Introduction: Ischemia/reperfusion (I/R) injury is a considerable source of morbidity in replantation, resulting in decreased function and sometimes in loss of the replanted tissue. Pathology in I/R involves alterations in the microcirculatory environment not amenable to direct surgical repair. Possible sources of micropathology include edema, capillary plugging, free radical damage, vasospasm, and inflammatory cell recruitment. Nitric oxide (NO) is involved in each of these processes, whether as a pathological entity or a protector against damage. The role of NO is difficult to directly ascertain as it is a short-lived molecule undergoing many routes of local metabolism. Complicating the study of NO in vivo is the presence of at least three isoforms of NO synthase (NOS), which differentially regulate NO production in various locations in a particular organ. In skeletal muscle, endothelial (eNOS) and neuronal (nNOS) forms are constitutively produced, mediating vascular tone and regulating contractility, respectively. Inducible NOS (iNOS) is not detectable in normal skeletal muscle but is highly expressed in reperfused muscle. Studies using NOS inhibitors have produced conflicting results because the inhibitors lack sufficient isozyme selectivity. Recently, iNOS inhibitor 1400W was introduced for laboratory use; it exhibits 5000-fold and 200-fold selectivity for iNOS over that for eNOS and nNOS, respectively. In this study, we examined the effects of 1400W on contractile function and regulation of iNOS production in reperfused rat skeletal muscle.

Methods (approved by IACUC): Right hindlimb extensor digitorum longus (EDL) muscles in 91 female rats weighing 225-250g were divided into seven groups (n=13 each). In group 1 (sham operation), EDLs were surgically exposed, closed, and harvested 24 hrs later, corresponding to 3 h ischemia (I) and 24 hrs reperfusion (R). 3 mg/kg 1400W was injected subcutaneously 5 min prior to the start of sham reperfusion. Ischemia was produced in the remaining groups by exposing the EDL, isolating its vascular supply, ligating tendinous blood flow, and placing microclamps on the anterior tibial artery and vein, proximally and distally to its EDL branches, for a 3 hr period. Five minutes prior to reperfusion, 1400W or water (vehicle control) was injected s.c. Animals then underwent 3 or 24 hrs reperfusion. Group 2 received water and underwent 3 h R; group 3, 3 mg/kg 1400W, 3 h R; group 4, 10 mg/kg 1400W, 3 h R; group 5, water, 24 h R; group 6, 3 mg/kg 1400W, 24 h R; and group 7, 10 mg/kg 1400W, 24 h R. In each animal, the left EDL served as a normal control. Eight EDLs from each group were harvested at the end of reperfusion for contractile testing in an organ bath to test maximal twitch force produced, average force during tetanic stimulation at several frequencies, peak force during fatigue, and maintenance of force in fatigue. Data were expressed as percent of performance by contralateral (control) EDL.

Five EDLs from each group were harvested at the end of reperfusion and snap-frozen for Western blotting. Total protein was extracted from homogenized muscle, and concentration of each sample was determined by spectrophotometric assay. 50 mg total protein from each sample was separated using 7.5% SDS-PAGE and transferred to nitrocellulose. Mouse monoclonal antibody to iNOS was used for primary labeling, followed by detection with horseradish peroxidase-labeled secondary antibody. Films of ECL-detected blots were analyzed using band densitometry. Data were expressed as percent of enzyme level in contralateral (control) EDL. All data were analyzed using one-way ANOVA. Results: Contractile testing—After 24 hrs R, 1400W significantly improved contractile function at 3 mg/kg and 10 mg/kg doses (Fig. 1). EDLs in the sham group showed the greatest functional recovery. High dose 1400W (10 mg/kg) was most protective in three tests, achieving 58% of normal force in twitch compared to 36% for control (p<.01); 60% in tetanic testing at 40 Hz stimulation compared to 24% for control (p<.0001), and 54% at 70 Hz compared to 31% for control (p<.0001). Low dose 1400W (3 mg/kg) was most protective at 100 Hz, with an average recovery of 66%, compared to 49% for control (p<.01); and at 120 Hz, with recovery of 71% versus 54% for control (p<.02). Treatment with 3 mg/kg 1400W vs. 10 mg/kg produced similar functional recovery except at 40 Hz, where the higher dose group performed significantly better (p<.001). After 3 hrs R, no significant differences in contractile performance were noted between drug treated groups and controls.

Discussion: The results showed that highly specific inhibition of iNOS with 1400W resulted in significant functional recovery in skeletal muscle following 3- or 24 hr I/R, but not after 3 hrs R. iNOS expression was significantly attenuated in 1400W-treated groups after 3 hrs R and even more so after 24 hrs R. The effects of NO in I/R have been examined in many studies using various NOS inhibitors or NO donors, but conflicting findings have limited their acceptance. Our work represents the first use of a highly selective inhibitor of iNOS, 1400W, in a skeletal muscle I/R model, to clarify the role of this isozyme. Other available inhibitors have exhibited variable selectivity between isoforms that is enough to give different results with different inhibitors, but not enough to separate effects of each isoform.

iNOS is found primarily in neutrophils and macrophages, where its activity in rapid bursts contributes to lipid peroxidation and death of infected or damaged cells as part of the inflammatory process. Our finding that iNOS inhibition is protective in I/R is supported by our previous work showing the protective role of dexamethasone and of 21-aminosteroids, potent anti-inflammatory agents, in reperfused skeletal muscle. iNOS-mediated NO production has also been implicated in I/R in brain, gut, and cardiac muscle. Conclusions: 1) I/R magnifies iNOS mRNA expression in reperfused skeletal muscle, which was significantly down-regulated by a highly selective iNOS inhibitor 1400W. 2) Treatment with 1400W improves contractile function of skeletal muscle in late (24 hrs) reperfusion, suggesting that increased iNOS production during I/R may involve functional damage of reperfused skeletal muscle. Thus, inhibition of iNOS may find clinical application in replantations to protect against I/R injury.