AMPKα is essential for acute exercise-induced gene responses but not for exercise training-induced adaptations in mouse skeletal muscle

Joachim Fentz,1 Rasmus Kjøbsted,1 Caroline Maag Kristensen,2 Janne Rasmus Hingst,1 Jesper Bratz Birk,1 Anders Gudiksen,2 Marc Foretz3,4,5* Peter Schjerling,6 Benoit Viiolet,3,4,5 and Jørgen F. P. Wojtaszewski1

1Section of Molecular Physiology, the August Krogh Centre, Department of Nutrition, Exercise and Sports, University of Copenhagen, Copenhagen, Denmark; 2Centre of Inflammation and Metabolism, Department of Biology, University of Copenhagen, Copenhagen, Denmark; 3Institut National de la Sante et de la Recherche Medicale, U1016, Institut Cochin, Paris, France; 4CNRS, UMR8104, Paris, France; 5Université Descartes, Sorbonne Paris Cité, Paris, France; 6Institute of Sports Medicine, Department of Orthopedic Surgery, Bispebjerg Hospital and Center for Healthy Aging, Faculty of Medical and Health Sciences, University of Copenhagen, Copenhagen, Denmark

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Exercise training has the potential to prevent or offset the development of many health disorders, including the rapidly growing lifestyle-related disease type 2 diabetes (6). Augmentation of muscle angiogenesis, mitochondrial biogenesis, and metabolic enzyme expression are important mechanisms in the improved regulation of metabolic homeostasis with exercise training (63). A single bout of exercise increases the mRNA content of metabolic proteins in skeletal muscle (40, 50). It is believed that if physical exercise is repeated regularly for a prolonged period (i.e., exercise training), each single stimulus accumulates to create a measurable increase in protein content (56). However, the signaling pathways responsible for mediating the adaptive response of skeletal muscle to a single exercise bout and to exercise training are poorly defined.

Evidence indicates that the energy sensor 5′-AMP-activated protein kinase (AMPK) is important in conveying the exercise stimulus to induce biochemical adaptations in skeletal muscles. The regulation of metabolic genes by AMPK is achieved by downstream modulation of deacetylases, transcription factors, and transcriptional coactivators. Phosphorylation of histone deacetylase 5 (HDAC5) by AMPK increases HDAC5 binding to 14-3-3 protein, and the subsequent nuclear export of the complex allows transcription factor-mediated regulation of glucose transporter 4 (GLUT4) gene expression (41). The gene encoding the glucose-metabolizing protein hexokinase II (HKII) contains a cAMP response element in its promoter region (49), and AMPK regulates the activity of the transcription factor cAMP response element-binding protein through direct phosphorylation (53). Experiments performed in vitro and in cell culture have shown that AMPK phosphorylates peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (23), which is known as a major transcriptional regulator of genes encoding oxidative proteins in skeletal muscle (36). It has been suggested that phosphorylation of PGC-1α allows its subsequent deacetylation by silent information regulator T1 (SIRT1) (8), which is required for PGC-1α-mediated gene expression of fatty acid utilization proteins (16). Interestingly, SIRT1 activity is increased through an AMPK-mediated increase in NAD+/NADH ratio (7). Additional studies support an AMPK-PGC-1α pathway mediating mitochondrial and metabolic gene expression/adaptations; both AMPK and PGC-1α regulate gene expression of mitochondrial proteins (cytochrome c (cyt c), cytochrome c oxidase I [COX-I]; subunit of electron transport chain [ETC] complex IV), citrate synthase (CS), ATP-synthase, δ-aminolevulinate synthase, mitochondrial transcription factor A) (22, 25, 35, 62), metabolic...
AMPKα IN SKELETAL MUSCLE EXERCISE ADAPTATIONS

and angiogenic proteins (GLUT4, VEGF) (25, 33, 43, 66), and PGC-1α itself (19). A single injection of the AMP-mimetic compound 5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) has been shown to activate AMPK and increase PGC-1α, HKII, and VEGF mRNA levels in skeletal muscles of wild-type (WT) but not AMPKα2-deficient mice (27, 66). Also, mice that carry an activating AMPKβ mutation (R225Q) display increased basal (15, 37, 46) and exercise-induced mRNA levels (2) along with increased basal protein content of metabolic proteins in skeletal muscle (15). Thus, AMPK signaling seems to control skeletal muscle gene expression by direct and indirect regulation of transcription factors.

Chronic activation of AMPK by AICAR or the ATP-depleting creatine analog β-guanadionpropionic acid (β-GPA) results in numerous biochemical adaptations that are also induced by exercise training in skeletal muscle of rodents. These include increased protein content of GLUT4 and HKII in addition to mitochondrial enzyme content and activity (3, 20, 25, 32, 58, 65), as well as increased running endurance in mice (45). Importantly, the β-GPA- and AICAR-induced biochemical adaptations are not observed in mice lacking AMPKα2 catalytic activity (25, 65), proposing that AMPKα2 is essential in mediating these effects.

Acute exercise increases AMPK activation in human (9, 14, 61) and rodent skeletal muscle (57), and electrical stimulation of rodent muscle also potently increases the activity of AMPK (26, 55). Contraction and exercise primarily increase activation of the AMPKα2 isoform rather than the AMPKα1 isoform (14, 26, 25, 57, 61). However, both exercise-induced regulation of metabolic mRNA levels and exercise training-induced regulation of metabolic proteins are essentially normal in AMPKα2-deficient mice (1, 25, 27). A heightened contraction-induced activity of AMPKα1 in AMPKα2 knockout (KO) mouse muscle could suggest that AMPKα1 may compensate for the lack of AMPKα2 to induce normal mRNA and protein responses (26). Supporting this, mice lacking LKB1 (serine/threonine kinase 11) in skeletal muscle seem less capable of adapting to acute exercise and exercise training (52). Thus, a more complete ablation of AMPK activity, either by deleting the primary upstream kinase LKB1 or by disrupting both β-isozymes, appears to effectively inhibit muscle adaptations to contractile activity.

We recently revealed that skeletal muscle-specific AMPKα1 and -α2 knockout (mdKO) mice have reduced mitochondrial oxidative capacity and reduced capacity for fatty acid utilization in skeletal muscle during exercise (13, 29). This suggests that the AMPKα subunits have important roles in acute regulation of metabolism. To investigate whether AMPKα subunits are also involved in adaptations to exercise training by regulating gene and protein expression, AMPKα WT and mdKO mice were subjected to either an acute bout of exercise or voluntary running wheel exercise training.

METHODS

Mice

Female mice with skeletal muscle-specific deletion of AMPKα1 and AMPKα2 (mdKO; AMPKα1<sup>fl/fl</sup>, AMPKα2<sup>fl/fl</sup>, and HSA-Cre<sup>+/−</sup>) and their WT (AMPKα1<sup>+/+</sup>, AMPKα2<sup>+/−</sup>, and HSA-Cre<sup>−/−</sup>) littermates were used in the study (mean age ± SD: 14 ± 2 wk). The generation of the mice as well as the quality and specificity of the KO has been described previously (13, 29). Offspring were obtained from mating AMPKα1 mdKO males with WT females to prevent any potential adverse effects that a pregnant mdKO female could have on the offspring during the gestation period. The mice were tested for genotype by PCR on tail DNA using the following primers: AMPKα1<sup>fl/fl</sup> CTCCTCAACTCTGTACATCTAACT and GCA-GAGGAAGGCACAGCCAGAC; AMPKα2<sup>fl/fl</sup> CTTGCTTGCAG and GGGCGGAAAGTCTTACATCTTCTA; AMPKα2<sup>+/−</sup> GTATCAACCTCAATAATCCAA and CGTCCTAAAAGCAGAC; AMPKα2<sup>+/+</sup> AAATGTACATGCACCA; AMPKα2<sup>−/−</sup> TATAGCATACATT and AATCGTCTGCTTGCAGTTCCA; HSA-Cre, ACGGAGAGAAGGTTTTTGGG; and CGCGATAGTCGAAAGGTATG. Only the last two primer sets (AMPKα2<sup>+/−</sup> and HSA-Cre) were used once it was established that the breeding mice were all AMPKα1<sup>+/−</sup> and AMPKα2<sup>+/−</sup>, so only the presence of HSA-Cre was a variable. Mice were kept on a 12:12-h light-dark cycle (lights on from 6 AM to 6 PM, room temperature 22°C) with free access to water and standard rodent chow at all times (no. 1319F; Altromin, Lage, Germany). All experiments were approved by the Danish Animal Experimental Inspectorate and complied with the European Union convention for protection of vertebrate animals used for scientific purposes (Council of Europe 123, Strasbourg, France, 1985).

Study Design

Two cohorts of AMPKα WT and mdKO mice were randomly assigned to a sedentary control group or a voluntary exercise training group (design shown in Fig. 1). One cohort (n = 13–15) was terminated after 4 wk of exercise training (basal cohort [BC]) to obtain tissues for analysis of biochemical adaptations. The second cohort (n = 10–15) continued the exercise training in parallel with undergoing running performance tests to evaluate physiological adaptations to the exercise training [running test cohort (RTC)]. This design allowed for assessing skeletal muscle biochemical adaptations to exercise training in the BC while at the same time avoiding any confounding effects of the running test regimes. However, glucose tolerance was examined in both cohorts after 3 wk.

The RTC mice were adapted to treadmill running on 3 consecutive days (starting on day 26), followed by 1 day of recovery before undergoing a maximal running speed test (described below) on day 30. On day 33 they were refamiliarized with the treadmill, and on day 35 they performed a running endurance test (described below).

To investigate adaptive mRNA responses to exercise, a separate group of untrained AMPKα WT and mdKO mice performed an acute bout of treadmill exercise (described below).

In Vivo Protocols

Morphological analyses, MRI scan. The mice were weighed at the beginning of the study and thereafter once weekly along with registration of food consumption. The body composition of the mice was analyzed using magnetic resonance imaging (EchoMRI 4-in-1; EchoMRI, Houston, TX) before and after the 4 wk of exercise training.

Exercise training. AMPKα WT and mdKO mice were housed in single cages without (sedentary) or with (training) access to a running wheel (Techniplast activity cage, wheel Ø: 23 cm; Techniplast, Buguggiate, Italy). During the 4-wk exercise training period, voluntary running distance and duration were monitored with cycle computers (BC 1400; Sigma Sport, Neustadt, Germany). The running wheels were locked at 6 PM on the day before the mice were euthanized. Guttation was removed at 6 AM, mice were euthanized by cervical dislocation 6–8 h later, and tissues were removed, instantly frozen in liquid nitrogen, and kept at −80°C until they were processed further.

Oral glucose tolerance test. After 3 wk of voluntary running wheel exercise training, the mice were subjected to an oral glucose tolerance
test (OGTT). Running wheels of the exercise training groups were locked at 6 PM the day before the OGTT. On the experimental day the mice were fasted for 6 h (starting at 6 AM) before they were given an oral gavage of glucose (2 g/kg body wt) dissolved in a 0.9% saline solution (0.3 mg glucose/µl). Blood glucose concentration was measured in tail blood (Contour XT; Bayer, Leverkusen, Germany) shortly before mice were gavaged and again at 20, 40, 60, 90, and 120 min after the gavage.

**Exercise capacity tests.** The RTC mice underwent two exercise capacity tests. The tests were spaced at least 3 days apart to ensure proper time for recovery. At each occasion the running wheels were locked the evening before the test (at 6 PM). Maximal running speed of sedentary and trained AMPK mice was determined as described previously (13). In short, on **days −4, −3, and −2**, mice were acclimatized to treadmill running (5 min at 7.2 m/min, followed by 5 min at 9.6 m/min) before the test (**day 0**). On the test day, treadmill speed started at 4.8 m/min and increased by 2.4 m/min every 2 min until exhaustion. The incline was kept constant at 5°. The running endurance test was conducted at 75% of individual mouse maximal running speed (as determined above). The mice ran at a 10° incline at their individually predetermined speed until exhaustion [time to exhaustion (TTE)]. Before and immediately after each test, blood glucose concentration was measured from tail blood using a glucometer (Contour XT; Bayer). In addition, blood lactate concentration was measured prior to and after the running endurance test (Accutrend Delta; Roche, Basel, Switzerland).

**Acute exercise.** A separate group of untrained AMPK WT and mdKO mice were randomized into either a rest or an exercise group (n = 14–17). All mice were acclimatized to treadmill running and performed a maximal running speed test as described above. After 3 days of recovery, mice were refamiliarized with the treadmill once, using the acclimation protocol described above. Five days after the maximal running speed test, mice in the exercise groups performed an acute exercise bout consisting of 30 min of treadmill running up a 5° incline at 60% of individual mouse maximal speed. After exercise, mice recovered for 3 h before they were euthanized by cervical dislocation, and tissues were harvested and instantly frozen in liquid nitrogen. Work performed was calculated as follows; (J) = body weight (kg) × vertical ascent (m) × 9.8 m/s². Vertical ascent was determined using Pythagoras’ theorem vertical ascent (m) = [sin ([5°/360°]) × 2π] × running speed (m/min) × running time (min).

**Muscle Analyses**

**Muscle homogenization.** Whole quadriceps muscles crushed in liquid nitrogen and whole soleus and EDL muscles were homogenized in ice-cold homogenization buffer (10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES, 1% NP-40, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mM Na3VO4, and 3 mM benzamidine, final buffer adjusted to pH 7.5) with steel beads in a TissueLyser II (Qiagen) at 30 Hz for 2 × 1 min. Next, the samples were rotated end over end for 60 min at 4°C and were then centrifuged at 18,230 × g for 20 min at 4°C to obtain lysates (i.e., the supernatants).

**Analysis of muscle protein and protein phosphorylation.** Analysis of total protein concentration and preparation of lysates for SDS-PAGE were performed as described previously (13). Samples and controls were loaded onto self-cast Tris-HCl polyacrylamide gels (Mini-PROTEAN Tetra Cell Casting Module; Bio-Rad, Hercules, CA) or precast Tris-HCl gels (Mini-PROTEAN; Bio-Rad) in equal amounts, as described (13). The sample protein amount loaded onto the gels was ensured to be within the linear range of the assays when the specified antibodies were used. Control samples were loaded at each end of the gels to be able to correct for variations in gel-to-gel and gel-to-membrane transfer efficiency. Resolved proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P Membrane; Millipore, Billerica, MA) by semidry blotting. The membrane was blocked in a washing buffer [Tris-buffered saline-Tween 20 (TBST)] containing 2–5% skimmed milk and incubated with primary antibodies overnight at 4°C (TBST with 2–5% skimmed milk). The following primary antibodies were used: anti-cluster of differentiation 36 (CD36; no. AF2519; R&D Systems, Minneapolis, MN), anti-FABPpm (plasma membrane fatty acid-binding protein; kindly donated by Dr. Calles-Escandon, Wake Forest University, Winston-Salem, NC), anti-cyt c (no. 556433; BD Pharmingen, Franklin Lakes, NJ), anti-COX-I (no. 459600; Thermo Scientific), anti-CD31 (no. ab28364; Abcam, Cambridge, UK), anti-ubiquitin, and anti-ubiquinol cytochrome c reductase core protein 1 (UQRC1; complex III; no. A21362; Molecular Probes, Invitrogen). Secondary antibody incubation and chemiluminescence detection were performed as de-
Table 1. Primer and probe sequences used for real-time PCR

<table>
<thead>
<tr>
<th>Gene (Primer)</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>PGC-1α</td>
<td>Forward 5'-AGCCAAACCAACACATTTATCTCTTC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TCAGGGTGCTCCGAACTGGATG-3'</td>
</tr>
<tr>
<td></td>
<td>Probe 5'-AGACTGCCCAAGAGGTTGCC-3'</td>
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<tr>
<td>GLUT4</td>
<td>Forward 5'-CCCGGGGGCTCTTTAAGGAGTAC-3'</td>
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<td></td>
<td>Reverse 5'-AGGCCACCCCGAAGGATG-3'</td>
</tr>
<tr>
<td></td>
<td>Probe 5'-TGCCGGAATACCGATGAC-3'</td>
</tr>
<tr>
<td>HKII</td>
<td>Forward 5'-CTGGTCTACAAGAAGGATCCATT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GACGCGCGTCACTATACG-3'</td>
</tr>
<tr>
<td></td>
<td>Probe 5'-GATGTCAGGGGCTGTGCagara-3'</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Forward 5'-TGCCCAATTGCTGGACACTGT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CAGAGGCTGCACAGAATAAC-3'</td>
</tr>
<tr>
<td></td>
<td>Probe 5'-AGGAAGCACAGATCCGACCA-3'</td>
</tr>
<tr>
<td>COX-I</td>
<td>Forward 5'-TCGACACTTACACCGAGATATAA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-ATGGATCGTGAACAGATCTGA-3'</td>
</tr>
<tr>
<td></td>
<td>Probe 5'-CTCTGAACCCGATTTTACCTCGATCCCA-3'</td>
</tr>
<tr>
<td>CD36</td>
<td>Forward 5'-CCCAATGAAGATGACAGATTCAAT-3'</td>
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<tr>
<td></td>
<td>Reverse 5'-CGTGGATGCACCCGTAACA-3'</td>
</tr>
<tr>
<td></td>
<td>Probe 5'-AGACTGGGACCCCAACATGTTACCTACCA-3'</td>
</tr>
<tr>
<td>VEGF</td>
<td>Forward 5'-ACCTGCTGCCTTACGGCTTACT-3'</td>
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<tr>
<td></td>
<td>Reverse 5'-CTCTGGATCTGGTCTTGCTCTT-3'</td>
</tr>
<tr>
<td></td>
<td>Probe 5'-AAAGTGTGTCGGCTGCACCCGAC-3'</td>
</tr>
</tbody>
</table>

PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1a; GLUT4, glucose transporter 4; HKII, hexokinase II; cyt c, cytochrome c; COX-I, cyt c oxidase subunit I; CD36, cluster of differentiation 36.

scribed previously (13). VEGF protein was measured in muscle lysates using an ELISA kit (MMV00; R & D Systems).

**Enzyme activities.** The maximal activity of CS and β-hydroxycetyl-CoA dehydrogenase (β-HAD) was measured in muscle lysates (1 μg protein) as NADH production by spectrophotometric determination of NADH changes at 340 nm at 37°C, pH 7.0, using an automatic analyzer (Hitachi automatic analyzer 912; Roche, Basel, Switzerland). Acetyl-CoA and oxaloacetate were used as substrates for the CS-mediated conversion of NAD to NADH and acetoacetyl-CoA as substrate for the β-HAD-mediated production of NADH (38).

**mRNA isolation.** Approximately 25 mg of crushed quadriceps muscle was homogenized (Qiagen), and RNA was isolated by a guanidinium thiocyanate phenol-chloroform method, as described previously (11, 50). The Superscript II RNase system (Invitrogen) was used for reverse transcription of 3 μg of total RNA using 0.5 μg of oligo(dT), as described by Pilegaard et al. (50).

**Real-time PCR.** Fluorescence-based real-time PCR (ABI PRISM 7900 Sequence Detection System; Applied Biosystems) was used to determine mRNA content of specific genes as described (39). The mRNA content was analyzed using Taqman probes and forward and reverse primers as listed (Table 1), except for FABPpm (Mm00494703_m1), fatty acid transport protein (FATP1) (Mm0049511_m1), and FATP4 (Mm01327405_m1) mRNA, which were analyzed using premade kits (Applied Biosystems). Unknown samples were determined in triplicate and related to a standard curve made by a serial dilution of a representative pooled sample. The target gene mRNA content of each sample was normalized to the single-stranded DNA content measured by Oligreen as described (39).

**Statistics**

All data are presented as means ± SE. Statistical evaluation was performed by an unpaired t-test or by two- or three-way ANOVA with or without repeated measurements, as specified in the figure legends. If significant ANOVA interactions were detected, specific differences were identified by Student-Newman-Keuls post hoc analysis. Statistical significance was accepted at P < 0.05. In the PGC-1α gene data set, heteroscedasticity persisted (Levene’s test) despite data transformation (square, natural log, reciprocal, exponential, and square root transformations were attempted); thus these data have been analyzed using multiple i-tests, applying Bonferroni correction to the P values.

Data were analyzed using SigmaPlot (version 13.0; Systat Software, San Jose, CA) and SPSS (version 20; IBM, Armonk, NY).

Due to differences in total running distance of AMPKα WT and mdKO mice, an additional comparison of protein adaptations was made. The comparison was made in subgroups of AMPKα WT and mdKO mice, having performed an equal exercise volume [equal running distance (ERD)] during the 4 wk of exercise training. The inclusion criteria were defined as follows; the AMPKα WT mouse that ran the shortest distance set the lower limit of which mdKO mice to include (ERD-mdKO, n = 6; Fig. 7A). The AMPKα mdKO mouse that ran the longest distance set the upper limit of which WT mice to include (ERD WT, n = 8). The resulting mean running distances were analyzed by an unpaired t-test, showing no difference between the two ERD subgroups (Fig. 7B). These ERD training groups were statistically analyzed together with the full sedentary AMPKα WT and mdKO groups.

**RESULTS**

**Exercise-Induced mRNA Responses in Skeletal Muscle of AMPKα WT and mdKO Mice**

To examine the role of AMPKα on the mRNA response to a single bout of exercise, we determined the mRNA content of proteins important in skeletal muscle metabolism and energy production. Mice were either rested or subjected to an acute bout of exercise, as described in METHODS, after which they recovered for 3 h before muscles were removed (maximal running speed; WT rested group, 33.4 ± 1.2 m/min; mdKO rested group, 21.5 ± 0.8 m/min; WT exercised group, 35.0 ± 1.7 m/min; mdKO exercised group, 21.0 ± 0.9 m/min; main effect of genotype, P < 0.001; work performed during the 30-min exercise bout at 60% of maximal running speed; WT rested group, 11.4 ± 0.5 J; mdKO rested group, 7.1 ± 0.3 J; WT exercised group, 12.1 ± 0.6 J; mdKO exercised group, 7.1 ± 0.4 J; main effect of genotype, P < 0.001).

A decreased mRNA content was found in many [PGC-1α, cyt c, GLUT4, CD36, FABPpm, FATP1, VEGF, and HKII (P = 0.054, power = 0.369)] but not all [COX-I (complex IV) and FATP4] metabolic markers in muscle of AMPKα mdKO relative to WT (Fig. 2, A–F). Three hours after the acute exercise bout, an increase in mRNA content of COX-I, GLUT4, and VEGF was observed in AMPKα WT but not mdKO muscle (Fig. 2, B, C, and F), and the PGC-1α mRNA 3 h after exercise was significantly higher in WT than in mdKO muscle (Fig. 2A).

Furthermore, the acute exercise bout increased the mRNA content of CD36 and FATP1 similarly in muscle of AMPKα WT and mdKO mice (Fig. 2, D–E), although a tendency toward a larger increase in CD36 mRNA (P = 0.096, power = 0.253) was detected in muscle of WT mice compared with mdKO.

These data suggest that AMPKα is implicated in regulation of a diverse array of metabolic protein-encoding genes at basal and in response to exercise. A lack of gene responses to acute exercise in AMPKα mdKO mice could
Morphological Data of Sedentary and Trained AMPK

Exercise training.

AMPK increases in protein expression. To test this hypothesis, potentially result in the absence of exercise training-induced increases in protein expression. To test this hypothesis, AMPK WT and mdKO littermates underwent 4 wk of exercise training.

Morphological Data of Sedentary and Trained AMPK WT and mdKO Mice

There was no difference in weight gained between groups over the 4 wk of exercise training (Table 2). To compare body morphology between genotypes, lean body mass, fat mass, and the respective changes over time were expressed as percentage of body weight, correcting for a slightly higher body weight (~0.5 g) of AMPK mdKO than WT mice. Lean mass percentage was not different between groups, although the relative gain in lean mass tended toward being higher in the trained groups. Fat mass percentage was also similar in all groups, whereas there was a significantly larger decrease in fat mass in the trained groups than in the sedentary groups. Daily food intake related to body weight was higher in trained AMPK WT mice (P < 0.001) and tended toward being higher in trained mdKO mice (P = 0.077, power of interaction n = 0.550) than in the sedentary control mice. Lean mass gain (%BW) 3.2; fat mass gain (%BW) 2.4; daily food intake, g 3.5; daily food intake (%BW) 15.7; daily food intake, g 22.2; daily food intake, g 2.3.

Table 2. Morphological parameters of sedentary and trained AMPK WT and mdKO mice

<table>
<thead>
<tr>
<th></th>
<th>Sedentary</th>
<th>Training</th>
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<tr>
<td></td>
<td>WT (n = 30)</td>
<td>mdKO (n = 30)</td>
</tr>
<tr>
<td>BW, day 0, g</td>
<td>22.2 ± 0.3</td>
<td>22.4 ± 0.3</td>
</tr>
<tr>
<td>BW gain, g</td>
<td>0.5 ± 0.2</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Lean mass, day 0 (%BW)</td>
<td>81.6 ± 0.5</td>
<td>82.2 ± 0.5</td>
</tr>
<tr>
<td>Lean mass gain (%BW)</td>
<td>3.2 ± 0.7</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>Fat mass, day 0 (%BW)</td>
<td>9.3 ± 0.4</td>
<td>9.3 ± 0.3</td>
</tr>
<tr>
<td>Fat mass gain (%BW)</td>
<td>-1.4 ± 0.5</td>
<td>-1.2 ± 0.3</td>
</tr>
<tr>
<td>Daily food intake, g</td>
<td>3.5 ± 0.1</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>Daily food intake (%BW)</td>
<td>15.7 ± 0.5</td>
<td>16.2 ± 0.5</td>
</tr>
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</table>

Data are presented as means ± SE (n = 28–30). WT, wild type; mdKO, muscle-specific AMPKα1 and -α2 double-knockout; BW, body weight. Statistical test: 2-way ANOVA. Significant effect of exercise training, *P < 0.05, **P < 0.001; significant effect of genotype, #P < 0.05; ( ) indicates borderline significance 0.05 < P < 0.10. NS, nonsignificant; NA, not applicable.
respective sedentary groups. Within the trained groups, AMPKα WT mice had a higher food intake than mdKO mice ($P < 0.05$).

**Reduced Running Distance and Speed During Voluntary Running Wheel Exercise Training in AMPKα mdKO Mice**

Over the 4 wk of voluntary exercise training, AMPKα WT mice ran $135\pm 7$ km, as opposed to the mdKO mice, which only ran $56\pm 7$ km ($P<0.001$; Fig. 3, A and B). Although running distance increased over time in both groups, the AMPKα mdKO mice did at no time catch up with the WT mice (Fig. 3A). Also, when exercising in the running wheel, AMPKα mdKO mice ran at a $\sim 19\%$ ($P<0.001$) lower running speed than the WT mice (Fig. 3, C and D).

An OGTT was performed after 3 wk of voluntary exercise. This revealed that glucose tolerance was slightly impaired in AMPKα mdKO compared with WT mice. Exercise training did not improve glucose tolerance in any of the genotypes (Fig. 3, E and F).

**AMPKα Does Not Regulate Exercise Training-Induced Improvements in Running Performance**

To investigate whether lack of skeletal muscle AMPKα affects physiological adaptations to exercise training, AMPKα WT and mdKO mice were subjected to a maximal running speed test and a running endurance test on a motorized treadmill. Maximal running speed of sedentary AMPKα mdKO mice was $\sim 34\%$ lower than that of sedentary WT mice (WT sedentary $30.4\pm 2.0$ m/min, mdKO sedentary $20.2\pm 0.8$ m/min; Fig. 4A). Exercise training increased the maximal running speed of both genotypes comparably (WT trained, $32.8\pm 1.4$ m/min; mdKO trained, $24.6\pm 1.8$ m/min).

Mice underwent a running endurance test at which they ran until exhaustion at a constant speed set to $75\%$ of individual mouse maximal speed. Overall, the AMPKα WT mice ran for a longer time than the mdKO mice before reaching exhaustion, and exercise training did not improve TTE of either WT or mdKO mice (Fig. 4B). For unknown reasons, TTE data variations were particularly large in the AMPKα WT groups, decreasing the power of the statistical analysis (power of
interaction = 0.05). Thus, despite being nonsignificant, it is worth noting that the TTE of trained AMPKα mdKO mice was increased by ~125% compared with sedentary mdKO mice. At exhaustion, blood lactate concentration had increased similarly in the two genotypes, and the increase was independent of training status (Fig. 4C). Blood glucose concentration decreased with endurance exercise in the AMPKα WT mice, whereas it increased in the mdKO mice (Fig. 4D). This is in line with previous observations in AMPKα mdKO mice (13).

In summary, the data suggest that the lack of skeletal muscle AMPK does not prevent exercise training-induced improvements in running performance. Nonetheless, embryonic knockout of AMPKα greatly reduces exercise capacity regardless of training status.

Effects of Exercise Training on the Expression of Mitochondrial, Cytosolic, and Angiogenic Proteins

Molecular adaptations of skeletal muscles to exercise training were examined mainly in quadriceps muscle and for some proteins also in the more oxidative soleus muscle and in the glycolytic EDL muscle.

Content of mitochondrial proteins SDHA (a subunit of ETC complex II), cyt c, and COX-I (a subunit of ETC complex IV) was generally reduced in muscles of sedentary AMPKα mdKO mice compared with WT mice, whereas CS, β-HAD, ND6 (a subunit of ETC complex I), and UQCRCL1 (a subunit of ETC complex III) activity/protein content was similar between genotypes (Figs. 5, A and B, and 6, A–C). Exercise training increased the activity/content of CS, UQCRCL1, and cyt c in quadriceps muscle of AMPKα WT mice only (Figs. 5, A and B, and 6A). In contrast, the activity of β-HAD and the protein content of SDHA and COX-I muscle were similarly induced in quadriceps of trained AMPKα WT and mdKO mice (Figs. 5, A and B, and 6A).

A similar protein content of GLUT4, HKII, CD36, and FABPpm was found in quadriceps muscle of sedentary AMPKα WT and mdKO mice (Fig. 6, D and G). The increase in HKII and FABPpm protein content with exercise training was larger in AMPKα WT than in mdKO mouse muscle, whereas exercise training increased GLUT4 and CD36 comparably in quadriceps muscle of both genotypes (Fig. 6, D and G).

To evaluate the role of AMPK in angiogenesis, protein content of VEGF and CD31 (marker of vascular endothelium) was measured in quadriceps muscle. The level of VEGF protein was lower in sedentary AMPKα mdKO mice than in WT mice (Fig. 5C), whereas the CD31 protein level was similar in the two genotypes (Fig. 5D). A tendency toward a larger effect of exercise training on muscle VEGF protein content was observed in AMPKα WT compared with mdKO mice (P = 0.066, power = 0.33), whereas exercise training induced a similar increase in CD31 protein in WT and mdKO mice (Fig. 5, C and D).

In soleus muscle of sedentary mice, GLUT4 protein content was higher (Fig. 6E), whereas HKII, CD36, and FABPpm content was lower (Fig. 6, E and H) in AMPKα mdKO mice than in WT mice. The extensor digitorum longus (EDL) muscle of sedentary AMPKα mdKO mice also displayed lower levels of CD36 and FABPpm protein than WT (Fig. 6I), whereas there were no differences in EDL muscle GLUT4 and HKII content between genotypes (Fig. 6F). Exercise training did not induce increased protein expression of GLUT4, HKII, CD36, or FABPpm in soleus and EDL in any of the genotypes (Fig. 6, E, F, H, and I).
Because we have previously shown that acute voluntary running wheel exercise increases AMPKα1 and AMPKα2 activity (also in 4-wk-trained animals) (25), these data suggest that AMPKα subunits play a role in exercise training-induced adaptations in quadriceps muscle.

Comparison of Exercise Training Effects on Protein Content in a Subgroup of AMPKα WT and mdKO Mice Having Performed an Equal Training Volume

Because the total training volume of AMPKα WT and mdKO mice differed, potential differences in muscle protein adaptations may simply be an effect of training volume rather than genotype. Taking this into account, we further performed a comparison in a subgroup of trained mice in which total running distance was equal [ERD: WT, 110±9 km (n = 8); mdKO, 107±9 km (n = 6); Fig. 7, A and B]. All mice from the sedentary groups have been included for this secondary comparison.

In the ERD subgroups, the mean voluntary running speed tended to be reduced in AMPKα mdKO mice compared with WT mice (P = 0.053, power = 0.506; Fig. 7C). Morphological characteristics of the ERD subgroups (Table 3) showed only a few subtle differences compared with the full groups of mice (Table 2).
Fig. 6. Exercise training-induced adaptations in content of metabolic proteins in skeletal muscle of AMPKα WT and mdKO mice. Protein content of cyt c and COX-I was analyzed in quadriceps (A), soleus (B), and extensor digitorum longus (EDL) muscles (C) of sedentary and trained AMPKα WT and mdKO mice. Protein content of GLUT4 and HKII in quadriceps (D), soleus (E), and EDL muscles (F), and protein content of CD36 and FABPpm in quadriceps (G), soleus (H), and EDL muscles (I) of the sedentary and trained AMPKα WT and mdKO mice. Representative immunoblots are shown in A–I, bottom. Data are presented as means ± SEM (n = 12–15). Statistical test: 2-way ANOVA (A–I). Significant effect of exercise training: *P < 0.05, **P < 0.01, and ***P < 0.001; significant effect of genotype: #P < 0.05, ##P < 0.01, and ###P < 0.001; () indicates borderline significance 0.05. Horizontal lines above the bar graphs indicate main effect.
The analyses of protein adaptations in the ERD subgroups of mice showed that UQRC1 (complex III) protein expression increased exclusively in trained AMPKα WT mice (Fig. 7, D–P), suggesting that AMPK is an essential regulator of UQRC1 in response to exercise training. The remaining markers found to increase with exercise training in a seemingly AMPK-dependent manner in the full groups of mice (CS, cyt c, HKII, and FABPpm) increased similarly in AMPKα WT and mdKO mice of the ERD subgroups. Thus, for the majority of exercise training-induced adaptations of metabolic proteins, AMPKα is likely not essential.

**DISCUSSION**

The present study reveals that AMPKα is necessary for exercise-induced mRNA increments of several metabolic markers in mouse skeletal muscle [GLUT4, COX-I (complex IV) and VEGF]. Furthermore, exercise training-induced increases in protein activity/content of CS, UQRC1 (complex III), cyt c, HKII, and FABPpm in quadriceps muscle of AMPKα WT mice are partly or fully ablated in AMPKα mdKO mice. However, lack of AMPKα1 and α2 subunits in skeletal muscle does not compromise exercise training-induced improvements in running performance. Thus, although some molecular defects are evident in AMPKα mdKO mice, physiological improvements in exercise capacity are not compromised.

Both AMPK and PGC-1α are activated in response to exercise. Furthermore, both have been proposed to regulate GLUT4 expression. In myotubes that inherently express little or no PGC-1α and GLUT4, adenoviral expression of PGC-1α can drive the expression of GLUT4 in a myocyte enhancer factor 2(MEF2)-dependent manner (43). In addition, the binding of MEF2 to the GLUT4 promoter is mediated by an AMPKα2-dependent mechanism (17), and release of HDAC5 repression of the GLUT4 promoter is achieved by AMPK phosphorylation of HDAC5 directing it for nuclear export (41). The present observations that the PGC-1α mRNA level in skeletal muscle was lower in AMPKα mdKO mice than in WT after acute exercise and that GLUT4 mRNA increased only in WT mice support the view of a coordinated AMPKα–PGC-1α pathway regulating GLUT4 gene expression in response to acute exercise. Interestingly, two previous studies in AMPKα KO and AMPKα2 kinase-dead (KD) mice did not report impaired induction of GLUT4 mRNA with exercise in the AMPKα2-deficient mice (21, 27). Common to all three models (AMPKα2 KO, AMPKα2 KD, and AMPKα mdKO) is the almost total absence of AMPKα2 catalytic activity in skeletal muscle. However, the AMPKα2 KO mouse shows a compensatory increase in AMPKα1 protein content (26), whereas the AMPKα2 KD transgenic approach results in overexpression of a kinase dead AMPKα2 subunit leading to a severe reduction in endogenous AMPKα2 protein but only a modest reduction in endogenous AMPKα1 protein (31, 44). Thus, both the AMPKα2 KO and KD mice do express AMPKα subunits in skeletal muscle, whereas the AMPKα mdKO mice lack any form of AMPKα expression in skeletal muscle (29). Hence, this may suggest that catalytic activity of AMPKα2 is not necessary for an exercise-induced increase in GLUT4 mRNA. Alternatively, the AMPKα2 KO and KD mice may have developed different mechanisms to compensate for disrupted AMPKα signaling during embryogenesis and/or in the early postnatal period, whereas the deletion of both AMPKα subunits in the AMPKα mdKO mouse may have prevented such successful compensatory mechanisms from developing.

In parallel with GLUT4, the VEGF and COX-I (complex IV) mRNA was also upregulated with an acute bout of exercise in AMPKα WT but not in mdKO mice. Exercise-induced angiogenesis and VEGF content have previously been shown to be controlled by PGC-1α (10, 33). However, in AMPKα2 KD mice, exercise results in higher VEGF mRNA levels than in WT mice (66). On the contrary, the increase seen in VEGF mRNA of WT mice with electrical stimulation is fully ablated in muscle of skmLKB1 KO mice (52). Again, the present results using the AMPKα mdKO mouse model in which both catalytic subunits of AMPK are ablated show that AMPKα is indeed required for exercise-induced increases in skeletal muscle VEGF mRNA.

The present observations in AMPKα mdKO mice suggest that AMPKα is important for maintaining basal protein expression of cyt c, COX-I (complex IV), and SDHA (complex II), which is in line with previous observations in AMPKα2 KO mice (25) and with findings of reduced respiratory complex capacity in AMPKα2 KD mice (28, 30). Recently, we also reported a repressed respiratory capacity of complex I in AMPKα mdKO mouse muscle (29). Because the present results show a normal content of ND6 (complex I) protein in mdKO mice, the functional defect in complex I respiration may not be due to reduced protein expression but rather related to acute regulation of complex activity.

CS activity is thought to be a good marker of mitochondrial content, and exercise training increases both factors (4). The normal basal activity of CS in AMPKα mdKO compared with WT mice is in agreement with our previous data in these mice (29) and previous data from AMPKα2 KD mice (1). However, a decreased basal CS activity has been found in AMPKα2 KO (25), AMPKα2iITG (51), AMPKβ1/2M-KO (48), LKB1 MKO (24, 54), and skmLKB1-KO mice (52). Thus, although the majority of studies suggest that altered AMPK signaling plays a role in basal CS activity, it is clearly not the sole determinant. Habitual locomotor activity seems not to be an explanatory factor either in that it is normal in AMPK/LKB1-deficient mouse models that at the same time show either unaltered (13) or decreased (48, 52) levels of basal CS activity.

Exercise training induced a higher CS activity and protein content of cyt c, HKII, and FABPpm in quadriceps muscle of AMPKα WT than in mdKO mice, although mRNA content of these markers was similarly regulated with acute exercise in WT and mdKO mice. Recognizing the unique temporal responses of different genes to acute exercise (34), the current analysis of a single postexercise time point may have missed later potential genotype differences in gene expression. This limitation may also overlook later compensatory effects in mRNA responses to exercise. Thus, it could perhaps also explain the discrepancy between the AMPKα-dependent increase in exercise-induced GLUT4, VEGF, and COX-I (complex IV) mRNA levels and the AMPKα-independent increase in exercise training-induced GLUT4, VEGF, and COX-I protein content. It has recently been shown that skeletal muscle of AMPKα2 KD mice shows a compensatory increase in protein kinase D (PKD) Ser516 phosphorylation with exercise and that this may help to preserve regulation of HDAC5 phosphoryla-
exercise training increased maximal running speed similarly in inferior voluntary and forced running capacity. However, exercise training increased maximal running speed similarly in AMPK WT and mdKO mice. This may suggest that even though AMPK may be involved in exercise-induced regulation of some metabolic proteins, it is not essential for improving running speed.

The running endurance test showed no improvements in TTE with exercise training in either genotype, although it should be noted that trained AMPKα mdKO mice ran for twice as long as sedentary mdKO mice. An increase in TTE in the trained AMPKα mdKO mice may have been detected if the statistical power of the analysis had been higher. If true, this would suggest that the AMPKα mdKO mice are highly responsive to exercise training. Still, the AMPKα mdKO mice would not have attained the same overall exercise capacity as the WT mice, as the absolute speed of the running endurance test was lower in both untrained and trained mdKO than in the corresponding WT groups.

Despite the fact that the AMPKα mdKO mice ran less during the 4 wk of training, they appear to run at the same or even higher relative intensity as WT mice when exercising voluntarily. This is indicated by the fact that forced maximal running speed was ~29% lower in AMPKα mdKO mice than in WT mice (mean difference of AMPKα WT and mdKO untrained and trained mice, respectively), whereas the voluntary running speed of the mdKO mice was only ~19% lower than WT mice. If the running distance and speed of the AMPKα mdKO mice during the 4 wk of voluntary exercise training actually corresponds to a similar relative work load (speed and distance) compared with WT mice, it would suggest that comparable adaptive responses of the genotypes to the exercise training could be expected. At least for PGCG-1α, an exercise bout of a certain relative intensity induces a similar mRNA response despite differences in absolute exercise intensity in humans (12, 47). Whether the same conditions are valid for the remaining genes analyzed in the present study and for the increases in exercise training-induced protein content needs further investigation.

Exercise training volume is an important determinant of the adaptive response to exercise training (4). Thus, because of the large difference in total running distance between the AMPKα WT and mdKO training groups, the results on protein adaptation could potentially be biased. In an attempt to circumvent this, two subgroups of mice within the AMPKα WT and mdKO training groups, which had performed an equal running distance/exercise training volume during the 4 wk (ERD subgroups), were compared. However, this approach decreases statistical power and may introduce unknown selection bias in that the AMPKα WT mice that ran the least and the mdKO mice that ran the most are compared. Because none of

### Table 3. Morphological parameters of sedentary and trained (ERD subgroups) AMPKα WT and mdKO mice

<table>
<thead>
<tr>
<th></th>
<th>Sedentary</th>
<th>Training (ERD Subgroups)</th>
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<tbody>
<tr>
<td></td>
<td>WT (n = 30)</td>
<td>mdKO (n = 30)</td>
</tr>
<tr>
<td>Body weight, day 0, g</td>
<td>22.2 ± 0.3</td>
<td>22.4 ± 0.3</td>
</tr>
<tr>
<td>Body weight gain, g</td>
<td>0.5 ± 0.2</td>
<td>0.8 ± 0.2</td>
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<td>Lean mass, day 0 (%BW)</td>
<td>81.6 ± 0.5</td>
<td>82.2 ± 0.5</td>
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<td>Lean mass gain (%BW)</td>
<td>3.2 ± 0.7</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>Fat mass, day 0 (%BW)</td>
<td>9.3 ± 0.4</td>
<td>9.3 ± 0.3</td>
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<tr>
<td>Fat mass gain (%BW)</td>
<td>−1.4 ± 0.5</td>
<td>−1.2 ± 0.3(#)</td>
</tr>
<tr>
<td>Daily food intake, g</td>
<td>3.5 ± 0.1</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>Daily food intake (%BW)</td>
<td>15.7 ± 0.5</td>
<td>16.2 ± 0.5</td>
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Data are presented as means ± SE (n = 6–30). ERD, equal running distance. Statistical test: 2-Way ANOVA. Significant effect of exercise training, *P < 0.05, **P < 0.01, ***P < 0.001; significant effect of genotype, #P < 0.05; () indicates borderline significance 0.05 < P < 0.10.

### Fig. 7. Activity and content of mitochondrial, metabolic, and vascular endothelium-associated proteins in AMPKα WT and mdKO sedentary and trained equal running distance (ERD) subgroups. A: individual distances covered by 28 days of voluntary running wheel exercise training in AMPKα WT and mdKO mice (only mice from BC, as no protein data on TTE mice exist). Observations that lie within the box are those that have been included in the AMPKα WT and mdKO ERD subgroups. B: mean running distances of the ERD subgroups. C: mean running speed of the ERD subgroups. D: enzyme activity of CS and β-HAD in quadriceps muscle. E: protein content of ND6 (complex I), SDHA (complex II), and UQRC1 (complex III) in quadriceps muscle. Protein content of cyt C and COX-I in quadriceps (F), soleus (G), and EDL muscles (H). Protein content of GLUT4 and HKII in quadriceps (I), soleus (J), and EDL muscles (K). Protein content of CD36 and FABP (L) in quadriceps (M), soleus (N), and EDL muscles (O). Protein content of VEGF (O) and CD31 (P) in quadriceps muscle of sedentary and trained AMPKα WT and mdKO mice. Data are presented as means ± SE (A, n = 13–14; B and C, n = 6–8; D–P, n = 6–15). Statistical tests: unpaired t-test (B and C) and 2-way ANOVA (D–P). Significant effect of exercise training: *P < 0.05, **P < 0.01, and ***P < 0.001; significant effect of genotype: #P < 0.05, ##P < 0.01, and ###P < 0.001; () indicates borderline significance 0.05 < P < 0.10. Horizontal lines above the bar graphs indicate main effect.
the comparison options are optimal, we have chosen to show both the analysis of the full groups and that of the ERD subgroups. The major difference between the two analysis results is that exercise training-induced increases in CS activity and protein expression of GLUT4, cys ε, and UQRC1 (complex III) are dependent on AMPK when all mice are included, whereas only the increase in UQRC1 protein content with exercise training is dependent on AMPK when the ERD subgroups are analyzed. Because a considerable number of studies support a role for AMPK in regulation of exercise adaptations, the present study may suggest that redundant and/or compensatory mechanisms adequately make up for the loss of AMPKα1 and AMPKα2 in skeletal muscles. Whether such mechanisms develop during embryogenesis/early postnatal life and whether they involve stabilization/increased transcription of mRNA or upregulation of other pathways engaged by exercise will remain for future studies to answer.

Interestingly, UQRC1 (complex III) has been identified to interact with muscle RING finger 1 (MuRF1) in yeast two-hybrid screens of skeletal muscle cDNA libraries (59, 60), and we have recently reported MuRF1 mRNA content to be repressed in AMPKα mdKO mice (29). Because MuRF1 is known to induce muscle atrophy by ubiquitination of target proteins (5), a higher protein content of UQRC1 could be expected in AMPKα mdKO mice than in WT mice. However, because we found a normal basal protein content of UQRC1 in AMPKα mdKO muscle, additional studies will be required to elucidate the specific relationship between AMPK, MuRF1, and UQRC1 expression in skeletal muscle. It may further be appealing to investigate whether the reduction in MuRF1 mRNA in skeletal muscle of AMPKα mdKO mice slows the degradation of other metabolic proteins.

In summary, the data suggest that the AMPKα subunits mediate increases in skeletal muscle mRNA of key metabolic proteins in response to an acute bout of exercise. However, the catalytic subunits of AMPK are redundant to the majority of exercise training-induced adaptations in protein expression.

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DISCLOSURES

The authors have nothing to disclose. J. F. P. Wojtaszewski is the guarantor for this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

AUTHOR CONTRIBUTIONS


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