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SHORT COMMUNICATION

Moderate dependence of reactive oxygen species production on membrane potential in avian muscle mitochondria oxidizing glycerol 3-phosphate

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Abstract Mitochondria are a major source of reactive oxygen species production in cells, and the production level is sensitive to the magnitude of the membrane potential ($\Delta\Psi$). The present study investigated the level of superoxide production in mitochondria oxidizing glycerol 3-phosphate (GP) and its dependence on $\Delta\Psi$ in isolated avian muscle mitochondria. The levels of superoxide produced in mitochondria oxidizing GP were lower than those obtained with succinate and were similar to those obtained with NADH-linked substrates (glutamate/malate/pyruvate). The dependence of superoxide production on $\Delta\Psi$ in mitochondria oxidizing GP was lower than that of mitochondria oxidizing succinate, and a weak dependence of GP-supported superoxide production on $\Delta\Psi$ was observed in the presence of NADH-linked substrates or succinate. These results suggest that the levels of superoxide generated in response to GP are quantitatively low, but they are unsusceptible to changes in $\Delta\Psi$ in avian muscle mitochondria.

Keywords Mitochondrial glycerol 3-phosphate dehydrogenase · Mitochondrial membrane potential · Carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazine

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Introduction

Mitochondria are a major source of reactive oxygen species (ROS) production in most cells [1–3]. The primary ROS generated by mitochondria is superoxide as the result of a one-electron reduction of an oxygen molecule at the electron transport chain (ETC). Superoxide is a reactive molecule, but it can be converted to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (MnSOD) and then converted to oxygen and water by glutathione peroxidase within the mitochondria. Meanwhile, the levels of superoxide generation are dependent on the mitochondrial membrane potential ($\Delta\Psi$) [4]. Mitochondria can reduce superoxide formation by dissipating $\Delta\Psi$ after activation of uncoupling proteins. Mitochondrial superoxide that evades the above systems forms highly reactive radicals such as hydroxyl radicals and hydroperoxyl radicals, which cause irreversible molecular damage to biomolecules in cells. Mitochondria-derived superoxide production may increase muscle atrophy [5, 6], apoptosis [7, 8], autophagy [9], and hyperthermia-induced ubiquitin-proteasome-dependent protein degradation in avian muscle cells [10].

Superoxides generated in mitochondria need to be released outside of this organelle to act as signal messengers in the cytosol because superoxides are not able to cross the mitochondrial inner membrane. Although there are several sites of superoxide production in mitochondria [11], only a few are able to release superoxide outside of the inner membrane. Glycerol 3-phosphate dehydrogenase (mGPDH) is one of the enzymes that have the above ability [12], and several studies have reported the role of mGPDH as a potent superoxide generator [12, 13]. However, no published information is available about the superoxide production associated with mGPDH and its substrate, glycerol 3-phosphate (GP) in avian muscle mitochondria.

Therefore, the present study determined the level of superoxide production in avian muscle mitochondria oxidizing GP and compared it with the levels obtained with other mitochondrial substrates such as glutamate, malate, pyruvate, and succinate. Furthermore, this study evaluated the dependence of superoxide production on $\Delta\Psi$ in mitochondria oxidizing GP to explore the bioenergetic characteristic of mGPDH/GP-mediated superoxide production in avian muscle mitochondria.

Materials and methods

Ethics statement

The Animal Care and Use Committee of the Graduate School of Agricultural Science, Tohoku University, approved all procedures, and every effort was made to minimize the pain or discomfort to animals.

Animals and experimental design

Ten 0-day-old male chicks (Ross strain, *Gallus gallus domesticus*) were obtained from a commercial hatchery (Economic Federation of Agricultural Cooperatives, Iwate, Japan). They were housed in electrically heated batteries under continuous light and provided ad libitum access to water and a corn and soybean meal-based standard diet for meat-type chicks. At 3 weeks of age, all birds were killed by decapitation. *Gastrocnemius* muscles were rapidly excised, and a sample was placed in ice-cold isolation medium, comprising 100 mM KCl, 50 mM Tris/HCl (pH 7.4), and 2 mM EGTA, for mitochondrial isolation (see below).

Isolation of skeletal muscle mitochondria

Muscle mitochondria were isolated by homogenization, protein digestion, and differential centrifugation at 4 °C, as described previously [14]. The protein concentration of the isolated mitochondria was determined using the bicinchoninic acid assay, with bovine serum albumin (BSA) as the standard. All mitochondria were freshly prepared on the day of the experiment. Each sample of mitochondria was isolated from the pooled muscles of two birds, such that five mitochondrial samples were prepared from the ten birds. The respiratory control ratio (RCR) of each mitochondrial sample was measured by using 73 μM ADP to examine mitochondrial quality, as previously described [15]. The RCR is a ratio of the mitochondrial O_2 consumption rate in the presence of ADP (ADP phosphorylation: state 3) to its absence (non-phosphorylation: state 4). The RCR values for succinate-supported respiration were approximately 3.1–3.5 in all mitochondrial samples, meaning that the isolated mitochondria were of good quality for functional evaluation.

Measurements of mitochondrial membrane potential

Mitochondrial $\Delta\Psi$ was measured using a potential-dependent triphenylmethyl phosphonium cation ($TPMP^+$) probe [16, 17]. Briefly, mitochondria were incubated at 38 °C in assay medium [115 mM KCl, 10 mM KH_2PO_4, 3 mM HEPES (pH 7.2), 1 mM EGTA, 2 mM $MgCl_2$, 0.1 μM nigericin, 1.0 μg/ml oligomycin, and 0.3 % (*w/v*) defatted BSA], which was assumed to contain 402 nmol atomic oxygen per milliliter [18]. First, the $TPMP^+$ electrode was calibrated with sequential additions of 0.5 up to 2.0 μM $TPMP^+$. Next, NADH-linked substrates [glutamate (Glu), 10 mM; malate (Mala), 2.5 mM; pyruvate (Pyr), 10 mM] or $FADH_2$-linked substrates [succinate (Suc), 4 mM; glycerol 3-phosphate (GP), 10 mM] were added to initiate mitochondrial respiration. Thereafter, an incremental amount of carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (three additions of 0.1 μM) was added to dissipate the $\Delta\Psi$. The electrode linearity was routinely checked by the addition of 1.0 μM FCCP, which completely dissipated $\Delta\Psi$ and released TPMP back into the medium. This allowed for the correction of any small electrode drift. $\Delta\Psi$ values were calculated from the distribution of $TPMP^+$ across the mitochondrial inner membrane, using a binding correction factor of 0.45 mg protein/μl [19].

Determination of mitochondrial superoxide production

Mitochondrial superoxide generation rates were determined as the H_2O_2 generation rate, which was fluorometrically measured by the oxidation of 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red, Invitrogen) coupled to enzymatic reduction by horseradish peroxidase (HRP), as described previously [16]. Mitochondria were incubated in the assay medium supplemented with the NADH- or $FADH_2$-linked substrates in the absence of ADP, and superoxide released from mitochondria was converted into H_2O_2 by SOD (30 U/ml), which was detected by 50 μM Amplex® Red in the presence of 6 U/ml HRP. The rate of H_2O_2 production was spectrofluorimetrically determined by the change in fluorescence at excitation and emission wavelengths of 544 and 590 nm, respectively. FCCP (0.1 μM) was also added to the medium (three times) in a similar manner to that used for $\Delta\Psi$ measurement. The assay was carried out on a computer-controlled spectrofluorimeter, with appropriate correction for background

and use of a standard curve with commercially available H_2O_2.

Statistical analysis

All data are presented as the mean ± standard error (SE) of five individual mitochondrial preparations. Data were analyzed by the nonparametric Kruskal-Wallis test followed by the Steel-Dwass multiple comparison test. $^{a–f}$Values labeled with different letters are statistically different for the comparison of the mitochondrial superoxide production levels. Values of $P < 0.05$ were considered to indicate statistical significance in each test.

Results

The levels of NADH- and $FADH_2$-supported H_2O_2 production in avian muscle mitochondria

We first evaluated maximal H_2O_2 production in avian muscle mitochondria oxidizing NADH- and $FADH_2$-linked substrates under state 4 conditions (non-ADP-phosphorylating conditions). As illustrated in Fig. 1, mitochondrial H_2O_2 production was gradually enhanced with the supply of each NADH-linked substrate ($^{d–f}P < 0.05$). Suc-supported H_2O_2 production was significantly higher than Glu/Mala/Pyr-supported H_2O_2 production, while no difference in H_2O_2 production was observed between mitochondria oxidizing GP and Glu/Mala/Pyr. Mitochondria oxidizing Suc/GP showed the highest level of H_2O_2 production among the substrates tested, and the level was equal to the sum of the H_2O_2 production levels with either Suc or GP

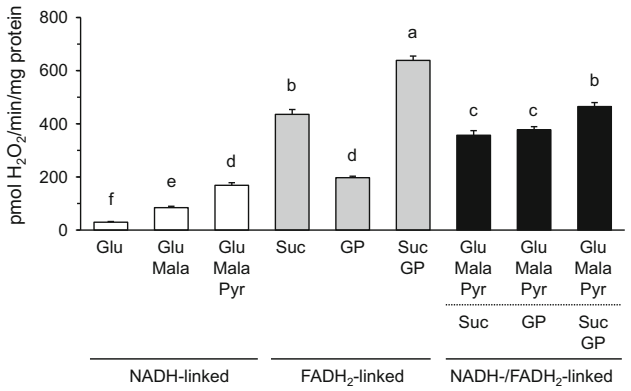

Fig. 1 ROS production in avian muscle mitochondria oxidizing a NADH-linked substrate (Glu, 10 mM; Mala, 2.5 mM; Pyr, 10 mM), a $FADH_2$-linked substrate (Suc, 4 mM; GP, 10 mM) or a combination thereof. ROS production was fluorometrically determined using Amplex® Red. Values are the mean ± SE of data from five mitochondrial preparations. $^{a–f}P < 0.05$, values with different superscripts are mutually significantly different

alone. These results suggest that Suc- or GP-supported H_2O_2 production may occur in separate places of the mitochondria in which mGPDH may be implicated.

H_2O_2 production in mitochondria oxidizing Glu/Mala/Pyr was significantly increased by either Suc or GP ($^{c,d}P < 0.05$). No difference in H_2O_2 production was observed between mitochondria oxidizing Glu/Mala/Pyr plus Suc and those oxidizing Glu/Mala/Pyr with GP (Fig. 1). H_2O_2 production in mitochondria oxidizing Glu/Mala/Pyr plus Suc and GP was significantly higher than in mitochondria oxidizing Glu/Mala/Pyr with either Suc or Glu ($^{b,c}P < 0.05$). The level of H_2O_2 production with Glu/Mala/Pyr/GP was equal to the sum of H_2O_2 production in the presence of Glu/Mala/Pyr alone and GP alone. A similar additive effect was not seen when Glu/Mala/Pyr was combined with Suc. The results suggest that, in avian muscle mitochondria, the site of GP-supported H_2O_2 production may be different not only from the site of Suc-supported superoxide production, but also from that of Glu/Mala/Pyr-supported H_2O_2 production.

The dependence of H_2O_2 production on ΔΨ in avian muscle mitochondria

The response of mitochondrial H_2O_2 production to ΔΨ was evaluated next. For each graph in Fig. 2, the furthest right-hand point in the kinetic curve is taken as representing the state 4 condition, in which mitochondrial superoxide production represented in Fig. 1 was measured. As seen in Fig. 2, for all of the substrate combinations tested, H_2O_2 production gradually decreased with reductions in ΔΨ. Mitochondria oxidizing NADH-linked substrates showed a low gradient of H_2O_2 production versus ΔΨ, indicating a weak dependence of H_2O_2 production on ΔΨ (Fig. 2a). Compared with these gradients, the gradient of H_2O_2 production versus ΔΨ in mitochondria oxidizing Suc or Suc/GP was much higher than in mitochondria oxidizing NADH-linked substrates, while the gradient observed in mitochondria oxidizing GP alone was similar to that of NADH-linked substrates (Fig. 2b). The difference between Suc- and Suc/GP-supported H_2O_2 production was little different at all tested ΔΨ values. The results also suggest that Suc- or GP-supported H_2O_2 production may occur in separate parts of the mitochondria and imply that GP-supported mitochondrial H_2O_2 production is insensitive to changes in ΔΨ.

As seen in Fig. 2c, the addition of Suc to mitochondria oxidizing Glu/Mala/Pyr resulted in significant increases in H_2O_2 production at any given ΔΨ (Fig. 2a closed circles versus 2C open diamonds), while the gradient of H_2O_2 production vs. ΔΨ with Glu/Mala/Pyr plus Suc was similar to that of Suc alone. While the addition of GP to mitochondria oxidizing Glu/Mala/Pyr significantly increased

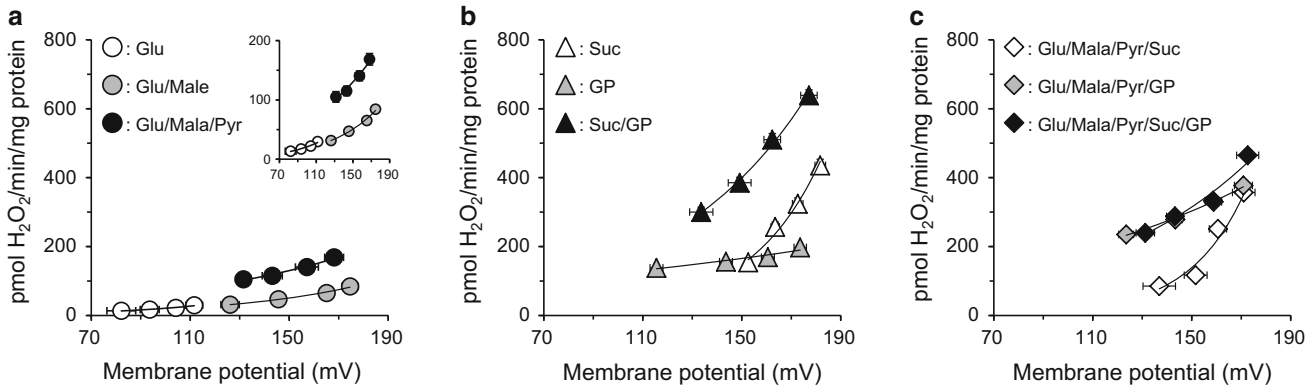

Fig. 2 Relationship between ΔΨ and ROS production in avian muscle mitochondria oxidizing NADH-linked substrates (Glu, 10 mM; Mala, 2.5 mM; Pyr, 10 mM), $FADH_2$-linked substrates (Suc, 4 mM; GP, 10 mM), or a combination thereof. Mitochondria (0.5 mg protein/ml) were energized with the above set of substrates, to which incremental amounts of FCCP were added (three additions of 0.1 μM were made; shifts in the symbols from *right* to *left* represent an increasing concentration of FCCP). Values are mean ± SE of data from five mitochondrial preparation

H_2O_2 production at any given ΔΨ (Fig. 2a closed circles versus 2C gray diamonds), the gradient of this H_2O_2 production vs. ΔΨ was less than was seen for mitochondria oxidizing Glu/Mala/Pyr plus Suc. The gradient of H_2O_2 production vs. ΔΨ in mitochondria oxidizing Glu/Mala/Pyr plus Suc/GP was similar to that due to Glu/Mala/Pyr plus GP.

Discussion

The present study characterized GP-supported superoxide production in avian muscle mitochondria, and the levels of this superoxide production were relatively low among the substrates tested (Fig. 1). The present study also found that the supply of GP may have additive effects on the superoxide production in mitochondria oxidizing NADH-linked substrates or Suc (Fig. 1). This should be considered the reason why the additive effect was not seen when Glu/Mala/Pyr was combined with Suc in NADH-/$FADH_2$-linked superoxide production. Mitochondrial ETC complex I (NADH: ubiquinone oxidoreductase) and complex III (ubiquinol: cytochrome *c* oxidoreductase) are involved in the superoxide production in mitochondria oxidizing Glu/Mala/Pyr. In mitochondria oxidizing Suc, superoxide are mainly generated at complex I via reverse electron flow from ubiquinol [1, 3]. Based on these findings, it is conceivable that the levels of complex I-related superoxide generation might be altered through the redox perturbation of this cite by supplementing both Glu/Mala/Pyr and Suc. Therefore, the additive effect was not seen in these substrates conditions.

The present study revealed that mitochondria oxidizing GP showed moderate dependence of the ROS production on ΔΨ compared with that of Suc-supported superoxide production (Fig. 2b) and that this bioenergetic characteristic was partly retained in the presence of NADH-linked substrates or Suc (Fig. 2c). These results suggest that a treatment that leads to a dissipation of ΔΨ may have only a small effect on the suppression of the superoxide generated in the presence of GP. One can assume that the insensitivity of GP-supported superoxide production on ΔΨ might be due to the localization of mGPDH in mitochondria. A previous investigation reported that mGPDH is located on the outer leaflet of the inner membrane [20], which in turn suggests that mGPDH may not be susceptible to changes in ΔΨ of the inner membrane. It remains unclear whether the insensitivity of mGPDH/GP-mediated superoxide production to ΔΨ is a unique phenomenon in avian muscle mitochondria. While several independent investigators have studied the functions and roles of mGPDH in superoxide production [21–24], there is no information on the sensitivity of mGPDH-dependent superoxide production to ΔΨ in vertebrate mitochondria as far as we know.

It is important to evaluate the physiological effect of mGPDH/GP-mediated superoxide production on cellular oxidative balance. Given that mGPDH and GP are components that interlink cytosolic and mitochondrial energy transduction pathways, some physiological stimuli that could activate this pathway might also induce mGPDH/GP-mediated superoxide production. A few studies have reported that high-intensity training altered mitochondrial functions in skeletal muscle [25, 26], and the training augmented GP-supported mitochondrial superoxide production [26]. We recently found that hyperthermia induced GP-supported superoxide production in avian muscle mitochondria [27]. Additional experiments would be needed to evaluate the extent to which mGPDH/GP-mediated

superoxide production is associated with the oxidative disturbance in several physiological conditions.

In conclusion, our data demonstrated that the levels of mGPDH/GP-mediated superoxide production were relatively low and that their dependence on $\Delta\Psi$ was weak in avian muscle mitochondria.

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Compliance with ethical standards

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Conflict of interest The authors declare no conflict of interest.

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