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AMP-activated protein kinase and metabolic regulation in cold-hardy insects

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ABSTRACT

Winter survival for many insects depends on cold hardiness adaptations as well as entry into a hypometabolic diapause state that minimizes energy expenditure. We investigated whether AMP-activated protein kinase (AMPK) could be involved in this adaptation in larvae of two cold-hardy insects, *Eurosta solidaginis* that is freeze tolerant and *Epiblema scudderiana* that uses a freeze avoidance strategy. AMPK activity was almost 2-fold higher in winter larvae (February) compared with animals collected in September. Immunoblotting revealed that phosphorylation of AMPK in the activation loop and phosphorylation of acetyl-CoA carboxylase (ACC), a key target of AMPK, were higher in *Epiblema* during midwinter whereas no seasonal change was seen in *Eurosta*. Immunoblotting also revealed a significant increase in ribosomal protein S6 phosphorylation in overwintering *Epiblema* larvae, and in both *Eurosta* and *Epiblema*, phosphorylation of eukaryotic initiation factor 4E-binding protein-1 dramatically increased in the winter. Pyruvate dehydrogenase (PDH) E1α subunit site 1 phosphorylation was 2-fold higher in extracts of *Eurosta* larvae collected in February versus September while PDH activity decreased by about 50% in *Eurosta* and 80% in February *Eurosta* larvae compared with animals collected in September. Glycogen phosphorylase phosphorylation was 3-fold higher in *Epiblema* larvae collected in February compared with September and also in these animals, triglyceride lipase activity increased by 70% during winter. Overall, our study suggests a re-sculpting of metabolism during insect diapause, which shifted to a more catabolic poise in freeze-avoiding overwintering *Epiblema* larvae, possibly involving AMPK.

1. Introduction

Winter survival for many species of insects living in seasonally cold environments includes a radical remodeling of metabolism. This frequently includes the development of cold hardiness, entry into a hypometabolic state of diapause, and a switch from feeding to the catabolism of stored body fuel reserves to support energy needs (Lee and Denlinger, 2010). All of these are closely coordinated, particularly in univoltine species where a specific life stage overwinters and entry into that stage is often tied to the initiation of cold hardiness adaptations and an obligate diapause. Winter cold hardiness has been well-studied in the larvae of two species of gall-forming insects that live on goldenrod – the goldenrod gall fly, *Eurosta solidaginis* Fitch (Diptera, Tephritidae) that is freeze tolerant and the goldenrod gall moth, *Epiblema scudderiana* Clemens (Lepidoptera, Olethreutidae) that uses the freeze avoidance strategy of deep supercooling (reviewed by Storey, 1990; Storey and Storey, 2010). The last instar larvae of both species build up high glycogen reserves during early autumn feeding (September, October) and then convert these into massive pools of cryoprotectants as the autumn progresses, producing glycerol in *Epiblema* (concentrations >2 M) or glycerol plus sorbitol in *Eurosta* (Storey and Storey, 1986; Rickards et al., 1987). Synthesis is modulated by autumn environmental factors including plant senescence, photoperiod, and decreasing environmental temperatures. The larvae also enter a period of obligate diapause that lasts for 3–4 months until about February in the Ottawa area. Subsequently, the larvae pass into a period of quiescence where development can continue towards the pupal transition, with both the speed of development and the rate of degradation of the winter cryoprotectants increasing over time and with rising environmental temperatures.

Hence, distinct metabolic states can be defined at different seasons. In early autumn (mid-September) larvae are still feeding,

Abbreviations: 4E-BP1, eukaryotic initiation factor 4E-binding protein-1; ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; (adipose) (A)TGL, (adipose) triglyceride lipase; eEF2, eukaryotic elongation factor-2; GDH, glutamate dehydrogenase; GP, glycogen phosphorylase; LDH, lactate dehydrogenase; (m)TORC1, (mammalian) target of rapamycin complex 1; p70S6K, p70 ribosomal protein S6 kinase; PDH, pyruvate dehydrogenase; PP2A, protein phosphatase-2A; rpS6, 40S ribosomal protein S6.

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body mass is increasing with the deposition of both carbohydrate and lipid reserves (Storey and Storey, 1986; Rickards et al., 1987), and the metabolic machinery is in place and poised to support high flux unidirectional synthesis of cryoprotectants when triggered by environmental cues. For example, when larvae are transferred from moderate to cold temperatures at this time, a rapid glycerol synthesis occurs that is not reversed when animals are returned to the higher temperature (Storey and Storey, 1983; Churchill and Storey, 1989). By late winter (February), however, the metabolic poise of the system has changed – cryoprotectant levels begin to fall as environmental temperatures warm, only about half of glycerol carbon reappears as glycogen if larvae are warmed, and subsequent cold exposures produce a progressively muted response in glycerol synthesis (Churchill and Storey, 1989). The fate of glycerol carbon that is not returned to glycogen is not fully known but possibilities include glycerol use as a substrate for aerobic metabolism and/or for biosynthesis supporting the pupal transition, or conversion to another fuel reserve (lipids, amino acids) that can be used during non-feeding pupal or adult life (Storey and Storey, 1991). Note, however, that in *Eurosta* sorbitol interconversion with glycogen remains intact throughout as a reversible response to cooling/warming (Storey and Storey, 1986).

Various regulatory controls can be applied to alter the metabolic poise of a system. A key player in the control of cellular energy homeostasis in eukaryotes is the AMP-activated protein kinase (AMPK). AMPK is a heterotrimer consisting of a catalytic α subunit and two regulatory subunits, β and γ. AMPK can be activated by changes in the intracellular AMP:ATP ratio, due to a drop in energy charge as occurs, for example, under hypoxia/anoxia, or as a result of other metabolic stresses (Hardie et al., 1998). Once activated, AMPK decreases ATP-consumption and stimulates ATP-producing processes (Hardie et al., 1998; Hardie, 2007). For example, its best known substrate is acetyl-CoA carboxylase (ACC). AMPK-mediated phosphorylation inactivates ACC to inhibit lipogenesis and stimulate fatty acid oxidation under energy stress situations (i.e. increased AMP levels). In addition, AMPK activation stimulates glucose uptake in skeletal muscle and heart. AMPK activation also inhibits glycogen synthase in order to favor glycolysis (Hardie et al., 1998; Hardie, 2007), and decreases protein synthesis in liver (Horman et al., 2002) and heart (Horman et al., 2003), but not in skeletal muscle (Miranda et al., 2008), via phosphorylation-induced activation of the eukaryotic elongation factor-2 (eEF2) kinase, which in turn phosphorylates and inactivates eEF2.

It is now becoming apparent that AMPK plays a role in multiple forms of animal hypometabolism where animals strongly reduce metabolic rate to achieve long-term survival under conditions of severe environmental stress (Rider, 2008). AMPK has been implicated in metabolic rate depression during freezing (Rider et al., 2006) and anoxia (Bartrons et al., 2004; Rider et al., 2006) in frogs as well as during anoxia in carp (Stensløkken et al., 2008), goldfish (Jibb and Richards, 2008) and turtles (Rider et al., 2009), but probably not during mammalian hibernation (Horman et al., 2005). In diapausing *Caenorhabditis elegans* dauers, AMPK was shown to directly phosphorylate and inactivate adipose triglyceride lipase (ATGL), thereby limiting energy expenditure to ensure survival in the dormant stage (Narbonne and Roy, 2009). The present study was therefore undertaken to determine whether AMPK might also have a role to play in insect diapause and cold hardiness.

2. Materials and methods

2.1. Materials

Routine chemicals were from Sigma, Boehringer or from sources previously cited (Horman et al., 2005; Rider et al., 2006; Miranda et al., 2008; Rider et al., 2009). Anti-rat phospho Ser14 glycogen phosphorylase (GP) antibody, anti-human phospho Ser293 (site 1), anti-human phospho Ser300 (site 2), anti-total human pyruvate dehydrogenase (PDH) E1α subunit (Pilegaard et al., 2006), and anti-phospho ACC antibody against the AMPK site of the *Drosophila melanogaster* protein (all polyclonal anti-peptide antibodies raised in sheep) were kindly provided by Prof. Grahame Hardie (University of Dundee). Anti-rat phospho Ser79 ACC1 antibody (polyclonal anti-peptide antibody raised in rabbit) was from Upstate. Anti-human phospho Thr172 AMPK α-subunit (rabbit monoclonal anti-peptide antibody), anti-human phospho Ser235/236 rpS6 and anti-mouse phospho Thr37/46 (polyclonal anti-peptide antibodies raised in rabbit) were from Cell Signaling. Anti-human liver GP polyclonal antibody raised in rabbit against the immunogenic peptide VVAATLQ DIIRRFKASKFGSTRGAGTVFDAFPDQVAIQLNDTHPALAIPELMRIFVDIE KLPWSKAWELTQKTFAYTNHTVLPEALERWPVDLVEKLLPRHLEIIYEIN QKHLDRIVALFPKDVDRLRRMSLI was from Sigma. Most of the antibodies (except the *Drosophila* anti-phospho ACC antibody) are routinely used in the Rider laboratory to study signaling in rat hepatocytes, rat adipocytes and rat cardiomyocytes where relevant signals are obtained in protein immunoblotting experiments. Moreover, the antibodies have been used to study AMPK signaling in the frog, *Rana sylvatica* (Rider et al., 2006) and in the turtle, *Trachemys scripta elegans* (Rider et al., 2009) by Western blot.

Streptavidin-Agarose, pigeon liver acetone powder and glyceryl trioleate were from Sigma, 4-aminoazobenzene-4′-sulfonic acid was from Alfa Aesar, [9,10-^{3}H(N)] triolein was from PerkinElmer and the "BAY" isoxazolone hormone-sensitive lipase inhibitor (Lowe et al., 2004; Claus et al., 2005) was a kind gift from Stefan Hallén (AstraZeneca, Mölndal, Sweden).

2.2. Preparation of tissue extracts

Galls containing final instar larvae of *Eurosta* and *Epiblema* were collected from fields around Ottawa, Ontario, Canada in September or February. Galls were briefly held in the lab in an incubator set to the current outdoor temperature and were then quickly opened and the larvae (50–80 mg body mass) were flash frozen in liquid nitrogen. Samples were air-freighted to Belgium on dry ice and stored at −80 °C until use. September animals had not yet been exposed to cold temperatures in nature, had minimal levels of cryoprotectants and were not yet cold hardened. February midwinter animals were fully cold-hardy with maximal cryoprotectant levels having experienced subzero temperatures for many weeks.

Samples of larvae (n = 4 samples for each condition, each sample consisting of a pool of four individuals with total mass 200–300 mg) were taken from −80 °C storage but were not allowed to thaw. Samples were quickly homogenized (Ultra-Turrax) in 1.3 ml of ice cold extraction buffer containing 50 mM Hepes, pH 7.4, 250 mM sucrose, 20 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 5 μg/ml pepstatin, 1 mM benzamidine–HCl, and 1 mM phenylmethanesulphonyl fluoride as described previously (Rider et al., 2006). Extracts were centrifuged in an Eppendorf microfuge at full speed for 10 min at 4 °C and then supernatants were removed and stored at −80 °C prior to measurements of AMPK activity and immunoblotting. Protein concentrations in the extracts estimated with bovine serum albumin as a standard (see below) did not show significant seasonal differences (*Eurosta* 12.9 ± 0.3 mg/ml in September versus 13.4 ± 1.5 mg/ml in February; *Epiblema* 18.6 ± 1.9 mg/ml in September versus 17.6 ± 1.3 mg/ml in February; n = 4 for each measurement).

2.3. AMPK assay

Aliquots of supernatants (0.25 ml) were mixed with an equal volume of 20% (w/v) polyethylene glycol 8000 in extraction buffer.