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Upregulation of the creatine synthetic pathway in skeletal muscles of mature *mdx* mice

Warren C. McClure [a], Rick E. Rabon [a], Hirofumi Ogawa [b], Brian S. Tseng [a,*]

[a] *Departments of Pediatrics, Neurology and Cell & Developmental Biology University of Colorado Denver Health Science Center, The Children's Hospital Fitzsimons Campus, Mail Stop 8108, PO Box 6511, Aurora CO 80045, USA*

[b] *Department of Biochemistry, Toyama Medical and Pharmaceutical University, Faculty of Medicine, 2630 Sugitani Toyama 930-0194, Japan*

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Abstract

Duchenne muscular dystrophy (DMD) is a fatal neuromuscular human disease caused by dystrophin deficiency. The *mdx* mouse lacks dystrophin protein, yet does not exhibit the debilitating DMD phenotype. Investigating compensatory mechanisms in the *mdx* mouse may shed new insights into modifying DMD pathogenesis. This study targets two metabolic genes, guanidinoacetate methyltransferase (GAMT) and arginine:glycine amidinotransferase (AGAT) which are required for creatine synthesis. We show that GAMT and AGAT mRNA are up-regulated 5.4- and 1.9-fold respectively in adult *mdx* muscle compared to C57. In addition, GAMT protein expression is up-regulated at least 2.5-fold in five different muscles of *mdx* vs. control. Furthermore, we find GAMT immunoreactivity in up to 80% of mature *mdx* muscle fibers in addition to small regenerating fibers and rare revertants; while GAMT immunoreactivity is equal to background levels in all muscle fibers of mature C57 mice. The up-regulation of the creatine synthetic pathway may help maintain muscle creatine levels and limit cellular energy failure in leaky *mdx* skeletal muscles. These results may help better understand the mild phenotype of the *mdx* mouse and may offer new treatment horizons for DMD. Published by Elsevier B.V.

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1. Introduction

Duchenne Muscular Dystrophy (DMD) is the most common, severe and lethal progressive muscular dystrophy to affect children. It is also the most common inherited lethal defect worldwide (1/3500 liveborn males) [1]. There are a number of animal models of DMD including dog, cat, and mouse [2–4]; which all display varying degrees of histopathologic muscle features consistent with muscular dystrophy. The *mdx* mouse has been a valuable animal model of DMD because it lacks dystrophin protein [1], has elevated serum creatine kinase (CK) and elevated intracellular calcium [5,6] which are all similar to that of human DMD. In addition, *mdx* skeletal muscle has abnormal contractile properties [7,8] and exhibits pathologic dystrophic features [9,10] particularly in the diaphragm [11].

Despite the histopathologic similarities between the *mdx* and humans with DMD, we postulate the *mdx* mouse has made unique compensatory adaptations to dystrophin-deficiency to enable a relatively benign phenotype. The *mdx* mice exhibit cage activity indistinguishable from control mice [12], live a near normal life span [13] and can run in voluntary wheels at distances near those run by age matched control mice [13–16]. In spite of the histopathology seen in the

* Corresponding author. Address: Harvard Mass Gen Hospital, Dept of Neurology, Charlestown Naval Yard B-114, Room 2600, 16th St., Charlestown, MA 02129, USA. Tel.: +1 303 861 6895; fax: +1 617 643 0141.

E-mail address: briantseng@partners.org (B.S. Tseng).

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diaphragm at 6 months of age [16], *mdx* mice do not suffer respiratory failure at that age and can still exercise at levels equivalent to the levels of control mice even up to 11 months of age [13,15].

The question of how the *mdx* mouse achieves this relatively benign functional phenotype is extremely important, when considering the severe phenotype displayed in human DMD where the same protein, dystrophin, is absent. Some explanations for the phenotypic variations in species such as differences in gait and life-span are readily apparent. It seems likely that there are many other compensatory molecular mechanisms contributing in combination. Since the *mdx* mouse is not severely crippled [12], has less fibrosis and more central nuclei than human DMD [10,17,18], we postulated that there may be additional compensatory molecular pathways or modifier genes in the *mdx* mouse that warrant further investigation. Disease-modifying factors implicated in the *mdx* mouse have been described [19], such as extra-cellular matrix alterations [20–23], naturally occurring and experimental up-regulation of utrophin [24–27], myostatin inhibition [28,29], calcium protein-handling protein(s) [30–32] and enhanced satellite cell function and regeneration [33–37]. While some of these modifiers are likely important in making the *mdx* phenotype relatively benign, many of these same *mdx* changes are occurring in parallel with DMD [2,12,38,39] and cannot fully account for the clear discordant phenotypic severity. Our overarching hypothesis is that there are compensatory pathways activated via modifier genes expressed in the *mdx* mouse that are not activated in the boys with DMD.

Several large-scale expression profiling or microarray studies of the mature *mdx* mouse hind-limb muscles have been published [23,40–43]. These studies were reviewed in an effort to find the most reproducible gene expression differences and then compare to three human DMD studies [44–46]. A gene whose mRNA expression moved in parallel or the same direction (up or down) in both *mdx* mouse and human DMD were eliminated leaving only the genes that were differentially expressed (manuscript in preparation). Of these genes, two in the same metabolic pathway guanidinoacetate methyltransferase (GAMT) and arginine:glycine amidinotransferase (AGAT) were found in multiple microarray studies to be up-regulated in *mdx* vs control mice while both were down-regulated in DMD (vs human control). In mammals, GAMT and AGAT are the only two enzymes required for creatine synthesis [47,48]. We were further intrigued by this novel finding given a prior study reported upregulated creatine kinase (CK) adaptations [49]. We were struck with magnetic resonance spectroscopy (MRS) studies that showed near normal intramuscular creatine levels in *mdx* mice [50], yet intramuscular creatine levels in boys with DMD were 20% of control boys [51]. This present study reports both GAMT and AGAT upregulation for *de novo* creatine synthesis in mature *mdx* muscle, which may help limit the cellular energy failure associated with the absence of dystrophin.

2. Materials and methods

2.1. Animals, care, specimen collection and preparation

Adult control (C57BL/10ScSn) ($n = 10$) and *mdx* (C57BL/10ScSn-mdx) mice were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were housed and handled in accordance with guidelines and procedures approved by Institutional Animal Care and Use Committee. Prior to being euthanized, 16 week mature mice and young mice postnatal 2 weeks, 4 weeks and 5 weeks of age were given intraperitoneal (IP) injections of pentobarbital sodium solution (100 mg/kg).

C57 ($n = 10$) and *mdx* ($n = 12$) mice age-matched at 9–11 weeks were housed with $4\frac{1}{2}$ inch running wheels (Super Pet Mini Run-Around) adapted with bicycle odometers (Sigma Sport BC 401). Weekly running distances and weights were recorded over a six-week period. Average daily running distances were determined for each mouse. These values were averaged for *mdx* and control mice for an overall value. Statistical differences between means were analyzed using Student's *t*-test.

Blood was collected via retro-orbital eye bleed. Muscle and liver specimens were excised and frozen in liquid nitrogen for homogenization. Contralateral muscles were removed, mounted in Tissue-Tek O.C.T. compound (Miles Lab, Elkhart, IN) and frozen in isopentane cooled to −160 °C in liquid nitrogen. Eight micron tissue cross-sections were obtained with a Microm cryostat at −22 °C. C57 and *mdx* tissue sections were collected onto Superfrost Plus Gold slides from Fischer Scientific and stained with H&E. Serial sections were used for immunolabelling studies (see below) and succinate dehydrogenase (SDH) histochemical stain as reported [52].

2.2. mRNA analysis

Total RNA was acid/phenol extracted from age 14–16 week old male control and *mdx* gastrocnemious mouse muscle using Trizol Reagent (Invitrogen). The RNA concentrations and purity from each sample were determined (Beckman DU 640 Spectrophotometer). Oligos (dT12–18) were used to synthesize cDNA from equal concentrations of RNA using SuperScript III Rnase H-Reverse Transcriptase (Invitrogen). Real time quantitative PCR was performed using Taq-Man reagents and ABI 7000 Prism Sequence Detection System. PCR Applied Biosystems Inc. (#377215) commercially available GAMT primers and probes, and