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Contribution to the study of insect stress hormones

Doctoral Dissertation

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SUMMARY

The present study considers the versatile role of adipokinetic hormones (AKHs). Using the firebug *Pyrrhocoris apterus* as a model insect, the hypothesis that AKHs mediate stress response mechanisms was explored. The outcome indicated that there is a positive feedback regulation between an oxidative stressor action and the level of AKH in insect body, and that AKHs might be involved in the activation of antioxidant protection mechanism. Further results revealed a functional homology between AKH and the mammalian hormone glucagon. The possible effects of glucagon on mobilisation of energy reserves and on elicitation of an antioxidant response to oxidative stress were investigated. As a result, glucagon-immunoreactive material was detected for the first time in the firebug central nervous system and gut. Antioxidant mechanisms are elicited after glucagon treatment but it did not involve mobilization of energy reserves or AKH level changes. As a complement, the existence of a feedback between juvenile hormone and AKH was investigated by topical application of the juvenile hormone analogue methoprene, which influenced the release of AKH from the central nervous system into the haemolymph and induced a partial reduction of lipid content in haemolymph.

1. INTRODUCTION TO THE STUDY OF STRESS

The response to the effect of stressors is a very complex and dynamic process, required for adaptation and evolution of practically all living beings. Furthermore, the stress response can be identified and studied at different levels of biological organization, that is, molecules, organelles, cells, organs, organisms and even populations. This broad range imposes the difficulty of discovery a general mechanism of stress[1]. At this point, the study of stress in insects might bring a broader understanding. At the population level, these studies may contribute to solve not only fundamental problems in ecology and evolutionary biology but also they are of applicable significance for insect pest control[2]. Nevertheless, it becomes essential to integrate the underlying processes at the organism and cellular level. Under the effect of various stressors the insects employ several strategies but the most common are: synthesis of protective substances and functional enzymes, compensatory reactions at the level of metabolism, modulation of membranes[1]. In most of the cases, mechanisms to combat oxidative stress and provide antioxidant defence mechanisms. The scope of this work is directed mainly at such level.

1.1 Stress

Recently, stress was defined as a state of threatened homeostasis. The adaptive response to stress reflects the activation of specific central circuits and is genetically and constitutionally programmed and constantly modulated by environmental factors. [3]

The classic description of neuroendocrine activation during stress, for mammals, includes: increased secretion of epinephrine, norepinephrine, cortisol, growth hormone, glucagon and decreased secretion of insulin. The hypothalamus is critical for the integration and coordination of those autonomic efferent responses[4]..

Although the *hypothalamo–pituitary–adrenal axis* (HPA) is the most explored neuroendocrine regulatory pathway in the stress response, other neuroendocrine organs also constitute important regulatory circuits that are involved in the organization of responses to stressful stimuli[3]. For instance the *hypothalamic-gonadal axis* and *immune system*[5]. Research regarding the biochemical mechanisms involved in stress response has extended not only to different groups of vertebrates, invertebrates and even plants and single-celled organisms.

1.2 Oxidative Stress

Reactive oxygen species (ROS) originated as a byproduct of aerobic metabolism[6]. ROS are highly reactive due to the presence of unpaired valence shell electrons. Therefore, detoxification of ROS is one of the prerequisites for all aerobic life forms, and multiple levels of enzymatic and nonenzymatic defenses have evolved to form what has been termed the oxidant defence network. [7]

The imbalance between production of reactive oxygen species (ROS) and the biological system's ability to readily detoxify the reactive intermediates and/or easily repair the resulting damage is known as oxidative stress. Most ROS are oxygen-centred or related radicals, such as: superoxide $\mathbf{O_2^-}$ and hydroxyl radicals (•OH). Interestingly, some ROS such as hydrogen peroxide (H_2O_2) are not free radicals, since H_2O_2 does not have unpaired electrons in the outer molecular orbit, a typical characteristic of free radicals. [8]

The toxicity of $\mathbf{O_2^-}$ can generate potent oxidant •OH in the presence of H_2O_2 and iron. The generation of •OH causes extensive protein and lipid oxidations. Furthermore, $\mathbf{O_2^-}$ can attack iron sulfur clusters, resulting in the release of ferrous iron, which can bind DNA. Thus, catalyzed by the iron bound in DNA molecule, the •OH would cause further damage and possible mutagenesis of DNA. [9]

Under normal circumstances, the rate of generation of superoxide from mitochondria is rather low and does little damage, simply because it is efficiently removed by the superoxide dismutases (SOD).

The •OH is highly reactive. It causes peroxidative damage to proteins, lipids and DNA.

The physiological fate of the H_2O_2 generated on either side of the mitochondrial membrane is to be processed by glutathione peroxidase (GPX) to water in a reaction that converts reduced glutathione (GSH) to oxidized glutathione (GSSG). Another enzyme, the catalase (CAT), reduces hydrogen peroxide to water and oxygen[10]. CAT is a low-affinity but high capacity enzyme, perfectly suited for scavenging high amounts of H_2O_2 that occur in the mitochondria and peroxisomes. Both SOD and CAT are principally intracellular enzymes but their activities, which reflect the gross rate of ROS turnover, were also recorded in insect gut. [11]

In general, cells have numerous defence systems for maintaining the cellular redox state and repairing oxidatively damaged proteins, DNA and lipids. These systems include the glutathione- and thioredoxin-dependent reduction systems and methionine sulphoxide reductases, all of which ultimately require reductive equivalents from NADPH (ß-nicotinamide adenine dinucleotide phosphate, reduced form) for their function[12]. Each of these systems protects against superoxide- and/or peroxide-mediated oxidative stress, as demonstrated in gene-deletion studies in organisms such as yeast[12-14]. The glutathione-S-transferases, cytochrome P450s and carboxyl/ cholinesterases have been directly implicated in the detoxification of xenobiotics. In insects these three superfamilies are heavily involved in insecticide metabolism and they contribute to the great majority of mutations conferring metabolic resistance to chemical insecticides[15-17]. Insects also possess ascorbate peroxidase and the glutathione S-transferase with peroxidase-like activity. [18-20]

A portion of ROS is scavenged by dietary antioxidants such as ascorbate (vitamin C), α – lipoic acidand, carotenoids (lycopene, lutein, astaxantin, violaxanthin, zeaxanthine, α-, β-, γ-carotene, and β- carotene-5,6-epoxide.) and vitamin E (α –tocopherol, α –T). But most of the ROS are eliminated by the suite of antioxidant enzymes already mentioned. [8]

1.3 How to elicit oxidative stress experimentally?

This brief description will be based in a substance that is commonly used to experimentally induce oxidative stress conditions in insect models including *Pyrrhocoris apterus*.

Paraquat (1,1' -dimethyl-4,4'-bipyridinium) and its dichloride salt (1,1', dimethy l-4, 4'-bipyridinium dichloride) are a broad-spectrum contact weed killer and herbage desiccant that is used widely in agriculture and horticulture. It was formulated in 1882, but its herbicidal properties were not discovered until 1955. Since its introduction in the early 1960's, Paraquat (PQ) has been used extensively in about 130 countries, on a wide variety of agricultural crops. [21 & 22]

Several studies have shown that terrestrial invertebrates display varying degrees of sensitivity to PQ. In honeybees, PQ produced toxic signs[23]. Adsorbed PQ may be ingested by soil invertebrates,. Two species of springtails (Collembola; *Folsomia candida*, *Tullbergia granulata*) survived without measurable adverse effects. However, higher dietary levels were associated with

decreased survival, lengthier instar development, decreased egg production, and decreased egg viability[24]. Adults and larvae of the German cockroach (*Blattella germanica*) died after consuming diets containing PQ 1,000 mg/kg. Also, PQ was lethal to two species of mites (*Tetranychus urticae, Typhlodromus sp*.) at concentrations below recommended field application rates[23]. The toxic effects of PQ have also been studied in vertebrate animal models, especially those related to neurodegenerative damage. [25]

Most authorities agree that free radical pathology is the most likely mechanism by which PQ is cytotoxic[26-32]. The biochemical mechanism of its toxicity is due to the cyclic oxidation and reduction in tissues, leading to production of $\mathbf{O_2^-}$, singlet oxygen, hydroxyl radicals and eventually, the highly destructive H_2O_2. They all are capable of initiating the peroxidation of membrane lipids, causing tissue damage and death. [25]

2. ADIPOKINETIC HORMONES AND THEIR FUNCTIONS

2.1 Adipokinetic hormones

Stress conditions are metabolic demanding, the requirements of animals in these situations are essentially similar: they must mobilise their energy stores to eliminate or at least to minimize the stress impact on their physiological functions. Insect metabolism and especially its energetic part is controlled predominantly by adipokinetic hormones (AKHs), which are synthesised, stored and released by neurosecretory cells from the *corpora cardiaca* (CC), a neuroendocrine gland connected with the brain.

With some simplification, an analogy can be drawn between the brain (neurosecretory cells) and CC secretory system in insects, and the hypothalamus and the pituitary (adenohypophysis and neurohypophysis) gland system in vertebrates. In both cases, brain neurosecretory cells synthesize neurohormones that are released from remote neurohaemal areas: the CC (neurohaemal lobe) in insects, and the pituitary gland (neurohypophysis) in vertebrates. Furthermore, both the CC and the pituitary (adenohypophysis) synthesize and secrete their own intrinsic hormones. [33]

Although the CC are the major source of AKHs, the brain of some insects also contains AKH-like material[34-36]. AKHs comprise eight to ten amino acids except for an unusual AKH of the butterfly *Vanessa cardui*[37] comprising an 11-mer. The hormones have been isolated from representatives of many insect orders[38 & 39]. To date, more than 40 insect AKHs have been characterized. Together with one crustacean representative recently found also in bugs (Heteroptera)[40], a chromatophorotropin – red pigment concentrating hormone (RPCH)[41], they form an AKH/RPCH peptide family.

2.2 Functions of adipokinetic hormones in energy mobilisation

A major function of AKHs is the control of energy metabolism. As typical stress hormones the AKHs stimulate catabolic reactions (e.g. mobilise lipids, carbohydrates and/or certain amino acids) making energy more available; at the same time they inhibit synthetic reactions. The mobilisation of energy reserves enables the organism to combat the immediate stress problems and to suppress processes that are momentarily less important and could, if the stress effect persists, even draw on the mobilised energy. Insect fat body is the main target tissue for AKHs action; there the signal transduction at the cellular level is well documented. [42 & 43]

Recently AKH receptors have been cloned from the fruit fly *Drosophila melanogaster* and the silkworm *Bombyx mori*[44 & 45]. The receptors are G protein-coupled and are, structurally and evolutionarily, related to the gonadotropin releasing hormone receptors from vertebrates. Generally, in *hypertrehalosemia*, AKHs bind their receptors and activate phospholipase C (PLC) increasing inositol 1,4,5-trisphosphate (IP3) levels. This induces the release of Ca^{2+} from intracellular stores which leads to the initiation of the capacitative Ca^{2+} entry into the cytosol. The increased Ca^{2+} concentration results into phosphorylation and activation of glycogen phosphorylase by phosphorylase kinase[43]. In *Periplaneta americana*, production of diacylglycerol (DAG) along with IP3 has been proposed. DAG in conjunction with Ca^{2+} then activates protein kinase C (PKC), which, in turn, activates glycogen phosphorylase by phosphorylation[46 & 47]. During *hyperlipemia*, binding of AKH may lead to a conformational change in a G_s protein which, in turn, activates an adenylate cyclase, resulting in an increase of intracellular cyclic AMP levels. Cyclic AMP stimulates lipase activity, most likely via activation of a protein kinase A. The influx of extracellular Ca^{2+} is also essential for the adipokinetic effect. In moths, release of Ca^{2+} from IP3-insensitive intracellular stores causes an increase in the hemolymph lipid titers[48]. The mode of action of AKHs during *hyperprolinemia* appears to be similar to that during hyperlipemia. It seems that AKHs bind to the receptor to cause a conformational change of a G_s-protein which, subsequently, activates an adenylate cyclase. The increase in cyclic AMP levels might activate triacylglycerol lipase and consequently triacylglycerol (TAG) breakdown to release fatty acids. AKHs seem to activate Ca^{2+} release from intracellular stores and also the capacitative Ca^{2+} entry into the cytosol. [43]

AKH functions in energy metabolism are accompanied with a number of supporting actions that increase the affectivity of mobilised energy. For example inhibition of anabolic processes such as RNA[49], protein[50] and lipid syntheses[51] ensure that mobilised energy is directed to cover the energy demands. The inhibition of total RNA synthesis by AKHs was shown in *L. migratoria* fat body incubated *in vitro*. The inhibition is dose-dependent, with potency decreasing in the order Locmi-AKH-III > AKHII > AKH-I. Studies in which the significance of the inhibition of protein synthesis by AKHs were investigated, are concerned with vitellogenin and arylphorin syntheses in

the fat body[52]. The inhibition of lipid synthesis by extracts of CC was demonstrated by Gokuldas *et al*.[51]. The inhibitory effect of AKH on fatty acid synthesis was confirmed in a series of experiments with dispersed fat body cells from *L. migratoria* prepared by chymotrypsin treatment. [53 & 54]

On the other hand AKH function in mobilisation of nutrients is supported by AKH elicited stimulation of heart beat[55] which helps to their quicker transport.

Special attention is paid to stimulation of locomotion. Hormonal control of insect visceral and skeletal muscle activity is a well-documented phenomenon. Stimulatory effects of AKHs on insect (skeletal) muscle activity are demonstrated in *P. apterus*[56 & 57], *Gryllus bimaculatus*[58] *P. americana*[59] and *D. melanogaster*[60 & 61], in all injections of the hormone increase locomotor activity significantly. The AKH modulation might be dual;metabolic and neuromodulatory. [56]

2.3 Other functions not associated with energy metabolism

Recently, it was found that AKHs participate in stress reactions that do not include rapid production and subsequent consumption of energy[62]. Stress-induced elevation of the AKH titre occurs in *Schistocerca gregaria* and *P. apterus* challenged with an insecticide[63 & 64], excessive KCl[65], photophase interruption[66], or exposure to constant darkness[67].

AKHs play a complex role in the formation of insect eggs which was proven by Lorenz in the cricket *G. bimaculatus*. Crickets injected with Grybi-AKH show a significantly reduced lipid and protein content in the fat body; and a significant reduction of the ovary mass due to the retarded maturation of the oocytes as well as a lower number of terminal oocytes[68]. The complex role of AKHs in two of the most energy-demanding events in the adult insect life (i.e. egg production and insect locomotion) prove their crucial position in insect metabolism and in adjusting to stress situations.

An interesting role of AKHs as multifunctional anti-stress coordinators was proven by their participation in insect humoral and cellular immune system. The prophenoloxidase cascade in the haemolymph of *L. migratoria* is activated when laminarin is injected, and this activation is prolonged when Locmi-AKH-I is co-injected with immunogen[67]. The injection of bacterial lipopolysaccharide (LPS) from *Escherichia coli* stimulates the formation of nodules but does not increase the phenoloxidase activity in the haemolymph; on the other hand, co-injection of Locmi-AKHI and LPS results in a greater number of nodules being formed, and activates also the prophenoloxidase cascade[68 & 69]. It is suggested that these two immunogens must activate the prophenoloxidase cascade by quite distinct pathways, that are probably not based on rapid changes in the energy rich metabolites; although, changes in the lipophorins and the apolipoprotein-III coincident with immune challenge point to a participation of lipids in this process. [70]

Application of PQ into the insect body increases the titre of AKH in both the CC

(*Leptinotarsa decemlineata*) and haemolymph (*L. decemlineata* and *P. apterus*)[71& 72](see page 8). Multiple increases in the titre of AKH occur in *L. decemlineata* fed on genetically modified potatoes that express *Bacillus thuringiensis* toxin or *Gallanthus nivalis* lectin[71]. Interestingly, these stressors also cause oxidative stress similar to that induced by PQ[73]. As both PQ and the toxins from the genetically modified potatoes have similar effects on the AKH levels, it is likely that the mechanism is similar. Moreover, an injection of exogenous AKH mobilises anti-oxidative mechanisms that reduce failures incurred by oxidative stressors: protein carbonylation, decrease of glutathione levels and attenuation of total antioxidant activity in haemolymph. These observations indicate that a feedback regulation might operate between the stressor and AKHs, and the last could elicit antioxidant protection mechanisms. The possible mode of action is not known in detail, but oxidative stress could be a causative factor accelerating synthesis or release of AKH in/from the CC. [72]

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Adipokinetic hormone-induced enhancement of antioxidant capacity of *Pyrrhocoris apterus* hemolymph in response to oxidative stress

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Abstract

The *in vivo* effects of oxidative stress on adipokinetic hormone (AKH) titer in short-winged (brachypterous) males of the firebug *Pyrrhocoris apterus* were tested using paraquat (PQ), a bipyridilium herbicide. PQ undergoes a cyclic redox reaction with oxygen during microsomal and electron transfer reactions forming free radicals in the insect body. Oxidative insult (40 pmol PQ) resulted in enhanced protein carbonylation (a biomarker for oxidative stress) and a depletion of glutathione (GSH) pool in the hemolymph. Interestingly, AKH titer was significantly enhanced in hemolymph at 4 h post inoculation of PQ, while its content in CNS (brain with corpora cardiaca) showed non-specific changes in comparable period. Co-injection of AKH with PQ (40 pmol each) reversed these effects by decreasing protein carbonyl formation, increasing reduced GSH levels, and enhancing the total antioxidant capacity of cell free plasma. Our results indicate that there is a positive feedback regulation between an oxidative stressor action and the level of AKH in insect body, and that AKHs might be involved in the activation of antioxidant protection mechanism.

Keywords: Adipokinetic hormone; Antioxidant activity; Oxidative stress; Paraquat

1. Introduction

Adipokinetic hormones (AKHs) are a family of structurally related neuropeptides that occur widely in insects. These short peptides of 8–10 amino acids regulate various aspects of insect intermediary metabolism and exert a wide range of effects. All AKHs contain an N-terminus pyroglutamate residue and a C-terminus amide, and possess tryptophan in position 8 and phenylalanine or tyrosine in position 4. AKHs are synthesized and stored in endogenous neurosecretory cells of the corpora cardiaca (CC), but small quantities are also found in the brain (Moshitzky et al., 1987a,b; Bray et al., 1993; Kodrík et al., 2003). The "classical" function of AKH peptides is the mobilization of energy substrates for flight activity, but, like true multifunctional and pleiotropic hormones, they are also involved in a large number of well-described actions such as cardiostimulation, inhibition of the synthesis of RNA, fatty acids and proteins in the fat body, stimulation of biosynthesis of mitochondrial cytoheme a+b, including the induction of gene expression for a cytochrome P-450 enzyme, and stimulation of oxidation of substrates by the flight muscles (Gäde, 2004; Gäde et al., 1997). AKHs also function as stress responsive hormones to insecticide treatment (Samaranayaka, 1974; Candy, 2002; Kodrík and Socha, 2005) photophase interruption (Kodrík et al., 2005) as well as infection by pathogens (Goldsworthy et al., 2002, 2003a,b, 2005; Mullen and Goldsworthy, 2003). Recently, we also demonstrated a multiple increase of AKH content in CC and hemolymph of the Colorado potato beetle *Leptinotarsa decemlineata* when fed genetically modified potatoes expressing *Bacillus thuringiensis* and *Galanthus nivalis* toxins concomitant with oxidative stress in gut tissues presumably because of survival challenge (Kodrík et al., 2007). The results were mimicked by the application of paraquat (PQ), a bipyridilium herbicide that creates conditions of oxidative stress (OS) by undergoing cyclic redox reactions with oxygen during microsomal and electron transfer reactions (Hassan, 1984). PQ has most often been implicated as a potential neurotoxicant

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3. THE ROLE OF GLUCAGON

3.1 Glucagon peptides

The well known vertebrate hormone glucagon and glucagon-like peptides are produced in the gut, pancreas, and brain, and exert multiple biological actions converging on energy homeostasis via activation of distinct G protein-coupled receptors. Glucagon, liberated from islet α-cells of the pancreas, promotes glucose homeostasis via control of glucose production and glycogenolysis. Glucagon-like peptide 1 (GLP-1) and glucagon-like peptide 2 (GLP-2) are secreted from gut endocrine cells. They regulate energy disposal and the functional integrity of the gastrointestinal epithelium. In humans, the actions of these peptides and related analogues are relevant to the treatment of disordered energy homeostasis as exemplified by diabetes, obesity, and intestinal insufficiency. [74]

The proglucagon gene is expressed as a single messenger RNA transcript in α –cells of the endocrine pancreas, the enteroendocrine L-cells of the small and large intestine, and in brain stem neurons within the central nervous system (CNS) [75]. Although very little is known about the factors responsible for regulating proglucagon gene expression in CNS[76].

Proglucagon mRNA transcripts are translated into a single 160 amino acid precursor in the pancreas, intestine, and brain. Subsequently it undergoes tissue specific posttranslational processing mediated by a prohormone convertase to generate structurally related, yet distinct, proglucagon-derived peptides (PGDPs)[75]; these PGDPs in turn activate specific seven transmembrane G protein-coupled receptors and play important roles in modifying nutrient intake, absorption and assimilation. [77]

3.1.2 Glucagon

In pancreatic α -cells, the action of the prohormone convertase-2 liberates glucagon, a hormone that is important for regulating carbohydrate metabolism. Glucagon is a 29 amino acid peptide whose effects converge on hepatic glucose output and oppose those of insulin. As a result, glucagon elevates blood glucose levels primarily by stimulating glucose output via enhancement of glycogenolysis and promotion of gluconeogenesis in the liver. The injection of glucagon into the CNS has a potent inhibitory effect of food intake and may also play a role in regulating meal size. Lower levels of glucagon receptor mRNA are present in the stomach and intestine. [75]

Glucagon receptors (GluR) are widely expressed in multiple mammalian tissues including liver, heart, kidney, spleen, ovary, pancreatic islets, thymus, stomach, adrenals, intestine, thyroid, skeletal muscle, adipose tissue, lung and brain. Therefore, their effects also include stimulation of lipolysis in adipose tissue, elevation of heart rate and blood pressure, and regulation of renal functions[77]. The binding of glucagon to its receptor leads to the activation of adenylyl cyclase, elevation of intracellular cyclic AMP, and activation of protein kinase A (PKA). Glucagon can also

activate phospholipase C and increase intracellular levels of IP3 and Ca^{2+}. Structure–function studies of the glucagon receptor indicate that sequences in the N-terminal extra cellular domain, in particular aspartic acid residue 64, as well as the first extra cellular loop and the third and fourth transmembrane domains are essential for ligand binding. *op. cit.*

3.1.3. Proglucagon-derived peptides in the intestine and brain

In the L-cells of the intestine, proglucagon is processed by the prohormone convertase 1/3 to glicentin, oxyntomodulin, GLP-1, GLP-2, and a peptide of unknown function designated intervening peptide[77]. Although receptors for the PGDPs have been best characterized in mammalian species[78] they have also been cloned from different vertebrate representatives. [78 - 80]

3.2 Glucagon related peptides in insects

Early studies in insects focused on the existence of metabolic hormones resembling those known in vertebrates. Several insect neurosecretory cells showed immunoreactivity to antisera raised against mammalian neuropeptides. Essentially, peptides of gastro-entero-pancreatic type have been detected also in insect nervous tissue[81 & 82]. But in insects, the regulation of glycaemic metabolism is mainly conferred to AKH family members. Almost all members of this family present a hyperglycaemic activity, but no close structural homology with the glucagon family was detected. The existence of glucagon family molecules has been reported in insects, but their involvement in glycaemia regulation may not be predominant: Tager *et al.*[83] reported the presence of glucagon related molecules in CC and *corpora allata* (CA) extracts of *M. sexta*. Kramer *et al*.[84] also detected a glucagon-like molecule in the haemolymph of larvae and pupae of *M. sexta*. El Sahly *et al*.[85] confirmed these results and suggested the CNS as the origin of this molecule; this factor may then be transported to CC and CA for storage or release. Thorpe and Duve[86] also reported immunological staining of secretory cells in the nervous system of *Calliphora vomitoria*. The data presented in this thesis, confirm the presence of immunoreactive glucagon material in the CNS and gut of the firebug *P. apterus* (see page 12). All these results attested to the existence of glucagon-like molecules in insects, probably produced by neurosecretory cells and stored in a neurohemal organ before release.

Maier *et al*. confirmed via experimental injections the involvement of glucagon-like peptide in the regulation of glycemia in the honeybee *Apis millifica*[87]. But our experiments showed, in accordance with Ziegler's findings in *M. sexta*[88], that porcine glucagon does not elicit mobilisation of energy supplies such as AKHs.

3.3 AKH and glucagon functional homologies

Different studies suggest that AKHs bear glucagon-like functions[89 - 91]. The main function of

glucagon is similar to that of AKHs: mobilization of energy reserves, mainly glucose; and participation in the control of glucose level in the blood. Although, the control of carbohydrate level in insect haemolymph is less accurate since insects can tolerate substantial fluctuations of these compounds[92]. Vertebrate glucagon activity is directed mainly to the liver, whereas AKH function is targeted mainly in the fat body, an insect analogue of the liver tissue. Apart from this tissue, glucagon and AKH are also found in peptidergic neurons.

Such as glucagon in pancreatic islet α- cells, AKH is synthesized as a pre-hormone and subsequently processed and stored in dense core vesicles[91 & 92]. Although structurally there are not close homologies, some authors have considered some sequence similarity with the N-terminus of glucagon and AKH peptides[93]. Our results in MegAligment showed only 37.5% similarity between *P. apterus* AKH and porcine glucagon. Similar to mammalian glucagon activity in liver, AKH has been demonstrated to bind a G-protein-coupled transmembrane receptor[94]. Nevertheless, it must be taken into account that AKH binds G-protein coupled receptors from the A family (Rhodopsin-like), wheras glucagon binds B family members, also called Secretin receptor family. And the fact that mammalian glucagon appears to activate different transduction mechanisms depending on hormone concentration. [95]

One more functional homology was suggested: apparently, the relationship between *D. melanogaster* insulin-like peptides and AKH is homologous to the antagonism between insulin and glucagon in vertebrates.[90 & 96]

3.4 Glucagon and oxidative stress in insects and vertebrates

There are indications that glucagon could play a regulatory role in activation of antioxidant mechanisms in vertebrates to protect the organism from oxidative stress. Lu *et al*.[97] reported that glucagon-mediated signal transduction pathways lead to a down regulation of hepatic GSH synthesis while promoting the efflux of GSH to the blood plasma in rats. Based on these findings, it was suggested that the amelioration of stress markers might be under regulation of certain hormones, at least those which act through the cyclic AMP second messenger cascade. [97]

Our studies showed no mobilisation of lipids and no changes on AKH levels after glucagon treatment in the insect model. However, glucagon stimulated antioxidant mechanisms after induction of oxidative stress by PQ. In both cases, the coupling of either AKH or glucagon with the stressor PQ prompted protective mechanisms against oxidative stress but by different pathways.

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Activation of insect anti-oxidative mechanisms by mammalian glucagon

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ABSTRACT

Resembling the main function of insect adipokinetic hormones (AKHs), the vertebrate hormone glucagon mobilizes energy reserves and participates in the control of glucose level in the blood. Considering the similarities, the effect of porcine glucagon was evaluated in an insect model species, the firebug *Pyrrhocoris apterus*. Using the mouse anti-glucagon antibody, presence of immunoreactive material was demonstrated for the first time in the firebug CNS and gut by ELISA. Mammalian (porcine) glucagon injected into the adult bugs showed no effect on hemolymph lipid level or on the level of AKH in CNS and hemolymph, however, it 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 levels in hemolymph, decreasing both protein carbonyl and protein nitrotyrosine levels in CNS. Additionally, when co-injected with paraquat, glucagon partially eliminated oxidative stress markers elicited by this redox cycling agent and oxidative stressor. This indicates that glucagon might induce an antioxidant defense in insects, as recently described for AKH. Failure of glucagon to alter AKH level in the bug's body indicates employment of an independent pathway without involving the native AKH.

1. Introduction

Glucagon is a 29-amino acid peptide well-known as a vertebrate hyperglycaemic hormone. It is synthesized mainly in the A-cells present at the periphery of the islets of Langerhans in the pancreas, but is also detected in some specific cells of stomach, intestine as well as specialized neurones (Mayo et al., 2003). In insects, immunochemically similar glucagon-like peptides were reported in the hemolymph of the tobacco hornworm *Manduca sexta* (Kramer et al., 1980), midgut endocrine cells of cockroaches (Žitňan et al., 1993) and neurosecretory system of several insect species (Howard et al., 1976; El-Salhy et al., 1983; Raabe, 1985). In *Mytilus edulis*, glucagon-like peptides have been immunocytochemically demonstrated in the cerebral ganglia (Kellner et al., 2002). On testing the effects of vertebrate (porcine) glucagon in this gastropod they postulated that the glucagon-like peptide may be implicated in the regulation of glucose metabolism of bivalves. Bioactive peptides immunochemically similar to glucagon have also been demonstrated in the hemocytes of the snail *Viviparus ater* (Ottaviani et al., 1992) and in the gut of rainbow trout, *Salmo gairdneri* (Holmgren et al., 1982) In mammals, in addition to the hormone glucagon, glucagon-like peptides have also been demonstrated (designated GLP-1) and have been implicated in stress response (Frezza and Wachtel, 2007). Mice lacking GLP-1 receptor have an altered response to stress (MacLusky et al., 2000). GLP-1 changes the corticosterone response in isolated adrenocortical cells to adrenocorticotropic hormone (ACTH), suggesting a link between GLP-1, the stress response, and the hypothalamic-pituitary-adrenal axis (Andreis et al., 1999). In insects, however, despite certain structural similarities to vertebrate glucagon and some promising indications of an influence on the carbohydrate level (Tager et al., 1976; Maier et al., 1990), the claims for general metabolic actions of the glucagon-like peptide failed to be reproducible, and its function remains unknown. In addition, mammalian glucagon fails to mobilize insect energy stores in *M. sexta* (Ziegler, 1979).

At the functional level glucagon resembles insect adipokinetic hormones (AKHs)—short peptides of 8–10 amino acids, synthesized, stored and released by neurosecretory cells in the corpora cardiaca, a neuroendocrine gland which together with the neurosecretory cells in brain and the corpora allata forms the main regulatory centre of neuro-endocrine functions in insects. The AKHs are sometimes designated as insect glucagon (Goldsworthy, 1994), because they mobilize energy reserves (lipids, carbohydrates and/or certain amino acids) (Gäde et al., 1997) in stress situations and suppress processes that are momentarily unimportant and could even draw on the mobilized energy (like e.g. synthetic reactions). However, these peptides are pleiotropic, with a number of functions in addition to their metabolic role (Kodrík, 2008).

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4. INTERACTIONS OF ADIPOKINETIC AND JUVENILE HORMONES

4.1 AKHs and juvenile hormones

Similarly, as AKHs and perhaps some other groups of insect hormones, also juvenile hormones (JHs) are pleiotropic in their functions, controlling many aspects of insect life, but their principal roles are regulating metamorphosis and, in many species, vitellogenin synthesis and deposition in the oocytes of adult females[98]. On the other hand, the adipokinetic peptides are controlling predominantly energy metabolism, but as mentioned previously, many other processes including synthesis of basic nutrients (inhibition of RNA, protein (vitellogenin) and lipid syntheses) that interfere with actions not only of energy metabolism, but also with processes like oxidative stress, humoral and cellular immune response or reproduction[62]. Therefore, it is evident that, functions of juvenile and adipokinetic hormones can intersect in some processes and a possible feedback between them is logical.

In this last chapter the coupling action of JH is analysed in terms of AKH characteristics already described for the insect model *P. apterus* (see page 15). As the natural *P. apterus* JH has not been identified yet, in spite of a prolonged and intensive effort of several researchers, the juvenile analogue (JH-a) methoprene was used to mimic the JH action.

4.2 Juvenile hormone

JH is a sesquiterpenoid lipid-like hormone secreted by the *corpora allata* (CA), endocrine glands of the head situated behind the brain[98-103]. Insects produce at least eight forms of JH-like compounds (0, I, II, III, JH3 bisepoxide [JHB3], 4-Methyl-JH, 80-OH-JH III, 120-OH-JH III), JH III being the most common type. [98 - 100, 102, 103]

While the molecular details underlying JH action remain poorly understood, JH is known to respond to various internal (e.g. hormonal, genetic) and external (e.g. temperature, nutrition, photoperiod) factors, to regulate and coordinate the expression of entire gene batteries, and to simultaneously affect multiple phenotypes[98, 104, 105]. Remarkably, JH affects a vast array of phenotypic traits and physiological or developmental processes including: pre-adult development, imaginal disc proliferation, organ looping, metamorphosis, ovarian development, sexual maturation, pheromone production, locomotor and courtship behaviour, diapause regulation, migration, various morphological polyphenisms, division of labour in social insects, neuronal architecture, memory, learning, immune function, stress resistance and ageing. [98 – 100, 104-113]

JH is present throughout late embryonic and larval development and serves a ‘status quo’ function coordinating with other basic methamorphosis hormones, ecdysteroides. JH thus determines whether the insect molts to a larva, pupa or adult. Metamorphosis occurs only when 20-hydroxy-ecdysone acts in the absence of JH[98 & 112]. In many insects, experimentally induced excess

of JH during larval development delays metamorphosis, whereas withdrawal leads to precocious metamorphosis[98].

The main function of JH in adults concerns the control of reproductive processes. It affects the synthesis and secretion of vitellogenins in fat body, yolk proteins in ovarian follicle cells and their uptake by developing oocytes[112, 114 - 116]. The treatment with either ecdysteroids or the JHa methoprene, upregulates transcription of proteins in the fat body[112 & 116]. In response to stressful environmental conditions, adult insects can enter a state of reproductive diapause (also called quiescence or dormancy), characterized by (1) reduced metabolism, (2) arrested oogenesis, mating and egg production, (3) increased stress resistance, and (4) enhanced survival. [117 - 121]

Interestingly, JH also suppresses stress resistance and innate immunity[119, 121 - 124]. Reproductively dormant *Drosophila* with down regulated JH exhibit greater resistance to heat and oxidative stress than nondiapausing flies[119], and methoprene treatment of female *D. melanogaster* increases the number of vitellogenic oocytes, while decreasing resistance to oxidative stress and starvation resistance[122]. In the mealworm beetle (*Tenebrio molitor*), the level of phenoloxidase, an antimicrobial agent, is reduced by mating but the application of the JH inhibitor fluvastatin increases the immune function[124]. By increasing reproduction at the expense of stress resistance, immunity and longevity, JH may thus be an important proximate mechanism underlying evolutionary trade-offs between reproductive and survival functions. [125]

4.3. AKH interaction with methoprene

An idea of possible interaction(s) of AKH with JH and/or JH-a comes from the studies of Kodrík and Socha and their co-workers on *P. apterus*[36, 56, 126 - 129]. Recently, we have shown that methoprene induced significant reduction of haemolymph lipids after the treatment, however, did not reduced significantly the ability of the AKH to mobilise fat body lipids. The same methoprene treatment elicited a significant increase of AKH content in the CNS (brain, *corpora cardiaca* and *corpora allata* complex) of experimental bugs after the JH-a application. A significant decrease of the AKH level in the haemolymph was recorded at the same times and under the same experimental conditions. It suggests that methoprene may reduce AKH release from the CNS, while an increase of the AKH content in the CNS could be a result of greater hormonal accumulation rather than the stimulation of AKH synthesis (see page 15).

4.4 Juvenile hormone analogues and their practical usage as biorationale pesticides

The idea that insects would be unable to develop resistance against JH if it was used as a control agent was one of the driving principles behind the impetus to develop this hormone as an insecticide[130]. The difficulty and cost of synthesizing such a complex molecule with a labile epoxide

moiety and susceptibility to degradation delayed the realization of this concept. However, it soon became apparent that several synthetic analogues of JH, many of them several fold more active than the native hormone, could be used as control agents. Nowadays, several hundreds or perhaps thousands of compounds, obtained by natural or synthetic processes, are known to act as JH-as. They are of practical importance, mainly to control insect pest populations, due to their negative effect on embryonic or larval development, or on adult reproduction altering the hormonal levels in the insect body. Methoprene, a well known JH-a, was used in our study (see below).

Methoprene is perhaps one of the best known terpenoids developed for pest control. Extensive data collected over several years have shown that this JHa is relatively nontoxic to most non-target organisms. It has been used as a mosquito larvicide and for controlling many coleopterans, dipterans, homopterans, and siphonopterans[131]. Data obtained from a mutant strain of *D. melanogaster* that was tolerant to methoprene (Met flies) showed that methoprene resembles better JH III, JH B3, and several JHas but not many other insecticides. [132]

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Methoprene modifies adipokinetic hormone characteristics in the firebug *Pyrrhocoris apterus* (L.)

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CONCLUSION REMARKS

The three papers that constitute the backbone of this thesis brought about the following conclusions:

1. A treatment of *P. apterus* adults by paraquat (PQ), a compound that creates conditions of oxidative stress, resulted in **(A)** a significant elevation of the AKH level in haemolymph without changing it in CNS. The PQ treatment resulted also in **(B)** significantly enhanced carbonyl contents and a depletion of glutathione pool in the haemolymph, but the co-injection of AKH and PQ reversed these effects by decreasing protein carbonyl formation, increasing reduced glutathione levels, and enhancing the total antioxidant capacity of cell free plasma. The results indicate **(C)** a positive feed back regulation between an oxidative stressor action and the level of AKH in insect body, and that AKHs might be involved in the activation of antioxidant protection mechanism.

2. In search for a functional homology between AKH and glucagon **(A)** the presence of immunoreactive glucagon material was demonstrated in the firebug CNS and gut. The injection of mammalian glucagon showed **(B)** no effect on haemolymph lipid level or on the level of AKH in CNS and haemolymph, but **(C)** glucagon elicited the antioxidant response by significantly increasing glutathione and decreasing protein carbonyl levels in haemolymph, and decreasing both protein carbonyl and protein nitrotyrosine levels in CNS. Additionally, **(D)** when co-injected with PQ, glucagon partially eliminated the oxidative stress markers. This implies that **(E)** glucagon might induce an antioxidant defense in insects, as mentioned above for AKH.

3. The treatment of *P. apterus* adults with the juvenile hormone analogue methoprene **(A)** induced reduction of haemolymph lipids, but did not reduce the ability of AKH to mobilise fat body lipids. The methoprene treatment elicited **(B)** a significant increase of the AKH content in CNS and a significant decrease of it in haemolymph. **(C)** Similar results were observed when the production of AKH, from the CNS, was evaluated under *in vitro* conditions. These results suggest that **(D)** methoprene may reduce AKH release from the CNS, while an increase of the AKH content in this tissue could be the result of a greater hormonal accumulation rather than the stimulation of AKH synthesis.

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Curriculum vitae

EDUCATION

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PUBLICATIONS

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