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Analysis of the effect of the mitochondrial prohibitin complex, a context-dependent modulator of longevity, on the *C. elegans* metabolome☆

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ABSTRACT

The mitochondrial prohibitin complex, composed of two proteins, PHB-1 and PHB-2, is a context-dependent modulator of longevity. Specifically, prohibitin deficiency shortens the lifespan of otherwise wild type worms, while it dramatically extends the lifespan under compromised metabolic conditions. This extremely intriguingly phenotype has been linked to alterations in mitochondrial function and in fat metabolism. However, the true function of the mitochondrial prohibitin complex remains elusive. Here, we used gas chromatography coupled to a flame ionization detector (GC/FID) and ^{1}H NMR spectroscopy to gain molecular insights into the effect of prohibitin depletion on the *Caenorhabditis elegans* metabolome. We analysed the effect of prohibitin deficiency in two different developmental stages and under two different conditions, which result in opposing longevity phenotypes, namely wild type worms and *daf-2(e1370)* insulin signalling deficient mutants. Prohibitin depletion was shown to alter the fatty acid (GC/FID) and ^{1}H NMR metabolic profiles of wild type animals both at the fourth larval stage of development (L4) and at the young adult (YA) stage, while being more pronounced at the later stage. Furthermore, wild type and the diapause mutant *daf-2(e1370)*, either expressing or not prohibitin, were clearly distinguishable based on their metabolic profiles, revealing changes in fatty acid composition, as well as in carbohydrate and amino acid metabolism. Moreover, the metabolic data indicate that *daf-2(e1370)* mutants are more robust than the wild type animals to changes induced by prohibitin depletion. The impact of prohibitin depletion on the *C. elegans* metabolome will be discussed herein in the scope of its effect on longevity. This article is part of a Special Issue entitled: Mitochondrial Dysfunction in Aging. Guest Editor: Aleksandra Trifunovic

1. Introduction

The nematode *Caenorhabditis elegans* has been extensively used to gain insights into the complex process of ageing and it has been extremely useful in the identification of mutations leading to increased longevity [1,2]. Research has led to remarkable progress in describing the molecular pathways that modulate ageing, including several signalling cascades, among them, the most prominent one being the insulin/insulin-like signalling (IIS) pathway [3]. One universal hallmark of ageing is the marked alterations in cellular energy metabolism [4]. Consistently, the biogenesis and function of mitochondria, the energy-generating organelles in eukaryotic cells, are primary longevity determinants [5]. Recently, the mitochondrial prohibitin complex was shown to differentially modulate longevity depending on intrinsic and extrinsic cues [6].

The mitochondrial prohibitin complex is composed of two proteins, PHB-1 and PHB-2, which bind to each other to form a heterodimer that is assembled into a ring-like macromolecular structure at the inner mitochondrial membrane [7]. These two subunits are interdependent for the formation of the complex, leading the absence of one of them to the absence of the whole complex [8–10]. Although different cellular functions have been suggested for both PHB-1 and PHB-2 in other cellular compartments, several evidences points to prohibitins functioning together within mitochondria (reviewed in [11,12]). Several roles have been proposed for the mitochondrial prohibitin complex, including a role as a membrane-bound chaperone, which holds and stabilizes newly synthesised mitochondrial-encoded proteins [13,14] and as scaffold proteins that recruit membrane proteins to a specific lipid environment [15,16]. Prohibitins have also been ascribed to different cellular processes such as cellular proliferation, cancer and ageing (reviewed in [12]). Prohibitin depletion delays development, reduces body size and gives rise to a wide range of somatic and germline defects, spanning

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from complete sterility to severely reduce brood sizes and a morphologically abnormal somatic gonad [6,8]. Intriguingly, prohibitin deficiency results in opposite ageing phenotypes depending on the metabolic status, being this phenotype evolutionarily conserved [6,17]. In particular, prohibitin deficiency shortens the lifespan of otherwise wild type nematodes, while it dramatically extends the lifespan of the already long-lived *daf-2(e1370)* insulin receptor mutants [6].

DAF-2, an orthologue of the insulin/insulin-like growth factor receptor, modulates the activity of the IIS pathway, through the FOXO transcription factor DAF-16 [18,19]. DAF-16 is required for reproductive growth and metabolism, as well as normal lifespan, responding to environmental cues, such as nutritional status or growth conditions [20,21]. Moreover, it regulates the entrance and exit into an alternative developmental stage, the diapause stage, characterised by extremely high resistance and survival to harsh conditions [20]. The diapause mutant *daf-2* is characterised by alterations in its metabolism, namely at the level of the glyoxylate shunt and gluconeogenesis [22,23]. Indeed, one plausible mechanism for the increased longevity of *daf-2* mutants involves its tuning of cellular metabolism towards maximal survival [23]. Nevertheless, how these metabolic shifts towards maximal survival occur, and how, if at all, they contribute, remains largely unknown. Interestingly, the modulation of lifespan by prohibitin is accompanied by alterations in the levels of fat content, as measured by different staining methods [6]. Additionally, prohibitin deficiency extends the lifespan of *nhr-49* mutant. NHR-49 is a key regulator of fat mobilization, modulating fat consumption and maintaining a normal balance of fatty acid composition [6]. In *C. elegans* up to 35% of the dry body mass is composed by lipids, from which 40–55%, depending on the diet and growth stage, are the fat stores triglycerides, esters derived from glycerol and three fatty acids [24,25]. Fat visualization using dyes, apart from the controversy on what these dyes are really staining [26], does not allow the distinction of the different lipid composition. Thus, a more direct way of examining the lipid composition upon prohibitin depletion and, in a broader perspective, a global view of the effect of prohibitin on the *C. elegans* metabolome is missing.

In recent years, the advances of different analytical platforms have allowed the development of the field of metabolomics. Metabolomics focus on the analysis of all the metabolites, the metabolome, of biological systems. However, the physicochemical diversity of the metabolites makes virtually impossible, even with all the advances made so far, to consider them all at the same time with a single strategy [27]. Instead, just to get near to its ultimate goal, a combination of different analytical methods and different metabolite extraction procedures must be used [27]. Application of a multitude and complementary metabolomics approaches will allow the gathering of molecular data that ultimately will led to a better understanding of the ageing process.

We present herein the first broad characterisation of the metabolic modifications induced by prohibitin depletion both in wild type and in *daf-2(e1370)* mutants. For that purpose, analysis of the fatty acid composition using gas chromatography coupled to a flame ionization detector (GC/FID) and ^{1}H NMR metabolic profiling of whole worms was conducted. Prohibitin deficiency was found to induce a broad reorganization of the metabolic network in both wild type and *daf-2(e1370)* mutants, and these findings will be discussed in the scope of the observed effect of prohibitin on the worm lifespan expectancy.

2. Material and methods

2.1. Strains, worm culture and sampling conditions

Nematodes were cultured and maintained according to standard methods at 20 °C. The strains N2, wild type Bristol isolate, and CB1370, *daf-2(e1370)III*, were used in this study. Synchronous animal populations were generated by hypochlorite treatment of gravid adults. Tightly synchronised embryos were obtained by allowing eggs to hatch and develop until L1 larval stage in S Basal during overnight incubation. The worms were then fed with HT115 (DE3) *Escherichia coli* bacteria (deficient for RNase-E), harboring the appropriate RNAi plasmids (pL4440 for control RNAi, pL4440 containing a *phb-1* or a *phb-2* genomic fragment for RNAi knockdown of either *phb-1* or *phb-2*, respectively). Each bacterial strain was inoculated, from an overnight pre-inoculum, in LB (carbenicillin (25 mg/l) (Sigma) and tetracycline (15 mg/l) (Sigma)) to an initial $OD_{600\ nm}$ of approximately 0.2 and grown until it reached an $OD_{600\ nm}$ of approximately 1.5 (37 °C). IPTG (Sigma) was then added to the bacterial culture (1 mM). The bacterial culture was incubated for 2 h (37 °C) and then harvested by centrifugation at 3200 ×*g* (4 °C) for 20 min. Pellets were washed with S Basal (4 °C) and harvested again (3200 ×*g*, 20 min and 4 °C). Finally, bacterial stocks were prepared by resuspending the pellets (30 g/l) in S Medium containing carbenicillin (25 mg/l), IPTG (1 mM) and cholesterol (5 mg/l) (Sigma), and kept at 4 °C at most 4 days before being used. The effectiveness of RNAi against *phb-1* or *phb-2* was checked phenotypically by the worm sterility (data not shown). To assess the effect of either *phb-1* or *phb-2* deficiency on the whole worm GC/FID and ^{1}H NMR profiles, synchronous populations of N2 wild type worms (approximately 2 worms/μl) were grown in liquid medium and sampled (approximately 30,000 worms) at either L4 larval or young adult (YA) stages. To compare the effect of prohibitin deficiency on the GC/FID and ^{1}H NMR profiles of wild type and *daf-2(e1370)* worms, synchronous populations were grown on agar plates seeded with the appropriate bacteria (control RNAi or *phb-1(RNAi)*) and sampled (approximately 10,000 worms) at the YA stage. Sampled worms were washed six times (three with S Basal, two more with double distilled water and a sixth time with MS-grade water (Fluka)), harvested after each washing step by centrifugation at 800 ×*g* for 1 min (20 °C) and the final worm pellet was snap-frozen in liquid nitrogen and kept in the freezer (−80 °C) until further use.

2.2. GC/FID analysis

To determine the overall fatty acid composition of the nematodes, lipids were extracted using an adaptation of the methyl-tert-butyl ether lipid extraction procedure [28]. Briefly, 1.5 ml of cold (−25 °C) MS-grade methanol (Sigma) was added to the frozen worm pellet (200 μl) and transfer to a 10 ml glass vial. Worms were broken up by sonication (SONOPLUS 2070; 20 cycles of 1 min at maximum power followed by a pause of 15 s to make sure the mixture was maintained cold) in a cold ethanol bath (between −20 °C and −30 °C). After, 5 ml of methyl tertiary-butyl ether (MTBE) (Sigma) was added to this mixture, vortexed slightly and incubated for 1 h at 20 °C in a shaker. Phase separation was induced by adding 1.25 ml of MS-grade water (Fluka), vortexed slightly and, upon 10 min of incubation (20 °C), centrifuged at 1000 ×*g* for 10 min (20 °C). The upper phase (organic phase) was collected, while the lower phase was re-extracted with 2 ml of MTBE/methanol/water (10:3:2.5, v/v/v). Both organic phases were then combined and kept in the freezer (−25 °C) until further use.

After lipid extraction, each sample was derivatised to make it amenable to be analysed by gas chromatography coupled to a flame ionization detector (GC/FID). First, the sample was evaporated in nitrogen atmosphere until dryness. To release the fatty acids of complex lipids and methylate them, 1 ml of hydrogen chloride (HCl)-methanol (1.25 M HCl) (Sigma) was added and kept in a bath at 80 °C for 1 h. Two phases were then obtained by the addition of 3 ml of hexane (Sigma) and 1.5 ml of Na_2SO_4 (6.7% m/v) (Sigma). Finally, the fatty acid methyl esters (FAMEs) were obtained by transferring the upper phase to another tube, where it was evaporated in the same conditions as before. In the end, 150 μl of heptane (Sigma) was added, FAMEs were resuspended and transferred to a vial with an insert for the GC/FID analysis and injected to the GC. The samples were analysed in a gas chromatograph (Perkin Elmer Clarus 500) equipped with a 0.2 μm × 60 m × 0.25 mm fused silica capillary column, hydrogen at 45 ml min^{-1} was employed as carrier gas and coupled to a flame

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