SUPPLEMENTARY DATA

Supplemental Research Design and Methods

Muscle triacylglycerol and diacylglycerol concentrations

Frozen muscle (30-40 mg) was rapidly homogenized in 1.0 ml ice-cold 0.9% saline, and lipids then extracted overnight at 4°C in a single-phase mixture of chloroform-methanol-aqueous homogenate (1:2:0.8, v/v/v) (1). Internal lipid markers for triacylglycerol (TAG), DAG, monoacylglycerol, non-esterified fatty acid (NEFA), phospholipid (PL), and cholesterol ester having fatty acid moieties of odd carbon number were added at the start of extraction, for subsequent purity and recovery determinations (Nu-Chek Prep Inc., Elysian, MN, USA; Avanti Polar Lipids Inc., Alabaster, AL, USA). TAG, DAG and PL markers were each homogenous in fatty acid content (e.g., $[C23:0]_3$-TAG). After lipid extraction, individual lipid species were eluted using previously described solvent mixtures (2). Column fractions were dried, and those containing either purified TAG or DAG were reconstituted in 100 µl toluene. Fatty acid methyl esters (FAMEs) were then generated via alkaline methanolysis, a transesterification process (2), by addition of 1.0 ml 0.2N NaOH in methanol having ultra-low H_2O content, in order to exclude hydrolysis. Individual FAMEs were purified by gas chromatography with capillary column (Agilent Technologies). FAMEs were detected by electron-impact mass spectrometry with selective ion monitoring, and quantified using FAME standards.

Muscle ceramide concentration

Analysis of skeletal muscle ceramide concentration was performed after lipid extraction via liquid chromatography-triple quadrupole mass spectrometry (LC-QQQ; Agilent Technologies 6410 Triple Quadrupole Mass Spectrometer). In brief, samples were extracted by a mixture of methanol, chloroform, and water (1) supplemented with internal ceramide standards. The lipid extract was dried under nitrogen gas and reconstituted in a 60:40 mixture of acetonitrile and isoproponal alcohol. The re-constituted lipid extract was analyzed by electrospray ionization LC-MS/MS on a tandem quadrupole instrument operating in multiple reaction monitoring mode (3). Ceramides were identified by retention time and MS/MS fragmentation parameters, and were quantified relative to the closest-matching internal standard.

Western blotting

An aliquot of the initial muscle sample homogenate in 0.9% saline was taken immediately after homogenization (described above in *Muscle triacylglycerol and diacylglycerol concentrations*), and supplemented to achieve the final buffer solution (20mM Tris-HCl pH 7.5, 150mM NaCl, 2mM Na_2EDTA pH 8.0, 20mM NaF, 10% (v/v) glycerol, 1% (v/v) NP-40, 2.5mM NaPP, 20mM β-glycerophosphate, and a mixture of protease inhibitors). Samples were vortexed vigorously, then centrifuged at 20,000 g for 10 min at 4°C. Supernatants were collected and tested for protein concentration. 30µg protein was separated by SDS-PAGE (8% gels) and transferred to nitrocellulose membranes. Equal loading was verified using Ponceau S staining (data not shown). Membranes were exposed to primary antibodies against glycerol-3-phosphate acyltransferase (GPAT1; 4613; ProSci Incorporated, Poway, CA, USA), diacylglycerol acyltransferase (DGAT1; NB110-41487; Novus Biologicals, Littleton, CO, USA), and DGAT2 (sc-66859; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) to assess the abundance of TAG synthesis enzymes, phosphorylated protein kinase C ($pPKC\beta^{Thr641}$; 07-873; EMD Millipore, Billerica, MA, USA), and inhibitor of NFκB-β (IκBβ; 9248; Cell Signaling Technology, Danvers, MA, USA) to assess proinflammatory stress activation, and phosphorylated insulin receptor substrate ($pIRS1^{Ser312}$; 2381; Cell Signaling Technology) to assess

inhibition of insulin signaling. Membranes were then incubated with appropriate secondary antibodies and developed using enhanced chemiluminescence (GE Healthcare). Bands of interest were imaged and then quantified via densitometry (AlphaEaseFC; Protein Simple, Santa Clara, CA, USA). All within-subject comparisons were made using the same blot.

Supplementary Table 1. Intensity, duration, and energy expended during the exercise sessions.

	EX50	**EX65**	***P*-value**
Intensity (% VO_2peak)	51 ± 0	66 ± 0	< 0.001
Duration (min)	70 ± 3	54 ± 2	< 0.001
Energy Expended (kJ)	1492 ± 11	1485 ± 6	0.511

Values are mean ± SEM. *P*-values are from paired Student's *t*-tests.

Supplementary Table 2. Fasting plasma insulin and substrate concentrations the day after exercise.

	CON	**EX50**	**EX65**	***P*-value**
Insulin (μU/ml)	27.5 ± 4.4	28.0 ± 4.1	26.7 ± 3.7	0.79
Glucose (mmol/L)	5.0 ± 0.1	5.2 ± 0.2	5.0 ± 0.1	0.26
Fatty acid (mmol/L)	0.48 ± 0.05	0.40 ± 0.04	0.45 ± 0.02	0.09
Triacylglycerol (mmol/L)	1.29 ± 0.18	1.40 ± 0.24	1.25 ± 0.19	0.24

Values are mean ± SEM. *P*-values are main effects from one-way, repeated measures ANOVA tests.

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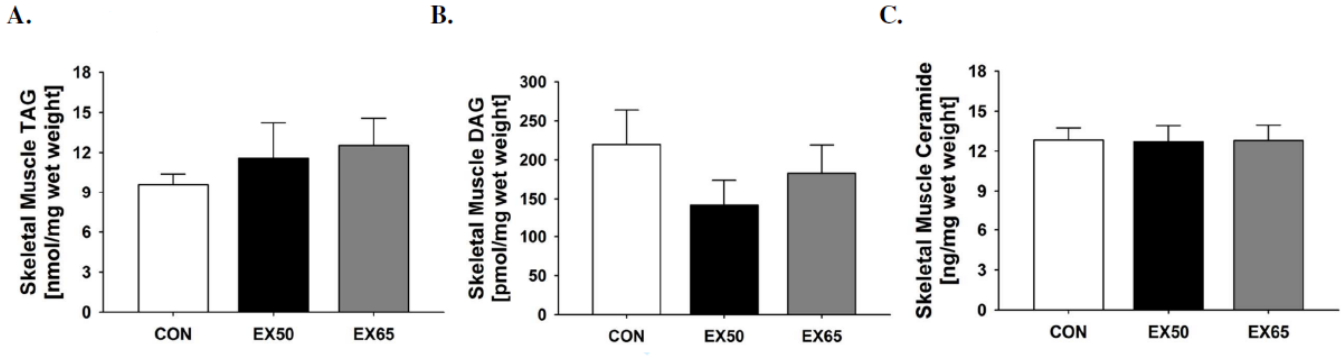
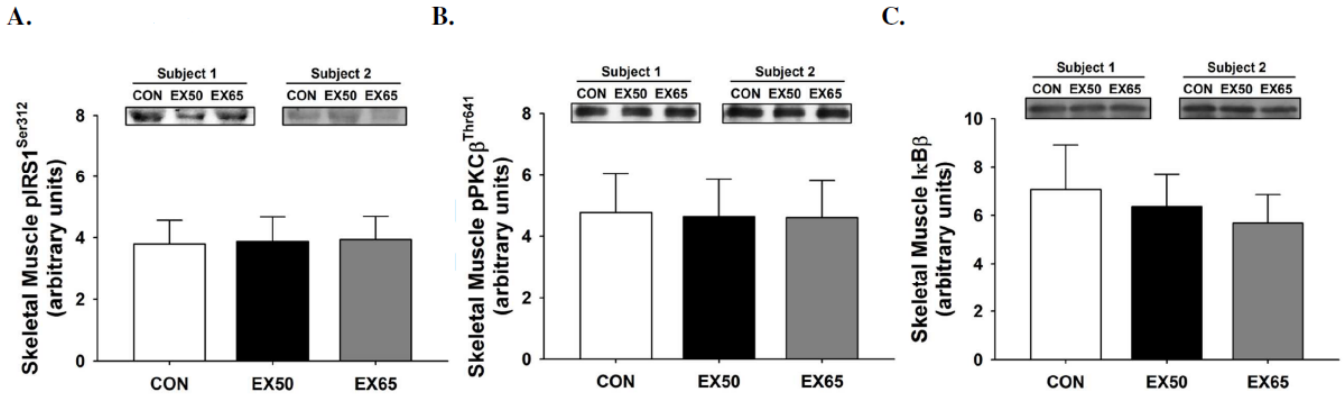
Supplementary Figure 1. Lipid content measured in skeletal muscle collected the day after exercise. A. Skeletal muscle triacylglycerol (TAG) concentration. B. Skeletal muscle diacylglycerol (DAG) concentration. C. Skeletal muscle ceramide concentration.



Supplementary Figure 2. Skeletal muscle insulin signaling and pro-inflammatory markers measured in skeletal muscle collected the day after exercise. The inset figure in each panel is representative blots for two subjects. The one-way, repeated measures ANOVA for (A) pIRS1^{Ser312} main effect P=0.72, (B) pPKCβ^{Thr641} main effect P=0.73, and (C) IκBβ main effect P=0.26.



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1. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-917

2. Bodennec J, Brichon G, Zwingelstein G, Portoukalian J: Purification of sphingolipid classes by solid-phase extraction with aminopropyl and weak cation exchanger cartridges. Methods Enzymol 2000;312:101-114

3. Kasumov T, Huang H, Chung YM, Zhang R, McCullough AJ, Kirwan JP: Quantification of ceramide species in biological samples by liquid chromatography electrospray ionization tandem mass spectrometry. Anal Biochem 2010;401:154-161