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# Knockdown of adipokinetic hormone synthesis increases susceptibility to oxidative stress in *Drosophila* — A role for dFoxO?

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ABSTRACT

Insect adipokinetic hormones (AKHs) are pleiotropic hormones known to play a protective role in response to oxidative stress (OS). 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 in its response to OS was studied in *Drosophila melanogaster*. AKH knockdown (AKH-RNAi) as well as AKH overexpression (AKH-oex) was achieved using the Gal-4/UAS system and controls were $w^{1118}$ (+/+), AKH-Gal4/+, UAS-AKH/+ and UAS-AKH-RNAi/+. 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*.



## 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, 2004a, 2004b; 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, 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, 2004a, 2004b), 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).

*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.

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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 (Mattila et al., 2009). 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 transcription 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 and Karin, 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

*D. 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 : 12-h dark (LD) cycles (with an average light intensity of 2000 lx). Stocks used in this study were the wild type $w^{1118}$, AKH-GAL4 (BL # 25683) (Lee and Park, 2004), UAS-AKH-RNAi where RNAi is induced by an expression of a TRiP line (using a short hairpin construct) (BL # 34960), 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 $w^{1118}$, AKH-Gal4/+, UAS-AKH/+ and UAS-AKH-RNAi/+. 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 they 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 h before being transferred to vials containing 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 h. HP was replenished once daily till the 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 h 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 a 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 h 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 (Livak and Schmittgen, 2001) with mRNA levels normalized to the gene *rp49*. Relative mRNA amplitude was calculated with respect to untreated control (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 4h exposure to HP stress. In parallel, flies from untreated groups were collected in a similar manner. About 25 flies were homogenized on ice in 50 mM phosphate buffer, sonicated, and centrifuged at 10,000 *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 1× 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