Stretch-induced oxidative damage to *mdx* muscle: the role of NADPH oxidase

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Duchenne muscular dystrophy (DMD) is a degenerative muscle disease caused by the absence of the protein dystrophin, which connects the cytoskeleton to the surface membrane. In the *mdx* mouse, an animal model of DMD,we have shown that increased stretch-activated channel (SAC) activity is the main source of Ca^{2+} influx following stretched contractions (Yeung *et al*., 2005). Canonical transient receptor potential 1 (TRPC1) is the putative SAC protein in mammalian cells (Maroto *et al*., 2005). It is known that some TRPC channels are activated by reactive oxygen species (ROS) and given that *mdx* muscles show evidence of oxidative stress, we postulated that stretch-induced ROS might activate SACs in dystrophic muscles. NADPH oxidase produces ROS in smooth muscle subjected to stretch (Grote *et al*., 2003), and so we also investigated whether this was a source of the stretch-induced ROS production in *mdx* skeletal muscle. The two hypotheses tested through these experiments were: 1) Stretch-induced ROS mediate Ca^{2+} influx through SACs and cause muscle damage; 2) NADPH oxidase is the main source of the stretch-induced ROS production in *mdx* muscle.

In the first experiments, we investigated whether the antioxidant N-acetylcysteine (NAC) could reduce stretch-induced muscle damage in *mdx* muscle. *mdx* and wild type (C57Bl/10ScSn) mice were euthanased and the *extensor digitorum longu* muscles removed. Muscles were perfused with or without 20 mM NAC. Solutions also contained 0.02% Evans Blue Dye (EBD) for assessment of membrane permeability. Muscles underwent 3 stretched (eccentric) contractions at 35°C. Tetanic force was measured before and 60 min after eccentric contractions and then muscles were frozen and sectioned for EBD uptake. Following the stretched contractions, force fell to 35 ± 3% for *mdx* muscles and NAC significantly improved force to 51 ± 2% ($p < 0.01$). As expected, force was much greater for wild-type muscles (69 ± 5%) and NAC had no additional effect. The area of EBD uptake was 8.6 ± 1.8% in *mdx* muscle cross-sections and this was significantly reduced by NAC to 2.6 ± 0.8% ($p < 0.01$). Wild-type muscles had a value of 1.8 ± 0.7%, which was not significantly different to NAC.

Secondly, we tested if ROS could activate SACs. *mdx* and wild type mice were killed by cervical dislocation and single muscle fibres from the *flexor digitorum brevis* were dissected. Fibres were loaded with the fluorescent Ca^{2+} probe Fluo-4 AM and subjected to 10 stretched contractions. Following the stretched contractions, there was a significant increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) up to 30 min for *mdx* fibres ($p < 0.001$) but not for wild type fibres. The increased $[Ca^{2+}]_i$ in *mdx* fibres was prevented by the antioxidant tiron (5 mM), which also reduced the force deficit. We then showed, in resting *mdx* fibres, that 10 μM hydrogen peroxide (H_2O_2), also increased $[Ca^{2+}]_i$, which could be returned to baseline levels by the SAC blocker, streptomycin. Taken together, these findings suggested that increased ROS production during stretched contractions activate SACs and allow Ca^{2+} entry in *mdx* muscle.

Finally, we explored whether NADPH oxidase was the main source of the enhanced ROS production during stretch. As with tiron, the increased $[Ca^{2+}]_i$ in *mdx* fibres following stretched contractions was significantly inhibited ($p < 0.01$) by 1 μM diphenyleneiodium (DPI), an NADPH oxidase inhibitor, and this was also accompanied by increased muscle force. In preliminary experiments, another NADPH oxidase inhibitor, apocynin, also decreased the $[Ca^{2+}]_i$ after stretched contractions.

The results of these experiments show that ROS are an important cause of stretch-induced damage to *mdx* muscle. As well as deleterious effects on proteins and muscle membranes, ROS also activate SACs, causing an influx of Ca^{2+} and the activation of Ca^{2+}-dependent damage pathways, such as calpains. We also provide evidence that NADPH oxidase is a primary source of ROS in stretched *mdx* muscle. We now aim to investigate why SACs are more sensitive to ROS in *mdx* muscle and to determine the key proteins targeted by ROS, which impair muscle function and contribute to dystrophic muscle damage.

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