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UCP4 expression changes in larval and pupal fat bodies of the beetle *Zophobas atratus* under adipokinetic hormone treatment

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ABSTRACT

We investigated the influence of adipokinetic hormone (AKH), an insect neurohormone, on uncoupling protein 4 (ZaUCP4) expression and activity in larval and pupal fat body mitochondria of the beetle *Zophobas atratus* in relation to intermediary metabolism. Homologous Tenmo-AKH was administered to the beetle larvae and pupae as either a single dose or as two doses of 20 pmol during a 24 h interval. In the larval and pupal fat bodies, downregulation of ZaUCP4 expression at the mRNA and protein levels was observed 24 h and 48 h after AKH treatment, respectively. In both developmental stages, ZaUCP4 activity was lowered in fat body mitochondria 48 h after AKH treatment. In the AKH-injected larvae, changes in ZaUCP4 expression were accompanied by the mobilization of carbohydrate reserves, no change in the concentration of total lipids and an increase in the free fatty acid level. In contrast, AKH had no effect on carbohydrate metabolism in the pupal fat body but induced lipid mobilization. It seems that AKH influences ZaUCP4 expression by triggering multiple events and that it has different physiological roles in controlling intermediary metabolism in the fat body of the beetle larvae and pupae.

1. Introduction

Uncoupling proteins (UCPs) are located in the inner mitochondrial membrane and uncouple the oxidation of fuels from ATP synthesis via the mitochondrial electron respiratory chain by mediating a proton leak into the mitochondrial matrix (Sluse et al., 2006). Five members of the UCP subfamily, UCP(1–5), have been identified and have distinct tissue distributions in mammals. UCP1 is present in brown adipose tissue and plays an important role in cold- and diet-induced thermogenesis (Nicholls and Locke, 1984). UCP2 (ubiquitously expressed in almost all mammalian tissues) and UCP3 (predominantly expressed in skeletal muscle) are implicated in physiological and pathological processes, such as body weight regulation, glucose and lipid metabolism, type 2 diabetes, inflammation, and heart failure (Echtay, 2007). The brain specific UCPs, UCP4 and UCP5, may play important roles in apoptosis, neuroprotection, neuronal differentiation and synaptic plasticity (Kim-Han and Dugan, 2005).

In addition to the brain (Mao et al., 1999), the UCP4 isoform was identified in the peripheral tissues of mammals (at low levels) (Lengacher et al., 2004, Smorodchenko et al., 2009), the frog *Xenopus laevis* (Keller et al., 2005), the nematode *Caenorhabditis elegans* (Iser et al., 2005), and the insects *Gromphadorhina coquereliana* and *Zophobas atratus* (Slocinska et al., 2011, 2012). The peripheral localization of UCP4 indicates its involvement in fat metabolism, oxidative stress or adipocyte insulin resistance (Gao et al., 2010; Ji et al., 2012). *C. elegans* UCP4 controls complex II-mediated oxidative phosphorylation through succinate transport (Pfeiffer et al., 2011). In the fat body and muscle mitochondria of the cockroach *G. coquereliana*, UCP4 activation results in a decrease in superoxide anion production, suggesting that protection against mitochondrial oxidative stress may be a physiological role of UCPs in insects (Slocinska et al., 2011). The different expression pattern and activity of the beetle *Z. atratus* UCP4 (ZaUCP4) during the larval–pupal transformation indicates an important role for UCP4 in fat body development and function during insect metamorphosis (Slocinska et al., 2012). Studies with UCP5 knockout flies indicated that *Drosophila melanogaster* UCP5 is important in maintaining metabolic homeostasis and that it may influence the hormonal control of metabolism and aging (Sanchez-Blanco et al., 2006). Flies without UCP5 had lower levels of body sugars, glycogen and triglycerides, indicating that UCP5 may affect the insulin-like peptide (ILP) or the adipokinetic hormone

Abbreviations: AKH, adipokinetic hormone; Tenmo-AKH, bioanalog of the AKH hormone family; UCP(1–5), uncoupling proteins, isoforms 1–5; ZaUCP4, uncoupling protein of *Zophobas atratus*.

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(AKH), the insect equivalent of vertebrate glucagon. AKH-producing cells express subunits forming a K_{ATP}-dependent channel homolog that causes these cells to be physiologically similar to mammalian islets cells ("fly pancreas") in the sensing and regulation of glucose homeostasis (Kim and Rulifson, 2004). Recent studies have shown that increased UCP5 activity in *D. melanogaster* insulin-producing neurons may attenuate insulin signaling and extend the lifespan (Fridell et al., 2009).

Hormones are important regulators of UCP expression. Several hormonal factors modulating UCP1, UCP2 and UCP3 expression are well described and include the thyroid hormone triiodothyronine (Lanni et al. 2003); pancreatic hormones, such as insulin (Chan et al., 1999; Zhang et al., 2001), the adipose-derived hormone leptin, and growth hormone (Ramsay and Rosebrough, 2005); and estrogens (Pedersen et al., 2001). Hormonal regulation of UCP4 and UCP5 is poorly understood.

Adipokinetic/hypertrehalosemic hormones (AKH/HrTH) are synthesized and stored by insects in the glandular lobes of the corpora cardiaca, a neuroendocrine gland that is physiologically equivalent to the pituitary gland of mammals (Gäde et al., 1997; Van der Horst et al., 2001). In adult insects, during energy demanding events such as flight, walking, egg production and molting, AKHs are released from the corpora cardiaca and induce the mobilization of energy stores in the fat body, leading to increased levels of diacylglycerol and trehalose in the insect hemolymph (Van der Horst et al., 2001; Lorenz and Gäde, 2009). In addition to the energy mobilizing function, AKHs inhibit anabolic pathways in the insect fat body, including the synthesis of lipids, proteins and glycogen (Gäde, 2004; Lorenz and Gäde, 2009). It was proposed that AKH plays a role in the regulation of insect development and reproduction by inhibiting the formation of energy stores and the synthesis of vitellogenins in the fat body. The majority of studies on energy homeostasis by AKH concerns adult insects. The possible role of AKH in larval and pupal stages has not been studied extensively. In insect larvae, AKH should be regarded as an important regulator of homeostasis leading to increased titers of sugar in the hemolymph (Ziegler et al., 1990; Woodring et al., 2002). Moreover, in the larval fat body of the cricket *Gryllus bimaculatus,* AKH effectively inhibits the synthesis of lipids and stimulates lipid mobilization by activating fat body triacylglycerol lipase (Anand and Lorenz, 2008). AKH fuels energy demanding processes such as the formation of the new cuticle and the emergence from the old one (Lorenz and Gäde, 2009). A possible engagement of AKH in diminishing oxidative stress in *Spodoptera littoralis* has recently been shown (Vecera et al., 2012).

The fat body is a major organ for nutrient storage and energy metabolism and undergoes developmental remodeling during insect metamorphosis, during which energy homeostasis must be tightly regulated (Arrese and Soulages, 2010). Because both AKHs, insect neurohormones, and UCPs, the main mitochondrial energy-dissipating proteins, significantly influence cellular metabolic and energy balance, we investigated the impact of AKH administration on ZaUCP4 expression and activity in the mitochondria of fat bodies of larvae and pupae, two metabolically different stages of the holometabolous beetle *Z. atratus*, with regard to lipid and carbohydrate metabolism. In our study, we used Tenmo-AKH, a bioanalog of the AKH/HrTH hormone family, which was also identified in the corpora cardiaca of *Z. atratus* adults (Gäde and Rosinski, 1990). We focused our interest on a long lasting effect of AKH, 24 h and 48 h after treatment, to observe possible correlations between the ZaUCP4 expression/activity and the larval and pupal intermediary metabolism.

2. Materials and methods

2.1. Experimental animals and injections

The larvae and pupae of the beetle *Z. atratus* were raised under laboratory conditions at a temperature of a 28 °C, in a 12/12 h light cycle, and with a relative humidity of 65–70%. Food, including lettuce, carrots, powdered milk, cooked chicken eggs, and water, was provided *ad libitum*. All experiments were performed using fat bodies or hemolymph from two developmental stages, larvae (between 8 and 10 instar) and pupae two days after molting. In adults, the vestigial fat body made mitochondria isolation and molecular studies impossible. Control insects received 4 μL of Ringer's solution (RS) (274 mM NaCl, 19 mM KCl, 9 mM $CaCl_2$, 5 mM glucose, and 5 mM HEPES, pH 7.0) per injection. Insects treated with hormone received 20 pmol of Tenmo-AKH (pELNFSPNWa) in 4 μl of RS per injection. This dose was previously determined as optimal to obtain a maximal hyperglycemic effect in vivo (Rosinski, 1995). Tenmo-AKH was synthesized by the classical solid-phase method according to the Fmoc-procedure (Fields and Noble, 1990).

Z. atratus larvae and pupae were divided into the following 3 groups: 1) a control group of insects injected with RS; 2) a 24hAKH group of insects injected with a single dose of AKH; fat body or hemolymph was collected for analysis 24 h after the injection in both groups; and 3) a 48hAKH group of insects injected twice with AKH at an interval of 24 h. The fat body or hemolymph was collected for analysis 24 h after the last injection. Although the response of insects to AKH treatment, which is reflected in carbohydrate and lipid mobilization, is very quick and usually takes only 1–2 h, a longer time is required to reveal changes in mRNA and protein expression. Moreover, multiple AKH-injections exert lasting effects on the general metabolism of the insect fat body or at least the enzymes involved in lipid synthesis (Lorenz, 2003). In the study, injections were performed through the intersegmental membrane between the second and the third abdominal segments in the direction of the head.

2.2. Glycogen concentration

Glycogen was extracted by the digestion of the control and AKH-injected larval and pupal fat bodies with 30% KOH using the Van Handel (1965) method. Glycogen was precipitated with absolute ethanol and centrifuged at 10,000 *g* for 5 min. The precipitate was dissolved in distilled water and was used for the quantitative determination of glycogen. The concentration of glycogen in the samples was estimated according to the sulfuric acid method (Dubois et al., 1956) using glycogen as the standard.

2.3. Hemolymph hyperglycemic response

The hyperglycemic activity of the Tenmo-AKH was evaluated in *Z. atratus* larvae and pupae according to a previously described method (Rosinski and Gäde, 1988). Hemolymph samples (2 μl) were collected 24 h after the last injection of hormone in the 24hAKH and 48hAKH groups of insects and after the last injection of RS in the control group. The samples were mixed with 500 μl of 70% ethanol and shaken for several minutes to obtain a complete extraction of the free sugar. The extracts were centrifuged at 10,000 *g* for 4 min and the supernatants were used for the free sugar concentration analysis using trehalose as a standard (Dubois et al., 1956).

2.4. Lipid composition

The lipids from the control and AKH-injected larval and pupal fat bodies were separated by methods described previously (Golebiowski et al., 2012). All lipids were separated using high performance liquid chromatography (HPLC) with a laser light scattering detector (LLSD) and were then analyzed by gas chromatography–mass spectrometry (GC-MS). The chemical compounds were identified based on their mass spectra.