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Nitrate consumption preserves HFD-induced skeletal muscle mitochondrial ADP sensitivity and lysine acetylation: A potential role for SIRT1

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ABSTRACT

Dietary nitrate supplementation, and the subsequent serial reduction to nitric oxide, has been shown to improve glucose homeostasis in several pre-clinical models of obesity and insulin resistance. While the mechanisms remain poorly defined, the beneficial effects of nitrate appear to be partially dependent on AMPK-mediated signaling events, a central regulator of metabolism and mitochondrial bioenergetics. Since AMPK can activate SIRT1, we aimed to determine if nitrate supplementation (4 mM sodium nitrate via drinking water) improved skeletal muscle mitochondrial bioenergetics and acetylation status in mice fed a high-fat diet (HFD: 60% fat). Consumption of HFD induced whole-body glucose intolerance, and within muscle attenuated insulin-induced Akt phosphorylation, mitochondrial ADP sensitivity (higher apparent K_m), submaximal ADP-supported respiration, mitochondrial hydrogen peroxide (mtH_2O_2) production in the presence of ADP and increased cellular protein carbonylation alongside mitochondrial-specific acetylation. Consumption of nitrate partially preserved glucose tolerance and, within skeletal muscle, normalized insulin-induced Akt phosphorylation, mitochondrial ADP sensitivity, mtH_2O_2, protein carbonylation and global mitochondrial acetylation status. Nitrate also prevented the HFD-mediated reduction in SIRT1 protein, and interestingly, the positive effects of nitrate ingestion on glucose homeostasis and mitochondrial acetylation levels were abolished in SIRT1 inducible knock-out mice, suggesting SIRT1 is required for the beneficial effects of dietary nitrate. Altogether, dietary nitrate preserves mitochondrial ADP sensitivity and global lysine acetylation in HFD-fed mice, while in the absence of SIRT1, the effects of nitrate on glucose tolerance and mitochondrial acetylation were abrogated.

1. Introduction

Inorganic nitrate is found mainly in green leafy vegetables, and, after intake, nitrate is serially converted by the commensal bacteria to nitrite and subsequently nitric oxide (NO). Accumulating evidence demonstrates the benefits of nitrate on the regulation of cardiometabolic function in high-fat diet (HFD)-induced insulin resistance models [6,11,14,34]. In particular, nitrate consumption reduces mitochondrial

Abbreviations: 4-HNE, 4-hydroxynonenal; ADP, adenosine diphosphate; AMPK, AMP-activated protein kinase; ANT, adenine nucleotide translocator; ATP, adenosine triphosphate; CTL, control; HFD, high-fat diet; JNK, c-Jun N-terminal kinase; K_m, Michalis-Menten constant; M-CoA, malonyl CoA; mtH_2O_2, mitochondrial hydrogen peroxide; mtROS, mitochondrial reactive oxygen species; NAD, nicotinamide adenine dinucleotide; NAPDH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; NO, nitric oxide; PPAR-γ, peroxisome proliferator-activated receptor gamma; PGC1α, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; PmFB, permeabilized muscle fibers; P-CoA, palmitoyl-CoA; PTM, post-translational modification; SIRT1, silent mating type information regulation 2 homolog; SOD-2, superoxide dismutase.

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hydrogen peroxide (mtH_2O_2) production and c-Jun N-terminal kinase (JNK) phosphorylation within epididymal adipose tissue following HFD feeding [6]. Within the liver, nitrate has been shown to attenuate HFD-induced increases in mtH_2O_2 and lipid content whereas it preserves AMP-activated protein kinase (AMPK) signaling and peroxisome proliferator-activated receptor gamma (PPAR-γ) coactivator 1-alpha (PGC-1α) expression [11,14]. Combined, these data suggest dietary nitrate affects mitochondrial function in diverse tissues, however, it remains to be determined if dietary nitrate also affects mitochondrial bioenergetics and cellular homeostasis within skeletal muscle.

In the context of obesity, compromised mitochondrial oxidative phosphorylation (OxPhos) and greater mitochondrial reactive oxygen species (mtROS) production have been causally linked to impaired insulin signaling [1,8,22,31,38] while conversely experimental models that preserve skeletal muscle mitochondrial content/function typically improve insulin sensitivity and glucose tolerance [26]. While mitochondrial content undoubtedly influences mitochondrial ADP sensitivity [15,45], we have recently demonstrated that skeletal muscle mitochondrial ADP sensitivity is reduced in diabetic and high-fat diet (HFD)-induced insulin resistance models in the absence of reductions in the abundance of mitochondrial proteins [7,31,39]. Combined, these data suggest posttranslational modification (PTM) play a role in HFD-induced mitochondrial dysfunction.

Lysine acetylation is a PTM found particularly on mitochondrial proteins, which are especially vulnerable to non-enzymatic acetylation due to their enrichment of lysine residues and the high acetyl-CoA concentration in the mitochondrial matrix [24]. Numerous studies have found that mitochondrial lysine acetylome is increased in the context of obesity and insulin resistance, due in part to lipid overload-induced accumulation of the mitochondrial acetyl-CoA pool [12,44]. Acetylation levels are controlled by the activity of sirtuins (SIRT1-7) [36], a class of NAD^+-dependent protein deacetylases considered to be central in the regulation of metabolism. Interestingly, SIRT1 and -3 have been shown to be decreased in insulin-resistance conditions such as those present in patients with T2DM and HFD-feeding [17,21,40]. In addition, while SIRT1 is activated by AMPK [9], several dietary interventions (i.e. resveratrol or caloric restriction) improve glucose tolerance in models of insulin resistance in parallel to SIRT1 activation or content [26,35]. Therefore, given the central role of the AMPK-SIRT1 axis in metabolism and maintenance of mitochondrial health, and the previously observed nitrate-mediated preservation of AMPK pathway in the liver [11], we hypothesized that dietary nitrate may improve skeletal muscle mitochondrial bioenergetics in an AMPK-SIRT1-dependent manner following HFD-feeding. Here, we show that chronic nitrate intake preserves skeletal muscle mitochondrial ADP sensitivity, attenuates mitochondrial H_2O_2 (mtH_2O_2) production and JNK phosphorylation while improving insulin-stimulated signaling events (p-Akt) in HFD-fed mice. Additionally, nitrate ingestion preserves mitochondrial lysine acetylation levels and SIRT1 content whereas the positive metabolic effects of nitrate on glucose homeostasis and mitochondrial acetylation profile were blunted in the absence of SIRT1.

2. Methods

Ethical approval

All experiments were performed under institutional guidelines and approved by the Animal Care Committee at the University of Guelph and the Paris-Saclay Université.

2.1. Mice

Male C57Bl/6N mice (15-20 weeks old) were randomly divided into three groups fed with chow diet (10% energy from fat, cat. Number D12450J), high-fat diet (HFD: 60% energy from fat, cat. Number D12492), and HFD + nitrate (4 mM nitrate via drinking water) for eight weeks. All diets were purchased from Research Diets (New Brunswick, NJ, USA). All mice were housed in the University of Guelph animal facility (22 °C) on 12 h light: dark cycle with 24 h access to food and water *ad libitum*. Of note, the wild-type mice used in this study are the same from a previous published research from our group investigating the effects of dietary nitrate on adipose tissue biology [6].

2.2. Metabolic characterization

After 7 weeks of intervention, resting oxygen consumption ($\dot{V}\ O_2$) and carbon dioxide production ($\dot{V}\ CO_2$) were monitored in metabolic caging (Columbus Instruments, OH) and used to calculate lipid and carbohydrate oxidation as previously described [32]. In a separate group of animals (n = 5–6 per group), mice were anesthetized using 2% isoflurane, and skeletal muscle tibial anterior was removed and rapidly frozen in liquid nitrogen before (Pre) and 15 min following (Post) an I. P. injection of insulin (Novorapid: 1U/kg body weight), as previously reported [6]. Blood glucose was determined before and after the insulin administration for internal control purposes. One control-fed animal did not respond to the insulin and was removed from the subsequent Western blotting analysis. All procedures were performed only after assurance of anesthesia depth checked by leg retraction after toe pinch, palpebral reflex, and movement of the whiskers.

2.3. Mitochondrial oxygen consumption

Mitochondrial respiration was determined in saponin-permeabilized red gastrocnemius skeletal muscle fibers in an Oxygraph high-resolution respirometer chamber with 2 mL MiR05 at 37 °C as previously described with minor modifications [7]. Briefly, red gastrocnemius was excised, immediately placed in ice-cold BIOPS (50 mM MES, 7.23 mM K_2EGTA, 2.77 mM CaK_2EGTA, 20 mM imidazole, 0.5 mM dithiothreitol, 20 mM taurine, 5.77 mM ATP, 15 mM PCr and 6.56 mM $MgCl_2{\cdot}H_2O$, pH 7.1) and fibers bundles were separated using fine-tipped forceps. Fibers were then transferred to 1.5 mL of BIOPS buffer and permeabilized with saponin (40 μg/mL) for 30 min at 4 °C with constant mixing. After permeabilization protocol, fibers were transferred to a 1.5 mL MiR05 buffer (0.5 mM EGTA, 3 mM $MgCl_2{\cdot}6H_2O$, 60 mM K-lactobionate, 10 mM KH_2PO_4, 20 mM HEPES, 20 mM taurine, 110 mM sucrose, 1 g/L FFA-free BSA; pH 7.1) and washed twice for 15 min. Thereafter, fibers were inserted into the chamber to determine rates of oxygen consumption by high-resolution respirometry (Oroboros Oxygraph-2k, Innsbruck, Austria). Mitochondrial respiration was assessed following the addition of 5 μM blebbistatin, 5 mM pyruvate and 1 mM malate, ADP was titrated in various concentrations (from 25 to 10000 μM), then 10 mM glutamate, 10 mM succinate, and 10 μM cytochrome *c* (to assess mitochondrial membrane integrity) were sequentially added. Experiments with cytochrome *c* responses greater than 10% were excluded from the analysis. Michaelis-Menten kinetic analysis was used to estimate mitochondrial ADP sensitivity (apparent K*m*) whereby O_2 consumption in the absence of ADP (only pyruvate + malate) was defined as V0 and analysis was constrained to maximal ADP-supported respiration (100% maximal mitochondrial respiration). All experiments displayed a curve-fit with an R^2 greater than 0.95.

Submaximal palmitoyl-CoA (P-CoA)-supported respiration was determined in the presence of 1 mM malate, 5 mM ADP, and 1 mM L-carnitine. 60 μM P-CoA was added, followed by the addition of 7 μM malonyl-CoA (M-CoA) to assess the inhibitory effects of M-CoA on carnitine palmitoyl transferase-I (CPT-I) mediated fatty acid transport.

2.4. Mitochondrial reactive oxygen species emission

Mitochondrial H_2O_2 emission was determined in red gastrocnemius permeabilized muscle fibers as previously described [31]. Briefly, saponin-permeabilized fibers were inserted into a cuvette containing 5

μM blebbistatin, 10 μM Amplex Red (Invitrogen, Waltham, MA, USA), 40 U/mL SOD, and 5 U/mL horseradish peroxidase in Buffer Z. Mitochondrial H_2O_2 emission rates were determined in the presence of 20 mM succinate in the absence and presence of ADP 100 μM. A standard curve was made with known concentrations of H_2O_2.

2.5. Isolated mitochondria

Skeletal muscle (quadriceps) mitochondria were isolated using differential centrifugation as previously described [19]. Tissues were harvested, minced in isolation buffer (100 mM sucrose, 100 mM KCl, 50 mM Tris- Cl, 1 mM KH_2PO_4, 0.1 mM EGTA, 0.2% BSA, and 1 mM ATP; pH 7.4), weighed, and homogenized using a motorized Teflon pestle (800 rpm). Mitochondria were centrifuged at 800 *g* for 10 min, resuspended in 4 mL of isolation buffer, and treated with 0.025 μg/mg tissue protease (Subtilisin A, Sigma-Aldrich) for 5 min. Thereafter, 10 mL of isolation buffer was added, and the sample immediately spun at 5000 *g* for 5 min. The pellet was repeatedly resuspended in isolation buffer and pelleted at 10,000 *g* for 10 min. Mitochondria were purified using a 60% Percoll gradient (1.25 M sucrose, 25 mM MOPS, 5 mM EDTA, 25 mM NaN_3, pH 7). For all experiments subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondrial subpopulations were pooled.

2.6. Western blots

Skeletal muscle tissue was homogenized as previously described [31], diluted to 1 μg/μL, and protein was loaded equally into a standard SDS-PAGE gel, transferred to PVDF membranes, and incubated for 1 h in the appropriate blocking solution according to the antibody. Antibodies included total-Akt (1:1000, Cell Signaling cat. no. 4691, Danvers, MA, USA), phosphorylated Akt-Ser473 (1:1000, Cell Signaling cat. no. 9271, Danvers, MA, USA), Akt-Thr308 (1:1000, Cell Signaling cat. no. 9275, Danvers, MA, USA), phosphorylated JNK 1/2 (1:1000, Cell Signaling cat. no. 4671, Danvers, MA, USA), total JNK 1/2 (1:1000, Cell Signaling cat. no. 9252, Danvers, MA, USA), catalase (1:5000, Abcam cat. no. 16731, Cambridge, MA, USA), superoxide dismutase 2 (SOD-2) (1:1000, Abcam cat. no. 13533, Cambridge, MA, USA), OXPHOS cocktail (1:500, MitoSciences cat. no. Ab110413, Eugene, OR, USA), SIRT3 (1:1000 Cell Signaling cat. no. C73E3, Danvers, MA, USA), SIRT1 (1:1000, Abcam cat. no. ab110304, Cambridge, MA, USA), acetylated lysine (1:1000 Cell Signaling cat. no. 9441S, Danvers, MA, USA), total AMPK (1:1000, Cell Signaling Technology cat. no. 2757), phosphorylated AMPK (1:2000, Cell Signaling Technology cat. no. 2535), total CaMK-II (1:1000, Cell Signaling Technology cat. no. 3362), phosphorylated CaMK-II (1:1000, Cell Signaling Technology cat. no. 12716), Caveolin-3 (BD Biosciences cat. no. 610421, 1:3000), SERCA-2 (Abcam cat. no. ab2861, 1:1000), VDAC-1 (Abcam cat. no. ab14734, 1:1000), GLUT-4 (1:1000 dilution, cat. no. AB1346, Chemicon International), COX-IV (Invitrogen cat. no. A21347, 1:1000), 4-HNE (Alpha Diagnostics cat. no. HNE11-S, 1:1000), Nitrotyrosine (Cayman Chemicals cat. no. 189542, 1:500), alpha-tubulin (1:5000, Abcam cat. no. ab7291, Eugene, OR, USA). All membranes were detected using enhanced chemiluminescence (Chem-iGenius2 Bioimaging System, SynGene, Cambridge, UK). Ponceau staining was used as a loading control.

2.7. Protein carbonylation

Protein carbonylation was detected using OxyBlot Protein Oxidation Detection Kit (1:150 in 1% BSA/PBST) according to the instructions of the manufacturer (Milipore, cat. no. S7150).

2.8. HFD-feeding in SIRT1$^{-/-}$ knock out mice

Male C57Bl/6J *Sirt1*$^{floxΔE4/floxΔE4}$ (*Sirt1f/f*) homozygous mice (kindly provided by David A Sinclair's group) were used in this study and bred as previously described [37]. While these mice are on a different background strain than those used in the original HFD + nitrate feeding experiments described above (C57Bl/6J vs N), we have previously shown that nitrate attenuates HFD glucose intolerance in both strains of mice [6,14] and therefore these animals represent an appropriate model to determine the necessity of SIRT1 in mediating the protective effects of nitrate. Briefly, exon 4 of *Sirt1* gene was flanked with two LoxP sites that were recognized and excised by tamoxifen-activated Cre recombinase driven by the alpha myosin heavy chain promoter (Amhc-MerCreMer). Male Sirt1$^{-/-}$ mice (*Sirt1*$^{f/f}$, *αMHC-MerCreMer*^{Pos}) were injected with tamoxifen (25 mg/kg i.p. daily for 3 days) at the age of 12–15 weeks to induce Sirt1 inactivation. Littermate Sirt$^{f/f}$ not carrying Cre transgene were subjected to the same tamoxifen treatment and were used as control mice. Four weeks after tamoxifen treatment, mice were submitted to an intraperitoneal glucose tolerance test, as previously described [6] to determine the effects of Sirt1 inactivation on whole-body glucose tolerance. Thereafter, Sirt1$^{-/-}$ mice were randomly divided into two groups, all receiving HFD (60% energy from fat, cat. Number D12492) while one group received nitrate (4 mM) via drinking water for eight weeks. All experiments were carried using the same diet, doses, and periods of treatment as the study in the wild-type animals. All mice were housed in the Université Paris-Saclay animal facility (22 °C) on 12 h light: dark cycle with 24 h access to food and water *ad libitum*. On the last week of treatment, mice were subjected to a glucose tolerance test after 4h fasting while a low-fat diet group was kept for internal control purposes. Three days later, mice were anesthetized with ketamine (50 mg/kg) and xylazine (8 mg/kg), skeletal muscle was removed, snap-frozen in liquid nitrogen, and kept in −80 °C freezer until further analysis.

2.9. Statistical analysis

Results are expressed as mean ± SD superimposed. Groups were compared using a one-way ANOVA followed by an LSD-Fisher post-hoc test where appropriate. For the Sirt1$^{-/-}$ experiments, unpaired students t-test was used. All statistical analyses were performed using Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Significance was assumed when $P < 0.05$.

3. Results

3.1. Nitrate consumption protects against HFD-induced glucose intolerance

We first measured the effects of HFD consumption on whole body weight gain, substrate preference, and insulin signaling within skeletal muscle (Fig. 1). As expected, HFD consumption increased body weight gain (Fig. 1A and B), caused glucose intolerance (Fig. 1C and D), increased whole-body lipid oxidation (Fig. 1E), and decreased carbohydrate oxidation (Fig. 1F). Importantly, the effects of HFD-consumption in whole-body substrate preferences were independent of the diurnal cycle (light/dark) (Fig. 1E and F). Within skeletal muscle, HFD-feeding decreased the ability of insulin to stimulate Akt-Thr308 and Akt-Ser473 phosphorylation (Fig. 1G). Altogether these data highlight that 8 weeks of HFD consumption caused obesity, alterations in whole-body substrate preference, and skeletal muscle insulin resistance. The consumption of dietary nitrate did not alter body weight gain (Fig. 1A and B) or whole-body substrate preference (Fig. 1E and F). Importantly, dietary nitrate partially prevented whole-body glucose tolerance (Fig. 1C and D) and mitigated HFD-induced reductions in insulin-stimulated Akt-Thr308 phosphorylation (Fig. 1G). Of note, nitrate consumption did not alter food intake (HFD-fed group = 18.45 ± 1.5 g/week/animal; HFD + Nitrate group 18.25 ± 1.7 g/week/animal, $p = 0.8$ unpaired two-tail Student's *t*-test) or water intake (HFD-fed group = 20.8 ± 2.9 mL/week/animal; HFD + Nitrate group 21.4 ± 1.7 ml/week/animal, $p = 0.6$ unpaired two-tail Student's *t*-test). These data suggest nitrate consumption partially protected skeletal muscle from

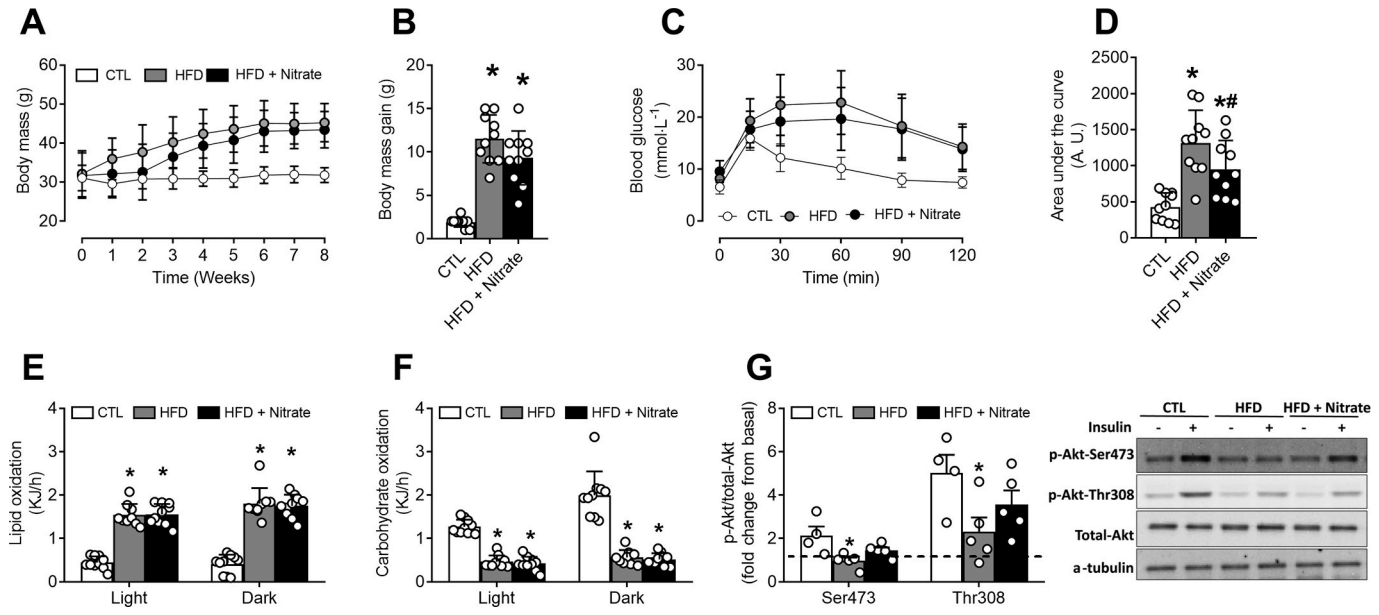

Fig. 1. – The effects of dietary nitrate on body weight, whole-body glucose tolerance and substrate utilization, and skeletal muscle insulin signaling. Body weight gain during the 8 weeks of HFD and nitrate consumption (A, n = 9–10/group), body mass gain at the end of the experimental protocol (B, n = 9–10/group), intraperitoneal glucose tolerance test (C, n = 6–7/group) and area under the curve calculated from ipGTT (D, n = 6–7/group), lipid (E, n = 9–10/group) and carbohydrate (F, n = 9–10/group) whole-body utilization, and insulin-stimulated skeletal muscle Akt phosphorylation levels (G, n = 4–5/group). White bars are control-fed group, gray bars are HFD-fed group, and black bars are HFD + Nitrate group. Data are expressed with individual values and mean ± SD superimposed. *p < 0.05 compared to control-fed group; #p < 0.05 compared to HFD-fed group. CTL – control group; HFD – high-fat diet; Statistical analysis: One-way ANOVA with Fisher's LSD *post hoc* test.

HFD-induced reductions in insulin signaling, which could contribute to the preservation of whole-body glucose homeostasis following nitrate consumption.

3.2. Skeletal muscle mitochondrial function

Given that nitrate consumption did not alter whole-body fuel preference or energy expenditure, we next investigated the effects of dietary nitrate on mitochondrial function in permeabilized muscle fibers (PmFB). While HFD increased indirect markers of mitochondrial content (i.e. subunits of proteins within the electron transport chain) (Fig. 2A), HFD-feeding did not alter state 2 mitochondrial respiration, maximal ADP-stimulated respiration, maximal oxidative capacity (glutamate + succinate), or the respiratory control ratio (Fig. 2B and C). The consumption of nitrate also did not affect these measurements. Moreover, regardless of the diet consumed, P-CoA-supported respiration (Fig. 2D) and the sensitivity to the inhibitory effects of M-CoA (Fig. 2E) were not different. Combined, these data suggest neither HFD nor nitrate affected various indexes of mitochondrial respiratory capacity.

3.3. Dietary nitrate preserves mitochondrial ADP sensitivity following HFD-feeding

Given the absence of effects of dietary nitrate on maximal mitochondrial respiration, we next determined the possible subtle effects of nitrate supplementation on mitochondrial respiration using non-saturating ADP concentrations. Similar to previous reports [31], mitochondrial ADP sensitivity was reduced by ~25% after 8 weeks of HFD-consumption (Fig. 3A and B). Interestingly, dietary nitrate totally prevented the impairment on mitochondrial submaximal ADP responsiveness caused by HFD-consumption (Fig. 3A and B). Accordingly, in the presence of physiological ADP concentrations (25 μM), the HFD-induced impairment on mitochondrial respiration was fully mitigated by nitrate consumption (Fig. 3C).

Since ADP utilizes the proton motive force to stimulate respiration, we also determined if increased ADP sensitivity would simultaneously decrease mtH_2O_2 production in PmFB after nitrate supplementation. While maximal mtH_2O_2 was not different between groups (Fig. 3D, left), there was a strong trend (P = 0.05) for mtH_2O_2 to be higher in the presence of submaximal (100 μM) ADP following HFD (Fig. 3D, right). As a result, the ability of ADP to suppress mtH_2O_2 emission was attenuated following HFD (Fig. 3E). In stark contrast, dietary nitrate prevented all of these responses. Redox imbalance has been suggested as one of the triggers for Nrf2-mediated antioxidant gene transcription. While SOD-2 or ANT-1 protein contents were consistent across diets, dietary nitrate prevented HFD-induced increases in catalase content (Fig. 3F). To further investigate the possibility that nitrate preserved cellular redox balance we examined several markers of oxidative stress. While HFD increased 4-HNE content, nitrosylation and protein carbonylation (Fig. 3G–I), nitrate consumption only normalized protein carbonylation (Fig. 3G–I). Importantly, measurements of lipid peroxidation (4-HNE) and NO-dependent covalent modifications (nitrosylation) following HFD + nitrate are confounded by greater substrate availability (lipids for oxidation and NO for nitrosylation) and therefore difficult to interpret. However, the reduction in mtH_2O_2, catalase content and protein carbonylation following nitrate consumption all suggest maintained redox balance.

3.4. Dietary nitrate preserves SIRT1 content and mitochondrial acetylation levels

Given that dietary nitrate preserved mitochondrial ADP sensitivity, reduced mtH_2O_2 emission in the presence of ADP, and prevented oxidative stress, we next investigated the mechanisms that could be linked to these functional outcomes. While HFD-feeding induced an increase in JNK1 and CaMK-II phosphorylation (Fig. 4A–C), a response dependent on increased mitochondrial ROS [20], dietary nitrate was able to prevent both JNK1 as well as CaMK-II phosphorylation (Fig. 4A–C). Since CaMK-II has several downstream protein targets, we examined the effects of the increase in CaMK-II phosphorylation on AMPK activation, a master regulator of metabolism. Neither HFD nor nitrate changed AMPK-Thr172 phosphorylation levels (Fig. 4D),

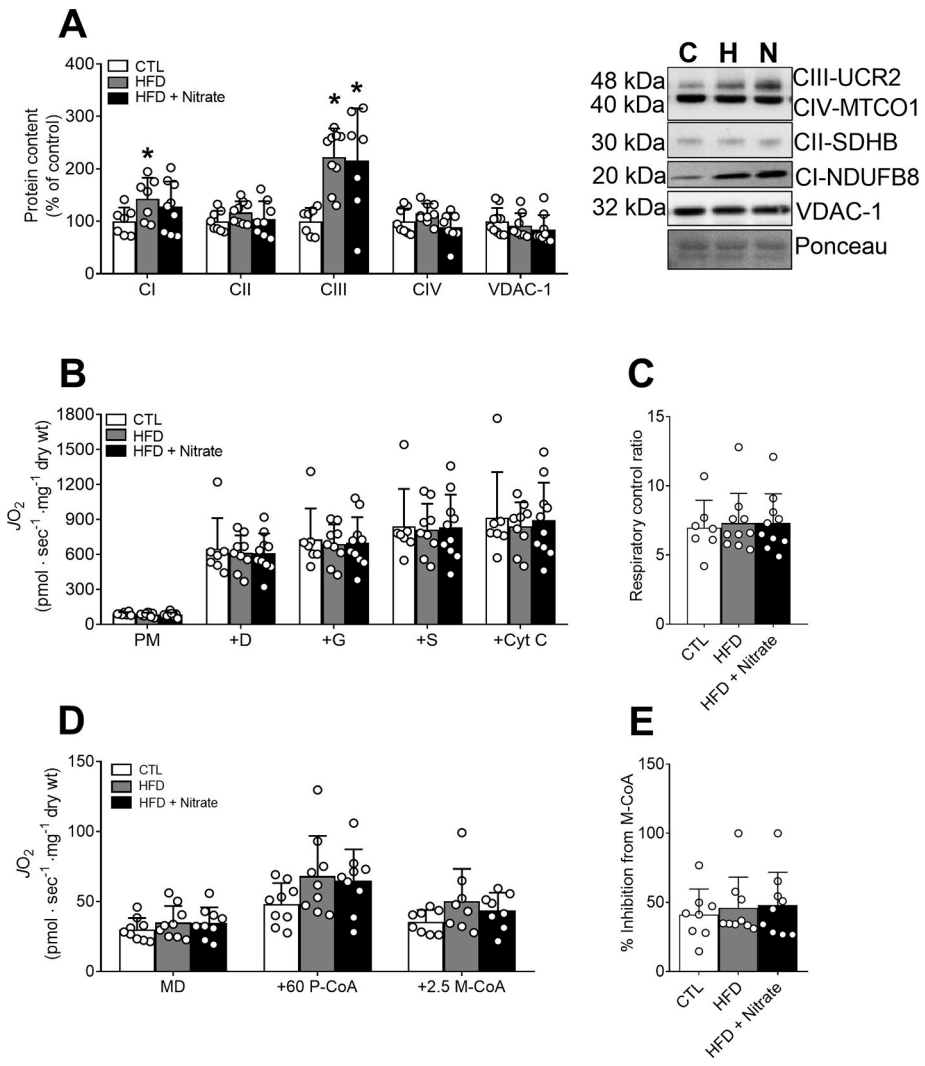

Fig. 2. – Nitrate consumption does not alter maximal mitochondrial capacity or lipid utilization. Mitochondrial OXPHOS subunits and VDAC content quantification (A, 7–9/group), mitochondrial respiration using a modified SUIT protocol (B, n = 9–10/group), respiratory control ratio (C, n = 9–10/group), lipid-supported respiration (D, n = 9–10/group), and percentage of M-CoA inhibition on P-CoA supported respiration (E, n = 8–9/group). White bars are control-fed group, gray bars are HFD-fed group, and black bars are HFD + Nitrate group. Data are expressed with individual values and mean ± SD superimposed. *p < 0.05 compared to control-fed group. P – pyruvate; M – malate; D – adenosine diphosphate; G – glutamate; S – succinate; P-CoA – palmitoyl-CoA; M-CoA – malonyl-CoA; CI – complex I subunit NDUFB8; CII – complex II subunit SDHB; CIII – complex III subunit UQCRC2; CIV – complex IV subunit MTCO1; VDAC1 – voltage-dependent anion channel 1; CTL – control group; HFD – high-fat diet; Statistical analysis: One-way ANOVA with Fisher's LSD *post hoc* test.

however, HFD-feeding decreased SIRT1 and SIRT3 content (Fig. 4E and F). Interestingly, nitrate consumption prevented the HFD-induced decrease in SIRT1 content (Fig. 4E).

Due to the deacetylase properties of SIRT1, we next investigated the protein acetylation profile in a whole-muscle preparation. While dietary nitrate prevented the reduction of SIRT1 content induced by HFD-consumption (Fig. 4E), this did not produce any effect on protein acetylation profile within the whole-lysate muscle (Fig. 5A). However, given the effects of dietary nitrate on mitochondrial function (ADP sensitivity and mtH_2O_2 emission), we reasoned that it might be more appropriate to examine protein acetylation profile in a sub-compartment manner, as mitochondria represent a small proportion of total cellular protein content. To address this, we isolated mitochondria from the skeletal muscle of mice fed our various diets. The purity of our mitochondrial preparation was verified by the enrichment of mitochondrial proteins (OXPHOS and SIRT3) and absence of proteins residing in plasma and sarco/endoplasmic reticulum membranes or the cytosol (Fig. 5B). Interestingly, while isolated mitochondria did not possess SIRT1 protein (Fig. 5B), we detected a 50% increase in the protein acetylation levels in the HFD-fed mice (Fig. 5C), a response totally prevented by the consumption of dietary nitrate (Fig. 5C).

3.5. *SIRT1 is required for the beneficial effects of dietary nitrate on glucose tolerance and mitochondrial acetylation levels*

Despite the absence of SIRT1 in mitochondria, given the protective role of dietary nitrate on cellular SIRT1 content and mitochondrial lysine acetylation, we next investigated if SIRT1 was required for the positive metabolic effects of nitrate with respect to glucose homeostasis and acetylation profile. Four weeks after tamoxifen injections, we verified that glucose tolerance was not altered in SIRT1$^{-/-}$ mice compared to wild-type controls (Fig. S1 A-B). Thereafter, we confirmed the effectiveness of tamoxifen injections as, compared to WT controls, SIRT1 protein content was decreased ~70% in SIRT1$^{-/-}$ mice following 8 weeks of HFD feeding in the presence/absence of dietary nitrate (Fig. 5D). Furthermore, 8 weeks of HFD-consumption caused whole-body glucose intolerance and adipose tissue accumulation in SIRT1$^{-/-}$ (Fig. S2 A-C). Interestingly, chronic nitrate consumption did not prevent HFD-induced glucose intolerance in SIRT1$^{-/-}$ mice (Fig. 5E and F). Additionally, lysine acetylation levels in isolated mitochondria were not altered by nitrate supplementation in SIRT1$^{-/-}$ mice (Fig. 5G). In summary, our data suggest that the positive effects of dietary nitrate on glucose homeostasis and mitochondrial lysine acetylation are dependent on the presence of cytosolic SIRT1.

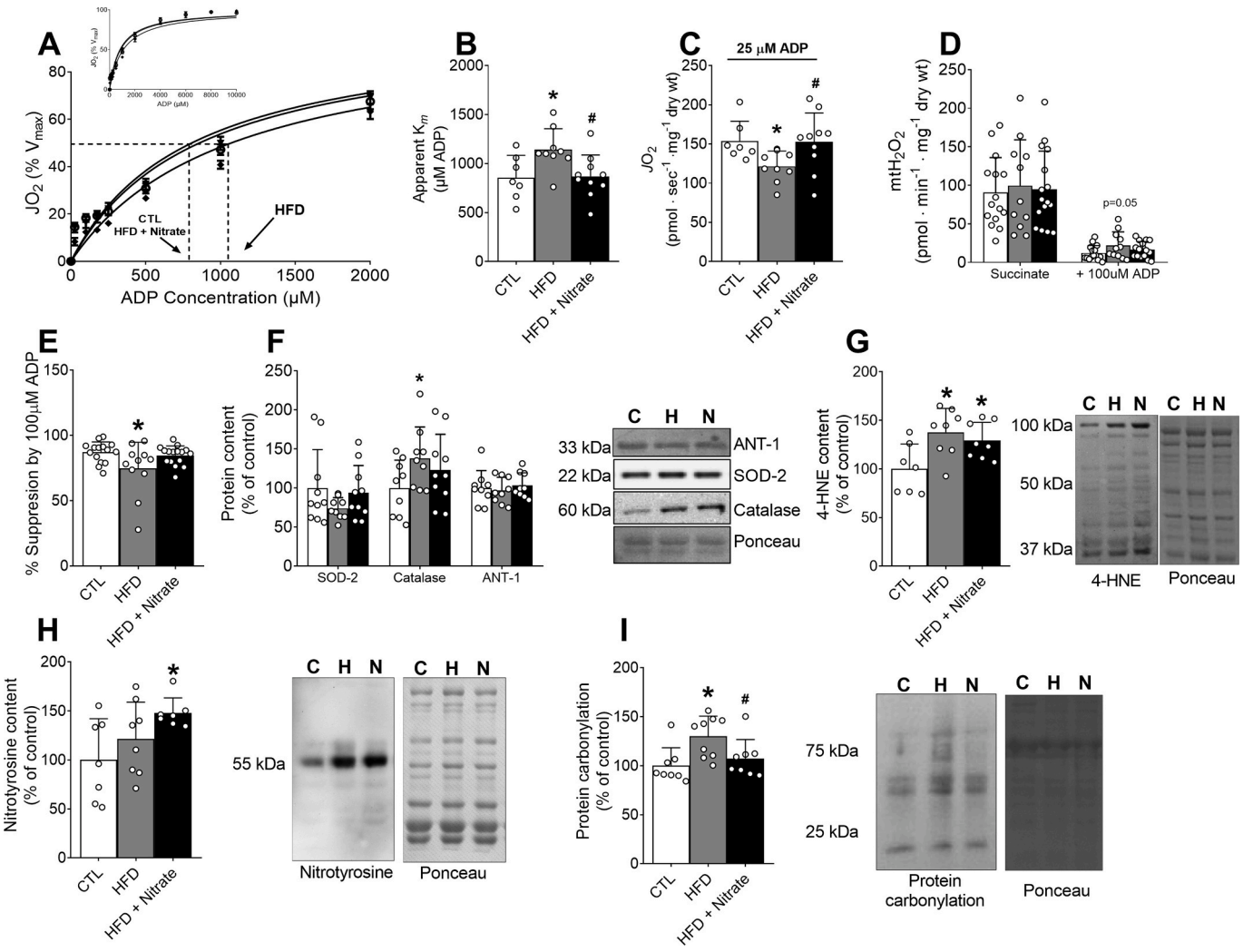

Fig. 3. – Dietary nitrate preserves mitochondrial ADP sensitivity in association with lower H_2O_2 production and JNK phosphorylation. Mitochondrial kinetics ADP-supported respiration (A, n = 7–10/group), ADP apparent K_m (B, n = 7–10/group), mitochondrial respiration with 25 μM ADP (C, n = 7–9/group), mitochondrial hydrogen peroxide emission (D, n = 11–16/group), % of suppression by 100 μM ADP (E, n = 11–16/group), SOD2, catalase, and ANT1 protein content (F, n = 8–9/group), 4-HNE (G, n = 7–8/group), nitrotyrosine content (H, n = 7–8/group), and protein carbonylation (I, n = 8–9/group). White bars are control-fed group, gray bars are HFD-fed group, and black bars are HFD + Nitrate group. Data are expressed with individual values and mean ± SD superimposed. *$p < 0.05$ compared to control-fed group; #$p < 0.05$ compared to HFD-fed group. CTL – control group; HFD – high-fat diet; ADP – adenosine diphosphate; K_m – Michaelis-Menten constant; mtH_2O_2 – mitochondrial hydrogen peroxide; SOD-2 – superoxide dismutase 2; ANT-1 – adenosine transporter 1; 4-HNE – 4-hydroxynonenal. Statistical analysis: One-way ANOVA with Fisher's LSD *post hoc* test.

4. Discussion

Although dietary intake of inorganic nitrate was first considered potentially harmful due to its biological conversion to carcinogenic nitrosamines, it has now emerged as a potential nutraceutical therapy due to its nitric oxide (NO)-like bioactivity [28]. Our data demonstrate that the positive metabolic effects of dietary nitrate are associated with the preservation of whole-body glucose homeostasis, skeletal muscle insulin-induced p-Akt, mitochondrial ADP sensitivity, mtH_2O_2 production, redox balance and global mitochondrial acetylation levels in HFD-fed mice. We further demonstrate that ablating SIRT1 prevents the ability of nitrate to improve glucose tolerance or mitochondrial acetylation status. Combined, these data suggest dietary nitrate exerts a beneficial effect on skeletal muscle mitochondrial bioenergetics, and this may occur in a SIRT1-dependent manner.

While the detailed effects of nitrate on cardiometabolic health are reviewed elsewhere [28], in insulin-target organs, nitrate consumption has been consistently shown to improve redox balance through a reduction in ROS production either from mitochondria [6] or other ROS-producing enzymes, namely NADPH oxidase [11,34]. Considered as the major site for insulin-stimulated glucose uptake, skeletal muscle plays a fundamental role in the maintenance of whole-body glucose and fatty acid homeostasis [13,23]. Here, nitrate-mediated preservation of whole-body glucose tolerance was associated with partial preservation of Akt-Thr308 phosphorylation upon insulin stimulation within skeletal muscle. Akt-Thr308 phosphorylation is under the direct regulation of the canonical insulin-mediated PI3K-PDK1 axis [3,29]. Since these signaling events are required for insulin-induced translocation of GLUT4 to sarcoplasmic membrane, the preservation of Akt-Thr308 phosphorylation following nitrate supplementation represents a possible mechanism by which nitrate improved glucose tolerance in the present study. However, it should be acknowledged that previous work has suggested that Akt phosphorylation levels do not linearly translate to functional glucose transport measurements [18], suggesting additional mechanistic work is required to solidify the interaction between nitrate and glucose transport.

While the mechanistic cause of the preserved glucose tolerance remains somewhat debatable, nitrate consumption improved the ability of

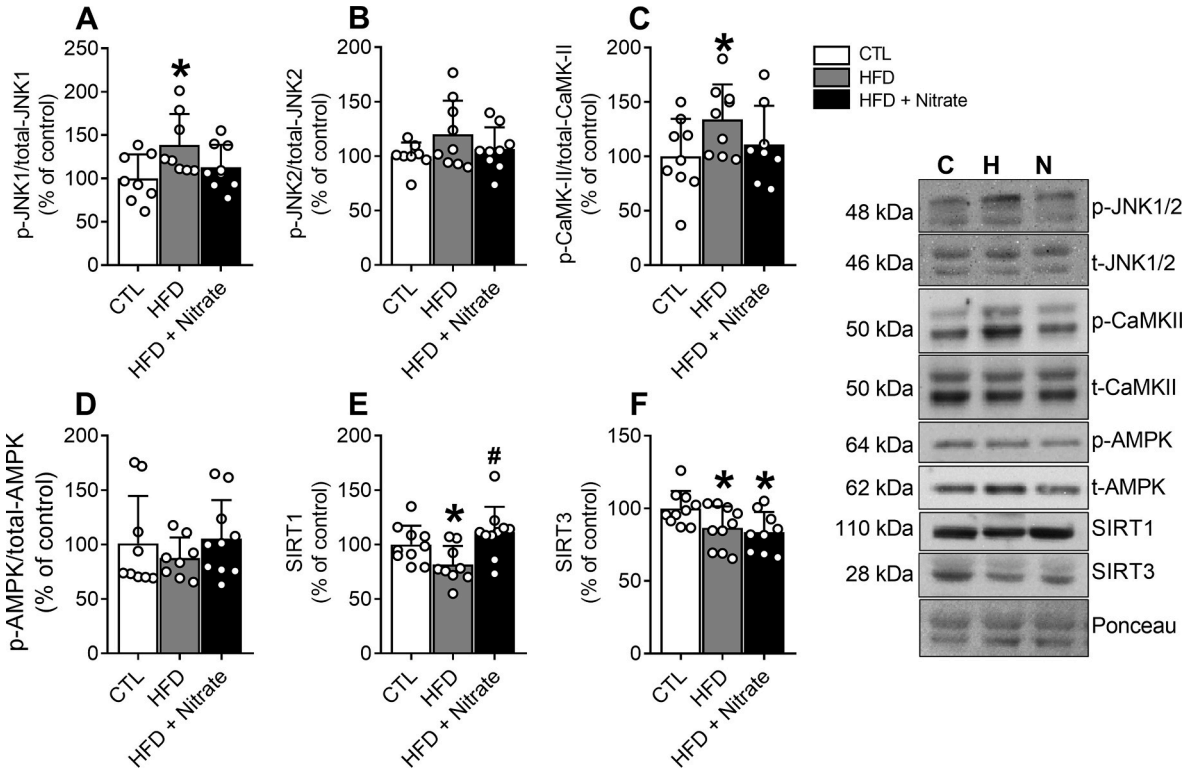

Fig. 4. – Nitrate supplementation preserves CaMK-II phosphorylation levels and rescue SIRT1 protein content. Quantification of western blotting for p-JNK1/total-JNK1 ratio (A, n = 8–9/group), p-JNK2/total-JNK2 ratio (B, n = 8–9/group), p-CaMK-II/total-CaMK-II ratio (C, n = 8–9/group), p-AMPK/total-AMPK ratio (D, n = 8–10/group), SIRT1 (E, n = 9–10/group), and SIRT3 content (F, n = 9–10/group). White bars are control-fed group, gray bars are HFD-fed group, and black bars are HFD + Nitrate group. Data are expressed with individual values and mean ± SD superimposed. *p < 0.05 compared to control-fed group; #p < 0.05 compared to HFD-fed group. CTL – control group; HFD – high-fat diet; CaMK-II – Ca^{2+}/calmodulin-dependent protein kinase II; AMPK - AMP-activated protein kinase; SIRT1 – sirtuin 1; SIRT3 – sirtuin 3. JNK – c-Jun N-terminal. Statistical analysis: One-way ANOVA with Fisher's LSD *post hoc* test.

ADP to decrease mtH_2O_2 emission and normalized p-JNK, catalase, and protein carbonylation levels. This suggests an improvement in redox stress may contribute to the attenuated development of glucose intolerance and reduced insulin-mediated Akt-Thr308 phosphorylation [1,31,43]. Although these findings support previous observations in liver and adipose tissue suggesting nitrate consumption improves redox homeostasis [6,11,14,34], this interpretation is confounded by the observation that nitrate increased total cellular nitrosylation. While nitrate-derived NO has been shown to compete with O_2 for binding on cytochrome *c* oxidase [4] and S-nitrosylation of thiol residues within complex I can reduce its activity [10], we did not observe an impairment in complex I-supported mitochondrial respiration or maximal O_2 consumption. Combined, these data suggest nitrotyrosine might not play a key role in the control of mitochondrial bioenergetics and insulin signaling, at least in our experimental model.

Along with redox imbalance, it has been proposed that lipid accumulation within skeletal muscle might be an underlying mechanism driving insulin resistance [5,25]. In this context, it is suggested that ectopic lipid accumulation arises from an inability of mitochondria to oxidize lipids [25], however, we did not observe any impairment in lipid oxidation capacity or the inhibitory effects of malonyl-CoA. Importantly, it has been shown that lipid accumulation within skeletal muscle is a result of lipid overload due to high-fat and high-caloric diet rather than an intrinsic mitochondrial dysfunction [19]. On the other hand, as previously demonstrated, mitochondrial ADP sensitivity is reduced in obesity/insulin-resistant pre-clinical models [31,39]. In the present study, submaximal (25 μM) ADP-supported respiration was lower after 8 weeks of HFD-feeding whereas absolute mtH_2O_2 emission exhibited a trend ($p = 0.05$) towards greater mtH_2O_2 production in the presence of 100 μM ADP. Interestingly, nitrate consumption mitigated all of these HFD-caused impairments in mitochondrial bioenergetics. Since mitochondrial OXPHOS content and ADP transport (i.e. ANT-1) proteins cannot explain the improvements observed in the nitrate-supplemented mice, post-translational modification emerges as a possible explanation. Our previous work suggested that impairments in mitochondrial ADP sensitivity caused by HFD consumption are related to reduced ANT activity [31], whereas lysine-23 acetylation is predicted to decrease ADP affinity and, as a result, reduce oxidative phosphorylation [30]. In the present study, while HFD consumption caused a ~50% increase in mitochondrial lysine acetylation levels, nitrate supplementation totally prevented this response. Therefore, it is tempting to speculate that the protection of mitochondrial ADP sensitivity observed in the nitrate-supplemented mice could be through maintenance of lysine acetylation levels, more specifically ANT-lysine-23, however, other studies are required to test this hypothesis.

Given that protein acetylation status is tightly controlled by a class of NAD^+-dependent deacetylases and those enzymes have been shown to be decreased in obesity/insulin resistance contexts [12,44], we wondered if the protection observed in the HFD + nitrate group would be associated with maintenance of SIRT content. Although HFD-consumption decreased both SIRT1 and -3 content within skeletal muscle, nitrate supplementation prevented only the HFD-induced decrease in SIRT1 protein levels, a cytosolic protein which could, in theory, affect transmembrane proteins like ANT directly. Interestingly, whereas it has been shown that nitrate can prevent p-AMPK in liver [11], dietary nitrate prevented the decrease in SIRT1 content even in the absence of any detectable change in AMPK phosphorylation levels. Even though it has been shown that AMPK activates SIRT1 [9], the interaction between these two enzymes might be reciprocal and it is not fully understood. Nonetheless, in the inducible whole-body $SIRT1^{-/-}$, dietary nitrate was not able to prevent HFD-induced glucose intolerance and mitochondrial lysine acetylation. Altogether, we provided evidence

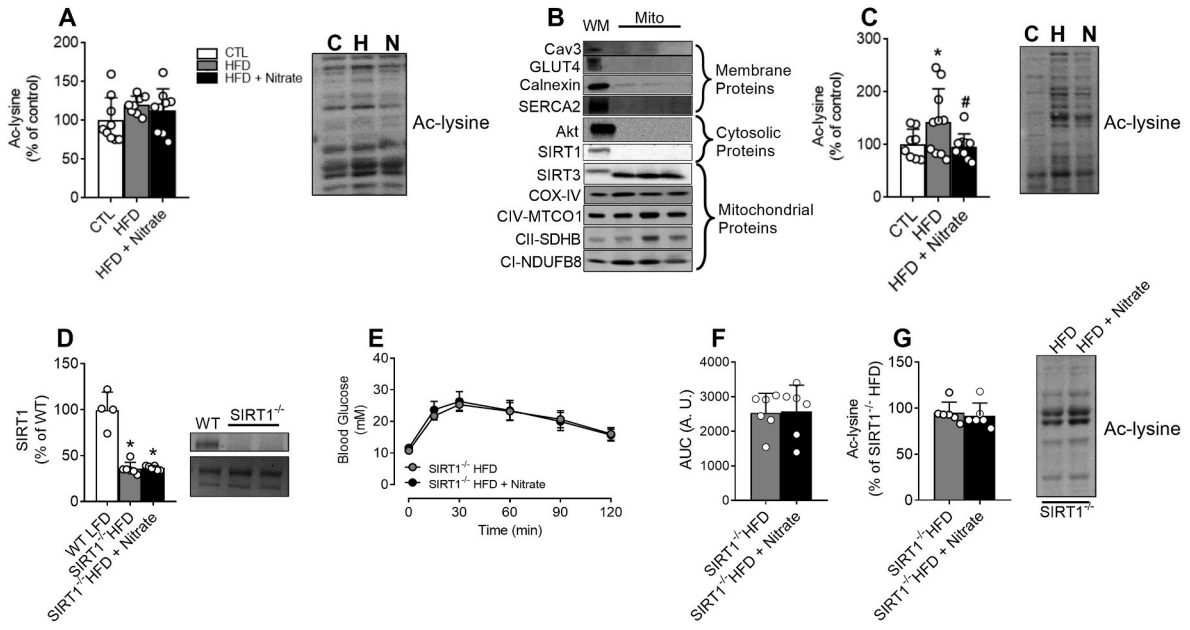

Fig. 5. – SIRT1 is required for positive metabolic effects and acetylation levels of nitrate. Quantification of acetylated lysine proteins in a whole-muscle lysate (A, n = 7–9/group), representative image of the mitochondrial isolation quality (B), quantification of acetylated lysine proteins in isolated mitochondria (C, n = 8–10/group), SIRT1 quantification in inducible SIRT1 knock out mice (D, n = 4–6/group), glucose tolerance test in HFD-fed SIRT1$^{-/-}$ mice (E, n = 6/group), area under the curve from ipGTT in HFD-fed SIRT1$^{-/-}$ mice (F, n = 6/group), quantification of acetylated lysine proteins in isolated mitochondria from gastrocnemius of HFD-fed SIRT1$^{-/-}$ mice (G, n = 6/group). Data are expressed with individual values and mean ± SD superimposed. *p < 0.05 compared to control-fed group; #p < 0.05 compared to HFD-fed group. CTL – control group; HFD – high-fat diet; Ac-lysine – acetylated lysine; Cav3 – caveolin 3; GLUT4 – glucose transporter 4; SERCA2 – sarcoendoplasmic reticulum (SR) calcium transport ATPase; COXIV – complex IV subunit COX; CIV – complex IV subunit MTCO1; CII – complex II subunit SDHB; CI – Complex I subunit NDUFB8; SIRT1 – sirtuin 1; SIRT3 – sirtuin 3; WT – wild-type; LFD – low-fat diet; AUC – area under the curve. Statistical analysis: One-way ANOVA with Fisher's LSD *post hoc* test for A, C, and D; Unpaired student's *t*-test for F and G.

regarding the necessity of SIRT1 for the positive metabolic effects of nitrate on preserving mitochondrial lysine acetylation levels.

While the present data strongly implicates SIRT1 in mediating the beneficial effects of nitrate, it remains debatable if the mechanism is muscle-specific since the present study utilized whole-body SIRT1 ablation, and skeletal muscle-specific SIRT1 overexpression does not protect mice from the deleterious effects of HFD-consumption [43] or improve skeletal muscle glucose uptake on mice fed with a standard diet [42]. Since dietary nitrate has been shown to act on other insulin-target organs, including the liver and adipose tissue [6,11,14,34], the present findings in whole-body SIRT1 knockout mice may be a result of the necessity of SIRT1 in these other nitrate-sensitive tissues and the consequent indirect effects on skeletal muscle. Additionally, it remains to be determined whether SIRT1 directly or indirectly regulates the acetylation of mitochondrial proteins. Since we could not detect SIRT1 in isolated mitochondria, SIRT1 can only directly affect outer mitochondrial membrane proteins and integral inner mitochondrial membrane proteins with residues on the outer leaflet/inter membrane space. While it is tempting to speculate that SIRT1 can regulate ANT in this manner to influence mitochondrial ADP sensitivity, it is estimated that over 60% of mitochondrial proteins carry one or more acetylation sites [2]. However, the majority of the mitochondrial acetylome is located within the matrix, and therefore unlikely to be directly regulated by a cytosolic sirtuin. Of note, although acetylation of mitochondrial proteins seems to present low stoichiometry [41] and genetic models of hyperacylation show marginal effects on overall mitochondrial bioenergetics [16], sirtuins appear to have a regulatory function in order to keep non-enzymatic acetylation levels under control, which is particularly important under lipid overload as found in HFD-feeding [27,44]. In this scenario, acetylation of specific residues in key proteins might have a prominent impact on mitochondrial function. For instance, in our experimental design, preservation of submaximal ADP-supported respiration and mtH_2O_2 production following nitrate consumption might be a result of preserved complex V and/or ANT activity, both proteins which have been shown to have lower activity upon high acetylation levels [16,30]. Nevertheless, given the necessity of SIRT1 in regulating the beneficial effects of nitrate on global mitochondrial acetylation, it is more likely that SIRT1 is mediating these effects indirectly through changes in cellular metabolism and redox balance, which may involve a number of key metabolic tissues. Regardless of these knowledge gaps, the present data implicates SIRT1 as a key protein mediating the beneficial metabolic responses to nitrate.

5. Conclusion

Altogether, the present data demonstrates an ability for nitrate consumption to preserve skeletal muscle insulin sensitivity, cellular SIRT1 content, mitochondrial acetylation, mitochondrial ADP sensitivity, and redox balance. Moreover, the positive effects of dietary nitrate on glucose tolerance and mitochondrial lysine acetylation profiles were prevented in the absence of SIRT1, suggesting that SIRT1 and acetylation balance might represent a mechanism-of-action for the beneficial effects of dietary nitrate. Combined, the present data suggests that the consumption of nitrate may attenuate the detrimental effects of overnutrition through the protection of skeletal muscle mitochondrial bioenergetics.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102307.

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