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Nitrite Activates AMP Kinase to Stimulate Mitochondrial Biogenesis Independent of soluble Guanylate Cyclase

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Abstract

Nitrite, a dietary constituent and endogenous signaling molecule, mediates a number of physiological responses including modulation of ischemia/reperfusion injury, glucose tolerance and vascular remodeling. While the exact molecular mechanisms underlying nitrite's actions are unknown, current paradigm suggests that these effects depend on the hypoxic reduction of nitrite to nitric oxide (NO). Mitochondrial biogenesis is a fundamental mechanism of cellular adaptation and repair. However, the effect of nitrite on mitochondrial number has not been explored. Herein, we report that nitrite stimulates mitochondrial biogenesis through a mechanism distinct from NO. We demonstrate that nitrite significantly increases cellular mitochondrial number by augmenting the activity of adenylate kinase, resulting in AMP kinase phosphorylation, downstream activation of sirtuin-1, and de-acetylation of PGC1, the master regulator of mitochondrial biogenesis. Unlike NO, nitrite-mediated biogenesis does not require the activation of soluble guanylate cyclase and results in the synthesis of more functionally efficient mitochondria. Further, we provide evidence that nitrite mediates biogenesis *in vivo*. In a rat model of carotid injury, two weeks of continuous oral nitrite treatment post-injury prevents the hyperproliferative response of smooth muscle cells. This protection is accompanied by a nitrite-dependent upregulation of PGC1 and increased mitochondrial number in the injured artery. These data are the first to demonstrate that nitrite mediates differential signaling than NO. They show that nitrite is a versatile regulator of mitochondrial function and number both *in vivo* and *in vitro*, and suggest that nitrite-mediated biogenesis may play a protective role in the setting of vascular injury.

Keywords

mitochondria; nitrite; nitric oxide; hypoxia; AMP kinase; biogenesis

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INTRODUCTION

Mitochondria, once thought solely to play a role in energy production, are now recognized as dynamic signaling entities within the cell, which not only generate ATP, but also modulate redox signaling and initiate apoptosis. Thus, mitochondrial number and function are tightly regulated and change rapidly based on cellular metabolic and signaling demand. Mitochondrial biogenesis, the genetic program by which new mitochondria are synthesized is integral in maintaining sufficient numbers of mitochondria and underlies the adaptive processes initiated by stimuli such as exercise training [1-3], hypoxia [4-6] and caloric restriction [7-9]. Central to the biogenesis signaling cascade is the activation of peroxisome proliferator-activated receptor-coactivator 1 (PGC1), whose activity is regulated by sirtuin-1 (SIRT1) and AMP Kinase (AMPK), two major metabolic sensors [10, 11]. Deficiencies in PGC1 lead to failure to adapt to cardiac pressure overload [12], decreased contractile function [13], and increased susceptibility to oxidative stress [14], highlighting the essential role of mitochondrial biogenesis in cellular adaptation and repair.

Nitrite (NO_2^-), a dietary constituent present in the blood ($0.1\text{-}0.3\text{ }\mu\text{mol/L}$) [15] and tissues ($1\text{-}20\text{ }\mu\text{mol/L}$) [16], is now established as an endocrine reservoir of nitric oxide (NO) and a mediator of hypoxic cell signaling [17]. Once thought to be physiologically inert, it is now recognized that NO_2^- is bioactivated along a decreasing physiological oxygen and pH gradient through its reaction with deoxygenated heme proteins, particularly myoglobin in the heart and smooth muscle [17-20]. Mechanistically, the reduction of NO_2^- to NO is thought to be the initial step in a number of vascular responses that NO_2^- mediates *in vivo*, including stimulation of angiogenesis [21], protection from ischemia/reperfusion injury [22-25] and reversal of intimal hyperplasia after vascular injury [26]. However, little is known about the events downstream of NO_2^- bioactivation that lead to tissue protection in these models. Furthermore, recent data suggesting that the reaction of NO_2^- with ferric heme can yield dinitrogen trioxide (N_2O_3) presents the possibility that NO_2^- may mediate cellular signaling through mechanisms distinct from those mediated by NO [27, 28].

NO is a well characterized regulator of mitochondrial function that stimulates mitochondrial biogenesis through the activation of soluble guanylate cyclase (sGC) and the production of cyclic guanosine monophosphate (cGMP) [9, 29]. While we and others have shown that NO_2^- modulates mitochondrial function through its modification of specific respiratory chain proteins, the role of NO_2^- in regulating mitochondrial number is not known [30, 31]. Herein, we demonstrate that NO_2^- increases the cellular number of functional mitochondria by stimulating mitochondrial biogenesis and show that the mechanism of nitrite's action occur through the activation of AMPK but are not dependent on sGC. We demonstrate that NO_2^- mediated biogenesis occurs both *in vitro* and *in vivo* and discuss the potential role of this pathway as a mechanism underlying physiological adaptation as well as the known therapeutic effects of NO_2^- .

MATERIALS AND METHODS

Materials

Reagents were obtained from Sigma-Aldrich unless otherwise noted.

Cell culture and staining

Primary rat aortic smooth muscle cells (RASMC) were treated every other day with either nitrite or NONOate. Adherent cells were treated with MitoTracker Green FM (Invitrogen; 100nmol/L ; 37°C ; 30 min). Fluorescence was quantified at Ex490nm/Em516nm. Nuclei were stained with crystal violet (0.25%; MP Biomedicals, Solon, OH) and absorption was read at 550nm.

Isolation of primary RASMC

Sprague-Dawley rats (6-8 weeks old) were anesthetized with an intraperitoneal injection of pentobarbital. The aorta was isolated from the aortic arch to the abdominal aorta, and immersed in 20% FBS-DMEM containing 1,000 U/ml of heparin. Fat and connective tissue was rapidly removed and the aorta was incubated in serum-free DMEM with collagenase type II (2mg/ml) for 45 min at 37 °C. After removal of the endothelium, the vessel was cut lengthwise, and the smooth muscle cells removed mechanically in gelatin as described in [32].

Transmission electron microscopy

Cells were fixed in 2.5% glutaraldehyde and post-fixed in 1% OsO₄/1% Fe₆CN₃ (1 h). They were subsequently dehydrated and embedded in Polybed 812 resin (Polysciences, Warrington, PA). Cross sections (60 nm) were obtained on a Riechart Ultracut E microtome, post-stained in 4% uranyl acetate and viewed on a JEOL JEM 1011 transmission electron microscope (JEOL, Peabody MA) at 80 KV. Images were taken using an AMT 2k digital Danvers, MA).

PCR

RNA was extracted with TRIzol (Invitrogen). Real time PCR using SYBR Green was performed on the ABI 7300 real time PCR system. Results were normalized against the gene GAPDH. The main primer pairs included: (1) PGC1 (5'-atgagagcgaggagtctga a-3', reverse: 5'-gcgggtctctcagttctgtcc-3'); (2) SIRT1 (5'-ccagatcctcaag ccattgtt-3', reverse: 5'-tgctgagttgctggattttg-3'); (3) Tfam (5'-ggagagc aaatggctgaag-3', reverse: 5'-cccaatcccaatgacaaactc-3'); (4) NRF-2 (5'-atgccagaaaccaaagtggac-3', reverse: 5'-tttgcattaacatcagcacc-3'); (5) GAPDH (5'-agacagccgcattcttctgt-3', reverse: 5'-tgatggcaacaaatgtccac t-3').

Complex activities

Activities of mitochondrial complexes I-IV and citrate synthase were measured spectrophotometrically in cell lysates as previously described [31].

Measurement of cGMP

RASMC treated with nitrite or NONOate (3 h, 1% O₂) were scraped and cGMP was measured according to the directions outlined in the cGMP kit (Cayman Chemicals; Ann Arbor, MI).

Measurement of NO, RSNO and Fe-NO by chemiluminescence

For the measurement of RSNO, nitrite treated RASMC were scraped in Triton X-100 (0.01%) and subjected to tri-iodide based reductive chemiluminescence in the presence and absence of acidified sulfanilamide (15% in 2M HCl) and mercuric chloride as described in [33]. Fe-NO was measured by injecting the treated cells into a solution of potassium ferricyanide (0.1M) to oxidize the heme iron and release the bound NO. The NO was detected by a Sievers Nitric Oxide Analyzer. Nitrite reduction to NO was measured by adding nitrite to cells in suspension in serum free media at 1% O₂ in a vessel connected in line to the Nitric Oxide Analyzer as described in [33].

Oxygen consumption rate (OCR)

RASMC (30×10³ cells/well) were treated in XF24 microplates. Media was changed to unbuffered DMEM after treatment and the plate incubated in a non-CO₂ incubator (37°C; 60

min) before running on the XF24 Analyzer (Seahorse Bioscience). Basal OCR was measured after which pre-warmed pharmacological modulators (see text) were injected into each well. Viability was assessed by crystal violet staining and OCR normalized to cell number.

ATP and ROS generation

ATP and H₂O₂ generation were assessed using the ATP determination kit and Amplex Red Hydrogen Peroxide assay kit respectively (Invitrogen). *NAD⁺/NADH ratio*: NAD⁺/NADH ratio was measured in cell extracts using the NAD⁺/NADH quantification kit (Biovision).

Adenylate kinase activity

Adenylate kinase activity was assessed by measuring luciferin-luciferase luminescence using the AK determination kit (PromoKine).

PGC1 acetylation

PGC1 was immunoprecipitated from RASMC using anti-PGC1 antibody (Santa Cruz). Immunoprecipitated PGC1 was electrophoresed and immunoblotted with antibody for acetylated lysine (Santa Cruz) and for PGC1.

Transfection of RASMC

RASMC were seeded in antibiotic free medium and transfected with siRNA to AMPK α_2 , PGC1 or sGC α_1 (100nmol/L; Santa Cruz) using the siRNA reagent system (Santa Cruz). After 5 hrs, fresh antibiotics were added to the medium and fetal bovine serum increased to 10% serum. Expression of proteins were assayed by Western blot 2 d later.

Carotid Artery Balloon Injury

All procedures were performed in accordance with the Institutional Animal Care and Use Committee of the University of Pittsburgh. Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN; 350-450 g) were anesthetized with intraperitoneal sodium pentobarbital (Nembutal; 45mg/kg) and supplemental inhalational isoflurane (Forane). The left carotid artery was injured as previously described [26]. Oral sodium nitrite supplementation (10.5mg sodium nitrite/L water) was delivered daily for 2 weeks post-injury. Carotid intima:media ratios, RT-PCR and microscopy were measured at 2 weeks.

Statistical Analysis

All results are expressed as means $\pm$ SEM; *n* represents the number of individual experiments. Single comparisons were tested for significance using a two-tailed Student's *t*-test. ANOVA followed by the Bonferroni post hoc test was used for multiple comparisons.

RESULTS

Nitrite increases mitochondrial number

To determine whether chronic NO₂⁻ treatment regulates mitochondrial number, rat aortic smooth muscle cells (RASMC) were treated every other day with or without NO₂⁻ (50 mol/L) in normoxia (21% O₂) or hypoxia (1% O₂), after which the cells were stained with crystal violet and Mitotracker green to quantify nuclear number and mitochondrial content respectively. After 6 days, Mitotracker fluorescence was increased in hypoxic NO₂⁻-treated cells compared to untreated cells, while no significant difference was observed in nuclear staining between these groups (Figure 1A-D), suggesting that NO₂⁻ increased cellular mitochondrial content. Calculation of the ratio of mitochondrial to nuclear staining

demonstrated that hypoxic NO_2^- treatment mediated a concentration-dependent biphasic increase in cellular mitochondrial mass, with a significant increase at 5 μM , a maximal effect occurring between 25 and 50 μM and an EC_{50} of 7.5 μM (Figure 1E). Notably, no change in mitochondrial content was observed with normoxic NO_2^- treatment, consistent with the hypoxic bioactivation of NO_2^- . This increase in mitochondrial number was further confirmed by electron microscopy (Figure 1F-I). To ensure that NO_2^- induced the synthesis of new mitochondrial proteins, the expression of respiratory complex IV and cytochrome *c* were measured and shown to increase 1.58 ± 0.06 and 1.80 ± 0.09 -fold respectively after hypoxic NO_2^- treatment compared to controls (Figure 1J). Collectively, these data corroborate that NO_2^- increased cellular mitochondrial content in hypoxia.

Nitrite mediates biogenesis through activation of PGC1, SIRT1, and AMPK

Activation of PGC1 leads to the downstream activation of nuclear respiratory factor 2 (NRF-2) and transcription factor A of the mitochondrion (Tfam), ultimately initiating the coordinated expression of nuclear and mitochondrial genes and the synthesis of mitochondrial proteins [34]. To determine whether NO_2^- activates PGC1, the mRNA and protein expression of PGC1 was measured in RASMC. After 1 day of hypoxic NO_2^- (50 μM) treatment, PGC1 mRNA was upregulated 1.79 ± 0.08 fold (Figure 2A), while its protein expression increased by approximately 2.5 fold after 6 d (data not shown). The mRNA expression of Tfam and NRF-2 also increased by $26.8 \pm 0.6\%$ and $21.0 \pm 1.5\%$ respectively (Figure 2A). Silencing of PGC1 using siRNA abolished the NO_2^- -mediated increase in mitochondrial number (Figure 2B), demonstrating that NO_2^- stimulates the classical PGC1 biogenesis pathway.

The activity of PGC1 is regulated by metabolic sensors including AMPK and the NAD^+ -dependent deacetylase SIRT1, which de-acetylates PGC1 to increase its activity [10]. We next determined whether nitrite activates AMPK and SIRT1. Hypoxic NO_2^- treatment of RASMC induced a 4-fold increase in the phosphorylation of Thr¹⁷² on the catalytic subunit of AMPK as early as 3h after treatment (Figure 2C). Additionally, NO_2^- increased the expression of SIRT1 mRNA (Figure 2A) as well as its de-acetylase activity (Figure 2D). To determine whether NO_2^- -mediated biogenesis was dependent on SIRT1 activity, the acetylation state of PGC1 was measured after NO_2^- treatment. Consistent with a role for SIRT1-dependent de-acetylation, hypoxic NO_2^- treatment significantly decreased the acetylation of PGC1 (Figure 2E). Additionally, the NO_2^- -induced upregulation of PGC1 mRNA (data not shown) and increase in mitochondrial number (Figure 2F) was inhibited by splitomycin (100 μM), a pharmacological inhibitor of SIRT1, demonstrating that NO_2^- -mediated biogenesis was dependent on SIRT1 activity.

We next determined the mechanism by which SIRT1 was activated. Phosphorylation of AMPK has been shown to activate SIRT1 by increasing cellular NAD^+/NADH ratios [10]. To determine whether NO_2^- -dependent AMPK phosphorylation regulates SIRT1 activation, the NAD^+/NADH ratio was measured in NO_2^- treated RASMC transfected with siRNA to the catalytic α_2 subunit of AMPK. In the presence of fully functional AMPK, NO_2^- increased the NAD^+/NADH ratio 1.41 ± 0.16 -fold compared to controls. However, this NO_2^- -induced increase was significantly attenuated in cells lacking AMPK α_2 (Figure 3A). Further, in the absence of AMPK α_2 NO_2^- was unable to increase PGC1 mRNA expression (Figure 3B). Consistent with these results, the NO_2^- -induced increase in mitochondrial number was inhibited in cells treated with Compound C, a pharmacological inhibitor of AMPK (Figure 3C). These data demonstrate that NO_2^- -mediated activation of SIRT1 and subsequent stimulation of PGC1 is dependent on AMPK phosphorylation.

Nitrite and NO-mediated activation of PGC1 are mechanistically distinct

NO is a known stimulator of mitochondrial biogenesis and activator of AMPK [9, 35]. While the exact mechanisms of NO-mediated stimulation of PGC1 is unclear, it is known that NO-mediated biogenesis is dependent on the activation of sGC and the production of cGMP [9, 29]. Thus, we next determined whether NO_2^- -mediated biogenesis occurs through the reduction of NO_2^- to NO and subsequent sGC activation. The extent of biogenesis was first compared in RASMC treated with either NO_2^- or the NO donor diethylaminiotriamine NONOate (NONOate). After 6 days of treatment, NONOate (12.5–25 μM) and NO_2^- (25–50 μM) both induced significant hypoxic mitochondrial biogenesis (Figure 4A). It was next investigated whether cGMP was required for NO_2^- -dependent biogenesis. Measurement of cGMP levels 3 h after hypoxic NO_2^- or NONOate treatment showed that both species resulted in a concentration dependent increase in cGMP, although NONOate was a much more potent inducer of cGMP production than NO_2^- (1114 $\pm$ 0.90 fmol/mg versus 102 $\pm$ 10.12 fmol/mg at 25 μM) (Figure 4B). Comparison of the relationship between cGMP produced and the extent of biogenesis stimulated at each concentration of NO/ NO_2^- was found to differ significantly between the species (Figure 4C). Figure 4C demonstrates that NO_2^- stimulated greater biogenesis at significantly lower levels of cGMP production (20–110 fmol/mg) in comparison to NO, while NO-mediated biogenesis did not occur until significantly higher concentrations of cGMP (~130 fmol/mg) accumulated. These data suggested that NO_2^- -induced biogenesis occurs through a mechanism at least partially independent of cGMP. To more definitively test the role of sGC in NO_2^- -mediated biogenesis, RASMC were transfected with siRNA to the α -subunit of sGC. This treatment inhibited cGMP production in response to NO/ NO_2^- . While the lack of sGC activity significantly inhibited the NO (25 μM)-mediated increase in PGC1 mRNA expression, it had no significant effect on the NO_2^- induced increase in PGC1 mRNA (Figure 4D). Further, the inhibition of sGC activity by siRNA or by the pharmacological inhibitor ODQ (1 M) had no significant effect on the NO_2^- -induced increase in the phosphorylation of AMPK (Figure 4E) or in the increase of the cellular NAD^+/NADH ratio (Figure 4F).

Nitrite mediated biogenesis is not dependent on the reduction of nitrite to NO

Since NO potentially stimulates mitochondrial biogenesis through mechanisms independent of sGC activation, we next sought to determine whether nitrite-mediated biogenesis was dependent on the reduction of nitrite to NO. The fate of nitrite in hypoxic RASMC was first assessed. Six days of hypoxic nitrite (50 μM) treatment produced significant levels of S-nitrosated (RSNO) and iron-nitrosyl (Fe-NO) proteins (Figure 5A), indicative of nitrite-dependent NO formation. To directly determine whether nitrite was reduced to NO in these conditions, a suspension of RASMC were treated with nitrite (1 mM) at 1% O_2 and NO generation measured by chemiluminescence. A significant rate of NO formation was measured that persisted for at least 30 min (Figure 5B). To determine the enzyme responsible for nitrite reduction, the cells were pre-treated with inhibitors of known nitrite reductase enzymes, including potassium ferricyanide, cyanide and Allopurinol to inhibit heme globins, mitochondrial complex IV and xanthine oxidoreductase respectively. Consistent with prior studies [26], treatment with Allopurinol (100 μM) inhibited over 97% of nitrite reduction to NO (Figure 5B–C). To test whether nitrite mediated biogenesis was dependent on the reduction of nitrite to NO, RASMC were treated with Allopurinol (100 μM) for 6 days (replenished daily) in the presence and absence of nitrite (50 μM). Consistent with the inhibition of nitrite reduction to NO, Allopurinol treatment abolished nitrite-dependent Fe-NO formation and significantly attenuated RSNO levels in the cells (Figure 5A). However, measurement of mitochondrial number in these cells showed that nitrite-mediated biogenesis was only inhibited by approximately 10% (Figure 5D). These data suggest that nitrite potentially activates signaling pathways independent of nitrite reduction to NO.

Nitrite increases adenylate kinase activity

Given that NO_2^- -mediated biogenesis was independent of sGC, but dependent on AMPK activation, we next determined the mechanism by which NO_2^- activates AMPK. Increases in AMP:ATP ratios are known to activate AMPK [36]. Cellular AMP levels are regulated predominantly by adenylate kinase 1 (AK1) and AMP deaminase, which catalyze the formation and metabolism of AMP respectively. While no change in AMP deaminase activity was observed 1 h after hypoxic NO_2^- treatment, AK1 activity increased by $34.1 \pm 6.0\%$ compared to untreated hypoxic cells (Figure 6A). Further, pre-treatment with Di(adenosine-5') Pentaphosphate pentaammonium (Ap5A; 50 M), a pharmacological inhibitor of AK1, abolished the NO_2^- -induced upregulation of SIRT1 mRNA, consistent with a regulatory role for AK1 in NO_2^- -mediated biogenesis (Figure 6B).

Nitrite-mediated biogenesis generates functional mitochondria

To determine whether the new mitochondria generated after NO_2^- treatment were functional, the specific activity of respiratory complexes I-IV in RASMC was measured after 6 days of hypoxic NO_2^- treatment. Consistent with the synthesis of mitochondrial proteins, the activities of all the respiratory complexes and the matrix enzyme citrate synthase were significantly increased (Figure 7A). To determine whether this increased activity resulted in increased function, oxygen consumption was measured in the intact cells (Figure 7B). After measurement of basal respiratory rate, RASMC were treated with the ATPase inhibitor oligomycin ($1 \mu\text{M}$) to measure the respiratory rate not linked to ATP generation (proton leak), followed by treatment with the respiratory uncoupler FCCP ($2.5 \mu\text{M}$) to measure maximal rate of uncoupled respiration, and rotenone ($10 \mu\text{M}$) to inhibit mitochondrial oxygen consumption. While normoxic NO_2^- treatment did not change bioenergetic function, cells treated with NO_2^- ($25 \mu\text{M}$) in hypoxia showed increased basal and maximal respiratory rate (234.0 ± 7.5 pmoles/min/cell, 396.2 ± 8.9 pmoles/min/cell) compared to hypoxic controls (181.4 ± 11.8 pmoles/min/cell, 241.7 ± 18.3 pmoles/min/cell), but no change in non-ATP linked respiration (Figure 7C). Consistent with necessity of AMPK phosphorylation for nitrite-mediated biogenesis, pharmacological inhibition of AMPK with Compound C ($5 \mu\text{M}$) inhibited the nitrite-mediated increase in oxygen consumption (Figure 7D). The lack of increase in non-ATP linked oxygen consumption by NO_2^- treated cells suggests that NO_2^- -mediated biogenesis generates not only greater numbers of mitochondria, but also more efficient mitochondria that have more coupling of oxygen consumption to ATP production (ie less proton leak). In addition to increasing respiratory rate, hypoxic NO_2^- treatment increased basal ATP generation rate, but did not increase H_2O_2 generation (Figure 7E, F). These data demonstrate that NO_2^- stimulates the production of new more functionally efficient mitochondria.

Nitrite stimulates biogenesis *in vivo*

Nitrite has recently been shown to protect against neointimal smooth muscle proliferation after balloon injury of the carotid artery [26]. To determine whether NO_2^- -dependent mitochondrial biogenesis occurs *in vivo*, rats were subjected to balloon injury in the left carotid artery and then treated with oral NO_2^- (10.5 mg/L supplemented in the drinking water daily). After 2 weeks neointimal proliferation was increased in the injured artery compared to the corresponding uninjured artery in the same animal. However, this smooth muscle proliferation was significantly inhibited by NO_2^- treatment (Figure 8A-C). Indicative of NO_2^- -dependent stimulation of the biogenesis pathway, expression of PGC1 mRNA was significantly increased in the NO_2^- treated injured vessel compared to the uninjured vessel or the non- NO_2^- treated vessel (Figure 8D). Additionally, quantification of mitochondrial volume in these vessels showed that the ratio of mitochondrial to tissue area in the NO_2^- treated vessel was significantly greater (0.061 ± 0.006) than in the untreated injured vessel (0.032 ± 0.008) (Figure 8E-G). These data demonstrate that NO_2^- stimulates

mitochondrial biogenesis *in vivo* and is associated with the protective mechanism mediated by NO_2^- after balloon injury.

DISCUSSION

The primary observation of this study is that NO_2^- activates the AMPK-SIRT1-PGC1 pathway to stimulate mitochondrial biogenesis *in vitro* and *in vivo*, leading to the production of new mitochondria with improved function. Further, the current study demonstrates that the actions of NO_2^- are distinct from those of NO, as NO_2^- does not rely on sGC activation to mediate biogenesis. It is unclear why separate pathways exist for NO and NO_2^- mediated biogenesis and which pathway dominates *in vivo*. It is likely that they work in parallel and that considerable overlap exists since NO is oxidized to NO_2^- rapidly in cells and at least a small portion of NO_2^- is reduced to NO in hypoxia. This is reflected in our data showing that inhibition of sGC does not completely abrogate NO mediated biogenesis, and likewise, inhibition of sGC also has a small inhibitory effect on NO_2^- -mediated biogenesis. Physiologically, endothelial nitric oxide synthase (eNOS) activity has been shown to be essential for biogenesis mediated by physiological stimuli such as exercise and caloric restriction [9]. Given that eNOS generates not only NO, but also NO_2^- (through NO oxidation), it is possible that these redundant pathways exist in the cell to ensure the ability to modulate mitochondrial number even if one or the other pathway is damaged.

Interestingly, our data show that while NO_2^- increases basal and uncoupled respiration, non-ATP linked respiration is unchanged. This suggests that NO_2^- not only increases mitochondrial number, but also modulates function to generate more coupled mitochondria (ie a larger percentage of basal respiration is linked to ATP generation). This is consistent with previous studies demonstrating that NO_2^- increases mitochondrial efficiency in healthy human subjects [30]. Larsen and colleagues showed that increased efficiency was associated with a decrease in the expression of the adenine nucleotide translocase and uncoupling protein-3 [30]. While we did not explicitly investigate the cause of increased coupling, further study is needed to determine whether NO_2^- -dependent increased coupling is linked to or independent of stimulation of the biogenesis pathway. Further, these data suggest that NO_2^- may provide added benefit over other inducers of biogenesis, which do not increase efficiency. This could be particularly beneficial in the treatment of genetic mitochondrial diseases, for which recent studies have shown that induction of mitochondrial biogenesis alleviates symptoms in patients and corrects biochemical deficiencies in animal models [37-39]. Many of these diseases are propagated by the inability to produce sufficient ATP. Thus, NO_2^- may be beneficial by not only increasing mitochondrial number but also ATP generating capacity.

Though our study demonstrates that NO_2^- -mediated biogenesis is distinct from NO and does not rely on the reduction of nitrite to free NO, the ability of NO_2^- to increase mitochondrial number only in hypoxia suggests that NO_2^- is bioactivated to another species. Nitrite can mediate protein nitration through its heme-catalyzed reaction with H_2O_2 [40, 41] or be converted to electrophilic nitrated lipids, which signal through PPAR as well as adduction to critical cysteine residues [42]. Additionally, recent reports show that through its reaction with ferric heme proteins in hypoxia, NO_2^- can be reduced to N_2O_3 [27]. Evidence of significant S-nitrosothiol formation, even in the presence of allopurinol, in our model suggests that this may be a plausible mechanism by which nitrite mediates biogenesis. Though we did not specifically investigate the target of S-nitrosation or the mechanism by which NO_2^- increases AK1 activity, we have previously shown that NO_2^- inhibits complex I activity by S-nitrosation [31]. Inhibition of oxidative phosphorylation in this manner could decrease ATP production, potentially resulting in increased AK1 activity.

While the benefits of increasing mitochondrial number and hence oxygen consumption in hypoxic conditions appears counter-intuitive, our findings are consistent with other studies demonstrating hypoxia-induced biogenesis [43-45]. Mitochondrial ROS production has been shown to be essential in the stabilization of hypoxia inducible factor-1, leading to hypoxic adaptation [46, 47]. Further, brief episodes of hypoxia induce preconditioning responses, including upregulation of mechanisms to generate ATP and increased antioxidant capacity, to protect against future hypoxic/ischemic episodes [5, 48]. It is possible that the benefits of these type adaptive responses outweigh the cost of increased oxygen consumption in our hypoxic model. Further, while hypoxia is used in the *in vitro* model presented here, the role of nitrite as a physiological inducer of biogenesis is likely more relevant in models of inflammation or disease, as shown in the *in vivo* model of vessel injury, in which hypoxia is a component.

While we demonstrate that NO_2^- mediates biogenesis *in vivo*, prior studies have reported a lack of NO_2^- -mediated biogenesis in other animal models [31, 49]. This discrepancy is likely due to the fact that previous studies have not measured biogenesis in an area of injury. Here we show that NO_2^- mediates biogenesis only in the injured vessel, while no change in mitochondrial number is observed in the uninjured vessel of the same animal, despite increases in systemic NO_2^- levels. This suggests that the injured tissue provides an optimal environment for NO_2^- bioactivation. This ability for NO_2^- to mediate biogenesis in an injured area suggests a potential therapeutic advantage over NO, which cannot be targeted to a specific site of injury.

Prior studies have demonstrated that the activation of AMPK inhibits smooth muscle proliferation through modulation of mTOR as well as cyclin dependent kinases [50]. Similarly, PGC1 expression and mitochondrial biogenesis have been associated with decreased neointimal formation after carotid injury [51]. While this study links the protective effects of NO_2^- to these signaling pathways, our data have implications beyond the reversal of intimal hyperplasia. Nitrite mediates a number of other therapeutic responses including increasing glucose tolerance [49], protection against myocardial infarction [23, 24] and reversal of pulmonary hypertension [52]. While the mechanisms underlying these phenomena remain unclear, the NO_2^- -dependent AMPK-SIRT1-PGC1 pathway described here may play a role in some of these protective effects of NO_2^- . For example, activators of AMPK increase insulin sensitivity as well as provide protection from pulmonary hypertension [53] and myocardial infarction [54]. PGC1 also mediates effects beyond biogenesis including muscle fiber type switching from glycolytic to oxidative fibers, which may play a role in NO_2^- -induced increased exercise tolerance [55].

In summary, these data expand the function of NO_2^- as a modulator of metabolism through the activation of AMPK and SIRT1. Further, this study extends the role of NO_2^- in regulating mitochondrial function from acute post-translational modulation of activity to more chronic changes in mitochondrial number and bioenergetics. Additionally, these data suggest a role for NO_2^- in mitochondrial regulation distinct from that of NO. Future studies will determine how these pathways and species harmonize to meet the dynamic bioenergetic and signaling demands of the cell.

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REFERENCES

- [1]. Holloszy JO. Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J Biol Chem.* 1967; 242:2278–2282. [PubMed: 4290225]
- [2]. Joseph AM, Pilegaard H, Litvintsev A, Leick L, Hood DA. Control of gene expression and mitochondrial biogenesis in the muscular adaptation to endurance exercise. *Essays Biochem.* 2006; 42:13–29. [PubMed: 17144877]
- [3]. Menshikova EV, Ritov VB, Fairfull L, Ferrell RE, Kelley DE, Goodpaster BH. Effects of exercise on mitochondrial content and function in aging human skeletal muscle. *J Gerontol A Biol Sci Med Sci.* 2006; 61:534–540. [PubMed: 16799133]
- [4]. Costa LE, Boveris A, Koch OR, Taquini AC. Liver and heart mitochondria in rats submitted to chronic hypobaric hypoxia. *Am J Physiol.* 1988; 255:C123–129. [PubMed: 2839034]
- [5]. Lynn EG, Lu Z, Minerbi D, Sack MN. The regulation, control, and consequences of mitochondrial oxygen utilization and disposition in the heart and skeletal muscle during hypoxia. *Antioxid Redox Signal.* 2007; 9:1353–1361. [PubMed: 17627469]
- [6]. Piel DA, Khan AR, Waibel R, Birbach M, Cohen MS, Spray TL, Deutschman CS, Gaynor JW, Levy RJ. Chronic hypoxemia increases myocardial cytochrome oxidase. *J Thorac Cardiovasc Surg.* 2005; 130:1101–1106. [PubMed: 16214526]
- [7]. Cerqueira FM, Laurindo FR, Kowaltowski AJ. Mild mitochondrial uncoupling and calorie restriction increase fasting eNOS, akt and mitochondrial biogenesis. *PLoS One.* 2011; 6:e18433. [PubMed: 21483800]
- [8]. Lopez-Lluch G, Hunt N, Jones B, Zhu M, Jamieson H, Hilmer S, Cascajo MV, Allard J, Ingram DK, Navas P, de Cabo R. Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency. *Proc Natl Acad Sci U S A.* 2006; 103:1768–1773. [PubMed: 16446459]
- [9]. Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, Carruba MO. Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science.* 2005; 310:314–317. [PubMed: 16224023]
- [10]. Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, Elliott PJ, Puigserver P, Auwerx J. AMPK regulates energy expenditure by modulating NAD⁺ metabolism and SIRT1 activity. *Nature.* 2009; 458:1056–1060. [PubMed: 19262508]
- [11]. Fulco M, Cen Y, Zhao P, Hoffman EP, McBurney MW, Sauve AA, Sartorelli V. Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt. *Dev Cell.* 2008; 14:661–673. [PubMed: 18477450]
- [12]. Lu Z, Xu X, Hu X, Fasset J, Zhu G, Tao Y, Li J, Huang Y, Zhang P, Zhao B, Chen Y. PGC-1 α regulates expression of myocardial mitochondrial antioxidants and myocardial oxidative stress after chronic systolic overload. *Antioxid Redox Signal.* 2010; 13:1011–1022. [PubMed: 20406135]
- [13]. Arany Z, He H, Lin J, Hoyer K, Handschin C, Toka O, Ahmad F, Matsui T, Chin S, Wu PH, Rybkin II, Shelton JM, Manieri M, Cinti S, Schoen FJ, Bassel-Duby R, Rosenzweig A, Ingwall JS, Spiegelman BM. Transcriptional coactivator PGC-1 α controls the energy state and contractile function of cardiac muscle. *Cell Metab.* 2005; 1:259–271. [PubMed: 16054070]
- [14]. Marmolino D, Manto M, Acquaviva F, Vergara P, Ravella A, Monticelli A, Pandolfo M. PGC-1 α down-regulation affects the antioxidant response in Friedreich's ataxia. *PLoS One.* 2010; 5:e10025. [PubMed: 20383327]
- [15]. Dejam A, Hunter CJ, Pelletier MM, Hsu LL, Machado RF, Shiva S, Power GG, Kelm M, Gladwin MT, Schechter AN. Erythrocytes are the major intravascular storage sites of nitrite in human blood. *Blood.* 2005; 106:734–739. [PubMed: 15774613]
- [16]. Bryan NS, Fernandez BO, Bauer SM, Garcia-Saura MF, Milsom AB, Rassaf T, Maloney RE, Bharti A, Rodriguez J, Feelisch M. Nitrite is a signaling molecule and regulator of gene expression in mammalian tissues. *Nat Chem Biol.* 2005; 1:290–297. [PubMed: 16408059]
- [17]. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov.* 2008; 7:156–167. [PubMed: 18167491]

- [18]. van Faassen EE, Bahrami S, Feelisch M, Hogg N, Kelm M, Kim-Shapiro DB, Kozlov AV, Li H, Lundberg JO, Mason R, Nohl H, Rassaf T, Samouilov A, Slama-Schwok A, Shiva S, Vanin AF, Weitzberg E, Zweier J, Gladwin MT. Nitrite as regulator of hypoxic signaling in mammalian physiology. *Med Res Rev.* 2009; 29:683–741. [PubMed: 19219851]
- [19]. Hendgen-Cotta UB, Merx MW, Shiva S, Schmitz J, Becher S, Klare JP, Steinhoff HJ, Goedecke A, Schrader J, Gladwin MT, Kelm M, Rassaf T. Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury. *Proc Natl Acad Sci U S A.* 2008; 105:10256–10261. [PubMed: 18632562]
- [20]. Totzeck M, Hendgen-Cotta UB, Luedike P, Berenbrink M, Klare JP, Steinhoff HJ, Semmler D, Shiva S, Williams D, Kipar A, Gladwin MT, Schrader J, Kelm M, Cossins AR, Rassaf T. Nitrite Regulates Hypoxic Vasodilation via Myoglobin-Dependent Nitric Oxide Generation. *Circulation.* 2012
- [21]. Kumar D, Branch BG, Pattillo CB, Hood J, Thoma S, Simpson S, Illum S, Arora N, Chidlow JH Jr, Langston W, Teng X, Lefer DJ, Patel RP, Kevil CG. Chronic sodium nitrite therapy augments ischemia-induced angiogenesis and arteriogenesis. *Proc Natl Acad Sci U S A.* 2008; 105:7540–7545. [PubMed: 18508974]
- [22]. Dezfulian C, Raat N, Shiva S, Gladwin MT. Role of the anion nitrite in ischemia-reperfusion cytoprotection and therapeutics. *Cardiovasc Res.* 2007; 75:327–338. [PubMed: 17568573]
- [23]. Duranski MR, Greer JJ, Dejam A, Jaganmohan S, Hogg N, Langston W, Patel RP, Yet SF, Wang X, Kevil CG, Gladwin MT, Lefer DJ. Cytoprotective effects of nitrite during in vivo ischemia-reperfusion of the heart and liver. *J Clin Invest.* 2005; 115:1232–1240. [PubMed: 15841216]
- [24]. Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc Natl Acad Sci U S A.* 2004; 101:13683–13688. [PubMed: 15347817]
- [25]. Bryan NS, Calvert JW, Elrod JW, Gundewar S, Ji SY, Lefer DJ. Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. *Proc Natl Acad Sci U S A.* 2007; 104:19144–19149. [PubMed: 18025468]
- [26]. Alef MJ, Vallabhaneni R, Carchman E, Morris SM Jr, Shiva S, Wang Y, Kelley EE, Tarpey MM, Gladwin MT, Tzeng E, Zuckerbraun BS. Nitrite-generated NO circumvents dysregulated arginine/NOS signaling to protect against intimal hyperplasia in Sprague-Dawley rats. *J Clin Invest.* 2011; 121:1646–1656. [PubMed: 21436585]
- [27]. Basu S, Grubina R, Huang J, Conradie J, Huang Z, Jeffers A, Jiang A, He X, Azarov I, Seibert R, Mehta A, Patel R, King SB, Hogg N, Ghosh A, Gladwin MT, Kim-Shapiro DB. Catalytic generation of N₂O₃ by the concerted nitrite reductase and anhydrase activity of hemoglobin. *Nat Chem Biol.* 2007; 3:785–794. [PubMed: 17982448]
- [28]. Hopmann KH, Cardey B, Gladwin MT, Kim-Shapiro DB, Ghosh A. Hemoglobin as a nitrite anhydrase: modeling methemoglobin-mediated N₂O₃ formation. *Chemistry.* 2011; 17:6348–6358. [PubMed: 21590821]
- [29]. Nisoli E, Falcone S, Tonello C, Cozzi V, Palomba L, Fiorani M, Pisconti A, Brunelli S, Cardile A, Francolini M, Cantoni O, Carruba MO, Moncada S, Clementi E. Mitochondrial biogenesis by NO yields functionally active mitochondria in mammals. *Proc Natl Acad Sci U S A.* 2004; 101:16507–16512. [PubMed: 15545607]
- [30]. Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Eklom B, Lundberg JO, Weitzberg E. Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab.* 2011; 13:149–159. [PubMed: 21284982]
- [31]. Shiva S, Sack MN, Greer JJ, Duranski M, Ringwood LA, Burwell L, Wang X, MacArthur PH, Shoja A, Raghavachari N, Calvert JW, Brookes PS, Lefer DJ, Gladwin MT. Nitrite augments tolerance to ischemia/reperfusion injury via the modulation of mitochondrial electron transfer. *J Exp Med.* 2007; 204:2089–2102. [PubMed: 17682069]
- [32]. Yu SM, Tsai SY, Guh JH, Ko FN, Teng CM, Ou JT. Mechanism of catecholamine-induced proliferation of vascular smooth muscle cells. *Circulation.* 1996; 94:547–554. [PubMed: 8759101]
- [33]. Curtis E, Hsu LL, Noguchi AC, Geary L, Shiva S. Oxygen Regulates Tissue Nitrite Metabolism. *Antioxid Redox Signal.* 2012

- [34]. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell*. 1999; 98:115–124. [PubMed: 10412986]
- [35]. Almeida A, Moncada S, Bolanos JP. Nitric oxide switches on glycolysis through the AMP protein kinase and 6-phosphofructo-2-kinase pathway. *Nat Cell Biol*. 2004; 6:45–51. [PubMed: 14688792]
- [36]. Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol*. 2007; 8:774–785. [PubMed: 17712357]
- [37]. Bonnefont JP, Bastin J, Behin A, Djouadi F. Bezafibrate for an inborn mitochondrial beta-oxidation defect. *N Engl J Med*. 2009; 360:838–840. [PubMed: 19228633]
- [38]. Viscomi C, Bottani E, Civiletto G, Cerutti R, Moggio M, Fagioli G, Schon EA, Lamperti C, Zeviani M. In vivo correction of COX deficiency by activation of the AMPK/PGC-1alpha axis. *Cell Metab*. 2011; 14:80–90. [PubMed: 21723506]
- [39]. Wenz T. PGC-1alpha activation as a therapeutic approach in mitochondrial disease. *IUBMB Life*. 2009; 61:1051–1062. [PubMed: 19859975]
- [40]. Baldus S, Eiserich JP, Mani A, Castro L, Figueroa M, Chumley P, Ma W, Tousson A, White CR, Bullard DC, Brennan ML, Lusis AJ, Moore KP, Freeman BA. Endothelial transcytosis of myeloperoxidase confers specificity to vascular ECM proteins as targets of tyrosine nitration. *J Clin Invest*. 2001; 108:1759–1770. [PubMed: 11748259]
- [41]. Castro L, Eiserich JP, Sweeney S, Radi R, Freeman BA. Cytochrome c: a catalyst and target of nitrite-hydrogen peroxide-dependent protein nitration. *Arch Biochem Biophys*. 2004; 421:99–107. [PubMed: 14678790]
- [42]. Baker PR, Schopfer FJ, O'Donnell VB, Freeman BA. Convergence of nitric oxide and lipid signaling: anti-inflammatory nitro-fatty acids. *Free Radic Biol Med*. 2009; 46:989–1003. [PubMed: 19200454]
- [43]. Hoppeler H, Vogt M, Weibel ER, Fluck M. Response of skeletal muscle mitochondria to hypoxia. *Experimental physiology*. 2003; 88:109–119. [PubMed: 12525860]
- [44]. Martinelli M, Winterhalder R, Cerretelli P, Howald H, Hoppeler H. Muscle lipofuscin content and satellite cell volume is increased after high altitude exposure in humans. *Experientia*. 1990; 46:672–676. [PubMed: 2373192]
- [45]. Reynafarje B. Effect of chronic hypoxia on the kinetics of energy transformation in heart mitochondria. *Cardiology*. 1971; 56:206–208. [PubMed: 5152743]
- [46]. Brunelle JK, Bell EL, Quesada NM, Vercauteren K, Tiranti V, Zeviani M, Scarpulla RC, Chandel NS. Oxygen sensing requires mitochondrial ROS but not oxidative phosphorylation. *Cell Metab*. 2005; 1:409–414. [PubMed: 16054090]
- [47]. Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. *J Biol Chem*. 2000; 275:25130–25138. [PubMed: 10833514]
- [48]. McLeod CJ, Pagel I, Sack MN. The mitochondrial biogenesis regulatory program in cardiac adaptation to ischemia--a putative target for therapeutic intervention. *Trends in cardiovascular medicine*. 2005; 15:118–123. [PubMed: 16039972]
- [49]. Carlstrom M, Larsen FJ, Nystrom T, Hezel M, Borniquel S, Weitzberg E, Lundberg JO. Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice. *Proc Natl Acad Sci U S A*. 2010; 107:17716–17720. [PubMed: 20876122]
- [50]. Ferri N. AMP-activated protein kinase and the control of smooth muscle cell hyperproliferation in vascular disease. *Vascular pharmacology*. 2012; 56:9–13. [PubMed: 22074809]
- [51]. Qu A, Jiang C, Xu M, Zhang Y, Zhu Y, Xu Q, Zhang C, Wang X. PGC-1alpha attenuates neointimal formation via inhibition of vascular smooth muscle cell migration in the injured rat carotid artery. *American journal of physiology. Cell physiology*. 2009; 297:C645–653. [PubMed: 19553562]
- [52]. Zuckerbraun BS, Shiva S, Ifedigbo E, Mathier MA, Mollen KP, Rao J, Bauer PM, Choi JJ, Curtis E, Choi AM, Gladwin MT. Nitrite potently inhibits hypoxic and inflammatory pulmonary arterial

hypertension and smooth muscle proliferation via xanthine oxidoreductase-dependent nitric oxide generation. *Circulation*. 2010; 121:98–109. [PubMed: 20026772]

- [53]. Agard C, Rolli-Derkinderen M, Dumas-de-La-Roque E, Rio M, Sagan C, Savineau JP, Loirand G, Pacaud P. Protective role of the antidiabetic drug metformin against chronic experimental pulmonary hypertension. *Br J Pharmacol*. 2009; 158:1285–1294. [PubMed: 19814724]
- [54]. Calvert JW, Gundewar S, Jha S, Greer JJ, Bestermann WH, Tian R, Lefer DJ. Acute metformin therapy confers cardioprotection against myocardial infarction via AMPK-eNOS-mediated signaling. *Diabetes*. 2008; 57:696–705. [PubMed: 18083782]
- [55]. Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, Spiegelman BM. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature*. 2002; 418:797–801. [PubMed: 12181572]

HIGHLIGHTS

- Nitrite activates AMP kinase, sirtuin-1 and PGC1 to mediate biogenesis
- Nitrite stimulates the synthesis of mitochondria with increased respiratory coupling
- Unlike NO, nitrite-mediated biogenesis does not require sGC activation
- Biogenesis associates with nitrite-mediated protection in a model of carotid injury

Figure 1. Nitrite increases mitochondrial number

Representative mitotracker (green) and nuclear (blue) staining of (A) Untreated 21% O₂, (B) NO₂⁻ (50 M) treated 21% O₂, (C) Untreated 1% O₂, and (D) NO₂⁻ (50 M) treated 1% O₂ cells. (E) Ratio of the intensity of mitotracker: nuclear staining in RASMTC treated with NO₂⁻ (0-100 M) at 21% or 1% O₂ for 6 d. (F-I) Representative electron micrographs (25,000X) of (F) untreated 21% O₂, (G) NO₂⁻ (50 M; 21% O₂) treated, (H) Untreated 1% O₂ and (I) NO₂⁻ (50 M; 1% O₂) treated cells. Arrows denote mitochondria. (J) Relative density of protein expression of complex IV, cytochrome *c* and -actin after 6 d treatment (1% O₂) with or without NO₂⁻ (normalized to -actin). Data are means ± SEM * *p* < 0.01 vs untreated. n > 5.

Figure 2. Nitrite activates AMPK, SIRT1 and PGC1

RASMC were treated with or without NO₂⁻ (50 M) at 1% O₂. (A) mRNA expression of PGC1, SIRT1, NRF2, and Tfam after 1 d. (B) Mitochondrial: nuclear ratio 4 d after nitrite treatment of cells transfected with an empty vector (open bars) or siRNA to PGC1 (filled bars). (C) Representative immunoblot and quantitation of the ratio of phosphorylated AMPK to total AMPK 3 h after treatment. (D) SIRT1 deacetylase activity 24 h after treatment. (E) Representative immunoblot of acetylated and total PGC1 protein with quantification 4 h after treatment. (F) Mitochondrial: nuclear ratio 6 d after treatment in the presence (filled bars) or absence (open bars) of splitomycin (100 M) *p<0.01, #p<0.05 vs. control. n>3

Figure 3. Nitrite-induced biogenesis is dependent on AMPK

RASMC were treated with or without NO_2^- (50 M) at 1% O₂. (A) NAD^+/NADH measured 6 h after treatment in cells transfected with an empty vector (control) or siRNA to AMPK₂. (B) PGC1 mRNA levels measured 1 d after treatment in cells transfected with an empty vector (control) or siRNA to AMPK₂. (C) The ratio of mitochondrial to nuclear staining measured 6 d after treatment in cells co-treated with or without Compound C (5 μM).

* $p < 0.01$, # $p < 0.05$. $n > 3$.

Figure 4. Nitrite induced biogenesis is not dependent on sGC

(A) Mitochondria:Nuclear content measured 6 d and (B) cGMP levels measured 3 h after treatment with NO_2^- (0-200 μM) or NONOate (0-100 μM) at 1% O_2 (C) Mitochondria:nuclear plotted as a function of cGMP produced at each concentration of NO_2^- or NONOate. (D-F) RASMC were treated with or without NO_2^- (50 M) at 1% O_2 after transfection with an empty vector, siRNA to sGC β_1 or ODQ (1 M). (D) PGC1 mRNA levels in sGC $\beta_1^{+/+}$ (open bars) and sGC $\beta_1^{-/-}$ (filled bars) cells, (E) phospho-AMPK levels in the presence (filled bars) or absence (open bars) of ODQ and (F) NAD^+/NADH measured 1 d, 3 h and 6 h after treatment respectively. * $p < 0.01$, # $p < 0.05$ vs control; $n > 3$.

Figure 5. Inhibition of nitrite reduction to NO does not abolish nitrite-mediated biogenesis
(A) Concentration of RSNO and Fe-NO generated in RASMC treated with nitrite (50μM) in the presence (open bars) or absence (filled bars) of Allopurinol (100μM) for 6 days. **(B)** Representative chemiluminescence trace of NO production by RASMC treated with nitrite (1mM) in the absence (black) or presence (gray) of Allopurinol (100μM). **(C)** Quantitation of NO production by RASMC treated with nitrite (1mM) and pre-treated with nothing (control), Allopurinol (100μM), Potassium ferricyanide (100 μM), or Potassium Cyanide (100 μM). **(D)** Mitochondrial to Nuclear ratio of RASMC treated with (open bars) or without (filled bars) nitrite (50 μM) in the presence of absence of Allopurinol (100μM). *p<0.01, #p<0.05. n>3.

Figure 6. Nitrite increases AK1 activity

(A) AK1 activity in RASMC treated with or without NO₂⁻ (50 M) at 1% O₂. (B) SIRT1 mRNA expression after co-treatment with or without Ap5A (50 M). #p<0.05 vs control; n>3.

Figure 7. Nitrite generates functional mitochondria

(A) Activity of complexes I, II, IV and citrate synthase 6 d after NO₂⁻ (50 M) treatment at 1% O₂. (B) Representative oxygen consumption (OCR) traces for RASMC treated with or without NO₂⁻ (50 M) at 1% O₂ for 6 d. Arrows denote addition of oligomycin (1 μM), FCCP (2.5 μM) and rotenone (10 μM). (C) Quantitation of several traces such as those shown in (B) as well as similar traces with NO₂⁻ treatment at 21% O₂. (D) Basal and uncoupled OCR of RASMC treated with nothing (open bars), Compound C (5 μM; hatched bars), NO₂⁻ (50 M; black bars), or NO₂⁻ and compound C (grey bars). (E-F) Rates of (E) ATP and (F) H₂O₂ generation in cells treated with NO₂⁻ (50 μM) for 6 d at 1% O₂. * *p*<0.01, #*p*<0.05 vs control. *n*>3.

Figure 8. Nitrite mediates biogenesis *in vivo*

Rats were treated with or without daily oral sodium NO₂⁻ (10.5 mg/L water) for 2 wks after balloon injury to the left carotid artery. Controls are uninjured artery of the same animal. (**A & B**) Representative H&E stain of a carotid artery 14 d after injury in the (**A**) absence or (**B**) presence of NO₂⁻ treatment. (**C**) Quantification of the ratio of intimal to media thickness in several arteries such as those in A/B. (**D**) PGC1α mRNA expression in injured or non-injured arteries from rats supplemented with NO₂⁻ or not. (**E&F**) Representative electron micrographs of a section of injured carotid artery from (**E**) untreated and (**F**) NO₂⁻ treated rats. (**G**) Quantification of the mitochondrial volume per tissue area for arteries such as those shown in E/F. *p<0.01, #p<0.05 vs control. n>3.