Exercise performance and peripheral vascular insufficiency improve with AMPK activation in high-fat diet-fed mice

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Periphereal Artery Disease (PAD) is estimated to be present in 8–10 million Americans and is especially prevalent in older individuals, occurring in nearly 5% of all persons over the age of 50 yr (10, 24, 40). PAD significantly impacts both the mortality and morbidity of affected patients, and substantial medical resources are directed toward treating its various complications. For example, there are roughly 260,000 revascularizations performed each year and 100,000 amputations, and nearly 4 million people with stable disease suffer from intermittent claudication, which is characterized by painful muscle cramps during walking that severely limit activity (16, 63). Although occlusive, extracoronary atherosclerosis in large vessels servicing the lower limbs, such as the iliac, femoral, popliteal, or tibial arteries, is one of the defining diagnostic features of PAD, tissue dysfunction at sites distal to the atherosclerotic stenosis is believed to play a prominent role in the development of claudication. For example, surgical correction of the primary flow deficit at the site of stenosis generally fails to fully reset functional performance (19, 53). Conversely, exercise training can delay the time until claudication during walking without fundamentally altering the status of upstream atherosclerotic lesions (49, 54). Thus, intermittent claudication is multifactorial, and approaches that only focus on alleviating large vessel stenosis, such as revascularization, may be insufficient in the context of downstream muscle and/or microvascular dysfunction. These observations have highlighted the potential role of endothelial and muscle dysfunction in the development of intermittent claudication (22, 31, 71) and have expanded the opportunities for developing therapeutic treatments directed toward skeletal muscle and vascular endothelial cells (ECs).

Numerous links exist between the biological mechanisms influenced by 5′-AMP-activated kinase (AMPK) and those known to be dysfunctional in PAD, suggesting that therapeutic modulation of this master regulatory protein might be beneficial in treating certain aspects of this disease, including the improvement of mitochondrial and endothelial function in skeletal muscle (71, 76). For example, exercise training can improve walking distance in patients with claudication (54, 65), and one of AMPK’s most important roles is to coordinate the acute and chronic adaptation of tissues in response to exercise, including the stimulation of local angiogenesis and mitochondrial biogenesis within skeletal muscle (22). 5-Aminoimidazole-4-carboxamid ribonucleotide (AICAR), which is an AMP analog and can activate AMPK, leads to increases in skeletal muscle oxidative enzyme activities in rodents indicative of mitochondrial biogenesis and/or improved efficiency (17, 70). In patients with PAD, skeletal muscle displays many characteristics of poor mitochondrial function, such as acylcarnitine accumulation, impaired electron transport chain activity, and delayed O2 utilization at the onset of exercise (7, 52). Similarly, AMPK can directly influence vascular tone through

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the activation of endothelial nitric oxide (NO) synthase (eNOS) within ECs (8, 71, 74, 76), reverse age-impaired endothelium-dependent vascular dysfunction (32), and increase skeletal muscle perfusion (6). Interestingly, NO bioavailability and subsequent endothelial function are compromised in patients with PAD (26, 69).

Currently, the most common preclinical approach for studying PAD is based on surgical ligation of the femoral artery in rodents. Although this model successfully creates ischemic conditions in the lower limbs, it falls short on several fronts (14, 75). First, this method is most often conducted in healthy, young animals, whereas PAD typically develops in older individuals with a history of smoking, hypertension, dyslipidemia, diabetes, metabolic derangement, obesity, and physical inactivity. Second, the initial drops in muscle blood flow after surgical ligation are severe and rapid, as opposed to the gradual loss of limb perfusion in humans with PAD. Third, rodents are exceptional at responding to surgical ligation with aggressive arteriogenesis and angiogenesis, thereby limiting the opportunity for chronic muscle dysfunction to develop and confounding attempts to conduct long-term longitudinal studies with in-depth functional assessments, including exercise performance. In studies where age and comorbidities have been factored into the research design, the primary end point has been perfusion of the feet, and exercise performance has not been considered (15, 33). Coincidentally, clinical trials aimed at reducing claudication use the 6-min walk test as the primary end point, and drugs that have improved the recovery of perfusion in the feet have not succeeded in delaying the time until claudication in clinical trials (75).

The disconnect between the recovery of perfusion after ligation in rodents and exercise performance in humans prompted us to explore alternative model systems more closely aligned with PAD in terms of evolution of the disease, metabolism, pathological sequence, comorbidities, and functional exercise deficits. In this regard, it is known that C57BL/6 mice with dietary-induced obesity (DIO) display many of the risk factors associated with the development of PAD, including type II diabetes and dyslipidemia (60, 66). Furthermore, there is limited evidence for the development of atherosclerosis (47), poor exercise performance (30), microvascular and endothelial dysfunction (11, 43), and impaired vasodilation of conduit arteries ex vivo in rodents (5). Therefore, we characterized aged, DIO mice in terms of exercise capacity and skeletal muscle perfusion as a potential model of peripheral vascular insufficiency. We hypothesized that a novel, small-molecule AMPK activator, R118, would improve exercise capacity and skeletal muscle perfusion in this model.

MATERIALS AND METHODS

Mice. C57BL/6 and apolipoprotein E gene (ApoE)-deficient (ApoE−/−) mice were purchased from Jackson Laboratories (Bar Harbor, ME). All mice were males except for the comparison of C57BL/6 and ApoE−/− mice. Mice were provided food and water ad libitum and housed in a room with a 12:12-h reverse light cycle (lights on from 7:00 PM to 7:00 AM). All mice were housed at 4 mice/cage for the duration of the study with the exception that the wheel-trained mice were singly housed. The Institutional Animal Care and Use Committee of Rigel Pharmaceuticals approved all procedures.

Dietary treatments. Mice were fed either standard rodent chow [normal diet (ND); no. 5001, LabDiet, St. Louis, MO] or a high-fat diet (HFD) consisting of 60% of kcal from fat (no. D12492, Research Diets, New Brunswick, NJ). The HFD was commenced at 6 wk of age. For the initial comparison of ND (n = 18) and HFD (n = 36), mice were tested monthly for exercise capacity and muscle perfusion from 9 to 46 wk of HFD. A larger, second study was conducted using 53-­wk-old mice fed either the ND (n = 34) or HFD (n = 36). Exercise-trained mice fed a HFD were used as positive controls, and they were individually housed with activity wheels (n = 18). Cilostazol is a phosphodiesterase 3A inhibitor that improves vasodilation (58) and was used as an active control drug that is currently approved for treatment of claudication. Two doses of cilostazol (Ontario Chemicals) were used: 550 mg/kg HFD (n = 36) and 733 mg/kg HFD (n = 18). These doses corresponded to 30 and 40 mg/kg body mass (48). AT-1015 is serotonin (5-HT)2A receptor antagonist that inhibits platelet aggregation and vasconstriction (28) that was previously developed for the treatment of claudication. This drug failed to improve walking time in clinical trials and was associated with antimuscarinic activities and a dose-dependent decrease in tolerability, so this drug served as a negative control for the exercise tests (23). Mice were dosed with AT-1015 (Tocris Bioscience) at 55 mg/kg HFD (n = 18).

Oral glucose tolerance test. ND (n = 34)-fed or HFD (n = 33)-fed mice were tested for glucose tolerance at 58 wk of age and after 52 wk of HFD. Mice were fasted for 6 h followed by a tail nick, and 1 drop of blood was analyzed for glucose using a glucometer (Breeze2, Bayer). A bolus of glucose was delivered via oral gavage at 2 g/kg body mass, and glucose was measured at 15, 30, 60 and 90 min after the glucose bolus. No anesthesia was used for blood collection.

Voluntary wheel running test. The rationale behind this test was to determine physical activity similar to a 6-min walk test in humans. Mice were removed from their home cages and placed individually in cages equipped with wireless activity wheels (ENV-044, Med-Associates, St. Albans, VT). Ventilated racks containing a total of 160 cages with wheels were set up in the same reverse light cycle room as the mice were housed to facilitate high-throughput voluntary exercise testing. Mice were run in two shifts at ∼9 and 11 AM each day during the active dark cycle, and treatment groups were equally split across the different time slots. The amount of activity was recorded for 60 min, but each mouse was allowed to run for ∼75–80 min to facilitate the transfer of mice back to their home cages. Only 60 min of activity was recorded instead of 24-h access to prevent positive exercise training adaptations and to mimic the short, timed nature of the 6-min walk test. Mice had ad libitum access to water but not to food. Wheel-trained mice also took part in the test, but their home wheels were locked for ~18 h to prevent exercise before the test. Mice were given 3 consecutive days for each test, and the activity across the 3 days was averaged to account for any acclimation effects. Wheel Manager software (Med-Associates) was used to collect activity wheel count data in 1-min bins. Rest time was also computed and was defined as the period of time (in 1-min intervals) where “0” counts/revolutions were recorded. Average speed was calculated only for the 1-min intervals where activity counts were >0. Preliminary data in a separate group of mice demonstrated good correlations between the first and second days of the test (n = 48, r =
animal was placed on its right side on a platform heated to 38°C. The left foot was taped to a footplate attached to a servomotor (model 305C, Aurora Scientific, Aurora, ON, Canada). Percutaneous electrodes were used to stimulate the sciatic nerve with a stimulator (model 701C, Aurora Scientific). Muscle contractions were elicited at a contraction frequency of 100 Hz, 0.15-ms square-wave pulses, and a train duration of 200 ms. The stimulator and dual-mode lever system (model 305C, Aurora Scientific) were interfaced with a PowerLab system (AD Instruments, Colorado Springs, CO) to control the stimulator and acquire data from the force transducer using LabChart software (AD Instruments). Maximal isometric torque was determined by increasing the stimulator voltage 0.2 V/min until maximum torque was reached. Muscle fatigue was measured by eliciting 1 contraction every 2 s for a total of 60 contractions. The amount of force at every contraction relative to the first contraction was used to assess the amount of fatigue.

Contrast-enhanced ultrasound. Mice were anesthetized with isoflurane (1.5–2%, 500 ml O2/min), and a 27-gauge catheter was placed into each mouse’s tail and kept in place with surgical glue. Each mouse was placed in a supine position on a platform heated to 38°C with each paw taped to a surface electrode to monitor ECG, heart rate, and respiratory rate. Contrast-enhanced ultrasound (Vevo 2100, Visualsonics, Toronto, ON, Canada) was performed at an imaging frequency of 18 MHz. The probe was placed on the medial side of the leg to image the lower hindlimb in a sagittal plane. A bolus injection of microbubbles (Vevo MicroMarker, Bracco, Geneva, Switzerland) were injected through the tail vein catheter according to the manufacturer’s instructions. A syringe pump delivered the bolus of 50 μl at an infusion rate of 17.33 μl/s. A cine loop was recorded at a frame rate of 20 frames/s for a total of 1,000 frames. Curve fit analysis was used to measure echo power over time. The difference in maximum and minimum video intensity was determined as the peak enhancement and was the variable used to determine muscle perfusion. Only samples with a quality fit of 90% or greater were used in the analysis.

Contrast-enhanced ultrasound before and after muscle contractions was performed similarly except for the following modifications. Each mouse was placed in a lateral position on its right side to facilitate placement of the left foot into the footplate of the servomotor. The probe was placed on the lateral side of the leg, but the leg was still imaged in a sagittal plane. Each mouse was imaged before muscle contractions with a 20-min washout period of the microbubbles before the second cine loop was recorded after muscle contractions. Muscle contractions were performed as described above, but there was a 1-min rest before a second set of fatiguing contractions was performed, making the entire contraction procedure 5 min. The microbubble injection and cine loop recording took place ~3 min after the fatiguing muscle contractions. Mice in the HFD + R118 group were switched to regular HFD the night before perfusion measurements to study the chronic effect of R118. Animals that did not demonstrate an increase in contraction-induced perfusion were removed from the analysis.

Cardiac ultrasound. Parasternal short-axis M-mode images were acquired using the Vevo 2100 system (Visualsonics) as previously described (56). Mice were anesthetized with 1–2% isoflurane, and heart rate was maintained between 450 and 500 beats/min for all measurements.

Micro-computed tomography. Micro-computed tomography (micro-CT) was used to detect atherosclerosis in the aorta and femoral arteries as well as the vasculature of the gastrocnemius muscle. Numira Biosciences (Salt Lake City, UT) generated images and raw data on dissected muscles and arteries. Mice were perfused with saline until blood was removed and then perfused with 5–10 ml of 10% formalin. Tissues were washed three times for 10 min in PBS with rocking and then fixed in 10% formalin for 1 wk. Samples were stained with a proprietary reagent to detect atherosclerosis and scanned using the μCT 40 desktop micro-CT scanner (SCANCO Medical, Zurich, Switzerland). Images were collected at 15-μm res-
olution for the aorta and 10-μm resolution for the femoral artery using image acquisition parameters as previously described (35). Given the average diameter of a mouse aorta and femoral artery to be 1,000 and 300 μm, respectively, this imaging modality can detect atherosclerotic plaques that occlude as little as 2–3% of the vessel.

A separate set of mice was perfused with heparinized saline (300 U/ml) at a flow rate of 4 ml/min using a syringe pump until blood was removed and then perfused with 5 ml of warmed AltumBlu (Numira Biosciences), a proprietory contrast agent that fills and provides a mold of the blood vessels. The contrast agent was allowed to cure at 4°C for 1 h. The gastrocnemius/plantarlis muscle group from both the right and left legs was dissected and fixed in 10% formalin at 4°C. Each muscle group was imaged at 6-μm resolution using similar imaging parameters as previously described (64). This imaging resolution allows the visualization of small arterioles and venules but not capillaries. Muscle samples were sent for analysis only if the contrast agent had perfused in all tissues.

**Immunofluorescence.** Frozen medial gastrocnemius muscles were sectioned (10 μm) with a cryostat and fixed in cold acetone for 10 min before immunofluorescent staining with anti-laminin (L3939, Sigma, 1:200 dilution) and anti-CD31 (AF5628, R&D Systems, 1:50 dilution) antibodies. Slides were digitally imaged with a ×4 objective using a Nikon Eclipse Ti fluorescent microscope equipped with an Andor Neo sCMOS camera with a 2,160 × 2,160 pixel field of view. Image analysis was performed with CellProfiler. Thresholding was performed using Otsu’s method minimizing weighted variance with three classes, and the middle class was assigned to background. Morphological techniques were applied to provide outlines for each fiber. Three serial sections per slide were analyzed and averaged for each animal.

**Skeletal muscle AMPK activation.** R118 was delivered via oral gavage at 2.5, 5, or 10 mg/kg body mass (n = 4 mice/group) to wild-type mice fed the ND at 11.5 wk of age. Mice were euthanized by CO₂, and gastrocnemius muscles were quickly dissected and flash frozen in liquid nitrogen 60 min after dosing. Phosphorylation of AMPK (Thr172, no. 2535) and phosphorylated (p)acetyl-CoA-carboxylase (ACC; Ser79, no. 3661) were detected via Western blot analysis using antibodies from Cell Signaling Technologies (Danvers, MA).

**NADH oxidation assay.** Mitochondrial lysate from purified mouse livers (330 μg/ml) was added to tubes containing 2 mM NADH and incubated for 20 min at room temperature. The conversion of NADH to NAD⁺ was calculated by measuring the change in absorbance at an optical density of 340 nm over the incubation period.

**RESULTS**

**Mice maintained on a HFD develop obesity, glucose intolerance, and microvascular insufficiency in skeletal muscle.** Similar to previous reports (60, 66), mice fed the HFD developed progressive obesity, and body mass exceeded ND-fed control mice by 22% after 10 wk and 52% after 46 wk of HFD (Fig. 1A). C57BL/6 mice with diet-induced obesity are known to develop glucose intolerance (1), and HFD-fed mice had clear reductions in glucose handling capabilities as measured with an oral glucose tolerance test (Fig. 1B). Despite the significant overlap that exists in the physical and metabolic profiles of mice with diet-induced obesity and patients with...
PAD, such as diabetes, dyslipidemia, and atherosclerosis (1, 60), assessments of lower limb skeletal muscle perfusion in DIO mice have not been previously described. We therefore used contrast-enhanced ultrasound imaging to chronicle lower limb muscle perfusion over several months of HFD in sedentary mice. Significant deficits in resting muscle perfusion were first noticeable as soon as 9 wk after the HFD (19% less than ND-fed control mice) and progressed to a stable nadir of 40% below ND-fed control mice by 29 wk as assessed by peak enhancement, which is a measure of relative blood volume at steady-state perfusion (Fig. 1, C–E). Cardiac function was also assessed in aged, obese mice, and cardiac output was 50% greater in HFD-fed mice than in ND-fed mice, suggesting that cardiac function was not impaired (Fig. 1F), and similar to previously published results using the same diet and age of mice (3). These data point to the importance of both obesity and age in the development of peripheral vascular insufficiency.

Significant and progressive exercise intolerance develops in aged, obese mice by the 29th wk of HFD. Patients with PAD (55) and mice fed a HFD (30) are known to develop exercise intolerance; therefore, we sought to longitudinally characterize exercise capacity in mice maintained on a HFD. When given a 1-h wheel running test, DIO mice voluntarily ran at least 50%
less than mice on standard chow after 17 wk of HFD, and this finding was replicated after 28 wk of diet (Fig. 2A). This impaired performance was due to both a higher frequency of rest periods (Fig. 2B) and a slower running speed during activity (Fig. 2C) (18). Similarly, DIO mice ran ~40% less during the treadmill test than mice fed normal chow, but this did not manifest until 29 wk of HFD (Fig. 2D). It is noteworthy that these decreases in treadmill exercise performance did not occur until at least 2–4 mo after the earliest perfusion deficits were first observed at 9 wk after the HFD (Fig. 1E), suggesting the sequential and gradual development of exercise intolerance secondary to initial alterations in muscle perfusion.

Decreased skeletal muscle perfusion in DIO mice occurs independent of large vessel atherosclerosis. Since decreased blood flow in patients with PAD occurs in the context of atherosclerotic occlusion of large vessels (37), we used 10- to 15-μm resolution CT imaging of both the aorta and femoral arteries of mice chronically maintained on a HFD to investigate whether or not occlusive atherosclerosis was present. For these experiments, C57BL/6 ApoE−/− mice were used as positive controls since they are known to develop atherosclerosis (35, 73). Additionally, exercise performance was also evaluated in ApoE−/− mice. Extensive atherosclerosis was detected in the aorta in five of five 65-wk-old ApoE−/− mice, but no atherosclerosis was detected in six of six wild-type C57BL/6 mice even after 49–73 wk of HFD (Fig. 3A). Similarly, atherosclerosis was also detected in the femoral arteries in three of five ApoE−/− mice examined, but there were no detectable plaques noted in the femoral arteries of wild-type C57BL/6 mice on either diet (Fig. 3B). Contrast-enhanced ultrasound imaging of the lower limb muscles of ApoE−/− mice from 2.5 to 8 mo of age demonstrated normal levels of perfusion relative to wild-type mice (Fig. 3C), indicating that significant occlusive disease and/or microvascular dysfunction was not present in these mice. In addition, functional exercise capacity in ApoE−/− mice was equal to or greater than C57BL/6 wild-type mice, as demonstrated in treadmill exercise tests (Fig. 3D). Thus, despite extensive atherosclerosis, ApoE−/− mice are not satisfactory as a model for exercise intolerance associated with PAD since they display no functional deficits in muscle perfusion or exercise capacity.

Vascular insufficiency in HFD-fed mice can be attributed to microvascular dysfunction within skeletal muscle. Since occlusive atherosclerosis was unlikely to account for the observed decreases in muscle perfusion in HFD-fed mice, we used 6-μm resolution CT imaging to anatomically assess the status of the vascular network within the lower limb muscles (Fig. 4A). As shown in Fig. 4A, mice maintained on the HFD had significantly fewer detectable vessels with inner radii below 12 μm relative to ND-fed control mice. This vessel range corresponds to second- and third-order arterioles and small venules (67). Since the frequency of branch points per length of vessel increases as arteries transition into second- and third-order arterioles before progressing into capillary beds (67), a relative lack of detectable small vessels above the 6-μm detection limit will also register as a decrease in the overall number of branches, and this was the case for HFD-fed mice compared with ND-fed control mice (Fig. 4C). Capillary density was similar between ND- and HFD-fed mice (Fig. 4, D and E). Collectively, these observations suggest that the poor muscle perfusion observed in aged, obese mice is related to a substantial increase in microvascular tone.

Endothelium-derived NO is critical for proper vascular tone, and patients with PAD have evidence of endothelial dysfunction associated with reduced levels of NO bioavailability (36, 68). We investigated whether similar mechanisms might con-

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**Fig. 2. Exercise capacity is impaired in male C57BL/6 mice fed a HFD.** Panel A: voluntary wheel running activity during a 1-h timed test in ND- and HFD-fed mice. Panel B: rest time (1-min periods with zero counts). Panel C: average speed calculated when mice were engaged with the wheel. Sample sizes for ND-fed mice were as follows: 17 wk (n = 16) and 28 wk (n = 18); sample sizes for HFD-fed mice were as follows: 17 wk (n = 32) and 28 wk (n = 29). Panel D: treadmill exhaustion time in ND- and HFD-fed mice. Sample sizes for ND-fed mice were as follows: 10–29 wk (n = 18) and 35 wk (n = 16); sample sizes for HFD-fed mice were as follows: 10–15 wk (n = 33), 19 wk (n = 17), 29 wk (n = 27), and 35 wk (n = 26). Only half of the HFD-fed mice were tested at 19 wk of HFD. Data are presented as means ± SE. Data were analyzed with two-tailed, independent t-tests between ND and HFD at each time point. **P < 0.01; ***P < 0.001.
AMPK AND PERIPHERAL VASCULAR INSUFFICIENCY

Fig. 3. Obesity-induced reductions in hindlimb perfusion are independent of large vessel atherosclerosis. A and B: representative images of atherosclerosis in the aorta (A) and femoral artery (B) from ND-fed (n = 5), HFD-fed (n = 6), and apolipoprotein E gene (ApoE)-deficient (ApoE−/−) mice (n = 5). Mice were assessed after 49–73 wk of HFD, and ApoE−/− mice were 65 wk old. C: C57BL/6 and ApoE−/− mice were also tested for skeletal muscle perfusion (n = 5–7 mice/group except at the age of 4 mo, where n = 3 mice/group). D: treadmill exercise capacity in C57BL/6 and ApoE−/− mice (n = 14–20 mice/group). Data are presented as means ± SE. Data were analyzed with independent t-tests between C57BL/6 and ApoE−/− mice at each time point. *P < 0.05; **P < 0.01; ***P < 0.001.

tribute to the lack of proper vascular tone in the muscles of aged, obese mice. NO is quickly converted to nitrate and nitrite in the plasma, and these byproducts can be used as an indicator of NO bioavailability. Total levels of nitrate and nitrite were 52% lower in aged, obese mice relative to ND-fed control mice (Fig. 4F). ADMA is an endogenous inhibitor of NOS, and circulating ADMA levels are associated with PAD symptom severity (69). In the present study, aged, obese mice had ADMA levels that were 37% higher than ND-fed control mice (Fig. 4G). Thus, decreased NO bioavailability may contribute to the overall decrease in muscle perfusion and small vessel detection.

R118 activates both AMPK and eNOS in vitro and in vivo. Since exercise training can significantly delay the time to claudication (54), and it is known to be a potential stimulator of AMPK (42), we hypothesized that pharmacological activation of AMPK might prove useful for the treatment of exercise intolerance associated with PAD. As part of a medicinal chemistry program directed toward activators of AMPK, we discovered a potent, small-molecule AMPK activator, R118 (21). AMPK activity is highly sensitive to shifts in the ratios of AMP, ADP, and ATP through allosteric mechanisms. R118 indirectly activates AMPK by influencing the production of these nucleotides through inhibition of mitochondrial complex I, as measured by O2 consumption rate cells or purified mitochondria, and NADH accumulation in mitochondrial lysates (Fig. 5, A–D). R118 has an EC50 of 270 nmol/l for AMPK activation in C2C12 myotubes. When R118 is administered in vivo, it dose dependently increases AMPK phosphorylation in gastrocnemius muscles of mice within 1 h at exposures as low as 690 nmol·h·l−1 (AUC24 h, Fig. 6, A and B).

AMPK is known to directly regulate eNOS activity through the phosphorylation of Ser1177 (8), and treatment of primary HuMECs with R118 potently induced the phosphorylation of eNOS (Fig. 6C). Similarly, phosphorylation of eNOS (Ser1177) was significantly induced in the aorta of mice treated with R118 (Fig. 6D). Thus, R118 effectively activated AMPK in muscle and both AMPK and its downstream substrate, eNOS, within vascular tissue.

Treatment of aged, obese mice with an AMPK activator increases voluntary wheel running distance and average running speed, decreases the frequency of rest periods, and prevents the progressive decrease in treadmill exercise capacity as early as 5 wk posttreatment. Using R118, the potential therapeutic benefit of AMPK activation for addressing vascular insufficiency and exercise intolerance was assessed in 53-wk-old DIO mice that had already progressed to a chronic disease state. Wheel running and treadmill tests were used to assess whether the treatment was capable of improving exercise performance. R118 dose dependently improved exercise capacity at both 5 and 15 wk posttreatment during the activity wheel test. Mice treated with the high dose of R118 (366 mg/kg HFD) increased their wheel running counts twofold (Fig. 7A), decreased the frequency of rest periods by 50% (Fig. 7B), and significantly increased their average running speed during periods of active wheel cycling (Fig. 7C). Estimated plasma exposures (AUC24 h) of R118-treated mice measured at the end of the study were 4,286 and 7,603 nmol·h·l−1 for...
the low (244 mg/kg HFD) and high doses, respectively, which are exposures sufficient to activate AMPK and eNOS in vivo (Fig. 6). In contrast, HFD mice that were untreated did not increase their wheel running counts despite small gains in rest time. Body masses were not different between untreated HFD and R118-treated mice during each wheel exercise test (data not shown).

Cilostazol, a phosphodiesterase 3A inhibitor, is currently used clinically to treat claudication (58), and there is limited evidence that part of its mechanism may involve weak activation of AMPK (61); therefore, cilostazol was used as a comparator throughout these experiments. Cilostazol did not improve wheel running counts, but average running speed transiently increased twofold at 5 wk posttreatment. The estimated exposures for the low (550 mg/kg HFD) and high (733 mg/kg HFD) doses of cilostazol were 9,665 and 35,112 nmol·h·l⁻¹, respectively. Importantly, the exposure obtained with the high dose of cilostazol was similar to that achieved commonly in humans during treatment of claudication (58). AT-1015 is a 5-HT₂A receptor antagonist that showed promise in other animal models of PAD (27–29) but failed to improve the time to claudication in the clinic (23). In the present study, treatment with AT-1015 did not lead to improvements in total wheel counts, average speed, or rest time, similar to HFD-fed control mice. Plasma levels of AT-1015 were also confirmed to verify drug delivery (AUC₂₄ h: 882 nmol·h⁻¹, n = 5). Regular exercise can also improve symptoms of claudication (54), and we therefore housed mice individually with activity wheels to generate an exercised control group. Daily exercise bouts averaged 0.9 km/day at the beginning of the study and reached a peak of 4.4 km/day after 10 wk. As predicted, mice that exercised regularly in the wheels improved their 1-h timed wheel running test and did so by substantially increasing their wheel running speed without decreasing their rest time.

As a further test of exercise capacity, a treadmill endurance test was conducted with all groups of mice (Table 1). As expected, exercise training was effective at improving treadmill performance. Interestingly, R118 treatment maintained treadmill run time with advancing age, but this was only effective in the low-dose group. A t-test was used to compare untreated and R118-treated mice, and R118-treated mice (low dose) ran ~45% farther at both the 6- and 16-wk assessment periods (P < 0.026). In contrast, mice treated with either dose of cilostazol failed to prevent the progressive decline in treadmill performance. These data demonstrate that AMPK activa-

Fig. 4. Microvascular dysfunction in skeletal muscle and nitric oxide (NO) bioavailability contribute to vascular insufficiency in aged, obese mice. A: representative micro-computed tomography (CT) images of AltaBlu-filled gastrocnemius/plantaris muscles delineating blood vessels after 73 wk of ND (n = 7) or HFD (n = 6) in male C57BL/6 mice. Scale bar = 200 μm. B: frequency distribution of vessel radius. C: frequency distribution of vessel branching. D: representative ×40 medial gastrocnemius muscle sections depicting capillary density (CD31⁺; green; laminin: red). Scale bar = 100 μm. Insets show higher-magnification images. E: quantification of capillary density. F and G: plasma total nitrate and nitrite concentration (F) and plasma asymmetric dimethylarginine (ADMA; G) after 46 wk of HFD (n = 8 mice/group). Data are presented as means ± SE. Data were analyzed with two-tailed, independent t-tests between ND and HFD. *P < 0.05; ***P < 0.001.
tion by R118 was effective at improving exercise performance in aged, obese mice that had already developed chronic peripheral microvascular insufficiency and exercise intolerance. Aged, obese mice demonstrate increased muscle weakness and fatigue during repetitive contractions, and these changes can be attenuated by R118. In addition to vascular dysfunction, skeletal muscle oxidative capacity and strength are also compromised in PAD (51, 55). Lower limb skeletal muscle strength was assessed in vivo by stimulating the sciatic nerve with electrodes inserted percutaneously to measure maximal isometric torque of the plantar flexors, similar to a calf raise exercise. Untreated HFD-fed mice had 13% less muscle strength than ND-fed mice (Fig. 8A), but R118 had no effect on absolute muscle strength when tested at least 17 wk posttreatment. Muscle fatigability was assessed by force loss during 60 consecutive in vivo plantar flexion contractions. Significant differences were detected between nonobese control and HFD-fed mice during contraction-induced muscle fatigue, and this was reversed with wheel running training (Fig. 8B). Treatment with the AMPK activator (Fig. 8C) and cilostazol (Fig. 8D) also significantly attenuated muscle fatigue, whereas AT-1015 had no effect, suggesting that both R118 and cilostazol could directly delay skeletal muscle fatigue during exercise. 

**R118 improves mitochondrial function.** Exercise, AICAR, and mild mitochondrial stress stimulate mitochondrial biogenesis through a process known as mitohormesis (17, 45, 70). Since complex I inhibition using R118 could regulate this process, we measured citrate synthase activity as a marker of mitochondrial function. Citrate synthase activity in the plantarflexed small vessels, we conducted a similar assessment of the vascular network within the lower limb muscles of R118-treated animals (Fig. 11A). Mice treated with either dose of R118 had a higher number of detectable small vessels (≤12
μm in radius) and more branching (≥3 branch points/mm) than mice fed the HFD (Fig. 11, B and C). There was a trend for cilostazol to improve small vessel detection, coinciding with the trend to improve perfusion, but this did not achieve statistical significance.

Given that that aged, obese mice have reduced NO bioavailability and R118 can induce the phosphorylation of eNOS in ECs in vitro and in vivo, we examined NO bioavailability in R118-treated mice. Total plasma nitrate and nitrite levels (Fig. 11D) were 40% greater and plasma ADMA levels (Fig. 11E) were 40% lower in R118-treated mice compared with untreated HFD-fed control mice. Both cilostazol- and AT-1015-treated mice had ADMA and total nitrate and nitrite levels similar to untreated HFD-fed control mice. Collectively, these data suggest that AMPK activation by R118 modulates microvascular tone by mechanisms that include increased NO bioavailability, contributing to the enhanced skeletal muscle perfusion in both the resting and exercising states.

DISCUSSION

PAD is a serious medical condition that significantly limits patient mobility and is independently related to increased mortality rates (59). More effective treatments are needed to improve exercise performance and walking ability. Much of the difficulty with current preclinical animal models arises from the fact that rodents undergo rapid and robust arteriogenesis in response to surgical reductions in blood flow, and measuring the recovery of blood flow in mice has not translated into new therapies for intermittent claudication (75). Thus,
alternatives to the current models are needed. The present study demonstrates numerous functional similarities between aged, obese mice and patients with PAD suffering from intermittent claudication, thereby suggesting this model as a suitable tool for exploring disease mechanisms and assessing the therapeutic potential of new treatments (Fig. 12). Evidence is provided to show that aged, obese mice progressively develop lower limb microvascular insufficiency, reduced strength, premature muscle fatigue, and a profound reduction in exercise performance that is accompanied by frequent bouts of rest. Importantly, these pathological changes occur gradually over time and are characterized longitudinally using multiple functional assessments. Furthermore, we demonstrate the presence of reduced muscle perfusion and impaired NO bioavailability independent of large vessel occlusive atherosclerosis. AMPK activation is identified as a promising therapeutic approach for

Table 1. Treadmill endurance capacity is maintained in mice treated with R118

<table>
<thead>
<tr>
<th></th>
<th>HFD Unt</th>
<th>HFD + Wheel Exercise</th>
<th>HFD + Low-Dose Cilostazol (550 mg/kg HFD)</th>
<th>HFD + High-Dose Cilostazol (733 mg/kg HFD)</th>
<th>HFD + AT-1015</th>
<th>HFD + Low-Dose R118 (244 mg/kg HFD)</th>
<th>HFD + High-Dose R118 (366 mg/kg HFD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of mice/group</td>
<td>26</td>
<td>12</td>
<td>30</td>
<td>17</td>
<td>14</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>746 ± 46</td>
<td>735 ± 24</td>
<td>818 ± 50</td>
<td>845 ± 86</td>
<td>640 ± 43</td>
<td>852 ± 50</td>
<td>842 ± 63</td>
</tr>
<tr>
<td>6 wk posttreatment</td>
<td>680 ± 41</td>
<td>5,216 ± 1,151†</td>
<td>725 ± 50†</td>
<td>674 ± 61</td>
<td>630 ± 53</td>
<td>927 ± 96</td>
<td>703 ± 42</td>
</tr>
<tr>
<td>16 wk posttreatment</td>
<td>595 ± 43†</td>
<td>4,913 ± 1,234†</td>
<td>647 ± 44†</td>
<td>644 ± 72</td>
<td>571 ± 43</td>
<td>826 ± 80</td>
<td>693 ± 52</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. The maximum time of the test was 9,000 s. HFD, high-fat diet. Data were analyzed by one-way ANOVA with repeated measures and Holm-Sidak’s post hoc test. *P < 0.05, †P < 0.01, and ‡P < 0.001 vs. pretreatment.
AMPK AND PERIPHERAL VASCULAR INSUFFICIENCY

H1139

Fig. 8. R118 improves skeletal muscle fatigue after in vivo plantar flexion contractions. The following groups of mice were evaluated: ND (n = 18), HFD-Unt (n = 15), HFD + wheel exercise (n = 15), HFD + cilostazol-high (n = 15), HFD + AT-1015 (n = 16), HFD + R118-low (n = 9), and HFD + R118-high (n = 16). A: maximal isometric torque. B–D: plantar flexion torque was measured during fatiguing contractions in ND, Unt, and wheel-exercised mice (B), R118-treated mice (C), and cilostazol- and AT-1015-treated mice (D). Exercise consisted of 60 plantar flexion contractions via stimulation of the sciatic nerve with percutaneous electrodes at 100 Hz, 200-ms train duration, and 0.15-ms pulse duration. One contraction was elicited every 2 s for 2 min. Data were collected 17–23 wk posttreatment. For maximal isometric torque, data are presented as means ± SE. For fatigue, only means are presented. The Untreated (Unt) group is regraphed in each Fig. and represents the same data set. Data were analyzed by 1-way or 2-way ANOVA with Holm-Sidak’s posthoc. Bars signify contractions that are different from HFD-Unt. *P < 0.05.

The treatment of intermittent claudication based on the ability of a small-molecule AMPK activator to improve NO bioavailability, normalize muscle perfusion, reduce fatigability, increase mitochondrial function, and significantly improve exercise performance.

Although others have documented altered ex vivo vasodilatory responses in isolated arteries of obese mice and rats (5, 11), this is the first study to show perturbed in vivo skeletal muscle perfusion in DIO mice. These differences developed relatively early in the course of the disease, suggesting that obesity can trigger microvascular dysfunction. These changes progressed gradually and then persisted over long periods of time, a situation akin to the development of PAD in humans (75). It is worth noting that perfusion was measured directly in limb muscles using contrast-enhanced ultrasound, and when this same approach is applied to patients with PAD, it is predictive of claudication (34). Furthermore, the magnitude of decreased muscle perfusion observed in aged, obese mice was very similar to that observed in patients with PAD symptomatic for intermittent claudication (40–50% below normal controls) (4). Importantly, these mice demonstrated clear impairments in the ability to mount reactive hyperemic responses during exercise, and this is one of the defining features of PAD in humans. The loss of perfusion observed in aged, obese mice was more gradual and moderate relative to surgical ligation-based models in which severe reductions are typically generated in first few days after surgery (80–90% reduction relative to baseline) (14).

In humans with PAD, atherosclerotic occlusions in peripheral arteries are not the only factor contributing to decreased muscle perfusion, and microvascular dysfunction is increasingly being recognized as an important aspect of this disease (9, 46, 68). In the present study, no evidence of occlusive atherosclerotic plaques could be found, even after 49–73 wk of HFD, in either the aortas or femoral arteries of aged, obese mice using micro-CT imaging. The resolution of this technique was sufficient to detect meaningful plaque deposits, since ApoE−/− mice had clear atherosclerosis as far down as the tibial-popliteal bifurcation of the femoral artery. Even rodents with verifiably high plaque loads, such as the ApoE−/− mice exam-
model is multifactorial. There is evidence for underlying endothelial dysfunction in patients with PAD (25, 36, 50, 72), and it has been hypothesized to be related to functional performance (25, 36, 50). Furthermore, cardiovascular risk factors predispose individuals to endothelial dysfunction, and if the cardiovascular risk factors are not controlled, endothelial dysfunction can directly contribute to the development of atherosclerosis (12, 62). One of the more surprising outcomes from clinical trials examining lower limb revascularization surgeries in patients with PAD was that exercise performance was not consistently restored (19). This suggests that other factors, especially those controlling microvascular function, might have a major role in determining functional performance in these patients (9, 46).

Interestingly, detectable decreases in treadmill exercise performance did not develop until 2–4 mo after the first documented decrease in perfusion. This suggests that an absolute or temporal threshold of microvascular insufficiency must be reached before exercise performance is impacted. The delayed decrease in exercise performance is consistent with prevailing theories on how intermittent claudication arises in PAD. For example, one hypothesis is that limitations in blood flow result in continuous cycles of ischemia-reperfusion injury during transient exercise episodes. The ensuing oxidative stress becomes cumulatively injurious over time to ECs, muscle tissue, and even distal motor axons (4, 36, 53). In exercise wheel tests, it was clear that performance decreases were largely the result of an increased frequency of rest periods and a slower overall pace. Although we cannot determine the exact mechanism behind exercise intolerance (e.g., claudication or fatigue), more rest periods could be reflective of lower limb discomfort, such as that experienced during claudication in patients with PAD. With advancing disease progression, DIO mice performed in both measures of exercise capacity at levels ~50% of control mice, and this drop is similar to the documented exercise reduction in patients with PAD experiencing intermittent claudication (2, 55). PAD is further associated with muscle weakness in the affected limb (55). Aged, obese mice also displayed decreased lower limb strength, measured in vivo by maximal isometric plantar flexion torque. This was accompanied by clear increases in fatigability within the same muscles during repeated serial contractions. Together, these observations demonstrate that aged, obese mice share many of the same comorbidities as patients with PAD, such as advancing age, obesity, dyslipidemia, and glucose intolerance, and are also functionally similar in the temporal development of reduced muscle perfusion, muscle dysfunction, and exercise intolerance. These similarities occurred despite the absence of large vessel occlusive atherosclerosis, providing evidence that microvascular dysfunction may prominently contribute to claudication.

To help further validate using aged, obese mice to test exercise intolerance, we treated mice with cilostazol or AT-1015 and also exercised mice in activity wheels. Cilostazol, at blood exposures comparable to clinical use in humans, was able to improve some of these parameters but not all. In contrast, AT-1015 failed to show meaningful improvement in any functional exercise assessments, similar to its performance in clinical trials (23). Not surprisingly, mice with unlimited access to an activity wheel made substantial improvements in voluntary and forced exercise tests. Exercise also offers alleviation of claudication symptoms in patients with PAD, al-

**fig. 9.** Skeletal muscle mitochondrial function is improved with R118. Plantaris muscles were collected 18 wk after treatment. A sample size of *n = 7–8 was used for all measurements, except the HFD + Wheel (*n = 5). Data are presented as mean ± SE. Data were analyzed by one-way ANOVA with Holm-Sidak’s posthoc (Untreated vs. all other treatments). *P < 0.05, ***P < 0.001 vs. HFD-Unt.
though the effectiveness of this type of intervention is much more pronounced in this study than in the human population.

The reason for the substantial improvements with exercise seen in this model (5- to 10-fold improvement) compared with humans (2-fold improvement) is most likely due to the volume of exercise performed (4 vs. 1 km/day) and the confounding effect of weight loss, as wheel-exercised mice experienced a 15% reduction in body mass not seen with any of the other treatments.

In our experiments, pharmacological activation of AMPK significantly improved exercise performance. Interestingly, in wheel exercise tests of mice treated with the AMPK activator R118, overall exercise capacity was improved by decreasing the frequency of rests taken during the test period and by running at a higher average speed during active episodes. The benefits of cilostazol were not as robust as those observed with R118, and the time needed for functional improvements was 10 wk longer relative to R118. We also subjected mice to a forced treadmill test, and mice treated with R118 were able to maintain their run time as all other mice regressed throughout the study. Since exercise tests measure global performance, we also sought to obtain a more focused assessment of in vivo muscle function during exercise using sciatic nerve stimulation to measure plantar flexion torque. Listed in order of the size

<table>
<thead>
<tr>
<th>Group</th>
<th>Rest</th>
<th>Exercise</th>
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<tr>
<td>ND</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Unt</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Wheel Exercise</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Cilostazol-high</td>
<td>15</td>
<td></td>
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<tr>
<td>AT-1015</td>
<td>15</td>
<td></td>
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<tr>
<td>R118-low</td>
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<td>R118-high</td>
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Fig. 10. R118 improves skeletal muscle perfusion at rest and after exercise. Mice were fed a ND (n = 18), HFD-Unt (n = 15), HFD + Wheel (n = 15), HFD + Cilostazol-high (n = 14), HFD + AT-1015 (n = 13), HFD + R118-low (n = 9), or HFD + R118-high (n = 16). Hind limb muscle perfusion was measured with contrast-enhanced ultrasound at rest and after exercise in the same limb. Exercise consisted of 120 plantar flexion contractions via stimulation of the sciatic nerve with percutaneous electrodes at 100 Hz, 200-ms train duration, and 0.15-ms pulse duration. One contraction was elicited every 2 s for 2 min, followed by a 1-min rest and a second set of 60 contractions. Data were collected 17–22 wk posttreatment. A: representative peak enhancement images before (rest) and after fatiguing muscle contractions (exercise). B and C: peak enhancement at rest (B) and after exercise (C). Data are presented as means ± SE. Data were analyzed by one-way ANOVA with Holm-Sidak’s post hoc test. *P < 0.05; **P < 0.01; ***P < 0.001 vs. HFD-Unt.
effect, exercise training, AMPK activation (R118), and cilostazol all significantly improved lower limb fatigue resistance.

There are many compelling reasons to explore the mechanism of AMPK activation in PAD, including its importance for muscle metabolism and mitochondrial function (17, 22, 70) and endothelial function through the regulatory control of eNOS (6, 8, 41, 74). The higher citrate synthase activity in skeletal muscle points to improved mitochondrial function with R118 treatment. This may be counterintuitive since acute dosing of R118 inhibits complex I. However, weak RNA

Fig. 11. R118 increases the number of detectable small vessels and branch points per vessel length in skeletal muscle, and this is related to NO bioavailability. The left leg was subjected to 120 plantar flexion contractions (exercise), and the contralateral limb served as a control (rest). There was no effect of exercise or an interaction, so data presented are the main effects of treatment (vessel radius: \( P = 0.034 \); vessel branching: \( P = 0.004 \)). A: representative micro-CT images of the gastrocnemius muscle from the unexercised leg from the ND \(( n = 14)\), HFD Unt \(( n = 12)\), HFD + cilostazol-high \(( n = 12)\), HFD + R118-low \(( n = 12)\), and HFD + R118-high \(( n = 12)\) groups. Muscles were collected 27–29 wk posttreatment. AT-1015-treated mice were not tested. Scale bar \(2 \text{ mm}\).

B: mean number of small vessels (\( \geq 12 \mu \text{m} \) in radius).

C: vessels with multiple branch points (\( \geq 3 \) branch points/mm).

D and E: plasma nitrate and nitrite \(( D)\) and plasma ADMA \(( E)\) from the ND \(( n = 18)\), HFD Unt \(( n = 18)\), HFD + cilostazol-high \(( n = 14)\), HFD + AT-1015 \(( n = 18)\), HFD + R118-low \(( n = 18)\), and HFD + R118-high \(( n = 18)\) groups. Plasma samples were collected 25 wk posttreatment. Data are presented as means ± SE. Data were analyzed by one- or two-way ANOVA with Holm-Sidak’s post hoc test. * \( P < 0.05 \); ** \( P < 0.01 \); *** \( P < 0.001 \) vs. HFD Unt.
interference of a complex I component demonstrates that skeletal muscles adapt to stress by increasing mitochondrial number and improving mitochondrial efficiency (45), a term known as mitohormesis. Other AMPK activators, such as exercise and AICAR, also improve mitochondrial function (17, 70), similar to the changes seen with R118. Exercise can also improve mitochondrial function, and in this study we saw comparable improvements in citrate synthase activity in wheel-trained mice but not in cilostazol-treated mice. These data suggest that improving mitochondrial function through AMPK activation may have a positive effect on walking time in patients with PAD.

The robust increases in skeletal muscle perfusion caused by AMPK activation, especially during exercise, stand out as one of the most striking findings of this study. This functional proof of improved perfusion was accompanied by anatomical evidence of increased small vessel density within hindlimb muscles, increased nitrate/nitrite, and decreased ADMA in plasma. Others have shown an important role for AMPK in arteriolar dilation (6, 13), but these assessments were not done in the context of overt disease, during exercise, or linked to functional performance. These improvements in microvascular function were not seen with cilostazol. Part of the improvement is exercise performance with AMPK activation may be related to the improvement in microvascular function of skeletal muscle. However, many systems affect exercise performance, and the relative amount of improvement in perfusion compared with exercise performance in exercise-trained mice suggest that additional physiological processes beyond AMPK activation and/or microvascular improvement contribute to exercise performance.

Preclinical animal models with relevancy to PAD present many challenges, and the limitations with current approaches have been widely commented upon (14, 75). While no single approach in animals can ever fully recapitulate the human condition, numerous aspects of functional measures, comorbidities, timescales, group sizes, controls, time of treatment (after onset of disease), and study design have been addressed here. These studies were well powered (18–36 animals/group) and included comparisons to molecules with clinical benefit (cilostazol) (58) or that have previously failed clinical trials assessing claudication (AT-1015) (23). Collectively, these data point to numerous functional and molecular overlaps between patients with PAD and aged, obese mice and highlight the therapeutic potential of AMPK activation for improving exercise tolerance in the context of underlying peripheral vascular insufficiency.

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AMPK AND PERIPHERAL VASCULAR INSUFFICIENCY

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DISCLOSURES
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AUTHOR CONTRIBUTIONS

REFERENCES


