The effects of increasing extramitochondrial calcium concentration on mitochondrial function in dystrophic muscle

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Duchenne Muscular Dystrophy (DMD) is a severe and fatal skeletal muscle wasting disease caused by the loss of the cytoskeletal protein dystrophin, which renders dystrophic muscle more fragile and susceptible to damage. This fragility increases calcium (Ca^{2+}) permeability which results in intracellular Ca^{2+} overload stimulating mechanisms of myofibril degeneration and the deposition of extensive connective and fatty tissue. The inability of dystrophic muscle to promote apt regeneration could be due to decreased adenosine triphosphate (ATP) availability as it has been shown that dystrophic muscle and mitochondria has a severe metabolic impairment that equates to a significant reduction in ATP content (Cole *et al.*, 2002). While it may be thought that this metabolic issue is a consequence of persistent Ca^{2+} overload, it appears that this metabolic dysfunction is an inherent feature of the disease, as dystrophic myoblasts present with metabolic issues prior to the time when dystrophin would be expressed (Onopiuk et al., 2009) and DMD carriers also exhibit metabolic anomalies (Barbiroli *et al.*, 1992). Furthermore, as Ca^{2+} has been shown to be a stimulator of oxidative phosphorylation (as reviewed in Gellerich *et al.*, 2010), it would be expected that dystrophic mitochondria should be increasing their ATP production. The aim of this study was to investigate the response of dystrophic mitochondria to increasing extramitochondrial Ca^{2+} (0 nM - 5 μM) to identify if dystrophic mitochondria do in fact respond in a similar fashion to healthy mitochondria.

All animal experimentation was approved by the Victoria University Animal Ethics Experimentation Committee and performed in accordance with the Australian Code of Practice for the Care and use of Animal for Scientific Purposes. Twelve week old male C57BL/10 and C57BL/10mdx (*mdx*) mice were deeply anaesthetised by an intraperitoneal injection of sodium pentobarbitone (10 $mg.kg^{-1}$) and the diaphragm excised. Mitochondria was isolated from the diaphragm and either given glutamate and malate (G+M) or succinate and rotenone (S+R) as substrates to stimulate either complex I and III or complex II-driven respiration of the ETC, respectively. Mitochondrial function (including oxygen consumption rate (OCR)) was tested by the addition of stimulators and inhibitors of oxidative phosphorylation and measured using the XF24 Analyser (Seahorse Bioscience) (control n = 11, *mdx* n = 12) while the mitochondrial membrane potential ($\Delta\Psi$) and mitochondrial swelling was measured using the Varioskan fluorimeter (ThermoFisher) (control n = 8, *mdx* n = 8).

A depressed basal OCR ($P < 0.05$) and depolarised $\Delta\Psi$ ($P < 0.001$) was observed in isolated *mdx* mitochondria respiring on G+M bathed in a Ca^{2+}-free (0 nM) environment, indicating a decreased drive for ATP synthesis compared to control mitochondria. Additionally, *mdx* mitochondria respiring on G+M in a Ca^{2+}-free environment were more uncoupled ($P < 0.005$) compared to control mitochondria, indicating less effective respiration. Furthermore, mdx mitochondria were observed to have a significantly reduced maximal respiration rate independent of substrate ($P < 0.005$) indicating that they have a blunted capacity to increase respiration when required. These metabolic impairments are observed in *mdx* mitochondria that have a swollen morphology prior to the addition of Ca^{2+}, and which continue to swell as extramitochondrial Ca^{2+} increases ($P < 0.001$). Together, these results suggest that *mdx* mitochondria have a reduced drive for ATP synthesis, especially during time of heightened demand where the ETC is unable to respond appropriately. As S+R respiration in mdx mitochondria is comparable to controls, it is likely that complex I is dysfunctional.

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