**Supplementary Methods and Materials**

**Intramuscular triglyceride analysis (Acute Study)**

The muscle mounted in Tissue-Tek was cut into 5 μm thick transverse sections with a cryostat at -25°C (Bright 5040, Bright Instrument Company; Huntingdon, England) and collected onto an uncoated glass slide and frozen immediately after sectioning. Each slide had 4 samples for a participant (pre- and post-exercise both trials) to decrease variation in staining intensity between muscle sections and slides were prepared and analyzed for each participant. For analysis, cryosections were removed from the freezer and fixed immediately in 3.7% formaldehyde for 60 min. Slides were then rinsed with distilled water (3 x 30 s) and treated for 5 min with 0.5% Triton-X100 in phosphate-buffered solution (PBS; 137 mmol·L-1 sodium chloride, 3 mmol·L-1 potassium chloride, 8 mmol·L-1 sodium phosphate dibasic, 3 mmol·L-1 potassium phosphate monobasic). The slides were washed (3 x 5 min in PBS) and incubated for 2 h at room temperature with anti-myosin heavy chain I antibody (MHCI; mouse IgM, Developmental Studies Hybridoma Bank: reference #A4.480) and anti-dystrophin antibody (mouse IgG2b, Sigma Aldrich: reference #D8168) in 5% goat serum diluted in PBS (1:1 PBS dilution). This was followed by washes in PBS (3 x 5 min), after which conjugated secondary antibodies [goat anti mouse (GAM) IgM conjugated to AlexaFluor 633 for MHCI; Thermo Fisher: reference #A21046; and GAM IgG2b conjugated to AlexaFluor 594 for dystrophin; Thermo Fisher: reference #A21145] were added and incubated at room temperature (30 min) followed by washes in PBS. Then, muscle sections were incubated in BODIPY 493/503 solution (Thermo Fisher: reference #D3922) for 20 min at room temperature in a dark room before washes (2 x 3 min in PBS). Stained sections were embedded in Mowiol 4-88 mounting medium (Fluka: reference #81381) and covered with a coverslip. Slides were left to dry overnight at room temperature before analysis by confocal microscope in duplicate (DMIRE2, Leica Microsystems; 40x oil objective; 1.25 NA). An argon laser 488 nm was used to excite BODIPY-493/503 (emission 510-652 nm), while a helium-neon 594 nm and 633 nm laser line were used to excite Alexa Fluor 594 (dystrophin, emission 6680698 nm) and AlexaFluor 633 (MHCI, emission 698-808 nm), respectively. Images were scanned in projection of 4 lines in 1024x1025 pixels format. Quantification of the lipid droplets was performed using Image J software and the intramuscular triglyceride (IMTG) content of each sample was calculated as the percent area of bodipy staining of the total fiber area ([BODIPY stained area [um2] / area of muscle [um2]\*100).

**Muscle glycogen analysis (Acute Study)**

For muscle glycogen concentrations, 10-15 mg of frozen tissue was powdered and transferred into a glass tube pre-cooled on dry ice. Thereafter, the samples were hydrolyzed by adding a 500 µl of 2M HCL and then incubated for 2 h at 95 ºC. After cooling to room temperature, 500µl 2M NaOH was added. Samples were centrifuged and the supernatant was analyzed for glucose concentrations using an ILAB 650 Clinical Chemistry Analyzer (Instrumentation Laboratory, Warrington, UK).

**Gene expression analysis (Acute Study)**

First, RNA was extracted from 20-40 mg of powdered muscle tissue using Tri reagent (1 mL, Sigma Aldrich, UK, T9424). After addition of chloroform (200 uL, Acros organics 268320025), tubes were incubated at room temperature for 5 min and centrifuged for 10 min (4 oC at 12 000 g). The RNA phase was mixed with an equal volume of ice cold 70% ethanol and RNA was purified on Reliaprep spin columns (Promega, USA, Z6111) as per manufacturer’s instructions. The LVis function of the FLUOstar Omega microplate reader was used to measure RNA concentrations to ensure all samples for each participant had the same amount of RNA (184 ng - 400 ng) and samples were reverse transcribed to cDNA using the RT2 First Strand kit (Qiagen, UK, 330401). Quantitative RT-PCR analysis was performed using custom designed 384-well RT2 PCR Profiler Arrays (Qiagen) and RT2 SYBR Green Mastermix (Qiagen) on a CFX384 Real-Time PCR Detection system (BioRad). 2.8 ng cDNA was added to each well. All primers that were used are commercially available (**shown below**). The absence of genomic DNA, the efficiency of reverse-transcription and the efficiency of the PCR assay were assessed for each sample and conformed to manufacturer’s limits. Relative mRNA expression was determined via the 2-∆∆CT method. Housekeeper genes (βactin [Refseq# NM\_001101]; ribosomal protein lateral stalk subunit P0 [Refseq# NM\_001002] and β-2-microglobulin [Refseq# NM\_004048] were controls.

**Western blotting analysis (Training Study)**

For western blots, 40 µg of protein was loaded for each sample and separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis on Tris-glycine SDS–polyacrylamide gels (15% for OXPHOS and CPT-1, 10% CD36 and GLUT4 and 8% for AMPK, CHC22, CHC17, AkT and AS160). Gels were electro-blotted (semi-dry transfer) onto a nitrocellulose membrane and were then washed in Tris-buffered saline (0.09% NaCl, 100 mM Tris–HCl pH 7.4) with 0.1% Tween 20 (TBS-T) and incubated for 30 min in a blocking solution (5% non-fat milk in TBS-T). Membranes were incubated overnight at 4 °C with primary antibodies against OXPHOS (Abcam: reference #ab110411), CPT-1 (Abcam: reference #ab134988), CD36 (Abcam: reference #ab133625), GLUT4 [self-raised rabbit polyclonal antibody against the C-terminus of GLUT4 (1)], CHC22 [SHL-KS, affinity purified self-raised rabbit polyclonal against the CHC22 C-terminus cross-absorbed against the CHC17 C-terminus (2)], CHC17 [TD.1 self-raised mouse monoclonal against CHC17 terminal domain (3)] AMPKα (Cell Signalling Technologies: reference #2532), Akt (Cell Signaling Technologies: reference #3063), AS160 (Millipore: reference #07-741). It should be noted that the AMPKα antibody recognises both α1 and AMPKα2 isoforms of the catalytic subunit and does not detect the regulatory AMPKβ or AMPKγ subunits (4). Following incubation with the primary antibodies, the membranes were washed in TBS-T and incubated for 60 min in a 1:4000 dilution of anti-species IgG horseradish peroxidase-conjugated secondary antibodies in the aforementioned blocking solution. After further washes, membranes were incubated in an enhanced chemiluminescence reagent and visualized (EpiChemi II Darkroom, UVP, Upland, USA). The band densities were quantified using Image Studio Lite software (Version 5.2; LI-COR, Nebraska, USA) and were normalized to either GAPDH (Proteintech: reference #60004-1-Ig) or Actin (Sigma Aldrich: reference #A2066), before the pre- to post-intervention change was calculated. Pre- and post-intervention samples from any given participant were included on the same gel.

**Phospholipid composition analysis (Training Study)**

Samples were freeze-dried, powdered under liquid nitrogen, and transferred into glass tubes containing 2 ml of methanol and butylated hydroxytoluene (0.01%) and heptadecanoic acid (as an internal standard), followed by the addition of 4 mL of chloroform and 1.5 mL of water, before lipids were extracted. The lipid containing fraction was transferred into thin-layer chromatography (TLC; Kieselgel 60, 0.22 mm, Merck, Darmstadt, Germany) silica plates and lipids were separated by TLC with a heptane: isopropyl ether: acetic acid (60:40:3, vol/vol/vol) resolving solution. Lipid bands were made visible by spraying the plates with a 0.2% solution of 3'7'-dichlorofluorescin in methanol and recognized under ultraviolet light using standards on the plates. Then the gel bands containing phospholipids were scraped off the plates, transferred into screw cap tubes and transmethylated with BF3/methanol. The fatty acid methyl esters (FAMEs) were then dissolved in hexane and analyzed by GLC. A Hewlett-Packard 5890 Series II gas chromatograph with Varian CP-SIL capillary column (100 m, internal diameter of 0.25 mm) and flame-ionization detector were used. In accordance with the retention times of standards, the individual long-chain fatty acids quantification was performed. The content of phospholipids was estimated as the sum of the total fatty acid species and expressed in nanomoles per milligram of dry mass.

**REFERENCES**

1. Satoh S, Nishimura H, Clark AE, Kozka IJ, Vannucci SJ, Simpson IA, Quon MJ, Cushman SW, Holman GD. Use of bismannose photolabel to elucidate insulin-regulated GLUT4 subcellular trafficking kinetics in rat adipose cells. Evidence that exocytosis is a critical site of hormone action. *Journal of Biological Chemistry*.1993;268(24):17820-17829.

2. Hoshino S, Sakamoto K, Vassilopoulos S, Camus SM, Griffin CA, Esk C, Torres JA, Ohkoshi N, Ishii A, Tamaoka A. The CHC22 clathrin-GLUT4 transport pathway contributes to skeletal muscle regeneration. *PloS one*.2013;8(10):e77787.

3. Näthke IS, Heuser J, Lupas A, Stock J, Turck CW, Brodsky FM. Folding and trimerization of clathrin subunits at the triskelion hub. *Cell*.1992;68(5):899-910.

4. Kjøbsted R, Hingst JR, Fentz J, Foretz M, Sanz MN, Pehmøller C, Shum M, Marette A, Mounier R, Treebak JT, Wojtaszewski JFP, Viollet B, Lantier L. AMPK in skeletal muscle function and metabolism. *FASEB J*.2018;32(4):1741-1777.

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| **Supplementary Table 1.** List of genes for mRNA expression | | |
| Gene | Qiagen catalogue number | Refseq# |
| *CD36* | Cat#PPH01356A | NM\_000072 |
| *SLC27A1* | Cat#PPH17902A | NM\_198580 |
| *SLC27A4* | Cat#PPH00471A | NM\_005094 |
| *FABP3* | Cat#PPH02460C | NM\_004102 |
| *FABP4* | Cat#PPH02382F | NM\_001442 |
| *ACSL1* | Cat#PPH19272A | NM\_001995 |
| *ACSL6* | Cat#PPH08013A | NM\_001009185 |
| *CPT1B* | Cat#PPH20905B | NM\_001145134 |
| *CPT2* | Cat#PPH15572A | NM\_000098 |
| *ACACA* | Cat#PPH02316A | NM\_000664 |
| *ACACB* | Cat#PPH02301A | NM\_001093 |
| *MLYCD* | Cat#PPH12795A | NM\_012213 |
| *HADHA* | Cat#PPH10000B | NM\_000182 |
| *GPAM* | Cat#PPH06361A | NM\_001244949 |
| *DGAT1* | Cat#PPH23420F | NM\_012079 |
| *PNPLA2* | Cat#PPH11403B | NM\_020376 |
| *LIPE* | Cat#PPH02383A | NM\_005357 |
| *PDK4* | Cat#PPH07615A | NM\_002612 |
| *PDK2* | Cat#PPH00810A | NM\_001199898 |
| *GYG1* | Cat#PPH13614A | NM\_001184720 |
| *GYS1* | Cat#PPH00988C | NM\_001161587 |
| *PRKAA1* | Cat#PPH00043B | NM\_206907 |
| *PRKAA2* | Cat#PPH15207A | NM\_006252 |
| *PRKAB2* | Cat#PPH09415B | NM\_005399 |
| *PRKAG1* | Cat#PPH07190A | NM\_001206709 |
| *PPARGC1A* | Cat#PPH00461F | NM\_013261 |
| *PPARA* | Cat#PPH01281B | NM\_001001928 |
| *PPARD* | Cat#PPH00455A | NM\_001171818 |
| *UCP3* | Cat#PPH06066A | NM\_003356 |
| *IL6* | Cat#PPH00560C | NM\_000600 |
| *SLC2A4* | Cat#PPH02326A | NM\_001042 |
| *IRS1* | Cat#PPH02328A | NM\_005544 |
| *IRS2* | Cat#PPH02297A | NM\_003749 |
| *AKT2* | Cat#PPH00289F | NM\_001243027 |

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| **Supplementary Table 2.** Postprandial plasma metabolite concentrations for the control (CON; *n*=9), breakfast-exercise (CHO-EX; *n*=12) and exercise-breakfast (EX-CHO; *n*=9) groups. | | | | |
|  | **Pre-intervention** | | **Post-intervention** |  |
| CON glucose AUC (mmol·L-1) | | 8.20 (1.36) | 8.43 (0.89) | *0.69* |
| CHO-EX glucose AUC (mmol·L-1) | | 8.26 (0.90) | 8.34 (1.35) | *0.78* |
| EX-CHO glucose AUC (mmol·L-1) | | 8.52 (1.05) | 8.14 (1.03) | *0.27* |
| ***CON insulin AUC (pmol·L-1)*** | | ***385 (221)*** | ***514 (382)*** | ***0.08*** |
| ***CHO-EX insulin AUC (pmol·L-1)*** | | ***268 (109)*** | ***303 (135)*** | ***0.16*** |
| ***EX-CHO insulin AUC (pmol·L-1)*** | | ***458 (441)*** | ***355 (254)*** | ***0.21*** |
| CON C-peptide AUC (ng*·*mL-1) | | 6.59 (2.57) | 7.32 (3.11) | *0.18* |
| CHO-EX C-peptide AUC (ng*·*mL-1) | | 5.25 (1.72) | 5.54 (1.87) | *0.34* |
| EX-CHO C-peptide AUC (ng*·*mL-1) | | 6.81 (4.40) | 6.03 (3.45) | *0.28* |
| ***CON NEFA AUC (mmol·L-1)*** | | ***0.17 (0.07)*** | ***0.19 (0.07)*** | ***0.52*** |
| ***CHO-EX NEFA AUC (mmol·L-1)*** | | ***0.20 (0.08)*** | ***0.16 (0.06)*** | ***0.08*** |
| ***EX-CHO NEFA AUC (mmol·L-1)*** | | ***0.14 (0.03)*** | ***0.13 (0.04)*** | ***0.48*** |
| CON OGIS (mL·min-1·m-2) | | 401 (39) | 372 (63) | *0.19* |
| CHO-EX OGIS (mL·min-1·m-2) | | 403 (31) | 380 (34) | *0.06* |
| EX-CHO OGIS (mL·min-1·m-2) | | 374 (56) | 399 (42) | *0.08* |
| Data are means and (SD). Abbreviations: NEFA = non-esterified fatty acid, OGIS = oral glucose insulin sensitivity, AUC = time-averaged area under the curve for the OGTT (120 min). | | | | |

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| **Supplementary Table 3.** Components of daily energy intake and daily energy expenditure pre- and post-intervention for the control (CON; n=9 [except for PAEE where *n*=8]), carbohydrate before exercise (CHO-EX; n=12 [except for PAEE where *n*=10]) and exercise before carbohydrate (EX-CHO; n=9) groups. | | | | | |
|  | Pre-intervention | | Post-intervention | Δ from pre-intervention | *time x group*  *interaction* |
| CON RMR (kcal·d-1) | | 1997 (225) | 2021 (232) | 24 (-56, 104) | F=1.791  p=0.19 |
| CHO-EX RMR (kcal·d-1) | | 1964 (167) | 2074 (208) | 110 (45, 174) |
| EX-CHO RMR (kcal·d-1) | | 1899 (228) | 1967 (194) | 68 (-13, 148) |
| CON TEF (kcal·d-1) | | 283 (61) | 292 (85) | 9 (-29, 48) | F=0.982  p= 0.38 |
| CHO-EX TEF (kcal·d-1) | | 292 (50) | 288 (42) | -4 (-19, 10) |
| EX-CHO TEF (kcal·d-1) | | 271 (55) | 258 (44) | -13 (-29, 4) |
| CON PAEE (kcal·d-1) | | 1144 (298) | 1077 (327) | -67 (-215, 81) | F=7.044  p<0.01 |
| CHO-EX PAEE (kcal·d-1) | | 986 (264) | 1190 (343) | 204 (56, 352) a |
| EX-CHO PAEE (kcal·d-1) | | 1006 (147) | 1357 (324) | 351 (126, 576) b |
| CON CHO intake (kcal·d-1) | | 1180 (357) | 1424 (600) | 244 (-29, 517) | F=0.977  p=0.39 |
| CHO-EX CHO intake (kcal·d-1) | | 1171 (317) | 1272 (220) | 101 (-51, 252) |
| EX-CHO CHO intake (kcal·d-1) | | 1133 (307) | 1214 (234) | 81 (-91, 252) |
| CON FAT intake (kcal·d-1) | | 1148 (326) | 1045 (311) | -104 (-257, 49) | F=0.347  p=0.71 |
| CHO-EX FAT intake (kcal·d-1) | | 1132 (307) | 1091 (238) | -41 (-156, 75) |
| EX-CHO FAT intake (kcal·d-1) | | 987 (320) | 882 (172) | -105 (-289, 79) |
| CON PRO intake (kcal·d-1) | | 429 (97) | 376 (89) | -52 (-89, 15) | F=1.517  p=0.24 |
| CHO-EX PRO intake (kcal·d-1) | | 511 (122) | 446 (59) | -65 (-118, -11) |
| EX-CHO PRO intake (kcal·d-1) | | 406 (97) | 399 (117) | -7 (-75, 62) |
| CON ALC intake (kcal·d-1) | | 72 (95) | 75 (154) | 3 (-145, 152) |  |
| CHO-EX ALC intake (kcal·d-1) | | 111 (118) | 69 (72) | -42 (-101, 17) | F=1.115 |
| EX-CHO ALC intake (kcal·d-1) | | 185 (168) | 90 (131) | -94 (-191, 2) | p=0.34 |
| Data are means and (SD) except for change scores which are means and (95% CI). Abbreviations: CHO = carbohydrate, PRO = protein, ALC = alcohol, RMR = resting metabolic rate, TEF = thermic effect of feeding, PAEE = physical activity energy expenditure.**a**denotes a difference in the change from pre-to post-intervention for CON *versus* CHO-EX and **b** for CON *versus* EX-CHO with *p*<0.05. | | | | | |

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| **Supplementary Table 4.** Skeletal muscle phospholipid composition in a control (CON; *n*=6), carbohydrate-exercise (CHO-EX; *n*=8) and exercise-carbohydrate (EX-CHO; *n*=5) groups. | | | | |
|  | Pre-intervention | | Post-intervention | *time x group interaction* |
| CON 14:0 (% of total) | | 1.3 (0.5) | 1.0 (0.3) | F=0.493  p=0.62 |
| CHO-EX 14:0 (% of total) | | 0.8 (0.1) | 0.7 (0.1) |
| EX-CHO 14:0 (% of total) | | 0.8 (0.4) | 0.7 (0.1) |
| ***CON 16:0 (% of total)*** | | ***24.8 (2.3)*** | ***24.3 (1.0)*** | ***F=1.537***  ***p=0.25*** |
| ***CHO-EX 16:0 (% of total)*** | | ***22.4 (0.6)*** | ***21.7 (1.2)*** |
| ***EX-CHO 16:0 (% of total)*** | | ***19.5 (1.5)*** | ***17.9 (1.4)*** |
| CON 18:0 (% of total) | | 14.2 (0.3) | 14.2 (0.5) | F=7.205  p<0.01 |
| CHO-EX 18:0 (% of total) | | 14.6 (0.4) | 15.2 (0.6) |
| EX-CHO 18:0 (% of total) | | 11.9 (0.8) | 13.0 (0.8) ***a*** |
| ***CON 20:0 (% of total)*** | | ***0.13 (0.05)*** | ***0.09 (0.02)*** | ***F=1.141***  ***p=0.34*** |
| ***CHO-EX 20:0 (% of total)*** | | ***0.08 (0.02)*** | ***0.08 (0.04)*** |
| ***EX-CHO 20:0 (% of total)*** | | ***0.10 (0.04)*** | ***0.10 (0.07)*** |
| CON 22:0 (% of total) | | 0.21 (0.07) | 0.20 (0.02) | F=0.666  p=0.53 |
| CHO-EX 22:0 (% of total) | | 0.20 (0.04) | 0.19 (0.04) |
| EX-CHO 22:0 (% of total) | | 0.20 (0.05) | 0.23 (0.10) |
| ***CON 24:0 (% of total)*** | | ***0.15 (0.07)*** | ***0.14 (0.04)*** | ***F=0.108***  ***p=0.90*** |
| ***CHO-EX 24:0 (% of total)*** | | ***0.12 (0.08)*** | ***0.11 (0.06)*** |
| ***EX-CHO 24:0 (% of total)*** | | ***0.16 (0.08)*** | ***0.13 (0.06)*** |
| CON 16:1 (% of total) | | 0.82 (0.34) | 0.70 (0.19) | F=1.254  p=0.31 |
| CHO-EX 16:1 (% of total) | | 0.66 (0.12) | 0.75 (0.26) |
| EX-CHO 16:1 (% of total) | | 0.66 (0.15) | 0.62 (0.17) |
| ***CON 18:1n9c (% of total)*** | | ***7.9 (3.0)*** | ***7.1 (1.5)*** | ***F=2.970***  ***p=0.08*** |
| ***CHO-EX 18:1n9c (% of total)*** | | ***6.3 (0.6)*** | ***6.8 (0.6)*** |
| ***EX-CHO 18:1n9c (% of total)*** | | ***6.6 (1.3)*** | ***6.6 (0.9)*** |
| CON 18:2n6c (% of total) | | 33.6 (5.0) | 34.5 (4.1) | F=0.250  p=0.78 |
| CHO-EX 18:2n6c (% of total) | | 37.2 (2.2) | 37.7 (2.5) |
| EX-CHO 18:2n6c (% of total) | | 29.2 (3.3) | 30.5 (0.8) |

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| **Supplementary Table 4 (con)** Skeletal muscle phospholipid composition in a control (CON; *n*=6), breakfast-exercise (CHO-EX; *n*=8) and exercise-breakfast (EX-CHO; *n*=5) groups. | | | | |
|  | Pre-intervention | | Post-intervention | *time x group interaction* |
| CON C18n3 (% of total) | | 0.23 (0.06) | 0.21 (0.05) | F=1.842  p=0.19 |
| CHO-EX C18n3 (% of total) | | 0.25 (0.05) | 0.29 (0.04) |
| EX-CHO C18n3 (% of total) | | 0.22 (0.03) | 0.26 (0.02) |
| ***CON 20:4n6 (% of total)*** | | ***14.4 (0.8)*** | ***15.0 (1.0)*** | ***F=1.862***  ***p=0.19*** |
| ***CHO-EX 20:4n6 (% of total)*** | | ***14.9 (1.8)*** | ***14.0 (1.4)*** |
| ***EX-CHO 20:4n6 (% of total)*** | | ***11.9 (1.3)*** | ***11.0 (1.7)*** |
| CON 20:5n3 (% of total) | | 0.66 (0.21) | 0.71 (0.18) | F=0.245  *p=0.79* |
| CHO-EX 20:5n3 (% of total) | | 0.71 (0.12) | 0.73 (0.12) |
| EX-CHO 20:5n3 (% of total) | | 0.64 (0.18) | 0.69 (0.18) |
| ***CON 20:0 (% of total)*** | | ***0.13 (0.05)*** | ***0.09 (0.02)*** | ***F=1.141***  ***p=0.34*** |
| ***CHO-EX 20:0 (% of total)*** | | ***0.08 (0.02)*** | ***0.08 (0.04)*** |
| ***EX-CHO 20:0 (% of total)*** | | ***0.10 (0.04)*** | ***0.10 (0.07)*** |
| CON 22:6n3 (% of total) | | 1.5 (0.4) | 1.6 (0.5) | F=0.077  p=0.93 |
| CHO-EX 22:6n3 (% of total) | | 1.6 (0.2) | 1.6 (0.2) |
| EX-CHO 22:6n3 (% of total) | | 1.3 (0.5) | 1.4 (0.3) |
| ***CON 24:1 (% of total)*** | | ***0.13 (0.08)*** | ***0.13 (0.05)*** | ***F=0.021***  ***p=0.98*** |
| ***CHO-EX 24:1 (% of total)*** | | ***0.10 (0.03)*** | ***0.10 (0.04)*** |
| ***EX-CHO 24:1 (% of total)*** | | ***0.14 (0.09)*** | ***0.13 (0.08)*** |
| Data are means and (SD). **a**denotes a difference in the change from pre-to post-intervention for CON *versus* EX-CHO. | | | | |