Musclin is an activity-stimulated myokine that enhances physical endurance

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Exercise remains the most effective way to promote physical and metabolic wellbeing, but molecular mechanisms underlying exercise tolerance and its plasticity are only partially understood. In this study we identify musclin—a peptide with high homology to natriuretic peptides (NP)—as an exercise-responsive myokine that acts to enhance exercise capacity in mice. We use human primary myoblast culture and in vivo murine models to establish that the activity-related production of musclin is driven by Ca^{2+}-dependent activation of Akt1 and the release of musclin-encoding gene (*Ostn*) transcription from forkhead box O1 transcription factor inhibition. Disruption of *Ostn* and elimination of musclin secretion in mice results in reduced exercise tolerance that can be rescued by treatment with recombinant musclin. Reduced exercise capacity in mice with disrupted musclin signaling is associated with a trend toward lower levels of plasma atrial NP (ANP) and significantly smaller levels of cyclic guanosine monophosphate (cGMP) and peroxisome proliferator-activated receptor gamma coactivator 1-α in skeletal muscles after exposure to exercise. Furthermore, in agreement with the established musclin ability to interact with NP clearance receptors, but not with NP guanyl cyclase-coupled signaling receptors, we demonstrate that musclin enhances cGMP production in cultured myoblasts only when applied together with ANP. Elimination of the activity-related musclin-dependent boost of ANP/cGMP signaling results in significantly lower maximum aerobic capacity, mitochondrial protein content, respiratory complex protein expression, and succinate dehydrogenase activity in skeletal muscles. Together, these data indicate that musclin enhances physical endurance by promoting mitochondrial biogenesis.

osteocrin | mitochondria | skeletal muscle | exercise | natriuretic peptide

The ability to sustain physical activity is necessary for both quality and longevity of life. Regular exposure to exercise is associated with reduced rates of all-cause mortality (1). There are multiple mechanisms by which physical activity promotes health; however, recently there has been an interest in defining the contribution of circulating proteins secreted by skeletal muscle, termed myokines (2, 3). Myokines are autocrine, paracrine, or endocrine stimuli that may guide local skeletal muscle remodeling, repair, and maintenance or steer systemic adaptation related to physical activity (2). Understanding the functional role and the signaling pathways of myokines, particularly as they relate to exercise, may reveal new therapeutic targets to promote health and augment the benefits of physical activity.

This study is focused on the recently discovered myokine musclin (4, 5). Two groups initially identified this peptide: one as bone-derived osteocrin (5) and the second as muscle-secreted musclin (4). Musclin mRNA expression has been linked to insulin-induced activation of protein kinase B (Akt) that phosphorylates forkhead box O1 transcription factor (FOXO1), causing it to be exported from the nucleus and thus releasing the musclin-encoding gene from transcriptional inhibition (4, 6). This pathway has been demonstrated to regulate musclin transcription in both cell culture and skeletal muscles (4, 6). Musclin contains two KKKR putative serine protease cleavage sites and a region homologous to members of the natriuretic peptide (NP) family (4, 5). However, musclin does not have two cysteine residues needed to form the Ω-like structure characteristic for NPs (4, 5). In line with these structural characteristics, it has been demonstrated that musclin binds to the NP clearance receptor, NPR_C, with affinity comparable to NPs, but exhibits only weak binding to NPR_A and NPR_B without activating the linked guanyl cyclase that is the primary effector of NP physiologic actions (7–9). Thus, it has been suggested that musclin function may be due to modulation of the action of NPs by competition with them for clearance via NPR_C binding (8, 9). Indeed, musclin overexpression in osteoblast-lineage cells has been shown to result in elongated bones and marked kyphosis (9), which is similar to the phenotype of mice transgenically overexpressing BNP (10) or CNP (11) or lacking NPR_C (12, 13). However, the physiological role of musclin production in skeletal muscles has remained elusive.

In this work, we demonstrate that musclin production by skeletal muscle is stimulated by physical activity and is paralleled by increased systemic musclin levels. Disruption of normal musclin

Significance

Skeletal muscle is increasingly recognized as a secretory organ. Revealing the identity and function of myokines can improve our understanding of skeletal muscle function under sedentary or exercise conditions, as well as its coordination with other organs, tissues, and overall body metabolism. This study identifies musclin as an exercise-responsive myokine critical for skeletal muscle adaptation to physical activity. We develop a musclin-encoding gene (*Ostn*) knockout mouse, which allows us to determine a previously unrecognized physiologic function of musclin in regulation of skeletal muscle mitochondrial biogenesis and physical endurance. The demonstrated molecular mechanism for musclin-dependent skeletal muscle adaptation to exercise also transforms the perspective on natriuretic peptide signaling, particularly as it relates to physical activity and exercise-induced remodeling in different tissues.

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signaling in mice by knockout of the musclin-encoding gene, *Ostn* (*Ostn*-KO), results in diminished exercise tolerance coupled with downgraded activity-related atrial NP (ANP)/cyclic guanosine monophosphate (cGMP)/PGC-1α–dependent skeletal muscle mitochondrial biogenesis. Thus, this work identifies a physiological role of musclin in enhancing skeletal muscle oxidative capacity and physical endurance.

Results

Musclin Production and Secretion into the Systemic Circulation Are Stimulated by Exercise. Normal skeletal muscle function requires tight coordination with the operation of other organs and systems. Such coordination has been attributed in part to the action of myokines. Specifically, "exercise factors," a subset of myokines whose production and secretion into systemic circulation are stimulated by physical activity, have been shown to modulate skeletal muscle and systemic metabolism, angiogenesis, growth, and inflammation (14).

To determine whether musclin is an exercise factor, the level of musclin peptide in skeletal muscle was probed in two groups of wild-type (WT) mice: one group that exercised on a moving treadmill for 45 min daily (exercise) and a sedentary control group. After 5 d of exercise or control treadmill exposure, mice were euthanized, and their tissues were harvested by rapid excision and freeze clamp. Proteins were extracted from gastrocnemius muscles and segregated by Western blot (Fig. 1*A*), showing that exercise was associated with a nearly 100% increase in skeletal muscle musclin over control conditions [1.15 ± 0.2 vs. 0.60 ± 0.1 arbitrary units (AU), $n = 5$ each, $P < 0.05$; Fig. 1*B*]. A similar increase was demonstrated in skeletal muscle musclin mRNA from tibialis anterior samples (0.75 ± 0.05 vs. 0.49 ± 0.05 AU, $n = 5$ each, $P < 0.05$; Fig. 1*C*), whereas musclin mRNA levels in femur were markedly (96–98%) lower and were unresponsive to exercise [0.02 ± 0.002 vs. 0.02 ± 0.003 AU, respectively, $n = 4$ each, P = not significant (NS) between exercise and sedentary, $P < 0.05$ compared with skeletal muscle mRNA]. The increased musclin production by skeletal muscle was paralleled by an increase in the plasma musclin level from 27.71 ± 5.54 pg/mL ($n = 3$) in sedentary control WT mice to 46.24 ± 4.69 pg/mL in WT mice after exercise ($n = 6$, $P < 0.05$; Fig. 1*D*). Furthermore, immunohistochemistry of gastrocnemius cross-sections demonstrated more intense staining for musclin when mice were after exercise vs. sedentary (Fig. 1*E*). Thus, musclin production and secretion into the systemic circulation are up-regulated in response to exercise, establishing musclin as an exercise factor.

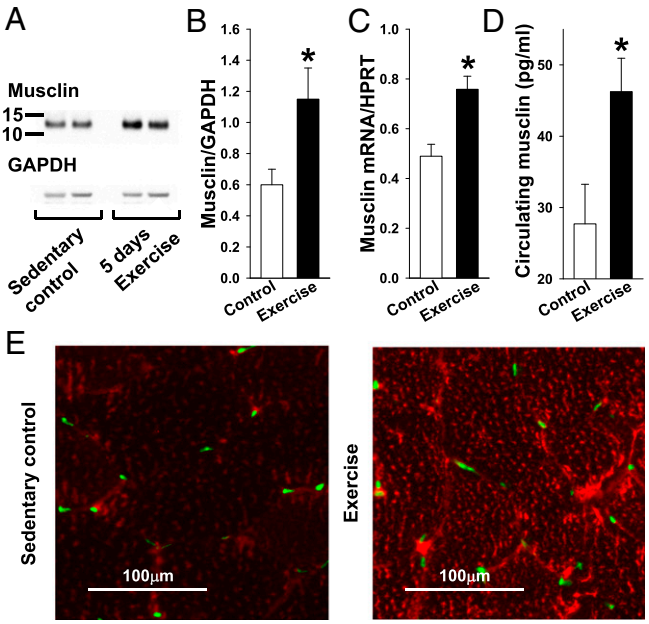

Fig. 1. Musclin expression is exercise-responsive. Musclin expression was tested in muscles of WT mice after 5 d of treadmill exercise vs. no exercise (control). (*A*) Representative Western blots for musclin and GAPDH in protein extracts from gastrocnemius. (*B–D*) Summary statistics for musclin protein expression normalized to GAPDH by densitometry of Western blots of protein extracts from gastrocnemius (*B*), tibialis anterior musclin mRNA normalized to hypoxanthine guanine phosphoribosyl transferase (HPRT) by quantitative RT-PCR (qRT-PCR) (*C*), and musclin peptide expression in plasma by custom ELISA (the *y* axis range begins at the lower limit for detection for this assay of 20 pg/mL; *D*). (*E*) Representative immunohistochemical stains of gastrocnemius cross-sections imaged by confocal microscopy. Red, musclin; green, nuclei. *$P < 0.05$ vs. control.

Activity-Induced Musclin Production Is Linked to Ca^{2+}-Dependent Activation of Akt. Regulation of musclin transcription has been linked to Akt activation (4, 6, 15, 16). Akt is a serine/threonine kinase that has emerged as a critical signaling component for the regulation of cellular metabolism, growth, and survival in multiple systems (17). Akt activity is increased in response to numerous stimuli, including a wide variety of growth factors and hormones activating phosphatidylinositol 3-kinase (PI3-kinase) (18–20). Akt can also be activated by mechanisms independent of PI3-kinase—for example, in response to increases in intracellular Ca^{2+} or cAMP, as occurs with increased muscle contractile activity (21–25).

Here we confirm Akt activation in our model of treadmill-exercised mice. Specifically, the levels of phosphorylated Akt (S473 and T308) and total Akt from gastrocnemius of WT mice were compared by Western blot (Fig. 2 *A–C*), showing a significant increase in phosphorylated Akt (0.48 ± 0.09 vs. 0.28 ± 0.01 AU, $n = 5$ each, $P < 0.05$ for S473; Fig. 2*E*; and 1.14 ± 0.02 vs. 0.68 ± 0.07 AU, $n = 5$ each, $P < 0.05$ for T308; Fig. 2*F*), but not total Akt (1.02 ± 0.05 vs. 1.21 ± 0.06 AU, $n = 5$ each, P = NS; Fig. 2*G*), in response to exercise vs. sedentary conditions. Akt is a known regulator of FOXO1 nuclear export (15, 26). Here, we find a significant increase in phosphorylated FOXO1 (1.473 ± 0.047 vs. 1.185 ± 0.056 AU, $n = 3$ each, $P < 0.05$), but not total FOXO1 (1.380 ± 0.046 vs. 1.337 ± 0.103 AU, $n = 3$ each, P = NS) normalized to GAPDH in gastrocnemius muscle from exercised vs. sedentary muscle (Fig. S1). Also, FOXO1 nuclear content quantification by Western blot (Fig. 2*D*) shows a dramatic reduction in response to exercise (0.16 ± 0.07 vs. 0.95 ± 0.29 AU, $n = 5$ each, $P < 0.05$; Fig. 2*H*). Because FOXO1 is known to inhibit musclin-encoding gene transcription in skeletal muscle (6), this exercise-related reduction in nuclear FOXO1 is consistent with our finding of increased musclin mRNA after exercise. Furthermore, we found no significant exercise-induced changes in musclin mRNA/hypoxanthine guanine phosphoribosyl transferase (HPRT) in Akt1-KO mice (0.47 ± 0.10 AU, $n = 5$ vs. 0.45 ± 0.06 AU, $n = 4$, P = NS).

This molecular cascade was verified in a cell culture model of primary skeletal myoblasts isolated from WT mice in which phosphorylation of Akt and FOXO1 were induced by application of a Ca^{2+} ionophore (A23187; Sigma Aldrich; Fig. 3 *A–C*; p-Akt/GAPDH 0.26 ± 0.008, $n = 2$ vs. 0.91 ± 0.05, $n = 4$, pFOXO1/GAPDH 0.12 ± 0.02, $n = 2$ vs. 0.39 ± 0.04, $n = 4$, pAkt/total Akt 0.21 ± 0.01, $n = 2$ vs. 0.66 ± 0.03, $n = 4$, pFOXO1/total FOXO1 0.07 ± 0.01, $n = 2$ vs. 0.27 ± 0.03, $n = 4$, for control and ionophore conditions, respectively, all in the presence of 1 mM Ca^{2+}; $P < 0.05$ for all comparisons). This activation of Akt by Ca^{2+} ionophore was paralleled by augmented musclin production (Fig. 3 *D* and *E*). Specifically, an increase in musclin mRNA was induced by Ca^{2+} ionophore in a dose-dependent manner (1.00 ± 0.08 AU no ionophore vs. 2.20 ± 0.06 AU for 0.5 μM ionophore vs. 3.60 ± 0.81 AU for 1 μM ionophore, all in presence of 1.0 mM Ca^{2+}, $n = 3$ each, $P < 0.05$ for each ionophore concentration vs. no ionophore or control; Fig. 3*D*)—a response that was eliminated when myoblasts were pretreated with Akt inhibitor-viii (Sigma-Aldrich; 1.09 ± 0.03 AU, $n = 3$, P = NS vs. control; Fig. 3*D*), or by removal of extracellular Ca^{2+} from the medium (0.96 ± 0.012 AU, $n = 3$ each, P = NS vs. control; Fig. 3*D*). The same musclin response to Ca^{2+} was

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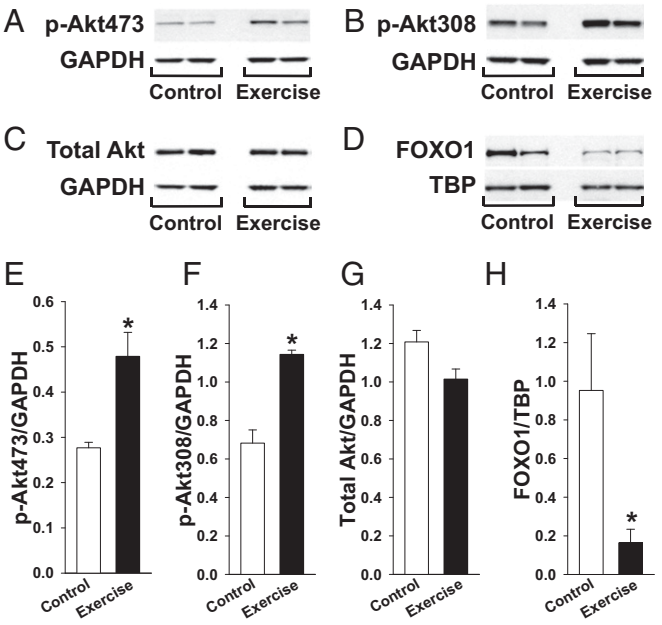

Fig. 2. Exercise promotes skeletal muscle Akt phosphorylation and FOXO1 nuclear export. Gastrocnemius of WT mice were assayed after 5 d of treadmill exercise vs. no exercise (control). (*B–D*) Representative Western blots of GAPDH and Akt phosphorylated at residue 473 (*A*), residue 308 (*B*), and total Akt (*C*) in muscle and representative Western blots of TBP and FOXO1 in nuclear extracts from muscle (*D*). (*E–H*) Summary statistics for expression of Akt phosphorylated at residue 473 (*E*), residue 308 (*F*), and total Akt normalized to GAPDH in muscle (*G*) and FOXO1 normalized to TBP in nuclear extracts from muscle by densitometry of Western blots (*H*). TBP, anti-TATA binding protein. **P* < 0.05 vs. control.

observed in a primary culture of myoblasts isolated from healthy human subjects (ZenBio; 1.0 ± 0.03 AU for no ionophore vs. 3.02 ± 0.23 AU for 0.5 μM ionophore vs. 3.95 ± 0.83 AU for 1 μM ionophore, all in presence of 1.0 mM Ca^{2+}, $n = 3$ each; $P <$ 0.05 for each ionophore concentration vs. no ionophore or control, values in Ca^{2+}-free buffer were 1.0 ± 0.05, 0.74 ± 0.18, and 0.79 ± 0.18 AU, respectively; $n = 3$ each, P = NS; Fig. 3*E*), confirming that this mechanism is not specific to mice. These findings all indicate that exercise-related musclin production is driven by the Ca^{2+}–Akt–FOXO1 signaling cascade.

Genetic Disruption of Musclin Production Causes Reduced Physical Endurance. To investigate the physiological significance and function of a physical-activity-induced increase in musclin production, we generated a mouse model with ubiquitous disruption of the musclin-encoding gene, *Ostn* (genOway) and confirmed the absence of musclin production in skeletal muscle of *Ostn*-KO mice vs. WT controls by Western blot (Fig. 4 *A* and *B*). The *Ostn*-KO mice, housed normally and fed standard chow, exhibited no skeletal deformities or differences in bone density and no growth abnormalities, blood pressure, or body composition changes (Fig. S2 and Table S1) compared with controls at 7–8 wk of age; however, they do demonstrate lower exercise tolerance than controls. Specifically, when challenged with a program of treadmill exercise with progressive increase in speed and incline (Fig. 4*C*), *Ostn*-KO mice demonstrate a significant deficit in exertional tolerance with respect to duration (71 ± 6 vs. 91 ± 6 min., $n = 6$ each, $P < 0.05$; Fig. 4*D*), distance (769 ± 102 vs. 1147 ± 121 m, $n = 6$ each, $P < 0.05$; Fig. 4*E*), and overall workload (34 ± 5 vs. 53 ± 4 J, $n = 6$ each, $P < 0.05$; Fig. 4*F*). Similarly, when mice were offered the opportunity for voluntary exercise on running wheels (Fig. 4*G*), *Ostn*-KO mice demonstrated significantly lower mean velocity (35 ± 4 vs. 52 ± 4 rotations per 5 min, $n = 6$ each, $P < 0.05$; Fig. 4*H*), duration (303 ± 38 vs. 383 ± 64 min., $n = 6$ each, $P < 0.05$; Fig. 4*I*), and distance (1,505 ± 231 vs. 2,218 ± 253 m, $n = 6$ each, $P < 0.05$; Fig. 4*J*) during the night when the vast majority of activity was recorded. To confirm that the observed phenotype is related to the absence of musclin in the systemic circulation, mice were implanted with osmotic pumps (Alzet Durect) loaded with saline or 50 μg of musclin. This dose resulted in musclin plasma levels of 69.7 ± 8.8 pg/mL ($n = 3$), comparable with levels observed in mice following exercise as presented in Fig. 1. Voluntary exercise on running wheels (Fig. 4*K*) was significantly increased in WT mice treated with musclin ($n = 5$) compared with WT mice treated with saline ($n = 4$), with respect to mean velocity (51 ± 7 vs. 16 ± 4 rotations per 5 min, $P < 0.05$; Fig. 4*L*), duration (336 ± 39 vs. 147 ± 10 min, $P < 0.05$; Fig. 4*M*), and distance (2,202 ± 280 vs. 696 ± 179 m, $P < 0.05$; Fig. 4*N*). Furthermore, treatment with musclin "rescued" the *Ostn-KO* mice ($n = 4$) because their exercise activity was equalized to that of musclin-treated WT mice ($n = 5$) in terms of night-time mean velocity (53 ± 15 vs. 51 ± 7 rotations per 5 min, P = NS; Fig. 4*L*), duration of running (333 ± 53 vs. 336 ± 39 min, P = NS; Fig. 4*M*), and distance run (2,290 ± 632 vs. 2,202 ± 280 m, P = NS; Fig. 4*N*). Thus, intact musclin production is critical for optimal exercise performance.

Musclin Boosts Activity-Related cGMP Production in Skeletal Muscle. To address the relationship between exercise, musclin, and ANP, we examined WT and *Ostn*-KO mice after exercise using the same protocol as in Fig. 1, which established a significant exercise-related musclin response in WT mice. We found a trend toward higher-plasma ANP levels in exercised WT ($n = 18$ mice in six groups) compared with *Ostn*-KO ($n = 15$ mice in five groups) mice, although it did not achieve statistical significance (140.4 ± 19.9 pg/mL vs. 98.2 ± 4.5, $P = 0.09$; Fig. 5*A*). Furthermore, when gastrocnemius muscles were assayed after

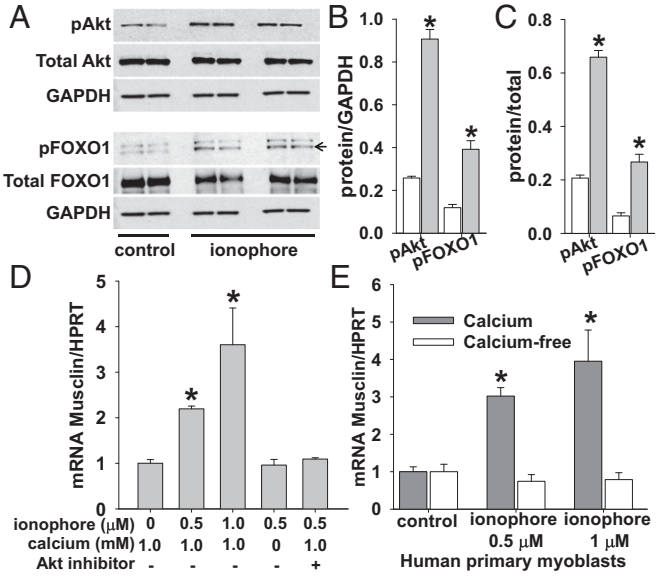

Fig. 3. Musclin production is stimulated by Ca^{2+}-dependent Akt phosphorylation. (*A*) Representative Western blots of Akt, FOXO1, and GAPDH from cultured murine primary myoblasts in 1 mM Ca^{2+} without ionophore (control) vs. with 1 μM ionophore A23187 (Sigma-Aldrich). (*B* and *C*) Summary statistics for phosphorylated Akt (pAkt) and phosphorylated FOXO1 (pFOXO1) normalized to GAPDH (*B*) and total Akt and total FOXO1 (*C*), respectively, with (gray) and without (white; control) 1 μM ionophore. **P* < 0.05 vs. control. (*D* and *E*) Summary statistics for musclin mRNA normalized to HPRT in murine cultured primary myoblasts exposed to various concentrations of ionophore, Ca^{2+} and Akt inhibitor-viii (*D*) and human cultured primary myoblasts exposed to no Ca^{2+} vs. 1.0 mM Ca^{2+} and various doses of ionophore, by densitometry of Western blots (*E*). **P* < 0.05 vs. no ionophore (*D*) or vs. Ca^{2+}-free (*E*).

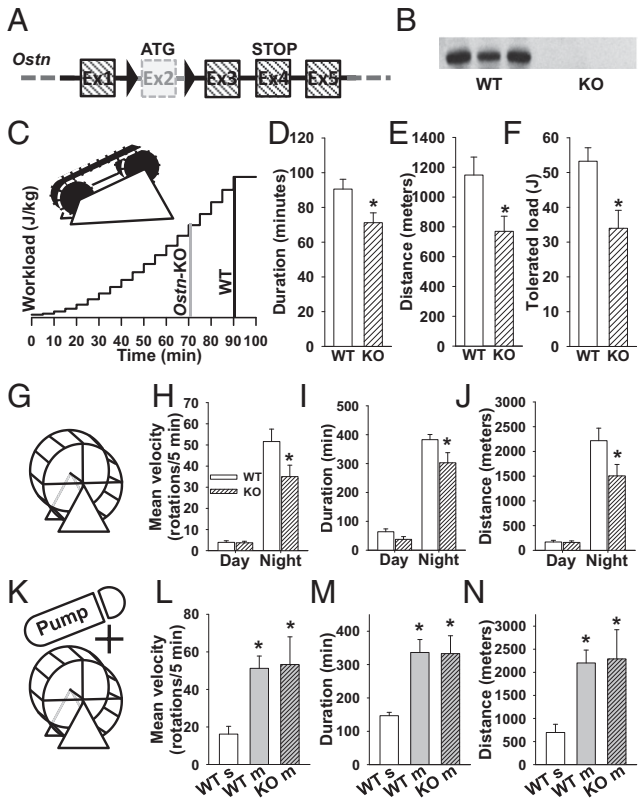

Fig. 4. Musclin supports physical performance. (*A*) Schematic of the modified *Ostn* gene indicating excision of the ATG-containing exon 2 to create the *Ostn*-KO mouse model. (*B*) Representative Western blot of musclin from gastrocnemius of WT and *Ostn*-KO mice. (*C*) Schematic of the treadmill exercise protocol. Vertical lines indicate mean time points of exercise dropout. (*D–F*) Summary statistics for treadmill exercise tolerance in terms of duration (*D*), distance (*E*), and tolerated workload: $E_k + E_p$ (*F*). *$P < 0.05$ *Ostn*-KO vs. WT. (*G*) Schematic of a running wheel. (*H–J*) Summary statistics for voluntary running-wheel exercise performance at day and night in terms of mean velocity (*H*), duration (*I*), and distance (*J*). *$P < 0.05$ *Ostn*-KO vs. WT. (*K*) Schematic of a running wheel and an osmotic pump loaded with musclin (m) or saline (s). (*L–N*) Summary statistics for voluntary running wheel performance at night in terms of mean velocity (*L*), duration (*M*), and distance (*N*). *$P < 0.05$ vs. WT saline. KO, *Ostn*-KO.

exercise, we detected significantly more cGMP in the muscle of WT compared with *Ostn*-KO mice (23.13 ± 0.88 fmol/mg vs. 20.62 ± 0.60 skeletal muscle tissue, $P < 0.05$; Fig. 5*B*). This ANP–musclin interaction with respect to cGMP signaling was further verified in a cell culture model. Specifically, we examined a skeletal myoblast culture exposed to various combinations and concentrations of these two peptides ($n = 3$ each; Fig. 5*C*). We found that, as expected, cGMP production was very low when no peptides were added (0.113 ± 0.003 fmol per μg of protein) or when musclin was added without ANP (0.104 ± 0.006 fmol/μg protein for 1 μM musclin and 0.117 ± 0.003 fmol/μg protein for 5 μM musclin; Fig. 5*C*). Also as expected, exposure to ANP resulted in a vigorous dose-dependent cGMP response (3.491 ± 0.057 fmol per μg of protein for 1 μM ANP and 6.046 ± 0.074 fmol per μg of protein for 5 μM ANP). Importantly, this response was augmented by the addition of musclin (6.840 ± 0.184 fmol per μg of protein for 5 μM ANP + 1 μM musclin, $P < 0.05$ vs. ANP 5 μM without musclin; Fig. 5*C*).

These data support a synergistic relationship between musclin and ANP and are consistent with the hypothesis that competition for NPR_C between musclin and ANP augments local ANP effects due to reduced clearance.

Normal Musclin Signaling Promotes Mitochondrial Biogenesis in Skeletal Muscle. NP/cGMP signaling is increasingly recognized as a key regulator of metabolic homeostasis, including effects on skeletal muscle mitochondrial biogenesis and oxidative phosphorylation potential (27–29). Maximal aerobic capacity ($\dot{V}O_{2max}$) is commonly used to estimate overall aerobic fitness based on cardiopulmonary function and oxidative phosphorylation potential (30). To determine whether musclin production impacts aerobic capacity as a potential mechanism underlying differences in exercise tolerance, we monitored exercise-trained *Ostn*-KO and WT mice on a metabolic treadmill equipped for indirect calorimetry (Columbus instruments). After 5 d of training, mice were placed on the stationary treadmill for 30 min and then were exposed to the protocol of escalating exercise workload to determine their $\dot{V}O_{2max}$. The resulting oxygen consumption recorded as a function of time reveals a significantly lower $\dot{V}O_2$ max for *Ostn*-KO compared with WT (7,629 ± 161 vs. 8,334 ± 212 mL/kg per h, $n = 5$ and 4, respectively, $P < 0.05$; Fig. 6*A*). Interestingly, after 3 wk of musclin infusion delivered via osmotic pumps (Alzet Durect), *Ostn*-KO and WT mice demonstrated comparable $\dot{V}O_{2max}$ (8,193 ± 100 vs. 8,034 ± 77 mL/kg per h, $n = 3$ and 4, respectively, P = NS; Fig. S3). These findings suggest musclin signaling may be tied to oxidative phosphorylation through mitochondrial density, size, or function. To assess this hypothesis, electron micrographs of longitudinal

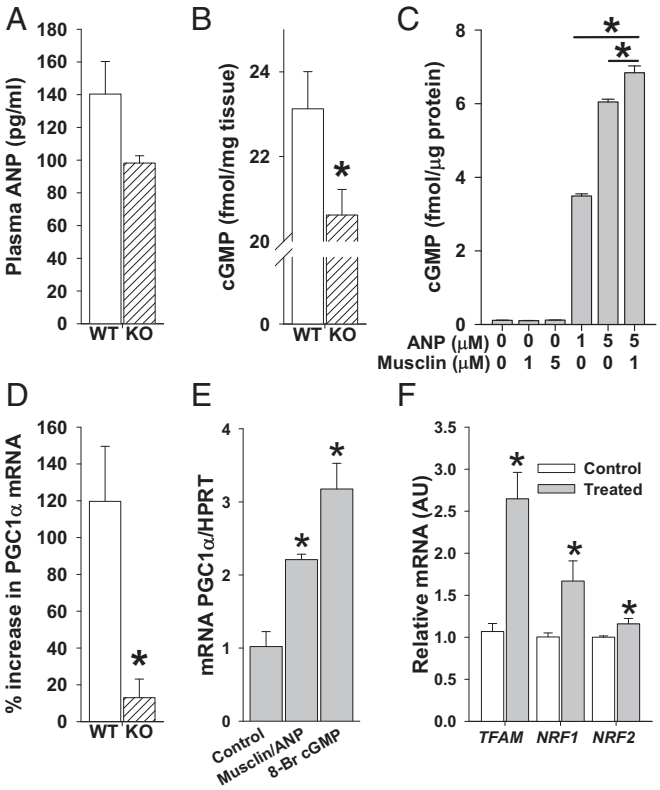

Fig. 5. Musclin augments ANP signaling in skeletal muscle. Mice were exercised on a treadmill for 5 d before undergoing assessment of ANP signaling. (*A*) Summary statistics for plasma ANP as assessed by ELISA. (*B*) Summary statistics for cGMP in gastrocnemius by enzyme immunoassay. *$P < 0.05$ *Ostn*-KO vs. WT. (*C*) Summary statistics for cGMP production in a culture of murine primary myoblasts exposed to various concentrations of ANP and musclin. *$P <$ 0.05 vs. columns indicated by bar. (*D*) Summary statistics for percent increase in peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1-α) mRNA over baseline in response to exercise in tibialis anterior by qRT-PCR. *$P <$ 0.05 vs. WT. (*E*) Summary statistics for primary myoblast culture mRNA of PGC1-α normalized to HPRT in response to musclin/ANP or to 8-Br-c-GMP. (*F*) Summary statistics for primary myoblast culture relative mRNA of *TFAM*, *NRF1*, and *NRF2* with and without musclin/ANP. KO, *Ostn*-KO.

sections through tibialis anterior of exercised mice were examined and appeared to show smaller mitochondrial size in the *Ostn*-KO compared with WT mice (Fig. 6*B*). This finding corresponds to a significantly lower mitochondrial protein content when normalized to the wet skeletal muscle weight (1.09 ± 0.04 vs. 1.33 ± 0.07 μg per mg of tissue, $n = 6$ each, $P < 0.05$; Fig. 6*C*), and respiratory complex expression (0.24 ± 0.02 vs. 0.31 ± 0.04 AU for CIII and 0.65 ± 0.08 vs. 0.91 ± 0.06 AU for CVI, $n = 3$ each, $P < 0.05$; Fig. 6 *D* and *E*) in gastrocnemius muscle homogenates, as well as succinate dehydrogenase (SDH) activity as assessed by percent of immunohistochemical staining in tibialis anterior cross-sections (43.33 ± 0.65 vs. 49.92 ± 2.09%, $n = 3$ each, $P < 0.05$; Fig. 6 *F* and *G*) from *Ostn*-KO vs. WT mice. Furthermore, disruption of normal musclin signaling appears to impact fiber type composition. Specifically, histologic examination of skeletal muscles from *Ostn*-KO vs. WT mice indicates a shift toward more pure glycolytic type IIb fibers in the KO (Fig. S4). Differences between sedentary WT and *Ostn*-KO mice were less marked (Fig. S5), consistent with the finding that exercise augments musclin signaling in the WT mice. Specifically, differences in oxygen consumption during treadmill testing ($n = 4$ each), mitochondrial content (0.83 ± 0.20, $n = 5$ vs. 0.71 ± 0.03 μg/mg tissue, $n = 6$, P = NS), and respiratory complex expression normalized to GAPDH in gastrocnemius muscle homogenates (0.64 ± 0.06 vs. 0.60 ± 0.02 for CII, 0.53 ± 0.04 vs. 0.57 ± 0.04 for CIII, 1.35 ± 0.08 vs. 1.36 ± 0.04 for CIV, 0.97 ± 0.07 vs. 0.86 ± 0.03 for CVI, $n = 4$ each, all P = NS) were not significantly different for sedentary WT *vs.* *Ostn*-KO mice, respectively, whereas SDH-positive staining of tibialis anterior cross-sections (45.33 ± 0.40 vs. 41.12 ± 1.24%, $n = 3$ each, $^*P < 0.05$) exhibited a smaller difference than that in exercise-trained WT *vs.* *Ostn*-KO mice. The pattern of distribution of fiber types from muscles of sedentary WT and *Ostn*-KO were similar to those of their exercise-trained counterparts, consistent with a slower adaptation of fiber type to exercise, except that there was a slightly more prominent component of type IIA fibers in exercised mice (Fig. S6; $n = 4$ each for tibialis anterior and $n = 3$ each for biceps femoris, $^*P < 0.05$).

To address the hypothesis that observed differences in skeletal muscle mitochondrial content of *Ostn*-KO vs. WT mice are driven by cGMP/peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1-α) signaling, we examined their tibialis anterior and found that, despite similar PGC1-α levels at baseline, (1.729 ± 0.44 vs. 1.125 ±0.1 AU, respectively, $n = 5$ and 7, P = NS) an exercise-related increase in PGC1-α over this baseline was significantly smaller in *Ostn*-KO than in WT mice (13.0 ± 0.1% vs. 119.7 ± 29.9% increase respectively, $n = 3$ each, $P < 0.05$; Fig. 5*D*). In a primary myoblast culture model, induction of cGMP by the combination of musclin and ANP (Fig. 5*C*) was associated with a significant increase in mRNA of PGC1-α (Fig. 5*E*) and its downstream targets linked to mitochondrial biogenesis, *TFAM*, *NRF1*, and *NRF2* (Fig. 5*F*).

These data indicate that exercise tolerance is influenced by musclin via an effect on ANP-dependent PGC1-α regulation of skeletal muscle activity-related mitochondrial biogenesis.

Discussion

This study establishes that production of the peptide musclin is up-regulated in skeletal muscle in response to physical activity and that musclin is secreted into the systemic circulation. Disruption of musclin signaling in *Ostn*-KO mice is associated with reduced oxidative phosphorylation potential and exercise tolerance that is corrected by musclin-replacement therapy. These findings indicate a previously unrecognized pathway for skeletal muscle metabolic adaptation to exercise.

Although we use forced treadmill exercise to demonstrate the responsiveness of musclin production and secretion to exercise, we found a phenotype of decreased endurance and trends toward lower mitochondrial content and higher presence of IIA glycolytic fibers, even in untrained *Ostn*-KO mice compared with WT controls. Importantly, these observed trends and changes in sedentary *Ostn*-KO became much more obvious and significant after exposure to the treadmill exercise protocol. We interpret these findings to indicate that musclin production and secretion, although more easily demonstrated in response to vigorous exercise, is physiologically relevant, even for routine daily activities.

Our data support the hypothesis that musclin modulates effects of cardiac NPs due to its ability to interfere with binding to NPR_C (8, 9). First, we demonstrate that musclin itself does not induce cGMP production in primary myoblasts, but, rather, potentiates ANP effects. Further, we confirm the significance of this signaling in vivo. Specifically, we demonstrate that WT mice have significantly higher muscle levels of cGMP after exposure to exercise compared with *Ostn*-KO mice. The higher level of muscle cGMP in WT *vs.* *Ostn*-KO mice is paralleled by a trend toward higher-plasma ANP. Elevation of ANP in plasma after physical activity has traditionally been linked to atrial wall stretch; however, our data suggest that increased production of musclin could also contribute to this phenomenon. Of note, it is possible that our measurement does not reach statistical significance due to significant variability of circulating ANP levels and the inability to use mice as their own controls, because the volume of plasma needed for testing precludes more than a single terminal blood draw per mouse.

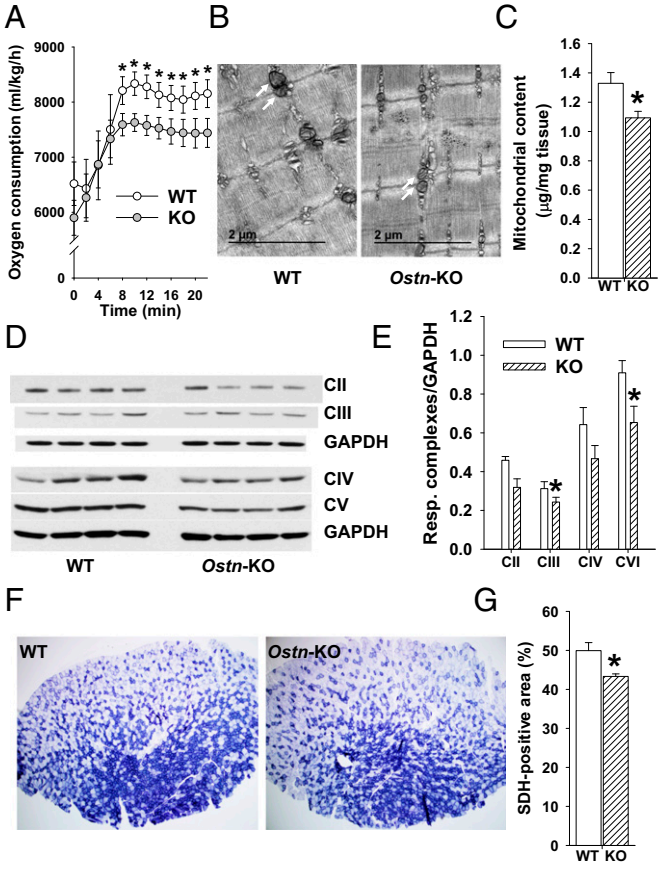

Fig. 6. Musclin signaling improves aerobic capacity and prompts mitochondrial biogenesis. (*A*) Summary statistics for trend of oxygen consumption over time of exercise-trained mice upon initiation of treadmill exercise at time 0. $^*P < 0.05$ *vs.* *Ostn*-KO. (*B*) Representative electron micrographs of longitudinal tibialis anterior sections from exercised mice. White arrows indicate mitochondria. (*C*) Summary statistics for mitochondrial content by weight in gastrocnemius isolates of exercised mice. $^*P < 0.05$ vs. WT. (*D* and *E*) Representative Western blots of respiratory chain enzymes and GAPDH (*D*) and summary statistics for respiratory complex expression normalized to GAPDH in gastrocnemius of exercise-trained mice (*E*). $^*P < 0.05$ vs. WT. (*F* and *G*) Representative stains for SDH activity of tibialis anterior cross-sections (*F*) and summary statistics for percent area of cross-sections stained for SDH activity (*G*) in exercise-trained mice. $^*P < 0.05$ vs. WT. KO, *Ostn*-KO.

We detected an order-of-magnitude lower production of musclin in the bone of our adult mice compared with that from skeletal muscle. This finding is consistent with original reports that indicate that high musclin expression during early bone development sharply declines in a time- and maturity-dependent manner in both mice (5) and humans (31). Furthermore, in contrast to skeletal muscle, no exercise-responsive increase in mRNA levels of musclin was observed in bones. Thus, it seems likely that the elevated systemic circulating musclin levels after exercise are largely supported by augmented production and secretion by skeletal muscle.

Identification of the exercise-responsive nature of musclin signaling, along with its systemic circulation, has important implications for our understanding of exercise-dependent NP signaling. Cardiac NPs are increasingly recognized as hormones with a wide spectrum of targets: In addition to traditional targets of vasculature and kidney, recently their effects on skeletal muscle mitochondrial biogenesis, angiogenesis, lipolysis, and adipose tissue remodeling (browning) have been reported (27–29, 32). Although the present study focuses on one aspect of this signaling network, intramuscular cGMP signaling and mitochondrial biogenesis, it is possible that other cardiac NP signaling targets are similarly affected. For example, musclin may be at least partially responsible for the beneficial effect of exercise on cardiac remodeling. Such targets should be the subject of future investigation.

cGMP signaling has been linked to PGC1-α–dependent mitochondrial biogenesis in many studies in different tissues and organs (33–35). cGMP production in skeletal muscles is typically linked to nitric oxide signaling (34–36), although recently a role for NPs in this process has been established (28). Our data support the importance NP signaling and its regulation by musclin in cGMP/PGC1-α–driven mitochondrial biogenesis: We confirm significantly greater mitochondrial quantity and function by multiple methods and demonstrate the in vivo functional importance by revealing a meaningful increase in the $\dot{V}O_{2max}$, a parameter that reflects many factors, including oxidative phosphorylation potential and cardiovascular and pulmonary functions critical for physical endurance, of mice with intact vs. disrupted musclin signaling.

Finally, our demonstration that musclin infusion rescues exercise and oxidative capacity in *Ostn*-KO mice, as well as enhances exercise and oxidative capacity in untrained WT mice, suggests a potential therapeutic role for musclin. Overexpression of musclin in chondrocytes has been linked to abnormal skeletal growth (9), but such changes may or may not occur with systemically delivered musclin. Thus, to better understand musclin's therapeutic potential, further studies will be required to determine the extent and durability of beneficial effects and whether there are accompanying long-term deleterious consequences. Such studies may be aided by conditional models.

In summary, this study defines musclin as an exercise-responsive factor promoting skeletal muscle mitochondrial biogenesis and exercise endurance.

Materials and Methods

All animal protocols conform to the *Guide for the Care and Use of Laboratory Animals* (37) generated by the Institute for Laboratory Animal Research, National Research Council of the National Academies. All animal protocols were approved by the University of Iowa Institutional Animal Care and Use Committee. See *SI Materials and Methods* for detailed methods. Results are expressed as mean ± SEM.

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