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Mode of action of adipokinetic hormone at the sub-cellular level in potentiating anti-oxidative responses in insects

Ph.D. Thesis

RNDr. BEDNÁŘOVÁ ANDREA

Supervisor: Prof. RNDr. Kodrík Dalibor CSc.,
Institute of Entomology, Biology Centre, AV CR

Co-supervisor: Dr. Natraj Krishnan
Department of Biochemistry, Molecular Biology, Entomology & Plant Pathology, Mississippi State University, USA

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Annotation

Adipokinetic hormones (AKHs) are neuropeptides from the arthropod AKH/RPCH (adipokinetic hormone/ red pigment concentrating hormone) family. The typical AKH is an octa-, nona- or decamer that is synthesized, stored and released by the neurosecretory cells of the *corpora cardiaca* (CC) connected to the brain and primarily involved in the mobilization of energy reserves from the fat body in insects. In addition to its well established role in energy metabolism, AKH has also been implicated to be involved in stress responses – specifically to oxidative stress. Oxidative stress induced elevation of AKH levels as well as a modulation of biomarkers of oxidative damage following exogenous application of AKH have been demonstrated. However, the discrete steps involved in the mode of action of AKH in triggering an anti-oxidative response is far from clear. Given the role of AKH as a neuroendocrine factor that mediates a response to oxidative stress, the mode of action of AKH at the sub-cellular level was investigated. Using isolated central nervous system (brain) as an *in vitro* model, we establish that AKH can potentiate an anti-oxidative response to oxidative stress. Further, we also demonstrate that AKH uses a conserved signal transduction mechanism involving both protein kinase C (PKC) and cyclic adenosine monophosphate (cAMP) and by mobilizing both intra as well as extra-cellular Ca^{2+} stores to elaborate its anti-oxidative response. Finally, using the genetically tractable fruit fly *Drosophila melanogaster*, we demonstrate through RNAi mediated knockdown of AKH synthesis as well as overexpression of AKH using the GAL4/UAS system, that the fork-head box transcription factor (*dFoXO*) might function downstream of AKH signaling in its stress responsive role. These results implicate AKH as a stress hormone while offering possibilities to further identify specific regulatory mechanisms and downstream effector molecules. Since stress signaling pathways are conserved, insights obtained from such studies on insects will offer some unique avenues for understanding stress responses and related pathologies in vertebrates including humans.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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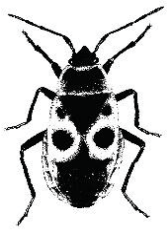

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. **Bednářová, A.**, Kodrík, D., Krishnan, N., 2013a. Unique roles of glucagon and glucagon-like peptides: Parallels in understanding the functions of adipokinetic hormones in stress responses in insects. *Comp. Biochem. Physiol. A.* **164**: 91-100. (IF = 2.371)

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II. **Bednářová, A.**, Krishnan, N., Cheng, I.C., Večeřa, J., Lee, H.J., Kodrík, D., 2013b. Adipokinetic hormone counteracts oxidative stress elicited in insects by hydrogen peroxide: *in vivo* and *in vitro* study. *Physiol. Entomol.* **38**: 54-62. (IF = 1.434)

CONTRIBUTION: Andrea Bednářová was instrumental in designing and conducting the work related to *in vitro* studies as well as wrote the first draft of the manuscript.

III. **Bednářová, A.**, Kodrík, D., Krishnan, N., 2013c. Adipokinetic hormone exerts its anti-oxidative effects using a conserved signal- transduction mechanism involving both PKC and cAMP by mobilizing extra- and intracellular Ca^{2+} stores. *Comp. Biochem. Physiol. C* **158**: 142-149. (IF = 2.829)

CONTRIBUTION: Andrea Bednářová designed and conducted all the work presented in this manuscript and was also responsible for writing the first draft of this manuscript.

IV. **Bednářová, A.**, Kodrík, D., Krishnan, N., 2015. Knockdown of adipokinetic hormone synthesis increases susceptibility to oxidative stress in *Drosophila* – a role for dFoxO? Communicated to: *Comp. Biochem. Physiol. C* (IF = 2.829).

CONTRIBUTION: Andrea Bednářová designed and conducted all the physiological and molecular work presented in this manuscript as well as was substantially involved in writing the manuscript.

STATEMENT ON CONTRIBUTION OF AUTHORS

The senior and corresponding authors of the main manuscripts included in this thesis, hereby confirm that Andrea Bednářová contributed significantly to these publications, according to the statements given in the previous page.

Prof. RNDr. Kodrík Dalibor CSc.

Dr. Natraj Krishnan

These papers are part of the thesis as supplemental publications and other scientific contributions:

V. **Bednářová, A.,** Kodrík, D., Krishnan, N., 2013d. Nature's timepiece – molecular coordination of metabolism and its impact on aging. *Int. J. Mol. Sci.* 14: 3026-3049. (IF = 2.339)

VI. Ivanchenko, M.G., den Os, D., Monhausen, G.,B., Dubrovsky, J.,G., **Bednářová, A.**, Krishnan, N., 2013. Auxin increases the hydrogen peroxide (H_2O_2) concentration in tomato (*Solanum lycopersicum*) root tips while inhibiting root growth. *Ann. Bot.* 112: 1107-1116. (IF = 3.295)

VII. Vinokurov, K., **Bednářová, A.,** Tomčala, A., Stašková, T., Krishnan, N., Kodrík, D., 2014. Role of adipokinetic hormone in stimulation of salivary gland activities: the fire bug *Pyrrhocoris apterus L*. (Heteroptera) as a model species. *J. Insect Physiol.* 60**:** 58-67. (IF = 2.5)

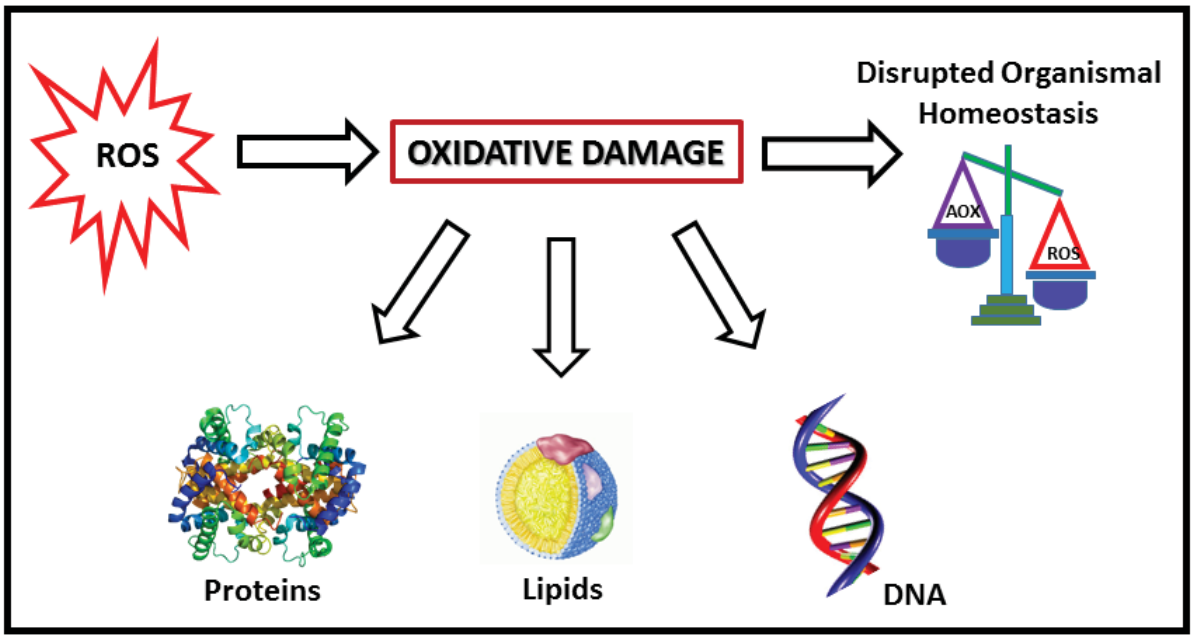
VIII. Wasielewski, O., Szczepankiewicz, D., Giejdasz, K., Wojciechowicz, T., **Bednářová, A.**, Krishnan, N., 2014. The potential role of adiponectin- and resistin-like peptides in the regulation of lipid levels in the hemolymph of over-wintering adult females of *Osmia bicornis*. *Apidologie* 45: 491-503. (IF = 2.16)

IX. Hanna, M., **Bednářová, A.**, Rakshit, K., Chaudhuri, A., O'Donnell, J., M., Krishnan, N., 2015. Perturbations in dopamine synthesis lead to discrete physiological effects and impact oxidative stress response in *Drosophila*. *J. Insect Physiol.*73: 11-19. (IF = 2.5)

CONTENTS

CHAPTER I

General Introduction



Schematic of reactive oxygen species induced oxidative damage.

1. Introduction

1.1. Overview of thesis

Insects along with other arthropods make up approximately 90% of all plant and animal species thus conferring upon them the status of the most dominant group of species in nature (Scheffers et al. 2012). Insects by themselves dominate the terrestrial ecosystem in terms of biodiversity and abundance (Scheffers et al. 2012). Their ubiquitous distribution in almost all ecological niches makes them subject to various environmental challenges which translates as a "stress factor" despite their unique adaptations. Climate change as well as various other environmental factors and variables are a source of stress to insects when the limits of their tolerance are exceeded. Like other living organisms, insects too respond to stress by elaborating specific physiological compensatory mechanisms that enable them to survive the stress situations. These compensatory responses are amply demonstrated on the life-history traits of insects, for example, in development, growth, aging, longevity, survival and reproduction. In biology, stress has become synonymous with the internal consequences of an external factor such that an anti-stress response is a cascade of internal changes triggered by stress. The stress faced by insects usually have the following basic properties: (a) it is usually transient (b) it involves a series of specific physiological responses and (c) it is accompanied by the induction of mechanisms that counteract its consequences. At the gross level, insects exhibit changes when specific traits are affected. At the cellular level however, certain specific stress responsive pathways are activated which combat the disturbance in cellular homeostasis brought about by stress. Thus, the study of these stress responses may ultimately reveal some very important information of how cellular processes are regulated. In this thesis, I examine the pathways at the sub-cellular level, by which a specific stress responsive hormone found in insects (the adipokinetic hormone) potentiates responses to counteract the effects of oxidative stress (OS) in the insect body.

In this introduction, I provide a general overview of reactive oxygen species (ROS) and free radicals and their involvement in the stress phenomenon. I also describe the endogenous ROS generation sites as well as the antioxidant systems which combat free

radical species. I provide a description of the phenomenon of OS, damage caused and the biomarkers of such damage and how OS could be experimentally induced. I then provide a brief description of the antioxidant response as well as other cellular defense mechanisms including the biochemical pathways triggered along with transcriptional and translational responses. I then describe the role of neurohormones as master regulators of stress responses and specifically the role of adipokinetic hormone as a stress responsive hormone with specific reference to OS.

1.2 Reactive oxygen species and free radicals – a general overview

Reactive oxygen species (ROS) and other radicals are generated in every organism living in aerobic environment; under physiological conditions the ROS are maintained at specific steady state levels. ROS are well recognized for playing a dual role as a beneficial as well as deleterious species. In the aerobic organism, ROS are involved in a variety of biological processes including aging and development, however, resulting in stress when produced in excess. Thus, the objective of this particular section of the Introduction is to describe the ROS species, and the origin and nature of free radicals, and to characterize their contribution to OS in insect body. I also would like to provide some basic information about the defense mechanisms of insects against these ROS, and describe some of the methodologies used to study OS in insects. The related terms - OS, oxidative damage, free radicals, and antioxidant - have become an integral part of the scientific vocabulary, and represent central themes of my thesis too.

Due to rising oxygen abundance in the atmosphere and favorable thermodynamic properties, oxygen was selected during the course of evolution as the terminal electron acceptor for the reduction of carbon-based fuels to generate adenosine 5'-triphosphate (ATP) by oxidative phosphorylation (Berner et al. 2007; Ma 2010). Oxygen is the primary oxidant in metabolic reactions designed to obtain energy from the oxidation of a variety of organic molecules. In other words, aerobic organisms use molecular oxygen to utilize chemical energy in the form of ATP that transforms "structure to function" in the cells. Moreover, oxygen is a critical component of O_2-dependent biosynthesis of many molecules

which are essential for many cellular structures and processes (Goldfine 1965). Although, aerobic respiration and O_2-dependent biosynthesis have significant advantages for life, at the same time the use of oxygen in aerobes comes with a price to pay. Thus, there are certain issues that cells encounter by using oxygen. For example OS itself results from the metabolic reactions that use oxygen. The generation of free radicals and ROS, both as by-products of oxygen metabolism or by specialized enzymes, potentially damages cellular components and may have a negative impact for the whole organism. The free radical is an oxygen containing molecule that has one or more unpaired electrons, making it highly reactive with other molecules. Oxygen by-products are relatively unreactive but some of these can undergo metabolism within the biological system to give rise to these highly reactive oxidants. Not all ROS are harmful to the cells and thus to the whole organism in general. Some of them are useful in killing invading pathogens or microbes (Vatansever et al. 2013). However, free radicals can chemically interact with cell components such as DNA, protein or lipid and steal their electrons in order to become stabilized. This, in turn, destabilizes the cell component molecules which then seek and steal an electron from another molecule, therefore triggering a large chain of free radical reactions (Dröge 2002).

Initially, free radicals were used to describe intermediate compounds in organic and inorganic chemistry, and several chemical definitions for them were suggested. Gersham & Gilbert (1954) postulated that free radicals are important players in biological environments and that they are responsible for deleterious processes in the cells. Two years after that, Harman (1956) proposed the “free radical theory” of aging, a fundamental life process, speculating that endogenous oxygen radicals are generated in the cells and result in a pattern of cumulative damage leading to aging. Following these postulations, lots of researchers got inspired and contributed significantly to our knowledge-base of the ROS and its involvement in biological reactions, cellular responses and clinical outcomes.

As was mentioned above, oxidation is a process in which a loss of electron occurs and chemically every compound, including oxygen that can accept electrons is an oxidant or oxidizing agent (Prior & Cao 1999). An oxidation process is always accompanied by a reduction processes in which there is usually a loss of oxygen, while in an oxidation process there is a gain of oxygen (Prior & Cao 1999; Hrbac & Kohen 2000; Schafer & Buettner

2001). Such reactions, called redox reactions, are the basis for numerous biochemical pathways and cellular chemistry, biosynthesis, and regulation (Shapiro 1972).

Nevertheless, first it has to be mentioned that in addition to recognized deleterious effects of ROS, cumulative evidence reveal that ROS have useful purposes in the body too (Thannickal 2009; Ma 2010). ROS can be generated in an endogenous process or is induced by exogenous agents. ROS generation within certain boundaries is essential for maintaining homeostasis. Moreover, ROS also play an important role as specific signaling molecules by themselves as well as regulatory mediators in signaling processes (Dröge 2002). Some of the pathways transmit the effect of ROS on cellular functions, such as regulating the proliferative response at low levels of oxidative stress, whereas other pathways represent the cellular strategies for detoxification of ROS and thus are essential for survival of living organisms exposed to high level of ROS (Finkel 1998; Nemoto et al. 2000; Nishikawa et al. 2000: Ma 2008; Ma 2010). Regardless of the origin of ROS, increased ROS production has two consequences: damage to cellular components, and activation of specific signal transduction pathways, both of which significantly impact on physiological and biochemical processes in the body. Mechanistically, many of these effects involve activation of specific transcription factors to control the transcription of a range of target genes. These genes encode specific proteins/enzymes to mediate biological responses to OS. In recent years, significant advances have been made in understanding the interaction between OS and the transcriptional machinery, in particular, the molecular mechanism of such interaction and its implications for stress responses. This topic has been discussed in detail in the section: 1.3.1: Important pathways and transcription factors involved in response to OS.

1.2.1 ROS generation sites and representatives of ROS

ROS can be generated in multiple compartments and by multiple processes in cells. In fact, most, if not all, enzymes that are capable of metabolizing oxygen are also capable of generating ROS either intentionally or accidentally (Ma 2010). Therefore mitochondria are the main location where the ROS are produced, because these organelles consume about 90% of the organism's oxygen to generate ATP by oxidative phosphorylation. However simultaneously 1-2% of the oxygen molecules consumed are converted to superoxide anions in mitochondria (Boveris & Chance 1973). So the majority of intracellular ROS is actually present in mitochondria and thus the mitochondrial DNA is very probably more susceptible to oxidative damage than the nuclear DNA. Oxidative phosphorylation in mitochondria uses controlled oxidation of NADH or $FADH_2$ to generate a potential energy for protons across the mitochondrial inner membrane. This potential energy is then used to phosphorylate ADP by the F1-F0 ATPase. Along the respiratory chain, electrons derived from NADH or FAD_2 can directly react with oxygen and generate free radicals. Production of superoxide radicals in mitochondria occurs primarily in complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochorme c reductase), with the latter being the major site of ROS production under normal metabolic conditions (Tunners 1997; Kohen & Nyska 2002; Ma 2010).

Among ROS generated, superoxide anion and hydroxyl radical eagerly interact with proteins, lipids and nucleic acids and as a consequence irreversibly destroy or at least alter the function of target molecules. ROS consist of a variety of oxygen-derived small molecules with diverse structures, including oxygen radicals such as above mentioned superoxide anion, hydroxyl radical, or certain non-radicals that are either oxidizing agents, or easily converted into the radicals, such as hypochlorous acid, ozone, singlet oxygen and hydrogen peroxide. More information on various free radicals is provided in **Table 1**.

Table 1: An overview of the various reactive oxygen species (ROS) and reactive nitrogen species (RNS) encountered in aerobic organisms. As depicted in the table, ROS and RNS can be either radicals or non-radicals and they are equally reactive and capable of causing significant damage to macromolecules.

Oxygen species	Description
•O^{2-} superoxide anion	One-electron reduction state of O^2, formed in many autoxidation reactions and by the electron transport chain. Undergoes dismutation to form H_2O_2 spontaneously or by enzymatic catalysis and is a precursor for metal-catalyzed •OH formation.
H_2O_2 hydrogen peroxide	Non-radical molecule- two-electron reduction state, formed by dismutation of $•O^{2-}$ or by direct reduction of O_2. Lipid soluble and thus able to diffuse across membranes.
•OH hydroxyl radical	Formed by Fenton reaction and decomposition of peroxynitrite. A principal actor in the toxicity of partially reduced oxygen species, very reactive with all kinds of biological macromolecules, producing products that cannot be regenerated by cell metabolism.
ROOH organic hydroperoxide	Formed by radical reactions with cellular components such as lipids and nucleobases.
RO • alkoxyl radical ROO • peroxyl radical	Oxygen centered organic radicals. Occurring during the oxidation of lipids or other organic molecules. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.
HOCl hypochlorous acid	Formed from H_2O_2 by myeloperoxidase. Lipid soluble and highly reactive. Will readily oxidize protein constituents, including thiol groups, amino groups and methionine.
ONOO- peroxynitrite	A strong oxidant that attacks protein cysteines and methionines. Formed in a rapid reaction between $•O^{2-}$ and NO•.
Nitrogen species	**Description**
NO• Nitric oxide	Abundant reactive radical that acts as an important oxidative biological signal in a large variety of diverse physiological processes. Produced by the cells of the immune system during the oxidative burst triggered during inflammatory processes.

Figure 1: A schematic representation of the balance between reactive oxygen species (ROS) and antioxidants (AOX). Any disequilibrium which results from excess of ROS or depleted AOX can result in oxidative stress (OS).

1.2.2 Oxidative stress (OS)

An organism must confront and control the presence of both free radicals and antioxidants continuously. The balance between these is tightly regulated and extremely important for maintaining vital cellular and bio-chemical functions (Shapiro 1972; Sies 1985; Hrbac & Kohen 2000; Jones et al. 2000; Schafer & Buettner 2001). This balance often referred to as the redox potential is specific for each organelle and biological site, and any interference of the balance in any direction might be deleterious for the cell and the whole organism in general. Changing the balance towards an increase in the pro-oxidant over the capacity of the antioxidant is defined as OS (Figure 1) and might lead to oxidative damage with irreversible impact for the whole organism.

OS results from the metabolic reactions that use oxygen, but can be intensified by environmental factors such as pesticides, chemicals, food sources, UV light, irradiation etc. The primary site of OS is in the mitochondria, during respiration by electron transport chain. Living organisms respond to exogenous and endogenous stress situations in various ways. Despite the diversity of responses to stress at both cellular and organismal level, the major task is a concerted attempt to negate the effects of the stress factors. Thus, it is necessary to establish cellular as well as organismal level homeostasis, thereby increasing the organism's resistance to stress.

One can even afford to describe OS as a double-edged sword: within the physiological range it is necessary for proliferative stimulation and perhaps the removal of aged cellular components, whereas extensive OS damages the structure and function of tissues via multiple mechanisms that include damaging mitochondrial respiration, increased ROS production, lipid peroxidation and depletion of antioxidants (He et al. 2007, 2008; Kasprzak 2002; Valko et al. 2005; Ma 2010). It is important to note that many oxidatively damaged macromolecules also act as regulatory molecules in cell-signaling pathways. For example, several lipid peroxidation products have been implicated in the activation of stress-response signal transduction pathways (Uchida et al. 1999; Leonarduzzi et al. 2000; Janssen et al. 2005). The continuous efflux of ROS from endogenous and exogenous sources results in continuous and accumulative oxidative damage to cellular components (Comporti 1989) and alters many cellular functions (Gracy

et al. 1999). Compared to its little known beneficial effects, much more is known about the deleterious consequences of OS. Among the biological targets most vulnerable to oxidative damage are proteinaceous enzymes (Halliwell & Gutteridge 1999; Levine & Stadtman 2001), lipidic membranes (Davis 1987; Halliwell & Gutteridge 1999) and DNA (Beckmen & Ames 1997; Halliwell & Gutteridge 1999). This is discussed in greater detail in the ensuing section.

1.2.3 Specific targets of oxidative damage

At high concentrations, ROS can be important mediators of damage to cell structures, nucleic acids and proteins (Valko et al. 2005). Typical DNA damages under OS are represented by altering purine and pyrimidine bases, by the chain breaks and/or by base modifications (Halliwell & Gutteridge, 1999). An important metabolic effect of DNA damage is also rapid induction of polyadenosine diphosphate ribose synthesis (ADP-ribosylation) in nuclei, resulting in extensive depletion of cellular nicotinamide adenine dilucleotide (reduced) (NADH) pools.

Proteins have many reactive sites that can be modified or damaged during OS. Modification of proteins then leads to the formation of carbonyl derivatives by direct oxidation of certain amino acid side chains and oxidation-induced peptide cleavage (Stadtman 1992, 2004). Mechanisms involved in the oxidation of proteins by ROS were elucidated in studies in which amino acids, simple peptides and proteins were exposed to ionizing radiations under conditions where hydroxyl radicals are formed. The side chains of all amino acid residues of proteins are susceptible to oxidation by the action of ROS/RNS (Stadtman 2004). Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups (-SH) and low molecular weight thiols, in particular, glutathione (GSH). The concentration of carbonyl groups, generated by many different mechanisms is a good measure of ROS-mediated protein oxidation.

It is known that generation of ROS also results in attack on cellular components involving polyunsaturated acid residues of phospholipids, which are extremely sensitive to oxidation (Siems et al. 1995). The modification of lipids by ROS and other radicals results

in lipid peroxidation. Lipid peroxidation of polyunsaturated lipids is a facile process. Peroxidation of cell membrane lipid seriously impairs membrane function. Once formed, peroxyl radicals ($ROO^{\bullet}$) can be rearranged via a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of peroxidation process being malondialdehyde (MDA) (Marnett 1999). The major aldehyde product of lipid peroxidation other than MDA is 4-hydroxy-2-nonenal (4-HNE). Both MDA and 4-HNE have been shown to be mutagenic and are major toxic products of lipid peroxidation.

1.2.4 Biomarkers of oxidative damage

Many approaches allow determination of the participation of ROS in biochemical events. The literature is replete with description of different methodologies for these purposes (Cao & Prior 1998; Llesuy et al. 2001). There is only one technique for direct detection of radicals - it is electron spin resonance which allows the detection of relatively stable radicals (Rice-Evans et al. 1991). Another technique is the spin trapping method in which a highly reactive radical, such as OH-, reacts with a trap molecule to produce a stable radical product that can be evaluated (Mason 1996). Other trapping procedures allow the radicals to react with a detector molecule to yield a stable product that can then be evaluated using a variety of techniques, such as hydroxylation of salicylic acid (Halliwel & Kaur 1997), the deoxyribose assay (Biaglow et al. 1997), and the cytochrome c reduction assay for detection of superoxide radicals by colored end-product compounds (Amano & Noda 1995).

It is important to distinguish between the measurement of particular ROS themselves and the assessment of damage that these ROS cause. Techniques for quantification of oxidative damage markers are often called fingerprinting methods by which specific end products resulting from the interaction with the ROS with biological macromolecules, such as DNA, protein, or lipids are measured. The appearance of these end products serves as a proof of the prior existence of ROS that left their footprints in the cell.

For work related to this dissertation I have been using techniques for detecting damage caused by OS in lipids and proteins. Damage to lipids deleteriously alters and modifies cellular membranes and therefore cellular function. Lipid peroxidation, important in mitochondrial functions, is a complex process consisting of three stages- initiation, propagation and termination (Halliwel & Gutteridge 1999). For each stage, there are many available methods to quantify the progress of the process and find evidence for its existence. One approach is to measure the peroxide formation during the process of lipid peroxidation. In the last stage of peroxidation process, peroxides are decomposed to aldehydes like MDA, which can be detected by thiobarbituric acid that gives a color signal which is easily measurable by different spectroscopic techniques, for example using HPLC. All of these are termed as thiobarbituric reactive substances (TBARS). This technique was employed in my dissertation work and its detailed description is given in Chapter III. Additionally, 4-HNE which is another major product of lipid peroxidation was also measured as a biomarker of ROS damage and is described in Chapter IV. On the other hand, evaluation of protein oxidative damage can be accomplished using the carbonyl assay (Levine et. al 2000). Carbonyls are produced from the attack of ROS on amino-acid residues in proteins. This technique was employed in my work as well, and its description is given in Chapter III.

1.2.5 Experimental induction of ROS production in the model organism

In experimental biology there are a number of chemicals which can be used to evoke oxidative stress in the model organism to study the responses to these kind of situations *in vivo*, or *in vitro*. In this section I will mention just two of them, which have been used as elicitors of OS throughout my thesis.

Paraquat (N', N'-dimethyl-4,4'-bipyridinium dichlore) is a typical chemical having structural moieties that undergo redox cycling in living cells to induce ROS production. Therefore, paraquat causes apparent toxicities in animals and humans in which OS is a prominent component of pathogenesis. Besides, paraquat is one of the most widely used herbicides in the world, it produces degenerative lesions in the lung after systematic

administration to animals and humans. Cyclic single electron reduction/oxidation of paraquat is critical in the development of pulmonatory toxicity (Bus & Gibson 1984). This cyclic leads to two potentially important consequences relevant to the toxicity: (1) Generation of ROS including superoxide anion, hydrogen peroxide, and hydroxyl radical, and (2) Oxidation and depletion of reducing equivalents (e.g.: NADPH, GSH). Both contribute to the induction of OS and damage to the tissues, and to the whole organism in general.

Hydrogen peroxide (H_2O_2) is, in biological systems, generated as a result of dismutation of superoxide radicals. There are also some enzymes that can produce H_2O_2 directly or indirectly. Although H_2O_2 molecules are considered reactive oxygen metabolites, they are not radical by definition, however, they are capable of causing damage to the cell at a relatively low concentration (10 μM). Hence, this compound is very often used to evoke OS in biological systems. Another reason is that the molecules of H_2O_2 are freely dissolved in aqueous solution and can easily penetrate biological membranes. Their deleterious chemical effects can be divided into the categories of direct activity, originating from their oxidizing properties, and indirect activity in which they serve as a source for more deleterious species. Direct activities of H_2O_2 include inactivation of enzymes; and oxidation of DNA, lipids, -SH groups, and keto acids (Kohen & Nyska 2002).

1.3 Antioxidant defense responses of cells against OS

Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms (Cadenas 1997). Defense mechanisms against free radical-induced oxidative stress involve: (i) preventative mechanisms, (ii) repair mechanism, (iii) physical defenses and (iv) antioxidant defenses. Antioxidants are molecules present in cells that prevent damaging reactions by donating an electron to the free radicals without becoming destabilized themselves. An imbalance between oxidants and antioxidants is the underlying basis of oxidative stress (**Figure 1**). The antioxidant defense system has a functional connection between its components and thus has been considered as a

physiological system (Blagojević & Grubor-Lajšić 2000). In this section, only the antioxidant defenses elaborated by insects have been dealt with since it is more germane to my dissertation. Insects possess the classical antioxidant system which includes **enzymatic scavengers**, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (Felton & Summers 1995). Glutathione S-transferase exhibiting a peroxidase-like activity (GPOx) has also been reported in insects (Ahmad et al. 1989). In addition to these enzymes, a number of **non-enzymatic** and small molecules are important in scavenging ROS. The role of glutathione ascorbic acid redox-cycle and the activity of thioredoxin reductase has also been reported to serve as a powerful antioxidant system in the gut of certain insects such as the Colorado potato beetle (Krishnan et al. 2009). The non-enzymatic small molecules include glutathione, vitamins C and E, pyruvate, flavonoids, carotenoids, urate and many plant-derived antioxidants. Some examples are given in the **Table 2**.

The overall stress response, meaning the array of reactions generated in most body compartments as a result of threat of the stressor, is common to all organisms. The flight-or-fight reactions can be seen as a common behavior from invertebrates to man and the necessary energetic supply and coordination of neural circuits for these reactions are also common patterns among the animals along the phylogenetic tree. Thus, OS triggers a range of physiological, pathological and adaptive responses in cells either as a result of cellular damage or through specific signaling molecules. These responses ultimately modulate transcriptional outputs to influence cell fate and disease processes. In the last couple of decades, a number of signaling pathways have been identified and delineated to mediate critical responses to OS. These examples demonstrate the importance as well as the complexity of how alterations in intracellular ROS are converted into discrete and reproducible alterations in enzymatic activity, gene expression, and ultimately disease or organismal survival outcomes. The transcriptional responses and signaling pathways triggered have been dealt with in the following section 1.3.1.

Table 2: List of some enzymatic and non-enzymatic antioxidants.

Enzymatic scavengers	Description
Superoxide dismutase SOD	Important enzyme that functions as a cellular anti-oxidant. It is present in cell cytoplasm (copper-zinc enzyme) and in mitochondria (manganese enzyme) in order to maintain a low concentration of superoxide anion. It catalyzes the dismutation of superoxide anion.
Catalase CAT	A heme protein that catalyzes the reaction in which H_2O_2 is detoxified: $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ It is usually found in peroxisomes. Catalase provides a protective role that is similar to that of glutathione peroxidase because both are important means of removing H_2O_2.
Glutathione peroxidase	A cytoplasmic and mitochondrial enzyme important for detoxifying H_2O_2 in most cells. Conteins a selenocysteine amino acid at the active site.
Glutathione reductase	Flavoprotein, which uses the reducing power for the pentose phosphate pathway (NADPH) to keep the glutathione pool in cell in a reduced state.
Ascorbate peroxidases APX	Enzymes that detoxify peroxides such as hydrogen peroxide using ascorbate as a substrate. The reaction they catalyse is the transfer of electrons from ascorbate to a peroxide, producing dehydroascorbate and water as products.
Peroxiredoxins	Reduce peroxides in the presence of thioredoxins.
Non-enzymatic scavengers	**Description**
Glutathione	One of the most important cellular antioxidants, contains a cysteine and thiol group in its cysteine moiety which is a reducing agent and can be reversibly oxidized and reduced.
Vitamin E *(tocopherols)*	Important antioxidant particularly effective in biological membranes and in lipid particles found in blood plasma.
Vitamin C *(ascorbic acid)*	A redox catalyst which can reduce, and thereby neutralize, ROS such as H_2O_2.In addition to its direct antioxidant effects, ascorbic acid is also a substrate for the redox enzyme ascorbate peroxidase, a function that is particularly important in stress resistance.
Melatonin	A powerful antioxidant which can easily crosses cell membranes and the blood–brain barrier.

1.3.1 Important pathways and transcription factors involved in response to OS

The OS responses ultimately modulate transcriptional outputs to influence cell fate and stress responsive processes. In the past two decades, a number of transcription factors and signaling pathways have been identified and delineated to mediate critical transcriptional responses to OS. These findings demonstrate the importance as well as complexity of how alterations in intracellular ROS are converted into discrete and reproducible alterations in gene expression, and ultimately organismal response outcomes (Ma 2010).

In this section I would like to mention only some of the transcriptional factors and signaling molecules involved in response to OS which are, to a certain point, connected with each other and which have a special significance in my work.

FoxO: The forkhead transcription factors are a family of conserved proteins that regulate the cellular response to various stimuli, such as energy deprivation, stress, as well as developmental cues. Members of the FoxO subfamily are emerging as a shared component among pathways regulating diverse cellular functions such as differentiation, metabolism, proliferation and survival. A number of mechanisms have been implicated in the activation of FoxO transcription factors by OS signals (Brunet et al. 2004; Furukawa-Hibi et al. 2005; Cartert & Brunet 2007). Under condition of oxidative stress, c-Jun N-terminal kinases (JNK) phosphorylates FoxO proteins to cause its nuclear translocation and so its activation. This is opposite in case of protein kinase A/ phosphoinositide kinase 3 (Akt/PI3) mediated phosphorylation of FoxO, which results in the export of FoxO factors from the nucleus to the cytoplasm and consequently inhibition of FoxO transcription activity. Nuclear translocation of FoxO has been implicated in cellular protection against OS via the transcriptional regulation of manganese superoxide dismutase (*MnSOD*) and catalase (*Cat*) gene expression (Glauser & Schelegel 2007). FoxO can also induce transcriptional activation of sestrin, which could lead to elevated levels of the energy sensor protein AMP-activated protein kinase (AMPK), which has an inhibitory effect on the transcript of the homolog of the target of rapamycin (TOR) (Lee et al. 2010).

Akt/PI3: Akt is a **serine/theorine kinases** which plays an important role in integrating cellular responses to growth factors and other extracellular signals (Kandel & Hay 1999). Akt is activated through **phosphoinositide 3-kinase** (PI3K) pathway. Akt is an important anti-apoptic protein and is activated in response to oxidant injury as well as stresses known to induce oxidative stress and toxicity (Ma 2010). The PI3K/Akt pathway transduces survival signals through phosphorylation dependent-suppression of apoptic factors including for example forkhead transcription factors (FoxO). In some other example PI3K/Akt and JNK pathways can interact and so regulate transcription and apoptosis (Kim et al. 2001).

PKC: Protein kinase C is a family of phospholipid-dependent serine/threonine kinases that are involved in a variety of biochemical pathways including those regulating cell growth, death, and stress responses (Nishizuka 1992; Ma 2010). PKCs contain unique structural features that are susceptible to oxidative modification. The N-terminal regulatory domain contains zinc-binding, cysteine-rich motifs that are readily oxidized by peroxide. When oxidized, the autoinhibitory function of the regulatory domain is compromised which subsequently elevate the PKC activity. The C-terminal catalytic domain contains several reactive cysteines that are targets for various antioxidants. Modification of these cysteines decreases cellular PKC activity (Gopalakrishna & Jaken 2000). Obviously, the two domains of PKCs respond differently to oxidants and antioxidants (Ma 2010). Individual PKC isoforms mediate distinct cellular responses, further complicating the role of PKCs in oxidative stress and related diseases. Nevertheless, as other protein kinases, so activated PKCs phosphorylate particular transcription factors to modulate gene transcription in oxidative stress responses.

AMPK: 5'-AMP-activated protein kinase is another kinases involved also in OS. This kinase is considered to be a sensor and regulator of energy balance at the cellular level, being able to respond to hormonal and nutrient signals. It is typically activated by cellular stress that results in ATP depletion and an increased AMP:ATP ratio (Kahn et al. 2005; Fuentes et al. 2013).

Sestrin: Sestrins are a family of highly conserved proteins that were originally discovered in mammals as antioxidants (Peeters et al. 2003; Budanov et al. 2004). However, it was found that they have an additional function that leads to the activation of AMPK, although the exact mechanism is not fully understood (Budanov & Karin 2008). Sestrin can aslo act as a feedback inhibitor of TOR that prevents age-related pathologies (Lee et al. 2010).

TOR: Target of rapamycin proteins are members of the phosphatidylinositol kinase-related kinase (PIKK) family and are highly conserved from yeast through insects to mammals. TOR proteins integrate signals from growth factors, nutrients, stress, and cellular energy levels to control cell growth (Inoki et al. 2005). TOR is also one of the player in FOXO-Sestrin-AMPK-TOR pathway in *Drosophila*. FoxO can induce transcriptional activation of sestrin, which could lead to elevated levels of the energy sensor protein AMPK, which has an inhibitory effect on TOR (Lee et al. 2010).

MAPKs (mitogen-activated kinases): It is well known that many OS signals activate protein kinase pathways that modulate gene transcription. Mitogen activated kinases (MAPKs) are one such example. They encompass a large number of serine/theorine kinases critically involved in the regulation of proliferation, differentiation, stress adaptation, and apoptosis (Chang & Karin 2001; Ma 2010). MAPKs include three structurally divergent subfamilies which are activated via independent, but sometimes overlapping, pathways to transduce signals to effect proteins, most notably transcription factors that regulate gene transcription. One of the subfamily is called c-Jun N-terminal kinases (JNK).

JNK: c-Jun N-terminal kinases are related to stress response signals, such as cytokines, radiation, osmotic shock, mechanical injury, heat stress and oxidative damage; therefore they are called stress-activated protein kinases (SAPK). It has been found out previously that phosphorylation of FoxO proteins due to the cellular stress of various origins (including OS) can be caused by c-JUN N-terminal kinase (JNK) (Essers et al. 2004; Wang et al. 2005). In other words, activation of JNK under the OS condition leads to the phosphorylation of FoxO factors and drive translocation of FoxO factors into the nucleus.

For instance, recent findings in mouse pancreatic β cells indicate that activation of JNK by oxidative stress result in FoxO translocation to the nucleus (Kawamori et al. 2006).

CREB: cAMP response element-binding protein is a cellular transcription factor. It binds to certain DNA sequences called cAMP response elements (CRE), thereby increasing or decreasing the transcription of the downstream genes. CREB is also involved in oxidative stress processes. It has been found out recently that CREB also functions as a pivotal upstream integrator of neuroprotective signaling against ROS-mediated cell death (Lee et al. 2009).

CYP 450: Cytochrome P450 is a superfamily of hemoproteins essential for the biotransformation of drugs. In other words, they are responsible for metabolism of most xenobiotics and are required for the efficient elimination of foreign chemicals from the body (Gonzales 2005). The cytochrome P450 enzymes are a class of heme-containing enzymes involved in phase I metabolism of a large number of xenobiotics. The CYP family member CYP2E1 metabolises many xenobiotics and pro-carcinogens (Leung et al. 2013). CYP450s are highly conserved across species implying that, in addition to their function in the metabolism of xenobiotics, these enzymes possibly exert broader physiological functions (Miksys & Tyndale 2009).

Thus, we are witnesses to a significant advance in understanding the signal transduction pathways of response to OS. With this has also come an appreciation for the complexity of the responses and awareness that the individual signaling pathways do not act in isolation. It works the other way around, it intersect with one another to mediate complex and many physiological, pathological and adaptive effects of ROS in dose and cellular-dependent manners. In this regard, regulation of gene transcription by ROS has been an evolutionally conserved strategy to convert alteration in the amount of intracellular ROS into discrete and reproducible alterations in gene expression and by employing different signaling pathways. This is tightly connected with the hormonal regulation of different signaling pathways and subsequent gene transcription and expression.

The next chapter deals with neurohormonal regulation of oxidative stress processes and with hormonal signals involved in the anti-oxidative responses.

1.4 Neurohormones and their role in insect stress responses

Living organisms respond to stressful changes of the environmental conditions in various ways, and in doing so, two basic levels of responses can be delineated: (a) cell-autonomous and (b) systemic (Johnson & White 2009). Cell-autonomous responses are operationally characterized as consisting of molecules within cells which enhance the individual cell's chance of survival. Systemic responses, on the other hand, increase the organism's chance of survival. However, the precise mechanism of regulation between these two stress-response pathways remains unclear and warrants continued investigations. Such investigations are likely to offer insight into the complex network of gene products with cell-context-dependent functions that assist in coordinating systemic responses. Hormones and especially those produced by neuro-endocrine system, are the most probable candidates for the coordination of the behavioral, physiological and cellular responses. Insects produce steroid hormones such as ecdysteroids and sesquiterpenes that include all the juvenile hormones as well as a number of peptide hormones. Additionally, there are also a number of biogenic amines, such as octopamine and serotonin, neurotransmitters which are derived from amino acids. In insects, the neurohormones are the master regulators of almost all life processes. Neurohormones are synthesized by specialized neurosecretory neurons from which they are transported along the axon to the *corpora cardiaca* (CC) and *corpora allata* (CA), and/or into other neurohemal organs in the nervous system. Neurohormones are then released into the hemolymph from these neurohemal organs, where they can exert their functions. Many of the hormones involved in mediating stress-induced changes in insects are homologous to vertebrate stress hormones. The stress response system in vertebrates has both central nervous system (CNS) and peripheral components (Tort & Teles 2011). The central tissue components of the stress system in mammals are located in the hypothalamus and also in the brainstem, and include a number of endocrine messengers with a principal role in mediating the

neuroendocrine stress response. The Corticotrophin Releasing Hormone (CRH) is the key peptide in this activation process along with many other steroids and peptides, however, some of the major stress hormones present in the vertebrates have no insect counterpart. The components of the stress system in vertebrates include the peripheral limbs of the hypothalamic-pituitary-adrenal (HPA) axis; the efferent sympathetic-adrenomedullary system; and components of the parasympathetic system. However, such systems have no counterpart in insect stress responsive pathways. A close analogy of such a system would be the brain-CC-CA axis in insects, although not very comparable. Despite this, insects do elaborate a very specific response to stress situations and there is a plethora of literature available, dealing with effects of extreme conditions (caused for instance by high or low temperature, starvation, or by insecticides or other kind of stressors) on the insect body and its responses to such situations (Johnson & White 2009). Various stressors of different intensity cause specific changes which induced specific responses mediated by specific neurohormones. And so the purpose of this particular section is to provide a brief review about the main neurohormones involved in the response to stress situations (with an emphasis on OS) in the insect body (**Figure 2**).

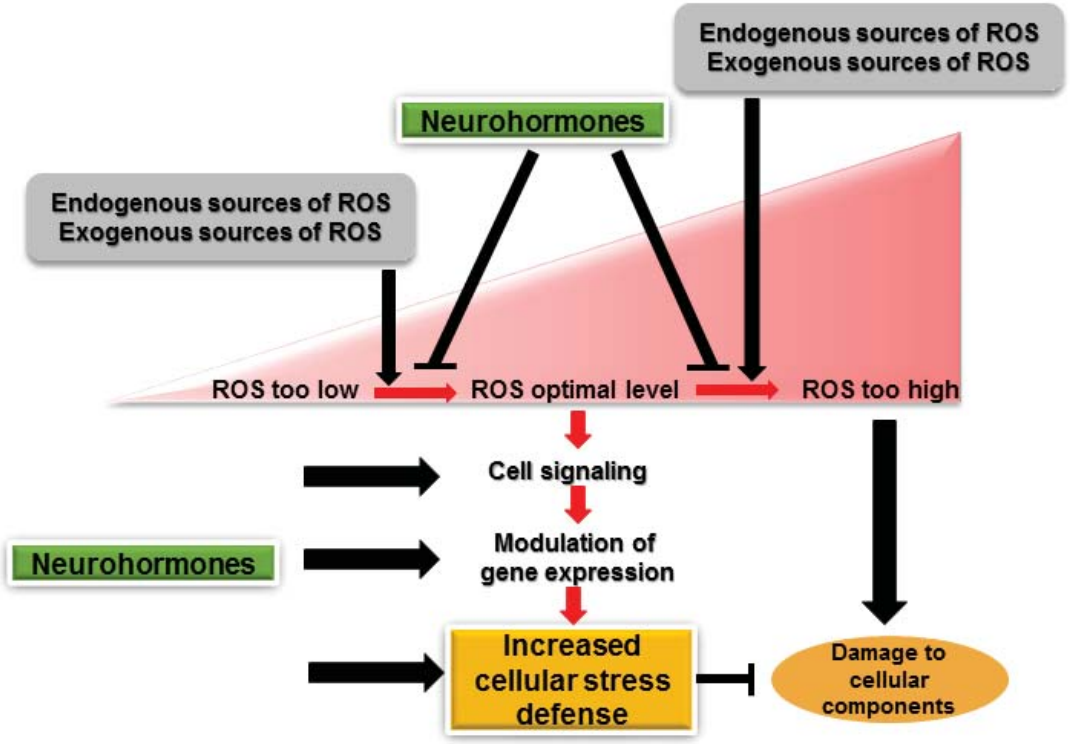


Figure 2: A hypothetical general schematic of role of neurohormones in regulating responses to oxidative stress created by reactive oxygen species.

1.4.1 Biogenic amines and their stress responsive roles

Biogenic amines are important neuroactive molecules in the central nervous system of both vertebrates as well as invertebrates, where they primarily work as neuromediators. Further, in vertebrates, biogenic amines control and regulate various vital functions, including circadian rhythms, endocrine secretion, and cardiovascular functions, but also ability of learning, memory and emotions (Hirashima & Morifuza 1993). In insects, biogenic amines are known to play an important role in regulation of energy metabolism and motor functions. The catecholamine dopamine (DA) is a highly conserved transmitter in both vertebrates and invertebrates which contributes to many different behavioral and physiological processes that are targets of stress modulation. DA elevates heart rate (Johnson et al. 1997) and its levels increase with the presentation of heat stress (Rauschenbach et al. 1993). In addition, the activity of the rate-limiting enzyme in DA synthesis, tyrosine hydroxylase (TH), decreases under several types of stresses in *Drosophila* (Gruntenko et al. 2004, 2005; Neckameyer & Weinstein 2005). In addition, selection experiments and QTL (Quantitative trait locus) analysis of potential loci impacting survivorship under various stressors further implicate DA as a critical insect stress response hormone. DA is also recognized as a critical factor for determining oxidative stress sensitivity. In *Drosophila*, dopaminergic neurons are lost during challenges from paraquat, and these results are paralleled by normal aging processes (Menzeis et al. 2005). There are multiple DA receptors encoded within the genomes of insect species, thus complicating the identity of specific receptor subtype(s) that are involved in mediating stress induced behavioral changes. Unfortunately, there has been little research investigating the signaling pathways that are elicited by stress via DA. DA is clearly a stress hormone with pleiotropic functions in insects, although the precise mechanism of DA action are unclear. I have addressed the issue of perturbations in DA synthesis and its impact on certain physiological characteristics and response to OS in *Drosophila* (see supplemental publication: Hanna et al. 2015). Further studies on the role of specific receptor subtype(s) as well as identification of the regulation and behavioral contributions of different DA pools (central or epidermal) will likely offer insights into the relationship between DA and stress responses.

Octopamine (OA) is another neurotransmitter synthesized from chemical modifications of the tyrosine amino acid. This appears to be an arthropod specific amine transmitter and is involved in a number of different behaviors and physiologies. The presence of OA has been detected in the nervous system, neuroendocrine system, and hemolymph of many insect species (Evans 1989; Hirashima & Morifuza 1993). It has been shown in the cockroach *Periplaneta americana* that various stressful factors elicit the increase in glucose and trehalose content in hemolymph (Wilson & Roundus 1972; Mathews & Downer 1973; Huang et al. 2011). Moreover, it has been confirmed that OA in the hemolymph of insect can function as neurohormone controlling carbohydrate and lipid metabolism as the primary response of the insect to the effect of the stressor (Downer et al. 1984). In the locust, OA directly regulates the release of adipokinetic hormone (AKH) from the *corpora cardiaca* (Pannabecker & Orchard 1986). OA levels have also been demonstrated to rise during stress in *Drosophila virilis* (Hirashima et al. 1999). In addition to OA's effects on behaviors targeted by stress, OA also interacts with other stress hormones. OA has been shown to stimulate the levels of juvenile hormone (JH) in many different insects (Kaatz et al. 1994; Hirashima et al. 1999). Thus, OA has been demonstrated to modulate the same behavioral and physiological repertoires altered by stress, OA levels have been shown to be modified by stress and OA regulates and is regulated by other stress hormones. Taken together, these results implicate OA as a stress responsive hormone in insects. Despite this, it is unclear as yet, the precise roles of OA within the neuroendocrinology of stress. Thus, biogenic amines are in general considered to be responsible for stressogenic changes in the energy metabolism of insects (Bicker & Menzel 1989; Ivanovic 1991; Roeder 1999, 2005; Gruntenko et al. 2004; Perić-Mataruga et al. 2006).

1.4.2 Insulin signaling – a stress responsive hormone regulating growth

Insulin-like peptides (ILPs) regulate development, growth, reproduction, metabolism, and aging as well as stress resistance in both vertebrates and invertebrates (Baker & Thummel 2007; Giannakou & Partridge 2007; Grönke et al. 2010; Antonova et

al. 2012). The first identification of insect ILPs were made in *Bombyx mori* (Nasagawa et al. 1986) and the migratory locust *Locusta migratoria* (Lagueux et al. 1990). The insulin signaling pathway is conserved in all multicellular organisms (Garofalo 2002; Teleman 2010) and has regulatory roles on diverse physiological functions such as growth, development, reproduction, metabolic homeostasis and lifespan (Brogiolo et al. 2001; Ikeya et al. 2002; Broughton et al. 2005). Several publications have demonstrated a role for insulin signaling in regulation of responses to stress such as extreme temperatures, starvation, desiccation, oxidative stress and infection. Ablation of brain insulin producing cells (IPCs) in *Drosophila* resulted in increased lipid and carbohydrate levels, reduced fecundity as well as increased lifespan and resistance to oxidative stress and starvation (Broughton et al. 2005). Apparently insulin signaling may target different pathways depending on type of stress. In general, diminished insulin signaling increases stress resistance and extends lifespan at the cost of growth and fertility. Manipulations of insulin signaling, like knockdown of neurotransmitter receptors in IPCs, have also been shown to affect stress responses (Enell et al. 2010; Luo et al. 2011; Söderberg et al. 2011). More needs to be learned about regulation of ILPs production and release, and also about the possibility of nutrient-sensing cells additional to the adipokinetic hormone (see below) producing cells and fat body cells (Kim & Rulifson 2004; Geminard et al. 2009), and their integration in stress responsive signaling.

1.4.3 Role of juvenile hormones and ecdysteroids in stress response

Juvenile hormones (JHs) and ecdysteroides are major insect developmental hormones, however they control also other aspects of insect physiology and behavior. JHs are synthesized in the *corpus allatum*, while ecdysteroids are synthesized primarily by the prothoracic glands and gonads (in certain developmental stages also by other tissues). JH performs pleiotropic functions, from (a) orchestrating metamorphosis in concert with the molt-inducing ecdysteroid hormones, (b) regulating female fertility by stimulating vitellogenin synthesis in the fat body and its uptake by the growing oocytes, up to the (c) generation of sophisticated polymorphisms in aphids and social insects. In this sense, JH is clearly a pleiotropic master hormone of insects which governs most aspects of their integration with the ecosystem and affects decisive life history parameters during the entire

life cycles. Given the pleiotropic nature of this hormone, it is expected that any stress which affects specific physiological functions governed by JH would also trigger a stress responsive role by JH.

In *Drosophila*, mutations in the *apterous* (ap) gene cause a JH deficiency and cause reproductive and behavioral phenotypes (Altaratz et al. 1991; Ringo et al. 1992). Since reproductive behaviors are affected by different stressors and also because JH performs vital functions in reproductive behaviors, it is expected that JH has a role in modifying these behaviors under stress. In *Manduca sexta*, JH is elevated during stress and effectively lengthens larval stages (Tauchman et al. 2007). In *Drosophila* adults, heat stress leads to a decreased activity of JH esterase (Rauschenbach et al. 2001). JH is also positively correlated with locomotion in some insects such as the cockroach (Lin & Lee 1998). With respect to JHs role in oxidative stress, this might be indirectly mediated through the regulation of biologically active proteins such as vitellogenins and transferrins (Jamroz et al. 1993; Harizanova et al. 2005). Vitellogenins were reported to protect honey bee workers against oxidative damage of paraquat as they were the preferred target of deleterious carbonylation among other hemolymph proteins (Seehus et al. 2006). Moreover, the survival rate was significantly increased in high-vitellogenin level bee phenotypes compared to low-vitellogenin ones after paraquat exposure indicating the importance of vitellogenins in OS prevention in insects.

Ecdysteroids are steroid hormones in insects that may act in conjunction with JH as well as participate and coordinate a plethora of different physiologies and behaviors (Riddiford 1993). Hence, in general, insect molting and metamorphosis are governed by ecdysteroids and JH, with ecdysone (E) and 20-hydroxyecdysone (20E) orchestrating the molting process and JH determining the nature of the molt. As in case of JH, ecdysteroids are also known to respond to stress. For example, flies heterozygous for a mutation in ecdysone receptor or for a gene involved in ecdysone biosynthesis exhibit increased lifespan as well as resistance to various stressors (Simon et al. 2003). Ecdysone has also been shown to be involved in mediating the up-regulation of a methionine sulfoxide reductase (MsrA) during challenges with hydrogen peroxide to cell lines that differ in the expression of the ecdysone receptor (Roesijadi et al. 2007). MsrA is thought to minimize the effects of oxidation on various proteins through its reducing action (Cabreiro et al.

2006). 20E was also found to be a potent antioxidant able to minimize the effects of oxidative stress induced by paraquat in *P. apterus* brains (Krishnan et al. 2007). In addition to the combinatorial interactions with JH, ecdysone may be integrally connected to other stress hormones.

1.4.4 Role of adipokinetic hormone (AKH) in stress response

The adipokinetic hormones (AKHs) belong to the family of one of the best-defined groups of neurohormones from the arthropod AKH/RPCH family (adipokinetic hormone/red pigment concentrating hormone family) (Gäde et al. 1997; Kodrík 2008). They are synthesized, stored and released by the neurosecretory cells of the *corpora cardiaca* (CC) connected to the brain. AKHs typically occur as octa-, nona-, or decapeptides with the N-terminus blocked by pyroglutamate and the C-terminus blocked by amidation, which make the peptide only accessible by endopeptidases. The presence of aromatic amino acids at positions 4 (Phe or Tyr) and 8 (Trp) are conserved as well. Most of the identified AKH peptides are uncharged, only some AKHs contain an aspartic acid at position 7, which gives the peptide a net negative charge. There is also ample evidence that AKHs possess secondary structures in the form of a β-strand and a β-turn (Zubrzycki & Gäde 1994; Cusinato et al. 1998; Nair et al. 2001). Within the insects there is a high diversity of the number and sequence of AKH peptides. For example, there are three different AKH hormones in the locust, *L. migratoria* (Bogerd et al., 1995), however in *Drosophila* only single member of the AKH peptide family was identified (Schaffer et al. 1990). First of all, these metabolic neuropeptides mediate the mobilization of energy substrates from the fat body of many insects (Gäde & Auerswald, 2003; Van der Horst 2003; Lorenz & Gäde 2009). Mobility in insects requires the presence of a fine-tuned energy metabolism to cope with the acquirement of energy during highly energy-demanding processes like flight. When energy is required, AKHs are released from the CC into the hemolymph. This will trigger a number of intracellular pathways which finally result in the mobilization of carbohydrate or lipid reserves and, in some insects, the release of proline. Oxidation of these energy rich substrates by muscle tissue will yield the necessary energy.

Thus, AKHs perform a significant function as stress responsive hormones by stimulating catabolic reactions (by mobilizing lipids, carbohydrates and/ or certain amino acids) thus making energy more available, while inhibiting synthetic reactions. These concerted series of actions are accompanied by the activation of physiological responses such as increased heartbeat (Scarborough et al. 1984), increase of muscle tonus (O'Shea et al. 1984), stimulation of general locomotion (Orchard et al. 1983; Socha et al. 1999), enhancement of immune response (Goldsworthy et al. 2002, 2003) in addition to certain other reactions (Steele 1985; Perík-Mataruga et al. 2006). On the other hand AKHs inhibit synthesis of RNA, fatty acids and proteins in the fat body (Kodrík 2008; Bednářová et al. 2013a (see also Chapter II)).

Recent studies of our laboratory have demonstrated also an active role of AKH in protection of insects against oxidative stress (OS) (Kodrík et al. 2007; Večeřa et al. 2007; Velki et al. 2011; Večeřa et al. 2012; Bednářová et al. 2013b (see also Chapter III). These results demonstrate that OS increases the level of AKHs in insect body, moreover, exogenous AKH modulates OS biomarkers in insect tissues (as well as in *in vitro* organ cultures) experimentally enhanced by application of the OS stressors. The role of AKH in anti-stress reactions not necessarily involved in mobilizing energy substrates and functioning primarily in reducing OS has been reviewed by Kodrík (2008), and by Krishnan and Kodrík (2012).

It is generally accepted that the release of AKH into the blood circulation triggers a cascade of reactions which are initiated by the binding of these neuropeptides to G-protein coupled receptors (GPCRs) located on the plasma membrane of target cells (Staubli et al. 2002; Caers et al. 2012). The GPCRs for AKH have been identified and characterized in the fruit fly *Drosophila melanogaster* and the silkworm *Bombyx mori* (Park et al. 2002; Staubli et al. 2002; Zhu et al. 2009) as well as in other insect species (Hansen et al. 2006; Kaufman & Brown 2006; Wicher et al. 2006; Kaufman et al. 2009; Zeigler et al. 2011). It has been demonstrated earlier that AKH triggers in the target tissues (mostly fat body), a classical intracellular cascade typical for peptide hormone signaling via the adenylyl cyclase (Spencer & Candy 1976) or phospholipase C (Vroenen et al. 1997) pathways, both involving presence of extracellular and/ or intracellular Ca^{2+} (Van der Horst et al. 2001; Gäde & Auerswald 2003). Details of the mechanism of AKH action at the sub-cellular

level during oxidative challenge are not known, nevertheless our recent experiments proved employing both above mentioned pathways in it (Bednářová et al. 2013c (see also Chapter IV)).

At the functional level, there is a similarity in the role of AKH and glucagon in stress situations and maintenance of homeostasis. The information generated on the role and mode of action of AKH in insects can inform us on the modes of action of glucagon and glucagon-like peptides in mammalian systems in stress situations and vice versa (Bednářová et al. 2013a (see also Chapter II)).

Thus, AKH has a multiplicity of roles in mediating behavioral responses to stress and many of these may be directly tied to the physiological effects of AKH signaling. Identification of specific regulatory mechanisms and downstream effector molecules will throw some insights into the modulation of specific physiological programs that are altered during stress and this is the main theme of my dissertation work.

1.5. HYPOTHESES, AIMS, AND OBJECTIVES

In insects AKH signaling has been demonstrated to have a major regulatory role in stress responses with particular reference to oxidative stress (OS). However, the precise mode of AKH action at the sub-cellular level is still unclear. *Therefore, the primary aim of my study was to unravel the mode of action of AKH in potentiating an anti-OS response in insects.* I have studied this topic in two different insect models – the firebug *Pyrrhocoris apterus* and the fruit fly *Drosophila melanogaster*. While in the former, I have employed an *in vitro* as well as an *in vivo* experimental strategy, in the latter I have utilized transgenic flies (RNAi) to knockdown AKH synthesis as well as used the GAL4/UAS system to overexpress AKH in AKH secreting cells in the adult *Drosophila* brain.

1. *Determine if AKH function in insects has any parallels with glucagon and glucagon-like peptides which performs a stress responsive role in vertebrates*.

It has been demonstrated that glucagon and glucagon-like peptides may modulate the response to stress in addition to performing its other physiological functions in vertebrates. It was hypothesized that AKHs might perform functions analogous to vertebrate glucagon and glucagon-like peptides in its stress responsive role in insects. Hence, an in-depth review that focused on studies on insect AKHs as well as parallel studies on glucagon and glucagon-like peptides in higher animals was conducted which suggested a close functional similarity between the two stress responsive hormones. These hormones perform similar unique roles in these two very different, yet physiologically comparable systems in an effort to maintain organismal homeostasis. This review has been presented in **Chapter II** (paper I).

2. *Determine if AKH can counteract oxidative stress elicited in in-vivo and in-vitro systems.*

Recent work suggested that AKH can mitigate the effects of OS in insects. To test this at both the *in vivo* and *in vitro* levels, the response to exogenous application of hydrogen peroxide, an important oxidative stressor, was conducted. Such studies can contribute to an understanding of the role of AKHs in the protection of the insect body from OS, and

also help to develop a robust and reliable system for replicating *in vitro* results obtained from *in vivo* studies, which could be utilized as a basis for studying in more detail the mode of action of AKH in relation to OS. Thus, the effectiveness of the use of an *in vitro* system to study the action of AKH at the sub-cellular level was established. The results of this study have been presented in **Chapter III** (paper II).

3. Determine if AKH employs a conserved signal transduction mechanism to exert its anti-oxidative stress reactions.

The involvement of members of the AKH family in regulation of response to OS has been established. However, their precise signaling pathways in their OS responsive role is unclear. To elucidate this, an *in vitro* assay, established in the previous study (paper II) was employed. Specific chemical antagonists and agonists were used to determine the importance of extra and intra-cellular Ca^{2+} stores as well as the involvement of protein kinase C (PKC) and cyclic adenosine 3′,5′-monophosphate (cAMP) pathways by which AKH can exert its anti-oxidative effects. The results of this study have been described in **Chapter IV** (paper III).

4. Determine if AKH can potentiate the FoxO-Sestrin-AMPK-TOR pathway in its stress responsive role.

It has been established that the transcriptional response to stress is affected by transcription factors such as forkhead-box O (FoxO) which induces transcriptional activation of antioxidant systems using the FoxO-Sestrin-AMPK-TOR pathway. Here, we have asked questions to study the possible relationship of AKH signaling to downstream effector molecules and signaling pathways by knocking down AKH synthesis (RNAi) as well ectopic overexpression of AKH synthesis (using GAL4/UAS system) on response to OS. We focused on the FoxO-Sestrin-AMPK-TOR pathway and established its link to AKH signaling during stress response. Results of this study were communicated as a paper and have been presented in **Chapter V**.

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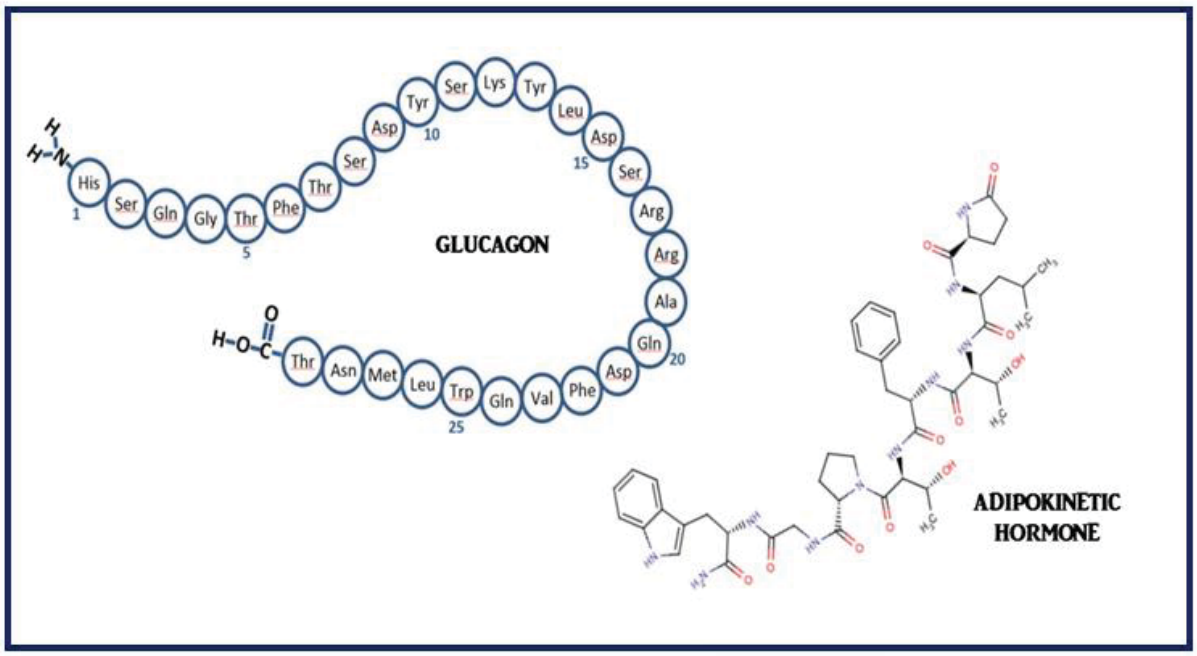
CHAPTER II

Unique roles of glucagon and glucagon-like peptides: Parallels in understanding the functions of adipokinetic hormones in stress responses in insects.



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Review

Unique roles of glucagon and glucagon-like peptides: Parallels in understanding the functions of adipokinetic hormones in stress responses in insects

Andrea Bednářová [a,b], Dalibor Kodrík [a,b], Natraj Krishnan [c,*]

[a] Institute of Entomology, Biology Centre, Academy of Science, Branišovská 31, České Budějovice, 370 05-CZ, Czech Republic
[b] Faculty of Science, South Bohemian University, Branišovská 31, České Budějovice, 370 05-CZ, Czech Republic
[c] Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA

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ABSTRACT

Glucagon is conventionally regarded as a hormone, counter regulatory in function to insulin and plays a critical anti-hypoglycemic role by maintaining glucose homeostasis in both animals and humans. Glucagon performs this function by increasing hepatic glucose output to the blood by stimulating glycogenolysis and gluconeogenesis in response to starvation. Additionally it plays a homeostatic role by decreasing glycogenesis and glycolysis in tandem to try and maintain optimal glucose levels. To perform this action, it also increases energy expenditure which is contrary to what one would expect and has actions which are unique and not entirely in agreement with its role in protection from hypoglycemia. Interestingly, glucagon-like peptides (GLP-1 and GLP-2) from the major fragment of proglucagon (in non-mammalian vertebrates, as well as in mammals) may also modulate response to stress in addition to their other physiological actions. These unique modes of action occur in response to psychological, metabolic and other stress situations and mirror the role of adipokinetic hormones (AKHs) in insects which perform a similar function. The findings on the anti-stress roles of glucagon and glucagon-like peptides in mammalian and non-mammalian vertebrates may throw light on the multiple stress responsive mechanisms which operate in a concerted manner under regulation by AKH in insects thus functioning as a stress responsive hormone while also maintaining organismal homeostasis.

Contents

1. Introduction

Glucagon is a 29-amino acid peptide hormone processed from proglucagon and plays a critical role in glucose metabolism *in vivo* in vertebrates including humans. The prohormone proglucagon is expressed in various tissues (mainly in brain, pancreas and intestine) and is proteolytically processed into several peptide hormones in a tissue specific manner. In vertebrates, both pancreas and intestine are known to actively process the proglucagon gene, and release a mixture of peptides, including GLPs with distinct physiological functions (Mommsen, 2000). The functional glucagon-like peptides (GLP-1 and 2) are processed by subtilisin-like proprotein convertases (PC1-3) in intestinal cells (Rouille et al., 1997a) and into functional glucagon by PC2 in the pancreatic α cells (Rouille et al., 1994, 1997b). Glucagon acts via a seven-transmembrane G protein coupled receptor which for example in rats consists of 485 amino acids (Jelínek et al., 1993). Glucagon binding sites have been identified in a number of tissues including

* Corresponding author. Tel.: +1 662 325 2978; fax: +1 662 325 8837.
E-mail address: nk260@msstate.edu (N. Krishnan).

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liver, brain, pancreas, kidney, intestine and adipose tissues (Christophe, 1995). There is also a large body of literature on glucagon as regulators of metabolism in fishes (reviewed by Mommsen and Plisetskaya, 1991; Plisetskaya and Mommsen, 1996). In general, in mammalian systems, glucagon is released into the blood stream when circulating glucose is low. It thus stimulates hepatic glucose output, thereby leading to increases in glycemia, which provides a counter regulatory mechanism to insulin in maintaining glucose homeostasis *in vivo* (Jiang and Zhang, 2003). This property of glucagon was instrumental in its nomenclature since it was identified as a compound contaminating pancreatic extracts that had quite the opposite effects of insulin (Kimball and Murlin, 1923). However, recent findings also indicate that glucagon actually increases energy expenditure (Habegger et al., 2010; Heppner et al., 2010) much like adipokinetic hormones (AKHs) in insects (Goldsworthy, 1994).

This is paradoxical since hypoglycemia is a state of energy deficiency, and glucagon actually increases energy expenditure, a situation which is quite in contrast to expectations. Glucagon-like peptides, as mentioned earlier, are one of several cleavage products from the precursor proglucagon. Two forms of GLP1, GLP-1 (7–37 amide) and GLP-1 (7–36 amide) are produced by the L-cells in the jejunum and colon in response to oral glucose or mixed meals (Creutzfeldt and Nauck, 1992). GLP-2 on the other hand, promotes growth of the intestinal mucosa (Estall and Drucker, 2006). GLP-1 increases glucose mediated insulin secretion by activating specific GLP-1 receptors on insulin secreting β cells in the pancreatic islets (Fehmann et al., 1994). The increase in insulin secretion is glucose dependent and originates from increases in cAMP and Ca^{2+} levels. This "incretin effect" of GLP-1 is one of its most important functions, however, in addition to this physiological function, GLP-1 has also been implicated in cardiovascular functions, such that, in the basal state, GLP-1 may inhibit contractility, but after cardiac injury GLP-1 has been shown to constantly increase myocardial performance both in experimental animals and patients (Nikolaidis et al., 2004a,b; Bose et al., 2005; Nikolaidis et al., 2005). GLP-1 also possesses neurotropic effects and has also been suggested as a therapeutic agent for neurodegenerative diseases (During et al., 2003; Perry and Greig, 2004). Also, while the functions of GLP-1 in fishes have been elucidated in some detail (Mommsen, 2000), yet, the biological actions of GLP-2 remain unclear in them. (For a comprehensive review of the glucagon-like peptides and the physiology of glucagon-like peptide-1 please see Kieffer and Habener, 1999; Holst, 2007). The stress responsive roles and modes of action of glucagon and GLPs could probably stem from a more generalized physiological response to stress as was also described in the case of AKH in insects (Kodrík, 2008), which is analogous to glucagon in mammals.

In insects, metabolism and particularly the generation of energy are regulated by AKHs, small neuropeptides belonging to the adipokinetic hormone/red pigment concentrating hormone (AKH/RPCH) peptide family (Gäde et al., 1997; Kodrík, 2008), which are synthesized, stored and released by neurosecretory cells of the *corpora cardiaca* an endocrine gland connected to the brain. AKHs predominantly perform roles as stress responsive hormones by stimulating catabolic reactions (mobilization of lipids, carbohydrates, and amino acids) which generate more energy while simultaneously inhibiting their synthesis (Kodrík, 2008). This they do by mobilizing the entire energy reserves to counter immediate stress situations while suppressing processes that would divert energy elsewhere, such as synthesis reactions. AKHs are usually octa or deca peptides but other forms of AKH have also been reported (Köllisch et al., 2000; Gäde et al., 2006). They possess a pyroglutamate residue blocking the N-terminus and amide group blocking the C-terminus. The amino acids tryptophan and glycine are at position 8 and 9 (when present); in addition to tryptophan the AKH molecule contains at least one more aromatic amino acid, most commonly phenylalanine at position 4 (Gäde et al., 1997). The insect fat body, an organ analogous to the vertebrate liver, is the main site of action of AKHs. The homeostatic regulation of blood sugar levels is a fundamental physiological process in both vertebrates and invertebrates. The fundamental endocrine regulation of homeostatic blood sugar levels is conserved in insects for e.g. an insulin-related peptide, bombyxin, lowers hemolymph sugar concentrations in a dose-dependent manner in the silkworm *Bombyx mori* and transgenic ablation of *dilp*-producing neurons results in the elevation of total blood sugar (Satake et al., 1997; Lee and Park, 2004). In the fruitfly *Drosophila*, insulin- and glucagon-like peptides have been reported and they are represented by seven *Drosophila* insulin-like peptides (DILPs), (Brogiolo et al., 2001; Cao and Brown, 2001) and AKH respectively (Wu and Brown, 2006). Moreover, dysregulation of glucose homeostasis is also observed when *akh*-expressing cells are ablated (Kim and Rulifson, 2004; Lee and Park, 2004). Also, microarray analysis of flies in which insulin-producing cells (IPCs) were ablated revealed a target gene, *target of brain insulin* (*tobi*), encoding an evolutionarily conserved α-glucosidase. *tobi* expression is increased by dietary protein and decreased by dietary sugar, which is reminiscent of mammalian glucagon secretion which also functions in a similar manner, and this suggests that *tobi* is regulated by a glucagon analog i.e. AKH. These findings strongly suggest that the insulin–glucagon system of mammals and the DILP-AKH system of *Drosophila* may have analogous roles in regulating metabolism (Buch et al., 2008). Also, similar to glucagon, AKHs mechanism of action includes signal transduction through membrane receptors linked to G-protein that activates multiple pathways leading to production of energy providing substrates such as trehalose, diacylglycerol or proline. Thus, at the functional level, AKHs resemble glucagon, whose main function (like AKH) is to mobilize energy reserves, mainly glucose and participation in glucose homeostasis in the blood. Despite an obvious lack of structural similarity, this functional similarity points to a wealth of knowledge one can obtain about the unique roles of glucagon and GLPs from emerging studies on AKHs in insect model systems and vice versa. This review focuses on studies on insect AKHs as well as parallel studies on glucagon and GLPs in higher animals which informs us and suggests a close functional similarity between the two stress responsive hormones, which perform similar unique roles in these two very different, yet physiologically comparable systems in an effort to maintain organismal homeostasis.

2. Glucagon and GLPs in insects

Since both insect AKHs and vertebrate glucagon perform similar functions, attempts have been made to discover glucagon or GLPs in insects and also to seek a role for mammalian glucagon in insect systems. Presence of a substance with hyperglycemic activity comparable to glucagon was first reported by Steele (1961, 1963) in the *corpus cardiacum* of *Periplaneta americana*. These observations were confirmed by further studies on this cockroach (Ralph and McCarthy, 1964; Brown, 1965; Natazili and Frontali, 1966; Natazili et al., 1970), and extended to many other insects: cockroaches *Blaberus discoidalis* (Bowers and Friedman, 1963) and *Leucophaea maderae* (Wiens and Gilbert, 1967); the locust *Locusta migratoria* (Goldsworthy, 1969; Highnam and Goldsworthy, 1972); the black blowfly *Phormia regina* (Friedman, 1967), the blowfly *Calliphora erythrocephala* (Normann and Duve, 1969; Vejbjerg and Normann, 1974; Normann, 1975); the bee *Apis mellifera* (Natazili and Frontali, 1966) and the moth *Manduca sexta* (Tager et al., 1975). All these earlier articles which were published before the isolation and identification of the first insect peptide hormones did not differentiate between glucagon and GLP effects, and the possible effect of AKH. On the other hand an immunochemically similar GLP was reported from the hemolymph of *M. sexta* (Kramer et al., 1980). However despite its structural similarity to vertebrate glucagon and some contradictory reports as to its influence on mobilization of energy reserves in insects (Tager et al., 1976; Ziegler, 1979), an unambiguous answer to the question of its actual role in insects was not forthcoming.

We demonstrated in our earlier paper (Alquicer et al., 2009) an immunological presence of glucagon in insect organs (gut, CNS) and suggested that glucagon may have a unique role in insects much like AKH which functions to ameliorate response to stress. The study also

indicated that injection of mammalian glucagon likely does not act as a uniform stimulant of insect stress-induced energy metabolism as do AKHs, since we could find no influence of it in the lipid levels of the model system we studied (the firebug *Pyrrhocoris apterus*). On the contrary we recorded certain positive effect of glucagon on *P. apterus* glycemia. However, this insect burns almost exclusively lipids and sugars play negligible role in its metabolism (Šula et al., 1998), so, the effects of mammalian glucagon in energy mobilization in this insect was disproved. Despite this, we could demonstrate that mammalian glucagon has the ability to potentiate an anti-oxidative response in this insect in response to oxidative stress (Alquicer et al., 2009). This suggests that glucagon in addition to its well accepted role as a hyperglycemic hormone (in vertebrates) can also function as a stress responsive hormone in response to oxidative stress (see also below).

3. The stress responsive nature of AKHs, glucagon and GLPs

Insect neurohormones are involved in most life processes including a participation in response to stress to maintain organismal homeostasis. They either directly regulate, or participate or potentiate response to stress factors that could prove to be of fundamental importance for the survival of the organism. As mentioned above, a critical role in hormonal control of anti-stress response in insects is played by AKHs (Gäde et al., 1997; Kodrík, 2008). Apart from their main role in energy metabolism these peptides have been implicated to also participate in stress situations that do not require rapid production and subsequent consumption of energy (although, the classical role of AKH is to perform exactly this function in the "*fight or flight*" response involving locomotory activity) (Gäde, 2004a). A series of reports informs us of this rather unique role of AKH in alternative stress situations. For example, when *Schistocerca gregaria* or *P. apterus* was challenged with an insecticide, an elevation of AKH titre was observed (Candy, 2002; Kodrík and Socha, 2005). Non-invasive stressors such as photophase interruption or exposure to constant darkness also resulted in increases in AKH titre (Kodrík and Socha, 2005; Kodrík et al., 2005). A several fold increase in AKH titre was recorded in the hemolymph as well as CNS (brain with *corpora cardiaca*) when the Colorado potato beetle, *Leptinotarsa decemlineatea* was fed on genetically modified potatoes expressing *Bacillus thuringiensis* toxin (Cry 3Aa) or *Galanthus nivalis* lectin (GNA) (Kodrík et al., 2007). AKH induced enhancement of antioxidant capacity in *P. apterus* hemolymph in response to exposure to paraquat (Večeřa et al., 2007) and induction of antioxidant by injection of exogenous *M. sexta* AKH (Manse-AKH) to *Spodoptera littoralis* fed with tannic acid (Večeřa et al., 2012) all point to the unique stress responsive roles played by these hormones. It was also demonstrated that AKH is involved in activation of the immune system when the insect (*L. migratoria*) is stressed by a bacterial infection. The mechanisms are not known, however, certain immune characteristics as prophenoloxidase cascade (Goldsworthy et al., 2002) or nodule formation (Goldsworthy et al., 2003a,b) are enhanced in the presence of external AKH. A comprehensive review of stress responsive actions of AKH has been dealt with elsewhere (Kodrík, 2008; Krishnan and Kodrík, 2012).

As in the case of AKH in insects, a stress responsive role for glucagon has been also reported in fishes. A significant elevation in plasma glucagon levels (2–3 folds) was observed in carp (*Cyprinus carpio*) after short-term temperature increases from 15 °C to 28 °C or long-term acclimatization to 28 °C (Blasco et al., 1988). The stress responsive actions of glucagon were further extended as a result of several intensive studies on mammalian systems (Jones et al., 2012). In mammalian systems, glucocorticoids and catecholamines are the best described stress hormones, since they are released in response to stress and help mediate adaptive responses to stress situations. Additionally, studies indicate that glucagon and GLPs also fit the criteria of stress responsive hormones. The role of glucagon as a physiological signal for satiety was reported by Geary et al. (1981). Subsequent work revealed that, this response could be mediated through neural signals relayed to the brain via the hepatic branch of the vagus nerve (Geary, 1990; Le Sauter and Geary, 1993; Geary and Guss, 1995). Further evidence for this rather unique role comes from a number of studies and improved assay techniques that report several fold increase in glucagon levels in response to stressful situations (Bloom et al., 1973; Freeman, 1975). High glucagon levels have also been reported from cases with a range of physiological stress states such as burns (Orton et al., 1975; Venter et al., 2007), trauma (Brockman and Manns, 1976), and sepsis (Rocha et al., 1973) among other pathological disease states (Segal and Esrig, 1973; Johnston et al., 1975; Oshima et al., 2010). A similar stress responsive role for GLP-1 in TNFα induced oxidative stress and inflammation in endothelial cells (Shiraki et al., 2012) and against reactive oxygen species (ROS) induced endothelial cell senescence (Oeseburg et al., 2010) has been reported. Also, the protective role of GLP-2 in intestinal injury in mice induced by tumor necrosis factor-alpha (TNFα) has been recently reported (Arda-Pirincci and Bolkent, 2011). Some of the principal stress responsive roles of AKH, glucagon and glucagon-like peptides (GLP-1 and GLP-2) in various organisms/model systems and their reporting authors have been presented in Table 1.

4. Mechanism of AKH, glucagon and GLPs release and mode of action in stress situations

4.1. Adipokinetic hormones in stress responses

The triggering of AKH release from the neurosecretory cells of the *corpora cardiaca* probably occurs at the level of appreciable changes in general and intermediary metabolism provoked by stress situations. These in turn stimulate neurons associated with release of the peptide from the neurosecretory cells. The main site of the AKH action is insect fat body that plays a key role in the entire process — while it has an essential role in energy storage and utilization; it is also an organ of great biosynthetic and metabolic activity. Most of the intermediary metabolism takes place in this organ. In order to perform multiple metabolic functions according to the changing physiological needs of the insect in its environment, the fat body must be able to integrate signals from other organs as well as have the capacity to initiate a "feed-back" regulation. Hence, several metabolic processes in the fat body must be tightly coupled to a number of metabolic pathways which in turn would be regulated by hormones. While the mode of action of AKH in energy metabolism has been extensively investigated and reviewed (Gäde et al., 1997; Gäde and Auerswald, 2003; Van der Horst, 2003; Gäde, 2004b), yet mechanisms that do not require energy mobilization, but which also trigger AKH release remain unclear. Despite this, once AKH is released through any of the stress stimulatory pathways, the next step is the binding of the AKH to their plasma membrane receptor(s) as a primary step to induce signal transduction events that ultimately lead to the activation at the transcriptional or post-translational level of key systems involved in stress responses (Fig. 1). As mentioned earlier, the AKHs constitute an extensively studied family of neurohormones and their actions have been shown to occur through G protein-coupled receptors (GPCRs) (reviewed in Van Marrewijk and Van der Horst, 1998; Vroemen et al., 1998), the general properties of which are very conserved during evolution (Vanden Broeck, 2001). GPCRs for AKH have been originally identified and characterized in the fruit fly, *Drosophila melanogaster* and the silkworm, *Bombyx mori* (Park et al., 2002; Staubli et al., 2002; Zhu et al., 2009), but later on also in a few other insect species: the American cockroach, *Periplaneta americana* (Hansen et al., 2006; Wicher et al., 2006), several mosquito species (Kaufmann and Brown, 2006; Kaufmann et al., 2009) and the tobacco hornworm, *M. sexta* (Ziegler et al., 2011). These receptors are typical G protein-coupled proteins structurally related to receptors of the vertebrate gonadotropin releasing hormone. Binding of AKH to these receptors triggers classical

Table 1
Examples of some principal stress responsive situations in which adipokinetic hormone, glucagon and glucagon-like peptides have been implicated.

Peptide	Stress situation	Model system	Reference
Adipokinetic hormone	Insecticide stress		
		S. gregaria	Candy (2002)
		P. apterus	Kodrík and Socha (2005)
			Velki et al. (2011)
			Kodrík et al. (2010)
	Photophase interruption or constant darkness	*P. apterus*	Kodrík and Socha (2005)
			Kodrík et al. (2005)
	Feeding on Bt toxin or GNA lectin	*Leptinotarsa decemlineata*	Kodrík et al. (2007)
	Oxidative stress responses	*P. apterus*	Večeřa et al. (2007)
		Spodoptera littoralis	Večeřa et al. (2012)
		Blatella germanica	Huang et al. (2012)
	Immune responses	*Locusta migratoria*	Goldsworthy et al. (2002, 2003a,b)
Glucagon	Noise, unpleasant stimuli	Primates	Bloom et al. (1973)
	Trauma	Sheep	Brockman and Manns (1976)
	Burns	Humans	Orton et al. (1975)
	Surgery	Humans	Russel et al. (1975)
	Sepsis	Humans, dogs	Rocha et al. (1973)
	Hemorrhage shock	Humans	Russel et al. (1977)
	Myocardial infarction	Humans	Segal and Esrig (1973)
	Feeding and hunger (satiety)	Rats	Geary et al. (1981)
			Geary (1990)
			Le Sauter and Geary (1993)
			Geary and Guss (1995)
	Cardiac arrest	Humans	Oshima et al. (2010)
	Inflammatory response (Oxidative stress)	Hepatocyte cultures	Lee et al. (2003)
	Thermal stress	Fishes	Blasco et al. (1988)
	Anti-oxidative stress response	Rat hepatocytes	Lu et al. (1990)
		Insects (*P. apterus*)	Alquicer et al. (2009)
Glucagon-like peptide 1	Feeding and hunger (satiety)	Rats	Punjabi et al. (2011)
	Anxiety responses	Rats	Kinzig et al. (2003)
	Interoceptive stress (LPS and drug treatment)	Rats	Rinaman (1999)
	Environmental, anesthetic and acoustic stress	Rats	MacLusky et al. (2000)
	Oxidative stress responses	Rats	Oeseburg et al. (2010)
		Endothelial cell cultures	Shiraki et al. (2012)
			Ding and Zhang (2012)
Glucagon-like peptide 2	Oxidative stress responses	Mice	Arda-Pirincci and Bolkent (2011)
	Short bowel syndrome stress	Humans	Jeppesen et al. (2001, 2005)
		Rats	Martin et al. (2004)
	Intestinal ischemia reperfusion stress	Rats	Zhang et al. (2008)

intracellular cascades typical for the peptide hormone signaling: the adenylyl cyclase or phospholipases C pathways depending on the activated substrates (and insect species) both involving presence of extracellular Ca^{2+}. In species using lipids or proline the adenylyl cyclase is activated (via G protein G_s) and the resulting cAMP switches on a cascade leading to activation of a triacylglycerol lipase. In insects burning carbohydrates the AKH activates a phospholipase C (via G protein G_q) and the resulting inositol 1,4,5 trisphosphate initiates Ca^{2+} release from internal stores (in addition to extracellular Ca^{2+} influx mentioned above). This stimulates the protein kinase cascade leading to activation of glycogen phosphorylase. In locusts this enzyme is activated via the cAMP pathway as well (for details see Gäde and Auerswald, 2003).

For processes that are not connected with the production of energy, the mode of action of AKH is unclear and demands intensive investigation. One can speculate that the biochemical pathways might be similar (e.g. increasing of stress resistance and stimulation of antioxidative responses), however, in some of them (e.g. stimulation of locomotion or immune response) neural or direct action cannot be completely excluded (Fig. 1).

4.2. Glucagon in stress responses

Predominantly in the vertebrate system the homeostatic regulation of blood sugar levels is a fundamental physiological process precisely hormonally controlled first of all by insulin and glucagon. In pancreatic α-cells, hypoglycemia stimulates increased intracellular calcium concentrations promoting glucagon secretion, whereas hyperglycemia inhibits these responses. The involvement of α1 and β-adrenoreceptors in mice has been documented in this process (Vieira et al., 2004). The stimulation of this particular pathway leading to glucagon release could being either through direct neuronal innervation or indirectly through catecholamines. The case for direct neuronal stimulation comes from studies revealing that direct projections exist between stress-sensing nuclei in the hypothalamus and pancreas (Jansen et al., 1997). Furthermore, stimulation of the splanchnic nerve in adrenalectomized calves results in hypergluconemia, however, this effect is mitigated following denervation of pancreas (Bloom and Edwards, 1975, 1980; Havel et al., 1996). Additionally, when the stress-responsive hypothalamic ventromedial nucleus is stimulated, it results in release of glucagon, whereas any lesions in this area inhibit its release (Shimazu and Ishikawa, 1981; Borg et al., 1994). Despite some tantalizing evidences to the neural activation, no direct mechanistic study has been done to firmly demonstrate that glucagon release is regulated primarily either through neuronal or systemic mechanisms or a combination of both.

Similar to AKH in insects glucagon in vertebrates signals through its receptor on the cell membrane (Fig. 2). The binding of glucagon to the glucagon receptor results in a conformational change in the latter leading to the activation of GPCRs. Two G protein namely G_s and G_q are involved in this process. Activation of $G_s\alpha$ results in activation of adenylyl cyclase with increase in intracellular cAMP which further activates protein kinase A (PKA). Additionally the activation of G_q leads to stimulation of phospholipase C, the production of inositol 1,4,5-triphosphate and subsequent release of intracellular Ca^{2+} (Burcelin et al., 1996). This process leads to the potentiation of glycogenolysis by PKA mediated phosphorylation of glycogen phosphorylase kinase thus activating glycogen phosphorylase. This in turn phosphorylates glycogen, leading to increased glycogen breakdown

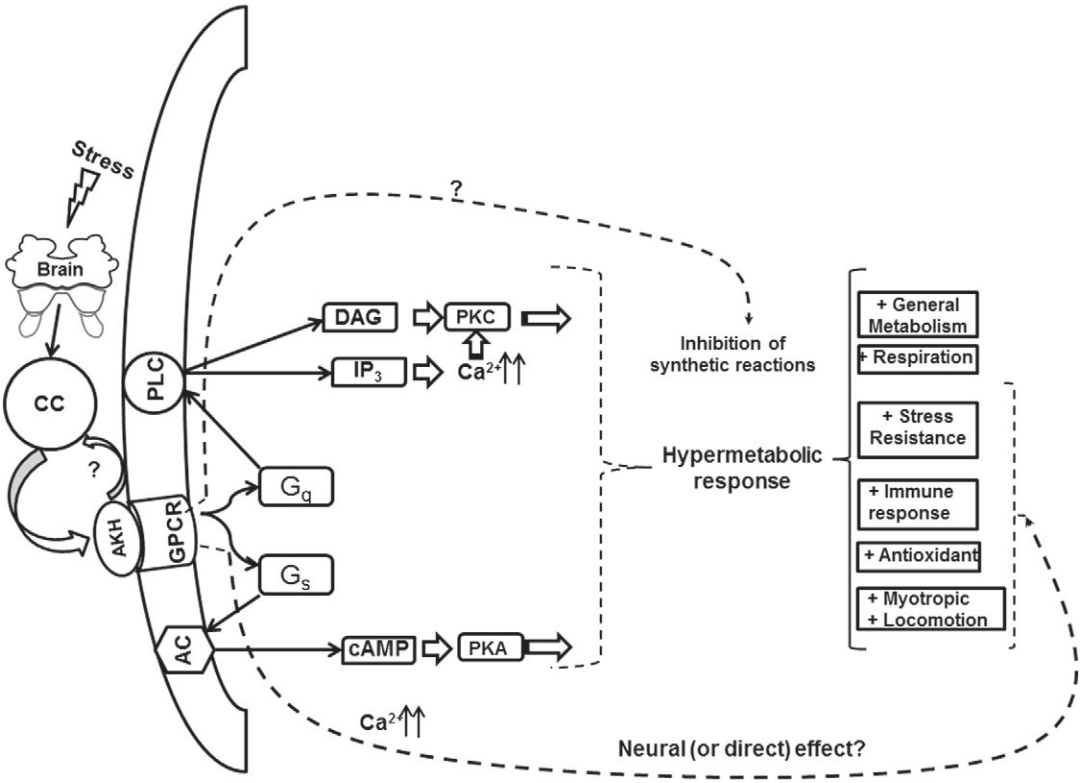

Fig. 1. A hypothetical scheme of release and action of adipokinetic hormone (AKH) in response to stress in insects. Stress registered by the insect brain triggers the release of AKH from the *corpora cardiaca* (CC) which then binds to specific G protein coupled receptors (GPCR) in cell membranes and specific G proteins such as G_q and G_s lead to activation of membrane bound phospholipase C (PLC) or adenylyl cyclase (AC) triggering the elevation in levels of inositol (1,4,5) phosphate (IP_3) or an elevation of cyclic adenosine monophosphate (cAMP) respectively. IP_3 elevation can raise the intracellular calcium levels, whereas PLC activation can also lead to an elevation of diacyl glycerols (DAG) and subsequently protein kinase C (PKC). On the other hand, elevation of cAMP levels activates protein kinase A (PKA). It is hypothesized that these can also activate and give rise to a generalized hypermetabolic response and hence mount a response to the stress situation. Dashed lines represent effects of AKH on inhibition of synthetic reactions as well as a neural (or direct) effect on certain responses which do not require energy production. Additionally, AKH released from CC can also have a neuro-modulatory effect on the central nervous system. Positive effects are preceded by a "+" symbol.

and the production of glucose-6-phosphate (G-6-P). G-6-P is subsequently converted into glucose by glucose-6-phosphatase (G-6-Pase), increasing the glucose pool for hepatic output. Thus, increasing glucose availability through stimulating hepatic glucose output in itself could serve as an adaptive response to stress situations such as the "*fight or flight*" response. Additionally, glucagon can also stimulate catecholamine (a stress responsive hormone) release through a potent feedback mechanism (Lawrence, 1967). Glucagon is also able to indirectly augment stress response pathways via other stress response hormones such as ACTH-induced (adrenocorticotropic hormone) cortisol release (Lawrence, 1967; Berg et al., 2010).

The glucagon mediated efflux of hepatic glutathione (GSH) leading to a significant increase in plasma GSH levels has been demonstrated in cultured rat hepatocytes (Lu et al., 1990). This indicates that under conditions of oxidative stress, hepatic GSH can be mobilized and transported through the blood plasma to areas where they would be required for combating stress and this is under regulation of glucagon. Induction of a "hypermetabolic state" following injury stress involves an altered metabolic state which features increased turnover of proteins, fatty acids and carbohydrates at the whole body level along with increased resting energy expenditure. The hypermetabolic state is mediated among others by hormones such as catecholamines, glucagon and insulin which are elevated following severe injury (Wilmore, 1974). The pathway through which this occurs is via the systemic activation and release of reactive oxygen species (ROS) from immune cells resident in various tissues. ROS and their products can also trigger cellular responses inducing hypermetabolism, e.g. oxidatively modified low-density-lipoproteins (LDL) can modulate the DNA-binding affinity of transcription factors that mediate cytokine and growth factor expression (Maziere et al., 1999). Other stress response pathways targeted by ROS include the mitogen activated protein (MAP) kinase cascade (Beltman et al., 1999) and nuclear factor κB (NFκB) activation (Wesselborg et al., 1997). Hence, ROS mediated effects also eventually trigger hormonal responses (stress responsive hormones) necessary for combating the stress situation resulting in the hypermetabolic state (Lee et al., 2003). The increased substrate availability in the form of glucose, or glutathione or other metabolites mediated through glucagon may be a very generalized and unique role of this stress responsive hormone, and it is likely that synergy with other stress hormones is also necessary and may exist.

Recently we have suggested that the main role of glucagon in insects might lie just in the antioxidant defense against the ROS (Alquicer et al., 2009). We have found that mammalian glucagon injected into the firebug *P. apterus* body activated an antioxidant response when oxidative stress was elicited by paraquat, a diquaternary derivative of 4, 4′ bipyridyl. Glucagon elicited the antioxidant response by increasing glutathione, and decreasing protein carbonyl and protein nitrotyrosine levels in the bug body. Additionally, when co-injected with paraquat, glucagon partially eliminated oxidative stress markers elicited by this redox cycling agent and oxidative stressor.

Glucagon also has a positive inotropic and chronotropic effect on the mammalian myocardium (Regan et al., 1964). This effect is recorded within a few minutes of administration of glucagon much like the cardio-acceleratory role for the representatives of the AKH family in insects. A long time ago, Scarborough et al. (1984) demonstrated a stimulatory activity of the *P. americana* cardio-acceleratory peptide I and II (Peram-CAH-I and -II) from the AKH/RPCH family on heart function in a bioassay using a semi-isolated heart preparation from the cockroach.

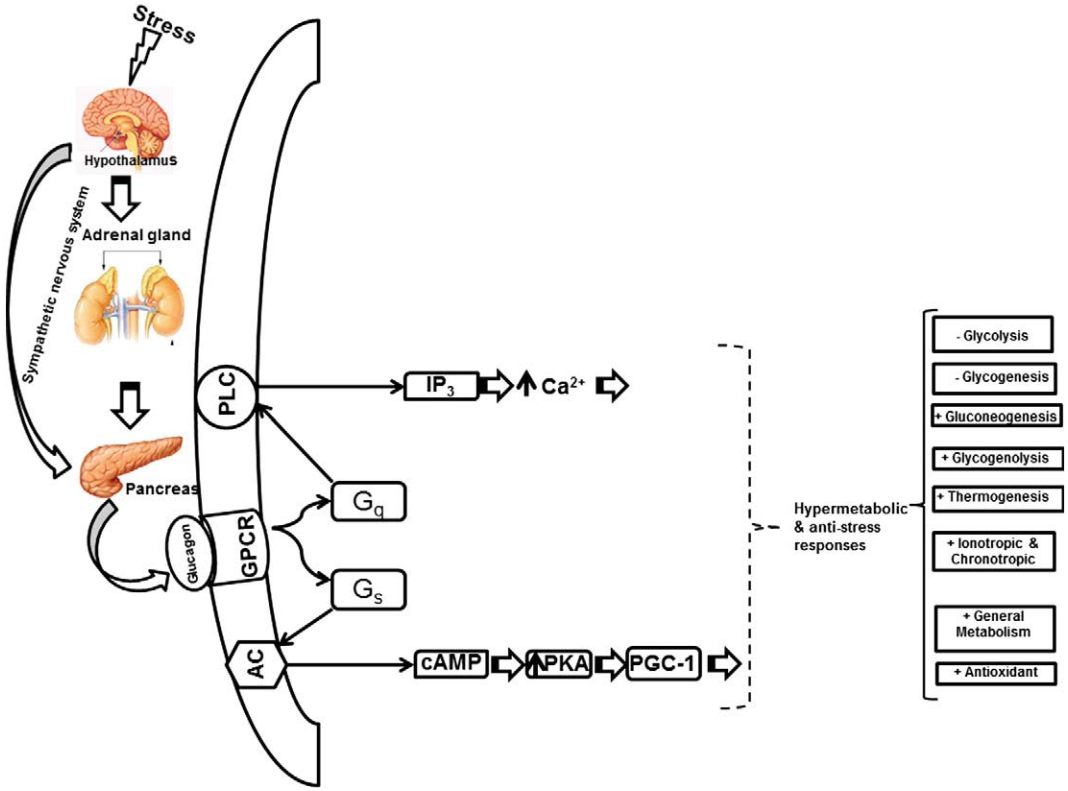

Fig. 2. A generalized model for the glucagon signaling pathway in response to stress. The pathways depicted herein could be specific to a particular tissue. Stress is perceived by the hypothalamus and neuronal (sympathetic nervous system) or hormonal (catecholamines) signals are transmitted to the adrenal glands thereby to the pancreas (or directly to the pancreas through the sympathetic nervous system or by stress sensing neurons innervation the pancreas) which result in release of glucagon. Glucagon binds to G protein coupled receptors (GPCR) in cell membranes and specific G proteins G_q and G_s lead to activation of membrane bound phospholipase C (PLC), or adenylyl cyclase (AC) triggering the elevation in levels of inositol (1,4,5) phosphate (IP_3) or an elevation of cyclic adenosine monophosphate (cAMP) respectively. IP3 activation triggers increase of intracellular Ca^{2+} whereas an elevation of cAMP leads to an increase of protein kinase C (PKC) and also activation of the transcription factor peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1). PGC-1 is known to activate a number of stress response genes which eventually lead to the hypermetabolic and anti-stress responses. Positive effects are preceded by a "+" symbol, whereas inhibitory effects are preceded by a "–" symbol.

Administration of the peptides increased the heart frequency by 60%. Stimulatory activities of the AKH representatives on other muscular insect organs including general locomotion were demonstrated also in a number of other studies (O'Shea et al., 1984; Socha et al., 1999; Kodrík et al., 2000; Lee and Park, 2004; Lorenz et al., 2004; Isabel et al., 2005; Wicher et al., 2006).

The glucagon receptor is expressed in cardiac myocytes, which operates by increasing its contractility through cAMP and consequent Ca^{2+} release (Mery et al., 1990). Acute stress can also result in high peripheral glucagon concentrations. Thus, glucagon has a key role in regulating cardiac function (Harney and Rodgers, 2008) much like the insect AKH (Scarborough et al., 1984).

4.3. Glucagon-like peptides (GLP-1and GLP-2) in stress responses

The glucagon-like peptide (GLP-1) activity is modulated through GPCR as well. Its receptor has been characterized extensively (Laburthe et al., 1996; Goke et al., 1997). The GLP-1 receptor is closely related to the glucagon receptor and hence the action of GLP-1 in vertebrates in the pancreatic islets via the cAMP system (Drucker et al., 1987) and it can also affect insulin secretion by raising Ca^{2+} levels (Fridolf and Ahren, 1993). The GLP-1 receptor is localized primarily in the β-cells in the pancreas, and to a lesser extent in the hypothalamus, adipose tissue and muscles (Drucker, 1998). The main actions of GLP-1 involve stimulation of insulin secretion and inhibiting glucagon secretion, thereby regulating circulating glucose levels. It also participates in inhibiting gastrointestinal motility and secretion and is also a physiological regulator of appetite and food intake. Since it is a brain-gut axis peptide, it can be categorized as an autonomic nervous system neurotransmitter (Perry et al., 2002). A number of evidences and recent literature point to a unique role of this peptide also in modulating stress response. While, data linking GLP-1 to inhibition of food intake is convincing, it is also important to consider a role for GLP-1 in the CNS response to aversive stimuli. A link between GLP-1 and the stress response was first suggested by Larsen et al. (1997) who demonstrated that intracerebroventricular (ICV) injection of GLP-1 activated hypothalamic cortocotropin-releasing hormone (CRH+) neuroendocrine neurons leading to increased corticosterone secretion in rats. GLP-1 containing neurons are hypothesized to have synaptic contacts with CRH neurons, facilitating GLP-1 action on the hypothalamo–pituitary–adrenal axis; this is supported by the presence of GLP-1 receptor mRNA in both hypothalamus and pituitary (Alvarez et al., 1996; Shughrue et al., 1996; Sarkar et al., 2003). It was also demonstrated that many of the aversive effects of lithium chloride administration in rats are blocked by ICV preadministration of exendin (9–39), the GLP-1 receptor agonist (Seeley et al., 2000). However, differential effects were also recorded in rats and mice on the role of GLP-1 in mediating the effects of visceral illness caused by lithium chloride indicating species specific responses to introceptive stress (Lachey et al., 2005). Some stressors increase the activity of GLP-1 containing neurons (Rinaman, 1999). Mice lacking GLP-1 receptor demonstrate an altered response to stress (MacLusky et al., 2000). GLP-1 has been reported to cause changes in the corticosterone response in isolated adrenocortical cells to ACTH, which indicates a link between GLP-1, the stress response and the hypothalamic–pituitary–adrenal axis (Andreis et al., 1999).

While the precise mode of action of GLP-1 in modulating stress responses is unclear, it is hypothesized that it could participate in such responses through the forkhead box (FOXO) family of transcription

factors (Glauser and Schlegel, 2007). FOXO factors have a key role in energy homeostasis and thus indirectly in actions that would also regulate cellular responses to stress. FOXO subtypes have been demonstrated to be expressed in β cells (mainly FOXO1) (Kitamura et al., 2002). The link between FOXO signaling and GLP-1 has been represented in Fig. 3. The PI3K-Akt pathway is the major upstream signaling molecule leading to the phosphorylation of FOXO factors and their exclusion from the nucleus. In β cells, FOXO is phosphorylated following the activation of the PI3K-Akt pathway by insulin, IGF1, glucose, GLP-1 or glucose dependent insulinotropic peptide (GIP) (Trumper et al., 2000, 2001; Wrede et al., 2002; Buteau et al., 2006). GLP-1 either released from the gut cells or from GLP-1 containing neurons potentiate glucose activation of insulin secretion. The secreted insulin would bind to insulin receptors (IRs) at the cell surface and lead to PI3K and then Akt (Ohsugi et al., 2005). However, the binding of GLP-1 to its receptor can also lead to direct activation of PI3K: through c-SRC-mediated activation of a membrane bound metalloprotease, which would cleave betacellulin membrane integral precursor and release betacellulin soluble ligand (BTC). BTC in turn would activate EGF receptor and PI3K (Buteau et al., 2003). Alternatively, GLP-1 can promote activation of PI3K by increasing expression of IRS-2, through G-protein-dependent activation of adenylyl cyclase, formation of cAMP, activation of PKA, and finally activation of CREB (cAMP-response element-binding) transcription factor (Holz and Chepurny, 2005). Activation of c-Jun NH(2)-terminal kinase (JNK) pathway (a member of an evolutionarily conserved sub-family of mitogen-activated protein (MAP) kinases) through oxidative stress (induced by elevated glucose levels) resulting in FOXO factor phosphorylation and translocation to nucleus also is a pathway that may discretely or simultaneously operate in response to cellular stress and both glucagon and GLP-1 would likely be involved in the entire process (Kawamori et al., 2006).

Another pathway likely to be involved in the stress responsive signaling pathway of GLP-1 is the endoplasmic reticulum (ER) stress pathway. ER stress is one of the molecular mechanisms underlying β-cell failure and this is caused by chronic exposure to saturated and to a lesser extent unsaturated free fatty acids (FFAs). Reports indicate that activation of the GLP-1R by agonists induce cAMP-PKA, extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase and phosphoinositide 3 kinase-Akt signaling, leading to protection of β-cells. This is achieved through increasing cellular defense mechanisms through induction of ER chaperone BiP and the anti-apoptotic protein JunB (Gurzov et al., 2008; Cunha et al., 2009).

Glucagon-like peptide-2 (GLP-2) is an intestinotrophic growth hormone which is implicated in promoting many aspects of intestinal function, including the enhancement of mucosal growth and promotion of nutrient absorption (for review see Drucker, 2002). A unique role of this growth hormone involves its ability to rapidly enhance mucosal barrier function, while reducing intestinal permeability of the epithelial barrier via both transcellular and paracellular routes (Benjamin et al., 2000). Stress-induced intestinal pathophysiology

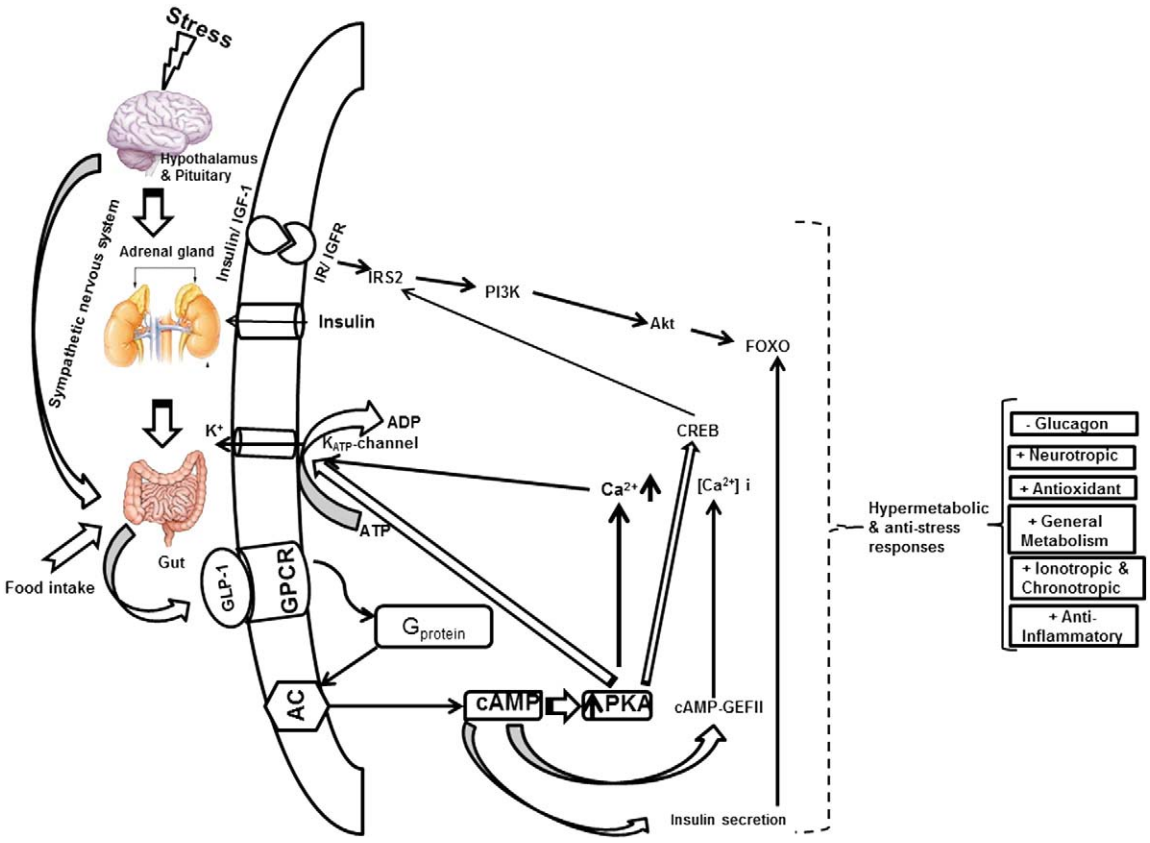

Fig. 3. A generalized scheme of the cellular actions of glucagon like peptide-1 (GLP-1) that lead to the stimulation of anti-stress responses. The pathways depicted herein could be specific to a particular tissue. Stress is perceived by stress-receptive neurons in the hypothalamus-pituitary body and the signals are either transmitted to the gut via hormones such as catecholamines released from adrenal glands or directly perceived through the sympathetic nervous system. Food intake (eventually the glucose intake) also plays a major role in triggering the release of GLP-1 by the gut. GLP-1 binds with the GLP-1 receptor a member of the G-protein coupled receptor family (GPCR) and via a stimulatory G protein adenylyl cyclase (AC) is activated leading to formation of cyclic adenosine monophosphate (cAMP). The subsequent activation of protein kinase A (PKA) and the cAMP regulated guanine nucleotide exchange factor II (cAMP-GEFII) leads to a cascade of events including altered ion channel activity, elevation of intracellular calcium concentrations and also insulin secretion and its exocytosis. PKA activation also leads to triggering the cAMP-response element-binding (CREB) transcription factor that increases expression of insulin receptor substrate-2 (IRS-2). These transmit signals from insulin bound to insulin receptors (IR) at the surface of β cells. An autocrine activation of phosphoinositide 3-kinase (PI3K) and a protein kinase B (Akt) leads to activation of the forkhead transcription factor (FOXO). All these discrete events eventually lead to the anti-stress responses as detailed in the figure and text. Positive effects are preceded by a "+" symbol, whereas inhibitory effects are preceded by a "–" symbol.

involves altered mucosal barrier function and GLP-2 has a role in preventing or ameliorating stress-induced mucosal pathophysiology (Cameron and Perdue, 2005). GLP-2 also has potent protective, antiapoptotic, proliferative and antioxidant effects against TNF-α/Act D-induced intestinal injury (Arda-Pirincci and Bolkent, 2011). It is possible that these antiapoptotic/growth-promoting effects of GLP-2 contribute to the amelioration of stress-induced effects by maintaining the integrity of the epithelial lining of the gut (Drucker et al., 1996; Tsai et al., 1997).

5. Conclusions and future perspectives

There are a lot of analogies between vertebrate and invertebrate endocrine systems and their hormones. One can be drawn between the brain (neurosecretory cells) and *corpora cardiaca* secretory system in insects as invertebrate representatives, and hypothalamus and the pituitary (adenohypophysis and neurohypophysis) gland system in vertebrates (Scharrer and Scharrer, 1944). Nevertheless, more analogies can be found on functional level where the comparison of glucagon and AKH roles in stress situations and maintenance of homeostasis represents a typical example. The hormonal regulation of response to stress situations is under intensive investigation and emerging evidence from diverse organism (both invertebrates and vertebrates) points to the conserved nature of the hormones involved at the functional level, if not at the structural level. Information generated on role of adipokinetic hormones in insects will inform us of likely modes of action of glucagon and GLP's (GLP-1 and GLP-2) in mammalian systems and vice versa. The unique roles of glucagon and GLP's (GLP-1 and GLP-2) in stress response in higher animals as well as a similar role of adipokinetic peptides in insects offer tantalizing glimpses of the evolutionary significance of stress responsive hormones and its likely conserved role at a physiological level despite evolutionary divergence more than ~900 million years ago. On the other hand the example of glucagon activities in insects suggests that functional role of the hormones can be modified or changed during the evolution. It is evident that glucagon in insects where the control of carbohydrate level in the hemolymph is less accurate and its substantial fluctuations are tolerated, probably focused on protection of insect body against the oxidative stress. Therefore a fuller understanding of the functions and the precise pathways by which these hormones operate in their respective systems will require intense multidisciplinary efforts from physiologists, molecular biologists, biochemists and anatomists. However, intensive research with more sophisticated instrumentation and precise methodologies will likely generate fundamental knowledge on aspects that will be important from the standpoint of human medicine. Future studies will not only unravel the precise role of stress responsive hormones in such diverse systems but also disclose the identity and cross-talk of additional pathways involved in the process of response to stress and organismal homeostasis.

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CHAPTER III

Adipokinetic hormone counteracts oxidative stress elicited in insects by hydrogen peroxide: in vivo and in vitro study.

Colony of Pyrrhocoris apterus.

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Adipokinetic hormone counteracts oxidative stress elicited in insects by hydrogen peroxide: *in vivo* and *in vitro* study

ANDREA BEDNÁŘOVÁ[1,2], NATRAJ KRISHNAN[3], I-CHENG CHENG[1,4], JOSEF VEČEŘA[1,2], HOW-JING LEE[5] and DALIBOR KODRÍK[1,2]

[1]Institute of Entomology, Biology Centre, Academy of Science, České Budějovice, Czech Republic, [2]Faculty of Science, South Bohemian University, České Budějovice, Czech Republic, [3]Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, Mississippi, U.S.A., [4]Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan and [5]Department of Entomology, National Taiwan University, Taipei, Taiwan

Abstract. The role of adipokinetic hormone (AKH) in counteracting oxidative stress elicited in the insect body is studied in response to exogenously applied hydrogen peroxide, an important metabolite of oxidative processes. *In vivo* experiments reveal that the injection of hydrogen peroxide (8 μmol) into the haemocoel of the firebug, *Pyrrhocoris apterus* L. (Heteroptera: Pyrrhocoridae) increases the level of AKH by 2.8-fold in the central nervous system (CNS) and by 3.8-fold in the haemolymph. The injection of hydrogen peroxide also increases the mortality of experimental insects, whereas co-injection of hydrogen peroxide with Pyrap-AKH (40 pmol) reduces mortality to almost control levels. Importantly, an increase in haemolymph protein carbonyl levels (i.e. an oxidative stress biomarker) elicited by hydrogen peroxide is decreased by 3.6-fold to control levels when hydrogen peroxide is co-injected with Pyrap-AKH. Similar results are obtained using *in vitro* experiments. Oxidative stress biomarkers such as malondialdehyde and protein carbonyls are significantly enhanced upon exposure of the isolated CNS to hydrogen peroxide *in vitro*, whereas co-treatment of the CNS with hydrogen peroxide and Pyrap-AKH reduces levels significantly. Moreover, a marked decrease in catalase activity compared with controls is recorded when the CNS is incubated with hydrogen peroxide. Incubation of the CNS with hydrogen peroxide and Pyrap-AKH together curbs the negative effect on catalase activity. Taken together, the results of the present study provide strong support for the recently published data on the feedback regulation between oxidative stressors and AKH action, and implicate AKH in counteracting oxidative stress. The *in vitro* experiments should facilitate research on the mode of action of AKH in relation to oxidative stress, and could help clarify the key pathways involved in this process.

Key words. Adipokinetic hormone, catalase, ELISA, hydrogen peroxide, malondialdehyde, oxidative stress, protein carbonyls.

Introduction

Oxidative radicals are generated during normal oxidative processes in the great majority of eukaryotic cells and extracellular fluids. However, when the production of free

Correspondence: Dr Dalibor Kodrík, Institute of Entomology, Biology Centre, Academy of Science, Branišovská 31, České Budějovice 370 05-CZ, Czech Republic. Tel.: +420 387 775 271; e-mail: kodrik@entu.cas.cz

radicals is enhanced and their scavenging systems are impaired, the situation results in oxidative stress. Oxidative stress may lead to membrane or the whole cell damage by lipid peroxidation and protein oxidation resulting in uncontrolled apoptosis. Eukaryotes, including insects, possess a suite of antioxidant enzymes that protects their cells from oxidative radicals (Ahmad, 1992).

Hydrogen peroxide (H_2O_2) is a stable by-product that is formed during the generation of oxidative radicals. It plays a crucial role in cellular signalling processes and can also potentiate anti-oxidative mechanisms but, if produced in excess, can have deleterious consequences (Veal *et al*., 2007). This radical is produced by superoxide dismutase from the $O_2^{\bullet -}$ radical in the creation of oxygen: $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$ (Fridovich, 1978). By contrast, hydrogen peroxide is quickly converted to water and oxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$) by catalase. Ascorbate peroxidase also scavenges hydrogen peroxide (ascorbic acid $+ H_2O_2 \rightarrow$ dehydroascorbic acid $+ 2H_2O$), although only at low concentrations (Clavaron-Mathews *et al*., 1997), which are not normally scavenged by catalase. Insects also possess a glutathione *S*-transferase with peroxidase-like activity that is effective in targeting hydroperoxides (Ahmad *et al*., 1991; Felton & Duffey, 1991; Ahmad, 1992) but not hydrogen peroxide.

Insect hormones are involved in activation of anti-oxidative enzymes (Krishnan *et al*., 2007; Alquicer *et al*., 2009; Krishnan & Kodrík, 2012) and the most important of these are adipokinetic hormones (AKHs) (Kodrík *et al*., 2007; Večeřa *et al*., 2007). The AKHs are a group of arthropod peptide neurohormones synthesized by neurosecretory cells from the *corpora cardiaca*, a neuroendocrine gland connected to the central nervous system (CNS) (Gäde *et al*., 1997). A major function of AKHs is the control of insect metabolism. Generally, they behave as typical stress hormones by stimulating catabolic reactions (mobilise lipids, carbohydrates and/or certain amino acids), making energy more available, at the same time as inhibiting synthetic reactions. They mobilize entire energy reserves to combat the immediate stress problems and suppress processes that are momentarily less important and could, if allowed to continue, even draw on the mobilized energy. However, these peptides are pleiotropic, with a number of functions in addition to their metabolic role, involving a response to stress situations that do not include rapid production and subsequent consumption of energy (Kodrík, 2008), which also include oxidative stress. Adipokinetic hormones mobilize anti-oxidative mechanisms that ameliorate damage incurred by oxidative stress: increased protein carbonylation, a decrease in the level of reduced glutathione, and impaired total antioxidant activity in haemolymph (Večeřa *et al*., 2007). These data indicate that there is a feedback regulation between oxidative stress and the actions of AKHs, and that AKHs might be involved in the activation of the anti-oxidative protective mechanisms, thus countering the effects of oxidative stress. The mode of action in this feedback regulation remains unclear, although oxidative stress could be a causative factor that accelerates the synthesis or release of AKH in or from the *corpora cardiaca* (Kodrík *et al*., 2007; Večeřa *et al*., 2007).

The present study aims to test the possibility that exogenous application of hydrogen peroxide, an important oxidative stressor, can contribute to an understanding of the role of AKHs in the protection of the insect body from oxidative stress, and also aims to develop a robust and reliable system for replicating *in vitro* the results obtained from *in vivo* studies, which could be utilized as the basis for studying in more detail the mode of action of AKH in relation to oxidative stress.

Materials and methods

Experimental insects

Laboratory stock cultures of the firebug *Pyrrhocoris apterus* L. (Heteroptera: Pyrrhocoridae) originating from the wild populations at České Budějovice, Czech Republic (48°59′N, 14°28′E) were used in the present study. All stages, from egg to adult, were reared under a long-day (LD 18 : 6 h) photocycle at a temperature of $26 \pm 1\,^{\circ}C$, allowing continuous breeding of the bugs. Larvae and adults were supplied with linden seeds (from *Tilia cordata* Miller) and water *ad libitum*. Further details on the breeding of this culture are provided elsewhere (Socha & Šula, 1996). To avoid possible complications from the ovarian cycle, only adult males (5–10 days old) were used for experiments.

Insect treatments

To study the effect of hydrogen peroxide on insect neurohormonal system and on activation of anti-oxidative stress mechanisms, a toxicity assay to hydrogen peroxide was first performed. The experimental insects were injected individually into the haemocoel with 2 μL of hydrogen peroxide solution in Ringer's saline, containing increasing doses of hydrogen peroxide (4.4–17.7 μmol). The Ringer's saline (NaCl 7.5 g, KCl 0.1 g, $CaCl_2.2H_2O$ 0.2 g, $MgCl_2.6H_2O$ 0.4 g, $NaHCO_3$ 0.2 g per 1 L) was prepared in accordance with a slightly modified Ephrussi & Beadle recipe (1937). The LD_{50} of hydrogen peroxide per insect (7.7 μmol) was determined from the numbers of test insects that died during the 24 h post-treatment ($n = 18–22$ per group) and calculated using EPA PROBIT, version 1.5 (U.S.A. Environmental Protection Agency, Cincinnati, Ohio). A similar dose (8 μmol) was then used to study interactions of hydrogen peroxide with the levels of AKH in the CNS and haemolymph, as well as the effects of hydrogen peroxide on oxidative stress markers (see below). In some experiments (e.g. investigating the effects of hydrogen peroxide on oxidative stress markers and on percentage mortality), hydrogen peroxide was also injected in combination with one of the two AKHs from *P. apterus* – Pyrap-AKH (Kodrík *et al*., 2000) at a dose of 8 μmol hydrogen peroxide + 40 pmol Pyrap-AKH, again in 2 μL of Ringer's saline. The other native AKH from *P. apterus* (Peram-CAH-II) (Kodrík *et al*., 2002b) was not tested in the present study because of its functional similarity with Pyrap-AKH, and a presumed similarity of action. Injections

of Ringer's saline only (2 μL) and Pyrap-AKH only (40 pmol; for selection of the dose, see Večeřa *et al*., 2007) were used as controls. The tissues (CNS and haemolymph), in which the level of oxidative stress biomarkers and also the level of AKH (see below) were determined, were sampled 24 h after the injection of test solutions.

Treatment of CNS with hydrogen peroxide and AKH in vitro

Groups of 10–15 CNS (brain with *corpora cardiaca* and *corpora allata* attached) were dissected from *P. apterus* and incubated in 200 μL of Elliot's buffer (129.7 mM NaCl, 5.44 mM KCl, 1.2 mM $MgCl_2 \bullet 6H_2O$, 4.2 mM $NaHCO_3$, 7.3 mM NaH_2PO_4, 20 mM HEPES, 63 mM sucrose, 1 mM $CaCl_2$) supplemented (in different experiments) with 2 μL of Ringer's saline only (control), 2 μL of Pyrap-AKH (40 pmol), 2 μL of hydrogen peroxide (8 μmol) or 2 μL of a combination of Pyrap-AKH (40 pmol) + hydrogen peroxide (8 μmol). After 4 h of incubation, the CNS from each treatment were assayed separately for biomarkers of oxidative stress.

Determination of AKH level in CNS and haemolymph

Methanolic extracts (80% methanol) of the CNS and/or haemolymph were used for determination of the AKH level by means of a competitive enzyme-linked immunosorbent assay (ELISA). Pre-purification by chromatography was essential for determination of the AKH level in the haemolymph (Goldsworthy *et al*., 2002). In the ELISA, 0.25 CNS equivalents and a 20-μL haemolymph equivalent were used, in accordance with the protocol described previously (Goldsworthy *et al*., 2002; Kodrík *et al*., 2003). Briefly, rabbit antibodies were raised commercially against Cys^1-Pyrap-AKH (Sigma Genosys Biotechnologies, Sigma-Aldrich Co. LLC, St Louis, Missouri) and the resulting antibody recognized both the *P. apterus* AKHs: Pyrap-AKH (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-amide; Kodrík *et al*., 2000) and Peram-CAH-II (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-amide; Kodrík *et al*., 2002b) that differ by one amino acid at position 3. The ELISA comprised precoating of the 96-well microtitre plates (high binding Costar; Corning Incorporated, Corning, New York) overnight with immunoglobulin G (dilution 1 : 10 000 for CNS and 1 : 5000 for haemolymph). A biotinylated probe prepared from Cys^1-Pyrap-AKH using Biotin Long Arm Maleimide (BLAM, Vector Laboratories, U.K.) was used in the assay (Kodrík *et al*., 2003). The synthetic Pyrap-AKH (Polypeptide Laboratories, Czech Republic) was used as a standard for AKH quantification.

Determination of oxidative stress markers

Protein carbonyl level in haemolymph: in vivo experiments. Total protein carbonyls were quantified after their reaction with 2,4-dinitrophenylhydrazine (DNPH) (Levine *et al*., 1990) with some modifications (Krishnan & Kodrík, 2006; Krishnan *et al*., 2007). Carbonyls were quantified spectrophotometrically at 370 nm in a microtitre plate reader. The results were expressed as nmol mg^{-1} protein using an extinction coefficient of 22 000 M^{-1} cm^{-1}. A bovine serum albumin standard curve was used to quantify protein concentrations in guanidine solutions measured at 280 nm. Protein carbonyl values were corrected for interfering substances by subtracting the A_{370}/mg protein measured without DNPH (controls).

Protein carbonyl level in CNS: in vitro experiments. Protein carbonyls were semi-quantified by western blots using the Oxyblot protein oxidation detection kit (Millipore, Billerica, Massachusetts) as described previously (Alquicer *et al*., 2009). In brief, CNS (approximately 15) that were incubated in various treatments were spun down, resuspended and homogenized using the Kimble-Kontes motorized pestle homogenizer in 200 μL of 50 mM KPO_4 buffer purged with nitrogen gas, followed by treatment with DNPH to derivatize the carbonyl groups in the protein side chains. The DNPH derivatized protein samples (approximately 25 μg) were separated by polyacrylamide gel electrophoresis in an 8–16% Tris-HCl precast ready gel (Bio-Rad, Hercules, California) followed by transfer onto polyvinylidene Immobilon fluoride membranes (Millipore) overnight at approximately 4 °C at a constant voltage of 10 V and then 25 V for 1 h. Membranes were blocked for 2 h with 5% skimmed milk in phosphate-buffered saline with Tween 20 and incubated overnight with primary antibody (dilution 1 : 150, anti-DNP antibody in rabbit) followed by goat anti-rabbit (IR680) secondary antibody (dilution 1 : 20 000) tagged with an infrared dye (Li-Cor Biosciences, Lincoln, Nebraska). The carbonylated proteins were visualized using fluorescence in an Odyssey CLx Infrared scanner (Li-Cor Biosciences). The results of the three separate Western blots were quantified using IMAGE STUDIO, version 2.0 (Li-Cor Biosciences) and averaged. Coomasie blue stained gels were run in parallel to check for equal protein loading (data not shown).

Lipid peroxidation in CNS: in vitro experiments. Lipid peroxidation was evaluated as the amount of malondialdehyde (MDA) adduct formed with thiobarbituric acid (TBA) in the CNS using fluorimetric detection (Uchiyama & Mihara, 1978). In brief, the CNS samples were homogenized in phosphoric acid (0.2% v/v), heated to 95 °C in the presence of TBA solution (0.6% TBA stabilized with 1 mM butylated hydroxytoluene in 50 mM NaOH) for 30 min and, after cooling, the TBA–MDA complexes were extracted by partitioning by *n*-butanol. The butanol phase was collected and the solvent removed in a vacuum evaporator. The residues were reconstituted in elution buffer (35% methanol in 50 mM KH_2PO_4-KOH, pH 7.0) and their purity verified using a Waters high-performance liquid chromatography (HPLC) system (Waters Corp, Milford, Massachusetts) with CLARITY (DataApex, Czech Republic) software using the reversed-phase HPLC C18 column (Purospher RP-18; Merck, Germany). The samples were run under isocratic conditions at a flow rate of 0.5 mL min^{-1} and fluorescence detection at λ_{Ex} 515 nm and

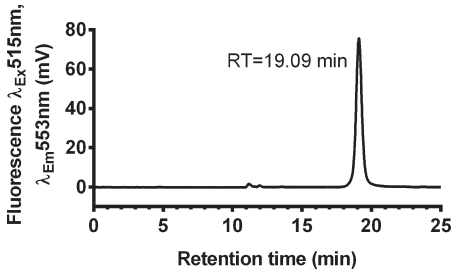

Fig. 1. Representative reversed-phase high-performance liquid chromatogram of a malondialdehyde-thiobarbituric acid [MDA-$(TBA)_2$] adduct from a lipid peroxidation assay of a control central nervous system sample of *Pyrrhocoris apterus*; for details, see Material and methods. RT, retention time.

λ_{Em} 553 nm: the MDA–$(TBA)_2$ adduct represented a single peak (retention time = 19.09 min; Fig. 1) that was quantified against a calibration curve prepared with MDA standard. It was established previously (Krishnan *et al*., 2009) that recovery using this process varied between 92% and 96% when some of the samples are spiked with known concentrations of MDA to assess loss during the extraction process. As a result of the high purity, the CNS samples were quantified in a Synergy 4 fluorimeter plate reader (BioTek, Germany) without employing HPLC using the same excitation and emission spectra and the results were compiled and expressed as MDA equivalents in nmol mg^{-1} protein.

Determination of antioxidative response

Catalase activity in CNS: in vitro experiments. Catalase activity was assayed in CNS homogenates from the various treatments using the Amplex Red Catalase Assay Kit (A22180) (Molecular Probes–Invitrogen Detection Technologies, U.K.) in accordance with the manufacturer's instructions. Catalase activity was expressed as mU mg^{-1} protein. Protein content in all assays was estimated using the BCA protein assay reagent (Stoscheck, 1990).

Statistical analysis

The results were plotted and statistical analyses were performed using the PRISM, version 5.0 (GraphPad Software, San Diego, California). The symbols or bars in graphs represent the mean ± SD. The significances of the difference between results were evaluated using one-way analysis of variance with Tukey's multiple comparison test because this test compares all treated groups including controls defined by a single factor.

Results

Dose–response of mortality after hydrogen peroxide treatment in P. apterus adults

A dose–response study of the effect of hydrogen peroxide on the mortality of *P. apterus* adults was conducted to obtain the

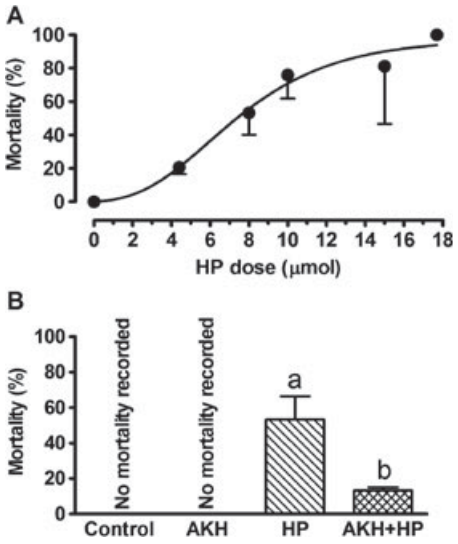

Fig. 2. The effect of the injection of hydrogen peroxide (HP) on the mortality of *Pyrrhocoris apterus* adults. (A) Dose–response effect on the mortality of hydrogen peroxide (4.4–17.7 µmol) in *P. apterus* adults. (B) Effects on mortality of Pyrap-adipokinetic hormone (AKH) (40 pmol) or hydrogen peroxide (8 µmol) injected separately, and Pyrap-AKH and hydrogen peroxide (40 pmol + 8 µmol) co-injected in *P. apterus* adults 24 hours post-treatment. Different letters above the columns represent significant differences at the 5% level computed using one-way analysis of variance with Tukey's multiple comparison test ($n = 5$ groups; 18–22 insects per group).

LD_{50} dose that would be used in subsequent studies. A range of doses of hydrogen peroxide from 4.4 to 17.7 µmol was injected into the haemocoel of *P. apterus* adults and mortality evaluated after 24 h. The data were subjected to Probit analysis, which revealed the LD_{50} dose to be 7.7 µmol (Fig. 2A). Hence, for all subsequent studies, a dose of 8 µmol of hydrogen peroxide was used to elicit oxidative stress *in vivo*, as well as *in vitro*. Mortality decreased significantly (to 13%) when the LD_{50} dose of hydrogen peroxide was co-injected with Pyrap-AKH (40 pmol) (Fig. 2B), whereas no mortality was recorded in the control and AKH-treated groups.

Effect of hydrogen peroxide on AKH titre in CNS and haemolymph of P. apterus adults

Injection of 8 µmol hydrogen peroxide into the body of adult female *P. apterus* stimulated a highly significant increase in AKH titres: 2.8-fold in CNS and 3.8-fold in haemolymph, respectively (Fig. 3).

Effect of hydrogen peroxide and Pyrap-AKH on protein carbonyl content in the haemolymph

Determination of the protein carbonyl levels in the haemolymph after treatment with 8 µmol hydrogen peroxide resulted in a significant (2.6-fold) increase in protein carbonyl levels compared with controls (Ringer's saline injection only),

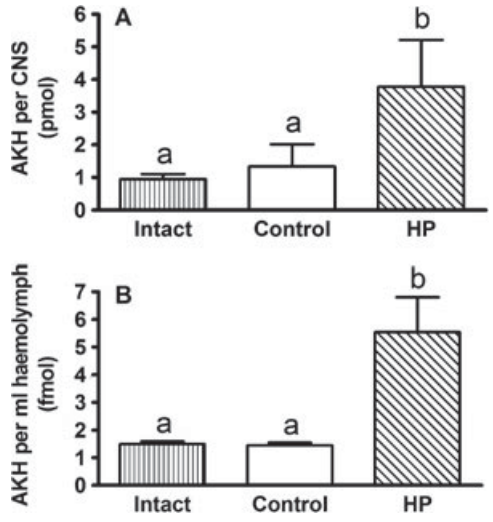

Fig. 3. The effect of hydrogen peroxide (HP) injection (8 µmol) on the levels of adipokinetic hormone (AKH) (Pyrap-AKH + Peram-CAH-II) in the *Pyrrhocoris apterus* adult (A) central nervous system and (B) haemolymph 24 h after treatment. Controls were injected with Ringer's saline only, and intact individuals were without any treatment. Different letters above the columns represent significant differences at the 5% level computed using one-way analysis of variance with Tukey's multiple comparison test ($n = 5–11$).

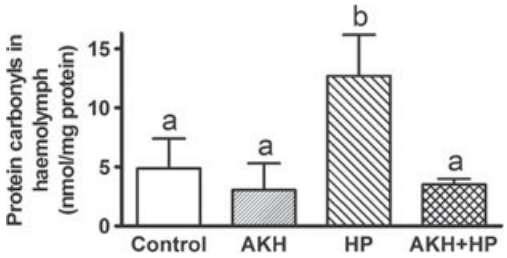

Fig. 4. The effect of the injection of Pyrap-adipokinetic hormone (AKH) (40 pmol) and hydrogen peroxide (HP) (8 µmol) into the haemocoel of the experimental *Pyrrhocoris apterus* adults on haemolymph protein carbonyl content 24 h after treatment. Different letters above the columns represent significant differences at the 5% level computed using one-way analysis of variance with Tukey's multiple comparison test ($n = 3$).

whereas injection of 40 pmol Pyrap-AKH had no significant effect. Interestingly, co-injection of hydrogen peroxide with Pyrap-AKH resulted in protein carbonyl levels that were not significantly different from Ringer's saline-treated controls (Fig. 4).

Effect of hydrogen peroxide and Pyrap-AKH on oxidative stress biomarkers in vitro

To confirm the effect of hydrogen peroxide and Pyrap-AKH on oxidative stress characteristics obtained *in vivo*, an *in vitro* approach was employed, wherein isolated CNS were used as target organs. The purpose of the *in vitro* studies was to develop a robust *in vitro* model system whereby various roles of AKH in the stimulation of anti-oxidative defence mechanisms, including their mode of action, could be studied in the future. Lipid peroxidation product thiobarbituric acid reactive substances, represented by MDA content (Fig. 5), and protein carbonyl levels (Fig. 6) were determined using the same treatment protocols (hydrogen peroxide and Pyrap-AKH) and their effect on isolated CNS was evaluated after 4 h of incubation. Co-incubation the CNS with hydrogen peroxide (8 µmol) resulted in a significant elevation of the deleterious oxidative stress markers: MDA content (Fig. 5) and protein carbonyl content (Fig. 6B). However, Pyrap-AKH treatment (40 pmol) alone either significantly reduced MDA levels (Fig. 5) or had no significant effect on protein carbonyl level (Fig. 6B), as in the case of *in vivo* assay (Fig. 4) However, the importance of Pyrap-AKH in activation of protective mechanisms was seen when the oxidative stressor was present (i.e. when Pyrap-AKH was co-incubated with hydrogen peroxide). In these experiments, co-treatment with Pyrap-AKH and hydrogen peroxide resulted in a significant reduction of oxidative stress markers compared with that elicited by hydrogen peroxide alone, and similar to control levels (Figs 5 and 6).

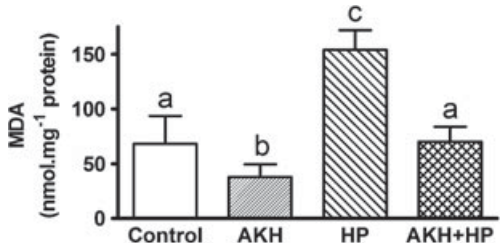

Fig. 5. The effect of Pyrap-adipokinetic hormone (AKH) (40 pmol) and hydrogen peroxide (HP) (8 µmol) applied to the incubation medium on the level of malondialdehyde-thiobarbituric acid [MDA-$(TBA)_2$] adducts in central nervous system samples of *Pyrrhocoris apterus* adults measured after 4 h of incubation *in vitro*. Different letters above the columns represent significant differences at the 5% level computed using one-way analysis of variance with Tukey's multiple comparison test ($n = 5–6$).

Effect of hydrogen peroxide and Pyrap-AKH on catalase activity

Incubation in the presence of 8 µmol hydrogen peroxide *in vitro* significantly impaired catalase activity in the CNS (Fig. 7), whereas co-incubation with hydrogen peroxide (8 µmol) and Pyrap-AKH (40 pmol) resulted in catalase levels that were not significantly different from the control level. As in previous experiments, incubation of the CNS in the presence of Pyrap-AKH alone (i.e. with no stressor) resulted in no discernible effect on the catalase activity.

Discussion

Reactive oxygen species such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$) or the hydroxyl radical ($^{\bullet}OH$) occur during

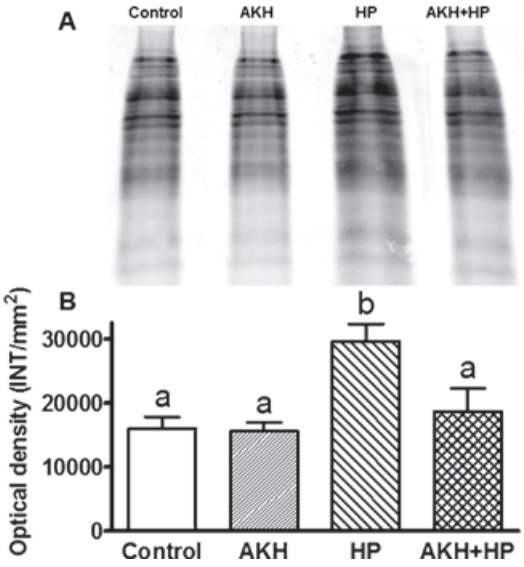

Fig. 6. The effect of Pyrap-adipokinetic hormone (AKH) (40 pmol) and hydrogen peroxide (HP) (8 µmol) applied to the incubation medium on protein carbonyl levels in central nervous system samples of *Pyrrhocoris apterus* adults determined after 4 h of incubation *in vitro*. (A) One of the protein carbonyl western blots and (B) quantification of the optical densities of the whole range of carbonylated proteins. Different letters above the columns represent significant differences at the 5% level computed using one-way analysis of variance with Tukey's multiple comparison test ($n = 3$).

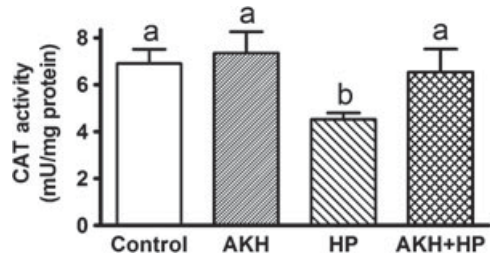

Fig. 7. The effect of Pyrap-adipokinetic hormone (AKH) (40 pmol) and hydrogen peroxide (HP) (8 µmol) on catalase (CAT) activity in central nervous system samples of *Pyrrhocoris apterus* adults measured after 4 h of incubation *in vitro*. Different letters above the columns represent significant differences at the 5% level computed using one-way analysis of variance with Tukey's multiple comparison test ($n = 5$).

incompatible electron transfer in all aerobic living organisms (Farr & Kogoma, 1991; Valko *et al*., 2007). Oxidative stress reflects the disruption of an intricate balance between the formation and clearance of highly-reactive free radicals in living organisms. The increased production of reactive oxygen species that exceeds the capacities of cellular defence systems leads to oxidative stress in the cell and to the oxidation of proteins, lipids and nucleic acids (Imlay, 2003; Stadtman & Levine, 2003). Insects are subjected to various environmental stressors that lead to the generation of deleterious reactive oxygen species (Felton, 1995). Cellular defence systems to counter the damaging effects of reactive oxygen species can be divided into three groups: (i) detoxification of harmful agents (e.g. by classical antioxidant enzyme systems such as catalase or superoxide dismutase); (ii) protection of macromolecules (e.g. carbonyl reductase); and (iii) repair or removal of damaged molecules (e.g. thioredoxin reductase, methionine sulfoxide reductase, etc.). However, the actual details of the coordinated regulation of these processes depend on the type of stressor, as well as the specific pathways that are activated in response to the stressor. Hydrogen peroxide is better known for its cytotoxic effects and, in recent years, it has also become established as an important regulator of eukaryotic signal transduction. In unicellular organisms, an important response to increased levels of hydrogen peroxide is the increased production of antioxidants and repair proteins that allow adaptation to these oxidative conditions (Storz & Tartaglia, 1992; Jamieson, 1998). Although, as a nonpolar molecule, hydrogen peroxide is able to diffuse across membranes, it is reported to be less effective at eliciting a signalling response when added exogenously than endogenously produced hydrogen peroxide (Choi *et al*., 2005). Consistent with this, the present study shows that it is possible to elicit oxidative stress in a model system by exogenous application of hydrogen peroxide, and to confirm that the neuropeptide AKH is indeed able to function as a stress responsive protective hormone.

Of the different organs and tissues, the CNS is one of the most sensitive to changes in the redox status, as well as the potential for oxidative damage, because of the high level of oxidative metabolism under normal physiological conditions and the abundance of post-mitotic highly differentiated cell elements that are hard to replace if damaged. The most common approaches to modelling oxidative stress in the CNS include the exposure of primary neural cell cultures, brain tissue explants and/or neural cell lines to pro-oxidative conditions. The methodological and practical aspects of *in vitro* based models of oxidative stress are discussed in several research reports and reviews (Gille & Joenje, 1992; Hirrlinger *et al*., 1999; Ricart & Fiszman, 2001; Halliwell, 2003). The objective of the present study is to investigate the involvement of AKH in regulating responses to oxidative stress. Hence, in the present study, after *in vivo* investigations, the entire isolated CNS is used as the model of choice for *in vitro* studies aiming to investigate adaptive cell responses.

The injection of increasing doses of hydrogen peroxide into the haemocoel reveals an adverse effect on survival of the experimental *P. apterus*. However, survival is increased when Pyrap-AKH is co-injected with hydrogen peroxide at the LD_{50} dose. This observation supports recent studies reporting that AKH is involved in the activation of anti-oxidative or protective mechanisms (Kodrík *et al*., 2007; Večeřa *et al*., 2007, 2012; Velki *et al*., 2011; Krishnan & Kodrík, 2012) and that it probably eliminates, or at least reduces, the toxic effect of injected hydrogen peroxide. The injection of Pyrap-AKH alone, at the concentrations tested, had no effect on the mortality of *P. apterus*.

There are many studies, mainly conducted in vertebrates, concerning a possible role of hydrogen peroxide in the

regulation of the hormonal response. In monkey cell cultures, hydrogen peroxide treatment alters the DNA binding of the glucocorticoid receptor, and thus represses glucocorticoid-induced effect within the cell. The response is prevented by the glutathione precursor *N*-acetylcysteine (Esposito *et al*., 1995). Additionally, the functioning of the oestrogen receptor is also repressed by hydrogen peroxide at micromolar concentrations in human breast cancer cells (Rao *et al*., 2009). By contrast, the insulin and glucagon responses are unaffected by moderate oxidative stress (Sutherland *et al*., 1997). It is also reported that, in O_2- sensitive PC-12 cells, the expression of tyrosine hydroxylase mRNA correlates inversely with hydrogen peroxide formation, and treatment with hydrogen peroxide reduces the level of tyrosine hydroxylase mRNA (Kroll & Czyzyk-Krzeska, 1998). Tyrosine hydroxylase is also present in insects and, because this enzyme is rate limiting in the synthesis of catecholamines, a similar redox regulation could influence the secretion of insect hormones such as AKH.

AKH participates also in stress reactions that do not include rapid production and subsequent consumption of energy (Kodrík, 2008). It appears that the injection of hydrogen peroxide into the body of *P. apterus* stimulates responses that fall into this category. The injection of hydrogen peroxide primarily increases the level of AKHs in the CNS and haemolymph. A similar stress-induced elevation of the AKH titre occurs also after an insecticide treatment not only in *P. apterus* (Kodrík & Socha, 2005), but also in *Schistocerca gregaria* (Candy, 2002). The AKH level in CNS and/or haemolymph is increased also after excessive KCl treatment (Candy, 2002), photophase interruption (Kodrík & Socha, 2005) or exposure to constant darkness (Kodrík *et al*., 2005). Significant increases in the titre of AKH occur also in the Colorado potato beetle *Leptinotarsa decemlineata* after feeding on the genetically modified potatoes expressing *Bacillus thuringiensis* toxin (Cry 3Aa) or *Gallanthus nivalis* lectin (GNA) (Kodrík *et al*., 2007). Interestingly, these stressors also cause oxidative stress similar to that induced by the herbicide paraquat, which is used commonly to elicit oxidative stress in animals, through redox-cycling reactions (Hassan, 1984). Paraquat applied to insects also increases the titre of AKH in both the *corpora cardiaca* (*L. decemlineata*) and haemolymph (*L. decemlineata, P. apterus*) (Kodrík *et al*., 2007; Večeřa *et al*., 2007). Interestingly, paraquat does not increase the level of AKH in the CNS of *P. apterus*, in contrast to that induced by hydrogen peroxide in the present study. The reasons for such a difference could be many but, essentially, it may be related to the timing used for the evaluation of the effects. The effects are evaluated after 24 h in the present study, whereas Večeřa *et al*. (2007) use a considerably shorter time interval of 4 h, which may be too short for the changes of AKH level in the CNS to be measurable.

Because the injection of hydrogen peroxide, paraquat and the toxins from genetically-modified potatoes have similar effects on AKH levels (at least in the haemolymph), it is likely that the mechanisms of AKH stimulation could be similar. Moreover, an injection of exogenous AKH mobilizes anti-oxidative mechanisms that ameliorate damage incurred by oxidative stressors: increased protein carbonyls, a decrease of reduced glutathione levels and attenuation of total antioxidant activity in haemolymph (Kodrík *et al*., 2007; Večeřa *et al*., 2007). The injection of the oxidative stress metabolite, hydrogen peroxide, results in a significant increase (2.6-fold) in protein carbonyl levels in the haemolymph within 24 h post-injection, which indicates a rapid oxidation of the haemolymph proteins. However, co-injection of hydrogen peroxide with Pyrap-AKH elicits a lower level of the protein carbonyl than injection of hydrogen peroxide alone (3.6-fold). Injection of Pyrap-AKH alone does not result in significant change in protein carbonyl levels, which are maintained at control levels.

Interestingly, the *in vitro* studies show that, even in an isolated organ, such as the CNS, treatment with Pyrap-AKH is only able to reduce levels of the lipid peroxidation product malondialdehyde to below control values. However, this is not so in the case of protein carbonyls, where Pyrap-AKH treatment alone exerts no effect. Despite this, co-incubation of isolated CNS with hydrogen peroxide and Pyrap-AKH together reduces MDA content and protein carbonyl levels to control levels. This phenomenon leads to the question: do AKHs act at the level of the cell membrane and prevent lipid peroxidation to the exclusion of other metabolic pathways by an as yet unknown mechanism? It is known that lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals and is an indicator of oxidative stress in cells and tissues. Lipid peroxides derived from polyunsaturated fatty acids are unstable and decompose to form a complex series of compounds. These include an abundant and highly-reactive three-carbon dialdehyde produced as a by-product of polyunsaturated fatty acid peroxidation, which combines readily with several functional groups on different molecules, including proteins, lipoproteins and DNA (Janero, 1990). It appears from the present *in vitro* studies that AKH has a role in inhibiting lipid peroxidation, although the mechanism by which it exerts this effect remains unclear.

The present study also examines whether AKH has a stimulatory effect on catalase, the principal enzyme involved in metabolizing hydrogen peroxide. However, AKH treatment alone does not bring about an elevation of catalase activity above control levels, whereas hydrogen peroxide inhibits catalase activity substantially. Interestingly, co-treatment of isolated brains with hydrogen peroxide and AKH does not restore catalase activity to control or AKH treatment levels, indicating that, at least for potentiation of a principal antioxidant system, AKH functions only in presence of a stressor. A phenomenon such as this is not completely unknown: a positive correlation between the hyperlipaemia that is elicited by Pyrap-AKH in *P. apterus* and the effect of the same hormone on locomotion is observed only when the hormone is applied by injection. In the case of the topical application of Pyrap-AKH, when an injury stressor is absent, the correlation is not observed and both responses are slower and more diffuse in time (Kodrík *et al*., 2002a).

Taken together, these observations confirm that there is a feedback regulation between an oxidative stressor (hydrogen peroxide) and the actions of AKH, and that AKHs might participate directly or indirectly in oxidative stress responses, either by detoxification of harmful radicals through enhancement of

antioxidant systems, or by stimulation of the repair of damaged molecules, or their removal. It could be that AKH performs these roles either simultaneously or in a mutually exclusive manner. The precise possible mode of action in this process is as yet unclear, although oxidative stress could be a causative factor that accelerates the synthesis of AKH or its release from the *corpora cardiaca* (Kodrík *et al*., 2007; Večeřa *et al*., 2007).

The findings of the present study also demonstrate that isolated organ cultures such as the CNS can serve as an excellent model *in vitro* for studying the mechanism of action of AKH in conferring protection against oxidative stress.

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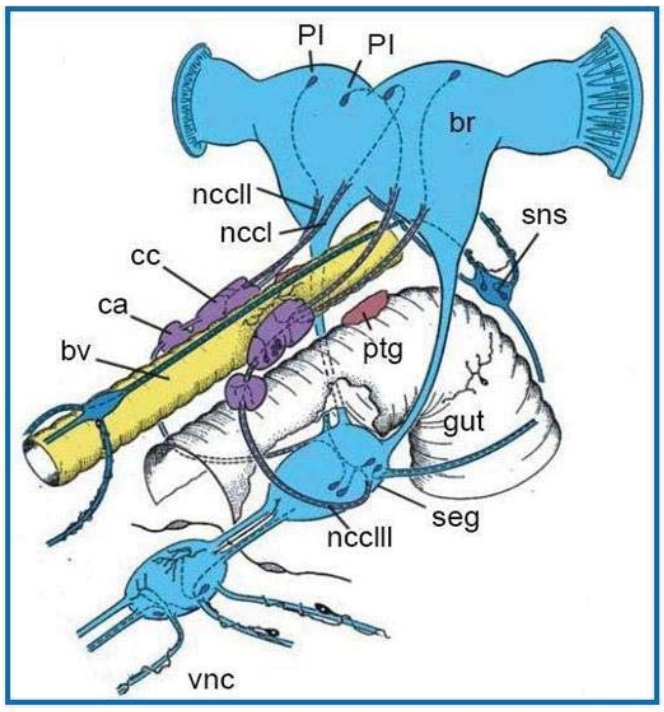
CHAPTER IV

Adipokinetic hormone exerts its anti-oxidative effects using a conserved signal- transduction mechanism involving both PKC and cAMP by mobilizing extra- and intracellular $Ca^{(2+)}$ stores.



Insect central nervous system, adapted from Hartenstein (2006) *J Endocrinol* 190: 555-570

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Adipokinetic hormone exerts its anti-oxidative effects using a conserved signal-transduction mechanism involving both PKC and cAMP by mobilizing extra- and intracellular Ca^{2+} stores

Andrea Bednářová [a,b,c], Dalibor Kodrík [a,b], Natraj Krishnan [c,*]

[a] Institute of Entomology, Biology Centre, Academy of Science, Branišovská 31, České Budějovice, 370 05-CZ, Czech Republic
[b] Faculty of Science, South Bohemian University, Branišovská 31, České Budějovice, 370 05-CZ, Czech Republic
[c] Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, MS 39762, USA

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ABSTRACT

The involvement of members of the adipokinetic hormone (AKH) family in regulation of response to oxidative stress (OS) has been reported recently. However, despite these neuropeptides being the best studied family of insect hormones, their precise signaling pathways in their OS responsive role remain to be elucidated. In this study, we have used an *in vitro* assay to determine the importance of extra and intra-cellular Ca^{2+} stores as well as the involvement of protein kinase C (PKC) and cyclic adenosine 3′,5′-monophosphate (cAMP) pathways by which AKH exerts its anti-oxidative effects. Lipid peroxidation product (4-HNE) was significantly enhanced and membrane fluidity reduced in microsomal fractions of isolated brains (CNS) of *Pyrrhocoris apterus* when treated with hydrogen peroxide (H_2O_2), whereas these biomarkers of OS were reduced to control levels when H_2O_2 was co-treated with Pyrap-AKH. The effects of mitigation of OS in isolated CNS by AKH were negated when these treatments were conducted in the presence of Ca^{2+} channel inhibitors ($CdCl_2$ and thapsigargin). Presence of either bisindolylmaliemide or chelyrythrine chloride (inhibitors of PKC) in the incubating medium also compromised the anti-oxidative function of AKH. However, supplementing the medium with either phorbol myristate acetate (PMA, an activator of PKC) or forskolin (an activator of cAMP) restored the protective effects of exogenous AKH treatment by reducing 4-HNE levels and increasing membrane fluidity to control levels. Taken together, our results strongly implicate the importance of both PKC and cAMP pathways in AKHs' anti-oxidative action by mobilizing both extra and intra-cellular stores of Ca^{2+}.

1. Introduction

Adipokinetic hormones (AKH) are one of the best-defined groups of neurohormones belonging to the arthropod AKH/RPCH family (adipokinetic hormone/red pigment concentrating hormone family) (Gäde et al., 1997; Kodrík, 2008). In general, a major function of these small peptides (octa-, nona- or decapeptides) that are synthesized and released from an endocrine retrocerebral gland – the *corpus cardiacum* (CC), is insect metabolism control. The release of AKH from CC into the hemolymph occurs whenever there is an energy requirement (Gäde and Auerswald, 2003; Van der Horst, 2003; Lorenz and Gäde, 2009) or in response to stress situations (Kodrík, 2008). The immediate trigger for the release of AKH from the neurosecretory cells probably occurs at the level of appreciable changes in general and/or intermediary metabolism provoked by both mentioned stimuli.

AKHs primarily function as stress responsive hormones by stimulating catabolic reactions (mobilization of lipids, carbohydrates, and/or certain amino acids), making energy more available, while inhibiting synthetic reactions. They mobilize entire energy reserves to mitigate the immediate stress problems and suppress processes that are momentarily less important and could, if allowed to continue, even draw on the mobilized energy. These biochemical reactions are accompanied by activation of physiological stress response that includes stimulation of heart beat (Scarborough et al., 1984), increase of muscle tonus (O'Shea et al., 1984), stimulation of general locomotion (Orchard et al., 1983; Socha et al., 1999), enhancement of immune response (Goldsworthy et al., 2002; Goldsworthy et al., 2003) as well as certain other reactions (Steele, 1985; Perić-Mataruga et al., 2006). An active role of AKH in protection of insects against oxidative stress (OS) was reported in a series of publications (Kodrík et al., 2007; Večeřa et al., 2007; Velki et al., 2011; Večeřa et al., 2012; Bednářová et al., 2013b). These results reveal that OS increases the level of AKHs in insect body, and that exogenous AKH influence OS biomarkers in insect tissues (as well as in *in vitro* organ cultures) experimentally enhanced by application of the stressors. The role of AKH in performing functions not necessarily involving the mobilization of energy stores and in controlling

Abbreviations: AKH, adipokinetic hormone; cAMP, cyclic adenosine monophosphate; 4-HNE, 4-hydroxy-2-nonenal; PKC, protein kinase C.

* Corresponding author. Tel.: +1 662 325 2978; fax: +1 662 325 8837.
E-mail address: nk260@msstate.edu (N. Krishnan).

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OS in insects has been reviewed by Kodrík (2008), and by Krishnan and Kodrík (2012).

The precise mechanism by which AKH exerts its anti-OS functions is unclear. It is generally accepted that release of AKH into the blood circulation from the CC results in a cascade of reactions initiated by the binding of these neuropeptides to G-protein coupled receptors (GPCRs) located on the plasma membrane of target cells (Staubli et al., 2002; Caers et al., 2012). GPCRs share a common molecular architecture and a common signaling mechanism involving interaction with G proteins (heterodimeric GTPases) to regulate various intracellular messenger cascades. GPCRs for AKH have been identified and characterized in the fruit fly, *Drosophila melanogaster* and the silkworm, *Bombyx mori* (Park et al., 2002; Staubli et al., 2002; Zhu et al., 2009) as well as in other insect species (Hansen et al., 2006; Kaufmann and Brown, 2006; Wicher et al., 2006; Kaufmann et al., 2009; Zeigler et al., 2011). It has been documented in a number of reports on signaling pathways employed by AKH in energy mobilizing reactions that the neurohormone triggers in the target tissues (mostly fat body) a classical intracellular cascade typical for peptide hormone signaling via the adenylyl cyclase (Spencer and Candy, 1976) or phospholipase C (Vroenen et al., 1997) pathways, both involving presence of extracellular and/or intracellular Ca^{2+} (Van der Horst et al., 2001; Gäde and Auerswald, 2003). Ion channels have two main signaling functions here: either they can generate second messengers or they can function as effectors by responding to such messengers. Their role in signal generation is mainly centered on the Ca^{2+} signaling pathway, which has a large number of Ca^{2+} entry channels and internal Ca^{2+} release channels, both of which contribute to the generation of Ca^{2+} signals. Like other cells, neurons use both extracellular and intracellular sources of calcium (Berridge, 1998).

In our previous study (Bednářová et al., 2013b), we demonstrated that in isolated brain with *corpora cardiaca* and *corpora allata* (CNS) of the adult firebug *Pyrrhocoris apterus,* the exogenous application of Pyrap-AKH (Kodrík et al., 2000) was able to counteract the effects of OS induced by hydrogen peroxide. In the present investigation, we extended the study to determine if both extracellular and/or intracellular Ca^{2+} stores are required, as well as if protein kinase C (PKC) and cAMP are involved in AKHs' action in its protective function against OS.

2. Materials and methods

2.1. Insect and hormones

Laboratory stock culture of the firebug *Pyrrhocoris apterus* (Heteroptera) originating from a wild brachypterous population collected at České Budějovice (Czech Republic, 48º59′N, 14°28′E) were used in the study. All stages from egg to adult were kept in plastic boxes supplied with linden seeds (*Tilia cordata,* Miller) and water *ad libitum*. Insects were reared at 26 ± 1 °C under long-day conditions (light: darkness 18:6 h). Males were separated from females on the first day after adult ecdysis; ten-day-old males (for reasons, see Večeřa et al., 2007) were used for experiments. In the firebug two different AKHs (Pyrap-AKH: pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-NH_2 (Kodrík et al., 2000) and Peram-CAH-II: pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2 (Kodrík et al., 2002) has been found. Because there is no apparent difference in activities between them (Kodrík and Socha, 2005) only Pyrap-AKH has been used in the experiments. The synthetic Pyrap-AKH was commercially prepared by Dr. Lepša, the Vidia Company (Praha, Czech Republic).

2.2. Dose–response study of Ca^{2+}-channel inhibitors

In order to determine the optimal concentrations of Ca^{2+} – channel inhibitors used in the study – the FluoForte Calcium Assay kit was employed (Enzo Life Sciences, USA). This assay provides a homogenous fluorescence-based test for detecting intracellular calcium mobilization across a broad spectrum of biological targets. A day prior to the experiment, ten CNS (with *corpora cardiaca* and *corpora allata* attached) from 10-day old adult males of *P. apterus*, for each inhibitor and dose used, were incubated in 1% collagenase (Sigma-Aldrich, St Louis, MO, USA; Type IV). The next day the cells were washed clean of collagenase and incubated in medium (Elliot's buffer – see next section for full details) with or without $CaCl_2$. Collagenase treatment of CNS was performed only to dissociate the cells for detecting Ca^{2+} mobilization in a plate reader. The dissociated neuronal cells were then processed according to manufacturer's instructions for Ca^{2+} mobilization assay. Cadmium chloride ($CdCl_2$), the universal calcium channel inhibitor, was applied in seven different doses in nmol range (0, 1, 4, 8, 12, 16 and 20 nmol, respectively) whereas the sarcoplasmic/endoplasmic reticulum calcium channel inhibitor – thapsigargin – was applied also in seven doses, but in pmol range (0, 1, 4, 8, 12, 16 and 20 pmol, respectively). Calcium flux within the cells was monitored in a Biotek Synergy H1M plate reader using an excitation wavelength of 490 nm and an emission wavelength of 525 nm. Based on the results of our dose–response study (Fig. 1) we chose to use doses 8 nmol of $CdCl_2$ and 4.8 pmol of thapsigargin, for subsequent experiments that were performed on intact CNS without collagenase (we found that absence of collagenase, did not prevent the mode of action of Ca^{2+} channel inhibitors, see also below).

2.3. In vitro treatments of CNS

In general, all chemicals used in the experiments were purchased from Sigma-Aldrich Co., St. Louis, MO, unless otherwise specified.

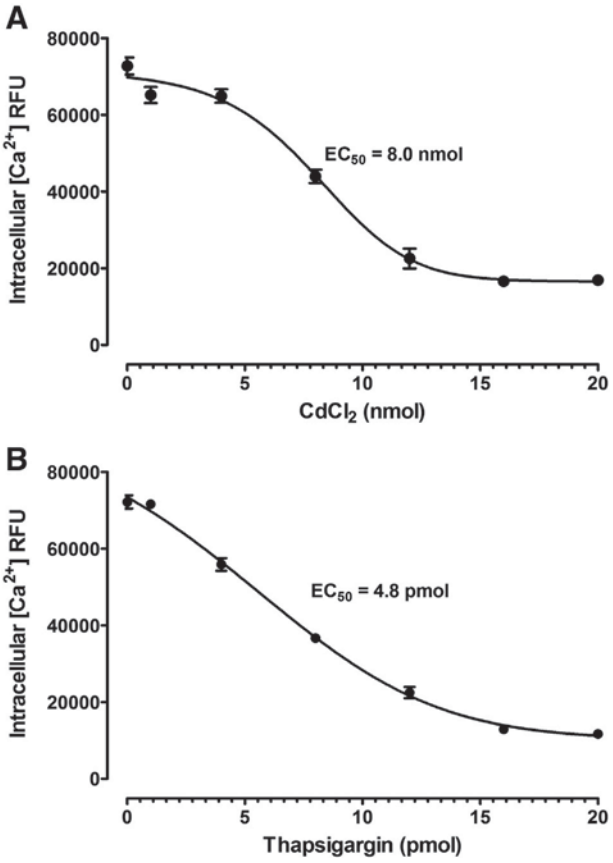

Fig. 1. The effect of increasing doses of cadmium chloride ($CdCl_2$) (**A**), and thapsigargin (**B**) on intracellular calcium mobilization in suspended cells of 10 CNS of *P. apterus* males incubated for 4 h *in vitro*. The values represent means ± SD of relative fluorescence units (RFU) depicting intracellular stores of Ca^{2+} following incubation of dissociated neuronal cells (for details see Materials and methods section).

Ten whole CNS – brain with *corpora cardiaca* and *corpora allata* attached – were dissected from insects and incubated in 200 μL of Elliot's buffer (129.7 mM NaCl, 5.44 mM KCl, 1.2 mM $MgCl_2$.6 H_2O, 4.2 mM $NaHCO_3$, 7.3 mM NaH_2PO_4, 20 mM HEPES, 63 mM saccharose, 1 mM $CaCl_2$). In a basic experimental set, where the effect of AKH and hydrogen peroxide (H_2O_2) on the oxidative stress was documented (see Fig. 2), and which served as a control set for the following "signaling" experiments (see below), the medium with CNS was supplemented separately with 2 μL of Ringer saline (control), 40 pmol of Pyrap-AKH in 2 μL (for selection of the dose see Večeřa et al., 2007) or 8 μmol of H_2O_2 in 2 μL (for selection of the dose see Bednářová et al., 2013b) and incubated for 4 h.

To prove participation of particular intracellular signal transduction pathways triggered by AKH in response to H_2O_2 induced OS we added into the incubation medium the following inhibitors or activators of critical intracellular steps using the same basic experimental set as above:

- 2 μL of 4 mM (8 nmol) $CdCl_2$ – as general inhibitor of calcium channels
- 2 μL of 2.4 μM (4.8 pmol) thapsigargin – as specific inhibitor of intracellular calcium channels (see also Section 2.2). In this case the medium used to incubate the CNS was without calcium.
- 2 μL of 1 mM (2 nmol) chelerythrine chloride or 2 μL of 1 μM (2 pmol) bisindolylmaliemide – to inhibit protein kinase C (PKC)
- 2 μL of 10 μM (20 pmol) 8-diethylamino-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) – to inhibit both PKC and intracellular Ca^{2+} mobilization together
- 2 μL of 1 μM (2 pmol) phorbol myristate acetate (PMA,) – to activate PKC
- 2 μL of 1 μM (2 pmol) forskolin – to increase cAMP level

The doses of the inhibitors and activators were based on our previous publication, Kodrík and Goldsworthy (1995).

2.4. Competitive ELISA for lipid peroxidation product 4-hydroxy-2-nonenal (4-HNE)

The lipid peroxidation product 4-hydroxy-2-nonenal (4-HNE) was assayed in the CNS by a competitive enzyme-linked immunosorbent assay (ELISA) as described by Satoh et al. (1999) with some slight modifications (Krishnan et al., 2009). Briefly, free hydroxynonenal (4-HNE, Alpha Diagnostic, San Antonio, TX, USA) was conjugated to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein. Wells in a 96-well high binding plate (Costar) were coated with 500 ng of HNE-GAPDH protein for 2 h at 4 °C, washed in PBS-Tween (PBST), and blocked with 1% BSA. A standard curve was developed from serial dilutions of HNE-GAPDH with polyclonal anti-HNE antibody (1:1000; Alpha Diagnostic). The CNS samples, from each treatment was homogenized separately using a Kimble Kontes motorized homogenizer followed by sonication for 30 s. Samples were centrifuged at 16,000 *g* for 15 min, at 4 °C. The protein content of supernatant (mitochondrial and cytosolic fractions) was estimated using bicinchoninic acid (BCA). Protein lysate (50 μg) from each treatment and biological replicate was mixed with 1:1000 polyclonal rabbit anti-HNE antibody and added to wells in triplicate. Plates were incubated for 1 h, washed with washing buffer (PBST), incubated with 1:5000 secondary anti-rabbit antibody conjugated with horse radish peroxidase, washed, mixed with detection buffer TMB (Alpha Diagnostic) and the reaction stopped with Stop solution (Alpha Diagnostic), and absorbance read at 450 nm in a Biotek Synergy H1M plate reader. 4-HNE values from samples were obtained from the interpolation of values in the standard calibration curve and expressed as pmol of 4-HNE per mg protein.

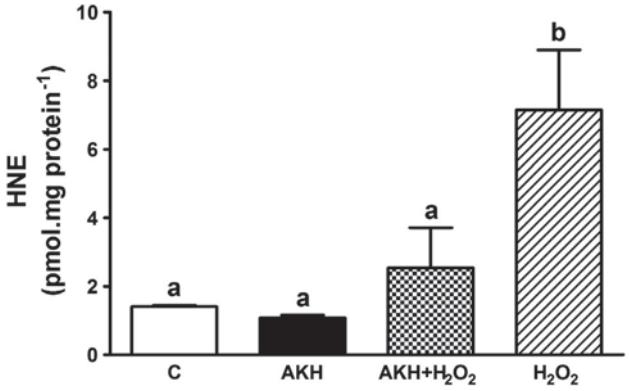

Fig. 2. The effect of Pyrap-AKH (40 pmol) or hydrogen peroxide (H_2O_2) (8 μmol) or both on the level of 4-HNE in 10 CNS of *P. apterus* males incubated for 4 h *in vitro*. Data represent mean ± SD. Different letters above the columns represent significant differences at the 5% level calculated by one-way ANOVA with Tukey's multiple comparison test.

2.5. Membrane fluidity measurements

The hydrocarbon 1,6-diphenyl-1,3,5-hexatriene was employed as a fluorescent probe for monitoring fluidity in the carbon chain of lipids by fluorescence depolarization (Krishnan et al., 2007). The probe (2 mM) in tetrahydrofuran was diluted 1000 times with phosphate buffered saline (PBS, pH 7.2) under rapid stirring for 2 h at room temperature. One hundred and fifty micrograms (protein) of CNS microsomal membrane preparation (19,000 *g* supernatant centrifuged at 100,000 *g* for 1 h in an Sorvall RC 5B centrifuge to obtain microsomal pellet, for details see Krishnan et al., 2007) in 150 μL of PBS was incubated at 25 °C with 150 μL of probe, resulting in 2 μM probe solution. Fluorescence polarization was measured at λ_{Ex} 362 nm and λ_{Em} 440 nm using the fluorescence polarization mode in a Biotek Synergy H1MF plate reader. A sharp decrease in the fluorescence signal indicative of probe penetration in the hydrocarbon membrane region leveled off in 30 min. The degree of polarization (P) was calculated by the formula: $P = (I_{0,0} - GI_{0,90})/(I_{0,0} + GI_{0,90})$ where $G = I_{90,0}/I_{90,90}$, where $I_{0,0}$ is the fluorescence intensity of the emitted light when the excitation and emission light polarizers are both vertical; $I_{0,90}$ is the fluorescence intensity when the excitation polarizer is vertical and the emission polarizer is horizontal; $I_{90,90}$ is the fluorescence intensity when the excitation and emission light polarizers are both horizontal; $I_{90,0}$ is the fluorescence intensity when the excitation polarizer is horizontal and the emission polarizer is vertical; and G is a correction factor. A smaller value of P demonstrates a greater fluidity of membrane lipids. The microviscosity of membranes η was calculated by the formula $\eta = 2P/(0.46\text{-}P)$.

2.6. Statistical treatment of data

For obtaining the EC_{50} values for Ca-channel inhibitors, the data from six independent experiments were processed with Graph Pad Prism version 5.01 (San Diego, CA, USA) using the asymmetric five parameter dose–response curve fit. For evaluation of significance of inhibiting and activating treatments employed in evaluation of lipid peroxidation product 4-HNE, as well as in membrane fluidity assays we used one-way ANOVA with Tukey's post-test (GraphPad Instat 3 and GraphPad Prism 5.01). Data were plotted using Graph Pad Prism 5.01 and represented as mean ± SD based on results from three independent experiments (each with 3 technical replicates) for each parameter studied.

Table 1
The effect of various inhibitors (Ca^{2+} channels and PKC) and activators (PKC and cAMP) on membrane fluidity characteristics – degree of fluorescence polarization (*P*) and microviscosity (η) – measured in microsomal fractions of the *P. apterus* male CNS incubated in presence of Pyrap-AKH (40 pmol) or hydrogen peroxide (H_2O_2) (8 μmol) or both for 4 h *in vitro*. Data represent mean ± SD (n = 3). Different letters in superscript above the values in a row (for a particular parameter) represent significant differences at the 5% level calculated by one-way ANOVA with Tukey's multiple comparison test.

Chemical	Target	Fluorescence polarization (*P*)				Microviscosity (η)			
		Control	AKH	AKH + H_2O_2	H_2O_2	Control	AKH	AKH + H_2O_2	H_2O_2
Control (No inhibitor/activator)-		$0.177^{a} \pm 0.003$	$0.133^{b} \pm 0.010$	$0.183^{a} \pm 0.017$	$0.367^{c} \pm 0.005$	$1.247^{a} \pm 0.01$	$0.816^{a} \pm 0.04$	$1.325^{a} \pm 0.08$	$7.857^{b} \pm 0.02$
Cadmium chloride ($CdCl_2$)	Universal Ca^{2+} channel inhibitor	$0.242^{a} \pm 0.006$	$0.233^{a} \pm 0.006$	$0.340^{b} \pm 0.012$	$0.360^{b} \pm 0.021$	$2.211^{a} \pm 0.028$	$2.044^{a} \pm 0.025$	$5.667^{b} \pm 0.057$	$7.200^{b} \pm 0.097$
Thapsigargin	Intracellular Ca^{2+} (sarco/endoplasmic reticulum) inhibitor	$0.197^{a} \pm 0.018$	$0.193^{a} \pm 0.007$	$0.363^{b} \pm 0.010$	$0.373^{b} \pm 0.005$	$1.494^{a} \pm 0.083$	$1.450^{a} \pm 0.032$	$7.517^{b} \pm 0.044$	$8.651^{b} \pm 0.024$
Bisindolylmaliemide	Selective inhibitor of protein kinase C (PKC)	$0.213^{a} \pm 0.007$	$0.220^{a} \pm 0.012$	$0.337^{b} \pm 0.011$	$0.350^{b} \pm 0.009$	$1.730^{a} \pm 0.032$	$1.833^{a} \pm 0.057$	$5.459^{b} \pm 0.049$	$6.364^{b} \pm 0.043$
Chelerythrine chloride	Cell permeable inhibitor of PKC	$0.257^{a} \pm 0.010$	$0.250^{a} \pm 0.009$	$0.357^{b} \pm 0.017$	$0.363^{b} \pm 0.010$	$2.525^{a} \pm 0.044$	$2.381^{a} \pm 0.043$	$6.903^{b} \pm 0.077$	$7.517^{b} \pm 0.044$
8-Diethylamino-octyl-3,4,5, trimethoxybenzoatehydrochloride (TMB-8)	Inhibitor of PKC as well as inhibits intracellular Ca^{2+}	$0.230^{a} \pm 0.009$	$0.217^{a} \pm 0.005$	$0.360^{b} \pm 0.005$	$0.370^{b} \pm 0.005$	$2.00^{a} \pm 0.043$	$1.781^{a} \pm 0.024$	$7.20^{b} \pm 0.021$	$8.222^{b} \pm 0.021$
Phorbol myristate acetate (PMA)	Activator of PKC	$0.240^{a} \pm 0.005$	$0.200^{b} \pm 0.012$	$0.237^{a} \pm 0.012$	$0.320^{c} \pm 0.005$	$2.182^{a} \pm 0.021$	$1.538^{a} \pm 0.057$	$2.119^{a} \pm 0.054$	$4.571^{b} \pm 0.021$
Forskolin	Activator of adenylyl cyclase increases intracellular cAMP	$0.137^{a} \pm 0.012$	$0.133^{a} \pm 0.010$	$0.143^{a} \pm 0.010$	$0.287^{b} \pm 0.014$	$0.845^{a} \pm 0.054$	$0.816^{a} \pm 0.044$	$0.905^{a} \pm 0.044$	$3.308^{b} \pm 0.066$

3. Results and discussion

3.1. AKH protects cell membranes from oxidative damage by preventing lipid peroxidation and maintaining membrane fluidity

In vitro treatment of isolated CNS with hydrogen peroxide (8 μmol) resulted in ~ 5 fold ($p < 0.005$) elevation of lipid peroxidation (4-HNE) (Fig. 2) compared to control (supplemented only with 2 μL Ringer saline). However the co-application of H_2O_2 with exogenous Pyrap-AKH (40 pmol) significantly reduced ($p < 0.05$) the 4-HNE level comparable to the control (no significant difference). Application of the Pyrap-AKH alone had no effect on lipid peroxidation.

Fluorescence polarization (*P*) of CNS microsomal membranes treated by H_2O_2 alone was about 2 fold higher ($p < 0.001$) than control treatment indicative of higher membrane rigidity. This was reflected in the microviscosity of the membranes (η) which was also significantly higher ($p < 0.001$) than the control one (Table 1). Furthermore, co-treatment of CNS with H_2O_2 and AKH resulted in significant lowering of fluorescence polarization (*P*) value as well as microviscosity (η) ($p < 0.001$) of microsomal membranes to those of control levels. Treatment of the CNS with AKH alone resulted in significant reduction of membrane rigidity ($p < 0.05$) compared to control treatment, however, a similar significant effect on microviscosity was not observed ($p > 0.05$, Table 1) despite the fact that the value was partially reduced.

Taken together, our results conclusively demonstrated that AKH has the capacity to counteract OS induced by H_2O_2, which was reflected in restricting lipid peroxidation and restoring membrane fluidity close to control values (Fig. 2, Table 1). In subsequent experiments, we tested the hypothesis whether extracellular or intracellular Ca^{2+} stores, and PKC and cAMP activities are involved in AKH mode of action in anti-OS reactions using various inhibitors and activators of cellular signaling pathways in the same experimental pattern.

3.2. Extracellular and intracellular stores of Ca^{2+} are involved in AKH mediated anti-oxidative response

To investigate the role of extracellular and intracellular stores of Ca^{2+} and their mobilization by AKH in its anti-oxidative role, we supplemented medium containing incubated CNS in presence of H_2O_2 and Pyrap-AKH (see the basic experimental pattern shown in Fig. 2) by cadmium chloride, $CdCl_2$ (8 nmol), as the universal Ca^{2+} channel blocker or by thapsigargin (4.8 pmol) as a specific inhibitor of intracellular Ca^{2+} from endoplasmic reticulum (Fig. 3). Interestingly, inhibition of Ca^{2+} channels with $CdCl_2$ resulted in a negation of the protective effects of AKH in lipid peroxidation (4-HNE production) in isolated CNS induced by H_2O_2 (Fig. 3A). Hence, in presence of $CdCl_2$ we observed no significant difference in 4-HNE levels in AKH + H_2O_2 co-treated CNS as compared with CNS treated by H_2O_2 only. As expected a significant difference ($p < 0.05$) was observed between the 4-HNE levels in control and H_2O_2 treated CNS, and between the AKH and H_2O_2 treated CNS. Besides, no significant difference in 4-HNE levels in any of the treatments was observed in presence of the inhibitor thapsigargin (Fig. 3B) suggesting that intracellular calcium from endoplasmic reticulum might play a more specific role than the extracellular one. One can also speculate that thapsigargin alone could also modulate OS (endoplasmic reticulum (ER) stress) because the levels of 4-HNE were higher in all treatments (Control, AKH, AKH + H_2O_2 and H_2O_2, Fig. 3B) compared to the levels in Fig. 2 or Fig. 3A.

These results were reflected in the membrane fluidity studies as well. Table 1 shows that the presence of $CdCl_2$ or thapsigargin removed the protective effects of AKH in AKH + H_2O_2 co-treatment, so, we recorded no significant change in the membrane rigidity (*P*) or microviscosity (η) of microsomal membrane fractions in CNS incubated in presence of H_2O_2 and AKH + H_2O_2. Moreover we also recorded no differences in the two studied parameters between the

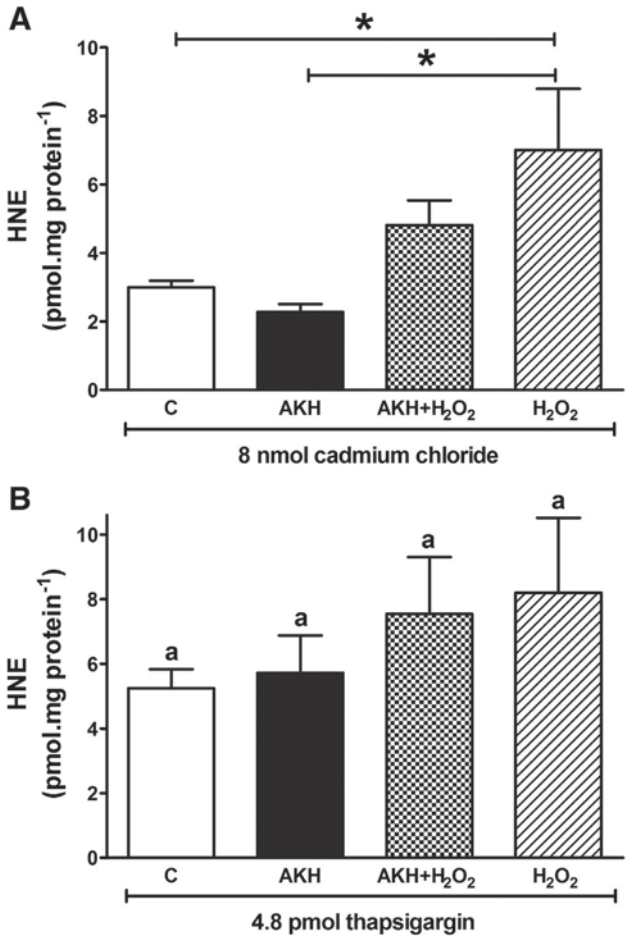

Fig. 3. The effect of Ca^{2+} channel inhibitors – cadmium chloride (**A**) and thapsigargin (**B**) – on the level of 4-HNE in 10 CNS of *P. apterus* males incubated in presence of Pyrap-AKH (40 pmol) or hydrogen peroxide (H_2O_2) (8 μmol) or both for 4 h *in vitro*. Data represent mean ± SD. Statistically significant differences at the 5% level among the columns calculated by one-way ANOVA with Tukey's multiple comparison test are indicated by asterisks (**A**); the letter "a" in (**B**) indicates no statistically significant differences.

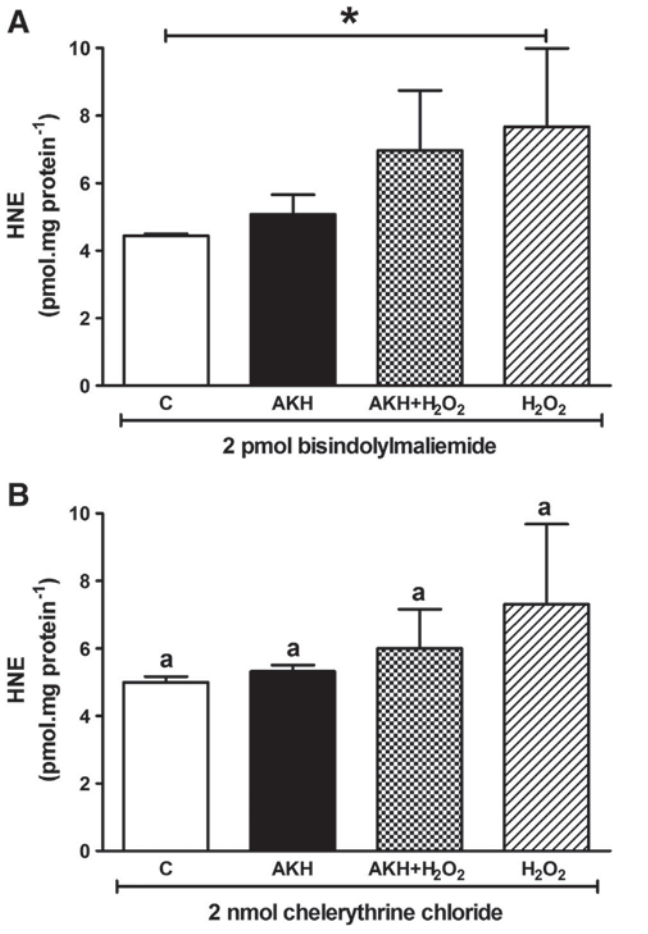

Fig. 4. The effect of PKC inhibitors – bisindolylmaliemide (**A**) and chelerythrine chloride (**B**) – on the level of 4-HNE in 10 CNS of *P. apterus* males incubated in presence of Pyrap-AKH (40 pmol) or hydrogen peroxide (H_2O_2) (8 μmol) or both for 4 h *in vitro*. Data represent mean ± SD. Statistically significant difference at the 5% level among the columns calculated by one-way ANOVA with Tukey's multiple comparison test is indicated by the asterisk (**A**); the letter "a" in (**B**) indicates no statistically significant differences.

control and AKH treated CNS, despite the fact that these values were lower than in presence of H_2O_2.

The involvement of both extracellular and intracellular Ca^{2+} stores thus appears to be imperative for AKH to exert its protective role against OS induced damage. Many different peptide hormones trigger an inositol 1,4,5-triphosphate (IP_3) induced release of sequestered calcium from a non-mitochondrial intracellular storage compartment (Berridge and Irvine, 1989). A potential mechanism for this phenomenon is calcium release from separate calcium pools (extracellular and intracellular), requiring coordinated cycles of emptying and refilling (Berridge, 1990; Meyer, 1991). In case of AKH, extracellular Ca^{2+} appears to play an essential role in signaling, whereas endogenous AKH as well as its exogenous application causes an immediate influx of extracellular Ca^{2+} *in vitro* (Auerswald and Gäde, 2001a). Incubation of fat body pieces of *Pachnoda sinuata* in Ca^{2+}- free medium, results in dramatically reduced elevation of cAMP induced by endogenous AKH, when compared with Ca^{2+} containing medium (Auerswald and Gäde, 2001b). Besides, the inhibitory activity of Locmi-AKH-I, -II and -III on *in vitro* RNA synthesis in *Locusta migratoria* fat body was recorded just when Ca^{2+} was present in the medium; when the calcium was absent the effect was missing (Kodrík and Goldsworthy, 1995). Similarly, Ca^{2+} from intracellular stores was also found necessary for AKH action (Auerswald and Gäde, 2001b). The precise role of Ca^{2+} from intracellular sources in AKH signaling is not sufficiently investigated, and knowledge is rather fragmentary. In moths, the injection of thapsigargin causes an increase in lipid release into the hemolymph which demonstrates that Ca^{2+} from IP_3-insensitive intracellular stores is also part of the adipokinetic signaling process (Arrese et al., 1999). In our present study, we demonstrate that both extracellular and intracellular stores of Ca^{2+} are required by AKH to exert its protective action against OS induced by H_2O_2. Hence, a "capacitative entry" as well as mobilization of intracellular Ca^{2+} stores is pre-requisite for AKH to successfully play its role in anti-stress actions.

3.3. Protein kinase C is essential for potentiating AKHs anti-oxidative role

In signal transduction systems, there is an enzyme that plays an important part, more particularly by its presence in most cellular types and by the number of pathway networks to which it belongs. This is the protein kinase C (PKC) discovered by Nishizuka (1995). It is a serine/threonine kinase, capable of phosphorylating proteins on serine or threonine residues, thus implicating its activation which leads to cellular response. In order to investigate if PKC is involved in AKH signaling process in its anti-oxidative function, we used two specific inhibitors – bisindolylmaliemide and chelerythrine chloride – in separate experiments after elicitation of OS and application of exogenous Pyrap-AKH either alone or in combination with

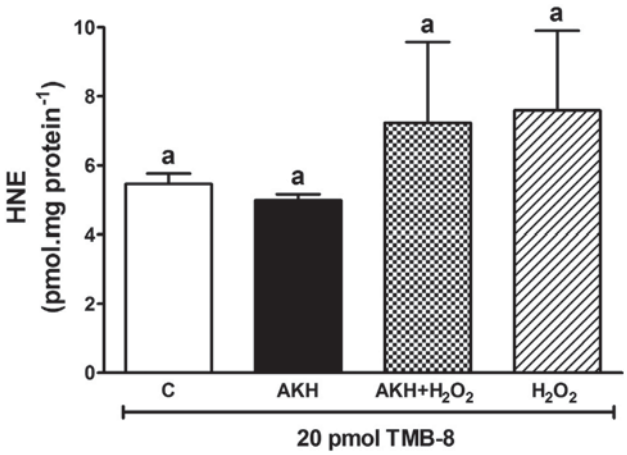

Fig. 5. The effect of PKC and intracellular Ca^{2+} stores inhibitor – TMB-8 – on the level of 4-HNE in 10 CNS of *P. apterus* males incubated in presence of Pyrap-AKH (40 pmol) or hydrogen peroxide (H_2O_2) (8 μmol) or both for 4 h *in vitro*. Data represent mean ± SD. The letter "a" indicates no statistically significant differences at the 5% level among the columns calculated by one-way ANOVA with Tukey's multiple comparison test.

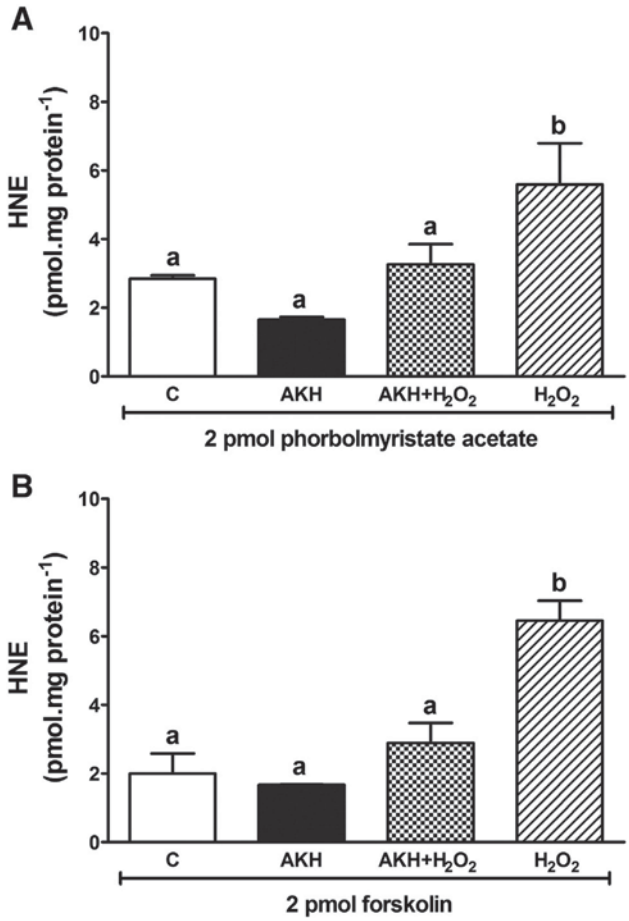

Fig. 6. The effect PKC activator – phorbol myristate acetate (**A**) – and adenylyl cyclase activator – forskolin (**B**) – on the level of 4-HNE in 10 CNS of *P. apterus* males incubated in presence of Pyrap-AKH (40 pmol) or hydrogen peroxide (H_2O_2) (8 μmol) or both for 4 h *in vitro*. Data represent mean ± SD. Statistically significant differences at the 5% level among the columns calculated by one-way ANOVA with Tukey's multiple comparison test are indicated by different letters.

H_2O_2. The result showed that AKH was not able to realize its protective role when the PKC activity was inhibited as demonstrated by just negligible non-significant differences in 4-HNE levels in CNS incubated in presence of Pyrap-AKH and/or H_2O_2 (Fig. 4). In presence of bisindolylmaliemide in incubating medium, a significant difference ($p < 0.05$) between control and H_2O_2 alone treatments were observed, however using chelyrythrine chloride (Fig. 4B), even this difference was negated leading us to the assumption that the latter compound could indirectly induce some amount of OS as in case of thapsigargin (Fig. 3B). A similar effect was also noticed when membrane fluidity served as an indispensable marker for PKC in AKHs anti-oxidative role (Table 1).

Classical PKC can be stimulated by calcium, various lipidic compounds like diacylglycerol (DAG) or phosphatidyl serine or by analogs like phorbol esters. Novel PKC can be activated by diacylglygerols whereas atypical PKCs require neither of the two co-factors (Newton, 2001). Inhibitors belonging to the bisindolemaliemide class either interfere with C_1- domain-mediated kinase activation (e.g. calphostin C) or directly block PKC kinase activity through ATP-competitive interaction with the nucleotide binding pocket. Chelerythrine chloride is a benzophenanthridine alkaloid that can inhibit PKC with a 200 fold lower concentration than other kinases. It exerts actions on PKC: primarily by the binding on the catalytic domain which leads to modification of the regulatory domain, preventing a normal binding of its inducer, and secondly it is able to methylate the cysteine of the protein, reducing binding with the ligand (Gopalakrishna et al., 1995). In our study we proved the inhibitory actions of both these inhibitors by demonstrating that in the presence of these inhibitors AKH does not perform its classical role as an anti-stress hormone.

To further elucidate if PKC as well as intracellular Ca^{2+} mobilization is required for AKH to perform its stress protective role, we used 8-diethylamino-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8) which can inhibit PKC as well as interfere with intracellular Ca^{2+} mobilization. We observed that in presence of TMB-8 co-application of AKH with H_2O_2 did not result in any significant decrease in 4-HNE levels nor were the membrane fluidity parameters significantly affected (Fig. 5, Table 1). Thus, this substantiates that both intracellular Ca^{2+} and PKC are involved in the signaling pathway of AKH in its anti-OS role. The lack of any significant difference in 4-HNE levels in control and H_2O_2 treated groups in presence of TMB-8 could probably be explained by its dual inhibition of both PKC as well as intracellular calcium channels thus creating a condition of ER stress much like thapsigargin (Fig. 3B). However, since membrane fluidity is a specific parameter, the effects of this stress was significantly less in control groups compared to H_2O_2 or AKH + H_2O_2 groups (Table 1).

3.4. Involvement of both PKC and cAMP in AKH signaling response to OS

To confirm that PKC, and to investigate if cAMP are both involved in AKH signaling response to OS, we used phorbol mysistate acetate (PMA) as a specific PKC activator and forskolin as an activator of adenylyl cyclase resulting in increased level of intracellular cAMP. Phorbol esters such as PMA bind with high affinity to the C_1-domain of classical and novel PKC isozymes and thereby induce kinase activity through the same membrane translocation mechanisms as the endogenous C_1-interacting ligand DAG. Use of 2 pmol of PMA was sufficient to potentiate AKH action and we recorded that in presence of PMA, co-treated by H_2O_2 with AKH significantly ($p < 0.05$) reduced lipid peroxidation (4-HNE) by almost 2 fold when compared to H_2O_2 treatment alone in presence of PMA (Fig. 6A). Similarly, when the diterpene forskolin (2 pmol) was applied, co-treatment of H_2O_2 with AKH significantly reduced lipid peroxidation product 4-HNE almost by 2.2 fold ($p < 0.05$) when compared to H_2O_2 treatment alone (Fig. 6B). In both cases, there was no significant difference between control, AKH alone or co-treatment (AKH + H_2O_2) groups in presence of either PMA or forskolin.

The fluorescence polarization in presence of PMA indicated that AKH treatment alone significantly improved membrane fluidity characteristics and also protected membrane from OS effects by restoring

the fluidity to control levels (Table 1). A similar effect was also observed in case of forskolin, though membrane fluidity characteristics of AKH treatment alone was not significantly different from either control or co-treatment groups. In both cases H_2O_2 treatment alone increased membrane rigidity by oxidation of lipids in the microsomal preparations.

Cyclic nucleotides regulate many cellular events, including hormonal signaling; however, it has been proven difficult to firmly establish the relationship of cAMP levels to physiological functions in intact cells, tissues and organisms. In part, this is due to lack of a satisfactory general activator for adenylyl cyclase in intact cells. Receptor-mediated activation of enzyme is highly specific and is further dependent on interactions of an intracellular guanyl nucleotide binding subunit with the catalytic subunit of adenylyl cyclase. Here, we demonstrated that the diterpene forskolin is a potent activator of adenylyl cyclase in membranes from brain of insects and also potentiates the protective role of AKH against OS.

To sum up the obtained results it seems evident that the studied Pyrap-AKH intracellular signaling pathways eliciting anti-OS responses use at least in part the signal-transduction mechanism involving both PKC and cAMP by mobilizing extra- and intracellular Ca^{2+} stores. Those conserved pathways were described in detail in fat body of several insect species, when the mode of AKH action in energy mobilizing processes was studied (Spencer and Candy, 1976; Vroenen et al., 1997; Van der Horst, et al. 2001; Gäde and Auerswald, 2003). From this point of view our results are not so surprising. On the other hand, the mode of action of AKH in inhibition of RNA synthesis in *L. migratoria* fat body does apparently involve neither cAMP and intracellular calcium nor protein kinase C. The precise mechanism by which AKHs control the stimulation of anti-OS responses is far from being fully elucidated, however, its detailed study can bring new interesting data.

4. Conclusions

Neuropeptides exert their physiological roles by triggering intracellular responses upon interacting with specific signal-transducing membrane receptors (Zupanc, 1996). Most of these receptors belong to the GPCRs, the largest family of cell surface proteins. Insect AKHs bind to GPCRs and recent studies indicate that the function of AKH is not restricted to energy releasing processes of locomotor activity alone, but that it acts as a general regulator of homeostasis, influencing all energy requiring processes (e.g. egg production, feeding behavior, larval growth, immune response, and anti-stress reactions) (Lorenz, 2003; Kodrík, 2008; Lorenz and Gäde, 2009; Krishnan and Kodrík, 2012; Bednářová et al., 2013b).

Calcium plays an important role in regulating a great variety of cellular processes. Like other cells, neurons in the brain use both extracellular and intracellular sources of calcium. Calcium stored within the endoplasmic reticulum of neurons represents an important source of signal Ca^{2+} that is released upon activation of inositol triphosphate receptors ($InsP_3Rs$). The phosphoinositide system is particularly well developed in the mammalian brain (Furuichi and Mikoshiba, 1995) and we hypothesize that a similar system may also exist in insect CNS. A large number of different receptors respond by stimulating the hydrolysis of phosphatidylinositol 4,5-biphosphate ($PtdIns[4,5]P_2$) to form second messengers DAG and $InsP_3$. The latter acts by releasing Ca^{2+} from ER with $InsP_3$ receptors, which are widely distributed throughout the brain (Furuichi and Mikoshiba, 1995). These released calcium stores mediate other biochemical pathways in cellular signaling.

cAMP, the first identified second messenger, has a fundamental role in the cellular response to many extracellular stimuli – neurotransmitters, hormones, inflammatory stimuli or stress. The cAMP intracellular signaling pathway is usually mediated by GPCRs and controls a diverse range of cellular processes including the kinase-A pathway and transcription of specific genes.

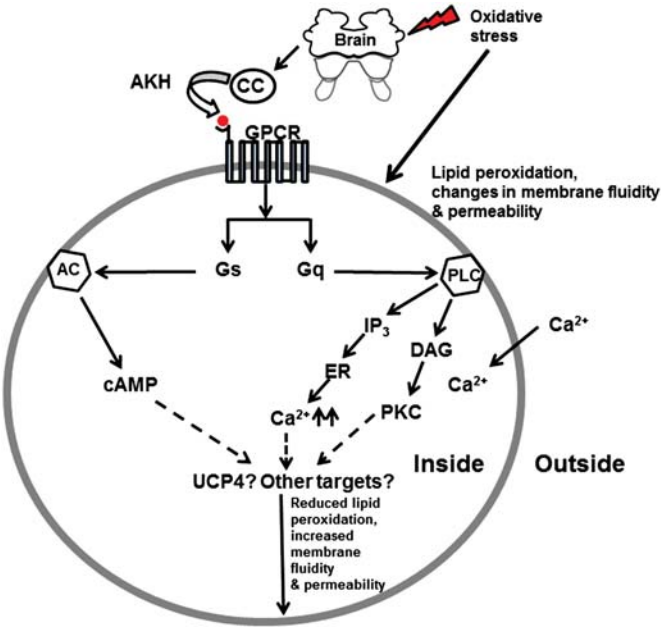

Fig. 7. A hypothetical model for the release and action of adipokinetic hormone in response to oxidative stress (OS) in insects. OS results in lipid peroxidation in the cell membranes leading to reduced membrane fluidity and permeability. OS also induces the insect brain to release AKH from the *corpora cardiaca* (CC) which binds to the specific G protein coupled receptors (GPCR) in cell membrane. Activation of specific G proteins such as G_q and G_s leads to activation of membrane bound adenylyl cyclase (AC) or phospholipase C (PLC). Activation of AC leads to an elevation of cyclic adenosine monophosphate (cAMP), while PLC activation leads to elevation in levels of inositol (1,4,5) phosphate (IP_3). The latter raises intracellular calcium levels from endoplasmic reticulum (ER) pools and can also lead to elevation of diacyl glycerols (DAGs) and subsequently protein kinase C (PKC). These events result in triggering a further cascade of reactions (represented by dashed lines) which are as yet unclear (involvement of uncoupling protein 4 (UCP4), Other targets), leading to protection of membrane from lipid peroxidation and retaining membrane integrity and fluidity.

While the precise pathways triggering the anti-OS reactions are as yet unclear, in this study we demonstrated that Ca^{2+} stores (extracellular as well as intracellular) are indispensable and that both PKC and cAMP are involved in the initial sequence of steps of the signal transduction process triggered by AKH. Hence, it is possible, that AKH potentiates anti-OS reactions using the classical/conserved signal-transduction process (Bednářová et al., 2013a, Fig. 7). Recently, the involvement of AKH in triggering the expression and activity of uncoupling protein 4 (UCP4) was demonstrated in the fat bodies of the beetle *Zophobas atratus* (Slocinska et al., 2013). It has also been reported that UCP4 activation lowers the reactive oxygen species formation in cockroach fat body mitochondria (Slocinska et al., 2011). This leads us to speculate that AKH may have a regulatory role in protection against mitochondrial OS through UCP4. However, the direct involvement of UCP4 as well as additional downstream pathways and genes in AKHs anti-oxidative role remain to be elucidated.

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CHAPTER V

Knockdown of adipokinetic hormone synthesis increases susceptibility to oxidative stress in Drosophila – a role for dFoxO?

Fly Pushing

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Knockdown of adipokinetic hormone synthesis increases susceptibility to oxidative stress in *Drosophila* – a role for dFoxO?

Andrea Bednářová[a,b,c], Dalibor Kodrík[a,b] and Natraj Krishnan[c]*

[a] Institute of Entomology, Biology Centre, Academy of Sciences, Branišovská 31, 370 05 České Budějovice, Czech Republic

[b] Faculty of Science, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

[c] Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA.

*Corresponding author: Phone: +1-662-325-2978, Fax: +1-662-325-8837

E-mail: nk260@msstate.edu

Abstract

Insect adipokinetic hormones (AKHs) are pleiotropic hormones known to play a role in oxidative stress (OS) response. However, the precise signaling pathways are unclear. We present evidence that AKH may primarily employ the Forkhead box class O transcription factor (FoxO) to exert this effect. The impact of knocking down AKH synthesis or its over-expression on response to OS was studied in *Drosophila melanogaster*. AKH knockdown was achieved using a transgenic RNAi fly line whereas AKH was overexpressed (AKH-oex) using the Gal-4/UAS system and controls were UAS/+ and AKH-Gal4/+. Exposure to 80 μM hydrogen peroxide (HP) revealed that AKH-RNAi flies showed significantly higher mortality than AKH-oex or the respective control lines. This susceptibility was evidenced by significantly enhanced levels of protein carbonyls – a biomarker of OS, in AKH-RNAi flies compared to controls and AKH-oex flies. Interestingly, AKH-oex flies had the least amount of protein carbonyls. AKH-RNAi flies had significantly less *dFoxO* transcript and translated protein compared to control and AKH-oex flies in un-challenged condition as well as when challenged with HP. Sestrin – a major antioxidant defense protein and one of the targets of dFoxO was also significantly down-regulated (both at mRNA and protein level) in AKH-RNAi flies (both unchallenged and challenged with HP) compared to control flies and flies with over-expressed AKH. These findings imply that dFoxO may act downstream of AKH as a transcription factor to mediate response to OS in *D. melanogaster*.

Key words: Adipokinetic hormone; Drosophila; Hydrogen peroxide; Oxidative stress; FoxO

Abbreviations: AKH, adipokinetic hormone; AKT, protein kinase B; AMPK, 5' adenosine monophosphate-activated protein kinase; FoxO, Forkhead box proteins; HP, hydrogen peroxide; OS, oxidative stress; TOR, target of rapamycin.

1. Introduction

Insect adipokinetic hormones (AKHs) are produced in the neurosecretory glands – the *corpora cardiaca* near the brain. AKHs are pleiotropic and they perform a number of actions other than their primary role in flight and energy metabolism (Gäde, 2004; Kodrík, 2008, Bednářová et al., 2013a). Most of these secondary functions are stress responsive in nature whereby AKHs stimulate catabolic reactions (mobilization of lipids, carbohydrates and/or certain amino acids), modulating energy availability, while inhibiting synthetic reactions. An active role for AKH in modulating responses to oxidative stress (OS) in insects has been proposed (Kodrík et al., 2007; Večeřa et al., 2007; Velki et al., 2011; Huang et al., 2012; Večeřa et al., 2012; Bednářová et al., 2013b; Bednářová et al., 2013c). These studies implicate AKH in potentiating a cascade of reactions resulting in effectively countering the action of the stressor (in this instance an elicitor of OS) in insects. While the mode of action of AKH in energy metabolism has been intensively investigated and reviewed (Gäde et al., 1997; Gäde and Auerswald, 2003; Van der Horst, 2003; Gäde, 2004), the pathways triggered by AKH in its anti-OS roles are only being recently uncovered (Bednářová et al., 2013c; Slocinska et al., 2013).

The fruit fly *Drosophila melanogaster* has emerged as a favorable model system for understanding how AKH functions at the cellular level to regulate physiology, behavior and response to OS because of the excellent genetic tools available that allow cell-type specific expression and inactivation. It is possible that given the conserved mode of action of AKH in insects, insights obtained from *Drosophila* may be also applicable to other insects. The neuropeptide control of behavior (Nassel, 2002; Taghert and Veenstra, 2003; Ewer, 2005) and energy-dependent modulation of signaling by AKH (Braco et al., 2012) has already been described in *Drosophila*, where a single AKH (octamer) is present (Noyes et al., 1995). This AKH expression is restricted to a small group of cells representing the *corpus cardiacum* part of the ring gland. The ablation of AKH secreting cells results in resistance to starvation and concomitant changes in carbohydrate metabolism (Lee and Park, 2004; Isabel et al., 2005).

Other than stress responsive hormones such as AKH, the transcriptional response to stress is also affected by transcription factors such as the forkhead-box O (FoxO). FoxO transcription factors are a family of conserved proteins that regulate the cellular response

to various stimuli, such as energy deprivation, stress, as well as developmental cues. FoxO proteins are also important mediators of the response to OS. Members of the FoxO subfamily are emerging as a shared component among pathways regulating diverse cellular functions such as differentiation, metabolism, proliferation and survival; in *D. melanogaster dFoxO* is expressed predominantly in fat body (Zheng et al., 2007). These transcription factors are negatively regulated as a result of phosphorylation by protein kinase B (Akt) in response to insulin/insulin-like growth factor (IGF) signaling. In contrast to insulin signaling, OS generated by treatment with H_2O_2 can also induce the activation of FoxO (Essers et al., 2004). This is because of phosphorylation of sites other than those induced by Akt and elicited by stress inducible kinases that promote nuclear localization of FoxO. Nuclear translocation of FoxO has been implicated in cellular protection against OS via the transcriptional regulation of manganese superoxide dismutase (*MnSOD*) and catalase (*Cat*) gene expression (Glauser and Schelegel, 2007). FoxO can also induce transcriptional activation of *sestrin (dSesn)*, which could lead to elevated levels of the energy sensor protein AMP-activated protein kinase (AMPK), which has an inhibitory effect on the transcript of the *Drosophila* homolog of the target of rapamycin (*dTOR*) (Lee et al., 2010). The sestrins are a family of highly conserved proteins that were originally discovered in mammals as antioxidants (Peeters et al., 2003; Budanov et al., 2004). However, it was found that they have an additional function that leads to the activation of AMPK, although the exact mechanism by which sestrin activates AMPK is not fully understood (Budanov et al., 2008).

Our hypothesis was that AKH could also potentiate the FoxO-Sestrin-AMPK-TOR pathway in its stress responsive role. To test this, we used fly lines with impaired AKH synthesis (RNAi) as well as ectopically over-expressed AKH in *Drosophila*. Insights obtained from this study reveal that FoxO might operate downstream of AKH signaling to mediate response to OS in *D. melanogaster*.

2. Materials and Methods

2.1 Drosophila stock and husbandry

Drosophila melanogaster were reared on 1% agar, 6.25% cornmeal, 6.25% molasses, and 3.5% Red Star yeast at 25 °C in 12-h light per 12-h dark (LD) cycles (with an average light intensity of 2000 lux). Stocks used in this study were the AKH-RNAi where RNAi is induced by an expression of a TRiP line (using a short hairpin construct) (BL # 34960), AKH-GAL4 (BL # 25683) (Lee and Park, 2004), UAS-AKH (BL # 27343) all procured from the Bloomington Stock Center in Indiana, USA. All transgenes were backcrossed to the w^{1118} background for five generations. Controls were AKH-Gal4/+ and UAS-AKH/+. Since RNA transcript levels (Suppl. Fig. S1) and their response to OS was not significantly different (data not shown), the data were pooled and henceforth will be referred to as controls. Only male flies (F1 generation after cross) were used in this study, since female flies have altered physiological status because of reproductive development and respond differently from male flies.

2.2 Mortality in response to oxidative stress

To test the resistance of experimental fly lines, adult males (5 days old) were starved for 6 hrs before being transferred to vials containing a 22 mm filter paper disks soaked with 200 µl of 80 µM hydrogen peroxide (HP) (Krishnan et al., 2008). Untreated controls were exposed to 200 µl of water. The number of dead flies was scored in 72 hrs. HP was replenished once daily till end point of the experiment. Each genotype contained 25 flies per vial and was tested in 3 replicates.

2.3 Total protein carbonyl content assay

The amount of protein carbonyls was quantified in whole body homogenates (25 flies in each replicate in three bioreplicates) of all fly lines 4 hour after exposure to HP stress (80 µM) (this treatment period has been proven to be a potent elicitor of OS and the exposure time is optimal (Krishnan et al., 2008)). Samples were derivatized after reaction with 2,4 dinitrophenylhydrazine (DNPH) as described before (Krishnan et al., 2007). Results were expressed as nmol mg^{-1} protein using an extinction coefficient of 22,000 M^{-1}

cm^{-1} at absorbance maxima of 370 nm in BioTek H1M Synergy plate reader. Bovine serum albumin (BSA) standard curve was used for protein concentrations in guanidine solutions (Abs 280 nm). Protein carbonyl values were corrected for interfering substances by subtracting the Abs 370 nm mg^{-1} protein measured in control samples.

2.4 Quantitative real-time polymerase chain reaction

Three independent bioreplicates of flies (5 days old) were collected following 4-hour exposure to HP from each genotype. In parallel, flies from untreated groups (exposed to 200 µl of water only) were also collected in a similar manner. Total RNA was extracted from 25 flies using Tri Reagent (Sigma, St. Louis, MO, USA). The samples were treated with Takara Recombinant DNase I (Clontech Laboratories Inc., Mountain View, CA, USA). Synthesis of cDNA was achieved with the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Quantitative real-time PCR (qRT-PCR) was performed on the Eppendorf realplex^2 Mastercycler (Eppendorf, USA) under default thermal cycling conditions, with a dissociation curve step. Every reaction contained Power SYBR Green (Applied Biosystems), 10 ng cDNA, and 400 nM primers. Primer sequences are given in Suppl. Table T1. Data were analyzed using the $2^{-\Delta\Delta CT}$ method with mRNA levels normalized to the gene *rp49*. Relative mRNA amplitude was calculated with respect to untreated controls (see Section 2.1) flies whose expression for a particular gene was set as 1.

2.5 Western blotting

Three independent bioreplicates of 5-day-old males of different genotypes were collected following 4-h exposure to HP stress. In parallel, flies from untreated groups were collected in a similar manner. About 25 flies were homogenized on ice in 50mM phosphate buffer, sonicated, and centrifuged at 10000 g for 10 min at 4 °C. The protein content was equalized to ensure equal protein loading using the bicinchoninic acid method (Smith et al., 1985). Samples were then separated by polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% resolving gel (Laemmli, 1970) followed by transfer onto PVDF Immobilon membranes (Millipore Billerica, MA, USA) and incubated in 1X TBST (10 mM Tris, 0.15 M NaCl, 0.1% Tween-20, pH 7.5) + 5% milk for 2 h. Then the membranes

were incubated overnight at 4 °C with primary antibody (1:1000 for Akt procured from Cell Signaling Technology) and 1:1500 for FoxO (kind gift from Dr. M. Tatar, Brown University, USA) and sestrin (kind gift from Dr. J. H. Lee, University of Michigan, USA) in blocking buffer. Membranes were treated for 2 h with 1:20,000 goat anti-rabbit IRDye680 (LI-COR Biosciences, Lincoln, NE, USA). Blots were scanned using the LI-COR Odyssey Infrared Imaging System (CLx) and quantified with imaging software (Image Studio, v. 3.0, LI-COR Biosciences, Lincoln, NE, USA).

2.6 Statistical analysis

Statistical analysis of mortality to oxidative stress (the percentage data of dead flies were transformed prior to statistical analysis), protein carbonyl content, gene expression and Western blot analysis post-quantification, was conducted using one-way ANOVA with Tukey's post hoc tests (GraphPad Instat v 3.0). Graphs were developed in GraphPad Prism v 5.01 (GraphPad software Inc. San Diego, CA).

3. Results

3.1 Impairment of AKH synthesis increases susceptibility to oxidative stress induced by hydrogen peroxide.

In response to 80 μM H_2O_2 (HP) stress, the AKH-RNAi flies showed significantly increased mortality (~ 97%) compared to control flies (UAS/+ or AKH-Gal4/+) (~ 71%). However flies where AKH synthesis was ectopically over-expressed (AKH-oex), showed significantly reduced mortality (~ 57%) (Fig. 1).

3.2 Impairment of AKH synthesis significantly increases protein carbonyl content following exposure to hydrogen peroxide.

Exposure of fly lines to 80 μM HP stress for a period of 4 h revealed that AKH-RNAi flies showed significantly increased levels of protein carbonyls (~ 1.8 fold) compared to similarly treated controls and AKH-oex flies (~3.7 fold) (Fig. 2). Interestingly, AKH-oex flies showed the least among of protein carbonyl accumulation unpon challenge with HP compared to either Controls or AKH-RNAi flies (Fig. 2).

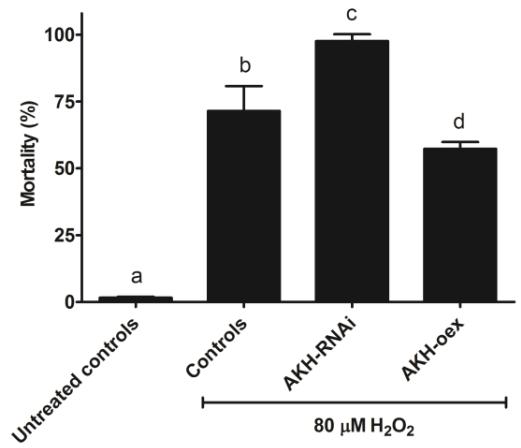

Fig. 1: The mortality test of the experimental flies exposed for 72 hours to 80 μM H_2O_2. Values represent mean mortality (%) ± SD. Bars with different superscripts are significantly different at $p<0.05$ using one-way ANOVA followed by Tukey's multiple comparison test.

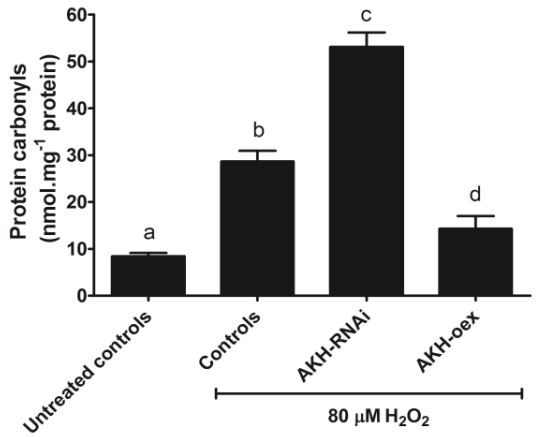

Fig. 2: Total protein carbonyl levels in flies exposed to 80 μM H_2O_2 for 4 h. Values represent mean ± SD of assays from three independent bioreplicates. Bars with different superscripts are significantly different at $p<0.05$ using one-way ANOVA followed by Tukey's multiple comparison test.

3.3 Differential responsiveness at the transcriptional level of some key signaling moieties in flies with impaired AKH synthesis in response to hydrogen peroxide stress.

To investigate if AKH signaling does indeed involve the FoxO-Sestrin-AMPK-TOR pathway, we checked the gene expression of all the major players in this pathway. The levels of mRNA expression of some key members of the hypothesized AKH signaling pathway under the OS elicited by HP are summarized in Fig. 3. The results revealed that *dFoxO* mRNA expression was significantly down-regulated in AKH-RNAi flies (both control and HP treated) when compared to controls (UAS/+ or AKH-Gal4/+) or AKH-oex flies (Fig. 3A). While in controls *dFoxO* was significantly up-regulated upon HP exposure, this up-regulation was not significantly different from untreated and HP-treated levels in AKH-oex flies. Thus, basal transcript levels (untreated) of *dFoxO* in AKH-oex flies were higher than untreated levels in control flies. Also, the up-regulation of *dFoxO* in AKH-oex flies upon challenge with HP was not significantly different from comparable untreated ones (Fig. 3A).

The stress responsive gene *dSesn* (sestrin) was enhanced in all fly lines exposed to HP compared to untreated controls, however, just in AKH-oex and controls (UAS/+ or AKH-Gal4/+) this up-regulation was significant (Fig. 3B). AKH-RNAi flies showed significantly lower transcript levels of dSesn in unchallenged conditions compared to AKH-oex and controls. Upon challenge with HP there was a marginal increase in dSesn transcript levels in AKH-RNAi flies compared to unchallenged flies but this increase was non-significant. In general, for both unchallenged and challenged groups, expression of *dSesn* in AKH-RNAi flies was significantly lower when compared to similarly treated controls or AKH-oex flies (Fig. 3B).

The gene expression for the energy sensor protein AMPK was significantly up-regulated by HP treatment in both AKH-oex and control flies, while being marginally down regulated in AKH-RNAi compared to untreated controls (Fig. 3C). Interestingly, the AMPK expression levels were significantly lower in groups (both treated and untreated) with AKH synthesis inhibited (RNAi) compared to AKH-oex or control flies (Fig. 3C).

Finally, the gene expression for the target of rapamycin protein (*dTOR*) was significantly down-regulated in control and AKH-oex flies when exposed to HP compared to untreated comparable controls (Fig. 3D). However, the level of expression of *dTOR* was

significantly higher in HP-treated AKH-RNAi flies compared to similarly treated control and AKH-oex flies. This up-regulation, however was not significantly different when compared to untreated RNAi group (Fig. 3D).

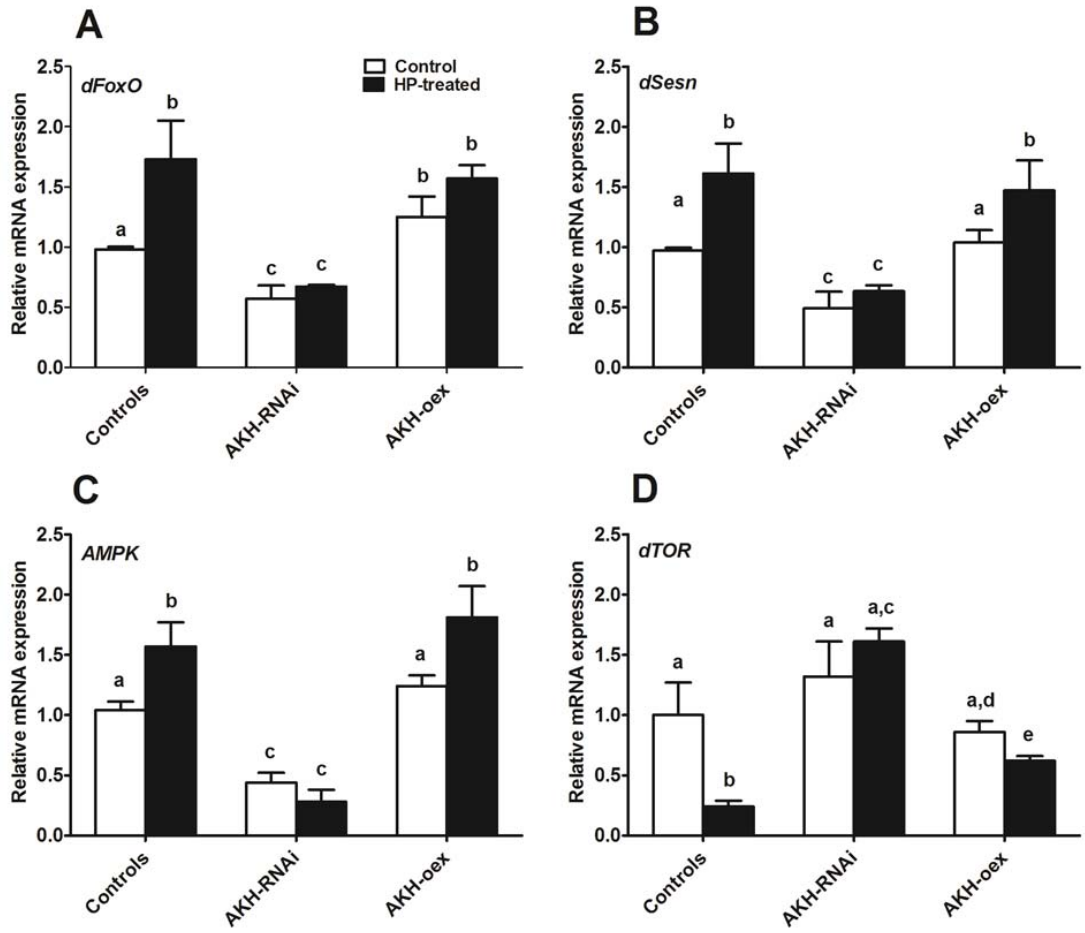

Fig. 3: Quantitative-Reverse Transcription PCR of key signaling members: (**A**) *dFoxO*, (**B**) *dSesn*, (**C**) *AMPK* and (**D**) *dTOR*, after 4 hours exposure to 80 µM H_2O_2. Relative mRNA expression of different fly lines was quantified using controls (AKH-Gal4/+ or UAS-AKH/+) lines (without H_2O_2 exposure-control) which was set as reference (= 1). Values represent mean ± SD of three independent biological replicates. One-way ANOVA with Tukey's post-hoc test was conducted to separate out the means. Values with different superscripts are significantly different at $p<0.05$.

3.4 Flies with impaired AKH synthesis exhibit significantly decreased FoxO and sestrin protein levels both in unchallenged and challenged conditions.

To check if indeed FoxO protein levels were affected by a lack of AKH (RNAi) as observed from gene expression analysis, we performed western blots to demonstrate the FoxO protein levels. We found that FoxO protein was markedly reduced in AKH-RNAi flies compared to AKH-oex and control fly lines (for both treated and untreated groups) (Fig. 4A). A relative quantitation of the blots revealed no significant differences in untreated and treated groups within a genotype (Fig. 4B).

Sestrin protein levels were significantly up-regulated in both controls and AKH-oex flies upon challenge with HP (Fig. 5A, B) and also the basal unchallenged sestrin levels were significantly higher in these two groups compared to AKH-RNAi flies. The latter group showed the least levels of sestrin proteins which were marginally enhanced upon stress with HP (Fig. 5A, B). However, both in unchallenged and unchallenged conditions the sestrin levels were significantly low in AKH-RNAi flies compared to similarly treated controls and AKH-oex flies.

3.5 Akt transcript and protein levels were markedly decreased following exposure to hydrogen peroxide.

Interestingly there was no significant difference in the transcript levels of Akt in unchallenged conditions among all the genotypes examined (Fig. 6A). Following exposure to HP a marked decrease in Akt transcript levels was recorded in all genotypes but this decrease was not significant among the genotypes tested. A similar pattern was recorded for the proteins levels of Akt (Fig. 6B, C)

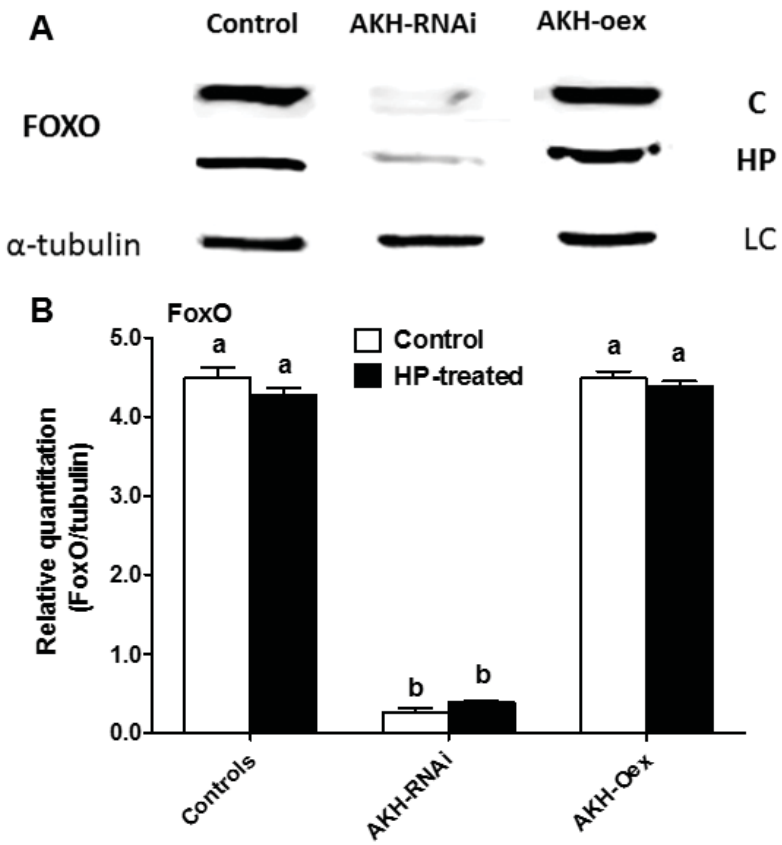

Fig. 4: AKH-RNAi flies revealed significantly depleted levels of dFoxO compared to controls and AKH-oex flies. Western blots were performed for (**A**) FoxO, both with (HP) and without (C) exposure to hydrogen peroxide. Alpha-tubulin was used as the loading control. Figures are representative of one of three independent blots. (**B**) Blots were quantified by mean grey scale value relative to tubulin and bars are represented as mean ± SD of three independent blots. One-way ANOVA with Tukey's post-hoc test was conducted to separate out the means. Values with different superscripts are significantly different at $p<0.05$.

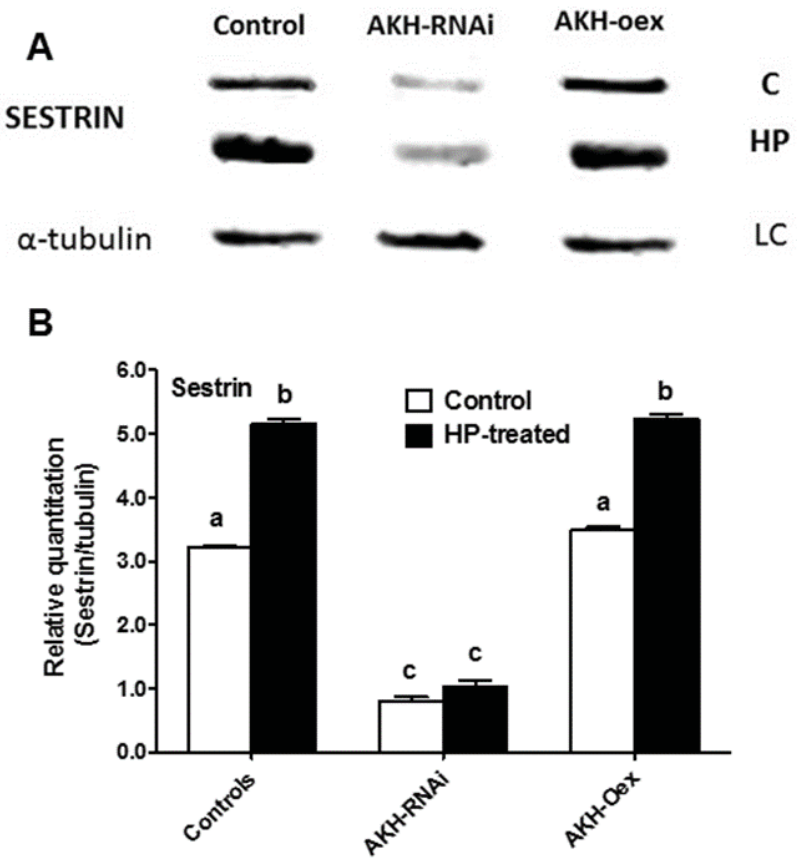

Fig. 5: Sestrin protein levels are significantly depleted in AKH-RNAi flies compared to controls and AKH-oex flies. Western blots were performed for (**A**) Sestrin, both with (HP) and without (C) exposure to hydrogen peroxide. Alpha-tubulin was used as the loading control. Figures are representative of one of three independent blots. (**B**) Blots were quantified by mean grey scale value relative to tubulin and bars are represented as mean ± SD of three independent blots. One-way ANOVA with Tukey's post-hoc test was conducted to separate out the means. Values with different superscripts are significantly different at $p<0.05$.

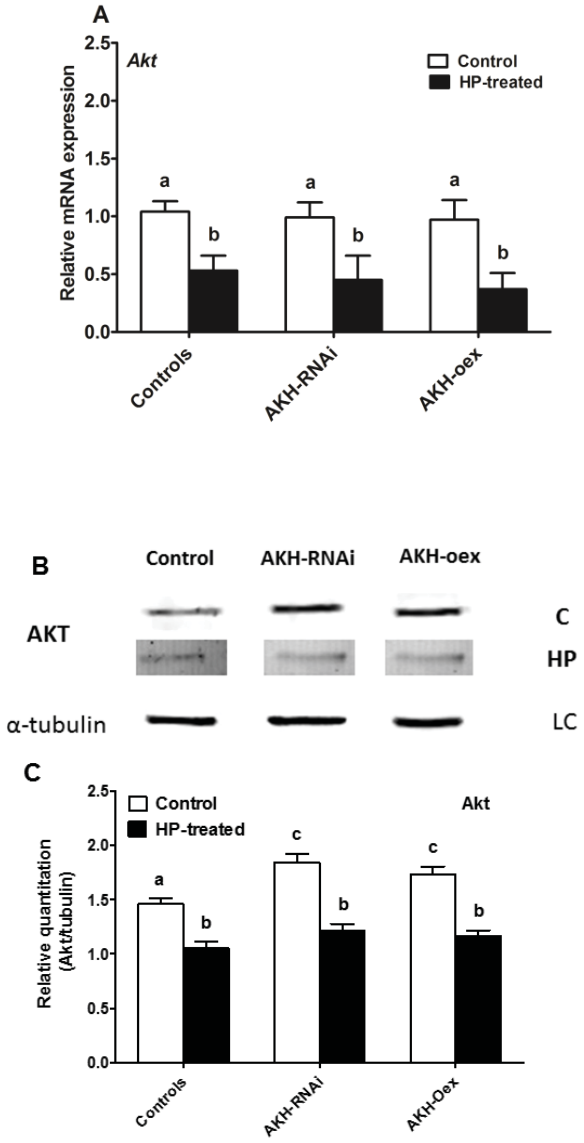

Fig. 6: Akt transcript as well as protein levels were down-regulated markedly across all genotypes tested. (**A**) transcript levels of Akt both with and without exposure to HP. Relative mRNA expression for Akt in different fly lines was quantified using controls (AKH-Gal4/+ or UAS-AKH/+) lines (without H_2O_2 exposure-control) which was set as reference (= 1). Values represent mean ± SD of three independent biological replicates. One-way ANOVA with Tukey's post-hoc test was conducted to separate out the means. Values with different superscripts are significantly different at $p<0.05$. Western blots were performed for (**B**) Akt both with (HP) and without (C) exposure to hydrogen peroxide. Alpha-tubulin was used as the loading control. Figures are representative of one of three independent blots. (**C**) Blots were quantified by mean grey scale value relative to tubulin and bars are represented as mean ± SD of three independent blots. One-way ANOVA with Tukey's post-hoc test was conducted to separate out the means. Values with different superscripts are significantly different at $p<0.05$.

4. Discussion

In subjecting the flies with altered AKH synthesis to OS (with H_2O_2) it was observed that the AKH-RNAi flies were far more susceptible to this kind of stress than the AKH-oex or the control flies. This strongly suggests that AKH is necessary for anti-OS responses. The knockdown efficiency of AKH-RNAi flies used in this study demonstrated a significant 84% reduction in AKH mRNA levels compared to controls whereas AKH-oex flies showed almost a 2 fold increase in AKH synthesis (Suppl. Fig. S1). Interestingly, AKH-RNAi flies showed significantly reduced transcript as well as protein levels of FoxO compared to controls or AKH-oex flies. In *Drosophila*, FoxO proteins are important mediators of the insulin signaling pathway that adjust growth and metabolism to nutrient availability (Puig et al., 2006). We hypothesized that the insulin signaling pathway and AKH-stimulated cAMP signaling pathway may be linked, or they use similar mechanisms to orchestrate the organism response to both nutritional conditions and stress. Our finding that in flies with impaired AKH synthesis, there is both a decreased transcription of *dFoxO* and its translated protein product (dFoxO), is consistent with the idea that there may be a link between these two signaling pathways when facing a stress situation. However it has been demonstrated previously that FoxO is required for activation and expression of the hypertrehalosemic hormone (HTH), a member of the AKH-family in cockroaches in response to starvation (Suren-Castillo et al., 2014). In insects, AKHs might be the main hormones that counteract the action of insulin. Thus, one can hypothesize that there could be a feed-back regulation between AKHs and FoxO, such that, under conditions of OS, AKHs may trigger FoxO to combat OS. Central to FoxO's role in regulating transcriptional changes in response to stress is its localization in cytosol (thereby terminating its transcriptional function), to being translocated to nucleus where it can act as a transcription factor. PI3-Akt pathway is a major upstream signaling module leading to the phosphorylation of FoxO factors and their exclusion from the nucleus (Kops et al., 1999). FoxO transcription factors, across species, have highly conserved phosphorylation sites that are phosphorylated by Akt. This phosphorylation takes place in nucleus and creates the 14-3-3 binding site to FoxO that mask the nuclear localization signal and prevents nuclear translocation, thereby inhibiting the activities of FoxO (Hay, 2011). In this study we found that Akt levels were significantly down-regulated by OS in all genotypes studied

and there was no difference in the transcript levels in untreated or treated levels across genotypes. The decrease in protein levels of Akt was more marked in AKH-oex flies when compared to AKH-RNAi or controls (see Fig. 5 B, C). AMPK is another candidate as a regulator of FoxO function. FoxO can indirectly activate AMPK, which in turn activates the tuberous sclerosis complex 2 (TSC2) and inhibits TORC1 activity. The activation of AMPK by FoxO occurs through the transcriptional up-regulation of sestrin (Hay, 2011). Notably, FoxO itself is subjected to the regulation by the energy status of cells, as AMPK has been shown to phosphorylate FoxO and to facilitate its nuclear localization (Hay, 2011). Therefore, the FoxO-sestrin-AMPK axis could be further augmented by AMPK through a feed-forward mechanism. This pathway, by which FoxO inhibits TORC1 in flies, manifests under OS conditions (Hay, 2011). In our study we observed that *dSesn* levels were only markedly up-regulated in control and AKH-oex flies upon challenge with HP and not in AKH-RNAi flies, a similar result was also recorded for transcript levels of sestrin. In consonance, the transcript levels of the energy sensor protein AMPK were significantly down-regulated in AKH-RNAi flies compared to AKH-oex and controls. Finally, the FoxO inhibitory *dTOR* was markedly elevated in AKH-RNAi flies but not in controls or AKH-oex flies. This implies that AKH probably employs the FoxO-sestrin-AMPK-TOR pathway in response to OS. FoxO transcription factors, across species, have been reported to promote resistance to OS, premature aging, and cellular senescence (Eijkelenboom and Burgering, 2013). Further, Jünger et al. (2003) reported that the adult dFoxO mutant flies were hypersensitive to OS conditions: when placed on hydrogen-peroxide-containing food, dFoxO mutant flies displayed a significant reduced survival time compared to control flies. This is consistent with our finding that AKH-RNAi flies showed reduced dFoxO levels and are more susceptible to OS. The mechanism by which FoxO confers OS resistance is not yet known. Glauser and Schlegel (2007) proposed in their work that genes like manganese superoxide dismutase (*MnSOD*), and catalase (*Cat*) might be employed by FoxO too, and therefore they partially mediate the protective effect of dFoxO. Thus, the activities of FoxO were attributed largely to their ability to induce the expression of antioxidants genes.

The precise pathway by which FoxO signaling may be activated, and its relationship to AKH remains hypothetical at this point of time. Little is also known about the genomic

targets through which AKH signaling acts. Our present findings about the possible involvement of dFoxO leads to the assumption that this might be the general mechanism of AKH action in response to OS in other insects as well. It is also plausible that in fruit flies insulin-like peptide signaling and AKH signaling are closely linked (Buch et al., 2008), and such signaling by AKH might involve FoxO as a mediator particularly in its role in anti-OS response as demonstrated in this study.

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Supplemental Table 1: List of primers and their sequences used in this study.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
dAKH	ATG-AAT-CCC-AAG-AGG-GAA-GTC-CTC	CTA-CTG-GGG-GTG-CTT-GCA-GTC-CAG
rp49	ACG TTG TGC ACC AGG AAC TT	CCA GTC GGA TCG ATA TGC TAA
dFoxO	AGG CGC AGC CGA TAG GAC GAA	TGC TGT TGA CCA GGT TCG TGT TGA
dSesn	CTC GAC TCG ATC CCC TCC	CAG GTC ATC GAG CTC GTC C
dAkt1	GCA GAG AAA TTC AGC TGG CAG CAA	TGA GTC TGT TCC GTA AGC GCA TGA
dAMPK	CAT CCG CAC ATC ATC AAG TT	TTC TCT GGC TTC AGG TCT CG
dTOR	CAG GTT ATC CCG CAG CTT ATT	GCG GGT GAT TCT TTC CTA TGT

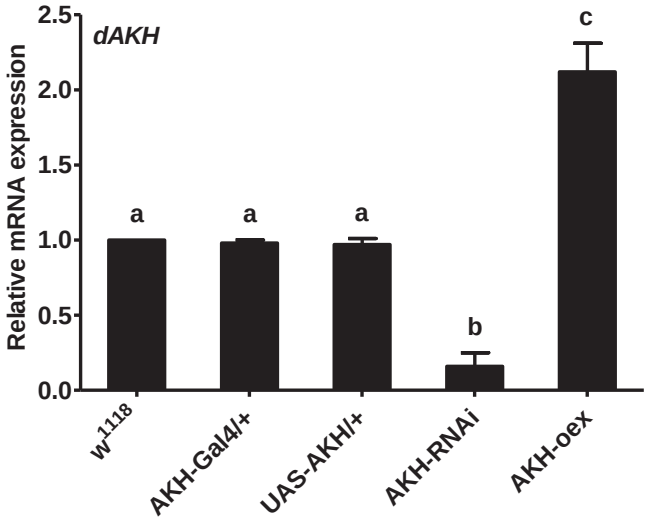
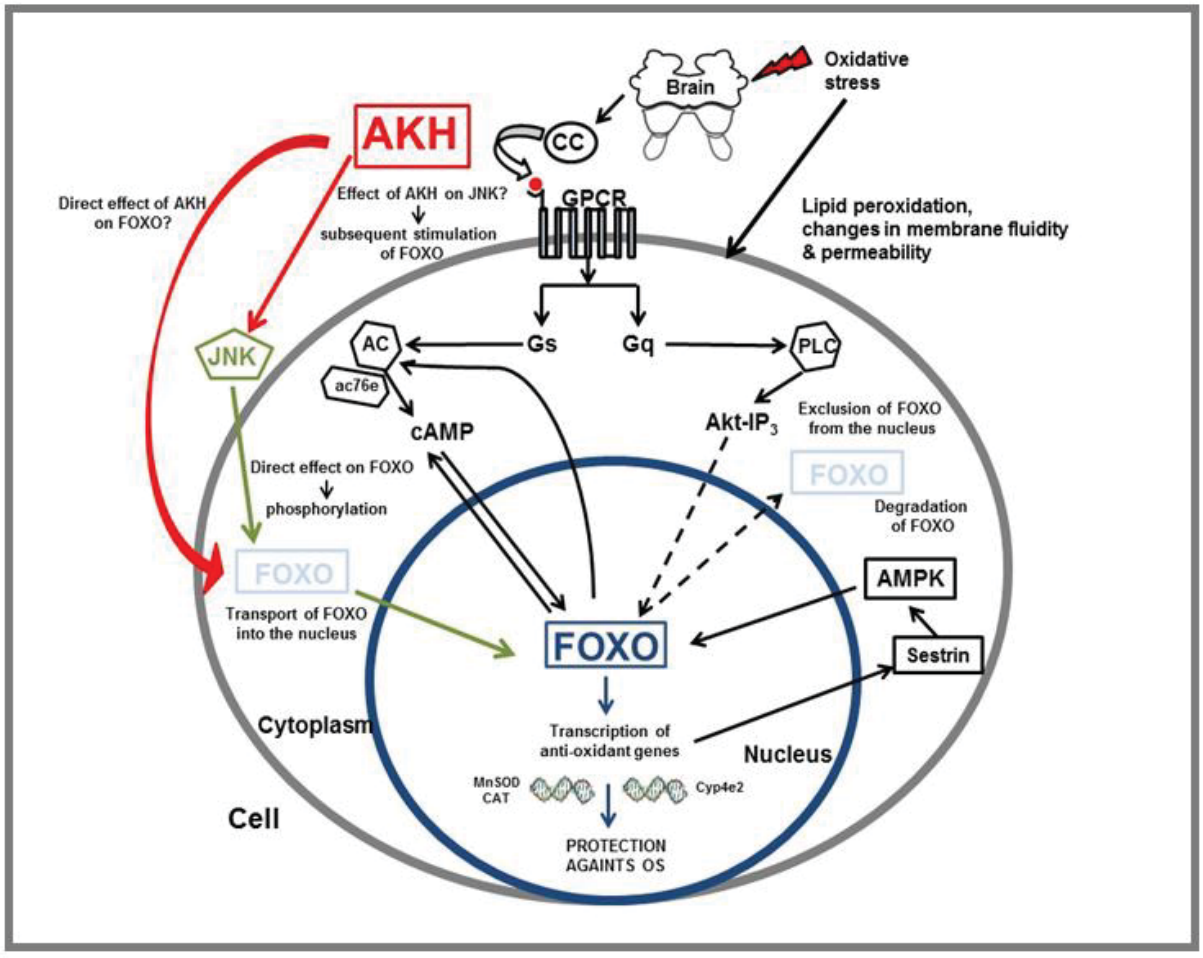


Fig. S1: Quantitative-reverse transcription PCR of *dAKH* transcript in different control lines as well as AKH-RNAi and AKH-overexpressing flies. Relative mRNA expression of different fly lines was quantified using w1118 which was set as reference (=1). Values represent mean ± SD of three independent biological replicates. One-way ANOVA with Tukey's post-hoc test was conducted to separate out the means. Values with different superscripts are significantly different at $p<0.05$.

CHAPTER VI

Conclusion and future perspectives



A schematic hypothetical mode of action of AKHs in oxidative stress response.

CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis focused on the role and mode of action of AKH at the subcellular level in its protective functions in response to OS in insects. The major conclusions emanating from the series of publications which form a part of this thesis are as follows:

1. There are analogies between AKH and glucagon and glucagon-like peptides (GLP-1 and GLP-2) at the functional level in stress situations and maintenance of homeostasis.
2. Using both *in vivo* as well as *in vitro* experimentation it was demonstrated that OS could be elicited effectively in both conditions and also the neuropeptide AKH is able to function as a stress protective hormone.
3. It was further demonstrated that Ca^{2+} stores (extracellular as well as intracellular) are indispensable and both protein kinase C and cyclic AMP are involved in the initial sequence of steps of the signal transduction process triggered by AKH in response to OS. Hence, it was concluded that AKH potentiates its protective functions against OS using the classical / conserved signal transduction process that it employs for its prominent roles in energy metabolism.
4. Finally using the fruit fly *Drosophila melanogaster* as a model system and by using the Gal4-UAS system as well as using specific RNAi lines it was possible to specifically knockdown the synthesis of AKH as well as over-express AKH. The experimental data strongly suggests a role for dFoxO that may act downstream of AKH as a transcription factor to mediate response to OS in *D. melanogaster*.

Stress in general has many different physiological effects in insects, however, the manifestations of stress and its responses are conserved and likely to be similar at the molecular level. It appears that we still know relatively little about the regulation of AKH signaling in its multiplicity of roles. Insight into its precise signaling events might however be afforded by a comprehensive analysis of signaling targets. For the future, it would be interesting to identify further downstream elements of AKH signaling and also the precise signaling that regulate AKH release. This would help us to better comprehend how AKH functions as a stress responsive hormone.

CHAPTER VII

Scientific contributions (supplementary publications)

Int. J. Mol. Sci. **2013**, *14*, 3026-3049; doi:10.3390/ijms14023026

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Review

Nature's Timepiece—Molecular Coordination of Metabolism and Its Impact on Aging

Andrea Bednářová [1,2,3], Dalibor Kodrík [1,2] and Natraj Krishnan [3,*]

[1] Institute of Entomology, Biology Centre, Academy of Science, Branišovská 31, České Budějovice 370 05-CZ, Czech Republic; E-Mails: bednarovaandrea@centrum.cz (A.B.); kodrik@entu.cas.cz (D.K.)

[2] Faculty of Science, South Bohemian University, Branišovská 31, České Budějovice 370 05-CZ, Czech Republic

[3] Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA

***** Author to whom correspondence should be addressed; E-Mail: nk260@msstate.edu; Tel.: +1-662-325-2978; Fax: +1-662-325-8837.

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Abstract: Circadian rhythms are found in almost all organisms from cyanobacteria to humans, where most behavioral and physiological processes occur over a period of approximately 24 h in tandem with the day/night cycles. In general, these rhythmic processes are under regulation of circadian clocks. The role of circadian clocks in regulating metabolism and consequently cellular and metabolic homeostasis is an intensively investigated area of research. However, the links between circadian clocks and aging are correlative and only recently being investigated. A physiological decline in most processes is associated with advancing age, and occurs at the onset of maturity and in some instances is the result of accumulation of cellular damage beyond a critical level. A fully functional circadian clock would be vital to timing events in general metabolism, thus contributing to metabolic health and to ensure an increased "health-span" during the process of aging. Here, we present recent evidence of links between clocks, cellular metabolism, aging and oxidative stress (one of the causative factors of aging). In the light of these data, we arrive at conceptual generalizations of this relationship across the spectrum of model organisms from fruit flies to mammals.

Annals of Botany Page 1 of 10
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Auxin increases the hydrogen peroxide (H_2O_2) concentration in tomato (*Solanum lycopersicum*) root tips while inhibiting root growth

Maria G. Ivanchenko[1,*], Désirée den Os[2,3], Gabriele B. Monhausen[2], Joseph G. Dubrovsky[4], Andrea Bednářová[5,6] and Natraj Krishnan[5]

[1]*Department of Botany and Plant Pathology, Oregon State University, 2082 Cordley Hall, Corvallis, OR 97331, USA,* [2]*Biology Department, Penn State University, 208 Mueller Lab, University Park, PA 16802, USA,* [3]*University of Groningen, Ecophysiology of Plants, Centre for Ecological and Evolutionary Studies, 9700 CC Groningen, The Netherlands,* [4]*Departamento de Biologia Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de México, A. P. 510-3, 62250 Cuernavaca, Morelos, México,* [5]*Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA and* [6]*Institute of Entomology, Biology Centre, Academy of Science, and Faculty of Science, South Bohemian University, Branišovská 31, České Budějovice, 370 05-CZ, Czech Republic*
** For correspondence. E-mail ivanchem@science.oregonstate.edu*

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• *Background and Aims* The hormone auxin and reactive oxygen species (ROS) regulate root elongation, but the interactions between the two pathways are not well understood. The aim of this study was to investigate how auxin interacts with ROS in regulating root elongation in tomato, *Solanum lycopersicum*.
• *Methods* Wild-type and auxin-resistant mutant, *diageotropica* (*dgt*), of tomato (*S. lycopersicum* 'Ailsa Craig') were characterized in terms of root apical meristem and elongation zone histology, expression of the cell-cycle marker gene *Sl-CycB1;1*, accumulation of ROS, response to auxin and hydrogen peroxide (H_2O_2), and expression of ROS-related mRNAs.
• *Key Results* The *dgt* mutant exhibited histological defects in the root apical meristem and elongation zone and displayed a constitutively increased level of hydrogen peroxide (H_2O_2) in the root tip, part of which was detected in the apoplast. Treatments of wild-type with auxin increased the H_2O_2 concentration in the root tip in a dose-dependent manner. Auxin and H_2O_2 elicited similar inhibition of cell elongation while bringing forth differential responses in terms of meristem length and number of cells in the elongation zone. Auxin treatments affected the expression of mRNAs of ROS-scavenging enzymes and less significantly mRNAs related to antioxidant level. The *dgt* mutation resulted in resistance to both auxin and H_2O_2 and affected profoundly the expression of mRNAs related to antioxidant level.
• *Conclusions* The results indicate that auxin regulates the level of H_2O_2 in the root tip, so increasing the auxin level triggers accumulation of H_2O_2 leading to inhibition of root cell elongation and root growth. The *dgt* mutation affects this pathway by reducing the auxin responsiveness of tissues and by disrupting the H_2O_2 homeostasis in the root tip.

Key words: Auxin, ROS, hydrogen peroxide, root elongation, tomato, *Solanum lycopersicum*, *diageotropica*, *dgt*.

INTRODUCTION

Plant root growth depends on production of new cells in the root apical meristem and cell elongation once cells leave the meristem. Cell expansion generally depends on turgor pressure and modulations of the cell-wall extensibility. Plant organ growth and cell length are targets of the hormone auxin, which seems to act differentially depending on concentration, specific tissues and growth conditions. For example, auxin promotes shoot growth and root hair formation and elongation (Pitts *et al.*, 1998; Rahman *et al.*, 2002) but strongly suppresses root growth when applied even at relatively low doses. When applied at nanomolar concentrations (30 nM and 100 nM, respectively), the natural auxin indol-3-acetic acid (IAA) and the synthetic form 1-naphthaleneacetic acid (NAA) have been reported to inhibit root growth in *Arabidopsis thaliana* by reducing the length of the root elongation zone (Rahman *et al.*, 2007). However, these treatments did not significantly decrease cell division in the root apical meristem, as judged from expression level of the cell-cycle marker construct *At-CyclinB1;1:GUS* (Rahman *et al.*, 2007). In contrast, when applied at micromolar concentrations (10 μM for 40 h) NAA was shown to cause an almost complete consumption of the tomato root apical meristem and replacement of the meristem by developing lateral root primordia (Ivanchenko *et al.*, 2006). These examples illustrate the ability of auxin to regulate root growth and development in a concentration-dependent manner.

In addition to hormones, the regulation of root growth has been strongly linked to the generation of reactive oxygen species (ROS). The superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the highly reactive and unstable hydroxyl radical ($^{\bullet}OH$) are produced during normal plant metabolism and in response to biotic and abiotic stresses (Neill *et al.*, 2002; Dietz *et al.*, 2006; Gapper and Dolan, 2006). Compared with other ROS, H_2O_2 is relatively more stable. H_2O_2 is also able to diffuse freely among cellular compartments and between cells facilitated by movement through specific aquaporin membrane channels (Henzler and Steudle, 2000; Bienert *et al.*, 2007).

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Role of adipokinetic hormone in stimulation of salivary gland activities: The fire bug *Pyrrhocoris apterus* L. (Heteroptera) as a model species

Konstantin Vinokurov [a,1], Andrea Bednářová [a,b,d,1], Aleš Tomčala [c], Tereza Stašková [a], Natraj Krishnan [d], Dalibor Kodrík [a,b,*]

[a] Institute of Entomology, Biology Centre, Academy of Sciences, Branišovská 31, 370 05 České Budějovice, Czech Republic
[b] Faculty of Science, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic
[c] Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Flemingovo Sq. 2, 166 10 Praha 6, Czech Republic
[d] Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA

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ABSTRACT

The effect of adipokinetic hormone (Pyrap-AKH) in stimulating the function of insect salivary glands (SGs) in extra-oral digestive processes was studied in the firebug, *Pyrrhocoris apterus* L. (Heteroptera). The analyses were performed on samples of SGs and extracts of linden seeds, a natural source of the bug's food. The SGs from 3-day old *P. apterus* females (when the food ingestion culminates), primarily contained polygalacturonase (PG) enzyme activity, whereas the level of lipase, peptidase, amylase and α-glucosidase was negligible. The transcription of PG mRNA and enzymatic activity were significantly increased in SGs after Pyrap-AKH treatment. The piercing and sucking of linden seeds by the bugs stimulated the intrinsic enzymatic cocktail of seeds (lipase, peptidase, amylase, glucosidase), and moreover the activity of these enzymes was significantly enhanced when the seeds were fed on by the Pyrap-AKH treated bugs. Similarly, a significant increase in PG activity was recorded in linden seeds fed on by hormonally-treated bugs or when injected by SG extract from hormonally treated ones as compared to untreated controls. The mechanism of AKH action in SGs is unknown, but likely involves cAMP (and excludes cGMP) as a second messenger, since the content of this compound doubled in SGs after Pyrap-AKH treatment. This new and as yet undescribed function of AKH in SGs is compared with the effect of this hormone on digestive processes in the midgut elucidated earlier.

1. Introduction

The insect alimentary tract provides a complex physical and chemical environment for processing of ingested food, and its morphology and physiology reflects the character of intaken food. The salivary glands (SGs) play an important role in digestive processes of many insect species. The saliva secreted by the SGs moistens the food, adjusts its pH and ionic content, and often initiates the oral or extra-oral digestion. The enzymatic cocktail frequently contains carbohydrases and peptidases, as described in the saliva of some phytophagous and predaceous bugs: e.g. α-amylase has been reported in *Lygus hesperus* and *Lygus lineolaris* (Zeng and Cohen, 2000; Cooper et al., 2013) and serine peptidase in *Oncopeltus fasciatus* (Francischetii et al., 2007); interestingly Woodring et al. (2007) also reported the presence of a cysteine peptidase in salivary glands of the latter species. In a number of insects, SGs produce specific enzymes which facilitate disruption of cell protective barriers to reach the food source: the saliva of phytophagous bugs *Pyrrhocoris apterus*, *L. hesperus* or representatives of the Miridae family contains pectinase polygalacturonase (PG; the enzyme degrading polygalacturonan present in the cell walls of plants by hydrolysis of the glycosidic bonds) (Courtois et al., 1968; Frati et al., 2006; Allen and Mertens, 2008; Walker and Allen, 2010); the saliva of predaceous and mycophagous species often contains chitinases (Chapman, 1998). The salivary secretion of plant-sucking Hemiptera is ample in oxidases (peroxidase, catechol oxidase) which upon being injected into the plant tissue, participate in detoxification of phytochemicals (terpenes, alkaloids) and together with other components of saliva forms the sheath material surrounding stylets during its passage through the leaf (Peng and Miles, 1988; Miles and Peng, 1989).

A paracrine hormonal regulatory mechanism is proposed to control the synthesis and release of insect digestive enzymes produced in the midgut (Lehane et al., 1996). The available data indicate that most of the midgut digestive processes are under control of specialized midgut endocrine cells expressing genes for

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

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Original article

The potential role of adiponectin- and resistin-like peptides in the regulation of lipid levels in the hemolymph of over-wintering adult females of *Osmia bicornis*

Oskar Wasielewski[1], Dawid Szczepankiewicz[2], Karol Giejdasz[1], Tatiana Wojciechowicz[2], Andrea Bednářová[3,4], Natraj Krishnan[3]

[1]Institute of Zoology, Poznań University of Life Sciences, Wojska Polskiego 71C, 60-625 Poznań, Poland
[2]Department of Animal Physiology and Biochemistry, Poznań University of Life Sciences, Wojska Polskiego 35, 60-637 Poznań, Poland
[3]Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State MS 39762, USA
[4]Institute of Entomology, Biology Centre, Academy of Science and Faculty of Science, South Bohemian University, Branišovská 31, České Budějovice 370 05-CZ, Czech Republic

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Abstract – The presence and potential role of adiponectin- and resistin-like peptides in mobilizing free lipids of hemolymph during over-wintering was studied in females of the European solitary red mason bee *Osmia bicornis* L. (Hymenoptera: Megachilidae). The levels of both peptides (as demonstrated both by RIA/ELISA and Western blots) were highest in fat body tissue homogenates during early pre-wintering (September) followed by a gradual and significant decline during wintering and post-wintering months (November–March). There was a gradual reduction of the lipid levels in hemolymph and adiponectin-like and resistin-like peptide content in fat body. Thus, the total lipid content in hemolymph and the adiponectin-like and resistin-like peptides in fat body homogenates was positively correlated. Our experiments also demonstrated that injections of various concentrations of fat body extracts as well as various doses of adiponectin and resistin increased the lipid levels in hemolymph in *O. bicornis* females at the three different periods of over-wintering time. In particular, injections of fat body extract and adiponectin resulted in the strongest mobilization of lipids especially in the first two periods of over-wintering: pre-wintering and wintering. Resistin also elicited an increase of lipid levels in hemolymph, but its effectiveness was lower compared to fat body extract and adiponectin. Taken together, our results strongly suggest the presence of adiponectin-like and resistin-like peptides in the fat body of *O. bicornis* and postulate a dynamic physiological role for these peptides during the process of over-wintering.

adiponectin / diapause / free lipids / hemolymph / *Osmia bicornis* / over-wintering / resistin / solitary bee

1. INTRODUCTION

Diapause is a strategy to survive seasons with environmental conditions that are inadequate for sustaining continuous development or maintenance of the organism (Denlinger 2002). Depending on the species, diapause can occur in one or sometimes several developmental stages: as eggs, larvae, pupae or adults (Tatar and Yin 2001). In insects, an adult diapause is a unique type of quiescence observed which involves the arrest of reproductive development.

Corresponding author: O. Wasielewski, oskarw@au.poznan.pl
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Perturbations in dopamine synthesis lead to discrete physiological effects and impact oxidative stress response in *Drosophila*

Marley E. Hanna [a], Andrea Bednářová [a,b], Kuntol Rakshit [c], Anathbandhu Chaudhuri [d], Janis M. O'Donnell [e], Natraj Krishnan [a,*]

[a] Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Mississippi State, MS 39762, USA
[b] Institute of Entomology, Biology Centre, Academy of Sciences and Faculty of Science, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic
[c] Department of Physiology and Biomedical Engineering, Mayo Clinic School of Medicine, Mayo Clinic, 200 First St. SW, Rochester, MN 55905, USA
[d] Department of Natural Sciences, Stinson Mathematics and Science Building, 3601 Stillman Blvd, Stillman College, Tuscaloosa, AL 35043, USA
[e] Department of Biological Sciences, The University of Alabama, Tuscaloosa, AL 35487, USA

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ABSTRACT

The impact of mutations in four essential genes involved in dopamine (DA) synthesis and transport on longevity, motor behavior, and resistance to oxidative stress was monitored in *Drosophila melanogaster*. The fly lines used for this study were: (i) a loss of function mutation in *Catecholamines up* (*Catsup*^{26}), which is a negative regulator of the rate limiting enzyme for DA synthesis, (ii) a mutant for the gene *pale* (*ple*^{2}) that encodes for the rate limiting enzyme tyrosine hydroxylase (TH), (iii) a mutant for the gene *Punch* (*Pu*^{Z22}) that encodes guanosine triphosphate cyclohydrolase, required for TH activity, and (iv) a mutant in the vesicular monoamine transporter (*VMAT*$^{\Delta 14}$), which is required for packaging of DA as vesicles inside DA neurons. Median lifespans of *ple*^{2}, *Pu*^{Z22} and *VMAT*$^{\Delta 14}$ mutants were significantly decreased compared to *Catsup*^{26} and wild type controls that did not significantly differ between each other. *Catsup*^{26} flies survived longer when exposed to hydrogen peroxide (80 μM) or paraquat (10 mM) compared to *ple*^{2}, *Pu*^{Z22} or *VMAT*$^{\Delta 14}$ and controls. These flies also exhibited significantly higher negative geotaxis activity compared to *ple*^{2}, *Pu*^{Z22}, *VMAT*$^{\Delta 14}$ and controls. All mutant flies demonstrated rhythmic circadian locomotor activity in general, albeit *Catsup*^{26} and *VMAT*$^{\Delta 14}$ flies had slightly weaker rhythms. Expression analysis of some key antioxidant genes revealed that glutathione S-transferase Omega-1 (*GSTO1*) expression was significantly up-regulated in all DA synthesis pathway mutants and especially in *Catsup*^{26} and *VMAT*$^{\Delta 14}$ flies at both mRNA and protein levels. Taken together, we hypothesize that DA could directly influence *GSTO1* transcription and thus play a significant role in the regulation of response to oxidative stress. Additionally, perturbations in DA synthesis do not appear to have a significant impact on circadian locomotor activity rhythms *per se*, but do have an influence on general locomotor activity levels.

1. Introduction

Dopamine (DA) is a catecholamine that modulates fast neurotransmission in the central nervous systems of both vertebrates and invertebrates. In insects such as the fruit fly, *Drosophila melanogaster*, DA has several roles in neural functions, from modulation of locomotor behaviors and arousal states, to appetitive and aversive learning and memory (Restifo and White, 1990; Barron et al., 2010; Waddell, 2013). The dopaminergic system in *Drosophila* is highly rhythmic, as supported by rhythmicity in responsiveness to DA agonists, and by the rhythmic transcription of the rate-limiting enzyme tyrosine hydroxylase (TH) encoded by the gene *pale* (*ple*) (Hirsh et al., 2010). The *pale* mutants have been reported to show decreased locomotor activity (Pendleton et al., 2002). DA has been implicated in promoting arousal in *Drosophila* as well as promoting higher nocturnal activity in the *Clk*^{Jrk} clock mutant (Kumar et al., 2012; Kume et al., 2005). In rats, DA regulates the expression of the clock protein PER2 (Hood et al., 2010). However, precise links between DA synthesis levels and circadian locomotor activity behavior are unclear. It has been reported that disruptions

Abbreviations: BH4, tetrahydrobiopterin; Cat, catalase; *Clk*, *clock*; Cu/Zn SOD, copper–zinc superoxide dismutase; DA, dopamine; DD, constant darkness; FFT, Fast Fourier Transform; GSTO1, glutathione S-transferase Omega-1; GTPCH, guanosine triphosphate cyclohydrolase; HP, hydrogen peroxide; MLS, median life span; Mn SOD, manganese superoxide dismutase; *ple*, *pale*; PQ, paraquat; *Pu*, *Punch*; TH, tyrosine hydroxylase; VMAT, vesicular monoamine transporter.

* Corresponding author. Tel.: +1 662 325 2978; fax: +1 662 325 8837.
E-mail address: nk260@msstate.edu (N. Krishnan).

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Appendix

Curriculum Vitae

Personal details:

Date of Birth: May 21st, 1986

Citizenship/Nationality: Czech

Education:

Doctor of Science (PhD) 2015 – in the field of Physiology and Developmental Biology.

- University of South Bohemia, České Budějovice, CZ & Department of Biochemistry and Physiology, at the Laboratory of Insect Physiology, Institute of Entomolgy, Biology Centre AS CR, CZ.

Rerum naturalium doctor (RNDr.) 2013 – Graduated RNDr. in Experimental Biology.

- University of South Bohemia, České Budějovice, CZ & Department of Biochemistry and Physiology, at the Laboratory of Insect Physiology, Institute of Entomolgy, Biology Centre AS CR.

Master of Science (MSc.) 2011 – Graduated MSc. in Parasitology.

- University of South Bohemia, České Budějovice, CZ, & Department of Parasitology, Institute of Parasitology, Biology Center, AS CR.

Bachelor of Science (BSc.) 2008 – Graduated BSc. in Biology.

- University of South Bohemia, České Budějovice, CZ

Work Experience:

May 2012; November 2012 - April 2013; May - August 2014:
Research stay at the Mississippi State University, College of Agriculture and Life Sciences, Dept. of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Laboratory of Insect Biochemistry and Molecular Biology, MS 39762. (Mentor: Dr. Natraj Krishnan)

2011 – till date: Research Assistant and PhD. student at the Department of Biochemistry and Physiology, at the Laboratory of Insect Physiology, Institute of Entomolgy, Biology Centre AS CR, České Budějovice, CZ (Supervisor: Prof. Dalibor Kodrík, co-supervisor: Dr. Natraj Krishnan)

2010 August-September: Scientific expedition to India to collect and study plant (including population biology and ecophysiology).

2009, 2010: Part- time job in Šumava National Park (Monitoring of the status and dynamics of the natural regeneration of forest ecosystems).

2009 July-August: Scientific expedition to Svalbard Arctic archipelago to study littoral biocenoses and host-parasite relationships in extreme conditions.

2006 – 2011: Research Assistant at the Laboratory of Helminthology, Institute of Parasitology, Biology Centre AS CR, České Budějovice, Czech Republic.

Teaching experience:

Teaching Assistant in practical course of "Medical parasitology and diagnostic methods", "Physiology of animals", "Insect Physiology", "Developmental Biology" (Faculty of Science, University of South Bohemia, CZ).

Grants and honors:

2012: Travel grant from the Czech grant agency: Nadání, Josefa, Marie a Zdeňky Hlávkových for the stay at the Mississippi State University, College of Agriculture and Life Sciences, Dept. of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Laboratory of Biochemistry and Molecular Biology.

2013: Travel grant from the Czech grant agency: Nadace Český literární fond for the stay at the Mississippi State University, College of Agriculture and Life Sciences, Dept. of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Laboratory of Biochemistry and Molecular Biology.

2014: Grant agency of the University of South Bohemia (GAJU): project title: Mode of action of Adipokinetic hormone at the sub-cellular level in its response to oxidative stress processes in the insect body. Principal Investigator, 1 year grant, ~ 10,000 USD

PEER-REVIEWED PUBLICATIONS (in chronological sequence):

1. **Bednářová A.,** Kodrík D., Krishnan N. (2013) Unique roles of glucagon and glucagon-like peptides: Parallels in understanding the functions of adipokinetic hormones in stress responses in insects. *Comp. Biochem. Physiol. A.* 164: 91-100.

2. **Bednářová A.,** Krishnan N., Cheng I.C., Večeřa J., Lee H.J., Kodrík D. (2013) Adipokinetic hormone counteracts oxidative stress elicited in insects by hydrogen peroxide: in vivo and in vitro study. *Physiol. Entomol.* 38: 54-62.

3. **Bednářová A.,** Kodrík D., Krishnan N. (2013) Nature's timepiece – molecular coordination of metabolism and its impact on aging. *Int. J. Mol. Sci.* 14: 3026-3049.

4. **Bednářová A.,** Kodrík D., Krishnan N. (2013) Adipokinetic hormone exerts its anti-oxidative effects using a conserved signal- transduction mechanism involving both PKC and cAMP by mobilizing extra- and intracellular Ca^{2+} stores. *Comp. Biochem. Physiol. C* 158: 142-149.

5. Ivanchenko M.G., den Os D., Monhausen G.B., Dubrovsky J.G., **Bednářová A.,** Krishnan N. (2013) Auxin increases the hydrogen peroxide (H_2O_2) concentration in tomato (*Solanum lycopersicum*) root tips while inhibiting root growth. *Annals of Botany* 112: 1107-1116.

6. Vinokurov K., **Bednářová A.,** Tomčala A., Stašková T., Krishnan N., Kodrík D. (2014) Role of adipokinetic hormone in stimulation of salivary gland activities: the fire bug *Pyrrhocoris apterus* L. (Heteroptera) as a model species. *J. Insect Physiol.* 60: 58-67.

7. Wasielewski O., Szczepankiewicz D., Giejdasz K., Wojciechowicz T., **Bednářová A.,** Krishnan N. (2014) The potential role of adiponectin- and resistin-like peptides in the regulation of lipid levels in the hemolymph of over-wintering adult females of *Osmia bicornis*. *Apidologie* 45: 491-503.

8. Hanna, M., **Bednářová, A.,** Rakshit, K., Chaudhuri, A., O'Donnell, J.M., Krishnan, N. (2015) Perturbations in dopamine synthesis lead to discrete physiological effects and impact oxidative stress response in *Drosophila*. *J. Insect Physiol.* 73: 11-19.

MANUSCRIPTS in communication:

1. **Bednářová, A.**, Kodrík, D., Krishnan, N., (2015) Knockdown of adipokinetic hormone synthesis increases susceptibility to oxidative stress in *Drosophila* – a role for dFoxO? Communicated to: *Comp. Biochem. Physiol. C*

Conference presentations:

1. *15th Helminthological days*, Ředkovec near Světlá nad Sázavou, Czech Republic, May 14-18, 2007. Bednářová, A.: Seasonal changes in host exploitation by digenean parasites: Are cercariae in summer larger and more numerous? (Oral presentation).

2. *3rd Workshop on Bird Schistosomes and cercarial dermatitis*, Rejčkov-Kouty, Czech Republic, July 6-10, 2009.

3. *26th Conference of European Comparative Endocrinologists, Zurich, Switzerland, August 21-25, 2012.* Bednářová A., I-Cheng C., Večeřa J., Lee H.J., Kodrík D. (2012) Adipokinetic hormone enhances insect anti-oxidative mechanisms elicited by hydrogen peroxide. *Abstract No. P 14-3*: p. 128. (Presented by poster).

4. *17th Congress of Comparative Endocrinology, Barcelona, Spain, July 15-19, 2013.* Bednářová, A., Kodrík D., Krishnan N.. Adipokinetic hormone exerts its anti-oxidative effects using a conserved signal- transduction mechanism involving both PKC and cAMP by mobilizing extra- and intracellular Ca^{2+} stores. (Oral presentation).

5. *27th Conference of European Comparative Endocrinologists, Rennes, France, August 25-29, 2014.* Bednářová A., Kodrík D., Rakshit, K., Krishnan N. (2014) Downstream signalling by adipokinetic hormone to counter oxidative stress in Drosophila melanogaster – Is FoxO involved? *Abstract No. PO 15, p. 104.*

6. *Howard Hughes Medical Institute Undergraduate summer research symposium, University of Alabama, Tuscaloosa, AL, USA, August 2014.* Caver, E., Bednářová A., Krotzer, M., J., O'Donnell, J., Krishnan, N., Chaudhuri, A. Myo-Inositol rescues alcohol induced locomotor behavior: Is alcohol addiction linked to dopamine synthesis pathway? (Poster presentation)

7. *61st Annual Meeting of the Mississippi Entomological Association, Mississippi State University, October 21st and 22nd, 2014*. Hanna, M., Bednářová A., Rakshit, K., Krishnan, N. Dopaminergic regulation of circadian locomotor activity and resistance to oxidative stress in *Drosophila melanogaster*. (Poster presentation)

8. *61st Annual Meeting of the Mississippi Entomological Association, Mississippi State University, October 21st and 22nd, 2014.* Fleiming, D., Bednářová A., Krishnan, N., Musser, F. Biological rhythm of polygalacturonase enzyme in the salivary glands of *Lygus lineolaris*. (Oral presentation).

Professional Associations:

2011- till date: Member of the **European Society of Comparative Endocrinologists**

2014 – till date: Member of the **Entomological Society of America**

Hobbies and extra-curricular interest:

Languages: English – (fluent in written and spoken); Spanish – beginner.

Standard driving licence, SCUBA diving licence.

Social skills: I have experience with team work as well as with leading and organization of public sport's events.

Other Interests:
Diving, swimming, running, getting to know new places and cultures- travelling the world.

e-mailová adresa: bednarovaandrea@centrum.cz

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University of South Bohemia in České Budějovice
Faculty of Science Branišovská 1760
CZ-37005 České Budějovice, Czech Republic

Phone: +420 387 776 201
www.prf.jcu.cz, e-mail: sekret-fpr@prf.jcu.cz