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Fasting promotes functional changes in liver mitochondria

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ABSTRACT

Overnight fasting of rodents is commonly adopted in protocols to obtain isolated liver mitochondria, but the effects of fasting itself on mitochondrial function are poorly characterized. In this study we show that overnight fasting (15 h) promotes a shift in the liver mitochondrial bioenergetic profile, with a reduction in ADP-stimulated and maximal respiration, lower membrane potentials and lower resistance to Ca^{2+}-induced mitochondrial permeability transition. Short term fasting (4 h) promoted similar changes, suggesting that this is a physiological shift in mitochondrial function associated with fasting, but not torpor. Our results suggest that the widely adopted liver mitochondrial isolation technique using fasted animals should be reconsidered, and also uncover physiological changes in bioenergetic function associated to nutritional status.

1. Introduction

Overnight fasting is commonly adopted prior to the isolation of liver mitochondria for use in functional studies [1–3]. The use of fasted animals is justified by the need to control excess fat and glycogen in homogenates and avoid variations in hormonal signaling. However, the effects of fasting itself on isolated liver mitochondrial functions are, surprisingly, not well characterized.

Fasting and the consequent decrease in blood nutrients lead to decreased insulin levels as well as increased glucagon, thus stimulating glycogenolysis and gluconeogenesis, while suppressing lipogenesis and glycolysis [reviewed in [4]]. At the mitochondrial level, fatty acid oxidation is enhanced [5,6] due to increased CPT1 expression (the main regulator of mitochondrial fatty acid oxidation [7]), while in the fed state mitochondria contribute toward lipogenesis [4]. However, the effects of overnight fasting on isolated mitochondrial electron transport chain activity and oxidative phosphorylation remain largely unknown. Understanding the effects of fasting on liver mitochondrial function is not only relevant for improved isolated mitochondrial protocols, which very commonly adopt substrates other than fatty acids, but also to gain insight into metabolic adaptations during fasting and liver function control by hormonal action and nutrient availability.

Interestingly, studies testing the effects of glucagon on liver mitochondrial function showed a clear increase in respiration [8–10] and calcium retention capacity [11]. This suggests that fasting may be associated with a boost in mitochondrial function, since glucagon signaling is present. Later studies showed that the increase observed in calcium retention capacity was indirectly related to the classical glucagon signaling pathway, acting through its cellular Ca^{2+} mobilizing effects [12]. Indeed, Ca^{2+} release from the endoplasmic reticulum is also known to increase Ca^{2+} concentrations in the mitochondrial matrix and stimulate oxidative phosphorylation [reviewed in [13]]. Notably, these studies with glucagon administration did not assess the effects of physiological hormonal signaling on mitochondrial function, but rather were conducted by administrating glucagon to fed mice, which promotes simultaneous high levels of insulin and glucagon, as well as high nutrient availability.

In this study, we examined the effects of 15 h (overnight) and short term (4 h) fasting on the function of isolated liver mitochondria. We surprisingly show that both overnight and short-term fasting result in less functional mitochondrial preparations, reducing ADP-stimulated (state 3) and maximal respiration, respiratory control ratios, membrane potentials, and calcium retention capacity. These results suggest that fasting promotes a physiological shift in liver mitochondrial

Abbreviations: CRC, calcium retention capacity; ER, endoplasmic reticulum; MCU, mitochondrial calcium uniporter; RCR, respiratory control ratio; CS, citrate synthase

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bioenergetics toward an overall decrease in oxidative phosphorylation efficiency and capacity, with possible physiological implications for liver energy metabolism.

2. Materials and methods

2.1. Animals and fasting

Male, adult, 8 week old C57BL/6N mice were caged individually and randomly divided into “fasted” or “fed” groups. Mice in the fasted group were moved to new cages 15 or 4 h before the experiments without any access to food, while the mice from the “fed” group were moved to new cages and given chow *ad libitum*. Under fasting conditions, torpor typically occurs starting at 8 h of fasting [14,15]. All experiments were conducted starting around 10 AM. Animals were kept at a controlled temperature of 22 °C in a 12 h night/day cycle, with lights turning on at 7 AM and going off at 7 PM. Usage of the mice was approved by the local animal care and use committee and followed National Institutes of Health guidelines for humane treatment of animals.

2.2. Mitochondrial isolation

Liver mitochondria were isolated and used for oxygen consumption and calcium uptake experiments employing the same methods described previously by our group [16]. Briefly, fed and fasted animals were euthanized prior to the isolation procedure and had their livers removed, finely minced and homogenized in an isolation buffer containing 250 mM sucrose, 10 mM HEPES and 1 mM EGTA, pH 7.2 (K^+) using a glass potter to obtain liver homogenates. A sample of the homogenates was separated and stored with protease inhibitors at −80 °C for later use. The remaining volume was used to obtain isolated mitochondria by differential centrifugation, with two centrifugation steps at 700*g*, collecting the supernatant, followed by two centrifugation steps at 10,000*g*, collecting the pellet containing concentrated isolated mitochondria. After these procedures, the isolated mitochondrial pellet was resuspended in a buffer containing 75 mM D-mannitol, 25 mM sucrose, 5 mM KH_2PO_3, 20 mM Tris-HCl, 100 mM KCl and 0.1% BSA, 1 mM EGTA and 0.5 mM EDTA, pH 7.4 (K^+). Experiments were carried out in the same media devoid of EGTA and EDTA, at 37 °C, with 2 μM rotenone and 5 mM succinate as the substrate, which provides better respiratory rates. Similar results to those shown in Fig. 1A and B were observed using malate plus glutamate (1 mM each) as substrates (results not shown). Protein quantification in the samples was performed using the Bradford method.

2.3. Oxygen consumption

Oxygen consumption was measured using a high-resolution Oroboros oxygraph. Mitochondria (250 μg/mL) were suspended in the experimental medium described above. State 3 respiration was measured by adding 1 mM ADP, oligomycin-insensitive respiration by adding 1.5 μg/mL oligomycin and maximal respiration was promoted by successive additions of 0.5 μM CCCP until maximal O_2 consumption was observed. Respiratory control ratios were determined as State 3 (ADP)/Oligomycin rates.

2.4. Ca^{2+} uptake assays

Mitochondria (500 μg protein) were incubated in 2 mL experimental buffer containing 0.1 μM Ca^{2+} Green 5 N and 1 μM $MgCl_2$. Ca^{2+} Green fluorescence was measured at 506 nm excitation and 532 emission using a F2500 Hitachi Fluorimeter at 37 °C with continuous stirring. Ca^{2+} additions (50 μM for Fig. 1, conducted in the presence of 2 mM ATP, and 10 μM for Fig. 4) were made where indicated in the figures. Using Ca^{2+} uptake traces, fluorescence values (F) were converted into $[Ca^{2+}]$ using the formula $[Ca^{2+}] = Kd\ (F - F_{min})/(F_{max} - F)$. The Kd value was experimentally estimated as the value which corresponds to a 10 μM increase in $[Ca^{2+}]$ after each addition, since the presence of magnesium in the media interferes with the theoretical Kd. F_{max} was obtained by adding 100 μL of a saturated Ca^{2+} solution until no increase in fluorescence was observed, and F_{min} was estimated by doing the same with an EGTA solution. Calcium retention capacity was calculated as the total amount of Ca^{2+} taken up by mitochondria before the onset of mitochondrial permeability transition (nmol Ca^{2+}/mg protein), as seen by the lack of further Ca^{2+} accumulation and increase in Ca^{2+} Green fluorescence due to Ca^{2+} release from mitochondria.

2.5. Mitochondrial membrane potential assay

Membrane potentials were quantified in isolated mitochondria (500 μg/mL) suspended in the same experimental buffer described supplemented with 5 μM safranine O. Uptake was measured by following safranine O fluorescence at 496 nm excitation and 586 nm emission in a F4500 Hitachi fluorimeter. A calibration curve was made for each preparation by incubating mitochondria in a sodium-based potassium-free media with valynomicin, followed by the addition of known KCl concentrations and fitting the resulting membrane potential values (obtained through the Nernst equation) with the observed fluorescence intensity [17].

2.6. Western blots and antibodies

Western blots of mitochondrial samples were carried out by resolving them using SDS-PAGE (20% gels) with 30 μg of protein (quantified by the Bradford method) in each lane, and then transferring the proteins to nitrocellulose membranes. Membranes were stained with Ponceau solution and scanned for later use as a loading control. Primary antibodies and dilutions used were HSP60 (1:5000; Abcam), TOM20 (1:200, Santa Cruz Biotechnology) and VDAC (1:500; Cell Signaling). Band intensities were quantified using ImageJ and then normalized by the intensities of total protein indicated by Ponceau staining (also quantified using ImageJ).

2.7. Phospholipid quantification

Total phospholipids were extracted from liver homogenates by the method of Bligh & Dyer [18]. Samples (10 μL) were used to quantify phosphorous by the malaquite green method [19]. Phospholipids were analyzed by two-dimensional thin layer chromatography using chloroform/methanol/H_2O/NH_4OH (aq 30%) (60:37.5:3:1) as the mobile phase for the first dimension and a solution of chloroform/methanol/acetic acid/H_2O (170:25:25:6) for the second dimension. Phospholipids were qualitatively visualized by iodine staining, then cardiolipin bands were scraped from silica plates, dissolved in 50 μL chloroform/methanol (1:1) and used for phosphorous quantification by the malaquite green method [19] normalized to protein content.

2.8. Citrate synthase activity

Citrate synthase activity was measured in total liver homogenates and in isolated mitochondria following the increase in 5,5′-dithiobis(2-nitrobenzoic acid) absorbance at 412 nm in the presence of 10 mM acetyl-CoA as described in [20].

2.9. Statistical analysis

Statistical analysis was carried out using two-tailed, unpaired, Student's *t*-tests. Errors are presented as the standard error of mean (SEM).

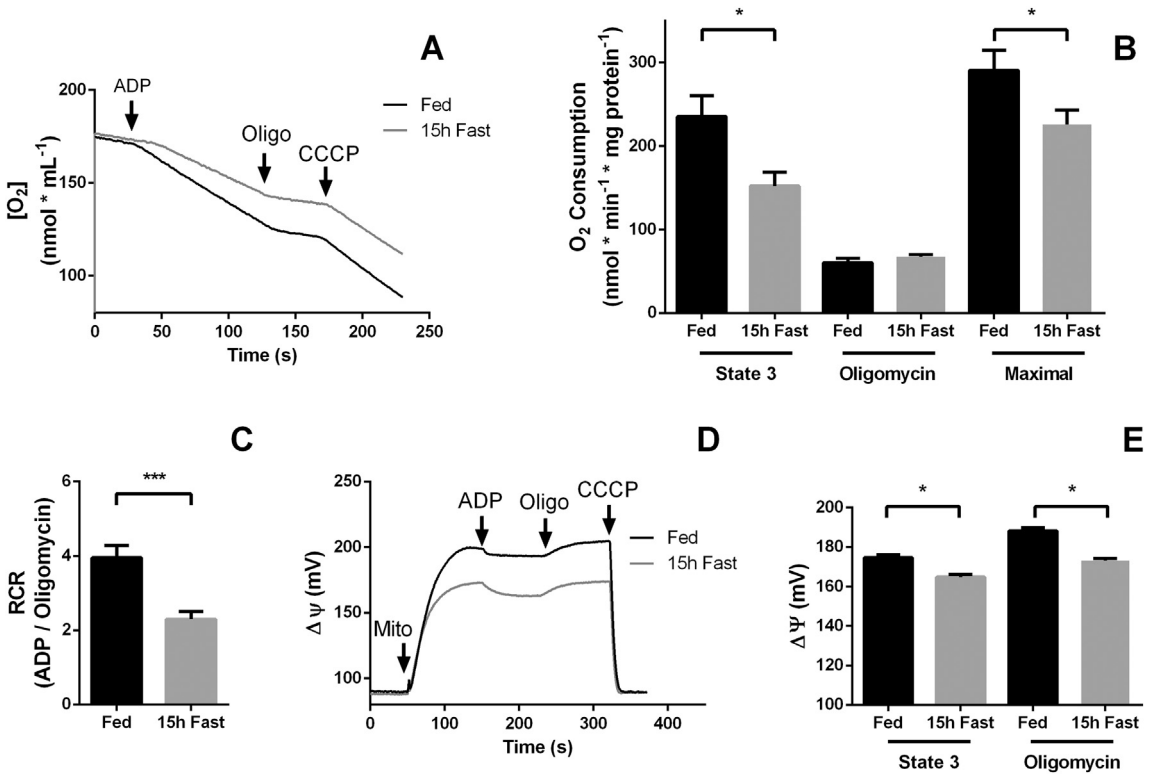

Fig. 1. Overnight fasting decreases oxidative phosphorylation capacity, coupling and membrane potentials in isolated liver mitochondria. (A) Representative oxygen consumption traces. Arrows indicate the addition of ADP (to induce state 3), oligomycin (to block ATP synthase) and CCCP (to induce maximal respiration). (B) Oxygen consumption per mg mitochondrial protein from experiments such as those shown in Panel A. (C) Respiratory control ratios (state 3/oligomycin). (D-E) Representative traces and quantifications of mitochondrial membrane potentials. *, $p < 0.05$; ***, $p < 0.001$.

3. Results

Overnight fasting reduces mitochondrial oxidative phosphorylation capacity, respiratory control ratios and membrane potentials.

In order to evaluate the effects of overnight fasting on mitochondrial function, mitochondria from the livers of fed (*ad libitum* food access) and overnight (15 h) fasted mice were isolated and used in oxygen consumption and membrane potential experiments, as described in Materials and Methods. Mitochondria from overnight fasted animals consistently displayed an overall decrease in all measured mitochondrial oxidative phosphorylation parameters (Fig. 1). More specifically, fasting lead to a reduction in both ATP synthesis-linked state 3 and maximal uncoupled respiration (Fig. 1A and B, measured in the presence of added ADP and FCCP, respectively) as well as reduced respiratory control ratios (Fig. 1C), or the relationship between ADP-stimulated respiration and that in the presence of the ATP synthase inhibitor oligomycin. Indeed, a significant decrease in membrane potentials (Fig. 1D and E) was experimentally measured, confirming a loss in oxidative phosphorylation efficiency. Interestingly, although oligomycin-insensitive oxygen consumption was not significantly altered by fasting (suggesting the same absolute amount of proton leak), membrane potentials measured after oligomycin were lower in 15 h fasted mitochondria (Fig. 1E), suggesting similar amounts of proton leak for a lower membrane potential. Since the proton leak is expected to be exponentially increased relative to the membrane potential [21], the presence of similar amounts of proton leak under a condition in which the membrane potential is lower suggests a leakier membrane. Overall, these results clearly demonstrate that fasting promotes a decrease in oxidative phosphorylation efficiency in liver mitochondria.

3.1. Overnight fasting reduces mitochondrial calcium retention capacity

The amount of Ca^{2+} mitochondria are able to accumulate is an indicator of their vulnerability to pathological over-accumulation of this ion [22,23]. Indeed, mitochondrial permeability transition, a non-selective form of inner membrane permeabilization induced by excessive Ca^{2+} and oxidants, participates in many pathological conditions including liver ischemia-reperfusion and acetaminophen-induced damage [16,22–25].

We measured mitochondrial Ca^{2+} accumulation and retention capacity by adding multiple pulses of Ca^{2+} at fixed time intervals and following free Ca^{2+} concentrations in the media using the fluorescent Ca^{2+} probe Calcium Green 5 N, which was not taken up by mitochondria (Fig. 2A). The arrows in Fig. 2A indicate Ca^{2+} additions, leading to an immediate increase in medium [Ca^{2+}]; the subsequent downward deflection of the fluorescence curve over time indicates mitochondrial Ca^{2+} uptake, decreasing the fluorescence signal of the membrane-impermeable probe. Ca^{2+} additions were continued until a spontaneous increase in free [Ca^{2+}] in the media was observed (Fig. 2A), promoted by the release of accumulated Ca^{2+} by mitochondria after permeability transition. Our data show a large decrease in calcium retention capacity in mitochondria isolated from the livers of mice fasted overnight (Fig. 2A and B), once again indicating that fasting decreases the overall quality of isolated mitochondrial preparations.

3.2. Liver mitochondrial preparation purity is unchanged by overnight fasting

Since experiments described above were carried out with the same amounts of mitochondrial protein, we questioned if the observed increases in oxygen consumption and calcium retention capacity could be attributed to different contamination of the samples with components

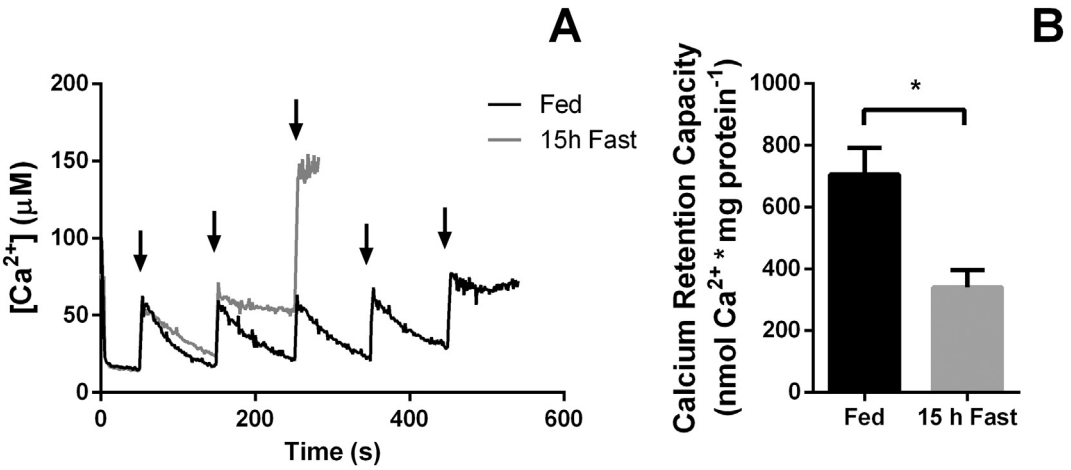

Fig. 2. Overnight fasting decreases mitochondrial calcium retention capacity. (A) Representative Ca^{2+} uptake assay traces; each arrow indicates a bolus Ca^{2+} addition. (B) Maximal calcium retention capacity quantification before the onset of permeability transition. *, $p < 0.05$.

from other sources (such as microsomes and peroxisomes). To assess this possibility, we performed western blots for three widely accepted marker proteins in our isolated mitochondrial samples: HSP60, TOM20 and VDAC (Fig. 3A and B). None of the markers showed any differences between the samples of the two groups when normalized to total protein, indicating similar mitochondrial purity in the fed and fasted samples. The mitochondrially-specific lipid cardiolipin was also present in equal amounts in mitochondria from the livers of both groups (results not shown), showing that fasting does not lead to a higher abundance of membranes from other sources, which could cause differential contamination. Mitochondria from fasted livers also showed no significant changes in citrate synthase activity, although a tendency toward a decrease was noted (Fig. 3C, $p = 0.10$). Interestingly, changes in citrate synthase enzymatic content or activity normalized to mitochondrial mass have been observed elsewhere [26,27]. This suggests that the levels of this specific mitochondrial protein are not necessarily constant in the mitochondrial population under different conditions, and brings to light the necessity to use multiple mitochondrial markers as indicators of mitochondrial mass. In total liver homogenates, citrate synthase activity was also not significantly different (Fig. 3D), although the dispersion of data was large, leading us to measure a second mitochondrial marker in the homogenate. Cardiolipin quantities relative to total protein in homogenates were slightly higher in fasted animals (Fig. 3E), suggesting liver mitochondrial mass increases with fasting, which may be a compensatory mechanism for the loss of oxidative phosphorylation capacity observed.

3.3. Short term fasting has similar effects

Our results up to now indicate clearly that, although it does not change the purity of isolated liver mitochondrial samples, fasting promotes changes in mitochondrial function, with a decrease in respiratory capacity and oxidative phosphorylation, as well as higher susceptibility to permeability transition. The fasting protocol used involved the typical overnight fasting almost universally used for isolated liver mitochondrial studies. This, however, leads to the question if this decrease in mitochondrial function is an effect of fasting alone or of torpor, a physiological hypometabolic state which mice often enter after many hours without food [14,15]. Indeed, Brown and Staples [14] have described changes in mitochondrial oxygen consumption and coupling in torpid rodents.

In order to verify if the mitochondrial changes observed were secondary to fasting alone or fasting-induced torpor, oxygen consumption and calcium uptake assays were repeated with a separate group which was fasted for only 4 h, less time than necessary for the induction of the first bout of torpor [14,15], but sufficient time to change hormonal levels. As shown in Fig. 4A, B and C, mitochondria from the 4 h fasted group showed essentially the same changes in oxidative phosphorylation as the 15 h group, with a reduction in ADP-stimulated, maximal uncoupled respiration and respiratory control ratios. Calcium retention capacity was also decreased when compared to the fed group (Fig. 4D and E). These results show that the functional changes observed in mitochondria from fasted mice are not the result of stress responses to poor nutrient availability or torpor-induced metabolic suppression, but rather a physiological change related to the fasted state itself.

4. Discussion

The results presented in this paper show that mice submitted to short or overnight fasting periods undergo a shift in liver mitochondrial bioenergetics characterized by a decrease in maximal respiratory capacity and oxidative phosphorylation. These results are compatible with those of Brown and Staples [14], who found a decrease in oxidative phosphorylation and coupling in fasting-induced torpid mice, and thus suggested this could be a metabolic suppression to save energy in the torpid state. However, we found that only 4 h of fasting, a time which is much lower than needed to induce the first bout of torpor [14,15], was sufficient to promote the same changes, indicating they are induced by fasting itself, not torpor. Indeed, a decrease in mitochondrial respiratory control ratios and membrane potentials such as that observed is not compatible with metabolic suppression, as it could promote more wasteful usage of the available substrates [28].

These results have an immediate practical implication for isolated mitochondrial studies, in which fasting was typically adopted to date, purportedly to avoid excess fat in the liver homogenate. Fasting has also been justified to be useful in mitochondrial isolation to avoid differences in hormonal and nutrient levels that may affect preparation homogeneity. However, we noted no difference in heterogeneity between mitochondrial preparations conducted in fasted *versus* fed animals to justify this hypothesis. Overall, our data suggest that, for most isolated mitochondrial studies, using fed animals may be not only a better ethical choice, but also one that yields higher quality preparations.

Interestingly, we find that not only electron transport and oxidative phosphorylation are augmented in fed animals, but also the ability to take up and store calcium, limiting Ca^{2+}-induced mitochondrial permeability transition [22]. In this line, fasting has been shown to potentiate liver damage induced by acetaminophen [29], a type of liver damage that is related to the mitochondrial permeability transition [25]. This suggests that the increased resistance to permeability

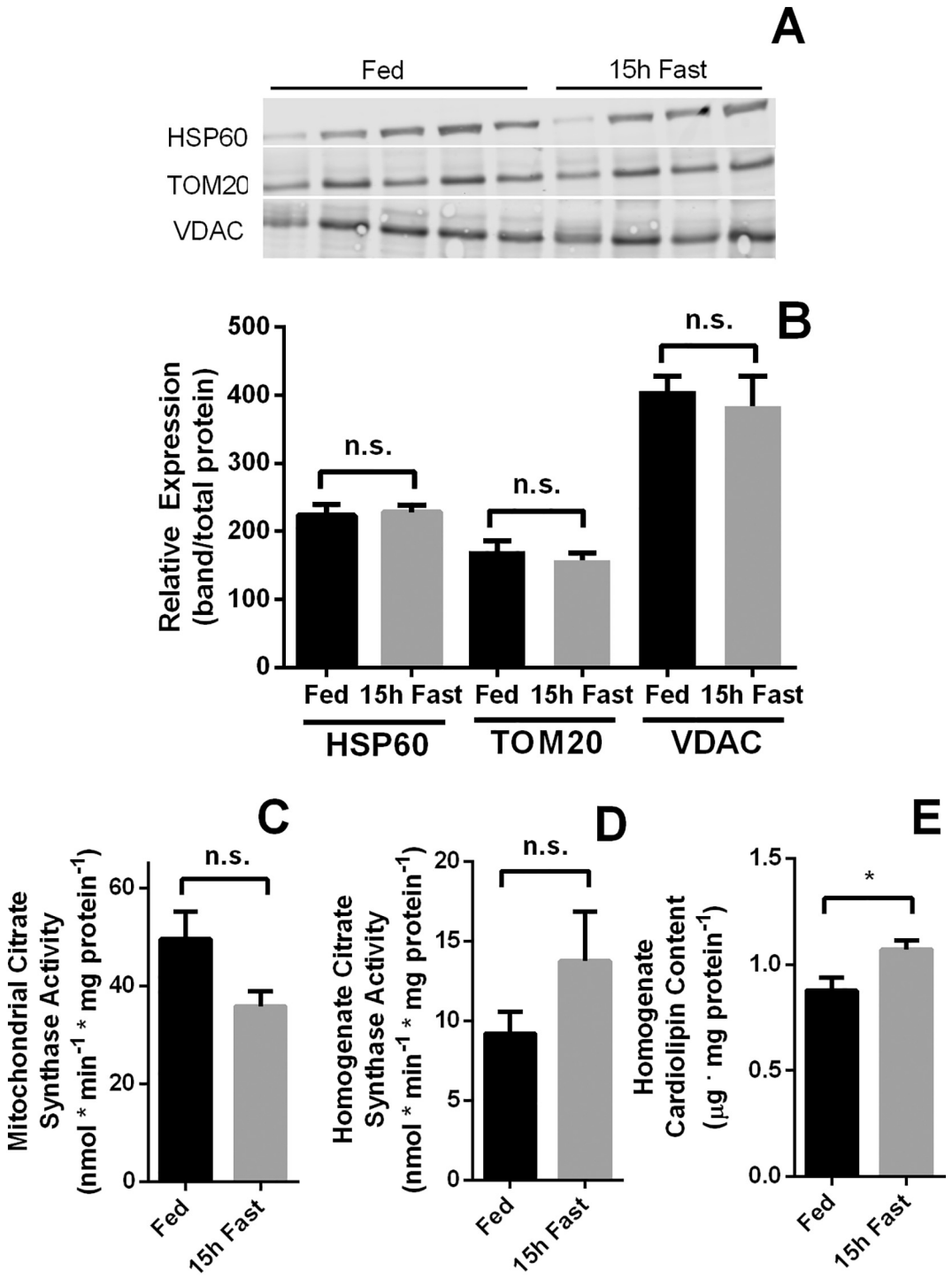

Fig. 3. Purity of isolated mitochondrial samples are unchanged by fasting. (A) Western blot for mitochondrial mass markers HSP60, TOM20 and VDAC in isolated mitochondria from fed or 15 h fasted mice. (B) Band intensity quantification (C) Citrate synthase activity in isolated mitochondria. (D) Citrate synthase activity in total liver homogenates (E) Cardiolipin content (a mitochondrial marker) in total homogenates. n.s. = non-significant; *, $p < 0.05$.

transition seen in fed animals occurs not only *in vitro*, but also *in vivo*, and further supports our isolated mitochondrial results. Of note, decreased Ca^{2+} accumulation capacity is seen in acute short and long-term fasting (as described here), but not in chronic daily caloric intake limitation (such as in caloric restriction), which promotes increased resistance against mitochondrial permeability transition and liver injury [16].

The mechanism in which respiration, membrane potentials and Ca^{2+} uptake are improved in fed *versus* fasted animals remains to be determined. Based on results in perfused livers, in which glucagon administration in the presence of high nutrient levels promoted increases in mitochondrial respiration and calcium retention capacity [8–11], we suggest that the effects of fasting seen here are not attributable to increased glucagon signaling alone. One contributing factor for the effects of fasting may be nutrient levels and their combined influence with hormonal signaling on the subtypes of mitochondria present. It should also be noted that fasting is a potent inducer of autophagy and mitophagy [30], which, associated with mitochondrial

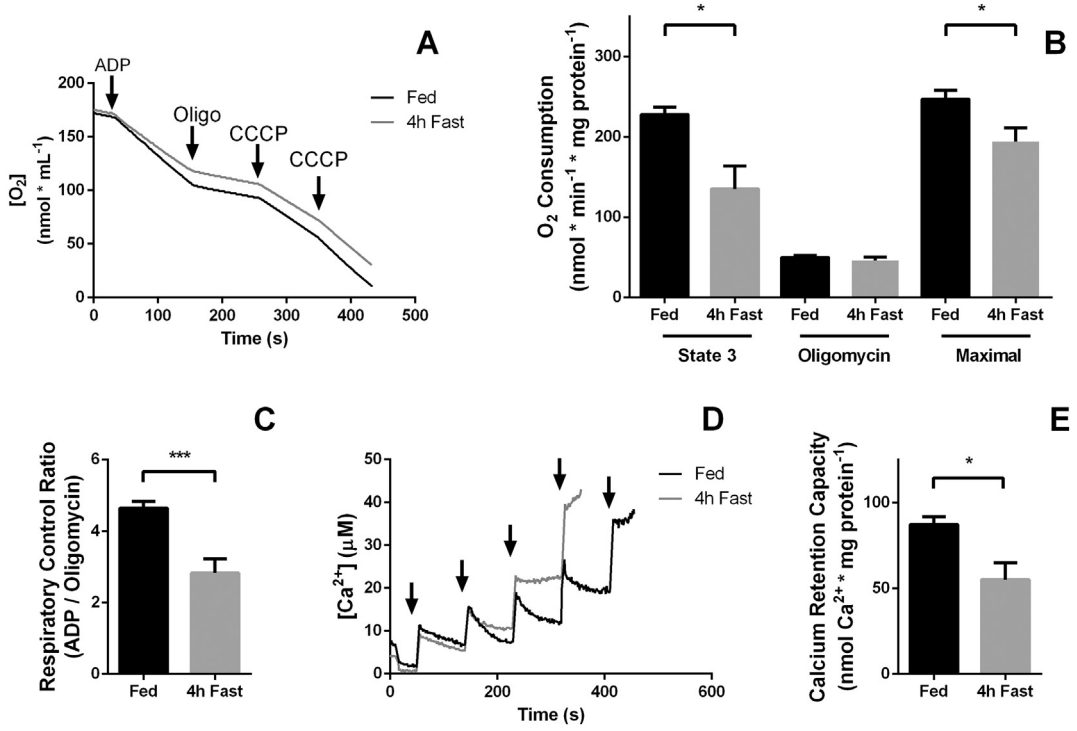

Fig. 4. Short term fasting promotes similar changes in function to overnight fasting. Experiments showed in Fig. 1 were repeated comparing fed animals to animals fasted for 4 h. (A) Representative oxygen consumption traces. The arrows indicate the addition of ADP, oligomycin and CCCP titrations. (B) Oxygen consumption quantification. (C) Respiratory control ratios. (D) Representative Ca^{2+} uptake traces in which each arrow indicates a bolus Ca^{2+} addition. (E) Maximal Ca^{2+} retention capacity before the onset of permeability transition. *, $p < 0.05$; ***, $p < 0.001$.

biogenesis, could promote replacement of part of the mitochondrial population with a distinct bioenergetic profile. A recent paper from Shirihai's group [27] shows that mitochondria associated to lipid droplets in the brown adipose tissue constitute a separate population with a distinct bioenergetic profile, high coupling and focused on fueling substrates for fatty acid synthesis, while the remaining mitochondria in the brown adipose tissue cells are poorly coupled and associated with fatty acid oxidation [27]. Interestingly, the bioenergetic characteristics of lipid droplet-associated mitochondrial populations shows similar features to the mitochondria from fed animals in our study, namely higher state 3 and maximal respiration, higher mitochondrial coupling and increased citrate synthase activity when compared to the fasted group [27]. Since fatty acid synthesis (lipogenesis) is upregulated by insulin and normally occurs in the fed state [reviewed in [4]], it is tempting to hypothesize that the liver, similarly to brown adipose tissue, may present more efficient mitochondria when synthesizing lipids in the fed state. Further studies uncovering the mechanisms regulating these shifts would be of interest to understand metabolic fluctuations in liver bioenergetics, both in physiology and pathology.

In brief, our data show that mitochondria isolated from fasted animals show clear differences in their bioenergetic profile compared to mitochondria from fed animals, which could reflect physiological adaptations to hormonal signaling and nutrient availability. This finding has both implications for understanding bioenergetic regulation in different nutritional states and for standard laboratory mitochondrial isolation protocols, which we suggest should no longer use animals fasted overnight.

Conflict of interest

The authors have no conflict of interest to declare.

Transparency document

The Transparency document associated with this article can be found in online version.

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