Depressed protein synthesis and anabolic signaling potentiate ACL tear resultant quadriceps atrophy

APPENDIX

Western Blot: Human and Mouse

All muscle samples were placed in ice-cold homogenization buffer[2] containing a variety of protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Single-Use Cocktail; Thermo) and homogenized via bead milling using a Bullet Blender tissue homogenizer (Next Advance).[1,4] Two fractions of protein homogenate were collected from murine quadriceps for downstream analysis: whole homogenate for puromycin and ubiquitin assays and a supernatant for intracellular signaling (protein kinase B [Akt], ribosomal protein S6 [RPS6], etc). The supernatant was obtained after centrifugation of the whole homogenate at 6000g for 10 minutes. For human quadriceps assessment, only the supernatant fraction was used for the determination of intracellular signaling. Protein concentration of all fractions was assessed with the Bradford Assay. Fifty micrograms of supernatant or whole homogenate was then diluted 1:1 in sample buffer (1610737EDU, 2× Laemmli Sample Buffer; Bio-Rad), boiled at 100°C for 3 minutes, loaded onto a 4%-20% gradient gel (5678094, TGX Stain-Free Protein Gels; Bio-Rad), and run electrophoretically at 150 V until the protein bands reached the bottom of the gel. After electrophoretic separation, stain-free gels were activated on a ChemiDoc MP for 1 minute under UV illumination. Protein was then transferred to polyvinylidene difluoride (PVDF) membranes at 50 V for 1 hour in ice-cold transfer buffer. Equivalent loading and efficient transfer were verified through stain-free imaging of the PVDF membranes. Representative images of human and mouse membranes can be seen in Figure A1. Membranes were subsequently blocked for 1 hour at room temperature in a 2% bovine serum albumin solution and then incubated in primary antibody overnight with gentle agitation: rabbit polyclonal Ser51 phospho eukaryotic initiation factor 2 eIF2a (9721S; Cell Signaling Technologies), rabbit polyclonal total eIF2a (9722S; Cell Signaling Technologies), rabbit

polyclonal Ser235/236 phospho-RPS6 (2211S; Cell Signaling Technologies), rabbit monoclonal total RPS6 (2217S; Cell Signaling Technologies), rabbit monoclonal Ser473 phospho-AKT (4060S; Cell Signaling Technologies), rabbit monoclonal total AKT (4685S; Cell Signaling Technologies), rabbit polyclonal ubiquitin (3933S; Cell Signaling Technologies), rabbit polyclonal MuRF1 (MP3401; ECM Biosciences), rabbit polyclonal atrogin-1 (AP2041; ECM Biosciences), and mouse monoclonal IgG2a puromycin (MABE343; EMD Millipore). After a brief wash, membranes were incubated in a horseradish peroxidase– or fluorophore-conjugated secondary antibody for 1 hour at room temperature. Chemiluminescent solution (32132; Thermo Scientific) was applied to each blot and incubated for 5 minutes. Membranes were imaged by optical density measurements on a ChemiDoc MP (Bio-Rad) and quantified with Image Lab software (Bio-Rad).

Quantitative Reverse Transcriptase Polymerase Chain Reaction: Mouse

One microgram of RNA was reverse transcribed using iScript reaction mix (170-8890; BioRad) and a MasterCycler Gradient thermocycler (EP-MC; Marshall Scientific). cDNA was then diluted 1:7 in nuclease-free water and pipetted into a 96-well plate with mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 4352339E), MuRF-1 (4331182), and atrogin-1 (4331182) primer probes, as well as TaqMan Fast Advanced Master Mix (4444556; Thermo Fisher). The quantitative polymerase chain reaction (qPCR) was performed on the QuantStudio 3 Real-time PCR System (Bio-Rad), and data were analyzed using the $2^{-\Delta\Delta CT}$ method with GAPDH as a reference gene[3] after verification that it was stable across time points and between limbs in mouse quadriceps.

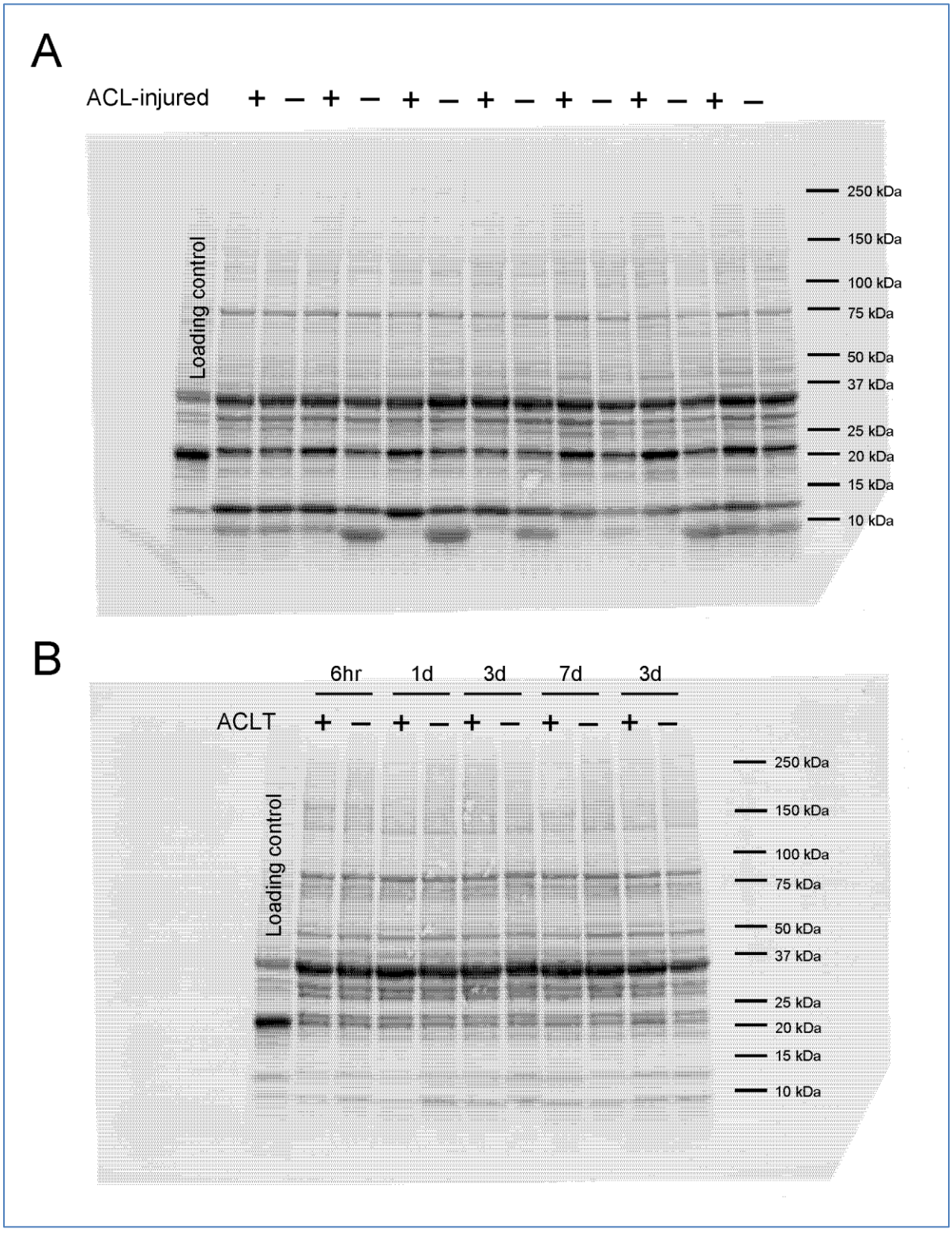

Figure A1. Representative stain-free membrane images. Equal protein loading (50 µg) across lanes in (A) human and (B) mouse polyvinylidene difluoride membranes. Expression of proteins of interest are normalized to an intermembrane loading control (left-most lane). (A) +, ACL-injured limb; –, healthy contralateral limb. (B) +, ACL-transected limb; –, healthy contralateral limb. ACL, anterior cruciate ligament; ACLT, anterior cruciate ligament tear.

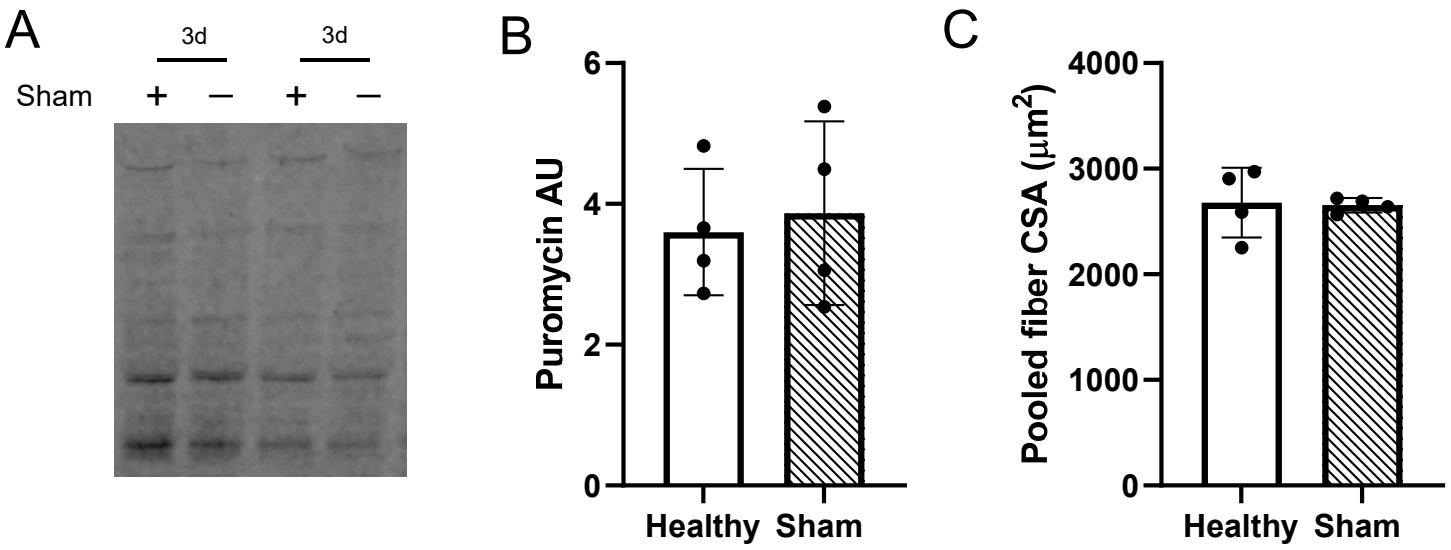

Figure A2. ACL sham transection surgery does not induce deficits in quadriceps protein synthesis or fiber size. (A) Representative image of whole quadriceps muscle homogenate showing puromycin-labeled peptides. B) Quantification of puromycin band intensity in mouse quadriceps at 3 days post ACL sham surgery. C) Pooled muscle fiber cross-sectional area at 7 days post ACL sham surgery. Values are presented as mean ± SD with individual points overlaid. n=4 mice per time point. "+" denotes sham surgery limb, "-" denotes healthy contralateral limb.

References

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