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Membrane potential and delta pH dependency of reverse electron transport-associated hydrogen peroxide production in brain and heart mitochondria

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Abstract

Succinate-driven reverse electron transport (RET) is one of the main sources of mitochondrial reactive oxygen species (mtROS) in ischemia-reperfusion injury. RET is dependent on mitochondrial membrane potential (Δψₘ) and transmembrane pH difference (ΔpH), components of the proton motive force (pmf); a decrease in Δψₘ and/or ΔpH inhibits RET. In this study we aimed to determine which component of the pmf displays the more dominant effect on RET-provoked ROS generation in isolated guinea pig brain and heart mitochondria respiring on succinate or α-glycerophosphate (α-GP). Δψₘ was detected via safranin fluorescence and a TPP⁺ electrode, the rate of H₂O₂ formation was measured by Amplex UltraRed, the intramitochondrial pH (pHₖₑₜₜ) was assessed via BCECF fluorescence. Ionophores were used to dissect the effects of the two components of pmf. The K⁺/H⁺ exchanger, nigericin lowered pHₑₜₜ and ΔpH, followed by a compensatory increase in Δψₘ that led to an augmented H₂O₂ production. Valinomycin, a K⁺ ionophore, at low [K⁺] in increased ΔpH and pHₑₜₜ, decreased Δψₘ, which resulted in a decline in H₂O₂ formation. It was concluded that Δψₘ is dominant over ΔpH in modulating the succinate- and α-GP-evoked RET. The elevation of extramitochondrial pH was accompanied by an enhanced H₂O₂ release and a decreased ΔpH. This phenomenon reveals that from the pH component not ΔpH, but rather absolute value of pH has higher impact on the rate of mtROS formation. Minor decrease of Δψₘ might be applied as a therapeutic strategy to attenuate RET-driven ROS generation in ischemia-reperfusion injury.

Keywords Reactive oxygen species · Mitochondria · Proton motive force · Membrane potential · Reverse electron transport · Nigericin · Valinomycin · Succinate · Alpha-glycerophosphate

Highlights

Reverse electron transport (RET)-evoked ROS production is dependent predominantly on Δψₑₜₜ.
RET-evoked ROS formation is dependent more on absolute value of pH than on ΔpH.
Higher extramitochondrial pH is followed by enhanced RET-evoked ROS production.

Abbreviations

α-GP alpha-glycerophosphate
α - alpha-glycerophosphate dehydrogenase
GPDH
CI complex I (NADH:ubiquinone oxidoreductase)
CIi complex II (succinate dehydrogenase; succinate:coenzyme Q reductase)
CIii complex III (coenzyme Q:cytochrome c oxidoreductase)
ΔpH transmembrane pH gradient
Δψₑₜₜ mitochondrial membrane potential
mtROS mitochondrial reactive oxygen species
O₂⁻ superoxide
pHₑₜₜ extramitochondrial pH
pHₑₜₜ intramitochondrial pH
pmf proton motive force
Q ubiquinone

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QH$_2$ ubiquinol  
RET reverse electron transport  
ROS reactive oxygen species  
SDH succinate dehydrogenase, Complex II, CII  
SQ$^-$ semiquinone  
TPP$^+$ tetraphenylphosphonium cation  
TPP$^+Cl^-$ tetraphenylphosphonium chloride  
UCP uncoupling protein

**Introduction**

There is a large body of experimental evidence demonstrating pathologically enhanced mitochondrial reactive oxygen species (mtROS) production in several diseases such as diabetes, neurodegenerative conditions including Alzheimer’s and Parkinson’s diseases, diabetes, and ischemia-reperfusion injury; for review see (Beal 1996; Giacco and Brownlee 2010; Chouchani et al. 2014). Respiratory Complex I (CI) is a primary source of mtROS and its dysfunction is thought to be pathologically relevant (Cadenas et al. 1977, Grivennikova and Vinogradov 2006, Treberg et al. 2011). In isolated mitochondria CI-mediated mtROS generation can be initiated under the following conditions: 1) with NADH-linked substrates (such as glutamate and malate), which generate mtROS at a relatively low rate; 2) with NADH-linked substrates in the presence of a CI inhibitor, like rotenone producing high rate of ROS; 3) with FADH$_2$-linked substrates like succinate or (Lambert and Brand 2004; Zoccarato et al. 2004; Treberg et al. 2011; Orr et al. 2012) alpha-glycerophosphate (α-GP) (Tretter et al. 2007b, c). In hyperpolarized non-phosphorylating mitochondria, FADH$_2$-linked substrates generate mtROS at a higher rate by supporting a reverse electron transport (RET) which occurs from Complex II (CII) or alpha-glycerophosphate dehydrogenase (α-GPDH) to CI via the Q-junction (Treberg et al. 2011). According to prior reports, succinate-driven mtROS production appears to have the highest rate in isolated murine mitochondria in the absence of ADP compared to NADH-linked substrates initiated mtROS (Korshunov et al. 1997; Kwong and Sohal 1998; Votyakova and Reynolds 2001; Liu et al. 2002; Zoccarato et al. 2011); this is attributed primarily to RET towards CI and partially to the forward electron transport (FET) towards CIII (Grivennikova and Vinogradov 2006; Treberg et al. 2011; Zoccarato et al. 2011; Quinlan et al. 2013). Upon succinate oxidation, in the absence of ATP synthesis, FET secures the energy demands of RET.

Rate of RET-associated ROS production is thought to be dependent on pmf which comprises mitochondrial transmembrane potential ($\Delta$$\psi_m$) and mitochondrial transmembrane pH gradient ($\Delta$$p$H) (Liu 1997; Votyakova and Reynolds 2001; Lambert and Brand 2004). It is a well-known phenomenon that high pmf, such as the one measured in the absence of ADP, is required for maintenance of RET. It has been shown that succinate- or α-GP-fueled RET is very sensitive to minor changes in $\Delta$$\psi_m$ in isolated mammalian (Tretter and Adam-Vizi 2007) and Drosophila (Miwa and Brand 2003) mitochondria.

More specifically, a 10% decrease in $\Delta$$\psi_m$ (caused by an uncoupler agent) gave rise to a 90% decrease in succinate-driven ROS production in rat heart mitochondria (Korshunov et al. 1997). The other component of pmf, $\Delta$$p$H, also appears to have a regulating effect on mtROS formation. Upon acidification of the matrix, mtROS generation is decelerated, which can be explained by the stabilisation of the semiquinone radicals (SQ$^-$) (Selivanov et al. 2008). The question arises as to which component of pmf plays the key role in the control of mtROS production. According to Lambert and Brand (Lambert and Brand 2004), succinate-driven ROS production is more dependent on $\Delta$$p$H than on $\Delta$$\psi_m$, as detected in mitochondria isolated from rat skeletal muscle. On the contrary, Selivanov and co-workers (Selivanov et al. 2008) revealed that mtROS generation is significantly affected by the actual value of pH itself (extramitochondrial pH; pH$_{ext}$ and intramitochondrial pH; pH$_m$), and not much influenced by $\Delta$$p$H or $\Delta$$\psi_m$, as measured in rat brain mitochondria.

The aim of the present study was to clarify which of the two components of pmf has a predominant role in the control of mtROS formation and to assess whether absolute pH value modulates RET-dependent mtROS production. We also aimed to test whether the effect of $\Delta$$\psi_m$, $\Delta$$p$H, and absolute pH values on ROS formation is different in brain compared to heart muscle mitochondria. $\Delta$$\psi_m$ and $\Delta$$p$H usually change in the same direction; for example, uncoupling depolarisation (decrease of $\Delta$$\psi_m$) is generally followed by a decrease in $\Delta$$p$H as well. With ionophores, like valinomycin and nigericin, it is possible to dissect the two components of pmf: $\Delta$$\psi_m$ and $\Delta$$p$H can be varied in a different direction. Nigericin decreases pH$_m$ (Rottenberg and Lee 1975) and hyperpolarises $\Delta$$\psi_m$ (Selivanov et al. 2008), whilst valinomycin elevates pH$_m$ (Selivanov et al. 2008) and depolarizes $\Delta$$\psi_m$ (Selivanov et al. 2008) under specific conditions. In the present study, $\Delta$$\psi_m$, pH$_m$, and H$_2$O$_2$ production were measured systematically and $\Delta$$p$H was calculated. To scrutinize Selivanov’s theory, pH dependence of the above-mentioned parameters was examined. In contrast to Lambert and co-workers (Lambert and Brand 2004), we concluded that the succinate-driven RET-evoked ROS production is more dependent on $\Delta$$\psi_m$ and less influenced by $\Delta$$p$H in both guinea pig brain and heart mitochondria. Furthermore, we showed, in agreement with Selivanov and colleagues, that absolute pH rather than $\Delta$$p$H itself modulates succinate- and α-GP-driven RET. Our results suggest that lowering $\Delta$$\psi_m$ might be an effective solution to reduce the RET-provoked mtROS load in conditions like ischemia-reperfusion where oxidative stress and high $\Delta$$\psi_m$ prevail.
Materials and methods

Chemicals

Standard laboratory reagents, except ADP, were obtained from Merck (Darmstadt, Germany). ADP was purchased from Sigma (St. Louis, MO, USA). Biochemicals were used for the determination of mitochondrial protein concentration according to the guidelines for animal experiments (Waltham, MA, USA).

Preparation of mitochondria

Mitochondria were prepared from albino guinea pig brain cortex using a Percoll gradient (Rosenthal et al. 1987, Tretter and Adam-Vizi 2007) and from whole heart using differential centrifugation (Mela and Seitz 1979, Korshunov et al. 1997), as previously described. Animal experiments were performed in accordance with the guidelines for animal experiments (Semmelweis University). A modified centrifugation protocol of Korshunov and co-workers (Korshunov et al. 1997) was used to determine mitochondrial protein concentration (Bradford 1976).

Brain mitochondria

The brain was rapidly homogenized in Buffer A (in mM: 225 mannitol, 75 sucrose, 5 HEPES, pH 7.4) and centrifuged for 3 min at 1300 g. The supernatant was centrifuged for 10 min at 20,000 g, and the resulting pellet was resuspended in 15% Percoll and layered on a discontinuous gradient consisting of 40 and 23% Percoll. This was centrifuged for 8 min at 30,700 g using no brake. After resuspension of the lower fraction in Buffer A, centrifugation was applied at 16,000 g for 10 min. Pellet was resuspended in Buffer A and centrifuged at 6300 g for 10 min. Subsequently, supernatant was discharged, and the pellet was resuspended in Buffer B (in mM: 225 mannitol, 75 sucrose, 5 HEPES, pH 7.4). All operations above were performed either on ice or at 4 °C (Komary et al. 2008).

Heart mitochondria

Mitochondria from heart were isolated following the modified protocol of Korshunov and co-workers (Korshunov et al. 1997). The heart was repeatedly washed in homogenisation buffer. All operations above were performed on ice or at 4 °C.

Buffers

Depending on the requirement of K⁺ of the applied ionophore (nigericin or valinomycin), one of the following media was applied in the relevant experiments:

- Standard medium A (high K⁺ content for nigericin; in mM): 125 KCl, 20 HEPES, 2 KH₂PO₄, 0.1 EGTA, 1 MgCl₂, and 0.025% BSA.
- Standard medium B (low K⁺ content for valinomycin to avoid mitochondrial swelling; in mM): 240 saccharose, 10 Tris, 2 KH₂PO₄, 4 KCl, 0.1 EGTA, 1 MgCl₂, and 0.025% BSA. pH of the respiratory media was adjusted prior to the measurements, in the absence of mitochondria, with HCl or NaOH to 6.4, 6.8, 7.0, 7.2, 7.4, 7.6 or 8.0. Addition of mitochondria suspended in buffered solution and addition of high concentrations of respiratory substrates (succinate or α-GP) could shift the pH of the incubation medium slightly. In order to calculate an accurate ΔpH, pH_extr, measured in the presence of mitochondria and respiratory substrate was applied in this study.

Measurement of mitochondrial membrane potential (ΔΨₘ)

Measurement with safranine-O

ΔΨₘ was assessed using safranine-O, a lipophilic cationic fluorescent dye, which accumulates in the mitochondrial membrane upon hyperpolarisation resulting in fluorescence quenching (Akerman and Wikstrom 1976). Safranine (2 μM) fluorescence (495 nm for excitation, 585 nm for
emission) was detected using a Hitachi F-4500 spectrofluorimeter (Hitachi High Technologies, Maidenhead, UK). All measurements were carried out at 37 °C in standard medium A or B, as previously described.

**Measurement with TPP⁺ electrode**

$\Delta \psi_m$ was estimated via the distribution of the tetraphenylphosphonium ion (TPP⁺). TPP⁺ was detected using a custom-made TPP⁺-selective electrode (Kamo et al. 1979), as described previously (Trettet al. 2007a). $\Delta \psi_m$ was calculated using the Nernst equation and the reported binding correction factor for brain mitochondria, as previously described (Rottenberg 1984; Rolfe et al. 1994). The calculation was performed according to Rottenberg and co-workers (Rottenberg 1984) assuming that the matrix volume of the mitochondria is 1 μl/mg protein (D.G. Nicholls, personal communication). The sensitivity of the TPP⁺ electrode was found to be decreased at low $\Delta \psi_m$ (less than ~120 mV) (Starkov and Fiskum 2003).

**Measurement of the intramitochondrial pH (pHᵢ)**

pHᵢ of isolated mitochondria was measured with the acetoxymethyl ester form of 2,7-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF/AM) (Jung et al. 1989), as described earlier (Sipos et al. 2005). Briefly; 100 μl mitochondria (35–40 mg/ml protein) were incubated with 50 μM BCECF/AM in Buffer C (in mM: 225 mannitol, 75 sucrose, 5 HEPES, 0.1 EGTA, pH 7.4) for 10 min at 25 °C. Ice-cold Buffer C (325 μl) was supplemented with 0.1 mM ADP (in order to prevent permeability transition pore opening). Loaded mitochondria were centrifuged for 2 min at 13000 g, the supernatant was removed, the pellet was resuspended in 450 μl Buffer C, and this was centrifuged for 2 min at 13000 g. The new pellet was resuspended in 450 μl Buffer C minus ADP, left standing for hydrolysis (10 min), and then centrifuged for 2 min at 13000 g. All centrifugation steps were performed at 4 °C. The supernatant was discharged. The pellet was supplemented with 13 μl Buffer C. BCECF-loaded mitochondria were used within 90 min. For fluorescence measurements, 3 μl aliquots of mitochondria were diluted in 2 ml of standard medium A or B. Fluorescence ratios were determined using the PTI Deltascan fluorescence spectrophotometer (440 or 505 nm for excitation, 540 nm for emission). Leaching of BCECF from mitochondria was determined by measuring the fluorescence of the supernatant of the centrifuged loaded mitochondria. Corrections were made by subtracting the fluorescence values of the supernatant from those of the experimental values. For calibration, the external and internal $[H^+]$ were equilibrated at varying $pH_{\text{extra}}$ values by the addition of a mixture of 8 μM nigericin ($K^+$/H⁺ antiporter), 2.5 μM gramicidin (Na⁺/K⁺ ionophore), and 8 μM monensin (Na⁺/H⁺ antiporter), as previously described (Sipos et al. 2005).

**Statistical analysis**

The statistical differences in multiple comparisons were evaluated with ANOVA (SigmaPlot™, Version 11, Systat Software, Inc., San Jose, CA, USA). Values of $p < 0.05$ were considered to be statistically significant.

**Results**

In order to dissect $\Delta \psi_m$ and ΔpH, the two components of $pmf$, ionophores were introduced throughout the experiments. The standard media A contained 2 mM $K_2HPO_4$ and 125 mM KCl, whilst standard medium B was supplemented with 2 mM $K_2HPO_4$ and 4 mM K⁺. ADP was absent providing a high $\Delta \psi_m$ to support RET in succinate- or α-GP-energised mitochondria. At the end of each experiment the uncoupler FCCP was given to eliminate any $\Delta \psi_m$ and abolish the succinate- or α-GP-driven RET.

**Effects of nigericin on pHᵢ, ΔpH, Δψᵢ, and mtROS production in brain mitochondria at medium pH 7.0**

Nigericin, a $K^+$/H⁺ antiporter, allows the electroneutral transport of these two ions in opposite directions across the mitochondrial inner membrane following the $K^+$ concentration gradient (Henderson et al. 1969; Rottenberg and Lee 1975). As displayed in Fig. 1, nigericin (20 nM) decreased pHᵢ (Fig. 1a) at $pH_{\text{extra}} = 6.84 ± 0.01$ (medium pH = 7.0) by 0.13 ± 0.04 pH unit and ΔpH from 0.23 ± 0.06 to 0.089 ± 0.02 (Fig. 1c). In addition, nigericin increased $\Delta \psi_m$ by 7.78 ± 2.5 mV; $\Delta \psi_m$ could not be increased any further by subsequent additions of nigericin. In contrast to Lambert and co-workers (Lambert and Brand 2004), we found that nigericin increased the rate of $H_2O_2$ generation by 52 ± 11% (from 1894 ± 169 to 2871 ± 169 pmol/min/mg protein) in succinate-respiring brain mitochondria (Fig. 1e). We can conclude that in succinate-supported mitochondria, nigericin decreased ΔpH and induced mitochondrial hyperpolarization, simultaneously elevating $H_2O_2$ production.

In order to gain a deeper insight into the effects of nigericin on RET, α-GP was also applied as a respiratory substrate. Unlike succinate, α-GP does not enter the mitochondria, it is oxidized by α-GPDH on the outer surface of the inner mitochondrial membrane and does not form NADH. Addition of rotenone diminished the $H_2O_2$ production both in succinate and α-GP energised mitochondria, which points to a CI-related ROS production, likely RET (Votyakova and Reynolds 2001). Both respiratory substrates upon their oxidation by succinate dehydrogenase (SDH) or α-GPDH reduce...
the coenzyme Q (Q; ubiquinone)-junction bypassing CI. Similarly to that observed with succinate, nigericin decreased pH$_{in}$, increased Δψ$_{m}$ (data not shown), and stimulated H$_2$O$_2$ production (Fig. 2c) in succinate-energised brain mitochondria. Mitochondria (0.05 or 0.1 mg/ml) were incubated in different standard media as described under Materials and Methods. Succinate (5 mM), FCCP (250 nM), valinomycin (0.25 nM), nigericin (20 nM) and cocktail (gramicidin, monensin, nigericin) were given as indicated. ΔpH (a, b) values were calculated from the difference between pH$_{in}$ and pH$_{extra}$. In A and B each experiment was calibrated by KOH. In E and F results (slope) are expressed in pmol/min/mg protein and each experiment was calibrated by 100 pmol H$_2$O$_2$. For (a, b, c, d, e, f) traces are representative of at least three independent experiments.

**Effects of valinomycin on pH$_{in}$, ΔpH, Δψ$_{m}$, and mtROS production in brain mitochondria at pH 7.0**

Valinomycin is a K$^+$ ionophore transporting K$^+$ along its electrochemical gradient across the mitochondrial inner membrane. In succinate-supported mitochondria, valinomycin (0.25 nM) increased pH$_{in}$ by 0.38 ± 0.04 pH unit (Fig. 1b), ΔpH from 0.39 ± 0.001 to 0.75 ± 0.04, and depolarized Δψ$_{m}$ in a dose-dependent manner (Fig. 1d). We found that valinomycin decreased the rate of H$_2$O$_2$ generation by 44.5 ± 4% when mitochondria were supported by succinate (Fig. 1f, trace b). Valinomycin displayed similar effects on α-GP-respiring brain mitochondria. At pH$_{extra}$ = 7.22 ± 0.01 (Fig. 2d), valinomycin alkalized the mitochondrial matrix by 0.26 ± 0.02 pH unit, while ΔpH was increased from 0.32 ±
0.01 to 0.59 ± 0.02 (Fig. 3d) with α-GP. Simultaneously, a decreased rate of the α-GP-evoked H$_2$O$_2$ production (by 45 ± 14%) was measured, similarly to that observed in succinate-supported mitochondria.

**Effects of pH$_{\text{extra}}$ on H$_2$O$_2$ production, pH$_{\text{intr}}$, ΔpH, and Δψ$_m$ in succinate- and α-GP-respiring brain mitochondria**

To examine the influence of changes in pH$_{\text{extra}}$ on ΔpH and H$_2$O$_2$ production, experiments were carried out in standard media A (nigericin) or B (valinomycin) varying pH from 6.4 to 8.0 (see Materials and Methods).

**H$_2$O$_2$ production** As seen in Fig. 2, upon increasing pH$_{\text{extra}}$ a sharp increase of succinate- and α-GP-related H$_2$O$_2$ generation was observed both in the absence (Fig. 2a, c, *black circles*) and presence (Fig. 2a, c, *white circles*) of nigericin. The nigericin treatment of succinate-supported mitochondria elevated the rate of H$_2$O$_2$ production significantly between pH$_{\text{extra}}$ = 6.45 ± 0.004 and 7.03 ± 0.02 (Fig. 2a). Similarly, in α-GP-respiring mitochondria nigericin increased the rate of H$_2$O$_2$ formation by 48 ± 3% at pH$_{\text{extra}}$ = 6.81 ± 0.01, 42 ± 4% at pH$_{\text{extra}}$ = 7.05 ± 0.05, and 21 ± 11% at pH$_{\text{extra}}$ = 7.45 ± 0.02 (Fig. 2c). The addition of valinomycin to succinate- and α-GP-supported mitochondria significantly reduced the rate of H$_2$O$_2$ formation at all different pH$_{\text{extra}}$ values (Fig. 2b, d).

Δψ$_m$ Measuring Δψ$_m$ by a TPP$^+$ electrode, it was concluded that nigericin always increased the Δψ$_m$ approximately to the same level (∼−195 to −200 mV), even at different pH$_{\text{extra}}$ values in brain mitochondria. At pH$_{\text{extra}}$ = 6.45 ± 0.004,
nigericin hyperpolarized the membrane by 12.5 mV, at pH\textsubscript{extra} = 6.84 ± 0.013 by 19 mV, and at pH\textsubscript{extra} = 7.30 ± 0.047 by 8.5 mV. Taken together, these data show that Δψ\textsubscript{m} and the rate of H\textsubscript{2}O\textsubscript{2} production were the highest when nigericin was present and the medium was the most alkaline.

pH\textsubscript{in} and ΔpH As shown in Fig. 3, upon elevation of pH\textsubscript{extra}, ΔpH was concomitantly decreased in both succinate- and α-GP-respiring mitochondria. The addition of nigericin was followed by acidification of the mitochondrial matrix, resulting in a drop of ΔpH (Fig. 3a, c). At the most alkaline pH\textsubscript{extra} (7.45 ± 0.02), in the presence of succinate, nigericin could neither decrease pH\textsubscript{in} nor ΔpH. However, in α-GP-respiring mitochondria, nigericin reduced both pH\textsubscript{in} and ΔpH at all measured pH\textsubscript{extra} values (Fig. 3c). Valinomycin treatment of both succinate- and α-GP-respiring brain mitochondria caused alkalinization of the mitochondrial matrix and a corresponding elevation of ΔpH (Fig. 3b, d).

Heart mitochondria. Effects of nigericin and valinomycin on mitochondrial parameters

Detecting RET is also relevant in organs other than the brain, like heart, regarding their exposure to oxidative stress under pathological conditions, like ischemia-reperfusion (Chouchani et al. 2014). To deepen our understanding on RET in heart mitochondria, effects of ΔpH and Δψ\textsubscript{m} on...
succinate-supported \( H_2O_2 \) production were investigated applying the above-mentioned ionophores.

Similarly to brain, in heart mitochondria nigericin hyperpolarized the membrane at various pH\(_{\text{extra}}\) values. In the absence of nigericin, \( \Delta \psi_m \) of succinate-supported, non-phosphorylating mitochondria was similar at all pH\(_{\text{extra}}\) values analogously to brain. In contrast to that observed in brain mitochondria, in heart, the addition of nigericin led to an increase of the rate of succinate-evoked \( H_2O_2 \) generation only between pH\(_{\text{extra}}\) = 6.46 ± 0.005 and 7.03 ± 0.008 (Fig. 4a). At a more alkaline pH (pH\(_{\text{extra}}\) = 7.54 ± 0.002), nigericin decreased the rate of \( H_2O_2 \) formation by 22 ± 8% (Fig. 4a, white circles). In the absence of nigericin, upon elevation of \( \text{pH}_{\text{extra}} \), the rate of the succinate-initiated \( H_2O_2 \) generation was steeply increasing (Fig. 4a, black circles). In the absence of nigericin, \( \Delta \text{pH} \) decreased with incrementing \( \text{pH}_{\text{extra}} \) (pH\(_{\text{extra}}\) from 6.46 ± 0.005 to 7.03 ± 0.008) until pH\(_{\text{extra}}\) 7.03 ± 0.008; at pH\(_{\text{extra}}\) above such value, \( \Delta \text{pH} \) increased (Fig. 4b, black circles). Contrary to this, in the presence of nigericin, \( \Delta \text{pH} \) was slightly ascending upon \( \text{pH}_{\text{extra}} \) elevation (Fig. 4b, white circles) and at pH\(_{\text{extra}}\) = 7.54 ± 0.002 there was no statistically significant difference between \( \Delta \text{pH} \) in the presence of nigericin compared to \( \Delta \text{pH} \) in its absence.

Discussion

There is a lack of consensus regarding the role of \( \Delta \text{pH} \) and \( \Delta \psi_m \) on mtROS generation (Lambert and Brand 2004; Selivanov et al. 2008), therefore, in our study, we aimed to clarify the dependence of succinate- and \( \alpha \)-GP-driven \( H_2O_2 \) production on components of \( \text{pmf} \). The results presented above allow the conclusion that \( \Delta \psi_m \) displays a stronger influence on the succinate- or \( \alpha \)-GP-supported, RET-initiated \( H_2O_2 \) production than \( \Delta \text{pH} \). In this study, we did not only measure \( H_2O_2 \) production and \( \Delta \psi_m \), but we also detected matrix pH (pH\(_{\text{in}}\)) with the fluorescent dye BCECF and calculated \( \Delta \text{pH} \). Under most physiological conditions depolarization of the inner membrane (decrease of the absolute value of \( \Delta \psi_m \)) is associated with a decrease of \( \Delta \text{pH} \) and an elevation of matrix [\( H^+ \)]. It is unfeasible to create conditions where one of the components of \( \text{pmf} \) is maintained constant whilst the other one is independently altered. With ionophores however, these two parameters can be changed in opposite directions. In order to increase \( \Delta \psi_m \), nigericin was applied, which decreased \( \Delta \text{pH} \) and increased \( H_2O_2 \) production (Fig. 1) suggesting that mtROS production is directly proportional to \( \Delta \psi_m \). If \( \Delta \text{pH} \) was the dominant factor of the RET-initiated \( H_2O_2 \) formation, then \( H_2O_2 \) production should have been decreased. To increase \( \Delta \text{pH} \), valinomycin was added, which simultaneously depolarized the inner membrane and decreased the rate of \( H_2O_2 \) generation which changed in accordance with \( \Delta \psi_m \) values. If \( \Delta \text{pH} \) had been the major player in \( H_2O_2 \) production, then \( H_2O_2 \) production should have been higher in the presence of valinomycin than in its absence.

Our measurements were carried out not only in brain but also in heart mitochondria, both displaying similar effects. Based on these observations, we can exclude the tissue specific modification of RET– supported \( H_2O_2 \) generation in these tissues.

In summary, our studies with the two ionophores showed that RET-evoked \( H_2O_2 \) production always varied in accordance with changes of \( \Delta \psi_m \), which leads to the conclusion that \( \Delta \psi_m \) has a greater influence on mitochondrial RET-initiated \( H_2O_2 \) formation than \( \Delta \text{pH} \). In addition, we also showed that elevation of \( \text{pH}_{\text{extra}} \) resulted in increased \( H_2O_2 \) generation, a finding that suggests a clear correlation between absolute pH and \( H_2O_2 \) production.

Nigericin

Nigericin, as a K\(^+\)/H\(^+\) antiporter, is responsible for the electroneutral exchange of K\(^+\) and H\(^+\) (Henderson et al. 1969; Bernardi 1999). In our preliminary experiments, the dose-dependent effects of nigericin on \( \Delta \psi_m \) were studied, and the lowest possible concentration was used which created a maximal mitochondrial hyperpolarization measured by safranin fluorescence (data not shown). Contrary, Lambert and colleagues (Lambert and Brand 2004) as well as Selivanov’s group (Selivanov et al. 2008) applied 100 nM nigericin, which in our hands did neither increase \( \Delta \psi_m \) further, nor dissipate \( \Delta \text{pH} \) completely but established a new equilibrium with lower pH\(_{\text{in}}\), \( \Delta \text{pH} \), after administration of 100 nM nigericin, could be decreased further by addition of 250 nM FCCP and mixture of ionophores (see Materials and Methods). To eliminate confounding factors that could have influenced ROS production (e.g. succinate transport or further metabolism of succinate in the tricarboxylic acid cycle), not only succinate, but also \( \alpha \)-GP was used to energize mitochondria and support RET-mediated ROS production. Results with \( \alpha \)-GP were qualitatively equivalent to those obtained in succinate-supported mitochondria (Figs. 2 and 3). The stimulating effect of nigericin on \( H_2O_2 \) generation was more pronounced at acidic pH\(_{\text{extra}}\). Interestingly, in heart mitochondria, at alkaline pH\(_{\text{extra}}\), nigericin decreased the rate of \( H_2O_2 \) release (Fig. 4a). It appears that in heart mitochondria, the diminution in the rate of \( H_2O_2 \) production at alkaline pH cannot be explained by depolarisation of the mitochondrial membrane.

Valinomycin

In the presence of valinomycin, the mitochondrial membrane is permeable to K\(^+\); its effect is highly dependent on the K\(^+\) concentration of the medium and the applied valinomycin concentration. High K\(^+\) concentrations in the presence of 2 mM KH\(_2PO_4\) and valinomycin lead to high amplitude
mitochondrial swelling (Ligeti and Fonyo 1977; Bernardi 1999), therefore, 4 mM KCl was used in valinomycin experiments. It is well known that in isolated mitochondria the highest ΔpH can be achieved at low K⁺ concentration (Mitchell and Moyle 1968; Nicholls 1974; Nicholls 2005). It is noteworthy that ΔpH in low K⁺ medium is about 0.6–0.8 pH unit, but at high K⁺ medium it is only 0.3 pH unit. In our experiments valinomycin caused matrix alkalization and concomitant ΔpH elevation. This observation can be explained by the fact that the valinomycin-induced entry of K⁺ into the mitochondrial matrix usually triggers H⁺ extrusion and Pᵢ/ OH⁻ exchange (Garlid and Paucek 2003). The H⁺ extrusion generally mediates a compensatory decrease in Δψₑᵢ and an elevation of respiration both in the succinate- or α-GP-supported mitochondria. Valinomycin-caused depolarisation led to inhibition of RET-supported H₂O₂ production.

**Effects of pH<sub>extra</sub> on H₂O₂ production**

In agreement with the observations of Selivanov (Selivanov et al. 2008), in non-phosphorylating mitochondria, the acidification of the mitochondrial matrix is followed by an elevation in ΔpH and a decrease in the succinate- and α-GP-driven H₂O₂ production. There is an inverse proportionality between ΔpH and H₂O₂ formation, which weakens the notion of Lambert and Brand that ΔpH would exhibit a stronger effect on RET than Δψₑᵢ (Lambert and Brand 2004). Our measurements of pHₑᵢ with BCECF have shown that ΔpH is greater at lower pH and varies with pH<sub>extra</sub>. Banh and Treberg observed an analogous pattern in glutamate and malate-respiring, non-phosphorylating, rat skeletal muscle mitochondria, where the H₂O₂ generation was enhanced upon alkalization (Banh and Treberg 2013).

**What mechanisms are behind the effects of Δψₑᵢ and ΔpH on mitochondrial H₂O₂ production?**

To understand the effects of Δψₑᵢ and ΔpH on the RET-evoked H₂O₂ generation, we need to be aware of the production of superoxide (O₂⁻) by the CI. CI predominantly generates O₂⁻ (Ohnishi et al. 2005; Grivennikova and Vinogradov 2006). Two mechanistic models exist for the explanation of mtROS production by the CI: (1) the one-site model states that the O₂⁻ production site, during both FET and RET, is ultimately the reduced flavin (Galkin and Brandt 2005; Pryde and Hirst 2011), whereas (2) the two-site model suggests that during FET, the flavin of CI is responsible for O₂⁻ formation, while, under RET, the SQ⁻ species, synthetized at the ubiquinone-binding Q-site (Q-binding site) of CI, are liable for the elevated O₂⁻ release (Brandt 2010; Treberg et al. 2011). Both theories agree that the greatest drop in redox potential in the CI occurs between the N2 subunit and the ubiquinone (Q), whose interaction initiates conformational changes that are coupled to the proton translocation (Treberg et al. 2011).

Δψₑᵢ: There are speculations that the above mentioned conformational changes of the CI might also depend on Δψₑᵢ (Brandt 2006; Dlaskova et al. 2008). When Δψₑᵢ is adequately high, it decelerates the proton pumping activity of the CI, which may favour SQ⁻ formation and hence O₂⁻ generation.
Our results do not support the hypothesis that ΔpH would influence the RET-initiated ROS production to a higher degree than Δψm. The theory that tries to explain the influence of absolute pH on the H2O2 formation assigns a potential role to SQ2⁻ formation at the Q-site of the CI (Ohnishi et al. 2005; Treber et al. 2011). At the Q-site, Q is reduced by a single electron to SQ⁻. SQ⁻ can react further in two possible ways (Selivanov et al. 2008): (1) with a single electron plus two H⁺ to form ubiquinol (QH₂) (SQ⁻ + e⁻ + 2 H⁺ ↔ QH₂), or (2) with O₂ to form the highly reactive O₂⁻ (SQ⁻ + O₂ ↔ Q + O₂⁻). At acidic pH, the first reaction is shifted towards QH₂ formation according to the Le Chatelier’s principle (Selivanov et al. 2008).

**Potential significance of our results: Mild uncoupling**

In succinate-respiring mammalian mitochondria, mild uncoupling lowers Δψm and consequently also the rate of ROS generation (Skulachev 1996; Korshunov et al. 1997; Miwa and Brand 2003). Mild uncoupling is a special condition where oxidative phosphorylation occurs at a relatively higher conductance of the inner mitochondrial membrane, this results in lowered pmf and a minor stimulation of respiration (Skulachev 1996; Brand et al. 2004). Our results support the notion that a minor decrease in Δψm leads to a diminution of the succinate-evoked, RET-initiated H2O2 release. Uncoupling proteins (UCP; like UCP1–3) and the adenine nucleotide transporter are also involved in mild uncoupling processes (Andreyev et al. 1988; Ježek 2002). Interestingly, O₂⁻ can activate UCPs in the matrix with the contribution of fatty acids resulting in mild uncoupling (Echtay et al. 2002) and consequently a slower ROS production. Although it is likely that in vivo, under physiological conditions, ATP synthesis caused depolarisation of Δψm is sufficient to decrease ROS generation (Votyakova and Reynolds 2001; Starkov and Fiskum 2003), effects on mtROS of mild uncoupling and of Δψm are possibly relevant to pathological states.

In fact, it has been hypothesized that initiation of mild uncoupling might be beneficial in oxidative stress-related diseases characterized by high Δψm such as in ischemia-reperfusion injury (Kadenbach et al. 2011). This hypothesis has been corroborated by a report showing that under ischemia, succinate can accumulate in mouse heart owing to the reversal of SDH (Chouchani et al. 2014). In reperfusion, SDH returns to oxidize the accumulated succinate and this has been claimed to result in an enhanced RET-mediated mtROS formation (Chouchani et al. 2014).

In summary, data from our laboratory provided evidence that the succinate- or α-GP-evoked, RET-initiated H2O2 production is more dependent on Δψm than on ΔpH. Our findings have helped elucidating mechanisms underpinning mtROS production and support consideration of the therapeutic applications of mild uncoupling, which can be initiated by e.g. mitochondria-targeted antioxidants.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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