Correlated NOS-Ιμ and myf5 expression by satellite cells in mdx mouse muscle regeneration during NOS manipulation and deflazacort treatment

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Abstract

Satellite cells, muscle precursor cells in skeletal muscle, are normally quiescent and become activated by disease or injury. A lack of dystrophin and changes in the expression or activity of neuronal nitric oxide synthase (NOS-I) affect the timing of activation in vivo. Nitric oxide synthase inhibition delays muscle repair in normal mice, and worsens muscular dystrophy in the mdx mouse, a genetic homologue of Duchenne muscular dystrophy. However, the potential role of activation and repair events mediated by nitric oxide in determining the outcome of steroid or other treatments for muscular dystrophy is not clear. We tested the hypothesis that the extent of repair in dystrophic muscles of mdx mice is partly dependent on NOS-Ιμ expression and activity. Myotube formation in regenerating muscle was promoted by deflazacort treatment of mdx dystrophic mice \((P, 0.05)\), and improved by combination with the nitric oxide synthase substrate, l-arginine, especially in the diaphragm. NOS-Ιμ mRNA expression and activity were present in satellite cells and very new myotubes of regenerating and dystrophic muscle. Deflazacort treatment resulted in increased NOS-Ιμ expression in regenerating muscles in a strong and specific correlation with myf5 expression \((r = 0.95, P, 0.01)\), a marker for muscle repair. Nitric oxide synthase inhibition prevented the deflazacort-induced rise in NOS-Ιμ and myf5 expression in the diaphragm without affecting the diameter of non-regenerating fibres. These in vivo studies suggest that gains in NOS-Ιμ expression and nitric oxide synthase activity in satellite cells can increase the extent and speed of repair, even in the absence of dystrophin in muscle fibres. NOS-Ιμ may be a useful therapeutic target to augment the effects of steroidal or other treatments of muscular dystrophy.

Keywords: Dystrophy; Regeneration; c-met; c-fos; NG-nitro-L-arginine methyl ester hydrochloride

1. Introduction

Nitric oxide (NO) mediates early activation of satellite cells, the muscle precursor cells in skeletal muscle, and has important impact on skeletal muscle repair after injury in vivo [1]. NO is also required for activation of cultured quiescent satellite cells from older animals [2] and for activation of quiescent satellite cells on single fibres in culture [3]. Activation defines the first step in repair or growth of skeletal muscle. During activation, quiescent satellite cells are mobilized and recruited to cycle. NO is produced in skeletal muscle by a muscle-specific isoform of nitric oxide synthase, NOS-Ιμ, that is expressed and stabilized by the dystroglycan complex [4,5]. Once activated, muscle precursors subsequently fuse to form new fibres and contribute toward repairing damaged fibres.

The only factor other than NO that is known to induce mouse and rat satellite cell activation both in vivo and in vitro and lead to muscle cell proliferation is hepatocyte growth factor, the ligand for the c-met receptor [6,7].

In Duchenne muscular dystrophy (DMD), corticosteroid treatments such as prednisone and deflazacort (an oxalazine derivative of prednisone) are used commonly as treatments to reduce disease progression [8]. Clinical studies were previously modelled by our laboratory using mdx mice, a genetic homologue of DMD. Changes in fibre damage during mdx mouse muscular dystrophy, fibre growth in injured-regenerating and dystrophic muscles, and muscle strength were reported in detail, using indices of pathology, repair, metabolism and function [9–14]. Deflazacort treatment reduced the severity of dystrophic damage in limb, diaphragm and cardiac muscle, improved muscle strength and accelerated muscle repair [9–12]. As well, there was a positive relationship among effective repair, myf5
expression and the proliferation of muscle precursors expressing the muscle regulatory gene myf5 [11,13]. The positive effect of NO in differentiation and repair both in vitro and in vivo [1,14] suggests the possibility of treating muscular dystrophy by changing NOS expression and/or NOS activity. While some reports have shown that oxidative damage and the progression of dystrophy in mdx mice are independent of NOS-I expression [15–17], contrasting studies on mdx mice over-expressing NOS-I suggested a large benefit from NO in sparing muscle from dystrophic damage [18]. However, neither study specifically examined the early time course and outcome of repair in relation to the extent or location of NOS-I expression.

Since changes in satellite cell activation induced by NO inhibition are known to reduce repair by normal and dystrophic muscles [1], we investigated the possibility that NO may mediate some additional effects of deflazacort treatment on muscle repair in mdx mice. We tested the hypothesis that the extent of repair induced by crush injury, and the progression of dystrophy in mdx dystrophic mice are related in part, to the expression of NOS-I.

Experiments used deflazacort treatment in combination with L-arginine, the NOS substrate, or a NOS inhibitor in mdx mice. Repair was first assessed using standard techniques of histology, immunostaining and morphometry on sections of non-injured muscles, scoring for myotube numbers, measuring fibre diameter, and calculating the central nucleation index (CNI) that marks regenerated muscle fibres. Myf5 and c-met expression in crush-injured muscles (2 or 4 days after injury) were determined as representing actively regenerating muscle and the population of muscle precursors, respectively [6,13,19,20]. Expression of muscle-specific NOS-I was localized by in situ hybridization and measured from total RNA extracts of muscle tissue. C-fos expression was also examined, as it is reported to respond rapidly to mechanical signals and injury [21–23]. Results showed expression of NOS-I by dystrophin-deficient satellite cells and the earliest myotubes, and a strong, positive correlation between NOS-I and myf5 expression in regenerating muscles of mdx mice. NOS manipulation (either increase or decreased activity) was found to affect the extent of muscle regeneration in deflazacort-treated mdx mice, since l-arginine treatment significantly augmented the benefits of deflazacort treatment on dystrophy in the mdx mouse diaphragm.

2. Methods

2.1. In vivo experiments on dystrophic mice

Dystrophic mdx mutant mice (C57Bl/10ScSn-Dmd, Jackson Laboratories, Bar Harbor, ME) were bred and maintained at the University of Manitoba under protocols regulated by the Canadian Council on Animal Care. For studies of deflazacort and NO manipulation, dystrophic animals 3 weeks-of-age were housed for 12 days and weighed daily. Forty mdx mice were assigned to one of four groups (n = 10) as follows. A placebo group received a daily subcutaneous injection of methyl cellulose vehicle (50 µl). Treated mice received deflazacort (1.2 mg/kg per day) [9] and were subdivided into three groups. One group received deflazacort alone. One group received deflazacort plus drinking water containing L-arginine (3.75 mg/ml, Sigma), and the last group received deflazacort plus N\(^\text{G}\)-nitro-L-arginine methyl ester hydrochloride (L-NAME, 0.125 mg/ml in drinking water, Sigma) [1]. After 12 days, mice were anaesthetized and the right tibialis anterior (TA) muscle received a crush injury to stimulate synchronized regeneration [24]. Animals recovered 2 or 4 days and were euthanized. Body weight gain was calculated (mean ± SEM). Diaphragm and left and right TA (LTA, RTA) were removed and bisected. Muscle was embedded for frozen sections, or snap frozen and stored (−80 °C) prior to RNA isolation.

2.2. Muscle histopathology

Sections were stained in haematoxylin and eosin (H&E) and coded. Using coded sections of the left (non-injured) TA and diaphragm muscles, fibres were counted for central or peripheral nuclei by making counts across the largest diameter of the muscle belly from each of two sections, collected 100–120 µm apart. The central nucleation index (CNI) was calculated as the proportion of the sample of 60–120 fibres that displayed central nuclei in the non-injured muscles (left TA and diaphragm). The mean diameter of fibres in LTA muscles was calculated from diameter measurements (from 100 to 180 fibres per muscle section) collected as previously reported [9].

Regenerating muscles 4 days after crush injury were scored on a four-point Likert scale from 1 (low) to 4 (high) for the number of centrally-nucleated myotubes, confirmed using phase contrast optics and immunostaining for desmin or devMHC as previously reported. Two sections from each regenerating muscle were used to score myotubes, which were easily visible under 200× magnification in the region of inflammatory cells produced as a result of the crush injury. Numbers of myotubes were scored over two non-overlapping fields adjacent and distal to the centre of the crushed region from each muscle section, as defined earlier [24]. Representative sections of non-injured and regenerating muscles were immunostained using an antibody against desmin (with diamobenzidine/nickel chloride detection) or developmental myosin heavy chain (devMHC, with immunofluorescent detection) as reported [9,26]. Immunostained sections and phase contrast optics were used to confirm the identification of regenerating myotubes.

CNI, fibre diameter, body weight gain and myotube score were compared among groups with analysis of variance (ANOVA) and post hoc Duncan’s tests, or non-parametric Kruskal–Wallis ANOVA (myotube score) using NWA
mRNAs were 1.7 kb (myf5), 9 kb (c-met), 10 kb (NOS-I) [22, 31]. Antisense, digoxigenin-labelled riboprobes were synthesized following Boehringer Mannheim protocols. cDNA for c-fos was obtained as a gift from Dr. R.L. Prewitt (c-met), 0.12 kb (NOS-I), 0.12 ng of NOS-I probe, 1.3 ng of c-met probe) in hybridization buffer containing 80% formamide, 40 mM PIPES (pH 6.7), 0.4 M NaCl, and 1 mM EDTA following standard protocols (Boehringer Mannheim). Hybridized samples were digested with a mixture of RNase A and RNase T1 for 1 h at 30 °C [33], loaded on thick MOPS–formaldehyde 1.0% agarose gels, and run for 2 h at 25 V (BioRad, Model 1000/500). RNA was transferred by concentration gradients overnight at 4 °C onto nylon membrane (Boehringer Mannheim) according to standard protocols, and fixed (120 °C, 15 min). Northern blots for myf5 and c-fos were prepared from the same samples of total RNA, and were hybridized overnight at 68 °C, again with an excess of riboprobe (100 ng/ml). Hybridization was detected using alkaline phosphatase-conjugated anti-digoxigenin antibody and CSPD substrate (Boehringer Mannheim) exposed to X-ray film (Fuji, Mississauga, ON). The lower limit of RNA detection was determined in advance to be far less than 0.01 ng (myf5), 0.05 ng (c-met), 0.025 ng (NOS-I), and 0.034 ng (c-fos), standardized (in the linear range) against dot blot serial dilutions of control digoxigenin-labelleia RNA (760 bp, Boehringer Mannheim).

From the pooled tissues (ten mice per group), sufficient RNA was isolated to allow measures of expression using RNase protection assays for NOS-I (n = 2) and c-met (n = 1), and Northern blots for myf5 (n = 2) and c-fos (n = 1 or 2). In every case, hybridization was determined by subtracting background density from scans made using exactly the same window in each lane, and band density was standardized against muscle weight represented by the loaded RNA and the intensity of the 18S band on gels stained with ethidium bromide before transfer. Density (arbitrary units) was tabulated as a ratio to the placebo lane on the same gel. Gels, blots and scans for each probe were always run using complete sets of the different muscles and treatment groups at the same time to minimize variation.

3. Results:

3.1. Histopathology in mdx mice in vivo

Deflazacort treatment reduced the extent of inflammation, fibre damage and CNI in the diaphragm (Fig. 1) and non-injured LTA, and increased fibre diameter in sections of LTA (Table 1), as previously reported [9]. During treatment, body weight gain was higher (P < 0.01) with deflazacort (1.30 ± 0.03) or deflazacort plus l-arginine treatments (1.45 ± 0.03) than in mice treated with placebo (1.17 ± 0.04) or deflazacort plus l-NAME (1.20 ± 0.03). In regenerating muscles (Fig. 1), myotubes were very prominent in the region affected by the crush injury, as shown by mononuclear inflammatory cell infiltration and removal of necrotic debris. Myotubes were especially notable in the TA muscles regenerating after deflazacort...
treatment, as identified using routine histology, phase contrast (not shown) and immunostaining for desmin and devMHC.

In mdx mice treated with deflazacort plus l-arginine, inflammation in diaphragm and LTA, and CNI in the diaphragm muscle were further reduced compared to deflazacort alone. The mean diameter of LTA fibres after combined deflazacort and l-arginine treatment did not differ from the deflazacort-treated group. In regenerating muscles from this group of mdx mice, myotubes were observed earlier (at 2 days after injury) compared to muscles regenerating in mdx mice treated
with deflazacort alone, and by 4 days after injury appeared larger than after deflazacort treatment. Changes in the activity of NOS significantly affected the numbers of myotubes observed and scored in regenerating muscles \( (P < 0.05, \text{Kruskal–Wallis statistic} = 13.1) \). After treatment with deflazacort plus the NOS inhibitor, \( \text{L-NAME} \), the number and diameter of new myotubes observed in regenerating muscles was smaller compared to muscles of deflazacort-treated \( \text{mdx} \) mice. Branched profiles of new myotubes were infrequent (< 5% of myotubes) and were observed only in muscles after treatment with \( \text{L-NAME} \) and deflazacort. In non-injured LTA muscles, the diameter of differentiated fibres and the calculated CNI were not different from those observed with deflazacort treatment alone (Table 1).

The observations of muscle histology suggest that \( \text{l-arginine} \) augmented the action of deflazacort to reduce the damage and inflammation characteristic of dystrophy in the \( \text{mdx} \) mouse especially in the diaphragm, and also advanced the rate of new fibre differentiation and muscle repair.

### 3.2. Expression of NOS-\( \text{I-}\mu \), myf5, c-met and c-fos

Molecular biology studies of gene expression were used to assess whether regeneration (marked by myf5 expression) was related to the extent or location of NOS-\( \text{I-}\mu \) expression (Fig. 2). NOS activity and NOS-\( \text{I-}\mu \) expression in fibres of \( \text{mdx} \) mouse muscle were absent compared to normal muscle. Surprisingly, the in situ hybridization signal for NOS-\( \text{I-}\mu \) transcripts was present, and strongly localized in satellite cells, small myotubes and a few mononuclear cells between fibres. The NOS-\( \text{I-}\mu \) signal was specific, since expression was absent in smooth muscle, in endothelial and gut epithelial cells that had showed NOS activity by histochemical staining.

Myf5 expression was observed in activated satellite cells and myotubes (Fig. 2I). Myf5 expression in regenerating muscle was increased by deflazacort treatment alone or in combination with \( \text{l-arginine} \) (Table 2). In the diaphragm, deflazacort increased myf5 expression relative to placebo, and this was prevented by adding \( \text{L-NAME} \) treatment. Regenerating muscle from the three groups of mice treated with deflazacort (either alone or combined with \( \text{l-arginine} \) or \( \text{L-NAME} \)) had higher NOS-\( \text{I-}\mu \) expression than the placebo group (Table 2). In the diaphragm, treatment with \( \text{L-NAME} \) and deflazacort reduced the expression of NOS-\( \text{I-}\mu \) compared to deflazacort alone (deflazacort: 1.08 \pm 0.01 vs. deflazacort plus \( \text{L-NAME} \): 0.28 \pm 0.03). NOS-\( \text{I-}\mu \) expression in muscles after 2 or 4 days of repair was highly correlated with myf5 expression \( (r = 0.95, \text{df} = 6) \) and not significantly different from 1.0 (Fig. 3).

C-met mRNA was localized in satellite cells and some mononuclear cells (likely myoblasts) between fibres (Fig. 2K,L). C-met expression was very low in early myotubes and absent from fibres. In muscles regenerating during deflazacort treatment alone (or with \( \text{l-arginine} \) or \( \text{L-NAME} \)), the expression of c-met was approximately twofold higher (1.6–2.6-fold) compared to placebo. NOS-\( \text{I-}\mu \) and c-met expression in regenerating muscles were not significantly correlated \( (r = 0.45, P > 0.05, \text{Fig. 3C}) \).

An intense signal for c-fos transcripts in regenerating muscles and diaphragm was localized in satellite cells and new myotubes close to foci of dystrophy (Fig. 2M,N). The level of c-fos expression determined by Northern blot was roughly doubled after \( \text{l-arginine} \) and deflazacort treatment compared to deflazacort alone (diaphragm: 1.3 \pm 0.2 vs. 0.7 \pm 0.01; regenerating muscle (one blot only), 2.5 vs. 1.2).
ing muscles. Surprisingly, NOS-I\(\mu\) mRNA was expressed by satellite cells and very early myotubes in muscles regenerating in \textit{mdx} mice, despite the absence of dystrophin. NOS-I\(\mu\) expression was not observed in other cells of \textit{mdx} or normal tissues that showed NOS activity as detected using histochemistry, or in satellite cells of NOS-I(\textit{−/−}) muscle. These observations strongly suggest that the regulation of NOS-I\(\mu\) expression in mononuclear muscle cells is distinct from that in fibres with a mature or genetically deficient cytoskeleton. The effects of deflazacort were typical in that treatment alleviated dystrophy, reduced the central nucleation index and increased fibre diameter, effects that did not change during NOS inhibition. However, NOS inhibition did reduce the formation of myotubes in regenerating muscles. NOS inhibition also reduced or blocked the increases in c-met and myf5 expression in

Fig. 2. Studies of NOS activity using enzyme histochemistry (A–E) and expression of NOS-I\(\mu\) (F–I), myf5 (J), c-met (K,L), and c-fos (M,N) using in situ hybridization in muscle from normal (A,B,F), NOS-I(\textit{−/−}) (C), and \textit{mdx} mice from regenerating tibialis anterior or diaphragm muscle (D–N). (A,B) Normal muscle with NOS activity at the periphery of fibres, in vascular smooth muscle cells (top right) and a satellite cell (arrow). (C) A low level of NOS activity is observed in a satellite cell of muscle from a NOS-I(\textit{−/−}) mouse (arrow) presumably from activity of endothelial and/or inducible NOS isoforms. NOS activity was not noted in adjacent fibres (dashed outline) and NOS-I\(\mu\) transcripts were not identified in muscle satellite cells or fibres of NOS-I(\textit{−/−}) mice (not shown). (D) NOS activity in a satellite cell and myotube (E) of regenerating \textit{mdx} muscle 4 days after injury. (F) NOS-I\(\mu\) expression visualized by in situ hybridization (NOS ISH) in a satellite cell from normal muscle. (G) NOS-I\(\mu\) expression in mononuclear cells (likely myoblasts) in regenerating \textit{mdx} muscle. (H,I) NOS-I\(\mu\) transcripts in new myotubes in \textit{mdx} diaphragm and regenerating muscle (arrow). (J) Myf5 transcripts (myf5 ISH) are dispersed within cytoplasm of relatively large myotubes (central nuclei are unstained). (K,L) C-met transcripts (c-met ISH) are localized to an \textit{mdx} satellite cell (arrow), but are not present in a myotube with central nuclei (outlined, open arrow). (M,N) C-fos expression (c-fos ISH) in satellite cells of diaphragm muscle fibres, mononuclear cells and new myotubes adjacent to recent fibre necrosis. Bar: 10 \textmu m (A–D,F–H,K–N) or 20 \textmu m (E,L,J).
Fig. 3. (A) A graph showing data for NOS-Iµ expression as a function of myf5 expression in regenerating muscles at 2 days (circles) and 4 days (diamonds) after injury in the four treatment groups. There was a strong correlation ($r = 0.95, P < 0.01$) between NOS-Iµ and myf5 expression ($n = 8$). (B) NOS-Iµ expression as a function of c-met expression in the same muscles after 2 days (circles) and 4 days (diamonds) of regeneration showed no significant correlation ($r = 0.45, P > 0.05$).

mdx mouse diaphragm muscle during deflazacort treatment. Regeneration was assessed according to standard histology indices including myotube formation (morphology, timing and numbers, desmin and devMHC immunostaining), and myf5 expression which is related to the success of repair in skeletal muscle [13]. Finally, the level of expression of NOS-Iµ in regenerating mdx mouse muscles was strongly correlated with myf5 expression. The results of these in vivo experiments strongly support the hypothesis that increased NOS-Iµ expression in regenerating muscle, which was localized only in satellite cells and very early myotubes in the absence of dystrophin, gave rise to an improved outcome of deflazacort treatment in mdx mouse muscular dystrophy.

NOS-Iµ expression increased in response to deflazacort treatment, and responded appropriately to changes in NOS activity. Corticosteroids are commonly used to treat Duchenne muscular dystrophy [8], and have been reported to either up- or down-regulate NOS-Iµ expression in neurons [34–36]. In our in vivo studies NOS-Iµ expression also increased during treatments with the NOS substrate and a NOS inhibitor. The apparently paradoxical rise in NOS-Iµ mRNA during NOS inhibition may be compensatory for lost NOS-Iµ activity, as is required during muscle repair. The weakly positive NOS activity observed in some satellite cells of muscle from NOS(−/−) mice may indicate activity by other isoforms of NOS, possibly induced or increased in the absence of NOS-I. Earlier reports that NOS (or NOS-Iµ) activity is required for myotube formation, is restricted to the membrane region during fibre differentiation, and is down-regulated by the loss of dystrophin [14,37,38] attest to the importance of NOS-Iµ expression and activity. Present in vivo observations now support the idea that increased NOS-Iµ expression (and presumably higher concentrations of NO) in muscle precursors and new fibres can help attenuate inflammation and dystrophic fibre damage, and accelerate muscle repair in mdx mice. The observation is consistent with studies of mdx mice that over-express a NOS-I transgene in mature muscle fibres and show a significant alleviation of dystrophy [18]. The present finding suggests that satellite cell-derived NO also can directly affect regeneration even in the absence of dystrophin, in addition to the role of NO in satellite cell activation reported previously [1,2]. It is possible that greater NOS-Iµ expression during early repair may help to stabilize expression during early differentiation of the cytoskeleton in growing myotubes. A similar idea was proposed for the transcriptional regulation of utrophin [39,40] and restoration of dystrophin-associated proteins as dystrophin diffused into dystrophin-negative domains in ‘hybrid’ myotubes [41]. Other influences of NO that augment the vascular responses to exercise [42,43], reduce macrophage cytotoxicity [18], or may contribute to increasing extrajunctional utrophin [44] would further contribute to improve muscle repair and reduce dystrophic damage, as were observed here during treatment with deflazacort and l-arginine, and in previous experiments with l-arginine alone [1].

The in situ hybridization studies and a strong correlation between myf5 and NOS-Iµ expression in regenerating muscle suggest myf5 and NOS-Iµ are co-expressed by activated satellite cells and new fibres. Satellite cells also expressed c-fos mRNA, a standard indicator of rapid responses in other tissues expressing NOS-I or NOS-III (e.g. Refs. [21,45]). Whether a population of satellite cells or activated muscle precursors shows a uniform level of expression for c-met, NOS-Iµ, or c-fos is not known. However myf5 expression represents repair, and persists during differentiation of myotubes [13,46–48]. Therefore tandem study of NOS-Iµ and myf5 expression in dystrophic muscles may be useful indices for treatment effects on repair, and c-fos is a potential early marker of satellite cell activation.

Perhaps not surprisingly, NOS inhibition during deflazacort treatment did not change CNI or fibre growth (diameter) in comparison to deflazacort alone. A complex regulation of fluid balance, contraction, and the expression and activity of NOS-I contributes to the robust effects of steroids [34,36,49–52]. As well, our novel observations of NOS-Iµ expression in satellite cells and early myotubes highlight a feature that is specific to adult muscle regeneration, since developmental NOS-I expression switches to NOS-Iµ as myotubes first form [27]. NOS inhibition can affect how steroids act on energy use,
vascular flow and macrophage functions [53,54], which are consistent with the observed reduction in muscle repair during L-NAME treatment of mdx mice. While NOS inhibition studies in vivo serve to illustrate one part of a so-called 'two hit' hypothesis of muscle necrosis [55], the correlation between NOS-1 expression and myf5 expression, localized in satellite cells and myotubes, adds muscle regeneration to the roles of NO signalling in inflammation, apoptosis, vascular perfusion and satellite cell activation [1,18,56,57]. Whereas dystrophin and NOS-1 are absent from mature fibres in dystrophic muscle, the new finding of NOS-1 expression in satellite cells and very new fibres in vivo, strongly underlines a potential for NO-based therapies to improve muscle repair.

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