Nitric oxide signalling pathway in Duchenne muscular dystrophy mice: up-regulation of L-arginine transporters

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INTRODUCTION

DMD (Duchenne muscular dystrophy) is an incurable rapidly worsening neuromuscular degenerative disease caused by the absence of dystrophin. In skeletal muscle a lack of dystrophin disrupts the recruitment of neuronal NOS (nitric oxide synthase) to the sarcolemma thus affecting NO (nitric oxide) production. Utrphin is a dystrophin homologue, the expression of which is greatly up-regulated in the sarcolemma of dystrophin-negative fibres from mdx mice, a mouse model of DMD. Although cardiomyopathy is an important cause of death, little is known about the NO signalling pathway in the cardiac muscle of DMD patients. Thus we used cardiomyocytes and hearts from two month-old mdx and mdx:utrophin−/− (double knockout) mice (mdx:utr) to study key steps in NO signalling: L-arginine transporters, NOS and sGC (soluble guanylate cyclase). nNOS did not co-localize with dystrophin or utrophin to the cardiomyocyte membrane. Despite this nNOS activity was markedly decreased in both mdx and mdx:utr mice, whereas nNOS expression was only decreased in mdx:utr mouse hearts, suggesting that utrophin up-regulation in cardiomyocytes maintains nNOS levels, but not function. sGC protein levels and activity remained at control levels. Unexpectedly, L-arginine transporter expression and function were significantly increased, suggesting a novel biochemical compensatory mechanism of the NO pathway and a potential entry site for therapeutics.

Key words: cardiomyopathy, cationic amino acid transporter (CAT), mdx mouse, neuronal nitric oxide synthase (nNOS), soluble guanylate cyclase (sGC), utrophin.

Abbreviations used: CAT, cationic amino acid transporter; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein; DAPI, 4′,6-diamidino-2-phenylindole; DGC, dystrophin–glycoprotein complex; DMD, Duchenne muscular dystrophy; eNOS, endothelial nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HRP, horseradish peroxidase; IBMX, isobutylmethylxanthine; L-Arg, L-arginine; L-Lys, L-lysine; L-NAME, N′-nitro-L-arginine methyl ester; 7-NINA, 7-nitroindazole; nNOS, neuronal nitric oxide synthase; RT, reverse transcription; sGC, soluble guanylate cyclase; UMDNJ, University of Medicine and Dentistry of New Jersey; WGA, wheat-germ agglutinin; WT, wild-type.

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such as premature ventricular contractions. These observations point to a corrective role for nNOS in DMD and prompted us to assess the status of the NO signalling pathway in cardiac muscle from mdx and mdx:utrophin mice. Specifically, we focused on the first three members of this pathway: CATs (cationic amino acid transporters) that supply the NO substrate L-Arg [24–26], NOS-mediated NO production and the ‘NO receptor’ sGC (soluble guanylate cyclase), a key mediator of vascular relaxation, cardiac remodelling and contractility [27,28].

In cardiac myocytes, which lack the enzymes for the de novo synthesis of L-Arg or its recycling from citrulline [29,30], the NO signalling pathway must begin with the transport of L-Arg across the plasma membrane. L-Arg transport is mediated by a family of glycosylated proteins known as system y+ [25]. Members of this family (termed CATs) are highly selective for cationic L-amino acids such as L-Arg, L-Lys (L-lysine) and L-Orn (L-ornithine). System y+ includes the high-affinity CAT-1, CAT-2B and CAT-3 (K_0.5 = 100–250 μM) as well as the low-affinity CAT-2A (K_0.5 = 2–10 mM) [25]. Within a given isoform all three amino acids are transported with similar apparent affinities and transport capacities. Previously we have identified high- (CAT-1) and low- (CAT-2A) affinity L-Arg transporters that function in parallel in rat ventricular cardiomyocytes and cardiac sarcosomial vesicles [24,26]. Particularly, because of its high capacity, the activity of the low-affinity transporter was found to be physiologically relevant as it accounts for >50% of total transport at normal plasma levels of cationic amino acids [26]. Moreover, L-Arg transport is inhibited by NOS-derived NO in cardiac muscle cells through a negative-feedback mechanism by which NO acutely regulates its own production [31].

We report in the present paper that although nNOS levels are not significantly affected in mdx mice, nNOS function is dramatically decreased. Conversely, CAT-2A activity is significantly enhanced, highlighting a novel potential compensatory mechanism at the biochemical level of the NO pathway.

EXPERIMENTAL

Animal husbandry

WT (wild-type) C57BL/10 mice were purchased from Jackson Laboratories and used as strain-specific controls. Hemizygous C57BL/10ScSn-mdx/J (mdx is the muscular dystrophy gene localized to the X-chromosome; also known as Dmd) males and homozygous C57BL/10ScSn-mdx/J (mdx) females were purchased from Jackson Laboratories. The colony was maintained by crossing mdx males with mdx females. Mdx:utrophin−/− (double knockout) mice were generated by crossing mdx:utrophin−/− males with mdx:utrophin+/− females. The mdx:utrophin−/− mice were kindly provided by Dr R. Grange (Virginia Tech, Blacksburg, VA, U.S.A.). PCR analysis to determine utrophin-knockout mice was performed with puReTaq Ready-To-Go PCR beads (GE Healthcare). This PCR uses a reverse primer complementary to exon 7 of mouse utrophin (5'-CTTACTAGGCAACAAACCTAC-3') and forward primers complementary to either intron 7 (5'-CTGATCTGAATAATGACGT-3') or to the PGK promoter located within the Neo-knockout cassette (5'-ATCCATATGGCTATGCAATGCCG-3'). Reactions were carried out on genomic DNA for 35 cycles under the following conditions: 94°C, 30 s; 57°C, 30 s; and 72°C, 25 s. Mdx:utrophin−/− mice exhibit phenotype abnormalities beginning at 3 weeks of age, including kyphosis, diminished body mass, loss of ambulation and hypertrophy of the upper body. All animal experiments were approved by the IACUC (Institutional Animal Care and Use Committee) of the UMDNJ (University of Medicine and Dentistry of New Jersey).

Tissue collection

Male and female mice were injected with Rodent Cocktail (100 mg/ml ketamine and 100 mg/ml xylazine), 0.2–0.4 ml/100 g intraperitoneally, in accordance with institutional guidelines. The hearts were removed under complete anaesthesia and processed as detailed in the assays described below.

NOS protein levels and IHC (immunohistochemistry)

For immunofluorescence, hearts were collected from mice killed at the desired age (2–19 months), Tissue-Tek OCT embedded, frozen in liquid nitrogen-cooled 2-methylbutane and cryosectioned (10 μm). After blocking using the appropriate 5% serum, serial sections were stained adjacentely utilizing antibodies reactive with NOS1 (nNOS), (anti-rabbit, 1:2000 dilution, sc-648, Santa Cruz Biotechnology), WGA (wheat-germ agglutinin; fluorophore-conjugated, 1:100 dilution, W11262, Life Technologies) and utrophin [anti-guinea pig, 1:1000 dilution, kindly supplied by Dr J.M. Fritschi (University of Zurich, Zurich, Switzerland)] overnight at 4°C. Nuclei were identified with DAPI (4′,6-diamidino-2-phenylindole; H-1500, Vector Labs). Confocal images of nNOS, WGA and DAPI distribution (×60 magnification) were obtained using a Zeiss LSM 510 microscope on a Zeiss Axiovert 100M Base and processed using NIS Elements (Nikon). Individual images were generated in excitation wavelengths corresponding to blue (DAPI), green (nNOS) and red (WGA) emission spectra, and were processed and compiled to generate tri-colour overlays.

For protein analysis, hearts were collected and snap-frozen in liquid nitrogen. Tissue was homogenized in homogenization buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM NaVO_3, 1 mM NaF, 1 mM Na_2P_2O_7, 1 mM benzamidine and 1% protease inhibitor (P8340, Sigma). A 4–20% gradient acrylamide gel and high-molecular-mass protein standards (HiMark, Invitrogen and Precision Plus, Biolab respectively) were used. Proteins were transferred on to a nitrocellulose membrane and transiently stained with Ponceau so that the membranes could be cut horizontally on the basis of protein size. This technique allows for simultaneous, yet separated, blotting with different antibodies for proteins of different sizes that were transferred to a single membrane. In the case of nNOS and eNOS (endothelial NO synthase), separate nitrocellulose membranes were used due to similarities in protein size. Membranes were blocked with 5% dried skimmed milk powder and probed overnight in 1% dried skimmed milk powder at 4°C using antibodies reactive with nNOS (see above), eNOS (anti-mouse, 1:2500 dilution, 610296, BD Transduction Laboratories), utrophin (anti-mouse, 1:100 dilution, Mancho3 Developmental Studies Hybridoma Bank) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a loading control (anti-mouse, 1:10 000 dilution, G8795, Sigma). Membranes were washed in PBS containing 0.5% Tween and then incubated independently with appropriate secondary antibodies: eNOS, utrophin and GAPDH membrane strips were probed with HRP (horseradish peroxidase)-conjugated anti-mouse secondary antibody and nNOS membranes were probed with HRP-conjugated anti-rabbit secondary antibody for 1 h at 23°C and visualized with a Thermo Scientific SuperSignal West Dura Substrate kit. The intensities of the Western blot exposures were quantified using Quantity One software on a GS800-Densitometer.
(Bio-Rad Laboratories). Relative protein levels were normalized using GAPDH bands within the same linear range of detection.

**Fluorescence measurements**

Single ventricular myocytes were enzymatically isolated from 2-month-old mouse hearts with a Langendorff perfusion system following methods published previously [32]. Briefly, hearts were removed from anaesthetized WT, mdx and mdx:utrophin−/− mice and perfused retrogradely in Langendorff fashion for 8 min at 37°C with nominally calcium-free Langendorff solution containing ~1.4 mg/ml collagenase (Type II). After washing out the enzyme solution, the hearts were removed from the perfusion apparatus and teased apart in a culture dish. The cardiomyocytes thus obtained were suspended in 1.5 ml of Langendorff solution containing 135 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 0.2 mM CaCl2, 0.33 mM NaH2PO4 and 10 mM sodium Hepes (pH 7.2) at 23°C, and incubated for 1 h at 23°C with 10 μM of the dye DAF-FM (4-amino-5-methylamino-2′,7′-difluorofluorescein) diacetate in aluminium-foil-wrapped glass tubes. The supernatant was removed and cells were incubated in Langendorff solution for 15 min at 37°C. After supernatant removal, cells were resuspended in 2 ml of Langendorff solution and 225 μl aliquots were distributed in 96-well plates. We have successfully sequenced RNA for final identification.

**RNA isolation, reverse transcriptase reaction and real-time PCR**

RNA was isolated from mouse hearts as described previously [26], except that a Fibrous Tissue Midi kit (Qiagen) was used, following the manufacturer’s protocol. Sense and antisense oligonucleotide primers were synthesized in the UMDNJ Molecular Resource Facility. The primer pairs and optimal PCR amplification conditions used in the present study have been described previously [26], except for: sense nNOS, 5′-ACCCACAGCTCATTTCTGTC-3′; antisense nNOS, 5′-AAGGTGCTTCCAGGTGTGTT-3′; sense eNOS, 5′-GACCCCTACCCGTACAACAT-3′; and antisense eNOS, 5′-CTGGGCTTCTGCTTATTTC-3′. The cycles were performed under the following conditions. For nNOS, denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 25 s. For eNOS, denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 60 s. RT (reverse transcription)–PCR was performed on mRNAs using a Light Cycler real-time PCR instrument (Roche), and the original number of PCR templates was normalized to the amount of protein in each well determined by real-time RT–PCR in the hearts of 2-month-old NOS mice [26].

**Reagents**

L-[U-14C]lysine monohydrochloride, specific activity >300 mCi mmol−1, was from MP Biomedicals. L-Lys and L-Arg (monohydrochloride salts), L-NAME (Nω-nitro-L-arginine methyl ester) and IBMX were from Sigma–Aldrich. DAF-FM was from Invitrogen, 7-NINA (7-nitroindazole) was from Alexis Biochemicals and DEA-NONOate was from Axxora. Collagenase type II was obtained from Worthington Biochemical. Salts and reagents were of analytical reagent grade.

**RESULTS**

The status of the NO signalling pathway in terms of L-Arg transporters, NOS-mediated NO production and sGC was determined by real-time RT–PCR in the hearts of 2-month-old muscular dystrophy mice.
mdx and mdx:utr mice. nNOS expression was significantly decreased only in mdx:utr mice, whereas eNOS remained at WT control levels (Figure 1A). Likewise, densitometric analysis of Western blots showed that nNOS protein levels were decreased only in mdx:utr mice compared with the WT and mdx mice of the same age (Figure 1B), whereas eNOS protein levels remained unmodified in mdx and mdx:utr mice compared with the WT controls (Figure 1C). Immunohistochemical studies revealed that nNOS (Figure 1D, green) was profusely distributed in WT hearts, but, oppositely to skeletal muscle, its distribution was punctated and did not co-localize with dystrophin at the myocyte membrane (Figure 1D inset, red). Heart sections from mdx mice also showed the presence of punctated non-plasma-membrane cardiac nNOS in the absence of dystrophin, as evidenced by staining with the plasma membrane marker WGA (Figure 1D, red). Finally, the joint absence of dystrophin and utrophin in heart
sections of mdx:utr mice resulted in a severe reduction in nNOS immunostaining. To determine whether the levels of utrophin and NOS isoforms remain constant with age in mdx mice, Western blotting and immunohistochemical studies were also performed with 19-month-old mdx mice. The results show that nNOS and eNOS are still present in the dystrophic hearts of 19-month-old mice (Figure 1E), along with utrophin (Figures 1E and 1F).

Measurements of NOS activity were performed in acutely isolated disaggregated cardiac myocytes (Figure 2A) that were loaded with the NO-sensitive fluorescent dye DAF-FM. Changes in fluorescence (i.e. NO production) were detected following application of extracellular L-Arg. Considering that L-Arg had to be transported into cardiomyocytes before triggering NO synthesis, CAT and NOS activities were assessed together in these assays. Increases in fluorescence were observed at millimolar L-Arg concentrations, suggesting an important role for the low-affinity member of the \( \gamma^+ \) family, CAT-2A. Cardiac myocytes from 2-month-old WT mouse hearts generated NO in a dose-dependent manner in the presence of 1, 5 or 10 mM L-Arg (Figure 2B). The shape of the curves related to the irreversible nature of NO–DAF-FM interactions [31]. Curves showed an initial fast component (not resolved in the 30-s sampling interval after adding L-Arg) followed by a lower rate of NO production. The secondary slope decreased as the concentration of L-Arg

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**Figure 2** L-Arg transport and NO production in cardiac myocytes

(A) WT cardiac ventricular myocytes from 2-month-old mice were acutely isolated by collagenase treatment with a Langendorff perfusion system. (B) Extracellular L-Arg concentration dependence of NO production. Myocytes were loaded with the fluorescent dye DAF-FM, placed in well plates in a spectrophotometer and exposed (arrow) to 1 (\( \downarrow \), 5 (\( \uparrow \)) or 10 (○) mM L-Arg. Background fluorescence was recorded for 5 min and subtracted from each trace. NO production was normalized to the amount of protein present in each well. (C) Involvement of NOS. Myocytes were incubated for 8 min (left-hand arrow) with either Langendorff buffer (○) or 1 mM of the general NOS inhibitor L-NAME (●). Both treatment groups were then exposed to 10 mM L-Arg (right-hand arrow). (D) Specificity for L-Arg in NO production. Myocytes were exposed (arrow) to either 10 mM L-Arg (○) or 10 mM L-Lys (●). (E) NO production in cardiac myocytes from all three groups: DAF-FM-loaded myocytes from 2-month-old WT (●), mdx (○) and mdx:utr (▲) mice were exposed to 10 mM L-Arg (arrow). (F) L-Arg concentration dependence of NO production. Changes in fluorescence were determined as the first data-point resolved in time after application of 1, 5 or 10 mM L-Arg to cardiomyocytes isolated from WT (●), mdx (○) and mdx:utr (▲) mice. Symbols represent means ± S.E.M. from three experiments performed in triplicate for each condition. Lines through the data points are best-fitting hyperbolic functions. All experiments were performed at 23°C.
was increased, consistent with the negative feedback mechanism previously described for NO modulation of L-Arg transport in rat cardiomyocytes [31]. The involvement of NOS activity in these experiments was demonstrated by pre-incubating the myocytes with the NOS inhibitor L-NAME, which completely prevented an increase in fluorescence upon addition of 10 mM L-Arg (Figure 2C). Likewise, replacing L-Arg with L-Lys, which is equally efficiently transported by CATs but is not a NOS substrate, resulted in no production of NO (Figure 2D).

Similar manipulations were performed on cardiomyocytes from mdx and mdx:utr mice. Figure 2(E) shows the NO production by all three experimental groups in the presence of 10 mM L-Arg. NO production was diminished ~75% in mdx mice and close to 90% in mdx:utr mice with respect to the WT controls. A statistical account of NO released as a function of L-Arg concentration is shown in Figure 2(F). Hyperbolic fitting analysis in all three curves yielded similar $K_m$ values (8–10 mM), which are consistent with the activity of CAT-2A in the absence of an imposed membrane potential [24,26,31,35]. The values of $V_{max}$, on the other hand, were dramatically decreased in mdx and even more so in mdx:utr mice.

A pertinent question is whether the low levels of NO production that are still observed in mdx and mdx:utr cardiomyocytes (Figure 2F) represent residual nNOS activity or the functioning of eNOS, also highly expressed in cardiomyocytes. To address this question, we took a pharmacological approach. The NOS inhibitor 7-NINA blocks nNOS in vitro with an $IC_{50}$ of 0.47 μM [36], and competitively inhibits L-Arg binding to this NOS isoform with a $K_i$ of 2.8 μM [37] without affecting eNOS activity [38]. Thus, to determine if other NOS isoforms were responsible for the residual NO production, cardiac myocytes from 2-month-old WT mouse hearts were loaded with DAF-FM and exposed to 10 mM L-Arg after a 5 min incubation with either Langendorff solution or 20 μM 7-NINA. The results in Figure 3(A) show that 7-NINA blocked more than 80% of the NO production. On the other hand, similar experiments performed with 2-month-old mdx mice showed that NO production was largely insensitive to 7-NINA (Figure 3B). A summary of three experiments with the WT and mdx groups is shown in Figure 3(C). The 7-NINA-resistant WT NO levels were similar to the total NO production that remained in mdx myocytes. This residual NO production, in turn, is largely resistant to 7-NINA treatment. In fact, normalizing NO levels to the values of WT and mdx cardiomyocytes, the 7-NINA-resistant portions were found to be 15.5 and 74.3% respectively (Figure 3D). Thus the residual NO production observed in mdx cardiomyocytes appears to be the result of eNOS activity.

**CATs**

A decrease in the number of L-Arg transporters on the membrane of mdx and mdx:utr cardiomyocytes might be a concomitant cause for the reduced NO production values shown in Figure 2. Since this is an unexplored field, and because reliable antibodies are not available for CATs, we used real-time RT–PCR to measure CAT-1 and CAT-2A mRNA transcripts as an indicator of protein levels.
Unexpectedly, mRNA copies for both transporters were increased in 2-month-old hearts from mdx and mdx:utr mice, compared with the WT control (Figure 4A). Densitometric analysis showed that mRNA levels for the high-affinity CAT-1, normalized to β-actin and expressed as a percentage of the WT value, were increased by an average 12% and 32% in mdx and mdx:utr mice respectively (Figure 4B). Likewise, the low-affinity CAT-2A also displayed increased mRNA levels, with average values of 18% and 45% over the WT control for mdx and mdx:utr mice respectively (Figure 4B).

To determine whether these larger mRNA transcript levels translate into a larger number of functional transporters on the cardiomyocyte membrane, we measured L-Lys uptake in giant sarcolemmal vesicles isolated from hearts of all three experimental groups at 2 months of age, as previously described for rat hearts [26]. The L-Arg analogue L-[14C]lysine was used in these radiotracer studies taking advantage of the fact that L-Lys is not a NOS substrate, thus avoiding a potential reduction in uptake levels due to feedback inhibition of NO on L-Arg transport [31]. Experiments performed in the presence of 10 mM L-Lys to test the low-affinity component showed a significant increase in cationic amino acid uptake for both DMD groups with respect to the WT control, doubling the uptake levels in the case of mdx:utr mice (2.8 ± 0.6 compared with 5.6 ± 0.7 nmol·mg of vesicle protein−1·min−1; Figure 4C). Thus increased CAT-2A mRNA levels are in qualitative agreement with an augmented low-affinity transporter activity in cardiac muscle from mdx and mdx:utr mice, suggesting that the observed increase in transport capacity is the result of a larger number of transport units on the cell membrane. Both CAT-2A mRNA levels and transport activity were more dramatically increased in mdx:utr mice. Given the large number of animals required to perform these uptake experiments, we did not determine CAT-1 activity. Nonetheless, it is anticipated that high-affinity uptake will also correlate with the increase in CAT-1 mRNA levels shown in Figure 4(B). This novel up-regulation of CATs appears to be indicative of a compensatory mechanism set in place by DMD cardiac muscle cells to oppose the effects of nNOS reduction.

sGC

On the basis of the results described above and the reported beneficial effects of sildenafil [22,23], the status of the enzyme sGC, the immediate downstream member of the nNOS canonical (GC-dependent) pathway, was determined in mdx and mdx:utr mice. Heart extracts from 2-month-old mice of all three groups were subjected to Western blotting with antibodies against the α1 and β1 subunits of sGC and against β-actin. The results in Figure 5(A) show that both of the sGC subunits have similar levels throughout all of the experimental groups. Densitometric analysis was performed on four such experiments with the WT and mdx mice, and three experiments with mdx:utr mice. The ratios of total sGC (α1 + β1) to β-actin normalized to that of the WT control were not statistically different as calculated by a one-way ANOVA test (Figure 5B).

To assess sGC function, the hearts from 2-month-old WT, mdx or mdx:utr mice were treated with 100 μM of the NO-donor DEA-NONOate and cGMP production as well as cAMP content were quantified by radioimmunoassay. cGMP production was normalized to cAMP content, which is a good indicator of the amount of tissue used in the assay and is independent of NO donor stimulation. A summary of these studies is shown in Figure 5(C), where the results of five experiments performed on each group show no significant differences in cGMP production between the WT, mdx or mdx:utr mice (one-way ANOVA, P > 0.05). Therefore sGC protein levels and activity (under maximal NO stimulation) were not affected in mdx or mdx:utr mice.

Figure 4 Expression and activity of L-Arg transporters

(A) The PCR products for CAT-1 and CAT-2A determined in the heart lysates from WT, mdx and mdx:utr mice. Shown are ethidium bromide-stained 1.2% agarose gels for CATs and β-actin (marker) PCR products obtained from the same tissue within each group. (B) Summary of the RT–PCR experiments. Densitometric measurements for CAT-1 and CAT-2A were normalized by area and then by β-actin, and values for mdx (black) and mdx:utr (white) mice were plotted as the percentage of the WT. Results are means ± S.E.M. for seven experiments for each individual condition, including two RT and three PCR preparations. All differences were statistically significant (P < 0.05). (C) L-Lys (10 mM) uptake in cardiac sarcolemmal vesicles from WT (black), mdx (light grey) and mdx:utr (dark grey) mice. Experiments were performed with L-[14C]Lys at 37 °C as described previously [26]. Results are means ± S.E.M. for three experiments performed in quintuplicate for each condition. All values were statistically different with respect to the control (*P < 0.05).
Figure 5  Protein levels and activity of sGC

Figure 5  Protein levels and activity of sGC

(A) sGC protein levels. Proteins extracted from the WT, mdx or mdx:utr mouse hearts were subjected to Western blotting with antibodies against the α1 and β1 subunits of sGC and against β-actin, which was used as a loading control. The blot shown is from one membrane and representative of blots for WT (n = 4), mdx (n = 4) and mdx:utr (n = 3) hearts. Molecular masses are given on the right-hand side in kDa. (B) Densitometric analysis. The ratios of total sGC (α1 + β1) to β-actin normalized to that of the WT control (black) are plotted for mdx (light grey) and mdx:utr (dark grey) mice. (C) NO-stimulated cGMP production. Hearts from five WT (●), mdx (○) and mdx:utr (▼) mice were incubated with IBMX (500 μM) and treated with DEA-NONOate (100 μM) for 10 min at 37°C. cGMP production and cAMP content were quantified by RIA. Displayed are all five experimental results for the three groups plus the 25th–75th percentile range (grey boxes) and the median for each set of data.

DISCUSSION

The present study demonstrates that the loss of nNOS activity in cardiac muscle cells from mouse models of muscular dystrophy is accompanied by the up-regulation of upstream members of the NO pathway (CATs) while having no significant effect on downstream signalling steps (sGC). Our work also depicts a bifurcated system in which the lack of dystrophin in mdx mice impairs nNOS functionality, whereas the concomitant absence of utrophin in mdx:utr mice decreases nNOS expression.

Consistent with previous findings [12], we observed no evidence indicating that dystrophin and nNOS co-localize at the membrane of the WT mouse cardiomyocytes. This implies that the physical proximity of dystrophin and nNOS is not a factor in nNOS regulation and suggests an indirect although efficient role for dystrophin in modulating nNOS activity. Although the pattern of nNOS expression merits further study, our observation of punctated nNOS is consistent with recently discovered nNOS isoforms that do not co-localize to dystrophin at the sarcolemma of skeletal muscle [39,40]. Similarly, our results support previous observations whereby nNOS levels remain unchanged in the mdx mouse heart relative to the WT [21]. We found that the additional absence of utrophin in mdx:utr mice results in a significant decrease in nNOS expression and protein levels. This strengthens the notion that, unlike in the skeletal muscle where dystrophin sustains nNOS expression and localization, the presence of utrophin sustains nNOS levels in cardiac muscle. It remains to be elucidated whether this effect of utrophin on nNOS levels is direct or indirect. Contrastingly, invariance of eNOS expression and protein levels in mdx and mdx:utr hearts compared with the WT mice suggests that the presence of dystrophin or utrophin regulates nNOS only. The fact that nNOS mRNA transcripts and protein levels decline similarly in mdx:utr mice suggests that regulation of this isoform is primarily at the RNA level.

The production of NO in response to the application of 10 mM extracellular L-Arg decreases to less than 20% in cardiomyocytes lacking dystrophin, and is further decreased to 10% when utrophin is also absent. According to the observed L-Arg concentration dependence of NO production, this effect reflects changes in the V_{max} value. Possible causes for a decrease in the V_{max} value include: a significant decrease in NOS expression, an increase in NOS removal and degradation, and kinetic effects due to either post-translational modifications that inhibit NOS enzymatic activity or the limited availability of NOS cofactors [3,4]. Although the significant decrease in nNOS expression and protein levels observed in mdx:utr mice would explain the large drop in the V_{max} value, this is not the case for mdx cardiomyocytes, where nNOS levels are similar to the WT control. Thus, at least in the absence of dystrophin, a diminished NOS function mediated by a dystrophic environment may explain the reduction in the V_{max} value for NO production. Whether these kinetic effects are due to NOS phosphorylation, S-nitrosation, or to the lack of cofactors involved in NO synthesis, remains an open question.

Our experiments with the selective nNOS blocker 7-NINA suggest that eNOS accounts for the remaining minimal NO levels in both the WT and dystrophic mice. It is tempting to speculate that despite appropriate expression and protein levels, eNOS has little access to L-Arg pools or cofactors and, thus, is not the main source of NO in WT mouse ventricular cardiomyocytes.

The dystrophic environment that is likely to affect nNOS function in mdx cardiac muscle also affects nNOS expression and protein levels in the additional absence of utrophin. The special 'dystrophic stress' produced by the simultaneous absence of dystrophin and utrophin might enhance nNOS targeting for removal and degradation. Thus, in muscular dystrophy, the greatly diminished NO production may lead to the absence of normal NO physiological effects, probably resulting in abnormal cardiac function. This view is in line with the finding that a transgenic nNOS expression in the mdx mouse myocardium prevents the development of cardiomyopathy [21]. The results of the present study support the notion that although dystrophin and/or utrophin regulate nNOS in skeletal and cardiac muscle, the mechanism of regulation differs between these two tissues.

Cardiac myocytes rely on plasma membrane transporters to deliver the L-Arg required for NO production. Although a functional relationship between the DGC complex and L-Arg transporters has not been established, we anticipated the scarce NO production in mdx mice to be attributed to a down-regulation of CATs. In contrast, we found that the high-
low-affinity CATs CAT-1 and CAT-2A display augmented levels of mRNA transcripts in mdx hearts. In the case of the low-affinity component, the increase in mRNA copies was paralleled by a significant increase in amino acid uptake by cardiac sarcolemmal vesicles. These effects were more pronounced in the additional absence of utrophin. Thus, unlike its effect on nNOS, the presence of utrophin decreases both expression and activity of L-Arg transporters towards levels closer to those of the WT mice. The fact that CATs are up-regulated is particularly interesting in the context of mdx mice. Although mdx:utr mice develop early myopathy and die at 2–4 months of age, mdx mice develop a late myopathy (12–15 months) and yet CAT activity and expression are dysregulated in mdx mice well before the disease becomes apparent. Considering that nNOS, eNOS and utrophin protein levels are maintained in the dystrophic heart, CAT dysregulation may be regarded as a predictor of future cardiac disease. Alternatively, the dystrophic environment brought about by the early skeletal muscle disease might be the cause of the up-regulation of CATs. This novel up-regulation of CATs may represent the attempt of mdx mice cardiomyocytes to compensate for the loss of nNOS function by recruiting additional L-Arg, albeit unsuccessfully. This is consistent with the concept that nNOS function may be affected by the presence of a dystrophic environment [41]. However, since cardiac-specific nNOS transgenic expression is ameliorative in mdx mice, this result suggests the interesting possibility that further enhancement of L-Arg transport beyond endogenous compensation may boost NO production to ameliorate the disease.

The downstream member of the NO signalling pathway, sGC, is fully functional under optimal NO stimulation in hearts from mdx and mdx:utr mice. In line with the beneficial effect of sildenafil reported in mdx mice [22,23], our results suggest that reduced sGC activity may solely be the result of suboptimal NO production by residual nNOS activity. A summary of our findings in DMD mice is shown in Table 1.

<table>
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<tr>
<th>Protein</th>
<th>WT mice</th>
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<td>Dystrophin</td>
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<td>Utrophin</td>
<td>(+)</td>
<td>(−)</td>
<td>(−)</td>
</tr>
<tr>
<td>nNOS</td>
<td>(+)</td>
<td>(+)</td>
<td>(−)</td>
</tr>
<tr>
<td>eNOS</td>
<td>(+)</td>
<td>(+)</td>
<td>(−)</td>
</tr>
<tr>
<td>CATs</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>sGC</td>
<td>(+)</td>
<td>(+)</td>
<td>(−)</td>
</tr>
</tbody>
</table>

*Although nNOS protein is present, nNOS-mediated NO production is minimal.

### AUTHOR CONTRIBUTION

Diego Fraidenraich and R. Daniel Peluffo co-ordinated the research design. Annie Beuve, Diego Fraidenraich and R. Daniel Peluffo participated in experimental design. Joel Schneider, Jayalakshmi Ramachandran, James Gonzalez and Lai-Hua Xie collected tissue samples and isolated myocytes. Joel Schneider, James Gonzalez, Ruifang Zheng, Jayalakshmi Ramachandran and Pierre-Antoine Crassous performed the experiments. Jayalakshmi Ramachandran, Joel Schneider, Pierre-Antoine Crassous, Annie Beuve, Diego Fraidenraich and R. Daniel Peluffo analysed the data. R. Daniel Peluffo wrote the paper with contributions to writing and editing, prior to submission, from Jayalakshmi Ramachandran, Joel Schneider, Pierre-Antoine Crassous, James Gonzalez, Annie Beuve and Diego Fraidenraich. Annie Beuve, Diego Fraidenraich and R. Daniel Peluffo provided administrative support.

### ACKNOWLEDGEMENTS

We thank Dr Eldo Kuzhikandathil for kindly sharing his Light Cycler real-time PCR equipment, Dr Robert Grange (Virginia Tech, Blackledge, VA, U.S.A.) for providing mdxutr+/− mice and Luke Fritsky for help with confocal imaging.

### FUNDING

This work was supported by the Northeast Consortium for Minority Faculty Development (to R.D.P. and D.F.), the UMDNJ-NJMS Hispanic Center of Excellence (to R.D.P. and D.F.), the Josiah Macy Jr. Foundation (to R.D.P.), the Founders Affiliate of the American Heart Association (postdoctoral fellowship to P.A.C.), the National Institutes of Health, National Institute of General Medical Sciences [grant number R01 GM067640 (to A.B.)], the National Heart, Lung and Blood Institute [grant numbers T32 HL069752 (to J.S.S.) and R01 HL076392 (to R.D.P.)] and the Muscular Dystrophy Association [grant number 200037 (to D.F.)].

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