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Adenylosuccinic acid: a novel inducer of the cytoprotectant Nrf2 with efficacy in Duchenne muscular dystrophy

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ABSTRACT

Adenylosuccinic acid (ASA) modifies Duchenne muscular dystrophy (DMD) progression in dystrophic *mdx* mice and human DMD patients. Despite an established role for ASA in augmenting metabolism and cellular energy homeostasis, our previous data suggests an undiscovered ulterior mode of action capable of modifying DMD disease course. Here, we identify ASA as a novel inducer of nuclear factor erythroid 2-related factor-2 (Nrf2), master regulator of the antioxidant and cytoprotective response to cell stress.

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Duchenne Muscular Dystrophy (DMD), an incurable neuromuscular disorder, is fatal in all cases due to progressive skeletal and cardiac muscle wasting in mice¹ and humans². Standard-of-care corticosteroid treatment has prevailed for two decades and, combined with nocturnal ventilation, has increased the lifespan of amenable DMD patients significantly³. However, childhood loss of ambulatory function has persisted as a relatively unmodifiable prognostic event which severely impacts patient (and family) quality of life. Recently approved gene therapies represent promising progress in therapeutic development despite efficacy and long-term safety being equivocal³. With high unmet clinical need, the hunt for efficacious therapeutics that improve both patient quality and quantity of life is crucial.

In clinical trials conducted by the late Dr. Charles Bonsett (1980–1990s), adenylosuccinic acid (ASA) was identified as a candidate therapeutic with strong translational potential as it attenuated DMD progression². ASA is made endogenously by ASA synthetase from inosine in the purine nucleotide cycle (PNC), which is augmented during metabolic stress to recover degraded purines and stimulate the mitochondria to re-balance energy homeostasis⁴. Since DMD was considered a metabolic disease during that era (i.e. pre-discovery of DMD's genetic origin), ASA's mode of action (MOA) was attributed to its metabolic activity. Due to ASA's high production cost, infusion difficulties, and the eventual inability to source sufficient quantities for long-term treatment, these trials were discontinued despite clinical data suggesting persistent efficacy long after treatment cessation².

Recently, we confirmed that ASA can also attenuate murine (*mdx*) DMD¹. Despite profound benefits on skeletal muscle histopathology, we saw only modest effects of ASA on metabolic and mitochondrial function parameters. These data suggested to us an ulterior MOA for ASA aside from metabolic regulation, which appears more influential on mitigating the severity and progression of DMD. We suspected activation of nuclear factor erythroid-2 related factor-2 (Nrf2) since ASA: (1) attenuated the superoxide content of DMD myoblasts¹ and; (2) is a fumarate-generating compound and pharmacological fumarate donors, such as dimethyl fumarate (DMF), are well-established Nrf2 activators⁵. Herein, we assessed Nrf2 protein expression in skeletal muscles from our previous study to confirm whether induction of this master cytoprotective regulator was the putative MOA. Nrf2 was quantified by immunoblotting (rabbit, 1:1000, Gene Signaling, #12721) in quadriceps muscles from 12-week-old male C57Bl/10ScSn (healthy CON) and C57Bl/10*mdx* (dystrophic *mdx*) mice treated with/without ASA (3–3000 µg/mL) for 8-weeks¹.

Nrf2 is an important facilitator of hormesis: the adaptive response to cellular stress which promotes survival. Endogenously, Nrf2 elicits a graded cytoprotective response proportional to the scale and repetition of cytotoxic insult. Thus, basal Nrf2 expression reflects the current cellular stress climate. Basal Nrf2 expression was comparable between CON and *mdx* quadriceps consistent with stabilised disease in *mdx* mice of this age (Figure 1(A)). The *mdx* mouse recapitulates a milder disease course and disease stabilisation from ~1 to 12 m age. In contrast, oxidative stress, muscle damage and

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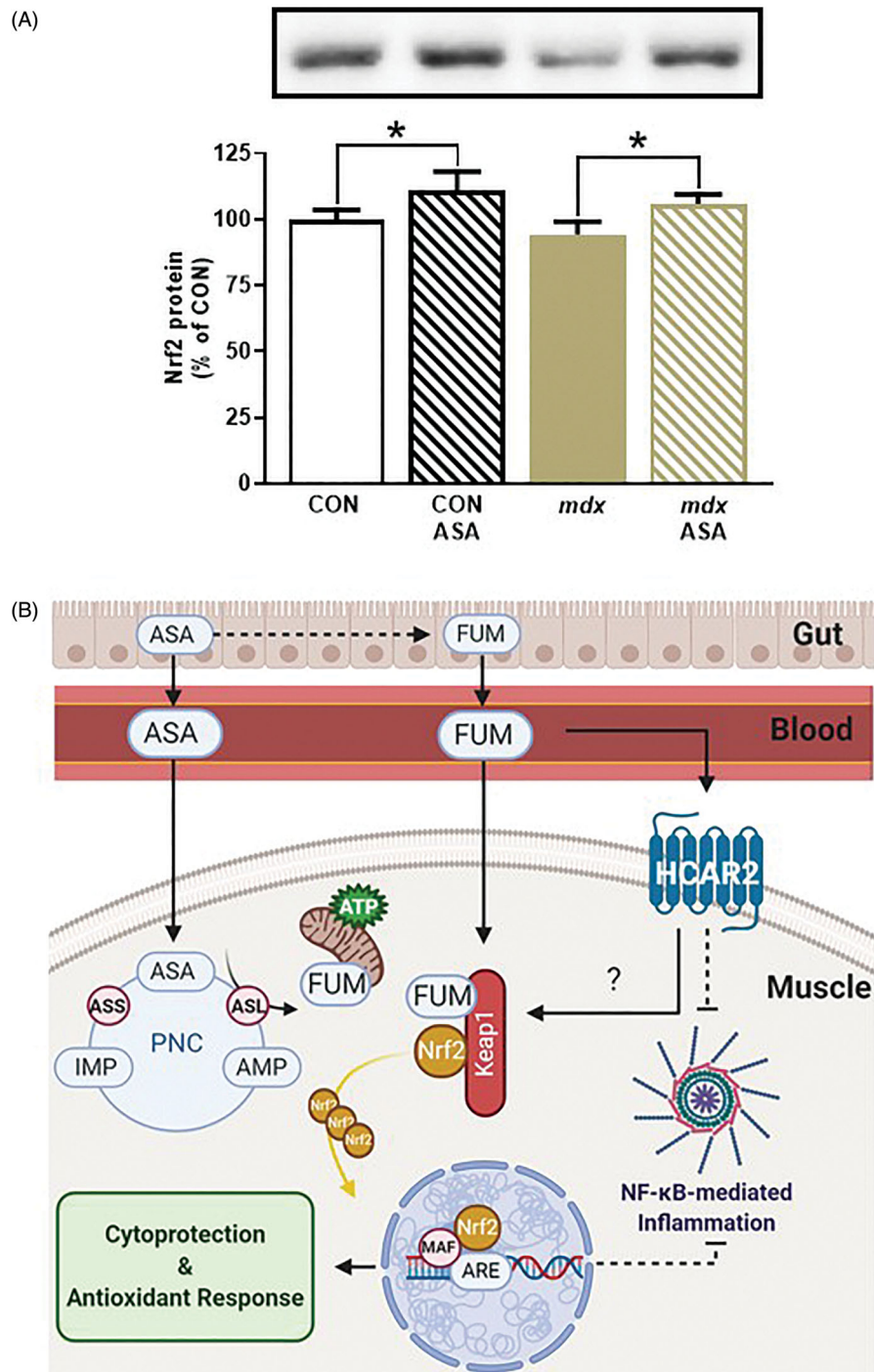


Figure 1. Adenylosuccinic Acid (ASA) is a novel inducer of nuclear factor erythroid 2-related factor 2 (Nrf2). Basal Nrf2 protein expression is comparable but ASA treatment induces Nrf2 in CON and *mdx* quadriceps ($p < .05$ treatment effect (A)). Data is mean Nrf2 protein normalized to the total protein signal in in each lane \pm SEM with representative bands; $n = 6-8$. In a proposed mechanism of action (B), ASA is converted to bioactive fumarate (FUM) either in the gut epithelium or in target tissues which express adenylosuccinate lyase (ASL). ASL converts ASA to FUM and recovers inosine monophosphate (IMP) to adenosine monophosphate (AMP) in the purine nucleotide cycle (PNC). ASA is endogenously re-synthesized through adenylosuccinate synthase (ASS). FUM is shuttled into the mitochondria to stimulate oxidative phosphorylation or binds cytosolic Kelch-like ECH-associated protein-1 (Keap1) to interrupt Nrf2 repression. This prevents Nrf2 ubiquitination and degradation leading to protein accumulation (A&B). Nrf2 translocates nuclearly where it heterodimerizes with MAF proteins to transcribe the antioxidant response element (ARE) and induce the cytoprotective and antioxidant response. Circulating FUM can also activate the hydroxycarboxylic acid receptor 2 (HCAR2) which, like Nrf2, exerts strong anti-inflammatory activity through the inhibition of nuclear factor kappa B (NF- κ B). Created with BioRender.com.

inflammation is rampant in skeletal muscles from juvenile (0–1.5 m) and aged (>12 m) mice. Basal Nrf2 expression patterns are currently uncharacterised in severe/progressive murine DMD.

In DMD patients, Nrf2 expression and activation paradoxically declines with age as weight-bearing activity and

disease severity escalates⁶. While the mechanisms concerning this apparent failure of hormesis are unclear, a similar scenario is portrayed in sarcopenic muscle wasting⁷. In aged skeletal muscle at least, suppressed Nrf2 expression/activity are amenable to exercise stimuli⁷ suggesting that pharmacological/metabolic Nrf2 targeting could be beneficial to re-

establish hormetic cytoprotection in DMD muscles. Our data demonstrate that ASA potentiates Nrf2 expression in both healthy and dystrophic muscles (Figure 1(A)). Nrf2 is negatively regulated by Kelch-like ECH-associated protein-1 (Keap1) which, under basal conditions, binds cytosolic Nrf2 stimulating its degradation⁵ (Figure 1(B)). Chemical modification of Keap1 by inducers (i.e. fumarate, endogenous free radicals⁵) precludes Keap1-dependent Nrf2 degradation causing cytosolic Nrf2 accumulation. Thus, increased Nrf2 protein content is synonymous with Keap1 cleavage and Nrf2 activation. Our new data highlight ASA as a novel inducer of Nrf2 in this regard and explain the efficacy we have described previously¹.

Following dissociation from Keap1, Nrf2 translocates nuclearly where it binds the antioxidant response element (ARE) enhancer, initiating transcription of downstream target genes and the antioxidant and cytoprotective response⁵ (Figure 1B). Nrf2 also modulates the transcription of mitochondrial biogenesis genes (i.e. mitochondrial transcription factor A (TFAM))⁸, and thus directly regulates mitochondrial pool maintenance. These Nrf2-dependent modifications reflect our published data in ASA-treated myoblasts derived from DMD and healthy patients, in which superoxide levels diminished and mitochondrial content increased¹. Our data are consistent with the known cytoprotective effects of Nrf2 activation.

ASA likely induces Nrf2 through driving PNC-mediated synthesis of endogenous fumarate (Figure 1(B)). This MOA assumes that ASA is bioavailable and able to penetrate the sarcolemma. That ASA treatment decreases adenosine monophosphate-activated protein kinase (AMPK) phosphorylation in healthy muscles suggest both bioavailability and PNC integration sufficient to re-balance energy levels (i.e. increase the ATP/AMP ratio)¹. Alternatively, ASA may be converted to fumarate in gut epithelium and circulated in this bioactive form. Fumarate donors are well-established Nrf2 activators through multiple modalities. Monomethyl fumarate, the bioactive form of the approved Multiple Sclerosis drug, DMF, induces Nrf2 both directly through fumarate modulation of Keap1 and indirectly through the hydroxycarboxylic acid receptor 2 (HCAR2)⁵. Both pathways exert potent anti-inflammatory effects through nuclear factor kappa B (NF-κB) inhibition⁵. Anti-inflammation could be highly influential in ASA-dependent modification of the severity and progression of human and murine DMD^{1,2}.

Here, we highlight ASA as a novel inducer of the Nrf2-mediated cytoprotective response through long-term supplementation, and Nrf2 as a feasible drug target to treat DMD. Further studies, e.g. using luciferase-based reporters of ARE transcription, will confirm the downstream antioxidant/cytoprotective response elicited by ASA.

Transparency

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