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Sodium nitrate supplementation alters mitochondrial \( \text{H}_2\text{O}_2 \) emission but does not improve mitochondrial oxidative metabolism in the heart of healthy rats

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Monaco CM, Miotto PM, Huber JS, van Loon LJ, Simpson JA, Holloway GP. Sodium nitrate supplementation alters mitochondrial \( \text{H}_2\text{O}_2 \) emission but does not improve mitochondrial oxidative metabolism in the heart of healthy rats. *Am J Physiol Regul Integr Comp Physiol* 315: R191–R204, 2018. First published March 7, 2018; doi:10.1152/ajpregu.00275.2017.—Supplementation with dietary inorganic nitrate (NO3−) is increasingly recognized to confer cardioprotective effects in both healthy and clinical populations. While the mechanism(s) remains ambiguous, in skeletal muscle oral consumption of NaNO3 has been shown to improve mitochondrial efficiency. Whether NaNO3 has similar effects on mitochondria within the heart is unknown. Therefore, we comprehensively investigated the effect of NaNO3 supplementation on in vivo left ventricular (LV) function and mitochondrial bioenergetics. Healthy male Sprague-Dawley rats were supplemented with NaNO3 (1 g/l) in their drinking water for 7 days. Echocardiography and invasive hemodynamics were used to assess LV morphology and function. Blood pressure (BP) was measured by tail-cuff and invasive hemodynamics. Mitochondrial bioenergetics were measured in LV isolated mitochondria and permeabilized muscle fibers by high-resolution respirometry and fluorometry. Nitrate decreased \( P < 0.05 \) BP, LV end-diastolic pressure, and maximal LV pressure. Rates of LV relaxation (when normalized to mean arterial pressure) tended \( P = 0.13 \) to be higher with nitrate supplementation. However, nitrate did not alter LV mitochondrial respiration, coupling efficiency, or oxygen affinity in isolated mitochondria or permeabilized muscle fibers. In contrast, nitrate increased \( P < 0.05 \) the propensity for mitochondrial \( \text{H}_2\text{O}_2 \) emission in the absence of changes in cellular redox state and decreased the sensitivity of mitochondria to ADP (apparent \( K_m \)). These results add to the therapeutic potential of nitrate supplementation in cardiovascular diseases and suggest that nitrate may confer these beneficial effects via mitochondrial redox signaling.

bioenergetics; heart; hemodynamics; mitochondria; nitrate

INTRODUCTION

The ubiquitous gaseous signaling molecule nitric oxide (NO) is critical for regulating various physiological processes, including vasodilation, muscle contractility, calcium homeostasis, and mitochondrial bioenergetics (reviewed in Refs. 16, 27). Historically, it was believed that NO was generated exclusively through the oxidation of \( \text{L} \)-arginine by the oxygen-dependent NO synthases (NOS1–3); however, the nitrate-nitrite-NO pathway has recently emerged as an additional source of NO. Nitrate (NO3−) is an inorganic anion found naturally in our diet, particularly in green leafy vegetables such as spinach, lettuce, and beetroot. Upon ingestion, ~25% of dietary nitrate is serially reduced to nitrite (NO2−) by bacteria in the oral cavity (28) and then to NO via enzymatic and nonenzymatic pathways in the body, creating a readily bioavailable NO storage pool, which is potentiated under hypoxic and acidic conditions (30, 32).

The oral consumption of nitrate in humans, in the form of inorganic salts (NaNO3 and KNO3) or a natural food product (e.g., beetroot juice), has been shown to reduce blood pressure (BP) (19, 24, 50), prevent endothelial dysfunction (50), and improve arterial compliance (3). Moreover, there is emerging evidence that nitrate also confers beneficial cardiovascular effects in certain clinical populations. Specifically, nitrate supplementation has recently been shown to reduce BP in drug-naïve hypertensive patients (10, 18) and improve vascular function in patients with peripheral artery disease (20) and heart failure (52). Collectively, from a therapeutic aspect, these findings suggest that augmentation of NO via nitrate supplementation is a low-cost intervention for reducing the burden of cardiovascular diseases.

In addition to modulating cardiovascular function, nitrate supplementation in humans has been shown to reduce the oxygen cost of submaximal exercise (4, 5, 9, 23, 25, 26, 47, 51) and enhance exercise performance (4, 9, 23). Although the mechanism(s) behind these effects is not completely understood, improved skeletal muscle mitochondrial coupling efficiency (25), reduced ATP cost of muscle contraction (4), and increased skeletal muscle contractile force (11) in association with improved calcium handling (12) have been proposed as potential explanations within skeletal muscle. However, whether these beneficial effects extend to other muscle tissues remains relatively unknown, given the paucity of research at this time. One study reported that nitrate supplementation improved mitochondrial function in the heart; however, this was only found in doxorubicin-treated mice, whereby complex I and III activity and respiration under state IV conditions (not representative of in vivo conditions) were preserved in isolated mitochondria with nitrate (53). While this may indicate that nitrates improve mitochondrial respiratory function in the heart, alternatively, the preservation of mitochondrial function...
in doxorubicin-treated mice may reflect an attenuation of pathological disease progression as opposed to a direct influence on mitochondrial bioenergetics. However, similar to skeletal muscle, a recent study found that nitrate supplementation increases in vitro cardiac contractility in association with enhanced intracellular calcium handling in healthy mice (39), which is also consistent with a previous report of findings in older mice with age-related cardiac dysfunction (41) (i.e., nitrate restored contractile function). Therefore, while it is tempting to speculate that the heart and skeletal muscle respond similarly to nitrate, the effect of nitrate supplementation on mitochondrial bioenergetics within the heart, particularly mitochondrial efficiency, remains to be established. This is particularly important, as the ability of nitrate supplementation to improve mitochondrial coupling efficiency has been challenged (15, 51), and nitrate has also been shown to increase the propensity for mitochondrial reactive oxygen species (ROS) production within skeletal muscle (51), an effect that would be detrimental in pathological conditions in which levels of ROS are already elevated (49).

Therefore, in the current study we investigated the ability of nitrate supplementation to 1) improve in vivo cardiac function in healthy rats under basal conditions and 2) determine whether altered cardiac mitochondrial bioenergetics could be implicated in this process.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were bred on-site at the University of Guelph and housed in a temperature-controlled room on a 12:12-h light-dark cycle. Water and a standard rodent chow diet were provided ad libitum. All experiments were approved by the University of Guelph Animal Care Committee and conformed with the guidelines of the Canadian Council on Animal Care as set out in the National Institutes of Health Guide to the Care and Use of Experimental Animals.

Experimental design. At 20 wk of age, the rats were randomly assigned to a control group or a nitrate group. The nitrate group was supplemented with NaNO3 in the drinking water (1 g/l) for 7 days, similar to previous studies (13, 53). This dose equates to ingestion of ~1.0 mM·kg−1·day−1 of NaNO3 and represents a concentration that is achievable through nitrate-rich supplements (e.g., beetroot juice). On day 8, animals were weighed and anesthetized with pentobarbital sodium (60 mg/kg ip). Blood was carefully collected from the inferior vena cava (plasma was frozen at −80°C); immediately thereafter, the heart was excised and the left ventricle (LV) was isolated.

Plasma nitrate/nitrite measurements. Frozen plasma samples were thawed at 4°C and analyzed for nitrate + nitrite concentrations fluorometrically using a commercially available nitrate/nitrite assay kit (Cayman Chemical, Ann Arbor, MI). First, samples were ultrafiltered using a centrifuge filter tube (Millipore, Amicon Biosperations, Billerica, MA) with a molecular weight cutoff of 10 kDa and centrifuged at 14,000 g for 10 min. This procedure removes interfering agents, particularly hemoglobin, which can drastically reduce the intensity of the fluorescence. The filtered samples were diluted 10-fold with assay buffer [20 mM KH2PO4 (pH 7.4)], loaded in triplicates, and incubated with nitrate reductase and the enzyme cofactor for 3 h at room temperature to convert all plasma nitrate to nitrite. After incubation, 2,3-diaminonaphthalene was added followed by sodium hydroxide, which enhances detection of the fluorescent product, 1H-naphthotriazole. Fluorescence intensity was measured with a spectrofluorometer (SpectraMax M2e) using an excitation wavelength of 365 nm and emission wavelength of 430 nm. The standard curve of nitrate + nitrite (NOx) was plotted, and individual sample concentration was determined as follows

\[ \text{NOx (} \mu \text{M)} = \frac{\text{Fluorescence - y-intercept}}{\text{slope}} \times \text{dilution} \]

Measurement of noninvasive hemodynamics. Conscious, restrained rats were placed on a heating pad in a dark, temperature-controlled (22°C) room in the early morning, and systolic BP, diastolic BP, and heart rate were measured using a CODA 2 tail-cuff system (Kent Scientific, Torrington, CT). Rats were habituated to the procedure for ≥7 days before the study. After habituation, the rats underwent 5 acclimation cycles followed by 2 sets of 10 cycles for a total of 25 measurements. Heart rate was continuously monitored during the experiments. The last 10 cycles were averaged and used for measurements. Mean arterial pressure was calculated from systolic and diastolic BP.

LV echocardiography. Echocardiographic examination of the LV was performed 24 h before experiments (on day 7) in the early morning using the Vevo770 imaging system (VisualSonics, Toronto, ON, Canada), as previously described (40). Briefly, the animals were anesthetized with isoflurane-oxygen (4%, 1 l/min), secured to a heated platform in the supine position, and maintained under anesthesia (1.5–2%, 1 l/min) via nose cone. Body temperature was maintained between 37.0 and 37.8°C during the entire examination. Once heart rates and body temperature had stabilized (~5 min), a transducer (model MS-250, MicroScan) was used to obtain parasternal long-axis two-dimensional B-mode and M-mode images of the LV. The M-mode images (cross section taken at the widest point in the ventricle) were analyzed using the M-mode measurement tools provided in the manufacturer’s software (Vevo 2100 Workstation Software package). Heart rate, LV end-diastolic dimension, and LV end-systolic dimension were measured and used to calculate stroke volume, cardiac output, end-diastolic volume, end-systolic volume, ejection fraction, and fractional shortening.

Measurement of LV invasive hemodynamics. Rats were anesthetized with isoflurane-oxygen (4%, 1 l/min), and a catheter was inserted via the right carotid artery into the LV. Hemodynamic signals were recorded 15 min later by a computer using Labscribe2 analytic software (iWorx, Dover, NH).

Mitochondrial isolation. Differential centrifugation was used to isolate subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria from the LV, as described previously (33). Briefly, the LV was dissected from the heart and cut in half: one half was immediately snap-frozen in liquid nitrogen for Western blotting, and the other half was weighed, minced with scissors, diluted in ice-cold mitochondrial isolation buffer [100 mM sucrose, 100 mM KCl, 50 mM Tris-HCl, 1 mM KH2PO4, 0.1 mM EGTA, 1 mM ATP, and 2 mg/ml fatty acid-free BSA (pH 7.4)], and homogenized on ice at 750 rpm using a Polytron equipped with a tight-fitting Tetlon pestle. The homogenate was centrifuged at 800 g for 10 min at 4°C to separate the SS mitochondria from the myofibrils (IMF pellet). The supernatant (containing the SS mitochondria) was placed on ice, while the IMF pellet was resuspended in isolation buffer and treated with protease (0.025 µl/mg muscle) for exactly 5 min. Then the IMF pellet was diluted 10-fold in isolation buffer to slow the reaction and centrifuged again at 5,000 g for 5 min. The IMF pellet was resuspended in isolation buffer and centrifuged at 800 g for 10 min. The supernatant (now containing the IMF mitochondria) was pelleted at 10,000 g for 10 min along with the supernatant containing the SS mitochondria. The pellets were washed in Mg2+-free Mir05 buffer [0.5 mM EGTA, 60 mM K-lactobionate, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, and 1 mg/ml fatty acid-free BSA (pH 7.1)] and centrifuged again at 10,000 g for 10 min. The final pellets for SS and IMF isolations were resuspended in Mg2+-free Mir05 buffer before oxygen affinity, respiration, and H2O2 emission measurements. Protein content was quantified spectrophotometrically using the Bradford protein assay.
Preparation of permeabilized muscle fibers. Permeabilized muscle fibers (PmFBs) were prepared as previously described in our laboratory (6). Briefly, the LV was dissected and immediately placed in ice-cold BIOPS ([in mM]: 50 MES, 7.23 K₂EGTA, 2.77 CaK₂EGTA, 20 imidazole, 0.5 DTT, 20 taurine, 5.77 ATP, 15 phosphocreatine (PCr), and 6.56 MgCl₂·H₂O (pH 7.1)) on ice. Muscle bundles (3–4 mm long, ~1 mm diameter) were cut from the endocardial surface along the fiber orientation to avoid mechanical damage of the cells (44). Each bundle was carefully and gently separated along the longitudinal axis using a pair of sharp fine-tipped needle forceps under magnification (MX6 Stereoscope, Zeiss Microsystems, Wetzlar, Germany). After separation, fiber bundles were incubated in BIOPS containing 50 µg/ml saponin and agitated for 30 min at 4°C on a rotor. Saponin is a mild cholesterol-specific detergent that selectively permeabilizes the sarcolemmal membrane due to its high cholesterol content but leaves intracellular membrane structures (mitochondria and endoplasmic reticulum, which contain very little cholesterol) intact (22, 48). PmFBs were subsequently washed for 10 min at 4°C in MiR05 respiration buffer ([0.5 mM EGTA, 3 mM MgCl₂·H₂O, 60 mM lactobionic acid, 10 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, and 1 mg/ml fatty acid-free BSA (pH 7.1)]) for respiration analysis or buffer Z ([105 mM K-MES, 30 mM KCl, 1 mM EGTA, 10 mM KH₂PO₄, 5 mM MgCl₂·H₂O, 0.005 mM glutamate, and 0.002 mM malate with 5 mg/ml BSA (pH 7.4)]) for H₂O₂ emission measurements. PmFBs were washed two more times for a total of 30 min to ensure complete removal of saponin, metabolites, and trace amounts of free ADP.

Mitochondrial respiration and oxygen affinity measurements. Mitochondrial respiration was measured in isolated mitochondria by high-resolution respirometry (Oroborus Oxygraph-2k, Innsbruch, Austria) at 25°C and room air saturated oxygen tension, as previously described (33). Briefly, basal respiration was determined in the presence of 10 mM pyruvate and 2 mM malate. Mitochondrial efficiency [amount of ADP consumed per oxygen atom consumed (P/O ratio)] was determined in the presence of submaximal ADP (100 µM), while maximal respiration (state III) was determined in the presence of saturating ADP (5 mM). Maximal complex I- and complex II-supported respiration was determined in the presence of glutamate (10 mM) and succinate (10 mM), respectively. Mitochondrial oxygen affinity experiments were performed in both SS and IMF mitochondria (0.2 mg/ml): the mitochondria were allowed to respire until controlled respiration was determined in the presence of glutamate (10 mM) and succinate (10 mM) (AJP-Regul Integr Comp Physiol). PmFBs. In the PmFB experiments, 50 µM ADP was also added at the end of the reaction. Addition of ADP dissipates the proton motive force and, thus, in theory, decreases H₂O₂ emission. The rate of H₂O₂ emission was calculated from the slope (absorbance/s) after subtraction of the background from a standard curve established with the same reaction conditions. H₂O₂ emission rates were normalized to fiber dry weight or protein content.

Western blotting. Whole muscle from the LV was homogenized in lysis buffer, and 5 µg of protein were loaded equally and separated by SDS-polyacrylamide gel electrophoresis at 150 V for 1 h, transferred to a polyvinylidine difluoride membrane at 100 V for 1 h at 4°C, and incubated in blocking solution, primary antibody, and the corresponding secondary antibody as specified by the supplier. Commercially available antibodies were used to detect α-tubulin (Abcam), superoxide dismutase 2 (SOD2; Abcam), catalase (Abcam), cytochrome c oxidase subunit IV (COXIV; Invitrogen), oxidative phosphorylation cocktail (OXPHOS; Mitosciences), 4-hydroxynonenal (4-HNE; Alpha Diagnostics), N³-nitrosylation (Cayman Chemical), mitochondrial creatine kinase (miCK; Abcam), adenosine nucleotide translocase 1 (ANT1; Mitosciences), and adenine nucleotide translocase 2 (ANT2; Abcam). Membrane proteins were visualized by Western Lightning Plus-ECL (Perkin Elmer, Woodbridge, ON, Canada), using a FluorChem HD2 imager and quantified using Alpha Innotech software (Fisher Scientific, Ottawa, ON, Canada).

Glutathione measurements. Approximately 30 mg of wet muscle were homogenized in 7% perchloric acid-phenanthroline [1:10 (wt/vol)] homogenization medium, incubated for 10 min, and centrifuged at 1,000 g at 4°C. The supernatant was added to 0.4 M iodoacetic acid (Sigma Aldrich, Zwijndrecht, The Netherlands) and neutralized by addition of excess NaHCO₃. An internal standard [G3640 (25 mg), Sigma Aldrich] was added, and samples were incubated at room temperature for 1 h in a darkened room, vortexed following addition of dinitrofluorobenzene, and incubated at room temperature in darkness for a further 8 h. Finally, samples were injected into a high-performance liquid chromatograph (model LC-20AD, Shimadzu, Kyoto, Japan) coupled with a Microsorb 100×5 NH₂ 250×4.6 mm high-performance liquid chromatography column (Agilent Technologies, Santa Clara, CA) and measured at a flow rate of 0.5 ml/min at 350 nm. Retention times were 18.5, 21, and 23 min for the internal standard, GSH, and GSGG, respectively.

Statistical analysis. Values are means ± SE. Prism (version 6.2, GraphPad Software) was used for statistical analysis and calculation of the apparent Kₘ values for ADP titrations, as described previously (37). Control versus nitrate measures were compared using a two-tailed Student’s unpaired t-test, except for BP analyses, for which a one-tailed t-test was used. P < 0.05 was considered significantly different.

RESULTS

Effect of nitrate supplementation on plasma levels of nitrate and nitrite, morphology, and BP. We first established the effect of 7 days of nitrate supplementation on plasma levels of NOx. As shown in Table 1, plasma NOx significantly increased by ~20-fold after 7 days, indicating that the dose-and-supplementation regimen was effective. Importantly, no differences in food or water intake were observed between the control and nitrate groups during the entire supplementation period. In addition, there were no statistical differences in final body weight, whole heart weight, and whole heart weight-to-tibia length ratio between the two groups after 1 wk of supplemen-
Nitrate supplementation, indicating that nitrate does not affect gross morphology in 20-wk-old healthy rats (Table 1).

Next, we measured BP by noninvasive hemodynamics (tail-cuff method) to ascertain that the rise in plasma NOx concentrations coincided with the expected BP-lowering effects of nitrate supplementation previously reported in rodents (13, 38, 50) and humans (45). Systolic BP was significantly reduced in the nitrate-supplemented animals (Fig. 1A; \( P = 0.04 \)), while mean arterial pressure tended to decrease (Fig. 1B; \( P = 0.11 \)). In contrast, diastolic BP was not significantly different but tended to be lower with nitrate supplementation (Fig. 1C; \( P = 0.15 \)). There was no significant difference in heart rate between the two groups (Fig. 1D; \( P = 0.31 \)).

**Effect of nitrate supplementation on in vivo cardiac function.**

Given that nitrate supplementation has been shown to increase cardiac contractility in vitro (39), we next aimed to determine if nitrate supplementation altered cardiac function in vivo under resting conditions. First, we used echocardiography to noninvasively examine LV function and chamber dimensions in sedated rats. The results for echocardiographic analysis are shown in Table 2. Briefly, there were no significant alterations \( (P > 0.05) \) in any of the parameters measured between the two groups, including cardiac output, stroke volume, and LV dimensions. These findings indicate that nitrate supplementation for 7 days does not cause structural alterations of the LV of healthy rats and suggest that nitrate does not improve in vivo cardiac function under basal conditions.

However, echocardiography is an indirect measure of cardiac function and may not be sensitive enough to detect subtle changes within the heart. Therefore, we next performed invasive LV hemodynamics to obtain a direct assessment of in vivo cardiac pressure in both groups of animals. Nitrate supplementation significantly decreased maximal LV pressure (Fig. 2A; \( P = 0.005 \)) and LV end-diastolic pressure (Fig. 2B; \( P = 0.01 \)). Surprisingly, however, these decreases in LV pressure were not

### Table 1. Plasma NOx concentrations and morphological characteristics in control and nitrate-supplemented rats

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control</th>
<th>Nitrate</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma NOx, ( \mu M )</td>
<td>9.5 ± 2.1</td>
<td>196.8 ± 40.6</td>
<td>0.02</td>
</tr>
<tr>
<td>Anthropometrics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final body wt, g</td>
<td>465 ± 13</td>
<td>456 ± 11</td>
<td>0.60</td>
</tr>
<tr>
<td>Heart wt, g</td>
<td>1.32 ± 0.05</td>
<td>1.29 ± 0.03</td>
<td>0.66</td>
</tr>
<tr>
<td>Tibia length, cm</td>
<td>4.2 ± 0.1</td>
<td>4.3 ± 0.1</td>
<td>0.10</td>
</tr>
<tr>
<td>Heart wt/tibia length, g/cm</td>
<td>0.31 ± 0.01</td>
<td>0.30 ± 0.01</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 4 \) rats/group for plasma concentration and \( n = 8 \) rats/group for anthropometric data. NOx, nitrate; LV, left ventricle.

### Table 2. LV echocardiography analysis of control and nitrate-supplemented rats

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control</th>
<th>Nitrate</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac output, ml/min</td>
<td>85 ± 2</td>
<td>84 ± 2</td>
<td>0.96</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>360 ± 5</td>
<td>359 ± 6</td>
<td>0.90</td>
</tr>
<tr>
<td>Stroke volume, ( \mu L )</td>
<td>235 ± 5</td>
<td>238 ± 6</td>
<td>0.69</td>
</tr>
<tr>
<td>End-systolic dimension, mm</td>
<td>4.1 ± 0.1</td>
<td>4.3 ± 0.1</td>
<td>0.71</td>
</tr>
<tr>
<td>End-diastolic dimension, mm</td>
<td>7.7 ± 0.1</td>
<td>7.8 ± 0.1</td>
<td>0.38</td>
</tr>
<tr>
<td>End-systolic volume, ( \mu L )</td>
<td>77 ± 6</td>
<td>80 ± 5</td>
<td>0.59</td>
</tr>
<tr>
<td>End-diastolic volume, ( \mu L )</td>
<td>318 ± 15</td>
<td>322 ± 11</td>
<td>0.75</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>76 ± 1</td>
<td>74 ± 1</td>
<td>0.37</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>46 ± 1</td>
<td>45 ± 1</td>
<td>0.36</td>
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</table>

Values are means ± SE; \( n = 11–12 \) rats/group. Two-dimensional echocardiographic imaging of the left ventricle (LV) was performed after 7 days of nitrate supplementation in sedated rats.

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![Fig. 1. Blood pressure measurements by noninvasive hemodynamics in control and nitrate-supplemented rats.](image-url)
accompanied by increases in maximal rates of pressure development (dP/dt<sub>max</sub>; Fig. 2C) or pressure decline (dP/dt<sub>min</sub>; Fig. 2D), indexes of cardiac contractility and relaxation, respectively (P = 0.53 and P = 0.32, respectively). In addition, dP/dt during isovolumic LV contraction (dP/dt<sub>LVP40</sub>) and the time constant of isovolumic LV relaxation (τ; Weiss method) were not significantly different with nitrate supplementation (Table 3), suggesting that nitrate supplementation does not improve systolic or diastolic function in vivo. However, dP/dt values are load-dependent indexes of cardiac function, and nitrate supplementation decreased BP, thereby confounding this interpretation. Indeed, our invasive hemodynamic measurement of BP showed that nitrate supplementation caused significant reductions of mean arterial pressure and systolic BP by ~8 mmHg (P < 0.0001) and ~9 mmHg (P = 0.003), respectively, and strongly trended (P = 0.05) toward a ~4-mmHg reduction of diastolic BP (Table 3), coinciding with our noninvasive measurements of BP reported above. Therefore, we normalized dP/dt<sub>min</sub> to mean arterial pressure, which revealed a trend (P = 0.13) for increased LV rates of relaxation normalized to mean arterial pressure (dP/dt<sub>min</sub>/MAP). Values are means ± SE; n = 8–11 rats/group. *P < 0.05 vs. control.

Table 3. Invasive hemodynamic measurements of control and nitrate-supplemented rats

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Nitrate</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>328</td>
<td>332</td>
<td>0.44</td>
</tr>
<tr>
<td>dP/dt&lt;sub&gt;LVP40&lt;/sub&gt;, mmHg/s</td>
<td>6.024</td>
<td>6.064</td>
<td>0.98</td>
</tr>
<tr>
<td>τ, ms</td>
<td>10.74</td>
<td>10.63</td>
<td>0.71</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td>112</td>
<td>102</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>121</td>
<td>113</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>96</td>
<td>92</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8–11 rats/group. dP/dt<sub>LVP40</sub>, rate of pressure development during isovolumic LV contraction; BP, blood pressure; τ, time constant of isovolumic LV relaxation (Weiss method).

Fig. 2. Left ventricular (LV) pressure characteristics measured by invasive hemodynamics in control and nitrate-supplemented rats. A–D: nitrate supplementation significantly decreased maximal LV pressure (LVP<sub>max</sub>) and LV end-diastolic pressure (LVEDP) but did not alter maximal rates of pressure development (dP/dt<sub>max</sub>) or maximal rates of pressure decline (dP/dt<sub>min</sub>). E: nitrate trended to increase maximal rates of pressure decline normalized to mean arterial pressure (dP/dt<sub>min</sub>/MAP). Values are means ± SE; n = 8–11 rats/group. *P < 0.05 vs. control.

Nitrate supplementation does not increase LV mitochondrial oxidative metabolism. Since cardiac relaxation is an ATP-dependent process, we next investigated the effect of nitrate supplementation on various indexes of mitochondrial bioenergetics within the heart. First, we measured mitochondrial...
respiration in SS and IMF mitochondria isolated from the LV. Nitrate supplementation did not significantly alter \((P > 0.05)\) basal mitochondrial respiration (state IV; absence of ADP) or maximal respiration (state III; ADP-stimulated) across a range of complex I (pyruvate + malate + glutamate) and complex II (succinate) substrates in SS (Fig. 3A) or IMF (Fig. 3C) mitochondria. Similarly, the respiratory control ratio, a quality-control check of the functional integrity of the mitochondria, was not altered \((P > 0.05)\) with nitrate supplementation in either mitochondrial subpopulation (Fig. 3, A and C). Next, we measured mitochondrial coupling efficiency by calculating the P/O ratio and found no significant differences in SS \((P = 0.63)\) or IMF \((P = 0.41)\) mitochondria with nitrate supplementation (Fig. 3, B and D). Together, these findings indicate that nitrate supplementation for 7 days in healthy rats does not lead to improvements in mitochondrial respiration, oxidative capacity, or coupling efficiency within the LV of the heart.

While isolation of mitochondria allows study of both subpopulations separately, the process of isolating mitochondria inevitably requires mechanical homogenization and a series of differential centrifugation steps that disrupt the structural integrity of the mitochondria. Therefore, we repeated these measures in LV PmFBs (mitochondria still structurally intact). Similar to isolated mitochondria, nitrate supplementation did not significantly alter \((P > 0.05)\) basal respiration, maximal respiration, or the respiratory control ratio (Fig. 4A). We also measured leak respiration, as this has been shown to be decreased with nitrate supplementation in skeletal muscle (25), but we found no significant differences \((P = 0.81)\) between the two groups in cardiac muscle (Fig. 4B). Moreover, the content of the various proteins of the electron transport chain was not altered with nitrate supplementation (Fig. 4C; \(P > 0.05\)), which further supports the lack of difference in maximal respiration. Together, these findings provide compelling evidence that 7 days of nitrate supplementation does not directly lead to improvements in mitochondrial oxidative metabolism in the heart of healthy rats.

LV mitochondrial oxygen affinity is not altered with nitrate supplementation. While NO has been shown to bind competitively to cytochrome c oxidase in the heart of rats (7), reduction of mitochondrial oxygen affinity (sensitivity of the mitochondria to oxygen) would conceivably compromise cardiac function. Therefore, we aimed to determine whether chronic nitrate supplementation improves oxygen sensitivity, perhaps through posttranslational modifications (i.e., structural alterations) that would offset the detrimental effects of increased NO bioavailability. We allowed SS and IMF mitochondria to respire until anoxia in the presence of saturating

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**Fig. 3.** Nitrate supplementation does not lead to increases in left ventricular (LV) mitochondrial oxidative capacity or coupling efficiency in subsarcolemmal (SS; A and B) and intermyofibrillar (IMF; C and D) mitochondria. Mitochondrial respiration was measured in state IV respiration [addition of 10 mM pyruvate + 2 mM malate (PM) in the absence of ADP (-ADP)] followed by state III respiration [addition of 5 mM ADP (+ADP)] with final sequential additions of glutamate (10 mM; PMG) and succinate (10 mM; PMGS). Maximal complex I- and II-supported respiration was measured in SS (A) and IMF (C) mitochondria. Mitochondrial coupling efficiency (P/O ratio) was calculated following an initial submaximal bolus of ADP (100 μM) in SS (B) and IMF (D) mitochondria. \(J_{O_2}\), mitochondrial oxygen flux; RCR, respiratory control ratio (state III/state IV). Values are means ± SE; \(n = 9–14\) rats/group.
Nitrate supplementation does not alter left ventricular (LV) mitochondrial oxidative capacity or leak respiration in permeabilized cardiac muscle fibers (PmFBs). A: mitochondrial respiration was measured using the protocol used for isolated mitochondria. B: oligomycin (oligo; 2 μg/ml) was added during state IV respiration [10 mM pyruvate + 2 mM malate (PM) in the absence of ADP]. C: protein content of oxidative phosphorylation cocktail (OXPHOS), expressed as percentage of control, with representative blots of OXPHOS and cytochrome c oxidase subunit IV (COXIV-4), and with α-tubulin as loading control (right). Con, control; JO2, mitochondrial oxygen flux; Nit, nitrate; PM, state IV respiration with 10 mM pyruvate + 2 mM malate in the absence of ADP (+ADP); PMGS, 10 mM pyruvate + 2 mM malate + 10 mM glutamate + 10 mM succinate in the presence of maximal ADP (+ADP); RCR, respiratory control ratio (state III/state IV). Values are means ± SE; n = 8 rats/group for PmFBs and 7–8 rats/group for Western blot data.

Nitrate supplementation decreases LV mitochondrial ADP sensitivity. Next, we investigated the sensitivity of the mitochondria to ADP, as NO has been shown to reduce mitochondrial ADP sensitivity by inhibiting miCK in cardiac PmFBs (17). We performed ADP titrations in PmFBs, but most importantly, with PCr and Cr (PCr/Cr) either absent (no Cr) or present (+PCr/Cr) in the respiration medium, since manipulation of the concentration of these metabolites provides mechanistic insight into the regulation of energy production in situ (36) (i.e., recruitment of miCK for energy transfer). As shown in Fig. 6, A and D, a typical Michaelis-Menten curve was observed in both groups, and this curve was used to calculate the apparent ADP K_m (ADP sensitivity) and maximal ADP-stimulated respiration (V_max). In the absence of PCr/Cr, nitrate supplementation did not alter the apparent K_m (Fig. 6B; P = 0.68) or V_max (Fig. 6C; P = 0.94). In contrast, when miCK was recruited (+PCr/Cr), the apparent K_m significantly increased by ~30% in the nitrate-supplemented group (Fig. 6E; P = 0.03). Importantly, this occurred without detectable changes in V_max (Fig. 6F; P = 0.61), indicating a decrease in mitochondrial ADP sensitivity with nitrate supplementation when miCK is recruited for energy transfer. The change in mitochondrial ADP sensitivity was not due to differences in the protein expression of miCK or ANT1 and ANT2 (Fig. 6, G and H; P > 0.05). Therefore, these data suggest that the decrease in ADP...
sensitivity with nitrate supplementation in the heart is the result of altered regulation of ADP kinetics, likely posttranslational modifications on miCK.

**Nitrate supplementation increases LV mitochondrial H$_2$O$_2$ emission.** Because nitrate supplementation has been found to decrease mitochondrial ADP sensitivity and provision of ADP to mitochondria is critical for redox homeostasis (21, 46), we next examined whether nitrate supplementation increases the capacity of mitochondria to generate ROS (measured as H$_2$O$_2$ emission potential) in cardiac muscle. In the absence of ADP, rates of succinate-induced H$_2$O$_2$ emission increased by ~21% (Fig. 7A; $P = 0.06$) in the nitrate group. H$_2$O$_2$ emission was decreased by ~50% after addition of a submaximal bolus of ADP (50 $\mu$M) in the control group but remained significantly elevated in the nitrate group (Fig. 7A; $P < 0.05$). However, this was simply the result of H$_2$O$_2$ emission levels already being elevated before addition of ADP, as the percent difference in H$_2$O$_2$ emission after the addition of submaximal ADP was the same between the two groups (data not shown). The majority of H$_2$O$_2$ emission appeared to be generated from SS mitochondria, as the rate of succinate-induced H$_2$O$_2$ emission increased by ~30% with nitrate supplementation in this subpopulation (Fig. 7B; $P = 0.04$), whereas there was no significant difference in the IMF subpopulation (Fig. 7B; $P = 0.42$). This increase in mitochondrial H$_2$O$_2$ emission potential occurred independent of detectable differences in the protein content of the antioxidant enzymes catalase ($P = 0.21$) and SOD2 ($P = 0.12$) (Fig. 7C) and did not result in overt oxidative stress, given the lack of significant difference in lipid peroxidation (4-HNE; $P = 0.50$) and nitrotyrosine residues (N$_3$; $P = 0.96$) in LV homogenate (Fig. 7E). Moreover, there were no alterations ($P > 0.05$) in the ratio of reduced to oxidized glutathione and total GSH and GSSG, further supporting the lack of change in the cellular redox state (Fig. 7, E–G). Together, these data demonstrate that nitrate supplementation increases the propensity for mitochondrial ROS production in the heart of healthy 20-wk-old rats without causing overt oxidative stress.

**DISCUSSION**

In the present study the effects of nitrate supplementation on in vivo cardiac function and in vitro cardiac mitochondrial bioenergetics were investigated. We demonstrate that nitrate supplementation in the form of a sodium salt for 7 days in healthy rats decreased BP and maximal LV pressure (afterload), as well as LV end-diastolic pressure (preload) in vivo without altering LV mitochondrial coupling efficiency, oxygen affinity, or oxidative capacity in vitro. Furthermore, LV mitochondrial ADP sensitivity was decreased, while the propensity for ROS production was increased, in the absence of change in cellular redox state following nitrate supplementation. Together, these findings demonstrate beneficial effects of nitrate supplementation.
supplementation on various indexes of cardiovascular “health” under basal healthy conditions that occur in the absence of intrinsic improvements in LV mitochondrial oxidative metabolism.

It is well recognized that nitrate supplementation is an important and alternative source for NO bioavailability in the body due to its NO-like effects following ingestion. Indeed, among the plethora of pathways regulated by NO, of particular interest from a therapeutic aspect is the ability of NO to influence contractility and relaxation within the heart (reviewed in Ref. 42). However, we found that chronic supplementation with NaNO3 in healthy rats does not robustly improve LV contractile function. Specifically, we did not observe a change in indexes of cardiac contractility (dP/dt max) or relaxation (dP/dt min) after 7 days of supplementation. This is similar to previous findings in doxorubicin-treated mice (53) but is in stark contrast to the recent report of Pironti et al. where NaNO3 supplementation increased peak rates of force.

Fig. 6. Nitrate supplementation alters mitochondrial ADP kinetics in cardiac permeabilized muscle fibers (PmFBs). A and D: ADP titrations in the presence of 10 mM pyruvate and 2 mM malate generated typical Michaelis-Menten kinetics in the absence of creatine (Cr; A) and in the presence of phosphocreatine (PCr) and Cr (D) in PmFBs. B and E: apparent K_m remained unchanged in the absence of PCr/Cr (B) but was increased in the presence of PCr/Cr (E) with nitrate supplementation. C and F: maximal respiration (V_max) was not different in any group. G: increase in the apparent K_m occurred in the absence of changes in protein content of adenosine nucleotide translocase (ANT) and mitochondrial creatine kinase (miCK), expressed as percentage of control, with α-tubulin as a loading control. H: representative blots. Con, control; JO2, mitochondrial oxygen flux; Nit, nitrate. Values are means ± SE; n = 7–8 rats/group. *P < 0.05 vs. control.
**Sodium Nitrate and Cardiac Mitochondrial Bioenergetics**

**A**
- Control: Open bars
- Nitrate: Filled bars

**H₂O₂ Emission**
- Succinate: 0.0, 0.5, 1.0, 1.5 pmol·sec⁻¹·mg⁻¹ dry weight
- Succinate + ADP: P = 0.06

**B**
- SS: 0, 50, 100, 150 pmol·sec⁻¹·mg⁻¹ mito protein
- IMF: 100 ± 20 pmol·sec⁻¹·mg⁻¹ mito protein

**C**
- Con: Control
- Nit: Nitrate
- Catalase
- SOD2
- α-tubulin

**D**
- 4-HNE
- N3

**E**
- GSH:GSSG
- Control: 10, 15 pmol·mg tissue⁻¹
- Nitrate: 15, 20 pmol·mg tissue⁻¹

**F**
- GSH
- Control: 1500, 2000 μmol·mg tissue⁻¹
- Nitrate: 1500, 2000 μmol·mg tissue⁻¹

**G**
- GSSG
- Control: 150, 200 μmol·mg tissue⁻¹
- Nitrate: 150, 200 μmol·mg tissue⁻¹

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Development (contractility) and relaxation in Langendorff-perfused hearts from healthy mice (39). Indeed, these opposing findings are likely the result of methodological differences, as Langendorff-perfused hearts allow for the direct assessment of contractile function independent of hormonal/neral and hemodynamic influences, parameters that cannot be controlled with invasive hemodynamics. Given the well-documented vasodilatory properties of nitrates (45) and our observations of reductions in BP following nitrate supplementation, it seems very likely that the effects of nitrate on the vasculature in the present study masked improvements in cardiac contractile function. In support of this idea, when we normalized dP/dt_{max} to mean arterial pressure, as relaxation rates are influenced by pressure, we found that nitrate trended (P = 0.13) to increase rates of LV relaxation, which coincided with the reduction (P = 0.01) of LV end-diastolic pressure (preload). Alternatively, the lack of detectable differences in rates of contractility (dP/dt_{max}) and relaxation (dP/dt_{min}) in the present study could be explained by the timing of the dosing regimen (39). Together, our findings add to the growing body of evidence that suggests that nitrate supplementation improves indexes of cardiac hemodynamics, even under healthy conditions.

Although nitrate supplementation has been demonstrated to increase cardiac contractility via improvements in intracellular calcium-handling properties (39), an increase in calcium release would inevitably place a greater energetic demand on the heart (e.g., myosin and calcium ATPase activity), making increased mitochondrial content or improved mitochondrial coupling efficiency (i.e., improved capacity for energy production) likely adaptations. However, in the current study we did not observe differences in the protein expression of the individual complexes of the electron transport chain, mitochondrial coupling efficiency (P/O ratio), expression of the uncoupling proteins ANT1 and ANT2, basal/maximal respiration, or measures of true “leak” respiration (state IV respiration in the presence of oligomycin). While the lack of statistical difference in the P/O ratio differs from a previous report of work in skeletal muscle (25), it nonetheless corroborates more recent studies in skeletal muscle (15, 51) and suggests that nitrate supplementation does not improve indexes of mitochondrial function in the heart. Furthermore, titration of ADP unexpectedly revealed an increase in the apparent K_{m} for ADP following nitrate supplementation when the miCK shuttle was recruited (+PCr/Cr), indicating a decrease in mitochondrial ADP sensitivity. Indeed, this finding seems counterintuitive, considering the implications to mitochondrial ATP production; however, clearly, contractile function was not compromised in these animals (e.g., conserved cardiac output and stroke volume). Previous work has shown that ablation of miCK does not result in heart failure (29, 34); therefore, the functional consequence of this observation remains unclear. Nevertheless, the present data do not suggest that NaNO3 improves indexes of cardiac hemodynamics via increases in the intrinsic capacity for mitochondrial oxidative metabolism.

Previous work in skeletal muscle showed an increased capacity for mitochondrial ROS production with nitrate supplementation (51), and in the present study, increased rates of mitochondrial ROS production (measured as H2O2 emission) were found in cardiac PmFBS and isolated SS mitochondria following nitrate supplementation. These data are in contrast to a previous report in the heart (53); however, different substrates were used to estimate ROS production. In particular, we found that nitrate supplementation caused a significant increase in the propensity for mitochondrial H2O2 emission via succinate, which is primarily associated with reverse electron flow to complex I, while the absence of a response appears to occur with complex I (glutamate + malate)- and complex III (rotenone + succinate)-derived H2O2 emission (53). Together, these data may indicate that nitrate increases the propensity for mitochondrial ROS production, specifically at the ubiquinone binding site on complex I. Interestingly, NO has been recently shown to interact with the ubiquinone-cytochrome b site (complex III), causing antinmycin-like effects that, in turn, increased cardiac mitochondrial H2O2 production (14). Nevertheless, because our data also revealed a preserved redox state [no change in glutathione ratios, lipid peroxidation (4-HNE), and nitrosylation (N3)], despite an increased potential for mitochondrial ROS emission, nitrate may exert improvements in cardiac function via mitochondrial redox-induced signaling. Consistent with this idea, increased mitochondrial H2O2 production has been shown to alter calcium handling in cardiac cells (54) and has been implicated in the response of cardiomyocytes to β-adrenergic stimulation (2). Clearly, more studies are warranted to better understand and elucidate how nitrate alters contractile function and, in particular, if increased mitochondrial ROS and the associated redox signaling play a part.

Limitations of the study. In the present study we provide evidence that nitrate consumption does not improve mitochondrial oxidative metabolism, suggesting that alterations in mitochondrial bioenergetics are not a likely explanation for the enhancement of cardiac hemodynamics. However, a limitation to our study is the fact that we measured the effect of nitrate supplementation on cardiac mitochondrial bioenergetics in vitro, which may not accurately reflect effects at the whole body level, as chronically elevated NO could be regulating mitochondrial bioenergetics in vivo through its reversible interaction with complex IV (7, 8). Therefore, while the acute effects of nitrate are likely lost in our assessments of mitochondrial bioenergetics, it is unlikely that NO-mediated inhibition of complex IV would result in improved cardiac con-

Fig. 7. Nitrate supplementation increases mitochondrial reactive oxygen species (ROS) production in cardiac permeabilized muscle fibers (PmFBS) and isolated mitochondria but does not lead to oxidative stress. A: succinate (10 mM)-supported H2O2 emission potential was increased in the nitrate group and decreased with addition of 50 μM ADP to PmFBS in both groups. B: the majority of increased H2O2 emission potential with nitrate stemmed from subsarcolemmal (SS) mitochondria, as this remained unchanged in intermyofibrillar (IMF) mitochondria. C: nitrate supplementation did not reduce protein content of the antioxidant enzymes catalase and SOD2, expressed as percentage of control; representative Western blots for catalase and SOD2 with α-tubulin as a loading control are shown. D: nitrate supplementation did not alter markers of overt of oxidative stress, expressed as percentage of control; representative blots of lipid peroxidation (4-HNE) and nitrosylation (N3) are shown. E-G: redox state, measured as reduced glutathione (GSH)-to-oxidized glutathione (GSSH) ratio, was not altered with nitrate supplementation. Con, control; Nit, nitrate. Values are means ± SE; n = 7 rats/group for PmFBS, 10–14 rats/group for isolated mitochondria, 7–8 rats/group for Western blot data, and 5 rats/group for GSH-GSSG. *P < 0.05 vs. control.
tractile function. In addition, previous work showed that nitrate supplementation increases peak rates of force development (contractility) and relaxation in a Langendorff-perfused system (39), which, given the absence of nitrate in the perfusate and the short half-life of NO, also represents a model that would be expected to minimize the acute effects of nitrate. Nevertheless, the present data should be interpreted within this context and, as such, only suggest that chronic nitrate supplementation does not alter the intrinsic function of mitochondrial oxidative metabolism.

**Perspectives and Significance**

The current study demonstrates that, in healthy rats, 7 days of NaNO3 supplementation causes reductions in BP and LV end-diastolic pressure and has a tendency to increase rates of LV relaxation when measured in vivo. While the exact mechanism(s) of action for the beneficial effects of nitrate on cardiac tissue remains to be elucidated, our current data indicate that intrinsic changes in LV mitochondrial oxidative metabolism are not required for nitrate to confer improvements in cardiac contractile function. However, we do provide evidence that mitochondrial redox-induced signaling may contribute to the beneficial responses associated with nitrate consumption. While this finding appears paradoxical, considering the previous finding that increased levels of ROS and oxidative stress are common denominators in the pathophysiology of cardiovascular disease (49), transient increases in intracellular ROS are increasingly recognized as an essential signal for cellular adaptations to external stimuli (e.g., exercise) (43). Since the cellular redox state was not altered within cardiac tissue in the present study, these data suggest that the dose and timing of nitrate supplementation may have been sufficient to induce "physiological doses" of ROS required for nitrate to induce benefits on cardiovascular health, as opposed to pathological responses. While, therefore, the present data extend the therapeutic potential of dietary nitrate supplementation in cardiovascular diseases, future research should delineate whether mitochondrial-induced redox signaling is required for nitrate-mediated improvements in contractile function in the heart and also how this may manifest under diseased conditions.

**REFERENCES**


