

Characterization of neutral sphingomyelinase activity and isoform expression in rodent skeletal muscle mitochondria

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Abstract

Skeletal muscle is composed of fiber types that differ in mitochondrial content, antioxidant capacity, and susceptibility to apoptosis. Ceramides have been linked to oxidative stress-mediated apoptotic intracellular signalling and the enzyme neutral sphingomyelinase (nSMase) is, in part, responsible for generating these ceramides through the hydrolysis of sphingomyelin. Despite the role of ceramides in mediating apoptosis, there is a gap in the literature regarding nSMase in skeletal muscle mitochondria. This study aimed to characterize total nSMase activity and individual isoform expression in isolated subsarcolemmal (SS) mitochondria from soleus, diaphragm, plantaris, and extensor digitorum longus (EDL). Total nSMase activity did not differ between muscle types. nSMase2 content was detectable in all muscles and higher in EDL, soleus, and plantaris compared to diaphragm whereas nSMase3 was undetectable in all muscles. Finally, total nSMase activity positively correlated to nSMase2 protein content in soleus but not the other muscles. These findings suggest that nSMase associated with SS mitochondria may play a role in intracellular signalling processes involving ceramides in skeletal muscle and nSMase2 may be the key isoform, specifically in slow twitch muscle like soleus. Further studies are needed to fully elucidate the specific contribution of nSMase, along with the role of the various isoforms and mitochondrial subpopulation in generating mitochondrial ceramides in skeletal muscle, and its potential effects on mediating apoptosis.

1.1 Introduction

Skeletal muscle is a highly dynamic and metabolically active organ. It is composed of various muscle fibers, which differ in oxidative capacity, mitochondrial content (Delp and Duan, 1996; Schiaffino and Reggiani, 2011; Schiaffino and Serrano, 2002; Tavi and Westerblad, 2011), and more importantly, susceptibility to apoptosis (Ciciliot et al., 2013). Skeletal muscle can undergo apoptosis as a result of aging (Dirks and Leeuwenburgh, 2002), prolonged periods of disuse (Powers et al., 2012), denervation, cancer, cachexia, starvation (Schwartz, 2008), and muscle myopathies such as Duchenne muscular dystrophy (Imbert et al., 1995; Millay et al., 2009; Robert et al., 2001).

There are two forms of apoptosis, extrinsic and intrinsic, with activation originating at the cell surface or the mitochondria, respectively (D'Arcy, 2019). The intrinsic pathway of apoptosis can be triggered by a variety of signals, such as high mitochondrial calcium, oxidative stress, and mitochondrial membrane permeability (D'Arcy, 2019). Ceramides, a central sphingolipid, have been extensively linked to increased mitochondrial permeability prior to apoptosis (Novgorodov et al., 2005; Rego et al., 2012; Siskind et al., 2006; Turpin et al., 2006). The hydrolysis of sphingomyelin is believed to be a primary way of generating ceramides, which is facilitated by neutral sphingomyelinase (nSMase).

Currently, four main isoforms of nSMase have been identified, nSMase1, 2, 3, and mitochondrial-associated (MA)-nSMase (Airola and Hannun, 2013). nSMase1 does not appear to possess *in vivo* SMase activity (Sawai et al., 1999; Tomiuk et al., 2000) and nSMase1 KO mice have no apparent metabolic or phenotypic defects or abnormalities (Zumbansen and Stoffel, 2002). nSMase2 has been the most studied isoform and previous research has demonstrated that nSMase2 is primarily found on the Golgi which translocates to the inner leaflet of the plasma membrane in response to oxidative stress (Airola and Hannun, 2013; Levy et al., 2006) and is highly expressed in the brain with limited mRNA and protein expression in skeletal muscle (Hofmann et al., 2000; Wu et al., 2010a).

Interestingly, despite limited expression in skeletal muscle, its absence (nSMase2 knockout) attenuated muscle degeneration and improved muscle function in the rodent model of Duchenne muscular dystrophy (Matsuzaka et al., 2020). In contrast, nSMase3 mRNA is highly expressed in skeletal muscle (Krut et al., 2006; Moylan et al., 2014) and appears to be involved in oxidant signalling (Ferreira et al., 2010; Moylan et al., 2014). Finally, MA-nSMase is a mitochondrial specific nSMase highly expressed in brain, testis, pancreas, and epididymis but not skeletal muscle (Wu et al., 2010b) and contains a specific sequence of amino acids which tethers the protein to the outer mitochondrial membrane, and the catalytic subunit of the enzyme is on the cytosolic side, which suggests that this enzyme plays a role in generating ceramides on the outer mitochondrial membrane (Rajagopalan et al., 2015).

Fiber type isoform expression and mitochondrial localization of nSMases can serve as an important factor in ROS mediated signalling and apoptotic cell death. Importantly, different skeletal muscle types have been shown to differ in their ability to defend against oxidative stress, as slow oxidative muscles are equipped with a better antioxidant defense system to counteract ROS compared to fast glycolytic (Loureiro et al., 2016; Pinho et al., 2017). Previous research has demonstrated that total nSMase in skeletal muscle at the whole tissue level did not differ between muscle types (Błachnio-Zabielska et al., 2011; Moylan et al., 2014) and nSMase 2 and 3 mRNA were detected in mixed skeletal muscle, but nSMase activity and isoform expression had not been examined at the level of the mitochondria. This leads to question whether ceramide generating enzymes such as nSMase in the mitochondria of skeletal muscle play a role in the differential susceptibility to apoptosis experienced by muscle with varying fiber type compositions. Thus, the purpose of the study was to characterize total nSMase activity and individual isoform content, with a focus on nSMase2 and 3 given their putative roles in skeletal muscle, in mitochondria isolated from skeletal muscle that represent the spectrum of fiber type compositions.

1.2 Methods

1.2.1 Animals, tissue collection, and mitochondrial isolation

Nine male Wistar rats were purchased from Charles River (14 weeks of age). Animals were housed in a climate and temperature-controlled room with access to food and water *ad libitum*. This study was approved by the Animal Care Committee at Brock University. Animals were anaesthetized using isoflurane (2-5% at 1-2 L/min flow rate). Soleus, plantaris, extensor digitorum longus muscles were dissected from each hind limb, and diaphragm was removed after all other muscle were dissected. Subsarcolemmal (SS) mitochondria from each muscle were isolated as per previous reported (Peters et al., 2001; Stefanyk et al., 2010).

1.2.2 Neutral sphingomyelinase activity

nSMase activity was assessed by measuring the conversion of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) to the fluorescent by-product resorufin as previously reported (Moyle et al., 2014) with the following modification to ensure maximal activity. Isolated SS mitochondria were diluted to 1µg/µL in water, and a total of 20µg of mitochondrial protein was mixed in a 96-well plate with 0.4U horseradish peroxidase, 1.3U choline oxidase, 1.6U alkaline phosphatase, 0.05mM ADHP and brought up to a total volume of 200µL with 1x reaction buffer (0.1mM Tris-HCl, 10mM MgCl₂, pH 7.0). Triton-X was omitted from the mixture, as it has been previously found to interfere with nSMase activity (Moyle et al., 2014). The reaction was incubated at 37°C and fluorescence (530 nm excitation, 590 nm emission) was monitored in the absence (background) and presence of 0.5 mM sphingomyelin (SM). Slopes were calculated as the change in fluorescence over time minus background.

1.2.3 Western blot analysis

Western blot analysis was conducted on isolated SS mitochondria to determine the protein content of nSMase2 and nSMase3. Isolated SS mitochondria were diluted to 1 μ g/ μ L in Laemmli buffer (Laemmli, 1970) and electrophoretically separated using 7.5% standard glycine-based SDS-PAGE. Separated proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad, Mississauga, ON) using a semi-dry Trans-Blot Turbo system (Bio-Rad). Membranes were immunoprobed with primary antibodies directed against nSMase2 and nSMase3 (ECM Biosciences, Versailles, KY) diluted in 5% (w/v) milk in tris-buffered saline then anti-rabbit horseradish peroxidase conjugated secondary antibody (Jackson Immuno Research Labs, West Grove, PA), and visualized with Clarity Western ECL substrate (Bio-Rad Inc) and the Li-Cor imaging system (LI-COR Biosciences, Lincoln, NE). Ponceau stain (Bioshop, Burlington, ON) was used to normalize protein loading.

1.2.4 Statistical analysis

All results are expressed as the mean \pm standard error. All statistical analyses were performed using SPSS Statistics for Windows, version 25 (SPSS Inc., Chicago, Ill., USA). Comparisons among isolated SS mitochondria from the four different muscles were made using one-way ANOVA. A Tukey's multiple comparison post hoc test was used to compare the means, with significance accepted at $p \leq 0.05$. Associations between total nSMase activity and nSMase2 protein content were determined using one-tailed Pearson r correlation analyses.

1.3 Results and discussion

To our knowledge, this is the first study to examine neutral sphingomyelinase activity and protein content in SS mitochondria isolated from skeletal muscle of varying fiber type compositions. Due to intrinsic differences between muscle fiber types, specifically type II fibers having greater

sensitivity to apoptosis and oxidative stress, it was hypothesized that nSMase content and activity would be highest in SS mitochondria isolated from predominantly type II fibers compared to predominantly type I. The major findings of the study were 1) total nSMase activity was not found to be significantly different in SS mitochondria isolated from the various muscles when expressed per mg mitochondrial protein content, 2) nSMase2 was the predominate isoform detected in isolated SS mitochondria in all muscles examined whereas nSMase3 was not detected, and 3) although not statistically significant, total nSMase activity was positively correlated with nSMase2 protein content in SS mitochondria isolated from soleus and negatively correlated in plantaris and EDL and to a lesser degree in diaphragm.

1.3.1 Total nSMase activity was not dependent on muscle fiber type predominance

Total neutral sphingomyelinase activity expressed per mg of mitochondrial protein in SS mitochondria did not differ between muscle types (Fig 1). Despite the novelty of examining nSMase activity at the level of mitochondria, these findings are consistent with previously published results examining whole muscle homogenates demonstrating that nSMase activity did not differ between soleus, red gastrocnemius, and white gastrocnemius (Błachnio-Zabielska et al., 2011) or between soleus, extensor digitorum, and diaphragm (Moyle et al., 2014). The lack of observed differences could stem from a variety of factors. First, the regulation of nSMase activity, specifically nSMase2 being the most studied isoform, appears to be highly coordinated and independent processes. nSMase activity can be increase by changing its oligomeric state to more monomers (Dotson et al., 2015), binding of the co-factor Mg^{2+} (Ago et al., 2006) or anionic phospholipids such as phosphatidylserine, phosphatidic acid, and cardiolipin (Hofmann et al., 2000; Marchesini et al., 2003; Shanbhogue et al., 2019), serine phosphorylation (Filosto et al., 2012), and enhanced membrane anchoring mediated by palmitoylation of cysteine residues (Tani and Hannun, 2007). Second, SS mitochondria are not

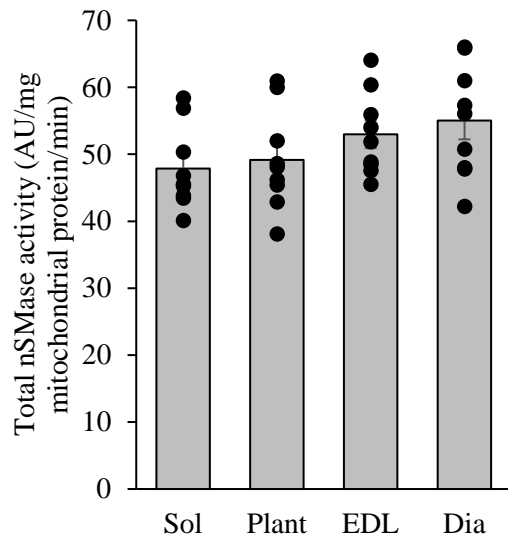


Figure 1. Total nSMase activity in isolated subsarcolemmal mitochondria. Values are expressed as mean \pm standard error (n=9). Sol, soleus; Plant, plantaris; EDL, extensor digitorum longus; Dia, diaphragm.

localized proximally to calcineurin, a putative regulator of nSMase activity (Filosto et al., 2010; Goldkorn and Filosto, 2010) which is found primarily along the contractile units and transverse tubules (Frey and Olson, 2002; Frey et al., 2000; Ortenblad et al., 2018; Torgan and Daniels, 2006) where intermyofibrillar (IMF) mitochondria are found (Ogata and Yamasaki, 1985). Finally, SS mitochondria do not appear to be as susceptible to ROS mediated apoptosis compared to their IMF counterpart (Adhiketty et al., 2007), suggesting SS mitochondria may experience greater control on enzymes that promote pro-apoptotic processes, such as nSMase. Future research should examine nSMase activity in the different mitochondrial subpopulations and in the face of apoptotic triggers such as oxidative stress.

1.3.2 nSMase2 is expressed in all muscles examined and is higher in SS mitochondria from limb muscles compared to diaphragm

nSMase2 protein content was detectable in all muscles examined (Fig 2). This is a novel finding as previous research did not detect nSMase2 in whole homogenate of mixed muscle (Hofmann et al., 2000). In addition, and somewhat supportive of no difference in total nSMase activity between SS mitochondria isolated from muscles with different fiber type compositions, nSMase2 protein content was also similar between limb muscles but significantly lower in diaphragm. Although not statistically significant, a higher nSMase2 protein content in fast twitch EDL may have biological significance. A well known by product of contraction is the generation of free radicals and resulting oxidative stress (Powers et al., 2011). Compared to slow twitch, fast twitch muscles have a greater capacity to generate free radicals (Loureiro et al., 2016; Pinho et al., 2017) but have lower levels of antioxidants (Pereira et al., 1994; Picard et al., 2008; Qaisar et al., 2016), making them more susceptible to oxidative stress-mediated apoptosis (Kannan and Jain, 2000). Previous research has shown that nSMase2 plays an important role in apoptotic cell signalling mediated by excessive oxidative stress (Filosto et al., 2010). As such, nSMase2 may be the key isoform contributing to oxidative stress-mediated apoptotic cell

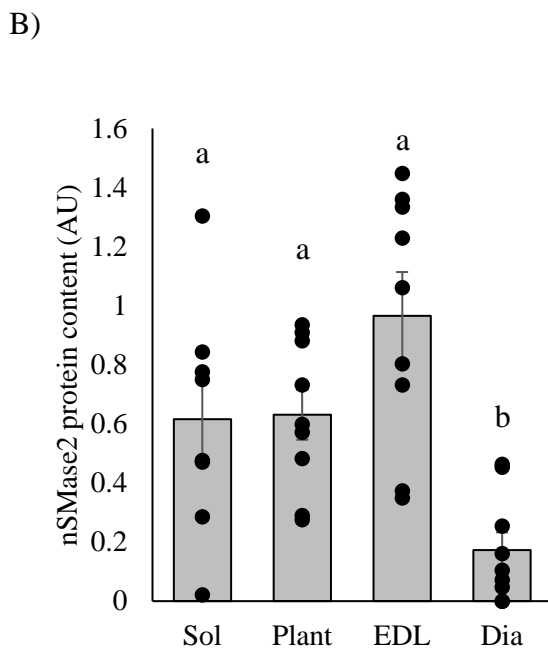
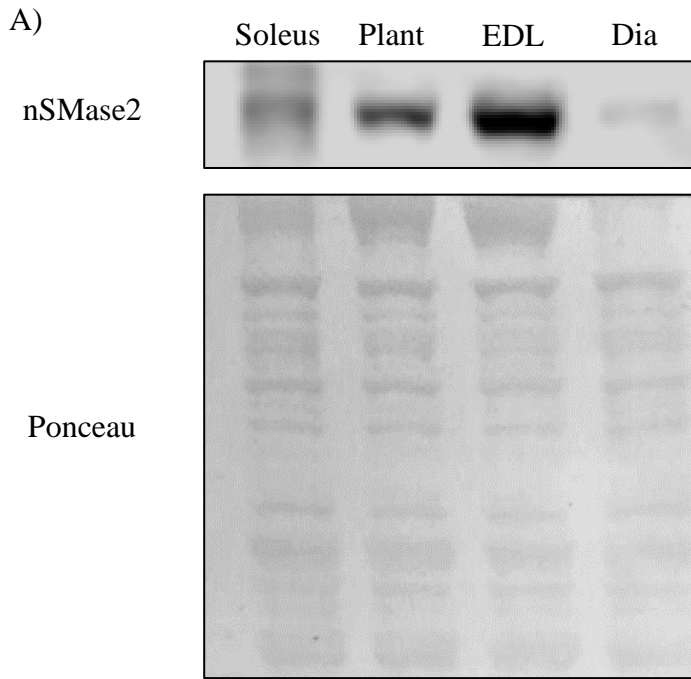


Figure 2. nSMase2 protein content in subsarcolemmal mitochondria isolated from various muscle groups. A) Representative western blot of nSMase2 (predicted molecular weight of 80 kDa) and Ponceau stain with each lane being loaded with 25 μ g of mitochondrial protein. B) Mean arbitrary units (AU) \pm standard error (n=9) standardized to total protein using Ponceau staining. Values with different letters indicate significant difference from each other ($p < 0.05$). Sol, soleus; Plant, plantaris; EDL, extensor digitorum longus; Dia, diaphragm.

signalling in fast twitch muscle. In contrast, ROS production and oxidative stress in diaphragm is more common during heat and hypoxic stress, and respiratory diseases (e.g. COPD)(Zuo et al., 2015). As a result, lower nSMase2 protein content in diaphragm compared to limb muscles may be related to a reduced reliance on ceramide-mediate cell signalling associated with oxidative stress or the use of alternative pathways. Future research should examine the role of nSMase2 in skeletal muscle by examining nSMase activity in the presence of exacerbated oxidative stress and if the response is attenuated in the recently generated nSMase2 knockout mouse (Matsuzaka et al., 2020).

1.3.3 nSMase3 is not detectable in SS mitochondria isolated from skeletal muscle

nSMase3 was below the level of detection in isolated SS mitochondria from all muscles examined (Fig 3). This finding was interesting as previous research has shown nSMase3 mRNA to be highly expressed in skeletal muscle (Krut et al., 2006; Moylan et al., 2014). Compared to nSMase2, nSMase3 is less studied with some question regarding a clear biological role (Airola and Hannun, 2013), it is thought to be primarily localized to the mitochondria associated membranes in various cell types, including skeletal muscle (Airola and Hannun, 2013; Corcoran et al., 2008; Krut et al., 2006; Moylan et al., 2014). SS mitochondria are more closely associated with the sarcolemma whereas IMF mitochondria are found between the myofibrils and have been shown to physically interact with SR in skeletal muscle (Boncompagni et al., 2009; Eisner et al., 2014). Given the detection of nSMase3 in whole muscle homogenates, due to potential colocalization, nSMase3 may co-localize with IMF rather than SS mitochondria, preventing detection in the SS mitochondrial fraction. Future research should examine IMF mitochondria and nSMase3 protein content.

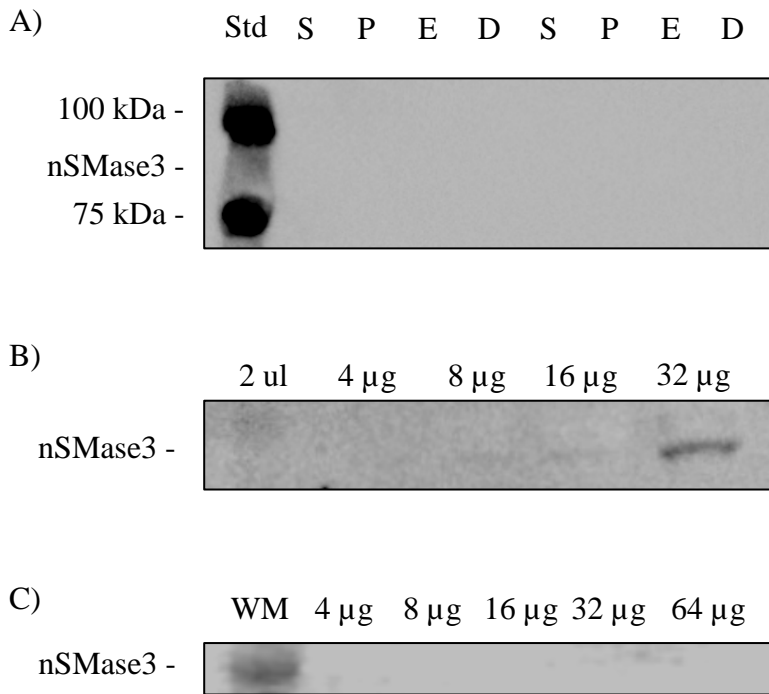


Figure 3. nSMase3 content in whole muscle and subsarcolemmal mitochondria isolated from various muscle groups. A) nSMase3 was not detectable in 25 μ g of isolated subsarcolemmal mitochondrial protein in all muscles examined. B) nSMase3 in whole muscle homogenates was detectable in as low as 8 μ g of loaded protein, where the rabbit polyclonal antibody raised against the C-terminal region detected nSMase3 at 85 kDa as per the supplier. C) When compared to 25 μ g of loaded protein from whole muscle homogenates, nSMase3 was not detectable in subsarcolemmal mitochondria isolated from diaphragm up to 64 μ g of mitochondrial protein. Std, molecular weight standard; S, soleus; P, plantaris; E, extensor digitorum longus; D, diaphragm; WM, whole muscle.

1.3.4 nSMase2 content does not correlate with total nSMase activity

Given that nSMase2 was the only isoform examined that was detected in SS mitochondria isolated from various muscles, correlation analysis revealed that nSMase2 protein content did not correlate with total nSMase activity in any of the muscles examined (Fig 4). This finding may be due to nSMase2 not being the only functional nSMase isoform in skeletal muscle SS mitochondria. Due to previous research suggesting other nSMase isoforms either not having *in vivo* SMase activity or not being expressed in skeletal muscle, future research will have to reassess these findings and determine which nSMase isoform is contributing to total nSMase activity. Alternatively, as previously stated, future research could examine this relationship by using the nSMase2 knockout mouse (Matsuzaka et al., 2020).

Conclusion

This study is the first to report total nSMase activity and nSMase2 protein expression in SS mitochondria isolated from skeletal muscle. The role that nSMase plays in mitochondrial mediated apoptosis through its ability to generate ceramides is still largely unknown, however this study provides a foundation for future research. Further studies are needed to fully elucidate the specific contributions of nSMase2&3 isoforms in generating mitochondrial ceramides in skeletal muscle, and its potential effects on mediating apoptosis.

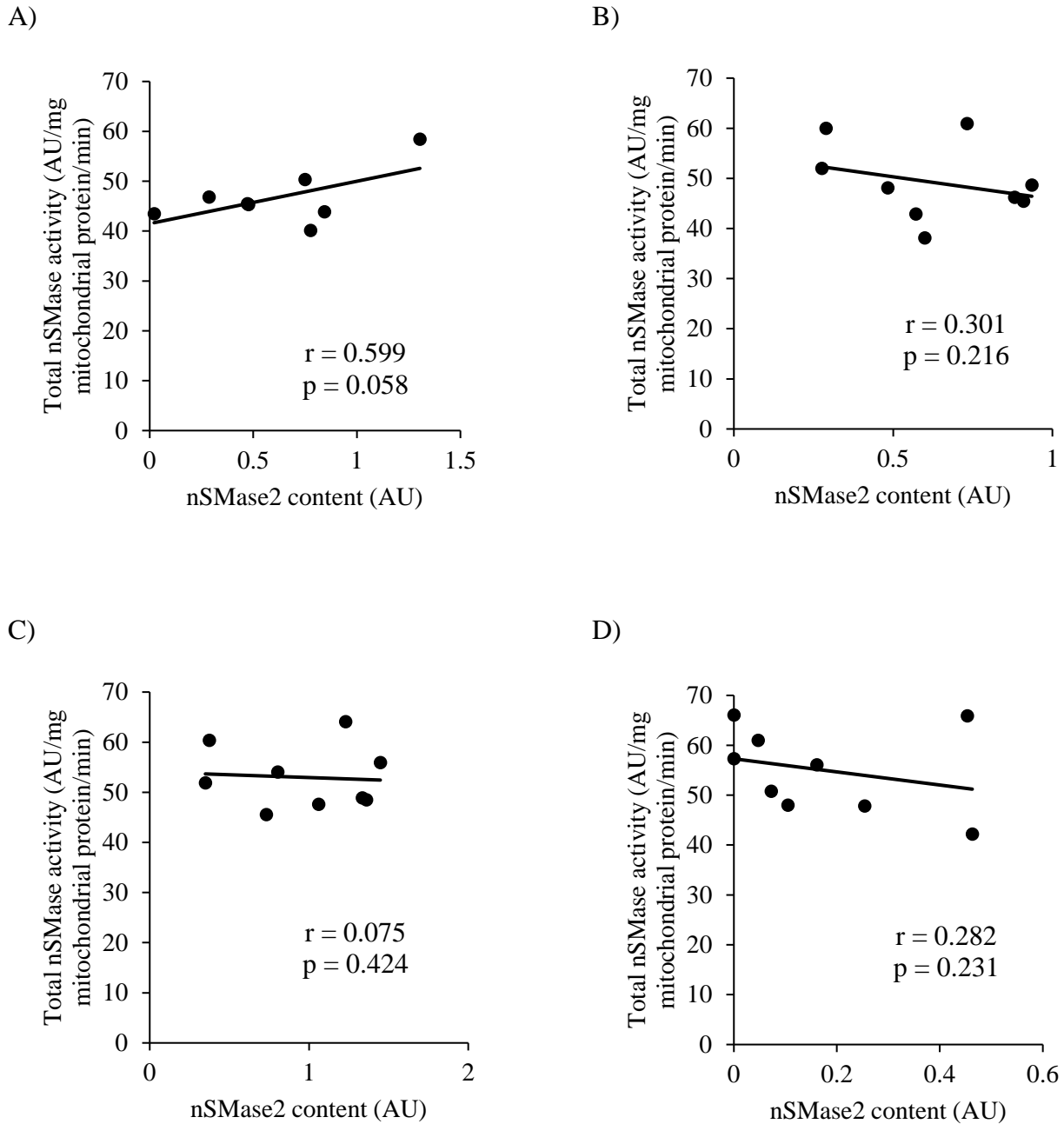


Figure 4. Correlation between total nSMase activity and nSMase2 protein content in subsarcolemmal mitochondria isolated from different muscle groups. A non-significant positive correlation was observed between total nSMase activity and nSMase2 protein content in subsarcolemmal mitochondria isolated from A) soleus (p=0.058), but not B) plantaris, C) EDL, or D) diaphragm.

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Conflict of Interest

The authors have none to declare.

Authors Contributions

SS performed the experiments, analyzed the data, and wrote the manuscript. JAW assisted in working up the peroxidase activity assay and edited the manuscript. PJJ developed the overall study, analyzed the data, and wrote and edited the manuscript.

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