Electronic Supplementary Material (ESI) for Food & Function.

Beneficial effects of dark chocolate on exercise capacity in sedentary subjects: Underlying mechanisms

A double blind, randomized, placebo controlled trial

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Online supplemental material

Online supplemental methods

Electron microscopy

As previously reported [1], muscle samples were fixed in 2% paraformaldehyde plus 2.5% glutaraldehyde (Ted Pella, Redding, CA, USA) in 0.1 M sodium cacodylate (pH 7.4) on ice for 24 h and processed for EM. A stereological analysis to ascertain the ratio of mitochondrial volume to cytoplasmic volume was performed with Adobe Photoshop. Point counting was used to determine the mitochondrial volume densities by overlaying a grid on each digitized image. Mitochondria and cytoplasm lying under intercepts were counted. The relative volume of mitochondria was expressed as the ratio of intercepts coinciding with this organelle to the intercepts coinciding with cytoplasm. Mitochondrial membrane surface areas were measured using ImageJ software (http://www.nih.gov).

Reduced (GSH) and oxidized (GSSG) glutathione

SkM samples (25 mg) were homogenized with a polytron in 250 µL of cold buffer (50 mM potassium phosphate, pH 7, containing 1 mM EDTA), centrifuged at 10,000 x g for 15 min at 4°C [2]. Supernatants were deproteinated as per manufacturer instructions. Briefly, samples were mixed with an equal volume of 1.25 M metaphosphoric acid and centrifuged at 2,000 x g for 2 min. Supernatants were recovered and incubated with triethanolamine 4 M, then samples were used to measure GSH and GSSG by a colorimetric detection kit according to the manufacturer's instructions (Cayman Chemicals, intra-assay coefficient of variation of 1.6%). All samples were tested in duplicates and measured at room temperature.

Protein carbonylation

Approximately 50 mg of SkM was rinsed with PBS pH 7.4 [2]. Tissue was homogenized in 1 ml of cold buffer (50 mM MES pH 6.7, containing 1 mM EDTA). Homogenates were centrifuged at 10,000 g for 15 min at 4 °C then, supernatants were recovered and incubated at room temperature for 15 min with streptomycin sulfate at a final concentration of 1%. Samples were centrifuged at 6,000 g for 10 min at 4 °C. Supernatant was recovered and an aliquot used to measure protein content using a Bradford assay (Bio-Rad). Carbonyl assay kit (Cayman Chemicals) relied on protein carbonyl functional group derivatization with 2,4-dinitrophenyldrazone forming a Schiff base to produce hydrazone, which was measured in a spectrophotometer (μQuant, Bio-Tek Instruments, Inc.) at 360-385nm.

Western blotting

Approximately 25 mg of SkM were homogenized with a polytron in 500 μl lysis buffer (1% Triton X-100, 20 mM Tris, 140 mM NaCl, 2 mM EDTA, and 0.1% SDS) with protease and phosphatase inhibitor cocktails (P2714 and P2850, Sigma-Aldrich), supplemented with 0.15 mM phenylmethylsulfonyl fluoride, 5 mM Na_3VO_4 and 3 mM NaF [1]. Homogenates were sonicated 10 min at 4◦C and centrifuged 12,000 g for 10 min. Total protein content was measured in the supernatant using the Bradford method. A total of 40 μg of protein was loaded onto a 4-15% precast polyacrylamide gel (Bio-Rad), electrotransferred to a polyvinylidene difluoride membrane using a semidry system. Membranes were incubated 1 h in blocking solution (5% non-fat dry milk in TBS plus 0.1% Tween 20 [TBS-T]), followed by a 3 h incubation at room temperature with primary antibodies. To examine mitochondrial biogenesis/cell metabolism related proteins we evaluated, LKB1 and phospho-LKB1 (Abcam), peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α), 5' AMP-activated protein kinase (AMPK),

phospho-AMPK (Cell Signaling), porin (membrane protein) and mitofilin (cristae protein) in Western blot of total lysates from SkM tissue. GAPDH (Cell Signaling) was used as a loading control. All primary antibodies were diluted 1:1000-2000 in blocking solution. Membranes were washed (3 X for 5 min) in TBS-T and incubated 1 h at room temperature with specific HRP-conjugated secondary antibodies. Membranes were again washed 3 times in TBS-T, and the immunoblots developed using an enhanced chemiluminescence Plus detection kit (Amersham-GE). The band intensities were digitally quantified using ImageJ software (http://www.nih.gov).

Citrate synthase (CS) activity

As a measure of mitochondrial function we evaluated CS activity [3]. SkM tissue samples (25 mg) were homogenized with a polytron in 250 µL of cold extraction buffer (20 mM Tris-HCl, 140 mM NaCl, 2 mM EDTA, and 0.1 % sodium dodecyl sulfate) with protease inhibitors (P2714, Sigma-Aldrich), 5 mM Na_3VO_4, and 3 mM NaF. Homogenates were centrifuged at 10,000 x g for 15 min at 4°C. Supernatants were recovered and used to measure CS as the rate of production of the mercaptide ion based on conversion of acetyl-CoA and oxaloacetate into CoA-SH. CoA-SH in the presence of 5,5-disthiobis-2-nitrobenzoic acid produces mercaptide ion. Samples were analyzed in a Beckman DU 730 spectrophotometer (Beckman, Fullerton, CA, USA) at 412 nm. All samples were tested in duplicates and measured at room temperature.

References

1. P. R. Taub, I. Ramirez-Sanchez, T. P. Ciaraldi, G. Perkins, A. N. Murphy, R. Naviaux, M. Hogan, A. S. Maisel, R. R. Henry, G. Ceballos and F. Villarreal, *Clin Transl Sci*, 2012, 5, 43-47.
2. I. Ramirez-Sanchez, P. R. Taub, T. P. Ciaraldi, L. Nogueira, T. Coe, G. Perkins, M. Hogan, A. S. Maisel, R. R. Henry, G. Ceballos and F. Villarreal, *Int J Cardiol*, 2013, 168, 3982-3990.
3. L. Nogueira, I. Ramirez-Sanchez, G. A. Perkins, A. Murphy, P. R. Taub, G. Ceballos, F. J. Villarreal, M. C. Hogan and M. H. Malek, *J Physiol*, 2011, 589, 4615-4631.