

# Dietary Inorganic Nitrate Improves Mitochondrial Efficiency in Humans

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## SUMMARY

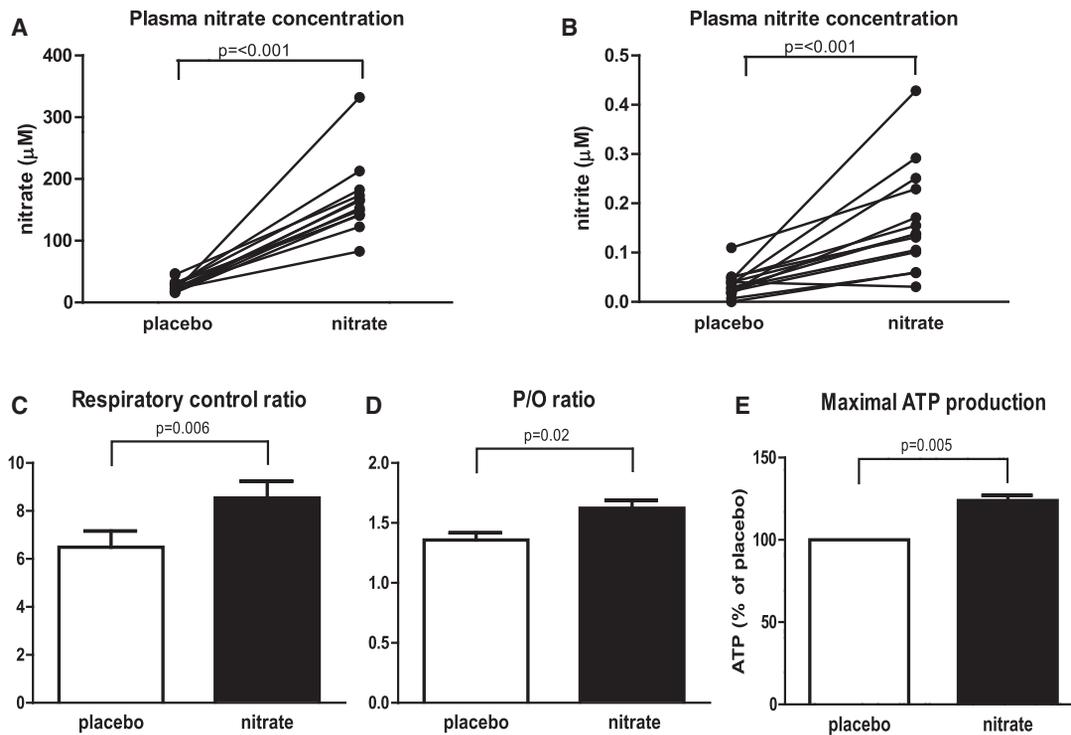
Nitrate, an inorganic anion abundant in vegetables, is converted *in vivo* to bioactive nitrogen oxides including NO. We recently demonstrated that dietary nitrate reduces oxygen cost during physical exercise, but the mechanism remains unknown. In a double-blind crossover trial we studied the effects of a dietary intervention with inorganic nitrate on basal mitochondrial function and whole-body oxygen consumption in healthy volunteers. Skeletal muscle mitochondria harvested after nitrate supplementation displayed an improvement in oxidative phosphorylation efficiency (P/O ratio) and a decrease in state 4 respiration with and without atractyloside and respiration without adenylates. The improved mitochondrial P/O ratio correlated to the reduction in oxygen cost during exercise. Mechanistically, nitrate reduced the expression of ATP/ADP translocase, a protein involved in proton conductance. We conclude that dietary nitrate has profound effects on basal mitochondrial function. These findings may have implications for exercise physiology- and lifestyle-related disorders that involve dysfunctional mitochondria.

## INTRODUCTION

The importance of nitric oxide (NO) in regulating a multitude of physiological processes has been well established since the initial discovery of this endogenous gaseous messenger more than two decades ago (Ignarro et al., 1987). To ensure NO production throughout the body, complex NO synthases (NOSs) generate NO by oxidation of the amino acid L-arginine. NO signaling is partly regulated by its rapid oxidation to the inorganic anions nitrite (NO<sub>2</sub><sup>-</sup>) and nitrate (NO<sub>3</sub><sup>-</sup>). Nitrate, which is also abundant in green leafy vegetables, has previously been considered merely as an inert end product of NO metabolism (Moncada and Higgs, 1993) or as a potentially toxic constituent in our diet (Tannenbaum and Correa, 1985). However, during the last decade its importance in biological processes has

been increasingly appreciated (Gladwin et al., 2005; Lundberg et al., 2008; van Faassen et al., 2009). Circulating nitrate, normally derived both from endogenous NO production and from dietary intake, is actively taken up by the salivary glands, excreted in saliva, and reduced to nitrite by commensal bacteria in the oral cavity (Lundberg et al., 2004). By this route nitrate intake elevates systemic nitrite levels (Lundberg and Govoni, 2004; Webb et al., 2008). Several mechanisms for further reduction of nitrite to NO and other reactive nitrogen intermediates (RNIs) have now been identified (Lundberg et al., 2008). This nitrate-nitrite-NO pathway represents an alternative and differently regulated system for NO generation that operates in parallel to the classical L-arginine-NOS-NO pathway. One important difference is that, in contrast to the NOSs, all known mechanisms by which nitrite is converted to NO are greatly facilitated during hypoxia (Castello et al., 2006; Nohl et al., 2000) and low pH (Modin et al., 2001; Zweier et al., 1995). Several lines of research indicate that the nitrate-nitrite-NO pathway is involved in regulation of blood flow, blood pressure (Larsen et al., 2006), cell signaling, glucose homeostasis (Carlstrom et al., 2010), and tissue responses to hypoxia (Gladwin et al., 2005; Lundberg et al., 2008; van Faassen et al., 2009).

We and others have recently reported a reduction in oxygen cost in exercising healthy individuals after short-term dietary supplementation with nitrate (Bailey et al., 2009, 2010; Larsen et al., 2007, 2010). This effect was highly surprising, since classical exercise physiology dictates minimal fluctuations in oxygen consumption for any individual at a given workload regardless of training status (Moseley et al., 2004), age or diet (Åstrand et al., 2003). The oxygen-sparing effect of nitrate occurred without any changes in circulating lactate concentrations and with maintained or even increased work performance, indicating a more efficient aerobic metabolism or improved mechanical efficiency. The former points toward the mitochondria as targets for the nitrate-induced effects and suggests that ATP production has become more efficient or that the oxygen consumption not directly related to ATP production has been reduced. Mitochondrial oxidative phosphorylation efficiency is classically measured as the amount of oxygen consumed per ATP produced, termed P/O ratio (Hinkle, 2005). Normally, a number of factors can negatively affect P/O ratio including proton leak (Rolfe et al., 1994), proton slip (Groen et al., 1990), energetic cost of metabolite transport (Klingenberg, 1970), or physiological uncoupling (Echtay et al., 2002).



**Figure 1. Dietary Nitrate Increases Circulating Nitrite and Improves Mitochondrial Efficiency**

(A and B) (A) Plasma levels of nitrate and (B) nitrite increased in healthy subjects after 3 days of dietary sodium nitrate supplementation ( $0.1 \text{ mmol kg}^{-1}, \text{ day}^{-1}$ ) compared to placebo (sodium chloride) ( $n = 14$ ).

(C and D) (C) RCR ( $n = 13$ ) and (D) oxidative phosphorylation efficiency (P/O ratio,  $n = 8$ ) in human skeletal muscle mitochondria were higher after nitrate supplementation compared to placebo.

(E) Maximal rate of ATP production increased relative to the rate during the placebo condition ( $n = 4$ ). Data are mean  $\pm$  SEM. See also Figure S1.

There are several possible interactions between nitrate, nitrite, NO, and the mitochondrion. Perhaps the most well-characterized effect of NO is its binding to cytochrome c oxidase (COX), the terminal electron acceptor in the electron transport system (ETS), leading to partial inhibition of mitochondrial respiration (Brown and Cooper, 1994). This binding, which is reversible and regulated by oxygen, may also serve to control reactive oxygen species (ROS) signaling and to regulate tissue oxygen gradients (Thomas et al., 2001). In addition, NO stimulates mitochondrial biogenesis through a cGMP-dependent mechanism (Nisoli et al., 2003). The effects of nitrite might be similar to those of NO, since proteins in the ETS are able to reduce nitrite to NO (Kozlov et al., 1999). Finally, nitrite may signal independent of NO formation to regulate tissue protein expression and activity (Bryan et al., 2005).

We hypothesized that oral supplementation with inorganic nitrate, in amounts readily achievable through the diet, would affect mitochondrial function in healthy human subjects. In a double-blind, placebo-controlled, crossover study, skeletal muscle biopsies taken in conjunction with bicycle ergometer exercise were used to study the effects of nitrate on mitochondrial respirometric and biochemical parameters as well as expression of proteins involved in proton conductance. The results obtained from isolated human mitochondria were correlated to the *in vivo* effects of nitrate on whole-body oxygen consumption.

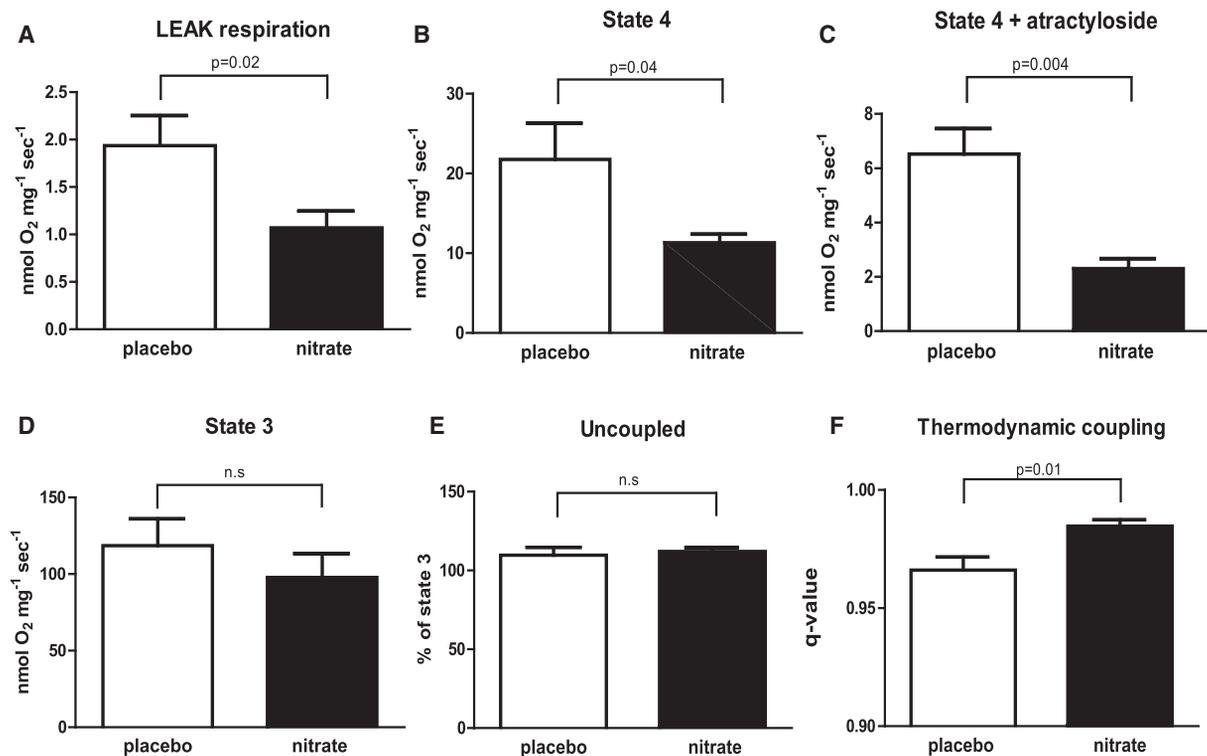
## RESULTS

### Plasma Nitrate, Nitrite, and cGMP

To ensure that the subjects adhered to the study protocol and to confirm systemic conversion of nitrate to nitrite, plasma levels were analyzed after each 3 day period of nitrate or placebo supplementation. As expected, plasma nitrate levels were markedly higher ( $169 \pm 18 \mu\text{M}$ ) after nitrate load compared to placebo ( $27 \pm 2.6 \mu\text{M}$ , Figure 1A). Plasma nitrite was  $35 \pm 7 \text{ nM}$  after placebo and was elevated after the nitrate supplementation ( $163 \pm 29 \text{ nM}$ , Figure 1B). Nitrate treatment did not significantly alter plasma levels of cGMP (see Figure S1 available online).

### Mitochondrial Characteristics

Skeletal muscle biopsies from vastus lateralis were immediately subjected to the isolation protocol and then first checked for quality by measurement of respiratory control ratio (RCR), which is the ratio between state 3 (respiration with saturating amounts of substrates and ADP) and state 4 respiration (respiration with substrates when all ADP has been phosphorylated to ATP). Surprisingly, the RCR value was higher ( $8.5 \pm 0.7$ ) in the mitochondria after nitrate supplementation compared to placebo ( $6.5 \pm 0.7$ ), indicating a better coupling between respiration and oxidative phosphorylation after nitrate treatment (Figure 1C).



**Figure 2. Dietary Nitrate Reduces Basal Mitochondrial Oxygen Consumption and Improves Thermodynamic Efficiency**

(A–C) (A) After 3 days of dietary nitrate supplementation or placebo, skeletal muscle mitochondria were subjected to respirometric analysis. Oxygen consumption during LEAK respiration (n = 9), (B) state 4 respiration (n = 14), and (C) state 4 respiration with atractyloside (n = 6) was lower after dietary nitrate compared to placebo.

(D and E) (D) Nitrate supplementation did not influence oxygen consumption (n = 14) or (E) uncoupled respiration achieved by optimal titration with FCCP (n = 6).

(F) Thermodynamic coupling (q value), calculated according to Equation 1 (see the Results), was improved by dietary nitrate compared to placebo (n = 6). Data are mean ± SEM.

### Nitrate Supplementation Increases P/O Ratio

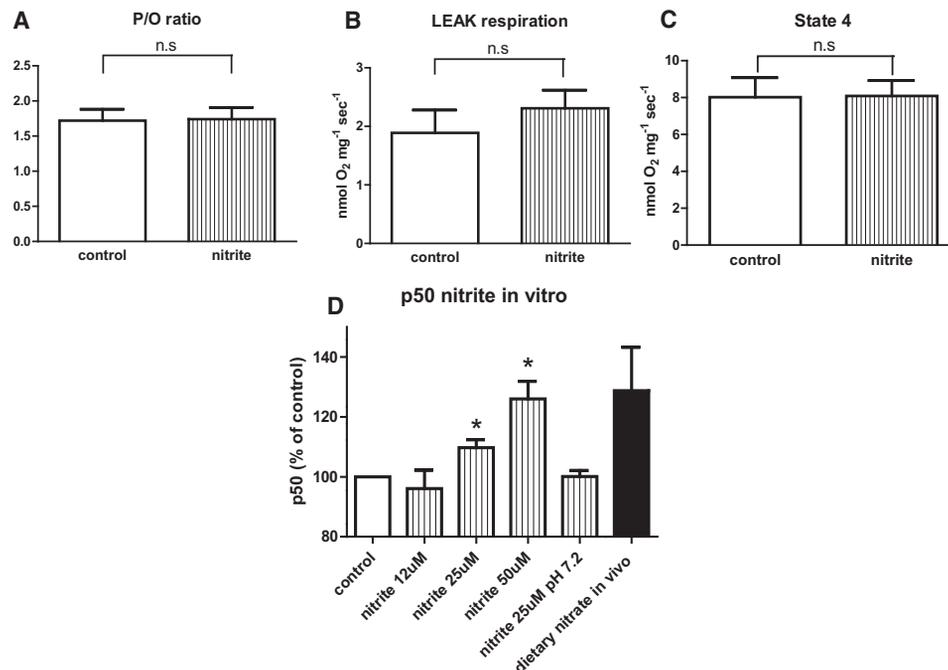
The reduced oxygen consumption at a given absolute mechanical work output after nitrate supplementation (Bailey et al., 2009, 2010; Larsen et al., 2007, 2010) indicates a more efficient conversion of oxygen and oxidation of substrates to yield ATP and/or an improved mechanical efficiency. To investigate if the mitochondrial P/O ratio had improved after nitrate supplementation, we used a controlled submaximal infusion of ADP to nonsaturating levels, which yields a lower mechanistic P/O ratio than the standard practice of pulse titrating to saturating levels of ADP (Gnaiger et al., 2000; Mogensen et al., 2006). This procedure was chosen because P/O ratio during submaximal respiration more closely resembles the metabolic state in vivo, where intracellular concentrations of ADP rarely are saturating (Kuznetsov et al., 1996). The ADP infusion rate was adjusted to approximately 50% of maximal state 3 respiration. The effective P/O ratio during submaximal ADP stimulation was increased by 19% after nitrate supplementation (from  $1.36 \pm 0.06$  to  $1.62 \pm 0.07$ , Figure 1D), suggesting an improved mitochondrial efficiency.

An improved P/O ratio and unchanged state 3 respiration indicates a higher maximal ATP production rate after nitrate supplementation. We decided to further investigate this by measuring maximal rates of ATP formation luminometrically by

the luciferine-luciferase reaction. Indeed, the maximal rate of ATP production was increased by 23% after nitrate supplementation (Figure 1E), which further supports an improvement in the P/O ratio after nitrate treatment.

### Basal Mitochondrial Oxygen Consumption Is Reduced after Nitrate Supplementation

An increased P/O ratio indicates that a larger part of the membrane potential is distributed toward ATP synthesis, and less is wasted through uncoupling actions like proton leak. Next, we wanted to explore if the improved P/O ratio after nitrate intake was due to changes in basal mitochondrial respiration characteristics. Mitochondrial respiration with substrates (in this case pyruvate and malate) but without added ADP, denoted LEAK respiration, is driven by back leakage of protons through the inner membrane. LEAK respiration was reduced by 45% after nitrate supplementation compared to placebo (Figure 2A). Also state 4 respiration, which was the oxygen consumption when exhaustion of ADP had occurred, was reduced by 48% after nitrate (Figure 2B). State 4 respiration can also be driven by ADP generated through intra- and extramitochondrial ATPases. To eliminate interference with extramitochondrial ATPase activity, we blocked the ADP/ATP translocase (ANT) with 0.5 mM atractyloside. This chemical inhibits the adenylate



**Figure 3. Sodium Nitrite Acutely Decreases Mitochondrial Oxygen Affinity In Vitro**

(A–C) In vitro nitrite (25  $\mu$ M) had no effect on (A) P/O ratio and (B) oxygen consumption during LEAK and (C) state 4 respiration. (D) Mitochondrial oxygen affinity (p50) was measured in skeletal muscle mitochondria taken after placebo treatment. Mitochondria were allowed to respire in state 3 until all oxygen in the chamber was consumed. The p50 is the oxygen tension where respiration is half-maximal. In vitro administration of sodium nitrite (12–50  $\mu$ M) dose-dependently increased p50 (striped bars). The effect of nitrite was abolished when raising pH to 7.2. The effects of in vitro nitrite were compared to p50 after in vivo nitrate supplementation (black bar).  $n = 5$ –12. Data are mean  $\pm$  SEM. \* $p < 0.05$  after Bonferroni correction.

transport through the ANT but not the proton conductance (Brand et al., 2005). After inhibition, the remaining oxygen consumption stems from intramitochondrial sources only. This type of respiration was also significantly reduced by nitrate (64%, Figure 2C). In contrast, nitrate supplementation did not affect state 3 respiration with or without the uncoupler carbonyl-cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Figures 2D and 2E). Taken together, these results suggest that the improved P/O ratio after nitrate supplementation involves a decreased proton leakage or slippage during basal and submaximal mitochondrial respiration.

Mounting data suggest that nitrite is an obligate intermediate in the bioactivation of nitrate (Govoni et al., 2008; Webb et al., 2008) and that ETS proteins are capable of reducing nitrite to NO (Nohl et al., 2000). Therefore, we wanted to test if nitrite added directly to the isolated mitochondria in vitro would mimic the effect on P/O ratio seen with dietary nitrate in vivo. Surprisingly, nitrite (25  $\mu$ M) had no acute effect on the P/O ratio, LEAK, or state 4 respiration (Figures 3A–3C). These negative findings indicate differential effects of nitrate given in vivo as compared to nitrite effects in vitro. One explanation could be that a 3 day dietary nitrate intervention is needed to induce structural modifications or changes in protein expression on mitochondrial proteins. This is supported by the findings presented below on the effects of nitrate on UCP-3 and ANT. Also, by necessity, the respiratory measurements were performed at oxygen tensions around 10 kPa, which is well above the intracellular oxygen tensions during normal physiological conditions.

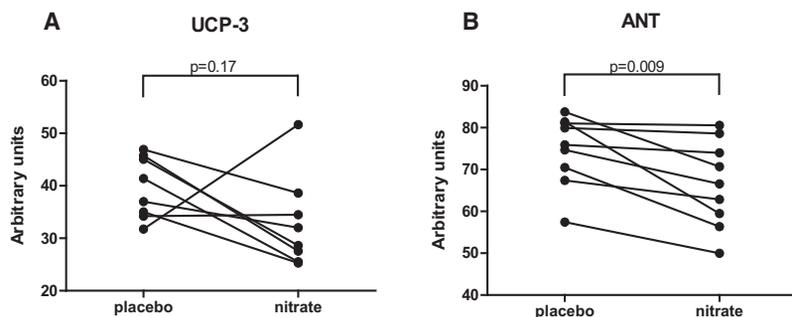
Nitrite is reduced to NO by mitochondria mainly under hypoxia (Kozlov et al., 1999), which also could explain the absence of effect on the respiratory parameters.

### Dietary Nitrate Increases Mitochondrial Thermodynamic Coupling

An alternative way to estimate mitochondrial respiratory efficiency is by applying a thermodynamic approach using the following equation:

$$q = \sqrt{1 - \left( \frac{\text{static head}}{\text{state3u}} \right)}. \quad (1)$$

The  $q$  value denotes thermodynamic coupling and is considered a combined measure of all enzymatic processes and free energy changes that take place in oxidative phosphorylation (Stucki, 1980). This contrasts the classic P/O ratio, which measures endpoint oxygen consumption and ATP production. Static head is equivalent to state 4 respiration in the presence of atractyloside, and state 3u is fully uncoupled state 3 respiration after titrating optimal concentrations of FCCP. Theoretically, a change in thermodynamic coupling is expected if there is a change in P/O ratio (Cairns et al., 1998). Indeed, the  $q$  value increased from 0.966 after placebo to 0.985 after nitrate supplementation (Figure 2F). Since there was no significant change in state 3u respiration, the improvement in thermodynamic efficiency was mostly driven by the lower basal respiration rate after nitrate supplementation.



**Figure 4. Effects of Dietary Nitrate on UCP-3 and ANT Expression in Skeletal Muscle**

(A) Western blot analysis of UCP-3 (n = 8) and (B) ATP/ADP translocase (ANT) from human skeletal muscle harvested after 3 days of dietary nitrate compared to placebo (n = 9). See also Figure S2.

From a thermodynamic perspective, an improved efficiency would inevitably reach a point where the rate of ATP production would start to decrease (Stucki, 1980). In the respirometric assay, the mitochondria already had high  $q$  values, which increased further after nitrate supplementation. With this improvement in thermodynamic coupling, a decreased maximal rate of ATP production would be expected. In contrast, we found an increase in ATP production during maximal state 3 respiration. However, by necessity, direct measurements of ATP production were performed during different assay conditions compared to the respirometric experiments, which makes comparative calculations hazardous.

#### Effects of Nitrate and Nitrite on Mitochondrial Oxygen Affinity

Next, we wanted to investigate the effects of *in vivo* nitrate and *in vitro* nitrite on mitochondrial  $p_{50}$ , which is the oxygen tension where half-maximal respiration occurs. The  $p_{50}$  is the apparent mitochondrial affinity for oxygen, and a high  $p_{50}$  indicates a low oxygen affinity and vice versa. In a series of experiments, mitochondria were allowed to respire in state 3 until all oxygen in the chamber was consumed. The average  $p_{50}$  in state 3 was  $0.042 \pm 0.002$  kPa after placebo supplementation, and a near significant increase was observed in mitochondria harvested after dietary nitrate supplementation ( $p = 0.08$ , Figure 3D). The mitochondria obtained after placebo treatment were used to study the acute effects of nitrite on  $p_{50}$ . Nitrite can be metabolized by mitochondria to form NO (Kozlov et al., 1999), which in turn reversibly inhibits COX (Brown and Cooper, 1994) in competition with oxygen. Thus, a small amount of NO generated from nitrite would likely decrease the oxygen affinity of mitochondria. When sodium nitrite was added to the medium, the  $p_{50}$  increased in a dose-dependent manner (Figure 3D). This effect was lost when pH in the mitochondrial respiration medium was increased from 6.7 to 7.2, which supports an NO-mediated effect, since earlier studies have showed that nitrite conversion to NO is greatly facilitated by low pH (Modin et al., 2001).

#### Effects of Dietary Nitrate on Protein Expression of UCP-3 and ANT

Mechanistically, we next wanted to investigate if our respirometric findings, which indicate a reduced proton leak by nitrate supplementation, could be related to decreased expression of UCP-3 or ANT, mitochondrial proteins believed to be responsible for a major part of the specific proton leak in mitochondria (Bevilacqua et al., 2010; Parker et al., 2008). Western blot

analysis on muscle homogenates did indeed reveal a significant downregulation of ANT protein levels, whereas the effect on UCP-3 did not reach statistical significance (Figures 4A and 4B, see also Figure S2). These results indicate that at least a part of the decrease in proton leak observed after nitrate supplementation is due to a reduction in ANT content.

#### Mitochondrial Biogenesis Is Unaffected by Dietary Nitrate

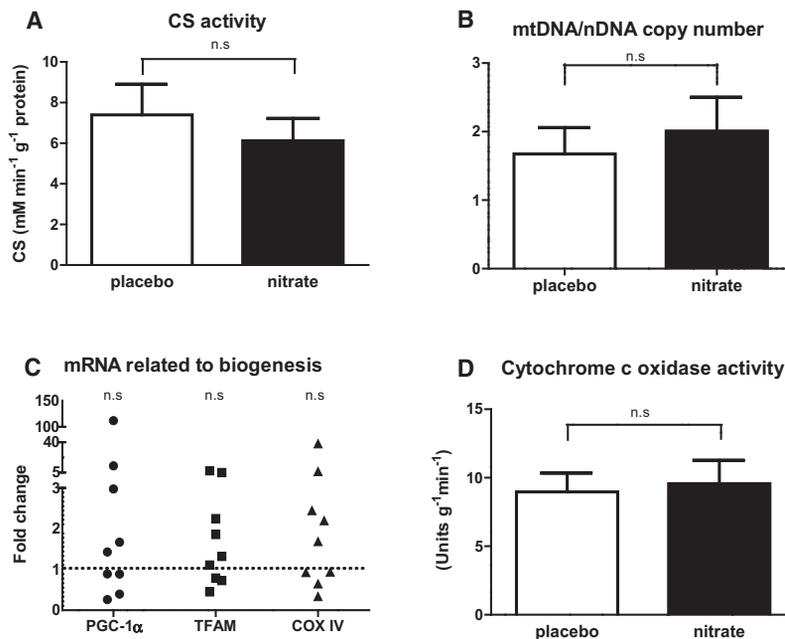
The downregulation of UCP-3 and ANT could theoretically be explained by a lower overall mitochondrial density or a decreased mitochondrial biogenesis after nitrate supplementation. To examine this in detail, we first measured citrate synthase (CS) activity in the skeletal muscle biopsies. There was no difference in CS activity per milligram protein in tissue homogenates (Figure 5A). Next we quantified the amount of mitochondrial DNA and nuclear DNA in the tissue samples with real-time PCR. The ratio of mitochondrial DNA to nuclear DNA (mtDNA/nDNA) is then directly related to the mitochondrial density. As can be seen in Figure 5B, there was no significant difference in mtDNA/nDNA ratio. Furthermore, we investigated the mRNA levels of peroxisome proliferator-activated receptor- $\gamma$  coactivator 1 $\alpha$  (PGC-1 $\alpha$ ), transcription factor A, mitochondrial (TFAM), and COX, which are genes linked to mitochondrial function and biogenesis. Again, we found no differences in the gene expression between any of these genes (Figure 5C). Finally, we also analyzed the activity of COX in muscle homogenates and found no change in the level of activity between placebo and nitrate conditions (Figure 5D). Overall, these results clearly suggest that mitochondrial density and biogenesis is not affected by a 3 day dietary intervention with nitrate.

#### Nitrate Does Not Increase Tissue Levels of Nitrotyrosine

A potential adverse effect of long-term dietary nitrate supplementation would be generation of peroxynitrite and other RNIs capable of nitration reactions. We therefore studied if nitrate treatment would lead to formation of nitrotyrosine, which is commonly used as a footprint of such reactions. Overall tyrosine nitration of skeletal muscle proteins was determined by western blot analysis in the molecular size 25–40 kDa. Although we did find a clear staining for nitrotyrosine on several proteins, there were no differences between treatments (Figure S2), indicating that nitrate does not increase nitration reactions under the conditions applied in the current study.

#### Dietary Nitrate Reduces the Oxygen Cost during Exercise

In an integrated approach, we next measured whole-body oxygen consumption during a submaximal workload after nitrate



**Figure 5. No Effect of Dietary Nitrate on Mitochondrial Biogenesis**

(A) Citrate synthase activity, (B) mitochondrial DNA/nuclear DNA copy numbers, (C) mRNA levels for three proteins involved in mitochondrial biogenesis and function, (D) and cytochrome c oxidase activity were measured in human skeletal muscle tissue, harvested after a 3 day dietary intervention with inorganic nitrate or placebo. In (C), each point denotes change in mRNA expression after dietary nitrate supplementation compared to placebo, and data points below the dotted line represent decreases in expression. Data are mean  $\pm$  SEM.

supplementation or placebo, in the same subjects from which we had obtained skeletal muscle biopsies. The absolute value of oxygen consumption was  $1.95 \pm 0.09$  l  $\text{min}^{-1}$  during the placebo trial and decreased to  $1.89 \pm 0.1$  l  $\text{min}^{-1}$  after nitrate supplementation (Figure 6A). The mechanical work output to oxygen uptake was  $66.5 \pm 2.1$  W  $\text{l}^{-1}$   $\text{min}^{-1}$  during placebo exercise and increased to  $69.3 \pm 2.5$  W  $\text{l}^{-1}$   $\text{min}^{-1}$  after nitrate supplementation (Figure 6B). These results are in agreement with recent studies (Bailey et al., 2009, 2010; Larsen et al., 2007, 2010) and indicate a more efficient metabolism after nitrate supplementation. Of note, we found a small but significant increase in respiratory exchange ratio (RER) (placebo  $0.883 \pm 0.01$  versus nitrate  $0.914 \pm 0.01$ , Figure 6C) that indicates a minute shift toward a relatively greater reliance on carbohydrate oxidation after nitrate supplementation. Carbohydrates are a slightly more efficient fuel than fatty acids, but the modest elevation in RER in this study does only correspond to a 1% reduction in energetic cost during exercise (Brouwer, 1957) and cannot fully explain the 3% reduction in oxygen consumption induced by nitrate.

#### The Effect of Nitrate on Whole-Body Oxygen Consumption Is Coupled to Mitochondrial Efficiency

Finally, we compared the results from the *in vitro* mitochondrial experiments with the actual whole-body oxygen consumption level during exercise in each individual after nitrate supplementation. Remarkably, there was a clear correlation between the increase in mitochondrial P/O ratio and the reduction in oxygen cost during exercise after nitrate supplementation (Figure 6D). Moreover, the increase in P/O ratio also correlated with the reductions in energy expenditure and increases in watt/ $\text{VO}_2$ , (Figures S3A and S3B). Thus, a greater reduction in oxygen consumption *in vivo* was reflected by increases in mitochondrial efficiency measured *in vitro*. This strongly suggests that the enhanced metabolic efficiency observed in our subjects after dietary nitrate supplementation is due to improved mitochondrial function.

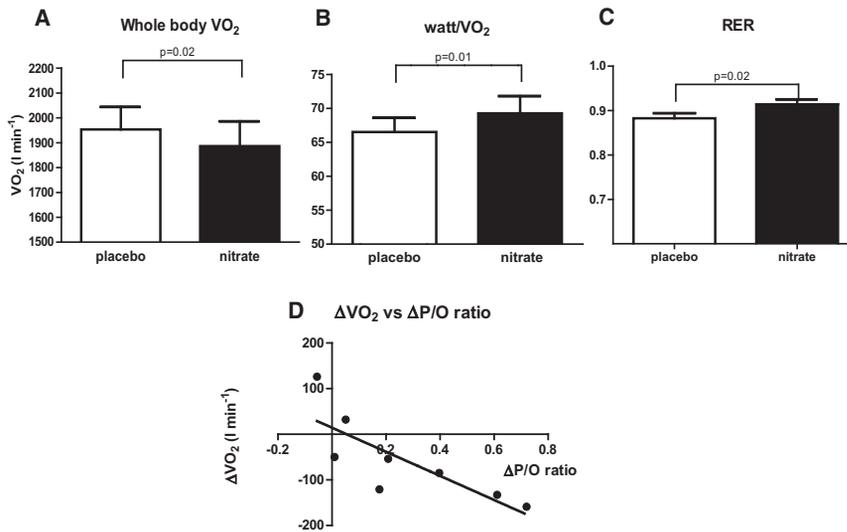
term dietary regimen can influence expression of important mitochondrial proteins may have profound impact on exercise physiology. Moreover, it may also have implications for metabolic and cardiovascular diseases in which dysfunctional mitochondria play a central role.

The mitochondrial oxidation of substrates generates electrons to enter the ETS, thereby creating the transmembrane proton gradient, which is finally used to produce ATP for cellular functions. However, the mitochondrial membrane potential is dissipated by other means than phosphorylation, and thus oxidation is not 100% coupled to production of ATP. Several of the findings in our study point toward a more efficient intrinsic mitochondrial function after nitrate intake. The effects of training status on P/O ratio are contradictory, with some studies showing no difference between trained and untrained subjects (Mogensen et al., 2006), while others show a decreased P/O ratio in trained subjects (Befroy et al., 2008). Acute high-intensity exercise does not seem to affect the P/O ratio (Tonkonogi et al., 1999). Surprisingly, in the current study we found a 19% increase in P/O ratio after nitrate supplementation. To our knowledge, there are no other dietary regimes described that have this effect. Intriguingly, there was a strong correlation between the reduction in whole-body oxygen consumption during cycling and the increase in P/O ratio, which clearly suggests that a large part of the improved exercise efficiency is taking place at the mitochondrial level.

The underlying mechanisms for the improved P/O ratio and thermodynamic coupling were investigated in detail by examining respiration under different conditions. The mitochondrial membrane potential that drives ATP synthesis can be dissipated in a number of ways. The inner mitochondrial membrane is slightly permeable to proton leakage, especially at high membrane potentials, and this back leakage of protons has been found to account for as much as 25% of resting energy expenditure (Rolfe et al., 1994). In this study, we have several

#### DISCUSSION

We show here that nitrate, a simple inorganic anion abundant in our everyday diet, has profound effects on basal human mitochondrial function as well as whole-body oxygen consumption during exercise. These effects were accompanied by a reduction in ANT, a major determinant of mitochondrial proton conductance. The fact that the relatively short-



**Figure 6. Dietary Nitrate Decreases Whole-Body Oxygen Consumption during Exercise**

(A) Oxygen consumption measured during exercise was reduced in healthy volunteers after 3 days of dietary supplementation with sodium nitrate ( $0.1 \text{ mmol kg}^{-1}, \text{ day}^{-1}$ ) compared to placebo (sodium chloride). On two separate occasions the subjects performed a submaximal workload on a cycle ergometer at 50% of  $\text{VO}_2\text{max}$  ( $n = 13$ ).

(B) The mechanical work output to oxygen uptake was increased after dietary nitrate supplementation ( $n = 13$ ).

(C) The RER increased significantly after nitrate supplementation ( $n = 13$ ). Data are mean  $\pm$  SEM.

(D) A significant correlation ( $R^2 = 0.64$ ,  $p = 0.02$ ) was seen between the nitrate-induced reduction in whole-body oxygen consumption during exercise ( $\Delta\text{VO}_2$ ) and the increase in P/O ratio measured in isolated skeletal mitochondria harvested from the same individuals ( $\Delta\text{P/O}$  ratio) ( $n = 8$ ). Data are mean  $\pm$  SEM. See also Figures S3A and S3B.

indications that the proton leak or proton slip is decreased after nitrate supplementation. Thus, the LEAK respiration and state 4 respiration with or without atractyloside were decreased after nitrate supplementation. Taken together, these results suggest that the nitrate-induced increase in mitochondrial efficiency can be explained by reduced leakage/slippage of protons across the inner mitochondrial membrane. This prompted us to investigate mitochondrial proteins known to be involved in proton conductance. Indeed, we found a decrease in the protein expression of ANT, which is responsible for a substantial part of proton leakage under physiological conditions (Brand et al., 2005). The effect of nitrate supplementation did not significantly alter UCP-3 expression, although a trend toward a downregulation is apparent. The role of UCP-3 in mitochondrial uncoupling in skeletal muscle is not fully determined. Up- or downregulation of UCP3 has not been shown to mediate basal proton conductance (Affourtit et al., 2007). However, a UCP3-mediated proton leak may be induced via fatty acids (Zackova and Jezek, 2002) or ROS products (Affourtit et al., 2007). The effect on ANT and possibly UCP-3 may at least partly explain the observed effects of dietary nitrate on mitochondrial function observed in this study. In addition, it may also explain the different effects on mitochondrial respiration by 3 days of in vivo nitrate administration compared to acute administration of nitrite in vitro. Nitrite is considered an obligate intermediate in the conversion of nitrate to NO and other bioactive nitrogen oxide species. Even so, it is unlikely that acute administration of nitrite would induce changes in expression of relevant proteins such as ANT, while 3 days of nitrate treatment would be a sufficient time to allow for such changes to occur. We cannot exclude that nitrate, or its reaction products, also induce posttranscriptional structural modifications of mitochondrial proteins, possibly by nitrosylation, as has been described earlier (Shiva et al., 2007) (Figure 7).

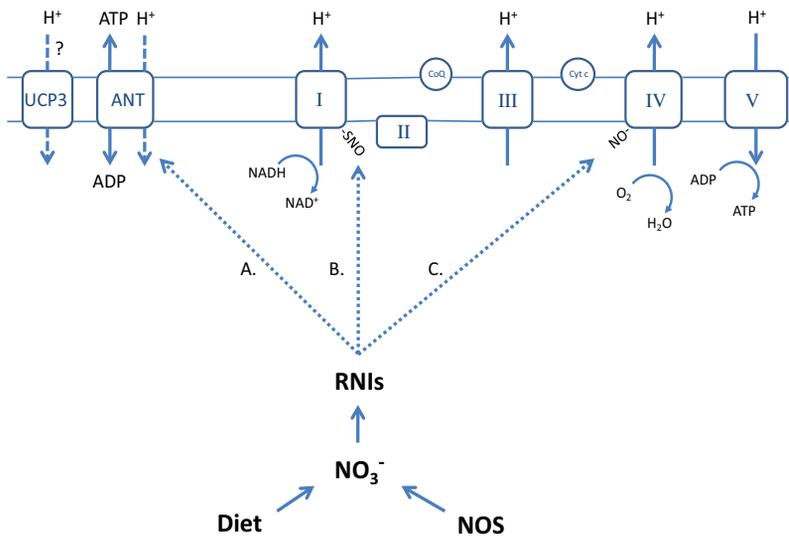
The increased P/O ratio after nitrate supplementation in this study is paralleled by an increased thermodynamic efficiency, as would be expected. The thermodynamic efficiency concept was originally postulated as a phenomenological theory (Stucki, 1980) but has since then been supported by experimental

evidence (Cairns et al., 1998; Iossa et al., 2004; Soboll and Stucki, 1985). The paradigm in this theory is that maximum efficiency and power cannot be achieved simultaneously (Cairns et al., 1998). In contrast, we find an increased rate of ATP production after nitrate supplementation (Figure 1E). However, it should be noted that the assay conditions are by necessity different from those during the respirometric conditions with saturated ADP and oxygen concentrations, and the mitochondrial suspension is also more diluted.

In our in vitro experiments with nitrite, the  $p50$  in placebo mitochondria was  $0.042 \pm 0.002 \text{ kPa}$  and increased in a dose-dependent manner by nitrite. The  $p50$  values presented here are close to what has been recorded in mitochondria from other species when using high-resolution respirometry (Gnaiger, 2003). The  $p50$  after in vivo nitrate supplementation also tended to be increased, but this fell just short of reaching statistical significance ( $p = 0.08$ ). Since the oxygen tension in working tissue is very low, it is of highest priority for the cell to keep  $p50$  substantially below the tissue  $p\text{O}_2$  to avoid that oxygen becomes limiting for respiration. Considering an intracellular oxygen tension of  $0.3 \text{ kPa}$  (Richardson et al., 2006) and a  $p50$  of  $0.042 \text{ kPa}$ , Equation 2 yields the following:

$$j'_{\text{O}_2} = \frac{p\text{O}_2}{p50 + p\text{O}_2} = \frac{0.3}{0.042 + 0.3} = 0.88. \quad (2)$$

$j'_{\text{O}_2}$  is the respiratory rate relative to the maximum rate at saturating oxygen. A value of 0.88 indicates that respiration rate is reduced by 12% due to oxygen limitation. A small increase in  $p50$  from  $0.042$  to  $0.052 \text{ kPa}$ , which is seen after nitrate supplementation, will result in an increased oxygen limitation  $j'_{\text{O}_2} = 0.85$  or 15% oxygen limitation. This 3% decrease is very close to the 2.7% reduction in  $\text{VO}_2\text{max}$  during whole-body exercise that we have recently reported (Larsen et al., 2010). Evidence leans toward the conclusion that the  $p50$  is regulated both by a kinetic and a thermodynamic part (Steinlechner-Maran et al., 1996). The kinetic part can be related to the interaction with competitive inhibitors of the ETS, such as NO (Aguirre et al., 2010). The thermodynamic part is illustrated by the decrease in  $p50$  when



**Figure 7. Modulation of Mitochondrial Function by Inorganic Nitrate**

Nitrate from endogenous (NO synthase) or dietary sources is metabolized *in vivo* to nitrite, NO, and other reactive nitrogen intermediates (RNIs). These RNIs can interact with mitochondrial oxidative phosphorylation efficiency. In this study we show that dietary nitrate downregulates the expression of adenine nucleotide translocase (ANT) in human skeletal muscle mitochondria (A). Since ANT is a well-known site of proton leak, this effect may explain why nitrate increases mitochondrial oxidative phosphorylation efficiency. Other potential mitochondrial targets of nitrate-derived RNIs include complex I (B) and complex IV (C). Previous studies have shown that nitrite can nitrosate (–SNO) complex I, which is associated with reduced ROS generation after ischemia reperfusion. In addition, nitric oxide (NO) derived from nitrate directly interacts with complex IV in competition with oxygen to partly inhibit respiration. The mechanism for the downregulation of ANT by nitrate (A) is not clear, but this effect might be secondary to the direct effects of nitrate-derived RNIs on complex I and IV, including modulation of ROS formation and signaling.

respiration is uncoupled with FCCP (Steinlechner-Maran et al., 1996). It is therefore likely that the increased p50 when adding nitrite *in vitro* is by direct action of NO on the COX. The effect of nitrite was abolished by increasing pH in the respiration medium from 6.7 to 7.2, which also indicates that the increased p50 could be due to NO formation, since elevated pH inhibits nitrite-dependent NO production (Castello et al., 2006; Modin et al., 2001). However, the trend toward increased p50 after nitrate supplementation *in vivo* could be related to the increased thermodynamic efficiency of respiration, since it is unlikely that a substantial amount of nitrite is still present in the mitochondria after the isolation procedure.

It is reasonable to assume that the effect of nitrate supplementation on state 4, LEAK, and static head mitochondrial respiration would also have a coupling to the subjects' basal metabolic rate. State 4 respiration has been proposed to be more closely linked to basal metabolic rate than state 3 respiration, since the intracellular amount of ADP is low during resting conditions. In this study we did not measure basal metabolic rate in the subjects. However, to detect a small reduction in basal metabolic rate would be very difficult without including a very large number of subjects, due to the substantial biological and methodological variations in this parameter.

Clearly further studies are needed to elucidate the molecular mechanism and signaling pathways by which nitrate affects the expression of mitochondrial uncoupling proteins such as ANT and UCP-3. An attractive speculation is that the acute effects of nitrite on oxygen affinity (p50) and the longer-term effects of nitrate on protein expression are coupled (Figure 7). Dietary nitrate generates low rates of nitrite and then NO over a prolonged period of time (Lundberg et al., 2008, 2009). NO would then partially inhibit respiration via binding to COX in competition with oxygen (Brown and Cooper, 1994; Palacios-Callender et al., 2007). This could be sensed by the cell as mild hypoxia, eliciting signaling pathways to downregulate ANT. A recent animal study actually lends some support to this theory; rats were exposed to hypoxia for several days, and this was paralleled by a marked decrease in ANT expression and activity (Li et al., 2006).

The importance of our everyday diet in health and disease is attracting immense scientific interest. Among the more consistent findings from nutritional research are the beneficial effects of a high intake of fruit and vegetables in protection against major disorders such as cardiovascular disease and diabetes (Liese et al., 2009; Willett, 1994). However, the underlying mechanism(s) responsible for these effects is still unclear, and trials with single nutrients have generally failed. It is tempting to speculate that boosting of the nitrate-nitrite-NO pathway may be one mechanism by which vegetables exert their protective effects. Recent research has revealed numerous beneficial NO-like effects of dietary inorganic nitrate, in amounts easily achieved by a diet rich in vegetables. The effects of nitrate include a robust reduction in blood pressure, protection against ischemia/reperfusion injury, inhibition of platelet aggregation, and preservation of endothelial function (Bryan et al., 2007; Larsen et al., 2006; Webb et al., 2008). We now add to this list a nitrate-induced improvement in intrinsic mitochondrial efficiency. Since mitochondria are essential for cellular function and signaling, it is evident that their functionality will have a major bearing on normal physiology and disease. Future research will reveal if an improved mitochondrial function by dietary nitrate could explain some of the beneficial effects hitherto shown with this anion.

#### EXPERIMENTAL PROCEDURES

This study was approved by the Regional Ethics Committee in Stockholm, Sweden. The study participants gave written informed consent before initiation of the study. For a more detailed description of experimental procedures, see the Supplemental Information.

#### Subject Characteristics

In this randomized, double-blind, and crossover study, 14 healthy, nonsmoking subjects were included (11 males, age 25 ± 1 years, weight 70 ± 2 kg, height 178 ± 2 cm, and VO<sub>2peak</sub> 56 ± 3 ml kg<sup>-1</sup> min<sup>-1</sup>). They were given either sodium nitrate (0.1 mmol kg<sup>-1</sup> day<sup>-1</sup>, divided in three doses) or an equimolar amount of sodium chloride (placebo) for 3 days prior to experiments, with a washout period of at least 6 days between tests. The subjects were instructed to avoid all nitrate-rich food products including vegetables, grapes,

tea, alcohol, and cured meats during each 3 day period that preceded the tests. Subjects were instructed to avoid physical exercise or heavy activity 36 hr before they reported at the laboratory. At each occasion, a muscle biopsy was taken, followed by a submaximal bicycle ergometer test. The last dose of nitrate (or placebo) was taken 90 min prior to the experiments.

#### Biopsies

Human biopsies (100–150 mg) were obtained under local anesthesia (Lidocaine, without epinephrine) from the subjects' vastus lateralis after a small incision (0.5–1 cm) through the skin and muscle fascia. A small portion (10–25 mg) of the biopsy was dissected and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  for later analysis of enzymatic activity and protein concentration.

#### Isolation of Mitochondria

Muscle tissue was immediately weighed and added to ice-cold isolation medium followed by the isolation protocol described in detail elsewhere (Tonkonogi and Sahlin, 1997) (Supplemental Experimental Procedures).

#### Mitochondrial Respiration and p50

The respiration rate was analyzed by high-resolution respirometry in a two-channel titration injection respirometer at  $37^{\circ}\text{C}$  (Oroboros oxygraph, Innsbruck, Austria) (Supplemental Experimental Procedures).

#### Protein Determination of UCP-3 and ANT

Portions of freeze-dried muscle were homogenized in ice-cold lysis buffer and protein concentration was determined. Western blot was performed as described in detail previously (Fernstrom et al., 2004) (Supplemental Experimental Procedures).

#### Analysis of Nitrate and Nitrite

Nitrate and nitrite were analyzed by chemiluminescence after reductive cleavage and determination of the NO released into the gas phase as previously described (Lundberg and Govoni, 2004).

#### Enzymatic Activity of CS and COX

CS activity was determined at  $25^{\circ}\text{C}$  in skeletal muscle homogenate extracted from freeze-dried tissue and isolated mitochondrial samples as previously described (Reisch and Elpeleg, 2007). COX activity was determined using Cytochrome C Oxidase assay kit from Sigma (CYTOCOX1) at  $25^{\circ}\text{C}$  and according to the manufacturers' instructions.

#### Nuclear and Mitochondrial DNA Quantification

DNA was extracted using the DNAeasy Blood & Tissue Kit (QIAGEN) following manufacturer's instructions. The mitochondrial DNA/nuclear DNA (mtDNA/nDNA) quantification was carried out by multiplex real-time PCR, using the mtDNA qkit (Genemore Italy srl, Modena, Italy). The signal deriving from probes for mtDNA and nDNA was detected using FAM and TexasRed filters, respectively. The DNA for the standard curves was provided by the kit, and consisted on five standards (10-fold dilutions) from 1 to 1/10,000. An ABI PRISM 7500 Sequence Detector (Applied Biosystems) was used for the amplification and the detection ( $50^{\circ}\text{C}$  2 min,  $90^{\circ}\text{C}$  3 min, followed by 45 cycles at  $95^{\circ}\text{C}$  15 s and  $60^{\circ}\text{C}$  for 30 s).

#### RNA Isolation and Quantitative RT-PCR

The RNA isolation and the RT-PCR were performed as described previously (Borniquel et al., 2010). Sequences of the primers used were hTFAM forward 5'-cagacagattttccagttttcc-3' and reverse 5'-ccaaaaagacctgttcagc-3'; hCOX IV forward 5'-acgagctcatgaaagtgttg-3' and reverse 5'-aatgcgatacaactgcattcttc-3'; hPGC-1 $\alpha$  forward 5'-ggcagtagatcctcttcaagatc-3' and reverse 5'-tcacacggcgcctcttcaattg-3'; and  $\beta$ -actin forward 5'-gctcctctgagcgc-3' and reverse 5'-gtggacagtagggccaggat-3'.

#### Measurement of Mitochondrial ATP Production

Mitochondrial rates of ATP production were assessed luminometrically according to Wibom et al. (2002). pH was set to 6.7 and mitochondria were diluted 1:62.5 in respiration medium. The substrates used were pyruvate (5 mM) and malate (2 mM). ATP:AMP phosphotransferase was inhibited by

p1,p5-diadenosine-5-pentaphosphate (DAPP). ADP was purified free from contaminating ATP according to Wibom et al. (2002) and mixed with AMP. The solution of ADP, AMP, and DAPP was used to initiate the reaction. ATP production was assessed in triplets as the linear rate of increase in [ATP]. The analog signals were sampled at 200 Hz by using an A/D converter (MP150 BioPac Systems, Inc.) and subsequently analyzed in Acqknowledge 3.7.3 software.

#### P/O Ratio

P/O ratio was determined in the presence of ATP (2 mM) by steady-state infusion of nonsaturating levels of ADP with a microdialysis pump (CMA 100, Solna, Sweden) through high-pressure hosing. This method has been described in detail elsewhere (Gnaiger et al., 2000). The ADP infusion rate was set to approximately 50% of maximal state 3 respiration and was initiated at an oxygen pressure of approximately 10 KPa. The effective P/O ratio was calculated as the rate of infused ADP divided by the oxygen consumed during steady state at the last minutes of infusion. Correction was made for the amount of oxygen added to the respiration medium by the infused ADP solution.

#### Thermodynamic Coupling

The degree of thermodynamic coupling ( $q$ ) can be calculated by (Cairns et al., 1998):

$$q = \sqrt{1 - \left( \frac{\text{static head}}{\text{state3u}} \right)}. \quad (1)$$

The static head condition corresponds to the respiration rate when the adenylate nucleotide translocator (ANT) is blocked with 0.5 mM atractyloside. The exchange of ADP and ATP is then zero, but a small rate of oxygen consumption is maintained due to basal mitochondrial oxygen consumption, mainly driven by proton leak. State 3u is fully uncoupled respiration during state 3 and is achieved by titrating optimal concentrations of FCCP. In the uncoupled state all frictional forces are dissipated and respiration proceeds at its unrestricted maximum rate.

#### Exercise Tests

The exercise tests were performed on a cycle ergometer (Monark 839E, Varberg, Sweden). Each individual performed a maximal exercise test to determine  $\text{VO}_2$  peak. At the two main tests, workload was chosen with respect to the fitness level of the subject and was set to 100, 120, or 150 W, and cadence was kept constant at 60 or 70 rpm throughout the tests. Subjects cycled for approximately 10 min at the chosen work rate until steady-state oxygen consumption was achieved. Heart rate was monitored with a Polar heart rate monitor (Polar Electro, Finland). Analysis of oxygen uptake ( $\text{VO}_2$ ), expired carbon dioxide ( $\text{VCO}_2$ ), and pulmonary ventilation (VE) were performed with a computerized metabolic system (Jaeger Oxycon Pro, Hoechberg, Germany), and gases were analyzed breath by breath with a flowmeter connected to a face mask. RER was calculated as  $\text{VO}_2/\text{VCO}_2$ .

#### Statistics

D'Agostino and Pearson omnibus normality test was performed to evaluate normality distribution. Student's paired t test was used to compare placebo and nitrate supplementation in normally distributed data, otherwise Wilcoxon signed-rank test was used. Values  $<0.05$  were considered significant. Bonferroni correction was applied when multiple comparisons were made. Pearson r was used in correlation analysis. Statistical analysis was performed in Graph-Pad Prism 5.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at doi:10.1016/j.cmet.2011.01.004.

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