INTRODUCTION: Nitric oxide (NO) and nitric oxide synthase (NOS) are thought to play a major role in the development of ischemia/reperfusion (I/R) injury in skeletal muscle. The roles of endothelial (eNOS) and inducible NOS (iNOS) have been studied extensively. However, neuronal NOS (nNOS) is the most abundant isozyme in rat and human skeletal muscle, but its function and regulation are poorly understood. The role of nNOS in the development of skeletal muscle I/R injury is even less clearly established. NOS has been shown to play a deleterious role in CNS I/R injury, but no role in myocardial I/R injury. Previous work in our laboratory has shown that the highly selective NOS inhibitor N[^6]-propyl-L-arginine (NPA) slightly improves contractile function in reperfused skeletal muscle. To further confirm the role of nNOS in I/R, this study was designed to observe the effects of NPA on skeletal muscle microcirculation during early reperfusion. We chose to use the denervated model because it more closely simulates clinical situations. To gain insight into the mechanism of NPA at a molecular level, NOS mRNA and protein levels of all three isoforms were measured following reperfusion.

METHODS: (Approved by IACUC) Denervated cremaster muscles from 60 rats weighing 90–120 g underwent 4 hours ischemia followed by 90 minutes of reperfusion. The rats were divided into 3 groups, receiving intravenous infusion of control phosphate buffered solution (PBS), low-dose (10 nmol/kg/hr), or high-dose (100 nmol/kg/hr) NPA starting 10 minutes prior to and for the duration of the reperfusion period. The left cremaster muscle was exposed and isolated, attached only by a neurovascular pedicle. The genitofemoral nerve was cut to render the muscle denervated. The muscle was moistened with warm lactated Ringer’s solution (34°C) and covered with a thin layer of oxygen impermeable plastic polymer to prevent diffusion of oxygen. Blood vessel diameters were measured in 10 rats per group using intravital microscopy with a video measure gauge and divided into 3 vessel categories (10-20 µm, 21-40 µm, and 41-70 µm). Blood flow into the cremaster muscle was measured with laser Doppler Flowmetry in 10 rats per group. Baseline values were recorded for vessel diameter and flow prior to ischemia, and recovery was measured at 10 min intervals during reperfusion and expressed as a percentage of baseline. Real-time PCR and western blot analysis were used to quantitate mRNA and protein levels of NOS isoforms in the cremaster muscle following reperfusion. An additional 8 rats were used to determine the systemic effects of NPA administration. Mean arterial pressure (MAP) and heart rate (HR) were monitored using a non-invasive blood pressure analysis system prior to and during NPA infusion. Data analysis was performed with two-way repeated-measures ANOVA and one-way ANOVA to compare the PBS and NPA-treated groups. A p-value of <0.05 was considered statistically significant.

RESULTS: During the 90 min reperfusion period, average vessel diameters in the 10-20 µm arterioles (Figure 1) were significantly greater in the low dose (89.3-97.8 % of baseline) and high-dose (84.1-97.5%) NPA groups compared to control (70.1-79.4%) between 40-90 minutes of reperfusion. Vessel diameters in the 21-40 µm and 41-70 µm arterioles showed similarly significant improvement in the two NPA groups between 40-90 minutes. Furthermore, total blood flow in the low-dose (65.2-107.2% of baseline) and high-dose NPA (77.6-106.4%) groups vs. control (44.4-82.0%) was also significantly improved between 40-90 minutes. Improvements in vessel diameter and blood flow were found in the two NPA groups prior to 40 minutes (Figure 1); however, the difference was not statistically significant compared to controls. In addition, there was no significant difference in vessel diameter or total blood flow between the two NPA groups. MAP and HR did not vary significantly from baseline with infusion of NPA at 10 nmol/kg/hr or 100 nmol/kg/hr.

Following reperfusion, mRNA expression of iNOS and eNOS had a 40-fold and 1.3-fold increase from normal muscle, respectively, but nNOS mRNA expression was reduced to only 60% of normal. iNOS protein levels were increased to over 500% of normal muscle while eNOS and nNOS levels were 80% of normal. For both mRNA and protein expression, there were no statistically significant differences between the controls and the drug treated groups.

DISCUSSION: Our results show that NPA significantly increased vessel diameter and improved total blood flow in denervated skeletal muscle during early reperfusion. These results correlate with our earlier studies showing improved contractile function with NPA treatment. There was no additional improvement in the high dose NPA group, suggesting an increase in NPA dosage is not beneficial. Our results also show that a combined downregulation of eNOS and nNOS protein and upregulation of iNOS protein contributes to injury during early reperfusion. Since mRNA and protein levels of NOS were not significantly different between controls and NPA treated groups (Figure 2), the mechanism of NPA appears not to involve altered NOS expression. One potential mechanism of improved microcirculation by NPA may be related to the inhibition of nNOS activity. It is possible that NPA may have an alternative mechanism unrelated to NO/NOS regulation in vivo, despite in vitro studies having shown NPA to be a highly selective NOS inhibitor. In conclusion, our results showed that NPA significantly improves microcirculation in reperfused skeletal muscle, suggesting a reduction in I/R injury.