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Dietary alpha-ketoglutarate promotes higher protein and lower triacylglyceride levels and induces oxidative stress in larvae and young adults but not in middle-aged *Drosophila melanogaster*

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ABSTRACT

Alpha-ketoglutarate (AKG) is involved in multiple metabolic and regulatory pathways. In this work, the effects of AKG-supplemented diets on selected physiological responses and metabolic processes, including metabolism of reactive oxygen species, was assessed in larvae and adult (both 2 and 24 days old) *Drosophila melanogaster*. Dietary supplementation with AKG resulted in dose-dependent effects on larval development, body composition and antioxidant status of third instar larvae. Larvae and young (2 days post-eclosion) adult females fed on AKG shared similar metabolic changes such as higher total protein levels, lower triacylglyceride levels and higher values for oxidative stress indices, namely lipid peroxides and low molecular mass thiols. The latter indicated the development of oxidative stress which, in turn, may induce adaptive responses that can explain the higher resistance of AKG-fed young females to heat shock and hydrogen peroxide exposure. In contrast to young flies, middle-aged females (24 days) on AKG-containing diet possessed higher total protein, glucose and triacylglyceride levels, whereas oxidative stress parameters were virtually the same as compared with control females of the same age. In parallel, females fed an AKG-supplemented diet showed lower fecundity, higher heat shock resistance but no change in oxidative stress resistance at middle age which in combination with levels of protein, glucose, and triacylglycerides can be considered as potentially beneficial AKG effects for aging organisms. To our best knowledge, this is the first study on age-matched AKG influence on animals' organism which shows that *Drosophila* may be used as a model for previous quick study in cost-efficient manner age-related AKG effects in mammals and humans.

1. Introduction

Alpha-ketoglutarate (AKG) is an important cellular metabolite that participates in energy production and amino acid metabolism. Oxidative deamination of glutamate via glutamate dehydrogenase and oxidative decarboxylation of isocitrate in the tricarboxylic acid (TCA) cycle are the main endogenous sources of AKG. Recent studies with mammals have indicated that AKG used as a dietary supplement can confer favorable effects on both injured and healthy organisms (Harrison and Pierzynowski, 2008; Zdzisińska et al., 2016).

Stimulation of protein synthesis, absorption/metabolism of amino acids, prevention of protein degradation in skeletal muscle and maintaining bone structure are the best studied health-promoting effects of AKG (Lambert et al., 2006; Harrison and Pierzynowski, 2008; Hou et al., 2010; Yao et al., 2012). These effects of AKG are mainly explained by its role as a precursor for biosynthesis of certain amino acids such as glutamate, glutamine, leucine, and proline (Lambert et al., 2006; Wu, 2009; Zdzisińska et al., 2016). Glutamine in combination with leucine stimulates protein synthesis via activation of the mammalian target of rapamycin (mTOR) signaling pathway (Durán et al., 2012; Yao et al., 2012). Therefore, AKG was proposed to be a compound that could activate the mTOR pathway and anabolic processes in animal tissues (Hou et al., 2010; Durán et al., 2012; Yao et al., 2012). By contrast, Chin et al. (2014) reported that the lifespan-prolonging effects of AKG supplements on nematodes, *Caenorhabditis elegans*, were due to inhibition TOR kinase and promotion of a state similar to that induced by dietary restriction.

Supplementation with AKG promoted higher levels of reactive oxygen species (ROS) in *C. elegans* (Chin et al., 2014). It seems that intensification of the TCA cycle is responsible for enhanced ROS production,

Abbreviations: AKG, alpha-ketoglutarate; KPi, potassium phosphate buffer; ROS, reactive oxygen species; L-SH, low molecular mass thiols; LOOH, lipid peroxides; IDH, isocitrate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase.

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since AKG is preferentially metabolized in this pathway (Lambert et al., 2006; Harrison and Pierzynowski, 2008). The increased metabolite flux trough TCA cycle can result in overload of the mitochondrial electron transport chain with reductive equivalents (NADH and $FADH_2$) which may lead to an increment in ROS generation. Enhanced ROS production may induce low intensity (mild) oxidative stress (Lushchak, 2014) that, in turn, can activate defense mechanisms and potentially provide sufficient protection against any subsequent strong oxidative or other stresses and thereby contribute to lessening functional decline of various processes with age (Lushchak, 2014; Sies, 2015). In line with this, supplementation with AKG was found to prevent an age-related increase in free radical damage to biomolecules in aged mice that was accompanied by modulation of antioxidant enzyme activities (Niemiec et al., 2011). Finally, one should not ignore the fact, that like all alpha-keto acids, AKG possesses antioxidant activity itself. AKG can react freely with H_2O_2 with the formation of succinate and CO_2. This nonenzymatic oxidative decarboxylation has been widely studied (Fedotcheva et al., 2006; Bayliak et al., 2016a) and, hence, some beneficial effects of AKG can be caused by its direct antioxidant action.

Thus, the facts described above suggest that dietary AKG can be advantageous for both young and older organisms by affecting energy/or protein metabolism and adaptive capacity. Some discrepancies revealed in different animal models of different ages, including opposite effects of AKG on TOR signaling and ROS production (Hou et al., 2010; Niemiec et al., 2011; Durán et al., 2012; Yao et al., 2012; Chin et al., 2014) prompt a need for deeper research on species-specific effects of AKG supplementation and its effects depending on duration of consumption and age of an organism. In the present study, we examined the effects of dietary AKG on some physiological functions and metabolic processes in the fruit fly *Drosophila melanogaster*, a model for studies of many aspects of animal nutrition (Lushchak et al., 2012; Rovenko et al., 2015; Reis, 2016). In particular, we examined AKG effects on *D. melanogaster* of different ages by analyzing various metabolic parameters and indicators of ROS homeostasis in larvae, and young (2 day post-eclosion) and middle-aged (24 day old) adult flies. The study also addressed the question whether changes in metabolic profiles increased adaptive capability of AKG-reared flies. In our previous work, we showed that an AKG-enriched diet promoted higher cold tolerance in a dose-dependent manner in young *D. melanogaster* Canton S flies with simultaneous enhancement of antioxidant potential and levels of cryoprotective molecules such as proline in sex-dependent manner (Bayliak et al., 2016b). Here, the resistance to starvation, heat shock and oxidative stress which is known to change with fly age (Gospodaryov et al., 2013, 2014) was tested in young and middle-aged animals. Since AKG could affect protein metabolism, the reproduction of flies which depends on protein intake was evaluated in flies reared on control and AKG-containing diets.

2. Materials and methods

2.1. Fly stock and rearing

D. melanogaster w^{1118} flies were used in all experiments. Stock flies were kindly provided by Bloomington Stock Center (Indiana University, USA). All fly cultures were kept at 25 °C, 55–60% humidity in a 12-h dark/light cycle. Parental populations of flies were maintained on yeast-corn-molasses media (Rovenko et al., 2014). Experimental fly cultures were reared on medium containing 5% sucrose, 5% yeast, 1% agar, 0.18% methylparaben to inhibit mold growth, and different concentrations of disodium salt of alpha-ketoglutarate (AKG). About 200 eggs laid by parental flies within a 6 h time period were put in each 250 ml glass bottle with 25 ml of the experimental medium.

The dynamics of fly development on the different experimental diets was assessed by counting the number of pupae formed once per day, starting 96 h after egg deposition. The pupation at each day was expressed as the percentage of eggs which reached the pupa stage over 9 days.

Newly eclosed flies were transferred onto fresh food of the same composition and held for 2 or 24 days. The food was changed every other day. Two-day old and 24-old day flies were separated by sexes and females were used for physiological tests or quickly frozen in liquid nitrogen for further biochemical analyses. In one series of experiments, third-instar larvae reared on control and experimental diets were also used for biochemical analyses.

2.2. Assay of food intake

Food intake was measured as described earlier (Lushchak et al., 2011). Briefly, groups of 15 third instar larvae or 10 adult flies reared on different experimental diets were placed for 20 min or 15 min, respectively, on the same diets which also contained 0.5% erioglaucine dye (FD&C Blue No. 1, Brilliant Blue FCF). After feeding, larvae and flies were homogenized in 50 mM potassium phosphate buffer (KPi, pH 7.0) at a ratio 1:100 (mg/μl) and centrifuged at room temperature at 13,000*g* for 15 min. Supernatants were removed and absorbance was measured at 629 nm and compared against a calibration curve built with different concentrations of the dye (Lushchak et al., 2011).

2.3. Heat stress and starvation resistance assay

To evaluate resistance to heat stress, single females were transferred into small glass vials with cotton stoppers and placed in water baths set at 39, 40 and 41 °C (Lushchak et al., 2012). Full immobility of the fly was referred to as heat-induced paralysis, and the time taken to reach coma was recorded. After 30 min heat shock at 40 °C, vials with paralyzed flies were placed, without further disturbance, at room temperature (23–25 °C), to score the time taken for full recovery of locomotion (Gospodaryov et al., 2014). About 10–12 females were tested in each of three independent replicates. To measure starvation resistance, 10 females were transferred to 24-ml glass vials containing 1.25 ml of 1% agar and plugged with cotton in order to prevent desiccation. Three vials were prepared in each of four independent replicates. Dead fly number was registered every 24 h and values were expressed as the percentage of flies that survived.

2.4. Oxidative stress resistance assay

Ten females were transferred into empty vials for 2 h of starvation. After starvation, flies were transferred into vials containing folded and rammed strips (2.4 × 12 cm) of 4-layer cellulose filter paper soaked with 0.8 ml of 5% sucrose solutions additionally containing either 5% H_2O_2 or 20 mM menadione (Gospodaryov et al., 2013). Two vials were tested in each of three independent replicates. Survivors were counted after 48 h of exposure and values were expressed as the percentage of flies that survived.

2.5. Fecundity test

To assess fruit fly fecundity, one male and one female fly newly emerged on control or experimental food were then transferred in small vials (15 × 60 mm) with 0.7 ml of the same food (Gospodaryov et al., 2013). Food was changed every day. The number of eggs laid by individual females was determined at 24 h after fly transfer onto fresh food. Measurements were performed during the first 24 days of adult life. Eggs were counted every second day. Nine fly pairs were tested for each control and AKG-reared group.

2.6. Determination of wet body mass and water content

Twenty females were weighed with a balance WTW 2 ("Techniprot", Poland) and then transferred into ventilated vials. These were held in

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