Comparative Biochemistry and Physiology, Part C 155 (2012) 389–395

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Adipokinetic hormone-induced antioxidant response in *Spodoptera littoralis*

Josef Večeřa [a,b], Natraj Krishnan [c], Axel Mithöfer [d], Heiko Vogel [e], Dalibor Kodrík [a,b,*]

[a] Institute of Entomology, Biology Centre, Academy of Sciences, Branišovská 31, České Budějovice 370 05, Czech Republic
[b] Faculty of Sciences, University of South Bohemia, Branišovská 31, České Budějovice 370 05, Czech Republic
[c] Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, MS 39762, USA
[d] Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, 07745 Jena, Germany
[e] Department of Entomology, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, 07745 Jena, Germany

ARTICLE INFO

Article history:
Received 5 September 2011
Received in revised form 31 October 2011
Accepted 31 October 2011
Available online 7 November 2011

Keywords:
Adipokinetic hormone
Antioxidant response
Antioxidant enzymes
Glutathione
Oxidative stress
Protein carbonyls
Real-time PCR
Tannic acid

ABSTRACT

The antioxidative potential of the *Manduca sexta* adipokinetic hormone (Manse-AKH) in the last instar larvae of *Spodoptera littoralis* (Noctuidae, Lepidoptera) was demonstrated after exposure to oxidative stress (OS) elicited by feeding on artificial diet containing tannic acid (TA). Determination of protein carbonyls (PCs) and reduced glutathione (GSH) levels, monitoring of activity of antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione-S-transferases (GSTs), as well as measuring of the mRNA expression of CAT and SOD were used as markers of the OS. Injection of the Manse-AKH (5 pmol per individual) reversed the OS status by mitigation of PCs formation and by stimulation of glutathione-S-transferases (GSTs) activity. The CAT and SOD mRNA expression was significantly suppressed after the Manse-AKH injection while activity of these enzymes was not affected. These results indicate that diminishing of OS after the AKH injection might be a result of activation of specific enzymatic pathway possibly at the post-translational level rather than a direct effect on regulation of antioxidant marker genes at the transcriptional level.

1. Introduction

Herbivory has enabled insects to both detoxify and utilize various secondary metabolites in plants. Plant polyphenols and flavonols are examples of allelochemicals that can act either as prooxidants (Summers and Felton, 1994; Metadiewa et al., 1999) or as antioxidants (Hagerman et al., 1998), depending on chemical properties of the phenol (e.g. redox potential) and physico-chemical environment (e.g. pH and overall redox potential). The polyphenol tannin, ingested in plant or artificial diet, is well known for its capability to induce formation of reactive oxygen species (ROS) as well as formation of fatal lesions as a result of harmful oxidative stress (OS) conditions within insect gut (Barbehenn and Martin, 1994; Krishnan and Kodrík, 2006; Barbehenn and Constabel, 2011). Although tannins and other polyphenolic compounds are not able to penetrate membranes of gut epithelial cells, relatively small molecules as products of tannin hydrolysis or oxidation in insect gut lumen (such as quinones, hydrogen peroxides and organic peroxides) are more likely to evoke OS in midgut tissue (Canada et al., 1990). Defensive responses of insects to OS include activation of antioxidant enzymes as well as low-molecular-weight antioxidants such as thiols and ascorbate (Ahmad and Pardini, 1990; Timmerman et al., 1999; Barbehenn, 2003). The distribution of the ROS and the antioxidant enzymes that eliminate them are compartmentalized in the digestive tract of larvae of the Egyptian cotton leafworm *Spodoptera littoralis* after tannic acid (TA) feeding. This indicates that the generation of ROS in the foregut and the induction of antioxidant defense in the midgut are controlled processes and that their spatial separation is an important functional feature of the digestive tract (Krishnan and Sehnal, 2006).

In the last decade, a series of investigations confirmed the potential of several chemical and biological compounds to elicit OS conditions in insect models. The herbicidal diquaternary derivative of 4,4′ bipyridyl, paraquat, increased the level of protein carbonyls (PCs—products of oxidative damage to protein molecules) and decreased the level of reduced glutathione (GSH) after injection into the firebug *Pyrrhocoris apterus* (Večeřa et al., 2007) and into the Colorado potato beetle *Leptinotarsa decemlineata* (Kodrík et al., 2007). Increased PCs were also found in gut tissue of *L. decemlineata* fed on genetically modified

Abbreviations: AKHs, adipokinetic hormones; AsPx, ascorbate peroxidase; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; Cry 3Aa, *Bacillus thuringiensis* toxin; DNPH, 2,4 dinitrophenylhydrazine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GNA, *Galanthus nivalis* agglutinin; GR, glutathione reductase; GSH, reduced glutathione; GST, glutathione-S-transferase; Helze-HrTH, *Heliothis zea* hypertrehalosaemic hormone; Manse-AKH, *Manduca sexta* adipokinetic hormone; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; OS, oxidative stress; PCs, protein carbonyls; S8, supernatant after centrifugation at 8000*g*; SOD, superoxide dismutase; TA, tannic acid; WST-1, highly water-soluble tetrazolium salt; XO, xanthine oxidase.

* Corresponding author at: Institute of Entomology, Biology Centre, Academy of Sciences, Branišovská 31, České Budějovice 370 05, Czech Republic. Tel.: +420 387 775 271; fax: +420 385 310 354.
E-mail address: kodrik@entu.cas.cz (D. Kodrík).

1532-0456/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2011.10.009

potatoes containing either GNA (*Galanthus nivalis* agglutinin) lectin or Cry 3Aa (*Bacillus thuringiensis*) toxin (Kodrík et al., 2007). In *P. apterus*, insecticides endosulfan and malathion increased catalase activity and caused depletion of GSH in hemolymph (Velki et al., 2011).

It seems evident that in insects (and vertebrates as well) the endocrine system is involved in the activation of protective mechanisms against the OS (Krishnan and Kodrík, 2011). In this process a substantial role is played by adipokinetic hormones (AKHs). AKHs are small oligopeptides consisting of usually 8 to 10 amino acids. These neuropeptides behave as insect stress hormones by stimulating catabolic reactions (mobilize lipids, carbohydrates and/or certain amino acids), making energy more available, while inhibiting synthetic reactions (Kodrík and Goldsworthy, 1995; Vroenen et al., 1997). Despite this well-known function of AKHs in metabolic stress, there are many studies documenting their important role in processes that are not directly associated with mobilization and subsequent rapid consumption of energy (reviewed by Kodrík, 2008); this role includes e.g. stimulation of general locomotion (Socha et al., 1999), photophase interruption (Kodrík and Socha, 2005) and exposure to constant darkness (Kodrík et al., 2005). Further, the elevation of the AKH titer recorded in insects treated with insecticides (Candy, 2002; Kodrík and Socha, 2005) suggests involvement of this hormone also in "toxic" stress activities, although the mode of action is not known. AKHs obviously play a role also in enhancement of immune response (Goldsworthy et al., 2002, 2003), but the results suggest a complex interplay of factors and the underlying mechanisms are far from clear.

Application of the oxidative stressors (paraquat, GNA lectin, Cry 3Aa toxin, insecticides malathion and endosulfan) increased the level of AKHs in insect body, and additionally, exogenous AKHs reversed the OS status experimentally enhanced by application of the stressors (Kodrík et al., 2007; Večeřa et al., 2007; Velki et al., 2011). These results indicate that there is a feed-back regulation between oxidative stressors and AKH actions, and that AKHs are involved in the activation of antioxidant protective mechanisms in insects.

The aim of the present study was to demonstrate the antioxidative effects of the AKH neuropeptide during OS elicited by TA feeding in 6th larval instar of the cotton leafworm, *S. littoralis* using several OS-biomarker assays. This model insect seems to be highly convenient for such studies because two AKHs (Manse-AKH and Helze-HrTH) were identified in this species (Gäde et al., 2008), and both hormones are easily commercially available and suitable for physiological research. Moreover, the genomic database of the genus *Spodoptera* is continuously enriched and updated with new information which is useful for DNA-based methods including real-time PCR which has also been used in this work.

2. Material and methods

2.1. Insect and diet

The Egyptian cotton leafworm *S. littoralis* (Noctuidae, Lepidoptera, Insecta) larvae were reared on soybean flour-based Premix diet (Stonefly Industries, Inc., TX, USA) under the long day photoperiod (16 L:8 D) at 25 °C from eggs to the 6th larval instar. Diet was prepared either by mixing of the Premix diet with tap water (normal diet) or by adding of TA to the normal diet to reach 5% concentration (w/w) (TA-rich diet). One or two days old last (6th) instar larvae were used for the experiments. They were selected from the colony according to the size and feeding activity. Prior to each experiment, larvae were starved for 2 h (8:00 am–10:00 am) and then allocated to two groups, the controls were fed on the normal diet, and experimental insects fed on the TA-rich diet. Experimental feeding always started at 10:00 am and lasted 12 h until the larvae were dissected and the OS was evaluated using OS markers (see sections 2.3 to 2.6).

2.2. Hormone treatment, dissection and tissue homogenization

For the hormonal application, synthetic Manse-AKH (Bachem) was used. The hormone was dissolved in Ringer saline to obtain a dose of 5 pmol injected in 5 μL of the solvent; this dose was estimated to be optimal based on literature data (Ziegler, 1990; Kumari and Gokuldas, 2001) and on preliminary experiments where serially diluted doses 0.5–50 pmol were tested using selected OS biomarker assays (data not shown). The concentration of the hormone was verified by measurement of fluorescence ($\lambda_{Ex} = 280$ nm; $\lambda_{Em} = 348$ nm) of the hormonal solution due to the presence of tryptophan in the Manse-AKH molecule and serially diluted solutions of tryptophan as standard. Individual larvae were injected exactly in the middle of the feeding period (i.e. 6 h after the control or TA feeding). The Manse-AKH (or solvent only for the controls) was injected into the body by puncturing of the third abdominal segment with a steel syringe (Hamilton, 10 μL); after several muscle contractions, the syringe was carefully pulled out from the larva to avoid hemolymph bleeding. At the end of the feeding period (12 h), head and the last abdominal segment of the larva were cut off, the middle part of gut (midgut) was dissected and the content with a peritrophic membrane was separated from the midgut tissue devoid of Malpighian tubules. Midgut tissue was well-rinsed in the Ringer saline, blotted and homogenized in 60 mM potassium phosphate buffer (pH 7, 1:4 w:v) using a glass homogenizer. The crude homogenate was then transferred into a plastic tube and centrifuged at 8000*g* for 15 min at 4 °C. Supernatants (S8) were diluted as necessary prior to each experiment. Alternatively, the dissected and rinsed midgut tissue was frozen in liquid nitrogen and stored at −80 °C for processing later.

2.3. Reduced glutathione assay

GSH and its oxidized product (GSSG) were quantified according to Griffith (1980) with some modifications. The S8 sample homogenates (see above) were diluted 1:29 (for GSH) and 1:14 (for GSSG) in 60 mM K-PO_4 buffer (pH ~7). For GSSG assay, 2-vinylpyridine was added to derivatize GSH in sample, and the mixture was incubated 1 h at 30 °C. The samples were then vortexed, centrifuged and the supernatants used for the GSSG determination. The samples of both series (GSH and GSSG) were mixed in microtiter wells with 0.2 mM NADPH and glutathione reductase (0.5 U) to convert all GSSG in the samples into GSH. The mixture was incubated at 37 °C for 5 min with gentle shaking, and after that 6 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was added to react with GSH in sample. The change of absorbance was recorded for 5 min at 30-s intervals at 412 nm in a Spectramax microplate reader. Total glutathione (GSH + GSSG) content was quantified against a calibration curve of GSH standard (0 to 25 μM) using the kinetic method (slope) and expressed per mg of protein. The amount of GSSG was estimated from the calibration curve of the GSSG standard (0 to 25 μM) in the same way as mentioned for the total glutathione. The amounts of GSH were calculated by subtracting the GSSG content from that of the total glutathione and the results were used to demonstrate this OS marker (see below Fig. 2): it seems evident that expressing of results as GSH or as GSH/GSSG ratio serves as a better OS marker than the total glutathione pool since it differs between reduced and oxidized form and thus is more informative about changes in redox state of the cell/tissue under OS.

2.4. Antioxidant enzymes activities

Catalase (EC 1.11.1.6) (CAT) activity was measured according to Beers and Sizer (1952) with a few modifications. Ten microliters of S8 sample homogenate (see above) was mixed with 390 μL of 60 mM K-PO_4 buffer (pH ~7.0) and poured into the glass cuvette, then 400 μL of 21 mM H_2O_2 solution was added and mixed, and