposure (Fig. 1A).

We analyzed in total 31 samples by DGEP: 10 samples for iBAT, 12 samples for IWAT, and 9 samples for eWAT. For each time point three to five samples were sequenced. A total of ~8,000,000 tags per sample were obtained, and, after filtering, around 40% of the tags were mapped to mRNA within the CATG sites covering ~40% of all mouse genes.

To approach the difference between adipose depots and between time points, we did a principal component analysis (PCA) for all of the sequenced samples (Fig. 1B). In the plane composed of the first and third PC (PC1 and PC3, respectively), the global gene expression clearly separated and grouped the samples by tissues, indicating the distinct gene expression difference between adipose depots. In the Pearson correlation coefficient matrix, all samples positively correlated.

Fig. 1. Overview of gene expression in interscapular brown adipose tissue (iBAT), inguinal white adipose tissue (iWAT), and epididymal white adipose tissue (eWAT). Male C57BL/6J mice \((n = 8)\) were exposed to cold (4°C) for 0, 2, and 4 days. Samples \((n = 3–5)\) from each time point were sequenced for transcriptome profiling. A: total body weight and the weight of liver, iBAT, iWAT, and eWAT were measured. Data represent means ± SE \((n = 8)\). *Difference with statistical significance \((P < 0.05)\). B: principal component analysis (PCA). All samples were subjected to PCA, and first and third principal components were used to visualize the clustering of the different groups of adipose tissue. The ellipses are confidence ellipses with confidence level 0.99, and barycenter is true. C: Pearson correlation coefficient was calculated for all pairs of samples representing the relationship between the samples on the global gene expression level. D: expression levels of brown, brite/beige, and white adipocytes marker genes from the sequencing data. Bars represent means ± SE.
GLUCOSE METABOLISM IN BROWN ADIPOCYTES

Table 1. Selected genes upregulated in iBAT between day 0 and day 2

<table>
<thead>
<tr>
<th>Refseq ID</th>
<th>MGI Symbol</th>
<th>MGI Description</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_021273</td>
<td>Ckb</td>
<td>Creatine kinase, brain</td>
<td>20.08</td>
</tr>
<tr>
<td>NM_007703</td>
<td>Elovl3</td>
<td>Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3</td>
<td>13.07</td>
</tr>
<tr>
<td>NM_007559</td>
<td>Bmp8b</td>
<td>Bone morphogenetic protein 8b</td>
<td>11.89</td>
</tr>
<tr>
<td>NM_027976</td>
<td>Acyl5</td>
<td>Acyl-CoA synthetase long-chain family member 5</td>
<td>11.17</td>
</tr>
<tr>
<td>NM_010050</td>
<td>Dio2</td>
<td>Deinidase, iodothyronine, type II</td>
<td>10.37</td>
</tr>
<tr>
<td>NM_145572</td>
<td>Gsy2</td>
<td>Glycogen synthase 2</td>
<td>8.65</td>
</tr>
<tr>
<td>NM_008062</td>
<td>G6pdx</td>
<td>Glucose-6-phosphate dehydrogenase X-linked</td>
<td>5.80</td>
</tr>
<tr>
<td>NM_010634</td>
<td>Fabp5</td>
<td>Fatty acid-binding protein 5, epidermal</td>
<td>5.45</td>
</tr>
<tr>
<td>NM_175207</td>
<td>Ankrd9</td>
<td>Ankyrin repeat domain 9</td>
<td>5.43</td>
</tr>
<tr>
<td>NM_008828</td>
<td>Pgk1</td>
<td>Phosphoglycerate kinase 1</td>
<td>4.56</td>
</tr>
<tr>
<td>NM_181748</td>
<td>Ffar4</td>
<td>Free fatty acid receptor 4</td>
<td>4.55</td>
</tr>
<tr>
<td>NM_023418</td>
<td>Pgam1</td>
<td>Phosphoglycerate mutase 1</td>
<td>4.38</td>
</tr>
<tr>
<td>NM_001013780</td>
<td>Slc25a34</td>
<td>Solute carrier family 25, member 25, member 34</td>
<td>3.25</td>
</tr>
<tr>
<td>NM_001080940</td>
<td>Gm6484</td>
<td>Predicted gene 6484</td>
<td>3.93</td>
</tr>
<tr>
<td>NM_010271</td>
<td>Gpd1</td>
<td>Glycolate-phosphate dehydrogenase 1 (soluble)</td>
<td>3.93</td>
</tr>
<tr>
<td>NM_009204</td>
<td>Slc2a4</td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 4</td>
<td>2.94</td>
</tr>
<tr>
<td>NM_008509</td>
<td>Lpl</td>
<td>Lipoprotein lipase</td>
<td>2.94</td>
</tr>
</tbody>
</table>

Table 2. Selected genes upregulated in iWAT between day 0 and day 2

<table>
<thead>
<tr>
<th>Refseq ID</th>
<th>MGI Symbol</th>
<th>MGI Description</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_009463</td>
<td>Ucp1</td>
<td>Uncoupling protein 1 (mitochondrial, proton carrier)</td>
<td>22.81</td>
</tr>
<tr>
<td>NM_007703</td>
<td>Elovl3</td>
<td>Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3</td>
<td>14.91</td>
</tr>
<tr>
<td>NM_010174</td>
<td>Fabp3</td>
<td>Fatty acid-binding protein 3, muscle and heart</td>
<td>11.42</td>
</tr>
<tr>
<td>NM_010050</td>
<td>Dio2</td>
<td>Deinidase, iodothyronine, type II</td>
<td>10.32</td>
</tr>
<tr>
<td>NM_001013780</td>
<td>Slc25a34</td>
<td>Solute carrier family 25, member 34</td>
<td>6.86</td>
</tr>
<tr>
<td>NM_007702</td>
<td>Cidea</td>
<td>Cell death-inducing DNA fragmentation factor, subunit-like effectors A</td>
<td>6.72</td>
</tr>
<tr>
<td>NM_001080940</td>
<td>Gm6484</td>
<td>Predicted gene 6484</td>
<td>5.85</td>
</tr>
<tr>
<td>NM_175207</td>
<td>Ankrd9</td>
<td>Ankyrin repeat domain 9</td>
<td>5.84</td>
</tr>
<tr>
<td>NM_013820</td>
<td>Hk2</td>
<td>Hexokinase 2</td>
<td>4.79</td>
</tr>
<tr>
<td>NM_013743</td>
<td>Pk4</td>
<td>Pyruvate dehydrogenase kinase, isoenzyme 4</td>
<td>3.99</td>
</tr>
<tr>
<td>NM_008492</td>
<td>Ldhb</td>
<td>Lactate dehydrogenase B</td>
<td>3.85</td>
</tr>
<tr>
<td>NM_145942</td>
<td>Hmgcs1</td>
<td>3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1</td>
<td>3.63</td>
</tr>
<tr>
<td>NM_009338</td>
<td>Acat2</td>
<td>Acetyl-coenzyme A acetyltransferase 2</td>
<td>3.39</td>
</tr>
<tr>
<td>NM_010271</td>
<td>Gpd1</td>
<td>Glycolate-phosphate dehydrogenase 1 (soluble)</td>
<td>3.22</td>
</tr>
</tbody>
</table>

iBAT, interscapular brown adipose tissue.

iWAT, inguinal white adipose tissue.

to each other (data not shown) with correlation coefficients from 0.3 to 1.0 (Fig. 1C). The difference between adipose depots was distinct, especially comparing iBAT and eWAT, which represent classical BAT and browning-resistant WAT, respectively. In the browning-prone iWAT, the transcriptome of day 0 (thermoneutrality) samples was clearly different from that of iBAT, but after 48 h in the cold (day 2) the transcriptome of iWAT and iBAT became similar, whereas the iWAT and iBAT transcriptomes of samples from day 4 separated again. In contrast, the difference between iBAT and eWAT was evident at every time point. The eWAT day 4 sample b showed a remarkable difference from the other eWAT samples and was excluded in subsequent analyses (Fig. 1C).

Before analyzing differences between time points in each adipose depot, we compared expression, indicated as tags per million, of previously reported adipose depot-selective genes. Zinc finger protein of the cerebellum 1, which was reported as a brown adipocyte marker, was only detected in iBAT (Fig. 1D) (63). The bristle/beige adipocyte marker T-box 1 was expressed at high level in iBAT and at lower levels in iBAT and eWAT (69). Finally, expression of bristle/beige and white adipose tissue marker homeobox C8 and transcription factor 21 was consistent with previous reports (Fig. 1D) (63).

Differentially expressed genes in iBAT and iWAT during cold exposure. By the differential expression analysis using edgeR, we found 304 genes significantly changed [false discovery rate (FDR) < 0.05] in iBAT between control (thermoneutrality) and 2 days of cold exposure (Supplemental Table S2). Comparing day 2 with day 4 revealed no genes with an FDR < 0.05. One hundred and forty-five genes were upregulated and 159 genes were downregulated during the first 2 days in cold. Consistent with previous reports, creatine kinase, brain type increased ~20-fold (Table 1) (65). A number of additional established cold-responsive genes were found to be significantly induced in iBAT, e.g., deiodinase iodothyronine type II (Dio2) (35), elongation of very long chain fatty acids 3 (Elovl3) (60), and predict gene 6484/refeeding induced fat and liver/angiopoietin-like protein 8 (Gm6484/ANGPTL8/betatrophin) (Table 1) (42, 73).
Acta1; actinin, α3; Mylpf; and phosphoglycerate mutase 2 (Pgam2) (Fig. 2A and Supplemental Tables S2 and S3). Next, we searched for enriched GO terms among the genes significantly regulated from day 0 to day 2 in iBAT and iWAT (data not shown). Thirty-nine GO terms were shared by iBAT and iWAT among the upregulated genes, and 27 GO terms were shared among the downregulated genes (Fig. 2B). Thus, although iBAT and iWAT only shared 6 out of 197 (iBAT and iWAT in total) upregulated genes, corresponding to 3% of the significantly upregulated genes, the two adipose depots shared 39 out of 187 GO terms, equaling 21% of the enriched GO terms. Similarly, iBAT and iWAT shared 5 out of 230 downregulated genes (2.2%), but 27 out of 389 (6.9%) of the GO terms. In summary, we find that iBAT and iWAT share relatively few gene expression changes at the level of individual genes but display a more similar change when looking at biological processes, particularly for GO terms identified among the upregulated genes. One interpretation of the larger overlap in metabolic GO terms compared with individual genes can be that the same metabolic change is obtainable in different ways, e.g., by the two tissues having different rate-limiting enzymatic steps in a certain pathway. We have summarized the upregulated GO terms shared by iBAT and iWAT in Fig. 3 to show the most notable biological processes. Examples of metabolic GO terms among the shared upregulated GO terms were the "oxidation-reduction process," "glucose metabolism," "lipid biosynthesis," and "lipid catabolism."

Glycolytic genes were induced in iBAT during cold exposure. We noticed from the sequencing data that glucose transporter solute carrier family 2, member 4 (glucose transporter type 4, insulin-responsive and a number of genes encoding glycolytic enzymes were induced in iBAT and iWAT (Supplemental Tables S2 and S3). We confirmed these gene expression changes by qPCR (Fig. 4A). In general, the glycolytic genes were significantly increased in iBAT during cold exposure, indicating an increased glycolytic flux in activated BAT. Hexokinase 2 (Hk2), glucose phosphate isomerase 1 (Gpi1), phosphoglycerate kinase 1 (Pgk1), enolase 1, α, non-neuron, and Pkm were induced two- to threefold from day 0 to day 2 (Fig. 4A). An important regulatory step in glycolysis, the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate, is catalyzed by phosphofructokinase (PFK). Two of the Pfk isoenzymes (Pfk, liver, B type; and Pfk, platelet) were significantly upregulated in iBAT, whereas expression of Pfk, muscle was not significantly changed (Fig. 4A). Pgam1 was induced threefold, but expression of Pgam2 tended to decrease (Fig. 4A). Consistently, protein levels of GAPDH and PKM were significantly higher in iBAT from mice housed for 8 days at 4°C compared with mice housed at thermoneutrality (Fig. 5). In iWAT, cold exposure caused an induction of several glycolytic genes, e.g., Hk2, Gpi1, and Pgk1 (Fig. 4A). In eWAT, none of the glycolytic genes was significantly regulated in response to cold.

Because glycolytic gene expression was enhanced, we also measured expression of the enzymes acting in the malate-aspartate shuttle, a shuttle system for transferring reducing equivalents (NADH) produced during glycolysis to the mitochondrial matrix. Malate dehydrogenase 1, soluble (Mdh1) and the mitochondrial Mdh2 were both significantly induced in iBAT from day 0 to day 2 (Fig. 4B). Glutamate oxaloacetate transaminase 1, soluble (Got1) was significantly induced, whereas expression of the mitochondrial Got2 was unchanged (Fig. 4B).

Expression of enzymes acting in pathways linked to glycolysis. As a nonglycolytic route for glucose 6-phosphate catabolism, we measured genes involved in the PPP, a source of NADPH and ribose phosphate (Fig. 6A). The rate-limiting enzyme, glucose-6-phosphate dehydrogenase, X-linked (G6pdx), was significantly upregulated in iBAT. 6-Phosphogluconolactonase and 6-phosphogluconate dehydrogenase (Pgd) were also significantly induced from day 0 to day 2 in iBAT (Fig. 6A).

In addition to being an intermediate in glycolysis and PPP, glucose 6-phosphate is a substrate for glycogen synthesis. Gysl and Gys2 were induced 1.5- and 7-fold, respectively, in iBAT during cold exposure (Fig. 6B). Among the three isoenzymes of PYG, Pygl was induced in iBAT, whereas Pyg, brain was repressed and Pyg, muscle (Pygm) was unchanged (Fig. 6C). Protein levels of PYGL in iBAT were significantly higher in cold-exposed mice compared with mice housed at thermoneutrality, whereas no effect of cold was observed for GYS1/2 (Fig. 5).

GPD is a major link between glycolysis and TAG synthesis. The cytosolic GPD (encoded by the Gpd1 gene) catalyzes the
reduction of dihydroxyacetone phosphate to glycerol 3-phosphate, and the mitochondrial GPD (encoded by the \textit{Gpd2} gene) catalyzes the oxidation of glycerol 3-phosphate to dihydroxyacetone phosphate. Expression of both \textit{Gpd1} and \textit{Gpd2} was significantly induced in iBAT (Fig. 6B).

The pyruvate dehydrogenase complex (PDC), located in the mitochondrial matrix, converts pyruvate to acetyl-CoA by decarboxylation. Pyruvate dehydrogenase phosphatase (PDP) and pyruvate dehydrogenase kinase (PDK) are key positive and negative regulators of PDC. The expression level of \textit{Pdk2} was induced by cold from day 0 to day 2 in iBAT, whereas expression of \textit{Pdk4} was barely changed (Fig. 6D). Furthermore, \textit{Pdk2} and \textit{Pdk4} in iBAT were expressed at higher levels than in WAT.

LDH catalyzes the reversible conversion of pyruvate to lactate. \textit{Ldha} and \textit{Ldhb} mRNAs and LDHA protein were significantly induced by cold exposure in iBAT (Figs. 5 and 6E), indicating increased lactate production.

Finally, we also tested the key enzymes of glyceroneogenesis. The expression level of phosphoenolpyruvate carboxykinase 1 (\textit{Pck1}/\textit{Pepck}) was not changed in iBAT, whereas \textit{Pcx} was induced (Fig. 6E). Protein levels of PCX tended to be higher in iBAT from cold-acclimated mice (Fig. 5).

Of the genes regulated by cold in iBAT in Fig. 6, about half were also regulated in iWAT. Of notice, expression of three genes was significantly reduced by cold only in eWAT, \textit{Pygm}, \textit{Pdp1}, and \textit{Pck1}.

\textbf{β-Adrenergic stimulation of cultured brown adipocytes elicits an induction of enzymes acting in pathways linked to glycolysis.} To investigate if the gene expression changes described above were caused by a direct or an indirect effect of β-adrenergic stimulation of brown adipocytes, we treated cultured mature brown adipocytes with the pan-β-adrenergic receptor agonist isoproterenol for a relatively short period of time (6 h). Expression of a number of enzymes regulating glucose metabolism was significantly induced in response to β-adrenergic stimulation, e.g., \textit{Mdh1}, \textit{Got1}, \textit{G6pdx}, \textit{Gys2}, \textit{Pygl}, and \textit{Ldha} (Fig. 7). Induction of these genes was less pronounced than the induction of \textit{Ucp1}, the expression of which increased 96-fold after treatment with isoproterenol (Fig. 7). Two genes induced in iBAT from cold-exposed mice were oppositely regulated in the cultured brown adipocytes after the 6-h treatment with isoproterenol (\textit{Pgd} and \textit{Gpd1}; Fig. 7). Overall, these data suggest that at least some glucose metabolic enzymes are induced as a direct response to β-adrenergic stimulation.

\textbf{Gene expression changes of enzymes involved in lipid metabolism.} It is well established that cold exposure increases lipid uptake, lipolysis, and fatty acid β-oxidation in BAT. The DGEP and qPCR results confirmed that key regulators of lipid uptake, lipolysis, fatty acid activation, β-oxidation, and TAG

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{revigo_plot.png}
\caption{REVIGO scatterplot of upregulated GO terms shared by iBAT and iWAT. Upregulated GO terms from day 0 to day 2 shared by iBAT and iWAT were summarized and visualized using the REVIGO web server. The color of an individual bubble indicates the enrichment \textit{P} value of the GO term in iBAT. The size of the bubble representing the GO term shows the frequency of occurrence of that term in the underlying GO annotation database. Semantic space is based on the semantic similarity, which is the degree of relatedness between two entities by measuring the similarity of their annotation meanings. The list of enriched GO terms is subjected to redundancy reduction, based on the “most informative common ancestor” approach in REVIGO and is represented by cluster representatives in a scatterplot. The \textit{x}- and \textit{y}-axes of the scatterplot represent the distance between the cluster representatives.
}
\end{figure}
synthesis were induced in iBAT from cold-exposed mice, and only a subset of these changes was observed in iWAT (Supplemental Table S4).

Overall, our data are consistent with profound changes in both glucose and lipid metabolism in BAT of cold-exposed mice.

DISCUSSION

We have profiled the transcriptome of mouse iBAT, iWAT, and eWAT during cold exposure and provide a comprehensive view of changes in gene expression related to glucose metabolism in response to cold.

Glucose metabolism in activated BAT. The utilization of glucose is highly regulated at several steps: glucose uptake, glycolytic flux, and entry into the tricarboxylic acid (TCA) cycle. It is well established that cold exposure stimulates the uptake of glucose into brown adipocytes (3). The maximum activity of the key glycolytic enzymes, hexokinase and PFK, was increased twofold in BAT from cold-acclimated rats, whereas the activity was unaffected in muscle and liver (6). Consistently, expression of selected glucose metabolism-related genes, including Pfk, was shown to be induced in cold-acclimated mice (75). The glycolytic pathway as a GO term has previously been reported to be increased in BAT during cold acclimation (49). Consistently, our results demonstrated that expression of many glycolytic enzymes was significantly elevated by cold. Despite glucose uptake by brown adipocytes being induced by cold, the fate of the glucose taken up is unclear: how much is used for complete oxidation, for synthesis of glycerol, lactate, or fatty acids; and how much is oxidized by the pentose phosphate pathway? Moreover, the importance of glucose as a thermogenic

Fig. 4. Expression of genes related to glucose uptake, glycolysis, and the malate-aspartate shuttle in iBAT, iWAT, and eWAT. Male C57BL/6J mice (n = 8) were exposed to cold (4°C) for 0, 2, and 4 days. A: glucose transporter (GLUT) 4 and glycolytic gene expression. B: malate-aspartate shuttle gene expression. Gene expression was normalized to TATA box-binding protein (Tbp). Bars represent means ± SE. The lowercase letters a, b, and c above the bars represent different expression levels with statistical significance (P < 0.05). Statistics were performed separately in each tissue.
fuel remains to be established. Some studies, both in vivo and in vitro, have reported that glucose is a minor fuel for activated brown adipocytes (3, 20, 28). Expression of genes involved in the malate-aspartate shuttle (Mdh1, Mdh2, and Got1) was increased during cold exposure, consistent with the NADH produced by glycolysis being used for thermogenesis. It was also shown that the use of glucose carbon for de novo synthesis of fatty acids and glyceride glycerol is increased during cold exposure (55). In a study of the turnover of triglycerides and phospholipids in warm- and cold-acclimated rats labeled with [14C]glucose, Himms-Hagen demonstrated that in BAT of cold-acclimated animals, 71% of the total radioactive carbon in the lipids was in the glycerol portion and only 27% was in the fatty acids (19). The incorporation of glucose carbon into BAT glyceride glycerol was 30 times greater in cold- than in warm-acclimated rats (19).

In a study of BAT in rabbits, Knight and Myant reported that cold exposure of newborns led to an increase in the incorporation of glucose carbon into the glycerol of TAG and that incorporation into fatty acids was lower than into glycerol and was not consistently affected by cold (24). Moreover, Ma and Foster have shown that, of the glucose taken up, 33 and 88% were released as lactate and pyruvate at submaximal and maximal norepinephrine stimulation, respectively (28). Our data demonstrated an induction of Gpd1, Gpd2, Ldha, and Ldhb by cold in iBAT, consistent with increased synthesis of glycerol 3-phosphate and lactate. Based on previous studies and our gene expression data, it is likely that a considerable amount of the glucose taken up by activated iBAT was neither completely oxidized nor incorporated into fatty acids, but instead was converted to lactate and glycerol 3-phosphate followed by release into circulation or use for local TAG synthesis, respectively.

PDC catalyzes the key regulatory step linking glycolysis to the TCA cycle. PDC is also important for maintaining glucose flux to malonyl-CoA via production of mitochondrial citrate. PDC activity is negatively regulated by various factors such as acetyl-CoA, NADH, ATP, and by PDK-mediated phosphorylation. Four PDK isoenzymes (PDK1, PDK2, PDK3, and PDK4) are present in mammals. Among these, Pdk2 and Pdk4 have attracted the most attention due to their responsiveness to starvation and diabetes (21, 48, 56). We found that the expression of Pdk2, but not Pdk4, was induced by cold in iBAT. It has, however, been reported that Pdk4 expression was induced by β-adrenergic receptor agonists in brown adipocytes and BAT (33). The discrepancy might be due to the difference between systemic treatment with a β-adrenergic receptor agonist and selective sympathetic stimulation in iBAT during cold exposure (4). Although PDC activity has been reported to
increase in BAT from rats during cold exposure (29), the high expression of \textit{Pdk2} and \textit{Pdk4} in activated iBAT suggests that PDK activity might restrict the conversion of glucose to acetyl-CoA.

It has been reported that glyceroneogenesis is increased by cold exposure in iBAT, and it was proposed that a substantial part of the glyceride glycerol was synthesized via glyceroneogenesis and used mostly to esterify preformed fatty acids (34). In WAT, glyceroneogenesis has been reported to be the dominant source of glycerol in TAG in vivo (40). In BAT from cold-acclimated mice, \textit{Pcx} expression was increased. Although we did not detect an induction of \textit{Pck1} expression, it remains possible that \textit{Pck1} is regulated at the protein or activity level in BAT. In addition to \textit{Pcx}, we observed an induction of \textit{Mdh1} and \textit{Mdh2} expression, suggesting an increased potential for fatty acid synthesis through the citrate cleavage pathway, which in addition would secure an additional supply of NADPH (1).

GYS activity in BAT was reported to be significantly increased by cold acclimation (29), but the glycogen content in BAT decreased by cold exposure (10, 25). We observed an induction of \textit{Gys1} and \textit{Gys2} mRNA expression at days 2 and 4 of cold exposure, but no increase in GYS1/2 protein levels after 8 days. We did not measure the activity of GYS1/2. \textit{PYGL} in iBAT was significantly induced by cold exposure at both the RNA and protein level, suggesting a simultaneous increase in glycogen synthesis and degradation. Expression of both \textit{Gys2} and \textit{Pygl} was induced in brown adipocytes after \beta-adrnergic stimulation in vitro. The function of glycogen in BAT in general and the role of the apparent increase in glycogen turnover in activated brown adipocytes are unknown and await further investigation.
All three genes encoding enzymes of the oxidative phase of PPP were stimulated by cold exposure, indicating an increased flux through this pathway. PPP is a key provider of nucleic acid precursors and reducing equivalents in the form of NADPH. The percentage of glucose metabolized through PPP is believed to be relatively high in the lipogenic tissues, such as liver and adipose tissue (68). The induction of all genes of the oxidative phase by cold exposure indicates that the additional NADPH generated from PPP might support de novo fatty acid synthesis by providing reducing equivalents.

**Interactions between glucose and lipid metabolism.** Glucose and fatty acids are the principal direct fuels for respiration. The idea that substrates compete for their oxidation goes back to the beginning of the last century (44). Based on experimental reports, Randle et al. proposed the idea of “The glucose fatty acid cycle” (44). The principal components of this cycle explain the reciprocal relationship between glucose and fatty acid metabolism (43). According to the theory, fatty acid oxidation inhibits glucose utilization in at least three steps (glucose uptake, glycolysis, and PDC). In the unique metabolic situation of activated BAT, we speculate that glucose uptake and glycolytic flux coexist with highly active fatty acid oxidation, but complete glucose oxidation beyond pyruvate might be restricted by high PDK activity, the latter possibly caused by high acetyl-CoA levels from fatty acid oxidation. An interesting question is why, based on the gene expression changes, glucose uptake and glycolysis appeared being significantly induced by cold exposure instead of being inhibited by β-oxidation. We

---

**Fig. 7.** Effect of β-adrenergic stimulation of brown adipocytes on expression of glucose metabolic enzymes. Mature WT-1 brown adipocytes were stimulated with isoproterenol (0.1 µM) or vehicle for 6 h after which cells were harvested. Expression of selected genes (Ucp1, malate dehydrogenase (Mdh) 1, glutamate oxaloacetate transaminase 1, soluble (Got1), glucose-6-phosphate dehydrogenase, X-linked (G6pdx), 6-phosphogluconate dehydrogenase (Pgd), glycerol-3-phosphate dehydrogenase (Gpd) 1, Gys2, Pygl, and Ldh) was analyzed by real-time quantitative PCR. Gene expression was normalized to Tbp. Data represent means ± SE (n = 3). *Different expression levels with statistical significance (P < 0.05).

---

**Fig. 8.** Glucose metabolism and its interaction with lipid metabolism in activated brown adipocytes. Substrates are depicted in blue and enzymes are in green. Black words represent pathways. Broken lines and solid lines indicate direct and indirect flows, respectively. The red arrow lines and purple blunt end line indicate assumed activation and inhibition effects, respectively. The citrate cleavage pathway, which might supply cytosolic acetyl-CoA and NADPH for fatty acid synthesis, is not included. CPT1, carnitine palmitoyltransferase 1; G3P, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate.
speculate that glycolysis and PPP are important for promoting other metabolic pathways like fatty acid synthesis and conversion of pyruvate to lactate, and at the same time providing glycerol 3-phosphate for re-esterification of fatty acids.

In summary, based on our gene expression profiling and numerous previous studies, we propose a metabolic model for activated brown adipocytes (Fig. 8): 1) glucose uptake is elevated, glycolysis and PPP are induced; 2) β-oxidation of fatty acids restricts complete oxidation of glucose by causing phosphorylation of PDC by PDK; 3) enhanced activity of GPD and LDH catalyzes the conversion of glucose to glycerol 3-phosphate and lactate, respectively; 4) glycerol 3-phosphate is used for TAG synthesis; and 5) PPP provides NADPH for de novo fatty acid synthesis.

Cold-induced metabolic changes in subcutaneous WAT. Subcutaneous WAT, e.g., iWAT, undergoes browning and becomes thermogenic during prolonged cold exposure or treatment with β-adrenergic agonists. At the same time, WAT may contribute indirectly to thermogenesis by releasing fatty acids from lipolysis to the circulation from which brown adipocytes take them up for β-oxidation. The significant decrease in iWAT mass during cold exposure indicates that this might occur. However, our gene expression analyses show that lipases are not significantly induced by cold in WAT. Because mRNA levels do not necessarily reflect protein function, it is likely that lipase activity increases in WAT during cold exposure.

Interestingly, we measured increased expression of many glucose metabolic enzymes in iWAT during cold exposure. In comparison, very few of these genes were induced in eWAT. We speculate that the browning of iWAT is accompanied by a substantial increase in the capacity for glucose metabolism, which might in part explain the improved glucose homeostasis in mice with increased amounts of brite/beige adipose tissue (16, 22).

Since the reports documenting that adult humans have active BAT (8, 51, 61, 62), an increasing number of studies point to BAT (8, 51, 61, 62), an increasing number of studies point to

**REFERENCES**


60. Watanabe M, Yamamoto T, Mori C, Okada N, Yamazaki N, Kajimoto K, Kataoka M, Sinohara Y. Cold-induced changes in gene
expression in brown adipose tissue: implications for the activation of thermogenesis. **Biol Pharm Bull** 31: 775–784, 2008.