

# Nitric oxide synthase inhibition reduces muscle inflammation and necrosis in modified muscle use

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**Abstract:** The objective of this study was to determine the role of nitric oxide in muscle inflammation, fiber necrosis, and apoptosis of inflammatory cells in vivo. The effects of nitric oxide synthase (NOS) inhibition on the concentrations of neutrophils, ED1<sup>+</sup> and ED2<sup>+</sup> macrophages, apoptotic inflammatory cells, and necrotic muscle fibers in rats subjected to 10 days of hindlimb unloading and 2 days of reloading were determined. Administration of NOS inhibitor N<sup>w</sup>-nitro-L-arginine methyl ester (L-NAME) significantly reduced the concentrations of neutrophils, ED1<sup>+</sup> and ED2<sup>+</sup> macrophages, and necrotic fibers in soleus muscle relative to water-treated controls. The concentration of apoptotic inflammatory cells was also significantly lower for L-NAME-treated animals compared with water-treated controls. However, the proportion of the inflammatory cell population that was apoptotic did not differ between L-NAME-treated and control animals, suggesting that L-NAME treatment did not decrease inflammatory cell populations by increasing the frequency of apoptosis. Thus, nitric oxide or one of its intermediates promotes muscle inflammation and fiber necrosis during modified muscle use and plays no more than a minor role in the resolution of muscle inflammation by inducing apoptosis of inflammatory cells. *J. Leukoc. Biol.* 64: 427–433; 1998.

**Key Words:** apoptosis · muscle injury · macrophage · neutrophil

## INTRODUCTION

Skeletal muscle injury resulting from modified muscle use is associated with degenerative events that include sarcolemma damage, sarcomeric disruption, and reduced contractility [1–4]. These disruptions to the normal structure and function of muscle are apparent within minutes after injury and may become more extensive during the following hours to days [2, 4–6]. Muscle injury also typically causes edema and the extravasation of neutrophils followed by ED1<sup>+</sup> macrophages and later the proliferation of resident ED2<sup>+</sup> macrophages [2, 5, 6].

Inflammation of injured muscle has been interpreted as indicating an early stage of muscle regeneration because of the ability of neutrophils and ED1<sup>+</sup> macrophages to phagocytose cellular debris and ED2<sup>+</sup> macrophages to stimulate the proliferation and fusion of muscle precursor cells [7–9]. However, the

ability of neutrophils and macrophages to induce injury in other cells in vitro [10–12] suggests that these early stages of muscle inflammation may also involve damage to muscle fibers caused by the invading cells. This possibility is supported by the ability of neutrophils to induce skeletal muscle injury during tissue reperfusion after ischemia [13, 14].

A potential mechanism by which inflammatory or other cells could induce muscle injury after modified loading is through reactive metabolites of nitrogen and oxygen, such as nitric oxide (NO). NO, synthesized by a family of nitric oxide synthases (NOS) from L-arginine and oxygen, can function as a pro-inflammatory molecule by activating cyclooxygenases and thereby increasing prostaglandin production [15], which can promote inflammation and muscle proteolysis [16, 17]. Cell damage caused by NO or other injurious molecules may also promote inflammation by causing the release of cytosolic molecules that can attract or activate inflammatory cells. For example, platelet-derived growth factor and other cytosolic proteins that are released by muscle after crush injury [18] can act as chemoattractants to inflammatory cells [19] and contribute to inflammatory cell activation [20]. NO has also been shown to promote motility [21] and chemotaxis [22] of neutrophils, which may contribute to their ability to invade injured tissues.

NO can function as an anti-inflammatory molecule through several mechanisms. For example, NO can inhibit leukocyte adhesion to vascular endothelium [23], thereby preventing their entry into injured tissue. NO may also function as an anti-inflammatory molecule through its ability to inhibit the synthesis of superoxide anions by neutrophils [24] to scavenge superoxide [25] and to inhibit macrophage Ia<sup>+</sup> expression [26]. Anti-inflammatory actions of NO may also result from the induction of apoptosis of inflammatory cells [27, 28]. A previous investigation showed that ED1<sup>+</sup> macrophages undergo apoptosis during the stage of muscle inflammation when their concentration declines [29], which indicates that apoptosis of ED1<sup>+</sup> macrophages is a negative regulator of their population during muscle inflammation in vivo. However, it is unknown whether NO plays a role in regulating ED1<sup>+</sup> macrophage apoptosis.

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Abbreviations: NOS, nitric oxide synthase; NO, nitric oxide; PBS, phosphate-buffered saline; TdT, terminal deoxynucleotide transferase; NSAIDs, non-steroidal anti-inflammatory drugs.

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Most NO-mediated effects that are expected to influence inflammation are based on *in vitro* studies. Because of the potentially opposing effects of NO as a promoter or inhibitor of processes associated with inflammation, it is difficult to determine whether NO will function *in vivo* as a proinflammatory or an anti-inflammatory molecule based solely on the findings of *in vitro* studies. This determination has practical importance in view of the potential use of NO donors and NOS inhibitors as therapeutic agents for the treatment of disease and trauma [30, 31]. In this study, we tested whether modifying NO production *in vivo* affects muscle inflammation and necrosis or influences the occurrence of inflammatory cell apoptosis during modified muscle use. Rats were subjected to periods of muscle unloading followed by reloading, which has been shown previously to cause muscle inflammation and necrosis [2, 5, 6] and inflammatory cell apoptosis [29]. Experimental manipulations were performed on animals to which NOS inhibitors were administered, and the concentrations of neutrophils, macrophages, necrotic fibers, and apoptotic inflammatory cells were compared to control animals not receiving NOS inhibitors. Through this analysis, we were able to determine whether the net effect of NO synthesis *in vivo* during muscle injury is to function as a pro-inflammatory or anti-inflammatory molecule.

## MATERIALS AND METHODS

### Experimental design

Forty-two female Wistar rats (Charles River Laboratories) were housed individually and maintained on a 12-h light-dark cycle and given food and water *ad libitum*. Treatment of animals followed procedures approved by the UCLA Animal Research Committee. The hindlimbs of 28 rats were unloaded for 10 days through the use of a tail harness attached to a swivel at the top of the cage that allowed 360° rotation [6, 32]. After 10 days of hindlimb unloading, the tail harnesses were removed and the rats experienced muscle reloading during 2 days under normal cage activity. The reloaded rats were randomly assigned to *N<sup>ω</sup>*-nitro-L-arginine methyl ester treated (L-NAME), *N<sup>ω</sup>*-nitro-D-arginine methyl ester treated (D-NAME), or water treated. L-NAME and D-NAME (0.5 mg · mL<sup>-1</sup>) were provided continuously in the drinking water beginning at 9 days of suspension until 2 days of reloading. The remaining rats served as hindlimb-suspended controls that were killed after 10 days of suspension or ambulatory controls that were not suspended and had normal cage activity for 10 days. All rats were killed by intraperitoneal injection of 100 mg · kg<sup>-1</sup> pentobarbital sodium. Both L-NAME and D-NAME inhibit NOS activity, although L-NAME is a more effective inhibitor than D-NAME [33, 34].

### Tissue preparation and immunohistochemistry

Soleus muscles were excised and prepared for immunohistochemistry following previously published protocols [6]. Frozen sections were immunolabeled for neutrophils (anti-W3/13, Sera-lab; diluted 1:75), ED1<sup>+</sup> macrophages (anti-ED1; Serotec; 1:100), and ED2<sup>+</sup> macrophages (anti-ED2, Serotec; 1:100). Negative controls received phosphate-buffered saline (PBS) instead of primary antibody. Biotinylated anti-mouse IgG (Vector Laboratories; 1:200) was used as a secondary antibody. After secondary antibody incubation, sections were incubated with avidin D horseradish peroxidase and developed with 3-amino-9-ethylcarbazole to produce a red reaction product.

### TdT-mediated dUTP labeling of apoptotic DNA

Apoptotic nuclei were detected using terminal deoxynucleotide transferase (TdT)-mediated dUTP-biotin nick end labeling [35]. Tissue sections were processed using procedures described by Spencer et al. [36]. Positive control sections of young mouse thymus were treated in parallel with soleus sections.

Negative controls were prepared identically, except TdT was eliminated from the reaction mixture.

### Quantification of inflammatory cells and apoptotic nuclei

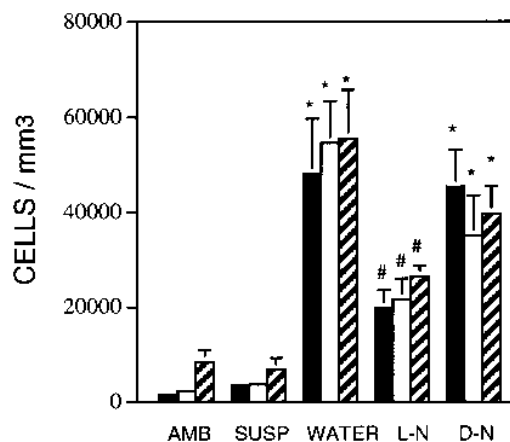
The number of inflammatory cells and apoptotic nuclei in two sections were counted by light microscopy and the total area of the section was measured using previously described techniques [37]. The number of labeled nuclei located in the connective tissue between muscle fibers and the apoptotic cells located within fibers were counted separately. The volume of muscle sampled was calculated as the product of the cross-sectional area of the section and the section thickness (10 μm). The concentrations of inflammatory cells and apoptotic nuclei were expressed as number per cubic millimeter. The number of ED1<sup>+</sup> macrophage-invaded fibers, an indicator of fiber necrosis, was counted using the same morphometric techniques as previously described except that the number of invaded fibers was expressed relative to the total area of the section (mm<sup>2</sup>).

### Statistical analyses

A one-way analysis of variance was used to analyze the treatment effect for the concentrations of inflammatory cells and apoptotic inflammatory cell nuclei. Body mass was analyzed using a two-way repeated measures analysis of variance to determine the main effects [treatment and time (pre- and post hindlimb unweighting and 48 h of reloading)] and the interaction effect. If the observed *F* ratio was statistically significant, the Fisher least significance difference test was used to locate the differences.

## RESULTS

Ten days of hindlimb unloading followed by two days of reloading resulted in an increase in the concentrations of neutrophils and ED1<sup>+</sup> and ED2<sup>+</sup> macrophages (Fig. 1; Table 1), and fiber necrosis (Fig. 2) relative to ambulatory and suspended only animals, which is consistent with previous observations [6]. Novel findings of this study were that NOS inhibition by L-NAME significantly reduced the concentrations



**Fig. 1.** Concentrations of inflammatory cells in rat soleus muscles after 10 days of unloading followed by 2 days of reloading. Solid bars, neutrophil concentrations; white bars, ED1<sup>+</sup> macrophage concentrations; hatched bars, ED2<sup>+</sup> macrophage concentrations. AMB, ambulatory controls; SUSP, suspended animals experiencing unloading without reloading; WATER, reloaded animals provided water without NOS inhibitor; L-N, reloaded animals provided water containing L-NAME; D-N, reloaded animals provided water containing D-NAME. \*Significant difference from AMB and SUSP (*P* < 0.05); #significant difference from WATER and D-N; \$, significant difference from WATER only. Standard errors are shown.

TABLE 1. Concentration of Leukocyte Populations in Rat Soleus Muscle and Measurement of Muscle Necrosis After Experimental Treatments

Treatment	Neutro/mm <sup>3</sup>	ED1/mm <sup>3</sup>	ED2/mm <sup>3</sup>	Invaded fibers
AMB. CONT. (n = 5)	1599 (430)	2280 (741)	8433 (5592)	0.1 (0.15)
SUSP. (n = 7)	3579 (1601)	3662 (1650)	6806 (9350)	0.76 (0.30)
WATER (n = 10)	48,138 (36,308)*	54,579 (27,560)*	55,460 (32,424)*	6.48 (6.39)*
L-NAME (n = 11)	19,840 (12,943)†	21,680 (14,481)†	26,537 (7171)†	1.65 (1.03)‡
D-NAME (n = 9)	45,372 (22,063)*	35,071 (25,298)*	39,660 (17,501)*	3.51 (1.70)*

Standard deviation is shown in parentheses.

\* Values that differ significantly from suspended only muscle at  $P < 0.05$ .

† Significant difference from WATER and D-NAME.

‡ Significant difference from WATER only. AMB. CONT. group, ambulatory controls; SUSP, animals experiencing suspension for 10 days without a subsequent reloading period. All other animals were suspended for 10 days, followed by 24 h of reloading while provided with water only (WATER), or water containing L-NAME (L-NAME) or D-NAME (D-NAME). Neutro, neutrophils; ED1, ED1<sup>+</sup> macrophages; ED2, ED2<sup>+</sup> macrophages.

of inflammatory cells (Fig. 1) and fiber necrosis (Fig. 2) relative to animals experiencing reloading without NOS inhibition. In addition, the concentrations of inflammatory cells and necrotic fibers in NOS-inhibited animals were similar to ambulatory and suspended only animals. D-NAME-treated rats contained significantly more neutrophils, ED1<sup>+</sup> macrophages, and ED2<sup>+</sup> macrophages relative to L-NAME-treated animals, ambulatory, or suspended only animals. D-NAME-treated animals did not differ significantly from water-treated animals in the concentration of neutrophils, macrophages, or invaded fibers. However, administration of D-NAME showed a trend in reducing muscle inflammation and necrosis for all parameters assayed (Table 1). Although the trend was not significant at the confidence interval employed in this investigation ( $P < 0.05$ ), this result reflects the small inhibitory effect of D-NAME on NOS activity compared to that of L-NAME. This small inhibitory effect has been observed previously [33, 34].

ED1<sup>+</sup> macrophages in muscles experiencing reloading without L-NAME treatment were primarily found in clusters in the muscle that were usually located in the connective tissue surrounding muscle fibers, but were also observed in the cytoplasm of fibers they invaded (Fig. 3). Reloaded muscles of animals receiving L-NAME treatments also contained clusters of ED1<sup>+</sup> macrophages, although they were uncommonly found in invaded fibers and more often located near blood vessels. Neutrophils and ED2<sup>+</sup> macrophages were more uniformly

distributed through the connective tissue of the muscle (Figs. 4 and 5) and L-NAME treatment had no detectable effect on their distribution within the muscles, although their concentration in the reloaded muscle was greatly reduced by the L-NAME treatment. The intramuscular blood vessels of L-NAME-treated animals commonly showed neutrophils and ED1<sup>+</sup> macrophages closely apposed to the luminal surface of the vascular endothelium of reloaded animals (Fig. 6), so that they appeared to be adhering to the endothelium. Neither neutrophils nor macrophages were observed to be closely apposed to the vascular endothelium of reloaded animals receiving D-NAME or water only.

The distribution of apoptotic cells also tended to be clustered, and uncommon instances of apoptotic cells in necrotic muscle fibers were observed (Fig. 7). The concentration of apoptotic inflammatory cells was also significantly lower in L-NAME-treated animals relative to water-treated or D-NAME-treated animals, but did not differ significantly from muscles of ambulatory and suspended only animals (Fig. 8).

We tested the possibility that the frequency of occurrence of inflammatory cell apoptosis was lower in L-NAME-treated animals than in water- or D-NAME-treated animals by comparing the ratio of apoptotic cells to the total number of neutrophils and ED1<sup>+</sup> macrophages in the sample. The number of apoptotic

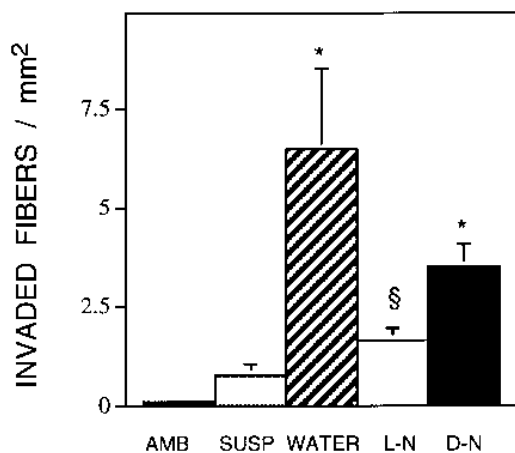
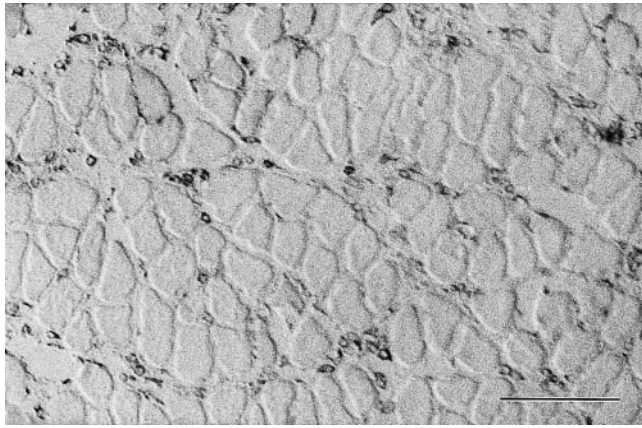


Fig. 2. Histogram showing the number of necrotic fibers/unit cross-sectional area of rat soleus muscle. Labels for bars and markers indicating significance of differences are defined in the legend for Figure 1.



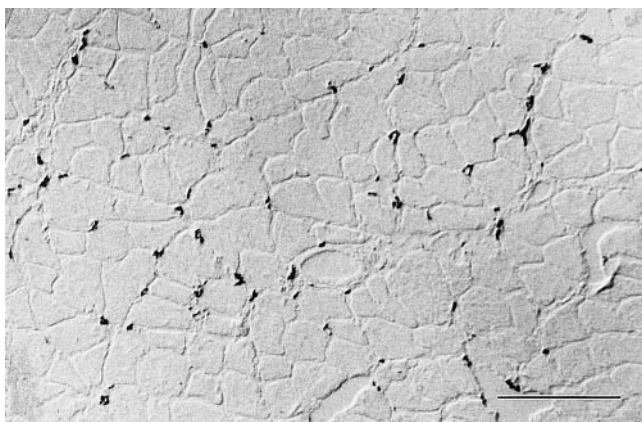
Fig. 3. Cross-section of rat soleus muscle collected after 10 days of hindlimb unloading followed by 2 days of reloading while receiving water without NOS inhibitor. Section was immunolabeled for ED1<sup>+</sup> macrophages. Dark staining mononucleated cells lying primarily in the connective tissue between fibers (arrowheads) and occasionally present within the cytoplasm of necrotic muscle fibers (arrows) are ED1<sup>+</sup> macrophages. Bar = 100  $\mu$ m.



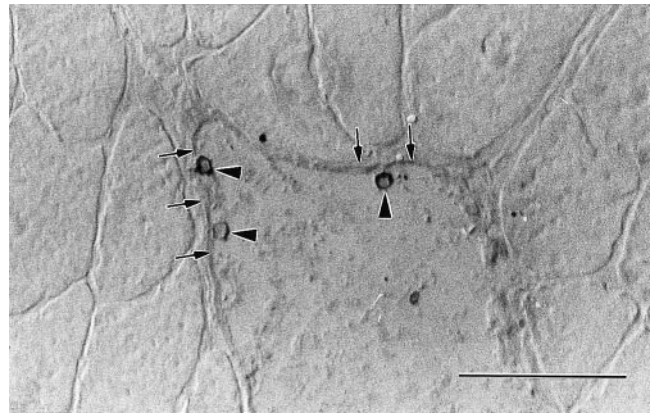
**Fig. 4.** Cross-section of rat soleus muscle collected after 10 days of hindlimb unloading followed by 2 days of reloading while receiving water without NOS inhibitor. Section was immunolabeled for neutrophils, which were observed primarily in the connective tissue between fibers. Bar = 100  $\mu$ m.

cells was expressed relative to neutrophil and ED1<sup>+</sup> macrophage populations because these cells experience a decrease in their concentration at these early stages of muscle reloading, whereas ED2<sup>+</sup> macrophage populations remain elevated for at least 7 days after commencement of reloading [6]. This analysis revealed that the ratio of the concentration of apoptotic inflammatory cells to the concentration of neutrophils and ED1<sup>+</sup> macrophages did not differ significantly between any experimental groups (**Fig. 9**). This indicates that L-NAME did not increase the frequency of occurrence of apoptosis of inflammatory cells.

The volume of water ingested from 9 days of hindlimb unloading throughout the 2 days of reloading was not significantly different between the treatments (mean  $\pm$  SE; water, 108  $\pm$  6.2; L-NAME, 89.7  $\pm$  5.9; D-NAME, 105.6  $\pm$  6.6 mL). The L-NAME- and the D-NAME-treated animals ingested 753.4  $\pm$  53 and 864.4  $\pm$  60  $\mu$ mol  $\cdot$  kg<sup>-1</sup> body mass, respectively ( $P > 0.05$ ). Body mass was not influenced by hindlimb unloading or by D-NAME or L-NAME administration.



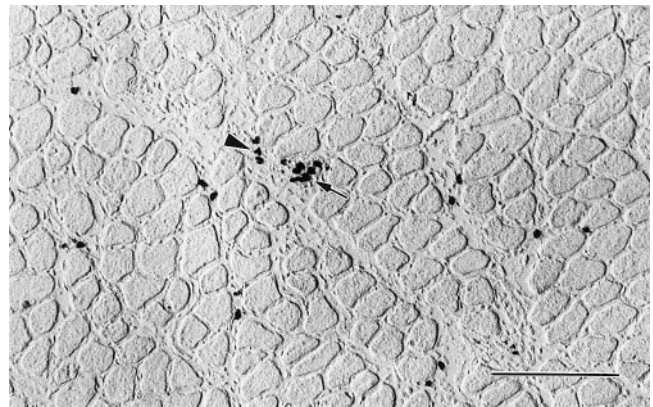
**Fig. 5.** Cross-section of rat soleus muscle collected after 10 days of hindlimb unloading followed by 2 days of reloading while receiving water without NOS inhibitor. Section was immunolabeled for ED2<sup>+</sup> macrophages, which were exclusively located in the connective tissue between fibers. Bar = 100  $\mu$ m.



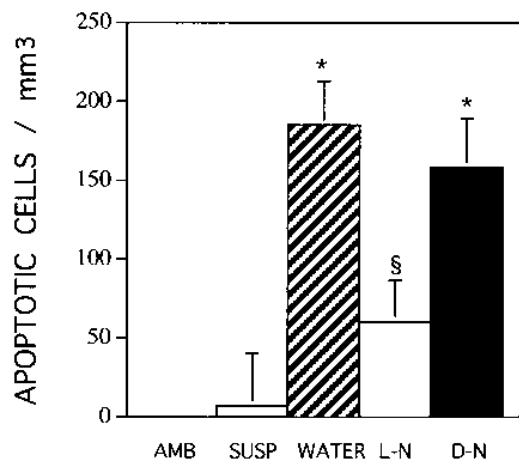
**Fig. 6.** Cross-section of rat soleus muscle collected after 10 days of hindlimb unloading followed by 2 days of reloading while receiving water containing L-NAME. Section was immunolabeled for neutrophils. L-NAME treatment resulted in the presence of neutrophils (arrows) lying on the luminal surface of vascular endothelia (arrowheads). Bar = 50  $\mu$ m.

## DISCUSSION

Previous investigations conducted *in vitro* have shown that NO can influence inflammatory cell functions as either a proinflammatory or an anti-inflammatory molecule [22–24, 27, 28]. The apparently opposing effects of NO *in vitro* make it difficult to predict the biological effects of NO because its role may vary with differences in the tissue in which the inflammation occurs [38–41], differences in the presence of other pro-inflammatory or anti-inflammatory molecules, and its concentration or rate of delivery in the tissue [42]. The possibility that NO production by muscle can influence the occurrence of muscle injury and inflammation has been suggested by the observation that mdx mouse muscles, which express very low levels of NOS [43, 44], experience extensive muscle inflammation and necrosis [45, 46]. Although it is not expected that NOS deficiency is a major cause of muscle inflammation and necrosis in dystrophic mdx muscle, it is feasible that the low levels of NO production by mdx muscle may exacerbate muscle inflammation if NO release by muscle serves an anti-inflammatory role.

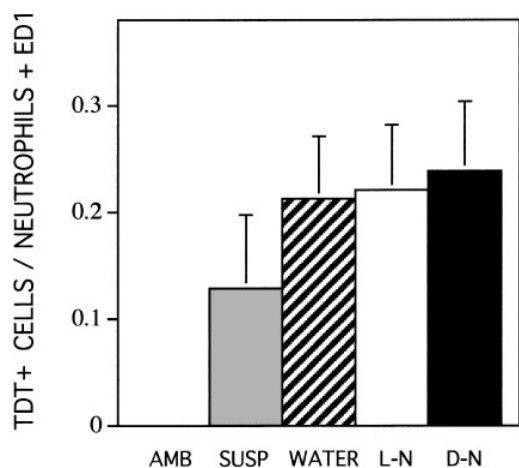


**Fig. 7.** Cross-section of rat soleus muscle collected after 10 days of hindlimb unloading followed by 2 days of reloading while receiving water containing no NOS inhibitors. Section was labeled for apoptotic nuclei. Dark staining nuclei of apoptotic mononucleated cells located in the connective tissue (arrowhead) or clustered in a necrotic fiber (arrow) are shown. Bar = 100  $\mu$ m.



**Fig. 8.** Histogram showing the concentration of apoptotic inflammatory cells in rat soleus muscle. Labels for bars and markers indicating significance of differences are defined in the legend for Figure 1.

In this investigation, we tested whether NOS inhibition had a positive or negative effect on muscle inflammation by administering NOS inhibitor to rats experiencing muscle reloading after periods of muscle unloading. Previous investigations have shown that this technique of modifying muscle loading results in significant muscle inflammation and necrosis [1, 2, 6, 37]. Our data show that NOS inhibition reduces muscle inflammation and necrosis during muscle reloading following periods of unloading to levels that do not significantly differ from ambulatory control animals, thereby indicating that NO plays a prominent proinflammatory role in at least this model of muscle inflammation. The anti-inflammatory effect of NOS inhibition was observed in all populations of inflammatory cells assayed, which suggests that NO may positively influence the concentration of each of these cell populations in reloaded muscles. Alternatively, NO may play a positive role in regulating the invasion or proliferation of one inflammatory cell type in muscle that is capable of affecting the subsequent increase of other inflammatory cell populations by NO-independent mecha-



**Fig. 9.** Histogram showing the ratio of the number of apoptotic nuclei expressed relative to the total number of neutrophils and ED1<sup>+</sup> macrophages in each group tested. This ratio was significantly higher ( $P < 0.05$ ) for all experimental groups relative to ambulatory controls, but there was no significant difference between experimental groups.

nisms. If this latter case exists, neutrophils would be the likely cell type on which NO could act directly as a proinflammatory molecule because they are typically the first inflammatory cells to invade injured tissue, including muscle [45].

NOS inhibition may cause a reduction in muscle inflammation by blocking several proinflammatory effects of NO that have been demonstrated in vitro. The activation of cyclooxygenases by NO [15] leads to the production of prostaglandins that typically function as proinflammatory molecules and can increase muscle proteolysis [16, 17]. NO can also act as a chemoattractant to neutrophils [22], which would promote inflammation. Furthermore, NO generated by activated inflammatory cells would be inhibited by the administration of L-NAME, which could thereby reduce inflammatory cell-mediated damage to the reloaded muscle. Thus, the reduction in muscle fiber necrosis in NOS-inhibited animals may be attributed both to a decrease in the concentration of inflammatory cells in the reloaded muscle and to inhibition of NO-mediated fiber injury.

Systemic inhibition of NOS by the administration of L-NAME at the concentrations used in this investigation has been shown previously to inhibit NO-mediated vasodilation [48–51] and to cause an increase in blood pressure [48]. Administration of NOS inhibitors, including L-NAME, either by intravenous injection or by addition to drinking water has also been found to reduce blood flow to muscle [51–53]. The effect of NOS inhibition on muscle blood flow varies with muscle type, with rat soleus muscle experiencing approximately a 30% decrease in blood flow after administration of L-NAME [52]. Thus, reduction of blood flow to the muscle during reloading may be a potentially important factor contributing to the reduction in muscle inflammation in NOS-inhibited animals, since this would be associated with a reduction in the number of neutrophils and ED1<sup>+</sup> macrophages circulating through the muscle during the reloading period.

The observation reported here that NOS-inhibited animals contained neutrophils and ED1<sup>+</sup> macrophages adhering to the vascular endothelium of intramuscular vessels, although no similar cell adhesion was observed in reloaded muscle of animals that did not experience NOS inhibition, is consistent with previous observations made in vitro. For example, application of L-NAME to neutrophils in vitro increases their adhesion to endothelial cells [54] and administration of the NOS substrate arginine inhibits monocyte adhesion to endothelial cells [55]. L-NAME application in vivo has also been shown to increase leukocyte rolling [49] and binding [49, 56] in situ. The L-NAME promotion of leukocyte rolling and binding is explicable by the observation that NO suppresses the expression of key cell adhesion molecules involved in leukocyte diapedesis, in particular P-selectin, ICAM, and VCAM [54, 55, 57]. These findings suggest that NOS inhibition would lead to an increase in inflammation of injured tissues because leukocyte rolling and adhesion that are mediated by selectins and CAM molecules are required for diapedesis that leads to injured tissue invasion by inflammatory cells. Furthermore, NOS inhibition by L-NAME increases vascular permeability during tissue injury [58], which would also be predicted to promote inflammatory cell invasion of the injured tissue. However, the findings of

this investigation show that increased leukocyte adhesion resulting from NOS inhibition is not associated with increased muscle inflammation during muscle reloading.

The ability of NOS inhibition to reduce muscle inflammation in this investigation does not exclude the possibility that NOS inhibition could also produce other proinflammatory effects in this system. For example, the observation that administration of NO to peritoneal macrophages or RAW 264.7 macrophages induced apoptosis *in vitro* [27, 59] suggests that NOS inhibition would reduce the proportion of the inflammatory cell population experiencing apoptosis. The finding that the ratio of apoptotic inflammatory cells to ED1<sup>+</sup> macrophages and neutrophils did not differ significantly in the reloaded muscles of animals treated with L-NAME, D-NAME, or water indicates that NOS inhibition did not affect the frequency of occurrence of apoptosis in these inflammatory cell populations, and thereby supports the conclusion that NO is not an important negative regulator of inflammatory cell populations in muscle via apoptosis in the model used here.

The anti-inflammatory effects of NOS inhibition that were identified in this investigation suggest that a portion of the anti-inflammatory effects of some drugs, such as nonsteroidal anti-inflammatory drugs (NSAIDs), may be mediated through NOS inhibition. Although the anti-inflammatory role of NSAIDs is largely attributable to inhibition of cyclooxygenases and thereby reducing synthesis of proinflammatory prostaglandins, recent findings show that aspirin, ibuprofen, sodium salicylate, and indomethacin also inhibit the expression of iNOS [60]. Thus administration of these NSAIDs would be predicted to diminish muscle inflammation in the model used here through the reduced expression of NOS, as well as by cyclooxygenase inhibition.

Although NO present in muscle during reloading can be generated by eNOS in endothelial cells, iNOS in inflammatory cells, and nNOS<sub>μ</sub> in skeletal muscle, both eNOS and nNOS<sub>μ</sub> can be logically excluded from being the primary sites of NO production that normally lead to muscle necrosis and inflammation during increased muscle use. NO synthesized by muscle is not expected to serve the proinflammatory role identified in the present investigation because recent findings [61] show that there is nearly a 50% decrease in rat soleus nNOS<sub>μ</sub> concentration after 10 days of hindlimb muscle unloading. Thus, non-inflamed ambulatory muscle would be capable of twice as much NO generation by nNOS<sub>μ</sub> as inflamed muscle experiencing reloading after 10 days of unloading. Similarly, eNOS expression in blood vessels in muscle is positively regulated by blood flow [60, 62], so that decreased blood flow to the hindlimb muscles during suspension would decrease the concentration of eNOS and thereby decrease the NO-generating capability of blood vessels in muscle upon reloading. Thus, inhibition of iNOS in animals at the time of increased muscle use is predicted to reduce muscle inflammation and necrosis, as shown in this investigation.

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## REFERENCES

1. Krippendorf, B. B., Riley, D. A. (1993) Distinguishing unloading- versus reloading-induced changes in rat soleus muscle. *Muscle & Nerve* 16, 99–108.
2. Riley, D. A., Ellis, S., Giometti, C. S., Hoh, J. F. Y., Ilyina-Kakeeva, E. I., Oganov, V. S., Slocum, G. R., Bain, J. L. W., Sedlak, F. R. (1992) Muscle sarcomere lesions and thrombosis after spaceflight and suspension unloading. *J. Appl. Physiol.* 73, 33S–43S.
3. Warren, G. L., Hayes, D. A., Lowe, D. A., Williams, J. H., Armstrong, R. B. (1994) Eccentric contraction-induced injury in normal and hindlimb-suspended mouse soleus and EDL muscles. *J. Appl. Physiol.* 77, 1421–1430.
4. Kasper, C. E. (1995) Sarcolemmal disruption in reloaded atrophic skeletal muscle. *J. Appl. Physiol.* 79, 607–614.
5. Riley, D. A., Ellis, S., Slocum, G. R., Sedlak, F. R., Bain, J. L. W., Krippendorf, B. B., Lehman, C. T., Macias, M. Y., Thompson, J. L., Vijayan, K., DeBruin, J. A. (1996) In-flight and postflight changes in skeletal muscles of SLS-1 and SLS-2 spaceflown rats. *J. Appl. Physiol.* 81, 133–144.
6. St. Pierre, B. A., Tidball, J. G. (1994) Differential response of macrophage subpopulations to soleus muscle reloading after rat hindlimb suspension. *J. Appl. Physiol.* 77, 290–297.
7. Papadimitriou, J. M., Robertson, T. A., Mitchell, C. A., Grounds, M. D. (1990) The process of new plasmalemma formation in focally injured skeletal muscle fibers. *J. Struct. Biol.* 103, 124–134.
8. Robertson, T. A., Maley, M. A. L., Grounds, M. D., Papadimitriou, J. M. (1993) The role of macrophages in skeletal muscle regeneration with particular reference to chemotaxis. *Exp. Cell Res.* 207, 321–331.
9. Massimino, M. L., Rapizzi, E., Cantini, M., Libera, L. D., Mazzoleni, F., Arsian, P., Carraro, U. (1997) ED2<sup>+</sup> macrophages increase selectively myoblast proliferation in muscle cultures. *Biochem. Biophys. Res. Commun.* 235, 754–759.
10. Weiss, S. J., Young, J., LoBuglio, A. F., Slivka, A. (1981) Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. *J. Clin. Invest.* 68, 714–721.
11. Weiss, S. J., LoBuglio, A. F., Kessler, H. B. (1980) Oxidative mechanisms of monocyte-mediated cytotoxicity. *Proc. Natl. Acad. Sci. USA* 77, 584–587.
12. Hibbs, J. B., Taintor, R. R., Vavrin, Z., Rachlin, E. M. (1988) Nitric oxide: a cytotoxin activated macrophage effector molecule. *Biochem. Biophys. Res. Commun.* 157, 87–94.
13. Carden, D. L., Smith, J. K., Korthuis, R. J. (1990) Neutrophil mediated microvascular dysfunction in postischemic canine skeletal muscle: role of granulocyte adherence. *Circ. Res.* 66, 1436–1444.
14. Korthuis, R. J., Granger, K. N., Townsley, M. I., Taylor, A. E. (1988) Leukocyte depletion attenuates vascular injury in postischemic skeletal muscle. *Am. J. Physiol.* 254, H823–H827.
15. Salvemini, D., Misko, T. P., Masferrer, J. L., Seibert, K., Currie, M. G., Needleman, P. (1993) Nitric oxide activates cyclooxygenase enzymes. *Proc. Natl. Acad. Sci. USA* 90, 7240–7244.
16. Rodemann, H. P., Goldberg, A. L. (1982) Arachidonic acid, prostaglandin E and F<sub>2a</sub> influence rates of protein turnover in skeletal and cardiac muscle. *J. Biol. Chem.* 257, 1632–1638.
17. Goldberg, A. L., Kettelhut, I. C., Furuno, K., Fagan, J. M., Baracos, V. (1988) Activation of protein breakdown and prostaglandin E<sub>2</sub> production in rat skeletal muscle in fever is signaled by a macrophage product distinct from interleukin 1 or other known monokines. *J. Clin. Invest.* 81, 1378–1383.
18. Chen, G., Birnbaum, R. S., Yablonka-Reuveni, Z., Quinn, L. S. (1994) Separation of mouse crushed muscle extract into distinct mitogenic activities by heparin affinity chromatography. *J. Cell Physiol.* 160, 563–572.
19. Deuel, T. F., Senior, R. M., Huang, J. S., Griffin, G. L. (1982) Chemotaxis of monocytes and neutrophils to platelet-derived growth factor. *J. Clin. Invest.* 69, 1046–1049.
20. Tzeng, D. Y., Deuel, T. F., Husang, J. S., Baehner, R. L. (1985) Platelet-derived growth factor promotes human peripheral monocyte activation. *Blood* 66, 179–183.
21. VanUffelen, B. E., deKoster, B. M., Van den Broek, P. J. A., VanSteveninck, J., Elferink, J. G. R. (1996) Modulation of neutrophil migration by exogenous gaseous nitric oxide. *J. Leukoc. Biol.* 60, 94–100.

22. Beauvais, F., Michel, L., Dubertret, L. (1995) Exogenous nitric oxide elicits chemotaxis of neutrophils in vitro. *J. Cell Physiol.* 165, 610–614.
23. Kubes, P., Suzuki, M., Granger, D. N. (1991) Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. USA* 88, 4651–4655.
24. Clancy, R., Leszczynska-Piziak, J., Abramson, S. (1992) Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J. Clin. Invest.* 90, 1116–1121.
25. Rubanyi, G. M., Ho, E. H., Cantor, E. H., Lumma, W. C., Botelho, L. H. P. (1991) Cytoprotective function of nitric oxide: inactivation of superoxide radicals produced by human leukocytes. *Biochem. Biophys. Res. Commun.* 181, 1392–1397.
26. Sicher, S. C., Vazquez, M. A., Lu, C. Y. (1994) Inhibition of macrophage Ia expression by nitric oxide. *J. Immunol.* 153, 1293–1300.
27. Albina, J. E., Cui, S., Mateo, R. B., Reichner, J. S. (1993) Nitric oxide mediated apoptosis in murine peritoneal macrophages. *J. Immunol.* 150, 5080–5085.
28. Sarih, M., Souvannavong, V., Adam, A. (1993) Nitric oxide synthase induces macrophage death by apoptosis. *Biochem. Biophys. Res. Commun.* 191, 503–508.
29. Tidball, J. G., St. Pierre, B. A. (1996) Apoptosis of macrophages during the resolution of muscle inflammation. *J. Leukoc. Biol.* 59, 380–388.
30. Moncada, S., Higgs, E. A. (1995) Molecular mechanisms and therapeutic strategies related to nitric oxide. *FASEB. J.* 9, 1319–1330.
31. Ogden, J. E., Moore, P. K. (1995) Inhibition of nitric oxide synthase—potential for a novel class of therapeutic agent? *Trends Biotechnol.* 13, 70–78.
32. Morey-Holton, E., Wronski, J. (1981) Animal models for simulating weightlessness. *Physiologist* 24, S45–S48.
33. Miller, M. J. S., Sadowska-Krowicka, H., Chotinaruemol, S., Kakkis, J. L., Clark, D. A. (1993) Amelioration of chronic ileitis by nitric oxide synthase inhibition. *J. Pharmacol. Exp. Ther.* 264, 11–16.
34. Li, Q., Bowmer, C. J., Yates, M. S. (1994) Diuretic effect of *N*<sup>ω</sup>-nitro-L-arginine methyl ester in the rat. *J. Pharm. Pharmacol.* 46, 510–512.
35. Gavrieli, Y., Sherman, Y., Ben-Sasson, S. A. (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119, 493–501.
36. Spencer, M. J., Lu, B., Tidball, J. G. (1997) Calpain II expression is increased by changes in mechanical loading of muscle in vivo. *J. Cell. Biochem.* 64, 55–66.
37. Tidball, J. G., Albrecht, D. E., Lokensgard, B. E., Spencer, M. J. (1995) Apoptosis precedes necrosis of dystrophin-deficient muscle. *J. Cell Sci.* 108, 2197–2204.
38. Billiar, T. R., Curran, R. D., Harbrecht, B. G., Stuehr, D. J., Demetris, A. J., Simmons, R. L. (1990) Modulation of nitrogen oxide synthesis in vivo: *N*-monomethyl-L-arginine inhibits endotoxin-induced nitrite/nitrate biosynthesis while promoting hepatic damage. *J. Leukoc. Biol.* 48, 565–569.
39. Hutcheson, I. R., Whittle, B. J., Boughton-Smith, N. K. (1990) Role of nitric oxide in maintaining vascular integrity in endotoxin-induced acute intestinal damage in the rat. *Br. J. Pharmacol.* 101, 815–820.
40. Mulligan, M. S., Hevel, J. M., Marletta, M. A., Ward, P. A. (1991) Tissue injury caused by deposition of immune complexes is L-arginine dependent. *Proc. Natl. Acad. Sci. USA* 88, 6338–6342.
41. Hughes, S. R., Williams, T. J., Brain, S. D. (1990) Evidence that endogenous nitric oxide modulates oedema formation induced by substance P. *Eur. J. Pharmacol.* 191, 481–484.
42. Tamir, S., Lewis, R. S., de Rojas Walker, T., Deen, W. M., Wishnok, J. S., Tannenbaum, S. R. (1993) The influence of delivery rate on the chemistry and biological effects of nitric oxide. *Chem. Res. Toxicol.* 6, 895–899.
43. Brenman, J. E., Chao, D. S., Xia, H., Aldape, K., Bretz, D. S. (1995) Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* 82, 743–752.
44. Chang, W.-J., Iannaccone, S. T., Lau, K. S., Masters, B. S. S., McCabe, T. J., McMillan, K., Padre, R. C., Spencer, M. J., Tidball, J. G., Stull, J. T. (1996) Neuronal nitric oxide synthase and dystrophin-deficient muscular dystrophy. *Proc. Natl. Acad. Sci. USA* 93, 9142–9147.
45. Torres, L. F. B., Duchon, L. W. (1987) The mutant mdx: Inherited myopathy in the mouse. *Brain* 110, 269–299.
46. Cullen, M. J., Jaros, E. (1988) Ultrastructure of the skeletal muscle in the X chromosome-linked dystrophic (mdx) mouse comparison with Duchenne muscular dystrophy. *Acta Neuropathol.* 77, 69–81.
47. Gilligan, D. M., Panza, J. A., Kilcoyne, C. M., Wacławiw, M. A., Casino, P. R., Quyyumi, A. A. (1994) Contribution of endothelium-derived nitric oxide to exercise-induced vasodilation. *Circulation* 90, 2853–2858.
48. Fukumura, D., Yuan, F., Endo, M., Jain, R. K. (1997) Role of nitric oxide in tumor microcirculation. Blood flow, vascular permeability, and leukocyte-endothelial interactions. *Am. J. Pathol.* 150, 713–725.
49. Gardiner, S. M., Compton, A. M., Bennett, T., Palmer, R. M. J., Moncada, S. (1990) Regional haemodynamic changes during oral ingestion of *N*<sup>G</sup>-monomethyl-L-arginine or *N*<sup>G</sup>-nitro-L-arginine methyl ester in conscious Brattleboro rats. *Br. J. Pharmacol.* 101, 10–12.
50. Hickner, R. C., Fischer, J. S., Ehsani, A. A., Kohrt, W. M. (1997) Role of nitric oxide in skeletal muscle blood flow at rest and during dynamic exercise in humans. *Am. J. Physiol.* 273, H405–H410.
51. Hirai, T., Visneski, M. D., Kearns, K. J., Zelis, R., Musch, T. I. (1994) Effects of NO synthase inhibition on the muscular blood flow response to treadmill exercise in rats. *J. Appl. Physiol.* 77, 1288–1293.
52. Wilson, J. R., Kapoor, S. (1993) Contribution of endothelium-derived relaxing factor to exercise-induced vasodilation in humans. *J. Appl. Physiol.* 75, 2740–2744.
53. Niu, X.-F., Smith, C. W., Kubes, P. (1994) Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. *Circ. Res.* 74, 1133–1140.
54. Adams, M. R., McCredie, R., Jessup, W., Robinson, J., Sullivan, D., Celermajer, D. S. (1997) Oral L-arginine improves endothelium-dependent dilatation and reduces monocyte adhesion to endothelial cells in young men with coronary artery disease. *Atherosclerosis* 129, 261–269.
55. Akimitsu, T., Gute, D. C., Korthuis, R. J. (1995) Leukocyte adhesion induced by inhibition of nitric oxide production in skeletal muscle. *J. Appl. Physiol.* 78, 1725–1732.
56. Gauthier, T. W., Davenpeck, K. L., Lefer, A. M. (1994) Nitric oxide attenuates leukocyte-endothelial interaction via P-selectin in splanchnic ischemia-reperfusion. *Am. J. Physiol.* 267, G562–G568.
57. Ialenti, A., Iannaro, A., Moncada, S., Di Rosa, M. (1992) Modulation of acute inflammation by endogenous nitric oxide. *Eur. J. Pharmacol.* 211, 177–182.
58. Von Knethen, A., Brüne, B. (1997) Cyclooxygenase-2: an essential regulator of NO-mediated apoptosis. *FASEB J.* 11, 887–895.
59. Aeberhard, E. E., Henderson, S. A., Arabolos, N. S., Griscavage, J. M., Castro, F. E., Barrett, C. T., Ignarro, L. J. (1995) Nonsteroidal anti-inflammatory drugs inhibit expression of the inducible nitric oxide synthase gene. *Biochem. Biophys. Res. Commun.* 208, 1053–1059.
60. Tidball, J. G., Lavergne, E., Lau, K. S., Spencer, M. J., T. Stull, J. T., Wehling, M. (1998) Mechanical loading regulates nitric oxide synthase expression and activity in developing and adult skeletal muscle. *Am. J. Physiol.* 275, C260–C266.
61. Sessa, W. C., Pritchard, K., Seyedi, N., Wang, J., Hintze, T. H. (1994) Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. *Circ. Res.* 74, 49–353.