SELECTIVE iNOS INHIBITOR, N-[3-(AMINOMETHYL) BENZYL]ACETAMIDINE ATTENUATES SKELETAL MUSCLE REPERFUSION INJURY IN EXTRACELLULAR SUPEROXIDE DISMUTASE DEFICIENT MOUSE
Jong Woong Park, M.D., Wen-Ning Qi, M.D., +Long-En Chen, M.D., James R. Urbaniak, M.D.
Orthopaedic Microsurgery Laboratory, Department of Surgery, Duke University Medical Center, Durham, North Carolina

INTRODUCTION:
Reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been considered as major contributors in the catastrophic Ischemia/reperfusion (I/R) injury. Excessively generated NO as a consequence of I/R readily reacts with superoxide anions (O?-) producing peroxinitrite (ONOOO) causing series of damages, including phospholipid peroxidation, loss of membrane integrity, intracellular calcium overload, and increase in capillary permeability. In 3 nitric oxide synthases (NOS), inducible NOS (iNOS) releases large amounts of NO2 and excessive NO results in cytotoxicity. Besides the NO regulation, excessive O?2 production is also limited by the superoxide dismutase (SOD), which dismutates O2? under the physiological condition. Among 3 isoenzymes of SOD, extracellular SOD (EC-SOD) is a major extracellular form especially in the vascular tissue. Recent evidence showed that EC-SOD knock-out (EC-SOD?) mice were significantly vulnerable to I/R injury suggesting increased O2? production due to EC-SOD deficiency as a principal cause. However, the relationship of NO and O2 in the skeletal muscle I/R injury was not defined clearly.

In this study, we hypothesize that selective inhibition of iNOS might attenuate the I/R injury in skeletal muscle of EC-SOD? mice by decreasing cytotoxic ONOOO level, a reaction product of O2? and NO. To verify this hypothesis, we observed the effects of a highly selective iNOS inhibitor, 1400W, on the skeletal muscle microcirculation in EC-SOD? mouse, which has deficient antioxidant activity against O2?.

MATERIALS & METHODS:
Male EC-SOD? mice weighing 25-30g were randomly divided into two groups, which received a subcutaneous injection of either 1400W (3 mg/kg, n=16) or the same volume of PBS (n=16), at 10 min before reperfusion.

Surgical procedure: The cremaster muscle flap only connected with its pedicle spread over a microscope stage. After the baseline data for the vessel diameter and blood flow were obtained, 4.5 h muscle ischemia was induced by clamping the pedicle. After the end of ischemia, the clamp was released and the vessel diameter and blood flow changes were measured at every 10 min intervals for 90 min of reperfusion.

Measurement of Vessel Diameter and Blood Flow: Intravital videomicroscope and Laser-doppler flowmetry were used. The internal vessel diameters of 10-70?m in selected areas were measured from the recorded image by a video-measuring gauge. For the blood flow measurement, doppler probe was positioned over the main arterial pedicle. At every 10 min from the start of reperfusion, the vessel diameter and total blood flow was measured and compared with the baseline value taken before the ischemia.

Histological Examination (Conventional and Immunohistochemistry): After the functional tests, the cremaster muscles were prepared for H & E staining. To determine the amount of ONOOO, immunohistochemistry was performed to detect nitrotyrosine, a marker of peroxynitrite. The cryotomed specimens treated with 0.3% hydrogen peroxide were incubated with monoclonal mouse anti-nitrotyrosine antibodies followed by biotinylated goat anti-rabbit antibody. The antibody binding sites were visualized using a DAB substrate kit. Negative controls were incubated with nonspecific serum rabbit IgG.

RESULTS
Vessel diameter and blood flow changes: The mean diameter of 10-20 ?m arterioles in 1400W-treated EC-SOD? mice was 86.2±2.5% of baseline at 10 min of reperfusion and increased to the maximum level of 115.1±2.6% at 90 min. PBS-treated EC-SOD? mice showed 53±4.5% of baseline at 10 min and 85.7±1.0% at 90 min. The 1400W-treated EC-SOD? mice showed significantly better and faster recovery than the PBS-treated EC-SOD? mice (Fig.1). Similar results were observed in 21- to 40 ?m and 41- to 70 ?m arterioles (p<0.001).

The mean blood flow measured at 10 min was 65.8±4.5% of base line in 1400W-treated EC-SOD? mice, and 16.4±3.4% in PBS-treated EC-SOD? mice. The blood flow was gradually increased and completely recovered reaching to 100.±4.6% in 1400W-treated EC-SOD? mice, while this value of PBS-treated EC-SOD? mice reached to 71.5±3.3% at the end of reperfusion. The blood flow recovery rate of 1400W-treated EC-SOD? mice was significantly different between the two groups at each time point (p<0.001).

DISCUSSION
This study revealed two major findings concerning skeletal muscle I/R injury. First, 1400W treatment decreases skeletal muscle I/R injury in EC-SOD? mice. This finding is well agreed with our previous results in which skeletal muscle I/R injury increases iNOS mRNA and protein levels and inhibition of iNOS improves functional recovery after I/R. Second, I/R injury results in excessive NO production, which is best demonstrated by the expression of nitrotyrosine (Fig. 2), a well-known marker of ONOOO interaction with tissue. ONOOO is a highly cytotoxic oxidant capable of causing lipid peroxidation and nitration of tyrosine residues on cellular proteins. The elevated levels of ONOOO might be expected in the situation in which the formation of both NO and O2? is increased. In the present study, augmented O2? production mediated by absence of EC-SOD in EC-SOD? mice might synergistically react with iNOS-induced excessive NO to generate ONOOO, thereby making profound I/R injury. Inhibition of iNOS, however, blocks excessive NO production to react with O2? consequently resulting less ONOOO formation.

In conclusion, our results suggest that ONOOO is a main cause of skeletal muscle I/R injury and selective iNOS inhibition can improve microcirculation in reperfused skeletal muscle. Decreased NO production from iNOS promotes this result perhaps via limiting cytotoxic ONOOO generation, a reaction product of NO and O2?.