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Powiązanie produkcji reaktywnych form tlenu ze stopniem redukcji koenzymu Q w mitochondriach

The interplay between reactive oxygen species formation and the coenzyme Q reduction level in mitochondria

Praca doktorska wykonana pod kierunkiem
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Spis treści

1. STRESZCZENIE

Koenzym Q (Q) jest kluczowym nośnikiem elektronów w mitochondrialnym łańcuchu oddechowym oraz ważnym przeciwutleniaczem chroniącym przed uszkodzeniami oksydacyjnymi, obecnym we wszystkich błonach komórki. Z jednej strony zredukowany Q (ubichinol, QH_2) zapobiega powstawaniu wolnych rodników, z drugiej strony niepełna redukcja Q prowadzi do tworzenia rodnika semichinonowego, będącego źródłem powstawania reaktywnych form tlenu (RFT) w łańcuchu oddechowym, które mogą prowadzić do stresu oksydacyjnego i uszkodzeń oksydacyjnych.

Celem niniejszej rozprawy doktorskiej było zbadanie powiązania produkcji mitochondrialnych RFT (mRFT) ze stopniem redukcji mitochondrialnego Q (mQ) w warunkach fosforylujących (podczas syntezy ATP) i niefosforylujacych. Materiałem użytym w pracy były mitochondria izolowane z komórek organizmu jednokomórkowego, ameby *Acanthamoeba castellanii* a także różne tkanki szczura, tj. płuca, mózg, serce i wątroba, oraz izolowane z nich mitochondria. Wykorzystanie różnych organizmów, różnych tkanek zwierzęcych oraz badanie wpływu treningu wytrzymałościowego na poziomie tkankowym i mitochondrialnym pozwoliło na szeroką, wielopoziomową analizę powiązania produkcji mRFT z poziomem redukcji mQ.

Przeprowadzona w mitochondriach *A. castellanii* szczegółowa analiza kinetyczna po raz pierwszy pokazała, że produkcja mRFT jest bezpośrednią funkcją poziomu redukcji mQ. Zwiększeniu poziomu redukcji mQ towarzyszy większa produkcja mRFT i odwrotnie. Zależność tworzenia mRFT od poziomu redukcji mQ jest różna dla dwóch dróg utleniających mQH_2, tj. drogi cytochromowej (kompleks III i IV) oraz oksydazy alternatywnej. Zaproponowano, że poziom redukcji endogennej puli mQ może być użytecznym wskaźnikiem pozwalającym oszacować całkowity poziom produkcji RFT w mitochondriach.

Następnie badano wpływ treningu wytrzymałościowego na produkcję mRFT pod kątem tkankowej i mitochondrialnej puli Q, zwłaszcza QH_2. Badając wpływ treningu wytrzymałościowego w płucach szczura wykazano odwrotną regulację Q na poziomie tkankowym i mitochondrialnym. Zwiększeniu poziomu Q jako przeciwutleniacza w komórkach płuc towarzyszył spadek mQ jako nośnika elektronów w łańcuchu oddechowym mitochondriów, prowadząc (wraz z innymi zmianami na poziomie łańcucha oddechowego) do zmniejszenia produkcji mRFT.

Po raz pierwszy badano zmianę w produkcji mRFT, która następuje w wyniku przejścia z warunków niefosforylujących do warunków fosforylujących w mitochondriach różnych tkanek szczura o różnej zawartości zredukowanego mQ. Zmiana ta, obrazowana przez zaproponowany przez nas nowy parametr, tj. kontrolę oddechową produkcji RFT (KO_{RFT}), pokazuje zakres zmian produkcji mRFT podczas funkcjonowaniu mitochondriów, gdy łańcuch oddechowy nie jest hamowany. Różnice obserwowane w tkankach szczurów i ich mitochondriach w wielkości puli zredukowanego Q, w tym mQ, odzwierciedlają różne poziomy produkcji mRFT, a zatem mogą wskazywać różne zapotrzebowanie na zredukowany Q jako przeciwutleniacz.

Badając wpływ treningu wytrzymałościowego na zawartość Q oraz tworzenie mRFT w tkankach szczura o dużym zapotrzebowaniu na energię, tj. w sercu, wątrobie i mózgu, stwierdzono, że trening może indukować różną odpowiedź tkankową i mitochondrialną związaną z Q działającym jako przeciwutleniacz i nośnik elektronów w łańcuchu oddechowym. Obserwowane po treningu zmiany w produkcji mRFT w mitochondriach poszczególnych tkanek mogą być związane ze zmianami w aktywności i ilości poszczególnych komponentów systemu fosforylacji oksydacyjnej oraz jego molekularnej organizacji, jak również z wielkością puli utlenionego mQ, działającego jako nośnik elektronów w łańcuchu oddechowym.

Wyniki pracy doktorskiej podkreślają istotną rolę Q jako nośnika elektronów w mitochondrialnym łańcuchu oddechowym oraz jako komórkowego przeciwutleniacza. Poziom produkcji mRFT zależy od szeregu wzajemnie powiązanych czynników, które wpływają na poziom redukcji mQ, m. in. od ilości Q w mitochondriach i tkankach, aktywności łańcucha oddechowego (w tym szlaków utleniających mQH_2 i redukujących mQ) a także poziomu stresu oksydacyjnego. Homeostaza redoks mQ jest kluczowym czynnikiem w modulowaniu produkcji mRFT.

2. ABSTRACT

Coenzyme Q (Q) is a key electron carrier in the mitochondrial respiratory chain and an important antioxidant against oxidative damage, present in all cell membranes. On the one hand, reduced Q (ubiquinol, QH_2) prevents the formation of free radicals, on the other hand, incomplete reduction of Q leads to the formation of a semiquinone radical, which is the source of reactive oxygen species (ROS) formation in the respiratory chain, resulting in oxidative stress and oxidative damage.

The aim of this dissertation was to investigate the relationship between the production of mitochondrial ROS (mROS) and the reduction level of mitochondrial Q (mQ) under phosphorylating (during ATP synthesis) and nonphosphorylating conditions. In this study, we used mitochondria isolated from the unicellular organism, amoeba *Acanthamoeba castellanii* as well as various rat tissues, i.e., lung, brain, heart and liver, and mitochondria isolated from them. The use of various organisms, various animal tissues and the study of the impact of endurance training at the tissue and mitochondrial levels allowed for a broad, multi-level analysis of the relationship between mROS production and the mQ reduction level.

A detailed kinetic analysis in *A. castellanii* mitochondria showed for the first time that mROS production is a direct function of the mQ reduction level. Increasing the level of mQ reduction is accompanied by greater production of mROS and vice versa. The dependence of mROR formation on the mQ reduction level is different for two mQH_2-oxidizing pathways, i.e., the cytochrome pathway (complex III and IV) and the alternative oxidase pathway. It has been proposed that the reduction level of the endogenous mQ pool may be a useful reporter to estimate the total level of ROS production in mitochondria.

The effect of endurance training on mROS production was then investigated in terms of tissue and mitochondrial Q pools, especially QH_2. When examining the effect of endurance training in rat lung, Q was shown to be inversely regulated at the tissue and mitochondrial levels. The increase in the level of Q as an antioxidant in lung cells was accompanied by a decrease in mQ as an electron carrier in the mitochondrial respiratory chain, leading (along with other changes at the level of the respiratory chain) to a decrease in mROS production.

For the first time, the change in mROS production, which occurs as a result of a transition from nonphosphorylating conditions to phosphorylating conditions in the mitochondria of various rat tissues with different content of reduced mQ, was investigated. This change, illustrated by proposed new parameter, i.e., respiratory control of ROS production

(RCR_{ROS}), shows the extent of changes in mROS production during mitochondrial function when the respiratory chain is not inhibited. The differences observed in rat tissues and their mitochondria in reduced Q pool size, including mQ, reflect different levels of mROS production and may therefore indicate a different need for reduced Q as an antioxidant.

By examining the effect of endurance training on Q content and the formation of mROS in rat tissues with high energy requirements, i.e., in the heart, liver and brain, it was found that training can induce a different tissue and mitochondrial response associated with Q acting as an antioxidant and an electron carrier in the chain respiratory. The changes in mROS production in the mitochondria of individual tissues observed after training may be related to changes in the activity and amount of individual components of the oxidative phosphorylation system and its molecular organization, as well as the size of the oxidized mQ pool, acting as an electron carrier in the respiratory chain.

The results of the presented dissertation emphasize the important role of Q as an electron carrier in the mitochondrial respiratory chain and as a cellular antioxidant. The level of mROS production depends on a number of interrelated factors that affect the level of mQ reduction, including the amount of Q in mitochondria and tissues, the activity of the respiratory chain (including mQH_2-oxidizing and mQ-reducing pathways), and the level of oxidative stress. Redox homeostasis of mQ is a key factor in modulating mROS production.

3. WYKAZ PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

Publikacja 1:

Dominiak K*, Koziel A*, Jarmuszkiewicz W (2018) The interplay between mitochondrial reactive oxygen species formation and the coenzyme Q reduction level. *Redox Biology* 18, 256-265 (IF_{5y} 12,038)

Publikacja 2:

Jarmuszkiewicz W, **Dominiak K***, Galganski L*, Galganska H, Kicinska A, Majerczak J, Zoladz JA (2020) Lung mitochondria adaptation to endurance training in rats. *Free Radical Biology Medicine* 161, 163-174 (IF_{5y} 7,934)

Publikacja 3:

Dominiak K, Jarmuszkiewicz W (2021) The relationship between mitochondrial reactive oxygen species production and mitochondrial energetics in rat tissues with different contents of reduced coenzyme Q. *Antioxidants*, 10, 533. doi.org/10.3390/antiox10040533 (IF_{5y} 6,649)

Publikacja 4:

Dominiak K*, Galganski L*, Budzinska A, Woyda-Ploszczyca A, Zoladz JA, Jarmuszkiewicz W (2022) Effects of endurance training on the coenzyme Q redox state in rat heart, liver, and brain at the tissue and mitochondrial levels: implications for reactive oxygen species formation and respiratory chain remodeling. *International Journal of Molecular Sciences* 23 (2), 896; doi.org/10.3390/ijms23020896 (IF_{5y} 6,132)

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4. WPROWADZENIE I CELE PRACY

Koenzym Q (Q) jest rozpuszczalną w tłuszczach cząsteczką obecną we wszystkich błonach komórki, głównie w wewnętrznej błonie mitochondrialnej (Dominiak i Jarmuszkiewicz 2019)[#]. Zredukowana forma Q (ubichinol, QH_2) jest ważnym błonowym przeciwutleniaczem w komórce, który hamuje peroksydację lipidów i zapobiega oksydacyjnym modyfikacjom DNA i białek, powodowanym przez wolne rodniki, w tym reaktywne formy tlenu (RFT). QH_2 regeneruje inne przeciwutleniacze w komórce, takie jak witamina E czy witamina C, oraz działa bezpośrednio na wolne rodniki lub utleniacze, w tym RFT, redukując je i neutralizując. Rośnie zainteresowanie zastosowaniem Q i jego związków pokrewnych w leczeniu zaburzeń, w których dochodzi do uszkodzeń oksydacyjnych.

Mitochondrialny Q (mQ) jest kluczowym niebiałkowym nośnikiem elektronów łańcucha oddechowego, który uczestniczy w produkcji ATP w mitochondriach (Dominiak i Jarmuszkiewicz 2019)[#]. Niepełna redukcja mQ prowadzi do powstania rodnika semichinonowego ($QH^{\bullet}$), który wchodząc w reakcję z tlenem tworzy anionorodnik ponadtlenkowy ($O_2^{\bullet-}$), będący prekursorem innych RFT. Zatem, mQ jako nośnik elektronów w łańcuchu oddechowym jest zaangażowany w produkcję mitochondrialnych RFT (mRFT), które powstają jako produkt uboczny metabolizmu tlenowego lub w warunkach stresu oksydacyjnego. Wzmożona produkcja mRFT może prowadzić do uszkodzeń oksydacyjnych, które leżą u podstaw starzenia się komórek czy szeregu chorób. Oprócz tego, mRFT pełnią rolę cząsteczek sygnałowych. Za główne miejsca produkcji mRFT uważane są nośniki elektronów mitochondrialnego łańcucha oddechowego, przede wszystkim kompleksy białkowe łańcucha związane z mQ (kompleksy I i III). Biorąc pod uwagę inne mitochondrialne dehydrogenazy, mQ jest bezpośrednio zaangażowany w tworzenie mRFT w czterech miejscach wiążących mQ, tj. w trzech miejscach redukujących mQ [kompleksu I (I_Q), mitochondrialnej dehydrogenazy glicerolo-3-fosforanu (G_Q) i dehydrogenazy dihydroorotanowej (D_Q)] oraz w jednym miejscu utleniania mQH_2 kompleksu III (III_{Qo}). Zmiany w poziomie mQ mogą wpływać na produkcję mRFT a także na aktywność łańcucha oddechowego i wydajność procesu fosforylacji oksydacyjnej (OXPHOS), co może skutkować chorobami mitochondrialnymi, sercowo-naczyniowymi, neurodegeneracyjnymi czy miopatiami.

[#]Dominiak K, Jarmuszkiewicz W (2019) Różne oblicza mitochondrialnego koenzymu Q (Different faces of the mitochondrial coenzyme Q). *Postępy Biochemii* (*Advances in Biochemistry*) 65(4) 271-277.

Celem niniejszej rozprawy doktorskiej było zbadanie powiązania produkcji RFT ze stopniem redukcji Q w mitochondriach w warunkach fosforylujących (podczas syntezy ATP) i niefosforylujacych (przy braku syntezy ATP). Materiałem użytym w pracy były mitochondria izolowane z komórek organizmu jednokomórkowego, ameby *Acanthamoeba castellanii* (Publikacja 1) a także różne tkanki szczura, tj. płuca, mózg, serce i wątroba, oraz izolowane z nich mitochondria (Publikacje 2-4).

W ramach pracy realizowano następujące zadania badawcze:

1) zbadanie powiązania pomiędzy produkcją mRFT a poziomem redukcji puli mQ w mitochondriach ameby *A. castellani* przy różnym poziomie zaangażowania drogi redukującej mQ (kompleksu II) oraz dróg utleniających mQH_2, tj. drogi cytochromowej (kompleksu III i IV) oraz oksydazy alternatywnej (AOX) (Publikacja 1),

2) zbadanie adaptacji mitochondriów płuc szczura do treningu wytrzymałościowego, ze szczególnym uwzględnieniem zmian w poziomie mQ i produkcji mRFT (Publikacja 2),

3) zbadanie powiązania pomiędzy wytwarzaniem mRFT a aktywnością bioenergetyczną mitochondriów w tkankach szczura o różnej zawartości zredukowanego Q, tj. w płucach, mózgu, wątrobie i sercu (Publikacja 3),

4) zbadanie wpływ treningu wytrzymałościowego na stan redoks Q w sercu, wątrobie i mózgu szczura na poziomie tkankowym i mitochondrialnym w powiązaniu z produkcją RFT (Publikacja 4).

5. PODSUMOWANIE WYNIKÓW

Publikacja 1:

Dominiak K, Koziel A, Jarmuszkiewicz W (2018) The interplay between mitochondrial reactive oxygen species formation and the coenzyme Q reduction level. *Redox Biology* 18, 256-265 (IF_{5y} 12,038)

W pracy tej badano powiązanie pomiędzy szybkością produkcji H_2O_2 a poziomem redukcji mQ (mQ9) w mitochondriach ameby *A. castellanii* przy różnym poziomie zaangażowania drogi redukującej mQ (dehydrogenazy bursztynianowej, kompleksu II) oraz dróg utleniających mQH_2, tj. drogi cytochromowej (kompleks III i IV) oraz AOX. Poziom redukcji mQ (mQH_2/mQtot) był zwiększany poprzez stopniowe hamowanie aktywności dróg utleniających mQH_2 przy użyciu inhibitorów kompleksu III (antymycyny A), kompleksu IV (cyjanku), AOX (kwasu benzohydroksamowego, BHAM) lub OXPHOS tj. syntazy ATP (oligomycyny) i translokazy ATP/ADP (karboksyatraktylozydu, CATR). Z kolei poziomu redukcji mQ był obniżany w wyniku stopniowego hamowania przepływu elektronów przez kompleks II przy użyciu malonianu (hamowanie drogi redukującej mQ) lub stopniową stymulację aktywności dróg utleniających mQH_2 w warunkach rozprzęgających w obecności karbonylocyjanku-p-trifluorometoksyfenylohydrazonu (FCCP) (aktywacja drogi cytochromowej) lub podczas aktywacji AOX przez GMP. Mierzono poziom redukcji mQ (mQH_2/mQtot) i szybkość tworzenia H_2O_2 w określonych warunkach zużycia tlenu i potencjału błonowego ($m\Delta\Psi$) mitochondriów *A. castellanii*. W przypadku badania zaangażowania drogi cytochromowej w produkcję mRFT, otrzymane wyniki były zestawiane jako zależność zużycia tlenu, $m\Delta\Psi$ lub szybkości produkcji H_2O_2 od stopnia redukcji mQ oraz jako zależność zużycia tlenu i szybkości produkcji H_2O_2 od $m\Delta\Psi$. W przypadku badania zaangażowania AOX w produkcję mRFT otrzymane wyniki były zestawiane jako zależność zużycia tlenu lub szybkości produkcji H_2O_2 tylko od stopnia redukcji mQ, gdyż pomiary prowadzono w obecności inhibitora drogi cytochromowej, przy braku $m\Delta\Psi$.

Przeprowadzona analiza kinetyczna wskazuje na bezpośrednią zależność tworzenia mRFT od poziomu redukcji puli mQ w przypadku obu dróg utleniających mQH_2, dla wszystkich badanych warunków energetycznych. Zwiększeniu poziomu redukcji mQ towarzyszyła większa produkcja mRFT, natomiast obniżeniu poziomu redukcji mQ towarzyszyła mniejsza produkcja mRFT. Przy braku modulatorów aktywności łańcucha oddechowego i OXPHOS, w warunkach fosforylujacych (w obecności ADP, stan 3)

obserwowano znacznie większą produkcję mRFT i znacznie większy poziom redukcji mQ (oraz większy mΔΨ) niż w warunkach niefosforylujących (stan 4).

W mitochondriach ameby, w przypadku drogi cytochromowej produkcja mRFT zależy nieliniowo od poziomu redukcji mQ, przy czym zależność ta jest silniejsza przy wartościach poziomu redukcji mQ wyższych od wartości obserwowanych dla stanu fosforylującego (stanu 3) (powyżej ~35%). Droga cytochromowa jest zaangażowana już od ~10% redukcji mQ, ale produkcja mRFT silniej zależy od poziomu redukcji mQ w zakresie 40-80% redukcji mQ. Powyżej wartości progowej (~35% redukcji mQ), niewielkie zwiększenie zredukowanej puli mQ prowadzi do dużego zwiększenia wytwarzania mRFT. Poniżej wartości progowej, zależność pomiędzy produkcją mRFT a poziomem redukcji mQ pokrywa się dla stanu fosforylującego (stanu 3) oraz stanu niefosforylującego (stanu 4) i jest nieliniowa.

Należy podkreślić, że przeprowadzona analiza kinetyczna wykazała, że produkcja mRFT jest bezpośrednią funkcją poziomu redukcji mQ ale nie mΔΨ, jak się powszechnie uważa. Zależność pomiędzy produkcją H_2O_2 a mΔΨ uzyskana podczas miareczkowania drogi cytochromowej przy użyciu antymycyny A i/lub cyjanku, gdy zmniejszeniu mΔΨ towarzyszy zwiększenie poziomu redukcji mQ, nie pokrywa się z zależnością otrzymaną dla pozostałych warunków, w których niższym wartościom mΔΨ towarzyszą niższe poziomy redukcji mQ. Wykazano więc, że mΔΨ nie jest bezpośrednią funkcją poziomu redukcji mQ. Nie zawsze obniżenie poziomu redukcji mQ oznacza obniżenie mΔΨ.

Badając zależność szybkości produkcji mRFT od stopnia redukcji mQ przy różnym zaangażowaniu AOX wykazano, że AOX jest aktywna dopiero przy większym poziomie redukcji puli mQ (powyżej 40%), gdy wzrasta produkcja mRFT przez drogę cytochromową. Zależność produkcji mRFT od stopnia redukcji mQ przy zaangażowaniu AOX jest liniowa i obejmuje zakres poziomu redukcji mQ pomiędzy 40-80%. Aktywacja AOX przez GMP istotnie zmniejsza poziom redukcji mQ i tworzenie mRFT.

Ponadto wykazano nieliniową zależność szybkości produkcji mRFT od stopnia redukcji mQ przy różnym zaangażowaniu drogi redukującej mQ (kompleksu II), przy czym punkty pomiarowe otrzymane dla stanu 3 pokrywały się z punktami pomiarowymi otrzymanymi dla stanu 4 przy niższych wartościach poziomu redukcji mQ.

Podsumowując, na przykładzie mitochondriów izolowanych z komórek ameby *A. castellanii* po raz pierwszy dokonano kinetycznej analizy zależności produkcji mRFT od poziomu redukcji endogennej puli mQ, miareczkowanej przy użyciu modulatorów

mitochondrialnego łańcucha oddechowego i OXPHOS. Nasze wyniki wskazują, że produkcja mRFT jest bezpośrednią funkcją poziomu redukcji mQ a nie mΔΨ. Produkcja RFT w mitochondriach zależy nie tylko od zaangażowania miejsc produkcji mRFT ale także od zaangażowania dróg utleniających mQH_2. Produkcja mRFT przy zaangażowaniu drogi cytochromowej (kompleks III i IV) jest silniej zależna od poziomu redukcji mQ niż w przypadku aktywności AOX. Proponujemy, że poziom redukcji endogennej puli mQ może być użytecznym wskaźnikiem, który pozwala oszacować całkowity poziom produkcji RFT w mitochondriach.

Publikacja 2:

Jarmuszkiewicz W, **Dominiak K**, Galganski L, Galganska H, Kicinska A, Majerczak J, Zoladz JA (2020) Lung mitochondria adaptation to endurance training in rats. *Free Radical Biology Medicine* 161, 163-174 (IF_{5y} 7,934)

Celem pracy było zbadanie wpływu 8-tygodniowego treningu wytrzymałościowego na metabolizm tlenowy, w tym biogenezę mitochondriów, aktywność oddechową i ilość Q w płucach szczurów. Ponadto badano zmiany wywołane treningiem wytrzymałościowym na poziomie izolowanych mitochondriów płuc poprzez pomiar aktywności oddechowej mitochondriów, $m\Delta\Psi$, wydajności OXPHOS, rozprzęgania, produkcji mRFT, ilości i poziomu redukcji mQ oraz analizę molekularnej organizacji superkompleksów i kompleksów systemu OXPHOS.

Wykazano, że w płucach szczurów trening wytrzymałościowy wywołuje zwiększoną pojemność oddechową mitochondriów (zwiększoną aktywność oksydazy cytochromu *c* i syntazy cytrynianowej) oraz zwiększoną biogenezę mitochondriów (zwiększony poziom białek markerowych), podczas gdy aktywność dehydrogenazy mleczanowej ulega zmniejszeniu. Obserwacje te wskazują na zwiększony udział oddychania tlenowego w oddychaniu komórkowym płuc trenowanych zwierząt. Ponadto trening wytrzymałościowy poprawia wydolność mitochondrialnego systemu energetycznego a tym samym korzystnie wpływa na radzenie sobie z dodatkowym zapotrzebowaniem na energię wywołanym intensywnym wysiłkiem fizycznym.

Ponadto wykazano, że trening wytrzymałościowy zwiększa systemy antyoksydacyjne (ilość Q9 i Q10 oraz poziom dysmutazy ponadtlenkowej, SOD) na poziomie tkanki płucnej, ale zmniejsza je (i dodatkowo poziom białka rozprzęgającego 2, UCP2) na poziomie mitochondriów płuc. Zwiększone zapotrzebowanie na systemy antyoksydacyjne (w tym Q) na poziomie tkankowym może wynikać ze zwiększonej ilości i aktywności mitochondriów w płucach trenowanych szczurów.

W mitochondriach płuc trenowanych szczurów obserwowano obniżoną ilość mQ (mQ9 i mQ10), zmniejszoną aktywność kompleksu I oraz zwiększoną aktywność drogi cytochromowej (kompleksu III i IV). Zmiany te mogą prowadzić do obniżonego poziomu redukcji mQ, co z kolei wpływa na zmniejszoną produkcję H_2O_2 obserwowaną w mitochondriach trenowanych szczurów zarówno podczas utleniania bursztynianiu (substratu kompleksu II), jak i jabłczanu (substratu kompleksu I), w warunkach fosforylujących (stan 3) i

niefosforylujacych (stan 4). Porównując utlenianie tych substratów oddechowych w mitochondriach płuc trenowanych i nietrenowanych szczurów w obu stanach energetycznych, obserwowano zwiększone utlenianie bursztynianu przy zwiększonym mΔΨ stanu 4 oraz obniżone utlenianie jabłczanu przy zmniejszonym mΔΨ stanu 4. Zmianom tym towarzyszył zwiększony poziom kompleksu II i III oraz zmniejszony poziom kompleksu I w łańcuchu oddechowym mitochondriów płuc trenowanych szczurów. Na poziomie molekularnej organizacji superkompleksów systemu OXPHOS obserwowano obniżony poziom kompleksu I we wszystkich jego superkompleksach oraz podwyższony poziom kompleksu III w superkompleksie dimeru kompleksu III i kompleksu IV (III_2+IV). Pomimo zaobserwowanej rearanżacji na poziomie łańcucha oddechowego, wydajność OXPHOS (wyrażana parametrami sprzężenia mitochondriów, kontrolą oddechową oraz stosunkiem ADP/O) podczas utleniania poszczególnych substratów oddechowych nie uległa zmianie.

W mitochondriach płuc szczura, potwierdzono wykazaną wcześniej dla mitochondriów ameby *A. castellanii* (Publikacja 1), bezpośrednią zależność produkcji mRFT od stopnia redukcji mQ w warunkach fosforylujacych i niefosforylujących. Analiza kinetyczna wskazuje, że w mitochondriach płuc trenowanych szczurów, podczas utleniania poszczególnych substratów oddechowych, zmniejszony poziom redukcji mQ towarzyszy zmniejszonej produkcji mRFT.

W płucach trenowanych szczurów obserwowano zwiększony poziom ekspresji czujników tlenu, czynnika indukowanego hipoksją 1α (HIF1α) (marker niedotlenienia i stresu oksydacyjnego) i lizyno-specyficznej demetylazy 6A (KDM6A) (komórkowy czujnik tlenu regulujący chromatynę). Obserwacje te wskazują na niedotlenienie w tkance płucnej towarzyszące intensywnemu treningowi wytrzmałościowemu. Również zmiany na poziomie mitochondriów płuc trenowanych szczurów, tj. mniejsza aktywność kompleksu I, zwiększona aktywność kompleksu I, zwiększone utlenianie glutaminianu oraz obniżony poziom mQ, wskazują na niedotlenienie wywołane intensywnym treningiem. Tak więc wydaje się, że trening wytrzymałościowy powoduje stres oksydacyjny w płucach szczurów, prowadzący do adaptacji metabolicznej obejmującej odpowiedź mitochondrialną, w tym zmiany w ilości i poziomie redukcji mQ.

Podsumowując, w płucach szczura obserwowano odwrotną regulację Q wywołaną treningiem wytrzymałościowym na poziomie tkankowym i mitochondrialnym. Zwiększeniu poziomu Q jako przeciwutleniacza w komórkach płuc towarzyszył spadek mQ jako nośnika elektronów w łańcuchu oddechowym ich mitochondriów prowadząc (wraz z innymi zmianami

na poziomie łańcucha oddechowego) do zmniejszenia produkcji mRFT. Obserwacje te wskazują na istotną rolę Q w komórkach płuc, który jest nie tylko zaangażowany w produkcję RFT w mitochondriach, ale jest także ważnym błonowym przeciwutleniaczem komórkowym. Nasze wyniki sugerują, że mRFT i Q mogą odgrywać istotną rolę regulacyjną w utrzymaniu homeostazy oksydoredukcyjnej płuc oraz w adaptacji płuc do treningu wytrzymałościowego, obejmującej zwiększenie biogenezy mitochondriów. Oba czynniki mogą poprawić funkcjonowanie płuc i ich mitochondriów podczas wysiłku fizycznego.

Publikacja 3:

Dominiak K, Jarmuszkiewicz W (2021) The relationship between mitochondrial reactive oxygen species production and mitochondrial energetics in rat tissues with different contents of reduced coenzyme Q. *Antioxidants*, 10, 533. doi.org/10.3390/antiox10040533 (IF_{5y} 6,649)

Następnie przeprowadziliśmy badania mające na celu zbadanie wytwarzania mRFT w różnych tkankach szczurów, tj. w sercu i wątrobie (tkanki o dużej zawartości Q) oraz w mózgu i płucach (tkanki o małej zawartości Q), w warunkach niefosforylujących (przy braku ADP) i w warunkach fosforylujących z aktywną OXPHOS (w obecności ADP). Badaliśmy produkcję mRFT pod kątem zawartości komórkowej (tkankowej) puli Q (Q9 i Q10) i mitochondrialnej puli Q (mQ9 i mQ10), zwłaszcza pul zredukowanych Q ($Q9H_2$ i $Q10H_2$).

Na początku, aby poznać zapotrzebowanie na Q jako przeciwutleniacz, zbadaliśmy zawartość zredukowanej puli Q ($Q9H_2$ i $Q10H_2$) na poziomie tkankowym i mitochondrialnym w warunkach utleniających (przy braku substratów redukujących mQ). Podobnie jak całkowita pula Q, zawartość zredukowanej puli Q była różna w badanych tkankach. Największą redukcję obu homologów Q, Q9 i Q10, obserwowaliśmy w wątrobie zarówno na poziomie tkankowym (~90%), jak i mitochondrialnym (Q10 ~90% i Q9 ~50%). W sercu poziom redukcji obu homologów Q był podobny i wynosił ~25-29% w tkankach i ~15-19% w mitochondriach. Co ciekawe, w płucach prawie całą pulę zredukowanego Q stanowił Q10, zarówno na poziomie tkankowym i mitochondrialnym (100% redukcji). Najniższy poziom redukcji obu homologów Q zaobserwowano w mózgu (brak detekcji w tkankach, ~10% redukcji w mitochondriach). A zatem, największe zapotrzebowanie na zredukowaną pulę Q obserwowano w wątrobie a najmniejsze w mózgu.

Poziom produkcji H_2O_2 w homogenatach badanych tkanek i izolowanych z nich mitochondriów w warunkach aktywacji OXPHOS (w obecności substratów oddechowych kompleksu I i II oraz ADP) był podobny i niezależny od wielkości puli zredukowanego Q. Natomiast w mitochondriach, podobnie jak w homogenatach tkankowych, w warunkach niefosforylujących (w obecności substratów oddechowych, bez ADP), produkcja mRFT była istotnie zależna od wielkości puli zredukowanego Q ($Q9H_2$ i $Q10H_2$). Mianowicie, w mitochondriach mózgu i płuc, mała produkcja H_2O_2 była powiązana z małą pulą zredukowanego mQ, podczas gdy w mitochondriach wątroby i serca wyższa produkcja H_2O_2 odpowiadała większej puli zredukowanego mQ. Uzyskane wyniki pozwalają stwierdzić, że tkanki szczurów, które mogą wytwarzać więcej mRFT mają większe całkowite i zredukowane pule Q (Q9 i Q10), a wytwarzanie RFT wydaje się proporcjonalne do puli zredukowanego Q.

Zaobserwowane różnice wskazują na różne zapotrzebowanie na pulę zredukowanego Q jako przeciwutleniacza, zarówno na poziomie tkankowym, jak i mitochondrialnym.

Ponadto w mitochondriach badanych tkanek szczura porównywano poziom produkcji mRFT podczas utleniania substratów oddechowych kompleksu I (jabłczan + glutaminian), kompleksu II (bursztynian) oraz ich mieszaniny w warunkach fosforylujących (stan 3) i niefosforylujących (stan 4). Dla wszystkich typów badanych mitochondriów i kombinacji substratów oddechowych, produkcja H_2O_2 w stanie 3 była znacznie niższa niż w stanie 4, podobnie jak to wcześniej obserwowaliśmy w mitochondriach *A. castellanii* (Publikacja 1) i mitochondriach płuc (Publikacja 2). Zaproponowaliśmy zatem stosowanie nowego parametru do badania izolowanych mitochondriów, tzn. kontrolę oddechową produkcji RFT (KO_{RFT}), będącą stosunkiem tworzenia mRFT w warunkach niefosforylujących (w stanie 4) i fosforylujących (w stanie 3). Parametr ten obrazuje maksymalne zwiększenie mitochondrialnej produkcji RFT osiągane po ufosforylowaniu ADP. W przypadku badanych mitochondriów, najwyższe wartości KO_{RFT} obserwowano w mitochondriach serca podczas utleniania bursztynianu (~5,5) i mieszaniny substratów kompleksów I i II (~4,5). W przypadku utleniania jabłczanu plus glutaminianu, największą wartość KO_{RFT} obserwowano w mitochondriach płuc (~3,2).

W warunkach niefosforylujących, niezależnie od substratu oddechowego, uwalnianie H_2O_2 było największe w mitochondriach wątroby, natomiast najmniejsze w mitochondriach mózgu. W warunkach fosforylujących produkcja H_2O_2 była najwyższa w mitochondriach serca w obecności bursztynianu oraz jego mieszaniny z jabłczanem i glutaminianem, natomiast najmniejsza w mitochondriach mózgu niezależnie od utlenianego substratu. Uzyskane wyniki wskazują także, że dla wszystkich typów badanych mitochondriów, uwalnianie H_2O_2 przy zaangażowaniu obu kompleksów oddechowych (kompleksu I i II), niezależnie od stanu oddechowego, nie przekracza uwalniania H_2O_2 przy zaangażowaniu jednego najlepszego wejścia elektronów na łańcuch oddechowy (podczas utleniania najlepszego substratu oddechowego). Świadczy to o istnieniu maksymalnej pojemności łańcucha oddechowego w produkcji mRFT, czyli górnej granicy dla stanu 4 i dolnej granicy dla stanu 3. Ponadto, pomiar $m\Delta\Psi$ podczas utleniania różnych substratów oddechowych w badanych mitochondriach szczura wskazuje, że maksymalna pojemność łańcucha oddechowego w produkcji mRFT może być powiązana z kontrolą OXPHOS za pośrednictwem $m\Delta\Psi$.

Podsumowując, po raz pierwszy badaliśmy zmianę w produkcji mRFT, która następuje w wyniku przejścia mitochondriów z warunków niefosforylujących do warunków fosforylujących w mitochondriach różnych tkanek szczura o różnej zawartości zredukowanego mQ. Zmiana ta, obrazowana przez nowy parametr (KO_{RFT}), pokazuje zakres produkcji mROS podczas funkcjonowaniu mitochondriów, gdy łańcuch oddechowy nie jest hamowany. Porównując różne tkanki stwierdziliśmy, że produkcja mRFT w warunkach niefosforylujących była silnie zależna od ilości zredukowanego mQ. Różnice obserwowane w tkankach szczurów i ich mitochondriach w wielkości puli zredukowanego Q, w tym mQ, odzwierciedlają różne poziomy produkcji mRFT, a zatem mogą wskazywać różne zapotrzebowanie na zredukowany Q jako przeciwutleniacz.

Publikacja 4:

Dominiak K, Galganski L, Budzinska A, Woyda-Ploszczyca A, Zoladz JA, Jarmuszkiewicz W (2022) Effects of endurance training on the coenzyme Q redox state in rat heart, liver, and brain at the tissue and mitochondrial levels: implications for reactive oxygen species formation and respiratory chain remodeling. *International Journal of Molecular Sciences* 23 (2), 896; doi.org/10.3390/ijms23020896 (IF_{5y} 6,132)

Celem ostatniej części pracy doktorskiej było wyjaśnienie wpływu 8-tygodniowego treningu wytrzymałościowego na zawartość Q oraz tworzenie RFT w tkankach szczura o dużym zapotrzebowaniu na energię, tj. w sercu, wątrobie i mózgu. Badano produkcję RFT oraz zmiany wielkości puli Q (Q9 i Q10) zredukowanej i utlenionej na poziomie tkankowym (w homogenatach tkanek) oraz izolowanych mitochondriów. Dodatkowo badano zmiany wywołane treningiem wytrzymałościowym w biogenezie mitochondriów oraz na poziomie komponentów systemu OXPHOS i enzymów antyoksydacyjnych w izolowanych mitochondriach badanych tkanek szczura.

Obserwowane zwiększenie poziomu białek mitochondrialnych (kanału anionowego zależnego od napięcia, VDAC1 oraz syntazy cytrynianowej) oraz białka markerowego biogenezy mitochondriów (PGC1α), wskazuje, że trening wytrzymałościowy zwiększa biogenezę mitochondriów we wszystkich badanych tkankach. Zmianom tym towarzyszył wzrost całkowitej, utlenionej i zredukowanej puli Q9 i Q10 we wszystkich badanych tkankach. W przypadku serca i mózgu trenowanych szczurów obserwowano wzrost stanu redoks Q (QH_2/Q) (odpowiednio o ~35% i ~28%), co wskazuje na podwyższony poziom QH_2 działającego jako komórkowy przeciwutleniacz. Ponadto, trening wytrzymałościowy zwiększył pulę mQH_2 w mitochondriach serca i wątroby, ale nie mózgu.

Pomiary RFT na poziomie tkankowym wykazały zwiększenie uwalniania H_2O_2 jedynie w sercu trenowanych szczurów w warunkach inaktywacji OXPHOS. Obserwowany w sercach trenowanych szczurów obniżony poziom SOD1 wskazuje na wzrost produkcji RFT do bezpiecznego poziomu. Z kolei podwyższony poziom SOD1 w mózgu trenowanych szczurów może pomagać w obniżaniu poziomu RFT.

Wyniki pomiarów mRFT potwierdzają obserwację, że serca trenowanych szczurów mogą mieć zwiększone zapotrzebowanie na QH_2, czyli przeciwutleniacz. Mianowicie, trening wytrzymałościowy zwiększał produkcję mRFT w mitochondriach serca podczas utleniania bursztynianu w warunkach niefosforylujących, podczas gdy utlenianie słabszych substratów (substratów kompleksu I) prowadziło do obniżonej produkcji mRFT. W mitochondriach

wątroby trenowanych szczurów obserwowano ogólny spadek produkcji mRFT, natomiast w mitochondriach mózgu nie obserwowano zmian w produkcji mRFT.

Obserwowane po treningu zmiany w produkcji mRFT w mitochondriach poszczególnych tkanek mogą być związane ze zmianami, które zaobserwowaliśmy w aktywności i ilości poszczególnych elementów systemu OXPHOS oraz jego molekularnej organizacji, jak również z wielkością puli utlenionego mQ, działającego jako nośnik elektronów w łańcuchu oddechowym.

W mitochondriach serca trenowanych szczurów analiza Western blot wykazała istotny statystycznie wzrost poziomu ekspresji wszystkich kompleksów OXPHOS, z wyjątkiem kompleksu IV. Dodatkowo, analiza Blue-Native PAGE (BN-PAGE) i testy aktywności w żelu wykazały wzrost aktywności kompleksu II, kompleksu V i wszystkich superkompleksów kompleksu I. Obserwowano także zwiększony poziom wszystkich superkompleksów kompleksu III (oprócz superkompleksu III_2+IV). Zmiany te wraz ze zwiększoną pulą utlenionego mQ, wskazują na zwiększony poziom OXPHOS w mitochondriach serca trenowanych szczurów. Zmiany te mogą prowadzić do obserwowanej zmniejszonej produkcji mRFT podczas utleniania substratów kompleksu I.

W mitochondriach wątroby trenowanych szczurów obserwowano obniżenie poziomu białka i aktywności dehydrogenaz (kompleksu I i II) oraz kompleksu V (syntazy ATP) a także zwiększenie poziomu kompleksu III we wszystkich jego superkompleksach (oprócz superkompleksu III_2+IV). Ponadto obserwowano podwyższony poziom UCP2. Wszystkie te zmiany wraz ze zwiększeniem puli zredukowanego mQ mogą prowadzić do obserwowanego ogólnego zmniejszenia produkcji mRFT.

W mitochondriach mózgu trenowanych szczurów nie obserwowano zmian w puli zredukowanego i utlenionego mQ, ani zwiększenia produkcji mRFT. Zwiększenie poziomu i/lub aktywności kompleksu II i superkompleksów kompleksu I i III (I+III_2+IV i I+III_2) może prowadzić do braku wzrostu produkcji mRFT i prawdopodobnie zwiększonej wydajności OXPHOS, ponieważ aktywność kompleksu V była zwiększona. Ponadto podwyższony poziom UCP2 po treningu wytrzymałościowym obserwowany w mitochondriach mózgu może obniżać poziom produkcji mRFT.

Podsumowując, otrzymane wyniki pokazują, że trening wytrzymałościowy może indukować różną odpowiedź tkankową i mitochondrialną związaną z Q działającym jako przeciwutleniacz i nośnik elektronów w łańcuchu oddechowym. Wyniki te podkreślają rolę puli

zredukowanego Q (QH_2) w obronie antyoksydacyjnej oraz rolę puli utlenionego mQ (redukowanej przez łańcuch oddechowy) w adaptacji systemu OXPHOS i modulacji produkcji mRFT w odpowiedzi na trening wytrzymałościowy. Zmiany wywołane przez trening na poziomie mitochondrialnej puli Q i produkcji mRFT mogą odgrywać istotną rolę w metabolizmie energetycznym, homeostazie redoks oraz wpływać na poziom stresu oksydacyjnego w komórce.

6. WNIOSKI

Po raz pierwszy wykazaliśmy istotną zależność pomiędzy produkcją mRFT a poziomem redukcji mQ. Zaproponowaliśmy stosowanie poziomu redukcji mQ jako endogennego markera, który umożliwia oszacowanie całkowitej produkcji mRFT w izolowanych mitochondriach. Zaproponowaliśmy także stosowanie nowego parametru do badania izolowanych mitochondriów, tzn. kontrolę oddechową produkcji RFT (KO_{RFT}), która obrazuje maksymalne zwiększenie produkcji mRFT osiągane po ufosforylowaniu ADP.

Wyniki pracy doktorskiej podkreślają istotną podwójną rolę Q jako nośnika elektronów w mitochondrialnym łańcuchu oddechowym oraz jako komórkowego przeciwutleniacza. Poziom produkcji mRFT zależy od szeregu wzajemnie powiązanych czynników, które wpływają na poziom redukcji mQ, m. in. od ilości Q w mitochondriach i tkankach, aktywności łańcucha oddechowego (w tym szlaków utleniających mQH_2 i redukujących mQ) a także poziomu stresu oksydacyjnego. Homeostaza redoks mQ jest kluczowym czynnikiem w modulowaniu produkcji mRFT. W zależności od aktywności szlaków utleniających mQH_2 i redukujących mQ, zmniejszona ilość mQ może prowadzić do zwiększonego poziomu redukcji mQ (zwiększenia stosunku mQH_2/mQtot), a tym samym do zwiększonej produkcji mRFT lub do obniżonego poziomu redukcji mQ (zmniejszenia stosunku mQH_2/mQtot), a zatem zmniejszonej produkcji mRFT.

Badania przeprowadzone na różnych tkankach szczura tj. serca, wątroby, mózgu i płuc wykazują znaczące różnice w wielkości puli Q, które odzwierciedlają różne poziomy produkcji mRFT, a także zapotrzebowanie na przeciwutleniacz, czyli pulę zredukowanego Q zarówno na poziomie tkankowym, jak i mitochondrialnym.

Badania wpływ treningu wytrzymałościowego w różnych tkankach szczura prowadzą do wniosku, że zmiana wielkości puli mQ i jej stanu redoks może być ważną, zależną od rodzaju tkanki, adaptacją fizjologiczną. Na przykład, w płucach trening wytrzymałościowy wywołuje zmiany adaptacyjne związane z poziomem Q (w tym mQ) i produkcją mRFT, które mogą być spowodowane niedotlenieniem i stresem oksydacyjnym. W przypadku płuc obserwowano odwrotną regulacją poziomu Q w odpowiedzi na trening na poziomie tkankowym (zwiększenie puli Q) i mitochondrialnym (obniżenie puli mQ). A zatem zwiększenie przeciwutleniacza w tkance, któremu towarzyszył spadek mQ jako nośnika elektronów w mitochondriach może prowadzić do obniżenia poziomu mRFT. W przypadku mitochondriów serca, trening wytrzymałościowy prowadzi do zwiększenia zarówno puli utlenionego mQ, jak i mQH_2,

wskazując na zwiększone zapotrzebowanie zarówno na obronę antyoksydacyjną i utrzymanie wydajnej pracy łańcucha oddechowego. Wydaje się zatem, że odpowiedź metaboliczna na zmienioną pulę mQ zależy od tego, czy ważniejsze jest utrzymanie wydajnej OXPHOS (za co odpowiedzialna jest redukowana przez łańcuch oddechowy pula mQ), czy też ważniejsza jest obrona przed stresem oksydacyjnym (za co odpowiedzialne są komórkowe i mitochondrialne pule QH_2. Analiza wpływu treningu wytrzymałościowego na stan redoks Q w różnych tkankach szczura wskazuje na zmiany w mitochondriach, które mogą odgrywać istotną rolę w metabolizmie energetycznym, homeostazie redoks czy poziomie stresu oksydacyjnego w komórkach. Uzyskane wyniki podkreślają rolę puli zredukowanego Q, zarówno na poziomie tkankowym, jak i mitochondrialnym, w obronie przed wzmożoną produkcją RFT oraz puli utlenionego Q w regulacji produkcji mRFT i utrzymaniu prawidłowego działania łańcucha oddechowego i OXPHOS w odpowiedzi na stres oksydacyjny.

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1. The interplay between mitochondrial reactive oxygen species formation and the coenzyme Q reduction level (2018) *Redox Biology* 18, 256-265
Dominiak K.*, Kozieł A.*, Jarmuszkiewicz W.

   Udział doktorantki w badaniach opisanych w tej publikacji obejmował:
   - uczestniczenie w przygotowaniu koncepcji badań,
   - izolację mitochondriów,
   - przeprowadzenie pomiarów zużycia tlenu, potencjału błonowego, ilości i poziomu redukcji koenzymu Q, produkcji H_2O_2,
   - analizę danych, pomoc w przygotowaniu manuskryptu.

2. Lung mitochondria adaptation to endurance training in rats (2020) *Free Radical Biology Medicine* 161, 163-174
Jarmuszkiewicz W., **Dominiak K.***, Galganski L.*, Galganska H., Kicinska A., Majerczak J., Zoladz A. J.

   Udział doktorantki w badaniach opisanych w tej publikacji obejmował:
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   - przygotowanie homogenatów i izolację mitochondriów,
   - przeprowadzenie pomiarów zużycia tlenu, potencjału błonowego, aktywności oksydazy cytochromu *c*, ilości i poziomu redukcji koenzymu Q, produkcji H_2O_2,
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3. The relationship between mitochondrial reactive oxygen species production and mitochondrial energetics in rat tissues with different contents of coenzyme Q (2021) *Antioxidants*, 10, 533. doi.org/10.3390/antiox10040533
Dominiak K., Jarmuszkiewicz W.

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   - przeprowadzenie pomiarów potencjału błonowego, ilości i poziomu redukcji koenzymu Q, produkcji H_2O_2,
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4. Effect of endurance training on the coenzyme Q redox state in rat heart, liver and brain at the tissue and mitochondrial levels: implications for reactive oxygen species formation and respiratory chain remodelling (2022) *International Journal of Molecular Sciences* 23 (2), 896; doi.org/10.3390/ijms23020896
Dominiak K.*, Galganski L.*, Budzinska A., Woyda-Ploszczyca A., Zoladz A. J., Jarmuszkiewicz W.

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   - przeprowadzenie pomiarów ilości i poziomu redukcji koenzymu Q, produkcji H_2O_2,
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Dominiak K, Koziel A, Jarmuszkiewicz W (2018) The interplay between mitochondrial reactive oxygen species formation and the coenzyme Q reduction level. *Redox Biology* 18, 256-265

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polegał na uczestniczeniu w wykonaniu analizy immunologicznej, BN-PAGE i pomiarze aktywności enzymów w żelu (in-gel activity) oraz w opracowaniu wyników tych doświadczeń.

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Oświadczam, że w pracy opublikowanej przez Dominiak K., Koziel A., Jarmuszkiewicz W.w 2018 r. w *RedoxBiology* (18, 256-265) pt. „The interplay between mitochondria reactive oxygen species formation and the coenzyme Q reductionlevel", mój udział polegał uczestniczeniu we wszystkich doświadczeniach i opracowaniu wyników.

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polegał na pomocy w obliczeniu wyników pomiaru produkcji H_2O_2 z trzech doświadczeń.

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9. WYKAZ POZOSTAŁYCH PUBLIKACJI AUTORA ROZPRAWY DOKTORSKIEJ

Zoladz JA, Koziel A, Broniarek B, Woyda-Ploszczycab AM, **Ogrodna K**, Majerczak J, Celichowski J, Szkutnik Z, Jarmuszkiewicz W (2017) Effect of temperature on fatty acid metabolism in skeletal muscle mitochondria of untrained and endurance-trained rats. *PLoS ONE* 12(12): e0189456. https://doi.org/10.1371/journal.pone.0189456

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Research Paper

The interplay between mitochondrial reactive oxygen species formation and the coenzyme Q reduction level

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ABSTRACT

Our aim was to elucidate the relationship between the rate of mitochondrial reactive oxygen species (mROS) formation and the reduction level of the mitochondrial coenzyme Q (mQ) pool under various levels of engagement of the mQ-reducing pathway (succinate dehydrogenase, complex II) and mQH_2-oxidizing pathways (the cytochrome pathway and alternative oxidase pathway, (AOX)) in mitochondria isolated from the amoeba *Acanthamoeba castellanii*. The mQ pool was shifted to a more reduced state by inhibition of mQH_2-oxidizing pathways (complex III and complex IV of the cytochrome pathway, and AOX) and the oxidative phosphorylation system. The mQ reduction level was lowered by decreasing the electron supply from succinate dehydrogenase and by stimulating the activity of the cytochrome or AOX pathways. The results indicate a direct dependence of mROS formation on the reduction level of the mQ pool for both mQH_2-oxidizing pathways. A higher mQ reduction level leads to a higher mROS formation. For the cytochrome pathway, mROS generation depends nonlinearly upon the mQ reduction level, with a stronger dependency observed at values higher than the mQ reduction level of the phosphorylating state (~ 35%). AOX becomes more engaged at higher mQ pool reduction levels (above 40%), when mROS production via the cytochrome pathway increases. We propose that the mQ pool reduction level (endogenous mQ redox state) could be a useful endogenous reporter that allows indirect assessment of overall mROS production in mitochondria.

1. Introduction

Mitochondrial coenzyme Q (mQ, ubiquinone) is an essential electron carrier that plays a central role in the mitochondrial electron transport respiratory chain [1,2]. Mitochondrial Q shuttles electrons between dehydrogenases and the oxidizing pathway(s) of the mitochondrial respiratory chain and is also involved in the formation of superoxide from semiubiquinone radicals by the respiratory chain, which can lead to mitochondrial oxidative damage [3–5]. It is widely accepted that mitochondrial reactive oxygen species (mROS) production depends on the level of reduction of mitochondrial electron carriers, especially on mQ. Surprisingly, there are only few data relating mQH_2/Q ratio [6] or mQ reduction level [7] with mROS generation.

The amoeba *Acanthamoeba castellanii* is a nonphotosynthesizing free-living protozoan belonging to the group that diverged from the animal/fungal line after the split from plants [8]. As an opportunistic pathogen that can cause serious diseases in humans, *A. castellanii* is an evolutionarily and medically important amoebozoan. This species presents features, including mitochondrial physiology, that are common to plants, fungi and animals. In addition to several dehydrogenases, the plant-type respiratory chain of *A. castellanii* mitochondria contains two ubiquinol (mQH_2)-oxidizing pathways, namely, the classical antimycin A- and cyanide-sensitive cytochrome pathway and the alternative benzohydroxamate- and propyl gallate-sensitive ubiquinol oxidase (AOX) pathway [9–11]. The mQ pool plays a central role in the respiratory chain; respiratory substrate-oxidizing dehydrogenases reduce mQ to mQH_2, and the two oxidizing pathways convert mQH_2 to mQ. Electron transfer via the AOX pathway does not result in proton pumping and is therefore not coupled to the mitochondrial production of ATP. The study of mitochondrial respiration of succinate (complex II substrate) in *A. castellanii* allows investigation of the kinetics of two mQH_2-oxidizing pathways; one proton electrochemical gradient

Abbreviations: AOX, alternative oxidase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; mROS, mitochondrial reactive oxygen species; mQ, mitochondrial coenzyme Q, ubiquinone; mQox, oxidized Q; mQH_2, reduced Q (ubiquinol); mQtot, total pool of endogenous Q in the inner mitochondrial membrane; mQH_2/mQtot, reduction level of Q; $m\Delta\Psi$, mitochondrial membrane electric potential; OXPHOS, oxidative phosphorylation; $\Delta\mu H^+$, proton electrochemical gradient

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($\Delta\mu H^+$)-generating pathway, consisting of the two proton-pumping complexes III and IV (the cytochrome pathway), and one $\Delta\mu H^+$-independent pathway (the AOX pathway). Because mROS production depends on $\Delta\mu H^+$ [4], investigation of *A. castellanii* mitochondria enables the determination of the relationship between mROS formation and the mQ reduction level at different mitochondrial membrane potential ($m\Delta\Psi$) values depending on the engagement of the two mQH_2-oxidizing pathways.

The aim of our work was to elucidate the relationship between mROS formation and the reduction level of the mQ pool under a variety of mitochondrial respiration conditions, i.e., at varying degrees of engagement of mQ-reducing the pathway (succinate dehydrogenase, complex II) and mQH_2-oxidizing pathways (the cytochrome pathway and AOX) in isolated *A. castellanii* mitochondria. The mQ reduction level was increased by decreasing electron flow out of the mQ pool via inhibition of the mQH_2-oxidizing pathways (complex III, complex IV, or AOX) or inhibition of the oxidative phosphorylation (OXPHOS) system (ATP synthase or ATP/ADP antiporter). The mQ pool was shifted to a more oxidized state by decreasing the electron supply from complex II via inhibition of the mQ-reducing pathway (substrate dehydrogenase) or by stimulation of the activities of the mQH_2-oxidizing pathways under uncoupling conditions (the cytochrome pathway) or under GMP activation (AOX). We measured the mQ reduction level under given mitochondrial oxygen consumption and mitochondrial membrane potential ($m\Delta\Psi$) conditions in terms of H_2O_2 formation.

2. Materials and methods

2.1. Acanthamoeba castellanii cell culture and isolation of mitochondria

Trophozoites of the *A. castellanii* strain Neff (ATCC®30010TM) were cultured as described previously [10]. Cells from 72-h cultures were inoculated (time 0) to a final density of approximately $2.5 \pm 0.4 \times 10^5$ cells $\times$ ml^{-1}. After approximately 40 h of exponential growth with a generation time (cell doubling time) of 8 h, the amoeba cultures reached the intermediate growth phase and then the stationary phase, the latter preceding transformation into cysts within a few hours. In this study, trophozoites of *A. castellanii* were harvested 48 h after inoculation, in the intermediate phase ($6.8 \pm 0.5 \times 10^6$ cells $\times$ ml^{-1}). Mitochondria were isolated in an isolation medium containing 0.25 M sucrose, 10 mM Tris/HCl (pH 7.4), 0.5 mM EGTA, and 0.2% bovine serum albumin (BSA) and then purified on a self-generating Percoll gradient (28%) for 45 min at 40,000 *g* [9]. Purified mitochondria were washed in isolation medium without BSA and EGTA. Protein concentrations of the isolated mitochondria were determined using the biuret method.

2.2. General measurement conditions

All measurements were performed in a standard incubation medium (28 °C) containing: 120 mM KCl, 20 mM Tris/HCl (pH 7.4), 3 mM KH_2PO_4, 8 mM $MgCl_2$, 1 mM EGTA, and 0.2% BSA with continuous stirring. Mitochondria (0.33 mg of protein/ml) were incubated with succinate (5 mM) as an oxidizable substrate of complex II in the presence of rotenone (2 μM) to block electron input from complex I. Respiratory rate, $m\Delta\Psi$, mQ reduction levels, and mROS formation were measured under (i) resting (nonphosphorylating, State 4) conditions, i.e., in the absence of exogenous ADP, and (ii) phosphorylating (State 3) conditions, i.e., in the presence of 1–2 mM ADP. The mQ-reducing pathway was titrated with increasing concentrations of malonate (an inhibitor of succinate dehydrogenase, complex II). The activity of a major mQH_2-oxidizing pathway (the cytochrome pathway) was varied with increasing concentrations of (i) antimycin A or cyanide (inhibitors of complex III and complex IV of the cytochrome pathway, respectively), (ii) oligomycin or carboxyatractyloside (inhibitors of ATP synthase and ATP/ADP antiporter, respectively), or (iii) uncoupler (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, FCCP) in the presence of AOX inhibitor. Parameters of the cytochrome pathway were measured in the presence of benzohydroxamate (1 mM) (respiratory rate, $m\Delta\Psi$, and mQ reduction level) or propyl gallate (3 μM) (H_2O_2 formation). The activity of the alternative mQH_2-oxidizing pathway (AOX) was varied with increasing concentrations of GMP (an allosteric activator of AOX, [12,13]) in the presence of 0.65 mM cyanide or 90 nM antimycin A (to exclude the activity of the cytochrome pathway). The relationships between the studied respiratory chain parameters (respiratory rate, $m\Delta\Psi$, mQ reduction levels, and H_2O_2 formation) and increasing concentrations of the respiratory chain and OXPHOS system modulators are shown in the Supplementary materials (Figs. S1–S6).

2.3. Mitochondrial oxygen consumption and mΔΨ measurements

Oxygen uptake was measured polarographically with a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) in 3.0 ml of incubation medium (28 °C) with 1 mg of mitochondrial protein. Only high quality *A. castellanii* mitochondrial preparations, i.e., those with ADP/O values of ~1.40 (with succinate as a respiratory substrate) and respiratory control ratios of ~3.5, were used in all the experiments. For OXPHOS control, phosphorylating respiration (State 3) was measured after an ADP pre-pulse (50 μM) using 150 μM ADP as the main pulse. The total amount of oxygen consumed during phosphorylating respiration was used to calculate the ADP/O ratio. The $m\Delta\Psi$ measurements allowed fine control of the duration of phosphorylating respiration. Values of O_2 uptake are given in nmol O_2 $\times$ min^{-1} $\times$ mg $protein^{-1}$.

The $m\Delta\Psi$ was measured simultaneously with oxygen uptake using a tetraphenylphosphonium (TPP^+)-specific electrode as described previously [14–16]. The TPP^+-specific electrode was calibrated with three sequential additions (0.8, 0.8, and 1.6 μM) of TPP^+. After each run, 0.5 μM FCCP was added to release TPP^+ for baseline correction. For calculation of the $\Delta\Psi$ value, the matrix volume of the amoeba mitochondria was assumed to be 2.0 μl $\times$ mg^{-1} protein. The calculation assumes that the TPP^+ distribution between the mitochondria and medium followed the Nernst equation. The values of $\Delta\Psi$ are given in mV.

2.4. Assay of H_2O_2 production by isolated mitochondria

The mitochondrial H_2O_2 production rate was measured by the Amplex Red-horseradish peroxidase method (Invitrogen) [17]. Horseradish peroxidase (0.1 U $\times$ ml^{-1}) catalyzes the H_2O_2-dependent oxidation of nonfluorescent Amplex Red (5 μM) to fluorescent resorufin red. Fluorescence was kinetically followed for 10 min at 545 nm (excitation), 590 nm (emission), and gain 150 using an Infinite M200 PRO Tecan multimode reader with 24-well plates. Mitochondria (0.17 mg of mitochondrial protein) were incubated in 0.5 ml of the standard incubation medium (see above) with 5 mM succinate as an oxidizable substrate in the presence of rotenone (2 μM). Because, at high concentrations, AOX inhibitors, benzohydroxamate and propyl gallate, are known to inhibit horseradish peroxidase activity and scavenge free radicals [18–21], H_2O_2 formation related to the cytochrome pathway activity was measured in the presence of a low concentration of propyl gallate (1.5 μM), which completely inhibits AOX activity. Because cyanide inhibits horseradish peroxidase activity [22], the H_2O_2 formation rate related to AOX activity was measured in the presence of 90 nM antimycin A to inhibit cytochrome pathway activity. Therefore, titration of H_2O_2 formation related to cytochrome pathway activity was performed only in the presence of increasing concentrations of antimycin A not cyanide. Reactions were monitored with constant stirring at 28 °C and calibrated with known amounts of H_2O_2 in the absence or presence of 1.5 μM propyl gallate. H_2O_2 production rates were determined from slopes calculated from readings obtained from several repeated 10-min measurements. Values of H_2O_2 production are given in

pmol H_2O_2 × min^{-1} × mg $protein^{-1}$.

2.5. Determination of the mQ reduction level

The reduction level of mQ (mQH_2/mQtot), i.e., the ratio of reduced mQ (ubiquinol, mQH_2) to the total endogenous pool of mQ in the inner mitochondrial membrane under steady-state respiration was determined by extraction followed by HPLC detection [23]. Mitochondria (1–2 mg of mitochondrial protein) in the steady state were chemically quenched with 4 ml of 0.65 M $HClO_4$ in methanol (0 °C), and mQ was subsequently extracted with 3 ml of petroleum ether. Detection of the oxidized and reduced forms of mQ (at 275 nm and 290 nm, respectively) was performed by HPLC with a reverses-phase Lichrosorb 10 RP 18 column (4.6 mm × 250 mm). A completely oxidized extract was obtained during incubation in the absence of a reducing substrate using an evaporation/ventilation step. A completely reduced extract was obtained upon anaerobiosis and in the presence of respiratory substrate (5 mM succinate), 1 mM cyanide and 1 mM benzohydroxamate. As previously determined, the endogenous Q in *A. castellanii* mitochondria is Q_9 [12]. Commercial Q_9 (Sigma) was used for peak calibration. The reduction level of mQ is expressed as the percentage of total mQ (mQH_2/mQtot).

3. Results

Cytochrome pathway activity was measured in the presence of AOX inhibitor (1 mM benzohydroxamate or 1.5 μM propyl gallate). The activity of the alternative mQH_2-oxidizing pathway (AOX) was measured in the presence of 0.65 mM cyanide or 90 nM antimycin A to exclude cytochrome pathway activity.

3.1. The relationship between mROS formation and the reduction level of the mQ pool under various degrees of engagement of the cytochrome pathway

At first, the mQ pool was progressively shifted to a more oxidized state by stimulating the activity of the mQH_2-oxidizing pathway (the cytochrome pathway) by uncoupling with increasing concentrations of FCCP (up to 500 nM) (Figs. 1, S1). Fig. 1 and B show changes in respiratory rate (progressively increasing from ~ 55 to ~ 195 nmol O_2 × min^{-1} × mg $protein^{-1}$) and H_2O_2 formation rate (progressively decreasing from ~ 100 to ~ 390 pmol H_2O_2 × min^{-1} × mg $protein^{-1}$) when the mΔΨ of nonphosphorylating mitochondria was gradually decreased (from ~ 189 to ~ 160 mV) by the uncoupler. The uncoupler-induced changes in the respiratory and H_2O_2 formation rates were accompanied by a gradual decrease in the mQ reduction level from ~ 69 to ~ 28% (Fig. 1C–E). A linear dependence of mΔΨ on the mQ reduction level for the cytochrome pathway under the uncoupling conditions was observed (Fig. 1E).

Next, the mQ reduction level of the cytochrome pathway under phosphorylating conditions was gradually increased by inhibition of the OXPHOS system with increasing concentrations of carboxyatractyloside (up to 325 nM) or oligomycin (120 nM) (Fig. S2). Fig. 2 shows the relationships between the respiratory rate and H_2O_2 formation versus mΔΨ (Fig. 2A, B) and mQ reduction level (Fig. 2C, D) when the cytochrome pathway activity of phosphorylating mitochondria (in the presence of 1 mM ADP) was gradually varied with the inhibitors of ADP/ATP antiporter and ATP synthase. During carboxyatractyloside/oligomycin titration, an increase in mΔΨ from ~ 160 mV (uninhibited phosphorylating state) up to ~ 192 mV (fully inhibited phosphorylating state) was accompanied by an increase in mQ reduction from ~ 30% (uninhibited phosphorylating state) to ~ 72% (nonphosphorylating state in the presence of OXPHOS inhibitors). A linear dependence of mΔΨ on the mQ reduction level for the cytochrome pathway under phosphorylating conditions (during the carboxyatractyloside/oligomycin-induced State 3 to State 4 transition) is presented in Fig. 1E. Moreover, a progressive decrease in the respiratory rate from ~ 195 to ~ 50 nmol O_2 × min^{-1} × mg $protein^{-1}$ and a progressive increase in H_2O_2 formation from 110 to ~ 440 pmol H_2O_2 × min^{-1} × mg $protein^{-1}$ accompanied the carboxyatractyloside/oligomycin-induced State 3 to State 4 transition (Fig. 2A–D).

Rates of H_2O_2 formation were elevated up to 4 times in the resting State 4 (in the absence or presence of OXPHOS inhibitors) compared to the rates in the phosphorylating State 3 or uncoupled state (Figs. 1, 2), because higher (up to ~ 30 mV) mΔΨ and higher (up to 40%) mQ reduction levels were observed.

Furthermore, the mQ reduction level of the cytochrome pathway was gradually increased by decreasing the electron flow out of the mQ pool via inhibition of complex III by increasing the concentrations of antimycin A (up to 60 nM) and complex IV by increasing the concentrations of cyanide (up to 0.4 mM) (Fig. S3). Measurements were performed under phosphorylating (State 3) and nonphosphorylating (State 4) conditions. Because cyanide inhibits peroxidase activity, measurements of H_2O_2 formation were performed only during antimycin A titrations. Fig. 3 shows the relationships between respiratory rate and H_2O_2 formation versus mΔΨ (Fig. 3A, B) and versus mQ reduction level (Fig. 3C, D) when the cytochrome pathway activity was gradually inhibited. The relationships obtained during the antimycin A and cyanide titrations were similar. A much steeper linear dependence of mΔΨ on the mQ reduction level was obtained for the cytochrome pathway under nonphosphorylating conditions than under phosphorylating conditions (Fig. 3E). During antimycin A/cyanide titration under nonphosphorylating conditions, a progressive decrease in mΔΨ from ~ 189 mV (uninhibited State 4) up to ~ 136 mV (fully inhibited State 4) was accompanied by a relatively moderate increase in mQ reduction level from ~ 69% (uninhibited State 3) to ~ 81% (fully inhibited State 3) (Fig. 1E). This 12% increase in the mQ reduction level was accompanied by an ~ 1.2-fold increase in the H_2O_2 formation rate (Fig. 3D). Under phosphorylating conditions, a progressive decrease in mΔΨ from ~ 160 mV (uninhibited State 3) up to ~ 141 mV (fully inhibited State 3) was accompanied by a progressive increase in mQ reduction from ~ 30% (uninhibited State 3) to ~ 77% (fully inhibited State 3) (Fig. 1E). This 47% increase in the mQ reduction level accompanying the inhibition of the cytochrome pathway in State 3 was accompanied by an ~ 4.2-fold increase in the H_2O_2 formation rate (Fig. 3D).

The results indicate a direct dependence of mROS formation on the reduction level of the mQ pool (and mΔΨ) under various levels of engagement of the cytochrome pathway. The higher mQ reduction level (and the larger mΔΨ) the bigger mROS production.

3.2. The relationship between mROS formation and the reduction level of the mQ pool under varying activity of complex II when the cytochrome pathway is engaged

The dependence of the rate of electron transfer through the mQ reducing enzyme (complex II) on the reduction level of the mQ pool (and mΔΨ) when the cytochrome pathway is active was studied by malonate titration under phosphorylating and nonphosphorylating conditions. Malonate titration enabled lowering of the mQ reduction level below 30% (reached with FCCP titration, Fig. 1C–E), leading to an ~ 10% reduction level for both respiratory states (Fig. 4C–E). At levels below the mQ pool reduction level (and mΔΨ) of uninhibited State 3, i.e., below 30% of the mQ reduction level (and below ~ 160 mV), the kinetic relationships between H_2O_2 formation versus mΔΨ (Fig. 4B) and versus mQ reduction level (Fig. 4D) overlapped for both respiratory states. Under both phosphorylating and nonphosphorylating conditions, H_2O_2 formation depends nonlinearly upon the mQ pool reduction level (and mΔΨ). The threshold values for the mQ pool reduction level and mΔΨ are slightly greater than the values for the uninhibited State 3, i.e., an ~ 35% reduction level of mQ and ~ 165 mV of mΔΨ. At values greater than these thresholds, a sharply increased dependence of

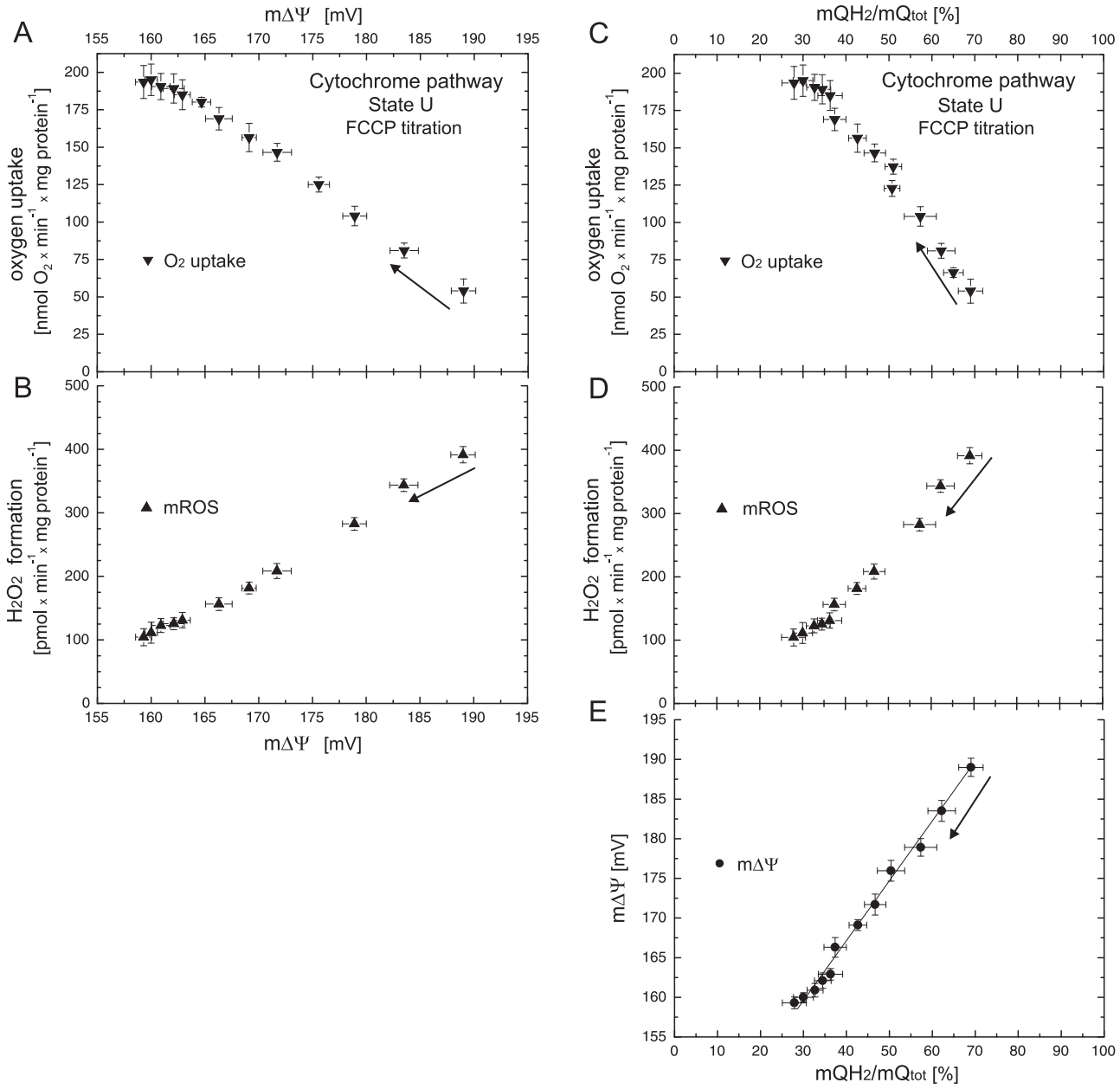

Fig. 1. The relationships between respiratory rate (A) and H_2O_2 formation (B) versus mΔΨ and the relationships between respiratory rate (C), H_2O_2 formation (D), and mΔΨ (E) versus mQ reduction level when the cytochrome pathway activity of nonphosphorylating mitochondria was varied with increasing concentrations of FCCP (up to 500 nM). Measurements were performed in the presence of 1 mM benzohydroxamate or 1.5 μM propyl gallate (H_2O_2 formation). Arrows indicate the starting point and direction of titrations.

H_2O_2 formation on the mQ reduction level (and mΔΨ) was observed (Fig. 4B, D); therefore, a small increase in the mQ reduction level (and mΔΨ) leads to a high rate of H_2O_2 production by mitochondria.

3.3. The relationship between mROS formation and the reduction level of the mQ pool under varying activity of AOX

When the cytochrome pathway was inactive (in the presence of high concentrations of cyanide or antimycin A), the activity of the unstimulated AOX pathway led to low levels of oxygen uptake (~ 20 mol $O_2 \times min^{-1} \times$ mg $protein^{-1}$) accompanied by very high mQ reduction levels (~ 77%) and H_2O_2 formation (~ 230 pmol $H_2O_2 \times min^{-1} \times$ mg $protein^{-1}$) (Fig. 5). The AOX-mediated mROS formation is independent of mΔΨ (and $\Delta\mu H^+$), since no proton pumping occurs in the respiratory pathway when AOX works with complex II. Increasing the concentration of GMP progressively stimulated AOX activity, leading to up to ~ 4.5 times higher respiratory rates, up to ~ 1.3 times lower H_2O_2 formation, and a considerable decrease in mQ reduction levels (up to ~ 59%). Fig. 5 shows the approximately linear relationships between respiratory rate (Fig. 5A) and H_2O_2 formation (5B) versus mQ reduction level (ranging from 59% to 77%) when the AOX activity was gradually stimulated by GMP.

3.4. The relationship between mROS formation and the reduction level of the mQ pool under varying activity of complex II when AOX is engaged

The dependence of the rate of electron transfer through the mQ-reducing enzyme (complex II) on the reduction level of the mQ pool when AOX is active was studied by malonate titration in the absence or presence of GMP (Fig. 6). A progressive decrease in respiratory rate and

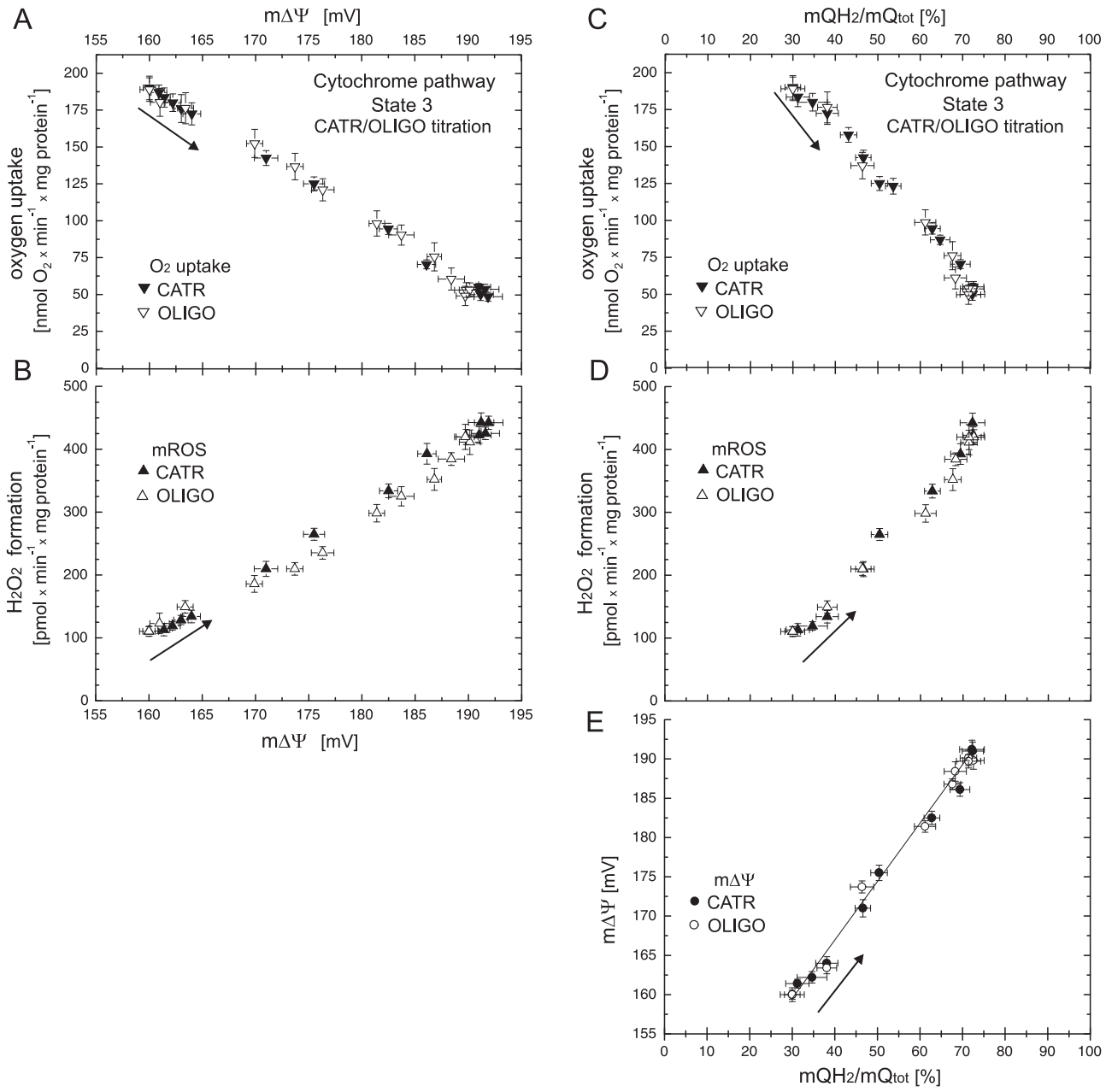

Fig. 2. The relationships between respiratory rate (A) and H_2O_2 formation (B) versus mΔΨ and the relationships between respiratory rate (C), H_2O_2 formation (D), and mΔΨ (E) versus mQ reduction level when the cytochrome pathway activity of phosphorylating mitochondria was varied with increasing concentrations of carboxyatractyloside (CATR, up to 350 nM) or oligomycin (OLIGO, up to 120 nM). Measurements were performed in the presence of 1 mM benzohydroxamate or 1.5 μM propyl gallate (H_2O_2 formation). Arrows indicate the starting point and direction of titrations.

H_2O_2 formation was observed with lowering of the mQ pool reduction level from ~77 to ~52% and from ~59 to ~42% for unstimulated and GMP-stimulated succinate-sustained AOX, respectively. The kinetic relationships between H_2O_2 formation versus mQ reduction level (Fig. 6B) overlapped for both conditions and were approximately linear in the range of the mQ reduction level of AOX during succinate oxidation. These results indicate that AOX becomes engaged at only high reduction levels of the mQ pool (greater than 40%).

3.5. Inactive mQ pool

In *A. castellanii* mitochondria, a completely oxidized mQ pool (0% mQ reduction level) was obtained after incubation of mitochondria in the absence of a reducing substrate. Upon anaerobiosis and in the presence of a respiratory substrate (5 mM succinate), 1 mM cyanide, and 1 mM benzohydroxamate, a completely reduced mQ pool with ~85% reduction was obtained. This result indicates that in *A. castellanii* mitochondria isolated from the intermediate phase of growth, ~15% of the mQ pool is unreducible.

4. Discussion

The mQ reduction shift that occurs when mitochondria switch from nonphosphorylating conditions (State 4, a more reduced state) to phosphorylating conditions (State 3, a more oxidized state) is of utmost physiological significance. In vivo, mitochondria can shift rapidly between these conditions inducing a change in the mQ reduction level and thereby in mROS formation. Our study combined quantitatively important mitochondrial parameters: mΔΨ, oxygen uptake, mROS formation, and mQ pool reduction level, with a special focus on the

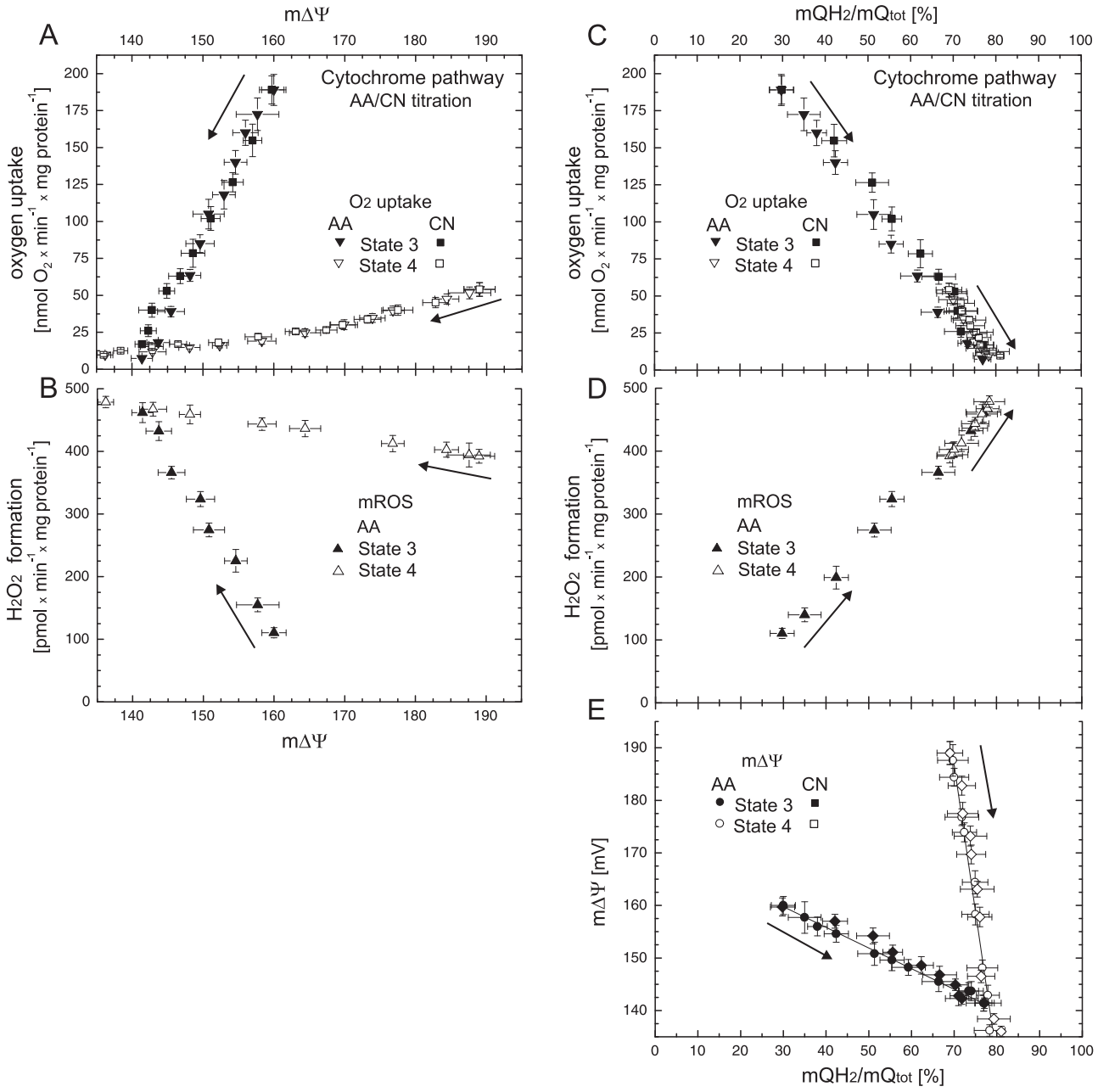

Fig. 3. The relationships between respiratory rate (A) and H_2O_2 formation (B) versus mΔΨ and the relationship between respiratory rate (C), H_2O_2 formation (D), and mΔΨ (E) versus mQ reduction level when the cytochrome pathway activity was varied with increasing concentrations of cyanide (CN, up to 0.4 mM) or antimycin A (AA, up to 60 nM). Measurements were performed under phosphorylating (State 3) and nonphosphorylating (State 4) conditions, in the presence of 1 mM benzohydroxamate or 1.5 μM propyl gallate (H_2O_2 formation). Arrows indicate the starting point and direction of titrations.

relationship between mROS formation and mQ reduction level. In *A. castellanii* mitochondria, during nonphosphorylating respiration, high mQ reduction levels were accompanied by increased mΔΨ values and mROS formation. In contrast, during phosphorylating respiration, the relative oxidation of the mQ pool was accompanied by decreased mΔΨ values and therefore by decreased mROS formation. In isolated mitochondria, the relationship between mROS formation and the mQ reduction level can be elucidated by varying the mQ reduction level (mQH_2/mQtot) by using agents that cause both stimulation and inhibition of respiratory chain electron transport.

In *A. castellanii* mitochondria, the respiratory activity of the cytochrome pathway is much higher than that of AOX. The cytochrome pathway becomes engaged at much lower mQ pool reduction levels (from ~ 10%) (Fig. 4C–E) than the AOX pathway (from ~ 40%) (Fig. 6). Comparing the relationships between mROS formation and the reduction level of the mQ pool for both mQH_2-oxidizing pathways (Fig. 7C), it is clear that at higher mQ reduction levels, the cytochrome pathway produces much more (even twice as much) mROS compared to AOX. At ~ 40% of mQ reduction level, the mROS formation is similar for both mQH_2-oxidizing pathways (~ 130 pmol H_2O_2 × mg protein^{-1}). The relationship between mROS formation and the mQ pool reduction level is much steeper for the cytochrome pathway than for AOX; for the cytochrome pathway, H_2O_2 formation depends strongly upon the mQ pool reduction level in the 40–80% range (Fig. 7C). Above the threshold value of the mQ pool reduction level, i.e., slightly above the value for the uninhibited State 3 (~ 35%), which corresponds to ~ 165 mV mΔΨ (Fig. 7A, B), a small increase in the mQ reduction level gives rise to a high rate of H_2O_2 production by mitochondria, with oxidation

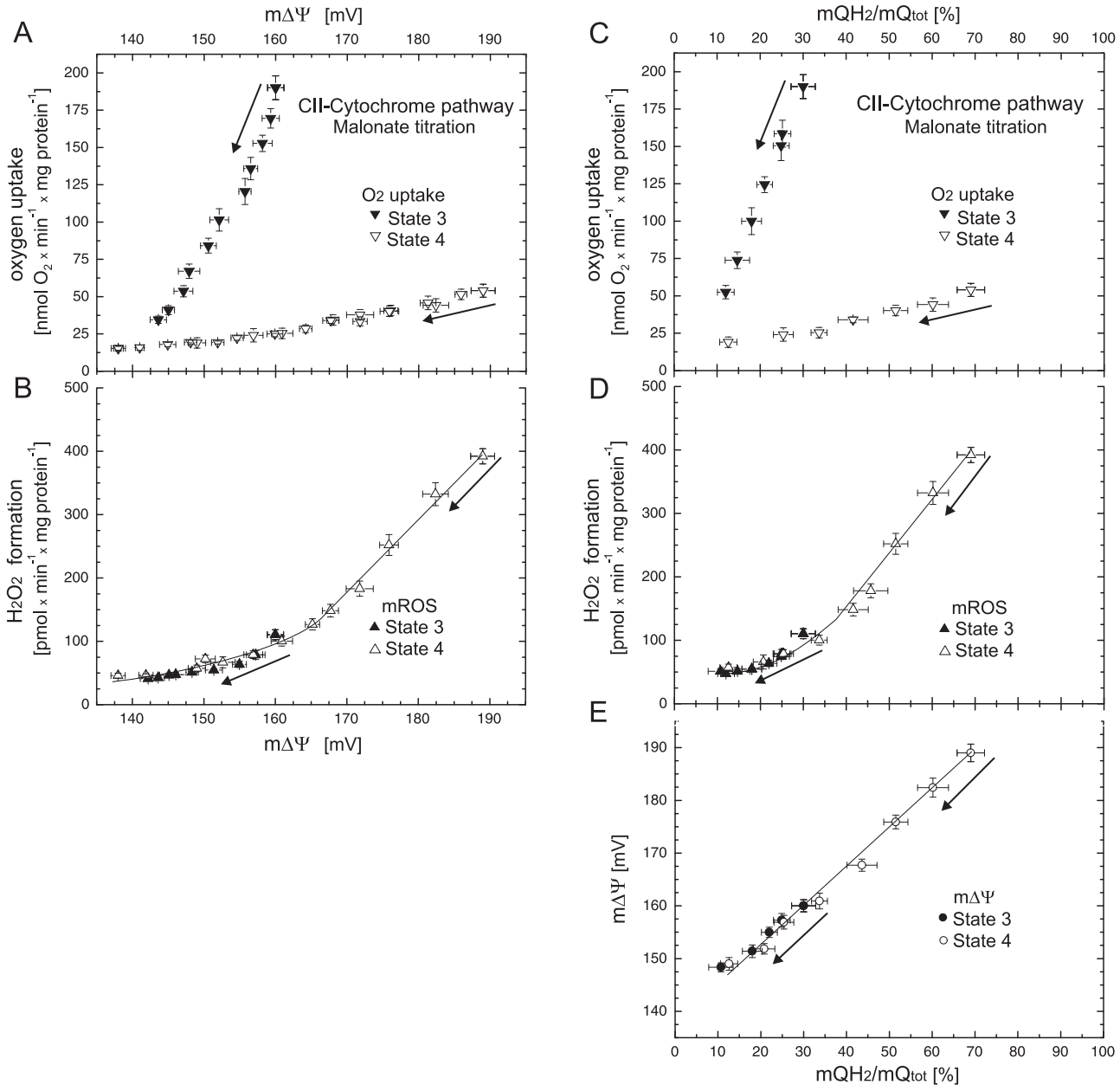

Fig. 4. The relationships between respiratory rate (A) and H_2O_2 formation (B) versus mΔΨ and the relationships between respiratory rate (C), H_2O_2 formation (D), and mΔΨ (E) versus mQ reduction level when the cytochrome pathway is engaged. Complex II (CII) activity was varied with increasing concentrations of malonate (up to 4 mM). Measurements were performed under phosphorylating (State 3) and nonphosphorylating (State 4) conditions, in the presence of 1 mM benzohydroxamate or 1.5 μM propyl gallate (H_2O_2 formation). Arrows indicate the starting point and direction of titrations.

occurring via the cytochrome pathway. It has been shown previously that in nonphosphorylating rat heart mitochondria, mROS generation strongly but nonlinearly depends upon mΔΨ, increasing at mΔΨ greater than that of State 3 [24]. Our results confirm this observation, however indicating the contribution of the mQ reduction level to mROS formation.

To date, at least 11 sites that produce superoxide anion and/or H_2O_2 have been identified in mammalian mitochondria [25]. Under our experimental conditions, during succinate oxidation, the flavin site of mitochondrial complex II (site II_F of complex II) produced mROS when AOX was active, while site II_F of complex II and the Q_o site of mitochondrial complex III (site III_{Qo}) participated in mROS production when the cytochrome pathway was active. The highest H_2O_2 formation was observed in the presence of antimycin A (Fig. 7A, C), a Q_i site inhibitor of complex III. It has been shown that contributions of specific sites of the mitochondrial respiratory chain to the production of ROS in mitochondria depend very strongly on the substrates being oxidized [26]. Our results indicate that the production of ROS in mitochondria depends not only on the engagement of mROS formation sites but also on the engagement of QH_2-oxidizing pathways.

There are only few studies relating mQ reduction level with mROS generation, which is likely due to difficulties in the measurement of the mQ reduction level. In different cell lines, it has been shown how the mitochondrial electron transport chain is optimized to better oxidize different fuels using the reducing status of mQ (mQH_2/Q ratio) as a metabolic sensor and mROS generated by complex I by reverse electron transport as an executor [6]. In rat heart mitochondria, mROS production by reverse electron transport at complex I is steeply dependent on mΔΨ and on mQ reduction level, indicating the sensitivity of superoxide anion production by this respiratory chain site to the two

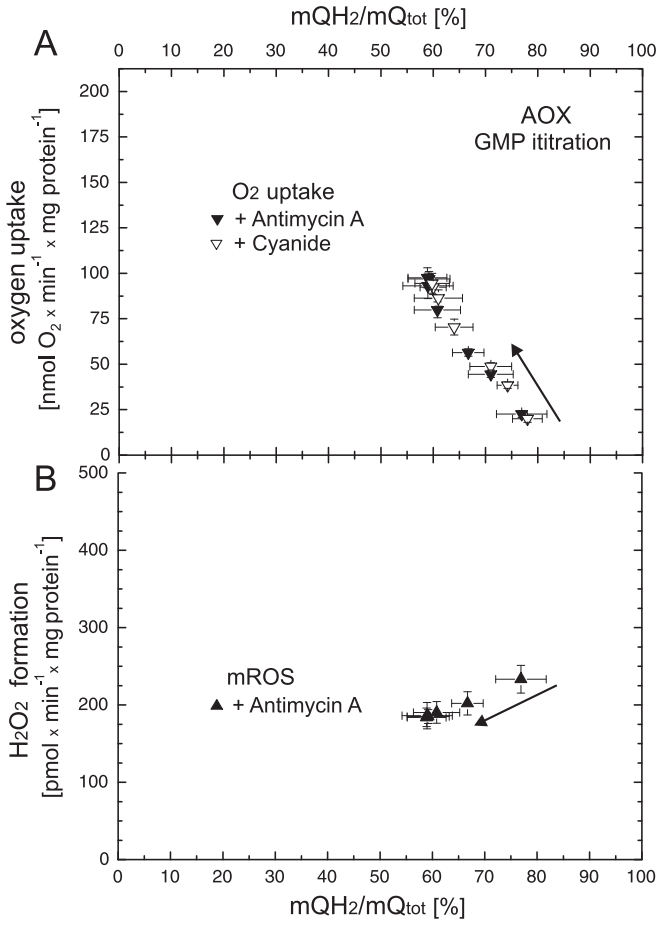

Fig. 5. The relationships between respiratory rate (A) and H_2O_2 formation (B) versus mQ reduction level when AOX activity was varied with increasing concentrations of GMP (up to 1 mM). Measurements were performed in the presence of 0.65 mM cyanide or 90 nM antimycin A. Arrows indicate a starting point and direction of titrations.

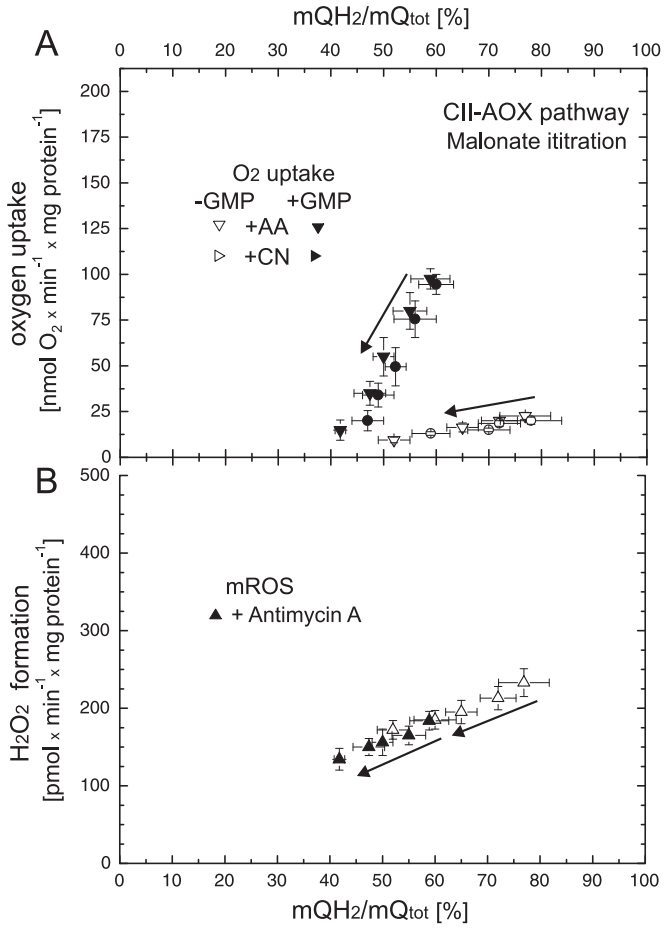

Fig. 6. The relationships between respiratory rate (A) and H_2O_2 formation (B) versus mQ reduction level when AOX is engaged in the absence or presence of 1 mM GMP. Complex II (CII) activity was varied with increasing concentrations of malonate (up to 4 mM). Measurements were performed in the presence of 0.65 mM cyanide or 90 nM antimycin A. Arrows indicate the starting point and direction of titrations.

physiological variables [7]. Marphy's group has shown that in the presence of antimycin A, the production of superoxide at the Q_o site is specifically linked to local accumulation of reduced cytochrome b_{566} when the Q_i site is inhibited by antimycin A, and this effect is independent of mΔΨ [7]. Our results indicate that mROS production is a direct function of the mQ pool reduction level and is independent of mΔΨ when the Q_i site is inhibited by antimycin A and when reverse electron transport is excluded by rotenone during succinate oxidation.

Complex I is believed to be a major site of mROS production in the mitochondrial electron transport chain, either from FAD- or NAD-driven electron flux [3]. It is also generally assumed that the generation of mROS when succinate is used as the substrate depends mostly on reverse electron transfer through complex I. Our study shows that during succinate oxidation, the production of mROS may be very high even when complex I is inhibited by rotenone. Further studies are needed to elucidate whether reverse electron transfer through complex I is the major source of mROS in *A. castellanii* mitochondria.

The present study indicates that in *A. castellanii* mitochondria, AOX becomes more engaged at higher mQ pool reduction levels, when mROS production via the cytochrome pathway increases (Fig. 7C). Activation of cyanide- and antimycin A-resistant AOX-mediated respiration by GMP significantly decreased mQ reduction levels and H_2O_2 formation (Figs. 5, 4), confirming the antioxidant role of the oxidase. It has been previously shown that in *A. castellanii* mitochondria, AOX may play a role not only in the energetic status of the cell by decreasing the yield of OXPHOS [12] but also in preventing the generation of mROS [27], which are maintained at a constant level throughout the growth cycle of amoeba batch culture [28]. In the present study, we found an ~15% unreducible mQ pool in mitochondria isolated from *A. castellanii* cells from an intermediate phase of growth. A question arises as to whether the size of the unreducible mQ pool in the inner mitochondrial membrane influences mQ reduction level-dependent mROS formation. Further studies are needed to answer this question. Our previous study indicates that, compared to *A. castellanii* cells from the intermediate phase of growth, decreased reducible mQ levels in intensively dividing cells (i.e., at the exponential phase of growth) accompanied by increased mQ reduction levels could lead to increased mROS formation and thereby to increased AOX activity (which depends on the mQ reduction level) [11,28].

Mitochondrial ROS production has been described to be a direct function of mΔΨ [4,29]. Fig. 7A is a summative description of the relationships between H_2O_2 formation versus mΔΨ under various levels of engagement of the complex II-supplied cytochrome pathway during titrations with carboxyatractyloside/oligomycin, FCCP, malonate, and antimycin A under nonphosphorylating and phosphorylating conditions. The relationship between H_2O_2 formation and mΔΨ obtained for antimycin A titration, in which a decrease in mΔΨ was accompanied by an increase in mQ reduction level, does not fit the relationship obtained for other conditions, in which lower mΔΨ values were accompanied by lower mQ reduction levels (Fig. 7A); this difference is also evident when mΔΨ is plotted against the mQ reduction level (Fig. 7B). Given these

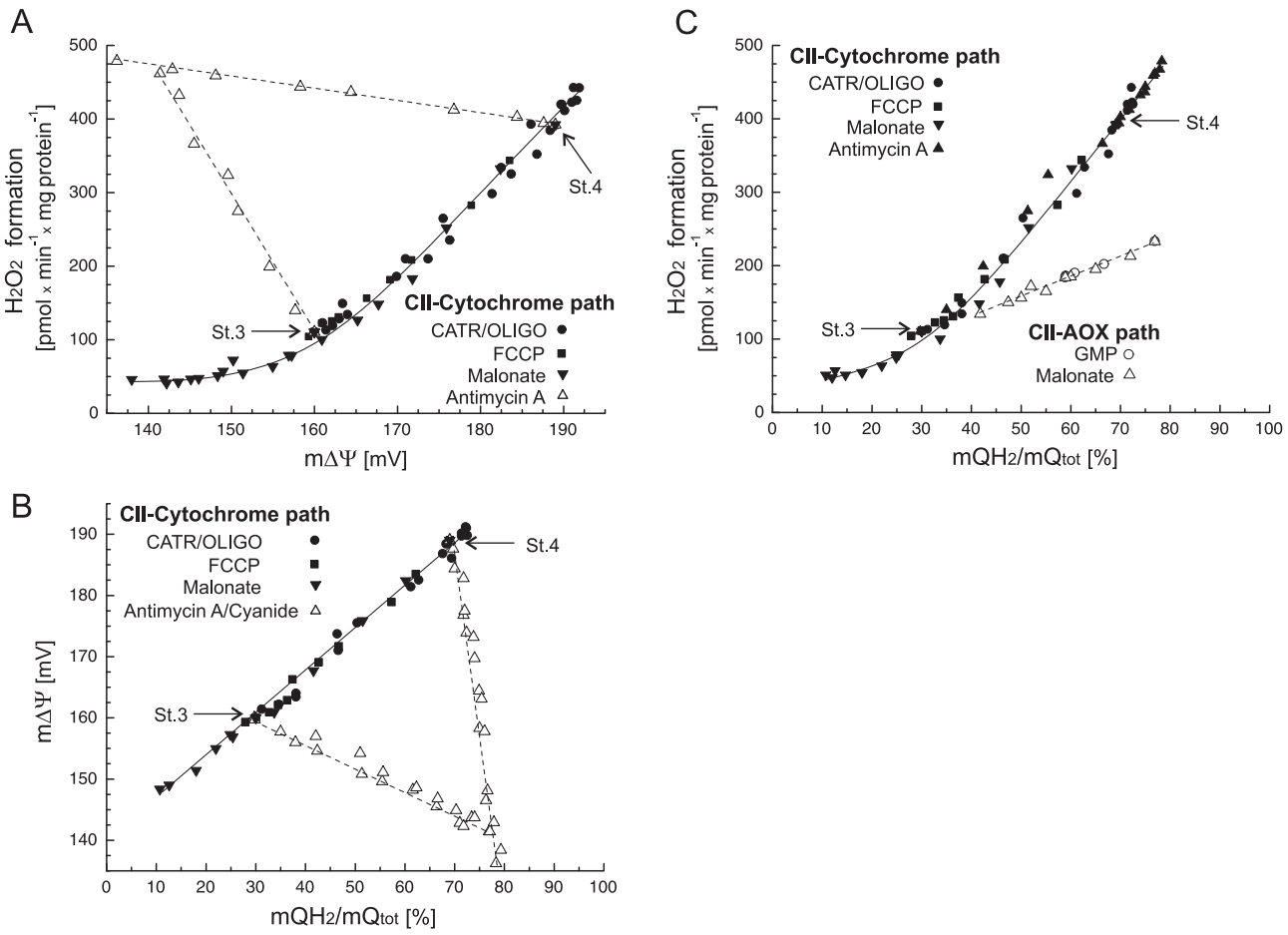

Fig. 7. Summative graphs. The relationships between H_2O_2 formation versus mΔΨ (A) and mΔΨ versus mQ reduction level (B) under various levels of engagement of the complex II (CII)-fueled cytochrome pathway. (A,B) Titrations with carboxyatractyloside (CATR), oligomycin (OLIGO), FCCP, malonate, and antimycin A/cyanide are all shown here. Measurements were performed under phosphorylating (St.3) and nonphosphorylating (St. 4) conditions, in the presence of 1 mM benzohydroxamate or 1.5 μM propyl gallate (H_2O_2 formation) to exclude AOX activity. (C) The relationships between H_2O_2 formation versus mΔΨ under various levels of engagement of the complex II (CII)-fueled cytochrome pathway (full symbols, solid curve) and the complex II (II)-fueled AOX (empty symbols, dashed line). For the cytochrome pathway, titrations (as in A and B) were performed in the presence of 1.5 μM propyl gallate to exclude AOX activity. For AOX, titrations with GMP and malonate (in the presence or absence of GMP) were performed in the presence of antimycin A to exclude the cytochrome pathway.

observations and the fact that AOX-mediated mROS formation is mΔΨ independent, we postulate that mROS production is a direct function of the mQ pool reduction level rather than of mΔΨ. The dependence of H_2O_2 production on the mQ reduction level is clearly evidenced in this study for both QH_2-oxidizing pathways (Fig. 7C). Therefore, the mQ pool reduction level (endogenous mQ redox state) could be a useful endogenous reporter that allows indirect assessment of overall mROS production in mitochondria. Most of the mitochondrial sites of superoxide anion/H_2O_2 formation are related to the reduction level of the mQ pool that connects the dehydrogenase and oxidase sides of the electron transport chain. For example, the rates of mitochondrial superoxide anion/H_2O_2 production from site I_F of complex I and site Q_o of complex III can be assessed indirectly by measuring endogenous reporters such as the mitochondrial NAD(P)H redox state and the cytochrome b_{566}, respectively [25,26]. Measurement of the endogenous mQ pool reduction level could provide insight into overall mROS production related to mitochondrial respiratory chain complexes. The present study analyses the dependence of H_2O_2 production on the mQ pool reduction level in mitochondria, by using *A. castellanii* mitochondria as an example, under conditions in which concentrations of agents that cause both stimulation and inhibition of respiratory chain electron transport (substrate, inhibitors and activators) were varied. In future studies, the measurements of the mQ pool reduction level under physiological conditions in the absence of inhibitors of electron transport may be helpful for the assessment of overall intrinsic production of mROS in mitochondria and the physiological role of these species as signaling molecules and in pathologies.

5. Conclusions

We elucidated for the first time the relationship between the rate of mROS formation and the reduction level of the endogenous mQ pool varied by agents that cause both stimulation and inhibition of the mitochondrial respiratory electron transport chain. Our results indicate that mROS production is a direct function of the mQ pool reduction level rather than of mΔΨ. The production of ROS in mitochondria depends not only on the engagement of mROS formation sites but also on the engagement of QH_2-oxidizing pathways. The reduction level of the mQ pool could be a useful endogenous reporter that allows indirect assessment of overall mROS production in mitochondria.

Funding

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Conflict of interest/declarations of interest

The authors declare no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2018.07.018.

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Supplementary figures

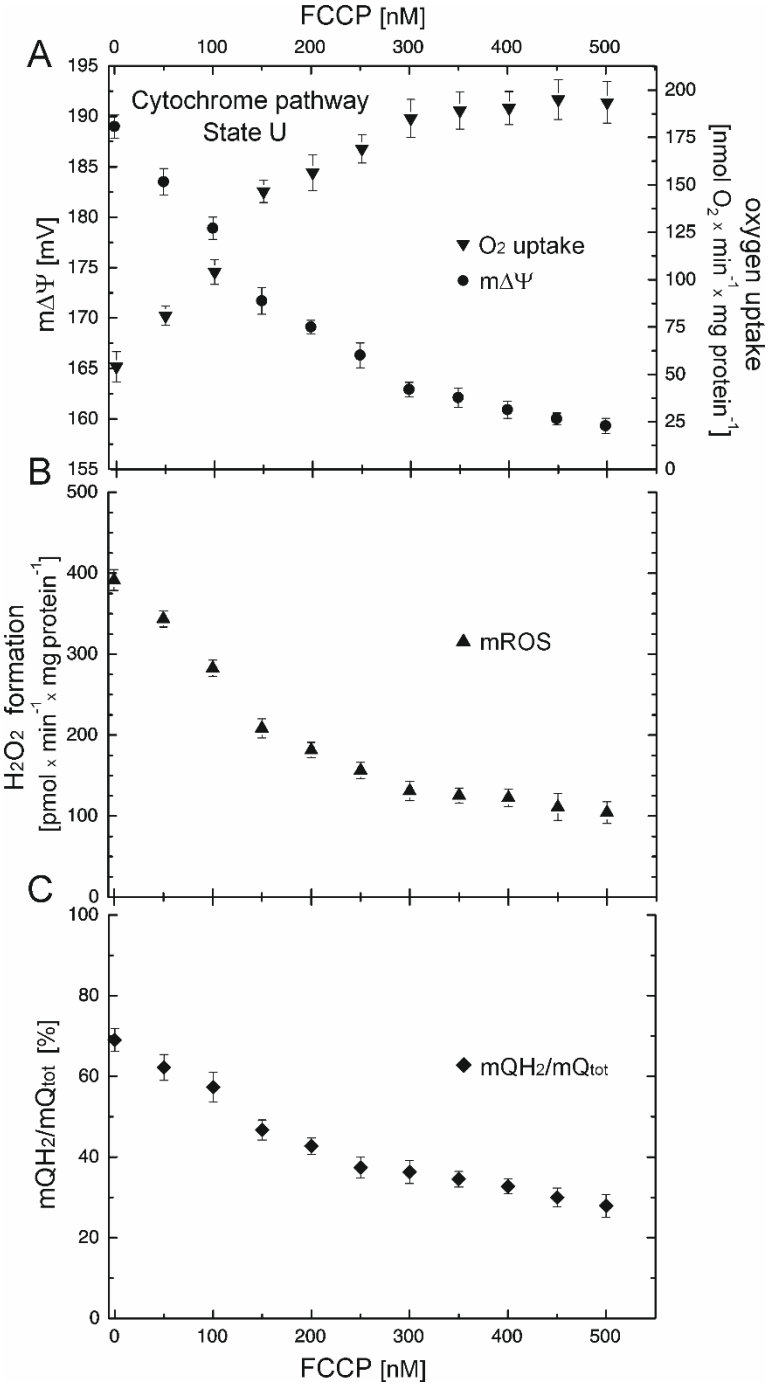

Fig. S1. Effects of increasing concentrations of FCCP (up to 500 nM) on mΔΨ and respiratory rate (A), H_2O_2 formation (B), and mQ reduction level (C) in nonphosphorylating *A. castellanii* mitochondria. To exclude AOX activity, measurements were performed in the presence of 1 mM benzohydroxamate or 1.5 μM propyl gallate (H_2O_2 formation measurements).

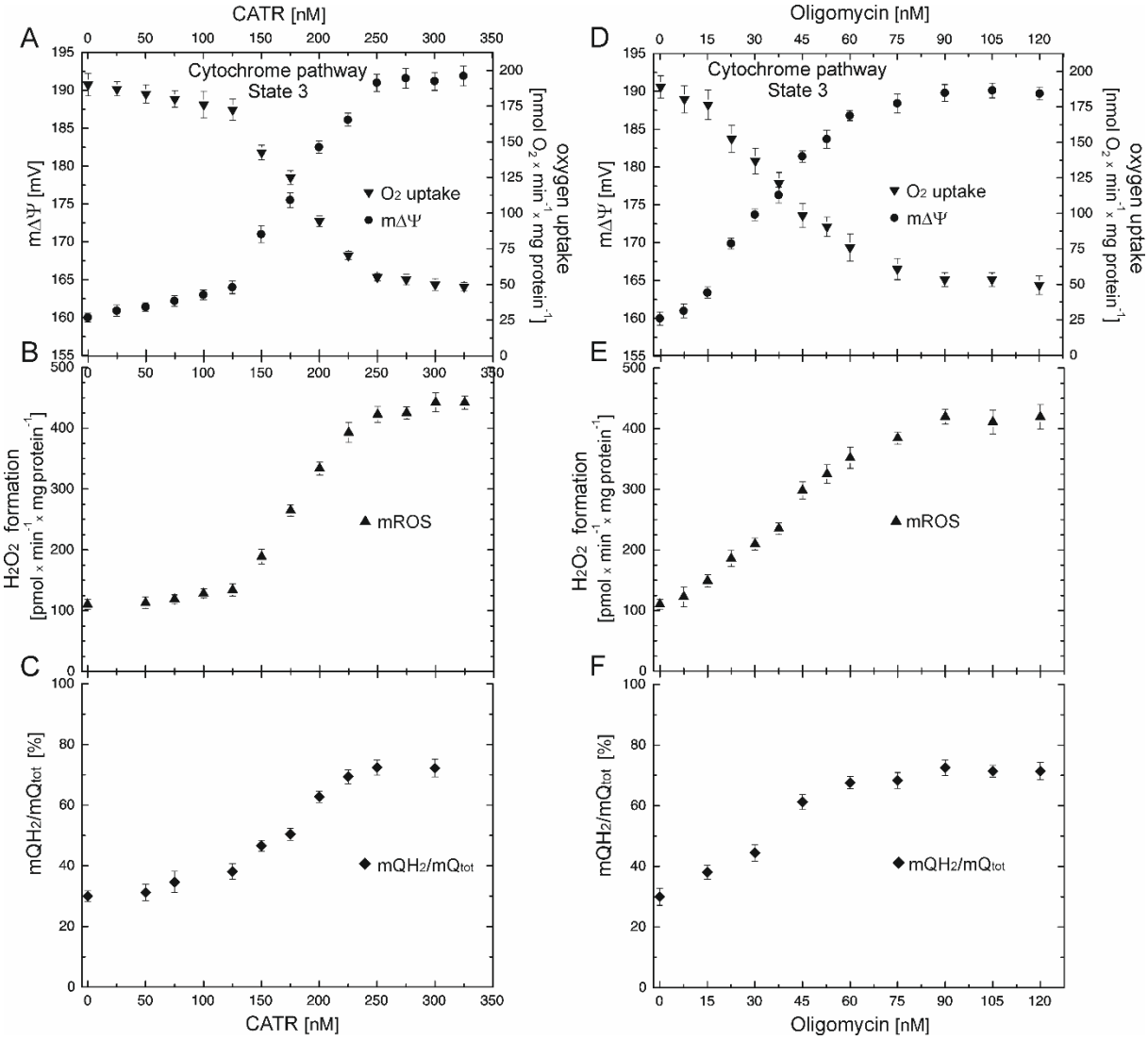

Fig. S2. Effects of increasing concentrations of carboxyatractyloside (CATR, up to 350 nM) and oligomycin (up to 120 nM) on mΔΨ and respiratory rate (A, D), H_2O_2 formation (B, E), and mQ reduction level (C, F) in phosphorylating *A. castellanii* mitochondria. To exclude AOX activity, measurements were performed in the presence of 1 mM benzohydroxamate or 1.5 μM propyl gallate (H_2O_2 formation measurements).

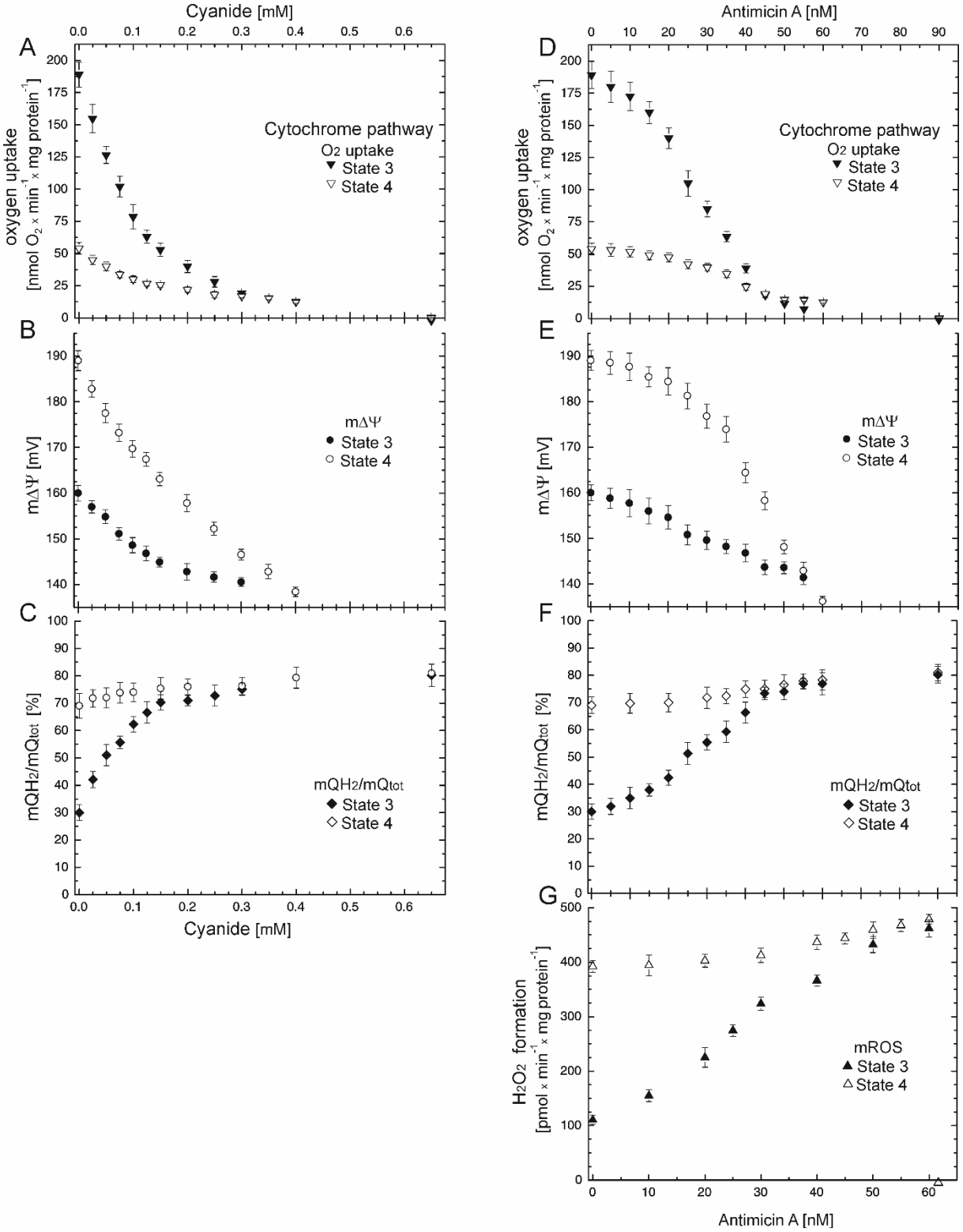

Fig. S3. Effects of increasing concentrations of cyanide (up to 0.4 mM) and antimycin A (up to 60 nM) on respiratory rate (A, D), mΔΨ (B, E), mQ reduction level (C, F), and H_2O_2 formation (for only antimycin A titration) (G) under phosphorylating (State 3) and nonphosphorylating (State 4) conditions. Effects of high concentrations of cyanide (0.65 mM) and antimycin (90 nm) are shown. To exclude AOX activity, measurements were performed in the presence of 1 mM benzohydroxamate or 1.5 μM propyl gallate (H_2O_2 formation measurements).

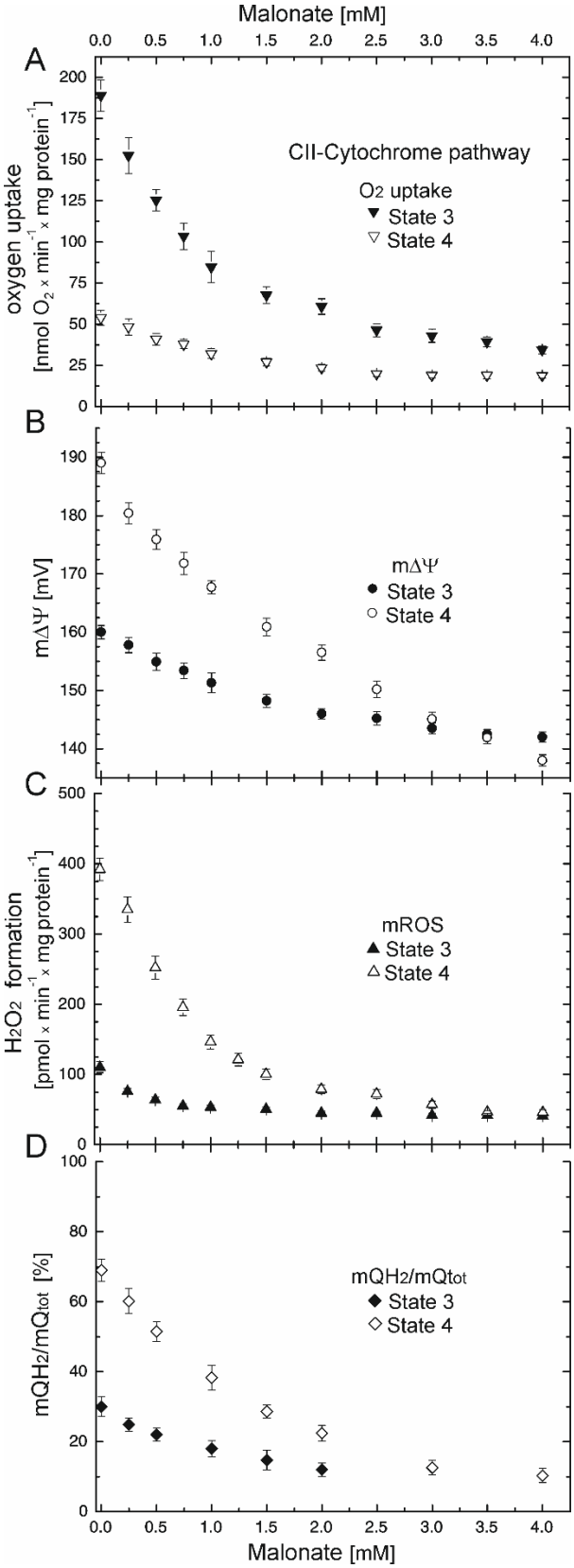

Fig. S4. Effects of increasing concentrations of malonate (up to 4 mM) on respiratory rate (A), mΔΨ (B), H_2O_2 formation (C), and mQ reduction level (D) under phosphorylating (State 3) and nonphosphorylating (State 4) conditions when the cytochrome pathway is engaged. To exclude AOX activity, measurements were performed in the presence of 1 mM benzohydroxamate or 1.5 μM propyl gallate (H_2O_2 formation measurements). CII, complex II.

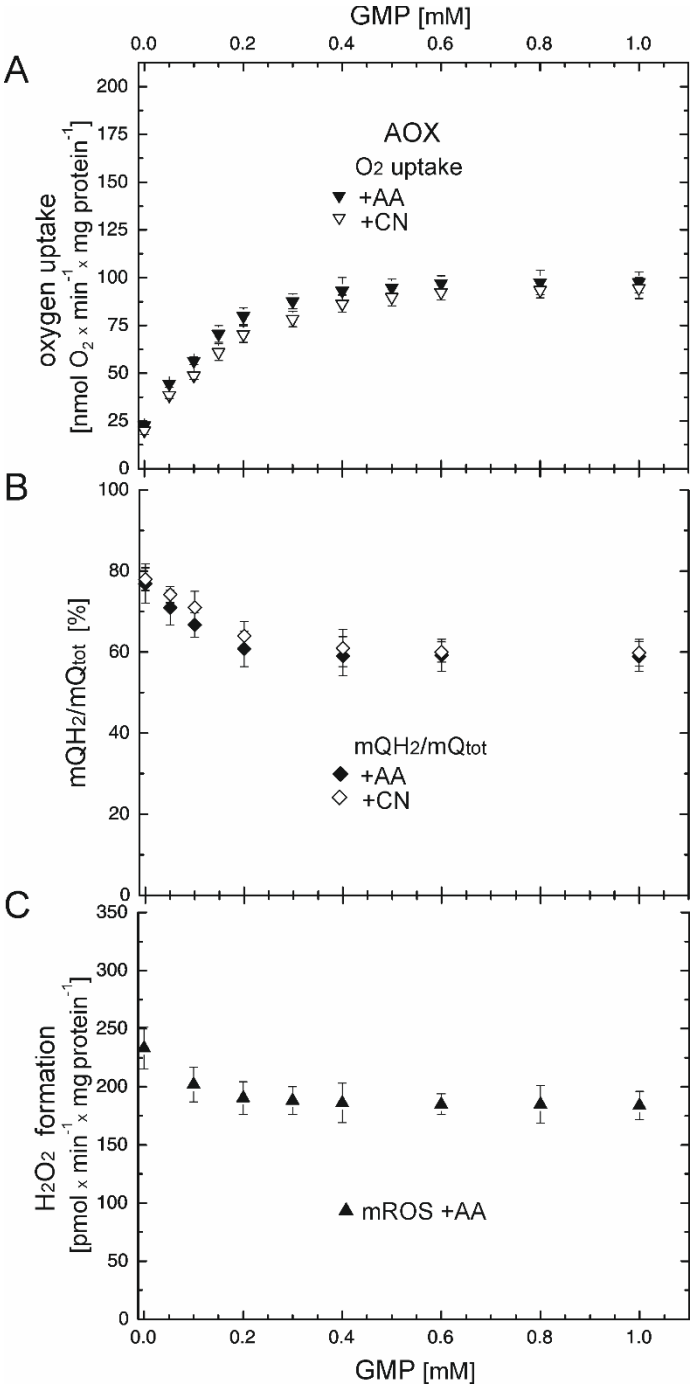

Fig. S5. Effects of increasing concentrations of GMP (up to 1 mM) on respiratory rate (A, C), mQ reduction level (B, D), and H_2O_2 formation (E). To exclude cytochrome pathway activity, measurements were performed in the presence of 0.65 mM cyanide (+CN) (A, B) or 90 nM antimycin A (+AA) (C-E).

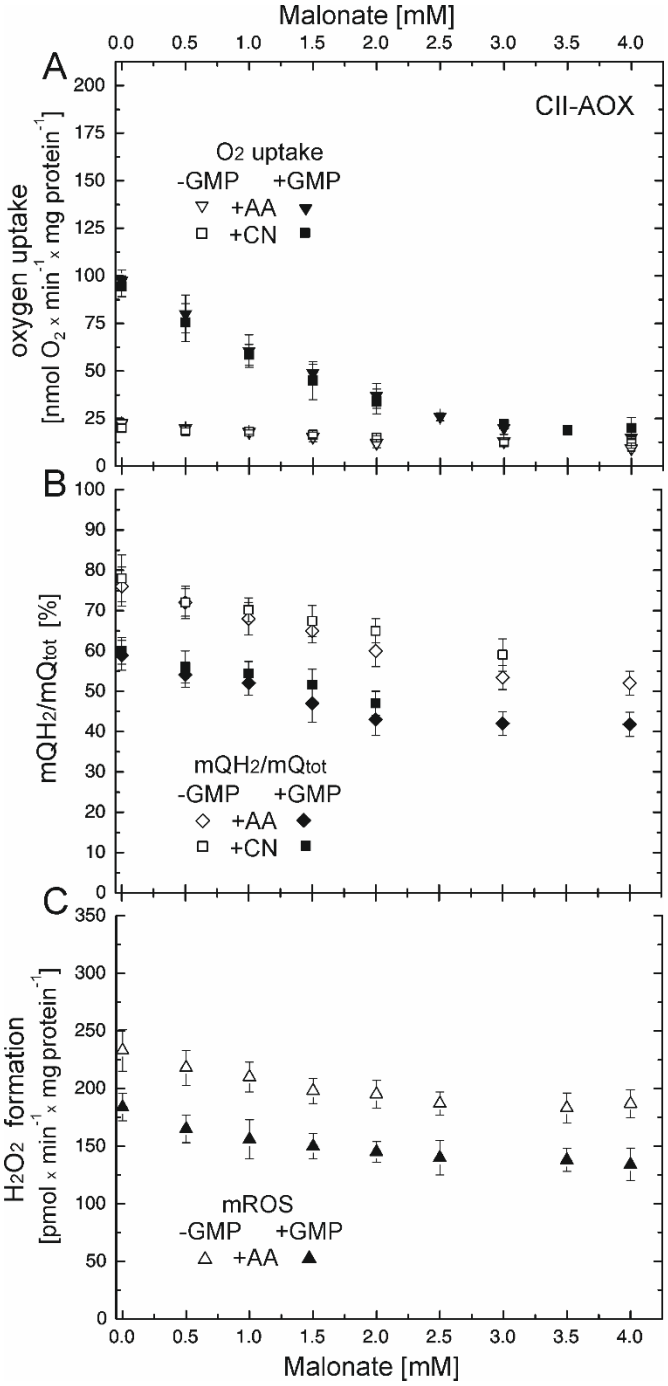

Fig. S6. Effects of increasing concentrations of malonate (up to 4 mM) on respiratory rate (A), H_2O_2 formation (B), and mQ reduction level (C) when AOX is engaged. To exclude cytochrome pathway activity, measurements were performed in the presence of 0.65 mM cyanide or 90 nM antimycin A. CII, complex II.

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Original article

Lung mitochondria adaptation to endurance training in rats

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ABSTRACT

We elucidated the impact of eight weeks of endurance training on the oxidative metabolism of rat lungs. Adult 3.5-month-old male rats were randomly allocated to a treadmill training group or a sedentary group as control. In the lungs, endurance training raised the expression level of the oxygen sensors hypoxia inducible factor 1α (HIF1α) and lysine-specific demethylase 6A (KDM6A) as well as stimulated mitochondrial oxidative capacity and mitochondrial biogenesis, while lactate dehydrogenase activity was reduced. Endurance training enhanced antioxidant systems (the coenzyme Q content and superoxide dismutase) in lung tissue but decreased them (and uncoupling protein 2) in lung mitochondria. In the lung mitochondria of trained rats, the decreased Q content and Complex I (CI) activity and the enhanced cytochrome pathway activity (CIII + CIV) may account for the diminished Q reduction level, resulting in a general decrease in H_2O_2 formation by mitochondria. Endurance training enhanced oxidation of glutamate and fatty acids and caused opposite effects in functional mitochondrial properties during malate and succinate oxidation, which were related to reduced activity of CI and increased activity of CII, respectively. In addition, endurance training downregulated CI in supercomplexes and upregulated CIII in the $CIII_2$+CIV supercomplex in the oxidative phosphorylation system. We concluded that the adaptive lung responses observed could be due to hypoxia and oxidative stress induced by strenuous endurance training.

1. Introduction

The lungs constitute an essential part of the gas exchange system, which plays a key role in maintaining body energy homeostasis both at rest [1] and especially during sustained physical exercise [2–4]. It is considered that the functional capacity of the respiratory system is overbuilt for gas exchange demands required even during intense exercise in young healthy, untrained people [5–7]. Paradoxically, however, in the case of highly trained endurance athletes, the lungs seem to limit the performance of athletes during strenuous physical exercise [7–9]. It is generally accepted that the functional capacity of the lungs in endurance athletes is greater than that in untrained people [10]. On the other hand, it is believed that the structural properties of the lungs and airways are rather resistant to physical training in elite athletes [6,8]. It is surprising, however, that the effect of physical training on lung tissue, especially on the mitochondrial content, mitochondrial respiratory capacity, and mitochondrial energy efficiency, is little known. However, it seems very likely that some stress factors to which the lung tissue is exposed by repeated intense physical exercise, such as exercise-induced hypoxemia, increased mechanical stress on the lungs, changes in blood

Abbreviations: CS, citrate synthase; COX, cytochrome *c* oxidase, Complex IV; CI–CIV, complexes of respiratory chain; LDH, lactate dehydrogenase; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; mROS, mitochondrial ROS; Q, coenzyme Q, quinone; mQ, mitochondrial Q; mQH_2/mQtot, mQ reduction level (reduced mQ/total mQ); UCP(2), uncoupling protein (2); mΔΨ, mitochondrial transmembrane electrical potential.

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acid-base balance, hyperthermia, tissue hypoxia, reactive oxygen species (ROS) formation, and other exercise-related factors, may cause some adaptive changes in lung mitochondria.

In addition to their vital function in energy production *via* oxidative phosphorylation (OXPHOS), lung mitochondria are essential for nutrient and oxygen sensing and for regulating critical cell processes, including cell death and inflammation [11,12]. Importantly, mitochondria are the main source of ROS and thus the main target of oxidative stress [13–16]. Under physiological conditions, including physical exercise, mitochondria-produced ROS (mROS) are signaling molecules involved in important signaling pathways [17]. Under pathophysiological conditions, mROS produced in excess contribute to oxidative stress, which is a key factor in the pathogenesis of lung diseases [12,18].

The purpose of this study was to investigate the effects of endurance training on oxidative metabolism, including mitochondrial biogenesis, respiratory capacities and the coenzyme Q (Q) content in rat lungs. In addition, we examined endurance training-induced alterations at the level of isolated lung mitochondria by measuring mitochondrial respiratory activities, transmembrane potential (m$\Delta\Psi$), OXPHOS efficiency, uncoupling, mROS production, mQ content and mQ reduction level, and the molecular organization of supercomplexes and complexes of OXPHOS components. Our results show that changes in pulmonary mitochondria caused by endurance training can be important for lung energy metabolism and redox homeostasis and thus exercise performance.

2. Material and methods

2.1. Chemicals

All chemicals were the highest available grade and were purchased from Sigma-Aldrich unless otherwise mentioned.

2.2. Animals and endurance training

Twenty eight adult 3.5-month-old male rats (Wistar) were randomly allocated to a training group ($n = 14$) or a sedentary group ($n = 14$) as control. The conditions for keeping animals are described in Supplementary material. Experimental protocols involving animals, their surgery and care were approved by the Local Ethics Committee on Animal Experimentation in Krakow, Poland (Permit Number: 197/2018), and were in compliance with the guidelines of the European Community Council Directive 2010/63/UE of September 22, 2010 on the protection of animals used for scientific purposes.

The applied training schedule, which was essentially adapted from Ref. [19] and similar to that previously used by our group [20,21], resulted in a significant increase in the mitochondrial biogenesis in the limb skeletal muscles. Rats from the trained group ($n = 14$) performed an eight-week endurance training (5 days per week) on a treadmill for rodents (Exer-6M Treadmill; Columbus Instruments, Ohio, USA) (for details, see Supplementary material). The total distance covered by the rats from the training group during the eight weeks of endurance training was 36.8 ± 12.8 km (SD). Twenty four hours after the end of the last training, exercising and sedentary rats were sacrificed by decapitation, and lungs were collected for testing. We made all efforts to minimize the suffering of the experimental animals.

A comparison of control and trained animals indicated that eight weeks of training caused a significant reduction in animal body weight and an increase in lung mitochondrial yield/kg/rat weight (Supplementary material, Table S1). No change in lung weight or mitochondrial yield per gram of lung was observed.

2.3. Lung homogenate and mitochondria preparation

All procedures were carried out at 4 °C. The collected lungs were placed in isolation medium A (pH 7.2) containing 50 mM Tris-HCl, 100 mM sucrose, and 0.5 mM ethylenediaminetetraacetic acid (EDTA) and washed several times. After removing the trachea, large airways and vessels, the lungs were minced over ice. The minced tissue was filtered through a strainer to remove remaining blood and then homogenized in isolation medium B containing 50 mM Tris-HCl, pH 7.2, 100 mM sucrose, 100 mM KCl, 1 mM KH_2PO_4, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 0.5 mM EDTA using a Teflon pestle. The strained homogenate was centrifuged at 850 *g* for 8 min. The resultant supernatant was filtered. Part of the filtered homogenate was used for enzymatic studies and the determination of protein levels. The greater part of the homogenate was supplemented with isolation medium B supplemented with 0.2% BSA and centrifuged at 17,800 *g* for 15 min. Mitochondrial pellet suspended in isolation medium B without BSA was centrifuged at 850 *g* for 8 min. The supernatant was filtered and again centrifuged at 17,800 *g* for 15 min. The final pellet of mitochondria was then resuspended in medium B (without BSA). Protein concentration of lung homogenates and mitochondria was determined by the Bradford method.

Mitochondrial yield was ~2.5 mg protein/g of lung for both trained and rats (Supplementary material, Table S1). The isolated lung mitochondria were stable for 6–7 h, and outer membrane of these mitochondria exhibited good integrity (97–98%).

2.4. Measurements of citrate synthase, cytochrome c oxidase and lactate dehydrogenase activities

The citrate synthase (CS) activity was determined spectrophotometrically at 412 nm by following the formation of 5,5′-dithiobis (2-nitrobenzoic acid)-coenzyme A (DTNB-CoA) with 100 mM DTNB [22,23]. Lactate dehydrogenase (LDH) activity was determined spectrophotometrically at 340 nm by tracking the oxidation of 150 μM NADH mixed with 10 mM pyruvate [24]. The activity of enzymes was measured in 50–70 μg of lung homogenate protein.

The maximal activity of cytochrome *c* oxidase (COX) was assessed polarographically [22]. The enzyme activity was assessed in 50–70 μg of homogenate protein or in 0.3 mg of mitochondrial protein with sequentially added 10 μM antimycin A, 7 mM ascorbate, 0.05% cytochrome *c* and up to 1.5 mM N,N,N'N'-tetramethyl-*p*-phenylenediamine (TMPD). The oxygen consumption rate measured after the TMPD addition reflects the maximum O_2 uptake by COX (Complex IV, CIV).

All measurements were carried out at 35 °C.

2.5. Respiration and membrane potential measurements in isolated mitochondria

Mitochondrial oxygen uptake was measured polarographically using oxygen electrodes [20,22] in either 3.0 ml or 0.5 ml of a standard incubation medium (pH 7.2) consisting of 75 mM sucrose, 225 mM mannitol, 5 mM KH_2PO_4, 10 mM KCl, 0.5 mM EGTA, 0.5 mM EDTA, 0.1% BSA, and 10 mM Tris/HCl with either 1.5 or 0.3 mg of mitochondrial protein (0.6 mg x ml^{-1}). m$\Delta\Psi$ was determined simultaneously with O_2 consumption using a tetraphenyl-phosphonium-specific electrode [20,22].

Phosphorylating (State 3) respiration was determined using 160 μM ADP (ADP/O measurements) or 2.5 mM ADP (maximal phosphorylating respiration). Uncoupled respiration was determined using up to 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). In the experiments we used only high-quality isolated mitochondria, i.e., with a respiratory control ratio of ~3.0–3.4 with succinate (with rotenone). Nonphosphorylating (resting state, State 4) respiration measurements were carried out without exogenous ADP. The respiratory substrate concentrations were as follows: 5 mM succinate (with 1.5 μM rotenone), 5 mM pyruvate, 5 mM malate, 5 mM glutamate, 20 μM palmitoylcarnitine (with 2 mM carnitine), and 2 mM duroquinol (with 1.5 μM rotenone). The phosphorylation rate of ADP was calculated

(phosphorylating respiration x ADP/O). mΔΨ measurements allowed accurate control of phosphorylating respiration duration.

Uncoupling protein (UCP)-mediated H^+ leak assessments (H^+ leak kinetics) during nonphosphorylating (State 4) respiration were carried out [22] with 5 mM succinate (with 1.5 μM rotenone) in the presence of 1.14 μg × ml^{-1} (2 μg/mg protein) oligomycin and 2 μM carboxyatractyloside, which block the activities of ATP synthase and ATP/ADP translocase, respectively. Both inhibitors eliminate ATP turnover-dependent H^+ leak, and carboxyatractyloside additionally eliminates inducible fatty acid-mediated H^+ leak *via* the ATP/ADP antiporter. To stimulate UCP activity, 10 μM linoleic acid was used. To block UCP activity, 1.5 mM GTP was added. To reduce the rate of the mQ-reducing pathway, succinate oxidation was titrated with up to 20 μM cyanide.

2.6. Assay of mitochondrial H_2O_2 production

The mitochondrial H_2O_2 production rate was measured by the Amplex Red assay [23,25]. The fluorescence kinetics was followed for 40 min at 545 nm/590 nm using a Tecan multimode reader (Infinite M200 PRO). Mitochondria (0.3 mg of protein) were incubated with 5 mM malate, 5 mM succinate (with 1.5 μM rotenone), or 5 mM malate and 5 mM succinate in the absence (nonphosphorylating conditions) or presence of 2.5 mM ADP (phosphorylating conditions). Reactions were calibrated with known H_2O_2 amounts.

2.7. Measurements of the tissue and mitochondrial Q concentrations and the mQ reduction level

Concentrations of tissue and mitochondrial Q9 (a dominant Q form in rat lungs) and Q10 (a less abundant Q form in rat lungs) were determined in lung homogenates and lung mitochondria by an extraction technique and HPLC detection [25,26]. For the quantification and calibration of the Q9 and Q10 peaks, commercial Q9 and Q10 were used.

The mQ reduction level (mQH_2/mQtot), i.e., the ratio of reduced mQ (mQH_2) to the total mQ in the lung mitochondria under steady-state respiration, was determined. Mitochondria (1.5 mg of protein) were incubated in 3.0 ml of standard incubation medium with 5 mM malate, 5 mM succinate (with 1.5 μM rotenone), or 5 mM malate and 5 mM succinate in the absence (nonphosphorylating conditions) or presence of 2.5 mM ADP (phosphorylating conditions). The reduction level of mQ is expressed as the percentage of total mQ (mQH_2/mQtot).

2.8. Immunodetection of protein levels

RIPA buffer was used to lyse the lung homogenates. The proteins were separated on 6–12% SDS-PAGE gels. The PageRuler Prestained™ Protein Ladder (Thermo Fisher Scientific) was used as a marker of molecular weight. The applied Abcam primary antibodies were raised against: CS (46 kDa) (ab96600), COX subunit II (COXII, 24 kDa) (ab110258), voltage-dependent anion channel 1 (VDAC1, 35 kDa) (ab14734), mitochondrial transcription factor A (TFAM, 28 kDa) (ab131607), nuclear factor erythroid 2-related factor (NRF2, 68 kDa) (ab137550), peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α, 92 kDa) (ab54481), superoxidase dismutase 1 (SOD1, 18 kDa) (ab13498), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 37 kDa) (ab9485), UCP2 (33 kDa) (ab97931), acyl-coenzyme A dehydrogenase (ACADS, 44 kDa) (ab154823), glutamate dehydrogenase (GDH, 61 kDa) (ab89967), mitochondrial Coenzyme Q-binding protein CoQ10 homolog B (CoQ10B, 46 kDa) (ab41997). Abcam total OXPHOS wb antibody cocktails (human, ab110411 and rodent, ab110413) were used, which contain antibodies against subunits of CI (20 kDa subunit NDUFB8), CII (30 kDa subunit SDHB), CIII (subunit Core 2, 48 kDa), CIV (COXII, 24 kDa or COXI, 40 kDa), and ATP synthase (CV) (subunit α, 57 kDa). We also used primary antibodies from other manufacturers raised against: hexokinase I (HK I, 120 kDa) (sc80978, Santa Cruz Biotechnology), LDH (35 kDa) (PA5-27406, Invitrogen), lysine (K)-specific demethylase 6A (KDM6A, 140 kDa) (PA5-68598, Thermo Fisher Scientific), hypoxia-inducible factor 1-alpha (HIF-1α, 115 kDa) (PA5-85494, Thermo Fisher Scientific), and mitochondrial Mn superoxide dismutase (SOD2, 25 kDa) (ADI-SOD, Enzo Life Sciences). As loading controls and for data normalization, the expression levels of GAPDH or β actin (42 kDa) (CP01, Calbiochem) (the homogenate fractions) and COX subunits or CS (the mitochondrial fractions) were used. Protein bands were digitally quantified using ImageJ software. Each protein separation on the gel had additional loading control by Ponceau staining (Supplementary Figs. S1, S2).

2.9. BN-PAGE and in-gel activity assays

After solubilization, mitochondrial proteins (50–200 μg) were separated on 1.5-mm-thick 3–11% gradient minigels. The activities of respiratory chain complexes CI, CII, CIV, and CV were detected [27,28]. To determine the OXPHOS complexes with immunoblotting, the BN-PAGE-separated proteins were transferred onto nitrocellulose membranes [23]. Anti-UQCRC2 antibody (against CIII, ab14745, Abcam) or the total OXPHOS rodent wb antibody cocktail (ab110413, Abcam) was used for immunodetection. Each OXPHOS complex separation on the BN-PAGE gel had a loading control by Coomassie staining (Supplementary Fig. S3).

2.10. Statistical analysis

The data is presented as the mean ± SD from at least three independent experiments (lung homogenate preparations and mitochondrial isolations). Each determination was carried out in at least duplicates. Significant differences were determined using unpaired *t*-tests or ANOVA (followed by Tukey's post hoc tests for $p < 0.05$). Differences were considered statistically significant if $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3. Results

3.1. In the lungs, endurance training upregulates mitochondrial oxidative capacities and mitochondrial biogenesis

We elucidated mitochondrial oxidative capacities by measuring the maximal activity of CS (a pace-making enzyme in the first step of the Krebs cycle) and COX (CIV of the respiratory chain) in lung homogenates of control and trained rats. In lung homogenates of trained rats, an over 40% increase in the activities of both enzymes was observed (Fig. 1A and B). CS and COX protein levels also increased (by ~20% and ~70%, respectively) (Fig. 1E).

Interestingly, augmented mitochondrial oxidative capacities were not accompanied by an alteration in the expression level of HK1, the rate-limiting enzyme of glycolysis (Fig. 1E). However, we observed a statistically significant reduction in the activity (~16%) and expression level (~10%) of LDH (Fig. 1C, E), an enzyme that converts pyruvate, the final product of glycolysis, to lactate. It should be noted, that many cells (mainly in muscles, heart, brain and liver) can use lactate as an oxidative metabolic fuel, and for this purpose lactate is converted back to pyruvate by LDH [29]. It is not known whether lactate is used continuously in pulmonary cells under fully aerobic conditions. In lung homogenates of trained rats, the ratio of COX activity to LDH activity was enhanced by more than 80% (Fig. 1D), indicating a significant increase in the contribution of aerobic respiration not associated with lactate as a potential fuel to lung cellular respiration.

The enhanced CS and COX activities and their protein levels (Fig. 1) indicate increased mitochondrial biogenesis in the lungs of trained animals. In addition, endurance training induced significantly higher expression levels of other mitochondrial biogenesis markers, including VDAC1, TFAM, NRF2, and PGC1α (Fig. 2).

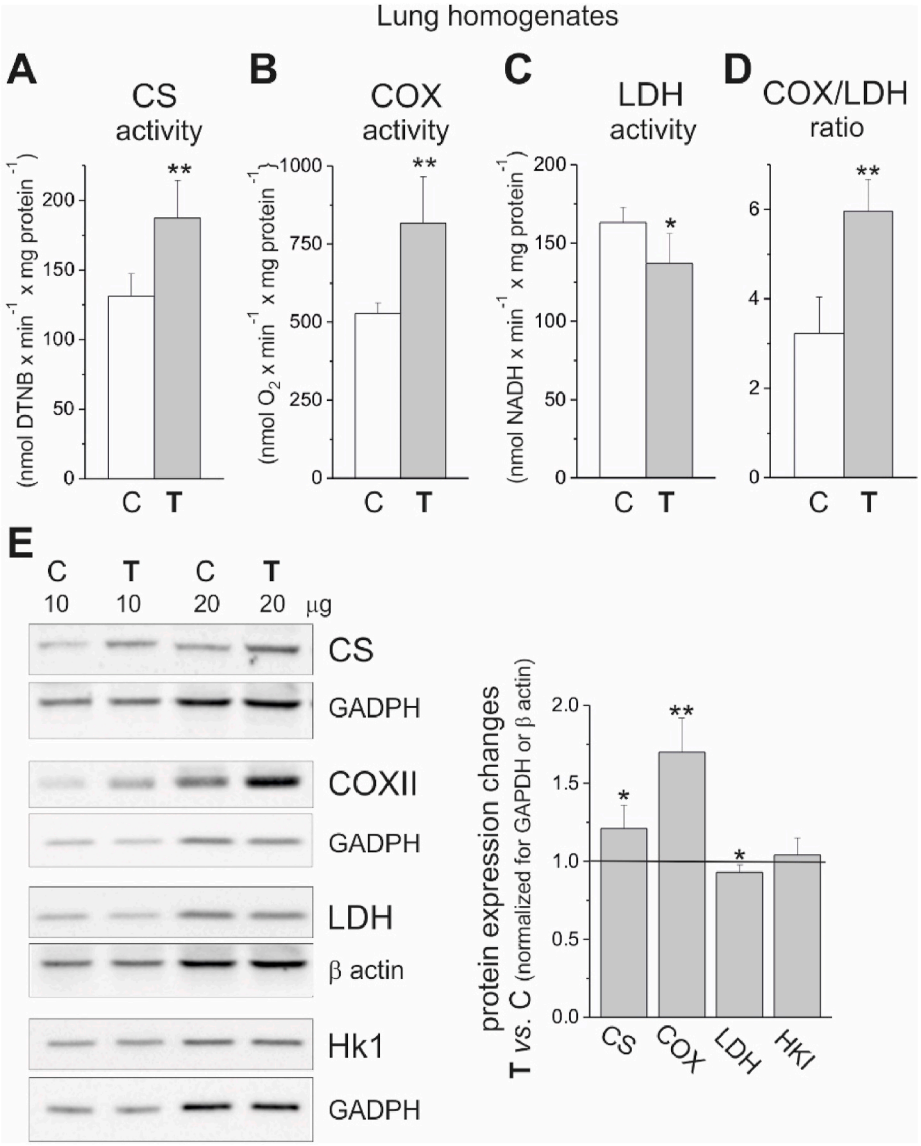

Fig. 1. Endurance training upregulates mitochondrial oxidative capacities. Maximal activities of key enzymes of aerobic (**A**, **B**) respiration and LDH (**C**), the ratio of COX activity and LDH activity (**D**), and representative western blots and analysis of protein expression (**E**) in lung homogenates from control (C) and trained (T) rats. DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); CS, citrate synthase; COX, cytochrome *c* oxidase; COXII, COX subunit II; LDH, lactate dehydrogenase; HKI, hexokinase I; loading controls: GAPDH, glyceraldehyde 3-phosphate dehydrogenase and β actin. Mean ± SD; $n = 5$. $p < 0.05$ (*), $p < 0.01$ (**), comparison *vs.* control values.

3.2. Endurance training increases antioxidant systems in the lungs and lowers them at the level of pulmonary mitochondria

Given the increased mitochondrial biogenesis and increased mitochondrial oxidative capacities in the lungs in response to endurance training and the fact that mitochondria are the main source of ROS, it was important to check the level of antioxidant systems. This level would indicate the need to attenuate the mROS excess. Interestingly, enhanced levels of Q (both Q9 and Q10 by over 50%) (Fig. 3A) and SOD1 expression (Fig. 3B) were observed in lung homogenates of trained rats. However, in contrast, reduced amounts of mQ (mQ9 by 16% and mQ10 by ~42%) (Fig. 3C) and slightly diminished expression levels of the antioxidant proteins SOD2 and UCP2 (Fig. 3D) were observed after eight weeks of endurance training in the lung mitochondria. In addition, UCP-mediated mitochondrial uncoupling determined from the H^+ conductance kinetics in the presence of OXPHOS inhibitors (Fig. 3E) was significantly reduced in the lung mitochondria of trained rats. Namely, the fatty acid-induced, GTP-inhibited H^+ leak determined at the highest common $m\Delta\Psi$ value (165 mV) was ~25% lower in the lung mitochondria from trained rats. Thus, eight weeks of endurance training decreased UCP2 level and activity in the lung mitochondria.

3.3. At the level of lung mitochondria, endurance training does not change the oxidation of pyruvate but increases the oxidation of fatty acids and glutamate

We determined the impact of eight weeks of training on respiratory activity at the mitochondrial level by measuring the maximal respiratory rate of isolated lung mitochondria with different respiratory substrates (Fig. 4A). Maximal oxidation (under uncoupling or phosphorylating conditions) of weaker reducing substrates was unchanged (with pyruvate) or slightly increased (with glutamate and palmitoylcarnitine) in the lung mitochondria from trained rats. The increased oxidation of both substrates was accompanied by increased expression of GDH, the enzyme that converts glutamate to α-ketoglutarate, which can be oxidized in the Krebs cycle (Fig. 4B). The expression level of ACADS, the enzyme catalyzing the first step β-oxidation of fatty acids, was also augmented in the lung mitochondria of the trained rats. These changes may indicate an increased contribution of glutamine and fatty acids as fuel sources for oxidative metabolism in the lungs of trained animals.

In lung mitochondria of trained and control rats, the highest maximal respiratory rate was observed with succinate alone (Fig. 4A), which appears to saturate the respiratory chain capacity. Surprisingly, in the mitochondria of trained rats compared to the mitochondria of sedentary

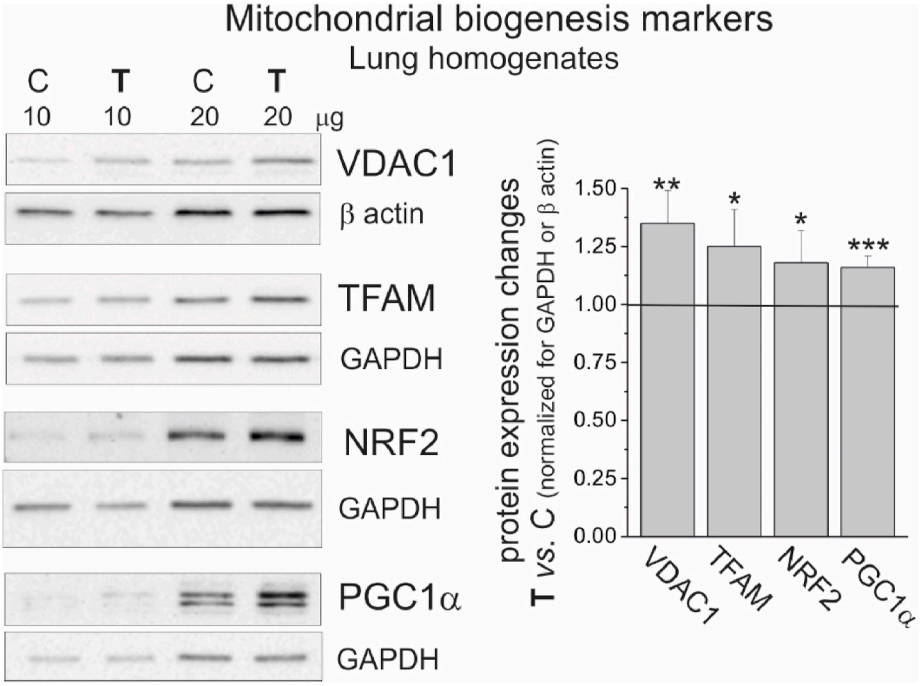

Fig. 2. Endurance training upregulates mitochondrial biogenesis. Representative western blots and analyses of protein expression of marker proteins in lung homogenates from control (C) and trained (T) rats. VDAC 1, voltage-dependent anion channel 1; TFAM, mitochondrial transcription factor A; NRF2, nuclear factor erythroid 2-related factor; PGC1α, peroxisome proliferator-activated receptor γ coactivator 1α; loading controls: GAPDH and β actin. Mean ± SD; $n = 5–8$. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), comparison vs. control values.

rats, opposite changes were detected in the maximal oxidation of CII substrate (succinate), which increased, and CI substrate (malate), which decreased. Therefore, we then examined the effects of these opposing changes on the mitochondrial ATP synthesis efficiency, mΔΨ, mROS production, and mQ reduction level.

3.4. Endurance training causes opposite effects on O_2 consumption, mΔΨ, the respiratory control ratio and the ADP phosphorylation rate during oxidation of malate (CI substrate) and succinate (CII substrate)

The functional parameters of lung mitochondria were compared during the oxidation of succinate alone (with rotenone), malate alone, and their mixture under nonphosphorylating (State 4) and phosphorylating (State 3) conditions. In the lung mitochondria of trained rats, an ~16% decrease in respiratory rates with malate and an ~19% increase in respiratory rates with succinate were observed under both respiratory conditions (Fig. 5A). In addition, under nonphosphorylating conditions, changes in respiration with the CI and CII substrates were accompanied by statistically significant changes (decrease and increase, respectively) in mΔΨ (Fig. 5D). For all tested substrates (Table 1), endurance training did not affect the ADP/O ratio, and thus OXPHOS efficiency. As a consequence, in the lung mitochondria of trained animals, the opposite effect on the phosphorylation rate was observed in the case of malate oxidation (19% decrease) and succinate oxidation (23% increase). During oxidation of succinate plus malate, the State 3 respiratory rate (Fig. 5A), phosphorylation rate, and respiratory control ratio (Table 1) were significantly elevated (by 22%, 29%, and 14%, respectively), indicating augmented ATP synthesis in lung mitochondria of trained animals with the involvement of both entrances (CI and CII) to the respiratory chain.

3.5. In the lung mitochondria, endurance training reduces H_2O_2 production resulting from a decreased mQ reduction level

Surprisingly (given the endurance training-induced opposite changes in succinate- and malate-sustained respiration), regardless of the substrate used, H_2O_2 production was always lower by 21–26% and 14–19% for nonphosphorylating and phosphorylating respiratory conditions, respectively in the lung mitochondria of trained rats (Fig. 5B). Consequently, in the lung mitochondria of trained rats, regardless of the substrate (malate alone, succinate alone or succinate plus malate), the reduction level of mQ always declined under both nonphosphorylating and phosphorylating conditions (Fig. 5C). The obtained relationship between H_2O_2 formation and the mQ reduction level during tested substrate oxidation under nonphosphorylating and phosphorylating conditions (Fig. 6) confirmed a direct dependence of mROS production on the mQ reduction level [25]. The points obtained for both types of lung mitochondria showed a single linear relationship (Fig. 6). Supplementary Fig. S4 presents disturbances in the relationship between mΔΨ and the reduction level of mQ, and between mitochondrial H_2O_2 production and mΔΨ as a result of changes occurring in pulmonary mitochondria under the influence of training.

To understand the general decrease in mROS production and the mQ reduction level observed in lung mitochondria of trained rats with the involvement of CI, CII or both, we looked at endurance training-induced changes in the activity and amount of individual components of the OXPHOS system and its molecular organization.

3.6. In the lung mitochondria, endurance training leads to an alteration in the molecular organization of the OXPHOS system: downregulation of CI in supercomplexes and upregulation of CIII in the $CIII_2$+CIV supercomplex

Western blot analysis showed that in the lung mitochondria of trained rats, the expression level of CI (subunit NDUFB8) was reduced by ~12%, while the expression of CII (subunit SDHB) was increased by ~13% (Fig. 7). From the remaining complexes of the OXPHOS system, the expression level of CIII (subunit Core 2) increased by ~14%, while the expression levels of CIV (subunits COXII and COXI) and ATP synthase (subunit α) remained unchanged. In addition, BN-PAGE followed by in-gel activity assays revealed that after endurance training, CII activity was enhanced and CV (ATP synthase) activity remained unchanged (Fig. 8A and B).

Interestingly, there was an ~18% decrease in CI activity in all CI-associated supercomplexes (I + III_2, I + III_2+IV, and I + III_2+IV_n) of lung mitochondria from trained rats (Fig. 8A and B). BN-PAGE followed by immunoblotting showed that in these supercomplexes, the amount of CIII was unchanged, although a statistically insignificant decrease was observed in the I + III_2 supercomplex. In addition, an ~27% statistically

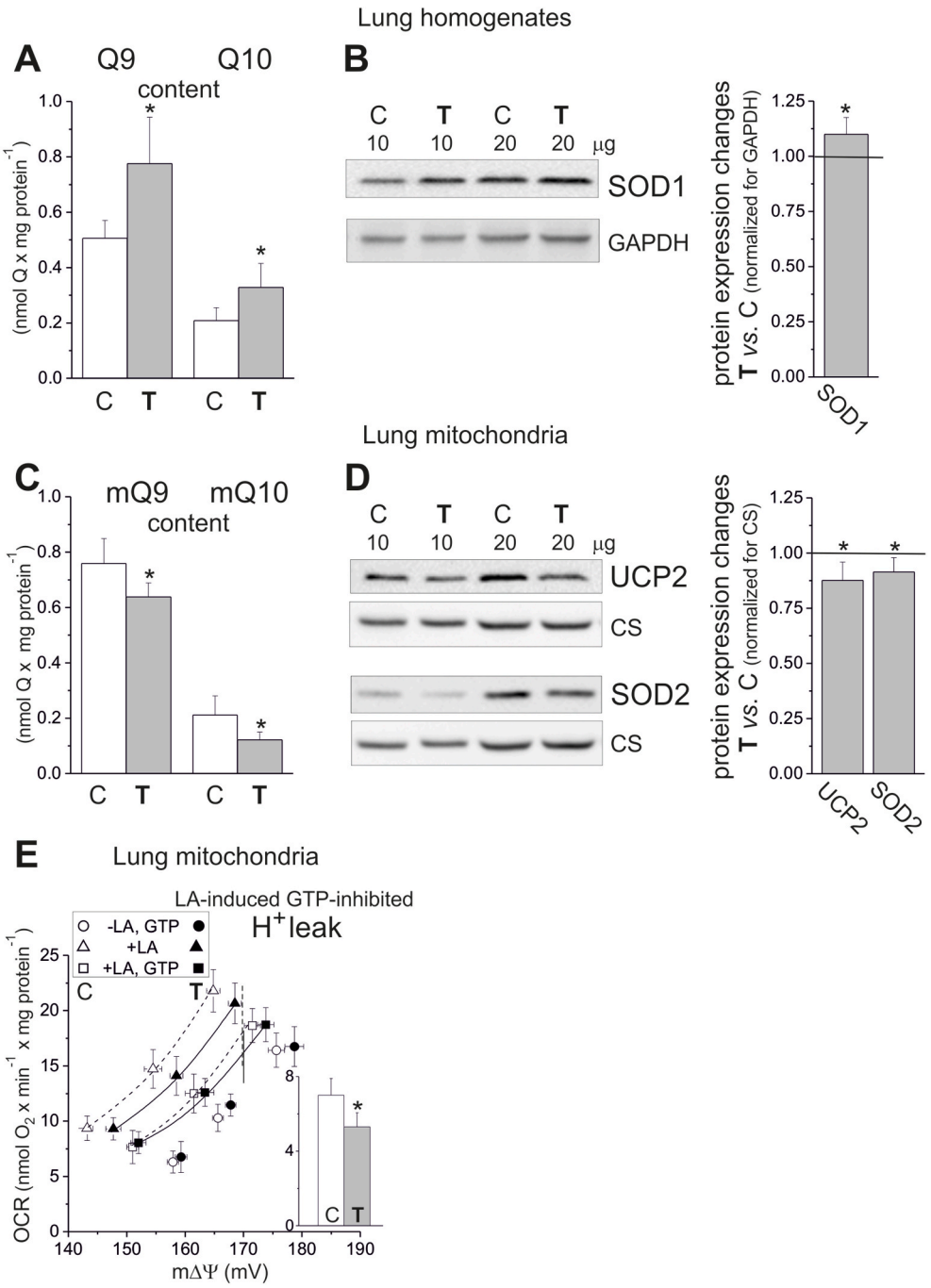

Fig. 3. Endurance training increases antioxidant systems in the lungs and lowers them at the level of mitochondria. Comparison of lung homogenates and mitochondria from control (C) and trained (T) rats. Coenzyme Q content in homogenates (Q9 and Q10) (**A**) and mitochondria (mQ9 and mQ10) (**C**). Representative western blots and analyses of protein expression (**B**, **D**). SOD1, SOD2, superoxide dismutase 1, 2; UCP2, uncoupling protein 2; loading controls: GAPDH or CS. Linoleic acid (LA)-induced GTP-inhibited UCP-mediated H^+ leak (**E**). The relationship between the O_2 consumption rate (OCR) and mΔΨ obtained from cyanide titration of succinate oxidation under nonphosphorylating conditions. The LA-induced GTP-inhibited H^+ leak (in nmol O_2 x min^{-1} x mg $protein^{-1}$) at the same mΔΨ (165 mV) (**E**, ***inset***). Mean ± SD; n = 5–8. $p < 0.05$ (*), comparison *vs.* control values.

significant increase in the amount of CIII was found in the lung mitochondrial III_2+IV supercomplex from trained rats. These observations are consistent with the slightly increased expression level of CIII observed after SDS-PAGE (Fig. 7) and with the increased CIII activity, measured during oxidation of duroquinol (an artificial substrate of CIII) (Fig. 8C) in intact lung mitochondria of trained animals. Considering that the CIV (COX) activity in intact mitochondria (Fig. 8C) and in BN gel (Fig. 8A and B) remained unchanged, it can be concluded that CIV was not a limiting factor in the lung mitochondria of trained rats, although the activity and amount of CIII increased.

The reduced activity of CI in all CI-associated supercomplexes shown by BN-PAGE analysis (Fig. 8) was accompanied by a reduced amount of mQ in the inner membrane of lung mitochondria of trained animals (Fig. 3A). In addition, a significant reduction in the expression level of mitochondrial Q-binding protein (CoQ10B) (Supplementary Fig. S2) required for mQ function in the respiratory chain was observed in the lung mitochondria of trained rats.

3.7. *In the lungs, endurance training can cause hypoxic conditions*

Some of the endurance training-induced alterations in lung mitochondrial properties observed in this study, i.e., reduced CI activity, increased CII activity, and increased glutamate oxidation, indicate a lung response to hypoxic conditions. An over 25% increase in the expression of HIF1α, a marker of hypoxia and oxidative stress, in the lungs of trained rats (Fig. 9) confirms this observation. Interestingly, a weak but statistically significant (11%) enhancement of the expression of histone demethylase KDM6A, a direct cellular oxygen sensor

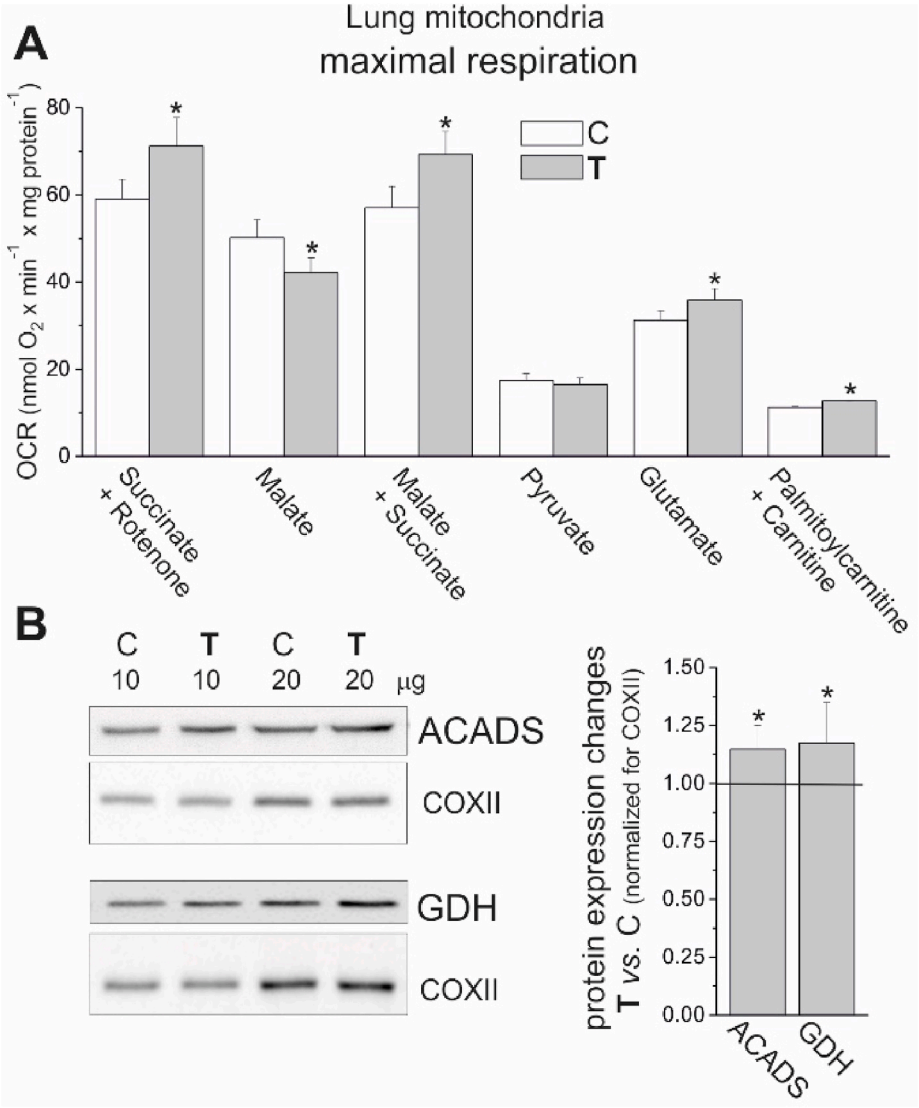

Fig. 4. Endurance training induces changes in the respiratory substrate oxidation. Comparison of lung mitochondria from control (C) and trained (T) rats. Maximal (phosphorylating or uncoupled) respiration with various respiratory substrates (**A**). OCR, oxygen consumption rate. Representative western blots and analyses of protein expression of ACADS, acyl-coenzyme A dehydrogenase, and GDH, glutamate dehydrogenase (**B**). Loading control: COXII, cytochrome *c* oxidase subunit II. Mean ± SD; $n = 4–10$. $p < 0.05$ (*), comparison *vs.* control values.

regulating chromatin [30,31], was also observed in the lungs of trained rats (Fig. 9).

4. Discussion

Physical exercise can modify the morphological and functional status of various body systems [32,33]. Despite the fact that lung function is intensified during all types of physical activities, surprisingly to the best of our knowledge, no studies have been performed showing the adaptation of lung tissue mitochondria during physical training. Our study indicates that eight-week endurance training, during which the animals covered an average of ~37 km, at a relatively high intensity, caused many alterations in bioenergetic functioning of mitochondria in the lungs.

For the first time, we have shown that strenuous endurance training enhanced mitochondrial biogenesis in the lungs, as indicated by a considerable increase (~40%) in the maximal activities of CS and COX, as well as the levels of these enzymes (Fig. 1) and other mitochondrial biogenesis protein markers (Fig. 2). In addition, in the lung mitochondria of trained rats, the eight-week endurance training caused a considerable increase (~30%) in the ADP phosphorylation rate (Table 1), resulting from augmented CII and CIII activity (Figs. 4A, 5A and 8), rather than the unchanged efficiency of mitochondrial OXPHOS (ADP/O ratio) (Table 1). Taking both elements together, the overall endurance training-induced enhancement of mitochondrial phosphorylation capacity in the lungs could be significantly greater. In addition, a significant enhancement of the contribution of aerobic respiration to cellular respiration was observed in the lungs after eight weeks of training. Thus, our study for the first time shows that endurance training, potent to modify the functional capacity of various body systems [32], also improves the ability of the mitochondrial energy system of the lung tissue. Our results suggest that endurance training has beneficial effects to help cope with additional energy requirements during prolonged exercise.

Our study for the first time shows that pulmonary mitochondria are sensitive to endurance training. Lung mitochondria predominantly oxidize glucose-derived substrates for oxidative energy production [11]. However, we have shown that endurance training increases the use of other energy sources, including fatty acids and glutamate, by lung mitochondria (Fig. 4A). It remains to be clarified whether these changes are related to the mobilization of energy reserves or, as in the case of increased glutamate oxidation, to a response to reduced oxygen concentration (hypoxia).

Little is known about the role of UCP2 in the lungs. Few studies have been performed regarding the role of UCP2 in lung pathogenesis, e.g., in cancer [34]. In our study, the endurance training-induced enhancement

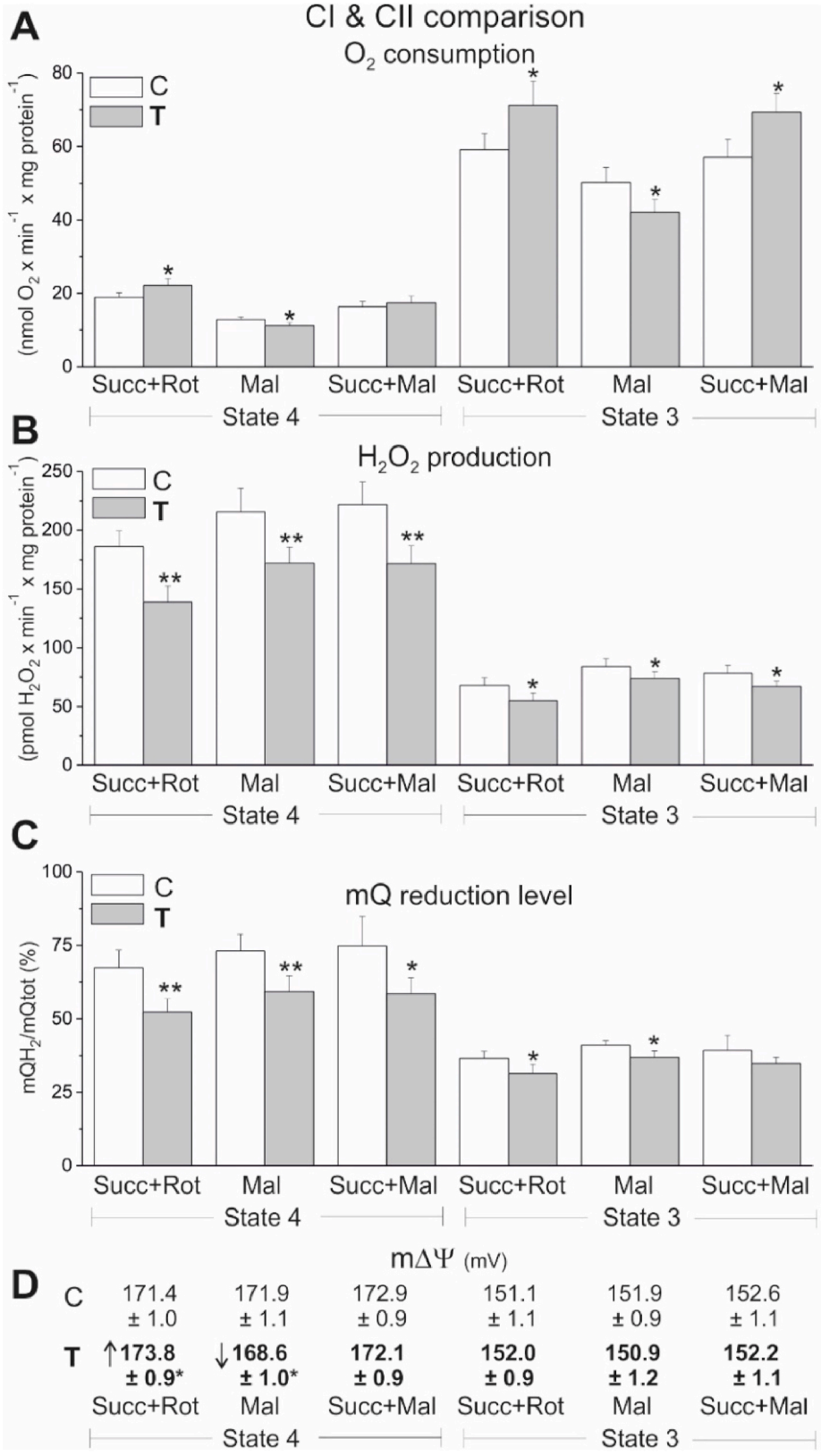

Fig. 5. Endurance training causes opposite effects during oxidation of CI substrate (malate) and CII substrate (succinate). Functional mitochondrial parameters during succinate (with rotenone) (Succ + Rot), malate (Mal), and a mixture of succinate and malate (Succ + Mal) oxidation under non-phosphorylating (State 4) and phosphorylating (State 3) conditions. O_2 consumption (**A**). H_2O_2 production rate (**B**). mQ reduction level (mQH_2/mQtot) (**C**). mΔΨ (**D**). Comparison of lung mitochondria isolated from control (C) and trained (T) rats. Mean ± SD; $n = 4–6$. $p < 0.05$ (*), $p < 0.01$ (**), comparison *vs*. control values.

of the ADP phosphorylation rate was accompanied by a significant decrease in mitochondrial UCP-mediated uncoupling and UCP2 protein levels (Fig. 3D and E). These results indicate a greater need for more efficient ATP synthesis than for the preservation of mROS formation in the lung mitochondria of trained rats. Moreover, training-induced rearrangements in the lung mitochondrial respiratory chain leading to a decline in the mQ reduction level (Fig. 5C) are not conducive to the activation of UCP2. Namely, it has previously been shown that a diminished reduction level of mQ favors the inhibition of UCP activity by purine nucleotides [35].

Table 1
Mitochondrial coupling parameters, i.e., the ADP/O ratio and the respiratory control ratio (RCR), and the rate of ADP phosphorylation (phosphorylating respiration × ADP/O) in the lung mitochondria of control (C) and trained (T) rats.

	Succinate + Rotenone		Malate		Succinate + Malate	
	C	T	C	T	C	T
RCR	3.13 ± 0.32	3.21 ± 0.23	3.91 ± 0.26	3.77 ± 0.27	3.50 ± 0.31	3.98 ± 0.25*
ADP/O	1.28 ± 0.10	1.31 ± 0.12	2.37 ± 0.19	2.30 ± 0.20	1.91 ± 0.17	2.02 ± 0.19
Phosphorylation rate	76 ± 6	93 ± 8*	119 ± 12	97 ± 11*	108 ± 9	140 ± 11*

Mean ± SD ($n = 12$). *$p < 0.05$ vs. control rats.

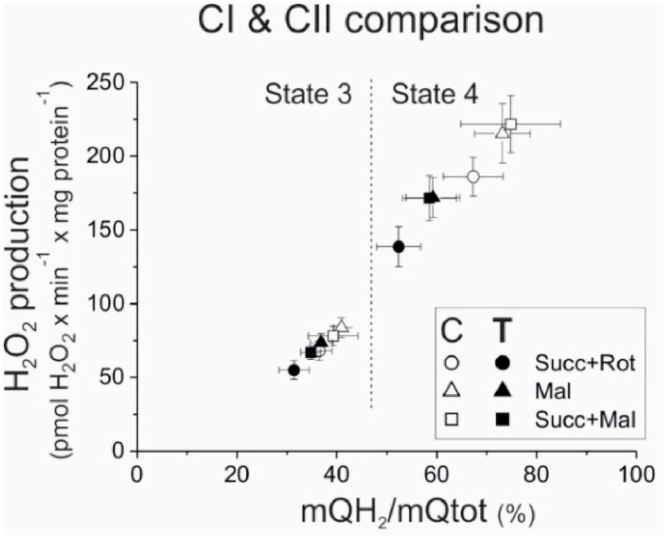

Fig. 6. The relationship between H_2O_2 formation and the reduction level of mQ during succinate (plus rotenone, Succ + Rot), malate (Mal), and a mixture of succinate and malate (Succ + Mal) oxidation under phosphorylating (State 3) conditions and nonphosphorylating (State 4) conditions of the lung mitochondria isolated from control (C) and trained (T) rats. Mean ± SD; $n = 4–6$.

In the lung mitochondria of trained rats, we observed the opposite changes in functional parameters during the oxidation of malate (CI substrate) and succinate (CII substrate) (Fig. 5, Table 1). Our study has shown that endurance training causes significant alternations at the level of the respiratory chain components of lung mitochondria, i.e., elevated expression and activity of CII, diminished expression and activity of CI, and reduced content of mQ9 and mQ10 (Figs. 3–5, 7). In addition, a considerable change in the molecular organization of the mitochondrial OXPHOS system was observed, i.e., downregulation of CI in supercomplexes and upregulation of CIII in the III_2+IV supercomplex (Fig. 8A and B). As a consequence of these changes, with the involvement of two main entries (CI and CII) into the mitochondrial respiratory chain, an increase in maximum respiration and the phosphorylation rate as well as a decline in the mQ reduction level and mROS production were observed in the lung mitochondria of trained rats. Namely, under both nonphosphorylating and phosphorylating conditions, a decline in the amount of mQ, a downregulation of CI, and an increase in the activity of the mQ-oxidizing pathway (CIII + CIV) may account for the diminished reduction level of mQ resulting in a general decrease in mROS formation in lung mitochondria of trained rats. Therefore, at the mitochondrial level, reduced mROS production was accompanied by a decrease in the antioxidant systems UCP2 and SOD2. Our study, for the first time, correlates changes in mQ content, mQ reduction level, and mROS production of lung mitochondria with endurance training.

The existence of mitochondrial respiratory supercomplexes is now widely accepted [36]. The subject of discussion is what functional or structural advantages, if any, they bring. Fedor and Hirst have recently demonstrated that supercomplexes do not facilitate rapid electron transfer between CI and CIII *via* mQ channeling, and that mQ functions as a shared, freely exchanging pool in mitochondria [37]. Thus, the mitochondrial respiratory complexes do not need to associate into supercomplexes in order to catalyze effectively. Our study shows that in rat lung mitochondria, the endurance training-induced decrease in mQ content (Fig. 3C) was accompanied by downregulation of CI in supercomplexes and upregulation of CIII in the $CIII_2$+CIV supercomplex (Fig. 8A and B). Considering the lack of mQ pool channeling in supercomplexes [37], our results indicate that changes in CI-associated supercomplexes may result from a reduced amount of CI (Fig. 7) rather than a reduced mQ availability (Fig. 3C). Our research, for the first time, describes changes in the composition of mitochondrial supercomplexes under physiological conditions of endurance training, leading to a decrease in the availability of mQ in the inner mitochondrial membrane (~22% decrease in the total mQ pool).

Given the increased mitochondrial oxidative capacities and increased mitochondrial biogenesis in the lungs in response to eight weeks of endurance training, it seems that the training could result in a substantial enhancement in overall mROS formation in the lungs, even when at the mitochondrial level, mROS formation is diminished. The need for antioxidants to reduce ROS excess was indicated by increased levels of cellular Q (both Q9 and Q10) (Fig. 3A) and SOD1 expression (Fig. 3B) observed in lung homogenates of trained rats. Thus, endurance training seems to cause excessive oxidative stress in the lungs, despite the adaptive changes in pulmonary mitochondria leading to a decrease in mROS formation by a given amount of mitochondria. In the studies described, we observed opposite regulation of Q in the lungs by training. There was an increase in Q as an antioxidant in lung tissue, accompanied by a decrease in mQ as an electron carrier in mitochondria, leading (along with other changes in the respiratory chain) to attenuation of mROS production. These results illustrate that Q is not only substantially involved in mROS formation but is also an important membrane-associated antioxidant in lung cells. Our results suggest that ROS and Q may play an essential regulatory role in maintaining lung oxidative homeostasis and in lung adaptation to endurance training, including an enhancement of mitochondrial biogenesis. Both factors can improve the function of the lungs and their mitochondria during exercise.

Although one of the most important lung functions is to maintain adequate oxygenation in the body, the lungs may be exposed to hypoxia in physiological and pathological situations [38,39]. To the best of our knowledge, no studies have been conducted to determine the effect of strenuous physical exercise on the availability of oxygen in rodent pulmonary cells to date. Based on our results, we hypothesized that lungs (at least some regions) may experience hypoxia during strenuous endurance training. Lung hypoxia can be caused by the insufficient redistribution of oxygen supply to working muscles during strenuous bouts of exercise [7–9]. To examine this possibility, we measured the level of some hypoxia markers in the lung tissue of rats subjected to intense endurance training. Namely, a significant increase in the expression of the ROS-evoked transcription factor HIF1α, a marker of hypoxia and oxidative stress, and KDM6A, a direct cellular oxygen sensor regulating chromatin, was observed in the lungs of trained rats (Fig. 9). For example, it has been shown recently that the histone demethylase KDM6A expression is significantly upregulated in the rat acute myocardial infarction model and in hypoxia induction [40]. It has been suggested that the coordinated increase in the expression of oxygen-dependent enzymes, including histone demethylase, by HIF may help compensate for diminished levels of oxygen under cellular hypoxia conditions [41]. In the present study, the alterations in activity and the expression level of specified mitochondrial enzymes (downregulation of CI and upregulation of CII and GDH) observed in the lung mitochondria

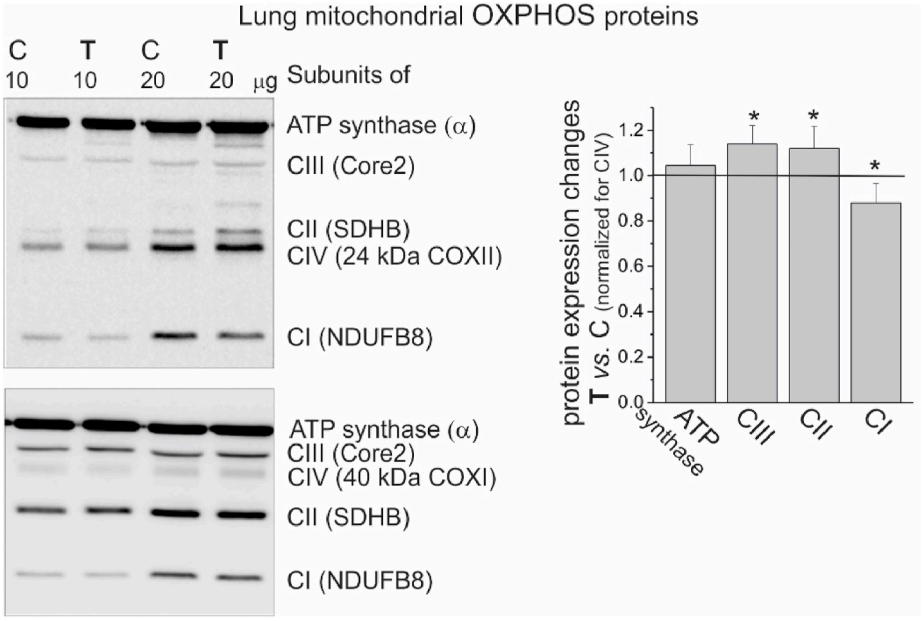

Fig. 7. Endurance training alters the expression level of oxidative phosphorylation (OXPHOS) proteins. Representative western blots and analysis of protein expression in mitochondria isolated from control (C) and trained (T) rats. CI–CIV, complexes of the respiratory chain. Two different total OXPHOS antibody cocktails were used, which differ in antibodies raised against the subunit of CIV (loading control). Mean ± SD; $n = 6$. $p < 0.05$ (*), comparison *vs.* control values.

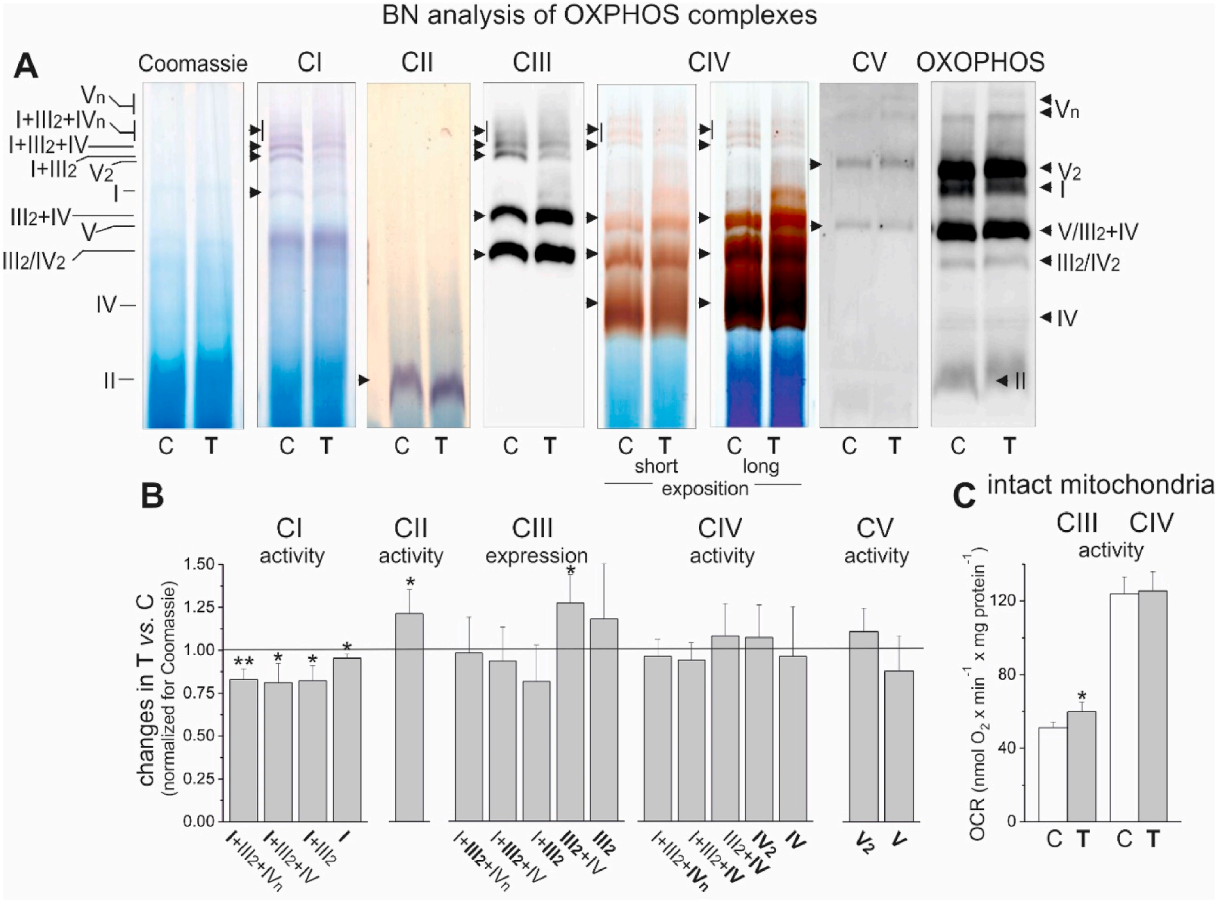

Fig. 8. Endurance training downregulates CI in supercomplexes and upregulates CIII in the $CIII_2$+CIV supercomplex. Representative BN-PAGE showing OXPHOS supercomplexes and complexes in the lung mitochondria from control (C) and trained (T) rats (**A**). Shown in sequence are Coomassie staining, CI and CII in-gel activity, CIII immunoblotting, CIV and CV in-gel activity, and total OXPHOS immunoblotting. CV, ATP synthase. Analysis of changes in in-gel activity or expression level of OXPHOS complexes (**B**). Maximal respiration with duroquinol (+rotenone) (CIII activity) and maximal CIV (COX) activity in the intact lung mitochondria (**C**). OCR, oxygen consumption rate. Mean ± SD; $n = 3$–6. $p < 0.05$ (*), $p < 0.01$ (**), comparison *vs.* control values.

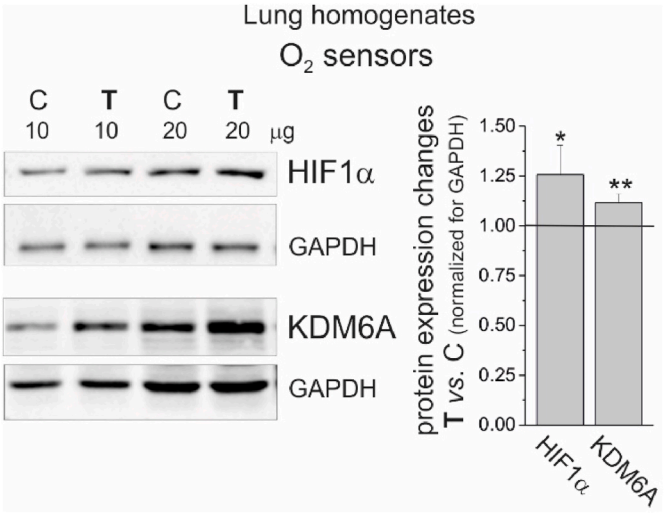

Fig. 9. Endurance training can cause hypoxic conditions. Comparison of lung homogenates from control (C) and trained (T) rats. Representative western blots and analyses of protein expression of lysine (K)-specific demethylase 6A (KDM6A) and hypoxia inducible factor 1 α (HIF1α). Loading control: GAPDH. Mean ± SD; $n = 5–6$. $p < 0.05$ (*),$p < 0.01$ (**), comparison *vs.* control values.

of trained rats (Figs. 4, 5 and 7) also confirmed a mitochondrial response to hypoxic conditions. For example, similar alterations in mitochondrial respiratory function have been observed in human endothelial cells exposed to chronic hypoxia [42]. In human lung cancer cells, hypoxia promotes mitochondrial glutamate oxidation by upregulating the expression of GDH through the HIF1α pathway [43]. In addition, exposure to hypoxia favors an increase in mitochondrial and non-mitochondrial ROS formation in the lungs [38,39]. In our study, given the significantly increased mitochondrial biogenesis and upregulation of antioxidant systems (SOD1 and Q) in the lungs of trained rats (Figs. 1–3), endurance training seems to have caused oxidative stress in this tissue. Thus, based on our findings, we hypothesize that endurance training induces hypoxia in the lung tissue, leading to metabolic adaptation involving the mitochondrial response. We postulate that hypoxia and the accompanying enhanced production of ROS may be one of the key factors initiating the described adaptive responses to the endurance training in the lung tissue.

5. Conclusions

In conclusion, we found that in rat lungs, an eight-week endurance training resulted in a significant increase in mitochondrial biogenesis and oxidative capacities, which was accompanied by several quantitative and qualitative alterations at the level of lung mitochondria. We showed that endurance training markedly enhanced respiratory function and diminished mROS production by lung mitochondria. Our results highlight the role of mitochondria in protecting lung cells from endurance training-induced oxidative damage. Our study indicates that the training-induced adaptive changes may be essential for maintaining lung cell energy and oxidative homeostasis during demanding exercise training. Adaptation of the lungs to endurance training is probably associated with an increase in lung cell energy supply, most likely necessary to increase lung functional capacity. We postulate that these adaptive responses in the lungs can be stimulated by exercise-induced hypoxia and a general increase in ROS production.

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Author contributions

Conceptualization, W.J. and J.A.Z.; Experimental design and supervision, W.J.; Performing experiments, K.D. (OCR, COX activity, mΔΨ, Q content, mQ reduction level, H_2O_2 production), L.G. (protein immunoblotting, BN-PAGE, in-gel activity assays), H.G. (protein immunoblotting), and A.K. (CS and LDH activities); Animal training and tissue dissection assistance, J.M.; Data analysis, K.D., L.G., J.M., and W.J.; Critical discussion, J.M, J.A.Z., and W.J.; Writing–original draft, review, W.J and J.A.Z.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2020.10.011.

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Methods – supplementary information

Animal endurance training – detailed protocol

During the experiment, the animals were kept in standard laboratory cages in a room with a 12 h/12 h light/dark cycle, controlled temperature (22 ± 2°C) and humidity (55 ± 10%). All rats had unrestricted access to standard rat feed (Altromin 1324) and tap water throughout the study period.

Training was conducted 5 days per week, at the same time each day for 8 weeks. Due to the nocturnal activity of the animals, a reversed day/night cycle was introduced in the animal room, and the training took place under weak red light. The applied endurance training program started with the lowest workload, which gradually increased in the subsequent weeks of training. In the 1st week of the training program, the rats underwent a habituation process to the treadmill running, which began with 5-10 min of slow running at a constant speed amounting to 5-10 m × min^{-1}, followed by 10-20 min of running at ~10 m × min^{-1} with 10-20 short bouts of faster running for 5-10 s, repeated every 60 s. The speed of running during these bouts of running was progressively increasing from the basic speed amounting to ~10 m × min^{-1} up to 30-40 m × min^{-1}. After this period, most of the rats learned how to run on the treadmill, although some reached this skill later, i.e., in the subsequent weeks of training. In the 2nd week, the animals performed a 40-min continuous run at a velocity of 30 m × min^{-1} with 30-s accelerations up to 35 m × min^{-1} conducted every 10 min. In the 3rd week of training, the duration of the continuous run was extended to 60 min, and the speed of the run was set at 30 m × min^{-1}. In the 4th week, the rats performed a 60-min continuous run at 30 m × min^{-1}, with acceleration every 10 min to 35 m × min^{-1} for 30-s intervals. In the 5-6th weeks of training, rats performed a 60-min continuous run at 30 m × min^{-1}, with a 30-s bout of running at 40 m × min^{-1}, repeated every 10 min. In the 7-8th weeks of training, the rats performed an 80-min continuous run at 25 m × min^{-1}, with a 30-s bouts of run at 30 m × min^{-1}, repeated every 13 min. In general, the animals tolerated this training program well, although some of them had problems maintaining the required speed and running time.

Table S1

During the 8 weeks of the experiment, the body mass of trained rats did not change, while the body mass of control animals increased significantly ($p < 0.001$). A comparison of trained and control animals indicates that 8 weeks of training led to a significant reduction in body mass and to a significant increase in lung mitochondrial yield per kg of rat body mass. No significant change in lung mass or mitochondrial yield per g of lung was observed.

	Control rats	Trained rats
Initial body mass (g)	426 ± 36	431 ± 36
Final (after 8 weeks) body mass (g)	484 ± 30	423 ± 37**
Lung mass (g)	1.50 ± 0.17	1.41 ± 0.19
Total lung mitochondrial yield (mg of protein/animal)	3.45 ± 0.21	3.58 ± 0.75
Mitochondrial yield per g of lung (mg of protein/g of lung)	2.30 ± 0.20	2.53 ± 0.25
Lung mitochondrial yield per g of animal (mg of protein/kg of animal)	7.13 ± 0.49	8.46 ± 0.59**

Data are represented as the mean ± SD ($n = 12$). **$p < 0.01$ vs. control rats.

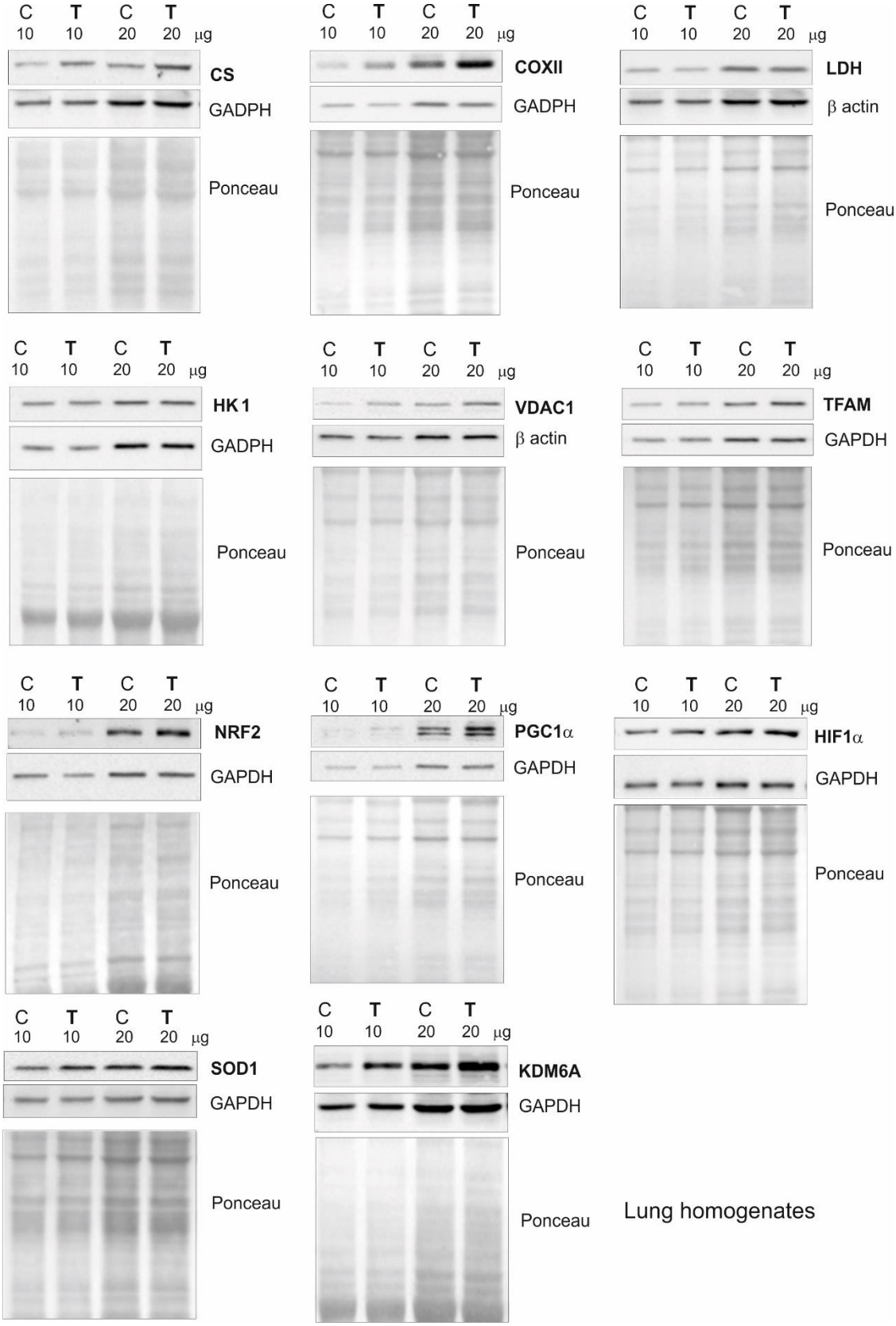

Fig. S1 Western blots of various proteins in the lung homogenates from control (C) and trained (T) rats. Corresponding loading controls, GAPDH (or β actin) immunodetection and Ponceau staining are shown. CS, citrate synthase; COX II, cytochrome *c* oxidase subunit II; VDAC1, voltage-dependent anion channel 1; TFAM, mitochondrial transcription factor A; NRF2, nuclear factor erythroid 2-related factor; PGC1α, peroxisome proliferator-activated receptor

γ coactivator 1α; SOD1, superoxidase dismutase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HKI, hexokinase I; LDH, lactate dehydrogenase; KDM6A, lysine (K)-specific demethylase 6A; HIF-1α, hypoxia-inducible factor 1-alpha.

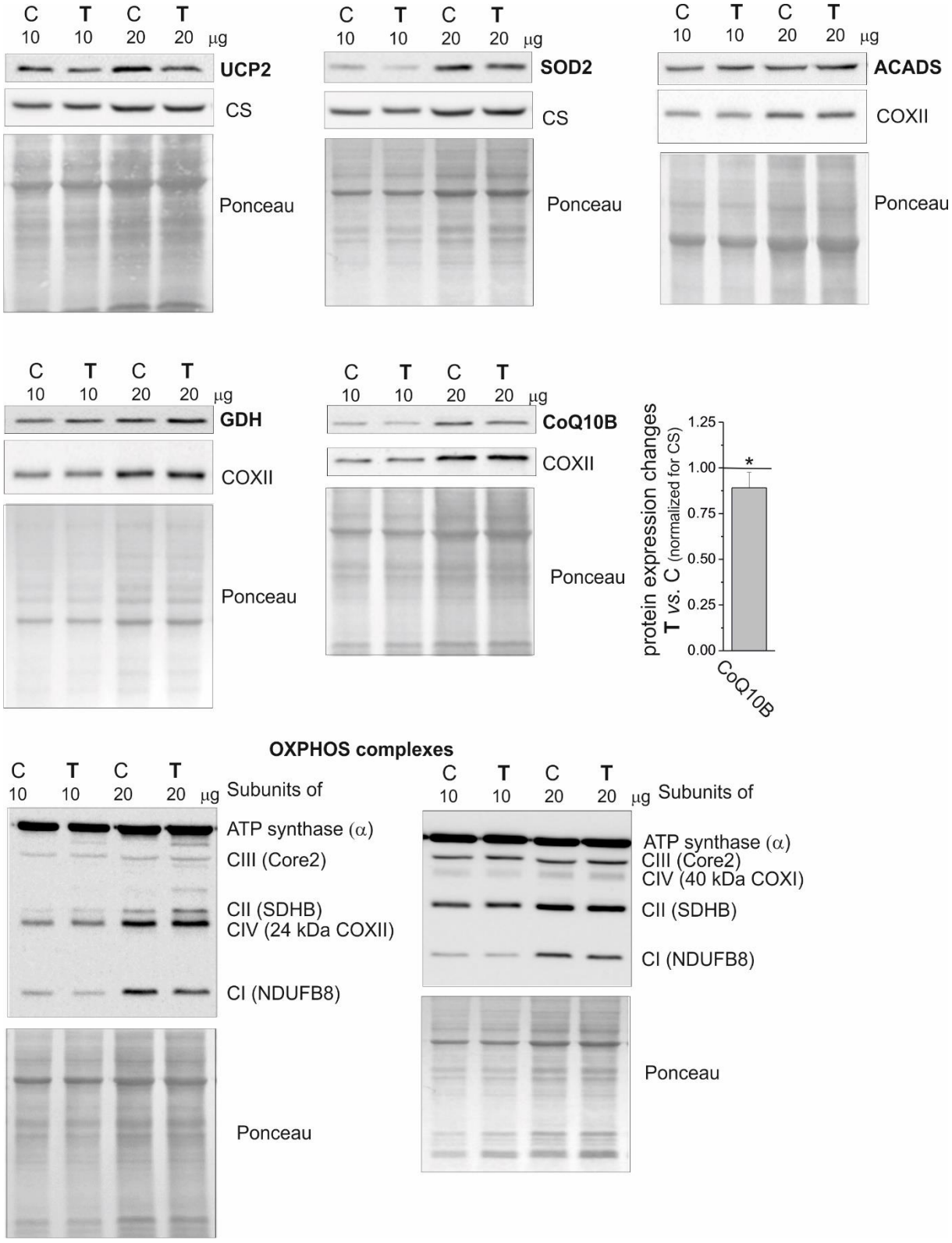

Fig. S2 Western blots of various proteins in the lung mitochondria from control (C) and trained (T) rats. Corresponding loading controls, CS or COXII immunodetection and Ponceau staining are shown. ACADS, acyl-coenzyme A dehydrogenase; GDH, glutamate dehydrogenase; CoQ10B, mitochondrial Coenzyme Q-binding protein COQ10 homolog B; SOD2, Mn superoxide dismutase; UCP2, uncoupling protein 2.

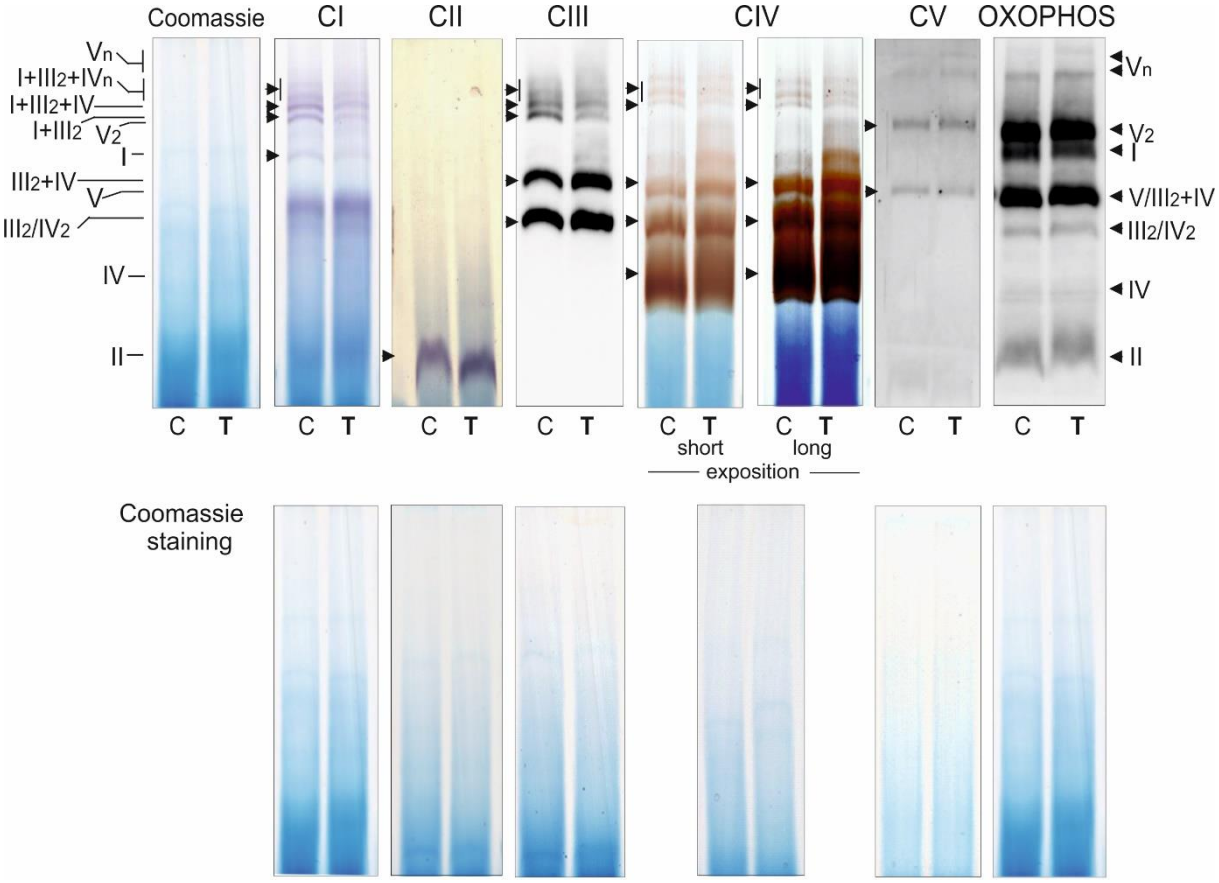

Fig. S3. Representative BN-PAGE showing OXPHOS supercomplexes and complexes in the lung mitochondria from control (C) and trained (T) rats (A). Shown in sequence are Coomassie staining (representative), CI and CII in-gel activity, CIII immunoblotting, CIV and CV in-gel activity, and total OXPHOS immunoblotting. CV, ATP synthase. Corresponding Coomassie staining (lower panel) is shown for each analysis (upper panel).

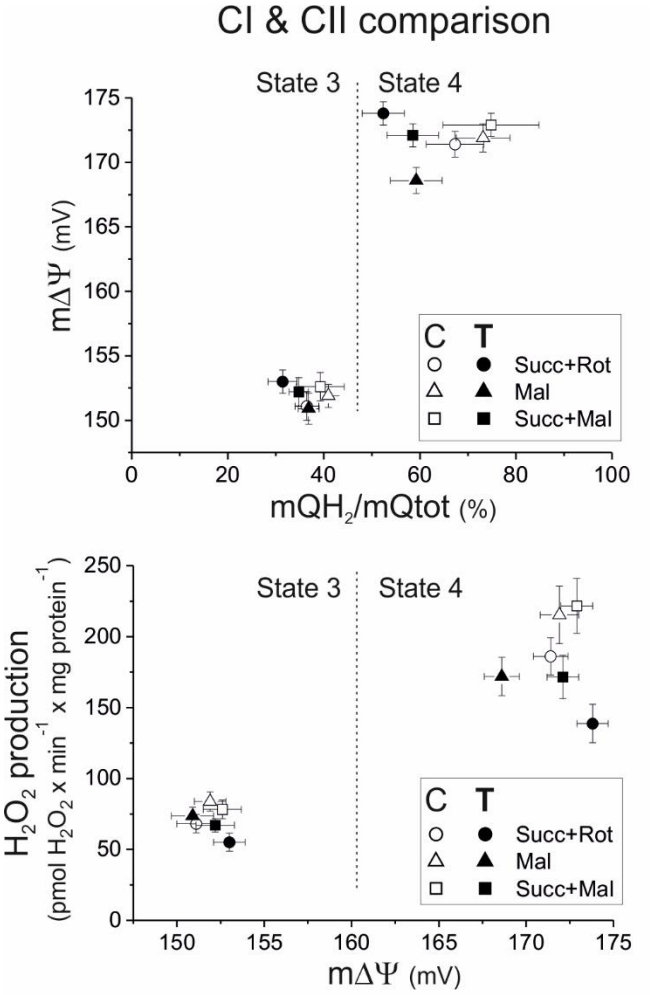

Fig. S4. The relationships between the mitochondrial membrane potential (mΔΨ) and coenzyme Q (mQ) reduction level (**A**), and between H_2O_2 formation vs. mΔΨ (**B**) during succinate plus rotenone (Succ+Rot) (complex II, CII, substrate), malate (Mal) (CI substrate) and a mixture of succinate and malate (Succ+ Mal) oxidation under nonphosphorylating (State 4) conditions (in the absence of ADP) and phosphorylating (State 3) conditions (in the presence of ADP) of lung mitochondria isolated from control (C) and trained (T) rats. Mean ± SD; *n* = 4-6.

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The Relationship between Mitochondrial Reactive Oxygen Species Production and Mitochondrial Energetics in Rat Tissues with Different Contents of Reduced Coenzyme Q

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Abstract: We investigated the relationship between mitochondrial production of reactive oxygen species (ROS) and mitochondrial energetics in various rat tissues with different contents of the reduced coenzyme Q (Q) pool (Q9 + Q10). Our results indicate that similar to the tissue level, mitochondrial H_2O_2 release under nonphosphorylating conditions was strongly dependent on the amount of the reduced Q pool. Namely, in brain and lung mitochondria, less H_2O_2 release corresponded to a less reduced Q pool, while in liver and heart mitochondria, higher H_2O_2 release corresponded to a more reduced Q pool. We can conclude that the differences observed in rat tissues in the size of the reduced Q pool reflect different levels of ROS production and hence may reflect different demands for reduced Q as an antioxidant. Moreover, differences in mitochondrial H_2O_2 release were observed in different types of rat mitochondria during the oxidation of succinate (complex II substrate), malate plus glutamate (complex I substrate), and their mixture under phosphorylating and nonphosphorylating conditions. Our results indicate the existence of a tissue-specific maximum respiratory chain capacity in ROS production, possibly related to the membrane potential-mediated control of oxidative phosphorylation. We propose the use of a new parameter for the study of isolated mitochondria, RCR_{ROS}, the ratio between the formation of mitochondrial ROS under nonphosphorylating and phosphorylating conditions, which represents the maximum factorial increase in mitochondrial ROS formation that can be achieved after all ADP is phosphorylated.

Keywords: mitochondrial reactive oxygen species; coenzyme Q; mitochondrial energetics

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1. Introduction

Mitochondria are key organelles for cellular energy (ATP) production and reactive oxygen species (ROS) formation. The energy and ROS produced by mitochondria play an important role in various physiological and pathophysiological processes. Several sites that produce superoxide anion ($O_2^{\bullet -}$) and/or hydrogen peroxide (H_2O_2) have been identified in mammalian mitochondria [1]. The predominant route of mitochondrial ROS (mROS) production by the electron transport chain is the premature leakage of electrons from complexes I, II, and III.

Coenzyme Q (Q) is a fat-soluble molecule present in all cell membranes, including the inner mito. hondrial membrane. Mitochondrial Q (mQ) plays a central role in the electron transport chain, transferring electrons between dehydrogenases and the oxidizing pathway (complex III and complex IV). Importantly, mQ is also involved in the formation of $O_2^{\bullet -}$ from semiubiquinone radicals at the Q-binding sites of complexes I and III [1]. However, Q is not only involved in mROS production through the mitochondrial respiratory chain, but also an important antioxidant in mitochondria and the entire cell [2–5]. Reduced Q (ubiquinol) recycles other antioxidants such as vitamin E or vitamin C, and directly acts on free radicals or oxidants, reducing and neutralizing them. Reduced Q via binding

free radicals inhibits lipid peroxidation and prevents oxidative modifications of DNA and proteins. Moreover, the Q redox state may be a useful marker of cellular oxidative stress [4].

The production of mROS depends on several factors, including the energetic status of the mitochondria, which affects the reduction of respiratory chain electron carriers [6–9]. Thus, mROS production is also dependent on the mitochondrial membrane potential ($m\Delta\Psi$), the major component of the proton electrochemical gradient that couples electron transport along the respiratory chain with ATP synthesis in the oxidative phosphorylation (OXPHOS) process. It should be emphasized that most studies on mROS production of isolated mitochondria concern nonphosphorylating (resting) conditions, which are accompanied by a large reduction of respiratory chain electron carriers and a high $m\Delta\Psi$. However, the change in mROS production that occurs as a result of mitochondrial transition from nonphosphorylating conditions (state 4 with high mROS production) to phosphorylating conditions (state 3 with lower $m\Delta\Psi$ and lower mROS production) is of great physiological importance. This change demonstrates the extent of mROS production that accompanies mitochondrial function when the respiratory chain is uninhibited.

The aim of our work was to investigate the production of mROS in various rat tissues, i.e., in the heart and liver (tissues with high Q content) and in the brain and lungs (tissues with low Q content) under nonphosphorylating conditions (in the absence of ADP) and under phosphorylating conditions with active OXPHOS (in the presence of ADP). We studied mROS production in the context of cellular (tissue) and mitochondrial Q pool (mQ9 and mQ10) content, especially the content of the reduced Q pool. Moreover, the relationship between mROS formation and $m\Delta\Psi$ was described for mitochondria isolated from the tested tissues.

2. Materials and Methods

2.1. Animals

The experiments were performed on adult 3- to 4-month-old male Wistar rats weighing 350–450 g. The animals were bred in the animal house at the AMU Advanced Technology Center, Poznan, Poland. They were given free access to water and pellet food and were housed under standard humidity and temperature conditions on a 12 h light/dark cycle. Experimental protocols involving animals, their surgery, and care were in compliance with the guidelines of the European Community Council Directive on the protection of animals used for scientific purposes. Animals were sacrificed by decapitation, and all efforts were made to minimize suffering. As no procedures were performed using live animals and as they were sacrificed for scientific purposes, no approval was needed for our study according to the Polish Animal Welfare Act.

2.2. Tissue Preparation and Mitochondria Isolation

All procedures were performed at 4 °C. To simultaneously isolate functional and intact mitochondria from rat heart, liver, brain, and lungs, the tissues were collected from one rat, placed in isolation medium A (pH 7.2) containing 50 mM Tris-HCl, 100 mM sucrose, and 0.5 mM ethylenediaminetetraacetic acid (EDTA) and washed several times. After removal of the connecting tissue and large vessels, the tissues were cut into small pieces over ice. The minced tissues were filtered through a strainer to remove the remaining blood and connecting tissue and then homogenized in isolation medium B containing 50 mM Tris-HCl (pH 7.2), 100 mM sucrose, 100 mM KCl, 1 mM KH_2PO_4, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 0.5 mM EDTA using a Teflon or glass pestle. The strained homogenates were centrifuged at 900× *g* for 10 min. The resultant supernatants were filtered. Part of the filtered homogenates was used for studies (measurements of H_2O_2 release and the Q content and reduction level). The greater part of the homogenates was supplemented with isolation medium B with 0.2% BSA and centrifuged at 17,800× *g* for 10 min. Mitochondrial pellets suspended in isolation medium B without BSA were centrifuged at 900× *g* for 8 min. The supernatants were filtered and again centrifuged at 17,800× *g* for 10 min. The final pellets of mitochondria were then

resuspended in medium C containing 10 mM Tris-HCl, pH 7.2, 75 mM sucrose, and 225 mM mannitol. The protein concentrations of homogenates and mitochondria were determined by the Bradford method.

All functional measurements were performed on freshly isolated mitochondria at 35 °C. Only mitochondria with good integrity of the mitochondrial outer membrane (97–100%) (measured as described in [10]) were used for the study.

2.3. Measurements of the Tissue and Mitochondrial Q Content

Concentrations of tissue and mitochondrial Q9 (a dominant Q form in rat tissues) and Q10 (a less abundant Q form in rat tissues) were determined in homogenates and mitochondria by an extraction technique and HPLC detection [9,11]. Both reduced (275 nm) and oxidized (290 nm) forms of Q9 and Q10 were detected. For the quantification and calibration of the Q9 and Q10 peaks, commercial Q9 and Q10 were used. The total and reduced Q9 and Q10 pools were determined in rat tissues and mitochondria under fully oxidizing conditions, i.e., in the absence of respiratory Q-reducing substrates. Before Q extraction, homogenates (7 mg) and mitochondria (3 mg) were incubated with gentle agitation for 20 min in 3 mL of standard assay medium, which comprised 75 mM sucrose, 225 mM mannitol, 10 mM KCl, 5 mM KH_2PO4, 0.5 mM EDTA, 10 mM Tris/HCl (pH 7.2), and 0.2% BSA.

2.4. Mitochondrial Membrane Potential Measurements in Isolated Mitochondria

$m\Delta\Psi$ was measured using a tetraphenyl-phosphonium (TPP+)-specific electrode [12,13] in 3 mL of standard assay medium with 3 mg of mitochondrial protein (1 mg $\times$ mL^{-1}). The TPP+-electrode was calibrated based on three sequential additions (1.6, 1.6, and 3.2 µM) of TPP+. After each run, 0.5 µM FCCP was added to release TPP+ to correct the baseline. To calculate the $m\Delta\Psi$ value, the volume of rat mitochondrial matrix was assumed to be 2.0 µL $\times$ mg^{-1} protein.

ADP (170 µM) was added to determine the $m\Delta\Psi$ of phosphorylating (state 3) respiration. The $m\Delta\Psi$ of nonphosphorylating (resting state, State 4) respiration was determined when ADP was exhausted or in measurements without exogenous ADP. The respiratory substrates were as follows: 5 mM succinate, 5 mM malate and 5 mM glutamate, and a mixture of 5 mM succinate, 5 mM malate, and 5 mM glutamate.

2.5. Assay of H_2O_2 Release

The H_2O_2 release rate was measured by the Amplex Red assay [9,14]. Horseradish peroxidase (0.14 U $\times$ mL^{-1}) catalyzes the H_2O_2-dependent oxidation of nonfluorescent Amplex Red (5 µM) to fluorescent resorufin red. The release of $O_2^{\bullet -}$ was also captured by the addition of exogenous superoxide dismutase (SOD, 5U $\times$ mL^{-1}) to convert $O_2^{\bullet -}$ to H_2O_2 in the assay medium. The fluorescence kinetics were followed for 40 min at 545 nm/590 nm using a Tecan multimode reader (Infinite M200 PRO) with 24-well plates. Homogenates (0.2 mg of protein) were incubated in 0.5 mL of standard assay medium with a mixture of 5 mM succinate, 5 mM malate, and 5 mM glutamate in the absence (nonphosphorylating conditions) or presence of 0.75 mM ADP (phosphorylating conditions). Mitochondria (0.26 mg of protein) were incubated in 0.5 mL of the standard incubation medium with 5 mM succinate, 5 mM malate, and 5 mM glutamate or a mixture of 5 mM succinate, 5 mM malate, and 5 mM glutamate in the absence (nonphosphorylating state 4 conditions) or presence of 1.5 mM ADP (phosphorylating state 3 conditions). H_2O_2 release of nonphosphorylating (resting state, state 4) respiration was determined after ADP depletion or in measurements without exogenous ADP. Reactions were calibrated with known amounts of H_2O_2.

Limitations resulting from the technique used to measure H_2O_2 release should be considered. The Amplex Red Assay does not take into account the portion of H_2O_2 produced in the mitochondrial matrix that is removed by matrix peroxidases. Furthermore, it is important to consider the limited diffusion of H_2O_2 from the matrix outside the mi-

tochondria in living cells [15]. Amplex Red is readily, independently of H_2O_2, converted into fluorescent resorufin by a carboxylesterase. Therefore, it would be worth carrying out measurements in the presence of phenylmethylsulfonyl fluoride, which inhibits this process [16].

2.6. Statistical Analysis

The results are presented as the means ± SD obtained from at least 4 independent homogenate preparations or mitochondrial isolations, in which each determination was performed at least in duplicate. Each isolation of lung, brain, liver, and heart mitochondria was performed from one animal. Significant differences were determined via unpaired *t*-tests or ANOVAs (followed by Tukey's post hoc comparisons for $p < 0.05$ from an ANOVA). Differences were considered to be statistically significant if $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***).

3. Results

3.1. Distribution of Q9, Q10 and Total Q (Q9 + Q10) in the Cells and Mitochondria of Different Rat Tissues

Four rat tissues were selected for the study, i.e., heart, liver, brain, and lungs, with different Q contents (Table 1). Our determinations of the total (cellular) amount of Q9, Q10 and total of Q9 + Q10 confirmed previous studies [5,17], indicating that in different rat tissues the level of both Q homologs varies, with Q9 being the dominant form. In 3- to 4-month-old male rats, the total amount of tissue Q content (Q9 + Q10) varied ~7-fold, with the highest in the heart and the lowest in the lungs (Table 1). Similarly, in the examined tissues, the total amount of mitochondrial Q content (Q9 + Q10) varied ~7-fold, again with the highest in the heart and the lowest in the lungs. Q9 was also the dominant form in mitochondria. The proportion between Q9 and Q10 varied between tissues and their mitochondria.

Table 1. Q9, Q10, and total Q (Q9 + Q10) content in different rat tissues and their mitochondria.

Rat Tissue	Tissue Content (nmol mg^{-1} Protein)			Mitochondrial Content (nmol mg^{-1} Protein)		
	Q9	**Q10**	**Q9 + Q10**	**mQ9**	**mQ10**	**mQ9 + mQ10**
Heart	2.32 ± 0.26	0.12 ± 0.01	2.44 ± 0.25	5.28 ± 0.66	0.54 ± 0.07	5.82 ± 0.74
Liver	1.41 ± 0.13	0.10 ± 0.01	1.51 ± 0.17	1.59 ± 0.18	0.19 ± 0.02	1.78 ± 0.19
Brain	0.48 ± 0.05	0.15 ± 0.01	0.63 ± 0.05	1.36 ± 0.14	0.27 ± 0.03	1.63 ± 0.19
Lung	0.25± 0.03	0.11 ± 0.01	0.36 ± 0.04	0.64 ± 0.07	0.20 ± 0.02	0.84 ± 0.09 [1]

[1] Mean ± SD, n = 4–6.

We also determined the levels of both oxidized and reduced Q pools of Q9 and Q10 in tissues and mitochondria (Table 2), as the reduced Q is the form that exerts an antioxidant effect. The percentage of reduced Q9 and Q10 also differed in different tissues. The highest reduction of both forms of Q was observed in the liver, both at the tissue and mitochondrial levels. Namely, in the liver ~90% of the Q9 and Q10 were reduced in tissues, as was Q10 in the mitochondria. Only mitochondrial Q9 showed a smaller but still high reduction (~50%) in liver mitochondria. In the heart, the level of reduction of both Qs was similar, i.e., ~25–29% in tissues and ~15–19% in mitochondria. Interestingly, almost the entire pool of reduced Q in the lungs consisted of Q10, both at the tissue and mitochondrial levels. The lowest level of reduction of both Qs was observed in the brain. No reduced Q pool was detected at the tissue level and was ~9% in mitochondria (for both Q9 and Q10). The differences in the size of the reduced Q pools in different tissues indicate different needs for reduced Qs as specific antioxidants at both the tissue and mitochondrial levels.

Table 2. Percentage of the reduced Q9 and Q10 pools in rat tissues and mitochondria under fully oxidizing conditions (no respiratory Q-reducing substrates).

Rat Tissue	Tissue % of Reduced Q Pool			Mitochondria % of Reduced Q Pool		
	Q9	Q10	Q9 + Q10	mQ9	mQ10	mQ9 + mQ10
Heart	28.7 ± 2.6	25.3 ± 2.9	28.5 ± 3.0	18.9 ± 2.0	20.1 ± 2.1	19.0 ± 2.1
Liver	95.1 ± 9.7	87.4 ± 9.1	92.1 ± 9.5	49.7 ± 5.2	93.5 ± 9.9	68.6 ± 7.2
Brain	nd (0)	nd (0)	0	9.3 ± 1.1	11.1 ± 1.5	9.6 ± 1.2
Lung	nd (0)	100	42	6.5 ± 0.7	100	28.9 ± 2.2 [1]

[1] Mean ± SD, n = 4. nd, not detectable.

3.2. The Relationship between H_2O_2 Formation and the Content of the Total and Reduced Q Pool (Q9 + Q10) in Rat Tissue Homogenates

We then measured the level of H_2O_2 release in tissue homogenates under mitochondrial respiratory chain activation conditions by adding succinate (substrate for complex II) and malate plus glutamate (substrate for complex I). The measurement was performed under OXPHOS activation conditions (in the presence of ADP) and in the absence of ADP. The obtained results were presented for the total (Figure 1a) and reduced (Figure 1b) Q (Q9 + Q10) pools.

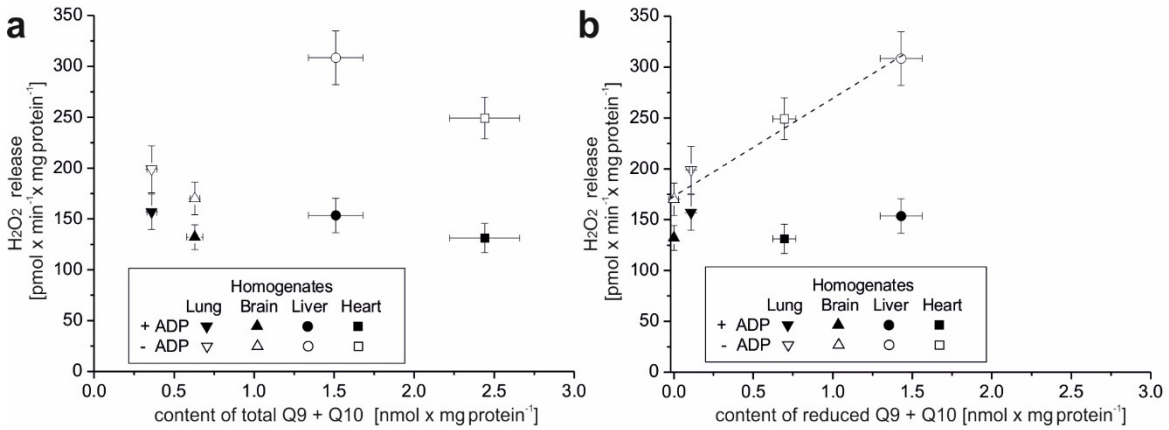

Figure 1. H_2O_2 release versus the content of total (**a**) and reduced (**b**) Q pools (Q9 + Q10) in rat tissue homogenates. H_2O_2 release measurements were performed in the absence or presence of ADP with succinate and malate plus glutamate as mitochondrial respiratory substrates. The total and reduced Q9 + Q10 pools were measured under fully oxidizing conditions (no respiratory Q-reducing substrates). Mean ± SD. (**b**) The linear regression between H_2O_2 release in the absence of ADP and the content of reduced Q9 + Q10 is shown (r = 0.988, p = 0.01).

Interestingly, in all four examined tissues a similar release of H_2O_2 (~130–155 pmol × min^{-1} × mg $protein^{-1}$) was observed under active OXPHOS conditions (Figure 1a,b). In the absence of ADP, the release of H_2O_2 was higher and was ~170–200 pmol × min^{-1} × mg $protein^{-1}$ for the brain and lungs, ~250 pmol × min^{-1} × mg $protein^{-1}$ for the heart, and ~310 pmol × min^{-1} × mg $protein^{-1}$ for the liver. These results indicate a greater ROS production capacity in the heart and liver tissues (the highest in the liver) than in the lung and brain tissues.

The increased H_2O_2 release (in the absence of ADP) in the heart and liver was accompanied by a much larger total Q (Q9 + Q10) pool than that in the lung and brain (Figure 1a). When comparing the release of H_2O_2 with the reduced Q pool (Figure 1b), a linear relationship was observed in the absence of ADP. Under these conditions, less H_2O_2 release corresponded to a less reduced Q pool and higher H_2O_2 release corresponded to a more reduced Q pool. Overall, our results indicate that rat tissues that can produce more

ROS have larger total and reduced Q pools (Q9 + Q10) and that tissue ROS production seems to be proportional to the reduced tissue Q pool.

3.3. Comparison of H_2O_2 Production in Mitochondria Isolated from Various Rat Tissues during the Oxidation of Succinate (Substrate for Complex II) and Malate Plus Glutamate (Substrate for Complex I)

The release of H_2O_2 in mitochondria isolated from different rat tissues was then compared. Mitochondrial H_2O_2 release was measured under the phosphorylating (state 4) and nonphosphorylating (state 4) conditions with succinate (substrate of complex II), malate, and glutamate (substrate of complex I) and a mixture of succinate and malate plus glutamate (two active electron inputs to the respiratory chain) (Figure 2).

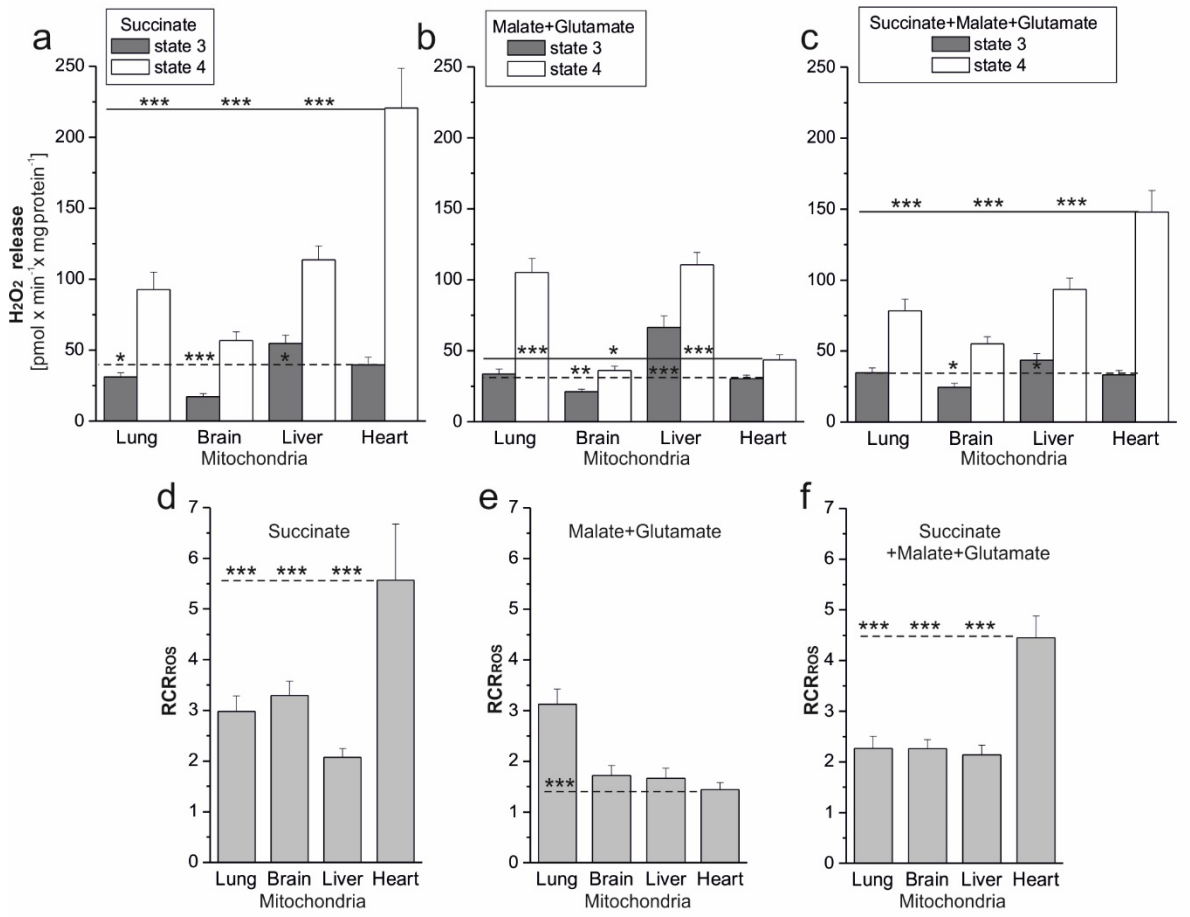

Figure 2. H_2O_2 release in isolated rat mitochondria oxidizing succinate (**a**), malate plus glutamate (**b**) and a mixture of succinate and malate plus glutamate (**c**) under phosphorylating (state 3) and nonphosphorylating (state 4) conditions. RCR_{ROS}, respiratory control ratio of mROS formation (state 4 H_2O_2 formation/state 3 H_2O_2 formation) with various respiratory substrates (**d**–**f**). Mean ± SD; $n = 5$. $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***), comparison vs. mean values for heart mitochondria.

For all tissues and combinations of respiratory substrates used, H_2O_2 release in state 3 was significantly lower than that in state 4. Figure 2d–f show the calculated ratio of H_2O_2 release in state 4, i.e., under conditions of highest ROS production when OXPHOS is inactive, to H_2O_2 release in state 3, i.e., when the respiratory chain is at maximum respiratory activity. We propose to call this ratio the respiratory control ratio of ROS formation (RCR_{ROS}). The ratio represents the maximum factorial increase in mitochondrial ROS formation that can be achieved after all ADP is phosphorylated. In the case of the examined mitochondria, the highest RCR_{ROS} values were observed in the heart mitochondria during the oxidation of succinate (~5.5) (Figure 2d) and the mixture of complexes I and II substrates

(~4.5) (Figure 2f). In the case of malate plus glutamate oxidation, the greatest increase in H_2O_2 release after ADP depletion was observed for lung mitochondria (~3.2) (Figure 2e).

Some properties can be observed when comparing H_2O_2 release in mitochondria from the examined rat tissues. Namely, regardless of the type of respiratory substrate, H_2O_2 release was the lowest in the brain mitochondria for both respiratory states. On the other hand, the highest H_2O_2 release was observed for succinate and a mixture of complex I and II substrates in heart mitochondria. In the case of lung and liver mitochondria, H_2O_2 release was similar for complex I substrate oxidation and for complex II substrate oxidation under both respiratory conditions. In the case of heart and brain mitochondria, H_2O_2 release under nonphosphorylating conditions was significantly higher during the oxidation of succinate (substrate for complex II) than during the oxidation of malate plus glutamate (substrate for complex I). Interestingly, for all types of mitochondria, in both respiratory states, the H_2O_2 release observed at the involvement of both electron inputs on the respiratory chain (oxidation of complex I and II substrates) did not exceed the H_2O_2 release observed with a single best substrate entry. This observation indicates the existence of a maximum respiratory chain capacity in ROS production, i.e., an upper limit in state 4 and a lower limit in state 3.

3.4. The Relationship between Mitochondrial H_2O_2 Formation and the Content of the Reduced mQ Pool (mQ9 + mQ10) and m$\Delta\Psi$

Figure 3 presents the relationship between H_2O_2 release and the content of the reduced mQ pool (mQ9 + mQ10) in various rat mitochondria. H_2O_2 formation was measured with a mixture of succinate and malate plus glutamate (complex II and complex I substrates) under phosphorylating (state 3) and nonphosphorylating (state 4) conditions. The reduced mQ9 + mQ10 pools were measured under fully oxidizing conditions (no respiratory Q-reducing substrates) (Table 2). As at the tissue level (Figure 1), H_2O_2 formation in mitochondria respiring in state 3 was not strongly associated with the amount of reduced Q pool (Figure 3). However, such a relationship (although not linear) can be observed in the case of mitochondria, which oxidize substrates under nonphosphorylating conditions. Under these conditions, in the brain and lung mitochondria, less H_2O_2 release corresponded to a less reduced mQ pool and in the liver and heart mitochondria, higher H_2O_2 release corresponded to a more reduced mQ pool. This relationship was much steeper for the amount of reduced mQ above 1 nmol$\times$ min^{-1} $\times$ mg protein^{-1}.

Figure 4 shows the relationships between H_2O_2 formation versus m$\Delta\Psi$ in various rat mitochondria oxidizing succinate, malate plus glutamate, and a mixture of succinate and malate plus glutamate under phosphorylating (state 3) and nonphosphorylating (state 4) conditions. The points obtained for all tested mitochondria and respiratory substrates form a single relationship clearly showing the nonlinear dependence of mROS formation on m$\Delta\Psi$. Up to ~180 mV, this relationship is not steep, with the slope clearly increasing between 180 and 200 mV. There is a threshold m$\Delta\Psi$ value (~200 mV) above which even a small increase in m$\Delta\Psi$ gives rise to a large stimulation of H_2O_2 release by mitochondria. The steepest relationship between H_2O_2 release and m$\Delta\Psi$ occurs for heart mitochondria, while for brain mitochondria this relationship is much less steep.

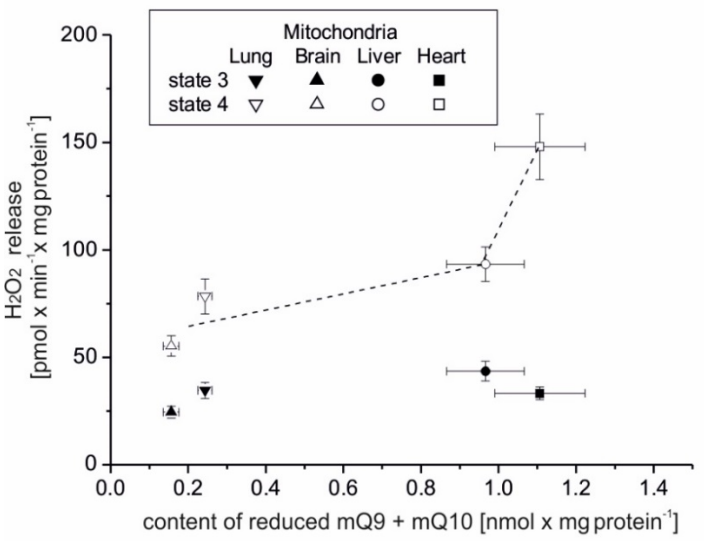

Figure 3. H_2O_2 release and the content of the reduced mQ pool (mQ9 + mQ10) in rat mitochondria isolated from various tissues. H_2O_2 formation measurements were performed in the absence (state 4) or presence (state 3) of ADP with a mixture of succinate and malate plus glutamate. The reduced mQ9 + mQ10 pools were measured under fully oxidizing conditions (no respiratory Q-reducing substrates). Mean ± SD; $n = 5$.

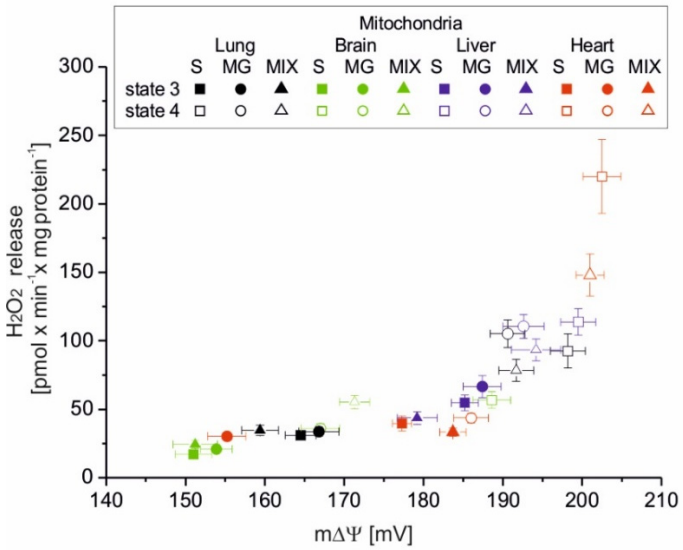

Figure 4. The relationship between H_2O_2 release and mΔΨ in isolated rat mitochondria oxidizing succinate (S), malate plus glutamate (MG), and a mixture of succinate and malate plus glutamate (MIX) under phosphorylating (state 3) and nonphosphorylating (state 4) conditions. Mean ± SD; $n = 5$.

4. Discussion

The present study focuses on the relationship between mROS generation and mitochondrial energetics in various rat tissues with different reduced Q pool contents. First, we found that rat tissues at the tissue and mitochondrial levels differ not only in the content of total Q pools (Q9, Q10, and Q9 + Q10) but also in the content of reduced Q pools (Q9, Q10, and Q9 + Q10). At the tissue level, we found a similar percentage reduction in the total Q9 + Q10 pool previously observed in various rat tissues [5]. However, reduced Q pools for the mitochondria of various rat tissues as well as the relationship of the total and reduced Q pools at the tissue and mitochondrial levels to ROS production have not been studied. Our results indicate that rat tissues that can produce more ROS (liver and

heart) in the absence of ADP have larger total and reduced Q pools (Q9 + Q10) than tissues producing less ROS (lung and brain). At the tissue level, ROS production seems to be proportional to the reduced tissue Q pool (Q9 + Q10). Similar to the tissue level, H_2O_2 release in mitochondria respiring under nonphosphorylating conditions (state 4) was strongly dependent (although nonlinearly) on the amount of reduced Q pool. Namely, in the brain and lung mitochondria less H_2O_2 release corresponded to a less reduced mQ pool, while in the liver and heart mitochondria, higher H_2O_2 release corresponded to a more reduced mQ pool. Thus, the observed differences in the size of the reduced Q pool (Q9 + Q10) reflect different levels of ROS production and hence may reflect different demands for reduced Qs as antioxidants at both the tissue and mitochondrial levels. It is well accepted that in their reduced form Qs have antioxidant activity, as scavengers of ROS or lipid radicals and regenerators of α-tocopherol from the α-tocopheroxyl radical [3–5]. Obviously, at the tissue level, the larger total and reduced Q pools are also related to the number of mitochondria in a given tissue. On the other hand, at the mitochondrial level, when the content of reduced mQ pool and mROS production are presented per mg of mitochondrial protein, increased production of mROS under nonphosphorylating conditions, combined with an increased reduced mQ pool, indicates a greater need for this antioxidant. However, further research, including measuring redox balance and other main antioxidant molecules such as glutathione, is necessary to elucidate the phenomenon described in this work. Our results also indicate that the Q redox state in a given tissue may be a useful marker of cellular oxidative stress. However, it must be remembered that Q levels decline with age in human and rat tissues [17]; hence, the levels of reduced Qs may also change.

Of great physiological importance is the change in mROS formation, which occurs during the transition of mitochondria from nonphosphorylating conditions (state 4, with increased mROS production) to phosphorylation conditions (state 3, with decreased mROS production). This change demonstrates the extent of mROS production that accompanies mitochondrial function when the respiratory chain is uninhibited. The parameter proposed by us, i.e., RCR_{ROS}, the ratio between mROS formation in state 4 versus mROS formation in state 3, represents the maximum factorial increase in mROS formation that can be achieved after the end of ADP phosphorylation. The RCR_{ROS} can be determined for isolated mitochondria. In vivo, mitochondria can shift rapidly between these conditions inducing a change in $m\Delta\Psi$ and thereby in mROS formation. In the present study, during nonphosphorylating respiration, high $m\Delta\Psi$ values were accompanied by increased mROS formation in tested rat mitochondria. In contrast, during phosphorylating respiration, the relative decrease in $m\Delta\Psi$ was accompanied by decreased mROS formation. It has been shown previously that in nonphosphorylating rat heart mitochondria, mROS generation strongly but nonlinearly depends upon $m\Delta\Psi$, increasing at $m\Delta\Psi$ greater than that of state 3 [18]. Our results obtained with heart mitochondria confirm this observation. In addition, we have shown that the measurements of H_2O_2 release and $m\Delta\Psi$ obtained for four types of rat mitochondria and different respiratory substrates form one relationship clearly showing the nonlinear dependence of mROS production on $m\Delta\Psi$. However, it should be remembered that mROS production is not a direct function of $m\Delta\Psi$ under the conditions of inhibition of the Q-oxidizing segment of the respiratory chain (complex III or complex IV) [9].

To date, at least 11 sites that produce $O_2^{\bullet-}$ and/or H_2O_2 have been identified in mammalian mitochondria [1,19]. The contributions of specific sites of the mitochondrial respiratory chain to the production of ROS in mitochondria depend very strongly on the substrates being oxidized [20]. Under our experimental conditions, during succinate oxidation, the flavin site of complex II (site II_F) and the Q_o and Q_i sites of complex III (site III_{Qo} and site III_{Qi}) participated in mROS production. Moreover, involvement of the flavin (IF site) and the Q site (IQ site) of complex I due to reverse electron transport cannot be ruled out. When the malate was oxidized, all mROS production sites mentioned were active. The purpose of our research was not to determine the contribution of individual mROS production sites but to determine the total mROS production when electrons enter

the respiratory chain via complex I and/or complex II under phosphorylating (state 3) and nonphosphorylating (state 4) conditions for the mitochondria of various rat tissues. As far as we know, there has been no such research before. Comparing mROS production in rat lung, brain, liver, and heart mitochondria, it can be seen that, regardless of the substrate used under phosphorylating conditions, H_2O_2 release was lowest in brain mitochondria and highest in liver mitochondria. Under nonphosphorylating conditions, H_2O_2 release was lowest in brain mitochondria (regardless of the substrate used) and highest in heart mitochondria (during the oxidation of succinate alone or in combination with malate plus glutamate). In the case of liver and lung mitochondria, no major quantitative differences in mROS production can be seen during the oxidation of the substrates of complex I and complex II. In the case of the heart and brain mitochondria, H_2O_2 release under nonphosphorylating conditions was significantly higher when complex II was involved compared to complex I. Interestingly, for all types of mitochondria, in both respiratory states, the H_2O_2 release observed at the involvement of both complex I and complex II did not exceed the H_2O_2 release observed with a single best substrate entry. This observation indicates the existence of a maximum respiratory chain capacity in ROS production, i.e., an upper limit in state 4 and a lower limit in state 3, possibly related to the OXPHOS control mediated by $m\Delta\Psi$. Measurements of mROS formation under physiological conditions in the absence of electron transport inhibitors may be helpful in assessing the overall intrinsic production of mROS in mitochondria and, in the longer term, the physiological role of these signaling molecules in mitochondrial dysfunction.

Comparing mitochondria isolated from different tissues is difficult due to possible different contaminants. Therefore, the data obtained should be interpreted with caution. In the case of liver mitochondria, the obtained results of the measurement of H_2O_2 release may be underestimated due to contamination with peroxisomal catalase. On the other hand, in the case of brain mitochondria, the data (H_2O_2 release, amount of mQ pool, and $m\Delta\Psi$) may be underestimated due to contamination with synaptosomes and myelin. Moreover, when interpreting the obtained results, one should take into account the limitations resulting from the applied technique of measuring H_2O_2 release, mentioned in Materials and Methods.

5. Conclusions

We studied the change in mROS production that occurs as a result of transition from nonphosphorylating conditions to phosphorylating conditions in mitochondria of various rat tissues with different contents of the reduced Q. This change, described by the RCR_{ROS} parameter, shows the extent of mROS production that accompanies mitochondrial function when the respiratory chain is uninhibited. We found that ROS production under nonphosphorylating conditions was strongly dependent on the amount of reduced Q and may reflect different requirements for reduced Q as an antioxidant.

Author Contributions: Conceptualization, W.J.; methodology, K.D., W.J.; carrying out experiments, K.D., W.J.; data analysis, K.D., W.J.; writing—original draft preparation, review and editing, W.J.; project administration, W.J.; funding acquisition, W.J. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This non-interventionary study involving animals was conducted according to the guidelines of the Declaration of Helsinki, and were in compliance with the guidelines of the European Community Council Directive 2010/63/UE on the protection of animals used for scientific purposes. As no procedures were performed using live animals and as they were sacrificed for scientific purposes, no approval was needed for our study according to the Polish Animal Welfare Act.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are openly available in Mendeley Data, V1 at doi:10.17632/sjtjy59ykv.1.

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Effects of Endurance Training on the Coenzyme Q Redox State in Rat Heart, Liver, and Brain at the Tissue and Mitochondrial Levels: Implications for Reactive Oxygen Species Formation and Respiratory Chain Remodeling

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Abstract: Sixteen adult, 4-month-old male Wistar rats were randomly assigned to the training group (n = 8) or the control group (n = 8). We elucidated the effects of 8 weeks of endurance training on coenzyme Q (Q) content and the formation of reactive oxygen species (ROS) at the tissue level and in isolated mitochondria of the rat heart, liver and brain. We demonstrated that endurance training enhanced mitochondrial biogenesis in all tested organs, while a significant increase in the Q redox state was observed in the heart and brain, indicating an elevated level of QH_2 as an antioxidant. Moreover, endurance training increased the mQH_2 antioxidant pool in the mitochondria of the heart and liver, but not in the brain. At the tissue and isolated mitochondria level, an increase in ROS formation was only observed in the heart. ROS formation observed in the mitochondria of individual rat tissues after training may be associated with changes in the activity/amount of individual components of the oxidative phosphorylation system and its molecular organization, as well as with the size of the oxidized pool of mitochondrial Q acting as an electron carrier in the respiratory chain. Our results indicate that tissue-dependent changes induced by endurance training in the cellular and mitochondrial QH_2 pool acting as an antioxidant and in the mitochondrial Q pool serving the respiratory chain may serve important roles in energy metabolism, redox homeostasis and the level of oxidative stress.

Keywords: coenzyme Q; reactive oxygen species; mitochondrial energetics; endurance training

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1. Introduction

Physical exercise provides a variety of metabolic, thermal and mechanical stimuli to various organs of the body, leading to a variety of adaptive responses [1]. Acute physical exercise has been shown to increase oxidative stress in skeletal muscles and other organs [2–5], which can be harmful to the body [2], but in small doses, can stimulate several adaptive responses in different organs [6–8]. It is well documented that endurance training has a strong effect on increasing the activity/amount of skeletal muscle mitochondrial enzymes [9–11], resulting in the improved metabolic stability of muscles during exercise [12,13] and resistance to fatigue [14]. Much less is known about the effects of endurance training on mitochondrial enzyme activity and the state of oxidants in other vital organs of the body, such as the brain, heart and liver. Acute exercise has been shown to increase the production of oxidants in the aged heart but not in the liver, thereby indicating that aging heart muscle is more susceptible to oxidative stress after heavy exercise than

the liver [15]. Moreover, the adaptive responses of the brain to oxidative stress induced by acute or chronic exercise differ significantly from those of the liver and heart as well as fast- and slow-twitch muscles [4]. Nevertheless, there are few studies involving the same animals that show the multiorgan response of mitochondria to endurance training—especially studies focused on the role of coenzyme Q (Q).

Mitochondria are organelles that are crucial for cellular energy production and the formation of reactive oxygen species (ROS) [16]. The reduced form of Q (QH_2) is an important antioxidant found in all cell membranes, including the mitochondria [17–19]. Additionally, the redox state of Q may be a useful marker of cellular oxidative stress. Mitochondrial Q (mQ) is a key nonprotein carrier of electrons in the respiratory chain that is also involved in the production of mitochondrial ROS (mROS), which arises as a byproduct of oxygen metabolism or under oxidative stress conditions [20,21], including acute or chronic/endurance training. However, little is known about the role of mQ in mitochondrial adaptation to endurance training.

The purpose of this study was to investigate the effects of 8-week endurance training on mitochondrial biogenesis, Q content and the formation of ROS in highly energy-dependent rat tissues such as the heart, liver and brain. Research on ROS production and changes in the amounts of the reduced and oxidized Q (Q9 and Q10) pools was performed at the tissue (homogenate) and isolated mitochondria levels. Additionally, we examined the changes induced by endurance training at the level of the components of the oxidative phosphorylation (OXPHOS) system and antioxidant enzymes in isolated mitochondria. Our results show that the endurance training-induced changes in the mitochondria of all examined tissues may serve a role in their energy metabolism, redox homeostasis and level of oxidative stress. Our research highlights the role of a reduced Q pool at both the cellular and mitochondrial levels in protecting against ROS overproduction. It also highlights the role of the oxidized (reducible via the respiratory chain) mitochondrial Q (mQ) pool in adapting the OXPHOS system and modulating mROS production in response to endurance training

2. Results

2.1. Endurance Training Increases Mitochondrial Biogenesis in the Heart, Liver and Brain

Elevated levels of citrate synthase (CS) and voltage-dependent anion channel 1 (VDAC1) (Figure 1) indicate increased mitochondrial biogenesis in all tissues tested in trained rats. As a result of an 8-week endurance training, the greatest increase in mitochondrial protein levels was observed in the liver (~30% mean increase), while this increase was smaller in the heart and brain (~17 and ~12%, respectively). Additionally, endurance training induced significantly higher expression levels of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), a transcriptional coactivator that regulates the genes involved in mitochondrial biogenesis.

In tissues with a greater increase in training-induced mitochondrial biogenesis (i.e., the heart and liver), a decrease in the level of superoxide dismutase 1 (SOD1) was observed. In contrast, the level of this antioxidant protein increased in the brain (Figure 1).

2.2. Endurance Training Increases the Reduced and Oxidized Pools of Q9 and Q10 in the Heart and Liver and Brain

In the tested tissues of both trained and untrained rats, the size of the total Q10 pool (reduced plus oxidized) appeared to be similar (Figure 2). However, there were significant differences in the size of the total pool of the dominant Q form (Q9), which was largest for the heart and the smallest for the brain. The sizes of the reduced pools of Q9 and Q10 were greatest in the liver and smallest in the brain, indicating a high need for reduced Q as an antioxidant in the liver and low need in the brain.

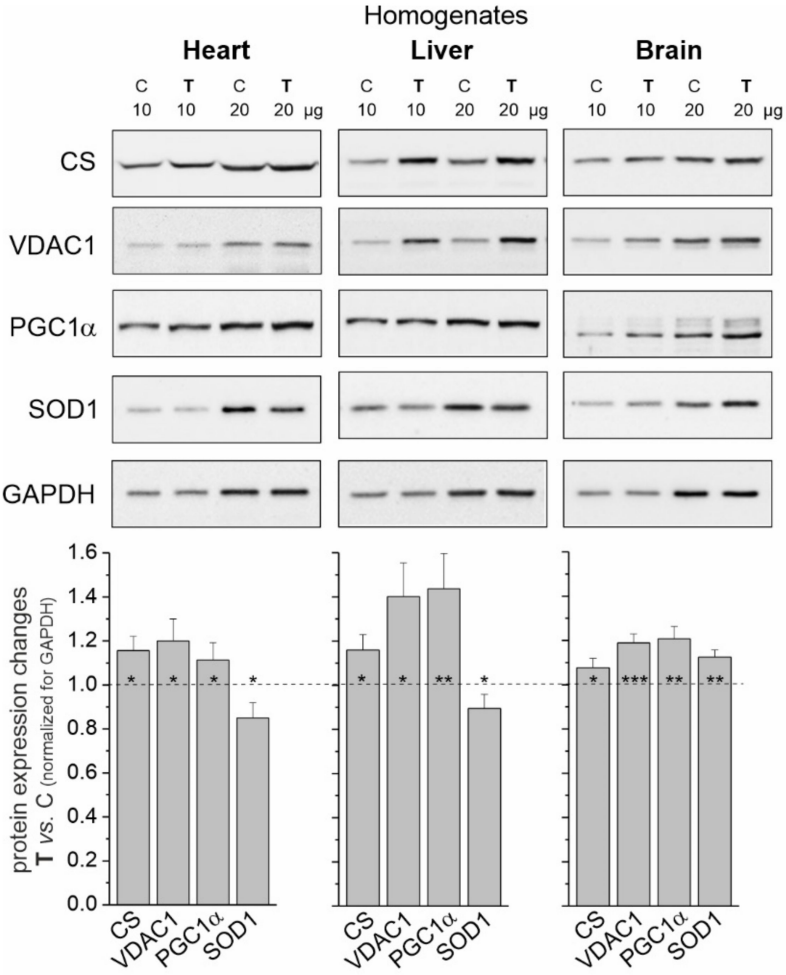

Figure 1. Representative Western blots and analyses of protein expression in rat heart, liver and brain homogenates from control (C) and trained (**T**) rats. CS, citrate synthase; VDAC 1, voltage-dependent anion-selective channel protein 1; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SOD1, superoxide dismutase 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Mean ± SD; n = 3–4 homogenate preparations (equal to the number of animals used in each group). $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), comparison vs. control values for a given tissue.

In all tested tissues, endurance training increased the total, reduced and oxidized pools of both Q forms (Figure 2). The total Q9 pool increased by ~35, ~45 and 17% in the heart, liver and brain, respectively. Moreover, the total Q10 pool increased by ~44, ~41 and ~34% in the heart, liver and brain, respectively. Reduced Q pools increased by ~77 (Q9) and ~190% (Q10) in the heart and by ~47 (Q9) and ~44% (Q10) in the liver. A significant increase in the reduced Q9 and Q10 pools was also observed in the brain. Since the reduced pools of both Qs were not detected in the brain before training, the percent increase could not be calculated. Given the difference in the size of the Q9 and Q10 pools, the amount of reduced Q9 increased the most in all examined tissues. Additionally, increases in the oxidized Q9 (~24, ~35 and ~11%) and Q10 (~12, ~30 and ~12%) pools in the heart, liver and brain, respectively, were observed after training.

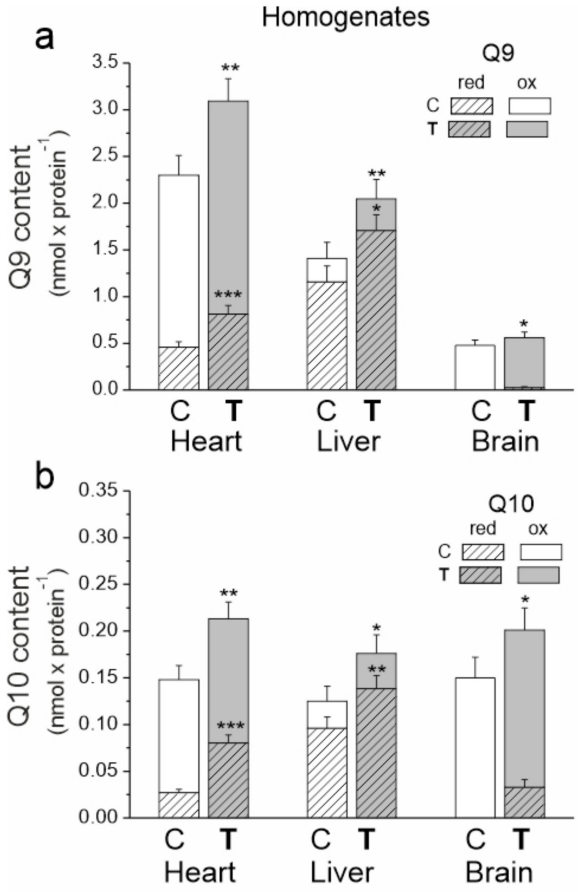

Figure 2. The content of coenzyme Q9 (Q9) (**a**) and coenzyme Q10 (Q10) (**b**) in the heart, liver and brain from control (C) and trained (**T**) rats. The total (Qred + Qox), reduced (Qred) and oxidized (Qox) Q pools were measured under fully oxidizing conditions (no respiratory Q-reducing substrates). Mean ± SD; n = 5–6 homogenate preparations (equal to the number of animals used in each group). $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), comparison vs. control values of Qred or Qtot for a given tissue.

2.3. Endurance Training Only Increases H_2O_2 Formation in the Heart (Tissue Homogenate)

We measured the rate of H_2O_2 release in tissue homogenates under mitochondrial respiratory chain activation in the presence of succinate and malate plus glutamate as respiratory substrates for complex II (CII) and complex I (CI), respectively (Figure 3). Measurements were made in the presence or absence of ADP (i.e., activation or inactivation of the mitochondrial OXPHOS).

Upon comparing the three examined tissues, the level of H_2O_2 production under OXPHOS activation conditions was similar for both trained and untrained rats (Figure 3). In the absence of ADP, the release of H_2O_2 was significantly higher when compared to the OXPHOS activation conditions, with the highest levels observed in the heart and liver homogenates from both trained and untrained rats. A statistically significant increase in H_2O_2 production was only observed in the heart homogenates under inactive OXPHOS conditions.

Further studies were conducted on the level of mitochondria isolated from the heart, liver and brain of trained and untrained rats.

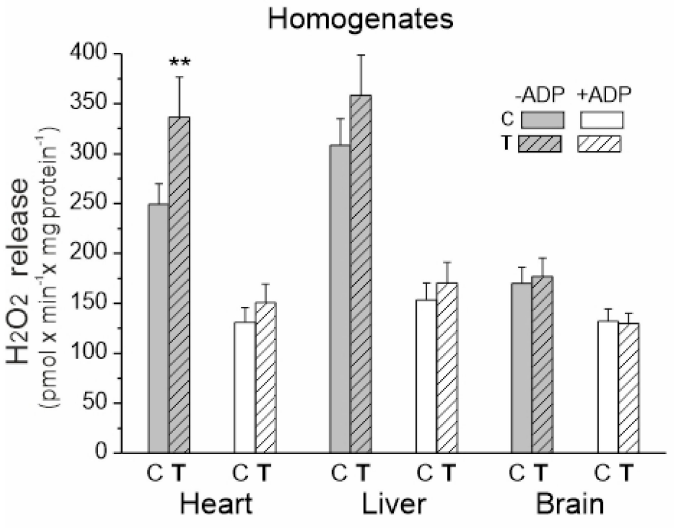

Figure 3. H_2O_2 release in heart, liver and brain homogenates from control (C) and trained (**T**) rats. Measurements were performed in the absence or presence of ADP with succinate and malate plus glutamate as mitochondrial respiratory substrates. Mean ± SD; n = 5–6 homogenate preparations (equal to the number of animals used in each group). $p < 0.01$ (**), comparison vs. control values for a given tissue.

2.4. Endurance Training (i) Increases the Reduced and Oxidized mQ Pools in the Heart Mitochondria, (ii) Increases the Total Reduced mQ Pool ($QH_2$9 + $QH_2$10) and Decreases the Total Oxidized mQ Pool (Q9 + Q10) in the Liver Mitochondria and (iii) Does Not Alter mQ Pools in the Brain Mitochondria

The heart mitochondria of untrained and trained rats had significantly greater amounts of mQ9 and mQ10 than the mitochondria of the other tested tissues (Figure 4). The liver mitochondria had the highest percentage of reduced mQ pools (~50% of the mQ9 pool and ~90% of the mQ10 pool).

The greatest response to endurance training at the mQ pool levels was observed in the mitochondria of the heart. After 8 weeks of training, significantly higher amounts of reduced mQ ($mQH_2$9 by ~66% and $mQH_2$10 by ~70%) and smaller increases in oxidized mQ (mQ9 by ~15% and mQ10 by ~30%) were observed in the heart mitochondria (Figure 4). Thus, while these changes indicate an increase in the oxidized Q pool required for respiratory chain function, they also indicate a much greater increase in the reduced Q pool that can serve as an antioxidant.

In the mitochondria of the liver, the importance of training-induced changes was evident when mQ9 and mQ10 pools were analyzed together. In the liver mitochondria of trained rats, the total reduced mQ pool ($mQH_2$9 + $mQH_2$10) increased by ~14%, while the total oxidized Q pool (mQ9 + mQ10) decreased slightly by ~10%. These changes indicate a decrease in the mQ pool available for the respiratory chain and an increase in the mQH2 pool acting as an antioxidant. No statistically significant changes were observed in the oxidized and reduced mQ pools in the brain mitochondria of trained animals.

Additionally, we investigated changes in the expression level of the mitochondrial Q-binding protein (CoQ10A) required for mQ function in the respiratory chain. In the heart mitochondria, where the pool of oxidized mQ increased after training, the amount of CoQ10A protein decreased significantly. In the mitochondria of the liver, where the pool of oxidized mQ decreased, the level of this protein increased (Figure 5a). In the brain mitochondria of trained rats, the lack of changes in the level of this protein was accompanied by a lack of changes in the pool of oxidized mQ.

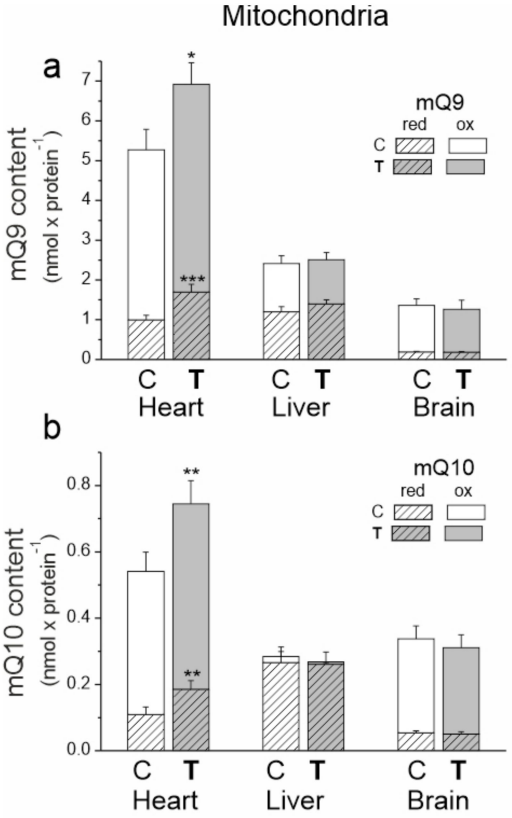

Figure 4. The content of coenzyme Q9 (Q9) (**a**) and coenzyme Q10 (Q10) (**b**) in the heart, liver and brain mitochondria from control (C) and trained (T) rats. The total (Qred + Qox), reduced (Qred) and oxidized (Qox) Q pools were measured under fully oxidizing conditions (no respiratory Q-reducing substrates). Mean ± SD; n = 5–6 mitochondrial isolations (equal to the number of animals used in each group). $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), comparison vs. values of Qred or Qtot of control mitochondria for a given type of tissue.

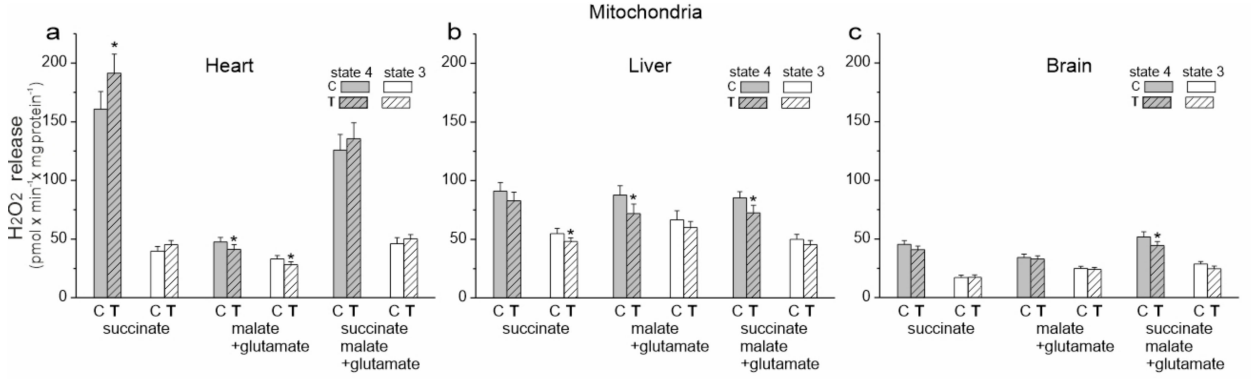

Figure 5. H_2O_2 formation release in heart, liver and brain mitochondria from control (C) and trained (T) rats. Measurements were performed with succinate (**a**), malate plus glutamate (**b**) and a mixture of succinate and malate plus glutamate (**c**) under nonphosphorylating (state 4) and phosphorylating (state 3) conditions. Mean ± SD; n = 5–6 mitochondrial isolations (equal to the number of animals used in each group). $p < 0.05$ (*), comparison vs. control mitochondria for a given tissue.

2.5. Endurance Training Does Not Increase mROS Production in the Liver and Brain Mitochondria but Changes mROS Production Depending on the Respiratory Substrate in Heart Mitochondria

We then examined the release of H_2O_2 by the isolated mitochondria of untrained and trained rats during the oxidation of succinate (CII substrate), malate plus glutamate (CI substrates) and a mixture of all of these substrates under phosphorylating (state 3) and

nonphosphorylating (state 4) conditions (Figure 5). The involvement of specific sites in the mitochondrial respiratory chain in mROS production is highly dependent on oxidized substrates [22]. Therefore, for a given type of mitochondria and a given respiratory state, a different production of H_2O_2 during the oxidation of different respiratory substrates was observed (Figure 5). Under our experimental conditions, during succinate oxidation, the flavin site of CII (site II_F) and the Q_o and Q_i sites of CIII (site III_{Qo} and site III_{Qi}) could participate in mROS production. Since no rotenone was used, reverse electron transfer from CII to Cl could occur during the oxidation of the succinate. When the malate (plus glutamate) was oxidized, all mROS production sites mentioned could be active, as well as the flavin-dependent site (I_F) and the mQ-binding site (I_Q) of CI. However, the purpose of our research was not to determine the contribution of individual mROS production sites but to determine the total mROS production when electrons enter the respiratory chain via CI and/or CII under phosphorylating and nonphosphorylating conditions for the mitochondria of various tissues from control and trained rats.

In the case of cardiac mitochondria among the tested substrates, the highest release of H_2O_2 was observed during succinate oxidation under nonphosphorylating conditions for both groups of rats (Figure 5a). Under these conditions, endurance training induced a statistically significant increase in H_2O_2 release. During the oxidation of CI substrates, a statistically significant reduction in H_2O_2 release in both energy states was observed in the cardiac mitochondria of trained rats. However, a slight non-statistically significant increase in H_2O_2 release was observed during the oxidation of the substrate mixture. These results indicate that mROS production could increase in the heart mitochondria of trained rats depending on the involvement of individual respiratory chain dehydrogenases. However, levels of mitochondrial antioxidant proteins such as superoxide dismutase 2 (SOD2) and uncoupling proteins (UCP2 and UCP3) remained unchanged (Figure 6a).

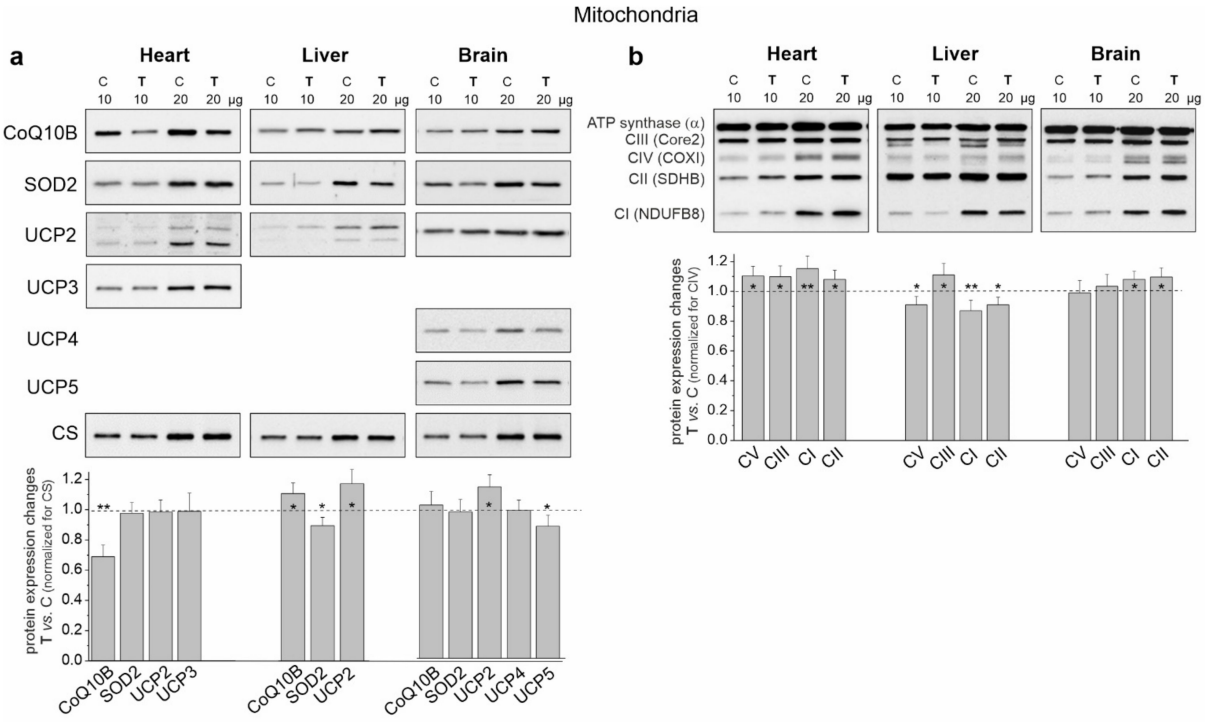

Figure 6. Representative Western blots and analyses of protein expression in the heart, liver and brain mitochondria from control (C) and trained (T) rats. (**a**) CoQ10B, mitochondrial coenzyme Q-binding protein CoQ10 homolog B; SOD2, superoxide dismutase 2; UCP2-5, uncoupling protein 2–5; CS, citrate synthase. (**b**) OXPHOS complexes: CI-CIV, complexes of the respiratory chain; CV and ATP synthase. Mean ± SD; n = 3–4 mitochondrial isolations (equal to the number of animals used in each group). $p < 0.05$ (*), $p < 0.01$ (**), comparison vs. control mitochondria for a given tissue.

An overall decrease in H_2O_2 release was observed in the liver mitochondria of the trained rats and it was statistically significant under phosphorylating conditions during succinate oxidation and under nonphosphorylating conditions during the oxidation of malate plus glutamate and the mixture of CI and CII substrates (Figure 5b). Additionally, although decreased SOD2 levels were observed in the liver mitochondria of trained rats, UCP2 levels increased (Figure 6a).

In the brain mitochondria of trained rats, no statistically significant changes in H_2O_2 release were observed during the oxidation of CI and CII substrates, which were administered separately (Figure 5c). However, during the oxidation of the mixture of these substrates, the level of H_2O_2 release was lower under nonphosphorylating conditions. Moreover, unchanged SOD2 levels and increased UCP2 levels were observed in the brain mitochondria of trained rats (Figure 6a).

To understand the changes in mQ pools and mROS production observed in the mitochondria of the heart, liver and brain of trained rats, we investigated at the changes induced by endurance training in the activity and quantity of individual components of the OXPHOS system and its molecular organization.

2.6. Endurance Training Leads to an Alteration in the Molecular Organization of the OXPHOS System in the Heart, Liver and Brain Mitochondria

In the cardiac mitochondria of trained rats, Western blot analysis showed a statistically significant increase in the expression level of all OXPHOS complexes except CIV (Figure 6b). Additionally, BN-PAGE followed by in-gel activity assays revealed that after endurance training, the activities of CII, CV (V) and all supercomplexes of CI increased (Figure 7a). Moreover, a Western blot of CIII supercomplexes separated by BN-PAGE showed increases in their levels except for III_2 + IV. All of these changes—along with the increase in the pool of oxidized mQ described earlier (Figure 4a)—indicate a general upregulation of the OXPHOS system in the heart mitochondria of trained rats. A greater increase in the activity of the I + III_2 + IV(n) compared to I + III_2 and I with a decrease in the level of III_2 + IV (Figure 7a) and an increase in the mQ oxidized pool (~23%) (Figure 4a) may lead to a decrease in mROS production during the oxidation of CI substrates (malate plus glutamate) (Figure 5a).

In the liver mitochondria of trained rats, an overall reduction in the protein level (Figure 6b) and activity (Figure 7b) of dehydrogenases (CI and CII) and ATP synthase (CV) was observed. Western blot of CIII separated by the SDS-PAGE (Figure 6b) and BN-PAGE (supercomplexes, Figure 7b) showed an elevation in its level, including all of the supercomplexes except for unchanged III_2 + IV. All of these changes, along with an increase in the pool of reduced mQs (Figure 4a), may lead to an overall decrease in mROS formation in the liver mitochondria of trained rats.

Although an increase in CI expression was observed (Figure 6b) in the brain mitochondria of the trained rats, the in-gel activity assay showed no statistically significant increase in the activity of individual supercomplexes (Figure 7c). Endurance training increased the level of protein and activity in CII (Figures 6b and 7c, respectively). Although the overall level of CIII did not change, increased levels of its supercomplexes containing CI were observed (I + III_2 + IV and I + III_2), possibly at the expense of III_2 and III_2 + IV. While the expression of total CV remained unchanged (Figure 6b), the activity of V2 and V increased slightly (Figure 7c).

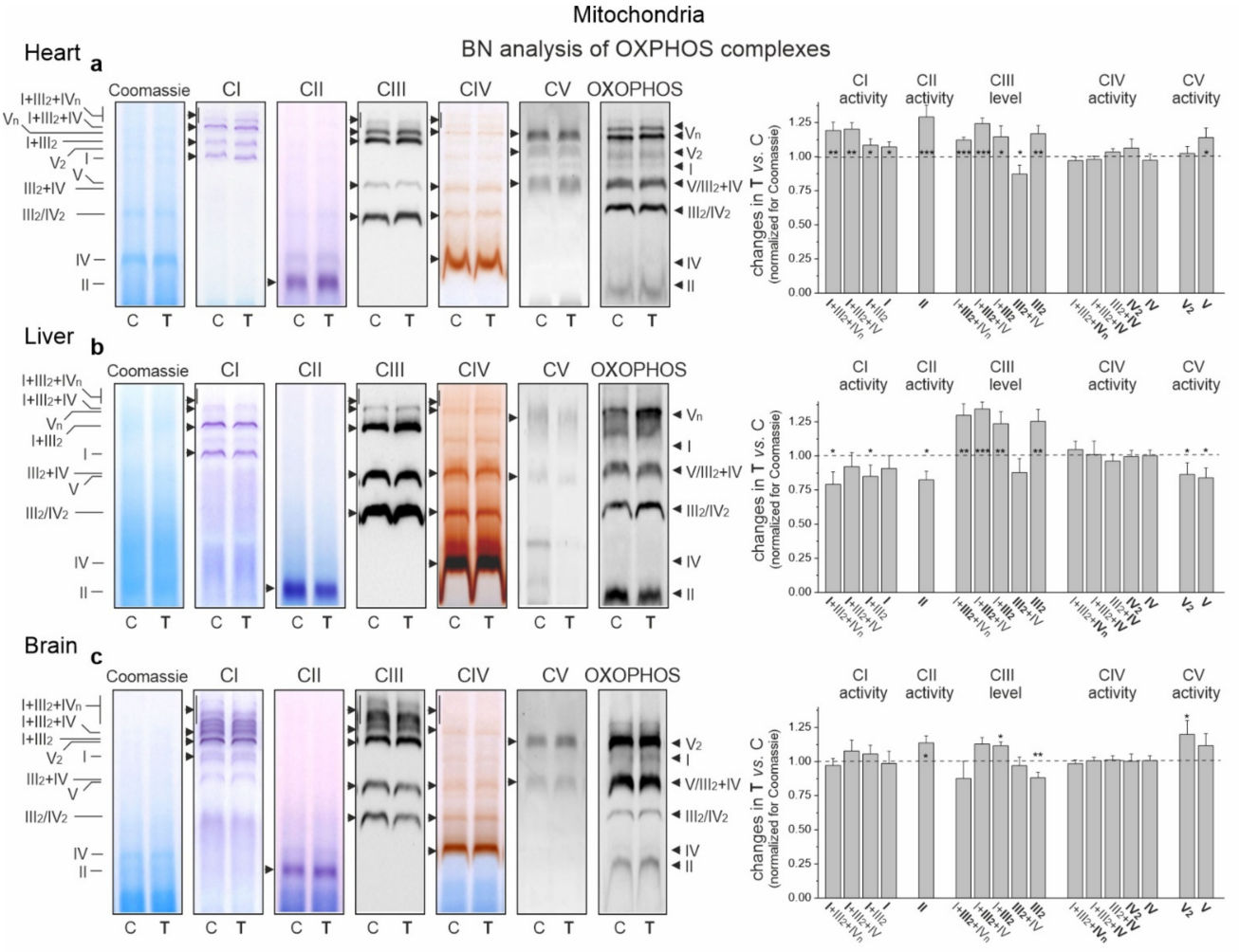

Figure 7. Representative BN-PAGE showing OXPHOS complexes and supercomplexes in mitochondria from the heart (**a**), liver (**b**) and brain (**c**) of control (C) and trained (T) rats. Shown in sequence are Coomassie staining/destaining (loading control), CI in-gel activity, CII in-gel activity, CIII immunoblotting, CIV in-gel activity, CV in-gel activity and total OXPHOS immunoblotting. OXPHOS, oxidative phosphorylation system; CI–CIV, respiratory chain complexes I–IV; CV, ATP synthase. Mean ± SD; n = 3–4 mitochondrial isolations (equal to the number of animals used in each group). $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), comparison vs. control values for a given tissue.

3. Discussion

One might expect that the amount of ROS produced during physical exercise should be higher in organs with a higher metabolic rate (oxygen consumption per gram of tissue × min^{-1}). It is well documented that the metabolic rates of the skeletal muscles, heart, brain and liver vary during exercise. Namely, the consumption of oxygen by human skeletal muscles during exercise can be over 80 times higher than at rest [23]. Moreover, it has been shown that oxygen consumption during exercise, when compared to resting level, increases by ~5–6 times in the heart [24], only ~2 times in the liver [25] and not more than ~0.5 times in the brain [26]. These results suggest that heart tissue should be much more exposed to exercise-induced oxidative stress than the brain and liver tissues. Moreover, as proposed by Liu et al. [4], it seems likely that susceptibility to oxidants, activation of antioxidant enzymes, antioxidant levels and other repair systems may vary from organ to organ. To date, no studies have been performed on the same animals to describe the multiorgan response of mitochondria to endurance training in terms of the role of mQ. Our research showed that 8 weeks of relatively intense endurance training resulted in many

changes important to energy metabolism, redox homeostasis and oxidative stress level. Notably, these changes differed among the studied tissues (i.e., heart, liver and brain).

We demonstrated that strenuous endurance training enhanced mitochondrial biogenesis in all tested organs, as evidenced by increased levels of mitochondrial proteins (VDAC1 and CS) and the mitochondrial biogenesis marker protein (PGC1α). Given the average increase in the mitochondrial proteins after endurance training, it can be estimated that the increases in mitochondrial biogenesis were ~17%, ~30% and ~12% in the heart, liver and brain, respectively. An increase in mitochondrial biogenesis after exercise or chronic training has previously been demonstrated in rodent heart [27], liver [28] and brain [29]. Moreover, endurance exercise-induced systemic mitochondrial biogenesis has been observed in many tissues, including the heart, liver and brain in mtDNA mutant mice [30].

The changes in mitochondrial biogenesis observed in this study were accompanied by larger changes in the total (reduced plus oxidized) Q9 + Q10 pool (~36, ~45 and ~21% in the heart, liver and brain, respectively), indicating that the nonmitochondrial Q pool also increased in all tissues. The tissue changes induced by training in the oxidized Q pool (reducible by the respiratory chain) can mainly be attributed to an increase in mitochondrial biogenesis, while changes to the reduced Q (QH_2) pool can be attributed to an increase in mitochondrial and nonmitochondrial antioxidants. In the heart, the total pool of reduced Qs ($QH_2$9 + $QH_2$10) increased by ~83%, while the total pool of oxidized Qs (Q9 + Q10) only increased by ~23%. Moreover, the increased total redox state of Q (QH_2/Q) increased by ~50% (from 0.25 to 0.37), indicating an upregulation of the antioxidant form of Q in a trained heart. In the liver, the total reduced and oxidized Q pools increased after endurance training by ~47 and ~36%, respectively. Meanwhile, the total Q redox state increased slightly (from 4.50 to 4.82), indicating no significant training-induced changes in the Q redox state. In the case of the brain, the total oxidized Q pool increased by ~12%. However, quantitative changes in the total reduced Q pool were difficult to quantify since QH_2 levels were not detected in control rats. However, it can be estimated that the Q redox state in the brain increased significantly after training. These results are consistent with earlier determinations of the ubiquinol/ubiquinone ratio after 8 weeks of treadmill running, which showed a ~40% and ~10% increase in this ratio within heart and brain tissues, respectively and no change in the liver [4]. Taken together, the response of QH_2 as an antioxidant pool to exercise varies from tissue to tissue.

While ROS are involved in redox signaling, they can lead to oxidative damage when present in excess. Therefore, the amount of ROS should be controlled. At the tissue (tissue homogenate) level, a statistically significant increase in H_2O_2 release was only observed in the heart and only under OXPHOS inactivation conditions. These observations indicate that endurance training may increase overall heart ROS production, but not likely above safe levels since SOD1 levels were even lower in the hearts of trained rats. In the liver and brain, endurance training did not increase oxidant formation. In the brain, elevated levels of SOD1 may help to remove excess ROS. These observations support the idea that the heart may be much more exposed to exercise-induced oxidative stress than the brain and liver, which may be due to a much greater increase in oxygen consumption during exercise compared to the level at rest (as previously discussed). In the brain, it is especially important to maintain a normal redox state due to the enhanced sensitivity to ROS [31].

Studies with mitochondria isolated from control tissues and trained rats provided insights into changes induced in the mQ pool, mROS formation and respiratory chain levels. Here, we demonstrated for the first time that endurance training can increase the mQH_2 antioxidant pool in the mitochondria of certain tissues. Endurance training increased the total reduced mQ pool ($QH_2$9 + $QH_2$10) in the heart and liver by ~70% and ~14%, respectively, while it did not significantly change this pool in the brain. These results suggest that the heart mitochondria of trained rats have a particularly high demand for this antioxidant. Liver mitochondria, which initially had the largest reduced mQ pool, increased the mQH_2 pool to a lesser extent in response to training, while brain mitochondria

did not use this molecule as an antioxidant under training conditions. Measurements of mROS in mitochondria isolated from control and training rats support these observations. Namely, endurance training did not increase the production of mROS in the mitochondria of the liver and brain. Depending on the involvement of individual dehydrogenases in the respiratory chain, it could increase this production in the mitochondria of the heart. In our study, no increase in the level of the antioxidant enzyme SOD2 was observed in the mitochondria of the heart, liver and brain of the trained rats.

Our research has shown that the mROS formation observed in the mitochondria of individual rat tissues after training may be associated with changes in the activity/amount of individual elements of the OXPHOS system and its molecular organization, as well as with the size of the oxidized pool of mQ acting as an electron-carrier in the respiratory chain. The enhanced energy requirements of the heart associated with endurance training performance (up to a ~5–6-fold increase in oxygen consumption from resting levels [24]) and a relatively small increase in mitochondrial biogenesis (~20%; this study) imply that major adaptive changes that can meet this metabolic challenge likely occur at the cardiac OXPHOS system level. In the heart mitochondria of trained rats, we observed an increase in the amount/activity of all components of the OXPHOS system (except the non-limiting CIV) as well as in the oxidized Q pool, which may indicate increased OXPHOS yield. Additionally, the observed rearrangement of supercomplexes in the respiratory chain of the mitochondria of trained rats, coupled with an increase in the oxidized mQ pool (~23%), may lead to a decrease in mROS production during the oxidation of CI substrates.

In the hepatic mitochondria of trained rats, rearrangement of the components of the respiratory chain and a concomitant increase in the pool of antioxidants (i.e., mQH_2) can lead to the observed overall reduction in mROS formation. Namely, as a result of training, a decreased level/activity of the mQ-reducing pathways (Cl and CII) and an increased level of the mQH_2-oxidizing CIII may lead to a decrease in the level of mQ reduction and thus the production of mROS. It has recently been shown that mROS formation is directly dependent on the level of mQ reduction (mQH_2/mQtot) in the active pool associated with the respiratory chain [8]. Moreover, the increased level of UCP2 in the liver mitochondria of trained rats may be involved in lowering mROS formation.

In the mitochondria of the brain, endurance training did not alter the total reduced and oxidized mQ pools or increase the level of mROS formation. As a result of training, increased levels and/or activity of CII and CI + CIII supercomplexes may lead to no increase in mROS formation and possibly an increased OXPHOS yield since CV activity is slightly increased. Moreover, the increased level of UCP2 after endurance training observed in the brain mitochondria may be involved in maintaining unelevated mROS levels. It has previously been observed that voluntary exercise induces UCP2 mRNA expression and mitochondria oxygen consumption in phosphorylating and nonphosphorylating respiratory states in the hippocampus of mice [32].

Our research has shown that in the mitochondria of the heart, liver and brain, endurance training triggered a different response to the redox state of mQ. Namely, the mQH_2/mQ ratio increased by 38% (from 0.24 to 0.33) and by 25% (from 1.19 to 1.49) in the heart and liver mitochondria, while remaining unchanged in the brain mitochondria (0.17). These results show for the first time that endurance training can affect mQ redox homeostasis in certain tissues. To date, it has been observed that after 8 weeks of endurance training, the total oxidized plus reduced mQ pool is lowered in lung mitochondria [8] and increased in skeletal muscle mitochondria [11].

Our results indicate that endurance training increased the nonmitochondrial and mitochondrial Q pools, especially the reduced Q pools, in all tissues tested. The question is whether Q10 supplementation could help meet the increased demand for Q in the heart, liver and brain of training animals. Perhaps increased endogenous Q synthesis in these tissues is sufficient. However, further research should clarify these points. There is also no convincing evidence that supplementation with Q10 as an antioxidant is effective and should be recommended to athletes [33]. Systemic functional measurements in several

tissues, including isolated mitochondria, are difficult and limited to perform. Our measurements do not describe the actual level of mQ reduction under the reducing pressure of the active mitochondrial respiratory chain. Moreover, it would be interesting to study the total respiration rate under resting and phosphorylating conditions during the oxidation of various respiratory substrates in order to compare the total bioenergetic efficiency with the partial parameters presented on the individual complexes. Therefore, more research is needed to elucidate the effects of endurance training on respiratory chain activity in relation to the functioning of mQ as an electron carrier in the mitochondria of the heart, liver and brain, as well as in other tissues where it has not been studied.

Taken together, our results show that endurance training can induce various tissue and mitochondrial adaptive remodeling related to Q acting as an antioxidant and electron carrier in the respiratory chain. Thus, these results emphasize the role of the QH_2 pool in protecting against excessive ROS levels and the role of the oxidized mQ pool (reduced by the respiratory chain) in the adaptation of the OXPHOS system and modulation of mROS production in response to endurance training. Several factors, including oxygen consumption, metabolic rate, susceptibility to oxidants, activation of antioxidant proteins and levels of other antioxidants, may influence organ-based differences in Q-related responses to endurance training.

4. Materials and Methods

4.1. Animals and Endurance Training

The study was conducted in 16 adult, 4-month-old male Wistar rats that were randomly assigned to the training group (n = 8) or the control group (n = 8). During the experiment, the animals had free access to food and water and were kept under standard conditions of humidity and temperature on a 12 h light/dark cycle. Experimental protocols involving animals, their care, training and surgery were approved by the Local Ethics Committee on Animal Experimentation in Poznan, Poland (Permit Number: 15/2013) and were in compliance with the guidelines of the European Community Council Directive on the protection of animals used for scientific purposes.

The 8-week training was conducted five times per week on a treadmill for small rodents (Exer 3/6 M Treadmill, Columbus Instruments, Columbus, OH, USA) as previously described [11]. During the first week of training, rats were introduced to treadmill running at different speeds (20–30 m $\times$ min^{-1}) during 20–30 min running sessions. At the end of the first week, the training session was extended to 40 min. In the first 2 weeks, the basal running speed was set at 30 m $\times$ min^{-1} and increased to 40 m $\times$ min^{-1} for 20 s every 10 min. After 4 weeks, the training sessions were extended to 1 h with a running speed of 30 m $\times$ min^{-1}, which increased to 40 m $\times$ min^{-1} after ~10 min. The duration of the higher speed was gradually increased from 20 s in the 6th week to 40 s in the final week of training. The day after the end of the final training session, the exercise and control rats were sacrificed by decapitation. Every effort was made to minimize suffering.

4.2. Tissue Preparation and Mitochondria Isolation

All steps of tissue preparation and mitochondria isolation were performed at 4 °C as previously described [19], with some modifications. To isolate mitochondria from rat heart, liver and brain (cortex), tissues were harvested and placed in isolation medium A (pH 7.2) containing 50 mM Tris-HCl, 100 mM sucrose and 0.5 mM ethylenediaminetetraacetic acid (EDTA) and washed several times. Blood cells were removed by decantation. Tissues were cleaned of connective tissue and large vessels, cut into small pieces on ice and then filtered. Tissues were homogenized in isolation medium B containing 50 mM Tris-HCl (pH 7.2), 100 mM sucrose, 1 mM KH_2PO_4, 100 mM KCl, 0.5 mM EDTA and 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) using a Teflon or glass pestle at different times and intensity. The filtered homogenates were centrifuged once or twice at 900$\times$ g for 10 min. Some of the filtered homogenate supernatants were used for measurements (H_2O_2 release, the Q content and reduction level and protein

immunoblotting). The remainder of the homogenates was supplemented with isolation medium B with 0.2% bovine serum albumin (BSA) and centrifuged at 17,800× *g* for 10 min. Mitochondrial pellets were suspended in isolation medium B without BSA and centrifuged at 900× *g* for 8–10 min. The supernatants were filtered and again centrifuged at 17,800× *g* for 10 min. The heart and liver mitochondria were washed and centrifuged again. The final mitochondrial pellets were then resuspended in a small volume of medium C containing 10 mM Tris-HCl (pH 7.2), 75 mM sucrose and 225 mM mannitol.

The Bredford method was used to determine the protein concentration in homogenates and mitochondria. All functional measurements were performed at 35 °C. Measurements of H_2O_2 release and Q content were normalized to GAPDH expression levels (homogenates) and cytochrome *c* oxidase (COX) activity or expression levels (isolated mitochondria).

4.3. H_2O_2 Release

The Amplex Red assay was used to measure the rate of H_2O_2 release as previously described [19]. Measurements were performed with horseradish peroxidase (HRP, 0.14 U × mL^{-1}), Amplex Red (5 µM) and exogenous superoxide dismutase (SOD, 5U × mL^{-1}) to convert superoxide anion to H_2O_2. As inhibitor of HRP-independent conversion of Amplex Red to resorufin, 100 µM PMSF was added to the experimental medium immediately prior to the measurement [34]. The fluorescence change was followed for 40 min at 545 nm/590 nm using a Tecan multimode reader (Infinite M200 PRO) with 24-well plates. Homogenates (50 or 100 µg of protein) were incubated in 0.5 mL of standard assay medium (75 mM sucrose, 225 mM mannitol, 5 mM KH_2PO_4, 10 mM KCl, 0.5 mM EDTA, 10 mM Tris/HCl (pH 7.2) and 0.2% BSA) with a mixture of 5 mM succinate, 5 mM malate and 5 mM glutamate under nonphosphorylating or phosphorylating conditions (in the presence of 0.5 mM ADP). Mitochondria (0.4 mg of protein) were incubated in 0.5 mL of the standard incubation medium with 5 mM succinate, 5 mM malate and 5 mM glutamate or a mixture of these three substrates in the absence (nonphosphorylating state 4 conditions) or presence of 0.5 mM ADP (phosphorylating state 3 conditions). H_2O_2 release of nonphosphorylating respiration was determined after ADP depletion or in the absence of exogenous ADP.

4.4. Q Content in Tissues and Mitochondria

Extraction and HPLC detection techniques were used to determine the concentration of oxidized (at 290 nm) and reduced (at 275 nm) forms of Q9 and Q10 in rat tissues and mitochondria as previously described [19]. The oxidized (Q9 and Q10) and reduced ($Q9H_2$ and $Q10H_2$) Q pools were determined in rat tissues and mitochondria under fully oxidizing conditions, i.e., in the absence of respiratory Q-reducing substrates. Before Q extraction, homogenates (3 mg) and mitochondria (0.8 mg) were incubated with gentle agitation for 30 min in 3 mL of the standard assay medium (at 35 °C) to obtain fully oxidizing conditions. The total oxidized and reduced Q9 + Q10 pool (Q + QH_2) and the total Q redox state (QH_2/Q) were then calculated.

4.5. Cytochrome c Oxidase Activity

The COX activity was assessed polarographically in 0.5 mL of the standard assay medium with 0.4 mg of mitochondrial protein with successively added 10 µM antimycin A, 7 mM ascorbate, 0.05% cytochrome *c* and up to 1.5 mM of N, N, N′N′-tetramethyl-*p*-phenylenediamine (TMPD).

4.6. Protein Level Immunodetection

Homogenate and mitochondrial proteins were separated on 6–12% SDS-PAGE gels with the PageRuler PrestainedTM Protein Ladder (Thermo Fisher Scientific) as a marker of molecular weight as previously described [8]. We used primary Abcam antibodies raised against: CS (46 kDa) (ab96600), VDAC1 (35 kDa) (ab14734), PGC1α (92 kDa) (ab54481), SOD1 (18 kDa) (ab13498), GAPDH (37 kDa) (ab9485), UCP2 (33 kDa) (ab97931), UCP3 (34 kDa) (ab3477) and CoQ10B (46 kDa) (ab41997). Abcam total rodent OXPHOS antibody

cocktails (ab110413) were used, which contained antibodies against subunits of CI (20 kDa subunit NDUFB8), CII (30 kDa subunit SDHB), CIII (subunit Core 2, 48 kDa), CIV (COXI, 40 kDa) and ATP synthase (CV) (subunit α, 57 kDa). We also used primary antibodies from other manufacturers raised against: SOD2 (25 kDa) (ADI-SOD, Enzo Life Sciences), UCP4 (25 kDa) (PA5-100668, Invitrogen) and UCP5 (33 kDa) (PA5-89394, Invitrogen). As loading controls, the expression levels of GAPDH (homogenates) and COX or CS (mitochondria) were used. Protein levels were digitally quantified using ImageJ software.

4.7. BN-PAGE and in-Gel Activity Assays

BN-PAGE separation of mitochondrial proteins (50–200 µg) and in-gel activity assays of complexes CI, CII, CIV and CV were performed as previously described [8,35,36]. To determine the OXPHOS complexes with immunoblotting, the BN-PAGE-separated proteins were transferred onto nitrocellulose membranes and immunodetected with anti-UQCRC2 antibody (against CIII, ab14745, Abcam) or the total OXPHOS rodent antibody cocktail (ab110413, Abcam).

4.8. Statistical Analysis

The means ± SD obtained from at least 3–6 independent homogenate preparations or mitochondrial isolations are presented. Each determination was performed at least in duplicate. Significant differences were determined via unpaired *t* tests or ANOVAs (followed by Tukey's post hoc comparisons for $p < 0.05$ from an ANOVA). Differences were considered to be statistically significant if $p < 0.05$ (* or $^{\#}$), $p < 0.01$ (** or $^{\#\#}$), or $p < 0.001$ (*** or $^{\#\#\#}$). Statistical analysis was performed with Origin v 8.5.1 software (OriginLab Corporation, Northampton, MA, USA).

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Local Ethics Committee for Animal Research in Poznan, Poland (15/2013).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are openly available in Mendeley Data, V1, doi:10.17632/gm4bj3mnrw.1.

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