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## Role of nSMase-3/Ceramide Signaling in Muscle Atrophy

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## **Introduction and Background**

Inflammatory diseases such as cancer and congestive heart failure (CHF) may be accompanied by cachexia, a progressive wasting of muscle mass (atrophy). The proinflammatory cytokine TNF  $\alpha$  is known to play a role in causing cachexia. Cytokines act as signaling molecules that communicate between cells. TNF  $\alpha$  can stimulate neutral sphingomyelinase-3 (nSMase-3), an enzyme that hydrolyzes phospholipids in the cell membrane to produce ceramide, a lipid-signaling molecule. TNF  $\alpha$  is also known to activate the protein complex NF- $\kappa$ B, which controls the transcription of DNA. Abnormal regulation of NF- $\kappa$ B has been associated with cancer and other inflammatory diseases. Our data show that inhibition of nSMase-3 activity is linked to a decrease in p38 MAPK activity. NF- $\kappa$ B and p38 MAPK are signaling molecules that promote protein breakdown.

It has been established that TNF  $\alpha$  causes muscle atrophy and stimulates nSMase-3. However, we do not know very much about the role of nSMase/ceramide signaling in the atrophy response. Would inhibiting nSMase-3 activity preserve muscle mass? Our data show that nSMase-3 promotes loss of muscle proteins, actin and myosin, suggesting that nSMase-3 is a key regulator in muscle wasting. Based on these findings, we hypothesized that increasing activity of nSMase-3 causes skeletal muscle wasting as evidenced by a decrease in myotube width and degradation of muscle proteins actin and myosin.

## **Experimental Methodology and Results**

A cell culture model of cachexia was used. Cell cultures of C2C12 myotubes were divided into four treatment groups as follows: 1.) wildtype control, 2.) nSMase-3 deficient control, 3.) wildtype + TNF  $\alpha$ , and 4.) nSMase-3 deficient + TNF  $\alpha$ . Experimental myotubes were treated with TNF  $\alpha$  once every 24 hours for three days. The wildtype control did not undergo any experimental intervention. The nSMase-3 deficient control had inhibited activity of nSMase-3.

### *Inhibiting expression of nSMase-3*

Small interfering RNA (siRNA) is a category of double stranded RNA molecules that can be used to inhibit gene expression. For the purposes of this study, siRNA that is nSMase-3 specific was used to inhibit gene expression, resulting in mRNA degradation. As a result, mRNA expression should decrease. Changes in mRNA expression were measured using real time PCR. Results indicated that mRNA expression had markedly decreased indicating that the knockdown of nSMase-3 was successful.

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## *Measuring nSMase-3 total protein content*

The nSMase-3 total protein content was also measured using Western blotting. Our results indicated that nSMase-3 total protein was lower in nSMase-3 knockdown myotubes compared to those myotubes that were not nSMase-3 deficient (i.e. wildtype control, wildtype + TNF  $\alpha$ ).

## *Measuring actin and myosin protein content*

Major muscle proteins actin and myosin were measured to determine the degree of muscle wasting in each of the treatment groups. Cultured C2C12 myotubes were transfected with nSMase-3 specific siRNA to knockdown gene expression. We used Western blot to measure protein content. Myosin and total protein were elevated compared to the treatment groups that were not nSMase-3 deficient (i.e. wildtype control, wildtype + TNF  $\alpha$ ). Myotubes were also transfected with pAcGFP fusion plasmid to induce overexpression of nSMase-3. Total protein and myosin proteins were decreased.

## *Measuring myotube width*

Measuring myotube width also serves as a direct method of investigating the extent of muscle atrophy. Myotube images were taken under a specialized microscope from each treatment group. From these images, myotube width was directly measured using image analysis software such as Image J. Myotube width is directly proportional to myosin and actin content. Cultured C2C12 myotubes were pretreated with nSMase-3 siRNA or nonsense siRNA then treated with vehicle (control) or TNF  $\alpha$ . Myotubes pretreated with nSMase-3 siRNA and then TNF  $\alpha$  showed an increase in myotube width compared to the control. On the other hand, myotubes pretreated with nonsense siRNA and then TNF  $\alpha$  showed a decrease in myotube width with respect to the control.

## **Conclusions**

The outcome of the research seems to be very promising. As expected, inhibiting expression of nSMase-3 showed an increase in myosin and total protein and overexpression of nSMase-3 resulted in a decrease in protein content. These findings further suggest that nSMase-3 may be a key regulator in muscle wasting. More experiments are being conducted to investigate catabolic signaling pathways associated with nSMase-3. For example, we are now measuring I $\kappa$ B total protein as well as p38 MAPK phosphorylation, both markers of catabolic signaling.