Increased hepatic lipogenesis in insulin resistance and Type 2 diabetes is associated with AMPK signalling pathway up-regulation in Psammomys obesus

Ali BEN DJOUDI OUADDA*†, Emile LEVY*‡, Ehud ZIV§, Geneviève LALONDE*‡, Alain T. SANÊ*‡, Edgar DELVIN*† and Mounib ELCHEBLY*†

*Research Centre, CHU-Sainte-Justine, 3175 Côte St-Catherine Road, Montreal, QC, Canada, H3T 1C5, †Department of Biochemistry, Université de Montréal, 2900 boul. Édouard-Montpetit, Montreal, QC, Canada, H3T 1J4, ‡Department of Nutrition, Université de Montréal, 2405 chemin de la Côte-St-Catherine, Montreal, QC, Canada, H3T 1A8, and §Diabetes Unit, Division of Internal Medicine, Hadassah University Hospital, Jerusalem, Israel

Synopsis

AMPK (AMP-activated protein kinase) has been suggested to be a central player regulating FA (fatty acid) metabolism through its ability to regulate ACC (acetyl-CoA carboxylase) activity. Nevertheless, its involvement in insulin resistance- and TD2 (Type 2 diabetes)-associated dyslipidaemia remains enigmatic. In the present study, we employed the Psammomys obesus gerbil, a well-established model of insulin resistance and TD2, in order to appreciate the contribution of the AMPK/ACC pathway to the abnormal hepatic lipid synthesis and increased lipid accumulation in the liver. Our investigation provided evidence that the development of insulin resistance/diabetic state in P. obesus is accompanied by (i) body weight gain and hyperlipidaemia; (ii) elevations of hepatic ACC-Ser79 phosphorylation and ACC protein levels; (iii) a rise in the gene expression of cytosolic ACC1 concomitant with invariable mitochondrial ACC2; (iv) an increase in hepatic AMPKα-Thr172 phosphorylation and protein expression without any modification in the calculated ratio of phospho-AMPKα to total AMPKα; (v) a stimulation in ACC activity despite increased AMPKα phosphorylation and protein expression; and (vi) a trend of increase in mRNA levels of key lipogenic enzymes [SCD-1 (stearoyl-CoA desaturase-1), mGPAT (mitochondrial isoform of glycerol-3-phosphate acyltransferase) and FAS (FA synthase)] and transcription factors [SREBP-1 (sterol-regulatory-element-binding protein-1) and ChREBP (carbohydrate responsive element-binding protein)]. Altogether, our findings suggest that up-regulation of the AMPK pathway seems to be a natural response in order to reduce lipid metabolism abnormalities, thus supporting the role of AMPK as a promising target for the treatment of TD2-associated dyslipidaemia.

Key words: acetyl-CoA carboxylase (ACC), AMP-activated protein kinase (AMPK), dyslipidaemia, insulin resistance, Psammomys obesus, Type 2 diabetes

INTRODUCTION

AMPK (AMP-activated protein kinase) is a phylogenetically conserved serine/threonine protein kinase that has been proposed to function as a cellular energy sensor [1]. It is broadly expressed as a heterotrimeric enzyme formed by a catalytic subunit (α) and two regulatory subunits (β and γ). Several isoforms have been identified for each subunit that can lead to the formation of different complexes. The combinations between these isoforms confer tissue specificity on the resulting AMPK complexes [2,3]. Both AMP-dependent and AMP-independent pathways regulate the physiological activity of AMPK. Accordingly, in response to the cellular stresses that deplete ATP and increase the intracellular AMP/ATP ratio (i.e. physical exercise, hypoxia and glucose deprivation), the AMPK activity is allosterically stimulated by AMP that binds to the cystathionine β-synthase tandem repeats in the γ-subunit [1,4–6]. Similarly, two adipocyte-derived hormones, leptin and adiponectin, are able to stimulate FA (fatty acid) oxidation and glucose uptake in peripheral tissues such as muscle and liver. In contrast, several signalling pathways are involved in the regulation of ACC activity and therefore in the control of FA synthesis. The AMPK/ACC pathway is known to be involved in the regulation of lipogenesis and FA oxidation. In the liver, AMPK is suggested to be a key regulator of hepatic lipid metabolism, through its ability to regulate the activity of ACC by phosphorylating it at Thr172 [1,7,8]. This modification leads to the dephosphorylation of ACC and therefore to the inhibition of FA synthesis. However, the role of AMPK in the regulation of FA metabolism in peripheral tissues is less clear. In muscle, AMPK is thought to play an important role in the regulation of FA oxidation [9,10]. However, in adipose tissue, the role of AMPK in the regulation of FA oxidation is less clear. It has been suggested that AMPK may regulate FA oxidation by activating carnitine palmitoyltransferase-I (CPT-I), the rate-limiting enzyme in the mitochondrial FA oxidation pathway [11]. In addition, AMPK may regulate FA oxidation by inhibiting the expression of FA synthase (FAS) and stearoyl-CoA desaturase-1 (SCD-1), key enzymes in FA synthesis [12]. In summary, AMPK is a key regulator of FA metabolism in both muscle and liver, but its role in the regulation of FA metabolism in adipose tissue is less clear.

Abbreviations used: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ACCKK, AMPK kinase; ChREBP, carbohydrate responsive element-binding protein; DTT, dithiothreitol; FA, fatty acid; FAS, FA synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HOMA, homoeostasis model assessment; mGPAT, mitochondrial isoform of glycerol-3-phosphate acyltransferase; PEG, poly(ethylene glycol); SCD-1, stearoyl-CoA desaturase-1; SREBP-1, sterol-regulatory-element-binding protein-1; TD2, Type 2 diabetes; TAG, triacylglycerol; VLDL, very-low-density lipoprotein.

1To whom correspondence should be addressed (email mounib.elchebly@umontreal.ca).
as skeletal muscle and liver by inducing AMPK activity via an AMP-independent pathway [7–9].

Activation of AMPK requires its phosphorylation at Thr\textsuperscript{172}, which resides in the activation loop of the α1- and α2-subunits [10]. Several upstream kinases mediate AMPK phosphorylation. For example, the tumour suppressor protein kinase LKB1 phosphorylates AMPK when AMP concentration rises in the cell and binds to the γ-subunit, thus transforming AMPK in an appropriate substrate for LKB1 [11–13]. Similarly, CaMKK (Ca\textsuperscript{2+}/calmodulin-dependent protein kinase kinase) phosphorylates and activates AMPK in the presence of an increased calcium concentration, independently of an increasing AMP concentration [14,15]. Once activated, AMPK phosphorylates a number of intracellular proteins and modulates the transcription of genes implicated in the regulation of energy metabolism to switch off energy-consuming biosynthetic pathways (e.g. lipogenesis) and switch on catabolic pathways that produce ATP (e.g. FA oxidation) [4].

Increasing evidence suggests AMPK as a central player regulating hepatic lipid metabolism [16]. In the short-term regulation, AMPK phosphorylates and inactivates ACC (acyetyl-CoA carboxylase), thus inhibiting FA biosynthesis. At the same time, inactivation of ACC decreases malonyl CoA concentration, which in turn de-represses CPT1 (carnitine palmitoyl transferase 1) and activates FA oxidation [4]. In the long term, AMPK is thought to regulate hepatic lipogenic gene expression by inhibiting transcription factors [17]. Indeed, AMPK has been found to down-regulate SREBP-1c (sterol-regulatory-element-binding protein-1c) [18] and inhibit ChREBP (carbohydrate responsive element-binding protein) [19].

Activation of AMPK can cause insulin sensitization and modulate plasma glucose level through its action on liver and muscle, which can have beneficial effects in TD2 (Type 2 diabetes) patients. AMPK activation also regulates both lipid oxidation and synthesis and thereby can be beneficial in controlling human dyslipidaemia. Therefore, over the past few years, AMPK received considerable attention as a promising target for the treatment of TD2 and dyslipidaemia. In line with these observations, several of the beneficial effects of rosiglitazone and metformin, two widely used antidiabetic drugs, have been shown to be mediated by indirect activation of AMPK [20].

The derangements of hepatic lipid homeostasis along with diabetic dyslipidaemia substantially contribute to the occurrence of cardiovascular diseases, especially in association with a ‘Westemized lifestyle’ (sedentary lifestyle and energy-dense diet) [21]. Our previous studies have focused on Psammomys obesus because it represents a useful experimental model for human nutrition-induced obesity and TD2, the so-called ‘diabetes’ syndrome. Our findings demonstrated various disturbances in plasma lipid profile and lipoprotein composition, as well as in liver lipid metabolism during the sequential development of insulin resistance and TD2 in P. obesus gerbils [22]. Furthermore, our observations pointed to an undoubtedly important role in the liver in the pathogenesis of metabolic disorders in the progression of nutritionally induced ‘diabetes’ in P. obesus since the development of these conditions triggered the whole intra-hepatocyte machinery, leading to lipoprotein assembly and favouring the oversecretion of apolipoprotein B-100-lipoproteins [23]. Beneficial effects of diets enriched with n-3 FA were noted on body weight, hyperglycaemia, hyperinsulinaemia and hyperlipidaemia, as well as on intestinal lipogenesis, lipid esterification and de novo apo B-48 synthesis [24,25]. Favourable n-3 FAs clearly impact on the over-production of intestinal TAG (triacylglycerol)-rich lipoproteins.

Despite the extensive previous research which has aimed to highlight the causes of lipid accumulation in the liver, additional studies are required to delineate the mechanisms triggered in insulin resistance and TD2. Although the role of AMPK/ACC in regulating hepatic lipogenesis has now been established, its involvement in the physiopathology of insulin resistance- and TD2-associated dyslipidaemia remains to be elucidated. Therefore, in the present study, we examined whether the P. obesus gerbils exhibit hepatic abnormalities in the AMPK/ACC signalling pathway when the animals develop states of insulin resistance and TD2.

**EXPERIMENTAL**

**Animals**

*P. obesus* gerbils (2.5–3.5 months old) from the Hebrew University colonies were obtained from Harlan (Jerusalem, Israel). They were housed in individual polypropylene cages in a temperature-controlled room with a 12 h light/12 h dark cycle. Water and *ad libitum* food were supplied as described previously [22–25]. All experimental procedures performed in the present study were authorized by the Institutional Animal Care Committee.

**Immunoblotting analysis**

Animals were anaesthetized using a ketamine-based rodent cocktail. Their livers were immediately collected, snap-frozen in liquid N\textsubscript{2} and stored at −80°C for further use. Later on, frozen liver tissues (50–100 mg) were homogenized on ice in lysis buffer [50 mM Hepes, pH 7.5, 150 mM sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 50 mM sodium fluoride, 1 mM phenethylalcohol and 0.001% Bromophenol Blue] and heated at 95°C for 5 min. Samples were then subjected to centrifugation at 16,000 g for 10 min. Protein concentrations were measured using a Bio-Rad protein assay kit. Subsequently, 20–60 μg of protein was combined with the appropriate amount of SDS sample buffer (100 mM Tris/HCl, pH 6.8, 20% glycerol, 4% SDS, 2% mercaptoethanol and 0.001% Bromophenol Blue) and heated at 95°C for 5 min. Samples were then subjected to SDS/PAGE and electrophoretically transferred to PVDF membranes (100 V for 70 min) as described previously [22–25]. The membranes were blocked for 60 min at room temperature (22°C) in 20 mM Tris/HCl (pH 7.6), 0.138 M NaCl and 0.1% Tween 20 (TBS-T) containing 1% non-fat dried skimmed milk powder or 1% BSA. Subsequently, the membranes were incubated with the indicated antibodies in TBS-T for 16 h at 4°C. Detection was performed using horseradish peroxidase-conjugated secondary
antibodies and enhanced chemiluminescence reagent (PerkinElmer). Finally, densitometric analyses of the bands were performed using ImageJ software (http://rsb.info.nih.gov/ij/).

**Purification of ACC from hepatic *P. obesus* tissues**

Before the *in vitro* ACC assay, hepatic tissues containing ACC were isolated as described previously [26,27]. Briefly, 200 mg of individual liver tissue was homogenized on ice with a Polytron homogenizer (~10–20 s at high speed) in 0.6 ml of a buffer (50 mM Tris/HCl, pH 7.5, 250 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT (dithiothreitol), 1 mM PMSF, 4 μg/ml soya-bean trypsin inhibitor and 1 mM benzamidene). Subsequently, the homogenates were centrifuged at 14000 g for 20 min. The resulting supernatants were recovered, adjusted to 2.5% PEG [poly(ethylene glycol)] with 25% (w/v) PEG-8000, agitated for 10 min at 4 °C and centrifuged at 14000 g for 10 min. Thereafter, 25% PEG was added to the recovered supernatants to bring the PEG final concentration to 6%. The mixtures were agitated for 10 min and centrifuged again at 10000 g for 10 min. The resulting precipitates were resuspended and washed with 1 ml of homogenization buffer containing 6% PEG. After a final centrifugation for 10 min at 10000 g, the precipitates were resuspended in a suspension buffer containing 100 mM Tris/HCl (pH 7.5), 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 4 μg/ml soya-bean trypsin inhibitor, 1 mM benzamidene, 0.02% sodium azide and 10% glycerol. Finally, the protein concentrations of the resulting preparation were measured using a Bio-Rad protein assay kit.

**ACC activity assay**

The hepatic ACC activity was assayed by following the incorporation of 14C from [14C]bicarbonate into acid-stable products [26,28]. The reaction was performed in a buffer consisting of 60.6 mM Tris/acetate (pH 7.5), 1 mg/ml FA-free BSA, 1.3 μM 2-mercaptoethanol, 5 mM magnesium acetate, 2.1 mM ATP, 1.1 mM acetyl-CoA, 6.6 mM glutamate, 18.2 mM sodium bicarbonate substrate (containing 1000 d.p.m/nmol of NaH14CO3) in a final volume of 167.5 μl. The ACC contained in the PEG fraction (25 μg of total protein) was added to the reaction buffer and incubated at 37 °C for 20 min. The reaction was triggered by the addition of 5 μl of the radioactive substrate and was stopped after incubation for 15 min by the addition of 30 μl of 10% HClO4. The samples were put on ice for at least 15 min before centrifugation at 12000 g for 20 min. The supernatants were recovered in vials and allowed to dry overnight in a fume hood to allow CO2 evaporation. Afterwards, the scintillation liquid (5 ml) was added to the vials and counting was carried out. The ACC activity was represented as the amount of radioactive 14C incorporated into malonyl-CoA expressed in terms of nmol/min per mg of total protein.

**RNA extraction and quantitative real-time PCR**

Total RNA was extracted from hepatic samples using TRIzol® reagent (Invitrogen) according to the manufacturer’s specifications and reverse-transcribed into cDNA using the Superscript First Strand Synthesis System (Invitrogen). The cDNA was used as a template for real-time PCR analysis. Primers against the genes of interest were designed using the available mRNA sequence information in the NCBI (National Center for Biotechnology Information) GenBank® Nucleotide Sequence Database. BLAST (NCBI; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was used for primer verification. Since the genes studied were not yet sequenced for the *P. obesus*, we designed primers based on the conserved regions in mRNA of the three well-characterized species: *Homo sapiens*, *Rattus norvegicus* and *Mus musculus*. The designed primers (Table 1) were verified for their specificity by classic RT–PCR (reverse transcription–PCR) using mouse liver tissue and human hepatoma cells (HepG2).

Quantitative real-time PCRs were performed using a Quantitect SYBR Green kit (Applied Biosystems, Foster City, CA, U.S.A.) in an ABI Prism® 7000 Sequence Detection System. The real-time PCR reactions were carried out in a 96-well plate with a final volume of 25 μl per well. A 12.5 μl portion of SYBR Green mix (2×) was added to a well containing 25 pmol of the forward and reverse primers and 0.5 μg of cDNA template in a total of 12.5 μl of DEPC (diethyl pyrocarbonate) water. Further, negative controls without cDNA were assessed. The reaction of amplification was carried out in ~40 cycles. To normalize the different cDNA sample amounts, we used the housekeeping gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) as a reference gene. The analyses were performed for each gene and for *GAPDH* in the same plate in triplicate for each sample. The relative mRNA fold changes between the three animal groups were calculated using the 2−ΔΔCt method (*Ct* is threshold cycle value) [29].

**Analytical procedures**

Plasma glucose was determined by the glucose oxidase method and insulin levels were assessed by RIA (Phadesph; Kabi Pharmac Diagnostics, Uppsala, Sweden). TAG, phospholipids and cholesterol levels were measured calorimetrically (Roche, Basel, Switzerland).

**Data analysis**

To assess differences in the parameters studied, results were statistically analysed by ANOVA. Differences between mean values were evaluated by a Student’s two-tailed *t* test.

**RESULTS**

**Body weight and biochemical parameters**

Body weight and different biochemical measured parameters of the studied animals are shown in Table 2. Measurements of plasma insulin and glucose were used to classify the animals into three distinct groups: group A, the control group with both normoglycaemia (<5 mM) and normoinsulinaemia (<100 pM);
Table 1 Primer sequences used in the present study to determine gene expression by quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer direction</th>
<th>Primer sequence</th>
<th>Amplicon (bp)</th>
<th>GenBank® accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPKα1</td>
<td>Forward</td>
<td>CTGTACCAGGCATCAGTACC</td>
<td>220</td>
<td>NM_006251, NM_019142, NM_001013367</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGTACACAGGTCTGTCTGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMPKα2</td>
<td>Forward</td>
<td>GCCAGTGAGTTCTACCTCGCCT</td>
<td>273</td>
<td>NM_006252, NM_023991, NM_178143</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGTGCTGGCTGGTGCTCATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACC1</td>
<td>Forward</td>
<td>AGTGGTGGTGCTGGTGCTCATG</td>
<td>341</td>
<td>NM_198834, NM_022193, NM_133360</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAGGAGGAGGAGACTCCGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACC2</td>
<td>Forward</td>
<td>TACAGATCGCCATGGGCGTGGC</td>
<td>307</td>
<td>NM_001093, NM_053922, NM_133904</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGGGTCCTCTCTCTGCGGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SREBP-1</td>
<td>Forward</td>
<td>CCACACTTCATCAAGGCAGACTC</td>
<td>287</td>
<td>XM_001005291, XM_001075680, NM_011480</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCCATCGCAAGGTCTACCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAS</td>
<td>Forward</td>
<td>GTCCACCCCAAGCAGGGACA</td>
<td>210</td>
<td>NM_004104, NM_017332, NM_007988</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCCATCGCAAGGTCTACCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCD-1</td>
<td>Forward</td>
<td>TTCTCTACAGGGGTGGTGCTG</td>
<td>114</td>
<td>NM_005063, NM_139192, NM_009127</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGGGTCCTCTCTCTGCGGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mGPAT</td>
<td>Forward</td>
<td>CCAGCTGTGCTACCTCTCTTC</td>
<td>354</td>
<td>NM_0020918, NM_017274, NM_008149</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCACGCGTCGTCGTCGATCCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChREBP</td>
<td>Forward</td>
<td>CCACAGGTGAAACTCAAGGA</td>
<td>176</td>
<td>NM_133552, NM_021455</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAGTCAAGAGGCCACGGTAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>CCTGACACCATCGACCTAGTC</td>
<td>248</td>
<td>NM_002046, NM_017008, NM_001001303</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTCCGGTACTCGTGACTGCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Anthropometric and biochemical parameters of *P. obesus* animals

Values are means ± S.E.M. for n = 13 in group A, n = 13 in group B and n = 8 in group C. *P < 0.01 compared with group A; **P < 0.001 compared with group A; ***P < 0.0001 compared with group A; #P < 0.01 compared with group B; ##P < 0.001 compared with group B; ###P < 0.0001 compared with group B.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>186.01 ± 5.09</td>
<td>216.12 ± 4.49**</td>
<td>211.76 ± 7.21*</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>6.14 ± 0.27</td>
<td>7.56 ± 0.39*</td>
<td>8.56 ± 0.52**</td>
</tr>
<tr>
<td>Liver weight (% of body weight)</td>
<td>3.30 ± 0.12</td>
<td>3.50 ± 0.17</td>
<td>4.04 ± 0.18*</td>
</tr>
<tr>
<td>Epididymal white adipose tissue (g)</td>
<td>2.41 ± 0.29</td>
<td>5.10 ± 0.32***</td>
<td>5.58 ± 0.72**</td>
</tr>
<tr>
<td>Epididymal white adipose tissue (% of body weight)</td>
<td>1.26 ± 0.13</td>
<td>2.35 ± 0.13***</td>
<td>2.59 ± 0.26**</td>
</tr>
<tr>
<td>Perirenal adipose tissue (g)</td>
<td>1.57 ± 0.15</td>
<td>3.18 ± 0.24***</td>
<td>3.52 ± 0.35***</td>
</tr>
<tr>
<td>Perirenal adipose tissue (% of body weight)</td>
<td>0.83 ± 0.09</td>
<td>1.46 ± 0.06***</td>
<td>1.59 ± 0.12***</td>
</tr>
<tr>
<td>Plasma glucose (mmol/l)</td>
<td>4.41 ± 0.22</td>
<td>4.80 ± 0.30</td>
<td>14.50 ± 0.93*** ***</td>
</tr>
<tr>
<td>Plasma insulin (pmol/l)</td>
<td>50.37 ± 7.47</td>
<td>931.23 ± 199.03**</td>
<td>2925.97 ± 562.47*** ***</td>
</tr>
<tr>
<td>HOMA index</td>
<td>1.43 ± 0.21</td>
<td>31.27 ± 8.02**</td>
<td>284.87 ± 61.92*** ***</td>
</tr>
</tbody>
</table>

Accordingly, the animals of group B developed hyperinsulinemia without elevations of glucose levels, whereas insulin in the diabetic group C failed to contain the progression of hyperglycaemia. To assess the insulin resistance state in groups B and C, we employed HOMA (homoeostasis model assessment) [30], which revealed a significantly higher index compared with the control group A, thereby confirming the severe insulin resistance condition described in our former studies [23]. In addition, the two animal groups B and C exhibited moderate weight gain compared with the controls, probably due to the adipose tissue magnitude.

Plasma and hepatic lipid profile

In human and several animal models of insulin resistance and TD2, the development of the insulin resistance state has been demonstrated to be associated with several lipid metabolism abnormalities. Therefore, to further characterize the *P. obesus* animals, we measured the plasma TAG and total cholesterol. Analysis...
Role of AMPK in Psammomys obesus hepatic lipogenesis

Table 3 Hepatic lipid contents in P. obesus animals

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>TAG (mg/g liver)</th>
<th>Total cholesterol (mg/g liver)</th>
<th>Phospholipids (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>44.6 ± 5.1</td>
<td>16.2 ± 2.3</td>
<td>82.8 ± 9.9</td>
</tr>
<tr>
<td>B</td>
<td>422.3 ± 63.3*</td>
<td>36.6 ± 2.4*</td>
<td>118.0 ± 6.2*</td>
</tr>
<tr>
<td>C</td>
<td>370.4 ± 28.2*</td>
<td>35.9 ± 4.2*</td>
<td>122.3 ± 6.9*</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for n = 6 per group. ***P < 0.001 compared with group A.

![Figure 1](image1.png)

Figure 1 Increased plasma TAG and total cholesterol levels in insulin-resistant (group B, hatched bars) and TD2 (group C, black bars) compared with control (group A, white bars) P. obesus animals

Plasma TAGs and total cholesterol were measured as described in the Experimental section. Results represent the means ± S.E.M. for n = 13 in groups A and B and n = 8 for group C. ***P < 0.0001 compared with group A.

![Figure 2](image2.png)

Figure 2 Increased hepatic ACC phosphorylation and protein content in insulin-resistant (group B, hatched bars) and TD2 (group C, black bars) compared with control (group A, white bars) P. obesus animals

Protein extracts from liver total cell lysates were resolved by SDS/PAGE and immunoblotted for ACC phosphorylation level (P-ACC-Ser79), total ACC and β-actin. A representative immunoblot (A) and corresponding densitometric analysis values (B) are shown. Results represent the means ± S.E.M. for n = 13 in groups A and B and n = 8 for group C. *P < 0.05 compared with group A, **P < 0.01 compared with group A, ***P < 0.0001 compared with group A.

![Table 3](image3.png)

Table 3 Hepatic lipid contents in P. obesus animals

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>TAG (mg/g liver)</th>
<th>Total cholesterol (mg/g liver)</th>
<th>Phospholipids (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>44.6 ± 5.1</td>
<td>16.2 ± 2.3</td>
<td>82.8 ± 9.9</td>
</tr>
<tr>
<td>B</td>
<td>422.3 ± 63.3*</td>
<td>36.6 ± 2.4*</td>
<td>118.0 ± 6.2*</td>
</tr>
<tr>
<td>C</td>
<td>370.4 ± 28.2*</td>
<td>35.9 ± 4.2*</td>
<td>122.3 ± 6.9*</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for n = 6 per group. ***P < 0.001 compared with group A.

of lipids in plasma disclosed substantial hypertriglyceridaemia and hypercholesterolaemia in groups B and C (Figure 1) along with significant differences in liver lipid contents, which is indicative of steatosis (Table 3).

Hepatic ACC phosphorylation, protein levels and isoenzyme expression

We first studied the impact of insulin resistance and TD2 on ACC, which catalyses the ATP- and biotin-dependent formation of malonyl-CoA, an essential substrate for FAS (FA synthase) and for the fatty acyl chain elongation system. Hepatic ACC phosphorylation and total protein contents were analysed by Western blots. Our results, expressed as a percentage of control values (Figure 2), showed a significant increase in ACC-Ser79 phosphorylation in groups B (151.2%, P < 0.0001) and C (177.7%, P < 0.0001). Similarly, total ACC levels were also markedly increased in groups B (92.5%, P < 0.01) and C (69.4%, P < 0.05). When the ratio of phosphorylated ACC to total ACC was calculated, it was significantly elevated in groups B (39.8%, P < 0.01) and C (75.3%, P < 0.001).

Since ACC comprises two major isoenzymes that have different physiological roles, it was important to examine their status in the liver of P. obesus. Unfortunately, there is no available antibody that can clearly distinguish between the two principal ACC isoenzymes. Therefore, to solve this technical problem, the gene expression of ACC1 and ACC2 was evaluated using the quantitative real-time PCR technique. The results are shown in Figure 3(A). Whereas ACC1 mRNA tended to be increased in group B (49%, P = 0.09), it was significantly enhanced in group C (144%, P < 0.05). Unlike ACC1, there were no changes in ACC2 isoform expression among the three P. obesus groups. Therefore our findings stress that the raised total ACC protein might mostly be due to the increased ACC1 isoform (Figure 3B).

Hepatic ACC activity

To assess the impact of increased ACC phosphorylation and protein content on hepatic ACC activity in insulin resistant and TD2, tissues were prepared from liver specimens of P. obesus animals. ACC activity was determined by following the incorporation of NaH14CO3 into malonyl-CoA. Our results (Figure 3C) demonstrated that group B displayed a trend of increase (62.3%, P = 0.08) in ACC activity, whereas group C exhibited a significant elevation (108.6%, P < 0.01). Therefore, despite the large proportion of phosphorylation, ACC activity persisted in insulin resistant and diabetic animals.

Gene expression of key lipogenic enzymes

According to our previous studies and the present lipid data, we put forward the hypothesis that the hepatic lipogenesis process...
might be increased in insulin resistant and diabetic *P. obesus* animals. To verify this hypothesis, we quantified the liver mRNA content of key lipogenic genes using the quantitative real-time PCR technique. The two experimental groups B and C exhibited a similar trend of increase in SCD-1 (stearoyl-CoA desaturase-1) and mGPAT (mitochondrial isoform of glycerol-3-phosphate acyltransferase) (Figure 4A). As for FAS, an upward tendency and a significant rise characterized groups B and C respectively (Figure 4A).

Next, we focused on the transcription factors ChREBP and SREBP-1, which emerged as central regulatory determinants of lipid synthesis in liver. As illustrated in Figure 4(B), the trend of increase in ChREBP and SREBP-1 in group B became statistically significant in group C.

**Hepatic AMPKα phosphorylation and expression**

The increased ACC serine phosphorylation in insulin resistant and TD2 *P. obesus* animals prompted us to explore the molecular status of AMPK, the principal ACC upstream kinase. Western-blot analysis (Figures 5A and 5B) showed a significant increase in AMPKα-Thr172 phosphorylation in groups B (58.8%, *P* < 0.05) and C (75.4%, *P* < 0.01) compared with group A. Similarly, total AMPK protein levels were significantly elevated in groups B (61.2%, *P* < 0.01) and C (66%, *P* < 0.01). In contrast with the calculated ratio of phosphorylated ACC to total ACC, which was significantly increased in groups B and C (illustrated in Figure 2), the calculated ratio of phospho-AMPKα to total AMPKα did not show any variation among the three groups (Figure 5B).

Finally, to discriminate between the principal AMPK isoforms that could increase the level of total AMPK protein in insulin resistant and TD2 animals, we examined the hepatic mRNA expression of AMPKα1 and AMPKα2 using the real-time PCR technique (Figure 5C). Our results revealed a significant increase in AMPKα1 isoform mRNA expression in group C (83%, *P* < 0.05) with a trend of decrease (65%) in group B. However, the mRNA expression of AMPKα2 isoform was significantly increased in group B (88%, *P* < 0.05) and remained unchanged in group C. Therefore our observations suggest a differential and an isoform-specific regulation of AMPK during the sequential development of insulin resistance and TD2.

**DISCUSSION**

The understanding of the cellular mechanisms involved in insulin resistance has become a very important aspect, not only to understand the various steps of its development, but also to comprehend the processes of dyslipidaemia and to establish approaches for the treatment and/or prevention of diabetes. In the present study, we
employed the *P. obesus* gerbil, a well-established model of TD2 and obesity, in order to appreciate the abnormal hepatic lipid synthesis along with the mechanisms implicated in conditions of insulin resistance and TD2. More specifically, we tested the hypothesis that dysregulation of AMPK and ACC might occur in the liver of insulin resistant and diabetic animals and contribute to the elevated plasma TAG levels and probably to the increased lipid accumulation in the liver. We provided evidence that the development of the insulin resistance/diabetic state in *P. obesus* is accompanied by (i) body weight gain and hyperlipidaemia; (ii) elevations of hepatic ACC-Ser^{79} phosphorylation and ACC protein levels; (iii) a rise in the gene expression of cytosolic ACC1 (the isoenzyme that participates in *de novo* lipogenesis) concomitant with invariable mitochondrial ACC2 (involved in negative regulation of mitochondrial β-oxidation); (iv) an increase in hepatic AMPKα phosphorylation and protein expression; (v) a stimulation of ACC activity despite increased AMPKα phosphorylation and protein expression; and (vi) an increase in mRNA levels of key lipogenic enzymes (FAS, SCD-1 and mGPAT) and of transcription factors (ChREBP and SREBP-1).

In the present study, the *P. obesus* gerbil developed hyperinsulinaemia, hyperglycaemia and dyslipidaemia along with a body weight gain, manifested by increased liver and abdominal fat pad mass. The use of HOMA, a model of interactions between glucose and insulin dynamics, confirmed the presence of insulin resistance previously reported by our laboratory and other groups [23,31]. Overall, numerous components of the metabolic syndrome characterize the *P. obesus* animal and amply justify its exploitation for the clarification of the intimate relationship that exists in lipid homoeostasis abnormalities. In particular, hepatic contributions through the amplification of insulin resistance, obesity and TD2 may be detrimental to lipid metabolism. A number of studies have already provided compelling evidence that the liver is among the major insulin-sensitive organs in glucose homoeostasis [32] and probably in lipid balance in other animal models.

A large fraction of glucose and fat absorbed from the small intestine is taken up by the liver. In hepatocytes, glucose is converted into glycogen and, after the saturation of the liver with glycogen, residual glucose is shunted into pathways leading to
the synthesis of FA, which will be esterified into TAG to be exported to the circulation as VLDLs (very-low-density lipoproteins). Furthermore, the removal of chylomicron remnants by the liver, which represents the final step in postprandial lipid metabolism, increased hepatic lipid content via recycling of chylomicron-remnant lipid components for the promotion of VLDL production. Finally, enhanced FA mobilization from the adipose tissue favours TAG formation in the liver. We have previously documented the potential lipid pathways contributing to the expansion of liver content, i.e. dietary FA mainly through the over-secretion of chylomicrons and hepatic lipogenesis in P. obesus animals [23]. In the present study, we attempted to elucidate the mechanisms behind the abnormally high lipogenesis in the hepatocyte. In fact, we could observe enhanced ACC protein levels and phosphorylation in both groups B and C characterized by insulin resistance and frank TD2 respectively. We also noticed, by mRNA expression analysis, that the major hepatic ACC isoform, ACC1, was up-regulated in these groups. Since ACC is inhibited by phosphorylation [33,34], we initially hypothesized that the activity of ACC could be decreased in insulin resistant and diabetic animals. Surprisingly, our findings demonstrated an increased trend of ACC activity in these animal groups, which is in agreement with previous results on JCR:LA-cp rats where ACC activity was elevated in the insulin resistance state [35]. The increased activity may be mainly explained by the high ACC protein levels, indicative of sustained transcription process. At the same time, the present study cannot rule out the contribution of altered intracellular metabolite concentrations that might also modulate ACC activity [36].

A previous study showed an increased AMPK expression in high-fat and high-sucrose diet-induced diabetic mice [37]. In our case, the up-regulation of AMPK could be an indirect consequence of hyperinsulinaemia and hyperglycaemia. This adaptive process could be triggered at least by two main mechanisms, i.e. stimulation through elevated plasma and liver-free FA concentrations and activation of hepatic lipogenesis in insulin resistance and diabetic P. obesus animals.

AMPK is activated allosterically by AMP and by the phosphorylation of Thr172 by upstream AMPKKs (AMPK kinases) [10,38]. In view of the elevated ACC phosphorylation in insulin resistant and diabetic P. obesus animals, we examined AMPK phosphorylation status and expression levels, since AMPK represents the principal ACC kinase. Our findings pointed out that increased ACC phosphorylation was associated with increased AMPK-Thr172 phosphorylation and protein expression in insulin resistance and diabetic states. Gene expression analysis indicated an up-regulation of AMPKα2 isoform in group B and AMPKα1 isoform in group C along with an upward trend of AMPKK, the LKB1 in both groups (results not shown).

AMPK activation aims to switch off intracellular biosynthesis processes. Here, we have reported the up-regulation of key lipogenic enzymes in group B and C, which is in line with the activation of de novo lipogenesis reported in our previous studies [22]. Since this process is highly ATP-consuming, it causes an increased concentration of AMP, leading usually to AMPK up-regulation necessary to restore the energy balance. According to our results, the protein expression and phosphorylation of AMPK were raised concomitantly with ACC phosphorylation, which could represent an adaptive mechanism aiming at inhibiting FA synthesis. It seems that ACC phosphorylation was insufficient for reducing the activity given the high ACC protein level.

The liver is responsible for the conversion of excess dietary carbohydrates into TAG through de novo lipogenesis. The transcription factor ChREBP has recently emerged as a major mediator of glucose action in the control of both glycolysis and lipogenesis in the liver [39]. Induction of lipogenic genes (ACC and FAS) is under the concerted action of ChREBP and the transcription factor SREBP-1c in response to glucose and insulin respectively [40]. Accordingly, the liver-specific inhibition of ChREBP decreased the rate of hepatic lipogenesis and improved hepatic steatosis and insulin resistance in obese ob/ob mice [41] in response to characteristic hyperglycaemia. Our findings showed a raised gene expression of ChREBP in the liver in response to the hyperglycaemia characterizing insulin resistance and diabetic P. obesus animals. Low rate of glucose transport and failure of insulin to suppress hepatic glucose production [42] may be implicated since the mechanism responsible for ChREBP activation at the post-translational level is thought to involve an increase in intracellular glucose metabolism [43].

In our studies, we could observe high levels of SREBP-1 mRNA. Importantly, studies using SREBP-1 gene knockout mice reported that SREBP-1 mediates insulin-stimulated lipogenic gene expression of ACC, FAS, SCD-1 and mGPAT and plays a crucial role in the induction of lipogenesis [44-46] despite a poor insulin signalling pathway [47].

Several studies have reported that AMPK activation inhibits lipogenesis by suppressing the expression of glucose-induced lipogenic genes such as FAS [18,48], ACC [49], mGPAT [50] and SREBP-1 [18]. In the present study, we demonstrated that AMPK activation/up-regulation in P. obesus is still associated with its direct and short-term effects (i.e. ACC phosphorylation) but not any more with its long-term effects on lowering lipogenic gene expression.

Collectively, our findings suggest that activation/up-regulation of the AMPK pathway might be a natural adaptive response to counteract lipid metabolism abnormalities associated with insulin resistance and TD2. Therefore the activation of AMPK, in particular, the isoform-specific activation of hepatic AMPK, might be a promising therapeutic approach for the treatment of TD2-associated dyslipidaemia.

ACKNOWLEDGEMENTS
We thank Shoohraya Spahis, Jean-Claude Lavoie and Therese Rouleau for their expert technical assistance.

FUNDING
This work was supported by the Canadian Institutes of Health Research [grant number MSH-63616], and the Canadian Foundation for Innovation. A.B.D.O. is the recipient of a graduate scholarship from the Sainte-Justine Foundation. E.L. holds the J.A. deSève
REFERENCES


A. Ben Djoudi Ouadda and others


Received 15 September 2008/30 September 2008; accepted 8 October 2008
Published as Immediate Publication 8 October 2008, doi 10.1042/BSR20080141