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Disruption of ATP-sensitive potassium channel function in skeletal muscles promotes production and secretion of musclin

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Abstract

Sarcolemmal ATP-sensitive potassium (K_{ATP}) channels control skeletal muscle energy use through their ability to adjust membrane excitability and related cell functions in accordance with cellular metabolic status. Mice with disrupted skeletal muscle K_{ATP} channels exhibit reduced adipocyte size and increased fatty acid release into the circulation. As yet, the molecular mechanisms underlying this link between skeletal muscle K_{ATP} channel function and adipose mobilization have not been established. Here, we demonstrate that skeletal muscle-specific disruption of K_{ATP} channel function in transgenic (TG) mice promotes production and secretion of musclin. Musclin is a myokine with high homology to atrial natriuretic peptide (ANP) that enhances ANP signaling by competing for elimination. Augmented musclin production in TG mice is driven by a molecular cascade resulting in enhanced acetylation and nuclear exclusion of the transcription factor forkhead box O1 (FOXO1) – an inhibitor of transcription of the musclin encoding gene. Musclin production/secretion in TG is paired with increased mobilization of fatty acids and a clear trend

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toward increased circulating ANP, an activator of lipolysis. These data establish K_{ATP} channel-dependent musclin production as a potential mechanistic link coupling “local” skeletal muscle energy consumption with mobilization of bodily resources from fat. Understanding such mechanisms is an important step toward designing interventions to manage metabolic disorders including those related to excess body fat and associated co-morbidities.

Keywords

action potential; calcium; natriuretic peptide; fatty acid; endurance

Introduction

Skeletal muscles account for about 40–50% of body mass and are major consumers of bodily energy. Recently we established that skeletal muscle energy efficiency is regulated by ATP-sensitive potassium (K_{ATP}) channels [1,2,3]. K_{ATP} channels control energy use through their ability to adjust membrane excitability and related cell functions in accordance with the metabolic status of the cell [1,2,3,4,5,6,7,8,9,10]. Specifically, sarcolemmal K_{ATP} channel opening limits skeletal muscle energy consumption, not only in response to high metabolic stress [5,6,7,8,9,10], but also in response to metabolic changes associated with moderate activity [1,2,3]. This function balances energy use and availability by preventing excessive muscle utilization of cellular energy resources, thereby enhancing energy efficiency and endurance [1,2,3]. Conversely, disruption of K_{ATP} channel function forces an increased cost of skeletal muscle performance, resulting in greater heat production, lower endurance and a lean phenotype [1,2,3]. Furthermore, mice with disrupted K_{ATP} channel function exhibit reduced adipocyte size and increased release of fatty acids into the circulation [2,3]. As yet, the molecular mechanisms underlying this link between skeletal muscle K_{ATP} channel function and adipose tissue mobilization have not been established.

Traditionally physical activity-related lipolysis has been linked to sympathetic activation [11,12]. However, recently, adipose tissue metabolic regulation was also attributed to the action of atrial natriuretic peptide (ANP), a hormone secreted predominantly by atrial myocytes at increased rates in response to hemodynamic and metabolic stress including exercise [13,14,15]. The biologic activity of ANP is mainly through natriuretic peptide receptor A (NPR_A) which has an intracellular guanylyl cyclase catalytic domain [13]. Binding of ANP to NPR_A activates guanylyl cyclase leading to increased intracellular cGMP [13]. ANP-dependent cGMP elevations linked to lipolysis were demonstrated both in isolated fat cells and *in vivo* [13,15,16,17,18,19]. Elimination of ANP from the plasma occurs mainly through binding the natriuretic peptide clearance receptor, NPR_C , resulting in internalization and degradation of ANP [14]. It was recently demonstrated that ANP levels are affected by competition between ANP and the structurally related signaling peptide secreted by skeletal muscle, musclin (AKA osteocrin), for binding to NPR_C [20,21,22,23]. Musclin binds to NPR_C with affinity comparable to NPs, but exhibits only weak binding to NPR_A and NPR_B and no activation of the linked guanylyl cyclase that is the primary effector of traditional NP physiologic actions [20]. Importantly, upregulation of ANP synthesis and secretion by atrial cardiomyocytes has been linked to cardiac K_{ATP} channel dysfunction

[24,25]. Whether there is a similar connection between skeletal muscle K_{ATP} channels and musclin production is unknown.

Here we establish that disruption of K_{ATP} channel function promotes production and secretion of musclin by skeletal muscles. This occurs in the setting of a molecular cascade that results in acetylation and nuclear exclusion of the transcriptional factor forkhead box O1 (FOXO1), known to inhibit musclin gene transcription [23,26]. Increased skeletal muscle musclin production and secretion in the presence of K_{ATP} channel deficit is associated with increased plasma ANP and fatty acid levels following physical activity. These data establish K_{ATP} channel-dependent musclin production and its effects on ANP signaling as a potential mechanistic link between skeletal muscle energy consumption and mobilization of fuel resources from fat. Understanding such molecular mechanisms is an important step toward designing interventions to manage metabolic disorders including those related to excess body fat and associated co-morbidities.

Materials and Methods

Mouse models

All animal protocols conform to the Guide for the Care and Use of Laboratory Animals generated by the Institute for Laboratory Animal Research, National Research Council of the National Academies, and are approved by the University of Iowa Institutional Animal Care and Use Committee.

Mice of either gender, aged 10–12 weeks, are used. The transgenic Tg[MyoD-Kir6.1AAA] model (TG) exhibits skeletal muscle-specific expression of the dominant-negative mutant pore-forming K_{ATP} channel subunit Kir6.1AAA [3]. TG mice are compared to green fluorescence protein-expressing controls (GFP) and wildtype (WT) mice of the same age and background [3]. Mice are fasted for 6 hours prior to exercise testing and blood/tissue collection.

For invasive procedures mice are anesthetized with isoflurane (5% induction, ~1.0–1.5% maintenance, Piramal Healthcare, Andhra Pradesh, India) *via* a nose-cone to maintain a respiratory rate ~50–60 breaths per minute.

Exercise—A multi-lane treadmill (Columbus Instruments, Columbus, OH) is used to simultaneously exercise model mice and controls. Mice are acclimated on the treadmill 20 min/day for 3 days at 3.5 m/min and 15° inclination. Mice are then exercised once at 10 m/min and inclination of 15° for 45 min immediately before sample (blood, tissue) collection.

Molecular Biology

RNA isolation, quantitative real time polymerase chain reaction (qRT-PCR), nuclear and whole cell protein extraction and western blot are performed as previously described [23]. Antibodies to HDAC4 are obtained from Cell Signaling, Danvers, MA.

Histology of skeletal muscles is as previously described [23].

Musclin and non-esterified fatty acids (NEFA) are measured as described [3,23]. ANP was measured by ELISA using a commercially available kit according to manufacturer instructions (RayBiotech, Norcross, GA).

Statistics

Results are expressed as mean±SEM—Comparisons between two groups are made using the 2-sided Student's t-test and between more than two groups using analysis of variance (ANOVA). A *p* value <.05 is considered significant. Sigma Plot 11 is used for all statistical analyses.

Results

Disruption of K_{ATP} channel function in skeletal muscles promotes musclin production

Stretch-dependent production of ANP by atria in the heart has been demonstrated to be inhibited by the activity of sarcolemmal K_{ATP} channels [24,25]. Here, we test whether skeletal muscle production of musclin is also under control of K_{ATP} channels. Mice with skeletal muscle-specific expression of a dominant-negative K_{ATP} channel pore-forming subunit (TG), which are previously confirmed to have absence of skeletal muscle sarcolemmal K_{ATP} channel activity [2,3], were compared to GFP-expressing littermates (GFP) and age matched wildtype (WT) controls, both of which have normal skeletal muscle K_{ATP} channel activity [2,3]. Previous characterization of the TG model indicates no changes in fiber type composition but a loss of skeletal muscle membrane/action potential adaptation to exercise with increased calcium release compared to controls [2]. These differences result in excess muscle caloric consumption from contraction and ion re-sequestration and a leaner phenotype compared to controls [2,3]. Here, we find that expression of musclin is significantly increased in limb muscles of TG mice, compared to GFP and WT, when assayed by western blot (1.44±.14, 1.48±.09, 2.05±.18 AU, n=5, 5, and 4 for TG, GFP and WT respectively, *p*<.05 for TG vs. GFP or WT, Fig. 1A, C), immunohistochemistry (28.5±1.73, 26.40±1.51, 53.45±2.46 AU, n=14 each for TG, GFP and WT respectively, *p*<.05 for TG vs. GFP or WT, Fig. 1B), or PCR for mRNA (1±.09, .89±.17, 2.63±.55 AU, n=6, 3, and 6 for TG, GFP and WT respectively, *p*<.05 for TG vs. GFP or WT Fig. 1E).

Disruption of K_{ATP} channel function relieves FOXO1 inhibition of musclin transcription

Regulation of musclin transcription has been linked to FOXO1 activity [21,23,27]. As demonstrated in cell culture and *in vivo*, nuclear export of FOXO1 releases transcription of the musclin gene from inhibition [23,26]. Here, we demonstrate that that disruption of K_{ATP} channel function is coupled to reduced FOXO1 in nuclei of skeletal muscles (Fig. 2A, D; .65±.05, .88±.12, .39±.06 AU, n= 5, 4, and 5 for WT, GFP and TG respectively, *p*<.05 for TG vs. WT or GFP). FOXO1 export from nuclei occurs with phosphorylation by AKT or through acetylation [26]. In K_{ATP} channel deficient skeletal muscles of TG mice, total FOXO1 is not different from controls (Fig. 2B, E; .72±.03, .69±.05, .67±.02 AU, for WT, GFP and TG respectively, n=4 each, *p*=NS for all comparisons), however acetylated FOXO1 is significantly increased (Fig. 2C, F; 1.37±.09, 1.16±.149, 2.07±.13 AU, n=12, 8 and 8 for WT, GFP and TG respectively, *p*<.05 for TG vs. WT or GFP). This shift to greater acetylation and nuclear export of FOXO1 in skeletal muscles of TG mice is paralleled by

decreased nuclear content of histone deacetylase 4 (HDAC4, Fig. 3A, D; $1.35 \pm .12$, $1.61 \pm .16$, $.87 \pm .08$ for WT, GFP and TG respectively, $n=4$ each, $p<.05$ for TG vs. WT or GFP) shown to diminish HDAC3-mediated deacetylation and activation of FOXO1 [28]. The nuclear export of FOXO1 in TG is also paralleled by an elevated presence of CaMKII activated by phosphorylation (p-CaMKII), a negative regulator of HDAC4 nuclear localization [29]. Specifically, phosphorylated CaMKII (p-CaMKII), but not total CaMKII (t-CaMKII) is significantly increased in nuclear extracts of limb muscle from TG compared to controls (p-CaMKII; Fig. 3B, E; $1.41 \pm .04$, $1.61 \pm .12$, $2.36 \pm .24$ for WT, GFP and TG respectively, $n=4$ each, $p<.05$ for TG vs. WT or GFP, t-CaMKII; Fig. 3C, F; $3.41 \pm .08$, $3.51 \pm .20$, $3.65 \pm .06$ for WT, GFP and TG respectively, $n=4$ each, $p=NS$ for all comparisons). These findings are consistent with prior studies indicating that K_{ATP} channel-deficient cardiac and skeletal myocytes, including those generated using the Kir6.1AAA transgene expressed in this study, have increased contractile calcium release and calcium loading [1,2,3,4,5,6,30,31,32].

Increased musclin production by K_{ATP} channel-deficient skeletal muscles is associated with greater mobilization of fatty acids

Mice with K_{ATP} channel-deficient skeletal muscles have increased energy expenditure [2,3] and therefore require greater energy resources to maintain muscle function. Musclin has been previously shown to regulate plasma ANP – a potent stimulator of lipolysis, particularly related to physical activity [15,16,18,19,20,23]. Here, after a single episode of low-intensity exercise, TG mice compared to controls display higher circulating levels of musclin (12.83 ± 1.42 $n=9$, 6.96 ± 1.06 $n=7$, 6.60 ± 2.08 pg/ml $n=4$, for TG, WT and GFP, respectively, $p<.05$ for TG vs. WT or GFP; Fig. 4A) and a clear trend toward elevated circulating ANP (60.70 ± 9.25 $n=5$, 36.36 ± 6.83 $n=5$, 40.57 ± 4.69 pg/ml $n=6$ for TG, WT and GFP, respectively, $p=.07$ for TG vs. GFP and $p=.07$ for TG vs. WT; Fig. 4B). When circulating ANP levels in TG mice are compared with combined controls, the difference is statistically significant (60.70 ± 9.25 $n=5$ vs. 38.66 ± 3.86 $n=11$, $p=.02$). These increases in musclin and ANP are associated with greater serum levels of non-esterified fatty acids (NEFA). Specifically, NEFA is greater in TG ($.82 \pm .07$ mmol/ml, $n=6$) compared with WT ($.51 \pm .05$, $n=6$, $p<.05$ vs. TG) and GFP controls ($.57 \pm .04$ mmol/ml, $n=4$, $p<.05$ vs. TG; Fig. 4C).

Discussion

K_{ATP} channels are membrane protein complexes vital for preservation of cellular energy balance under normal and disease conditions [1,2,3,5,8,10,24,25,30,31,32]. Here we use tissue-specific transgenic expression of a dominant-negative K_{ATP} channel subunit to selectively eliminate skeletal muscle K_{ATP} channel current. This intervention is associated with a blunted myofiber membrane/action potential adaptation to muscle contractions that results in increased intracellular calcium release [2]. We previously demonstrated that a calcium-dependent mechanism, protein kinase B (AKT) phosphorylation of FOXO1, underlies skeletal muscle exercise-dependent production of musclin [23]. In the current study, there are no significant differences in AKT phosphorylation between control and TG mice (data not shown). This could be due an actual absence of a role for AKT in increased

musclin production by TG mice, or to the short-lived nature of AKT phosphorylation such that the window for detecting significant differences is missed when collecting tissue at a single time point. However, another calcium-dependent mechanism for production of musclin is evidenced by CaMKII activation that can increase FOXO1 nuclear export by promoting its acetylation. This signaling cascade represents a previously unrecognized pathway for regulation of musclin transcription. We expect that the calcium homeostatic regulatory property of skeletal muscle K_{ATP} channels is an important link between muscle energetics and musclin production. Other mechanisms that regulate cellular calcium, including mitochondrial function, could also have significant effects.

Our previous work indicates that musclin production is associated with improved physical performance and enhanced mitochondrial biogenesis [23]. However, the TG model used here, despite its increased musclin production, has been characterized by decreased muscle energy efficiency and reduced exercise tolerance [2,3]. We interpret this to indicate that higher musclin production and secretion in TG functions to partially compensate for loss of K_{ATP} channel dependent control of myofiber energetics. For instance greater musclin production, with consequent mitochondrial biogenesis and adipose mobilization, may serve to generate the increased energy needed to support the function of calorically wasteful muscle [2,3].

The TG mouse model has also been previously demonstrated to manifest with lower body weight, reduced adipocyte size, and greater lipolysis [2,3]. Given the pattern of musclin, ANP and serum NEFA in TG compared with controls, the current study supports the hypothesis that musclin enhances ANP-driven lipolysis as a mechanism for fatty acid mobilization. Specifically, we hypothesize that increased musclin and ANP in the TG are related, due to reduced clearance of ANP as a result of musclin competing for binding to the NP clearance receptor [20,33]. Binding of ANP to NPR_A activates guanylyl cyclase leading to increased intracellular cGMP [13]. ANP-dependent cGMP elevations and lipolysis were demonstrated both in isolated fat cells and *in vivo* [15,16,18,19,34]. However, it is also possible that a musclin-induced rise in ANP stimulates lipolysis through a cGMP independent pathway. For instance, *in vivo* the vasodilatory and natriuretic properties of ANP favor sympathetic activation that could then result in fat mobilization [11,12,13]. Furthermore, it has been postulated that NPR_C could have its own signaling function [35] such that musclin binding to NPR_C may have a more direct effect on lipolysis than by boosting ANP levels. Further dissection of downstream lipolytic mechanisms promoted by musclin will be the aim of future studies.

In summary, data presented here introduce a mechanistic link between skeletal muscle energy consumption, lean phenotype, and adipose mobilization in a mouse model of skeletal muscle K_{ATP} channel deficit. More importantly, these data suggest a role for normal function of skeletal muscle sarcolemmal K_{ATP} channels not only in regulation of muscle energy consumption, as shown previously, but also as important regulators of a biochemical cascade responsible for delivery of nutrients to muscles through adipose mobilization. Examination of these mechanisms could uncover novel therapeutic targets for diseases of muscle and fat metabolism.

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Abbreviations

AKT	protein kinase B
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
CaMKII	calcium/calmodulin-dependent protein kinase II
FOXO1	forkhead box O1 transcription factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescence protein
HDAC	histone deacetylase
K_{ATP}	ATP-sensitive potassium
I_{KATP}	K _{ATP} channel current
NEFA	non-esterified fatty acids
NPR	natriuretic peptide receptor
TBP	TATA-binding protein
TG	transgenic
WT	wild-type

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Highlights

- ATP-sensitive K⁺ channels regulate musclin production by skeletal muscles
- Lipolytic ANP signaling is promoted by augmented skeletal muscle musclin production
- Skeletal muscle musclin transcription is promoted by a CaMKII/HDAC/FOXO1 pathway
- Musclin links adipose mobilization to energy use in K_{ATP} channel deficient skeletal muscle

Figure 1. Expression of musclin is increased in K_{ATP} channel deficient skeletal muscle

A) Representative western blots of musclin vs. GAPDH obtained from whole cell protein extracts of *gastrocnemius* muscle from WT, GFP and TG mice. **B)** Representative cross-sections of *gastrocnemius* muscle from WT, GFP and TG mice with labeling by immunofluorescent antibodies against musclin (red) and DAPI (blue). Summary statistics for musclin expression of WT, GFP and TG as probed by **C)** western blot of musclin protein normalized to GAPDH in whole cell protein extracts of *gastrocnemius* muscle, **D)** quantification of fluorescence by immunocytochemistry of musclin protein in muscle cross

sections of *gastrocnemius* muscle and **E**) PCR of musclin mRNA from *tibialis anterior* normalized to WT level (* $p < .05$). DAPI: 4',6-diamidino-2-phenylindole.

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Figure 2. Acetylation and nuclear exclusion of FOXO1 are increased in K_{ATP} channel deficient skeletal muscle

A) Representative western blots of FOXO1 vs. TBP in nuclear isolates from *gastrocnemius* muscle of WT, GFP and TG mice. **B)** Representative western blot of total FOXO1 vs. GAPDH and **C)** representative immunoprecipitation (IP) of FOXO1 with blot against acetylated lysine (Acetyl-Lys) in whole cell extracts of *gastrocnemius* muscle from WT, GFP and TG mice. Summary statistics of **D)** optical density of FOXO1 normalized to TBP in nuclear extracts from *gastrocnemius* muscle of WT, GFP and TG mice, **E)** optical density

of total FOXO1 normalized to GAPDH from western blots in whole cell protein extracts and **F)** immunoprecipitation of FOXO1 with blot against Acetyl-Lys in nuclear extracts (* $p < .05$).

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Figure 3. Phosphorylated CaMKII is increased and HDAC4 decreased in nuclei of K_{ATP} channel deficient skeletal muscles

Representative western blots of **A)** HDAC4, **B)** phosphorylated CaMKII (p-CaMKII) and **C)** total CaMKII (t-CaMKII) vs. TBP in nuclei of *gastrocnemius* muscles from WT, GFP and TG mice. Summary statistics for optical density of **D)** HDAC4, **E)** p-CaMKII and **F)** t-CaMKII normalized to optical density of TBP on western blots from nuclear extracts of *gastrocnemius* muscle of WT, GFP and TG mice (* $p < .05$).

Figure 4. Mice with absence of functional skeletal muscle sarcolemmal K_{ATP} channels exhibit increased serum NEFA following exercise

Summary statistics for **A)** plasma musclin, **B)** plasma ANP and **C)** serum NEFA measured after exercise in WT, GFP, and TG mice (* $p < .05$).