SUPPLEMENTAL MATERIAL

# METHODS

## *Metabolomics*

Metabolites from cell pellets from myotubes in control and enhanced dSL conditions were extracted with cold 5:3:2 methanol:acetonitrile:water, as previously described (1). Untargeted metabolomics data was acquired using a Thermo Vanquish UHPLC coupled online to a Thermo Orbitrap Exploris 120 mass spectrometer. Metabolites were separated using a 5 min C18 gradient for positive and negative ion modes as previously described in detail (2). Metabolites were annotated and integrated using Maven in conjunction with the KEGG database.

## *Phosphatidylserine LC-MS/MS analysis*

An internal standard cocktail containing phosphatidylserine (PS) 17:0/17:0 (300 pmol) was added and to cell homogenates from myotubes in control and enhanced dSL conditions and lipid extraction was performed according to Matyash et al. (3). For phospholipid analysis, samples were injected into an HPLC system connected to a triple quadrupole mass spectrometer (Sciex 3200, Framingham, MA) and normal phase chromatography was performed using a silica column (150x2 mm, Luna Silica 5 µm, Phenomenex). The mobile phase system consisted of solvent A (isopropanol/hexane/water (58/40/2, v/v)) and 35% solvent B (hexane/isopropanol/water (300/400/84, v/v/v) both containing 10 mM ammonium acetate. Mass spectrometric analysis was performed for 25 molecular species of PS in the negative ion mode using multiple reaction monitoring (MRM). This method monitors a set of collision-induced mass transitions

corresponding to the production of 16:0 (*m/z* 255.2), 16:1 (*m/z* 253.2), 18:0 (*m/z* 283.2), 18:1 (*m/z* 281.2), 18:2 (*m/z* 279.2), 20:4 (*m/z* 303.2), 20:5 (*m/z* 301.2), 22:4 (*m/z* 331.2), 22:5 (*m/z* 329.2), and 22:6 (*m/z* 327.2) carboxylate anions from the $[M-H]^-$ of PS molecular species. Quantitative results were determined using stable isotope dilution with standard curves for saturated and unsaturated PS molecular species and results were normalized to protein content.

*Thiobarbituric Acid Reactive Substances (TBARS) Assay*

TBARS assay was performed on myotubes in control and enhanced dSL conditions. The equivalent of about 2 x $10^7$ cells was homogenized in PBS on ice and lipid peroxidation was assessed by measuring malondialdehyde (MDA) cell homogenate content using a colorimetric (535 nm) TBARS Assay (Cayman Chemical), following manufacturer instructions.

RESULTS and CONCLUSION

We performed untargeted metabolomics profiling, with particular focus on central energy metabolism. Of the 160 named metabolites profiled (4), there were no significant differences between control and elevated dSL groups, after performing multiple comparison testing using the Holm-Šídák method. When we performed the same test on smaller metabolite groups, focused on specific pathways, the only metabolite that showed a significant difference was L-serine that was decreased, as expected, in the enhanced dSL group (2X L-alanine/glycine and 0.1X L-serine) ($p=0.0323$). A summary of the results from this analysis is shown using the Principal Components Analysis (PCA) plot in Figure S1. The principal components are overlapping and suggest that

altering L-alanine, glycine, and L-serine concentrations did not affect the global metabolite profile compared to control conditions.

We also conducted LC-MS/MS analysis of phosphatidylserines in myotube homogenates from cells in both control and enhanced dSL conditions. As shown in Figure S2, we could not detect any significant differences in the levels of these serine-containing phospholipids between the two treatments. These data indicate that the low L-serine concentration in our enhanced dSL condition was not low enough to alter the concentration of serine-dependent phospholipids.

Finally, when we performed a TBARS assay on cell lysates from myotubes manipulated *in vitro* to synthesize more dSL, we did not observe any significant differences in malondialdehyde (MDA) synthesis between cells in control conditions vs increased dSL concentrations (Figure S3), indicating that the increased dSL levels we observe in our *in vitro* protocol are not causing oxidative stress and lipid peroxidation.

To summarize, the data provided here show that the changes in the media amino acid concentrations we achieve in our *in vitro* experimental model are not significantly affecting the general myotube metabolic profile, phosphatidylserine levels, and cell oxidative stress. This further strengthens the feasibility of our protocol as a tool for investigating the role of elevated intracellular dSL levels in affecting skeletal muscle cell sensitivity to insulin.

FIGURES

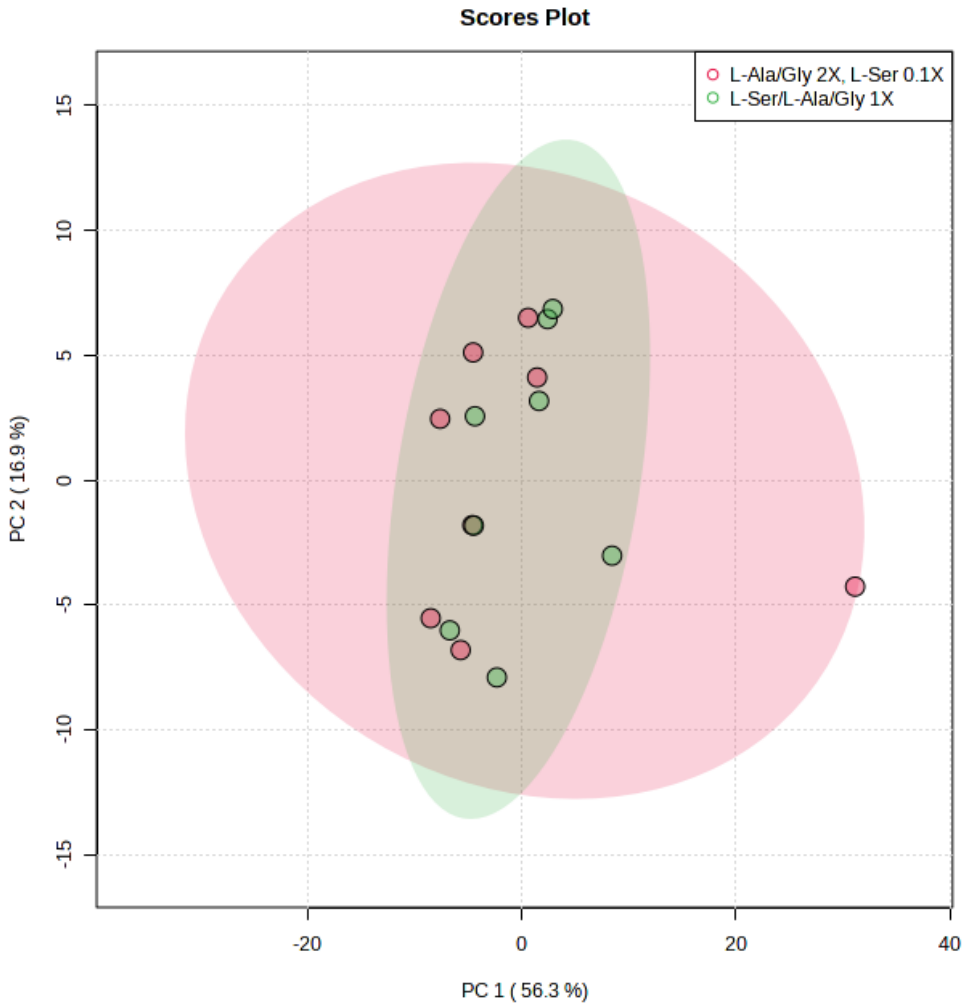


Figure S1: **Principal Component Analysis.** PCA plot summarizing the relationship between 160 different metabolites detected by metabolomics profiling in primary myotubes grown in control (1X L-serine/L-alanine/glycine) or enhanced (2X L-alanine/glycine and 0.1X L-serine) dSL conditions. n=4, with two technical replicates each.

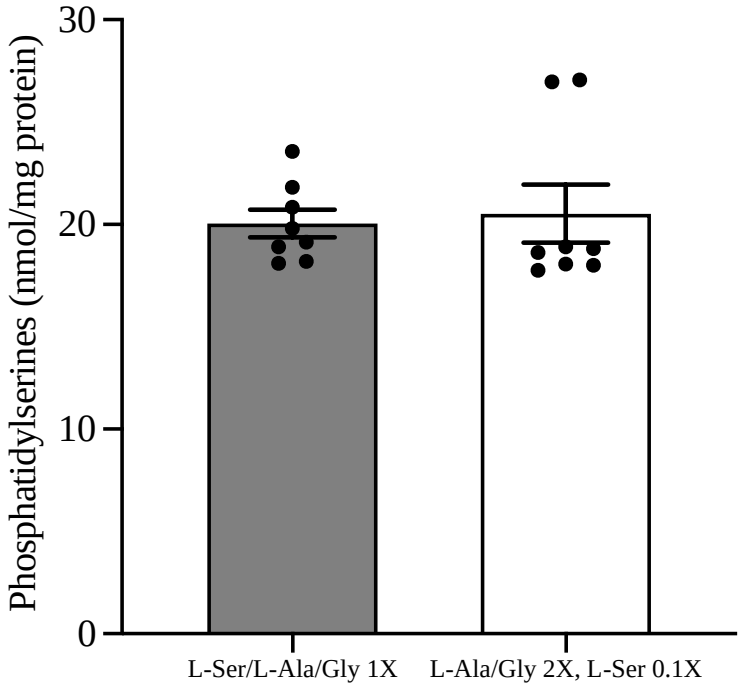


Figure S2: **Phosphatidylserines are not altered in myotubes grown in media manipulated to alter intracellular dSL content.** Effect of media amino acid content on total phosphatidylserine content in primary myotubes treated for 48 hours in differentiation media containing 1X L-serine/L-alanine/glycine or 2X L-alanine/glycine and 0.1X L-serine. Values are means ± SEM, n = 8.

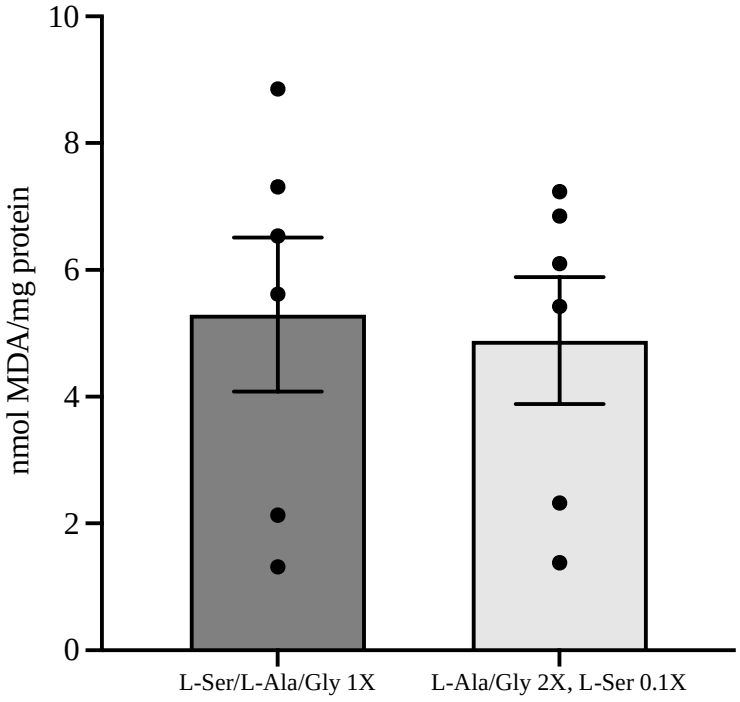


Figure S3: **Oxidative stress and lipid peroxidation are not altered in myotubes grown in media supplemented with different amino acid concentrations.** Effect of media supplementation with 1X L-serine/L-alanine/glycine or 2X L-alanine/glycine and 0.1X L-serine on primary myotube lipid peroxidation. MDA formation was measured using a TBARS assay. Values are means ± SEM, n = 6.

REFERENCES

1. Nemkov T, D'Alessandro A, Hansen KC: Three-minute method for amino acid analysis by UHPLC and high-resolution quadrupole orbitrap mass spectrometry. Amino Acids 2015;47:2345-2357
2. Nemkov T, Reisz JA, Gehrke S, Hansen KC, D'Alessandro A: High-Throughput Metabolomics: Isocratic and Gradient Mass Spectrometry-Based Methods. Methods Mol Biol 2019;1978:13-26
3. Matyash V, Liebisch G, Kurzchalia TV, Shevchenko A, Schwudke D: Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res 2008;49:1137-1146
4. Nemkov T, Hansen KC, D'Alessandro A: A three-minute method for high-throughput quantitative metabolomics and quantitative tracing experiments of central carbon and nitrogen pathways. Rapid Commun Mass Spectrom 2017;31:663-673