Skeletal Muscle Specific G Protein-Coupled Receptor Kinase 2 Ablation Alters Isolated Skeletal Muscle Mechanics and Enhances Clenbuterol-Stimulated Hypertrophy

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Running title: GRK2 and skeletal muscle physiology

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ABSTRACT

GRK2, a G protein-coupled receptor kinase, plays a critical role in cardiac physiology. Adrenergic receptors are the primary target for GRK2 activity in the heart; phosphorylation by GRK2 leads to desensitization of these receptors. As such, levels of GRK2 activity in the heart directly correlate with cardiac contractile function. Furthermore, increased expression of GRK2 following cardiac insult exacerbates injury and speeds progression to heart failure. Despite the importance of this kinase in both the physiology and pathophysiology of the heart, relatively little is known about the role of GRK2 in skeletal muscle function and disease. In this study we generated a novel skeletal muscle specific GRK2 knockout (KO) mouse (MLC-Cre:GRK2fl/fl) to gain a better understanding of the role of GRK2 in skeletal muscle physiology. In isolated muscle mechanics testing, GRK2 ablation caused a significant decrease in the specific force of contraction of the fast-twitch extensor digitorum longus muscle, yet had no effect on the slow-twitch soleus muscle. Despite these effects in isolated muscle, exercise capacity was not altered in MLC-Cre:GRK2fl/fl mice compared to wild-type controls. Skeletal muscle hypertrophy stimulated by clenbuterol, a β2-adrenergic receptor (β2AR) agonist, was significantly enhanced in MLC-Cre:GRK2fl/fl mice; mechanistically, this seems to be due to increased clenbuterol-stimulated pro-hypertrophic Akt signaling in the GRK2 KO skeletal muscle. In summary, our study provides the first insights into the role of GRK2 in skeletal muscle physiology, and points to a role for GRK2 as a modulator of contractile properties in skeletal muscle as well as β2AR-induced hypertrophy.

G protein-coupled receptors (GPCRs) comprise the largest family of membrane proteins in the genome. GPCRs regulate diverse processes throughout the body by responding to a vast array of extracellular stimuli including hormones, neurotransmitters and photons of light (1). Equally important to the stimulation and activation of GPCRs is the desensitization and ‘shutting-off’ the receptor. This task is primarily conducted by the GPCR kinase (GRK) family of proteins (2,3). GRK2 is ubiquitously expressed throughout the tissues of the body, perhaps most notably in the heart where its regulation of adrenergic receptors (ARs) is critical to physiological heart function. Cardiac overexpression of GRK2 in mice suppresses contractility, whereas cardiac overexpression of the GRK2 inhibitor βARKct (a C-terminal peptide that competes with GRK2 binding to Gβγ) enhances contractile function (4). Catecholamine overdrive during heart failure drives increased GRK2 expression in the cardiomyocyte, ultimately leading to excessive desensitization of βARs, loss of receptor density...
and a drop in inotropic reserve (5-7). Indeed, myocardial inhibition or deletion of GRK2 can prevent and even reverse heart failure in numerous animal models (4,8-13).

While we have a firm understanding of the role of GRK2 in the physiology and pathophysiology of the heart, relatively little is known about the function of this kinase in skeletal muscle. Many of the prominent effects of GRK2 in the heart are mediated by regulation of the $\beta_2$AR (2,14). This receptor is also expressed in skeletal muscle, and modulates various aspects of skeletal muscle physiology. $\beta$AR agonists have long been known to induce hypertrophy of skeletal muscle and have been studied as a potential therapeutic for muscle wasting diseases (15). In particular, clenbuterol administration has been shown to induce skeletal muscle hypertrophy via a $\beta_2$AR dependent mechanism (16). In addition, as is the case in the heart, $\beta_2$AR agonists can modulate the contractile properties of skeletal muscle (17).

Given the aforementioned roles of the $\beta_2$AR in skeletal muscle, and the fact that GRK2 is a regulator of the $\beta_2$AR we sought to determine the genetic requirement for GRK2 in skeletal muscle physiology. Specifically, using skeletal muscle specific GRK2 knock-out (KO) mice, we assessed exercise performance and contractile properties of isolated muscles. Furthermore we investigated whether GRK2 ablation in skeletal muscle would enhance the pro-hypertrophic effects of a $\beta_2$AR agonist.

RESULTS

Generation of a skeletal muscle specific GRK2 KO mouse – In order to study the role of GRK2 in skeletal muscle function and contractility we generated a skeletal muscle specific GRK2 KO mouse by crossing mice with loxP sites flanking exons 3-6 of GRK2 (GRK2 fl/fl) (18) with MLC-Cre mice. The MLC-Cre mice express Cre-recombinase under control of the myosin light chain 1f (MLC1f) promoter (19). In agreement with others, we find the greatest levels of target gene knockdown in the gastrocnemius and EDL muscles (~83% and 82% knockdown of GRK2 respectively), both of which contain a high proportion of type II slow fibers (Fig. 1A and 1B) (20,21). In contrast, the soleus muscle, which contains fewer type II fibers and a higher proportion of type I slow fibers showed around 65% GRK2 knockdown, reflecting lower MLC1f promoter activity in this muscle (Fig. 1C). To convince ourselves of the skeletal muscle specificity of the MLC-Cre:GRK2 fl/fl mice, we assessed GRK2 levels in heart tissue lysate by Western blot and found no change with respect to GRK2 fl/fl animals (Fig. 1D).

Skeletal muscle GRK2 knockdown does not impair exercise performance – GRK2 activity levels in the heart correspond directly with contractile function (4). To assess whether GRK2 ablation in the skeletal muscle has a direct functional impact we challenged GRK2 fl/fl and MLC-Cre:GRK2 fl/fl mice with involuntary treadmill running to exhaustion. Following a weeklong acclimatization period, mice were run using a protocol of increasing speed over time until they were unable to continue despite receiving a mild electrical shock from a platform at the rear of the treadmill. Using this protocol we found no difference in time taken to reach exhaustion between GRK2 fl/fl and MLC-Cre:GRK2 fl/fl mice (13.33 minutes and 12.52 minutes respectively) (Fig. 2A). Likewise we found no difference in the maximum speed attained during the treadmill protocol with GRK2 fl/fl mice reaching 25.56 m/min and MLC-Cre:GRK2 fl/fl mice reaching an average of 24.0 m/min (Fig. 2B). The average total distance run was also calculated for both groups and again we observe no difference between GRK2 fl/fl and MLC-Cre:GRK2 fl/fl mice, covering 267.84m and 250.39m respectively (Fig. 2C). Finally, we also kept tally of the number of times each mouse engaged the shock grid at the rear of the treadmill and once again found no difference between groups with GRK2 fl/fl mice engaging the grid an average of 2.75 times per minute and MLC-Cre:GRK2 fl/fl mice averaging 2.89 times per minute (Fig. 2D). In conclusion we find no effect of muscle GRK2 ablation on exercise capacity in four separate parameters of treadmill performance.

Skeletal muscle GRK2 knockdown differentially modulates ex vivo mechanics of isolated soleus and EDL muscles – To obtain a more detailed characterization of skeletal muscle function in the absence of GRK2, we next studied the ex vivo mechanics of soleus and EDL muscles isolated from GRK2 fl/fl and MLC-Cre:GRK2 fl/fl mice. Whole muscle mechanical measurements

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Skeletal muscle GRK2 knockdown does not alter Ca2+ transients in isolated myotubes – In the heart, βAR signaling modulates sarcolemmal calcium regulation, which, in turn, influences inotropy and chronotropy. We therefore next asked whether deletion of GRK2, a key regulator of βARs, could alter Ca2+ transients in skeletal muscle, and thus explain the altered contractility observed in GRK2 ablated EDL muscle (Fig. 3D). We isolated single skeletal muscle myotubes via enzymatic digestion of the fast-twitch flexor digitorum brevis (FDB) muscle. Myotubes, loaded with the fluorescent Ca2+ indicator Fluo-4, were electrically paced at 0.2Hz, and Cytosolic Ca2+ transients were assessed at baseline and following isoproterenol stimulation (100nM, 5 min), via fluorescence microscopy.

Baseline Ca2+ transients from GRK2fl/fl and MLC-Cre:GRK2fl/fl myotubes produced almost identical traces (Fig. 5A and B). Isoproterenol stimulation caused a significant increase in Ca2+ transient amplitude in both GRK2fl/fl and MLC-Cre:GRK2fl/fl myotubes, compared to their respective non-stimulated control groups (Fig. 5A-C). We found no statistical significance between the Ca2+ transient amplitudes of the isoproterenol treated groups, however, MLC-Cre:GRK2fl/fl myotubes trended higher than GRK2fl/fl. Further analysis found no significant difference between groups in the time to peak (Fig. 5D), or in the time rate of decay of Ca2+ transients (Fig. 5E). From these results, we conclude that neither baseline nor isoproterenol pretreated Ca2+ transients are significantly influenced by GRK2 deletion in skeletal muscle myotubes.

Clenbuterol stimulated skeletal muscle hypertrophy is enhanced by skeletal muscle specific GRK2 knockdown – Previous studies have identified clenbuterol administration as an effective way to stimulate muscle hypertrophy and inhibit atrophy (15). Furthermore, this effect of clenbuterol is β2AR dependent (16). Given that GRK2 mediated phosphorylation is instrumental in β2AR desensitization, we next asked whether ablation of GRK2 in the skeletal muscle would enhance clenbuterol-induced hypertrophy. GRK2fl/fl and MLC-Cre:GRK2fl/fl mice were administered clenbuterol or PBS continuously for 14 days via subcutaneous osmotic minipump. The effect of clenbuterol on body and muscle mass is shown in Table 1.

Chronic clenbuterol treatment (14 days) caused a significant increase in body weight in both GRK2fl/fl and MLC-Cre:GRK2fl/fl mice (2.17 ± 0.16g and 2.33 ± 0.14g respectively); though, the fold change in body weight, comparing PBS to...
Clenbuterol treatment was significantly greater in MLC-Cre:GRK2fl/fl compared to GRK2fl/fl mice (2.83 ± 0.17 fold and 2.11 ± 0.15 fold respectively). Clenbuterol treatment caused a significant increase in the soleus weight of both GRK2fl/fl and MLC-Cre:GRK2fl/fl mice (0.37 ± 0.01g and 0.41 ± 0.01g respectively). Clenbuterol treatment caused a significant increase in the soleus weight of both GRK2fl/fl and MLC-Cre:GRK2fl/fl mice (0.37 ± 0.01g and 0.41 ± 0.01g respectively). However, both the absolute weight gain and the fold change in soleus weight comparing PBS to clenbuterol treatment were significantly greater in MLC-Cre:GRK2fl/fl compared to GRK2fl/fl mice (1.31 ± 0.02 fold and 1.15 ± 0.03 fold respectively). In addition, clenbuterol treatment caused a significant increase in the TA and gastrocnemius weight relative to PBS treatment in MLC-Cre:GRK2fl/fl mice; whereas the same muscles in GRK2fl/fl mice trended towards a clenbuterol stimulated increase in mass, yet did not reach significance. As with the soleus, the fold change in gastrocnemius weight comparing PBS to clenbuterol treatment was significantly greater in MLC-Cre:GRK2fl/fl compared to GRK2fl/fl mice (1.16 ± 0.03 fold and 1.03 ± 0.03 fold respectively).

To support the muscle weight data, we analyzed fiber cross-sectional area of TA muscle from these mice (Fig. 6A and B). Clenbuterol treatment significantly increased cross-sectional area of both GRK2fl/fl and MLC-Cre:GRK2fl/fl muscle fibers compared to PBS treated groups. Importantly, the clenbuterol stimulated increase in fiber cross-sectional area was greater in MLC-Cre:GRK2fl/fl compared to GRK2fl/fl mice (1436.29 ± 88.70 µm² vs. 1166.57 ± 64.27 µm² respectively). Overall, we find GRK2 ablation increases clenbuterol induced hypertrophy of skeletal muscle.

Pro-hypertrophic Akt signaling is elevated in clenbuterol treated MLC-Cre:GRK2fl/fl mice – β2-adrenergic agonists are known to stimulate hypertrophy, in part via activation of the pro-hypertrophic Akt signaling pathway; we therefore hypothesized that increased Akt signaling contributes to the enhanced hypertrophy response in clenbuterol treated MLC-Cre:GRK2fl/fl mice.

We examined Akt signaling in soleus muscle harvested from mice following 4hr acute clenbuterol stimulation (1mg/mL). p-Akt levels were significantly increased in both GRK2fl/fl and MLC-Cre:GRK2fl/fl soleus muscle following clenbuterol stimulus (Fig. 7A and B), however, fold increase in p-Akt in clenbuterol treated mice relative to PBS controls was significantly greater in MLC-Cre:GRK2fl/fl mice (Fig. 7C). Levels of p-GSK3β, a downstream target of Akt, were likewise significantly increased in clenbuterol treated MLC-Cre:GRK2fl/fl mice compared to PBS controls (Fig. 7A and D). p-GSK3β levels trended higher in clenbuterol treated GRK2fl/fl mice, yet fell short of statistical significance (Fig. 7A and D). As with p-Akt, the fold increase in p-GSK3β levels in clenbuterol treated mice relative to PBS controls was significantly greater in MLC-Cre:GRK2fl/fl mice (Fig. 7E). In conclusion, clenbuterol stimulated Akt signaling is enhanced in GRK2 ablated skeletal muscle, which may in part explain the pro-hypertrophic phenotype in these mice.

DISCUSSION

Research over the last few decades has identified dichotomous roles for GRK2 in physiology and pathophysiology of the heart. GRK2 activity is instrumental in the physiological homeostasis of the heart by phosphorylating and thereby desensitizing activated GPCRs, predominantly βARs (2). As a consequence, GRK2 activity levels in the heart correlate with contractile function (4,22). However, following cardiac insult, increased GRK2 expression directly contributes to injury and progression to heart failure via excessive βAR desensitization and increased myocyte apoptosis (5-7,23).

Given the well-characterized roles of GRK2 in heart function and disease, in this study we sought insight into the role of GRK2 in skeletal muscle function. Though not identical in nature, skeletal muscle and cardiac muscle still share many features, including many of the same mechanisms and proteins required for excitation-contraction coupling. To examine the role of GRK2 in skeletal muscle we generated a skeletal muscle specific GRK2 KO mouse. To our knowledge this is the first study to directly investigate the role of GRK2 in skeletal muscle function. We studied the contractile properties of isolated soleus and EDL muscle and found that skeletal muscle GRK2 KO caused a decrease in specific force of contraction in the fast-twitch EDL muscle. Furthermore the twitch:tetanus ratio was significantly elevated in GRK2 ablated EDL. In contrast, we found no significant changes in the
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slow-twitch soleus mechanics from these animals; in fact, contrary to the EDL, the specific force trended higher in the soleus of skeletal muscle GRK2 KO mice. The EDL mechanics results were somewhat unexpected, since deletion of GRK2 increases isoproterenol stimulated contractility and calcium transients in isolated cardiomyocytes (24). In this study, we found no difference in baseline Ca\(^{2+}\) transients in GRK2 KO and WT myotubes. Isoproterenol pre-treatment increased Ca\(^{2+}\) transient amplitude in both GRK2 KO and WT myotubes, and though GRK2 KO amplitude values trended higher than those of WT cells, we ultimately found no statistical difference between isoproterenol treated groups. This would indicate that GRK2 KO alters EDL contractility independently of Ca\(^{2+}\) in the skeletal muscle. In GRK2 ablated cardiomyocytes, Raake et al. found that elevated Ca\(^{2+}\) transient amplitudes were caused, at least in part, by enhanced phosphorylation of the L-type Ca\(^{2+}\) channel (LTCC) by protein kinase A (PKA), a downstream effector of βAR activation (24). Phosphorylation of the LTCC increases Ca\(^{2+}\) influx through the channel, which, in turn, heightens calcium induced calcium release (CICR) through the ryanodine receptor (RyR). This GRK2 dependent effect is irrelevant in skeletal muscle, since RyR opening is regulated by direct physical interaction with the LTCC, and not by CICR. Furthermore, Raake et al. found that phosphorylation of phospholamban, a key regulator of sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) mediated Ca\(^{2+}\) re-uptake into the sarcoplasmic reticulum (SR), was decreased in GRK2 ablated cardiomyocytes, resulting in faster Ca\(^{2+}\) transient decay rates (24). Phospholamban is not expressed in type-II skeletal muscle fibers; hence any effect of GRK2 deletion on phospholamban phosphorylation is not relevant to this cell type. Given these key distinctions in Ca\(^{2+}\) handling machinery it is perhaps not surprising that isoproterenol mediated changes in the Ca\(^{2+}\) transients of GRK2 ablated cardiomyocytes are largely absent in the skeletal muscle myotube.

In the heart, βAR stimulation generates an increase in inotropy and chronotropy (25). Research into the effects of βAR stimulation on skeletal muscle contraction has yet to reach a consensus; results in this tissue seem to vary based upon species, muscle type and even dose of agonist (17). This may also help to explain why GRK2 deletion does not have the same effect on contractile properties in the EDL as it does in the heart. It is interesting to note however, that, similar to the heart, the soleus trended towards increased contractility following GRK2 ablation. In contrast to the fast-twitch EDL, the soleus is largely composed of mitochondria rich, type I muscle fibers, which are similar in nature to the slow oxidative fibers constituting the heart. It is therefore possible that the effects of GRK2 deletion on skeletal muscle contractile properties are dependent upon fiber type.

GRK2 can translocate to the mitochondria, and while the consequences of this localization are not completely understood, our lab has shown that GRK2 disrupts mitochondrial function in the cardiomyocyte by altering substrate utilization for ATP production (26). In addition, our lab and others have found GRK2 to be a negative regulator of glucose uptake (27,28). This may be of particular functional relevance to the glycolytic EDL muscle; it is possible that altering glucose homeostasis by GRK2 ablation in the skeletal muscle may result in a metabolic reprogramming, which in turn could affect energy production and muscle contractile properties as a consequence. Further studies examining this hypothesis are warranted.

We next conducted force frequency and fatigue tests on isolated soleus muscle to detect any physiological shift in the fiber type composition. Skeletal muscle GRK2 KO mice were indistinguishable from their WT counterparts in both tests, suggesting GRK2 expression in skeletal muscle has no effect on fiber type composition. Immunostaining and quantification of muscle fibers in soleus sections confirmed this finding. β\(_{2}\)AR agonists can promote slow-to-fast fiber type switching in skeletal muscle (29,30). Our data suggest that reducing the desensitization of β\(_{2}\)ARs by GRK2 ablation alone is not sufficient to promote a fiber type shift in the absence of an exogenous β-agonist.

The lack of fiber type shift or difference in soleus fatigability between GRK2KO and WT mice helps to explain why we also see no difference in involuntary treadmill running to exhaustion in these animals. However, given the apparent difference in EDL specific force generation between our mice, future studies may
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warrant additional experiments more suited to identifying differences in muscle strength, such as a grip strength test.

Chronic stimulation of βARs with β-agonists has long been known to cause a ‘repartitioning effect’, decreasing body fat while increasing skeletal muscle mass (15). This has led to extensive study of β-agonists as a potential therapeutic for muscle wasting diseases such as muscular dystrophy and cancer cachexia. The anabolic effect of clenbuterol on skeletal muscle is due to decreased protein degradation, while simultaneously increasing protein synthesis (31,16). The latter action seems to be primarily mediated by activation of Akt, a well-characterized mediator of muscle hypertrophy (32). In our study we found that the anabolic effects of chronic clenbuterol administration (14 days) were potentiated in skeletal muscle by the deletion of GRK2, most likely due to decreased desensitization of activated β2AR. Furthermore, this appears to be mediated in part by enhanced Akt activation that can occur when GRK2 activity is lowered via knockdown in skeletal muscle cells, though, other mechanisms, including gene regulatory changes downstream of the β2AR cannot be ruled out. Interestingly, in cardiomyocytes, GRK2 deletion prevents hypertrophy following injury; however, this can be explained by the lower levels of cardiomyocyte apoptosis and increased retention of contractile mass following injury in GRK2 KO hearts, reducing the necessity for compensatory hypertrophy (24). Overall, we find that inhibition of GRK2 may increase the effectiveness of β2AR-mediated strategies to grow skeletal muscle mass, which could have translational significance.

In summary, our findings provide novel insight into the function of GRK2 in skeletal muscle. These results highlight a role for GRK2 as a modulator of contractile properties in skeletal muscle. Furthermore, our data show that GRK2 deletion in skeletal muscle potentiates the anabolic effect of clenbuterol administration, in part by enhancing Akt activity. These observations may prove useful in the understanding of skeletal muscle physiology and in developing more effective therapeutic strategies for the treatment of skeletal muscle wasting diseases.

EXPERIMENTAL PROCEDURES

Mice - All animal studies were conducted with the approval of the Animal Care and Use Committee at Temple University. To obtain skeletal muscle specific GRK2 KO mice (MLC-Cre:GRK2fl/fl), MLC-Cre mice, expressing Cre-recombinase under control of the myosin light chain 1f (MLC1f) promoter (19) were crossed with GRK2fl/fl mice (18). GRK2fl/fl mice were used as wild-type (WT) controls for all experiments. All mice used were between 10 and 16 weeks of age.

Treadmill Exercise - Animals were acclimatized to treadmill running (Columbus Instruments) over 5 days starting with a 5-minute session at a speed of 6 m/min on day one, gradually increasing to a speed of 15 m/min by day 5. Mice were given two full days rest following acclimatization before undergoing the treadmill test to exhaustion. The test was conducted as follows: 3-min at 10 m/min, 3-min at 20 m/min, then increasing speed by 2 m/min every 3 minutes until a final speed of 28 m/min was reached. A 0% gradient was used for all treadmill running. Mice were considered to be exhausted when they engaged a platform at the rear of the treadmill for more than 5 seconds, despite receiving a mild electric shock. Running time, maximum speed reached, distance covered and number of times mice engaged the shock grid were recorded.

Muscle Functional Testing - Soleus and extensor digitorum longus (EDL) muscles were subjected to isolated mechanical measurements using a previously described apparatus (Aurora Scientific, Ontario, Canada) (33) and bathed in Ringer’s solution gas-equilibrated with 95% O2 / 5% CO2. Optimum muscle length (Lo) was determined with iterative manual adjustments of length to achieve maximum twitch force with supramaximal stimulation.

Maximum isometric twitch was measured in the muscles followed after 20 seconds by maximum isometric tetanus during a 500-ms stimulation. The maximum twitch and tetanus were measured a total of 3 times with 5 minutes intervals between tests, and the individual maximum value was used. The soleus was subjected to additional functional measures. First, a force-frequency test was employed using stimulation frequencies of 10, 20, 30, 50, 70, 90, and 100 Hz. Force generation at 90 and 100 Hz
did not differ in any muscle studied, and the force was normalized to the isometric tetanus at 100 Hz to generate force-frequency curves. Second, soleus muscles were subjected to a fatigue test, as previously described (34). Briefly, muscles were stimulated for once per second for 10 minutes (200 µsec pulse, 100 Hz, 330 msec duration) in order to determine resistance to fatigue. Upon completion of functional testing, muscles were blotted, weighed and rapidly frozen for subsequent analysis. Specific force was determined based on the physiological cross-sectional area (PCSA), using the following formula:

$$\text{PCSA} = \frac{m}{L_0 \cdot \left( \frac{L_f}{L_0} \right) \cdot \rho}$$

Where $m$ is muscle mass (m), $L_0$ is muscle length, $L_f/L_0$ is the ratio of fiber length to optimal muscle length, and the density of muscle is $\rho = 1.06$ g/cm$^3$. $L_f/L_0$ was 0.45 for EDL, and 0.69 for soleus.

**Immunoblotting** - After SDS-PAGE and transfer to nitrocellulose membranes, primary antibody incubations were performed overnight at 4°C. Rabbit-680 fluorescent secondary antibody was obtained from Li-Cor (#A21109) and mouse-800 fluorescent secondary antibody was obtained from Cell Signaling (#5257S). Membranes were scanned with the Odyssey infrared imaging system (LI-COR). Both GRK2 (#sc-562) and GAPDH (#sc-32233) primary antibodies used were sourced from Santa Cruz. Phospho-Akt (Ser473; #9271L), total-Akt (#2920S) and phospho-GSK3β (Ser9; #9336S) were sourced from Cell Signaling Technology. Total-GSK3β was sourced from Invitrogen (#44-610).

**Micro-osmotic Pumps** - Chronic infusion of clenbuterol (clenbuterol hydrochloride, Sigma) was done using Alzet 14-day micro-osmotic pumps (model 1002, DURECT Corporation). Pumps were filled following the manufacturer’s specifications with sterile PBS or clenbuterol (3mg/kg per day) and inserted as previously described (35). Briefly, Mice were anesthetized with isoflurane (2.5% vol/vol) and were implanted subcutaneously through a subcapsular incision, which was then closed using 4.0 silk suture (Ethicon). The contents of the pumps were delivered at a rate of 0.25 µl/hour for 14 days.

**Acute Clenbuterol Treatment** – Acute clenbuterol stimulation of mice was achieved via a single, subcutaneous injection of clenbuterol (1mg/kg) or sterile PBS (control). 4 hours post-injection muscles were isolated and rapidly frozen for subsequent analysis.

**Fiber Cross-Sectional Area** - TA muscles were paraffin embedded and 7µm sections were cut at the center of the muscle. Muscle sections were stained with H&E; images were taken using a Nikon DS-Ri1 and quantified in a blind manner using ImageJ. 100 fibers, across 5 separate fields were measured per mouse.

**Fiber Type Immunohistochemistry** - Following harvest, soleus muscles were immediately embedded in Tissue-Tek OCT compound (Sakura Finetek) and 10µm sections were cut using a cryotome. Sections were blocked in 5% BSA for 45 minutes prior to primary antibody incubation overnight at 4°C in a humid chamber. All sections were incubated with laminin primary antibody (Thermo, #RB-082-A0) in addition to one of the following fiber-type primary antibodies: MyHC1, MyHCIIa or MyHCIIb (all MyHC antibodies were sourced from the Developmental Studies Hybridoma Bank, #BF-F3, SC-71 and BA-F8). Secondary antibody (Life Technologies A21434, A21202 and Jackson Immuno Research Laboratories 715-545-140) was applied for 1 hour at room temperature. Sections were treated with mounting media containing DAPI and coverslipped. Sections were visualized using a Nikon-Ti fluorescence microscope.

**Isolation and Dissociation of Flexor Digitorum Brevis (FDB) muscle** – Single skeletal muscle myotubes were obtained via enzymatic digestion of FDB muscles. Following dissection, isolated FDB muscles were placed in 35mm dish containing dissociation media composed of DMEM, 2% fetal bovine serum and 2mg/mL Collagenase II (Worthington Biochemical Corporation), and incubated at 37°C, 5% CO$_2$, for 1.5-2 hours. Next, muscles were transferred to a new 35mm dish containing incubation media composed of DMEM, 2% fetal bovine serum and 1% penicillin/streptomycin. The muscles were gently triturated with a sterile, wide-bore P1000 pipette until single myotubes were dissociated. Myotubes were then seeded onto 35mm collagen coated, glass bottomed dishes (MatTek Corporation) at 50-60% confluence, and allowed to settle and adhere overnight in a 37°C, 5% CO$_2$ incubator. Experiments were conducted the following day.
Myotube Cytosolic Ca²⁺ transient measurements – Isolated myotubes were loaded with 5 µM Fluo-4 AM (Invitrogen) for 20 min at room temperature. Loaded myotubes were placed in a 37 °C heated chamber on an inverted microscope stage. Myotubes were perfused with a normal physiological Tyrode’s buffer (150 mM NaCl, 5.4 mM KCl, 1.2 mM MgCl₂, 10 mM glucose, 2 mM sodium pyruvate, and 5 mM HEPES, pH 7.4) containing 2 mM Ca²⁺. Myotubes were paced at 0.2 Hz and continuously recorded for Ca²⁺ transients using Zeiss Observer Z1 fluorescent microscope at 490/20ex and 535/50em. Ca²⁺ transients were measured at baseline and following pretreatment with isoproterenol (100nM, 5 min). For intracellular Ca²⁺ fluorescence measurements, the F₀ (or baseline) was measured as the average fluorescence of the myotube 100 ms before stimulation. The maximal Fluo-4 fluorescence (F) was measured for peak amplitude. Time to peak was calculated as the time from the beginning of the transient to peak amplitude. Tau was measured as the decay rate of the Ca²⁺ transient traces.

Statistics - All the values in the text and figures are presented as mean ± SEM. Statistical significance was determined by Student’s t-test or ANOVA. P values of <0.05 were considered significant.

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Author contributions: BPW designed and conducted most of the experiments, analyzed the results, and wrote the manuscript. MCW, TSL and LAG conducted some experiments, and helped with analysis and interpretation of results. DGT assisted in experimental design and analysis of results. JWE conducted some experiments and assisted in experimental design and analysis of results. WJK designed experiments, analyzed and interpreted results and assisted in writing the manuscript.

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FIGURE LEGENDS

TABLE 1. Clenbuterol stimulated skeletal muscle hypertrophy is enhanced by skeletal muscle specific GRK2 knockdown. PBS or Clenbuterol (3mg/kg per day) were administered continuously for 14 days via osmotic minipump. Mean ± standard error is reported for 10-11 mice per group. *P<0.05; **P<0.01 by ANOVA; PBS vs. Clenbuterol (same genotype). ●●P<0.01 by ANOVA; GRK2fl/fl Clenbuterol vs. MLC-Cre:GRK2fl/fl Clenbuterol. §P<0.05; §§P<0.01 by Student’s t-test; fold GRK2fl/fl vs. fold MLC-Cre:GRK2fl/fl.

FIGURE 1. Skeletal muscle specific knockdown of GRK2 in MLC-Cre:GRK2fl/fl mice. GRK2 protein levels were measured via Western blot in lysates from Gastrocnemius (A), Soleus (B), Extensor
GRK2 and skeletal muscle physiology

Digitorum Longus (EDL) (C), and Heart (D). GAPDH levels were assessed as a loading control. Bars depict the mean signal intensity of GRK2 normalized to GAPDH, and error bars represent standard deviation. **P < 0.01; ***P < 0.001 between GRK2fl/fl and MLC-Cre:GRK2fl/fl animals; n = 4 and 5 per group respectively.

FIGURE 2. Skeletal muscle GRK2 deletion does not impair exercise capacity. A-D, treadmill exercise performance. After an acclimatization period, mice were run on a treadmill with increasing speed until exhaustion was reached (engaging the shock grid for 5 seconds). The mean time to exhaustion (A), maximum speed reached (B), distance covered (C) and frequency per minute of shock grid engagement (D) were calculated from individual performances. Bars depict mean values and error bars represent standard deviation, n = 6 - 9 mice per group.

FIGURE 3. Contractile properties of soleus and EDL muscles from GRK2fl/fl and MLC-Cre:GRK2fl/fl animals. A-E, ex vivo muscle mechanics of soleus and EDL muscles isolated from GRK2fl/fl and MLC-Cre:GRK2fl/fl mice. Fiber cross-sectional area (CSA) (A), twitch (B), tetanus (C), specific force (D), and twitch:tetanus (E) are graphically represented (n = 6 mice per group). *P<0.05; **P<0.01 by Student's t-test. F, force frequency of soleus muscle isolated from GRK2fl/fl and MLC-Cre:GRK2fl/fl. Force frequency measurements were obtained at 10, 20, 30, 50, 70, 90 and 100Hz (n = 6 mice per genotype). G, percent fatigability of soleus muscle isolated from GRK2fl/fl and MLC-Cre:GRK2fl/fl mice following a fatigue protocol of 10 minutes (n = 6 mice per genotype). All graphs depict mean values and standard deviation of the mean.

FIGURE 4. Fiber type composition of soleus muscle is not altered by GRK2 ablation. A, muscle fiber type immunostaining of soleus cross-sections from GRK2fl/fl and MLC-Cre:GRK2fl/fl mice. Separate myosin heavy chain (MyHC) isoforms were immunostained in sequential sections, scale = 100µm. B, graphs depict the mean percentage contribution of each fiber type to the make-up of the entire muscle section, and error bars represent standard deviation; n = 3 mice per group.

FIGURE 5. Cytosolic Ca²⁺ transients in isolated mouse myotubes. Myotubes were isolated and loaded with the cytosolic Ca²⁺ sensor, Fluo-4 AM. Cytosolic Ca²⁺ was assessed in myotubes paced at 0.2 Hz, in the presence or absence of isopretanol stimulation (100nM, 5 min). A and B, representative traces of cytosolic Ca²⁺ transients from GRK2fl/fl and MLC-Cre:GRK2fl/fl mice respectively. C, amplitude of Ca²⁺ transients. D, time to peak of cytosolic Ca²⁺ transients. E, Time rate of decay. Boxes show the median and the 25th and 75th percentile values; whiskers depict minimum and maximum values. *P<0.05; **P<0.01 by ANOVA; control vs. Isoproterenol treated (same genotype). N = 11-56 myotubes per group.

FIGURE 6. Clenbuterol administration causes significant increase in muscle fiber area of MLC-Cre:GRK2fl/fl mice. A, hematoxylin and eosin staining of tibialis anterior cross-sections from GRK2fl/fl and MLC-Cre:GRK2fl/fl mice following two weeks of continuous PBS or clenbuterol (3mg/kg per day) administration via osmotic minipump, scale = 50µm. B, graphs depict the mean fiber cross-sectional area, and error bars represent standard deviation. *P<0.05; **P< 0.01 by ANOVA; n = 9-10 mice per group.

FIGURE 7. Enhanced Akt signaling in clenbuterol treated MLC-Cre:GRK2fl/fl mice. A, Western blot analysis of p-Akt (S473) and p-GSK3β (S9) levels in soleus muscle isolated from GRK2fl/fl and MLC-Cre:GRK2fl/fl mice, 4 hours following a single subcutaneous injection of PBS or clenbuterol (1mg/kg). B, bars depict the fold change in signal intensity of p-Akt/t-Akt relative to the GRK2fl/fl PBS treated group; error bars represent standard deviation. **P<0.01 by ANOVA; PBS vs. Clenbuterol (same genotype). C, bars depict the fold increase in signal intensity of p-Akt/t-Akt in clenbuterol vs. PBS treated animals; error bars represent standard deviation. §P<0.05 by Student's t-test. D, bars depict the fold change in signal intensity of p-GSK3β/t-GSK3β relative to the GRK2fl/fl PBS treated group; error bars represent standard deviation.
standard deviation. **P<0.01 by ANOVA; PBS vs. Clenbuterol (same genotype); *P<0.05 by ANOVA; GRK2fl/fl Clenbuterol vs. MLC-Cre:GRK2fl/fl Clenbuterol. E, bars depict the fold increase in signal intensity of p-GSK3β/t-GSK3β in clenbuterol vs. PBS treated animals; error bars represent standard deviation. §§§P<0.001 by Student's t-test. N = 6–10 mice per group.
### Table 1

#### Effect of clenbuterol on muscle and body weight in GRK2fl/fl and MLC-Cre:GRK2fl/fl mice

|                     | GRK2fl/fl          |                        | MLC-Cre:GRK2fl/fl |                        |
|---------------------|--------------------|------------------------|-------------------|------------------------|
|                     | PBS                | Clenbuterol            | Fold              | PBS                    | Clenbuterol            | Fold              |
| BW Gain (g)         | 1.03 ± 0.25        | 2.17 ± 0.16**          | 2.11 ± 0.15       | 0.82 ± 0.3             | 2.33 ± 0.14**          | 2.83 ± 0.17§§     |
| EDL Weight (mg/g BW)| 0.39 ± 0.01        | 0.39 ± 0.02            | 1.0 ± 0.05        | 0.38 ± 0.01            | 0.42 ± 0.01            | 1.11 ± 0.03       |
| TA Weight (mg/g BW) | 1.87 ± 0.04        | 2.02 ± 0.05            | 1.08 ± 0.03       | 1.87 ± 0.05            | 2.15 ± 0.04**          | 1.15 ± 0.02       |
| Soleus Weight (mg/g BW) | 0.32 ± 0.01    | 0.37 ± 0.01**          | 1.15 ± 0.03       | 0.32 ± 0.01            | 0.41 ± 0.01**          | # # 1.31 ± 0.02§§ |
| Gastroc Weight (mg/g BW) | 2.83 ± 0.06    | 2.91 ± 0.09            | 1.03 ± 0.03       | 2.74 ± 0.06            | 3.17 ± 0.08**          | 1.16 ± 0.03§§     |

Data (mean ± SEM) shown for 10-11 mice per group. *P<0.05; **P<0.01 by ANOVA; PBS vs. Clenbuterol (same genotype). # # P<0.01 by ANOVA; GRK2fl/fl Clenbuterol vs. MLC-Cre:GRK2fl/fl Clenbuterol. §§ P<0.01 by Student’s t-test; fold GRK2fl/fl vs. fold MLC-Cre:GRK2fl/fl.
Figure 1

A. Gastrocnemius

B. Soleus

C. EDL

D. Heart
Figure 3

A

B

C

D

E

F

G

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http://www.jbc.org/Downloaded from
**Figure 4**

**A**

|                  | MyHC-IIb | MyHC-IIa | MyHC-I |
|------------------|----------|----------|--------|
| GRK2^{fl/fl}     | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| MLC-Cre:GRK2^{fl/fl} | ![Image](image4) | ![Image](image5) | ![Image](image6) |

**B**

- **GRK2^{fl/fl}**
- **MLC-Cre:GRK2^{fl/fl}**

![Graph](image7)

Muscle Fiber Type (%)
Figure 5

A

GRK2^{f/f}

Baseline

Isoproterenol

Fluo-4 (F/F_0)

200 ms

B

MLC-Cre:GRK2^{f/f}

Fluo-4 (F/F_0)

200 ms

C

Amplitude (F/F_0)

GRK2^{f/f}

GRK2^{f/f} ISO

MLC-Cre:GRK2^{f/f}

MLC-Cre:GRK2^{f/f} ISO

D

T-Peak (ms)

GRK2^{f/f}

GRK2^{f/f} ISO

MLC-Cre:GRK2^{f/f}

MLC-Cre:GRK2^{f/f} ISO

E

Tau

GRK2^{f/f}

GRK2^{f/f} ISO

MLC-Cre:GRK2^{f/f}

MLC-Cre:GRK2^{f/f} ISO
Figure 6

A

PBS

Clenbuterol

GRK2^{fl/fl}

MLC-Cre:GRK2^{fl/fl}

B

■ PBS

○ Clenbuterol

Fiber Area (μm^2)

0 500 1000 1500 2000 2500

GRK2^{fl/fl} MLC-Cre:GRK2^{fl/fl}
Figure 7

A

|                | GRK2\textsuperscript{fl/fl} | MLC-Cre:GRK2\textsuperscript{fl/fl} |
|----------------|-----------------------------|--------------------------------------|
|                | PBS | Clenbuterol | PBS | Clenbuterol |
| GRK2           |     |             |     |             |
| p-Akt          |     |             |     |             |
| t-Akt          |     |             |     |             |
| p-GSK3β        |     |             |     |             |
| t-GSK3β        |     |             |     |             |
| GAPDH          |     |             |     |             |

B

Fold pAkt/Akt relative to GRK2\textsuperscript{fl/fl} PBS

C

Fold pAkt/Akt, Clenbuterol vs. PBS

D

Fold pGSK3\textsuperscript{β}/GSK3\textsuperscript{β} relative to GRK2\textsuperscript{fl/fl} PBS

E

Fold pGSK3\textsuperscript{β}/GSK3\textsuperscript{β} vs. PBS

§§§

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Skeletal Muscle Specific G Protein-Coupled Receptor Kinase 2 Ablation Alters Isolated Skeletal Muscle Mechanics and Enhances Clenbuterol-Stimulated Hypertrophy

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