Acute and chronic effects of exercise on mRNA expression in the skeletal muscle of two mouse models of peripheral artery disease

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Abstract

Endurance exercise improves walking performance in patients with peripheral artery disease (PAD), which is characterized by skeletal muscle dysfunction caused by lower extremity ischemia. Although transcriptional analyses of exercise-induced changes in normal animals and healthy volunteers have been reported, no detailed study has explored exercise-induced alterations in gene expression in PAD animal models. Here, we determined the acute and chronic effects of exercise on mRNA expression in the skeletal muscles of two mouse models of PAD. Three particular gene categories were investigated: known exercise-responsive genes (Pgc1a, Il6, Nr4a1, Nr4a2, and Nr4a3); myogenic and muscle regeneration-related genes (Myf5, Myogenin, Myomaker, and Myh3); and Gpr56 and its ligand Col3a1. PAD was induced by bilateral femoral artery ligation in normal C57BL/6 and diabetic KK-A'y mice. From 1 week after surgery, repetitive twice-weekly 30-min treadmill endurance exercise sessions were applied. Altered mRNA expression in the soleus muscles was measured in both the acute and chronic phases. In the acute phase, transcript levels of exercise-inducible genes showed significant increases in both C57BL/6 and diabetic KK-A'y PAD mice; levels of regeneration-related genes showed little alteration, and those of Gpr56 increased immediately and significantly after exercise in both models. In the chronic phase, transcript levels of Pgc1a, Myf5, Myogenin, Myomaker, Myh3, Gpr56, and Col3a1 were upregulated significantly in sedentary C57BL/6 PAD mice compared with that in sham-operated mice. Exercise training inhibited the upregulation of Col3a1, Myf5, and Myogenin significantly. In KK-A'y PAD mice, only Gpr56 mRNA levels increased significantly compared with those in sham-operated mice. RNA sequence analysis revealed 33 and 166 differentially upregulated, and 363 and 99 downregulated, genes after exercise training in C57BL/6 PAD and KK-A'y PAD mice, respectively. In summary, we detected significant alterations of skeletal muscle genes after exercise in PAD mouse models and characterized their expression patterns.
Introduction

Peripheral artery disease (PAD) is a disorder attributed to atherosclerotic occlusion in the lower extremities. PAD is characterized by skeletal muscular pain and dysfunction caused by insufficient blood supply [1]. A large population of patients with PAD is affected with concomitant coronary and cerebrovascular diseases, resulting in high mortality and morbidity in developed and developing nations [2]. The most common symptom of PAD is intermittent claudication (IC), characterized by exercise-induced discomfort that is relieved by rest [3]. The primary goal for patients with PAD and IC is to improve exercise performance, daily functional activity, and quality of life. Recent evidence revealed that exercise training has beneficial effects in extending the walking distance for patients with PAD and IC [4]. A randomized trial of exercise training added to optimal medical therapy, including cilostazol, demonstrated a clear additive benefit, even superior to that of aortoiliac revascularization [5]. Exercise training is considered to affect several machineries associated with clinical benefits, including alteration in skeletal muscle metabolism, conditioning underlying diseases, and improving endothelial function [5–8]; however the detailed mechanisms remain unknown. A better understanding of the molecular mechanism underlying the exercise-induced benefits is thus highly desirable.

There is accumulating evidence that exercise training can induce significant alterations in the expression levels of several genes in skeletal muscles; this effect is considered one of the most important underlying molecular mechanisms of exercise-induced benefits. For instance, interleukin-6 (IL-6), which is locally produced in skeletal muscles in response to exercise [9], is known to increase glucose availability and activate muscle satellite cells, contributing to muscle hypertrophy and regeneration [10, 11, 12]. NR4As (NR4a1, NR4a2, and NR4a3) are other genes that are reported to be induced by endurance exercise in human skeletal muscle [13] and are considered to play a role in exercise-induced benefits through improved muscle energy metabolism [14]. These considerations are largely based on the reported data from experiments on normal animals and healthy volunteers. To the best of our knowledge, to date, no detailed studies have described exercise-induced alterations in gene expression in PAD animal models and patients with PAD.

Bilateral ligation of the femoral arteries (FAL) is one of the most common methods to induce PAD in animal studies. Many investigators [15], including ourselves [16], have reported that several key characteristics of PAD, including muscle atrophy, apoptosis, fiber type switching, altered myosin heavy-chain expression, and muscle fiber denervation, can be induced in mice and rats using this surgery. In agreement with the fact that diabetes is an important risk factor for PAD patients [17], diabetic animals such as KK-AY mice have been reported to show more severe disease phenotypes [18,19].

Recently, White et al. reported that G protein-coupled receptor 56 (GPR56) might play an important role in muscle hypertrophy associated with resistance/loading-type exercise [20]. A murine model of overload-induced muscle hypertrophy is associated with increased expression of both Gpr56 and its ligand, collagen type III, whereas genetic ablation of Gpr56 expression attenuated overload-induced muscle hypertrophy and associated anabolic signaling. In addition, GPR56 expression was induced in humans by resistance exercise [20]. Although these new findings suggest GPR56 could be an interesting molecular target to mimic the molecular signaling related to exercise-induced clinical benefits, the expression profiles of GPR56 have not been reported in PAD animal models and patients with PAD.

The aim of the present study was to determine the acute and chronic effects of exercise on mRNA expression in the soleus muscles of two mouse models of PAD: normal C57BL/6 mice and diabetic KK-AY mice with FAL. In particular, we focused on three categories of genes: 1) known exercise-responsive genes (Pgc1a, Il6, Nr4a1, Nr4a2 and Nr4a3); 2) myogenic and
muscle regeneration-related genes (Myf5, Myogenin, Myomaker and Myh3); and 3) Gpr56 and its ligand Col3a1. In the present study, we also investigated whether diabetes could induce exercise-induced transcriptional alterations, by comparing the data obtained from the two PAD mouse models.

Materials and methods

Animals

Male C57BL/6 mice and KK-Ay/Ta mice (8 weeks old) were purchased from CLEA Japan (Tokyo, Japan). All animals were maintained on a laboratory chow diet (CE-2, CLEA Japan) and allowed free access to water and food before and during the experiments. The care and use of the animals and the experimental protocols used in this research were approved by the Institutional Animal Care and Use Committee of Takeda Pharmaceutical Co., Ltd. (Approval code: 00005751)

Bilateral femoral artery ligation (FAL)

C57BL/6 and KK-Ay mice underwent FAL at 12 and 10 weeks of age, respectively. All surgical procedures were performed under isoflurane anesthesia (1–5%) and buprenorphine analgesia (0.5–1 mg/kg, s.c.), and all efforts were made to minimize suffering. After skin incision, the proximal and distal ends of the femoral artery and the proximal profunda femoris artery in the groin were dissected and ligated for both legs. The intervening segments were excised and the dissected sites were sutured with sterile threads. After closing the incision using surgical sutures, the mice were allowed to recover for 7 days. In the sham-operated mice, the same surgical procedure was performed except for the ligation and excision of the artery.

Treadmill exercise training and tissue sampling

Before FAL surgery, all mice were subjected to a 10-min habituation to a treadmill (Panlab) three times, according to the following protocol: 3 m/min for 1 min and 9 m/min for 4 min, on an incline of 5°. Grouping was performed based on body weight for C57BL/6 mice, and on habituation running time, body weight, and plasma biochemical parameters (glutamic pyruvic transaminase, total cholesterol, triglyceride, glucose, and hemoglobin A1c) for the KK-Ay mice. For exercise training after FAL surgery, mice underwent a total of four to five 30-min treadmill exercise procedures twice a week from 1 week after surgery (exercise groups), according to the following protocol: 3 m/min, 4.2 m/min, 5.4 m/min, 6.6 m/min, and 7.8 m/min for 1 min each; 9 m/min for 5 min; 12 m/min for 10 min; and 15 m/min for 10 min, on an incline of 15°. To monitor the time course of recovery of their running performance, time to exhaustion in each exercise training session was measured. When mice were exhausted, they were allowed to recover (60–90 min), and then returned to the treadmill to achieve a total training time of 30 min. Our preliminary experiments revealed that these repetitive twice-weekly treadmill exercise sessions significantly prolonged time to exhaustion on the treadmill test at 3–4 weeks after FAL surgery in both C57BL/6 and diabetic KK-Ay PAD mice. In the sedentary groups, no mice were subjected to the treadmill exercise until the tissue sampling procedure. For acute assessment, mice were euthanized under pentobarbital anesthesia (50–100 mg/kg, ip) and the right soleus muscles were harvested at the indicated time points (described in the legends of each Fig). For chronic assessment, including individual transcript measurement and RNA seq analysis, the soleus muscles were harvested 4 days after the last exercise. In the present study, we used the soleus muscle, which consists predominantly of slow-twitch fibers, because our preliminary experiments indicated that more genes were
significantly altered in the soleus muscle than in the gastrocnemius muscle of these PAD models. The tissues were frozen immediately in liquid nitrogen and stored at –80˚C.

**Gene expression analysis**

Frozen tissues were dissociated using a GentleMACS dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) in 1 mL of RLT Plus buffer (Qiagen, Hilden, Germany) containing 0.04 M dithiothreitol. After standing at room temperature for 5 min, samples were centrifuged quickly and then 600 μL of each homogenate was digested using QIAshredder (Qiagen) and centrifuged (20,000 × g) for 2 min. The RNA-containing supernatants were purified using an RNeasy Mini kit (Qiagen) and reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) to obtain cDNA. The mRNA expression levels were then determined by TaqMan quantitative real-time reverse transcription PCR (qRT-PCR; Applied Biosystems, Foster City, CA), using the following predesigned primer/probe sets: *Rplp0*, Mm99999223_gH; *Ppargc1a (Pgc1a)*, Mm01184322_m1; *Il6*, Mm00446190_m1; *Nr4a1*, Mm01300401_m1; *Nr4a2*, Mm00443060_m1; *Nr4a3*, Mm0045074_m1; *Myf5*, Mm00435125_m1; *Myogenin*, Mm00446195_g1; *Tmem8c (Myomaker)*, Mm00481255_m1; *Myh3*, Mm01332463_m1; *Gpr56*, Mm00817704_m1; and *Col3a1*, Mm01254476_m1. Each expression value was normalized to the expression levels of *Rplp0* and calculated relative to the pre-exercise group for the acute phase study and to the sham-operated sedentary group for the chronic phase study.

**RNA sequence analysis**

Whole mRNA transcript expression analysis was conducted by Macrogen Inc. (Korea). Briefly, cDNA libraries were constructed using the Truseq RNA Sample Preparation kit (Illumina, San Diego, CA) and paired-end RNA sequencing was performed using the HiSeq2000 system (Illumina). RNA-seq data were processed using Array Studio 8.0.0.78 (OmicSoft Corp., Cary, NC) as follows. FASTQ files were mapped using mouse.B38 as the reference library, and then gene annotations were added to the read count values with ensembl.R78. After checking that the mapping rate was over 80%, the read counts of all samples were exported. Statistical analysis was conducted using the R language (https://www.r-project.org/). To calculate Trimmed Mean of the M-value (TMM) for normalization and to identify mRNAs differentially expressed among soleus muscle from diabetic KK-Ay or C57BL/6 mice, a procedure implemented in the edgeR package was applied. The count per million (cpm) was used in the analysis. Before statistical analysis, for genes with a cpm value greater than 1, at least one sample was retained for further analysis. Up- and downregulated genes were identified as having a p-value less than 0.01 and a fold change greater than 1.5. The BaseSpace Correlation Engine software (formally known as NextBio; https://japan.usc.informatics.illumina.com/c/nextbio.nb; Illumina, Cupertino, CA) was used to conduct Gene Ontology (GO) analysis.

**Measurement of hindlimb blood flow**

Mice were kept at 37˚C using a BWT-100A animal warming pad (Bioresearch Center, Nagoya, Japan) under isoflurane anesthesia (1–3%). The heart rate was measured by electrocardiography to maintain consistent anesthesia. The hindlimb blood flow was monitored at 8 and 10 min after the onset of warming, using a MoorLD12-2λsim laser Doppler imaging system (Moor Instruments, Devon, UK). Mice were then allowed to recover under normal conditions. For each measurement time point, the average blood flow perfusion units were calculated for both hindlimbs, and the average was calculated. The average of both time points was used for analysis. All procedures were performed in a blinded fashion.
Statistical analysis

The results are expressed as the mean ± SD. Statistical significance between two groups was determined by Student’s t-test for homogenous data and by Welch’s test for non-homogenous data. Statistical significance between groups of multiple time points was determined by a two-tailed Dunnett’s test. Two-way ANOVA was performed to evaluate statistical differences in the hindlimb blood flow by FAL surgery and by exercise training. Differences were considered significant if \( p < 0.05 \).

Results

Characterization of the two PAD mouse models

To characterize the disease phenotypes of normal C57BL/6 and diabetic KK-A\(^+\) PAD mice, their hindlimb blood flow was measured by laser Doppler perfusion imaging 3 weeks after FAL surgery (Fig 1A and 1B). A significant reduction in blood flow was confirmed in both PAD models. KK-A\(^+\) PAD mice showed a larger blood flow reduction (60–78% reduction vs. sham-operated mice) than C57BL/6 PAD mice (33–34% reduction). In addition, exercise training did not affect the blood flow. It should, however, be noted that the comparison of absolute flow units is a limitation of the study design, because laser Doppler flow assessments cannot be compared accurately mouse to mouse; limited effects of exercise training on the hindlimb blood flow should be confirmed using more suitable methods. Fig 1C shows the recovery time-course of the running performance of exercised C57BL/6 PAD and KK-A\(^+\) PAD mice after FAL surgery. KK-A\(^+\) PAD mice showed delayed recovery compared with C57BL/6 PAD mice. These results suggest a more severe disease phenotype for diabetic KK-A\(^+\) PAD mice than for C57BL/6 PAD mice, which is consistent with other studies [18,19].

Transcripts acutely altered by endurance exercise

Fig 2 shows the acute alterations of known exercise-responsive genes caused by our endurance exercise protocol in the soleus muscles of both C57BL/6 and KK-A\(^+\) PAD mice. The mRNA level of \( Pgc1a \), a known exercise-responsive gene, increased significantly and gradually after the exercise session in C57BL/6 PAD (2.1-fold at maximum) and KK-A\(^+\) PAD mice (1.7-fold). The mRNA level of another known exercise-responsive gene, \( Il6 \), also increased significantly immediately after the exercise session in both C57BL/6 PAD (3.4-fold) and KK-A\(^+\) PAD mice (5.7-fold). In addition, transcript levels of \( Nr4a1, Nr4a2, \) and \( Nr4a3 \) increased after endurance exercise and then gradually decreased in C57BL/6 PAD mice, peaking at 60–90 minutes after the onset of exercise (2.7, 8.5, and 3.8-fold, respectively; Fig 2C–2E). KK-A\(^+\) PAD mice also showed upregulation of \( Nr4a1, Nr4a2, \) and \( Nr4a3 \), with approximately 2-fold greater responses than C57BL/6 mice (7.2, 16, and 6.6-fold, respectively).

Fig 3 indicates the transcript levels of \( Myf5, Myogenin, Myomaker \) and \( Myh3 \), which are known markers of myogenesis and muscle regeneration, in the soleus muscles of both C57BL/6 and KK-A\(^+\) PAD mice up to 4 h after the cessation of the exercise. The mRNA levels of these genes showed little alteration during the observation period.

The mRNA levels of \( Gpr56 \) and its endogenous ligand, \( Col3a1 \), were determined using the same samples. \( Gpr56 \) transcripts increased immediately and significantly after the exercise session (Fig 4A) in both C57BL/6 PAD (1.9-fold) and KK-A\(^+\) PAD mice (1.8-fold). Although the \( Col3a1 \) transcript level showed an increasing trend in both models, the alteration did not reach statistical significance (Fig 4B).
In addition, no exercise-induced changes were observed in the transcript levels of angiogenesis-related genes such as Vegfa, Des, and Cx37 in both C57BL/6 PAD mice and KK-A′ PAD mice (data not shown).

**Transcripts chronically altered by endurance exercise**

Tables 1 and 2 summarize the transcript levels of several genes in the soleus muscles of sham-operated sedentary mice (sham sedentary), sedentary PAD mice (FAL sedentary), and chronically twice-weekly exercised PAD mice (FAL exercise), 3 weeks after FAL surgery.

In C57BL/6 sedentary PAD mice, transcript levels of Pgc1a, Gpr56, Col3a1, Myf5, Myogenin, Myomaker, and Myh3 were upregulated significantly compared to that in sham-operated mice.
Fig 2. Acute effects of treadmill exercise on the mRNA expression of known exercise-responsive genes. The soleus muscles of exercised C57BL/6 PAD mice (n = 4 per time point, total 24, left panels) and KK- Ay PAD mice (n = 6 per time point, total 30, right panels) were obtained before and after the fifth treadmill exercise session. The mRNA expression levels of *Pgc1a* (A), *Il6* (B), *Nr4a1* (C), *Nr4a2* (D), and *Nr4a3* (E) in the soleus muscles were determined by qRT-PCR, normalized to *Rplp0* expression level, and calculated relative to the pre-exercise group (0 min). The results are expressed as the mean ± SD. *p < 0.05, **p < 0.01 vs. 0 min (Dunnett’s test). Bold bars represent the timing of the fifth treadmill exercise.

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Fig 3. Acute effects of treadmill exercise on the mRNA expression of myogenic and muscle regeneration-related genes. The soleus muscles of exercised C57BL/6 PAD mice (left) and KK-4^y PAD mice (right) were obtained and the mRNA expression levels of Myf5 (A), Myogenin (B), Myomaker (C), and Myh3 (D) in the soleus muscles were determined, normalized, and calculated as described in the legend of Fig 2. The results are expressed as the mean ± SD. Bold bars represent the timing of the fifth treadmill exercise.

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Exercise training inhibited the upregulation of *Col3a1*, *Myf5*, and *Myogenin* significantly. No chronic upregulation by exercise was observed in the transcripts of *Il6*, *Nr4a1*, *Nr4a2*, or *Nr4a3*, which was consistent with the findings of acute and transient induction with rapid recovery after exercise (Fig 2B–2E).

In KK-*A*° PAD mice, no significant upregulation was observed in *Myf5*, *Myogenin*, or *Myomaker* mRNA levels compared with those in sham-operated mice, whereas the *Gpr56* and *Myh3* mRNA levels increased significantly (Table 2). Exercise training exerted little effect on the mRNA levels of the genes investigated; only *Myf5* mRNA was upregulated significantly by exercise in KK-*A*° PAD mice (Table 2).

To identify differentially expressed genes between sedentary and exercised PAD mice, RNA sequence analysis was also conducted using the same sedentary and exercised PAD mouse samples (Tables 3–6). RNA sequence analysis revealed 33 upregulated and 363 downregulated
genes in the soleus muscles of exercised C57BL/6 PAD mice (Tables 3 and 4). In KK-Ay PAD mice, 166 genes were upregulated and 99 genes were downregulated by the exercise training (Tables 5 and 6). Among these differentially expressed genes, only 37 genes overlapped between C57BL/6 PAD mice and KK-Ay PAD mice. Moreover, most (31 of 37) of these overlapped genes were downregulated in C57BL/6 PAD mice, but upregulated in KK-Ay PAD mice, showing a negative correlation.

Gene Ontology analysis was conducted for the differentially expressed genes identified between sedentary and exercised PAD mice (Tables 7 and 8). In the C57BL/6 PAD mice, extracellular matrix-related genes were ranked and downregulated (Table 7). In KK-Ay PAD mice, however, different types of genes, such as channels and transporters, were enriched (Table 8).

Table 1. Chronic effects of treadmill exercise on the mRNA expression in the soleus muscle of C57BL/6 PAD mice.

| Gene  | Sedentary sham-operated | Sedentary PAD | Exercised PAD |
|-------|-------------------------|---------------|---------------|
| Pgc1a | 1.00 ± 0.38             | 2.87 ± 0.64†† | 2.26 ± 1.03   |
| Il6   | 1.00 ± 0.54             | 1.41 ± 0.35   | 1.26 ± 0.38   |
| Nr4a1 | 1.00 ± 0.56             | 0.66 ± 0.56   | 0.80 ± 0.11   |
| Nr4a2 | 1.00 ± 0.37             | 1.71 ± 0.95   | 1.61 ± 0.39   |
| Nr4a3 | 1.00 ± 1.07             | 1.16 ± 0.21   | 0.85 ± 0.33   |
| Myf5  | 1.00 ± 0.15             | 1.79 ± 0.20†† | 1.46 ± 0.11‡‡ |
| Myogenin | 1.00 ± 0.35        | 3.18 ± 0.83†† | 1.63 ± 0.50‡‡ |
| Myomaker | 1.00 ± 1.08        | 11.7 ± 5.18†† | 5.91 ± 3.86   |
| Myh3  | 1.00 ± 0.42             | 12.7 ± 3.26†† | 10.9 ± 4.24   |
| Gpr56 | 1.00 ± 0.22             | 1.77 ± 0.50†  | 1.22 ± 0.40   |
| Col3a1| 1.00 ± 0.02             | 11.0 ± 1.78†† | 6.70 ± 0.98‡‡ |

Results are expressed as the mean ± SD; sedentary sham-operated mice (n = 3), sedentary PAD mice (n = 5), and exercised PAD mice (n = 4).

\[ p < 0.05 \]

\[ †† p < 0.01 \]

\[ † p < 0.05 \]

\[ ‡‡ p < 0.01 \]

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Table 2. Chronic effects of treadmill exercise on mRNA expression in the soleus muscle of KK-Ay PAD mice.

| Gene  | Sedentary sham-operated | Sedentary PAD | Exercised PAD |
|-------|-------------------------|---------------|---------------|
| Pgc1a | 1.00 ± 0.30             | 3.30 ± 1.96   | 2.21 ± 0.74   |
| Il6   | 1.00 ± 0.25             | 2.27 ± 1.38   | 1.55 ± 0.48   |
| Nr4a1 | 1.00 ± 0.60             | 1.29 ± 0.26   | 1.01 ± 0.23   |
| Nr4a2 | 1.00 ± 0.16             | 3.74 ± 2.76   | 2.90 ± 0.97   |
| Nr4a3 | 1.00 ± 0.46             | 5.92 ± 4.13   | 4.09 ± 2.05   |
| Myf5  | 1.00 ± 0.09             | 1.20 ± 0.38   | 2.31 ± 0.89‡  |
| Myogenin | 1.00 ± 0.10        | 2.20 ± 0.84   | 3.35 ± 1.24   |
| Myomaker | 1.00 ± 1.23        | 4.38 ± 5.23   | 9.81 ± 5.99   |
| Myh3  | 1.00 ± 0.49             | 4.62 ± 2.08†† | 8.80 ± 4.40   |
| Gpr56 | 1.00 ± 0.17             | 2.09 ± 0.56†† | 1.74 ± 0.21   |
| Col3a1| 1.00 ± 0.20             | 11.1 ± 9.44   | 9.74 ± 5.58   |

Results are expressed as the mean ± SD; sedentary sham-operated mice (n = 4), sedentary PAD mice (n = 4), and exercised PAD mice (n = 6).

\[ † p < 0.05 \] vs. sedentary sham-operated mice (Student’s or Welch’s t-test).

\[ † p < 0.05 \] vs. sedentary PAD mice (Student’s or Welch’s t-test).

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The directions of the alteration of these enriched genes were different between the two PAD models. The direction of all the top 10 enriched GO terms was downregulation in C57BL/6 PAD mice, while the direction of most (9 of 10) of the top 10 enriched terms was upregulation in KK-Ay PAD mice.

Discussion

In the present study, we detected significant acute and chronic alterations in the expression levels of several genes in the skeletal muscles of two different mouse models of PAD after exercise, and characterized the expression patterns of each gene. The main findings of this study are as follows: 1) exercise increased the mRNA expression of known exercise-responsive genes acutely and significantly in both C57BL/6 and KK-Ay PAD mice; 2) mRNA expression of skeletal muscle regeneration markers Myf5 and Myogenin was upregulated significantly in C57BL/6 PAD mice compared to that in sham-operated mice at 3 weeks after FAL surgery, and this upregulation was inhibited significantly by exercise training; 3) Gpr56 mRNA transcription was increased acutely and significantly by exercise in both PAD mouse models; and 4) diabetes did not inhibit the exercise-induced upregulation of Pgc1a, Il6, Nr4a1, Nr4a2, Nr4a3, or Gpr56 in KK-Ay PAD mice.

In the present study, exercise induced the mRNA expressions of Pgc1a, Il6, Nr4a1, Nr4a2, and Nr4a3 acutely in both C57BL/6 PAD and KK-Ay PAD mice. Several investigators have reported acute and chronic mRNA expression increases of PGC1A [21] and NR4A family genes [22] in exercised normal animals and healthy humans. The PGC1 and NR4A families play an essential role in the control of cellular energy metabolic pathways [23,24]; therefore, the exercise-induced upregulation of these nuclear receptors and nuclear receptor coactivators is considered an important molecular mechanism underlying exercise-induced benefits through improved muscle energy metabolism [14]. IL-6 is known as a "myokine", a protein produced and secreted by skeletal muscles to fulfill paracrine or endocrine roles in insulin-sensitizing effects following exercise [25]. IL-6 is also reported to play a role in muscle hypertrophy and regeneration through the activation of muscle satellite cell functions [10, 11, 12]. The present results showing the upregulation of these important exercise-responsive genes suggest that molecular signaling to induce these genes could remain functional in the compromised skeletal muscles of animals and patients with PAD.
Table 4. List of differentially downregulated genes in the soleus muscle of exercised C57BL/6 PAD mice.

| Gene Symbol | FC  | Gene Symbol | FC  | Gene Symbol | FC  |
|-------------|-----|-------------|-----|-------------|-----|
| Chga        | -41.09 | Ror2        | -2.80 | Gdf11       | -1.97 |
| Wnt16       | -29.94 | Fam83g      | -2.79 | Sult1       | -1.97 |
| Dlk1        | -26.98 | Cd109       | -2.79 | Adcy7       | -1.96 |
| Tnn         | -24.17 | Cpxm1       | -2.78 | 2610203C20Rik | -1.96 |
| Rspo2       | -23.43 | Snub1       | -2.77 | Has2        | -1.95 |
| Pr32        | -16.48 | Prc1        | -2.76 | Sylt2       | -1.95 |
| Rli1        | -15.48 | Enpp1       | -2.74 | Vgl13       | -1.94 |
| Lgr5        | -15.05 | Col6a3      | -2.73 | Scd2        | -1.93 |
| Arghgap36   | -14.34 | Slc37a2     | -2.67 | Il1r2       | -1.93 |
| Krt8        | -14.31 | Rbp4        | -2.67 | BC023105    | -1.93 |
| Mmp12       | -12.99 | Avpr1a      | -2.67 | Pthrh       | -1.92 |
| Dcx         | -12.88 | Crsp2       | -2.67 | Fam114a1    | -1.92 |
| Cldn2       | -12.21 | Ccl21a      | -2.66 | Rbp1        | -1.91 |
| Kr118       | -11.56 | Dkk3        | -2.62 | Khhl13      | -1.91 |
| Lrc15       | -11.08 | Pcdhgb1     | -2.62 | Myo5a       | -1.91 |
| Mest        | -10.79 | Peg3        | -2.61 | Arnt2       | -1.91 |
| C1qtnf3     | -10.05 | Ephb3       | -2.60 | Iltg11      | -1.90 |
| Nrk         | -10.04 | Adamts20    | -2.60 | Mfap5       | -1.90 |
| Mrg         | -9.65  | Anasel      | -2.60 | Frzb        | -1.90 |
| Rian        | -9.25  | Il20rb      | -2.59 | Rab31       | -1.90 |
| Meg3        | -8.99  | Basp1       | -2.59 | Col24a1     | -1.90 |
| Dsp         | -8.96  | Mspsd7a     | -2.58 | Rbms3       | -1.90 |
| Ptn         | -8.85  | Gldn        | -2.58 | Cdkn1a      | -1.89 |
| Ripply1     | -8.82  | E2f1        | -2.56 | Tlpi        | -1.89 |
| AW551984    | -7.95  | Cercam      | -2.54 | Bex1        | -1.89 |
| Myh4        | -7.67  | Tnfrsf11a   | -2.54 | Sorbs2      | -1.86 |
| Actc1       | -7.54  | Gamt        | -2.53 | Mtcp1       | -1.86 |
| Tro         | -7.47  | Ptprf       | -2.53 | Igtp        | -1.85 |
| Myl4        | -7.16  | Mdga1       | -2.52 | Mrc1        | -1.85 |
| Peg10       | -7.01  | Ilga9       | -2.51 | Boc         | -1.84 |
| Atp6v0d2    | -6.96  | Tlbs2       | -2.50 | Mir99ahg    | -1.84 |
| Fbn2        | -6.82  | Prkag3      | -2.50 | Npas2       | -1.84 |
| Postn       | -6.60  | Nr5a2       | -2.47 | Sfn         | -1.84 |
| Slc6a19     | -6.55  | Ccdc141     | -2.47 | Serf1       | -1.84 |
| Th          | -6.51  | Efs         | -2.45 | Gbp5        | -1.84 |
| Msln        | -6.23  | Kcnc4       | -2.45 | Gpc3        | -1.84 |
| Mybph       | -6.21  | Tdrd9       | -2.43 | Olfr558     | -1.83 |
| Spon1       | -5.74  | Bid         | -2.42 | Arghgap22   | -1.83 |
| Myh3        | -5.67  | Serpinb6b   | -2.41 | Dlg4        | -1.83 |
| Sfn4        | -5.34  | Vcan        | -2.40 | Lepre1      | -1.82 |
| Aldh3a1     | -5.25  | Adamts7     | -2.40 | Arghgap32   | -1.81 |
| Plagfl1     | -5.09  | Fndc1       | -2.39 | Sh3tc1      | -1.81 |
| Fjx1        | -4.99  | Srp2x       | -2.37 | Arm6cx6     | -1.80 |
| Kits14       | -4.92  | Igf1        | -2.37 | Selp        | -1.80 |
| Chtrc1       | -4.89  | Atp1b2      | -2.36 | Plat        | -1.80 |
| Gdnf        | -4.83  | Gatn        | -2.36 | Dupd1       | -1.80 |
| Grem2       | -4.78  | Eda2r       | -2.36 | Tnfaip3     | -1.79 |

(Continued)
Table 4. (Continued)

| Gene Symbol | FC  | Gene Symbol | FC  | Gene Symbol | FC  |
|-------------|-----|-------------|-----|-------------|-----|
| Glipr1      | -4.64 | Usp18     | -2.36 | Klhdc8b     | -1.78 |
| Gpr65       | -4.59 | Tubb6     | -2.35 | Bicc1       | -1.78 |
| Zfp365      | -4.38 | Ddah1     | -2.35 | Rap2b       | -1.77 |
| Tnnt2       | -4.34 | Orai2     | -2.34 | Irf8        | -1.77 |
| 6330403K07Rik | -4.33 | Pl15     | -2.33 | Klf10       | -1.77 |
| Spar        | -4.26 | Trnrsf23  | -2.33 | Musm1       | -1.76 |
| Adamts16    | -4.26 | Emilin1   | -2.33 | Vav3        | -1.76 |
| Igifn1      | -4.22 | Chst2     | -2.33 | Bir3        | -1.76 |
| Lair1       | -4.22 | Itgα4     | -2.32 | 9430073C21Rik | -1.76 |
| Tnc         | -4.19 | Nhs       | -2.32 | Nabp1       | -1.75 |
| Cemip       | -4.06 | Fbn1      | -2.30 | Morc4       | -1.75 |
| begin       | -3.97 | Kcnq1ot1  | -2.30 | Rcn1        | -1.75 |
| Ddc         | -3.95 | Fam109b   | -2.30 | Sh3p1       | -1.75 |
| Pmp2        | -3.90 | Parm1     | -2.29 | Zfp948      | -1.75 |
| A930003A15Rik | -3.89 | Anxa8     | -2.28 | Tlyh2       | -1.74 |
| Scn5a       | -3.88 | Lhpl2     | -2.28 | Zc2hc1a     | -1.74 |
| Oasl1       | -3.87 | Rtp4      | -2.27 | 4933439C10Rik | -1.74 |
| Prmd        | -3.81 | Sulf2     | -2.26 | Gpx7        | -1.73 |
| Col12a1     | -3.76 | Ccbe1     | -2.26 | Elfemp2     | -1.72 |
| Kcnk1       | -3.74 | Lyve1     | -2.26 | Fkbp14      | -1.72 |
| Sfn8        | -3.71 | Adamts3   | -2.25 | Pycr1       | -1.71 |
| Fam65c      | -3.69 | Ogn       | -2.24 | G0s2        | -1.71 |
| Mss51       | -3.68 | Osr2      | -2.24 | Rsad2       | -1.71 |
| Ankrd1      | -3.64 | Lsp1      | -2.24 | Stk17b      | -1.71 |
| Ifi3        | -3.54 | Primpol   | -2.22 | Junb        | -1.70 |
| Xir3b       | -3.53 | Vash2     | -2.22 | Malat1      | -1.70 |
| Dnm30s      | -3.51 | Traf4     | -2.21 | Htra1       | -1.69 |
| Map3k7cl    | -3.50 | Fat1      | -2.21 | Camk2d      | -1.69 |
| Ifit1       | -3.45 | A930004D18Rik | -2.21 | Mob3c       | -1.69 |
| Apo9b       | -3.42 | Igsf3     | -2.20 | Sdc2        | -1.68 |
| Fbn         | -3.42 | Mndal     | -2.20 | Man1a       | -1.68 |
| Nov         | -3.41 | Msc       | -2.17 | Igsf1       | -1.68 |
| Tceal7      | -3.39 | Ankrd35   | -2.17 | Angpt2      | -1.66 |
| Plezo2      | -3.37 | Igtbp4    | -2.16 | Col5a3      | -1.66 |
| Zmn1        | -3.36 | Trp63     | -2.16 | Atp10a      | -1.66 |
| Ccna2       | -3.33 | Plekha7   | -2.16 | Rln2        | -1.66 |
| Cpne2       | -3.31 | Gpr64     | -2.16 | Numb1       | -1.66 |
| Lax         | -3.31 | Rgs10     | -2.15 | Daam2       | -1.65 |
| Fcgr1       | -3.30 | Elovi6    | -2.14 | Tnfsf10     | -1.65 |
| Dcik1       | -3.30 | Rcor2     | -2.14 | Chst12      | -1.65 |
| Cx3cr1      | -3.29 | Trnrsf22  | -2.14 | Nes         | -1.65 |
| Pvalb       | -3.27 | Ckap4     | -2.14 | Dab2        | -1.64 |
| Pmr1        | -3.19 | Fap       | -2.14 | Ext1        | -1.63 |
| Actn3       | -3.17 | Matn2     | -2.12 | Mdm4        | -1.63 |
| Gadd45a     | -3.16 | Cd44      | -2.12 | Kdelc2      | -1.62 |
| Plod2       | -3.12 | Pdk3      | -2.11 | Chpt2       | -1.62 |
| Rec8        | -3.10 | Mrc2      | -2.10 | Tspan9      | -1.62 |

(Continued)
In C57BL/6 PAD mice, mRNA transcripts of myogenic and muscle regeneration-related Myf5, Myogenin, Myomaker, and Myh3 were upregulated significantly at 3 weeks after surgery, compared to that in sham-operated mice (Table 1). This result suggested that active muscle regeneration occurred in this model. In fact, previously, we reported the existence of regenerating myofibers, characterized by a central nucleus location, in the calf muscle of this model [16]. Treadmill exercise did not affect the expression of these myogenic and muscle regeneration-related genes acutely during the observation period (at and up to 4 h after cessation of 30-min exercise, Fig 3), whereas exercise training prevented the upregulation of Myf5 and Myogenin significantly in the chronic phase (Table 1). Yang et al. investigated the time-course activation of selected myogenic (Mrf4, Myf5, MyoD, and Myogenin) genes after an acute bout of 30-min treadmill running in the calf muscle of healthy subjects [26]. Similar to the present results, they failed to detect significant differences in Mrf4, Myf5, and Myogenin mRNA levels at 0–24 h post-running. However they noted significant upregulation of MyoD mRNA levels (by 8.0-fold) at 8 h post-running. These findings suggest that the optimal selection of time points and genes of interest are important to detect exercise-induced alterations in these myogenic and muscle regeneration-related genes. We speculate that the exercise training-induced

| Table 4. (Continued) |
|----------------------|
| Gene Symbol | FC  | Gene Symbol | FC  | Gene Symbol | FC  |
| Capn6      | -3.09 | Galnt16    | -2.10 | Ccdc102a   | -1.62 |
| Aspn       | -3.08 | Mybpc2    | -2.10 | Scai       | -1.61 |
| Zap70      | -3.07 | Tass1r1    | -2.10 | A1480526   | -1.60 |
| Hmnc1      | -3.06 | Cdk2      | -2.09 | Sesn2      | -1.59 |
| i830012O16Rik | -3.06 | Entpd7    | -2.09 | Gprc5c     | -1.59 |
| Oasl2      | -3.02 | Slc25a24  | -2.08 | Fam65b     | -1.59 |
| Oas1a      | -3.01 | Ztf760    | -2.08 | Fndc3b     | -1.59 |
| Col5a2     | -3.00 | Tmsb10    | -2.07 | Atp13a3    | -1.59 |
| Pbc2       | -3.00 | Sertad4   | -2.07 | Zip503     | -1.57 |
| Col7a1     | -2.97 | Abi3bp    | -2.06 | C130074G19Rik | -1.56 |
| Atx4       | -2.96 | Ili203    | -2.06 | Aebp1      | -1.56 |
| Myog       | -2.96 | Fbxl22    | -2.06 | Tnc2       | -1.56 |
| Lrc55      | -2.96 | Lox1      | -2.04 | Sort1      | -1.56 |
| C1ql3      | -2.96 | Myod1     | -2.04 | Ili122     | -1.55 |
| Sfrp2      | -2.95 | Spred3    | -2.04 | Ppp1r3d    | -1.55 |
| Mdx3a      | -2.93 | Dse       | -2.03 | Fmr1       | -1.55 |
| Cacnb3     | -2.92 | S100a4    | -2.02 | Iffo1      | -1.55 |
| Mmp3       | -2.92 | Cdkn1c    | -2.01 | Ctsz       | -1.54 |
| Ryr3       | -2.92 | Lgals3bp  | -2.01 | Pear1      | -1.52 |
| Mstn       | -2.91 | Atp1b4    | -2.01 | Ttc14      | -1.52 |
| Mmp23      | -2.90 | Ztp354c   | -2.00 | Mid1       | -1.52 |
| Gxylt2     | -2.90 | Mafa      | -2.00 | Tmem44     | -1.52 |
| Wisp1      | -2.89 | Adam19    | -2.00 | Mtap       | -1.52 |
| Marcksl1   | -2.88 | Gk5       | -2.00 | Lgals1     | -1.52 |
| Trim46     | -2.87 | Bbc3      | -1.99 | Txndc5     | -1.51 |
| D300019H16Rik | -2.85 | Hps1     | -1.98 | Dusp6      | -1.51 |
| Foxd3      | -2.82 | Pcdh18    | -1.97 | Creb3i2    | -1.50 |

FC, fold change.

Sedentary PAD mice (n = 4) and exercised PAD mice (n = 4). Differentially downregulated genes met an average FC criterion of <-1.5.

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In C57BL/6 PAD mice, mRNA transcripts of myogenic and muscle regeneration-related Myf5, Myogenin, Myomaker, and Myh3 were upregulated significantly at 3 weeks after surgery, compared to that in sham-operated mice (Table 1). This result suggested that active muscle regeneration occurred in this model. In fact, previously, we reported the existence of regenerating myofibers, characterized by a central nucleus location, in the calf muscle of this model [16]. Treadmill exercise did not affect the expression of these myogenic and muscle regeneration-related genes acutely during the observation period (at and up to 4 h after cessation of 30-min exercise, Fig 3), whereas exercise training prevented the upregulation of Myf5 and Myogenin significantly in the chronic phase (Table 1). Yang et al. investigated the time-course activation of selected myogenic (Mrf4, Myf5, MyoD, and Myogenin) genes after an acute bout of 30-min treadmill running in the calf muscle of healthy subjects [26]. Similar to the present results, they failed to detect significant differences in Mrf4, Myf5, and Myogenin mRNA levels at 0–24 h post-running. However they noted significant upregulation of MyoD mRNA levels (by 8.0-fold) at 8 h post-running. These findings suggest that the optimal selection of time points and genes of interest are important to detect exercise-induced alterations in these myogenic and muscle regeneration-related genes. We speculate that the exercise training-induced
Table 5. List of differentially upregulated genes in the soleus muscle of exercised KK-A\(^{y}\) PAD mice.

| Gene Symbol | FC  | Gene Symbol | FC  | Gene Symbol | FC  |
|-------------|-----|-------------|-----|-------------|-----|
| Tnn         | 47.79 | Sbk3        | 2.14 | Fbln7      | 1.66 |
| Dhrs9       | 9.29  | Mboat2      | 2.12 | Trl2        | 1.65 |
| Chrna9      | 7.20  | E2I2        | 2.12 | Agbl1       | 1.65 |
| Tmem8c      | 6.75  | Bex1        | 2.10 | Map1b       | 1.64 |
| Kcnf1       | 5.25  | Myh4        | 2.09 | Tcfp2l1     | 1.64 |
| 4930539E08Rik | 4.89 | Kcnk5       | 2.08 | Ilga9       | 1.64 |
| Igf2        | 4.58  | Vash2       | 2.06 | Lrch1       | 1.64 |
| Neu2        | 4.45  | Baiap2l1    | 2.04 | Soga1       | 1.62 |
| Zfp385c     | 4.37  | Myom2       | 2.04 | Lnx2        | 1.62 |
| Slc38a1     | 4.25  | Malat1      | 2.03 | Clcf1       | 1.62 |
| Myh8        | 3.97  | Ptgfr       | 2.02 | Ldlnrad3    | 1.61 |
| Dsp         | 3.75  | Mast4       | 2.00 | Col20a1     | 1.61 |
| Dhnx30      | 3.74  | Enox1       | 1.96 | Igl2r       | 1.60 |
| Mltus2      | 3.67  | Wnt2b       | 1.93 | Zc2hc1a     | 1.60 |
| Nxph4       | 3.59  | Klf5        | 1.93 | Wae1        | 1.60 |
| Sln         | 3.50  | Hipk4       | 1.92 | Ubash3b     | 1.60 |
| Myh3        | 3.42  | Fam129a     | 1.92 | Strip2      | 1.60 |
| Slc6a17     | 3.39  | Serpinb6a   | 1.90 | Cdkn1a      | 1.59 |
| Kcne1l      | 3.28  | Dclk1       | 1.89 | Gk5         | 1.59 |
| Zdb12       | 3.25  | Phida1      | 1.89 | 9330151L19Rik | 1.58 |
| 3632451O06Rik | 3.17 | B330095G15Rik | 1.87 | Agrpap      | 1.58 |
| Serpinb1a1  | 2.98  | Arhgap28    | 1.87 | Cx3c1       | 1.58 |
| Mamdc2      | 2.92  | 1110046J04Rik | 1.84 | Macf1       | 1.58 |
| 2310015K22Rik | 2.91 | Myoz3       | 1.83 | Mtap        | 1.57 |
| Rps6ka6     | 2.88  | Ire3        | 1.83 | Abca1       | 1.57 |
| Peg3        | 2.85  | Gtam        | 1.83 | Tceal7      | 1.57 |
| Rtm2        | 2.78  | Tnfrsf23    | 1.82 | Gnb3        | 1.57 |
| Ahnak2      | 2.74  | Pflkb3      | 1.82 | Akrb1b      | 1.57 |
| Zm1         | 2.72  | Kbtbd11     | 1.81 | Cdc25b      | 1.56 |
| H19         | 2.65  | Soot1       | 1.81 | Akrb        | 1.56 |
| Ngef        | 2.59  | Slc22a17    | 1.81 | Btnl9       | 1.56 |
| Piezo1      | 2.58  | Tmem30b     | 1.80 | Dnm3os      | 1.56 |
| Peg10       | 2.56  | Mvd         | 1.78 | Ocld2       | 1.55 |
| Map3k9      | 2.54  | Capn6       | 1.77 | Hcn2        | 1.54 |
| Pak1        | 2.54  | Sbk2        | 1.77 | Angptl2     | 1.54 |
| Gvin1       | 2.52  | Sh3rf1      | 1.74 | Tubb2b      | 1.54 |
| Ucp2        | 2.50  | Fgd3        | 1.74 | Col6a3      | 1.54 |
| Gnk3        | 2.48  | Myl4        | 1.74 | Mll         | 1.54 |
| Col7a1      | 2.45  | Fst         | 1.73 | Sec14l5     | 1.54 |
| Lgal3       | 2.45  | Per1        | 1.73 | Raph1       | 1.54 |
| Csnk2a1     | 2.43  | Hotair      | 1.72 | Acer2       | 1.54 |
| Ctxn3       | 2.43  | Lrc74b      | 1.71 | Sacs        | 1.53 |
| Cdk6        | 2.42  | Al838599    | 1.71 | Rfx5        | 1.53 |
| Frrs1       | 2.41  | Gcnt1       | 1.70 | Itm2a       | 1.52 |
| Maged2      | 2.40  | Galnt5      | 1.69 | Klg2        | 1.52 |
| Rin1        | 2.30  | Zfp568      | 1.69 | Fry1        | 1.52 |
| Ppnn13      | 2.21  | Meg3        | 1.69 | Cobl1       | 1.52 |

(Continued)
prevention of Myf5 and Myogenin mRNA upregulation might reflect exercise-induced acceleration of the muscle regeneration process; however, detailed time-course studies of mRNA expression of the optimally-selected gene set should be performed to confirm this speculation.

The present results support the involvement of GPR56 in exercise-related molecular signaling. Rapid upregulation of Gpr56 after the exercise session was observed in both C57BL/6 and KK-AY PAD mice, consistent with the finding that GPR56 expression was induced in humans by resistance exercise [19]. The mRNA expression level of Col3a1, an endogenous ligand of GPR56, also showed an upward trend after the treadmill exercise session. In addition, the time course of exercise-induced Gpr56 mRNA upregulation was similar to that of Pgc1a in both C57BL/6 and KK-AY PAD mice. This result agreed with the finding that Gpr56 is a transcriptional target of PGC-1α4, a splicing variant of PGC-1α [19].

Diabetic KK-AY PAD mice showed exercise-induced acute upregulation of Pgc1a, Il6, Nr4a1, Nr4a2, Nr4a3, and Gpr56, similar to that observed in non-diabetic C57BL/6 PAD mice (Figs 2 and 4). This result suggested that the molecular signaling to induce these genes can function in the diabetic skeletal muscle of this model. In the chronic phase, however, exercise exerted relatively weaker effects on mRNA expression in the KK-AY PAD mice compared with that in the C57BL/6 PAD mice (Table 2). RNA sequence analysis followed by Gene Ontology (GO) analysis also revealed different characteristics of exercise-related alterations in the chronic phase between KK-AY PAD mice and C57BL/6 PAD mice (Tables 3–8). Many reports have suggested that diabetic skeletal muscles have characteristics different from those of non-diabetic skeletal muscles, such as impaired metabolism [27], increased oxidative stress [28], chronic low-grade inflammatory profiles [29], and impaired extracellular matrix remodeling [30] and regeneration [31,32]. Further detailed studies are needed to clarify how diabetes (and the different characteristics of diabetic skeletal muscles) can affect exercise-induced transcriptional alterations acutely and chronically.

In the acute experiments, KK-AY PAD mice showed an apparent bimodal change of Il6 mRNA upregulation that peaked at 30 and 90 min (Fig 2B, right). However, it should be noted that the biological relevance of this bimodal change should be further confirmed, because of the relatively larger variation of the data at the 90-min time point in the present study.

In conclusion, the present study detected significant alterations in the expression of several genes in the skeletal muscles of two different mouse models of PAD upon exercise, and characterized the expression patterns of each gene. These data provide basic information about exercise-induced transcriptional alterations in the skeletal muscles in mouse PAD models.

### Table 5. (Continued)

| Gene Symbol | FC  | Gene Symbol | FC  | Gene Symbol | FC  |
|-------------|-----|-------------|-----|-------------|-----|
| C77080      | 2.21| Trim47      | 1.69| Tmbim1      | 1.52|
| Vwa3a       | 2.21| Slic25a24   | 1.68| Zbbt37      | 1.51|
| Cnr1        | 2.18| Galm        | 1.68| Grip2       | 1.51|
| Zfp697      | 2.18| Mamstr      | 1.68| Relb        | 1.51|
| Arhgf37     | 2.18| Calmi4      | 1.68| Zfp14       | 1.51|
| Prune2      | 2.18| Trp63       | 1.67| Cpd         | 1.51|
| Ky          | 2.17| Npnt        | 1.67| Cadm1       | 1.50|
| Eps8l2      | 2.15| Nkb2        | 1.67|             |     |
| Eda2r       | 2.14| Adam19      | 1.66|             |     |

FC, fold change.
Sedentary PAD mice (n = 4) and exercised PAD mice (n = 4). Differentially upregulated genes met an average FC criterion of >1.5.

https://doi.org/10.1371/journal.pone.0182456.t005
**Table 6. List of differentially downregulated genes in the soleus muscle of exercised KK-A^y PAD mice.**

| Gene Symbol | FC  | Gene Symbol | FC  | Gene Symbol | FC  |
|-------------|-----|-------------|-----|-------------|-----|
| Fcgbp       | -68.06 | 2310040G24Rik | -1.98 | Igpg        | -1.67 |
| Adamts8     | -5.27  | Cacna2d3     | -1.98 | Cuzd1       | -1.66 |
| BC048679    | -3.51  | Ppp1r1a      | -1.95 | Tiam1       | -1.66 |
| Cdhd22      | -2.85  | Mpped2       | -1.95 | Dpyd        | -1.64 |
| 9030617O03Rik | -2.53 | Dupd1        | -1.95 | Rspo3       | -1.64 |
| Lrtm1       | -2.52  | Ntsc1a       | -1.93 | Ephx2       | -1.63 |
| A930004D18Rik | -2.41 | Timp1        | -1.92 | Kit5c       | -1.63 |
| Crybb1      | -2.41  | H0dx3        | -1.92 | Krt222      | -1.63 |
| Amph        | -2.37  | Konk2        | -1.91 | Smco1       | -1.63 |
| Lypd6       | -2.36  | CS30008M17Rik | -1.90 | Fndc5       | -1.62 |
| Pde3b       | -2.35  | Ostpl6       | -1.89 | Galt        | -1.61 |
| Mchr1       | -2.35  | Ankrd23      | -1.88 | Mylk2       | -1.61 |
| Ankrd1      | -2.31  | 2310047D07Rik | -1.84 | St3gal5     | -1.60 |
| Dusp26      | -2.27  | Fbp2         | -1.82 | Apil        | -1.60 |
| Slc15a5     | -2.21  | Milf         | -1.82 | Incs        | -1.60 |
| Apobr       | -2.19  | Dhcr24       | -1.81 | Fgf1        | -1.60 |
| Dik2        | -2.18  | Dok5         | -1.81 | Lhlpl4      | -1.59 |
| Kcnq4       | -2.16  | A330094K24Rik | -1.81 | Col22a1     | -1.59 |
| Cd209f      | -2.16  | Barx2        | -1.76 | Mrtb2       | -1.58 |
| Cda         | -2.15  | Dapp1        | -1.75 | Cox7a1      | -1.58 |
| Ush1g       | -2.13  | Glns2        | -1.74 | Cux2        | -1.57 |
| Nog         | -2.11  | Sv2b         | -1.72 | Lyve1       | -1.57 |
| Tmem132d    | -2.09  | Fam81a       | -1.71 | Hif         | -1.56 |
| Rasl10b     | -2.08  | Tmem229b     | -1.71 | mt-Nd4l     | -1.56 |
| Tbc1d1      | -2.07  | Gata4        | -1.71 | Aldh1a2     | -1.56 |
| Gadd45b     | -2.06  | Hes1         | -1.70 | Plin5       | -1.55 |
| Cds1        | -2.05  | Cyt1         | -1.69 | Rasl2-9     | -1.54 |
| Retnla      | -2.05  | Wilkin2      | -1.69 | Tpm3        | -1.53 |
| Masp1       | -2.04  | Ccr2         | -1.69 | Sctr        | -1.53 |
| Kncn4       | -2.02  | Pde4a        | -1.69 | Nthl1       | -1.52 |
| Cacna2d2    | -2.01  | Pld4         | -1.67 | Slc8a1      | -1.52 |
| Dach2       | -2.00  | Boh1         | -1.67 | Tst         | -1.51 |
| Pdzd3       | -1.99  | Lamc3        | -1.67 | Ptgis       | -1.50 |

**FC**, fold change.

Sedentary PAD mice (n = 4) and exercised PAD mice (n = 4). Differentially downregulated genes met an average FC criterion of < -1.5.

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**Table 7. Enriched Gene Ontology (GO) terms for genes with altered expression induced by chronic treadmill exercise in the soleus muscle of C57BL/6 PAD mice.**

| Rank | Gene ontology (GO) terms                  | Direction | p-value       |
|------|-------------------------------------------|-----------|---------------|
| 1    | Extracellular matrix                       | down      | 1.50E-41      |
| 2    | Proteinaceous extracellular matrix         | down      | 2.20E-39      |
| 3    | Collagen                                  | down      | 6.90E-14      |
| 4    | Ossification                              | down      | 9.20E-14      |
| 5    | Cell migration                            | down      | 1.00E-12      |
| 6    | Muscle contraction                        | down      | 1.20E-12      |
| 7    | Urogenital system development             | down      | 1.50E-12      |
| 8    | Skeletal system development               | down      | 2.10E-12      |
| 9    | Contractile fiber                         | down      | 4.80E-12      |
| 10   | Regulation of cell growth                 | down      | 5.80E-12      |

‘down’ indicates downregulation

https://doi.org/10.1371/journal.pone.0182456.t007
Table 8. Enriched Gene Ontology (GO) terms for genes with altered expression induced by chronic treadmill exercise in the soleus muscle of KK-Ay PAD mice.

| Rank | Gene ontology (GO) terms                                      | Direction | p-value     |
|------|--------------------------------------------------------------|-----------|-------------|
| 1    | Contractile fiber                                           | up        | 3.20E-08    |
| 2    | Proteinaceous extracellular matrix                           | up        | 2.60E-07    |
| 3    | Regulation of ion transport                                  | down      | 3.50E-07    |
| 4    | Extracellular matrix                                        | up        | 6.90E-07    |
| 5    | Actin binding                                               | up        | 8.80E-07    |
| 6    | Cell leading edge                                           | up        | 2.10E-06    |
| 7    | Aging                                                       | up        | 2.30E-06    |
| 8    | Cell growth                                                 | up        | 2.30E-06    |
| 9    | Ion channel activity                                        | up        | 2.80E-06    |
| 10   | Passive transmembrane transporter activity                   | up        | 4.00E-06    |

*up* indicates upregulation; *down* indicates downregulation

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Supporting information

**S1 File. Data for Fig 1.**
(XLSX)

**S2 File. Data for Figs 2–4.**
(XLSX)

**S3 File. Data for Tables 1 and 2.**
(XLSX)

**S4 File. Data for Tables 3–6.**
(XLSX)

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References
1. Criqui MH, Aboyans V. Epidemiology of peripheral artery disease. Circ Res. 2015; 116: 1509–1526. https://doi.org/10.1161/CIRCRESAHA.116.303848 PMID: 25908725
2. Fowkes FG, Rudan D, Rudan I, Aboyans V, Denenberg JO, McDermott MM, et al. Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: a systematic review and analysis. Lancet. 2013; 382:1329–1340. https://doi.org/10.1016/S0140-6736(13)61249-0 PMID: 23915883
3. Hiatt WR, Armstrong EJ, Larson CJ, Brass EP. Pathogenesis of the limb manifestations and exercise limitations in peripheral artery disease. Circ Res. 2015; 116: 1527–1539. https://doi.org/10.1161/CIRCRESAHA.116.303566 PMID: 25908726
4. Fokkenrood HJ, Bendermacher BL, Lauret GJ, Willigendael EM, Prins MH, Teijink JA. Supervised exercise therapy versus non-supervised exercise therapy for intermittent claudication. Cochrane Database Syst Rev. 2013; 8: CD005263.
5. Murphy TP, Cutlip DE, Regensteiner JG, Cohen DJ, Reynolds MR, et al. Supervised exercise versus primary stenting for claudication resulting from aortoiliac peripheral artery disease: six-month outcomes from the claudication: exercise versus endoluminal revascularization (CLEVER) study. Circulation. 2012; 125: 130–139. https://doi.org/10.1161/CIRCULATIONAHA.111.075770 PMID: 22090168
6. Stewart KJ, Hiatt WR, Regensteiner JG, Hirsch AT. Exercise training for claudication. N Engl J Med. 2002; 347: 1941–1951. https://doi.org/10.1056/NEJMra021135 PMID: 12477945
7. Hiatt WR, Regensteiner JG, Wolfel EE, Carry MR, Brass EP. Effect of exercise training on skeletal muscle histology and metabolism in peripheral arterial disease. J Appl Physiol (1985). 1996; 81:780–788.
8. Hiatt WR, Wolfel EE, Meier RH, Regensteiner JG. Superiority of treadmill walking exercise versus strength training for patients with peripheral arterial disease. Implications for the mechanism of the training response. Circulation. 1994; 90: 1866–1874. PMID: 7922674
9. Pedersen BK, Feabraio MA. Muscle as an endocrine organ: focus on muscle-derived interleukin-6. Physiol Rev. 2008; 88: 1379–1406. https://doi.org/10.1152/physrev.90100.2007 PMID: 18923185
10. Serrano AL, Baeza-Raja B, Perdiguero E, Jardí M, Muñoz-Cánoves P. Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. Cell Metab. 2008; 7: 33–44. https://doi.org/10.1016/j.cmet.2007.11.011 PMID: 18177723
11. Toth KG, McKay BR, De Lisio M, Little JP, Tarnopolksy MA, Parise G. IL-6 induced STAT3 signalling is associated with the proliferation of human muscle satellite cells following acute muscle damage. PLoS One. 2011; 6: e17392. https://doi.org/10.1371/journal.pone.0017392 PMID: 21408055
12. Muñoz-Cánoves P, Scheele C, Pedersen BK, Serrano AL. Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword? FEBS J. 2013; 280: 4131–4148. https://doi.org/10.1111/febs.12338 PMID: 23663276
13. Mahoney DJ, Parise G, Melov S, Saldañar A, Tarnopolsky MA. Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. FASEB J. 2005; 19: 1498–1500. https://doi.org/10.1096/fj.04-3149fje PMID: 15985525
14. Zhao Y, Bruemmer D. NIFT4 orphan nuclear receptors: transcriptional regulators of gene expression in metabolism and vascular biology. Arterioscler Thromb Vasc Biol. 2010; 30: 1535–1541. https://doi.org/10.1161/ATVBHA.109.191165 PMID: 20631354
15. Lotfi S, Patel AS, Matlock K, Egginton S, Smith A, Modarai B. Towards a more relevant hind limb model of muscle ischaemia. Atherosclerosis. 2013; 227: 1–8. https://doi.org/10.1016/j.atherosclerosis.2012.10.060 PMID: 23177969
16. Sugio T, Terada M, Okawa T, Miyata K, Nishimura S, Kenjo E, et al. Development of antibody-siRNA conjugate targeted to cardiac and skeletal muscles. J Control Release. 2016; 237: 1–13. https://doi.org/10.1016/j.jconrel.2016.06.036 PMID: 27369865
17. Belch J, MacCuish A, Campbell I, Cobbe S, Taylor R, Prescott R, et al. The prevention of progression of arterial disease and diabetes (POPADAD) trial: factorial randomised placebo controlled trial of aspirin and antioxidants in patients with diabetes and asymptomatic peripheral arterial disease. BMJ. 2008; 337: a1840. https://doi.org/10.1136/bmj.a1840 PMID: 18927173

18. He S, Zhao T, Guo H, Meng Y, Qin G, Goukassian DA, et al. Coordinated Activation of VEGF/VEGFR-2 and PPARδ Pathways by a Multi-Component Chinese Medicine DHI Accelerated Recovery from Peripheral Arterial Disease in Type 2 Diabetic Mice. PLoS One. 2016; 11: e0167305. https://doi.org/10.1371/journal.pone.0167305 PMID: 27930695

19. Morimoto Y, Bando YK, Shigeta T, Monji A, Murohara T. Atorvastatin prevents ischemic limb loss in type 2 diabetes: role of p53. J Atheroscler Thromb. 2011; 18: 200–208. PMID: 21123956

20. White JP, Wrann CD, Rao RR, Nair SK, Jedrychowski MP, You JS, et al. G protein-coupled receptor 56 regulates mechanical overload-induced muscle hypertrophy. Proc Natl Acad Sci U S A. 2014; 111: 15756–15761. https://doi.org/10.1073/pnas.1417898111 PMID: 25336758

21. Norheim F, Langlie TE, Hjorth M, Holen T, Kielland A, Stadheim HK, et al. The effects of acute and chronic exercise on PGC-1α, irisin and browning of subcutaneous adipose tissue in humans. FEBS J. 2014; 281: 739–749. https://doi.org/10.1111/febs.12619 PMID: 24237962

22. Catoire M, Mensink M, Boekschoten MV, Hangelbroek R, Müller M, Schrauwen P, et al. Pronounced effects of acute endurance exercise on gene expression in resting and exercising human skeletal muscle. PLoS One. 2012; 7: e51066. https://doi.org/10.1371/journal.pone.0051066 PMID: 23226462

23. Finck BN, Kelly DP. PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. J Clin Invest. 2006; 116: 615–622. https://doi.org/10.1172/JCI27794 PMID: 16511594

24. Pearen MA, Muscat GE. Minireview: Nuclear hormone receptor 4A signaling: implications for metabolic disease. Mol Endocrinol. 2010; 24: 1891–1903. https://doi.org/10.1210/me.2010-0015 PMID: 20392876

25. Pai M, Febbraio MA, Whatham M. From cytokine to myokine: the emerging role of interleukin-6 in metabolic regulation. ImmunoCell Biol. 2014; 92: 331–339. https://doi.org/10.1038/icb.2014.16 PMID: 24751614

26. Yang Y, Creer A, Jemiolo B, Trappe S. Time course of myogenic and metabolic gene expression in response to acute exercise in human skeletal muscle. J Appl Physiol (1985). 2005; 98(5): 1745–1752.

27. Bianchi L, Volpato S. Muscle dysfunction in type 2 diabetes: a major threat to patient’s mobility and independence. Acta Diabetol. 2016; 53: 879–889. https://doi.org/10.1007/s00592-016-0880-y PMID: 27393005

28. Aragno M, Mastrocola R, Catalano MG, Brignardello E, Danni O, Boccuzzi G. Oxidative stress impairs skeletal muscle repair in diabetic rats. Diabetes. 2004; 53: 1082–1088. PMID: 15047625

29. McKay BR, Ogborn DI, Baker JM, Toth KG, Tarnopolsky MA, Parise G. Elevated SOCS3 and altered IL-6 signaling is associated with age-related human muscle stem cell dysfunction. Am J Physiol Cell Physiol. 2013; 304: C717–C728. https://doi.org/10.1152/ajpcell.00305.2012 PMID: 23392112

30. Gopinath SD, Rando TA. Stem cell review series: aging of the skeletal muscle stem cell niche. Aging Cell. 2008; 7: 590–598. https://doi.org/10.1111/j.1474-9726.2008.00399.x PMID: 18462272

31. Sala D, Zorzano A. Differential control of muscle mass in type 1 and type 2 diabetes mellitus. Cell Mol Life Sci. 2015; 72: 3803–3817. https://doi.org/10.1007/s00018-015-1954-7 PMID: 26091746

32. D’Souza DM, Al-Sajee D, Hawke TJ. Diabetic myopathy; impact of diabetes mellitus on skeletal muscle progenitor cells. Front Physiol. 2013; 4: 379. https://doi.org/10.3389/fphys.2013.00379 PMID: 24391596