Journal of Physiology and Biochemistry
https://doi.org/10.1007/s13105-022-00882-9

ORIGINAL ARTICLE

Oxidative damage and mitochondrial functionality in hearts from KO UCP3 mice housed at thermoneutrality

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Received: 15 September 2021 / Accepted: 21 February 2022

Abstract

The antioxidant role of mitochondrial uncoupling protein 3 (UCP3) is controversial. This work aimed to investigate the effects of UCP3 on the heart of mice housed at thermoneutral temperature, an experimental condition that avoids the effects of thermoregulation on mitochondrial activity and redox homeostasis, preventing the alterations related to these processes from confusing the results caused by the lack of UCP3. WT and KO UCP3 mice were acclimatized at 30 °C for 4 weeks and hearts were used to evaluate metabolic capacity and redox state. Tissue and mitochondrial respiration, the activities of the mitochondrial complexes, and the protein expression of mitochondrial complexes markers furnished information on mitochondrial functionality. The levels of lipid and protein oxidative damage markers, the activity of antioxidant enzymes, the reactive oxygen species levels, and the susceptibility to in vitro Fe-ascorbate-induced oxidative stress furnished information on redox state. UCP3 ablation reduced tissue and mitochondrial respiratory capacities, not affecting the mitochondrial content. In KO UCP3 mice, the mitochondrial complexes activities were lower than in WT without changes in their content. These effects were accompanied by an increase in the level of oxidative stress markers, ROS content, and in vitro susceptibility to oxidative stress, notwithstanding that the activities of antioxidant enzymes were not affected by UCP3 ablation. Such modifications are also associated with enhanced activation/phosphorylation of EIF2α, a marker of integrated stress response and endoplasmic reticulum stress (GRP778 BIP). The lack of UCP3 makes the heart more prone to oxidative insult by reducing oxygen consumption and increasing ROS. Our results demonstrate that UCP3 helps the cell to preserve mitochondrial function by mitigating oxidative stress.

Keywords UCP3 · Heart · Thermoneutrality · Oxygen consumption · Oxidative stress · Mitochondrial complexes · EIF2α · GRP78 BIP · Calnexin · Hsp 60

Key points
- UCP3 ablation increases ROS content and reduces mitochondrial respiration
- KO UCP3 mice mitochondria show a high susceptibility to oxidative stress
- UCP3 regulates the ROS level in the heart of mice kept at thermoneutrality

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Introduction

The heart, one of the organs which consume oxygen at the highest rate, requires a tuned balance between oxygen supply and consumption to face the physiological changes in workload and/or stresses such as hypoxia, ischemia, and excessive overload. At the same time, the production of reactive oxygen species (ROS) can oxidize and damage the cells, a process relevant particularly in the heart, since, compared to other tissues, it has a limited total antioxidant capacity [7]. Therefore, in the heart, other systems must be operative to counteract the conditions in which ROS production increases. Several experimental pieces of evidence suggest that cardiac uncoupling protein 3 (UCP3) can exert antioxidant action [4].

Published online: 03 March 2022

UCP3 belongs to the protein subfamily of the anion inner mitochondrial membrane transporters [29], including five proteins from UCP1 to UCP5. To date, a clear role in thermogenesis has been shown only for the UCP1 [32], while the physiological role played by the other UCPs is still under investigation. UCP3 expresses in muscles (cardiac and skeletal) and adipose organ (white and brown adipocytes). UCP3 could be involved in reducing oxidative stress for its ability to lower the mitochondrial proton-motive force, a key factor influencing mitochondrial superoxide release, by inducing mild mitochondrial uncoupling [4, 21]. Other studies indicate a role for UCP3 in mediating the extrusion of mitochondrial matrix lipid hydroperoxides (LOOH) [17], compounds able to interact with Fe^{2+} ions generating highly reactive alkoxy radicals (LO·). However, the role of UCP3 in protecting mitochondria from ROS-induced damage is still under investigation, and contrasting data are present in the literature. Indeed, even if UCP3 seems to regulate ROS production [22], the studies on mice lacking UCP3 have given discordant results. Some authors reported that skeletal muscle mitochondria from KO UCP3 mice showed increased ROS production [36] and elevated markers of oxidative stress [3], but other authors failed to show these effects [27].

In the heart, UCP3 plays a pivotal role in protecting the tissue from hypoxic or ischemic injury.

Indeed, UCP3 genetic deletion promotes a greater decline of mitochondrial function, increases ROS production, and reduces cell survival in the heart [28]. Moreover, after myocardial infarction, the infarct size is greater and the capacity of remodeling the cardiac tissue is reduced as well as the survival in KO UCP3 mice [30].

Until now, most of the studies on the effects of UCP3 gene ablation on the heart have been performed on mice kept at 21 ± 1 °C, which is a temperature away from their thermoneutral zone, i.e., the temperature at which basal metabolic rate generates enough heat to maintain a constant temperature [10]. The thermoneutral temperature of mice is high (about 30–32 °C) due to their high overall heat conductance, primarily stemming from the elevated body surface to volume ratio [10]. This implies that mice kept at the commonly used housing temperatures have considerably elevated metabolism with increased heart rate due to elevated sympathetic tone [6, 12, 35]. The low temperature also affects oxidative stress level in the heart, as suggested by the cold exposure-induced increase in oxidative stress marker levels [37]. Moreover, the low temperature affects mitochondrial oxidative capacity, ROS production, and modifies the relative contents of mitochondrial fractions obtained at different gravitational force in the heart [37]. In the present paper, we evaluated the impact of the absence of UCP3 on mitochondrial functionality, ROS levels, markers of lipid and protein oxidative damage, and activities of the main antioxidant enzymes in cardiac tissue and isolated mitochondria from mice housed at thermoneutrality (30 °C), to avoid the thermal stress, and the consequent influence on output results. We also evaluated if the eventual effect of UCP3 ablation on mitochondrial functionality and oxidative stress could elicit cellular stress response.

Materials and methods

Animals

UCP3-ablated Swiss Black mice (KO) were originally derived from Dr Reitman's lab [18]. KO and WT male mice (Swiss Black, Taconic, New York, USA) were housed at 30 ± 1 °C, with a 12/12-h light-dark cycle, and free access to food and water. Figure 1 reports a representative blot of the heart content of the protein UCP3 in WT and KO UCP3 mice. Eleven mice aged 3 to 4 months for each group (KO UCP3 and WT) were acclimated to thermoneutrality for 4 weeks. At the end of the acclimation period, they were anesthetized with pentothal (40 mg/100 g bw) and killed by decapitation. Hearts were immediately collected and processed or frozen in liquid nitrogen for later processing.

For homogenate preparation, the hearts of eight mice for each group were placed into ice-cold homogenization medium (HM) (220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.1% fatty acid-free albumin, 10 mM Tris, pH 7.4). The heart great vessels and valves were trimmed away, and the ventricles and atria were cut open and rinsed free of blood. The hearts were weighed, finely minced, washed with HM, and gently homogenized (20% w/v) in such a solution using a glass Potter–Elvehjem homogenizer set at a standard velocity (500 rpm) for 1 min.

This study was carried out according to the EU Directive 2010/63 for the Care and Use of Laboratory Animals. All animal protocols were approved by the Committee on the Ethics of Animal Experiments of the University of Naples Federico II and the Italian Minister of Health. Every effort was made to minimize animal pain and suffering.

Preparation of intact mitochondria

The homogenates, diluted at 10% (w/v) in HM, were centrifuged at 500 *g* for 10 min at 4 °C to remove cell debris

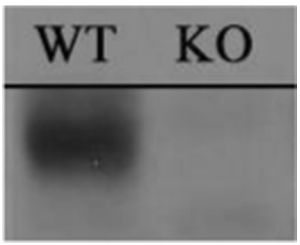

Fig. 1 Levels of UCP3 protein in the heart of wild type and KO UCP3 mice

and nuclei and, subsequently, the supernatants were centrifuged at 3,000 g for 10 min. The mitochondrial pellets were resuspended in washing buffer (WB) (220 mM mannitol, 70 mMsucrose, 1 mMEGTA, 20 mMTris, pH 7.4) and centrifuged again at 3,000 g for 10 min. This step was repeated twice, and mitochondria were resuspended in a minimal volume. Fresh mitochondria were used for oxygen consumption detections, and some aliquots were supplemented with antiprotease cocktail (Sigma-Aldrich) and immediately frozen. Mitochondrial protein content was measured by the biuret method.

Oxygen consumption and cytochrome oxidase (COX) activity

The rates of oxygen consumption in tissue homogenates and mitochondria were determined at 30° using an Hansatech respirometer in 1.0 mL of incubation medium (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.4) with 50 µL of 20% (w/v) homogenate or 0.25 mg of mitochondrial protein per mL. We used Succinate (10 mM), plus 5 µM rotenone, or pyruvate plus malate (10 and 2.5 mM, respectively) as substrates, in the absence (state 4) and in the presence (state 3) of 500 µM ADP. State 4 oxygen consumption of homogenates was measured by adding 2 µg/mL of oligomycin.

Complex IV (cytochrome c oxidase) activity was determined by a polarographical procedure [2] in 1.0 mL of buffer solution (30 µM Citc 3131, 10 mM Sodium Malonate, 75 mM Hepes, 4 µM rotenone, 0.5 mM 2.4-dinitrophenole, pH 7.4) containing 0.1 mg of mitochondrial proteins or 0.2 mg of mitochondria, after membranes solubilization with 1% Lubrol and in presence of a mixture of TMPD plus ascorbate (30 mM plus 400 mM, respectively).

Western blot analyses

Hearts and mitochondrial suspensions were lysed in a buffer containing 150 mM NaCl, 50 mM Tris–HCl, 0.5% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, (pH 8), and Tissue Protease Inhibitor Cocktail (Sigma-Aldrich, 1:500, v/v). After 15 min of incubation, lysates were centrifuged at 12,000 g for 30 min at 4 °C. Protein concentration in all lysates was assayed by the biuret method. Immunoblotting was performed as previously reported [38] using the following commercially available antibodies: UCP3 (ab3477, Abcam, Cambridge, UK); cytochrome c (SC-7159, Santa Cruz, San Diego, CA, USA); voltage-dependent anion channel1 (VDAC1, GTX114187, GeneTex, Inc. North America,); anti-Oxphos (Abcam, Cambridge, UK total Oxphos rodent WB antibody cocktail AB110413); EIF2α (L57A5, Cell Signaling Technology); EIF2αP (8B3A11, Cell Signaling Technology); GRP78 BIP (C50B12, Cell Signaling Technology); Calnexin (C5C9, Cell Signaling Technology); Hsp 60 (GTX110089, GeneTex); VDAC (sc-390996, Santa Cruz Biotecnology); and β-actin (A2066, Sigma-Aldrich, St. Louis, MO, USA). Secondary antibodies were purchased from Sigma-Aldrich (SC-2030, Santa Cruz, San Diego, CA, USA). Bands were visualized by the Excellent Chemiluminescent detection Kit (ElabScience, Microtech, Naples, Italy), according to the manufacturer's instructions. Quantitative densitometry of the bands was carried out by analyzing ChemiDoc images or digital images of X-ray films exposed to immunostained membranes, and the quantification of the signal was performed by Un-Scan-It gel software (Silk Scientific, UT, USA). To compare protein expression levels, a standard control sample was run on each gel, and all values were compared with the control sample to which was assigned the value 1.

Mitochondrial complexes activities

The activities of the first three complexes of the mitochondrial electron transport chain system were measured by spectrophotometric methods [31] using a Beckman (Fullerton, CA, USA) model DU 640. The fourth complex was determined as previously described.

Oxidative damage and in vitro susceptibility to oxidant

The levels of lipid hydroperoxides (HP) were used to measure the extent of the lipid peroxidative processes in tissue homogenates and mitochondria, according to Heath and Tappel [19]. The level of protein-bound (CO) carbonyl was used to determine protein oxidative damage to homogenates and mitochondria [34].

Susceptibility to oxidative stress of cardiac tissue and of the mitochondrial fraction were evaluated by the change in hydroperoxide levels following 10% tissue homogenate treatment, or 1 mg of mitochondrial proteins, with iron and ascorbate (Fe/As), at a concentration of 100/1000 µM, for 10 min at room temperature. The reaction was blocked by adding 0.2% 2, 6-di-t-butyl-p-cresol (BHT) and the hydroperoxide levels were evaluated as previously described [19].

Activities of the antioxidant enzymes GPX CAT and SOD

Glutathione peroxidase (GPX) activity was assayed according to Flohé and Günzler [13] with H_2O_2 as substrate. Catalase activity was determined by the method of Aebi [1]. Superoxide dismutase specific activity was measured spectrophotometrically (550 nm) at 25 °C, by monitoring the decrease in the reduction rate of cytochrome c induced by the superoxide radicals generated by the xanthine–xanthine

oxidase system. Mitochondria were incubated in a medium containing 0.1 mM EDTA, 2 mM KCN, 50 mM KH_2PO_4, pH 7.8, 20 mM cytochrome c, 5 mM xanthine, and 0.01 U of xanthine oxidase. One unit of SOD activity is defined as the concentration of enzyme that inhibits cytochrome c reduction by 50% in the presence of xanthine + xanthine oxidase [14].

Tissue and mitochondrial ROS content

The ROS content was measured following the ROS-induced conversion of 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA, nonfluorescent compound) in dichlorofluorescein (DCF, fluorescent compound) according to Driver et al. [9]. In brief, 12.5 µg of homogenate proteins or 20 µg of mitochondrial proteins in 200 µL of monobasic phosphate buffer 0.1 M, pH 7.4, were incubated for 15 min with 10 µM DCFH-DA. Then, 100 µM $FeCl_3$ was added, and the mixture was incubated for 30 min. The conversion of DCFH-DA to the fluorescent product DCF was measured using a multimode microplate reader (Synergy™ HTX Multimode Microplate Reader, BioTek) with excitation and emission wavelengths of 485 and 530 nm, respectively. Background fluorescence (conversion of DCFH to DCF in the absence of homogenate and mitochondria) was corrected with parallel blanks.

Data analysis

The data were expressed as the means ± standard error and analyzed by Student's *t*-test. Probability values (P) < 0.05 were considered significant. Eight animals were used for each group.

Results

Oxygen consumption and COX activity

We first evaluated if the absence of UCP3 could affect heart mitochondrial respiratory parameters, by evaluating uncoupled and coupled respiration (state 4 and state 3, respectively) and the respiratory control ratio (RCR, an index of the coupling between electron transport chain flux and ATP synthesis and of mitochondrial integrity) both in tissue homogenate and in isolated mitochondria (Fig. 2).

In the whole-tissue homogenates, oxygen consumption rates were lower in KO UCP3 than in the WT mice hearts

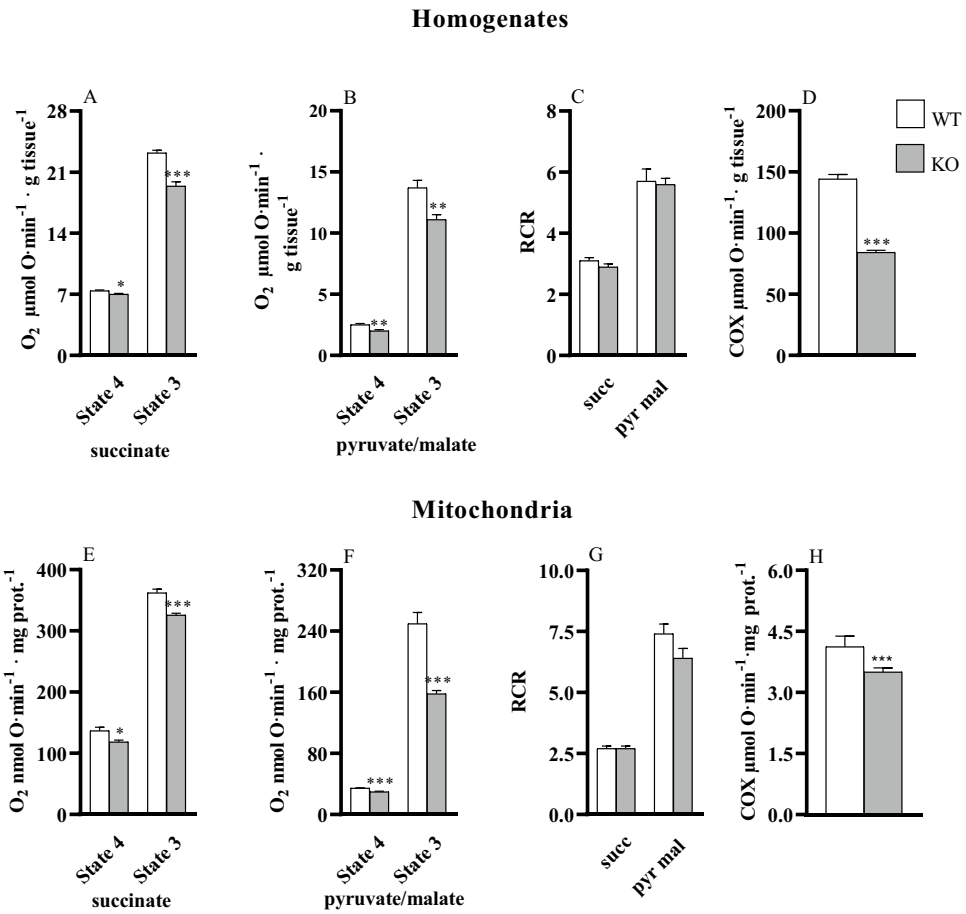

Fig. 2 Mitochondrial respiratory parameters and cytochrome oxidase activity detected in heart homogenates and isolated mitochondria from WT and UCP3 KO mice. State 3 and state 4 oxygen consumption and respiratory control ratio (RCR) detected in succinate and pyruvate plus malate supplemented homogenate (**A**, **B**, **C**) and mitochondria (**E**, **F**, **G**). Cytochrome oxidase activity detected in homogenate (**D**) e mitochondria (**H**). Values are means ± SEM of eight determinations from different mice. Each determination is the mean of three independent measures. Asterisks indicate significant differences (when at least $p < 0.05$ vs. WT controls) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

in both state 4 and state 3 respiration whatever the respiratory substrates, i.e., succinate plus rotenone, and pyruvate plus malate. RCR, obtained by the ratio between state 3 and state 4, did not change significantly between the two groups. Similar changes in state 4 and state 3 in KO UCP3 mice were also observed in isolated mitochondria.

Cytochrome oxidase/complex IV activity (COX), considered a limiting step of respiratory chain and its activity, and indicator of the oxidative capacity of the cells, was lower in both tissue and mitochondria from KO UCP3 than in WT mice. Our results indicate that the lack of UCP3 in heart determines a reduction in oxygen consumption.

Protein levels of VDAC, cytochrome c, and respiratory complexes subunits in heart lysates

To understand if the metabolic effects were due to changes in mitochondrial tissue content, we evaluated the levels of specific mitochondrial markers such as VDAC and cytochrome c and of the specific subunits of the five different mitochondrial complexes (I–V) (Fig. 3) were not modified by the absence of UCP3. These data indicate that there was not a reduction of mitochondria content in KO UCP3 mice.

Activity and abundance of the respiratory complexes in isolated mitochondria

To give further insight into the mechanism underlying the reduced respiration rate in UCP3 KO mice, we evaluated the activities of the mitochondrial respiratory complexes (I–IV) (Fig. 4, panel I), and their mitochondrial content (Fig. 4, panel II) are reported. The activities of the respiratory complexes were significantly lower in KO UCP3 mice than in WT ones. Such reduction cannot be attributed to changes in mitochondrial respiratory complexes abundance, since the levels of their specific subunits were unaffected by genotype. These data indicate that the reduced oxygen consumption in KO UCP3 mice depends on the reduced activities of the respiratory complexes rather than to a reduction in their content.

Lipid and protein oxidative damage, antioxidant enzyme activities, ROS content, and in vitro susceptibility to oxidative damages of heart homogenate and isolated mitochondria

To understand the factors involved in the reduction in complexes activities, we determined the level of oxidative stress.

In Fig. 5, homogenates and mitochondrial lipid hydroperoxides (panels A and A', respectively) and protein-bound carbonyls (panels B and B', respectively) are reported. Such markers significantly increased in the heart from KO UCP3 mice. These data indicate an increase of the oxidative stress in mice lacking UCP3.

Because oxidative stress can depend on reduced antioxidant capacities or increased ROS production, we measured the activities of the main antioxidant enzymes. The activities of the antioxidant enzymes GPX, catalase (CAT), and superoxide dismutase (SOD) detected in the homogenates and mitochondria from wild type and KO UCP3 mice are reported in Fig. 5 (GPX, C and C'; CAT, D and D'; SOD, E and E'). A slightly but significantly increase in the activity of GPX is found only in the mitochondria from KO UCP3 mice. No changes have been found for the other

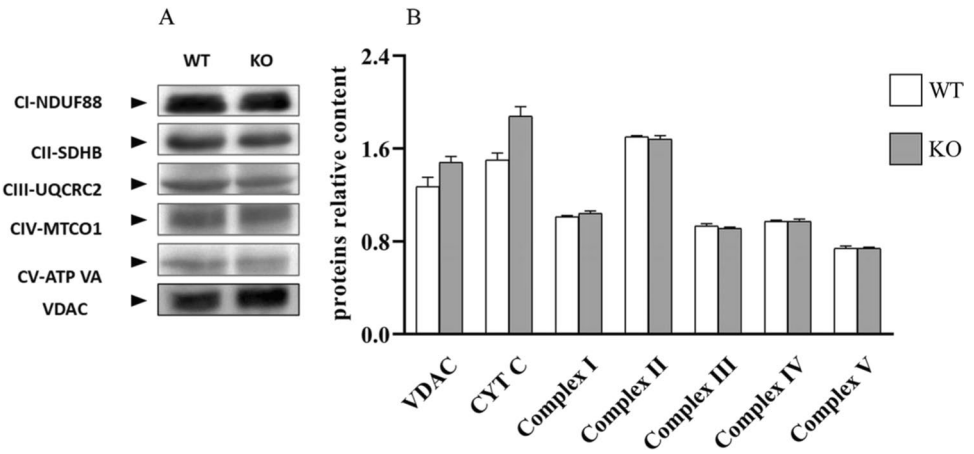

Fig. 3 Levels of mitochondrial protein markers detected in heart lysate from WT and UCP3 KO mice: voltage-dependent anion channel (VDAC), cytochrome c (Cit c), and subunits specific to the five respiratory complexes (CI subunit NDUFB8, CII-30 kDa, CIII-Core protein 2, CIV subunit I, and CV alpha subunit). **A** Representative Western blot and **B** quantification of data. Each line contained 30 µg of proteins. Bar charts show quantification of the signals. Actin protein expression is used as loading control. Values are means ± SEM of three determinations from different mice. Each determination is the mean of three independent measures

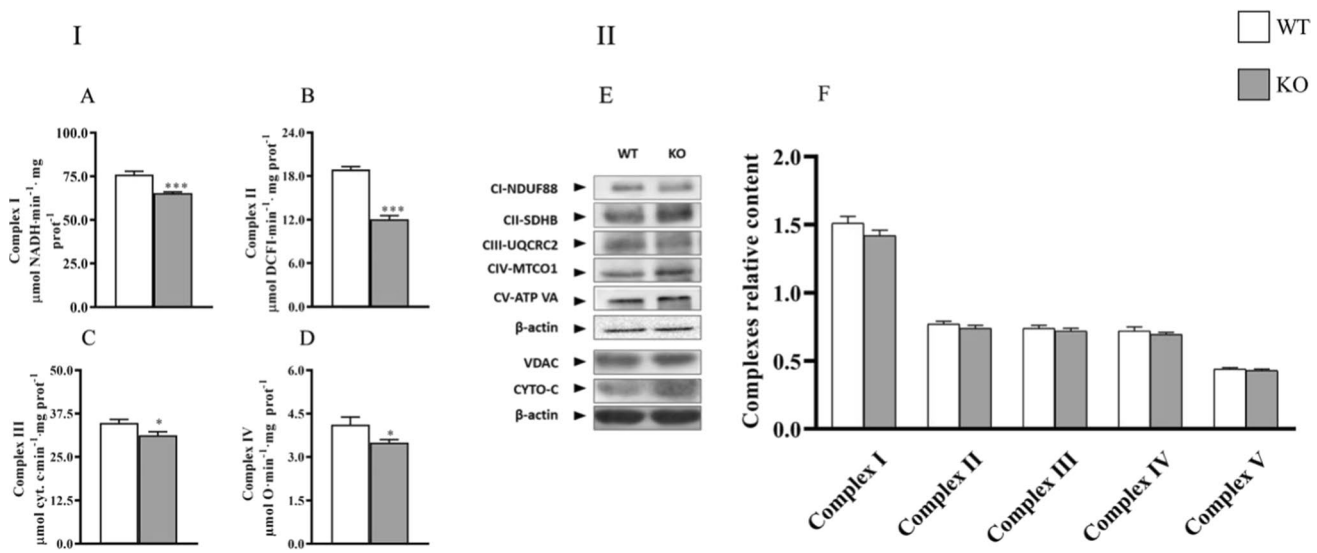

Fig. 4 **I** Mitochondrial respiratory complexes activities detected in isolated mitochondria from WT and UCP3 KO mice. Panel A, complex I; panel B, complex II; panel C, complex III; and panel D complex IV. Values are means ± SEM of eight determinations from different mice. Each determination is the mean of three independent measures. **II** Levels of subunits specific of the five mitochondrial complexes (CI subunit NDUFB8, CII-30 kDa, CIII-Core protein 2, CIV subunit I, and CV alpha subunit) detected in isolated mitochondria from WT and UCP3 KO mice. (E) representative Western blot and (F) quantification of data. Each line contained 30 µg of proteins. Bar charts show quantification of the signals. VDAC protein expression is used as loading control. Values are means ± SEM of three determinations from different mice. Each determination is the mean of three independent measures. Asterisks indicate significant differences (when at least $p < 0.05$ vs. WT controls). (*$p < 0.05$; ***$p < 0.001$)

enzymes, both when their activity has been detected in the tissue homogenate and isolated mitochondria. The lack of UCP3 does not seem to affect antioxidant capacities.

The levels of reactive oxygen species in the cardiac tissue and in the mitochondrial fraction are reported in Fig. 5 (F and F', respectively). In the presence of ROS, the nonfluorescent DCFH is rapidly oxidized to the highly fluorescent DCF. Several reactive intermediates may oxidize DCFH; therefore, it cannot be used to determine the presence of specific ROS, but it is a useful probe for assessing ROS production in biological systems [33]. The levels of DCF were not different between the wild type and KO UCP3 homogenates but was significantly higher in the mitochondrial fraction. These data suggest that mitochondrial but not tissue ROS content increases in the heart of KO UCP3 mice. To have information on the effects of such changes on the capacity of the heart preparation to counteract an oxidative insult, we stressed them with a mix of Fe^{2+} and ascorbate and evaluated the changes in the levels of lipid hydroperoxides.

In Fig. 5, the changes in the levels of lipid-bound hydroperoxides after in vitro Fe^{2+}-ascorbate-induced oxidative stress are reported. In both homogenates and mitochondria of KO UCP3 mice (G and G', respectively), the susceptibility to oxidative stress was higher than in control animals.

Effect of UCP3 ablation on the activation of integrated stress response and endoplasmic reticulum stress

Since mitochondrial dysfunction and enhanced oxidative stress could lead to the activation of integrated stress response and to changes in endoplasmic reticulum functionality, we evaluated if these processes could take place in heart from UCP KO mice.

We detected the levels of EIF2α as well as its activation since it is considered a crucial node in the integrated stress response signaling. EIF2α protein levels, that of its phosphorylated/activated form and EIF2α activation degree (the last evaluated by the ratio EIF2αP/EIF2α), increased in heart from KO UCP3 mice (Fig. 6, panel B), thus indicating the activation of stress response signaling in these mice.

Furthermore, we evaluated the levels of two markers of ER stress, such as GRP78 BIP and calnexin. Among the two, GRP78 BIP protein levels were significantly increased in sample from UCP3 KO mice compared to WT ones, indicating that the absence of UCP3 is associated to the occurring of ER stress in heart cells. Conversely, no changes were found in the level of the mitochondrial protein Hsp 60, involved in the mitochondrial protein quality control (Fig. 6 panel C).

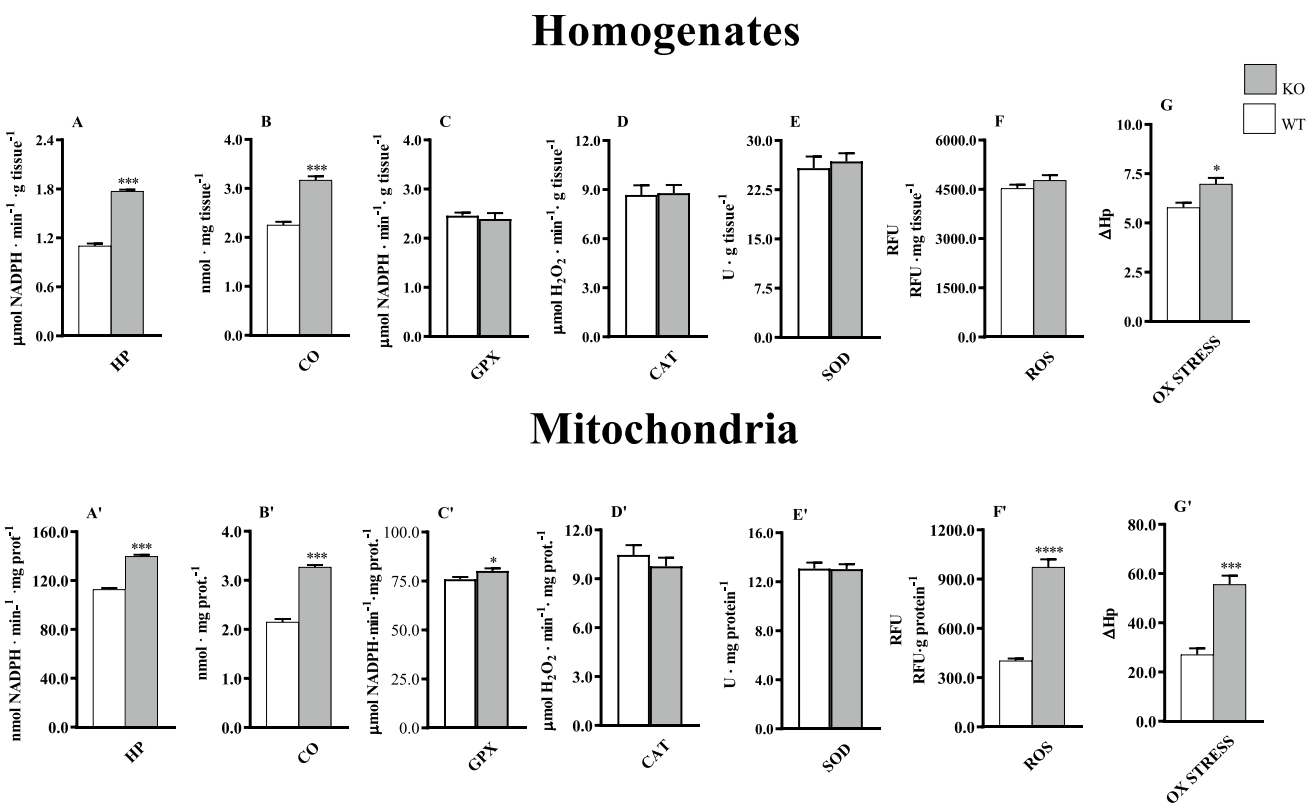

Fig. 5 Lipid (hydroperoxides, HP, A and A') and protein (protein-bound carbonyls, CO, B and B') oxidative damage markers; activities of antioxidant enzymes (glutathione peroxidase, GPX, C and C'; catalase, CAT, D and D'; superoxide dismutase, SOD, E and E'); total ROS content (F and F'); and in vitro response to oxidative stress (OX STRESS, G and G'), in heart homogenate (upper panels) and isolated mitochondria (lower panels) from WT and KO UCP3 mice. Values are means ± SEM of eight determinations from different mice. Each determination is the mean of three independent measures. Asterisks indicate significant differences (when at least $p < 0.05$ vs. WT controls). (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

Discussion

The total oxidative capacity of tissues depends on the content and functionality of their mitochondria. To determine if one or both causes are at the basis of a possible alteration of cardiac oxidative capacity depending on the presence/absence of UCP3, we first assessed whether there were differences in the mitochondrial content in KO mice vs. WT. For this purpose, as reported in the results, we measured some specific mitochondrial markers content. The lack of differences in their levels in KO and WT mice demonstrates that the differences found in cardiac oxidative capacity, respiratory rate, and cytochrome oxidase activity must be sought at the mitochondrial level.

We found that state 4 and state 3 of respiration were reduced in isolated mitochondria from KO UCP3 mice compared to wild type control, both by using substrates linked to complex I (pyruvate plus malate) and complex II (succinate plus rotenone). The respiratory control ratio is an index of the coupling between the electron transport system (ETS) and ATP synthesis other than mitochondrial integrity. Proton leak/proton conductance and the overall reactions involved in the oxidation of the substrates (including respiratory chain activity) affect state 4 respiration, while both the overall reactions involved in the oxidation of substrates and those involved in the synthesis and export of ATP affect state 3 respiration [33]. Our data show that the reduction in both state 3 and state 4 respiration, observed in KO UCP3, is not associated with significant variations in RCR, indicating that the lower state 4 respiration in KO UCP3 mice is not the result of a reduced proton conductance. This observation agrees with the generally accepted idea that UCP3 increases proton conductance only in the presence of activators, as also supported by data indicating that knockdown of UCP3 does not affect basal proton conductance of skeletal muscle and heart mitochondria [5, 24]. These reductions may involve alterations in the activity of the respiratory chain. Indeed, in mice lacking the UCP3, the activities of complexes from I to IV are significantly lower compared to those of the wild type control ones. Such effects do not depend on variations in the amount of the mitochondrial complexes

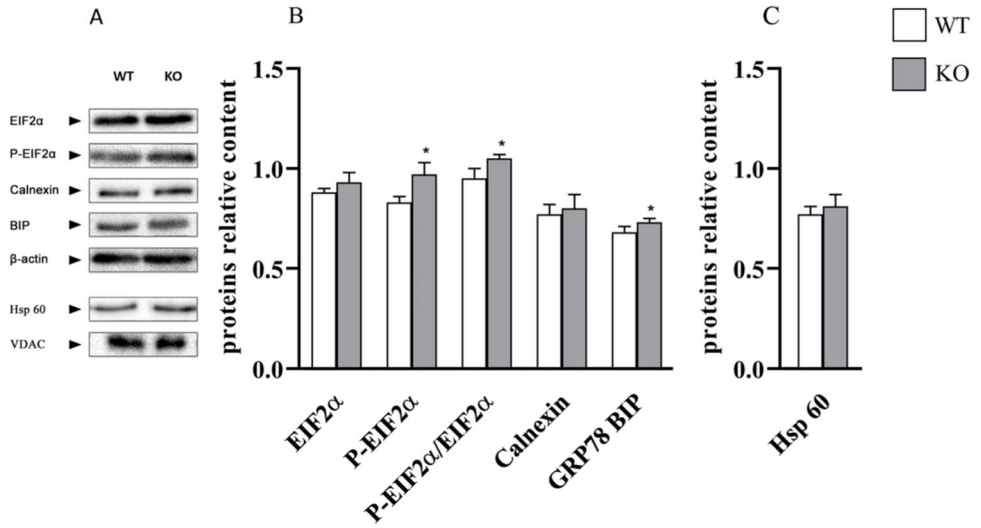

Fig. 6 Levels of markers involved in integrated stress response, endoplasmic reticulum, and mitochondrial UPR: eukaryotic translation initiation factor 2α (EIF2α), EIF2α phosphorylated (EIF2αP), the ratio between EIF2αP and EIF2α, calnexin, and ER chaperone 78-kDa glucose regulated protein also referred to as BIP (binding immunoglobulin protein (GRP78BIP), heat shock protein 60 (Hsp 60)). **A** Representative Western blot, **B** quantification of data in homogenate, and **C** quantification of data in mitochondria. Each line contained 30 μg of proteins. Bar charts show quantification of the signals. Actin protein expressions are used as loading control. Values are means ± SEM of three determinations from different mice. Each determination is the mean of three independent measures. Asterisks indicate significant differences (when at least $p < 0.05$ vs. WT controls) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

since the levels of specific subunits, representing the five respiratory complexes, did not differ between WT and KO UCP3 mice. These data also implicate post-translational mechanisms involvement, occurring in KO UCP3 mice, which negatively affect the complexes activity. Variations in the production/levels of ROS may be one of the factors. Some evidence showing that the mitochondrial electron-transport complexes are susceptible to oxidative damage seems to support this possibility [26].

In addition, we report here that in KO UCP3 mice, specific indexes of lipid and protein oxidative damage, such as the amounts of lipid hydroperoxides and the protein carbonyls, resulted significantly higher than in the control animals both in tissue and mitochondrial fraction. Other evidence also came from studies in the nematode *Caenorhabditis elegans*, where it was observed that increased oxidative stress reduced the activities of mitochondrial complexes and impaired electron chain flux [8].

An increase in oxidative damage could be the consequence of increased ROS production and/or decreased antioxidant capacity. In agreement with other studies [28], we found that higher levels of ROS were present in isolated mitochondria from KO UCP3 mice. This result suggests a role for UCP3 in mitigating ROS production and in preventing lipid hydroperoxides induced damage at mitochondrial level [3, 17].

Interestingly, in isolated mitochondria but not in whole-tissue homogenates, a significant difference in ROS content was detected between WT and KO UCP3 mouse samples.

This observation suggests that antioxidant systems, present outside mitochondria, are efficient in ROS detoxification. The involvement of the myoglobin/myoglobin reductase system, highly active in the heart, has been proposed [15].

To gain more insight into the heart's ability of KO UCP3 mice to cope with an oxidative insult, we stressed both homogenate and mitochondria with Fe^{2+}-ascorbate in vitro to assess changes in hydroperoxides levels. The susceptibility of a sample to Fe^{2+}-ascorbate-induced oxidative insult is supposed to be directly related to oxidative damage it suffers (that detected before the insult) and inversely related to sample antioxidant capacity [23]. Our data suggest that the hearts from KO UCP3 mice have a higher susceptibility to oxidants, greater in isolated mitochondria.

Although hydroperoxide levels increased by the same amount in heart tissue and mitochondria due to UCP3 ablation, in vitro stress with Fe^{2+}-ascorbate further increased these levels much more in mitochondria than in homogenates, showing that antioxidant capacity in heart tissue is less affected by UCP3 ablation than that of mitochondria.

The activities of the main antioxidant enzymes glutathione peroxidase, catalase, and superoxide dismutase were not affected by the absence of UCP3 in both homogenates and mitochondria. However, it should be emphasized that the total antioxidant capacity depends on the synergic actions of numerous factors, and it is also at least in part dependent on respiration [38]. Indeed, the activities of some enzymatic antioxidant mitochondrial systems, among which

the one involving GPX, known to remove hydrogen peroxide removal, need NADPH, which in mitochondria can be regenerated by NADH-supported NADP + reduction via energy-dependent trans-hydrogenation [20]. Therefore, the reduced oxygen consumption we found in UCP3 ablated mice may also contribute to the reduction of total antioxidant activity.

The increase in oxidative stress associated with the ablation of UCP3 is plausibly responsible for the bioenergetic decline of the heart, which could lead to relevant pathophysiological consequences. Among these, the increased heart susceptibility to ischemia–reperfusion is already observed in KO mice [30].

The increase in mitochondrial ROS content and protein carbonylation affects protein unfolding and upregulates the mitochondrial protein unfolding response [40]. At the same time, mitochondrial dysfunction can lead to consequences to the endoplasmic reticulum, whose functionality requires adequate ATP production and cell redox state, which undergoes stress. Many mitochondrial defects activate IRS response that is emerging as a common regulatory platform for the cell to deal with cellular stress, including mitochondrial dysfunctions [39], with EIF2α representing a crucial node. EIF2α activation by phosphorylation plays a relevant role in mediating the translation attenuation during mitochondrial dysfunction and ER stress finalized to reduce the influx of proteins into the two organelles [25, 40]. Increased activation of EIF2α associated with enhanced protein levels of the ER stress marker GRP78 BIP suggests that a similar condition can verify in the heart of KO UCP3 mice.

However, we do not find changes in the heat shock protein 60 (Hsp 60), the main protein involved in the mitochondrial protein quality control, which has been proposed to play a pivotal role in the regulation of protein folding and the prevention of protein aggregation [11]. This result agrees with the observation, reported in a mouse model for mitochondrial myopathy, that the cellular stress responses progress in temporal stages from early to chronic development of the disease [16], and marker proteins indicated that during the initial disease, the signs of ISR are present, while markers for UPRmt (increased mitochondrial chaperones and proteases) only were observed in the late disease stages in failing tissue [16].

These data strengthen the beneficial role of UCP3 in preserving mitochondrial performance and functionality of the endoplasmic reticulum, which is strictly influenced by the mitochondrial one.

Conclusions

In conclusion, our data show that in the heart, the presence of UCP3 plays an important role in mitigating oxidative stress and maintaining mitochondrial functionality and avoid the onset of cellular stress. Such factors are of crucial importance for the activity of this high energy supply-dependent relevant organ.

However, we must underline that since we did not use a heart-specific UCP3 KO mouse, rather we employed whole body KO UCP3 mice, we cannot exclude that factor released by other tissues expressing UCP3 could have contributed to influence heart functionality in our model. Further studies employing heart-specific KO UCP3 mice will help to clarify this aspect.

Author contribution N.G., L.A., G.F., and V.P. conceived and designed the experiments; F.G. and M.N. carried out all the animal studies and performed the experiments; G.N., G.F., L.A., and V.P. analyzed the data; G.F., L.A., and V.P. wrote the manuscript; all the authors revised the manuscript.

The authors declare that all data were generated in-house and that no paper mill was used.

Funding This work was supported by grants from the University of Naples Federico II [Ricerca Dipartimentale 2018–2020].

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval and consent to participate All animal protocols were approved by the Committee on the Ethics of Animal Experiments of the University of Naples Federico II and the Italian Minister of Health. Every effort was made to minimize animal pain and suffering.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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