

## SUMMARY OF RESEARCH (NAG2-1193)

### 1. Muscle unloading increases calpain activity and decreases NOS expression.

We have shown that modifications in muscle use result in changes in the expression and activity of calpains and nitric oxide synthase (NOS). Although muscle unloading for 10 days produced no change in the concentrations of calpain 1 or 2 and no change in calpain activation, muscle reloading produced a 90% increase in calpain 2 concentration. Muscle reloading caused no change in the activation index of calpain. However, this index is an expression of the proportion of the total mass of each calpain isoform that is autoproteolyzed, not a measure of the total mass of activated calpain present. Thus, there is approximately a 90% increase in activated calpain 2 during reloading which indicates that calpain may play an important role in muscle remodeling during reloading. Northern analysis showed a similar increase in calpain 2 mRNA during reloading, and in situ reverse polymerase chain reaction was used to show that the mRNA was located within the muscle fibers. We also examined changes in expression and concentration of molecules that regulate calpain activity and have found that mechanical loading is a positive regulator of NOS activity and expression. This is pertinent to understanding the regulation of calpain mediated proteolysis of muscle during modified muscle use because NO can inhibit calpain activity through S-nitrosylation of the active site in calpain.

### 2. Nitric oxide inhibits calpain activity.

We developed an in vitro model to test our hypothesis that nitric oxide can inhibit cytoskeletal breakdown in skeletal muscle cells by inhibiting calpain cleavage of talin. Talin was selected because it is a well-characterized calpain substrate and it is codistributed with calpain in muscle cells. C2C12 muscle cells treated with calcium ionophore showed increased talin proteolysis and evidence of talin release from the membrane into the cytosol. Nitric oxide prevented the proteolysis of talin and its release into the cytosol. In muscle cells given a more severe treatment with ionophore, NO inhibited cleavage of talin, loss of vinculin, cell detachment and loss of cellular protein. These results suggest that nitric oxide inhibition of calpain protected the cells from ionophore-induced proteolysis. Calpain inhibitor I and a cell permeable calpastatin peptide also protected the cells from proteolysis, confirming that ionophore-induced proteolysis was primarily calpain-mediated. In further experiments, the activity of purified m-calpain in a casein zymogram was inhibited by NO, and this inhibition was reversed by dithiothreitol. Prior incubation with the active site-targeted calpain inhibitor I prevented most of the sodium nitroprusside-induced inhibition of m-calpain activity. These data indicate that nitric oxide inhibited m-calpain activity via S-nitrosylation of the active site cysteine. The results of this study indicate that nitric oxide produced endogenously by skeletal muscle and other types of cells has the potential to inhibit m-calpain activity and cytoskeletal proteolysis.

### 3. Countermeasures to muscle mass loss during atrophy act in part through NO-dependent mechanisms.

We found that intermittent loading during hindlimb suspension that is sufficient to prevent muscle mass loss that occurs during muscle unloading is also sufficient to prevent the decrease in NOS expression that normally occurs during hindlimb unloading. This is consistent with our hypothesis that decreased NOS activity and expression that occurs during muscle unloading removes the negative inhibitory activity of NO on calpain, and thereby promotes calpain-mediated muscle wasting. The hypothesis was further tested by treating animals with NOS

inhibitors while they were subjected to countermeasures for muscle atrophy during hindlimb suspension. These experiments showed that NOS inhibition reduced the protective effect of countermeasures to muscle atrophy during unloading, which is consistent with the possibility that countermeasures to muscle mass loss during atrophy may act in part through NO-dependent mechanisms

### 3. Muscle wasting during unloading is mediated by calpain.

We generated a transgenic mouse with muscle specific overexpression of calpastatin, which is the endogenous inhibitor of calpains, and induced muscle atrophy by unloading hindlimb musculature for 10 days. Expression of the transgene resulted in increases in calpastatin concentration in muscle by 30 to 50-fold, and eliminated all calpain activity that was detectable on zymograms. Muscle fibers in ambulatory, transgenic mice were smaller in diameter, but more numerous, so that muscle mass did not differ between transgenic and non-transgenic mice. This is consistent with the role of the calpain/calpastatin system in muscle cell fusion that has been observed in vitro. Overexpression of calpastatin reduced muscle atrophy by 30% during the 10 day unloading period. In addition, calpastatin overexpression completely prevented the shift in myofibrillar myosin content from slow to fast isoforms, which normally occurs in muscle unloading. These findings indicate that therapeutics directed toward regulating the calpain/calpastatin system may be beneficial in preventing muscle mass loss in muscle injury, unloading and disease.

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