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Increased mitochondrial glycerol-3-phosphate acyltransferase protein and enzyme activity in rat epididymal fat upon cessation of wheel running

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Kump, David S., Matthew J. Laye, and Frank W. Booth. Increased mitochondrial glycerol-3-phosphate acyltransferase protein and enzyme activity in rat epididymal fat upon cessation of wheel running. Am J Physiol Endocrinol Metab 290: E480–E489, 2006.—Triacylglycerol synthesis in rat epididymal fat overshoots sedentary levels at 10, 29, and 53 h of physical inactivity after 21 days of wheel running. The purposes of the present study were to determine 1) whether this effect is also observed after an acute bout of physical activity and 2) what enzymatic changes might contribute to this effect. We show that more than one bout of physical activity, such as that which occurs with 21 days of wheel running, is necessary for palmitic acid incorporation into triacylglyceride (triglyceride synthesis) to overshoot sedentary values, which suggests that pretranslational mechanisms may be responsible for this overshoot effect. Ten hours after 21 days of wheel running, activity of the mitochondrial glycerol-3-phosphate acyltransferase-1 (mtGPAT1) isoform, a key regulator of triacylglycerol synthesis, overshoot sedentary values by 48% and remained higher than sedentary values at 29 and 53 h of reduced physical activity. The overshoot in mtGPAT1 activity was accompanied by an increase in mtGPAT protein levels. Cyclic AMP response element-binding protein-binding protein level was higher in sedentary animals at 29 and 53 h of reduced physical activity. The overshoot in mtGPAT1 activity was accompanied by an increase in mtGPAT protein levels. Cyclic AMP response element-binding protein-binding protein level was higher in sedentary animals at 29 and 53 h of reduced physical activity. These results support the hypothesis that more than one bout of physical activity is necessary for the regulation of triacylglycerol synthesis.

exercise; adipose tissue; triacylglycerol synthesis; adenosine 5′-monophosphate kinase; physical inactivity

THE ESCALATING PANDEMIC OF OBESITY has occurred concurrently with a precipitous decline in the total amount of daily physical activity (3, 8, 35, 36). In a recent report (16), we used a rat model of a decline in physical activity in young humans, where male rats that were physically active on voluntary running wheels for 21 days had their wheels locked for 5–53 h. During this time frame (between 5 and 53 h of reduced physical activity), epididymal fat mass increased 25% as a result of an increase in cell size. This increase in fat was accompanied by an ~3.5-fold increase in the incorporation of palmitic acid into triacylglycerol (an estimate of triacylglycerol synthesis) in epididymal fat homogenates that was observed at 10, 29, and 53 h after the reduction in physical activity. In contrast, when killed after 5 h of reduced physical activity, triacylglycerol synthesis was suppressed 80% relative to sedentary rats. It is unknown whether these same phenomena might be observed after shorter-term physical activity (such as after a single bout of treadmill exercise or after 24 h of wheel running in animals naive to running wheels) as they would after the 21 days of wheel running. Because the determination of whether the overshoot in triacylglycerol synthesis is from single or repeated daily bouts of physical activity would assist in understanding the underlying mechanisms, one purpose of this study was to determine whether an acute bout of physical activity would also result in suppression of triacylglycerol synthesis at 5 h and an overshoot of triacylglycerol synthesis at 10 h after activity.

Another question regarding the previously observed suppression and overshoot of triacylglycerol synthesis is which enzymatic steps might contribute to these observations. Two key regulatory enzymes in triacylglycerol synthesis are acyl-CoA:glycerol-sn-3-phosphate acyltransferase (GPAT) and 1,2-diacylglycerol:acyl-coenzyme A acyltransferase (DGAT). GPAT catalyzes the esterification of acyl coenzyme A to glycerol 3-phosphate, the initial and committed step in the synthesis of glycerolipids, which includes lysophosphatidic acid, phosphatidic acid, diacylglycerol, triacylglycerol, and membrane phospholipids. It is presumed that GPAT may be rate limiting for these processes (7, 31). Lewin et al. (18) proposed that changes in the regulation of the first mitochondrial isoform of GPAT (mtGPAT) might contribute to the etiology of diseases characterized by dysregulation of lipid metabolism, such as obesity and type 2 diabetes. DGAT, which converts diacylglycerol to triacylglycerol by the addition of an acyl-coenzyme A, catalyzes the terminal and only dedicated step that is exclusive to triacylglycerol synthesis. Mice deficient in DGAT1, one of two DGAT enzymes (20), are resistant to diet-induced obesity and have increased insulin and leptin sensitivity (5, 30). Another purpose of the present study was to test the possibility that changes in GPAT or DGAT activity might be associated with the physical inactivity-induced dysregulation of triacylglycerol synthesis and, if so, to investigate possible mechanisms regulating these enzymatic changes.

MATERIALS AND METHODS

Materials

[9,10-3H]palmitic acid was from Sigma (St. Louis, MO). 1-[2-3H(N)]glycerol 3-phosphate, [pal-9,10-3H]palmitoyl coenzyme A, and [γ-2-32P]adenosine 5′-triphosphate were from American Radiolabeled Chemicals (St. Louis, MO). mtGPAT antiserum was the generous gift of Dr. Rosalind Coleman (University of North Carolina, Chapel Hill, NC). Sterol regulatory element-binding protein-1 (SREBP-1) polyclonal antibody and cyclic AMP response element-binding protein-binding protein (c-EBP-β) antisera were gifts from Dr. Anthony C. O’Connor (University of Toronto, Toronto, Canada) and Dr. Harry W. Green (University of Colorado, Denver, CO), respectively. Antibody levels were quantified by an enzyme-linked immunosorbent assay (ELISA) as previously described (16). The ATP-γ-S assay was performed as described (16, 18) with minor modifications.

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binding protein-binding protein (CBP) and casein kinase-2α monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Nuclear factor Y-β polyclonal antibody was a kind gift from Dr. Roberto Mantovani (University of Milan, Milan, Italy). S1p monoclonal antibody was from BD Biosciences (San Jose, CA). AMP-activated protein kinase-α (AMPKα), AMPKα Thr172 phosphorylation-specific, and acetyl-coenzyme A carboxylase Ser79 phosphorylation-specific polyclonal antibodies were from Cell Signaling (Beverly, MA). The casein kinase-2 consensus target phosphorylation sequence peptide RRRDDDDDDDD was from Sigma Genosys (The Woodlands, TX). Real-time polymerase chain reaction (PCR) primers were purchased from Integrated DNA Technologies (Coralville, IA), and the probe was purchased from Applied Biosystems (Foster City, CA).

Twenty-One Days of Wheel Running

Details of the experimental design have been described previously (16). Briefly, 21- to 23-day-old Fischer-Brown Norway F1 generation male rats (Harlan) were allowed to acclimatize for 1 wk, after which they were randomly divided into six groups. Four groups were given access to voluntary running wheels for 21 days, after which the wheels were locked for 5 (WL5), 10 (WL10), 29 (WL29), or 53 (WL53) h before the rats were killed. Two other groups (SED5 and SED10) had only regular sedentary cage activity, with no access to voluntary running wheels, and were killed at the same time as WL5 and WL10 rats, respectively. Food was removed for either 5 (WL5, WL29, WL53, and SED5) or 10 (WL10 and SED10) h before death. The animals were anesthetized with 60 mg pentobarbital sodium/kg body mass, and epididymal fat pads were removed free of the spermatic vessels and used in experiments as described below. Plasma was collected via cardiac puncture, and the animals were exsanguinated. Animals used for 21 days of wheel running were the same as those previously used for measurement of triacylglycerol synthesis (16). There were 10 animals per group.

Short-Term (Acute) Physical Activity

Twenty-four hours of wheel running. Initially, rats of the same age as at the end of 21 days of voluntary wheel running (~7 wk) were used. The animals arrived at 21–23 days and were housed 5–6 animals per cage for 28 days. At the start of the light cycle, two groups were placed in individual cages containing voluntary running wheels, whereas two other groups were placed in individual cages without voluntary running wheels so that they were sedentary. After 24 h, the wheels were locked and food was removed from all groups. One group of runners and one sedentary group were killed 5 h after the wheels were locked, and the other two groups were killed 10 h after the wheels were locked. During the 24 h of wheel access, the rats averaged 1.50 ± 0.11 km (n = 9 per group), which is only about 25% of the running activity observed on either the first or last night of running as previously reported (16), when 28- to 30-day-old rats were given access to voluntary running wheels for 21 days. As we desired to approximate more closely the activity observed on the last night after 21 days of wheel running, two additional approaches were employed, one in which the rats were matched for the type of activity (voluntary wheel running) and another in which the rats were matched for age (7 wk).

We have observed that 28- to 30-day-old rats provided running wheels run 5–6 km during the first 24 h, which is similar to the distance observed during the last night after 21 days of wheel access (16). We therefore allowed 21- to 23-day-old rats to acclimatize to housing for 1 wk and then separated them into four groups as described above for the 7-wk-old animals. In agreement with our previous observation (16), the 4-wk-old rats averaged 5.05 ± 0.65 km during their 24 h of voluntary running wheel access. Wheels were locked and food was removed from all groups after the 24 h of access, and after either 5 or 10 h animals were killed and epididymal fat pads harvested as described above. There were eight animals per wheel-running group and six animals per sedentary group.

Acute treadmill running. To match rats of the same age at the time of death as those used for 21 days of wheel running, we employed a second method to approximate the last night of activity during 21 days of wheel running. Six-week-old rats were familiarized with running on a motorized treadmill (Quinton Instruments, Seattle, WA) at the start of the light cycle for 7 days. The familiarization protocol consisted of 10 min on the treadmill at 15 m/min, with the incline gradually adjusted to 6% on the 5th day. On the 8th day, at ~7 wk of age, the rats that were subjectively the most unwilling to run on the treadmill were assigned to a sedentary group, and the remaining animals were assigned to an acute treadmill-running group. Within each group, the animals were then randomly assigned to be killed either immediately (0 h) or 5 or 10 h after the last bout of treadmill running. At the start of the light cycle (0400), access to food was removed, and all animals were transported to the treadmill room. Animals assigned to the acute treadmill-running group ran 12 × 10-min bouts on the treadmill at 21 m/min on a 6% incline. There were 5-min rest periods between bouts, during which time the animals were returned to their cages and given access to water. Sedentary rats remained in their cages for the duration. Euthanasia of the 0-h groups were completed within 40 min after conclusion of the last activity interval, and the other animals were returned to their housing quarters until killed either 5 or 10 h later. The animals were killed and epididymal fat pads harvested as described above for 21 days of wheel running. There were 6–7 animals per group.

Enzyme Activity

All enzyme activity assays were performed on epididymal fat homogenates, which were prepared as described (16); all assays were linear with respect to time and amount of homogenate protein and were within the saturation range for all substrates and cofactors. Triacylglycerol synthesis was measured as the incorporation of radio-labeled palmitic acid into triacylglycerol, as previously described (16).

GPAT activity was measured as the incorporation of radio-labeled glycerol 3-phosphate into lysophosphatidic acid and phosphatidic acid (18). Forty microliters of a 2 μg protein/μl concentration of epididymal fat homogenate were incubated at 30°C for 15 min in a final volume of 400 μl of 75 mM Tris-HCl, 4 mM MgCl₂, 2 mg/ml free fatty acid-free BSA, 8 mM NaF, pH 7.4, 50 μM palmitoyl coenzyme A, and 300 μM [14C]glycerol 3-phosphate (5 μCi/ml) with or without 2 mM N-ethylmaleimide (NEM). The reaction was terminated by the addition of 3 ml of chloroform-methanol (1:2, vol/vol) and 600 μl of 10% trichloroacetic acid. After 10 min, 500 μl of chloroform and 500 μl of 10% trichloroacetic acid were added to each tube. Samples were then vortexed and centrifuged at 450 g for 2 min, the upper phase was aspirated and washed four times with 2 ml of 10% trichloroacetic acid, and 1 ml of the lower chloroform layer was evaporated in a rotary vacuum, reconstituted in chloroform-5% glacial acetic acid and separated using thin-layer chromatography by developing to 8 cm in chloroform-methanol-H₂O (65:25:4, vol/vol/vol) and then to 16 cm in hexane-ethyl ether-glacial acetic acid (80:20:2, vol/vol/vol). Standards for lysophosphatidic acid, phosphatidic acid, diacylglycerol, and triacylglycerol were run jointly, and the appropriate bands were scraped and subjected to scintillation counting. The amount of radioactive activity in the bands representing diacylglycerol and triacylglycerol was indistinguishable from background and is not included in the enzyme activity measurements. Total GPAT activity was determined in the absence of NEM, mtGPAT1 activity, which is resistant to inhibition by NEM (1), was determined in the presence of 2 mM NEM and subtracted from the total activity to yield the NEM-sensitive GPAT activity, representing the activity from the microsomal GPAT and mtGPAT2 (1, 19). Preliminary experiments indicated that 2 mM NEM was sufficient to inhibit all of the microsomal GPAT and mtGPAT2 activity.
DGAT activity was measured as the incorporation of radiolabeled palmitoyl coenzyme A into triacylglycerol (6). Twenty microliters of a 2 μg protein/μl epididymal fat homogenate (40 μg homogenate protein) were incubated at 37°C in a final volume of 200 μl of 175 mM Tris-HCl, 8 mM MgCl₂, 1 mg/ml free fatty acid-free BSA, pH 8.0, 200 μM dioleoylglycerol, and 30 mM [³²P]palmitoyl coenzyme A (1 μCi/ml) for 30 min. The reaction was terminated and samples were processed as described for triacylglycerol synthesis (16). This method does not distinguish the activities of the DGAT1 and DGAT2 isozymes.

The measurement of casein kinase-2 activity was adapted from the protocol of Shore et al. (29). Two-and-a-half microliters of epididymal fat homogenate at a concentration of 2 μg protein/μl (5 μg homogenate protein) were incubated in 30 μl of 50 mM HEPES, 150 mM NaCl, 11 mM MgCl₂, 60 mM β-glycerophosphate, and 1 mM [γ-³²P]adenosine 5'-triphosphate (1 μCi/ml) with or without 1 mg/ml RRREDDDDDDDD synthetic peptide at 37°C for 15 min. The reaction was terminated by spotting 15 μl onto Whatman P-81 paper discs, which were subsequently washed eight times for 5 min in 0.05% phosphoric acid, three times for 1 min in 95% ethanol, and air dried before scintillation counting. The specific incorporation of [³²P]ATP into the synthetic peptide was obtained by subtracting values determined in the absence of the peptide.

Immunobots

Immunobots were performed according to standard procedures, as previously described (17). Blots for AMPKα Thr172 phosphorylation were stripped by incubation in 62.5 mM Tris-HCl, pH 6.8/2% SDS/100 mM β-mercaptoethanol at 50°C, with light rocking for 30 min, and subsequently blocked and reprobed for AMPKα. All blots were corrected for a loading control as described (16).

Real-Time PCR

Isolation of total RNA from ~50 mg epididymal adipose tissue was accomplished using the RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, CA), and 700 ng of mRNA were reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA) with random hexamers. Real-time PCR for mtGPAT1 was performed on 25 ng of cDNA as described (26): the sequence for the forward primer was 5'-CAGTGCCGGAGTCTGACTCT-3', the sequence for the reverse primer was 5'-TCTCTCCGTCCTGGGAGA-3', and the probe sequence was FAM-AGAAGCTGACAGGTAC. All samples were assayed in duplicate, and statistics were performed on the mean cycle threshold value for each group using the ΔΔCT method (ABI User Bulletin No. 2). GAPDH served as the reference amplicon (P = 0.19 between groups); data are presented as the fold difference relative to WL5.

Plasma Assays

Plasma glucose, glycerol, triacylglycerol (Sigma), free fatty acids (Wako Chemicals, Richmond, VA), and insulin (Linco Research, St. Charles, MO) were measured using commercially available kits according to the manufacturer’s instructions.

Statistics

For 21 days of wheel running, comparisons between WL5, WL29, WL53, and SED5 were made using ANOVA. Comparisons between WL5, WL10, SED5, and SED10 were made using two-way ANOVA: activity (factor 1: WL = 21 days of wheel running or SED = sedentary) × time of death (factor 2: 5 or 10 h after running wheels were locked). For 28- to 30-day-old animals with 24 h of wheel running, comparisons were made using two-way ANOVA: activity (wheel running or sedentary) × time of death (5 or 10 h after running wheels were locked). For 7-wk-old animals undergoing acute treadmill running, comparisons were made using two-way ANOVA: activity (treadmill running or sedentary) × time of death (0, 5, or 10 h after the last activity bout on the treadmill). Significance level was set at P < 0.05, with the Neuman-Keuls post hoc method used for determining between group differences. All statistics were analyzed using SigmaStat (Systat Software, Point Richmond, CA).

RESULTS

Plasma Analytes After 21 Days of Wheel Running

Plasma insulin (pM) was higher in WL5 (220 ± 2) than in WL5 (209 ± 4; Fig. 1A). Plasma insulin for WL29 (215 ± 3)
and SED5 (212 ± 3) was intermediate between, but not different from, WL5 or WL53. Plasma triacylglycerol (µM) was greater in WL53 (205 ± 26) relative to all other groups (139 ± 14 for WL5, 145 ± 20 for WL29, and 120 ± 10 for SED5; Fig. 1B). Thus there are associations for increased plasma insulin and triacylglycerol, increased epididymal and omental fat masses (16), and decreased submaximal insulin-stimulated 2-deoxyglucose transport into the epitrochlearis muscle (17) in WL53 relative to WL5. Plasma glucose, free fatty acids, and glycerol were not different between groups (Fig. 1, C–E).

Triacylglycerol Synthesis After Acute Physical Activity

We (16) previously reported that triacylglycerol synthesis was at least three times greater at 10, 29, and 53 h after 21 days of voluntary wheel running compared with time-matched sedentary rats. A question posed in the present study was whether this response would occur after a single day of wheel running.

Twenty-four hours of wheel running: Triacylglycerol synthesis in the epididymal fat homogenates from 7-wk-old rats with 24 h of wheel running was not different from that in sedentary rats, which was likely due to the fact that they ran only 1.5 km during the 24 h of wheel access (data not shown). To increase the duration of the acute increase in physical activity, two additional methods were used to determine whether triacylglycerol synthesis is altered with 1 day of physical activity using 1) younger (28- to 30-day-old) rats with 24 h of wheel running to match the type of activity and 2) 120 min of intermittent treadmill running by 7-wk-old rats to match the 21-day wheel-running animals for age (see MATERIALS AND METHODS). When 28- to 30-day-old rats with 24 h of wheel running (during which time they ran ~5 km) were killed 5 h after running wheels were locked, triacylglycerol synthesis (544 ± 67 pmol·mg homogenate protein\(^{-1}\)·min\(^{-1}\)) was suppressed compared with age-matched sedentary rats (918 ± 52; Fig. 2, left). By 10 h after the running wheels were locked, triacylglycerol synthesis values were not different between animals with 24 h of wheel running (724 ± 91), and sedentary rats were killed at the same time point (722 ± 122).

Treadmill running, 7-wk-old rats that engaged in 120 total min of intermittent activity over a 3-h period on a motorized treadmill and then killed at either 0 or 5 h after the last bout of treadmill running had a suppression of triacylglycerol synthesis (249 ± 50 and 243 ± 58 pmol·mg homogenate protein\(^{-1}\)·min\(^{-1}\), respectively) relative to the sedentary group that was killed at the same time [545 ± 41 at 0 and 432 ± 35 at 5 h, respectively (Fig. 2, right)]. By 10 h after the last treadmill running bout, triacylglycerol synthesis values from animals that underwent treadmill running (392 ± 66) were not different from those of sedentary (397 ± 58) animals.

GPAT and DGAT Enzyme Activities After 21 Days of Wheel Running

mtGPAT1 activity (pmol·mg homogenate protein\(^{-1}\)·min\(^{-1}\)) was significantly lower in WL5 (34 ± 5) relative to SED5 (74 ± 6), significantly greater in WL10 (99 ± 8) relative to WL5 and SED10 (67 ± 8), and significantly greater in WL29 (107 ± 10) and WL53 (117 ± 12) relative to WL5 and SED5, respectively (Fig. 3A). Neither the total GPAT activity nor that of the NEM-sensitive isoforms differed among groups. DGAT activity was not significantly different among groups (Fig. 3B).

mtGPAT Protein Level

Twenty-one days of wheel running, mtGPAT protein level was significantly lower in SED5 than in WL5, WL29, or WL53, and it was lower in SED10 than in WL10 (Fig. 4A).

Short-term (acute) physical activity, mtGPAT protein level was not different between short-term runners and sedentary groups for either the 4-wk-old rats with 24 h of wheel running or 7-wk-old rats with 120 min of intermittent treadmill running (Fig. 4B).

mtGPAT1 mRNA After 21 Days of Wheel Running

mtGPAT1 mRNA level (normalized to GAPDH mRNA) in WL5 was 38 and 35% lower than in SED5 and WL10, respectively (Fig. 5), but WL5 was not significantly different from WL29 or WL53. SED5 and WL10 were not different from SED10. The lower mtGPAT1 mRNA in WL5, relative to SED5 and WL10, was also confirmed by normalizing the values to 18S ribosomal RNA (data not shown).

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Fig. 2. Triacylglycerol synthesis in epididymal fat homogenates after 24 h of wheel running (left) and 2 h of intermittent treadmill running (right). Triacylglycerol synthesis was measured as incorporation of radiolabeled palmitic acid into triacylglycerol. Time of death indicates hours after locking of voluntary running wheels for wheel-running groups (left) or time since the end of running for treadmill groups (right); n = 6–8/group. Groups connected by lines are significantly different: *P < 0.05; **P < 0.01; ***P < 0.001. See MATERIALS AND METHODS for group descriptions. Groups were compared using 2-way ANOVA (see Statistics).
SREBP-1, Nuclear Factor Y-β, CBP, and Sp1 Protein Levels

Twenty-one days of wheel activity. No significant differences were found for the protein levels of truncated (Fig. 6A), full-length (Fig. 6C), and total SREBP-1 (Fig. 6D) nor for the ratio of truncated SREBP-1 to total SREBP-1 (Fig. 6E). CBP protein levels were significantly higher in SED5 than in WL29 (Fig. 6F) and approached significance compared with WL5 and WL53 ($P = 0.09$ and 0.053, respectively). Protein levels of nuclear factor Y-β and Sp1 were not different among groups (Fig. 6, G and H).

Short-term (acute) physical activity. Truncated SREBP-1 protein levels were not different between short-term (acute) runners and sedentary groups (Fig. 6B).

AMPK Thr$^{172}$ Phosphorylation

Twenty-one days of wheel running. Protein levels for AMPKα (Fig. 7A), AMPKα Thr$^{172}$ phosphorylation (Fig. 7C), and acetyl-coenzyme A carboxylase Ser$^{79}$ phosphorylation (Fig. 7G) were not different among groups.

Short-term (acute) physical activity. AMPKα THR$^{172}$ PHOSPHORYLATION. AMPKα Thr$^{172}$ phosphorylation was significantly higher in 7-wk-old rats at 0 h after one bout of treadmill running relative to both sedentary and 5 and 10 h after one bout of treadmill running (Fig. 7B, right). AMPKα Thr$^{172}$ phosphorylation was not different for 28- to 30-day-old rats regardless of whether they had been physically active or sedentary (Fig. 7B, left).

TOTAL AMPKα PROTEIN LEVELS. Total AMPKα protein levels at 0, 5, and 10 h after a single bout of treadmill running in 7-wk-old rats were not different from respective time-matched sedentary animals (Fig. 7D, right). However, in 28- to 30-day-old animals with 24 h of wheel running, there was an effect of time of death (as determined by two-way ANOVA) that was independent of the activity level of the animals (either wheel running or sedentary), such that there was a significantly lower level ($P = 0.006$) of AMPKα protein between combined groups killed at 10 h compared with 5 h after the start of the light cycle. This significant difference between the 5- and 10-h groups was retained in the wheel-running groups ($P = 0.02$) and approached significance in sedentary ($P = 0.08$; Fig. 7D, left). The reason for the lower level of AMPKα protein at the 10-h time point is unclear.

RELATIVE AMPKα THR$^{172}$ PHOSPHORYLATION. As AMPKα Thr$^{172}$ phosphorylation did not change at 5 or 10 h after 24 h of wheel running, the decrease in total AMPKα protein at the 10-h post-running time point resulted in a significantly greater degree of Thr$^{172}$ phosphorylation per level of total AMPKα in
the wheel running groups killed at 10 h relative to 5 h (Fig. 7F, left). It should be noted that this increase in phosphorylation is observed only when the data are normalized to the total AMPKα protein levels, because the absolute level of phosphorylation was unchanged (data not shown). The degree of AMPKα Thr172 phosphorylation per level of total AMPKα was not significantly different in the acute treadmill running groups (P = 0.06 interaction effect; Fig. 7F, right).

**ACETYL-COENZYME A CARBOXYLASE SER79 PHOSPHORYLATION.**

Acetyl-coenzyme A carboxylase Ser79 phosphorylation was not different in the 28- to 30-day-old animals after 24 h of wheel running (Fig. 7H, left). Acetyl-coenzyme A carboxylase Ser79 phosphorylation was significantly higher at 0 h in the short-term (acute) treadmill running group relative to the sedentary group killed at the same time (Fig. 7H, right). Acetyl-coenzyme A is directly phosphorylated by AMPK (21), and the increase in acetyl-coenzyme A carboxylase Ser79 phosphorylation at this time point coincides with the increase in AMPKα Thr172 phosphorylation (Fig. 7F, right), providing additional indirect evidence of an increase in AMPK activity immediately after the treadmill running.

**Casein Kinase-2 Protein Level and Activity**

Casein kinase-2α protein levels and casein II kinase activity were not significantly different among groups in rats that engaged in 21 days of wheel running (Fig. 8).

**DISCUSSION**

This study demonstrates that repeated bouts of physical activity are necessary for the overshoot effect in triacylglycerol synthesis at 10 h of reduced physical activity, indicating that the essential mechanism might have some pretranslational component(s). Additional key findings from rats that had 21 days of wheel running in the present study are that 1) mtGPAT1 activity in epididymal fat was suppressed at 5 h and then elevated (actually overshooting sedentary values) at 10, 29, and 53 h following wheel running, a time course similar to the previously reported overshoot for triacylglycerol synthesis (16); and 2) increased mtGPAT protein levels in epididymal fat were associated with the overshoot in mtGPAT1 activity at 10, 29, and 53 h of reduced physical activity, but there was not a concomitant increase in mtGPAT1 mRNA at the time points measured.

**More Than a Single Day of Physical Activity Is Needed to Produce the Physical Inactivity-Induced Overshoot in Triacylglycerol Synthesis**

Short-term (acute) physical activity, either in the form of 24 h of wheel running or a 2-h duration of intermittent treadmill running, resulted in suppression of triacylglycerol synthesis (measured as the incorporation of [14C]palmitic acid into triacylglycerol) in epididymal fat ≤ 5 h after running. Triacylglycerol synthesis in epididymal fat has also been shown to be suppressed at 5 h after 21 days of wheel running (16). A potential interpretation of the “suppression effect” on triacylglycerol synthesis observed at 0–5 h after physical activity is that it involves suppression of enzyme activity by a posttranslational mechanism, because it occurred after both short-term (acute) physical activity and 21 days of voluntary wheel running and did not likely require the time needed to accumulate changes from pretranslational regulation.

A strikingly different comparison of acute and repeated daily physical activity exists for triacylglycerol synthesis at 10 h postactivity. Acute physical activity, either in the form of 24 h of wheel running or 2 h of intermittent treadmill running, resulted in no difference in epididymal triacylglycerol synthesis from sedentary animals at 10 h after physical activity (Fig. 2). By contrast, in rats that have undergone 21 days of voluntary wheel running, triacylglycerol synthesis overshoot sedentary values by more than threefold after 10 h of reduced physical activity (16). A potential interpretation is that the “overshoot effect” on triacylglycerol synthesis observed at 10, 29, and 53 h after 21 days of wheel running, which did not occur after short-term (acute) physical activity in naive rats, involves changes in gene expression of either enzyme involved in triacylglycerol synthesis or of kinases or other proteins that could modulate enzyme activity. Collectively, it is evident that one bout of activity is sufficient to suppress triacylglycerol synthesis in epididymal fat, but that more than a single day of increased physical activity is required to produce the overshoot in triacylglycerol synthesis that occurs 10 h after 21 days of wheel running.

**mtGPAT1 Activity and Protein Increase with 21 Days of Wheel Running, but mRNA Does Not Increase**

To begin to understand which enzymes involved in triacylglycerol synthesis might be involved in the observed “suppression” and “overshoot” effects, the activities of GPAT and DGAT in epididymal fat homogenates were determined. We found that after 21 days of wheel running, the pattern for mtGPAT1 activity was similar to that for triacylglycerol synthesis (Fig. 3A).

The next experiments were directed toward understanding how the observed suppression and overshoot of mtGPAT1 activity might be regulated. An increased epididymal fat mtGPAT protein level accompanied the increased mtGPAT1 protein levels, because the absolute level of phosphorylation was unchanged (data not shown). The degree of AMPKα Thr172 phosphorylation per level of total AMPKα was not significantly different in the acute treadmill running groups (P = 0.06 interaction effect; Fig. 7F, right).

**ACETYL-COENZYME A CARBOXYLASE SER79 PHOSPHORYLATION.**

Acetyl-coenzyme A carboxylase Ser79 phosphorylation was not different in the 28- to 30-day-old animals after 24 h of wheel running (Fig. 7H, left). Acetyl-coenzyme A carboxylase Ser79 phosphorylation was significantly higher at 0 h in the short-term (acute) treadmill running group relative to the sedentary group killed at the same time (Fig. 7H, right). Acetyl-coenzyme A is directly phosphorylated by AMPK (21), and the increase in acetyl-coenzyme A carboxylase Ser79 phosphorylation at this time point coincides with the increase in AMPKα Thr172 phosphorylation (Fig. 7F, right), providing additional indirect evidence of an increase in AMPK activity immediately after the treadmill running.

**Casein Kinase-2 Protein Level and Activity**

Casein kinase-2α protein levels and casein II kinase activity were not significantly different among groups in rats that engaged in 21 days of wheel running (Fig. 8).
activity at 10, 29, and 53 h after 21 days of wheel running (Fig. 4A), but an acute bout of running by rats naive to exercise did not increase mtGPAT protein at 0, 5, and 10 h after physical activity (Fig. 4B). Others have reported (13) that overexpression of mtGPAT1 in either Chinese hamster ovary or human embryonic kidney 293 cells results in preferential incorporation of fatty acids into triacylglycerol relative to phospholipids, indicating that the higher mtGPAT protein level at 10, 29, and 53 h of reduced physical activity after 21 days of wheel running could be contributing to the higher triacylglycerol synthesis.

Interestingly, the increased mtGPAT protein level was not accompanied by an increase in mtGPAT1 mRNA (Fig. 5). Indeed, 5 h after 21 days of wheel running, mtGPAT1 mRNA level was suppressed 54% relative to sedentary animals, but at 10 h, mtGPAT1 mRNA level had risen 150% so that it was not different from the sedentary animals. Importantly, the rebound increase of mtGPAT1 mRNA to sedentary levels occurred during an additional 5 h of fasting, during which plasma insulin might be expected to decrease. Because insulin increases mtGPAT1 mRNA (31), this indicates that the suppression of mtGPAT1 mRNA at 5 h after activity is likely a direct consequence of the physical activity and not due to fasting. Lewin et al. (18) have shown that, relative to other organs, in adipose tissue from sedentary rats there is a high level of mtGPAT1 mRNA expression but a low level of protein, which suggests potential translational or posttranslational regulation of mtGPAT1 protein levels. Thus the small increase in mtGPAT protein in the present study after 21 days of wheel running might be due to enhanced translational efficiency of mtGPAT1 mRNA, a lower mtGPAT protein degradation, or increases in mtGPAT1 mRNA earlier during the 21 days of voluntary wheel running that are not maintained after 21 days due to the adaptive increases in mtGPAT protein.
To determine whether changes in transcription factors might be responsible for the 54% suppression in mtGPAT1 mRNA 5 h after 21 days of wheel running, we determined the protein levels of SREBP-1, a principal regulator of lipogenesis.

SREBP-1 is known to increase the expression of multiple lipogenic genes (12), including mtGPAT1 (9, 11), which has three consensus SREBP-1 binding sites in the murine promoter (14) that are conserved in the rat promoter (accession no. Fig. 7. AMP-activated protein kinase-α (AMPKα) Thr172 phosphorylation (A and B), total AMPK protein levels (C and D), ratio of AMPKα Thr172 phosphorylation to total AMPK protein (E and F), and acetyl-coenzyme A carboxylase Ser79 phosphorylation (G and H). A, C, E, and G: after 21 days of wheel running; B, D, F, and H: after short-term (acute) physical activity, as indicated at bottom of figure. Time of death for short-term (acute) physical activity indicates hours after locking of running wheels for wheel-running groups (B, D, F, and H, left) or time since end of last bout of treadmill running for treadmill groups (B, D, F, and H, right). Levels are expressed relative to loading control, which consisted of epididymal fat homogenate protein from a nonexperimental animal that was loaded in triplicate on each blot. Representative immunoblot is located above most panels. Groups connected by lines are significantly different; *P < 0.05; **P < 0.005; ***P < 0.001; ###P = 0.01. Two-way ANOVA indicated significant effect of time of death (10 h > 5 h after running wheels were locked) for total AMPK protein level (not indicated on graph, P < 0.01; D) and ratio of AMPKα Thr172 phosphorylation to total AMPK protein (not indicated on graph, P < 0.001; F); Interaction effect between time of death (0, 5, or 10 h) and activity (treadmill or sedentary) approached significance (P = 0.06) for ratio of AMPKα Thr172 phosphorylation to total AMPK protein. Bars represent means ± SE. See MATERIALS AND METHODS for group descriptions. WL5, WL29, WL53, and SED5 (A, C, E, and G) were compared using ANOVA; WL5, WL10, SED5, and SED10 (A, C, E, and G) were compared using 2-way ANOVA; acute activity groups in B were compared using 2-way ANOVA (see Statistics).

Fig. 8. Casein kinase-2α protein level (A) and activity (B) after 21 days of voluntary wheel running. A: protein level is relative to loading control, which consisted of epididymal fat homogenate protein from a nonexperimental animal that was loaded in triplicate on each blot. Representative immunoblot is located above the graph. B: casein kinase-2α activity was measured as described in MATERIALS AND METHODS; n = 10/group. Bars for both graphs represent means ± SE. See MATERIALS AND METHODS for group descriptions. WL5, WL29, WL53, and SED5 were compared using ANOVA; WL5, WL10, SED5, and SED10 were compared using 2-way ANOVA (see Statistics).
AY-693775). Full-length SREBP-1 is located in the endoplasmic reticulum. In response to cellular signals, the NH2 terminus of SREBP-1 is cleaved and translocates to the nucleus, where it can bind to specific promoters and activate transcription (27). The antibody we used to detect SREBP-1 in immunoblots was raised against the NH2-terminal region and recognizes both the full-length and truncated forms of SREBP-1. However, there was no difference in the amount of truncated SREBP-1 after either 21 days of wheel running (Fig. 6A) or acute increases in physical activity in untrained rats (Fig. 6B).

CBP (2, 10, 23, 32, 34), nuclear factor Y-β (2, 15, 33), and Sp1 (2, 4, 15) are known to associate with truncated SREBP-1 and affect its nuclear stability and its ability to activate transcription. There were no changes in the protein levels of either nuclear factor Y-β (Fig. 6G) or Sp1 (Fig. 6H). However, at 29 h after 21 days of wheel running, there was a significantly lower level of CBP protein relative to sedentary animals, which approached significance (P = 0.053) at 53 h of reduced physical activity (Fig. 6F). This lower CBP protein level is associated with a similar decrease in the adipogenic transcription factor CCAAT/enhancer binding protein-α that we have previously reported at this same time point (16), although the physiological relevance is presently unclear.

**Posttranslational Regulation of mtGPAT1 Activity**

The increase observed in mtGPAT protein level was modest (9–19%) compared with the overshoot in enzyme activity (45–58%) for 10, 29, and 53 h after 21 days of wheel running relative to sedentary groups. As the antibody used against mtGPAT cannot distinguish between the two distinct mitochondrial isoforms (19), it might be that there are larger changes in mtGPAT1 that are obscured by a lack of change in mtGPAT2. Alternatively, the modest change in mtGPAT protein relative to mtGPAT1 activity may also indicate that, in addition to a higher level of mtGPAT protein, there might also be posttranslational regulation of GPAT activity involved in the overshoot effect. This idea is supported by a report showing that, relative to other organs, rat adipose tissue has a relatively low level of mtGPAT protein, but a high level of mtGPAT1 activity, indicating a potentially high degree of posttranslational modulation (18). MtGPAT1 activity has been shown to decrease in response to phosphorylation by AMPK (7, 22) and increase in response to phosphorylation by casin kinase-2 (24, 25). However, the overshoot in mtGPAT1 activity (Fig. 3A) was not associated with either AMPKα Thr\(^{172}\) phosphorylation or casin kinase-2 activity, because values for AMPKα Thr\(^{172}\) phosphorylation (Fig. 7A) and casin kinase-2 activity (Fig. 8B) are the same as they are in sedentary rats that do not have the overshoot in mtGPAT1 activity.

**Summary**

In summary, novel observations of the present study are that 1) although the suppression of triacylglycerol synthesis in epididymal fat of rats at 0–5 h after ending running is an acute response to a single bout of physical activity, the post-activity overshoot in triacylglycerol synthesis requires more than a single day of running; 2) changes in mtGPAT1 activity appear to account, at least partially, for the suppression effect on triacylglycerol synthesis at 5 h and the overshoot effect at 10, 29, and 53 h after 21 days of wheel running; 3) an increase in mtGPAT protein level is associated with the overshoot in enzyme activity at 10, 29, and 53 h after 21 days of wheel running compared with sedentary, although there is a paradoxical decrease in mtGPAT1 mRNA at 5 h and no difference in mtGPAT1 mRNA from sedentary levels at 29 and 53 h of reduced physical activity; and 4) CBP protein level is lower 29 h after 21 days of wheel running than in sedentary animals. These results demonstrate that an increase in mtGPAT protein and mtGPAT1 activity likely contribute to the overshoot in triacylglycerol synthesis after 21 days of wheel activity, which is consistent with survival mechanisms that support the maintenance of adipose triacylglycerol stores when there is greater energy expenditure because of regular physical activity.

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