Reduced skeletal muscle AMPK and mitochondrial markers do not promote age-induced insulin resistance

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Bujak AL, Blümer RM, Marcinko K, Fullerton MD, Kemp BE, Steinberg GR. Reduced skeletal muscle AMPK and mitochondrial markers do not promote age-induced insulin resistance. J Appl Physiol 117: 171–179, 2014. First published May 22, 2014; doi:10.1152/japplphysiol.01101.2013.—In both rodents and humans, aging-associated reductions in skeletal muscle AMP-activated protein kinase (AMPK) activity and mitochondrial function have been linked to the development of skeletal muscle insulin resistance. However, whether reductions in skeletal muscle AMPK and mitochondrial capacity actually precipitate the development of aging-induced insulin resistance is not known. Mice lacking both isoforms of the AMPK β-subunit in skeletal muscle (AMPK-MKO) have no detectable AMPK activity and are characterized by large reductions in exercise capacity, mitochondrial content, and contraction-stimulated glucose uptake making them an ideal model to determine whether reductions in AMPK and mitochondrial content promote the development of aging-induced insulin resistance. In the current study we find that a lack of skeletal muscle AMPK results in a life-long reduction in mitochondrial activity but does not affect body mass, body composition, glucose tolerance, or insulin sensitivity as measured by hyperinsulinemic-euglycemic clamp in mice of old age (18 mo). These data demonstrate that reductions in skeletal muscle AMPK and mitochondrial activity do not cause the development of age-induced insulin resistance.

mitochondria; obesity; AMPK-β1β2 muscle KO mice

In developed nations the prevalence of type 2 diabetes in the elderly (60–79 yr of age) is expected to increase beyond that of the middle-aged population (40–59 yr of age) (41). The development of insulin resistance is associated with an increased risk of type 2 diabetes (23). It is well established that the process of aging is associated with insulin resistance in both obese (29) and nonobese individuals (8, 38). Regardless of age, skeletal muscle has a predominant role in controlling insulin-stimulated glucose uptake, thereby contributing to whole body insulin sensitivity (15). Furthermore, evidence in humans suggests that a reduction in muscle glucose transport is a major factor in the onset of whole body insulin resistance and type 2 diabetes (37). Attesting to this, transgenic mice with ablated insulin-stimulated glucose uptake develop type 2 diabetes as early as 5 wk of age (7).

Impairments in skeletal muscle mitochondrial health are another consequence of aging that include the following: reduced mitochondrial density, reduced oxidative phosphorylation, increased mt-DNA mutations, and increased production of reactive oxygen species (18). One prominent hypothesis proposes that this muscle mitochondrial dysfunction is a primary cause for the development of age-induced insulin resistance (24). Reductions in skeletal muscle mitochondrial capacity with aging may promote the accumulation of lipotoxic intermediates including diacylglycerols and ceramides, which then results in impairments in insulin signaling (14, 30, 42, 47).

While there are certainly strong associations between a reduction in mitochondrial function and insulin resistance with aging, whether a reduction in skeletal muscle mitochondrial content is a sufficient cause of insulin resistance with aging is not fully understood.

An important regulator of mitochondrial content in skeletal muscle is the AMP-activated protein kinase (AMPK), and many studies have found AMPK protein or activity levels to be lower in aged vs. young humans (22, 25) and rodents (12, 33, 34), although it should be noted this is not observed in all studies (11, 35). AMPK is a heterotrimer composed of α-, β-, and γ-subunits (44). The β-subunit acts as a scaffold for the α- and γ-subunits and is essential for the stability and activity of the AMPK heterotrimer (13). AMPK-α2, -β2, and -γ1 are the predominant subunit isoforms found in skeletal muscle (3, 43, 46). We have recently developed AMPK-MKO mice that lack both isoforms of the β-subunit and are therefore completely deficient in skeletal muscle AMPK activity (28). AMPK-MKO mice have drastically reduced mitochondrial content and exercise capacity. While some mice with partial AMPK deficiencies have exhibited reductions in mitochondria and exercise capacity previously (2, 5, 16, 19, 21, 45), in all cases this phenotype is much less obvious than that observed in the AMPK-MKO mice.

Surprisingly, despite the low levels of mitochondrial content, young (8–12 wk old) AMPK-MKO mice showed normal insulin sensitivity (28). These data are in agreement with other reports from young chow-fed mice lacking either one or both of the AMPK α-subunits in skeletal muscle (2, 17, 20, 26). In addition, Frosig et al. (9) recently found 18-mo-old mice overexpressing the AMPK-α2 kinase dead (α2KD) isoform in skeletal muscle also had normal insulin sensitivity, although they had yet to develop aged-induced insulin resistance. Given the more pronounced phenotype of AMPK-MKO mice compared with previous AMPK-deficient models, in the current study we tested whether this exacerbated the development of aging-induced insulin resistance.
MATERIALS AND METHODS

Study design and generation of AMPK-MKO model. The McMaster University Animal Research Ethics Board approved all animal procedures employed. As both males and females develop similar degrees of insulin resistance with aging (32), female mice were used. Mice were housed in the McMaster University Central Animal Facility on a 12-h light/dark cycle. Mice were fed a normal chow diet (17% kcal fat, Teklad 22/5, Harlan Laboratories, Indianapolis, IN) with ad libitum access to food and water. Metabolic parameters as described below were assessed in young (2 mo), middle age (11 mo), and aged (18 mo) mice.

AMPK-MKO mice were generated as previously described by O’Neill et al. (28). Briefly, AMPK-β1 and -β2 floxed mice on a C57BL/6 background were crossed with mice having the Cre recombinase gene under control of the muscle creatine kinase promoter (MCK). The β1 fl/fl (β1-WT) and β1 MCK-Cre (β1-MKO) mice plus β2 fl/fl (β2-WT) and β2 MCK-Cre (β2-MKO) mice lines were bred to generate β1β2 fl/fl (WT) and the β1β2 MCK-Cre (AMPK-MKO) mice that were used in this study.

Metabolic techniques. Adiposity was evaluated with computed tomography at the McMaster Center for PreClinical and Translational Imaging as previously described (10). Metabolic parameters were analyzed over a 3-day period using the Oxymax Comprehensive Lab Animal Monitoring System (Columbus Instruments, Columbus, OH) as described previously (28). Briefly, mice were acclimatized to the cages for 12 h before measurements began. The volumes of oxygen consumption (\(\dot{V}_O_2\)) and carbon dioxide consumption (\(\dot{V}_C_0_2\)) were measured through indirect calorimetry. Respiratory exchange ratio was calculated as the ratio of \(\dot{V}_O_2\) into \(\dot{V}_C_0_2\). Activity levels were counted as infrared beam breaks along the x-axis of the cage.

Fasted blood glucose and insulin levels were measured following 11 h of fasting using an Accu-Chek Aviva blood glucometer and a rat/mouse insulin ELISA kit from Millipore, respectively. Clamped serum insulin levels were measured using a human insulin ELISA from Mercodia. Glucose and insulin tolerance were measured after 6 h of fasting, using 2 g glucose/kg or 1.15 U insulin/kg of body mass, respectively, in a saline solution as described previously (10).

Hyperinsulinemic-euglycemic clamps were performed as previously described (6, 39). Briefly, mice were acclimatized to the restrainers over an 8-day period before cannulation of the jugular vein. Five days following jugular vein cannulation and after a 6-h fast, mice were infused with a basal infusate (8% BSA, \(\delta^3\)H-glucose, and saline) at a constant rate of 7.5 µCi/h for 1 h using a syringe pump (KD Scientific; Holliston, MA). Basal hepatic glucose output (HGO) was measured as the basal glucose disposal rate (GDR) into peripheral tissues before the infusion of insulin. An insulin infusate (8% BSA, \(\delta^3\)H-glucose, insulin, and saline) with insulin concentration adjusted to 10 mU·kg\(^{-1}\)·min\(^{-1}\) was then infused at a constant rate of 7.5 µCi/h until the experiment was complete. Blood glucose levels were monitored every 10 min to effectively titrate glucose levels to reach the hyperinsulinemic-euglycemic state. Clamped HGO was calculated as the difference between the insulin-stimulated GDR and the amount of glucose consumed in the clamp.

Fig. 1. AMPK and mitochondrial enzyme activity in young and aged AMPK-β1β2 muscle knockout mice (AMPK-MKO). A and B: AMPK total and phosphorylated protein expression was undetectable in quadriceps muscle but normal in white adipose tissue (WAT) and liver tissue of AMPK-MKO mice (n = 7–11). C: a strong trend exists for reduced mitochondrial cytochrome c oxidase (COX) enzyme activity in young AMPK-MKO mouse quadriceps muscle vs. wild type (WT; P = 0.065), COX activity was significantly lower in aged WT vs. young WT, but aged AMPK-MKO were not significantly different from young AMPK-MKO mice (n = 5–11). Mitochondrial OXPHOS protein expression of mitochondrial subunits complex II–30kDA (CII-30), complex III-Core protein 2 (CIII-c2a), and complex V α-subunit (CV-α) were significantly lower in aged AMPK-MKO mice. CI-20, Complex I subunit NDUFB8; CIV-1, Complex IV subunit 1. Data are means ± SE. #P < 0.05 for young compared with aged mice (n = 6–11). *P < 0.05 for AMPK-MKO compared with WT.
insulin-stimulated glucose infusion rate. Following the insulin clamp, 100 μl of 2-deoxy-[14C]glucose (2-[14C]DG) were infused into circulation via the catheter. Blood was then collected from the tail at 10, 20, and 30 min for determination of the rate of 2-[14C]DG disappearance, and tissues were collected and snap frozen in liquid nitrogen.

**Analytical techniques.** Samples were homogenized with the Precellys 24 Homogenizer (Bertin Technologies, Paris, France), and lysates were collected after centrifugation at 16,000 g. To calculate the tissue-specific uptake of 2-[14C]DG, the radioactivity of a 50-μl aliquot of total lysate was determined (representing both phosphorylated and unphosphorylated 2-[14C]DG). A separate 100-μl aliquot of lysate was deproteinized in 350 μl Ba(OH)2, 350 μl ZnSO4, and 600 μl H2O to precipitate phosphorylated 2-[14C]DG. After centrifugation at 16,000 g, 1,000 μl of the supernatant (containing only extracellular unphosphorylated 2-[14C]DG) were then added to 4 ml scintillation fluid for counting. The radioactivity of the phosphorylated 2-[14C]DG was determined by subtracting the unphosphorylated from total radioactivity. The rate of glucose uptake was then determined using the amount of glucose uptake into the cell, the rate of disappearance from the blood, and the concentration of glucose during the clamped state. Half the volume of Ba(OH)2, ZnSO4, and H2O was added to the soleus, extensor digitorum longus, and adipose tissue to account for the reduced amount of cell lysis buffer used in these tissues compared with the gastrocnemius and quadriceps muscles.

SDS page was used to separate proteins within a 7.5% polyacrylamide gel using the Bio-Rad Mini Protean Tetra System (Bio-Rad Laboratories, Mississauga, ON, Canada). Gels were then transferred onto a polyvinylidene difluoride membrane and blocked in 5% BSA in 0.1% Tris-buffered saline with Tween 20 (TBST). Membranes were incubated overnight at 4°C in primary antibodies made in 5% BSA in 0.1% TBST. The MitoProfile total OXPHOS rodent antibody cocktail (ab110413) was purchased from Abcam. All other antibodies were purchased from Cell Signaling and included the following: AMPK-Thr172 (no. 2532), Akt (no. 9272), Akt Ser473 (no. 4058), AS160 (no. 2670), AS160 Thr642 (no. 4288), and GAPDH (no. 5174). All bands were quantified relative to the loading control GAPDH.

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**Fig. 2.** Weight and body composition of aged AMPK-MKO mice. A: body weights were not different from middle age to aged mice (n = 9–13). B: computed tomography imaging in middle age mice showed no differences in percent body fat (n = 11–22). C–E: cross-sectional areas (CSA) of adipocytes are comparable between AMPK-MKO mice and WT mice (n = 6). Data are means ± SE.
Table 1. Relative tissue weights

<table>
<thead>
<tr>
<th>Tissue</th>
<th>WT, mg/g Mean ± SE</th>
<th>AMPK-MKO, mg/g Mean ± SE</th>
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<tbody>
<tr>
<td>Liver</td>
<td>37.9 ± 1.3</td>
<td>41.4 ± 1.0*</td>
</tr>
<tr>
<td>Adipose</td>
<td>26.6 ± 5.6</td>
<td>30.5 ± 3.1</td>
</tr>
<tr>
<td>Heart</td>
<td>4.7 ± 0.3</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Quad</td>
<td>3.9 ± 0.2</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>Gast</td>
<td>3.8 ± 0.1</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>TA</td>
<td>1.2 ± 0.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Sol</td>
<td>0.26 ± 0.01*</td>
<td>0.32 ± 0.01*</td>
</tr>
<tr>
<td>EDL</td>
<td>0.28 ± 0.02</td>
<td>0.25 ± 0.03</td>
</tr>
</tbody>
</table>

Data are means ± SE for n = 3–7. Quad, quadriceps; Gast, gastrocnemius muscle; TA, tibialis anterior; Sol, soleus; EDL, extensor digitorum longus. *P < 0.05 for AMPK-β1β2 muscle knockout mice (AMPK-MKO) compared with wild type (WT).

Cytochrome c oxidase (COX) enzyme activity was measured in quadriceps muscle by incubating 20 μl of lysates with a solution of reduced cytochrome c (0.2 mg/ml) dissolved in 0.05 M potassium phosphate buffer. Activity was determined based on the change in absorbance at a wavelength of 550 nm every 10 s for 90 s in a 96-well plate.

Hemotoxylin and eosin staining was performed on paraffin-embedded adipose tissue at the John Mayberry Histology Facility at McMaster University. Cross-sectional area was determined by measuring the circumference of ~200 adipocytes per sample using Nikon Elements software (Nikon, Melville, NY) as previously described (31).

Statistical analysis. All data analysis was performed using GraphPad Prism Version 5.0. A two-way ANOVA was performed for measuring body weight, metabolic cage results, fasted/fed blood glucose and insulin levels, glucose tolerance tests, insulin tolerance tests (ITT), hyperinsulinemic-euglycemic clamp glucose infusion rate, and blood glucose curves. A two-tailed Student’s t-test was performed for measuring COX enzyme activity, computed tomography images, tissue weights, adipocyte cross-sectional area, hyperinsulinemic-euglycemic clamp GDR, basal HGO, clamped HGO, clamped serum insulin results, and 2-DG uptake. P < 0.05 was considered statistically significant. Data are means ± SE.

RESULTS

AMPK-MKO mice have reduced mitochondrial enzyme activity independent of age. The expression and phosphorylation of the catalytic site on the AMPK α-subunit (Thr172) were undetectable in the muscle of AMPK-MKO mice compared with WT controls but were unaltered in adipose tissue and liver (Fig. 1, A and B). Young AMPK-MKO mice have a strong tendency (P = 0.065) towards a reduction in quadriceps muscle mitochondrial enzyme activity independent of age.

Fig. 3. Activity and metabolic parameters of aging AMPK-MKO mice. A–C: activity levels were significantly reduced in aged vs. middle age mice in both AMPK-MKO and WT. Volume of oxygen consumption (V\textsubscript{O}_2; D–F) and respiratory exchange ratio (RER; G–I) were each normal in AMPK-MKO mice. Data are means ± SE for n = 4–18. *P < 0.05 for middle age compared with aged mice.

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muscle mitochondrial COX activity (~36%) and, as expected, young WT mice demonstrated substantially higher COX activity (~49%) than aged WT mice (P < 0.05). Importantly, the COX activity of aged AMPK-MKO mice was unaffected by the aging process and remained at the same reduced capacity that was observed in young AMPK-MKO mice (Fig. 1C). Despite the life-long reduction in COX activity, aged AMPK-MKO mice still had a further reduced mitochondrial protein expression of OXPHOS subunits complex II-30kDa (~37%, P < 0.05), complex III-Core protein 2 (~18%, P < 0.05), and complex V α-subunit (~14%, P < 0.05) compared with aged WT littermates (Fig. 1C).

**Aged AMPK-MKO mice have normal body composition.** There were no significant differences observed in body mass between middle age and aged mice over time (Fig. 2A). Similarly, the percent adiposity was not different (Fig. 2B). Consistent with similar body mass and adiposity, aged AMPK-MKO mice had normally sized adipocytes as determined by cross-sectional area (Fig. 2, C–E). Tissue weights were also comparable between genotypes with the exception of a small, but significant, increase in liver and soleus mass (P < 0.05; Table 1).

**Aged AMPK-MKO mice have normal metabolic parameters.** As expected, activity levels in both AMPK-MKO and WT littermates were significantly lower in aged vs. middle age mice, but there was no difference between genotypes (Fig. 3, A–C). Using indirect calorimetry, we found that the VO₂ (Fig. 3, D–F) and respiratory exchange ratio were comparable between both aging and genotype (Fig. 3, G–I).

**AMPK-MKO mice have normal age-induced reductions in glucose homeostasis and insulin sensitivity.** As expected, young AMPK-MKO and WT mice experienced a significant reduction in glucose tolerance and insulin sensitivity when compared at old age (P < 0.05; Fig. 4, A–D). Attesting to this, when the young group was given an ITT at the same dose (1.15 U insulin/kg) provided to the aged group, a majority of the mice became hypoglycemic by 60 min and required an injection of glucose to restabilize glycemia. AMPK-MKO mice had a very small but significant reduction in insulin sensitivity during the ITT. Blood glucose values during the fed and fasted
condition were comparable between aged AMPK-MKO mice and WT littermates (Fig. 4E). Additionally, serum insulin levels in the fed and fasted state were also similar between groups (Fig. 4F).

To examine whether there might be tissue-specific changes in insulin sensitivity that were masked by the whole body measure of glucose homeostasis and insulin sensitivity, we conducted hyperinsulinemic-euglycemic clamps in the aged mice. We found that the glucose infusion rate (Fig. 5A), blood glucose curve (Fig. 5B), and GDR (Fig. 5C) were all similar between genotypes indicating the maintenance of insulin sensitivity in the absence of skeletal muscle AMPK. Basal HGO (Fig. 5D), clamped HGO (Fig. 5E), and the percent suppression of HGO (Fig. 5F) were also not different between genotypes indicating hepatic insulin sensitivity remained intact with old age in AMPK-MKO mice. Importantly, clamped serum insulin levels were similar to those observed in the fed state for both AMPK-MKO and WT mice (Fig. 5G). Consistent with the other metabolic data indicating normal insulin sensitivity, 2-DG uptake during the clamp was not altered in all skeletal muscle types examined (gastrocnemius, quadriceps, extensor digitorum longus, and soleus; Fig. 5H). However, surprisingly, AMPK-MKO mice had an ~42% (P < 0.05) reduction in gonadal adipose tissue 2-DG uptake (Fig. 5H), but this did not affect the glucose disposal rate (Fig. 5C), consistent with the relatively small contribution adipose tissue plays in controlling insulin-stimulated glucose disposal.

We subsequently examined insulin signaling in the aged skeletal muscle, adipose tissue, and liver following the hyperinsulinemic-euglycemic clamp. Total protein expression levels of the insulin-signaling intermediates Akt and the Akt substrate of 160 kDa (AS160) were unchanged between the quadriceps

![Fig. 5. Hyperinsulinemic-euglycemic clamp of aged AMPK-MKO mice.](image)

There was no difference in glucose infusion rate (A), blood glucose curve during the insulin-clamp experiment (B), glucose disposal rate (GDR; C), basal hepatic glucose output (HGO; D), clamped HGO (E), and percent suppression of HGO (F; n = 7–13). Similarly, clamped serum insulin (G) and skeletal muscle 2-deoxy-glucose (2-DG) uptake (H) showed no differences between genotypes; however, glucose uptake was significantly lower in the adipose tissue of AMPK-MKO mice (n = 4–6). Data are means ± SE. *P < 0.05 for AMPK-MKO compared with WT.
molecules of AMPK-MKO and WT mice following the hyperinsulinemic-euglycemic clamp. There were also no differences observed in the phosphorylation of either Akt Ser473 or AS160 Thr642 in the quadriceps muscles (Fig. 6, A and B). Similar results were obtained in adipose tissue (Fig. 6, C and D) and liver (Fig. 6, E and F). These findings are consistent with the maintenance of insulin sensitivity in these tissues of AMPK-MKO mice.

**DISCUSSION**

The process of aging is associated with reductions in mitochondrial function, and this has been proposed to promote the development of insulin resistance (1, 32, 36). Skeletal muscle AMPK is reduced with aging in some (22, 25) but not all studies (11) and has been shown to be vital for maintaining mitochondrial content [for review, see O’Neill et al. (27)]. To establish whether a reduction in AMPK and mitochondrial content causes the development of aging-induced insulin resistance, we studied mice with undetectable levels of skeletal muscle AMPK and reduced mitochondrial function (28).

We find that while WT mice experience the normal age-associated decline in COX activity, AMPK-MKO mice experience reduced COX activity early (2 mo) and maintain this same basal level until 18 mo of age. The decline of COX activity we observe in aged WT mice (49%) parallels that seen in aging human skeletal muscles (43%) (4). The lower COX activity we have shown in young AMPK-MKO mice provides further evidence of the essential role AMPK plays in maintaining mitochondrial function, which has been shown previously in some (16, 20, 28) but not all (45, 48) studies of mice with a transgenic mutation of skeletal muscle AMPK. The finding that AMPK-MKO mice maintain a consistently low level of COX activity with aging is a novel finding not previously investigated and suggests that while AMPK is
required for full mitochondrial health, other cellular factors are capable of preventing further aging-induced reductions. Surprisingly, despite reduced mitochondrial enzyme activity with aging and AMPK deficiency, oxygen utilization as measured in the metabolic cages was not different between groups highlighting the large functional reserve of skeletal muscle to maintain resting/basal energy homeostasis.

Insulin sensitivity has been shown to decline in humans (22) and rats (12, 33) with age. Although the cause is presently unknown, one predominant hypothesis for the causation of age-induced insulin resistance attributes mitochondrial dysfunction as a primary factor in the decline of insulin sensitivity (40). Supporting this hypothesis, elderly lean individuals were shown to have lower skeletal muscle insulin sensitivity correlating with similarly reduced mitochondrial oxidative phosphorylation (32). In agreement with these previous reports, we found that aging indeed suppressed the ability of mice to efficiently regulate glucose homeostasis as aged mice experienced impaired glucose and insulin tolerance. Interestingly, a recent study on AMPK α2KD mice observed neither a genotype difference nor the expected increase in insulin resistance by 18 mo of age (9). A potential source for this discrepancy may be that our present study utilized young mice at 2 mo of age, whereas the young α2KD mice were not tested until ~4 mo of age. However, given that aged AMPK-MKO mice did not experience worsened insulin sensitivity compared with their aged WT controls, despite the presence of a life-long reduction in mitochondrial enzyme activity, this suggests that mitochondrial dysfunction is not the primary defect leading to the development of aging-induced insulin resistance. In addition, it was remarkable that the complete lack of skeletal muscle AMPK activity and life-long reduction in mitochondrial enzyme activity in muscle of AMPK-MKO mice did not alter body mass, adiposity, and insulin sensitivity at old age. Given the central role of AMPK in regulating numerous metabolic pathways, it is possible that AMPK-MKO mice have developed compensatory pathways to control insulin sensitivity not otherwise triggered by the gradual reduction of AMPK activity normally induced by aging.

We have shown that the maintenance of skeletal muscle AMPK and mitochondrial enzyme activity is not necessary for preserving insulin sensitivity with aging. Future investigations into the regulation of insulin sensitivity in aging populations may shed light on new therapies and will be a critical step in preserving insulin sensitivity with aging. Future investigations into the regulation of insulin sensitivity in aging populations may shed light on new therapies and will be a critical step in preserving insulin sensitivity with aging.

REFERENCES


AUTHOR CONTRIBUTIONS


DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).


