Early functional muscle regeneration after myotoxic injury in mice is unaffected by nNOS absence

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ABSTRACT

Nitric oxide (NO) is an important signaling molecule produced in skeletal muscle primarily via the neuronal subtype of nitric oxide synthase (NOS1, or nNOS). While many studies have reported NO production to be important in muscle regeneration, none have examined the contribution of nNOS-derived NO to functional muscle regeneration (i.e. the restoration of the muscle’s ability to produce force) after acute myotoxic injury. In the present study, we tested the hypothesis that genetic deletion of nNOS would impair functional muscle regeneration after myotoxic injury in nNOS<sup>−/−</sup> mice. We found that nNOS<sup>−/−</sup> mice had lower body mass, lower muscle mass, smaller myofiber cross-sectional area (CSA), and that their tibialis anterior (TA) muscles produced lower absolute tetanic forces than those of wild type littermate controls, but that normalized or specific force (sPo<sub>0</sub>) was identical between the strains. In addition, muscles from nNOS<sup>−/−</sup> mice were more resistant to fatigue than those of wildtype littermates (P<0.05). To determine whether deletion of nNOS affected muscle regeneration, TA muscles from nNOS<sup>−/−</sup> mice and wildtype littermates were injected with the myotoxin Notexin to cause complete fiber degeneration, and muscle structure and function were assessed at 7 and 10 days post-injury. Myofibre CSA of regenerating nNOS<sup>−/−</sup> mice was lower than wild type controls at both 7 and 10 days post-injury, however contrary to our original hypothesis no difference in force producing capacity of the TA muscle was evident between the two groups at either time point. Our findings reveal that nNOS is not essential for functional muscle regeneration after acute myotoxic damage.

Keywords: skeletal muscle injury, muscle repair, nNOS, contraction, fatigue
INTRODUCTION

Skeletal muscles are susceptible to damage from trauma, metabolic deficits, disease, and mechanical strains during lengthening (eccentric) contractions (4, 5, 20, 21). Although skeletal muscles have an impressive capacity to regenerate after injury, the repair is often slow and sometimes incomplete (11). The loss of muscle function can impact significantly on the lives of those affected and so there is a need to better understand the process of skeletal muscle regeneration in order to develop novel strategies and treatments that can promote muscle function after injury and improve quality of life for patients (29).

Nitric oxide (NO) is an important signaling molecule that plays a critical role in a number of biological processes. NO is synthesized from L-arginine by NO synthase (NOS) enzymes, of which three isoforms have been characterized: two ‘constitutive’ isoforms (nNOS/NOS1 and eNOS/NOS3) and an ‘inducible’ isoform (iNOS/NOS2). The primary isoform of NOS in skeletal muscle is the muscle-specific splice variant nNOSμ (32), although skeletal muscle also expresses some eNOS and, particularly during muscle inflammation, iNOS. The functional role of NO in skeletal muscle is complex. NO has been implicated in a number of aspects of muscle function such as contractility (7, 23), Ca^{2+} homeostasis (26), and muscle regeneration in conditions such as muscular dystrophy (2, 34). Previous studies examining the role of NO in regeneration from acute muscle injury have found that inhibition of NO production by all NOS isoforms with L-NAME inhibits successful muscle regeneration (1, 8, 9), although the contribution of nNOS-derived NO to this phenomenon is less clear. Furthermore, none of the studies on the role of NO in regeneration after acute injury examined arguably the most important facet of muscle regeneration; the restoration of muscle force producing capacity.
The present study sought to address this gap in existing knowledge by examining the functional regeneration of muscles in nNOS null mice after acute injury. We hypothesized that nNOS null mice would exhibit impaired muscle fiber regeneration after myotoxic injury, as evidenced by impaired muscle function and associated morphological changes compared with wildtype littermates.

MATERIALS AND METHODS

Animals. All procedures were approved by the Animal Ethics Committee of The University of Melbourne and conformed to the Australian code of practice for the care and use of animals for scientific purposes as stipulated by the National Health and Medical Research Council (Australia). Mice homozygous for targeted disruption of the nNOS gene (B6,129-NOS1tm1ph, nNOS−/−) (12) were purchased from Jackson Laboratories (Bar Harbor, ME, USA, stock no. 002633). A colony was then established by backcrossing the nNOS−/− onto a C57BL/6 background for at least six generations to obtain a colony of nNOS−/− and wild type littermate controls, with genotyping performed from tail clippings taken at day 21. Mice were housed in the Biological Research Facility at The University of Melbourne under a 12 h light-dark cycle, with drinking water and standard chow provided ad libitum. Male mice aged 8-9 weeks (n=20 nNOS−/−; n=24 nNOS+/+ littermates) were used.

Myotoxic injury. Myotoxic injury was induced in the tibialis anterior (TA) muscles of mice as described previously (31). Briefly, mice were anesthetized using a mixture of ketamine (76 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and a small portion of the TA muscle of the right hindlimb was surgically exposed by a single incision through the skin. The muscle was filled to its maximal holding capacity (~40 μl) via a single intramuscular injection with the myotoxin
Notexin (1 μg/ml, Latoxan) using a 29-gauge needle. The wound was closed with Michel clips (Aesculap, Tuttlingen, Germany) and the mice were allowed to recover from the myotoxic injury for 7 or 10 days before assessments of muscle structure and function.

**Muscle function.** Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg) and TA muscle function was assessed in situ as described previously (10). Briefly, TA muscles were stimulated by supramaximal 0.2 ms square wave pulses of 350 ms duration, delivered via two wire electrodes adjacent to the sciatic nerve. Optimal muscle length for contraction ($L_o$) was defined as the muscle length at which maximal isometric twitch force ($P_t$) was attained, and the absolute maximal isometric tetanic force ($P_o$) was determined from the plateau of the frequency-force relationship. Measurements of $P_o$ were normalized to the cross-sectional area of the muscle (CSA; calculated using $L_o$ and muscle mass) in order to determine maximum specific force ($sP_o$), a measurement that allows for relative comparisons of force production between muscles of different sizes. Immediately following the functional assessments, the muscles were carefully excised, trimmed of any adherent non-muscle tissue and tendons, and weighed. Muscles were then mounted in Tissue-Tek OCT embedding medium, frozen rapidly in thawing isopentane, and stored at -80°C for later histological and biochemical analyses. The mice were killed by surgical excision of the heart while they were still anesthetized deeply.

**Immunohistology.** Transverse sections (5 μM) were cut from the midbelly of each muscle on a cryostat microtome and serial sections were placed onto glass slides (Superfrost® Plus, Menzel-Gläser, Kensington, VIC, Australia). General muscle histology was determined by staining sections with hematoxylin and eosin (H & E) to visualize muscle fibers and areas of inflammatory cell infiltration post myotoxic damage. Fiber cross-sectional area (CSA) and
central nucleation (a distinguishing feature of regenerating muscle fibers) were determined immunohistochemically by probing for both the membrane protein laminin and nuclei using a polyclonal α-laminin primary antibody (Sigma-Aldrich, Castle Hill, NSW, Australia) and 4’6-diamidino-2-phenylindole (DAPI; Invitrogen, Mulgrave, VIC, Australia) as described previously (15). Macrophages were visualized after reacting sections with an F4/80 antibody (ab60343, Abcam, Sapphire Bioscience, Waterloo, NSW, Australia). The percentage of Type IIa fibres in the TA muscle was determined by probing sections with the antibody N2.261 (Developmental Studies Hybridoma Bank, The University of Iowa), and capillaries were visualized using α-CD31 antibody (Abcam, Sapphire Bioscience, Waterloo, NSW, Australia). Digital images of stained sections were obtained using a fluorescence microscope and camera (Carl Zeiss, Wrek, Göttingen, Germany) with associated imaging software (Axiovision V4.7.1.0). CSA and central nucleation were determined for at least 500 individual fibers from each section, from at least 4 muscles per experimental group.

Oxidative enzyme activity was determined from reacting sections for succinate dehydrogenase activity as described previously (3). The optical density of SDH reaction product was determined after 6 min for all samples and sections captured in full color using bright-field light microscopy. The images of SDH reactivity were converted to grayscale values, and the mean optical density of all pixels in a given myofiber was determined, then corrected for the mean optical density in a field of view containing no myofibers (14).

Determination of blood flow during fatigue. Changes in blood flow in the TA muscle during the fatiguing stimulation protocol were determined by infra-red laser Doppler assessment using a moorFLPI full-field blood perfusion imaging system (Moor Instruments, Devon, UK). The sub-surface vessels of the exposed TA muscle were imaged before stimulation and again
immediately after completion of the fatigue protocol. Blood flow (flux) was measured in arbitrary units by the associated moorFLPI software (Moor Instruments, Devon, UK).

**Quantitative RT-PCR.** Muscle samples were homogenized individually and mRNA extracted using an RNEasy™ fibrous tissue RNA extraction kit (Qiagen, Doncaster, VIC, Australia), according to manufacturer’s instructions. The concentration and quality of RNA in each sample was determined using a Nanodrop™ 2000 (Thermo Fisher Scientific, Scoresby, VIC, Australia) and the extracted mRNA was stored at -80°C. mRNA was transcribed into cDNA using the Superscript VILO cDNA synthesis kit (Invitrogen) according to manufacturer’s instructions, and stored at -20°C until further analysis. Quantitative RT-PCR was performed using an iCycler Thermal Cycler (Bio-Rad) with SYBR Green supermix (Quantace, Bioline, Alexandria, NSW, Australia). Primers for MyHCI, MyHCIIa, MyHCIIx, MyHCIIb, COXIV, calcineurin and PGC-1α have been published previously (22). Primers for nNOS, eNOS, myogenin, catalase, Cu²⁺/Zn²⁺-superoxide dismutase (SOD1), Peroxiredoxin 6 (PRDX6) and Hsp70 were designed using the Perfect Primer online program (Invitrogen) and are listed in Table 1. Due to the severity of the myotoxic injury caused by Notexin (25), we chose not to use the ΔCt method of analysis of mRNA expression as we could not be certain that the expression of any reference gene used would remain unchanged. We opted instead to measure the cDNA concentration of each sample using the Quant-iT OliGreen ssDNA Assay Kit (Molecular Probes, Eugene, OR) and to normalize the data to cDNA content as described previously (19, 22).

**Western blot analysis.** Western blots were performed as described previously (22). Briefly, muscle samples (20–30 mg) were homogenized in buffer of the following composition: 10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 1 mM EDTA; 1 mM EGTA; 10% glycerol; 1% Triton X-100; 0.1% SDS; 1mM NaF; 20 mM Na₄P₂O₇; 2 mM Na₃VO₄; 0.5% sodium deoxycholate; 1
mM PMSF; 0.1% protease inhibitor cocktail (Sigma Aldrich, Castle Hill, NSW, Australia); 0.1%
phosphatase inhibitor cocktail (Sigma Aldrich, Castle Hill, NSW, Australia). Samples were
centrifuged at 10,000 g for 10 min at 4°C, and the resulting supernatant was analyzed for total
protein content, heated at 95°C for 5 min with protein loading buffer (40% glycerol; 6% SDS;
250 mM Tris-HCl, pH 6.8; 0.04% bromophenol blue; and 415 nM DTT) and then size-
fractionated by SDS-PAGE. Blots were transferred to PVDF membrane in standard transfer
buffer for 18 h at 4°C and 100 mA, and then blocked in TBST containing 5% skim milk powder
or 5% BSA for 2 h at 25°C. After blocking, membranes were incubated with either anti-
calcineurin (1:1000; Abcam, Sapphire Bioscience, Waterloo, N.S.W., Australia), anti-COXIV
(1:5000; Cell Signaling Technology, Genesearch Pty. Ltd, Arundel, Queensland), or anti-
GAPDH (1:10,000; Sigma Aldrich, Castle Hill, NSW, Australia) for 2 h at 25°C, or anti-PGC-1α
(1:4000; Abcam, Sapphire Bioscience, Waterloo, N.S.W., Australia) overnight at 4°C. The
membranes were then washed, incubated with secondary antibody (anti-rabbit IgG conjugated to
horseradish peroxidase; Amersham Biosciences, GE Healthcare Life Sciences), and visualised
using enhanced chemiluminescence (ECL plus; Amersham Biosciences). The signal was imaged
using a ChemiDoc XRS machine (Bio-Rad, Gladesville, NSW, Australia) and blots were
quantified using Quantity One software (Bio-Rad), and normalized against GAPDH protein
expression.

Statistical analyses. All values except those for myofiber CSA, are expressed as mean ±
SEM. Groups were compared using either an unpaired Student’s t-test, or a 2-way ANOVA and
Bonferroni’s post hoc multiple comparison procedure where appropriate. Myofiber cross-
sectional area (CSA) is not normally distributed, and so medians were compared using either a
non-parametric Mann-Whitney test by ranks (for two groups) or a non-parametric Kruskal-
Wallis ANOVA by ranks (for greater than two groups). All statistical analyses were performed using Prism version 3 software (Graphpad Software Inc.). In all cases significance was defined as P<0.05.

RESULTS

Morphological and isometric contractile properties of uninjured muscles. We found that the mass of the TA muscle was significantly lower in nNOS−/− mice compared with nNOS+/+ mice (Table 2), but since body mass was also lower in these mice (26.5 ± 1.1g vs 30.3 ± 1.1g), there was no significant difference in muscle mass relative to body mass (1.73 ± 0.12 mg/g vs 1.79 ± 0.08 mg/g). Representative images of tibialis anterior (TA) muscle cross-sections from wild type (WT) and nNOS null (nNOS−/−) mice at 7 or 10 days post myotoxic injury, stained for hematoxylin and eosin (H&E, 20×) and reacted for F4/80 antibody (20×) to visualize macrophages, are presented in Fig. 1. Histological examination of myofiber cross-sectional area (CSA) in TA muscles from both strains revealed that the frequency distribution of myofiber CSA in nNOS−/− mice was shifted to the left compared with nNOS+/+ mice, and that the median CSA of myofibers from nNOS−/− mice was significantly lower than that for nNOS+/+ mice (Fig. 2a & b).

In uninjured muscles, both peak twitch force (P₁) and twitch half-relaxation time (1/2 RT) were significantly lower in nNOS−/− mice than in nNOS+/+ mice, but neither time-to-peak twitch tension (TPT) nor the rate of twitch force development (dP/dt) were significantly different between the two strains (Table 2). Absolute P₀ was also significantly lower in muscles from nNOS−/− mice compared with nNOS+/+ mice (Fig. 2c), but specific force (sP₀) was not significantly different between the two strains (Fig. 2d).
Uninjured TA muscles from nNOS \(^{-/-}\) mice were more resistant to fatigue and recovered from a fatiguing stimulation protocol more quickly than muscles from nNOS \(^{+/+}\) mice (Fig. 2e). Analysis of myosin heavy chain mRNA expression revealed that the TA muscles of nNOS \(^{-/-}\) mice had significantly higher expression of MyHC type I (Fig. 3a) but did not have altered expression of MyHC type IIa, IIx or IIb compared with nNOS \(^{+/+}\) mice (Fig. 3b). Furthermore, protein expression of the oxidative markers COXIV, calcineurin and PGC-1\(\alpha\) did not differ between the two groups of mice (Fig. 3c & d). Finally, myofiber succinate dehydrogenase activity did not differ between the TA muscles from the two groups (Fig 3e).

We then examined the hemodynamic properties of TA muscles from both strains. Immunohistological examination of uninjured muscles revealed no significant differences between nNOS \(^{-/-}\) mice and nNOS \(^{+/+}\) mice in the number of capillaries per myofiber (Fig. 4a) or in the number of capillaries per unit area (data not shown). Laser Doppler assessment of blood flow in the TA muscles before and after the fatigue protocol demonstrated that both nNOS \(^{-/-}\) mice and nNOS \(^{+/+}\) mice had increased blood flow after the contraction protocol, but there was no significant difference in the exercise-induced increase in blood flow between the two strains (Fig. 4b).

We also examined the mRNA expression of selected antioxidant and stress enzymes within the muscles. We confirmed that nNOS mRNA was not present in muscles from the nNOS \(^{-/-}\) mice, but found no significant differences in the mRNA expression of eNOS, catalase, Cu\(^{2+}/\)Zn\(^{2+}\)-superoxide dismutase (SOD1), Peroxiredoxin-6 (PRDX6) or heat-shock protein 70 (Hsp70) between muscles from the two strains (Fig 4c).

Muscle regeneration after myotoxic injury. We next examined muscle regeneration by subjecting the TA muscles of nNOS \(^{+/+}\) and nNOS \(^{-/-}\) mice to myotoxic injury with Notexin.
Analysis of myofiber cross-sectional area (CSA) in regenerating muscles from both strains revealed that the frequency distribution of fibers from nNOS\(^{-/-}\) mice was shifted to the left compared with that for nNOS\(^{+/+}\) mice at both 7 and 10 days post-injury (Fig. 5a & b), and that the median CSA of myofibers from nNOS\(^{-/-}\) mice was significantly lower than that of nNOS\(^{+/+}\) mice (Fig. 5c). However, there was no significant difference in the percentage of centrally-nucleated fibers between the two strains at any time point (Fig. 5d), nor was there any significant difference in myogenin mRNA expression between the two strains at 10 days post-injury (Fig. 5d).

Finally, we assessed the force-producing ability of the regenerating muscles. We found no difference in \(P_0\) between nNOS\(^{-/-}\) mice and nNOS\(^{+/+}\) mice at either 7 or 10 days post-injury (Fig. 6a). Furthermore, there was no significant difference in \(sP_0\) between strains at either 7 or 10 days post-injury (Fig. 6b).

**DISCUSSION**

The important role of nitric oxide (NO) in muscle repair has been the subject of a number of interesting studies (1, 2, 8, 27, 34). While the importance of NO production to the regenerative process after acute injury has been characterised, the relative contribution of the three isoforms of nitric oxide synthase (NOS) expressed in skeletal muscle remains unclear. Many previous studies examining the role of NO in regeneration have relied on pharmacological inhibition of nitric oxide production by L-NAME, however, L-NAME inhibits all three NOS isoforms and is therefore of limited use for determining the relative contributions of each. Although the primary isoform of NOS in skeletal muscle is nNOS (32), a specific pharmacological inhibitor of nNOS has yet to be developed, and the best model currently
available for examining the contribution of nNOS to muscle regeneration is the nNOS knockout (nNOS\textsuperscript{−/−}) mouse. Many studies have used this mouse model to examine the role of muscle nNOS in chronic degenerative conditions such as muscular dystrophy (2, 17, 18, 30, 33, 34), but only a few studies have examined the role of nNOS in muscle regeneration after acute myotoxic injury (1, 8, 9), and none have assessed arguably the most important facet of functional muscle regeneration, the return of the muscle’s force-producing capacity. To our knowledge, the present study represents the first assessment of the effect of genetic deletion of nNOS on the force-producing capacity of a muscle during regeneration after acute injury using the nNOS\textsuperscript{−/−} mouse model.

We first characterized the morphological and contractile properties of the TA muscles of our nNOS\textsuperscript{−/−} mice. Percival and colleagues (23, 24) examined the in vitro contractile characteristics of nNOS\textsuperscript{−/−} mice and found them to have lower body mass, lower muscle mass, smaller myofiber cross-sectional area (CSA), and to produce lower absolute P\textsubscript{o} than muscles from littermate controls, although sP\textsubscript{o} was identical between the strains. Our contractile data agree with those of Percival and colleagues and confirm that the muscles of nNOS\textsuperscript{−/−} mice do not exhibit significantly different contractile characteristics after accounting for differences in muscle size. We were surprised, however, to find that the TA muscles from our nNOS\textsuperscript{−/−} mice were more resistant to an in situ fatiguing stimulation protocol than muscles from their littermate controls, in contrast with the earlier study by Percival and colleagues where the muscles of nNOS\textsuperscript{−/−} mice were found to be less resistant to fatigue than wildtype littermates (23). Interestingly, when Percival and colleagues repeated their fatigue protocol on nNOS\textsuperscript{−/−} mice in the later study (24), they no longer observed the difference in muscle fatiguability, and postulated that this was due to the intervening back-crossing of the nNOS\textsuperscript{−/−} mice onto a C57BL/6
background rather than the B6129 background of their first study. The mice in our study were also back-crossed onto a C57BL/6 background, and so our data are more appropriately compared with the data from the latter study of Percival and colleagues (24).

In an effort to determine why our nNOS$^{-/-}$ mice were less susceptible to fatigue than wildtype littermates, we examined the expression of myosin heavy chain (MyHC) subtypes and the oxidative capacity of the muscles. We found no difference in the mRNA expression of MyHC type IIa or type IIb between nNOS$^{-/-}$ mice and wildtype littermates, but the expression of MyHC type I was increased significantly (albeit from a very low level in the predominantly fast fibered TA muscle). There was a (non-significant) trend for increased expression of the more fatigue resistant MyHC type IIx. When muscle oxidative capacity was assessed, we found no differences in protein expression of COXVI, calcineurin or PGC-1α, nor did we find any difference in succinate dehydrogenase activity between TA muscles from the two groups.

We then examined the hemodynamic properties of the muscle. Previous studies have suggested that a lack of nNOS enhances ‘functional ischemia' of skeletal muscle (33), and increases fatigue after exercise (13). We found no differences in either capillary density or blood flow between the muscles from nNOS$^{-/-}$ mice or wildtype littermates after a fatiguing protocol. It should be noted, however, that our blood flow measurements were determined by laser Doppler surface imaging of the exposed but otherwise intact TA muscle – an assessment of a single muscle and limited only to the blood flow through surface vessels due to the limited penetration of the infra-red laser. In contrast, Thomas et al. (33) measured blood flow through the exposed femoral artery, thereby obtaining an estimation of blood flow through all vessels of all muscles of the lower leg. Although aware of the limitations of our technique, we chose this method so that we could assess blood flow non-invasively during the fatigue protocol without
compromising muscle function. It is possible therefore that further studies examining whole-tissue blood flow either via Doppler assessment of the femoral artery (33), injection of labeled microspheres (16), or contrast enhanced ultrasound (28) could uncover functional ischemia in our nNOS\(^{-/-}\) mice during fatiguing stimulation. Nevertheless, even if functional ischemia in nNOS\(^{-/-}\) mice was apparent during the fatigue protocol, this would presumably have the effect of enhancing the muscle susceptibility to fatigue, rather than reducing it.

We also examined the expression of antioxidant and stress-related proteins in the uninjured muscles of nNOS\(^{-/-}\) mice. A recent study reported that the EDL muscles of nNOS\(^{-/-}\) mice have significantly higher mRNA and protein expression of a number of antioxidant enzymes when compared to wild type mice (6). We found no difference between nNOS\(^{-/-}\) mice and wild type controls in the mRNA expression of any of the antioxidant/cytoprotective proteins examined, although there was a (non-significant) trend for increased expression of all three antioxidant enzymes. Our results indicate that the enhanced resistance to fatigue of the TA muscles from nNOS\(^{-/-}\) mice in the present study cannot be explained by differences in myofiber oxidative capacity, antioxidant or heat shock protein expression, or hemodynamic changes; however, the increase in mRNA expression of MyHC type I and type IIx may in part explain the more fatigue resistant phenotype. The cellular mechanisms underlying this resistance to fatigue remain to be fully elucidated.

We then examined the return of force producing capacity to regenerating muscles of nNOS\(^{-/-}\) mice after acute myotoxic injury. We found that although the regenerating muscles of nNOS\(^{-/-}\) mice had a lower median myofiber CSA than wildtype littermates, there was no difference in muscle mass, the proportion of centrally-nucleated fibers in the muscle or myogenin mRNA expression between the groups. Finally, we found there was no difference in
force production by the regenerating muscle when measured as either absolute force ($P_o$) or normalized force ($sP_o$), suggesting that the return of force-producing capacity of the injured muscle was unaffected by the absence of nNOS. That nNOS$^{-/-}$ mice exhibited no deficit in force production during regeneration may seem difficult to reconcile with the observation that uninjured muscles from nNOS$^{-/-}$ mice produced lower absolute forces than their wildtype littermates. This suggests that the absence of nNOS does not affect the force-producing ability of muscles during the early stages of regeneration and myofibre formation, but exerts an effect on force production in the later stages of myofibre maturation. The equal force production in regenerating muscles from the two groups is even more difficult to reconcile with the significantly reduced myofiber CSA in the nNOS$^{-/-}$ muscles. This raises the possibility that at least in the early stages of muscle regeneration, morphological indicators such as myofiber CSA do not necessarily correlate with the force producing ability of the regenerating muscles. Further studies examining the effect of nNOS on the formation and maturation of myofibres in vivo following Notexin injury may elucidate this process more clearly.

Taken together our data indicate that, although NO produced by nNOS plays an important role in muscle repair in chronic conditions such as muscular dystrophy (2, 17, 18, 30, 33, 34), and indeed may play a role in some aspects of muscle repair after less severe forms of myotoxic injury, nNOS is not essential to functional recovery after Notexin injury. If, as reported previously, NO is necessary for satellite cell activation and muscle repair after injury (1,8,9), yet genetic deletion of nNOS did not impair regeneration in our study, then the isoform of NOS most important for generating NO during regeneration must therefore be either eNOS or iNOS. As any muscle injury (including the Notexin model used in the present study) will produce significant inflammation, it seems far more likely that iNOS is the source of NO
required for successful regeneration. Indeed, recent studies have suggested that iNOS is the crucial source of NO during repair after acute injury (8, 9). Further studies examining muscle regeneration in iNOS and eNOS knockout mice may shed light on the relative contribution of each isoform to muscle regeneration after acute injury.

**Perspectives and Significance**

While previous studies have established a role for nitric oxide in muscle regeneration, in the present study we found that nNOS is not essential for early functional regeneration after acute myotoxic injury. These data suggest that another NOS isoform, most probably iNOS, is likely to be responsible for influencing muscle regeneration after acute injury. Studies investigating muscle regeneration in iNOS and eNOS knockout mice may reveal how each isoform contributes to successful muscle fiber regeneration after injury. Furthermore, our results indicate that studies assessing muscle regeneration must not rely on morphological indicators alone, since these markers may not necessarily correlate with the restoration of contractile force.

**GRANTS**

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**DISCLOSURES**
No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


LEGENDS FOR FIGURES

**Figure 1.** Representative images of tibialis anterior (TA) muscle cross-sections from wild type (WT) and nNOS null (nNOS\(^{-/-}\)) mice at 7 or 10 days post myotoxic injury, stained for hematoxylin and eosin (H&E, 20×) and reacted for F4/80 antibody (20×) to visualize macrophages. While there were occasional pockets of greater inflammatory cell activity in some muscle sections from nNOS\(^{-/-}\) mice, these minor histological variations did not translate to differences in muscle function (see Results).

**Figure 2.** (a) Frequency distribution of myofiber cross-sectional area (CSA) in the TA muscles from nNOS\(^{-/-}\) mice (KO) and wildtype littermate controls (WT). (b) Median CSA of myofibers. Data pooled from >2000 fibers from at least 4 mice. *Significantly lower median CSA compared with WT, Mann-Whitney test by ranks, P<0.05. (c) Absolute tetanic force production in TA muscles. *Significantly lower tetanic force compared with WT, t-test, P<0.05, n=8. (d) Tetanic force production measured as force normalized to muscle CSA (sP\(_o\)) (n=8). (e) Fatigability of uninjured TA muscles from nNOS\(^{-/-}\) mice (KO) and wildtype littermate controls (WT). Force is expressed as % initial force. *KO significantly more resistant to fatigue than WT; strain main effect, 2-way ANOVA, P<0.05, n=8.

**Figure 3.** mRNA expression of (a) MyHC type I, and (b) MyHC type IIa, IIx, and IIb in uninjured TA muscles from nNOS\(^{-/-}\) mice (KO) and wildtype littermate controls (WT) as determined by real-time PCR. *Significantly altered expression compared with WT, t-test,
P<0.05, n=7. (c) Western blot of Calcineurin, COXIV, PGC-1α and GAPDH in both nNOS−/− mice (nNOS-) and wildtype littermate controls (nNOS+). (d) Quantification of protein expression of Calcineurin, COXIV and PGC-1α normalized to GAPDH expression. Data pooled from n=5 mice. (e) Assessment of oxidative capacity of uninjured TA muscles as determined by succinate dehydrogenase activity (see Methods). Data pooled from n=8 mice.

Figure 4. (a) Quantification of capillaries per muscle fiber as measured by immunohistochemistry of muscle sections (pooled data from n=3-4 muscles). (b) Blood flow in the uninjured TA muscles of nNOS−/− mice (KO) and wildtype littermate controls (WT) as measured by laser Doppler before and immediately after the in situ fatiguing stimulation protocol (n=3). (c) Relative mRNA expression of nNOS, eNOS, catalase, SOD1, PRDX6 and Hsp70 in uninjured TA muscles as determined by real-time PCR. *Significantly altered expression compared with WT, t-test, P<0.05, n=7.

Figure 5. Frequency distribution of myofiber cross-sectional area (CSA) from nNOS−/− mice (KO) and wildtype littermate controls (WT) at (a) 7 days post-injury or (b) 10 days post-injury. (c) Median CSA of myofibers from regenerating TA muscles at 7 or 10 days post-injury. *Significantly lower median CSA compared with WT, Kruskal-Wallis ANOVA by ranks, p<0.05. (d) Percentage of centrally-nucleated myofibers in regenerating muscles. Data pooled from >2000 fibers from at least 4 mice. (e) mRNA expression of myogenin in regenerating muscles at 10 days post injury as determined by real-time PCR (n=7-8).
Figure 6. Force production by regenerating TA muscles from nNOS<sup>-/-</sup> mice (KO) and wildtype littermate controls (WT) as measured by (a) absolute force, or (b) force normalized to muscle CSA (sP<sub>o</sub>) (n=5-8).
nNOS is not required for successful muscle regeneration

Table 1. Real time RT-PCR primers used.

<table>
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<tr>
<th>Gene</th>
<th>Genbank accession number</th>
<th>Primer Sequences (5’-3’)</th>
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| nNOS     | NM_008712                | 5’- TTT CTG TCC GTC TCT CTT CAA ACG CAA AGT GG-3’
|          |                          | 5’- GCG GGA GAC TGT TCG TTC TCT GAA TAC GGG-3’ |
| eNOS     | NM_008713                | 5’- TGA CCA GCA CAT TTG GCA ATG G-3’
|          |                          | 5’- CAT GAG CGC TGC TGC AAA GC-3’ |
| Myogenin | NM_031189                | 5’- CAC TCC CTT ACG TCC ATC GT-3’
|          |                          | 5’- CAG GAC AGC CCC ACT TAA AA-3’ |
| catalase | NM_009804                | 5’- CCA GTT GGC AAA GTG GTT TT-3’
|          |                          | 5’- GCC CTG AAG CTT TTT GTC AG-3’ |
| SOD1     | NM_011434                | 5’- TGG TGG TCC ATG AGA AAC AA -3’
|          |                          | 5’- GTT TAC TGC GCA ATC CCA AT -3’ |
| PRDX6    | NM_007453                | 5’- TCT CTG GTG ATG TCT GCC TG -3’
|          |                          | 5’- CCA TTA AAA GCA GGG ACC AA -3’ |
| Hsp70    | NM_010479                | 5’- GGC TGA TCG GCC GCA AGT T -3’
|          |                          | 5’- GGA AGG GCC AGT GCT TCA T -3’ |
Table 2. Selected morphological and isometric twitch contractile properties of regenerating tibialis anterior (TA) muscles from nNOS\(^{+/+}\) and nNOS\(^{-/-}\) mice.

<table>
<thead>
<tr>
<th></th>
<th>Uninjured</th>
<th>7 days post-injury</th>
<th>10 days post-injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nNOS(^{+/+})</td>
<td>nNOS(^{-/-})</td>
<td>nNOS(^{+/+})</td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>TA mass (mg)</td>
<td>51.1 ± 1.9</td>
<td>42.3 ± 1.2*</td>
<td>42.8 ± 2.2</td>
</tr>
<tr>
<td>(P_t) (mN)</td>
<td>497 ± 28</td>
<td>421 ± 21</td>
<td>105 ± 22</td>
</tr>
<tr>
<td>TPT (ms)</td>
<td>16.6 ± 0.9</td>
<td>15.3 ± 0.4</td>
<td>23.7 ± 6.1</td>
</tr>
<tr>
<td>½ RT (ms)</td>
<td>18.2 ± 1.1</td>
<td>15.0 ± 0.6*</td>
<td>18.1 ± 3.7</td>
</tr>
<tr>
<td>(dP_t/dt) (mN/ms)</td>
<td>71.5 ± 5.2</td>
<td>68.9 ± 2.8</td>
<td>22.5 ± 4.5</td>
</tr>
</tbody>
</table>

\(P_t\), peak twitch tension; TPT, time-to-peak twitch; ½ RT, one-half relaxation time; \(dP_t/dt\), maximum rate of twitch force development.

*Significant difference between nNOS\(^{+/+}\) and nNOS\(^{-/-}\) controls at given time points, \(P<0.05\).

N.B. Uninjured TA muscle mass relative to body mass was not different between nNOS\(^{+/+}\) and nNOS\(^{-/-}\) mice (1.67 ± 0.09 vs. 1.63 ± 0.09). Similarly, at 7 days post-injury there was no difference in TA muscle mass relative to body mass between nNOS\(^{+/+}\) and nNOS\(^{-/-}\) mice (1.60 ± 0.04 vs. 1.57 ± 0.02).
(a) Frequency distribution of CSA (μm²)

(b) Box plot of CSA (μm²) for WT and KO

(c) Comparison of initial Po (mN) for WT and KO

(d) Comparison of sPo (mN/m²) for WT and KO

(e) Fatigue and Recovery

- % initial Po over time (min)
  - WT (●)
  - KO (○)

* indicates statistical significance.
(a) mRNA expression (2^{−ΔΔCt} ng/ml × 10^{-13})

(b) mRNA expression (2^{−ΔΔCt} ng/ml × 10^{-9})

(c) Protein expression (AU)

(d) Reaction intensity (AU)
(a) WT and KO expression of Capillaries/fibre.

(b) WT and KO expression of Flux (AU) in Initial and Post-fatigue conditions.

(c) mRNA expression (fold change relative to WT) for nNOS, eNOS, Calalase, SOD1, PRDX6, and Hsp70.
(a) Maximum Force (mN)

Days post-injury

(b) Specific Force (kN/m²)

Days post-injury