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New metabolic activity of the nonsulfated sulfakinin Zopat-SK-1 in the insect fat body

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ABSTRACT

Insect sulfakinins are multifunctional neuropeptides homologous to vertebrate gastrin/cholecystokin (CCK) neuropeptide hormones. We investigated the action of the nonsulfated sulfakinin Zopat-SK-1 (pETSDDYGHLRFa) on the levels of chosen metabolites in the *Zophobas atratus* beetle fat body. Samples of fat body were collected 2 h and 24 h after hormone injection. The administration of 20 pmol of Zopat-SK-1 to feeding larvae significantly increased concentrations of lipids and proteins and decreased the content of glycogen in fat body tissue in the 24 h experimental group. In contrast, the only increase in total lipid concentration in prepupal fat bodies was observed 24 h after Zopat-SK-1 treatment. Simultaneously, changes in the quality and quantity of free sugars in the hemolymph were measured. In larval hemolymph, a marked increase in free sugar concentration and a decrease in glucose content were observed 24 h and 2 h after Zopat-SK-1 application, respectively. No changes in the prepupal stage were observed. For the first time we show potent metabolic activity of sulfakinin in the fat body tissue of an insect. Our findings imply a physiological function of the nonsulfated form of sulfakinin in energy storage and release processes in fat body tissue of larvae and prepupae was indicated. We suggest a role for sulfakinin signaling in the regulation of energy metabolism in insect tissues.

1. Introduction

Insect sulfakinins (SKs) are a family of neuropeptides homologous to the gastrin/cholecystokinin (CCK) peptide hormones in mammals. SKs were first found in the head extracts of the cockroach *Leucophera maderae* [25] and were later identified in many other insects, such as American cockroaches [35], locusts [38], flies [7,11], crickets [23] and beetles [21,37]. Members of the SK family typically contain the conserved *C*-terminal hexapeptide DYGHMFRamide and exhibit several biological activities. They increase the frequency and amplitude of gut contractions and heartbeat frequency in the cockroach. Similar to the mammalian homolog gastrin/CCK, sulfakinins reduce food consumption in locusts [38], cockroaches [20], crickets [23] and the red flour beetle *Tribolium castaneum* [41]. It has been observed that SKs stimulate release of the digestive enzyme alpha-amylase [12,24] and decrease carbohydrate ingestion [4].

SKs have been identified in several tissues, especially in the central and sympathetic nervous systems of various insect species [8,28], which indicates their role as neurotransmitters or neuromodulators [7,28]. In cockroaches and locusts, their expression near neurohemal sites suggest that they may be released into the hemolymph and act as hormones [7,8,29]. SKs were found to be expressed in all developmental stages, with high expression in the pupal stage [41]. The highest tissue expression of the SK gene was found in the insect head, from which SKs could be transported as neuropeptides to the other tissues. Intrestingly, different transcript levels of SKs were detected in the head, gut and other remaining tissues of the decapitated bodies of larvae and adult insects [41]. Recently Zels et al. reported a distribution profile of sulfakinin receptor transcripts in *T. castaneum* [43]. The authors showed that both receptors are primarily expressed in the central nervous system and optic lobes of the beetle but also in peripheral tissues (mainly fat body tissue). Limited expression was detected in testis and gut. This distribution pattern may indicate participation of SKs in the regulation of the brain-intestinal axis and the pleiotropic role of these peptides in signaling pathways.

Some time ago, it was suggested that sulfakinins require a sulfated tyrosine for activity [17]. However, further studies have

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shown that nonsulfated sulfakinin from *Drosophila melanogaster* (nsDSK) decreases the frequency of contractions of adult foregut, indicating a role for nsDSK in gut biology [26]. However, the nonsulfated form was approximately 3000-fold less potent than its sulfated form. This difference suggests the importance of the sulfate moiety for the binding of sulfakinins to their receptors [26]. It has been shown that nonsulfated and sulfated sulfakinins have different effects on odor preferences and insect locomotion [27]. Nonsulfated sulfakinin isolated from the brain of the *Zophobas atratus* beetle, Zopat-SK-1 (pETSDDYGHLRFa) [21], has been shown to stimulate contractions of the isolated hindgut of adult beetles but decrease contractions of the heart, ejaculatory duct and oviduct [22]. Sulfakinin activities and distributions suggest that sulfated and nonsulfated peptides play a diverse role in insect gastrointestinal tracts and neural systems [26]. Sulfakinin receptors (SKRs) are crucial components of the activities of SKs. In mammals, two CCK receptors, CCK1R, located in the gastrointestinal system and CCK2R, mainly located in the brain, have been determined to have different physiological functions [36]. In insects, SKRs were identified and characterized based on their sequence similarity to the CCK receptor in *D. melanogaster*. They have been named DSKR1 and DSKR2 [13,17]. Further studies have revealed the presence of genes for perisulfakinin receptor in *Periplaneta americana* [39] and of sulfakinin receptor 1 (SKR1) and sulfakinin receptor 2 (SKR2) genes in *T. castaneum* [14] and the pea aphid, *Acyrhosiphon pisum* [18]. The comparison of SK receptor sequences with homologous vertebrate cholecystokinin receptors revealed conserved regions for ligand binding and receptor activation [43]. SKR2 is considered an indispensable component in the regulation of food intake in *T. castaneum* [42].

The insect fat body, physiologically analogous to the mammalian liver and cytologically similar to adipose tissue, functions as a major organ for nutrient storage and energy metabolism. In the larval stage, the fat body fills almost the entire insect body. Taking into consideration the nature of the fat body and the role of sulfakinin in feeding regulation, it seems reasonable that this tissue might be the target of sulfakinin action or that it may at least play an intermediate role in sulfakinin transduction pathways. Thus, the aim of our studies was to elucidate the action of nonsulfated sulfakinin Zopat-SK-1 in carbohydrate, lipid and protein metabolism of the *Z. atratus* beetle fat body. We injected a synthetic analog of Zopat-SK-1 into intensively feeding larvae, which use nutrients from their diet as an energy source, and also into prepupae, which rely on the energy store built up during the feeding.

2. Materials and methods

2.1. Insects and injections

Larvae of the *Z. atratus* beetle were reared under laboratory conditions at a temperature of 28 °C and a relative humidity of 65–70%. Food, including lettuce, carrots, powdered milk and eggs, as well as water, were provided *ad libitum*. All experiments were performed on the fat bodies or hemolymph from two groups of insects: intensively feeding larvae and non-feeding-stage prepupae two days after molting. 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 the hormone received 20 pmol of Zopat-SK-1 in 4 μl RS per injection. The administered dose of 20 pmol of Zopat-SK-1 was determined experimentally based on a dose-response curve (not shown). The approximate blood volume of *Z. atratus* medium larva (weighed *ca.* 0.5 g) amounts to 50 μl. This means that the final concentration of Zopat-SK-1 in the haemolymph of the injected insect reached the value of around 0.4 pmol. The estimated dose evoked the maximal hyperglycemic effect in insect hemolymph *in vivo*, 2 h after only one hormone injection (not shown). The number of injections was evaluated with a series of preliminary experiments analyzing the efficacy of the peptide.

Samples were collected 2 h and 24 h after hormone injection. Zopat-SK-1 was produced by Fmoc solid-phase synthesis [9].

2.2. Determination of glycogen content in the fat body

Isolation of glycogen was carried out by hydrolysis of tissue in 30% KOH for 15 min at 90 °C according to the Van Handel procedure [34]. After tissue lysis, a saturated solution of Na_2SO_4 and 70% ethanol was added to precipitate the glycogen. Next, the samples were centrifuged at 10,000 × *g* for 5 min and washed twice with 70% ethanol. The pellet was dissolved in water and shaken for 10 min at 80 °C. This solution was used for the determination of glycogen content using Dubois' 5% phenol–sulfuric acid method [5]. Oyster glycogen was used as a standard.

2.3. Determination of total lipids in the fat body

The isolation of lipids from the fat body tissue was carried out according to Folch's method [10]. The tissue was homogenized in mixture of chloroform and methanol (2:1, v:v) and centrifuged at 10,000 × *g* for 10 min. The supernatant was washed three times with 0.29% NaCl, and the solvent was evaporated (at 30 °C). The pellet was dissolved again in the chloroform and methanol mixture. Aliquots of the mixture were taken, and lipid contents were measured gravimetrically.

2.4. Determination of soluble protein in the fat body

The fraction of soluble protein was isolated according to the method described by Lorenz [19]. The samples were homogenized in 75% methanol saturated with Na_2SO_4 and centrifuged at 10,000 × *g* for 10 min. Afterwards, the pellet was washed with Na_2SO_4-saturated 66% ethanol and again centrifuged (10,000 × *g*, 10 min). Finally, the pellet was hydrolyzed in 10% KOH for 15 min at 90 °C. The protein concentration in the pellet was determined by Bradford's procedure [2].

2.5. Determination of total free sugars of the hemolymph

The hemolymph (2 μl) was transferred into 500 μl of 70% ethanol and shaken for several minutes to obtain full extraction of free sugars. The samples were centrifuged at 10,000 × *g* for 4 min and the supernatant was used for determination of free sugar content by a colorimetric method using phenol–sulfuric acid [5]. As a standard, trehalose was used.

2.6. Qualitative and quantitative determination of free sugars in hemolymph

Hemolymph (5 μl) from larvae and prepupae was collected in 70% ethanol and kept at 4 °C. The samples were centrifuged (5 min, 10,000 × *g*), and the supernatants were used for analysis of sugars by reverse phase high performance liquid chromatography (RP-HPLC). Separations were performed using a Dionex Ultimate 2000 chromatographic system comprising a dual pump programmable solvent module and a Corona Charged Aerosol Detector (CAD). Supernatants were analyzed on APS-2 Hypersil 5 μl column (150 mm × 4.6 mm, Thermo Scientific). The samples were eluted with an isocratic gradient of 80% acetonitrile with a flow rate 1.5 ml/min for 15 min at 40 °C. Trehalose, glucose and glycerol (Merck, Germany) were used as standards.

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