UCP4C mediates uncoupled respiration in larvae of Drosophila melanogaster

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Abstract

Larvae of Drosophila melanogaster reared at 23°C and switched to 14°C for 1 h are 0.5°C warmer than the surrounding medium. In keeping with dissipation of energy, respiration of Drosophila melanogaster larvae cannot be decreased by the F-ATPase inhibitor oligomycin or stimulated by protonophore. Silencing of Ucp4C conferred sensitivity of respiration to oligomycin and uncoupler, and prevented larva-to-adult progression at 15°C but not 23°C. Uncoupled respiration of larval mitochondria required palmitate, was dependent on Ucp4C and was inhibited by guanosine diphosphate. UCP4C is required for development through the prepupal stages at low temperatures and may be an uncoupling protein.

Keywords Drosophila melanogaster; energy dissipation; mitochondria; respiration; uncoupling proteins

Introduction

The fruit fly Drosophila melanogaster originated in tropical Africa and colonized Europe only about 15,000 years ago (end of the last glaciation) eventually spreading to Australia and the Americas over the past few centuries [1]. This poikilotherm insect has become a cosmopolitan human commensal species, able to adapt to a wide range of environmental thermal conditions [2], yet the physiological bases for its climatic adaptations remain largely unknown. Moreover, while it is widely assumed that uncoupling proteins (UCPs) mediate mitochondrial thermogenesis in mammals, whether bona fide UCPs also exist in arthropods [13,14], C. elegans [15] and X. laevis [16]. Although a role as bona fide UCPs has not yet been demonstrated for any other UCP [6], it has been proposed that Arabidopsis UCP4 represents the ancestral UCP from which UCPs diverged [3]. Based on sequence homology, four UCPs have been identified in D. melanogaster, that is, UCP4A, 4B, 4C and 5 [3], and specific studies on Drosophila UCP5 have demonstrated that this species does not mediate uncoupling [17,18]. Here, we have studied mitochondrial respiration in body wall preparations (BWP) and isolated mitochondria from D. melanogaster instars II and III larvae after knockdown of Ucp4A, 4B and 4C.

Results and Discussion

Mitochondria of Drosophila larvae are uncoupled in situ

Body wall preparations from III instar Drosophila larvae exhibited a steady oxygen consumption rate (OCR) of about 350 pmol/min that was not stimulated further by the addition of glucose, indicating that trehalose (provided with the medium) is sufficient to generate adequate glucose and respiratory substrates (Fig 1A). However, respiration was not inhibited by oligomycin, suggesting that in BWP ATP is not produced by oxidative phosphorylation. In addition, OCR could not be stimulated by the uncoupler carbonyl cyanide-p-trifluoromethoxyphenyl hydrazine (FCCP) even when the latter was added before oligomycin (Fig 1B). Respiratory was largely inhibited by rotenone plus antymycin A, demonstrating its mitochondrial origin (Fig 1A and B), and was inhibited by both iodoacetate (inhibitor of glyceraldehyde 3-phosphate dehydrogenase) and 2-deoxyglucose (which cannot be metabolized) indicating that it is fueled by...
glycolysis (Fig 1C). Not a single larva underwent metamorphosis, and all larvae died, when trehalose in the growth medium was replaced by pyruvate, lactate, galactose plus pyruvate, carnitine, octanoate, acetate, β-hydroxybutyrate or alanine, indicating that glycolysis is essential for development and survival. Based on these unexpected findings, we then asked whether they reflect a peculiar biological condition of mitochondria in early developmental stages of Drosophila. To answer this question, we performed OCR measurements in Drosophila Schneider 2 Receptor plus cells (S2R+), which were derived from late embryonic stages and represent precursors to a variety of Drosophila tissues [19]. We found (as is usually seen with mammalian cells in culture) that respiration was inhibited by oligomycin and stimulated by FCCP (Fig 1D). Thus, lack of sensitivity to oligomycin and FCCP suggests that respiration of larvae is uncoupled.

**Larval mitochondria generate heat, while Ucp4C knockdown confers coupled respiration**

Genome analysis confirmed the existence of four putative Ucp genes in *D. melanogaster* that is, Ucp4A, Ucp4B, Ucp4C (which display a high homology with mammalian UCP4) [17] and Ucp5 (which displays a high homology with mammalian UCP5) [18]. Since it is already known that UCP4 does not mediate uncoupling in Drosophila [17,18], we knocked down individual Ucp4 genes using the GALA/UAS binary yeast system [20,21] and measured levels of silencing by qRT–PCR (Supplementary Fig S1) and respiratory features of BWPs derived from II instar larvae (Fig 2A–C). While downregulation of Ucp4 transcripts did not affect the basal OCR (Fig 2A–C), only knockdown of *Ucp4C* made respiration sensitive to inhibition by oligomycin and to stimulation by FCCP (Fig 2C, closed symbols). Only in Ucp4C-interfered larvae addition of FCCP before oligomycin caused a threefold increase in respiration (Fig 2C, open symbols, where indicated (arrows) 5 μM oligomycin, 1 μM FCCP and 5 μM rotenone plus 5 μM antimycin A were added (closed symbols); open symbols (C) denote OCR upon the addition of FCCP at the first arrow, and of rotenone plus antimycin A at the second arrow).

**Figure 1.** Oxygen consumption rate (OCR) of III instar larvae body wall preparations (BWPs) and of S2R+ cells. A, B Five micromolar oligomycin (Oligo), 1 μM carbonilcyanide-p-trifluoromethoxyphenyl hydrzone (FCCP) and 5 μM rotenone plus 5 μM antimycin A (Rot + AA) were added to BWPs. C Vehicle (circles), 20 mM deoxyglucose (2-DOG, triangles) or 5 mM iodoacetate (IA, squares) were added to BWPs. D 5 μM oligomycin, 1 μM FCCP and 5 μM rotenone plus 5 μM antimycin A were added to S2R+ cells.

Data information: In all panels, additions are marked by arrows. In (A–C), data are mean ± s.d. of 5 independent experiments with 20 BWPs each; in (D), data are mean ± s.d. of 3 independent experiments with 20 cell incubations each.

**Figure 2.** Effect of Ucp4 isoform silencing on oxygen consumption rate (OCR) of II instar body wall preparations (BWPs) and on heat production by III instar larvae. UAS fly strains v6162 (UAS-Ucp4A), v33128 (UAS-Ucp4B) and v100064 (UAS-Ucp4C) were used. A–C OCR of BWPs in Ucp4A- (A), Ucp4B- (B) or Ucp4C-interfered larvae (C). Where indicated (arrows) 5 μM oligomycin, 1 μM FCCP and 5 μM rotenone plus 5 μM antimycin A were added (closed symbols); open symbols (C) denote OCR upon the addition of FCCP at the first arrow, and of rotenone plus antimycin A at the second arrow. D Visible light (upper panels) and infrared thermography images (lower panels) of wild-type (left panels) and Ucp4C-interfered larvae (right panels). E Medium (filled bars) and larval temperature (open bars) determined from the thermography measurements (average of 27 wild-type and 30 Ucp4C-interfered larvae, error bar = 0.004°C).

Data information: In (A–C), data are mean ± s.d. of 5 independent experiments with 20 BWPs each.
interfered larvae) because in this case, ATP is not synthesized by mitochondria, and therefore, its levels are not affected by inhibition of F-ATP synthase by oligomycin. It should be noted that maximal respiration was higher in Ucp4C-interfered than in wild-type larvae, possibly the result of increased mitochondrial biogenesis—an issue that will require further work. It should also be mentioned that inhibition of transcription was particularly effective for Ucp4A and Ucp4B but not for Ucp4D mRNAs (Supplementary Fig S1), yet neither Ucp4A nor Ucp4B can be responsible for uncoupling, or else interference of Ucp4C mRNA should not have had a phenotypic effect. We also tested a different set of knockdown Ucp4 lines with superimposable results (Supplementary Fig S3). To assess whether uncoupled respiration is linked to heat production, we used infrared thermography to study body temperature of wild-type and of Ucp4C-interfered larvae. Larvae were reared at 23°C and switched to 14°C for 1 h, a protocol that does not affect development after larvae of either genotype are returned to 23°C. The temperature of wild-type larvae was 0.5°C warmer than that of the surrounding medium, while no thermal gradient was maintained by Ucp4C-interfered larvae (Fig 2D, E). Although we could not detect expression of Drosophila UCPs with antibodies against human UCP1, based on these results, it appears extremely likely that expression of Ucp4C mediates uncoupled respiration in Drosophila larvae and that UCP4C activity is associated with energy dissipation.

**UCP4C is essential for larval development at low temperatures**

To test whether expression of UCPs is essential for development, adult flies were mated and about 300 eggs (three replicates, each of about 100 eggs per genotype) from either wild-type or Ucp4A-, Ucp4B- or Ucp4C-interfered individuals were incubated at 23°C or 15°C, that is, close to the minimal temperature allowing progression in development of wild-type *D. melanogaster* [25]. About the same number of larvae and adults was recorded at 23°C irrespective of genotype, while the number of III instar larvae was drastically reduced—and not a single adult developed—in Ucp4C-interfered individuals at 15°C (Fig 3A–C). The OCR of BWPs from III instar Ucp4A- and Ucp4B-interfered larvae grown at 15°C displayed complete lack of response to oligomycin and FCCP (Fig 3D and E). Contrariwise, mitochondrial respiration was absent in Ucp4C-interfered larvae (Fig 3F; notice that the residual OCR was nonmitochondrial, as it was insensitive to rotenone and antimycin A). This finding indicates a severe metabolic impairment, which is consistent with lack of further development.

**Uncoupled respiration of larval mitochondria requires palmitate and is inhibited by GDP**

Isolated mitochondria from III instar larvae were not constitutively uncoupled; in the absence of palmitate, they underwent the expected oligomycin-sensitive stimulation of respiration with ADP and uncoupling with FCCP (Fig 4, trace a’). Like in brown fat mitochondria [5–7], respiration could be stimulated by palmitate, inhibited by the pan-UCP inhibitor guanosine diphosphate (GDP) and stimulated by FCCP (Fig 4, trace a). These findings suggest that the uncoupling effect of palmitate depends on an endogenous UCP, which we propose to be UCP4C, for four reasons. First, S2R+ cells that express only UCP4A (http://flyrnai.org) could not be stimulated by palmitate after permeabilization with digitonin (Fig 4, trace b) while they responded normally to ADP and FCCP (Fig 4, trace b’). Second, the uncoupling effect of palmitate was seen in wild-type mitochondria (Fig 4B) and after knockdown of Ucp4A (Fig 4C) or of Ucp4B (Fig 4D) while it was suppressed by knockdown of Ucp4C (Fig 4E); the effect of palmitate was insensitive to carboxyatractylate (Supplementary Fig S4), indicating that the adenine nucleotide translocator is not involved in the uncoupling effect [26]. Third, respiratory recoupling in BWPs was only observed after interference of Ucp4C mRNA (Fig 2D). Fourth, among Drosophila Ucp isoforms, Ucp4C displays the highest homology to UCP1, the gene encoding for the unique *bona fide* mammalian UCP1 [4,6] (Supplementary Fig S5).

It is apparent that uncoupled respiration in Drosophila requires a fatty acid, in keeping with results obtained in *C. elegans* [15,27], *Z. atratus* [14] and *G. coccuierla* [13] where palmitate was required to observe uncoupling. In Drosophila, the main source of fatty acids is triacylglycerol (TAG), which is hydrolyzed by activation of lipases, the most important being the Brummer TAG lipase, a homolog of the human adipocyte triglyceride lipase [28]. Larval fat body is composed essentially of TAG, while adult flies contain more phosphatidylglycerol [29]. TAG storage and mobilization are controlled by brain centers that regulate food intake and metabolism [30], suggesting that larval respiratory activity in vivo is modulated by hormonal regulation of lipases. The details of the Drosophila signaling pathway(s) required to activate lipolysis as well as the fatty acid(s) responsible for uncoupling remain to be determined. We note that the endogenous species may be different from palmitate, and this could explain why respiration is not stimulated maximally by added palmitate. Of note, muscle-specific knockdown of Ucp4C had the same recoupling effect as ubiquitous silencing (Supplementary Fig S6), suggesting that muscle mitochondria are effectors of uncoupled respiration in Drosophila larvae.

**Physiological implications of UCP4C expression in *D. melanogaster***

In temperate zones, *D. melanogaster* overwinters by entering an adult reproductive diapause characterized by a severe reduction in ovarian development [25,31,32]. The related physiological modifications allow adult flies to survive prolonged exposure to temperatures well below 12°C [32]. On the contrary, Drosophila larvae die in a few days at temperatures below 12°C [2,33] suggesting that larval stages represent a highly temperature-sensitive developmental window during Drosophila ontogeny. Moreover, larvae are way less mobile than adults and confined to their specific environmental niches. Uncoupled respiration could therefore have evolved as a transient adaptation favoring progression of larvae into more cold-tolerant developmental stages. It should be noted that UCP4C could also be required to allow fast turnover of the tricarboxylic acid cycle in order to provide biosynthetic intermediates required for growth, and/or to decrease production of reactive oxygen species, which is an exponential function of the mitochondrial membrane potential [34]. In summary, our data suggest that Drosophila metabolism during larval stages is fueled by glycolysis and uncoupled from ATP synthesis due to expression of UCP4C.
**Figure 3.** Effect of Ucp4 interference on egg-to-adult transition and oxygen consumption rate (OCR) of III instar larvae body wall preparations (BWPs).

A–C About 300 fertilized eggs (three replicates of n=100 each) were incubated at 23°C or 15°C, and larvae (closed bars) and adults (open bars) were counted. Y-axis, % of larvae or adults developing from eggs of UAS- or UAS-Gal4-interfered flies.

D–F OCR of BWPs from Ucp4A- (D), Ucp4B- (E) or Ucp4C-interfered larvae (F) grown at 15°C. Where indicated (arrows) 5 μM oligomycin, 1 μM carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP) and 5 μM rotenone plus 5 μM antimycin A were added.

Data information: In (D–F), data are mean ± s.d. of 3 independent experiments with 20 BWPs each.

**Figure 4.** Effect of palmitate on oxygen consumption rates of larval mitochondria and permeabilized S2R+ cells.

A Mitochondria (2 mg) isolated from larvae (traces a, a') or 2 × 10^7 S2R+ digitonin-permeabilized cells (traces b, b') were energized with 10 mM succinate (final volume 2 ml). Where indicated by arrows additions were palmitate (15 μM in trace a and 5 μM for each addition in trace b), 0.1 mM ADP, 2 mM GDP, 1 μg/ml oligomycin (Oligo) and 50 nM carbonylcyanide-p-trifluoromethoxyphenyl hydrazone (FCCP). Results are representative of at least three independent experiments.

B–E Experiments were carried out on larval mitochondria isolated from wild-type (B) or from Ucp4A- (C), Ucp4B- (D) or Ucp4C-interfered larvae (E). OCR refers to the values measured after addition of the indicated concentrations of palmitate (mean from 3 to 5 determinations ± s.d.). Maximal respiration (i.e. oxygen consumption after the addition of 50 nM FCCP) was 746 ± 16 ng atom O × per mg × per min (mean ± s.d. of all preparations used in B–D, n = 13).
Materials and Methods

Fly strains

UAS fly strains v6162, v102571, v33128, v33130, v100064 and v2647 were from the Vienna Drosophila RNAi Center (VDRC) [21]. The knockdown of Drosophila genes was achieved using the GAL4/UAS system to target the RNAi [20]. Act5C-Gal4 and 24B-Gal4 drivers (Bloomington Stock Center) were used to activate interference of UCPs. All flies were raised on standard cornmeal medium and were maintained at 23°C (70% relative humidity) on a 12 h light:12 h dark cycle.

Body wall preparations and cell cultures

A small portion of the tip was cut from larvae, internal organs were removed by gently squeezing from end to end, and the preparation was turned inside-out by rolling the cuticula along a holding tweezer. Each BWP was placed into one well of the Seahorse 24-well culture plate, held in place with a grid and covered with HL3A medium. Plate, characterized by a 480-× 640 pixel bolometric array (technical data available online at http://www.flirthermography.co.uk/cameras/camera/1079/). The thermal images acquired by the camera were post-calibrated on the basis of the acquisition distance, mean infrared reflectance of the observed object, reflected temperature and air temperature by means of FLIR QuickReport freeware package (download page: http://www.flir.com/thermography/eurasia/en/content/?id=11368). The images were subsequently exported as temperature matrices stored in ASCII files for processing and analysis in MATLAB.

Oxygen consumption measurements

Oxygen consumption rate was measured at 25°C either with a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA) (BWPs and intact cells) or with a Clark-type oxygen electrode (isolated mitochondria and permeabilized cells). The XF24 Extracellular Flux Analyzer measures the OCR through a sensor cartridge embedded with a fluorescent sensor for oxygen coupled to a fiber-optic waveguide. The waveguide delivers light at 532 nm and transmits a fluorescent signal through optical filters (650 nm for oxygen) to highly sensitive photodetectors [35]. The biosensor is much smaller than the BWPs and detects respiration in the region immediately below it, making the measurements independent of BWP size. S2R+ cells were seeded at 20,000 cells/well and cultured for 48 h, the medium being replaced with serum-free Schneider’s medium immediately before the measurements. Mitochondrial isolation from larvae and permeabilization of S2R+ cells were performed as described [36,37]. Respiratory measurements on mitochondria or permeabilized cells were performed in a medium containing 130 mM KCl, 10 mM MOPS-Tris, 5 mM succinate-Tris, 10 µM EGTA and 2 µM rotenone (final pH 7.4). Oligomycin, FCCP, rotenone and antimycin A were obtained from Life Technologies, and optimal concentrations were determined by titrations. Respiratory rates are average ± s.d. of at least 60 individual BWP per condition, or of three independent S2R+ and larval mitochondrial preparations.

RNA isolation and qRT-PCR experiments

Total RNA was isolated from 10 larvae with Trizol (Life Technologies) and further purified with 8 M LiCl. RNA samples were checked for integrity by capillary electrophoresis (RNA 6000 Nano LabChip, Agilent Technologies). One microgram of total RNA per sample was used for first-strand cDNA synthesis using oligo-dT and SuperScript II reverse transcriptase (Life Technologies), and PCR-amplified with SYBR Green chemistry (Promega). Gene-specific primers were designed using Roche Application Design Tool (Roche Applied Science), and qRT–PCR was performed in the Applied Biosystems SDS-7500 thermal cycler (Life Technologies). Primer sequences are reported in Supplementary Table S1. To calculate the relative expression ratio, we used the ΔΔCt method implemented in the software of the Applied Biosystems thermal cycler [38], and Rp49 was used as endogenous control. mRNA levels are expressed relative to the quantity of template in the sample (RQ).

Infrared thermography

Both wild-type and interfered larvae were reared at 23°C and switched to 14°C for 1 h before thermographic recording. The measurements were carried out with a FLIR T620 camera, which is characterized by a 480 × 480 pixel bolometric array (technical data available online at http://www.flirthermography.co.uk/cameras/camera/1079/). The thermal images acquired by the camera were post-calibrated on the basis of the acquisition distance, mean infrared reflectance of the observed object, reflected temperature and air temperature by means of FLIR QuickReport freeware package (download page: http://www.flir.com/thermography/eurasia/en/content/?id=11368). The images were subsequently exported as temperature matrices stored in ASCII files for processing and analysis in MATLAB.

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Author contributions

PB, RC and CDR conceived the study; MAZ, MZ and FN provided critical advice and suggested experiments; CDR and GT performed experiments; CDP designed qRT-PCR experiments; CDR, CDP and GT performed statistical analyses; PB, CDR and RC wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References


