Available online at www.sciencedirect.com
SCIENCE DIRECT
Comparative Biochemistry and Physiology, Part B 142 (2005) 374 – 382
CBP
www.elsevier.com/locate/cbpb

Evaluation of the role of AMP-activated protein kinase and its downstream targets in mammalian hibernation

Sandrine Horman [a], Nusrat Hussain [a], Stephen M. Dilworth [b], Kenneth B. Storey [c], Mark H. Rider [a,*]

[a] *Hormone and Metabolic Research Unit, Christian de Duve Institute of Cellular Pathology and University of Louvain Medical School, Avenue Hippocrate, 75, B-1200 Brussels, Belgium*
[b] *Tumour Cell Biology, Department of Metabolic Medicine, Imperial College Faculty of Medicine, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK*
[c] *Institute of Biochemistry and Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada K1S 5B6*

Received 27 May 2005; received in revised form 28 August 2005; accepted 29 August 2005
Available online 3 October 2005

Abstract

Mammalian hibernation requires an extensive reorganization of metabolism that typically includes a greater than 95% reduction in metabolic rate, selective inhibition of many ATP-consuming metabolic activities and a change in fuel use to a primary dependence on the oxidation of lipid reserves. We investigated whether the AMP-activated protein kinase (AMPK) could play a regulatory role in this reorganization. AMPK activity and the phosphorylation state of multiple downstream targets were assessed in five organs of thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) comparing euthermic animals with squirrels in deep torpor. AMPK activity was increased 3-fold in white adipose tissue from hibernating ground squirrels compared with euthermic controls, but activation was not seen in liver, skeletal muscle, brown adipose tissue or brain. Immunoblotting with phospho-specific antibodies revealed an increase in phosphorylation of eukaryotic elongation factor-2 at the inactivating Thr56 site in white adipose tissue, liver and brain of hibernators, but not in other tissues. Acetyl-CoA carboxylase phosphorylation at the inactivating Ser79 site was markedly increased in brown adipose tissue from hibernators, but no change was seen in white adipose tissue. No change was seen in the level of phosphorylation of the Ser565 AMPK site of hormone-sensitive lipase in adipose tissues of hibernating animals. In conclusion, AMPK does not appear to participate in the metabolic re-organization and/or the metabolic rate depression that occurs during ground squirrel hibernation.

Keywords: Metabolic rate suppression; Ground squirrels; Protein phosphorylation; Acetyl-CoA carboxylase; Elongation factor-2; Hormone-sensitive lipase

1. Introduction

Small mammalian hibernators, such as most ground squirrels, conserve energy over the winter months by entering a state of torpor, lasting for periods of days or weeks, during which the core body temperature (T_b) can drop to near 0 °C. Under these conditions, their metabolic rate is strongly suppressed, often to less than 5% of the normal euthermic resting rate, and their heart beat is reduced to just a fraction of normal, creating conditions that would constitute severe ischaemia in most mammals, but which are non-injurious to hibernators. The depression of metabolic rate in hibernation requires a well-coordinated control of metabolism, so that ATP-producing and ATP-consuming processes are balanced and energy homeostasis can be maintained at a much lower T_b (Storey, 2003; Storey and Storey, 2004).

The AMP-activated protein kinase (AMPK) is a highly conserved serine/threonine protein kinase that acts as a sensor of cellular energy status in eukaryotes (Hardie et al., 1998, 2003; Kemp et al., 1999; Carling, 2004). AMPK is a heterotrimer consisting of a catalytic α subunit and two regulatory subunits, β and γ. Each subunit exists as multiple isoforms (α1, α2, β1, β2, γ1, γ2, γ3) giving twelve possible combinations of holoenzyme with different tissue distribution

Abbreviations: ACC, acetyl-CoA carboxylase (EC 6.4.1.2); AMPK, AMP-activated protein kinase; B, brain; BAT, brown adipose tissue; GS, glycogen synthase (EC 2.4.1.11); eEF2, eukaryotic elongation factor-2; eEF2K, eukaryotic elongation factor-2 kinase; HSL, hormone-sensitive lipase (EC 3.1.1.79); L, liver; mTOR, mammalian target of rapamycin; PKA, cyclic-3′,5′-monophosphate-dependent protein kinase; p70S6K, p70 ribosomal protein S6 kinase; SKM, skeletal muscle; WAT, white adipose tissue; T_b, body temperature.

* Corresponding author. Tel.: +32 764 7485; fax: +32 764 7507.
E-mail address: rider@horm.ucl.ac.be (M.H. Rider).

1096-4959/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2005.08.010

and subcellular localization. AMPK is allosterically stimulated by AMP and is also regulated by phosphorylation via an upstream activating AMPK kinase (AMPKK) (Hawley et al., 1996). The major regulatory phosphorylation site is Thr172 in the activation loop of the α-subunits (Hawley et al., 1996) and phosphorylation at this site is both sufficient and necessary for AMPK activation. An upstream activating kinase phosphorylating Thr172 was recently identified as the Peutz–Jeghers syndrome protein LKB1 (Hawley et al., 2003; Hong et al., 2003). AMPK is activated by increases in the intracellular AMP : ATP ratio, as occurs during anoxia, and once activated it down-regulates ATP-consuming pathways and stimulates ATP-producing pathways (Hardie et al., 1998, 2003; Kemp et al., 1999; Carling, 2004). For example, fatty acid synthesis is inhibited via the phosphorylation and inactivation of acetyl-CoA carboxylase (ACC). As a consequence, malonyl-CoA concentrations fall which stimulates fatty acid oxidation, contributing to the maintenance of intracellular ATP levels. In addition, AMPK activation stimulates glycolysis by increasing glucose uptake in skeletal muscle (Merrill et al., 1997) and heart (Russell et al., 1999) and by activating 6-phosphofructo-2-kinase in heart (Marsin et al., 2000).

Ion-motive ATPases and protein synthesis are the dominant ATP-requiring processes in most cells, together accounting for up to 90% of oxidatively coupled ATP consumption (Boutilier, 2001). It is not surprising, therefore, that metabolic rate depression during hibernation includes strong inhibition of these processes (Storey and Storey, 2004). For example, Na^+/K^+-ATPase activity was decreased by 60% in muscle extracts from hibernating ground squirrels compared with euthermic controls (both assayed at 25 °C) (MacDonald and Storey, 1999). The rate of protein synthesis in vitro in extracts from hibernating versus euthermic animals was also reduced by 3-fold when assayed at constant temperature (Frerichs et al., 1998) and a similar inhibition was seen in kidney (Hittel and Storey, 2002). Furthermore, when assessed in vivo, the compounding effect of low T_b resulted in a virtual arrest of protein synthesis, with rates in brain, liver and heart of torpid squirrels (T_b=7.5 °C) being 0.1% or less of the value in euthermic animals (T_b=37 °C) (Frerichs et al., 1998). Interestingly, however, the capacity for protein synthesis of brown adipose tissue was unaltered when in vitro rates in tissue extracts from euthermic and hibernating animals were compared at the same temperature (Hittel and Storey, 2002).

Protein synthesis peptide chain elongation consumes at least 4 equivalents of ATP for each peptide bond synthesized and is inhibited via the phosphorylation of eukaryotic elongation factor-2 (eEF2) at Thr56 by a highly specific Ca^{2+} and calmodulin-dependent kinase called eEF2 kinase (eEF2K) (Redpath et al., 1993). Indeed, in livers and brains from hibernating ground squirrels, eEF2 Thr56 phosphorylation levels were increased (Chen et al., 2001), which could have been due to eEF2K activation. This protein kinase is subject to regulation by a variety of upstream protein kinases including AMPK and the p70 ribosomal protein S6 kinase (p70S6K) (Browne and Proud, 2002; Horman et al., 2003; Browne et al., 2004). The p70S6K phosphorylates and inactivates eEF2K (Wang et al., 2001) and the mammalian target of rapamycin (mTOR), which activates p70S6K, was shown to act as an ATP sensor in cells (Dennis et al., 2001). A reduction in ATP levels and a decrease in mTOR signalling could thus lead to an increase in eEF2K activity and subsequent phosphorylation of eEF2. In addition, AMPK activation inhibits p70S6K activation (Bolster et al., 2002; Dubbelhuis and Meijer, 2002; Krause et al., 2002) possibly via phosphorylation of the tuberous sclerosis complex TSC2 which potentiates its ability to inhibit phosphorylation of p70S6K by mTOR (Inoki et al., 2003). Also, AMPK activation leads to the phosphorylation of mTOR at Thr2446 which decreases p70S6K activation by insulin (Cheng et al., 2004). However, we and others showed that AMPK activation resulted in the phosphorylation of eEF2 and that this could be explained by the phosphorylation and activation of eEF2K by AMPK independently of an inhibition of mTOR signalling (Horman et al., 2002; McLeod and Proud, 2002; Horman et al., 2003; Browne et al., 2004).

Given that AMPK is a key sensor of cellular energy status and that hibernation involves a huge net suppression of ATP turnover as well as about a 50% decrease in cellular adenylate levels (MacDonald and Storey, 1999; English and Storey, 2000), we hypothesized that AMPK might have a key role in the restructuring and overall suppression of cellular energetics in the torpid state. We predicted that AMPK activation would be seen in association with increases in the extent of phosphorylation of targets involved in reorientating metabolism. AMPK activity in five tissues of hibernating ground squirrels was measured and compared with that in euthermic controls. In parallel, we studied the phosphorylation states of downstream targets of AMPK, with special emphasis on eEF2, by immunoblotting using a panel of phospho-specific antibodies.

2. Materials and methods

2.1. Materials

Anti-human eEF2, anti-rat p70S6K and anti-phospho Thr389 human p70S6K peptide polyclonal antibodies were from Santa Cruz. Anti-phospho Thr172 human AMPK α-subunit and anti-phospho Ser235/236 human S6 ribosomal protein peptide polyclonal antibodies were from Cell Signalling Technologies. These antibodies are routinely used in the Rider laboratory to study AMPK signalling in rat hepatocytes, rat cardiomyocytes and rat adipocytes where relevant signals are obtained in immunoblot experiments. Anti-phospho Thr56 eEF2 peptide polyclonal antibody was prepared as described (Wang et al., 2001). The following panel of anti-phospho peptide polyclonal antibodies — rat Ser79 ACC, rat Ser7 glycogen synthase (GS), rat/mouse Ser563 hormone-sensitive lipase (HSL) and rat/mouse Ser565 HSL were kindly provided by Prof. Grahame Hardie (University of Dundee) along with non-phosphospecific anti-rat α1-, α2-AMPK subunit, anti-rat/mouse GS and anti-rat/mouse/human HSL peptide polyclonal antibodies. These are Consortium Reagents

used by several different partners in an EU FP5/FP6 program on AMPK and have been thoroughly tested in rats, mice and humans to verify their specificity. Also, since most of the antibodies were raised against rat/human peptide sequences and since squirrels are also rodents/mammals, cross-reactivity would be expected with squirrel proteins. Mouse monoclonal antibodies were raised against overexpressed human p65 regulatory and p35 catalytic subunits of protein phosphatase 2A (PP2A) as described (Abraham et al., 2000). Other chemicals were from sources previously cited (Horman et al., 2002, 2003).

2.2. Animals

Tissue samples from thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*) were provided from experiments conducted by the laboratory of Dr. J.M. Hallenbeck, National Institute of Neurological Disorders and Stroke (NIH, Bethesda, MD, USA). Squirrels weighing 120 to 180 g were captured by humane trapping. Animals were housed in a holding room in individual shoebox cages maintained at 22–24 °C, with 60% relative humidity and 12/12 h light/dark cycle; free access was given to food and water. A thermal sensor chip was surgically implanted under the skin of each animal so that the body temperature of each animal could be monitored electronically. Individuals were weighed weekly to determine when they entered and finished the pre-hibernation phase of hyperphagia that maximizes body lipid reserves prior to hibernation. When animals had exhibited a rapid increase in body mass (to 220–240 g), some were placed in a dark chamber at 5–6 °C and 60% humidity to facilitate hibernation. Other animals were sacrificed as euthermic controls. After placing in the hibernation chamber, most animals entered a state of deep torpor within 3 to 8 days. Animals were sampled as hibernators after 3 to 7 days of continuous torpor, as determined from constant T_b recordings of 5–7 °C. For sampling, all animals were euthanized by intracardiac injection of sodium pentobarbital and tissues were rapidly excised, flash frozen in liquid nitrogen, transported to their destination on dry ice and then stored at −80 °C until use. Brown adipose tissue was taken from under the skin and over the muscle on the back of the animal. White fat was taken from the abdominal cavity.

2.3. Tissue extracts

Frozen tissues were partially thawed and homogenized on ice (Ultra-Turrax, 30 s) in the following wet weight : volume ratios — 1 : 5 (liver and skeletal muscle), 1 : 2 (brain) and 1 : 1 (white and brown adipose tissue) of ice cold extraction buffer containing 50 mM Hepes pH 7.6, 50 mM KCl, 50 mM KF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 mM sodium β-glycerophosphate, 4 μg/mL leupeptin, 1 mM benzamidine–HCl, 0.7 μg/mL pepstatin and 0.2 mM phenylmethanesulphonyl fluoride. Extracts were clarified by centrifugation (16,000 *g* for 15 min at 4 °C). Sample supernatants were then removed and stored at −80 °C prior to measurements of enzyme activity and immunoblotting.

2.4. Immunoblotting

Sample supernatant proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. The percentage of acrylamide used for the gels depended on the protein under study. Acrylamide concentrations were 12% (w/v) for PP2A, 10% (w/v) for AMPK, eEF2, GS, HSL and 6% (w/v) for ACC. Equal amounts of protein samples were loaded into each well of the gel, with one exception, as specified in the figure legends. The membranes were blocked in TBS (10 mM Tris–HCl, pH 7.4, 0.15 M NaCl) with 5% (w/v) low fat milk powder and incubated with various dilutions of primary antibody in the same buffer for 2–16 h (1 : 1000 for anti-phospho Thr172 AMPK, 1 : 10000 for anti-total α1/α2 AMPK, 1 : 1000 for anti-phospho Thr56 eEF2 and full-length eEF2, 1 : 1000 for the anti-p35 catalytic subunit of PP2A, 1 : 2500 for the anti-p65 regulatory subunit of PP2A, 1 : 4000 for anti-phospho Ser565 and anti-phospho Ser563 HSL, 1 : 1000 for anti-total HSL, 1 : 1000 for anti-phospho Ser7 GS and 1 : 1000 for anti-total GS). After extensive washing in TBS, the membranes were incubated for 1 h at room temperature with the appropriate secondary antibody coupled to horseradish peroxidase. After further extensive washing in TBS, the blots were developed for imaging by enhanced chemiluminescence (Amersham). Changes in phosphorylation states were assessed for statistical significance after quantification by densitometric scanning of films using the program "Image J 1.33" for Macintosh OS X.

2.5. Enzyme assays

For AMPK, about 300 μL of sample supernatants were precipitated with a final concentration of 10% (w/v) polyethylene glycol 6000 and resuspended in 100 μL of extraction buffer. Aliquots (corresponding to ~10 μg of protein) were assayed in a final volume of 50 μL containing 0.2 mM SAMS peptide, 0.2 mM AMP and 0.1 mM [γ-^{32}P]MgATP (specific radioactivity 500 cpm/pmol) for 10 min at 30 °C (Davies et al., 1989). One unit of enzyme activity is the amount that catalyses the incorporation of 1 nmol of ^{32}P into the SAMS peptide under the conditions of the assay. Total *sn*-glycerol 3-phosphate *o*-acyltransferase in 50 μL of centrifuged extracts was assayed with 0.5 mM [2-^{3}H] L-glycerol 3-phosphate and 50 μM palmitoyl-CoA (Rider and Saggerson, 1983).

2.6. Other methods

Protein was measured (Bradford, 1976) using bovine serum albumin as a standard. Statistically significant differences were assessed using an unpaired Student's two-sided *t*-test.

3. Results

3.1. AMPK activity and α-subunit phosphorylation and expression

In polyethyleneglycol fractions prepared from tissue extracts, AMPK activity was increased 3-fold in white adipose

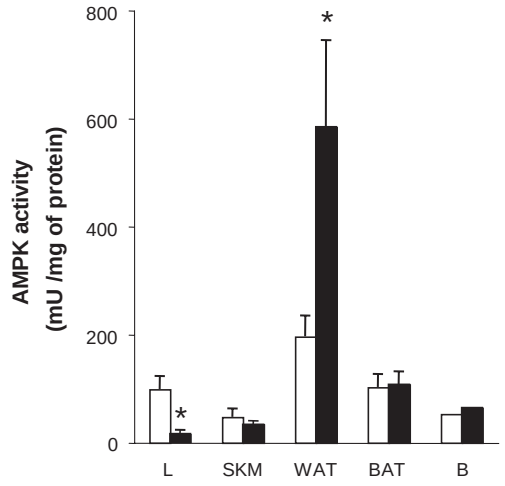

Fig. 1. AMPK activity in tissue extracts from euthermic and hibernating ground squirrels. AMPK was assayed in polyethylene glycol fractions of sample supernatants in the presence of 0.2 mM AMP as described in Materials and methods section. L: liver; SKM: skeletal muscle; WAT: white adipose tissue; BAT: brown adipose tissue; B: brain. Values are the means ± S.E. for $n=3$ (L), $n=4$ control and $n=5$ hibernator (SKM) $n=6$ (WAT, BAT) and means of 2 individuals (B), euthermics versus hibernators. *Indicates a statistically significant difference compared with the euthermic controls ($P<0.05$).

tissue from hibernating ground squirrels compared with the euthermic controls (Fig. 1). By contrast AMPK was significantly decreased during hibernation in liver to levels just 15% of the euthermic value, whereas activity was unchanged in the other tissues (Fig. 1). The extent of phosphorylation of Thr172 of the α1- and α2-subunits (which correlates with AMPK activation) was assessed by immunoblotting (Fig. 2). In these and all other immunoblotting experiments, equal amounts of protein from each cell-free extract from euthermic versus hibernating animals were separated on acrylamide gels prior to Western blotting. In white adipose tissue, Thr172 phosphorylation increased 2-fold in hibernators, whereas Thr172 phosphorylation in liver extracts decreased to about 10% of the euthermic value (Fig. 2), consistent with the changes in AMPK activity (Fig. 1). The expression levels of the α1- and α2-AMPK subunits were mainly unaffected in hibernators compared with controls, except in brown adipose tissue where a 2-fold increase in α2-AMPK subunit expression was observed and in skeletal muscle where a 60% increase in α1-AMPK subunit levels was seen (Fig. 3).

3.2. eEF2 Thr56 phosphorylation

The inhibition of protein synthesis in liver, heart and brain of hibernators was proposed by Chen et al. (2001) to be linked to Thr56 phosphorylation of eEF2. In agreement with these results, eEF2 phosphorylation was markedly increased in liver and brain from hibernating ground squirrels compared with euthermic controls (Fig. 4). In addition, eEF2 phosphorylation increased 2-fold in white adipose tissue from hibernators, but phosphorylation levels were unchanged in brown adipose tissue and skeletal muscle. No changes in overall protein expression levels of eEF2 were detected in hibernators compared with euthermic animals in any of the tissues screened (Fig. 4) and in most of the immunoblots, total eEF2 band intensities were used as loading controls to calculate relative band intensities for the anti-phospho antibodies.

We previously showed that AMPK activation leads to eEF2 phosphorylation in liver and heart (Horman et al., 2002, 2003). During hibernation, AMPK activation was only observed in white adipose tissue (Fig. 1) where it could be implicated in eEF2 phosphorylation, but in other organs different mechanisms must be involved. Therefore, we looked at hibernation effects on p70S6K, another kinase that could control eEF2 phosphorylation. Unfortunately, no changes in p70S6K could be demonstrated either by immunoprecipitation and direct assay or by immunoblotting with anti-phospho Thr389 antibody in extracts from liver and adipose tissues (data not shown). The anti-phospho Thr389 p70S6K antibody gave too

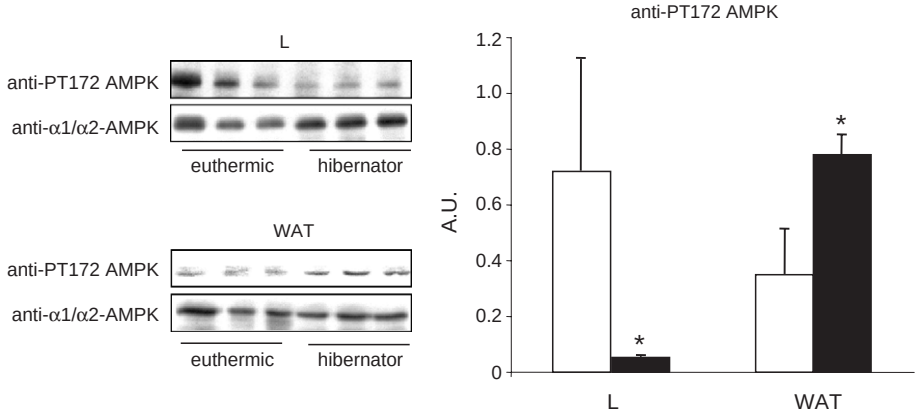

Fig. 2. Phosphorylation state of Thr172 of AMPK α-subunits in liver and white adipose tissue from hibernating ground squirrels. Sample supernatants (50 μg of protein in each lane) were subjected to SDS-PAGE and immunoblotted with anti-phospho Thr172 α-subunit AMPK antibody (upper panel). After stripping, the membrane was reprobed with anti-full length AMPK α-subunit antibody that recognizes both α1 and α2 forms but does not distinguish the phosphorylated protein (lower panel). The histogram shows mean arbitrary units for phospho-AMPK band intensities for tissue from three individual euthermic (white bars) and hibernating (black bars) animals relative to the band intensities of total eEF2 ± S.E. *Indicates a statistically significant difference compared with the euthermic controls ($P<0.05$).

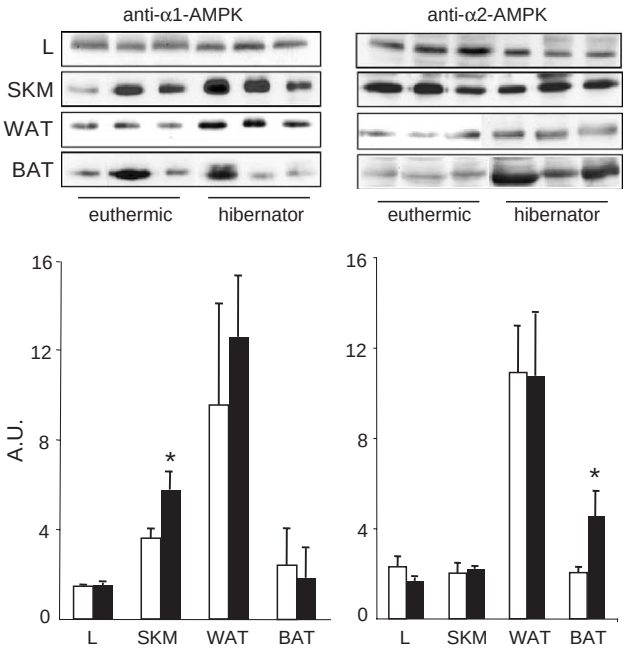

Fig. 3. Comparison of expression levels of the α1- and α2-AMPK subunits in tissues from hibernating ground squirrels compared with euthermic controls. Sample supernatants were subjected to SDS-PAGE and immunoblotted with either the anti-AMPK-α1 or anti-AMPK-α2-subunit antibody. Because the protein concentrations of the tissue extracts were different and because we used the maximum volume of sample that could be loaded into a well (30 μL) to optimize band detection, identical amounts of protein were not present in each lane. In this instance, band intensities were expressed relative to the protein content of each sample and blots for three individual euthermic (white bars) and hibernating (black bars) animals were used to construct the histograms showing mean arbitrary units of band intensities expressed per microgram of extract protein ±S.E. *Indicates a statistically significant difference compared with the euthermic controls ($P<0.05$).

many aspecific bands and our failure to assay p70S6K might have been due to loss of activity due to repeated freeze–thawing of extracts. Therefore, immunoblots with an anti-phospho ribosomal protein S6 antibody were performed on extracts of liver and brown adipose tissues from three individual control and hibernating animals, for which material was still available. A 70%–85% decrease in band intensity relative to total eEF2 was evident, however the decrease was not significant (results not shown). Therefore, a reduction in mTOR signalling via a drop in intracellular ATP levels might explain the increase in eEF2 phosphorylation in liver and perhaps in other tissues, where AMPK was not activated during hibernation.

Other mechanisms for increasing eEF2 phosphorylation are via inhibition and/or down-regulation of eEF2 phosphatase(s). However, in tissues from the hibernating ground squirrels studied here, p35 PP2A catalytic subunit and p65 regulatory subunit expression levels were unchanged compared with euthermic controls (Fig. 5).

3.3. ACC Ser79 phosphorylation

AMPK inactivates the two isoforms of ACC expressed predominantly in liver and adipose tissue (ACC1, 265 kDa) and in muscles (ACC2, 280 kDa) by phosphorylation at Ser79 in ACC1 (Davies et al., 1990) and Ser219 in ACC2 (Dyck et al., 1999). Immunoblotting with an anti-phosphopeptide antibody raised against a sequence of rat ACC2 containing phospho-Ser219, and which also recognizes phospho-Ser79, revealed a doublet in liver extracts from euthermic controls, which tended to decrease in hibernators (Fig. 6), in line with the decrease in AMPK activity (Fig. 1). In skeletal muscle from hibernators, no change in ACC phosphorylation (Ser219) was observed compared with the euthermic controls (Fig. 6). Surprisingly, the level of ACC phosphorylation increased 5-fold in brown adipose tissue, but no change was seen in white adipose tissue from hibernators compared with euthermic controls (Fig. 6).

3.4. GS Ser7 phosphorylation

The muscle isoform of GS is phosphorylated at Ser7 by AMPK leading to inactivation (Carling and Hardie, 1989). In skeletal muscle, white adipose tissue and brown adipose tissue of hibernators compared with euthermic animals, no changes in the levels of Ser7 GS phosphorylation were observed (data not shown).

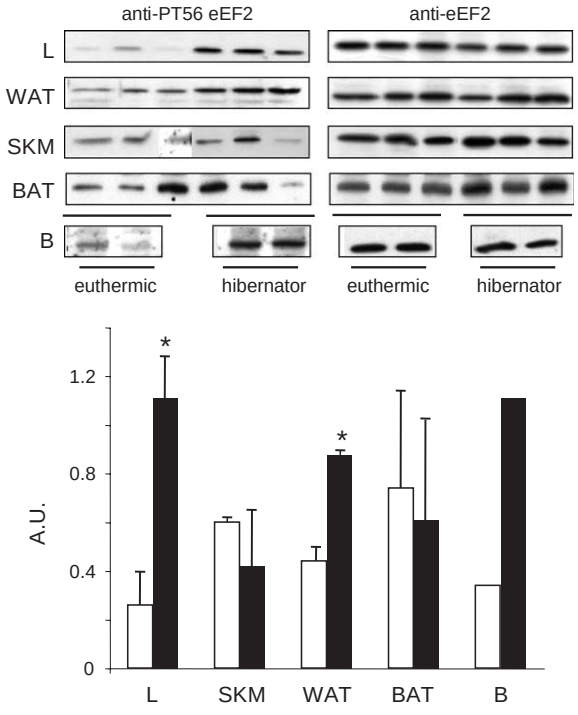

Fig. 4. Phosphorylation state of eEF2 Thr56 in hibernating ground squirrels. Sample supernatants (50 μg of protein in each lane) were subjected to SDS-PAGE and immunoblotted with anti-phospho Thr56 eEF2 antibody (left panels). The amount of eEF2 present in the extracts from euthermic and hibernating animals was the same as assessed by reprobing with anti-eEF2 antibody after stripping, except for brain where a separate gel was run (right panels). Blots for two (brain) or three (other tissues) individuals (euthermic versus hibernator) are shown. The histogram shows mean arbitrary units of phospho-eEF2 band intensities in euthermic (white bars) versus hibernating (black bars) animals relative to the band intensities for total eEF2±S.E. *Indicates a significant difference from the corresponding euthermic value, $P<0.05$.

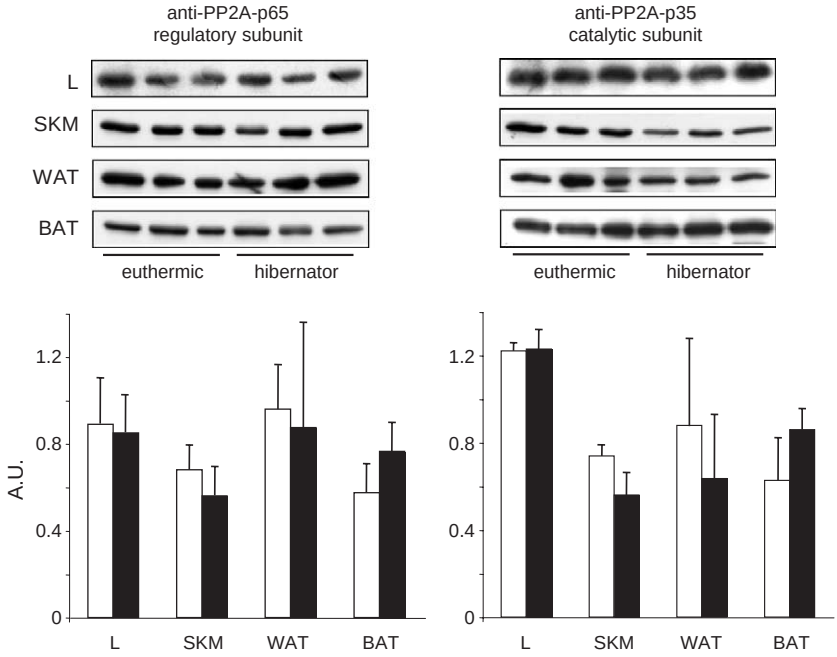

Fig. 5. Expression levels of the p65 regulatory subunit and p35 catalytic subunit of PP2A in tissues from hibernating and euthermic ground squirrels. Sample supernatants (20 μg of protein in each lane) were subjected to SDS-PAGE and immunoblotted with mouse monoclonal antibodies against the 65 kDa regulatory subunit of PP2A (left panels) or the p35 kDa catalytic subunit (right panels). The histograms show mean arbitrary units of PP2A band intensities for euthermic (white bars) versus hibernating (black bars) animals relative to band intensities for total eEF2. Values are the means ± S.E. for $n=3$ (L), $n=4$ control and $n=5$ hibernator (SKM) $n=6$ (WAT, BAT) individuals, euthermics versus hibernators.

3.5. HSL Ser563 and Ser565 phosphorylation

HSL is phosphorylated at two sites in rat adipocytes (Stralfors et al., 1984) that were identified as Ser563 (Garton et al., 1988) and Ser565 (Garton et al., 1989). In quiescent cells, only Ser565 is phosphorylated and stimulation by noradrenaline increased the phosphorylation of Ser563 with respect to Ser565 (Stralfors et al., 1984). In vitro, Ser563 is phosphorylated by the cyclic AMP-dependent protein kinase (PKA), which activates HSL, whereas Ser565 is phosphorylated by AMPK. Phosphorylation at Ser565 inhibits phosphorylation at Ser563 and thus was proposed to have an antilipolytic effect (Garton et al., 1989). In adipose tissues from hibernators, there was no change in phosphorylation state of either Ser563 or Ser565 of HSL (Fig. 7) or its total expression levels (not shown).

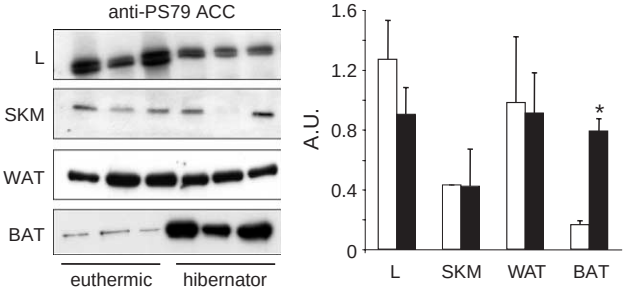

Fig. 6. Phosphorylation state of ACC Ser79 in hibernating and euthermic ground squirrels. Sample supernatants (50 μg of protein in each lane) were subjected to SDS-PAGE and immunoblotted with anti-phospho Ser79 ACC antibody. This antibody detects Ser219 of ACC2 in skeletal muscle and Ser79 of ACC1 in the other tissues. Blots for three individuals (euthermic versus hibernator) are shown and histograms show mean arbitrary units for band intensities of anti-phospho ACC relative to the band intensities for total eEF2 ± S.E. for euthermic (white bars) versus hibernating (black bars) individuals, $n=3$ L, SKM and $n=6$ WAT, BAT. *Indicates a significant difference compared with the corresponding euthermic value, $P<0.05$.

4. Discussion

In this paper, we have assessed the role of AMPK and phosphorylation of some of its downstream targets in the regulation of energy homeostasis during hibernation in ground squirrels. AMPK activation during hibernation was seen only in white adipose tissue, while in liver AMPK activity decreased (Fig. 1). In skeletal muscles from hibernators, the ATP : AMP ratio was reported to rise (el Hachimi et al., 1990) whereas others reported a fall in total muscle adenine nucleotide levels, but with no change in energy charge (MacDonald and Storey, 1999; English and Storey, 2000). Studies in skeletal muscle from 24 h versus 5–7 day hibernating mice suggested that transient disruptions of energetics might occur during entry into torpor, but disappear when steady-state torpor is achieved (Kelly and Storey, 1995). Therefore, AMPK-mediated suppression of some cell functions could occur in some tissues as a result of disrupted energetics on entry into torpor, but with the exception of white adipose tissue, we find no evidence of sustained AMPK activation in prolonged torpor.

The expression level of the α1-AMPK subunit in skeletal muscle and the α2-AMPK subunit in brown adipose tissue was increased in hibernating animals compared with the controls (Fig. 3), whereas no changes were observed in the other tissues.

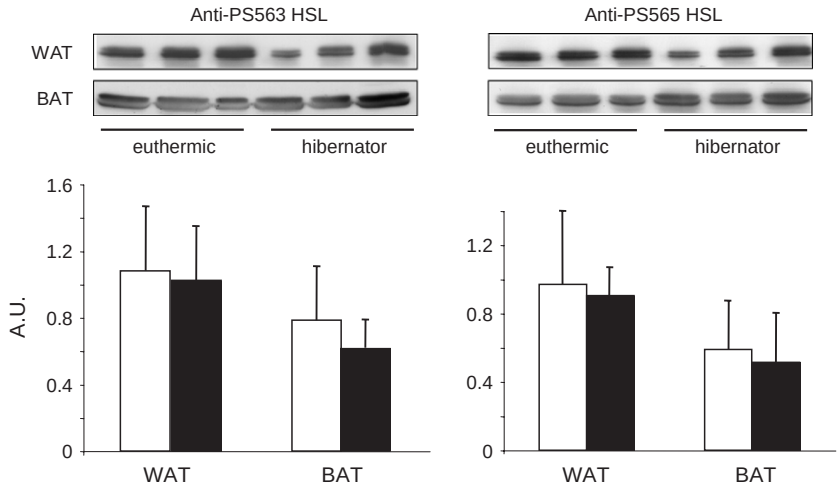

Fig. 7. Phosphorylation state of HSL at Ser563 and Ser565 in white and brown adipose tissue from hibernating and euthermic ground squirrels. Sample supernatants (50 μg of protein in each lane) from white and brown adipose tissue were subjected to SDS-PAGE and immunoblotted with anti-phospho Ser563 and anti-phospho Ser565 HSL antibodies. Blots for three individuals (euthermic versus hibernator) are shown. The histograms show mean arbitrary units of anti-phospho HSL band intensities relative to band intensities for total eEF2. Values are the means±S.E. for $n=5$ (anti-phospho Ser563) and $n=6$ (anti-phospho Ser565) individuals, euthermic (white bars) versus hibernating (black bars).

Expression levels of the α1- versus α2-AMPK subunits cannot be compared because recognition by the isoform-specific antibodies would not necessarily be the same. Unfortunately, we were unable to immunoprecipitate the α1- and α2-AMPK subunits to assess their contribution to overall activity, however this might have been due to loss of activity due to repeated freeze–thawing of extracts. The α2-AMPK isoform has been reported to be predominant in skeletal muscle and heart, whereas α1-AMPK expression is more widespread (Stapleton et al., 1996) and is predominant in white adipocytes (Daval et al., 2005). Interestingly, AMPK activation in white adipose tissue was seen in starved rats (Daval et al., 2005), as we observed here in hibernating ground squirrels. The precise role of the α1- and α2-AMPK subunits is unclear, although α2-AMPK has a greater AMP-dependence and localizes to the nucleus in certain cells (Salt et al., 1998). Also, the specificity of the α-subunit isoforms towards peptide substrates differs slightly (Woods et al., 1996).

In spite of the fact that AMPK activation was seen only in white adipose tissue, phosphorylation of its downstream targets was detected in other tissues, namely eEF2 (albeit indirect) in liver and brain, and ACC in brown adipose tissue, suggesting the operation of other mechanisms for increasing the phosphorylation of AMPK targets during hibernation. The lack of effect of hibernation on Thr56 eEF2 phosphorylation levels in brown adipose tissue might explain why protein synthesis rates in this tissue in vitro were unaffected in hibernators compared with euthermic controls (Hittel and Storey, 2002). A decrease in the activity of PP2A, a phosphatase that dephosphorylates eEF2 (Redpath and Proud, 1990), has been reported in livers and brains from hibernating ground squirrels (Chen et al., 2001). This was attributed to an increase in the level of inhibitor 2 of PP2A, but increases in expression of the p35 catalytic subunit of PP2A were also seen (Chen et al., 2001). We observed no decrease in expression levels of the p65 regulatory or p35 catalytic subunits in organs of hibernators to explain the observed increase in phosphorylation of AMPK targets (Fig. 5). However, we cannot exclude regulation of PP2A by a change in the level of one of the B subunits or by post-translational modifications. Unfortunately, we were unable to determine whether p70S6K activity was decreased in tissues during hibernation which could explain the increase in eEF2 phosphorylation. The reported increase in expression of eEF2K in liver and brain could also contribute towards an increase in eEF2 phosphorylation in these tissues during hibernation (Chen et al., 2001).

In white adipose tissue, where AMPK was activated during hibernation, surprisingly there was no increase in Ser79 ACC phosphorylation (Fig. 6). ACC is an extremely good substrate for AMPK and a small increase in AMPK activity via allosteric stimulation by AMP would lead to ACC phosphorylation. Such an increase in AMPK activity would not persist in a polyethyleneglycol fraction used for AMPK assay and thus would be missed. By contrast, in brown adipose tissue, ACC phosphorylation increased 5-fold in hibernators (Fig. 6). Although there was no increase in overall AMPK activity in brown adipose tissues of hibernators (Fig. 1), there was an increase in the amount of the α2-isoform (Fig. 3). The marked increase in ACC Ser79 phosphorylation seen in brown fat (Fig. 6) would stimulate fatty acid oxidation and inhibit lipogenesis. No increase in ACC phosphorylation was observed in skeletal muscle from hibernators to explain the expected increase in fatty acid oxidation (Fig. 6). In hearts from hibernating squirrels, ACC activity was reported to be decreased, due to a decrease in expression rather than a change in AMPK activity (Belke et al., 1998).

Plasma free fatty acid concentrations increase during hibernation (Wilson et al., 1992), which could be due to mobilization of triacylglycerols from adipose tissue, a decrease in esterification or a decrease in uptake into tissues. The total (microsomal plus mitochondrial) activity of *sn*-glycerol 3-phosphate *o*-acyltransferase, one of the control enzymes of

fatty acid esterification, was unchanged in liver, white and brown adipose tissue of hibernators compared with the euthermic controls (not shown). Therefore, we looked at the phosphorylation state of HSL in adipose tissues (Fig. 7). No increase in phosphorylation at the Ser563 PKA site in adipose tissues from hibernators was detected. Although AMPK was activated in white adipose tissue during hibernation, no change in phosphorylation of Ser565 of HSL was observed (Fig. 7). Phosphorylation at this site was shown to be increased in white adipocytes following AMPK activation and might inhibit lipolysis by preventing HSL translocation to the lipid droplet (Daval et al., 2005). Other PKA phosphorylation sites responsible for HSL activation (Ser659/Ser660 and not Ser563) have been reported (Anthonsen et al., 1998) and it remains to be seen whether phosphorylation at these new sites might explain the expected stimulation of fatty acid mobilization from adipose tissues during hibernation. Basal levels of HSL could be sufficient to supply the necessary levels of fatty acids during steady-state torpor and the major activation of HSL may occur only during arousal from torpor when noradrenaline-stimulated lipolysis provides fatty acids for thermogenesis in brown adipose tissue. Lastly, no change in expression levels of HSL was seen in white or brown adipose tissue from hibernators compared with euthermic controls (data not shown). This contrasts with the increase in HSL mRNA levels seen in white adipose tissue from hibernators (Wilson et al., 1992; Bauer et al., 2001).

In conclusion, AMPK activation in tissues of hibernators did not correlate well with phosphorylation of its downstream targets and AMPK does not seem to be involved in metabolic rate depression during hibernation. AMPK activation might occur on arousal which would be expected to stimulate fatty acid oxidation for thermogenesis. It is noteworthy in this respect, that AMPK is activated in white adipose tissue of transgenic mice overexpressing the mitochondrial uncoupling protein, UCP1 (Matejkova et al., 2004).

Acknowledgements

We thank Prof. L. Hue for his interest, Prof. Grahame Hardie for kindly donating antibodies and Dr. J. M. Hallenbeck for providing ground squirrel tissues. The work was supported by the Interuniversity Attraction Poles Program — Belgian Science Policy (P5/05), the Directorate General Higher Education and Scientific Research, French Community of Belgium, the Fund for Medical Scientific Research (Belgium), EU contract n°s QLG1-CT-2001-01488 (AMPDIAMET) and LSHM-CT-2004-005272 (EXGENESIS) and Natural Sciences and Engineering Research Council of Canada grant 6793.

References

Abraham, D., Podar, K., Pacher, M., Kubicek, M., Welzel, N., Hemmings, B.A., Dilworth, S.M., Mischak, H., Kolch, W., Baccarini, M., 2000. Raf-1-associated protein phosphatase 2A as a positive regulator of kinase activation. J. Biol. Chem. 275, 22300–22304.

Anthonsen, M.W., Ronnstraand, L., Wernstedt, C., Degerman, E., Holm, C., 1998. Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. J. Biol. Chem. 273, 215–221.

Bauer, V.W., Squire, T.L., Lowe, M.E., Andrews, M.T., 2001. Expression of a chimeric retroviral-lipase mRNA confers enhanced lipolysis in a hibernating mammal. Am. J. Physiol. 281, R1186–R1192.

Belke, D.D., Wang, L.C., Lopaschuk, G.D., 1998. Acetyl-CoA carboxylase control of fatty acid oxidation in hearts from hibernating Richardson's ground squirrels. Biochim. Biophys. Acta 1391, 25–36.

Bolster, D.R., Crozier, S.J., Kimball, S.R., Jefferson, L.S., 2002. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through downregulated mTOR signaling. J. Biol. Chem. 277, 23977–23980.

Boutilier, R.G., 2001. Mechanisms of cell survival in hypoxia and hypothermia. J. Exp. Biol. 204, 3171–3181.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Browne, G.J., Proud, C.G., 2002. Regulation of peptide-chain elongation in mammalian cells. Eur. J. Biochem. 269, 5360–5368.

Browne, G.J., Finn, S.G., Proud, C.G., 2004. Stimulation of the AMP-activated protein kinase leads to activation of eukaryotic elongation factor 2 kinase and to its phosphorylation at a novel site, serine 398. J. Biol. Chem. 279, 12220–12231.

Carling, D., 2004. The AMP-activated protein kinase cascade — a unifying system for energy control. Trends Biochem. Sci. 29, 18–24.

Carling, D., Hardie, D.G., 1989. The substrate and sequence specificity of the AMP-activated protein kinase. Phosphorylation of glycogen synthase and phosphorylase kinase. Biochim. Biophys. Acta 1012, 81–86.

Chen, Y., Matsushita, M., Nairn, A.C., Damuni, Z., Cai, D., Frerichs, K.U., Hallenbeck, J.M., 2001. Mechanisms for increased levels of phosphorylation of elongation factor-2 during hibernation in ground squirrels. Biochemistry 40, 11565–11570.

Cheng, S.W.Y., Fryer, L.G.D., Carling, D., Shepherd, P.R., 2004. T2446 is a novel mTOR phosphorylation site regulated by nutrient status. J. Biol. Chem. 279, 15719–15722.

Daval, M., Diot-Dupy, F., Bazin, R., Hainault, I., Viollet, B., Vaulont, S., Hajduch, E., Ferré, P., Foufelle, F., 2005. Anti-lipolytic action of AMP-activated protein kinase in rodent adipocytes. J. Biol. Chem. 280, 25250–25257.

Davies, S.P., Carling, D., Hardie, D.G., 1989. Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic-AMP-dependent protein kinase, studied a specific and sensitive peptide assay. Eur. J. Biochem. 186, 123–128.

Davies, S.P., Sim, A.T.R., Hardie, D.G., 1990. Location and function of three sites phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein kinase. Eur. J. Biochem. 187, 183–190.

Dennis, P.B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S.C., Thomas, G., 2001. Mammalian TOR: a homeostatic ATP sensor. Science 294, 1102–1105.

Dubbelhuis, P.F., Meijer, A.J., 2002. Hepatic amino acid-dependent signaling is under control of AMP-dependent protein kinase. FEBS Lett. 521, 39–42.

Dyck, J.R.B., Kudo, N., Davies, S.P., Hardie, D.G., Lopaschuk, G.D., 1999. Phosphorylation control of cardiac acetyl-CoA carboxylase by cAMP-dependent protein kinase and 5′-AMP activated protein kinase. Eur. J. Biochem. 262, 184–190.

el Hachimi, Z., Tijane, M., Boissonnet, G., Benjouad, A., Desmadril, M., Yon, J.M., 1990. Regulation of the skeletal muscle metabolism during hibernation of *Jaculus orientalis*. Comp. Biochem. Physiol. B 96, 457–459.

English, T.E., Storey, K.B., 2000. Enzymes of adenylate metabolism and their role in hibernation of the white-tailed prairie dog, *Cynomys leucurus*. Arch. Biochem. Biophys. 376, 91–100.

Frerichs, K.U., Smith, C.B., Brenner, M., DeGracia, D.J., Krause, G.S., Marrone, L., Dever, T.E., Hallenbeck, J.M., 1998. Suppression of protein synthesis in brain during hibernation involves inhibition of protein synthesis initiation and elongation. Proc. Natl. Acad. Sci. U. S. A. 95, 14511–14516.

Garton, A.J., Campbell, D.G., Cohen, P., Yeaman, S.J., 1988. Primary structure of the site on bovine hormone-sensitive lipase phosphorylated by cyclic AMP-dependent protein kinase. FEBS Lett. 229, 68–72.

Garton, A.J., Campbell, D.G., Carling, D., Hardie, D.G., Colbran, R.J., Yeaman, S.J., 1989. Phosphorylation of bovine hormone-sensitive lipase by the AMP-activated protein kinase. A possible antilipolytic mechanism. Eur. J. Biochem. 179, 249–254.

Hardie, D.G., Carling, D., Carlson, M., 1998. The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu. Rev. Biochem. 67, 821–855.

Hardie, D.G., Scott, J.W., Pan, D.A., Hudson, E.R., 2003. Management of cellular energy by the AMP-activated protein kinase system. FEBS Lett. 546, 113–120.

Hawley, S.A., Davison, M., Woods, A., Davies, S.P., Beri, R.K., Carling, D., Hardie, D.G., 1996. Characterization of the AMP-activated protein kinase from rat liver and identification of threonine 172 as the major site at which it phosphorylates AMP-activated protein kinase. J. Biol. Chem. 271, 27879–27887.

Hawley, S.A., Boudeau, J., Reid, J.L., Mustard, K.J., Udd, L., Makela, T.P., Alessi, D.R., Hardie, D.G., 2003. Complexes between the LKB1 tumor suppressor, STRAD α/β and MO25 α/β are upstream kinases in the AMP-activated protein kinase cascade. J. Biol. 2, 28.

Hittel, D., Storey, K.B., 2002. The translation state of differentially expressed mRNAs in the hibernating thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*). Arch. Biochem. Biophys. 401, 244–254.

Hong, S.-P., Leiper, F.C., Woods, A., Carling, D., Carlson, M., 2003. Activation of yeast Snf1 and mammalian AMP-activated protein kinase by upstream kinases. Proc. Natl. Acad. Sci. U. S. A. 100, 8839–8843.

Horman, S., Browne, G.J., Krause, U., Patel, J.V., Vertommen, D., Bertrand, L., Lavoine, A., Hue, L., Proud, C.G., Rider, M.H., 2002. Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis. Curr. Biol. 12, 1419–1423.

Horman, S., Beauloye, C., Vertommen, D., Vanoverschelde, J.-L., Hue, L., Rider, M.H., 2003. Myocardial ischemia and increased heart work modulate the phosphorylation state of eukaryotic elongation factor-2. J. Biol. Chem. 278, 41970–41976.

Inoki, K., Zhu, T., Guan, K.-L., 2003. TSC2 mediates cellular energy response to control cell growth and survival. Cell 115, 577–590.

Kelly, D.A., Storey, K.B., 1995. Glycolysis and energetics in organs of hibernating mice (*Zapus hudsonius*). Can. J. Zool. 73, 202–207.

Kemp, B.E., Mitchelhill, K.I., Stapleton, D., Michell, B.J., Chen, Z.P., Witters, L.A., 1999. Dealing with energy demand: the AMP-activated protein kinase. Trends Biochem. Sci. 24, 22–25.

Krause, U., Bertrand, L., Hue, L., 2002. Control of p70 ribosomal protein S6 kinase and acetyl-CoA carboxylase by AMP-activated protein kinase and protein phosphatases in isolated hepatocytes. Eur. J. Biochem. 269, 3751–3759.

MacDonald, J.A., Storey, K.B., 1999. Regulation of ground squirrel Na^+K^+-ATPase activity by reversible phosphorylation during hibernation. Biochem. Biophys. Res. Commun. 254, 424–429.

Marsin, A.S., Bertrand, L., Rider, M.H., Deprez, J., Beauloye, C., Vincent, M.F., Van den Berghe, G., Carling, D., Hue, L., 2000. Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia. Curr. Biol. 10, 1247–1255.

Matejkova, O., Mustard, K.J., Sponarova, J., Flachs, P., Rossmeisl, M., Miksik, I., Thomason-Hughes, M., Hardie, D.G., Kopecky, J., 2004. Possible involvement of AMP-activated protein kinase in obesity resistance induced by respiratory uncoupling in white fat. FEBS Lett. 569, 245–248.

McLeod, L.E., Proud, C.G., 2002. ATP depletion increases phosphorylation of elongation factor eEF2 in adult cardiomyocytes independently of inhibition of mTOR signalling. FEBS Lett. 531, 448–452.

Merrill, G.F., Kurth, E.J., Hardie, D.G., Winder, W.W., 1997. AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am. J. Physiol. 273, E1107–E1112.

Redpath, N.T., Proud, C.G., 1990. Activity of protein phosphatases against initiation factor-2 and elongation factor-2. Biochem. J. 272, 175–180.

Redpath, N.T., Price, N.T., Severinov, K.V., Proud, C.G., 1993. Regulation of elongation factor-2 by multisite phosphorylation. Eur. J. Biochem. 213, 689–699.

Rider, M.H., Saggerson, E.D., 1983. Regulation by noradrenaline of the mitochondrial and microsomal forms of glycerol phosphate acyltransferase. Biochem. J. 214, 235–246.

Russell, R.R., Bergeron, R., Shulman, G.I., Young, L.H., 1999. Translocation of myocardial GLUT-4 and increased glucose uptake through activation of AMPK by AICAR. Am. J. Physiol. 277, H643–H649.

Salt, I., Celler, J.W., Hawley, S.A., Prescott, A., Woods, A., Carling, D., Hardie, D.G., 1998. AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform. Biochem. J. 334, 177–187.

Stapleton, D., Mitchelhill, K.I., Gao, G., Widmer, J., Michell, B.J., The, T., House, C.M., Fernandez, C.S., Cox, T., Witters, L.A., Kemp, B.E., 1996. Mammalian AMP-activated protein kinase subfamily. J. Biol. Chem. 271, 611–614.

Storey, K.B., 2003. Mammalian hibernation: transcriptional and translational controls. Adv. Exp. Med. Biol. 543, 21–38.

Storey, K.B., Storey, J.M., 2004. Metabolic rate depression in animals: transcriptional and translational controls. Biol. Rev. 79, 207–233.

Stralfors, P., Bjorgell, P., Belfrage, P., 1984. Hormonal regulation of hormone-sensitive lipase in intact adipocytes: identification of phosphorylated sites and effects on the phosphorylation by lipolytic hormones and insulin. Proc. Natl. Acad. Sci. U. S. A. 81, 3317–3321.

Wang, X., Li, W., Williams, M., Terada, N., Alessi, D.R., Proud, C.G., 2001. Regulation of elongation factor 2 kinase by $p90^{RSK1}$ and p70S6 kinase. EMBO J. 20, 4370–4379.

Wilson, B.E., Deeb, S., Florant, G.L., 1992. Seasonal changes in hormone-sensitive and lipoprotein lipase mRNA concentrations in marmot white adipose tissue. Am. J. Physiol. 262, R177–R181.

Woods, A., Salt, I., Scott, J., Hardie, D.G., Carling, D., 1996. The alpha1 and alpha2 isoforms of the AMP-activated protein kinase have similar activities in rat liver but exhibit differences in substrate specificity in vitro. FEBS Lett. 397, 347–351.