Fiber hypertrophy and increased oxidative capacity can occur simultaneously in pig glycolytic skeletal muscle

T. L. Scheffler,1 J. M. Scheffler,1 S. Park,1 S. C. Kasten,1 Y. Wu,2 R. P. McMillan,2 M. W. Hulver,2 M. I. Frisard,2 and D. E. Gerrard1

1Department of Animal and Poultry Sciences, Virginia Tech, Blacksburg, Virginia; and 2Department of Human Nutrition, Foods, and Exercise, Virginia Tech, Blacksburg, Virginia

Submitted 3 January 2013; accepted in final form 1 December 2013

Scheffler TL, Scheffler JM, Park S, Kasten SC, Wu Y, McMillan RP, Hulver MW, Frisard MI, Gerrard DE. Fiber hypertrophy and increased oxidative capacity can occur simultaneously in pig glycolytic skeletal muscle. Am J Physiol Cell Physiol 306: C354–C363, 2014. First published December 4, 2013; doi:10.1152/ajpcell.00002.2013.—An inverse relationship between skeletal muscle fiber cross-sectional area (CSA) and oxidative capacity suggests that muscle fibers hypertrophy at the expense of oxidative capacity. Therefore, our objective was to utilize pigs possessing mutations associated with increased oxidative capacity [AMP-activated protein kinase (AMPK)-activated protein kinase (AMPK)*3R200Q] or fiber hypertrophy [ryanodine receptor 1 (RyR1)*R615C] to determine if these events occur in parallel. Longissimus muscle was collected from wild-type (control), AMPK*3R200Q, RyR1*R615C, and AMPK*3R200Q-RyR1*R615C pigs. Regardless of AMPK genotype, RyR1*R615C increased fiber CSA by 35%. In contrast, AMPK*3R200Q muscle exhibited greater citrate synthase and β-hydroxyacyl CoA dehydrogenase activity. Isolated mitochondria from AMPK*3R200Q muscle had maximal, ADP-stimulated oxygen consumption rate. Additionally, AMPK*3R200Q muscle contained more (~50%) of the mitochondrial proteins succinate dehydrogenase and cytochrome c oxidase and more mitochondrial DNA. Surprisingly, RyR1*R615C increased mitochondrial proteins and DNA, but this was not associated with improved oxidative capacity, suggesting that altered energy metabolism in RyR1*R615C muscle influences mitochondrial proliferation and protein turnover. Thus pigs that possess both AMPK*3R200Q and RyR1*R615C exhibit increased muscle fiber CSA as well as greater oxidative capacity. Together, our findings support the notion that hypertrophy and enhanced oxidative capacity can occur simultaneously in skeletal muscle and suggest that the signaling mechanisms controlling these events are independently regulated.

AMP-activated protein kinase; ryanodine receptor; mitochondria; calcium

Skeletal muscle, which comprises ~40% of body mass, plays fundamental roles in support, movement, and whole body energy metabolism. Functional and phenotypic diversity of skeletal muscle is ascribed to the heterogeneous composition of fibers, which vary in histological, biochemical, and structural characteristics. Fibers are typically classified according to contractile speed (slow or fast) and predominant type of energy metabolism (oxidative or glycolytic). Type I fibers are slow-contracting, highly oxidative fibers; type IIa (IIa, IIx, and IIb) fibers are considered fast-contracting fibers, yet they vary widely in their capacity for oxidative or glycolytic metabolism (42). Among these properties, oxidative capacity is strongly associated with muscle health and overall well being. Enhanced oxidative capacity affords protection against insulin resistance and metabolic dysregulation (3, 46), attenuates muscle loss during aging (48), and lessens energetic deficits associated with myopathies (33, 47). The protective effects of increased oxidative capacity in disease states are largely attributed to increased mitochondrial content and enhanced mitochondrial function, which increase fatty acid oxidation, augment ATP-generating capacity, and protect against cellular stress.

Muscle displays a remarkable ability to adapt its phenotype in response to environmental stimuli. Endurance training readily promotes increases in maximal oxygen consumption and metabolic adaptations that enhance oxidative capacity, whereas resistance training stimulates increases in muscle fiber size. Curiously, the combination of resistance and endurance training results in less adaptation than either type of training alone (26). The concurrent training phenomenon is not necessarily related to contractile phenotype, because mitochondrial biogenesis can be augmented without changes in myosin heavy chain (21, 22). Instead, oxidative capacity appears more closely associated with fiber size. Correspondingly, cross-sectional area (CSA) is inversely related to maximal rate of oxygen consumption of vastly different muscle preparations; in turn, maximal oxygen consumption is proportional to succinate dehydrogenase (SDH) activity and mitochondrial content (45). Thus, limiting fiber hypertrophy may be a means for maintaining optimal oxidative capacity.

Concomitant increases in size and oxidative capacity are likely limited by an interaction between intracellular signaling pathways. Specifically, an AMP-activated protein kinase (AMPK)-Akt “switch” may mediate specific adaptations to endurance or resistance exercise (2). The cellular energy sensor AMPK is a heterotrimeric serine/threonine kinase composed of a catalytic α-subunit and regulatory β- and γ-subunits; in response to low cellular energy status, AMPK inhibits energy-consuming pathways, including protein synthesis. In fact, AMPK attenuates growth signaling by inactivating the major signaling network for protein synthesis, the Akt-mammalian coactivator 1α (PGC1α), which coordinates expression of nuclear- and mitochondrial-encoded genes critical for mitochondrial biogenesis and increased oxidative capacity (52).

Thus activation of AMPK induces a shift to a more oxidative phenotype, while likely simultaneously limiting muscle growth.
The porcine R200Q mutation in the AMPK γ3-subunit and the equivalent mutation in the mouse (R225Q) result in constitutive AMPK activation; because AMPKγ3 is highly expressed in white skeletal muscle (35), this mutation contributes to increased mitochondrial protein content and enhanced oxidative capacity in glycolytic skeletal muscle (3, 21). While activated AMPK would be expected to limit growth, AMPKγ3R200Q does not appear to affect muscle or overall growth in pigs (9). Because pigs have a high potential for lean growth, it may be possible to increase fiber size and oxidative capacity in AMPKγ3R200Q pig muscle. We used a genetic approach to determine if we could stimulate fiber hypertrophy in conjunction with AMPKγ3R200Q. The porcine R615C mutation in skeletal muscle ryanodine receptor 1 (RyR1) increases muscle fiber size (17), although the exact mechanism is not known. The RyR1 protein assembles in tetramers to form Ca2+ release channels, which modulate the frequency and amplitude of Ca2+ release into the cytosol. Mechanistically, RyR1R615C increases sensitivity to agents that stimulate channel opening and enhances luminal Ca2+ activation of RyR1 (28, 37). Aberrant Ca2+ homeostasis profoundly influences muscle function and energy metabolism. In fact, RyR1R615C pig muscle exhibits lower resting glycogen and phosphocreatine levels and rapid glycolysis and ATP hydrolysis (29). Although increasing the oxidative capacity of RyR1R615C muscle might prevent hypertrophy, it would also be expected to improve energy homeostasis. Therefore, our objective was to utilize pigs possessing AMPKγ3R200Q and RyR1R615C mutations to investigate the relationship between fiber size and metabolic phenotype. We chose the longissimus muscle to evaluate these properties, because AMPKγ3 is more highly expressed in glycolytic muscle (35), and the highly glycolytic nature of longissimus muscle in pigs (19, 32) provides a large potential for augmenting mitochondrial biogenesis and oxidative enzyme activity. Here, we show that muscle fibers can hypertrophy in parallel with increased oxidative capacity.

MATERIALS AND METHODS

Animals. Animals were bred and reared at the Virginia Tech Swine Center, and all procedures were approved by the Virginia Tech Institutional Animal Use and Care Committee. Pigs heterozygous at the RyR1 and AMPKγ3 loci were bred to generate all possible genotype combinations. Female and castrated male pigs were reared under standard conditions and fed ad libitum. Pigs from this genetic background can be expected to reach compositional maturity at ~110–130 kg body wt and 5–7 mo of age. When the animals reached ~120 kg body wt, they were transported to the Virginia Tech Meat Science Center, and tissues were harvested. Immediately after exsanguination, muscle samples (~5–10 g) were collected from the lumbar region of the longissimus muscle. Samples were immediately frozen in liquid nitrogen and stored at −80°C until further analysis. Muscle samples for histology were mounted on cork with Tissue-Tek, placed in isopentane cooled by liquid nitrogen, and stored at −80°C.

Genotype determination. Genotypes were determined using the PCR restriction fragment length polymorphism technique. DNA was isolated from blood or tissue and used for PCR amplification. PCR products were digested with the appropriate restriction enzyme overnight, and fragments were separated on an agarose gel stained with ethidium bromide for visualization. Pigs were evaluated for RyR1 genotype (20) according to the procedures outlined by O’Brien et al. (38a). For determination of AMPKγ3 genotype, the primers (5′–3′) were AATAATGCAGACAGAGGTCTC (forward) and CCCACC-GAAGCTCTGCGT (reverse). AMPKγ3 products were digested with the restriction enzyme BsrBI. Those that were homozygous “normal” (wild-type) at both RyR1 and AMPK loci were considered control, while the pigs designated RyR1R615C were homozygous mutant. RyR1 heterozygotes were excluded, because they tend to exhibit an intermediate phenotype. In contrast, AMPKγ3 mutation is dominant, so both homozygous mutant and heterozygous (designated AMPKγ3R200Q) animals were utilized. Finally, pigs designated AMPKγ3-RyR1 mutants were either heterozygous or homozygous mutant at the AMPKγ3 locus and homozygous mutant at the RyR1 locus.

Histology. Skeletal muscle cross sections (10 μm) were placed on silane-coated microscope slides and stored at −80°C until analysis. Wheat germ agglutinin tagged with Alexa Fluor conjugate (Invitrogen, Carlsbad, CA) was used to label membranes for CSA. Mean fiber CSA was determined from ≥100 (range 100–400) fibers per pig. For SDH staining, sections were incubated at 37°C for 1 h in 0.2 M phosphate buffer containing sodium succinate and nitro blue tetrazolium. Sections were washed in water, treated with increasing concentrations of acetone, rinsed, and mounted. Images were captured with a Nikon Eclipse Ti inverted microscope and CoolSNAP HQ2 monochrome camera and analyzed using NIS Elements AR3.1 software.

Total longissimus muscle area. Carcasses were split down the midline and chilled for 24 h. To expose the total CSA of the longissimus muscle, carcasses were cut through the vertebrae (perpendicular to the vertebral) and between the 10th and 11th costae. A plastic grid was used to measure the area of the longissimus muscle to the nearest 0.32 cm2 (39).

Enzyme activity. Citrate synthase catalyzes the formation of citrate and CoASH from acetyl-CoA and oxaloacetate. CoASH reduces DTNB, and citrate synthase activity was determined from the reduction of DTNB over time. Briefly, 10 μl of a 1:5-diluted muscle homogenate were added, in duplicate, to 170 μl of a solution containing Tris buffer (0.1 M, pH 8.3), DNTB (1 mM, in 0.1 M Tris buffer), and oxaloacetate (0.01 M, in 0.1 M Tris buffer). After a 2-min background reading, the spectrophotometer (SpectraMax ME, Molecular Devices, Sunnyvale, CA) was calibrated, and 30 μl of 3 mM acetyl-CoA was added to initiate the reaction. Absorbance was measured at 405 nm at 37°C every 12 s for 7 min. Maximum citrate synthase activity was calculated and reported as nmoles per minute per milligram.

For the determination of β-hydroxacyl-CoA dehydrogenase (β-HAD), oxidation of NADH to NAD was measured. In triplicate, 35 μl of whole muscle homogenate were added to 190 μl of a buffer containing 0.1 M liquid triethanolamine, 5 mM EDTA tetrasodium salt dihydrate, and 0.45 mM NADH. The spectrophotometer was calibrated, and 15 μl of 2 mM acetoacetyl-CoA were added to initiate the reaction. Absorbance was measured at 340 nm every 12 s for 6 min at 37°C. Maximum β-HAD activity was calculated and reported as nanomoles per minute per milligram.

Mitochondrial respiration. Mitochondria were isolated by differential centrifugation. Briefly, freshly dissected longissimus muscle was placed in ice-cold homogenization buffer (5 ml/g fresh muscle; 100 mM sucrose, 180 mM KCl, 50 mM Tris, 10 mM EDTA, 5 mM MgCl2, and 1 mM K-ATP, pH 7.4), and visible connective tissue and fat were removed. Muscle (~300 mg) was finely minced in 1.5 ml of homogenization buffer, and after addition of protease (subtilisin A, 5 mg/ml), muscle was homogenized using a glass-Teflon homogenizer. Homogenate was diluted to ~6 ml with homogenization buffer, filtered through cheesecloth, and centrifuged at 450 g for 2 min at 4°C. Supernatant was filtered again and centrifuged at 6,000 g for 15 min at 4°C. The resulting mitochondrial pellet was resuspended in mannitol-sucrose medium (220 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, and 1 mM EGTA, pH 7.4). Protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Equal amounts of mitochondrial protein were plated in triplicate, and a flux analyzer (Seahorse Bioscience, North Billerica, MA) was used to measure mitochondrial oxygen consumption rate (OCR). After initial mixing and equilibration, OCR was measured to determine baseline rates.
Important properties relating to ATP-producing capacity of mitochondria include maximal ADP-stimulated respiration (state 3), basal state 4 respiration, and coupling between respiration and phosphorylation. The rate of oxidative phosphorylation is primarily determined by the need for ATP. Therefore, high concentrations of ADP stimulate maximal oxygen consumption (state 3), and greater ADP-stimulated respiration indicates a greater capacity for oxidative phosphorylation. State 3 (active, maximum phosphorylating) respiration through complex I was assessed in the presence of pyruvate (10 mM), malate (5 mM), and ADP (5 mM). Complex II-driven respiration was determined in the presence of succinate (20 mM) and ADP (5 mM), and rotenone (2 μM) was used to block complex I. β-Oxidation and electron transport chain function were evaluated in the presence of palmitoylcarnitine (40 μM) and malate (2 μM). State 4 nonphosphorylating, maximal leak-dependent respiration was quantified following the addition of oligomycin (2 μM). The state 3-to-state 4 ratio, referred to as respiratory control ratio (RCR), represents the coupling between respiration and phosphorylation. Maximum uncoupled respiration was determined following addition of FCCP (0.3 μM).

Mitochondrial DNA copy number. Total genomic DNA was isolated from longissimus muscle and treated with RNase. DNA concentration was determined using a spectrophotometer (NanoDrop, Thermo Scientific, Waltham, MA), and samples were diluted to yield equal DNA concentrations. Primers were designed using Primer-BLAST (National Center for Biotechnology Information) for mitochondrial DNA [mtDNA; cytochrome c oxidase subunit III (ID no. AJ002189.1)] and nuclear DNA [β-actin (ID no. DQ452569.1)]. Final reaction mix included 1× SYBR Green Master Mix (Applied Biosystems, Foster City, CA), each primer at 500 nM, and 5 ng of DNA. mtDNA was compared with nuclear DNA using relative expression, determined by $2^{-\Delta\Delta CT}$, where ΔCT represents $C_{T\text{target}} - C_{T\text{control}}$.

Transcript abundance using real-time PCR. Total RNA was isolated from longissimus muscle using TRIzol according to the manufacturer’s instructions (Life Technologies, Grand Island, NY). RNA was treated with recombinant DNase (Life Technologies), and RNA concentration was quantified using a NanoDrop spectrophotometer. RNA was reverse-transcribed to cDNA using reverse transcriptase (Promega, Madison, WI) according to the manufacturer’s instructions. Reactions (15 μl) were performed in triplicate in a 96-well format using the 7900 Fast Real-Time PCR system (Applied Biosystems). Final reaction mix included 1× Fast SYBR Green Master Mix (Applied Biosystems), each primer at 500 nM, and 2 ng of cDNA. Target specific primer sets were designed using Primer-BLAST (National Center for Biotechnology Information) for PGC1α (NM_213963), cytochrome c oxidase subunit 5B (COX5B; NM_001007517.1), SDH-A (XM_003362140.1), NADH dehydrogenase subunit 1 (ND1; GQ339894.1), and cytochrome B (GU937818.1). Genes were normalized to eukaryotic elongation factor 1α (XM_001097418). Relative standard curves containing 0.2–200 ng of cDNA were used to determine primer efficiency, which ranged from 97 to 106%. Relative expression was determined by $2^{-\Delta\Delta CT}$, where ΔCT represents $C_{T\text{target}} - C_{T\text{control}}$.

Western blotting. Longissimus muscle tissue was powdered in liquid nitrogen and homogenized in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 μg/ml each aprotinin, leupeptin, and pepstatin). Samples were centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was collected. Protein concentration was determined using a BCA protein assay kit, and samples were diluted to yield equal protein concentration and mixed with Laemmli buffer. Protein samples (25 μg) were separated using SDS-PAGE and transferred to polyvinylidene difluoride or nitrocellulose membranes. Equal loading was assessed using Ponceau S staining (0.1% Ponceau S in 5% acetic acid), and images were captured using the ChemiDoc imaging system (Bio-Rad, Hercules, CA). Membranes were blocked in BlockingStar blocking buffer (Thermo Scientific). Blots were probed with primary antibody [Akt, phosphorylated (Ser473) Akt, and Cox subunit IV (CoxIV, Cell Signaling, Beverly, MA) and SDH-A (Novus, Littleton, CO)] prepared in blocking buffer with 0.05% Tween and washed in Tris-buffered saline with 0.05% Tween. Then blots were incubated with the appropriate IRDye 680- or 800-conjugated anti-IgG antibody (LI-COR Biosciences, Lincoln, NE). Bands were visualized using the Odyssey infrared imaging system (LI-COR Biosciences) and quantified using the manufacturer’s software.

Statistical analysis. Data were analyzed using SAS JMP. Model analyzed the main effects of AMPK and RyR1 genotypes and the interaction (AMPK × RyR1). Main effect of sex (female and castrated male) was tested but was included in the model only if it was significant. For interactions, differences in least squares means were determined using Tukey’s adjustment for multiple comparisons. P < 0.05 was considered significant.

RESULTS

RyR1R615C contributes to muscle hypertrophy. First, we determined CSA of longissimus muscle fibers from control, AMPKγ3R200Q, RyR1R615C, and AMPKγ3R200Q/RyR1R615C pigs (Fig. 1A). RyR1R615C increased mean fiber CSA ~35% (P = 0.0003; Fig. 1B). A genotype interaction was not detected, supporting the notion that, regardless of AMPK genotype, RyR1R615C is associated with muscle fiber hypertrophy. Moreover, RyR1 is expressed in all skeletal muscle fibers, regardless of contractile or metabolic phenotype, suggesting that RyR1R615C should increase fiber size across the entire fiber population. In accord, RyR1R615C shifted the frequency distribution for CSA (Fig. 1C). Next, we examined if fiber hypertrophy resulted in overall increases in muscle size by determining total area of the longissimus muscle between the 10th and 11th costae. Curiously, the combination of AMPKγ3R200Q and RyR1R615C robustly increased muscle area (17%) compared with AMPKγ3R200Q alone, yet muscle area was not different in RyR1R615C compared with control pigs (AMPKγ3 × RyR1 interaction, P = 0.05; Fig. 1D). These results were similar if longissimus muscle area was evaluated relative to body weight. Together, these findings support the idea that RyR1R615C induces hypertrophy of fibers, but fiber hypertrophy does not necessarily correspond to overall increases in total muscle size.

Content and activation of Akt play an important role in muscle growth and protein synthesis signaling pathways (4, 40). However, AMPK activation attenuates protein synthesis signaling (44). AMPK and RyR1 genotype influenced Akt content (P = 0.001 and P = 0.03, respectively; Fig. 2A) in longissimus muscle. We did not detect differences in activation of Akt [phosphorylated (Ser473) Akt/total Akt; Fig. 2B], although control was numerically lower than in all other groups. AMPKγ3R200Q increases oxidative capacity. We employed histological staining for SDH activity as an indicator of mitochondrial content and activity (Fig. 3A). SDH activity staining appeared weakest in muscle from control pigs, whereas darker staining in AMPKγ3R200Q-containing muscle corroborated the idea that AMPKγ3R200Q enhances mitochondrial content and oxidative capacity. To confirm this, we utilized muscle homogenates to quantify the activity of citrate synthase and β-HAD, key enzymes in the tricarboxylic acid cycle and fatty acid β-oxidation, respectively. AMPKγ3R200Q increased citrate synthase activity ~40% (P = 0.002; Fig. 3B) and β-HAD activity ~50% (P = 0.02; Fig. 3C). Therefore, AMPKγ3R200Q enhances oxidative capacity irrespective of RyR1 genotype.
In addition, we used isolated mitochondria from longissimus muscle to assess functional aspects of mitochondria. AMPK\(\gamma_3^{R200Q}\) increased state 3 respiration, regardless of substrate, as evidenced by enhanced OCR per microgram of mitochondrial protein (Fig. 4). There also tended to be a genotype interaction, but interpretation of these data is limited by the few observations in the RyRR615C (\(n/11005\)) and AMPK\(\gamma_3^{R200Q}-RyRR615C\) (\(n/11005\)) groups. However, because of the response of AMPK\(\gamma_3^{R200Q}\) mitochondria, it seems that RyRR615C does not negatively influence mitochondrial function when it is present with AMPK\(\gamma_3^{R200Q}\). Overall, oligomycin greatly decreased oxygen consumption, indicating low proton leak and resulting in similar state 4 respiration among all genotypes (data not shown). RCRs (Table 1) were not significantly different, although values for genotypes followed a trend similar to OCR for state 3 respiration. In total, AMPK\(\gamma_3^{R200Q}\) increased capacity for oxidative phosphorylation in isolated mitochondria without significantly altering other functional parameters. Because mitochondrial function assays were conducted using the same concentrations of mitochondrial protein, we infer that AMPK\(\gamma_3^{R200Q}\) affects qualitative aspects of mitochondrial function.

**Mitochondrial content.** Gene dosage, or mtDNA content, is considered the primary regulatory mechanism governing mitochondrial gene and protein expression (51). We used the difference between copy number of mtDNA and nuclear DNA to examine differences in mitochondrial content between genotypes. AMPK\(\gamma_3^{R200Q}\) contributed to a \(\sim60\%\) increase in mtDNA content (Fig. 5). Unexpectedly, RyR1R615C enhanced mtDNA content by \(\sim40\%\).

Content of mitochondrial mRNA generally parallels increases in mtDNA and oxidative capacity (51). Therefore, we determined mRNA expression of activators of mitochondrial biogenesis and expression of nuclear- and mitochondrial-encoded genes. PGC1\(\alpha\) coordinates mitochondrial and nuclear gene expression to promote mitochondrial biogenesis; it interacts with a number of transcription factors and nuclear receptors to modulate gene expression and contributes to an autoregulatory loop that promotes its own expression (25). Genotype did not influence PGC1\(\alpha\) mRNA content (Fig. 6A). Because mitochondrial biogenesis requires coordinated expression of mitochondrial and nuclear genomes, we also evaluated mRNA expression of mitochondrial- and nuclear-encoded...
genes. Genotype did not alter expression of nuclear-encoded genes, Cox5B and SDH-A (Fig. 6B), nor was expression of mitochondrial-encoded cytochrome B affected (Fig. 5C). However, AMPK_γ_3[R200Q] increased transcript abundance of mitochondrial-encoded NADH dehydrogenase (complex I) subunit 1 (ND1) by ~30% (Fig. 6C). Overall, AMPK_γ_3 and RyR1 genotypes do not appear to alter transcription, indicating that translational mechanisms may make important contributions to mitochondrial content.

Improvements in oxidative capacity generally parallel increases in mitochondrial density and protein content (50). We employed SDH-A and Cox (complex IV) subunit IV (CoxIV) as markers of mitochondrial protein content (Fig. 7A). AMPK_γ_3[R200Q] augmented protein levels of SDH-A and CoxIV by ~45% (Fig. 7B and C). Curiously, RyR1[R615C] also increased mitochondrial protein content, although to a lesser extent (~20%). Mitochondrial protein content of RyR1[R615C] muscle is not consistent with oxidative capacity assessed by citrate synthase or β-HAD activity or state 3 respiration.

DISCUSSION

Enhanced skeletal muscle oxidative capacity protects against insulin resistance, sarcopenia, and energetic deficits (3, 33, ...
Associated with mutations in the pore of the Ca\textsuperscript{2+} type, and viability (5). While the most severe phenotype is differently influences energy homeostasis, muscle phenotype. Yet we clearly demonstrate that muscle fiber size and oxidative capacity can be increased simultaneously. RyR1R615C contributed to ~35% increase in fiber size, while AMPK\textsubscript{γ3}R200Q enhanced mitochondrial content and enzyme activity.

Porcine RyR1\textsuperscript{R615C} increases fiber size (17), yet RyR1 mutations in humans do not appear to have the ability to augment aerobic capacity may be limited by fiber size. Oxidative capacity and muscle fiber size are inversely related (45), and interaction between signaling pathways may prevent concurrent increases in fiber size and oxidative metabolism (2, 11). Yet we clearly demonstrate that muscle fiber size and oxidative capacity can be increased simultaneously. RyR1\textsuperscript{R615C} contributed to ~35% increase in fiber size, while AMPK\textsubscript{γ3}R200Q enhanced mitochondrial content and enzyme activity.

RyR1\textsuperscript{R615C} increases oxygen consumption rate (OCR) was measured in isolated mitochondria from longissimus muscle. Assessment of state 3 (active, maximum phosphorylating) respiration through complex I was determined in the presence of pyruvate, malate, and ADP. Complex II-driven respiration was determined in the presence of succinate and ADP, and rotenone was used to block complex I. β-Oxidation and electron transport function were evaluated in the presence of palmitoylcarnitine, malate, and ADP. Values are means ± SE [n = 3 (control), 5 (AMPK), 1 (RyR1), and 2 (AMPK-RyR1); no SE is shown for RyR1].

Over 300 mutations are identified in human RyR1, with most mutations being found in three separate regions (30). RyR1 mutations have disparate effects on Ca\textsuperscript{2+} release, which differentially influences energy homeostasis, muscle phenotype, and viability (5). While the most severe phenotype is associated with mutations in the pore of the Ca\textsuperscript{2+} release channel, the majority of mutations are mapped to interdomain and intersubunit interfaces (8). Several knockin mouse models of other RyR1 mutations have been developed, but they are not synonymous with RyR1\textsuperscript{R615C}, which is the only known porcine mutation (20). Whereas homozygosity for RyR1\textsuperscript{R163C} in mice causes embryonic lethality (18), RyR1\textsuperscript{R615C} homozygous pigs are stress-susceptible but viable, and heterozygous pigs exhibit an intermediate phenotype. Pigs with RyR1\textsuperscript{R615C} appear normal but are predisposed to malignant hyperthermia and respond to stress in much the same manner that humans with RyR1 mutations may respond to anesthetics: hypermetabolism, elevated body temperature, and muscle rigidity (34).

Mechanistically, RyR1\textsuperscript{R615C} increases sensitivity to agents that stimulate channel opening, contributing to enhanced Ca\textsuperscript{2+} release (36, 37); RyR1\textsuperscript{R615C} also lowers the threshold for store overload-induced Ca\textsuperscript{2+} release (28). This increased sensitivity and enhanced Ca\textsuperscript{2+} release are generally considered to contribute to the “work-induced hypertrophy,” or greater fiber CSA, in RyR1\textsuperscript{R615C} pig muscle. In mice, RyR1\textsuperscript{R163C} induces a slow-to-fast fiber transition, which is associated with a decrease in calcineurin relative to calcineurin inhibitor and activation of ERK1/2 signaling (23). RyR1\textsuperscript{R615C} is also implicated in increased content of fast myosin heavy chain type IIb, but we did not observe differences in ERK1/2 signaling (data not shown). Content of Akt is also positively related to muscle growth and fiber size (4). In accord, RyR1\textsuperscript{R615C} increased total Akt in longissimus muscle. However, AMPK\textsubscript{γ3}R200Q was also associated with elevated Akt content. In two previous studies, no change (16) or an increase (24) in Akt content was reported in AMPK\textsubscript{γ3}R200Q pig muscle, although both studies used younger, growing pigs subjected to an exercise program. Nevertheless, this suggests that increased Akt expression may be a means of compensating for downregulation of protein synthesis by activated AMPK.

Notably, the increase in fiber size in RyR1\textsuperscript{R615C} pig muscle did not contribute to an overall increase in longissimus muscle area. However, AMPK\textsubscript{γ3}R200Q-RyR1\textsuperscript{R615C} pigs possessed greater fiber size and greater muscle area than their AMPK\textsubscript{γ3}R200Q counterparts. This raises an intriguing possibility: RyR1\textsuperscript{R615C} pigs may possess fewer muscle fibers, and AMPK\textsubscript{γ3}R200Q in the presence of RyR1\textsuperscript{R615C} may partially ameliorate loss of fibers. We did not observe obvious histological defects or myopathic

Table 1. RCR values of isolated mitochondria from different pig genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>RCR</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>2.33 ± 0.5</td>
</tr>
<tr>
<td>AMPK</td>
<td>5</td>
<td>2.76 ± 0.4</td>
</tr>
<tr>
<td>RyR1</td>
<td>1</td>
<td>1.92</td>
</tr>
<tr>
<td>AMPK-RyR1</td>
<td>2</td>
<td>3.60 ± 0.6</td>
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Values are means ± SE. RCR, respiratory control ratio [ratio of O\textsubscript{2} consumption rate in the presence of ADP, pyruvate, and malate (state 3) to O\textsubscript{2} consumption rate after addition of the ATP synthase inhibitor oligomycin]; AMPK, AMP-activated protein kinase; RyR1, ryanodine receptor 1.
features in RyR1\textsuperscript{R615C} muscle that would suggest postnatal loss of fibers. Moreover, because muscle fibers are postmitotic, a developmental phenomenon could be responsible. Other quantitative trait loci associated with muscle development are linked with RyR1 (10), implying that RyR1\textsuperscript{R615C} may only partly explain changes in muscle development and/or fiber size.

Pigs with RyR1\textsuperscript{R615C} possess larger muscle fiber CSA but maintain a similar number of capillaries per fiber, resulting in lower capillary density than in wild-type animals (17). In turn, this could limit oxygen supply to the fibers and negatively affect oxidative capacity. Yet, oxidative enzyme activity in RyR1\textsuperscript{R615C} muscle is not different from that in control muscle, supporting the notion that oxidative capacity is not significantly impacted. In agreement, isolated mitochondria (7, 43) and permeabilized myofibers (49) from RyR1\textsuperscript{R615C} muscle exhibit respiratory activities similar to controls. While we observed that the RyR1\textsuperscript{R615C} genotype did not influence citrate synthase or β-HAD activity, the RyR1\textsuperscript{R615C} genotype increased mitochondrial proliferation and mitochondrial protein content. The reasoning for this is not clear, but it may be an attempt to compensate for energetic stress or oxidative damage. Pigs with RyR1\textsuperscript{R615C} are not easily distinguished from wild-type pigs until they are exposed to stress. However, selection emphasis on leanness led to increased frequency of this mutation in swine herds, supporting the notion that, even under “normal” circumstances, RyR1\textsuperscript{R615C} alters Ca\textsuperscript{2+} homeostasis and muscle phenotype. Enhanced Ca\textsuperscript{2+} release may contribute to increased ATP utilization, thereby triggering adaptive mechanisms. Additionally, Ca\textsuperscript{2+} amplitude is higher in RyR1\textsuperscript{R615C} mitochondria, and spikes in mitochondrial matrix Ca\textsuperscript{2+} increase generation of reactive oxygen species (5). Increased lipid peroxidation is associated with porcine malignant hyperthermia (15), indicating that RyR1\textsuperscript{R615C} may also promote oxidative damage of mtDNA and proteins. We quantified protein carbonyl content as an indicator of protein oxidation, but RyR1\textsuperscript{R615C} did not increase oxidative damage to proteins (data not shown).

In contrast, AMPK\textsubscript{γ3}\textsuperscript{R200Q}-induced increases in mRNA and protein content are associated with enhanced activity of oxidative enzymes. Therefore, AMPK\textsubscript{γ3}\textsuperscript{R200Q}-RyR1\textsuperscript{R615C} pigs possess increased oxidative enzyme capacity (conferred by the AMPK genotype), as well as an additive increase in mitochondrial proliferation and protein content (effect of AMPK + RyR1). Although energetic or oxidative stress may be a concern in AMPK\textsubscript{γ3}\textsuperscript{R200Q}-RyR1\textsuperscript{R615C} muscle, the increased oxidative capacity could provide several advantages. Most obviously, increased oxidative capacity augments ATP-generating ability, which may improve maintenance of resting cytosolic Ca\textsuperscript{2+} levels and membrane potentials. Additionally, mitochondria act as a high-capacity, low-affinity transient Ca\textsuperscript{2+} store (6), thereby providing a larger Ca\textsuperscript{2+} “sink” to help buffer fluctuations in cytosolic and mitochondrial matrix Ca\textsuperscript{2+}. Given that subsarcolemmal and intermyofibrillar populations of mitochondria exhibit different properties, functional improvements may be related to which mitochondrial population is affected. Intermyofibrillar mitochondria exhibit increased ADP-stimulated respiration (12) but are more sensitive to

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**Fig. 6. Transcript levels of key regulators of mitochondrial biogenesis and nuclear DNA- and mtDNA-encoded proteins.** Transcript levels of each gene were normalized to eukaryotic translation elongation factor 1α (eEF1α), the nuclear-encoded proteins cytochrome c oxidase subunit 5B (Cox5B) and succinate dehydrogenase A (SDH-A), and the mitochondrial-encoded proteins NADH dehydrogenase subunit 1 (ND1) and cytochrome B (Cyt B). Main effects of genotype were significant for ND1 and are indicated above the bars. Values are means ± SE (n = 7–12 pigs per genotype).
reactive oxygen species (1), while subsarcolemmal mitochondria are important for transport of metabolites and ions across the cell membrane (38).

AMPK plays a key role in modulating muscle metabolism in response to acute and chronic energy stress. It is intriguing that expression of the AMPK\(\gamma_3\)-subunit is not necessarily important for AMPK to influence adaptation (21); instead, \(\gamma_3\)-subunit mutations appear to have the most profound effects on energy substrates and metabolism in skeletal muscle. In addition to the aforementioned influence of AMPK\(\gamma_3\R^{200Q}\) in the pig, the AMPK\(\gamma_3R^{225W}\) mutation in humans confers a 90\% increase in muscle glycogen and a 30\% decrease in triglycerides (14), and the AMPK\(\gamma_3R^{225Q}\) mutation in mice increases glycogen nearly twofold and enhances fatty acid oxidation (3). These \(\gamma_3\)-subunit mutations are considered gain-of-function mutations that result in constitutive activity. We previously demonstrated that RyR1\(R^{615C}\) blunts AMPK phosphorylation in pigs with AMPK\(\gamma_3\R^{200Q}\) (41), suggesting that Ca\(^{2+}\)-induced abrogation of AMPK would prevent increases in oxidative capacity and glycogen content. Although content of the muscle-specific glucose transporter GLUT4 is decreased in RyR1\(R^{615C}\)-AMPK\(\gamma_3\R^{200Q}\) pig muscle (41), AMPK\(\gamma_3\R^{200Q}-RyR1\R^{615C}\) muscle still possesses dramatically increased glycogen (13). This verifies that the AMPK mutation may be more important than its expression or activity per se, further supporting the idea that we could expect fiber hypertrophy and increased oxidative metabolism in AMPK\(\gamma_3\R^{200Q}-RyR1\R^{615C}\) muscle.

AMPK\(\gamma_3\R^{200Q}\) clearly improved oxidative capacity, regardless of RyR1 genotype. Although mRNA levels were largely unaffected, we did detect differences in mitochondrial-encoded ND1. Because electrons enter the electron transport chain at complex I or complex II, it is possible that AMPK\(\gamma_3\R^{200Q}\) may improve mitochondrial quality by altering protein stoichiometry; that is, AMPK\(\gamma_3\R^{200Q}\) enhances function by preferentially increasing expression of certain metabolic enzymes. While mRNA expression appears fairly stable in AMPK\(\gamma_3\R^{200Q}\) muscle, mitochondrial protein content is increased. AMPK\(\gamma_3\R^{200Q}\) elicits similar effects on metabolic phenotype, regardless of RyR1 genotype, indicating that AMPK\(\gamma_3\R^{200Q}\) supports mitochondrial biogenesis and upregulation of fatty acid oxidation, irrespective of AMPK phosphorylation. It is possible the activating properties of the mutation impact cellular localization or how AMPK interacts with other proteins, but additional work is necessary to show this.

In summary, we have demonstrated that hypertrophy and oxidative adaptation occur in AMPK\(\gamma_3\R^{200Q}-RyR1\R^{615C}\) muscle. AMPK\(\gamma_3\R^{200Q}\) increases muscle oxidative capacity, evidenced by enhanced citrate synthase and \(\beta\)-HAD activity and state 3 respiration, as well as augmented mitochondrial content. Conversely, RyR1\R^{615C}\) contributes to muscle hypertrophy, which is corroborated by increased muscle fiber CSA. Although RyR1\R^{615C}\) also contributes to increases in mtDNA and mitochondrial protein, these changes are not associated with improvements in function. Nonetheless, increased oxidative capacity in RyR1\R^{615C}\) pigs possessing the AMPK\(\gamma_3\) mutation supports the notion that AMPK-induced mitochondrial and metabolic adaptations improve ATP-generating capacity, despite aberrant Ca\(^{2+}\) metabolism and increased fiber CSA. This highlights the important role of muscle mitochondrial content and quality and further substantiates the idea that oxidative capacity protects against energetic defects and is a critical determinant of muscle health and whole body metabolism. In total, this supports the idea that hypertrophy and enhanced oxidative capacity can occur simultaneously in skeletal muscle, and the signaling mechanisms controlling these events appear to be independently regulated.
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GRANTS

This work was supported by a grant from the US Department of Agriculture (National Institute of Food and Agriculture) Agriculture and Food Research Initiative.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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