Inorganic Nitrate Mimics Exercise-Stimulated Muscular Fiber-type Switching and Myokine and GABA Release

Short title: **Nitrate is an Exercise Mimetic**

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Abstract

Exercise is an effective intervention for prevention and treatment of type 2 diabetes. Skeletal muscle combines multiple signals contributing to the beneficial effects of exercise on cardiometabolic health. Inorganic nitrate increases exercise efficiency, tolerance and performance. The transcriptional regulator peroxisome proliferator-activated receptorγ coactivator1α (PGC1α) coordinates the exercise-stimulated skeletal muscle fiber-type switch from glycolytic fast-twitch (type IIb) to oxidative slow-twitch (type I) and intermediate (type IIa) fibers; an effect reversed in insulin resistance and diabetes. We find that nitrate induces PGC1α expression and a switch towards type I and IIa fibers in rat muscle and myotubes *in vitro*. Nitrate induces the release of exercise/PGC1α-dependent myokine FNDC5/irisin, and β-aminoisobutyric acid from myotubes, and muscle in rats and humans. Both exercise and nitrate stimulated, PGC1α-mediated, γ-aminobutyric acid (GABA) secretion from muscle. Circulating GABA concentrations were increased in exercising mice and nitrate-treated rats and humans, thus, GABA may function as an exercise/PGC1α-mediated myokine-like small molecule. Moreover, nitrate increased circulating growth hormone levels in humans and rodents. Nitrate induces physiological responses that mimic exercise training and may underlie the beneficial effects of this metabolite on exercise and cardiometabolic health.

Inorganic nitrate was considered a biologically inert metabolic end-product of nitric oxide (NO) metabolism (1). However, the discovery of the nitrate-nitrite-NO pathway in mammals, in which nitrate is reduced to the ubiquitous signaling molecule NO, has led to renewed interest in the physiological role of nitrate (2; 3). The discovery of anti-obesity and anti-diabetic effects of nitrate in rodents, has stimulated interest in nitrate as a therapeutic for the metabolic syndrome (4; 5).

NO has multiple effects on skeletal muscle phenotypes including mitochondrial biogenesis and fatty acid β-oxidation, glucose homeostasis, and contraction and fatigue (6; 7). Augmenting the nitrate-nitrite-NO pathway may be a means of increasing bioavailable NO to improve muscle function. Systemic concentrations of nitrate can be enhanced through dietary consumption of nitrate-rich foods such as green leafy vegetables (8). Following studies in rodents and humans, identifying that nitrate can improve exercise efficiency (9), tolerance (10), and performance (11), nitrate has gained popularity as a nutritional exercise supplement.

We recently demonstrated that dietary nitrate increases peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α) expression in white adipose tissue (WAT) (12). In skeletal muscle, PGC1α is a master transcriptional regulator of the adaptive response to exercise (13). Transgenic PGC1α expression in skeletal muscle increases exercise performance and transcription of genes for oxidative slow-twitch type I and intermediate type IIa fibers (13). Endurance exercise training, partially through PGC1α transcriptional activity, also increases slow-twitch type I and intermediate type IIa myosin heavy chains (MYH) within skeletal muscle (13; 14). These type I and IIa fibers are characterized by high mitochondrial content and facilitate resistance to fatigue (15). Skeletal muscle also mediates many of the signals contributing to the beneficial effects of exercise on health (16). Recently PGC1α was identified as a mediator of several non-muscle metabolic effects of exercise through the regulation of exercise-dependent myokine signals (17; 18).

Exercise is an effective intervention for both the prevention and treatment of type 2 diabetes mellitus (T2DM) (19). Diabetic myopathy, characterized by reduced muscle functional capacity, atrophy and a glycolytic fiber-type, is a major diabetic complication (20). Furthermore, the proportion of type I fibers in muscle is positively correlated with systemic insulin sensitivity (21); and decreased levels of type I fibers are associated with insulin resistant states including T2DM (22). Therefore, we employ transcriptional, protein and metabolomic analyses to determine the effects of inorganic nitrate on markers of the adaptive response to exercise in skeletal muscle, including fiber-type phenotype, and PGC1α and exercise-dependent myokine production. We demonstrate that nitrate induces physiological adaptations both in skeletal muscle, and systemically, that mimic exercise training and may underlie the beneficial effects of the small molecule on exercise tolerance, efficiency and performance, and cardiometabolic risk.

Research Design and Methods

Animal Experimentation. Male Wistar rats (6 weeks old) (269 ± 2 g) (Charles River) were weight matched and received either distilled water containing NaCl (0.7mM NaCl; n = 9) or water containing sodium nitrate ($NaNO_3$) (0.7 mM $NaNO_3$; n = 9) (Ultra-pure, Sigma-Aldrich) ad libitum for 18 days. Animals were housed in conventional cages at room temperature with a 12-hour/12-hour light/dark photoperiod. Morphological parameters are given in Table S1.

Muscle-specific PGC1α transgenic mice were generated and maintained as previously described (17). For the exercise experiments, 12-week-old B6 mice (Jackson Laboratory) were conditioned with 3 weeks of free wheel running (n = 6) (14; 17; 18). Controls were age matched sedentary littermates (n = 6).

All procedures were carried out in accordance with U.K. Home Office protocols by a personal license holder.

Blood and Tissue Collection. Rats and mice were euthanized (sodium pentobarbital 200 mg/ml, Vétoquinol UK). Blood was collected by cardiac puncture and centrifuged to obtain plasma. Soleus, gastrocnemius or quadriceps muscle were removed and flash frozen in liquid nitrogen.

C2C12 Culture. C2C12 myoblasts were grown to confluence in DMEM supplemented with 10% FBS. Differentiation was induced with DMEM containing 10% horse serum (HS, Sigma-Aldrich) and 850 nM insulin. During the 6 day differentiation, cells were cultured with either saline (control), 25 μM $NaNO_3$, or 500 μM $NaNO_3$ or, during the sodium nitrite studies ($NaNO_2$), with saline (control), 500 nM $NaNO_2$, 25 μM $NaNO_2$ or 500 μM $NaNO_2$ (Ultra-pure, Sigma-Aldrich). Differentiation of C2C12 cells is unaffected by $NaNO_3$ (23). Pharmacological agent studies utilized 2,2-Bipyridyl (100 μM), 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) (50 μM), N^G-nitro-L-arginine methyl ester (L-

NAME) (1 mM), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (1 μM), GW742 (100 nM) and sildenafil (1 μM) (Sigma-Aldrich). Cells were treated with 2,2-Bipyridyl, PTIO, L-NAME, or ODQ with and without 500 μM $NaNO_3$. $NaNO_3$ and pharmacological agents were added at day 1 of differentiation and maintained for 6 days with the exception of 2,2-Bipyridyl which was added 6h prior to the end of the experiment.

Primary Adipocyte Culture. Primary adipose stromal vascular cells were isolated, cultured and differentiated into adipocytes according to published methods (12; 17). During differentiation, cells were cultured with either saline (control), 500 μM $NaNO_3$ (Ultra-pure, Sigma-Aldrich), 125 ng/ml Irisin (BioVision Inc) or 125 ng/ml Irisin and 500 μM $NaNO_3$.

Small Interfering RNA PGC1α Knockdown. FlexiTube small interfering RNA (siRNA) against PGC1α, AllStars negative control siRNA, and HiPerFect Transfection Reagent were purchased from QIAGEN. C2C12 myotubes were transfected per the manufacturer's instructions (75 ng siRNA, 2 μL transfection reagent per well, 10 nmol/L final siRNA concentration) on days 2 and 4 of differentiation.

Metabolite Extraction. Metabolites were extracted from soleus muscle of nitrate treated rats, gastrocnemius and quadriceps muscle of exercise trained mice, plasma of nitrate treated rats, MCK-PGC1α transgenic mice, exercise trained mice and nitrate supplemented human volunteers, nitrate treated and PPARδ agonist treated C2C12 myotubes and culture media as previously described (12).

Gene Expression. Total RNA extraction from soleus and gastrocnemius muscle of nitrate treated rats, nitrate and irisin treated adipocytes and nitrate, GW742 PPARδ agonist, 2,2-Bipyridyl, PTIO, L-NAME, ODQ and sildenafil treated myocytes, cDNA conversion and quantitative RT-PCR was performed as per

published protocols (17). All data were normalized to 18SrRNA (mouse primary adipocytes, C2C12 myoblasts) or RLPL1 (rat muscle) and quantitative measures obtained using the Δ-Δ-C_T method.

Protein Analysis. PGC1α, MYH7and MYH2 were analyzed in nitrate treated rat soleus. Irisin was analyzed in nitrate supplemented human and rat plasma and nitrate, GW742 and sildenafil treated myocyte media. Rat and human growth hormone was measured in nitrate supplemented rat and human plasma, respectively. For all measurements analysis was performed using ELISA as per the manufacturer's instructions (PGC1α Kit SEH337Ra, MYH7 Kit SED418Ra, Cloud-Clone Corp. Houston, TX, USA) (MYH2 Kit, ABIN2093055, antibodies-online GmbH, Aachen, Germany) (Irisin Kit. K4761-100, BioVision Inc. Milpitas, CA, USA) (Rat Growth Hormone Kit E-EL-R0029, Elabscience, Beijing, China) (Human Growth Hormone Kit EK0578, Boster Biological Technology Co., Pleasanton, CA, USA).

Immunohistochemistry. Soleus and gastrocnemius cross sections from nitrate treated rats were prepared at a thickness of 10 μm using a cryostat. Immunohistochemical staining and fiber-type assessment was performed according to published methods (24). Antibodies were type I fiber, monoclonal antimyosin (skeletal, slow; clone NOQ7.5.4D; Sigma-Aldrich) and type II fiber, monoclonal antimyosin (skeletal, fast; alkaline phosphatase conjugate; clone MY-32; Sigma-Aldrich).

Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS and data analysis were performed on metabolites from nitrate treated rat soleus using the methods described previously (12).

Plasma Nitrate. Plasma nitrate from rats and humans was measured as described previously (12).

Liquid Chromatography-Mass Spectrometry (LC-MS). LC-MS analysis was performed on metabolites from the plasma of nitrate supplemented humans and rats, and MCK-PGC1α mice, soleus of

nitrate treated rats, nitrate and GW742 treated myotubes and culture media, and the gastrocnemius and quadriceps muscle of exercise trained mice using a 4000 QTRAP mass spectrometer (Applied Biosystems/Sciex), coupled to an Acquity UPLC (Waters Corporation, Manchester, UK) according to published methods (12; 17).

Human Study. A cross-over, randomized, placebo controlled, double-blind clinical trial of participants given either concentrated beetroot juice (2x70ml/day, 12.0 mmoles of nitrate) or nitrate-depleted beetroot juice (placebo, 2x70ml/day, 0.003 mmoles of nitrate) for 7 days was conducted. Nineteen participants completed the study. Mean age was 64.7 ± 3.0 years. Participants were healthy, non-obese volunteers (For BMI, sex distribution and age see table S2). Exclusion criteria included high resting blood pressure (Systolic>180mmHg and/or Diastolic >110mmHg), high physical activity level (>15000 steps per day), a weight change of more than 3.0kg in the last 2 months, diagnosis of metabolic, cardiovascular and inflammatory conditions or diabetes. Participants were randomized to: 1) Nitrate supplementation (beetroot juice 2x70ml/day, containing ~12.0 mmoles nitrate) or 2) Placebo (nitrate-depleted beetroot juice 2x70ml/day, containing ~0.003 mmoles nitrate) for 7 days. Nitrate-depleted beetroot juice was prepared according to published methods (25). After a 7 day wash-out period, the second 7-day intervention was conducted. Blood serum was collected at baseline and termination of each intervention and aliquoted. Participants were fasted for 12 hours prior, and avoided exercise 3 days prior, to each visit. Serum NOx concentrations were measured using GC-MS as previously described (26). The trial was approved by the North of Scotland Research Ethics committee (14/NS/0061).

Statistical Analyses. Error bars represent standard error of the mean. P-values were calculated by either t-test, one-way or two-way ANOVA as stated, with a Tukey's *post-hoc* test. Human study data was analyzed using paired t-test.

Results

Nitrate Promotes Fiber-type Switching in the Oxidative Soleus Muscle

PGC1α activation may underlie the effects of nitrate on exercise; therefore we analyzed the effect of nitrate on PGC1α expression in skeletal muscle *in vivo*. Wistar rats were treated with 0.7mM NaCl or 0.7 mM $NaNO_3$ in drinking water for 18 days. Plasma nitrate concentrations increased from 7.1 ± 1.0 μM in NaCl treated control rats to 12.8 ± 1.1 μM in rats treated with 0.7 mM $NaNO_3$ ($P \leq 0.001$). Water and food intake was not significantly different between groups (Table S1). Nitrate increased PGC1α expression in the soleus muscle of rats (Fig. 1*A*).

Hypoxia-inducible factor 2α (HIF2α) is a skeletal muscle-selective PGC1α transcriptional target that is positively regulated by exercise and has a key role in muscle function (14). HIF2α expression was increased in the soleus of nitrate treated rats (Fig. 1*B*). PGC1α and HIF2α coordinate to induce fiber-type switching from glycolytic type IIb fibers to intermediate type IIa and oxidative type I fibers as an adaptation to exercise training (13; 14). Nitrate also increased the expression of Myosin Heavy Chain 7 (MYH7), a fundamental constituent of type I muscle fiber, and Myosin Heavy Chain 2 (MYH2), an important component of type IIa fibers (Fig. 1*C*). Concomitantly, the expression of Myosin Heavy Chain 4 (MYH4), specific to fast-twitch type IIb fibers, was decreased. Expression of additional genes characteristic of slow-twitch muscle fibers such as Myoglobin (Mb) and Calmodulin2 (CALM2) was increased in the soleus by nitrate treatment (Fig. 1*C*).

To determine whether transcriptional changes in fiber-type specific genes translated into corresponding changes in protein levels, the concentrations of PGC1α, MYH2 and MYH7 protein in the soleus of nitrate treated rats were analyzed using ELISA. Nitrate increased the protein concentration of PGC1α (Fig. 1*D*), MYH7 and MYH2 (Fig. 1*E* and 1*F*).

An increase in type I fibers drives a switch in energetic fuel usage away from glycolytic metabolism. Therefore, we employed GC-MS-based metabolomics to analyze glycolytic intermediates from soleus muscle of nitrate treated rats. Nitrate decreased the concentrations of the key glycolytic intermediates glucose-6-phosphate (Fig. 1*G*) and 3-phosphoglycerate (Fig. 1*H*).

Consistent with these data, immunohistochemical analysis of soleus muscle from control and nitrate-treated rats confirms nitrate induces a decrease in constituent type II fibers and increase in type I fibers (Fig. 1*I* and 1*J*).

Nitrate Promotes Fiber-type Switching in the Glycolytic Gastrocnemius Muscle

To establish whether the effects in slow-twitch soleus were muscle type specific, the expression of genes determining fiber-type were examined in the mixed fiber-type gastrocnemius muscle of nitrate treated rats. Nitrate increased PGC1α and HIF2α expression in the gastrocnemius (Fig. 2*A*). The expression of the type I and type IIa fiber markers MYH7, Mb and MYH2 were increased to a greater extent in gastrocnemius than in soleus following nitrate treatment; possibly representing the greater capacity for gastrocnemius to undergo a switch towards oxidative fiber-types compared to the already highly oxidative soleus. Nitrate also decreased the expression of the type IIb fiber component, MYH4 (Fig. 2*A*).

Histological examination of the gastrocnemius muscle of nitrate treated rats confirms an increase in type I muscle fibers and a concomitant decrease in type II muscle fibers (Fig. 2*B* and 2*C*).

Taken together, these data suggest that nitrate is an inducer of muscle fibre-type switching, increasing the proportion of oxidative slow-twitch fibers to fast-twitch glycolytic fibers within muscle.

Nitrate Promotes an Exercise Training-like Phenotype in Myotubes via a NO-Mediated Mechanism

We next focused on establishing the mechanism underlying nitrate-induced fiber-type switching. C2C12 mouse myoblasts were differentiated into myotubes in the presence of nitrate to establish whether nitrate functions directly at the muscle to increase type I fiber-associated gene expression. Under normoxic conditions HIF2α protein is hydroxylated by prolyl hydroxylase domain containing enzymes (PHDs), ubiquitinated and degraded by the proteasome (27). HIF2α protein must be stabilized, following PGC1α-induced expression, to activate fiber-type switching (14); therefore the myotubes were also treated for 6 hours with 2,2-bipyridyl to inactivate PHDs and stabilize HIF2α. Expression of PGC1α was significantly and similarly increased in myotubes treated with either nitrate alone or nitrate in combination with 2,2-bipyridyl (Fig. 3*A*). Following HIF2α stabilization, treatment of myotubes with nitrate increased expression of type I and type IIa fiber-type genes and suppressed expression of type IIb fiber markers (Fig. 3*B*). Interestingly, nitrate in the absence of additional HIF2α stabilization also increased expression of type I and type IIa fiber-type markers, albeit to a lesser extent than nitrate in combination with 2,2-bipyridyl (Fig. 3*B*).

NO can be generated directly from nitrate via non-enzymatic processes and the enzymatic nitrate-nitrite-NO pathway *in vivo* (2; 12). NO increases PGC1α expression in skeletal muscle and inhibits HIF complex prolyl hydroxylases, stabilizing the HIF complex (6; 28). Therefore, C2C12 mouse myotubes were treated with a range of nitrate doses during differentiation (25 μM and 500 μM) to determine whether nitrate-induced fiber-type switching would occur in a dose-dependent manner, in the absence of additional HIF stabilization. The 500 μM nitrate dose corresponds to plasma concentrations in mice exhibiting improved metabolic phenotypes when chronically dosed with 0.1 mmol/kg $NaNO_3$ (4). Nitrate treatment alone significantly increased PGC1α and HIF2α expression (Fig. 3*C*). Also increased in expression in nitrate-treated myotubes were type I fiber-associated genes. The expression of type IIb

fiber-associated genes was decreased by nitrate. These data suggest nitrate-mediated induction of fiber-type gene expression occurs in skeletal muscle and does not require additional HIF stabilization.

We next examined whether the nitrate-stimulated expression of type I fiber-associated genes was NO-dependent. Myotubes were differentiated in the presence of nitrate and the NO scavenger PTIO. NO sequestration by PTIO abrogated nitrate-induced expression of type I and type IIa fiber-associated genes (Fig. 3*C*). Nitrite is the initial reduction product in the nitrate-nitrite-NO pathway and was also found to increase expression of type I fiber-associated genes in myotubes (Fig. 3*D*). The effect of nitrite was also found to be abrogated by NO sequestration (Fig. S1)

To eliminate the canonical L-arginine-nitric oxide synthase-NO pathway as the source of NO stimulating the fiber-type switching effect of nitrate, myotubes were co-treated with nitrate and the NOS inhibitor L-NAME. NOS inhibition did not prevent nitrate-stimulated expression of type I fiber-associated genes (Fig. S2). Therefore, the nitrate/NO-mediated fiber-type switching effect is independent of NOS.

Nitrate may augment the fiber-type switching effect observed in muscle following exercise training (29); therefore myotubes were treated with an exercise mimetic, the peroxisome proliferator-activated receptor δ (PPARδ) agonist GW742 (30), both alone and in combination with nitrate. Co-treatment of myotubes with nitrate and GW742 had an additive effect on type I fiber-associated gene expression (Fig. 3*E*). Thus, nitrate may augment muscle fiber-type switching in an exercise training background.

Nitrate-Induced Muscle Fiber-type Switching is mediated by PGC1α

The increases in PGC1α expression and protein concentration in skeletal muscle and myotubes likely mediates the nitrate-induced fiber-type switch. Therefore, the expression of PGC1α in C2C12 myotubes

was knocked down by ~60% using siRNA (Fig. 4*A*). Reduced PGC1α expression abrogated the nitrate-induced expression of type I and type IIa fiber genes in myotubes (Fig. 4*B*).

Nitrate-Mediated Muscle Fiber-type Switching Functions through Soluble Guanylyl Cyclase and is Enhanced by Sildenafil

We next sought to identify the downstream signaling cascade mediating nitrate/NO-induced muscle fiber-type switching. The principal downstream effector of NO is cGMP. NO activates cGMP signaling through stimulation of soluble guanylyl cyclase (sGC). The inhibitor of sGC, ODQ, abrogated the nitrate-induced expression of type I fiber-associated genes in myotubes (Fig. 4*C*).

The secondary messenger cGMP is degraded by Cyclic Nucleotide Phosphodiesterase 5 (PDE5). Interestingly, the expression of PDE5 is increased in nitrate treated myotubes, and may represent a counter-regulatory response (Fig. 4*D*). The PDE5 inhibitor, sildenafil, increases PGC1α expression in myocytes, reduces muscle fatigue and improves exercise efficiency (31; 32). Therefore, we hypothesized that PDE5 inhibition would enhance nitrate-mediated fiber-type switching. The expression of type I muscle fiber-associated genes in myotubes was increased by sildenafil; an effect further enhanced by co-treatment with nitrate (Fig. 4*E*).

Nitrate Activates Secretion of the PGC1α/Exercise-dependent Myokine FNDC5/irisin

Exercise-conditioned skeletal muscle is the source of hormone-like signals, known as myokines, which participate in organ cross-talk (17; 18). The myokine, fibronectin type III domain-containing 5/irisin (FNDC5/irisin) is expressed and secreted in a PGC1α-dependent manner (18). As nitrate increases PGC1α expression in skeletal muscle, we asked whether the nitrate-induced exercise training-like effect in myotubes increased irisin production and secretion. Nitrate increased FNDC5 expression in myotubes in an NO-dependent manner (Fig. 5*A*). Interestingly, FNDC5 expression in myotubes was increased by

PDE5 inhibition using sildenafil and further increased by co-treatment with both nitrate and sildenafil (Fig. 5*B*). The secretion of irisin from myotubes into serum-free culture media was increased following nitrate treatment, an effect also observed following treatment of myotubes with an exercise mimetic, GW742 (30) (Fig. 5*C*).

Having identified nitrate-stimulated expression and secretion of irisin from myotubes *in vitro*, we investigated whether this effect was translated *in vivo*. FNDC5 expression in the soleus (Fig. 5*D*) and gastrocnemius (Fig. 5*E*) of nitrate treated rats was increased compared to control animals. Increased irisin expression in the soleus and gastrocnemius muscles was reflected in a significant increase in the plasma irisin concentration of nitrate treated rats (Fig. 5*F*).

We then asked whether the effect of nitrate on plasma irisin concentrations in rodents translated into humans. A cross-over, randomized, placebo controlled, double-blind clinical trial of participants given either concentrated beetroot juice (2x70ml/day, 12.0 mmoles of nitrate) or nitrate-depleted beetroot juice (placebo, 2x70ml/day, 0.003 mmoles of nitrate) for 7 days was implemented (Table S2). Following a 7 day wash-out period, participants were crossed-over to the reciprocal intervention. Nitrate treatment significantly increased both the fasted serum nitrogen oxides (NOx) (Fig. 5*G*) and serum irisin concentrations (Fig. 5*H*) of the participants.

Nitrate and Irisin Function Additively to Induce Adipocyte "Browning"

Irisin induces a brown adipose-like phenotype in WAT depots in a PPARα-dependent manner (18). Nitrate activates WAT browning via a PPARα-independent nitrate-NO-cGMP signaling axis (12). Given their distinct signaling mechanisms, we hypothesized that irisin and nitrate may have an additive effect on brown adipocyte-associated gene expression in primary adipocytes. Murine primary adipocytes treated with a combination of both irisin and nitrate exhibited a greater expression of brown adipocyte-

associated genes than adipocytes treated with irisin or nitrate alone (Fig. S3). Therefore nitrate may enhance the exercise-induced, irisin-mediated browning of WAT.

Nitrate Induces the Secretion of the PGC1α/Exercise-dependent Myokine-like Small Molecule β-aminoisobutyric Acid

Exercise stimulates the release of β-aminoisobutyric acid (BAIBA) from skeletal muscle (17). BAIBA is an exercise training and PGC1α-dependent myokine-like small molecule signal with effects on both WAT and hepatic metabolism. Therefore, we employed LC-MS to profile metabolites in nitrate-treated myotubes and in serum-free conditioned cell media. Nitrate significantly increased the intracellular (Fig. 6*A*) and extracellular BAIBA concentrations (Fig. 6*B*). Nitrate also increased the expression of BAIBA biosynthetic enzymes acyl-CoA dehydrogenase short chain (ACADS) and hydroxyacyl-CoA dehydrogenase (HADHA) in myotubes in an NO-dependent manner (Fig. 6*C*).

We next used LC-MS to profile the metabolites in soleus muscle and plasma from nitrate treated rats. Nitrate significantly increased the BAIBA concentration (Fig. 6*D*) and the expression of BAIBA biosynthetic genes, ACADS and HADHA, in the soleus muscle of the nitrate treated rats (Fig. 6*E*). Plasma BAIBA concentration was also increased by nitrate treatment (Fig 6*F*).

Nitrate Increases Plasma γ-Aminobutyric Acid Concentrations in Rats and Humans

Interestingly, LC-MS profiling identified an increase in the plasma γ-aminobutyric acid (GABA) concentration of nitrate treated rats (Fig. 7*A*), with a concomitant decrease in the GABA metabolic precursor glutamine (Fig. 7*B*). This observation was reproduced in humans, with 7 day nitrate supplementation increasing serum GABA (Fig. 7*C*) and decreasing the serum glutamine concentration (Fig. 7*D*). GABA is also enriched in the media of transgenic PGC1α-expressing myocytes (17) and in the muscle of mice with muscle-specific transgenic expression of PGC1α (MCK-PGC1α) (33). We used

LC-MS to analyze the plasma GABA concentrations of MCK-PGC1α mice. The plasma GABA concentration was significantly increased by PGC1α forced expression in muscle *in vivo* (Fig. 7*E*).

Since nitrate increased PGC1α expression in myotubes, we applied LC-MS profiling to serum-free media, conditioned for 24 h on myotubes treated with nitrate or the exercise mimetic GW742 (30). GABA was significantly enriched in the media of both nitrate and GW742 treated myotubes (Fig. 7*F*). The intracellular GABA concentration was also increased in nitrate treated myotubes (Fig. 7*G*).

As GABA was increased in myocyte media by nitrate treatment and by forced PGC1α expression, and was increased in the plasma of nitrate treated rats and humans, we examined whether concentrations of GABA were increased following exercise training in the plasma (Fig. 7*H*), gastrocnemius (Fig 7*I*) and quadriceps (Fig 7*J*) muscles of mice conditioned with 3 weeks free wheel running (17; 18). Exercise training increased the GABA concentration in both muscle and plasma. These data suggest that PGC1α stimulates the biosynthesis and release of GABA from skeletal muscle during exercise training and nitrate treatment.

Dietary Nitrate Increases Plasma Growth Hormone Concentrations of Rats and Humans

Long-term exercise training, NO, and plasma GABA increase the plasma concentration of growth hormone (34-36). Nitrate increased the circulating growth hormone concentrations of nitrate treated rats (Fig. 7*K*) and nitrate supplemented humans (Fig. 7*L*). Together, these studies demonstrate that nitrate treatment mimics several physiological effects of exercise training both on skeletal muscle and systemically, and suggest GABA as a novel, PGC1α-mediated, exercise and nitrate-stimulated myokine-like small molecule signal.

Discussion

Exercise training demonstrates significant anti-diabetic effects (19). The ubiquitous dietary anion, nitrate, improves submaximal and high intensity exercise efficiency (9), tolerance (10), and performance (11). As a result, dietary nitrate supplementation is growing in popularity as an augmentation to exercise training. We show that nitrate increases PGC1α expression and a switch from fast-twitch type IIb towards oxidative slow-twitch type I and intermediate type IIa fibers in both the soleus and gastrocnemius muscle; a phenomenon typical of exercise training, and consistent with the effects observed on exercise, mitochondrial fatty acid oxidation and efficiency in muscle predominately consisting of type I and type IIa fibers (9; 23).

T2DM is accompanied by perturbations to muscle physiology, resulting in decreased mitochondrial content and abnormal lipid deposition, impairing the insulin-mediated switch between fat and carbohydrate metabolism (37). Dietary nitrate supplementation exhibits anti-obesity and anti-diabetic properties in rodents; improving homeostatic model assessment for insulin resistance and quantitative insulin sensitivity check index assessments in high-fructose diet-induced insulin resistant rats (5), and improving glucose tolerance in NOS-deficient mice (4); while reducing adiposity in both models. We have shown that nitrate increases muscle β-oxidation and mitochondrial biogenesis (23), and therefore may have utility in treating the intramuscular lipid deposition-induced lipotoxicity and insulin resistance associated with diabetes. However, diabetic myopathy is a major complication of diabetes and is characterized by muscle atrophy and reduced physical performance and muscle capacity (20). Skeletal muscle from diabetic patients exhibits a higher concentration of glycolytic fibers (38), and evidence of a switch towards a glycolytic phenotype (39). Indeed, the proportion of muscle type I fibers is positively correlated with insulin sensitivity (21); and decreased levels of type I fibers are associated with insulin resistance (22). Relative to glycolytic fibers, oxidative fibers are more resistant to atrophy following denervation or aging. Since, the concentrations of nitrate utilized in this study are achievable in humans

following dietary intervention (4); nitrate supplementation may provide a means of stimulating exercise-like fiber-type switching effects in the muscle of diabetic patients to treat perturbed muscle phenotypes. However, a small number of human trials assessing the anti-diabetic effects of short-term nitrate supplementation have thus far proved disappointing (40); it remains to be seen whether larger, longer-term studies in diabetic or pre-diabetic populations (in which disease is less advanced) will demonstrate the beneficial effects observed in rodent models.

Our data suggests that nitrate may be additive to the effects of exercise training on muscle phenotypes, complimenting studies by De Smet at al. showing sprint interval training, in combination with nitrate supplementation, increases the proportion of type IIa fibers in muscle in humans (29). Indeed, type I and type IIa muscle fibers are more efficient at performing, and have a greater capacity for, oxidative metabolism than type IIb muscle fibers (41; 42). A higher percentage of type I muscle correlates positively with exercise efficiency in exercise tests (43; 44); meaning that a higher percentage of type I muscle fibers improves endurance performance by significantly increasing the power output generated for a given rate of oxygen consumption and energy expenditure (43; 44). Therefore, the switching of muscle fibers from type IIb to types IIa and I (as we observe with nitrate supplementation) decreases the oxygen cost of endurance exercise, making our findings complementary to previous observations that dietary nitrate lowers the oxygen cost of exercise. Subsequently, nitrate-induced fiber-type switching likely contributes to the observed effects of nitrate on exercise.

Furthermore, we identify that the nitrate-mediated induction of fiber-type switching occurs, at least in part, through an NO-PGC1α-dependent mechanism. Rasbach et al. identified that PGC1α induced fiber-type switching requires functional, stabilized, HIF2α (14). Others have demonstrate that NO functions to stabilize HIF (6; 28). Since, we find that nitrate-induced fiber type switching occurs without extrinsic HIF stabilization, our work may suggest that NO generated as a result of nitrate reduction functions to

stabilize HIF2α during the nitrate-PGC1α-stimulated expression of type I and type IIa fiber-type markers.

It is worth noting that our *in vitro* mechanistic studies, implicating NO in nitrate-induced expression of fiber-type markers, were conducted in non-contracting myotubes. It will be of consequence for future studies to examine these mechanisms in models of exercising muscle. Moreover, while a physiological, enzymatic mechanism for the reduction of nitrate to NO in mammalian tissue has been suggested (2; 45), numerous proteins have been implicated in this reductive activity (2; 12; 46; 47). In addition, nitrate reduction has been observed in both hypoxia and normoxia (2; 12; 45). Therefore, the mechanisms underlying reduction of nitrate to NO in skeletal muscle, and the involvement of NO in the downstream effects of nitrate, remain to be fully determined.

Exercise-induced PGC1α expression in skeletal muscle contributes to the beneficial cardiometabolic effects of exercise through myokine signaling (17; 18). We find that nitrate regulates the exercise and PGC1α-dependent myokine FNDC5/Irisin and myokine-like small molecule BAIBA. BAIBA alters energy metabolism in adipose tissue and liver (17), whereas irisin stimulates the browning of adipose tissue (18). Here, nitrate and irisin are seen to function additively to induce brown adipocyte-specific gene expression in adipocytes; therefore nitrate supplementation on an exercise-trained background may augment the exercise-dependent activation of WAT browning. It is also reasonable to speculate that the nitrate-induced secondary release of myokine factors may contribute to the anti-diabetic effects of nitrate.

Unexpectedly, this study highlighted a PGC1α and exercise-induced mechanism mediating muscle production and release of GABA. Nitrate activated this pathway, increasing circulating GABA concentrations in rodents and humans. Peripheral GABA increases plasma growth hormone

concentration in humans (35). Independently, NO signaling has been implicated as a pathway mediating the exercise-stimulated increase in circulating growth hormone (48), and directly increases growth hormone release (36). Exercise is one of the most efficacious stimuli for growth hormone secretion, however the exact mechanisms remain to be defined. We observe that nitrate treatment increases the circulating growth hormone concentration in rats and humans. Therefore, GABA may function as a PGC1α-dependent myokine-like small molecule signal, with biosynthesis and secretion triggered by both exercise and nitrate. Our investigations highlight an interaction linking NO and PGC1α to muscle GABA secretion and peripheral GABA concentration, in a pathway that may contribute to exercise-stimulated growth hormone release in the adaptive response to exercise.

This investigation highlights that nitrate induces exercise training-like physiological adaptations both in skeletal muscle and systemically. GABA may function as an exercise and nitrate-stimulated, PGC1α-mediated, myokine-like small molecule, and the exercise mimetic effects of nitrate may contribute to the beneficial effects of nitrate on both exercise and cardiometabolic health.

Author Contributions

Dr. Lee Roberts is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors declare they have no conflicts of interest. LDR conceived, designed, and performed the experiments, and wrote the manuscript with input from all co-authors. TA performed animal and cell studies. BDM performed cell experiments. SAM performed GC-MS. BOF and MF performed nitrate measurements. RS assisted with animal studies. MS and EAW conceived, designed and performed the human experiments. AJM conceived and designed the animal studies. JLG contributed to the manuscript.

Acknowledgments

LDR is supported by the Diabetes UK RD Lawrence Fellowship (16/0005382) and the MRC-HNR Elsie Widdowson Fellowship. This work was supported by the BBSRC (Bb/H013539/2; bb/I000933/I), the British Heart Foundation and the MRC (UD99999906).

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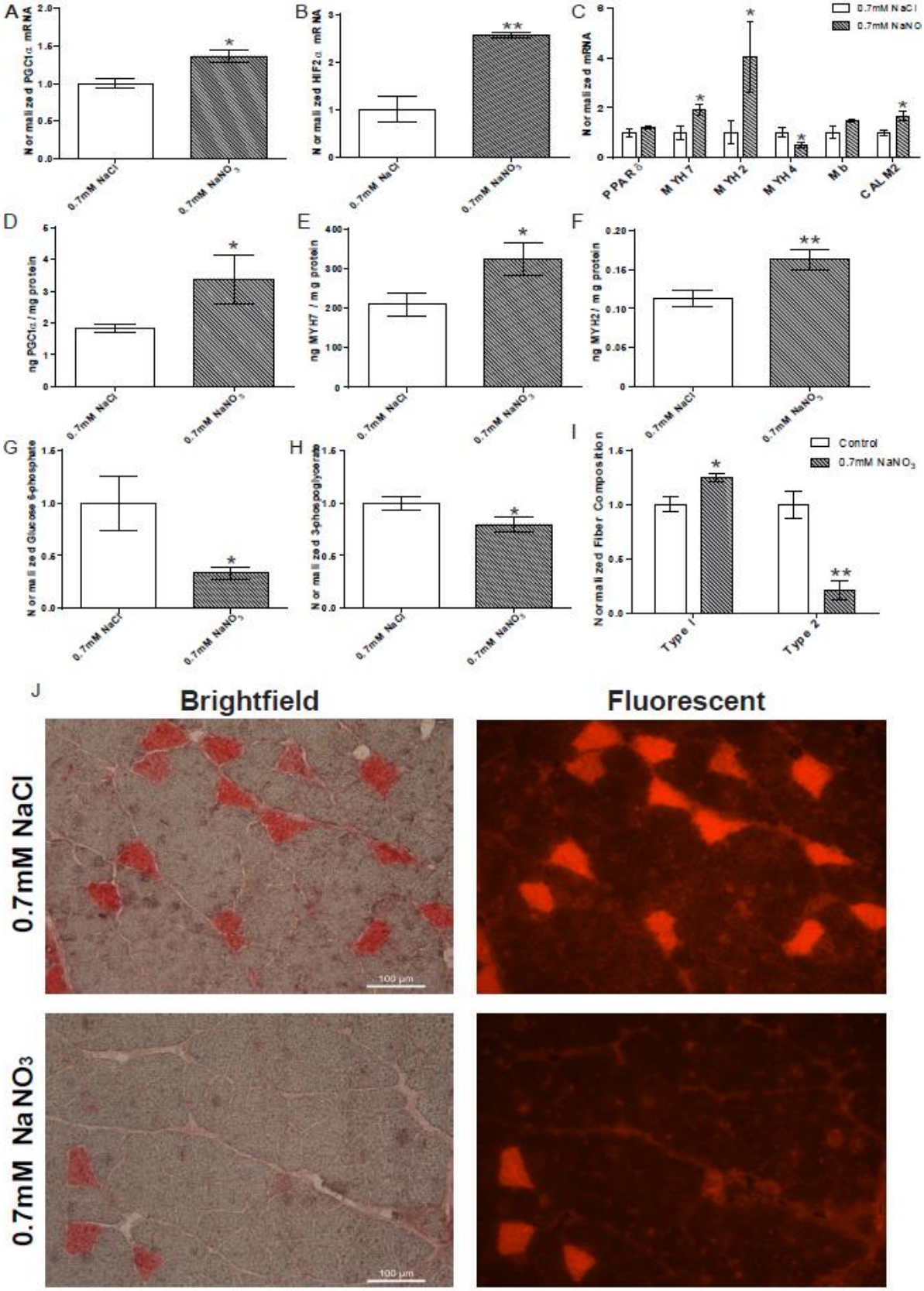
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Normalized PGC1α mRNA
0.7mM NaCl
0.7mM NaNO3
B
Normalized HIF2α mRNA
C
Normalized mRNA
0.7mM NaCl
0.7mM NaNO
PPARδ
MYH7
MYH2
MYH4
Mb
CALM2
D
ng PGC1α / mg protein
E
ng MYH7 / mg protein
F
ng MYH2 / mg protein
G
Normalized Glucose 6-phosphate
H
Normalized 3-phosphoglycerate
I
Normalized Fiber Composition
Control
0.7mM NaNO3
Type 1
Type 2
J
Brightfield
Fluorescent
0.7mM NaCl
0.7mM NaNO3
100 µm

Figure 1

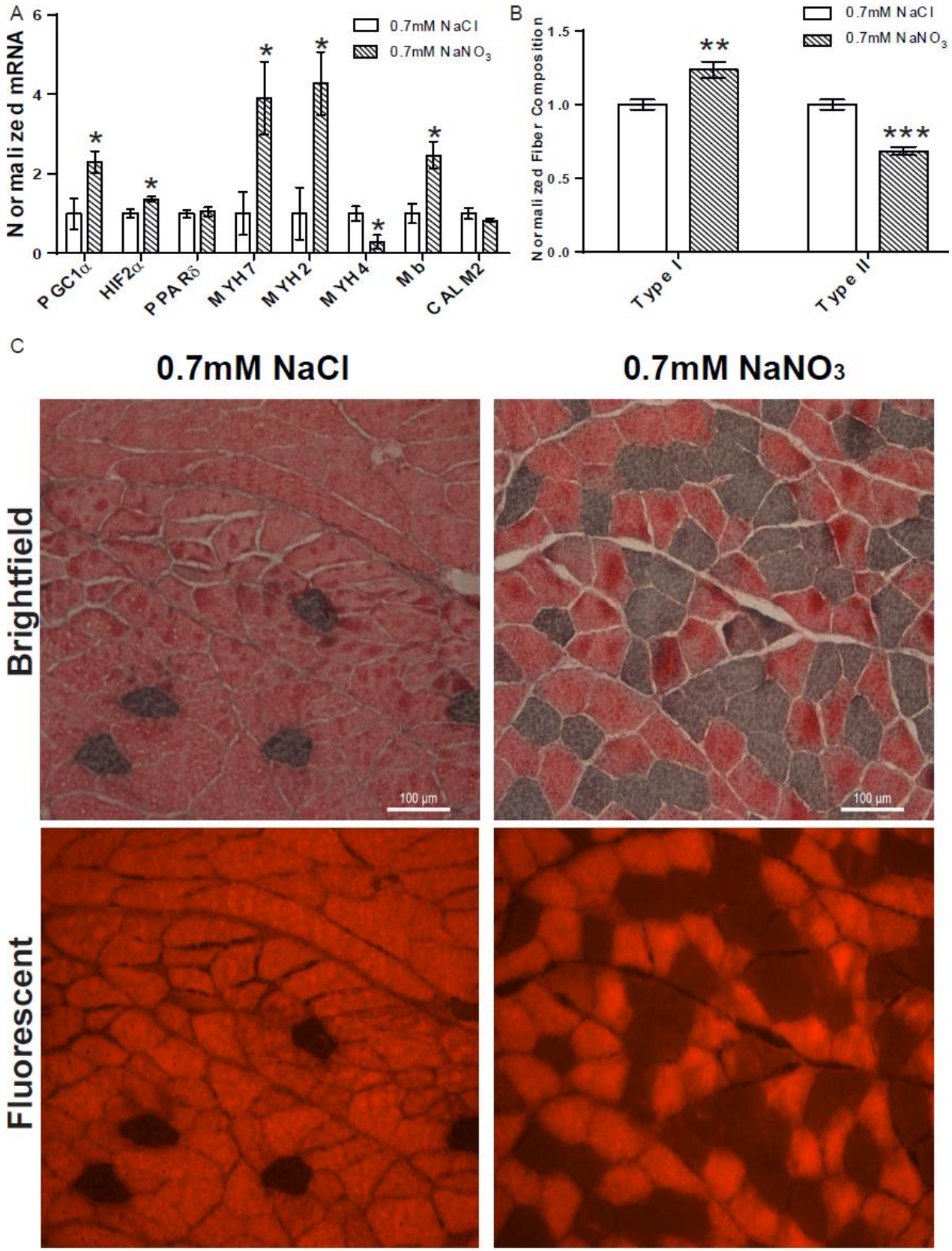
A
Normalized mRNA
0.7mM NaCl
0.7mM NaNO3
PGC1α
HIF2α
PPARδ
MYH7
MYH2
MYH4
Mb
CALM2
B
Normalized Fiber Composition
Type I
Type II
C
0.7mM NaCl
0.7mM NaNO3
Brightfield
Fluorescent
100 μm
100 μm

Figure 2

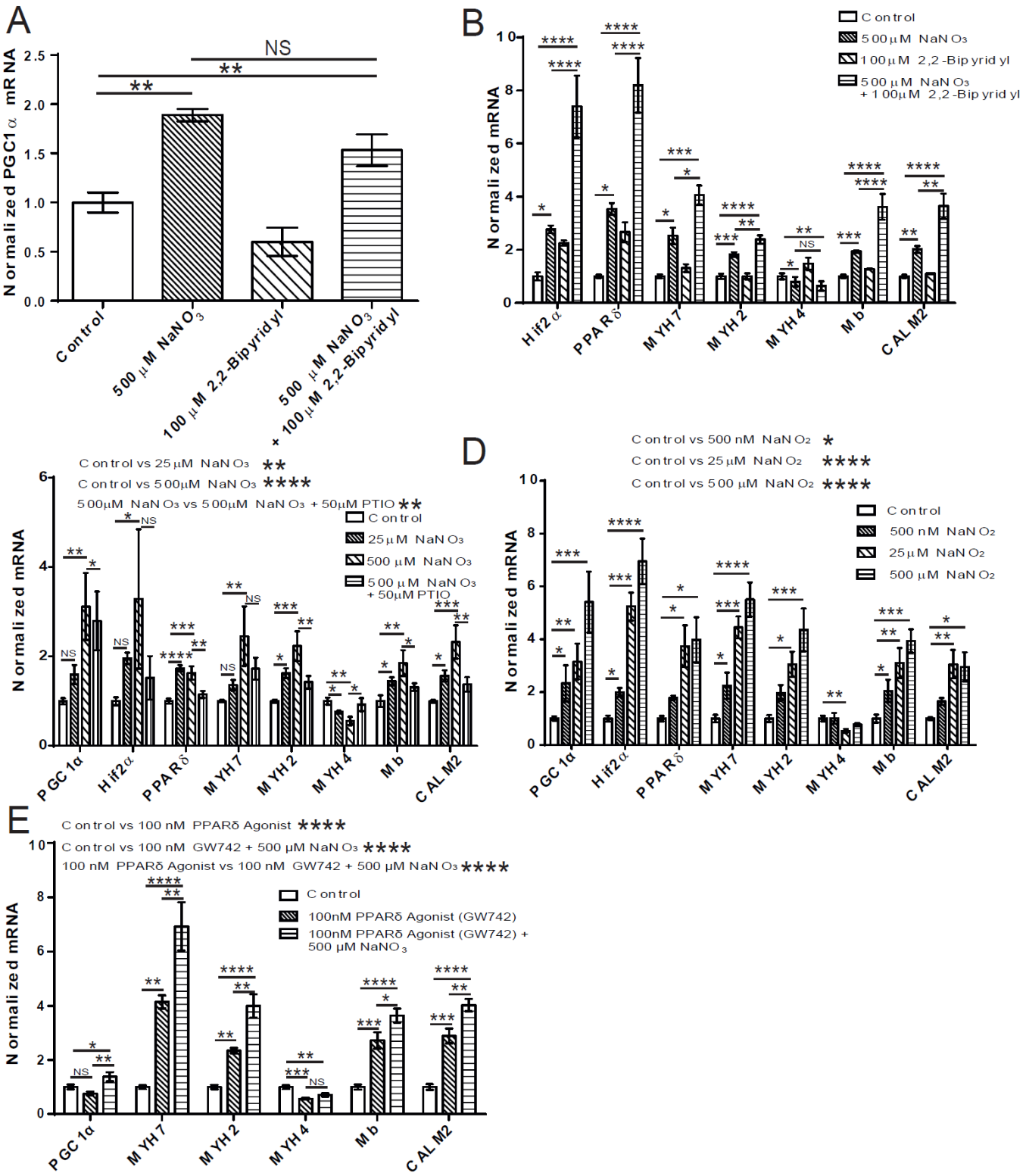
A
Normalized PGC1α mRNA
Control
500 μM NaNO3
100 μM 2,2-Bipyridyl
500 μM NaNO3 + 100 μM 2,2-Bipyridyl
B
Normalized mRNA
Control
500 μM NaNO3
100 μM 2,2-Bipyridyl
500 μM NaNO3 + 100 μM 2,2-Bipyridyl
Hif2α
PPARδ
MYH7
MYH2
MYH4
Mb
CALM2
C
Control vs 25 μM NaNO3 **
Control vs 500 μM NaNO3 ****
500 μM NaNO3 vs 500 μM NaNO3 + 50 μM PTIO **
Control
25 μM NaNO3
500 μM NaNO3
500 μM NaNO3 + 50 μM PTIO
PGC1α
Hif2α
PPARδ
MYH7
MYH2
MYH4
Mb
CALM2
D
Control vs 500 nM NaNO2 *
Control vs 25 μM NaNO2 ****
Control vs 500 μM NaNO2 ****
Control
500 nM NaNO2
25 μM NaNO2
500 μM NaNO2
E
Control vs 100 nM PPARδ Agonist ****
Control vs 100 nM GW742 + 500 μM NaNO3 ****
100 nM PPARδ Agonist vs 100 nM GW742 + 500 μM NaNO3 ****
Control
100nM PPARδ Agonist (GW742)
100nM PPARδ Agonist (GW742) + 500 μM NaNO3

Figure 3

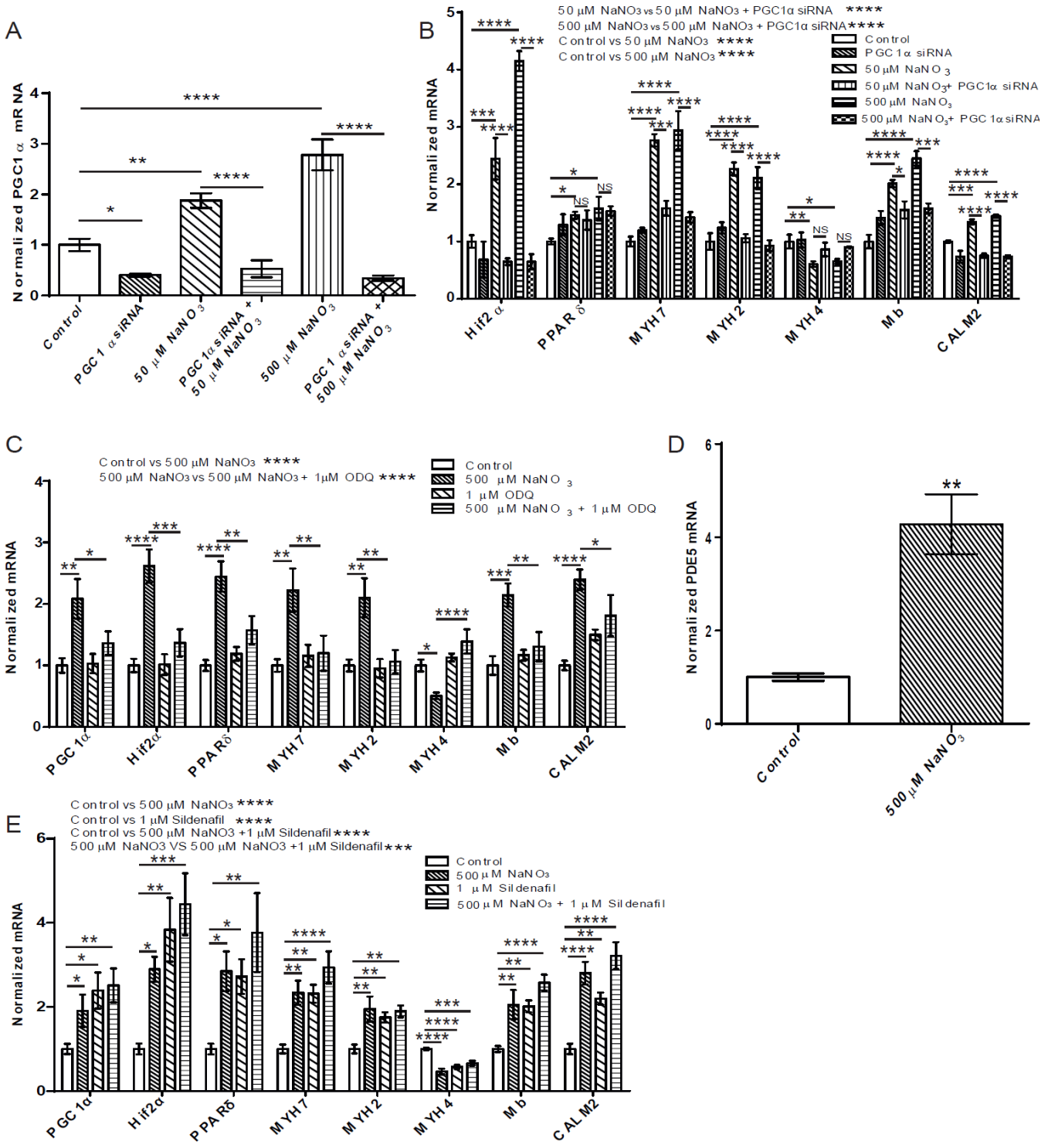
A
Normalized PGC1α mRNA
Control
PGC1α siRNA
50 μM NaNO3
PGC1α siRNA + 50 μM NaNO3
500 μM NaNO3
PGC1α siRNA + 500 μM NaNO3
B
50 μM NaNO3 vs 50 μM NaNO3 + PGC1α siRNA ****
500 μM NaNO3 vs 500 μM NaNO3 + PGC1α siRNA ****
Control vs 50 μM NaNO3 ****
Control vs 500 μM NaNO3 ****
Control
PGC1α siRNA
50 μM NaNO3
50 μM NaNO3+ PGC1α siRNA
500 μM NaNO3
500 μM NaNO3+ PGC1α siRNA
Normalized mRNA
Hif2α
PPARδ
MYH7
MYH2
MYH4
Mb
CALM2
C
Control vs 500 μM NaNO3 ****
500 μM NaNO3 vs 500 μM NaNO3 + 1μM ODQ ****
Control
500 μM NaNO3
1 μM ODQ
500 μM NaNO3 + 1 μM ODQ
Normalized mRNA
PGC1α
Hif2α
PPARδ
MYH7
MYH2
MYH4
Mb
CALM2
D
Normalized PDE5 mRNA
Control
500 μM NaNO3
E
Control vs 500 μM NaNO3 ****
Control vs 1 μM Sildenafil ****
Control vs 500 μM NaNO3 +1 μM Sildenafil ****
500 μM NaNO3 VS 500 μM NaNO3 +1 μM Sildenafil ***
Control
500 μM NaNO3
1 μM Sildenafil
500 μM NaNO3 + 1 μM Sildenafil
Normalized mRNA
PGC1α
Hif2α
PPARδ
MYH7
MYH2
MYH4
Mb
CALM2

Figure 4

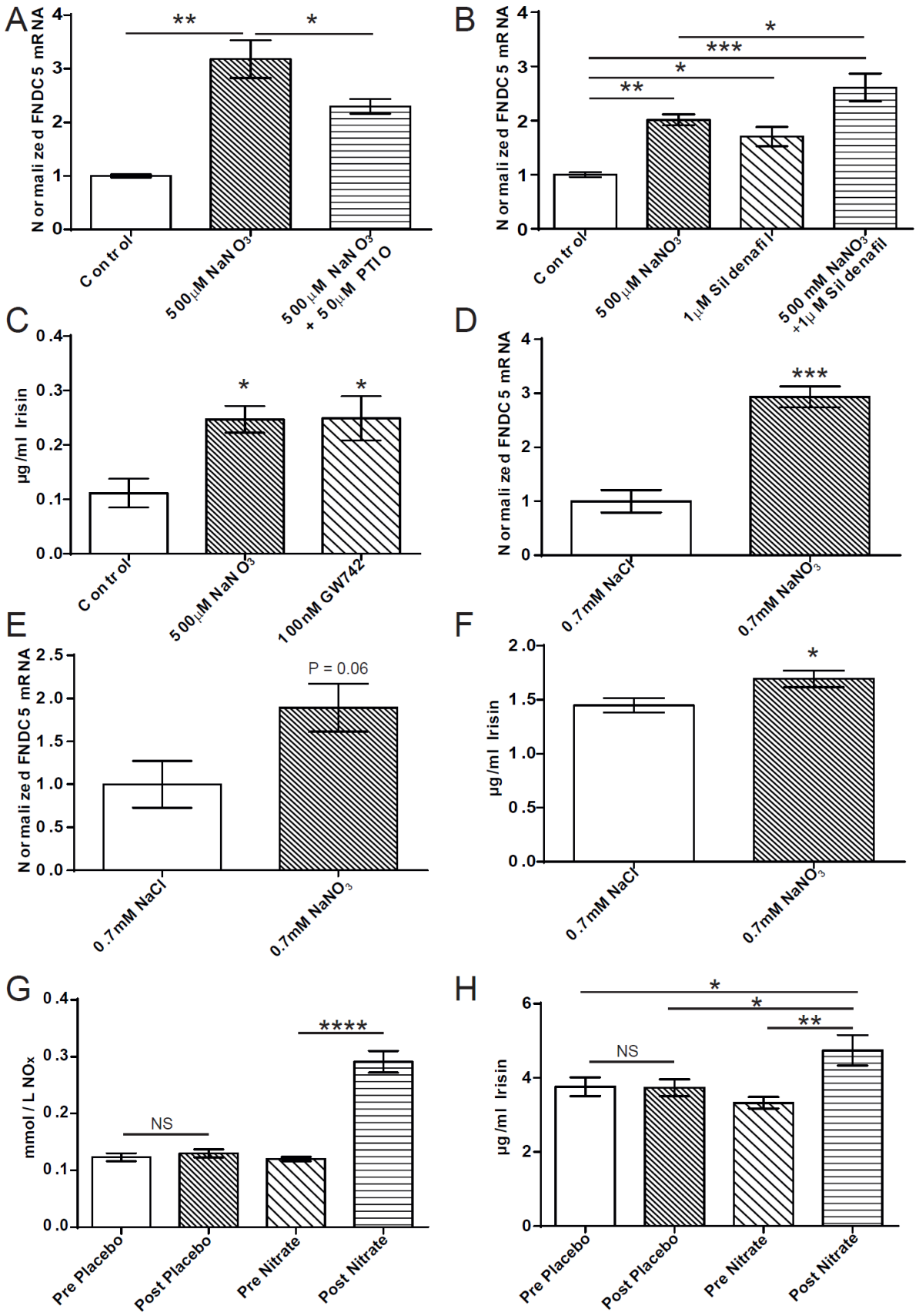
A
Normalized FNDC5 mRNA
Control
500μM NaNO3
500μM NaNO3 + 50μM PTIO
B
Normalized FNDC5 mRNA
Control
500μM NaNO3
1μM Sildenafil
500 mM NaNO3 +1μM Sildenafil
C
μg/ml Irisin
Control
500μM NaNO3
100nM GW742
D
Normalized FNDC5 mRNA
0.7mM NaCl
0.7mM NaNO3
E
Normalized FNDC5 mRNA
P = 0.06
0.7mM NaCl
0.7mM NaNO3
F
μg/ml Irisin
0.7mM NaCl
0.7mM NaNO3
G
mmol/L NOx
NS
Pre Placebo
Post Placebo
Pre Nitrate
Post Nitrate
H
μg/ml Irisin
NS
Pre Placebo
Post Placebo
Pre Nitrate
Post Nitrate

Figure 5

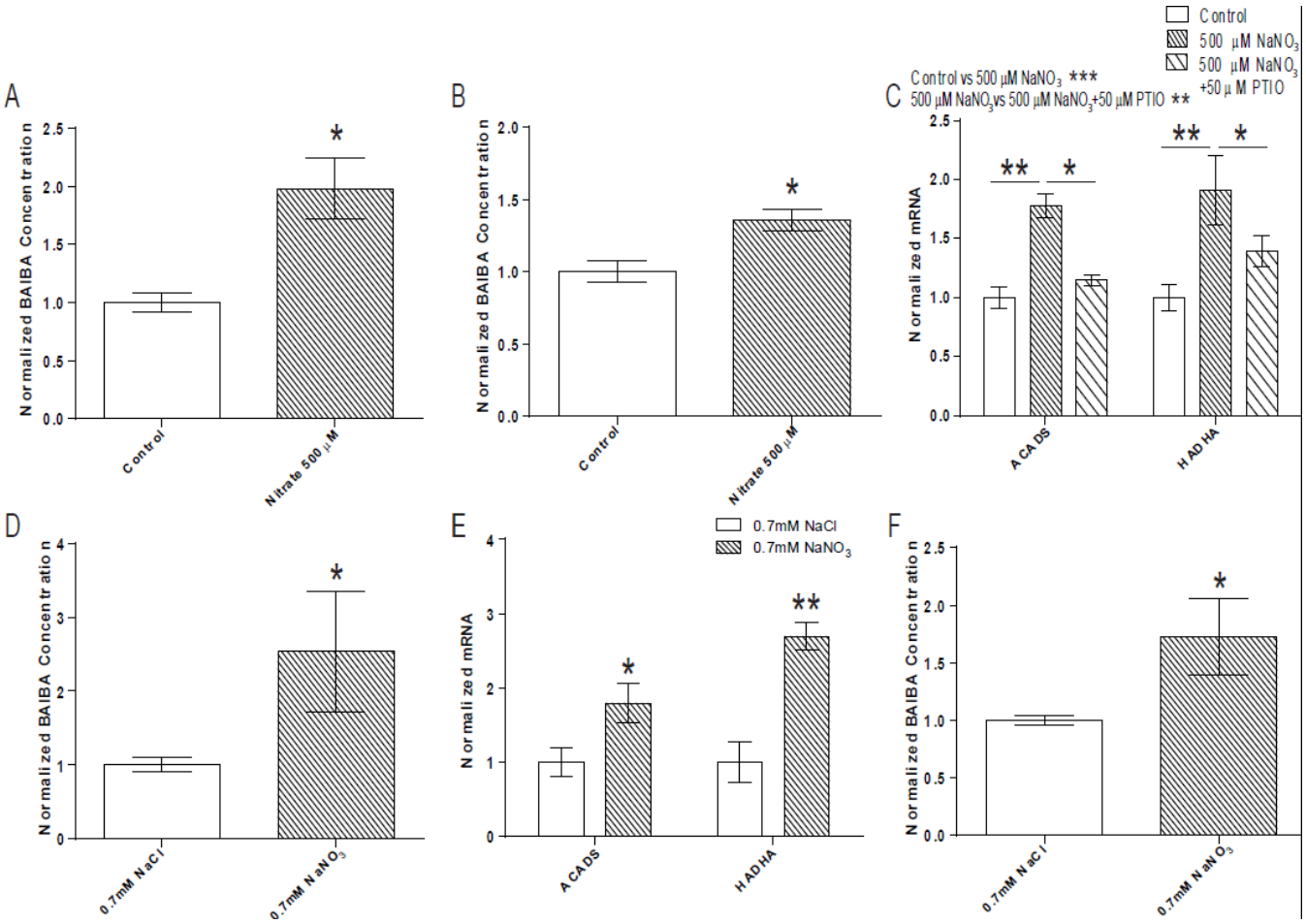
A
Normalized BAIBA Concentration
Control
Nitrate 500 μM
B
Normalized BAIBA Concentration
Control
Nitrate 500 μM
C
Control
500 μM NaNO3
500 μM NaNO3 +50 μM PTIO
Control vs 500 μM NaNO3 ***
500 μM NaNO3 vs 500 μM NaNO3+50 μM PTIO **
Normalized mRNA
ACADS
HADHA
D
Normalized BAIBA Concentration
0.7mM NaCl
0.7mM NaNO3
E
0.7mM NaCl
0.7mM NaNO3
Normalized mRNA
ACADS
HADHA
F
Normalized BAIBA Concentration
0.7mM NaCl
0.7mM NaNO3

Figure 6

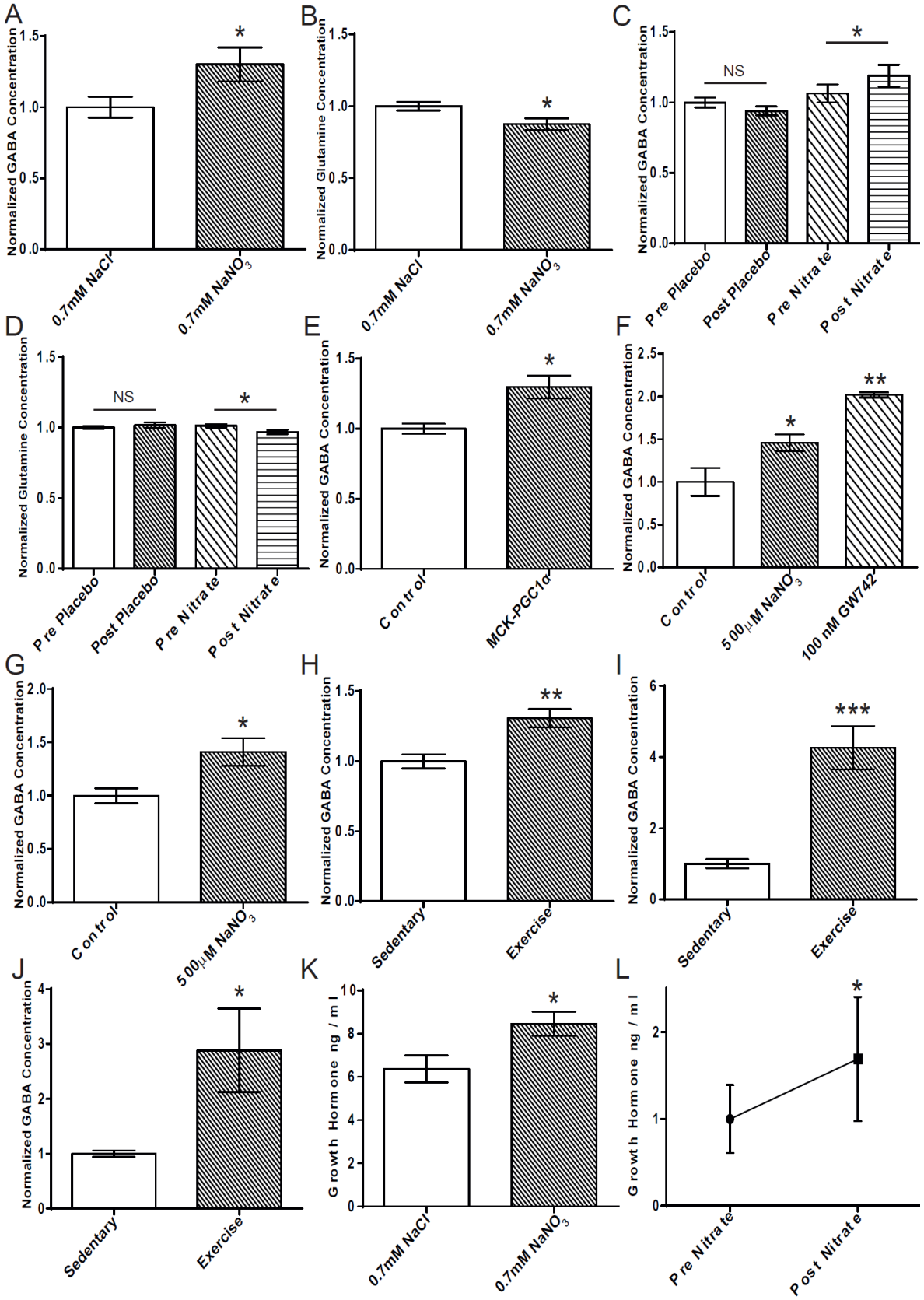
A
Normalized GABA Concentration
0.7mM NaCl
0.7mM NaNO3
B
Normalized Glutamine Concentration
0.7mM NaCl
0.7mM NaNO3
C
Normalized GABA Concentration
NS
Pre Placebo
Post Placebo
Pre Nitrate
Post Nitrate
D
Normalized Glutamine Concentration
NS
Pre Placebo
Post Placebo
Pre Nitrate
Post Nitrate
E
Normalized GABA Concentration
Control
MCK-PGC1α
F
Normalized GABA Concentration
Control
500μM NaNO3
100 nM GW742
G
Normalized GABA Concentration
Control
500μM NaNO3
H
Normalized GABA Concentration
Sedentary
Exercise
I
Normalized GABA Concentration
Sedentary
Exercise
J
Normalized GABA Concentration
Sedentary
Exercise
K
Growth Hormone ng / ml
0.7mM NaCl
0.7mM NaNO3
L
Growth Hormone ng / ml
Pre Nitrate
Post Nitrate

Figure 7

Figure Legends

Fig 1. Inorganic Nitrate Induces Muscular Fiber-type Switching in Oxidative Muscle (*A*) PGC1α mRNA in nitrate treated rat soleus (t-test, n = 5). (*B*) HIF2α mRNA in nitrate treated rat soleus (t-test, n = 5). (*C*) The expression of slow and intermediate-twitch muscle fiber genes, MYH7, MYH2, CALM2, and the fast-twitch fiber gene, MYH4, in nitrate treated rat soleus (multiple t-test, n = 5). (*D*) PGC1α protein in nitrate treated rat soleus (t-test, n = 5 / group). (*E*) MYH7 protein concentration in nitrate treated rat soleus (t-test, n = 5). (*F*) MYH2 protein concentration in nitrate treated rat soleus (t-test, n = 5). (*G*) Glucose-6-phosphate concentration in nitrate treated rat soleus (t-test, n = 9). (*H*) 3-phosphoglycerate concentration in nitrate treated rat soleus (t-test, n = 9). (*I*) Quantitation of fiber-types from cross-sections of soleus immunostained for myosin heavy chains I and II (n = 5). (*J*) Cross-sections of soleus immunostained for myosin heavy chains I (black/grey), II (red) (fluorescent red) (n = 5) Original magnification, × 20. Data are represented as Mean ± SEM. *, $P \leq 0.05$, **, $P \leq 0.01$.

Fig 2. **Inorganic Nitrate Induces Muscular Fiber-type Switching in Glycolytic Muscle** (*A*) The expression of slow and intermediate-twitch muscle fiber genes, PGC1α, HIF2α, MYH7, MYH2 and Mb, and the fast-twitch muscle fiber gene MYH4 in nitrate treated rat gastrocnemius (multiple t-test, n = 4). (*B*) Quantitation of fiber-types from cross-sections of gastrocnemius immunostained for myosin heavy chains I and II (n = 4). (*C*) Cross-sections of gastrocnemius were immunostained for myosin heavy chains I (black/grey), II (red) (fluorescent red) (n = 4). Original magnification, × 20. Data are represented as Mean ± SEM. *, $P \leq 0.05$, **, $P \leq 0.01$, *** $P \leq 0.001$.

Fig 3. Nitrate Induces Slow-twitch Muscle Fiber Gene Expression in Myotubes (*A*) PGC1α mRNA in myotubes treated with 500 μM $NaNO_3$ or 500 μM $NaNO_3$ and 100 μM 2,2-Bipyridyl (one-way ANOVA, n = 6). (*B*) Expression of slow, intermediate and fast-twitch muscle fiber genes in myotubes treated with 500 μM $NaNO_3$ or 500 μM $NaNO_3$ and 100 μM 2,2-Bipyridyl (one-way ANOVA, n = 6).

(*C*) The expression of transcription factors PGC1α, HIF2α and PPARδ and slow, intermediate and fast-twitch muscle fiber genes in myotubes treated with nitrate (25 μM $NaNO_3$, 500 μM $NaNO_3$), and nitrate and PTIO (500 μM $NaNO_3$ + 50 μM PTIO) (one-way ANOVA, n = 6). (*D*) The expression of slow-twitch muscle fiber genes is increased in myotubes treated with nitrite ($NaNO_2$) (500 nM, 25 μM and 500 μM) (one-way ANOVA, n = 6). (*E*) Expression of slow, intermediate and fast-twitch muscle fiber genes in myotubes treated with 100 nM PPARδ agonist GW742 or 500 μM $NaNO_3$ and 100 nM GW742 (one-way ANOVA, n = 4). Results of two-way ANOVA omnibus test are shown at the top of the graphs. Data are represented as Mean ± SEM. NS, not significant, *, $P \leq 0.05$, **, $P \leq 0.01$, *** $P \leq 0.001$, **** $P < 0.0001$.

Fig 4. Nitrate-mediated Muscle Fiber-type Switching Functions through PGC1α and Soluble Guanylyl Cyclase and is Enhanced by Sildenafil (*A*) PGC1α expression in C2C12 myotubes treated with negative control siRNA or siRNA against PGC1α with and without 50μM and 500 μM NaNO3 (one-way ANOVA, n = 3) (*B*) Expression of slow, intermediate and fast-twitch muscle fiber genes in myotubes treated with negative control siRNA or siRNA against PGC1α with and without 50 and 500 μM NaNO3 (one-way ANOVA, n = 3) (*C*) The expression of slow, intermediate, and fast-twitch muscle fiber genes in myotubes treated with the guanylyl cyclase inhibitor ODQ (1 μM) with and without 500 μM $NaNO_3$ (one-way ANOVA, n = 6). (*D*) The expression of PDE5 mRNA in nitrate treated C2C12 myotubes (t-test, n = 3). (*E*) C2C12 myotubes treated with the PDE5 inhibitor Sildenafil (1 μM) with and without 500 μM $NaNO_3$ (one-way ANOVA, n = 6). Results of two-way ANOVA omnibus test are shown at the top of the graphs. Data are represented as Mean ± SEM. NS, not significant, *, $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P < 0.0001$.

Fig 5. Nitrate Induces the Secretion of the PGC1α/Exercise-dependent Myokine Irisin (*A*) FNDC5/irisin expression in C2C12 myotubes treated with 500 μM $NaNO_3$ with and without the NO scavenger PTIO (one-way ANOVA, n = 3). (*B*) FNDC5/irisin expression in myotubes treated with 500

μM $NaNO_3$ with and without 1 μM Sildenafil (one-way ANOVA, n = 3). (*C*) Irisin protein concentration in the media of 500 μM $NaNO_3$ and PPARδ agonist GW742 treated myotubes (one-way ANOVA, n = 3). (*D*) FNDC5/irisin expression in nitrate treated rat soleus (t-test, n = 5). (*E*) FNDC5/irisin expression nitrate treated rat gastrocnemius (t-test, n = 4). (*F*) Plasma irisin concentration of nitrate treated rats (t-test, n = 9). (*G*) Concentration of nitrogen oxides (NOx) in the serum of human volunteers preceding and following 7 days of treatment with nitrate – containing beetroot juice (paired t-test, n = 18). (*H*) Irisin protein concentration in the serum of human volunteers preceding and following 7 days of treatment with nitrate – containing beetroot juice (paired t-test, n = 18). Data are represented as Mean ± SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P < 0.0001$.

Fig 6. Nitrate Induces the Synthesis and Secretion of the PGC1α/Exercise-dependent Myokine-like Small Molecule β-aminoisobutyric acid (*A*) BAIBA concentration in C2C12 myotubes treated with 500 μM $NaNO_3$ (t-test, n = 3). (*B*) BAIBA concentration in serum-free conditioned media from C2C12 myotubes treated with 500 μM $NaNO_3$ (t-test, n = 3). (*C*) The expression of BAIBA biosynthetic genes ACADS and HADHA in C2C12 myotubes treated with 500 μM $NaNO_3$ with and without the NO scavenger PTIO (one-way ANOVA, and two-way ANOVA omnibus test above graph, n = 3). (*D*) BAIBA concentration in soleus skeletal muscle of 0.7 mM $NaNO_3$ treated rats (t-test, n = 9). (*E*) The expression of BAIBA biosynthetic genes ACADS and HADHA mRNA in the soleus of nitrate treated rats (two-way ANOVA, n = 5). (*F*) Plasma BAIBA from 0.7 mM NaCl or 0.7 mM $NaNO_3$ treated rats (t-test, n = 9). Data are represented as Mean ± SEM. *, $P \leq 0.05$, **, $P \leq 0.01$, *** $P \leq 0.001$.

Fig 7. Nitrate Increases Circulating γ-Aminobutyric Acid and Growth Hormone Concentrations (*A*) Plasma GABA and (*B*) glutamine concentrations in 0.7 mM NaCl or 0.7 mM $NaNO_3$ treated rats (t-test, n = 9). (*C*) Serum GABA and (*D*) glutamine concentration of participants following 7 days of nitrate–containing beetroot juice supplementation (paired t-test, n = 18). (*E*) Plasma GABA

concentration from muscle specific PGC1α transgenic mice compared to age matched control mice (t-test, n = 5/group). (*F*) GABA concentration in the media of 500 μM $NaNO_3$ and 100 nM PPARδ agonist GW742 treated myotubes (one-way ANOVA, n = 3). (*G*) Intracellular GABA concentration in 500 μM $NaNO_3$ treated myotubes (t-test, n = 3). (*H*) Plasma, (*I*) gastrocnemius, and (*J*) quadriceps GABA concentrations of mice subjected to 3 weeks of free wheel running (t-test, n = 6) or sedentary controls (n = 6). (*K*) Plasma growth hormone concentration of nitrate treated rats (t-test, n = 9) (*L*) Serum growth hormone concentration of participants following 7 days of nitrate–containing beetroot juice supplementation (paired t-test, n = 18). Data are represented as Mean ± SEM. *, $P \leq 0.05$, **, $P \leq 0.01$, *** $P \leq 0.001$.