IMPACT OF HYPOXIC NITRATE RESPIRATION ON *MYCOBACTERIUM TUBERCULOSIS*

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An estimated one third of the world's population is infected with *Mycobacterium tuberculosis* (Mtb) and suppresses the pathogen's replication without eradicating it, thereby remaining at risk for developing active tuberculosis (TB). It is not clear how Mtb survives, potentially for decades, in a hostile host environment. Respiration of nitrate to nitrite could help Mtb to survive in hypoxic tissues, but was not thought to be significant at physiologic oxygen tensions, nor was the resultant nitrite considered consequential to Mtb's physiology. Here, we found that Mtb infecting human macrophages *in vitro* produces copious nitrite at physiologic oxygen tensions. The nitrite impacts Mtb in diverse ways, slowing its growth, reducing its consumption of ATP, regulating its survival following treatment with isoniazid and hydrogen peroxide, and remodeling its transcriptome in a manner distinct from that of nitric oxide. Thus, respiration of nitrate and adaptation to nitrite are likely to play a prominent role in Mtb's pathophysiology, whether or not the Mtb resides in hypoxic sites.

BIOGRAPHICAL SKETCH

Amy Christine Cunningham- Bussel graduated *magna cum laude* in biological sciences from Cornell University in 2005 where she majored in neurobiology and behavior. She is a student in the Tri-Institutional MD/PhD program of Weill Cornell Medical College, Rockefeller University and The Sloan-Kettering Institute.

She joined the laboratory of Dr. Carl Nathan in September of 2009 where her research focused on studying the impact of hypoxic nitrate respiration on *Mycobacterium tuberculosis*.

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LIST OF ABBREVIATIONS

CFU: Colony forming unit
ETH: Ethionamide
IFNγ: Interferon gamma
INH: Isoniazid
MCSF: Macrophage colony-stimulating factor
Mtb: *Mycobacterium tuberculosis*
NOS: Nitric oxide synthase
OD: Optical density
PIMO: Pimonidazole
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
TB: Tuberculosis
TNFα: Tumor necrosis factor

CHAPTER 1

INTRODUCTION

Mycobacterium tuberculosis (Mtb) is the second leading cause of death worldwide from a single infectious pathogen (1). Despite the induction of a potent immune response, Mtb is not eradicated and survives to establish lifelong disease. Furthermore, months of multidrug chemotherapy are required to treat patients with tuberculosis (TB) (2). How Mtb survives the arsenal of toxic small molecules that are products of an active immune response, and why months of multidrug chemotherapy are required to treat TB, remain active areas of investigation.

The toxic molecules produced by an active immune response.

Humans have co-evolved with Mtb over millennia and are its only known natural host (3, 4). Infection with Mtb begins with the inhalation of droplet nuclei containing Mtb, which arise from infected individuals and become airborne (5, 6). Within the lung alveoli of a newly infected host, Mtb is confined within the phagosomes of resident macrophages. Once activated by interferon gamma (IFNγ), these phagosomal compartments fuse with adjacent intracellular lysosomes and acidify (7). IFNγ also triggers the assembly and recruitment of enzymes responsible for the production of reactive nitrogen and oxygen species (RNS and ROS, respectively). Examples of RNS include, nitric oxide ($^{\bullet}NO$), nitrogen dioxide ($^{\bullet}NO_2$) and nitrite (NO_2^-) and examples of ROS are superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$)

(8, 9). Nitrate (NO_3^-) can give rise to the above RNS within humans, however, this primarily requires enzymatic reduction by nitrate reductase enzymes expressed by oral commensal bacteria (10). This is described in greater detail below.

Despite exposure to these potentially lethal host products, Mtb expresses enzymes that detoxify ROS and RNS directly, as well as enzymes that repair mycobacterial proteins and DNA damaged by these toxic molecules. For example, enzymes which degrade ROS and/or RNS include Mtb's catalase/peroxidase KatG, the superoxide dismutases SodA and SodC, an NADPH dependent peroxidase/peroxynitrite reductase encoded by *ahpC*, *ahpD*, *lpdC* and *dlaT* and the truncated hemoglobins trHbO and trHbN. The majority of these proteins have been shown to contribute to mycobacterial virulence (9, 11, 12). Methionine sulphoxide reductases, MsrA and MsrB, repair the ROS and RNS mediated oxidation of methionine residues and UvrB, a DNA repair protein contributes to resistance against both ROI and RNI *in vivo* (13, 14). Furthermore, mycobacteria synthesize mycothiol rather than glutathione, which serves as a dominant intracellular antioxidant. Mycobacteria unable to synthesize mycothiol are less able to survive treatment with ROS and RNS (15).

The host exploits the toxicity and essentiality of transition metals

Every living organism depends upon transition metals for growth, DNA replication and metabolic energy production. Moreover, these essential metals are required for the catalytic function of many of the enzymes involved in resistance to ROS and RNS mediated oxidative stress (16). Iron, the most abundant transition metal within human tissue,

is actively sequestered away from microbial pathogens by host proteins. Infected human phagocytes stimulated with IFNγ repressed the expression of transferrin and the intracellular iron storage protein, ferritin thus reducing the total concentration of intracellular iron and limiting intracellular bacterial replication (17, 18). The host encodes natural resistance- associated macrophage protein 1 (NRAMP1), which transports iron and manganese out of the phagosomal compartment. Though controversial, mutations in NRAMP1 have been demonstrated to confer resistance to tuberculosis in mice and humans (19-22).

In contrast, although transition metals are critical to cellular life, in abundance they are toxic. Copper accumulates within Mtb- associated pulmonary lesions. This is thought to be a strategy by which the immune system restricts mycobacterial growth *in vivo* (23)*9*. This is further supported by the observation that, in comparison to other bacteria, Mtb is particularly susceptible to copper *in vitro*. Moreover, Mtb lacking the copper transporter MctB accumulated 100 fold more intracellular copper *in vitro* and was markedly attenuated within guinea pigs (23). In addition, INFγ mediated transportation of copper into the phagolysosomes of activated macrophages was lethal to resident intracellular bacteria (24). This lethality of copper is attributed to the fact that copper disables critical cellular enzymes by dislodging catalytic iron atoms. In addition, copper participates in Fenton chemistry, which results in the production of toxic hydroxyl radicals and may potentiate RNS mediated oxidative stress (25). Thus, the host capitalizes on the dual essentiality and toxicity of transition metals to eradicate invading pathogens (16).

The availability of molecular oxygen regulates mycobacterial growth.

In the host, Mtb is thought to reside within oxygen-depleted tissue (26, 27). Individual macrophages, in which Mtb becomes sequestered, contain lower oxygen tensions than the extracellular space (28). These infected cells then aggregate forming avascular structures called granulomas within which oxygen tensions approach 1% (29-32). Although sudden exposure to anaerobiosis is lethal, Mtb can adapt to conditions in which oxygen is gradually depleted (33). In response to the gradual withdrawal of oxygen from the medium, Mtb carries out the orderly shut down of various cellular processes and enters into a nonreplicating persistent (NRP) state beginning once oxygen tensions reach 1%. NRP bacteria no longer divide or synthesize DNA and demonstrate limited RNA and protein synthesis (34). Furthermore, NRP Mtb are no longer sensitive to antibiotics that target these inactive cellular processes (27, 35).

Oxygen regulates the growth of Mtb within the host.

Before the advent of chemotherapy, surgical intervention to reduce Mtb's access to oxygen was a leading treatment for TB. Intentional lung atelectasis allowed for near-complete pathologic and clinical resolution of disease (36). While this suggests that mycobacterial growth requires access to oxygen *in vivo,* evidence that oxygen regulates the growth of Mtb within infected humans is, necessarily, indirect.

In order to investigate the *in vivo* oxygen dependence of Mtb, oxygen tensions were experimentally manipulated within the lung tissue of Mtb infected model organisms. Direct manipulation was achieved by

controlling the partial pressure of oxygen within inhaled air. Guinea pigs infected with Mtb and maintained in 10% oxygen contained reduced colony forming units (CFUs) within their lungs and spleens as compared to their Mtb-infected counterparts maintained under room air (21% oxygen) (37). Alternatively, indirect manipulation of oxygen by re-routing a percentage of the blood supply away from otherwise well-ventilated alveoli created an artificially elevated partial pressure of oxygen within the associated alveolar sacs, as oxygen was no longer carried off into the circulation by hemoglobin. Monkeys that received a surgical intervention to re-route blood from lung tissue prior to infection with Mtb, for example by pulmonary artery ligation, developed increased mycobacterial numbers within the associated lung tissue (38). Thus these initial experiments supported the centrality of oxygen in regulating mycobacterial growth *in vivo*. Further supporting evidence arose from the observation that TB-associated lesions tend to localize within lung tissue containing the greatest oxygen tension.

The diagnosis of TB in humans is made in part by capturing radiographic images of the lungs. This led to the observation that in adults, pathologic lesions associated with chronic pulmonary TB are often localized within the lung apices (39). As a result of gravity, the apices receive less blood flow than more inferior lung tissue, and therefore contain greater oxygen tensions, 132 mmHg as compared to 89 mmHg in more inferior tissue (40, 41). In order to determine whether the higher oxygen tensions of the lung apices contributed to the localization of TB-associated pathology, Medlar and Sasano infected rabbits with Mtb and maintained a subset of infected animals in a vertical position to simulate

the erect posture of humans. Typically, Mtb infected (horizontal) rabbits develop tissue pathology in the posterior dorsal basal lung segments that contain the highest oxygen tensions. However, vertically oriented rabbits developed Mtb-associated lesions in the lung apices (42). While the results of this seminal experiment supported a relationship between oxygen tension and progressive TB lung pathology, an alternative explanation posited that impaired lymphatic clearance of antigens within the lung apices could account for these observations (40).

Although these historic experiments suggested an association between oxygen and mycobacterial growth *in vivo*, it is not possible to conclude from them that Mtb infected hosts are hypoxic, or that oxygen regulates the growth of Mtb in the host. In the modern era, however, investigators have developed tools to directly address whether Mtb infected host tissues are hypoxic. Molecular markers of hypoxia, such as the nitroimidazoles, mycobacterial hypoxic response gene expression analysis and direct measurement of tissue oxygen tensions with an oxygen probe have been used to demonstrate that Mtb infected model hosts, such as the guinea pig, rabbit, macaque develop hypoxic tissue pathology (30). These same techniques have also indicated that the C3HeB/FeJ mouse model, but not BALB/c (43) or C57BL/6 (31, 32) mouse strains, develop hypoxic TB-associated lesions. Therefore, within model hosts that have demonstrated hypoxic, TB-associated tissue pathology, Mtb likely experiences hypoxia. That the availability of oxygen regulates mycobacterial growth *in vitro* is clear. Mycobacteria no longer replicate once oxygen tensions fall to, or below 1% (26, 27). Thus oxygen likely regulates mycobacterial replication *in vivo* as well.

When oxygen is scarce, Mtb respires the alternative electron acceptor nitrate.

In response to hypoxia, Mtb induces the expression of the nitrate/proton symporter *narK2*, which allows for the rapid entry of nitrate into the cell (44). Nitrate is subsequently reduced to nitrite by the constitutively active nitrate reductase encoded by *narGHJI* (45). This process reportedly supports hypoxic ATP synthesis and redox balancing (46, 47). Moreover, the upregulation of this alternative respiratory pathway enhances the survival of Mtb challenged with sudden anaerobiosis (48). Human tissues contain abundant quantities of nitrate, which arise primarily from the diet and nitric oxide synthase (NOS) enzymes. NOS enzymes produce nitric oxide that auto-oxidize to form both nitrate and nitrite (49). Over the course of infection with Mtb, patients accumulate increasing concentrations of plasma nitrite (50, 51). This likely arises from the two NOS isoforms, endothelial and inducible (eNOS and iNOS respectively) that are expressed in the granulomatous tissue of TB patients (52-54). Mycobacterial nitrate reduction, which is responsible for the production of 2.5 mM nitrite by axenic cultures of Mtb (55), may also contribute to this pool of plasma nitrite.

Mtb respires nitrate within primary human macrophages.

Our laboratory recently adopted an alternative protocol to differentiate primary human macrophages. There are several features that distinguish this protocol from others (56). In particular, monocytes are differentiated in 10% oxygen in the gas phase over 2 weeks in a medium

containing 40% human plasma, 60% RPMI and the cytokines GMCSF and TNFα. The resultant macrophages better restricted the replication of Mtb over a period of weeks (57), as compared to macrophages differentiated using more traditional protocols, which are incubated under room air oxygen tensions of a period of days, in a medium composed of DMEM, 10% fetal bovine serum and MCSF (56). Initial experiments revealed that, despite culture under non-hypoxic oxygen tensions, macrophage-resident Mtb produced significant amounts of nitrite. This was immediately of interest as nitrate and nitrite can be reduced to form nitric oxide *in vivo* (49).

The nitrate-nitrite-nitric oxide pathway begins with the reduction of nitrate to nitrite by oral commensal bacteria, which can then be further reduced to nitric oxide through an array of physiologic processes, many of which are non-enzymatic (49). The reduction of nitrate or nitrite to form nitric oxide has been shown to reduce blood pressure (58, 59), enhance the efficacy of mitochondrial oxidative phosphorylation (60), impair platelet aggregation (61), and to exert carcinogenic (62) and antimicrobial effects (49, 63, 64). These broad effects arise once nitrate or nitrite is reduced to nitric oxide. However, nitrite has also been proposed to directly react with hemes and thiols without the intermediary formation of nitric oxide (65).

Conclusions

In the context of tuberculosis, nitrite is generally overlooked as simply a degradation product of NOS enzymes. This is in part due to the fact that nitrate is not a component of the standard cell medium used to

culture Mtb axenically. In addition, in contrast to our protocol, traditional monocyte differentiation techniques typically involve the use of medium containing low levels of nitrate and so mycobacterial nitrate reduction is rarely appreciated in either axenic or co-culture experiments.

Although high concentrations of nitrite restrict the growth of Mtb without any associated lethality (55), this has remained mechanistically unexplained. Therefore we hypothesized that Mtb-derived nitrite might impact Mtb itself. Taken together, two central areas of investigation emerged from our observation that Mtb produced abundant amounts of nitrite within infected macrophages: first, what had prompted mycobacterial nitrate respiration, were macrophage-resident Mtb functionally hypoxic? Second, given the broad impact of the nitrate and nitrite on human physiology and the observation that nitrite impairs mycobacterial replication, we proposed to characterize additional consequences of Mtb-derived nitrite on the pathogen itself and to determine to what extent these effects resemble the well-documented effects of nitric oxide.

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CHAPTER 2

MYCOBACTERIUM TUBERCULOSIS GENERATES NITRITE IN HUMAN MACROPHAGES AT PHYSIOLOGIC OXYGEN TENSIONS: IMPACT ON MYCOBACTERIAL ATP CONSUMPTION AND GENE EXPRESSION*

Summary

In high enough concentrations, such as produced by inducible nitric oxide synthase (iNOS), reactive nitrogen species (RNS) can kill *Mycobacterium tuberculosis* (Mtb). Lesional macrophages in macaques and humans with tuberculosis (TB) express iNOS, and mice need iNOS to avoid succumbing rapidly to TB. However, Mtb's own ability to produce RNS is rarely considered, perhaps because nitrate reduction to nitrite is only prominent in axenic Mtb cultures at oxygen tensions ≤ 1%. Here we found that cultures of Mtb-infected human macrophages cultured at physiologic oxygen tensions produced copious nitrite. Surprisingly, the nitrite arose from the Mtb, not the macrophages. Mtb responded to nitrite by ceasing growth; elevating levels of ATP through reduced consumption; and altering the expression of 120 genes associated with adaptation to acid, hypoxia, nitric oxide, oxidative stress, and iron deprivation. The transcriptomic effect of endogenous nitrite was distinct from that of nitric oxide. Thus, whether or not Mtb is hypoxic, the host expresses iNOS, or hypoxia impairs the action of iNOS, Mtb *in vivo* is likely to encounter RNS by producing nitrite. Endogenous nitrite may slow Mtb's growth and prepare it to resist host stresses while the pathogen waits for

* Amy Cunningham-Bussel, Tuo Zhang, and Carl Nathan, "*Mycobacterium Tuberculosis* Generates

immunopathology to promote its transmission.

Introduction

The ability to produce RNS is widely distributed among animals, plants and eubacteria. The high-output pathway of RNS production by host iNOS contributes to control of diverse infections (1, 2), including tuberculosis (TB) (3, 4), via impacts on both the pathogen and the host (5). Macrophages in the lungs of humans (3, 6, 7) and macaques (8) with TB express functional iNOS. Correlative evidence suggests that the action of iNOS may contribute to human control of TB (1, 2, 9, 10).

Contributions of bacterial RNS production to host-pathogen interactions have only recently come under study. Bacterial NO synthases or nitrate reductases are sources of RNS that can contribute to antibiotic resistance (3, 4, 11, 12) and increase virulence (5, 13). Some commensal bacteria exploit host iNOS during colonic inflammation by using iNOS-derived nitrate (NO_3^-) as a terminal electron acceptor (3, 6, 7, 14). Nitrate, along with nitrite (NO_2^-), is an auto-oxidation product of nitric oxide ($^{\cdot}NO$), the product of iNOS.

In the host, Mtb is thought to survive over decades in hypoxic sites (8, 15, 16). When oxygen is scarce, Mtb reduces nitrate (NO_3^-) to nitrite (NO_2^-) as a means to maintain redox homeostasis and energy production (17). The mycobacterial respiratory nitrate reductase, encoded by *narGHJI*, is constitutively expressed (17), and functions at a low level under aerobic conditions when nitrate is taken up by passive diffusion (18). Hypoxia promotes the induction of *narK2*, a nitrate transporter and a member of the dormancy survival (*DOS*) regulon (17, 19-21), such that

Mtb cultured at ≤ 1% oxygen in the presence of nitrate rapidly produces nitrite up to a level of 2.5 mM (22). Further production is restricted in association with non-lethal cessation of bacterial growth (22).

Transcripts from *narG*, which encodes a subunit of the nitrate reductase, and *narX*, which lies downstream of *narK2* and encodes a non-functional nitrate reductase, were identified in granulomas from humans with TB (23, 24). This suggests that populations of mycobacteria residing within the human host are exposed to low oxygen tensions, and likely respire nitrate to adapt. Nitrate is a physiologic component of human body fluids, where it arises from the diet and from auto-oxidation of the NO produced not only by iNOS (NOS2) but also by the constitutively expressed enzymes NOS1 and NOS3. Studies in which nitrate was furnished *in vitro* revealed that Mtb's ability to respire nitrate allows it to better withstand acid, nitrosative stress (25) or sudden anaerobiosis (26). The evolutionary success of "modern" Mtb strains, which are more prevalent than "ancestral" strains, has been attributed to their enhanced nitrate reductase activity (27). Although nitrate and nitrite reproduce the bioactivity of nitric oxide *in vivo* in a wide array of physiologic processes (28), reduction of nitrate to nitrite is rarely considered in studies of the host-pathogen relationship in TB. In fact, nitrate was omitted when the standard growth media were formulated in which almost all *in vitro* experiments with Mtb are currently conducted.

During the course of modifying standard culture conditions for Mtb-infected human monocyte-derived macrophages (29), we observed abundant accumulation of nitrite in the supernatant. Herein, we report the identification of Mtb as both the source of the nitrite and a highly

responsive target.

Results

Mtb respires nitrate in human macrophages cultured at non-hypoxic oxygen tensions.

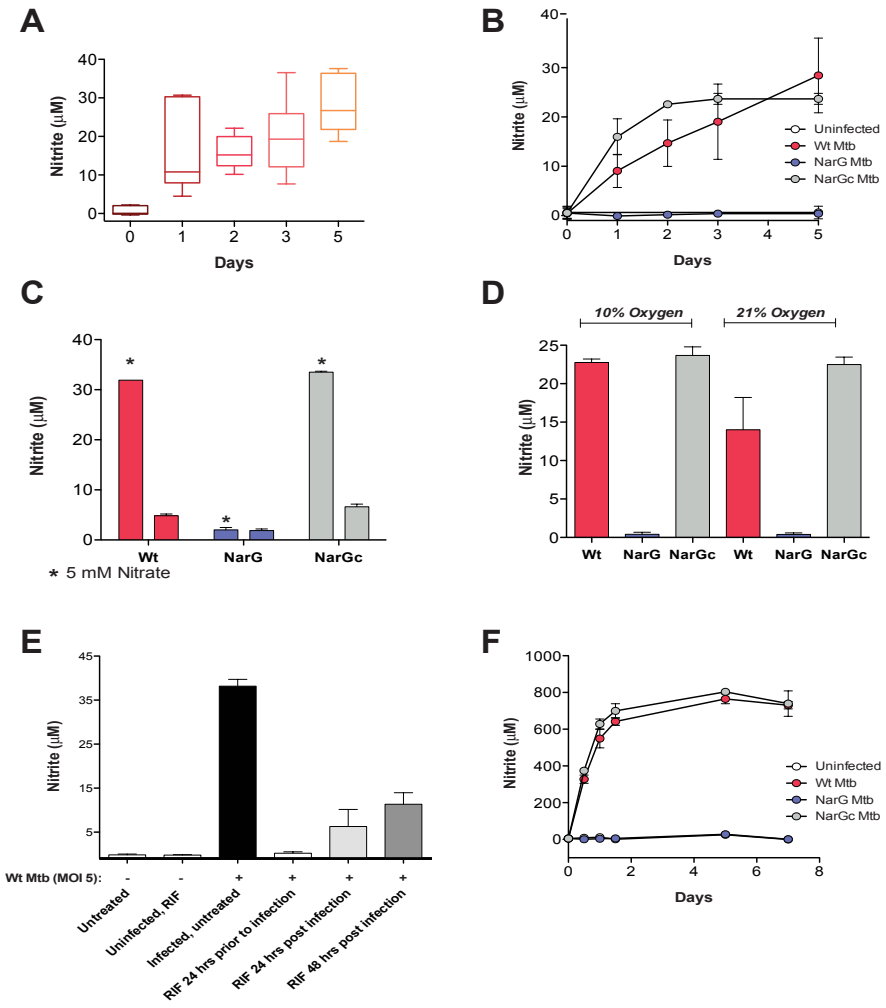
We differentiated normal donors' monocytes into macrophages under 10% oxygen for 2 weeks, activated them with interferon-g and infected them with Mtb, as described (29). Cultures of macrophages from multiple donors consistently accumulated nitrite in a time-dependent manner to a median level of 25 μM over 5 days (Figure 1a). This suggested that the macrophages may have expressed one of the three NOS isoforms. However, we detected no iNOS protein by immunoblot (Supplementary Figure S1, see Appendix 1) and no NOS1, NOS2 or NOS3 transcripts by microarray or RNA Seq (not shown). To see if Mtb might be the source, we infected macrophages with a strain of Mtb lacking *narG*. The mutant strain grows normally in aerobic culture and is as virulent as wild type Mtb in mice (15), but cannot respire nitrate. Macrophage cultures infected with Δ*narG* Mtb accumulated no nitrite, while infection with a mutant strain complemented with the wild type *narG* allele led to accumulation of as much nitrite as infection with wild type (WT) Mtb (Figure 1B). Accumulation of nitrite was nearly abolished when the cultures were washed gently to replace most of the standard culture medium with medium containing negligible nitrate, and was restored when the nitrate-deficient cultures were repleted with 5 mM nitrate (Figure 1C). Even when we cultured infected macrophages in conventional, hyperoxic levels of oxygen (21%), we observed *narG*-dependent nitrite accumulation in

the supernatant (Figure 1D). To further confirm that the resultant nitrite was a mycobacterial, rather than a host product, we infected macrophages with Mtb in the presence of the bactericidal antibiotic, rifampicin. Addition of rifampicin to macrophages 2 days prior to infection completely abolished nitrite production after infection. Treatment with rifampicin even 24 or 48 hours post infection markedly reduced the production of nitrite (Figure 1E).

We wondered what levels of Mtb-derived nitrite might be attained in tissue sites such as granulomas, where the number of macrophages per unit volume vastly exceeds the non-physiologic ratio attainable in monolayer culture. To better simulate the cell densities of tissues, we cultured macrophages on Cytodex microcarrier beads. The *narG*-dependent accumulation of nitrite in bead cultures reached 800 μM (Figure 1F). In both monolayer and bead culture, the colony-forming units (CFU) of Mtb recovered from the cultures were not significantly different for *narG*-deficient and WT Mtb (Cunningham-Bussel, *MicrobiologyOpen*, in press).

Figure 1. Nitrite generation by Mtb within primary human macrophages cultured in non-hypoxic levels of oxygen. (A) Accumulation of nitrite in supernatant of macrophages in monolayer culture in 10% oxygen infected with wild type (Wt) Mtb (MOI:5; $5x10^5$ bacteria) for the indicated times. Box and whisker plots depict median and 5^{th}-95^{th} percentiles of results from 6 donors. (B) As in (A), but macrophages were uninfected or infected with *narG*-deficient Mtb (NarG) or Wt or complemented (NarGc) strains. Results for uninfected macrophages and those infected with NarG Mtb overlap near zero. Means ± SD from one experiment representative of > 10. (C) Lack of accumulation of nitrite in nitrate-depleted medium. Macrophages were infected in the presence (*) or absence of 5 mM nitrate. Means ± SD for one experiment representative of 2. (D) As in (B), but with macrophages maintained under either 21% or 10% oxygen. Means ± SD for one experiment representative of 2. (E) Impact of rifampicin (RIF) on nitrite production. Where indicated, macrophages were infected with Wt Mtb (MOI: 5) for 3 days in 10% oxygen in the presence or absence of rifampicin (1 μg mL^{-1}). Rifampicin was added 2 days before, 24 hrs after or 48 hrs after infection. Uninfected macrophages were treated with rifampicin for the duration of the experiment. Nitrite was measured on day 3 after infection. Means ± SD, n= 2 experiments. (F) As in (B) but using ~20-fold higher numbers of macrophages grown on Cytodex surface microcarrier beads in 10% oxygen and then infected with Mtb (MOI: 5; 10^7 bacteria). Means ± SD, n= 2 experiments.

Figure 1 (continued)



Oxygen tension within Mtb-infected human macrophages functionally approximates 1%.

Abundant mycobacterial nitrate reduction within infected macrophages incubated under ambient oxygen tensions of 10% and 21% suggested that Mtb experienced an intracellular oxygen tension that approximated 1%. To test this, we compared the expression of *DOS* regulon members *hspX* and *fdxA* by Mtb cultured axenically or within infected macrophages. As reported (20), we found that Mtb cultured axenically in standard medium under 1% oxygen but not 21% oxygen induced both genes by ~100-fold (Figure 2A). Mtb residing within macrophages also highly induced the two *DOS* genes, particularly under 10% oxygen (Figure 2B) and less so under 21% oxygen (Figure 2C). To confirm this surprising finding, we treated Mtb-infected macrophages with pimonidazole. At oxygen tensions ≤ 1% pimonidazole, is reduced to a form that covalently modifies proteins, as detected by immunocytochemistry (30). Macrophages cultured at an oxygen tension of 10% showed intense pimonidazole staining, whether or not they were Mtb-infected (Figure 2D). Even under hyperoxic conditions (21% oxygen), considerable pimonidazole staining was detected (Figure 2D). Thus, despite incubation under ambient tensions of 10% or even 21%, intracellular oxygen tensions in the human macrophages functionally approximated 1%.

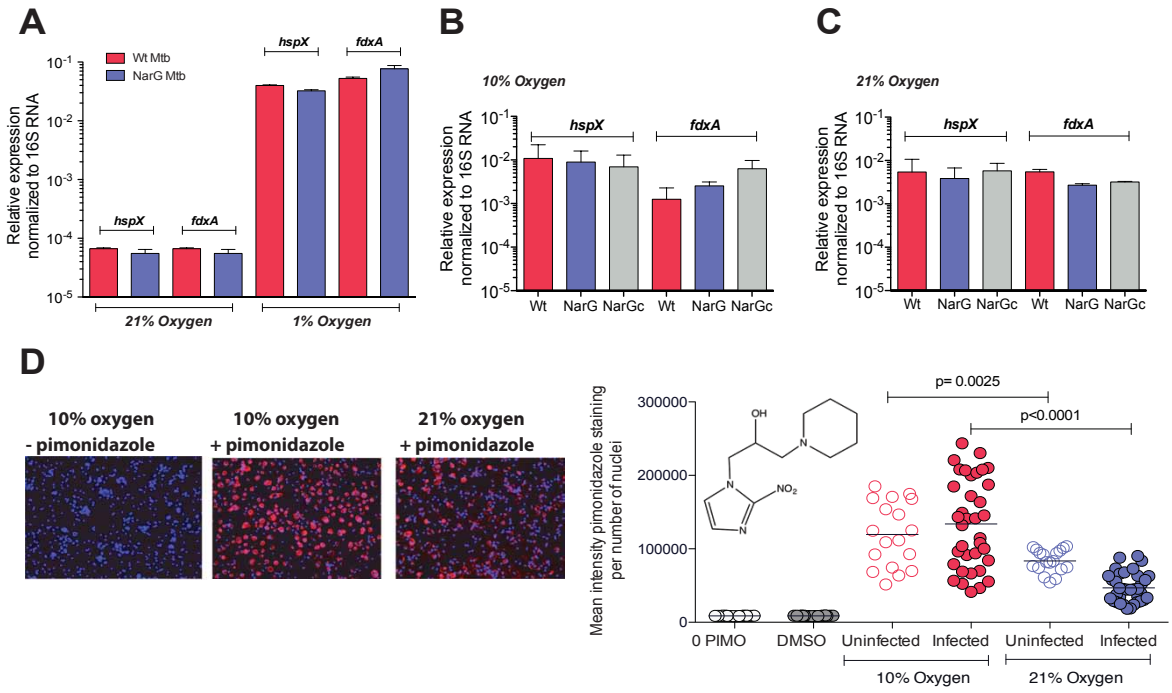


Figure 2. Functional evidence for intracellular hypoxia in primary human macrophages cultured at physiologic tissue-level (10%) or hyperoxic (room air) oxygen tensions. (A) Expression of DOS regulon genes *hspX* and *fdxA* in axenic cultures of Wt or *narG*-deficient (NarG) Mtb in 21% or 1% oxygen in standard medium without nitrate, OD_{580} 0.1 (means ± SD, n=2 experiments). (B, C) Expression of *hspX* and *fdxA* by Wt or NarG strains within human macrophages incubated in 21% or 10% oxygen for 10 hours (MOI: 40). Means ± SD, n=2 experiments. (D) Pimonidazole staining of macrophages infected or not with Wt Mtb (MOI: 5) and incubated at the oxygen tensions indicated. *Photomicrographs*: Representative images. Blue: DAPI- stained nuclei, Red: pimonidazole adducts. *Graph*: Quantification of pimonidazole staining intensity. 0 PIMO, uninfected cells treated with neither pimonidazole nor DMSO. DMSO, uninfected cells treated with only the vehicle. Each dot represents the average intensity in ~500 macrophages. Horizontal lines indicate the means of 2 independent experiments.

Endogenously generated nitrite represses mycobacterial growth and ATP consumption.

As noted by Wayne and Hayes (22), nitrite, whether endogenously produced or exogenously supplied, suppressed the growth of Mtb under 21% oxygen (Figure 3A). The concentration-dependence of this effect was similar for WT, *narG*-deficient and complemented Mtb (Figure 3A). In contrast, when we incubated the 3 strains in 1% oxygen for 3 days with 5 mM nitrate, followed by return to 21% oxygen, only the WT and complemented strains failed to resume growing, reflecting that they generated endogenous nitrite from nitrate, which *narG*-deficient Mtb could not (Figure 3B). The failure of cultures of WT Mtb to increase in turbidity after generating nitrite reflected bacteriostasis, not cell death, because the number of CFU recovered was unaffected (Figure 3C).

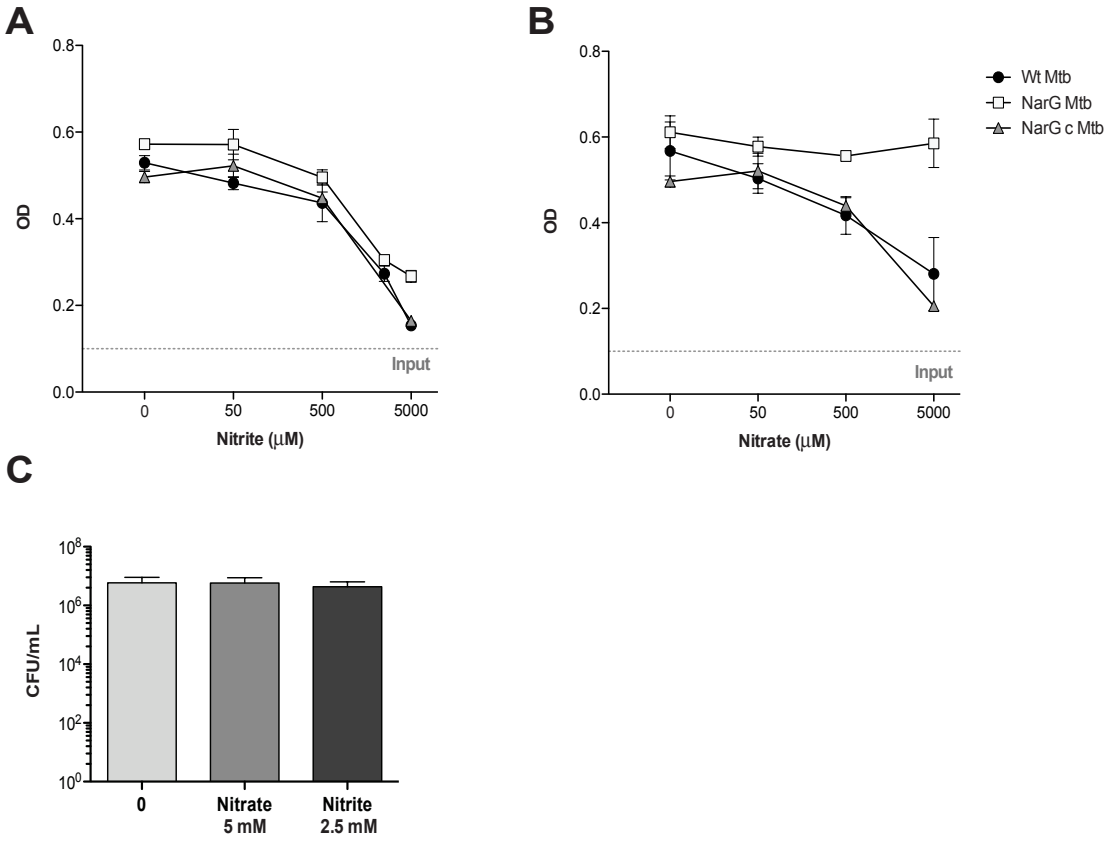


Figure 3. Nitrite represses mycobacterial growth. (A, B) Strains of Mtb designated as in Figure 1 (OD_{580} 0.1, as marked by horizontal dotted lines) were incubated in the presence of the indicated concentrations of nitrite (A) or nitrate (B) for 3 days in 1% oxygen and then transferred to 21% oxygen for 5 days before growth was assessed by measuring OD_{580}. Means ± SD, n=2 experiments. (C) As in (A) and (B), except that CFU were determined at the end of the 3-day incubation in 1% oxygen for wild type Mtb. Means ± SD, n=2 experiments.

It is not known how nitrite impairs aerobic mycobacterial growth. We suspected an impact of nitrate respiration on intrabacterial ATP, because nitrite has been shown to inhibit bacterial respiration and prevent ATP synthesis (31). To test this, we cultured WT, *narG*-deficient and

complemented strains of Mtb axenically for 3 days under 1% oxygen either at high density (OD: 0.1, ~ 5 x 10^7 cells mL^{-1}) or low density (OD: 0.01, ~ 5 x 10^6 cells mL^{-1}) in the presence of 5 mM nitrate and measured ATP in their lysates. As expected, in the low-density cultures the concentration of nitrite remained low. Under these conditions, ATP levels in WT and *narG*-deficient Mtb were not significantly different (Figure 4A). In contrast, high-density cultures of the WT and complemented strains contained significantly more ATP than the *narG*-deficient strain, confirming an observation of Tan et al. (25) (Figure 4A).

Tan et al. (25) attributed this result to increased ATP production by Mtb using nitrate as an electron acceptor when oxygen was limiting. However, when we added 2.5 mM exogenous nitrite to low-density cultures of Mtb in 1% oxygen, ATP levels were increased even farther than seen with WT Mtb given twice as much nitrate (Figure 4B). The ATP-elevating effect of exogenous nitrite was not dependent on the presence of *narG* or *nirBD*, which encodes a nitrite reductase (32). Thus, it appeared that nitrite, a product of nitrate respiration, led to accumulation of ATP in Mtb in a manner that did not depend on Mtb's donation of electrons to nitrate.

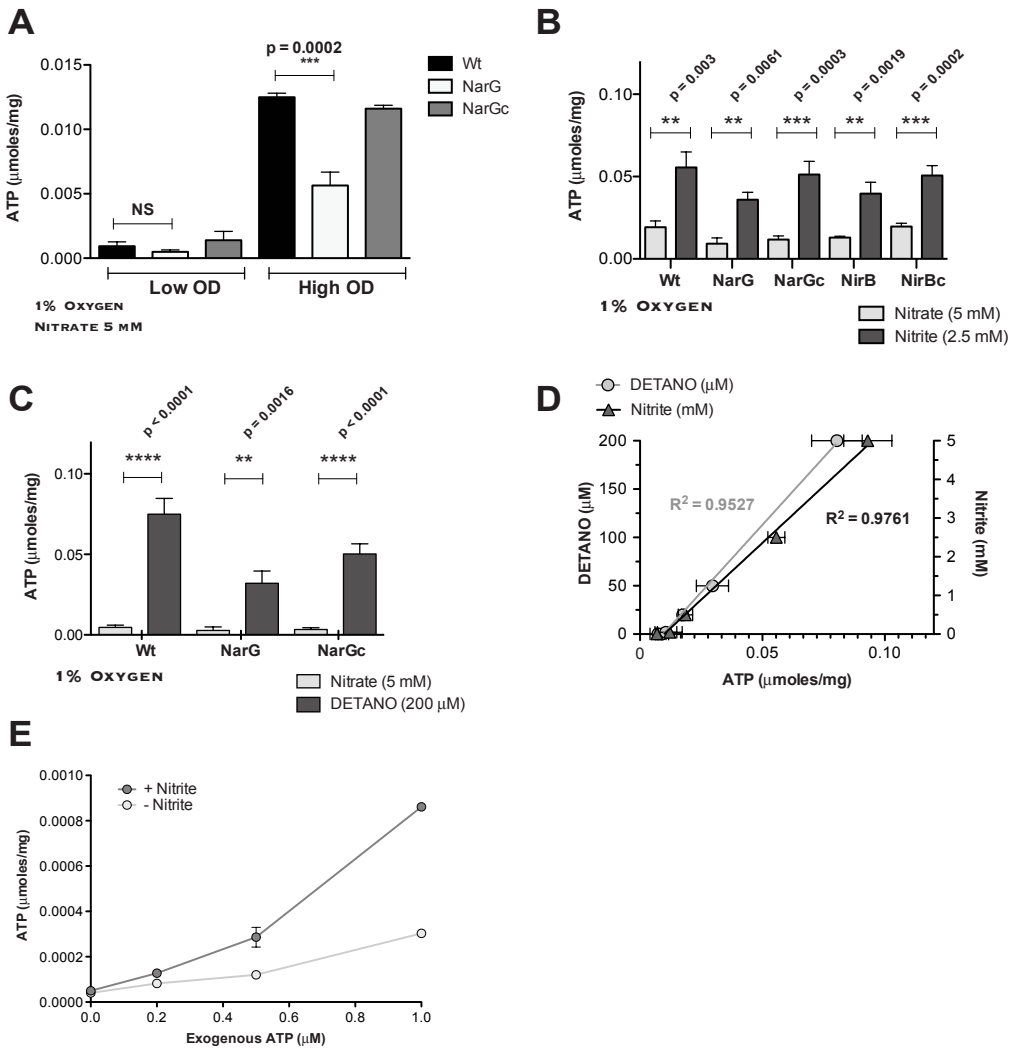
To test if nitrite itself was responsible for this effect, as opposed to nitric oxide, to which nitrite can be converted, we incubated low-density Mtb cultures with 5 mM nitrate as a control or with diethylenetriamine-NO adduct (DETANO), a compound that decomposes to release nitric oxide with a half-life of ~22 hours at pH 7.4. DETANO also elevated intrabacterial ATP levels in WT, *narG*-deficient and the complemented strains. Both nitrite and DETANO increased intrabacterial ATP in a

concentration-dependent manner. DETANO was ~25-fold more potent than nitrite (Figure 4C, D). Thus, either nitrite or nitric oxide potentially arising from it appeared to increase ATP.

We next considered whether nitrite or its possible conversion products might increase ATP production or decrease ATP consumption. Nitrite repressed the growth of Mtb; therefore, we considered it unlikely that nitrite enhanced the synthesis of ATP. Thus, we compared the consumption of exogenous ATP in lysates from nitrite-treated and untreated Mtb, after first demonstrating that neither nitrite nor nitric oxide interfered with the ATP assay (not shown). WT Mtb was incubated with or without nitrite as described above for 3 days in 1% oxygen. Whole cell lysates were prepared on ice, moved to room temperature and allowed to consume endogenous ATP until it was no longer detectable, which required less than 20 minutes. We then added various amounts of exogenous ATP to the lysates and immediately measured its concentration. We consistently recovered greater amounts of exogenous ATP in lysates from nitrite-treated Mtb than untreated Mtb (Figure 4E). Time-course measurements of the disappearance of exogenous ATP (Supplementary Figure S2, see Appendix 1) supported the interpretation that consumption of ATP was slower in lysates from nitrite-treated Mtb than from untreated Mtb.

Figure 4. Endogenously generated or exogenously supplied nitrite elevates ATP in Mtb and suppresses ATP consumption in its lysates. (A) Intrabacterial ATP content measured in wild type (Wt), *narG*-deficient (NarG) or complemented (NarGc) strains of Mtb incubated at high (0.1) or low OD_{580} (0.01) for 3 days in 1% oxygen in the presence of 5 mM nitrate. Results are expressed per mg protein in the lysate and are means ± SEM from 2 experiments.) (B) Impact of exogenous nitrite or nitrate on ATP content in Wt, *narG*-deficient (NarG), *nirB*-deficient (NirB) and respective complemented strains (NarGc, NirBc) of Mtb incubated for 3 days in 1% oxygen. (Means ± SEM, n=3 experiments). (C) As in (B), but comparing the impact of single concentrations of nitrate (5 mM) and DETANO (200 μM). Means ± SEM, n=3 experiments. (D) As in (C), but comparing the impact of varied concentrations of nitrate (y-axis on the right) and DETANO (y-axis on the left). Means ± SEM, n=2 experiments. (E) Recovery of exogenous ATP added to lysates from Wt Mtb that had been incubated in the presence or absence of 2.5 mM nitrite for 3 days at 1% oxygen. ATP was added only after cell lysates had consumed endogenous ATP. Means ± SEM, n=2 experiments. P values were determined by unpaired t tests.

Figure 4 (continued)



Endogenously generated nitrite regulates the Mtb transcriptome.

Having demonstrated an effect of nitrite on ATP metabolism, we characterized the impact of endogenously generated nitrite more broadly by examining its effect on Mtb gene expression as assessed by RNA Seq. Samples of Mtb were prepared following the same protocol that revealed that mycobacterial nitrate respiration repressed subsequent bacterial growth in air (Figure 3a-b). In 3 independent experiments, we treated axenic cultures of Mtb with or without 5 mM nitrate for 3 days at 1% oxygen to allow for the generation of endogenous nitrite and then transferred the cultures to 21% oxygen overnight. Figure 5 refers to these samples as "untreated" and "nitrate", respectively. Two further control cultures were prepared as above, except that one was treated with 2.5 mM nitrite in place of nitrate and the other was exposed neither to nitrate nor nitrite but its RNA was collected at the conclusion of the 3 day incubation in 1% oxygen, without an ~18 hour culture in 21% oxygen. Figure 5 refers to these two samples as "nitrite" and "untreated 1% oxygen," respectively. Ribosomal RNA was removed from the total RNA by oligonucleotide-based hybridization. Sequences determined in three separate sequencing runs were aligned to the Mtb H37Rv genome (Accession NC_000962.3). After culling sequences encoding ribosomal RNA, ~$3x10^6$ reads per sample were mapped to coding regions, covering ~99.9% of the coding genome. Gene expression was reported as reads per kilobase per million mapped reads (RPKM).

We found that 120 genes were significantly regulated by provision of nitrate. Those induced are listed in Supplementary Table S1 and those repressed are listed in Supplementary Table S2, see Appendix 1.

Inspection of the list suggested that the most informative comparisons would be with reports of Mtb's transcriptional adaptation to host-imposed stresses (33), such as hypoxia (20), iron restriction (34), acid (pH 5.5) (35), hydrogen peroxide (10 mM) (36), nitric oxide (50 mm) (21), and residence in mouse macrophages (35, 37)

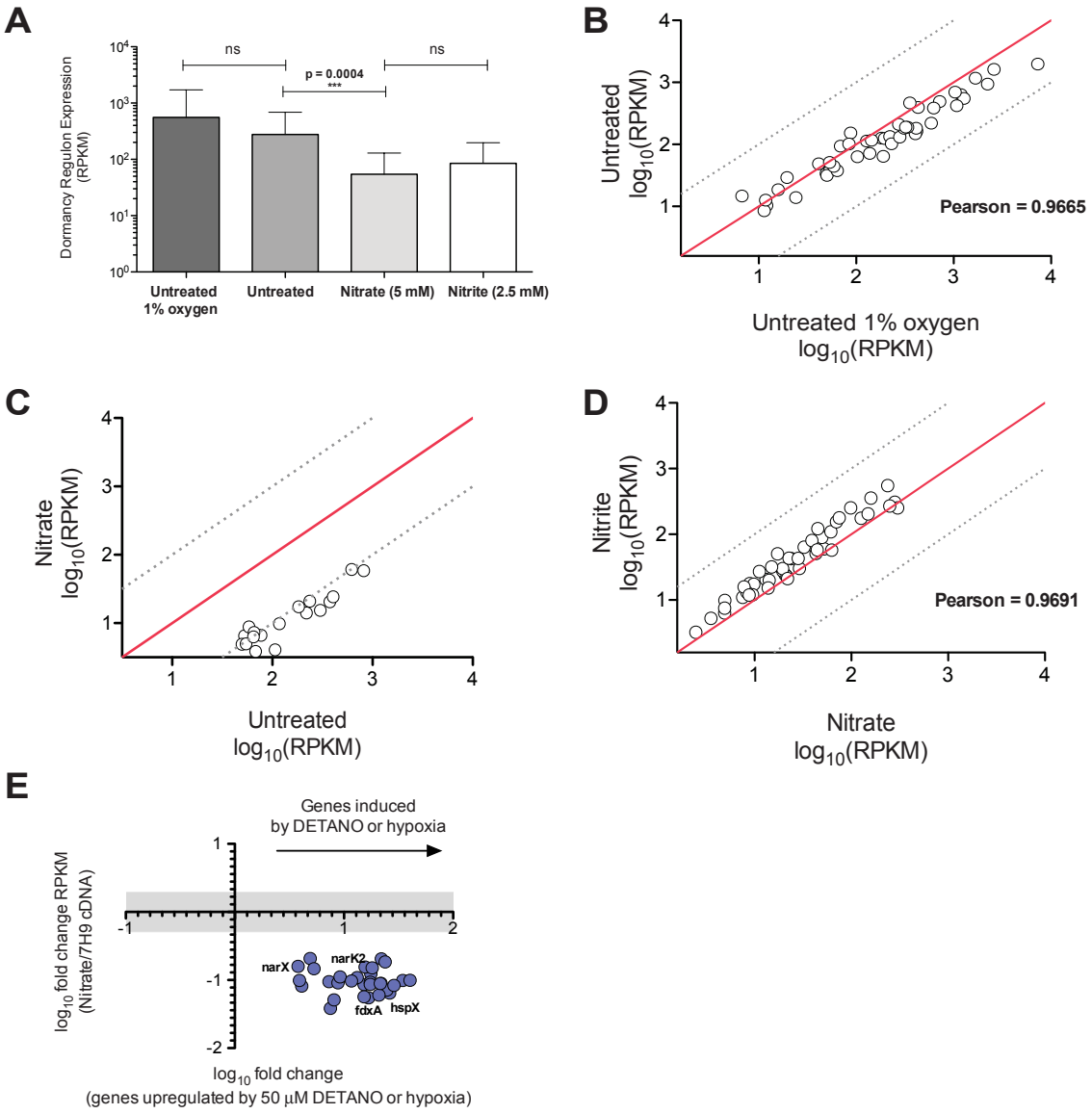
We first analyzed expression of the *DOS* regulon, which is induced by three of these conditions—hypoxia (20), nitric oxide (21), and residence in macrophages (35, 37). The average expression of all 47 *DOS* regulon genes was nearly the same for the "untreated" and "untreated 1% oxygen" samples (Figure 5A, first two columns). The close correspondence of *DOS* induction in these two conditions was also evident for each *DOS* gene considered individually (Figure 5B).

Surprisingly, in comparison to the untreated sample, the average expression of the *DOS* regulon was significantly repressed by provision of nitrate ($p < 0.0004$) (Figure 5A). Individually, 27 *DOS* genes were significantly repressed in nitrate-treated Mtb (Figure 5C). (To achieve statistical significance for all 47 *DOS* genes considered individually, we would likely need to sequence more samples.) This effect of nitrate could be attributed to generation of nitrite, because the effect was qualitatively and quantitatively the same when nitrite itself was provided (Figure 5D). Finally, the effect of nitrite could not be ascribed to conversion to nitric oxide, in that nitric oxide has the opposite effect: it induces the *DOS* regulon, rather than repressing it. This is shown in Figure 5E, which plots the present results for 28 *DOS* genes: 27 that were significantly repressed by nitrite and reported to be induced by hypoxia (20) and 28 that were significantly repressed by nitrite and reported to be induced by 50 mM

nitric oxide (21). Hypoxic cultures of Mtb have been reported to accumulate up to 2.5 mM nitrite and no more (22). These results offer an explanation: nitrite negatively regulates its own accumulation by limiting the expression of the DOS regulon and thus *narK2* (Supplementary Figure 3, see Appendix 1).

Figure 5. **Respiration of nitrate represses expression of the *DOS* regulon in Mtb.** (A-D) RPKM for *DOS* genes as determined by RNA Seq for Wt Mtb incubated in 1% oxygen for 3 days ("untreated 1% oxygen") or incubated in 1% oxygen for 3 days in the absence ("untreated") or presence of 5 mM nitrate ("nitrate") or 2.5 mM nitrite ("nitrite"), as indicated and transferred to 21% oxygen overnight. (A) The average expression of all 47 *DOS* genes across all samples (means ± SD) for the "untreated" and "nitrate" samples in 3 independent samples per condition, for the "untreated 1% oxygen " and "nitrite" samples (n=1 experiment per condition; see Methods). The p value was determined by an unpaired t test. (B) Gene-by-gene comparison of expression in the "untreated 1% oxygen" sample and the "untreated" sample. (C) Expression of the *DOS* genes that were significantly regulated by nitrate respiration compared to expression of each such gene in the untreated sample. (D) Expression of each *DOS* gene from Wt type Mtb treated with 5 mM nitrate as compared to a sample treated with 2.5 mM nitrite. (B-D) Red lines indicate 1:1 correlation; dashed lines indicate a 20% deviation from a 1:1 correlation. (E) Comparison of the expression of individual genes significantly regulated by nitrate in these experiments, with their reported regulation in hypoxia (20) or upon exposure to DETANO as a source of nitric oxide (21). The x-axis indicates the regulation of shared genes by the condition of interest and the y-axis indicates their regulation by nitrate respiration. All y values above or below the grey bar were regulated greater than 2-fold.

Figure 5 (continued)



Of the 120 genes that were significantly regulated by nitrate respiration, many overlapped with genes that were regulated by Mtb residing within activated mouse macrophages (35, 37) (Figure 6AB). In addition, Schnappinger *et al*. reported a subset of genes that were specifically regulated by iNOS. However, there was no correlation between regulation of genes by Mtb's respiration of nitrate and those regulated specifically by Mtb's residence in macrophages that expressed iNOS (37) (Figure 6A). This provided further evidence that the effect of endogenously generated nitrite was not solely mediated by conversion to nitric oxide.

Many Mtb genes regulated by nitrate respiration were reported to be similarly regulated in Mtb exposed to iron restriction (34) (Figure 6C), pH 5.5 (35) (Figure 6D), or treatment with 10 mM hydrogen peroxide (36) (Figure 6E). Table 1 presents the total number of genes regulated by nitrate respiration and reported to be regulated by these and other physiologic stimuli. After standardization to account for gene lists of differing size, the comparison indicates the number of genes expected to overlap by chance and whether the overlap is statistically significant. This analysis suggested that nitrate regulates the expression of genes that are also regulated when Mtb is exposed to several host-imposed stresses.

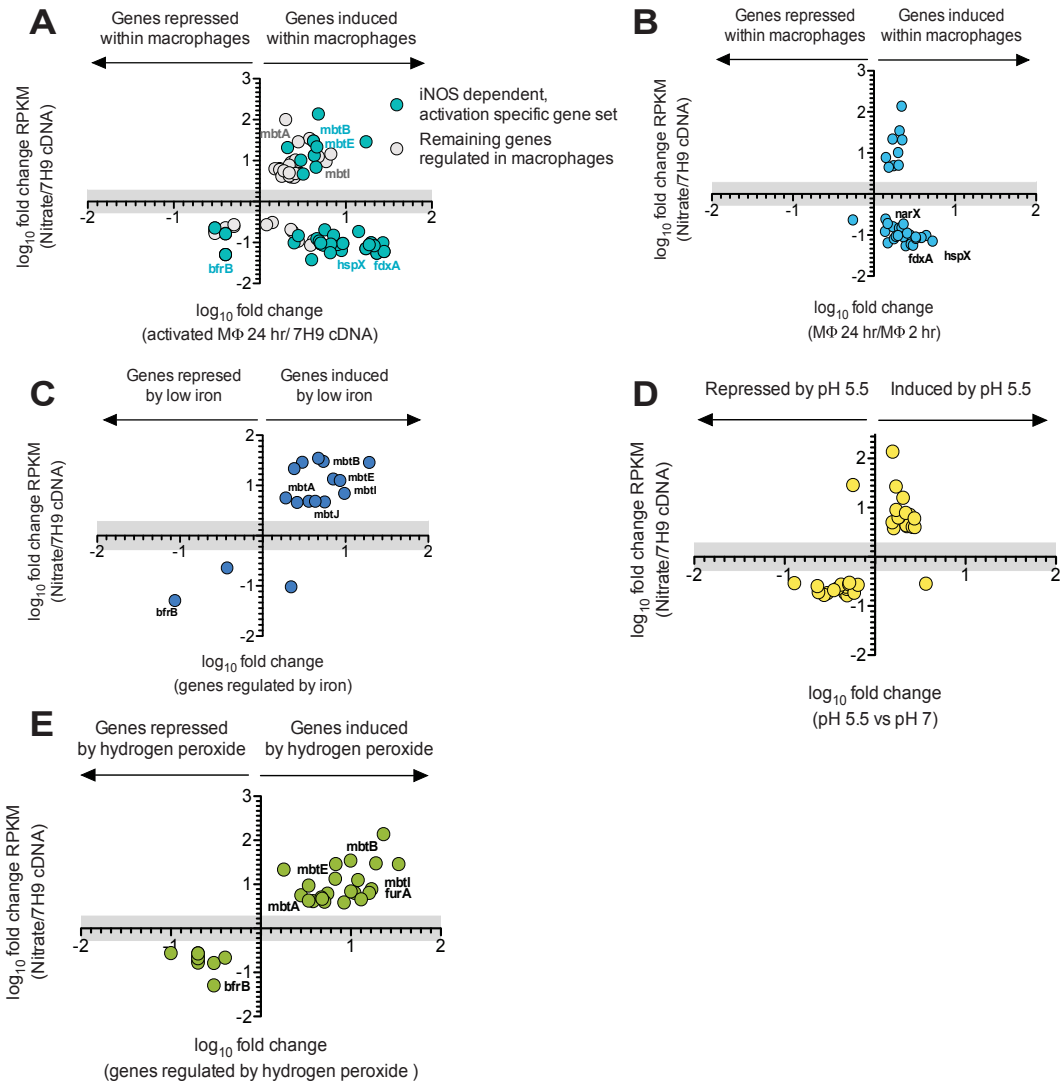


Figure 6. Comparison of the impact of nitrate respiration on the Mtb transcriptome with the impact of (A, B) residence within macrophages, (C) iron restriction, (D) exposure to pH 5.5, or (E) treatment with 10 mM hydrogen peroxide. Results of the present experiments and literature reports are graphed as in Figure 5E. The literature results were from the following studies. (A) (37). (B) (56). (C) (34) (D) (56). (E) reported by (36).

Table 1. Comparison of regulation of expression of Mtb genes by nitrate respiration observed here and by physiologic situations or stresses as reported in the literature. The total number of genes induced or repressed by nitrate respiration is listed along with the total number reported to be regulated by the indicated condition. The number regulated in common is compared to the number that would be expected to show a similar regulation by chance calculated by a Fisher's exact test. The columns labeled "number induced/repressed by nitrate respiration" refer only to the number of overlapping genes. "Percent of total gene list that overlaps" indicates the percent of all genes regulated by nitrate respiration that are also regulated by the condition of interest. The associated significance of this overlap, listed as a p value in the final column, was calculated using a Fisher's exact test. Of note, genes regulated by mitomycin C-induced DNA damage and starvation did not significantly overlap with genes regulated by nitrate respiration.

Table 1 (continued)

Condition	Regulation	Genes regulated by the indicated condition	Total number that overlap (by chance)		Number induced by nitrate respiration	Number repressed by nitrate respiration	Percent of total gene list that overlaps	Significance of the overlap, p value
Iron depletion[1] (Low iron culture: 2 μM $FeCl_3$ vs High iron culture: 50 μM $FeCl_3$, H37Rv)	Induced	66	14	(2)	13	1	11.67	4.17E-08
	Repressed	27	2	(1)	0	2	1.67	2.05E-01
Hypoxia[2] (2 hrs at 0.2% oxygen, H37Rv)	Induced	47	27	(1)	0	27	22.50	< 2.2e-16
Enduring Hypoxic Response[3] (Incubation at 0.2% oxygen over 4 days, H37Rv)	Induced	230	17	(7)	12	5	14.17	6.53E-04
Nitric oxide[4] (50 μM DETA/NO for 40 minutes, H37Rv)	Induced	48	28	(1)	0	28	23.33	< 2.2e-16
Hydrogen peroxide[5] (10 mM, 40 minutes, clinical isolate 1254)	Induced	166	22	(5)	22	0	18.33	1.11E-08
	Repressed	48	10	(1)	10	0	8.33	4.65E-06
pH 5.5[6] (pH 5.5 vs pH 7, H37Rv)	Induced	261	16	(8)	15	1	13.33	5.72E-03
	Repressed	375	18	(11)	1	17	15.00	3.27E-02
Intraphagosomal[7] (24 hrs, H37Rv)	Induced	454	65	(14)	35	30	54.17	< 2.2e-16
	Induced, iNOS dependent	60	32	(2)	9	23	26.67	< 2.2e-16
	Repressed	147	11	(4)	0	11	9.17	5.63E-03
	Repressed, iNOS dependent	8	3	(1)	0	3	2.50	3.31E-03
Intraphagosomal[8] (24 hrs, CDC 1551)	Induced	266	32	(8)	9	23	26.67	2.39E-11
	Repressed	111	1	(3)	0	1	0.83	9.65E-01
DNA damage[9] (Induced by mitomycin C, H37Rv)	Induced	113	1	(3)	1	0	0.83	9.67E-01
	Repressed	26	2	(1)	2	0	1.67	1.94E-01
Starvation[10] (24 hrs after growth in PBS, H37Rv)	Induced	252	6	(8)	6	0	5.00	7.69E-01
	Repressed	301	12	(9)	6	6	10.00	1.94E-01
Nitrate respiration (Genes regulated by nitrate respiration and no other physiologic stimuli reported above)	-	-	-	-	6	5	10.83	-

[1] Rodriguez 2002
[2] Sherman 2001
[3] Rustad 2008
[4] Voskuil 2003
[5] Voskuil 2011
[6] Rohde 2007
[7] Schnappinger 2003
[8] Rohde 2007
[9] Rand 2003
[10] Beste 2007

Discussion

Within the host, both host NOSs and mycobacterial nitrate reductase can serve as sources of nitrite. However, while mycobacterial nitrate reduction is markedly induced by hypoxia (17), NOSs require oxygen as a substrate (38), and at oxygen tensions of 1%, iNOS loses 80-90% of its activity (39). Therefore, the activities of host iNOS and mycobacterial nitrate reductase could be spatially and temporally segregated, depending on their access to molecular oxygen.

The studies reported here led to four inter-related findings. First, there can be a striking dissociation between the oxygen tension in culture and the functional oxygen concentration in macrophage phagosomes. Although a dissociation was previously noted between oxygen concentration in extracellular fluid and in mouse macrophage phagosomes (40), to our knowledge this is the first report that the level of oxygen in the phagosomes of primary human macrophages cultured at physiologic tissue oxygen levels of 10% or even 21% approximates 1% functionally, as reflected by Mtb's induction of *DOS* genes and copious reduction of nitrate. The low functional level of intramacrophage oxygen was not dependent on ingestion of Mtb. In agreement with James *et al*. (29, 40), we speculate that the steep gradient between atmospheric and intracellular oxygen reflects a rate of mitochondrial oxygen consumption that outpaces diffusion from the gas phase through the extracellular fluid and across the plasma membrane. If Mtb likewise experiences functional hypoxia within human macrophages at physiologic oxygen tensions *in vivo*, then Mtb's respiration of nitrate to nitrite is likely to be much more widespread than previously appreciated, and the convention of culturing Mtb in room air

levels of oxygen in the absence of nitrate is likely to be misleading with regard to Mtb's physiology.

Second, nitrite, whether endogenously produced or exogenously supplied, affects Mtb profoundly, although we may have only scratched the surface in characterizing its effects. Besides halting Mtb's growth, impairing its consumption of ATP and altering its transcriptome, mycobacterial nitrite also markedly reduces Mtb's susceptibility to isoniazid (Cunningham-Bussel, *MicrobiologyOpen*, in press). We do not know the major ATP-consuming pathway(s) in Mtb that function(s) in whole cell lysates and that nitrite inhibits. This contrasts with the reported effect of nitrite to reduce the amount of intrabacterial ATP in *P. aeruginosa* (31, 41).

Third, the effects of nitrite and nitric oxide partially overlap but in some respects are diametrically opposed. The effect of nitrite is also distinct from the effect of the respiration of nitrate, from which the nitrite arises. The ability of nitrite to affect biologic systems in its own right, rather than through conversion to more reactive forms, has been proposed (42, 43), and is supported by these results. For example, nitrite can oxidize ferrous iron to the ferric state and displace iron from iron-sulfur clusters (44, 45). The induced expression of mycobactin synthase genes suggests that Mtb-derived nitrite inactivated iron-containing enzymes, rendering the Mtb functionally iron-deficient despite the high iron content of the medium. *DOS* regulon expression is coordinated by the heme-containing kinases DosS and DosT, which function as redox and hypoxia sensors, respectively. Hypoxia, carbon monoxide and nitric oxide are physiologic ligands that induce DOS regulon expression by modulating

the oxidation and ligation states of the iron atoms in DosS and DosT (46). Nitrite may repress *DOS* regulon expression by oxidizing the iron of these sensor kinases in a manner that prevents their regulation by other physiologic ligands. Oxidation of iron in additional enzymes, such as ribonucleotide reductase, may also contribute to the ability of nitrite to slow or halt Mtb's growth (47-49).

Last, the shared regulation of genes by nitrate and by the potentially mycobactericidal conditions of iron restriction and exposure to acid and hydrogen peroxide suggests that nitrate respiration may benefit Mtb *in vivo* by preparing it to withstand the latter stresses. Nitrite is highly diffusible, as evidenced by its accumulation in the plasma of Mtb-infected individuals (50-52). Endogenously produced nitrite may diffuse within infected tissue and contribute to a pre-emptive induction of Mtb genes that benefit Mtb's survival in a hostile host environment.

To date, *in vivo* experiments to investigate the consequence of mycobacterial nitrate respiration have been limited to the mouse, where "granulomas" are areas of pneumonitic consolidation lacking the layered, fibrous-capped structure found in the granulomas of humans and non-human primates with TB. Mtb-infected granulomatous tissue in the mouse had a measured oxygen tension averaging ~40 mm Hg (or 2-9% oxygen) (15, 53) and did not stain with a hypoxia-sensitive 2-nitroimidazole probe (20, 54). Furthermore, Mtb in mice did not express the only DOS regulon member tested, *narK2,* until the onset of the adaptive immune response almost three weeks after infection, such that DOS regulon expression may have depended upon the activation of iNOS (55) rather than hypoxia. When mouse macrophages cultured in 21% oxygen were infected with

Mtb *in vitro, DOS* regulon induction depended on expression of iNOS (21, 37). In contrast, in the present studies, exposure of human macrophages to Mtb over an interval long enough to allow for phagocytosis but too brief to allow for nitrite accumulation led to marked induction of the *DOS* regulon in the absence of any NOS. Therefore, to the extent that *DOS* regulon induction is an indicator of hypoxia, human macrophages as cultured here appear to achieve a greater degree of functional intracellular hypoxia *in vitro* than mouse macrophages as usually cultured. The same may be true *in vivo*, insofar as iNOS expressed in mouse macrophages during Mtb infection appears to access sufficient oxygen to restrain Mtb growth (3, 37). Therefore, experiments with NarG-deficient Mtb in the mouse may not reflect the functional relevance of mycobacterial nitrate respiration in the human host.

RNS can act as double-edged swords, depending on their level (56, 57). The present results bring this truism into sharp relief with respect to Mtb. High levels of RNS can kill Mtb, but low levels may do the pathogen a service. First, nitrite of host or mycobacterial origin may induce genes with whose help Mtb can withstand other host stresses. Second, nitrite slows or stops Mtb's aerobic replication. Mtb may thus avoid killing a host that has not yet destroyed enough lung tissue to provide Mtb with a path back to the air. Third, nitrite increases intrabacterial ATP. Once Mtb is expelled from the host within aerosolized droplets, this reserve of metabolic energy may facilitate colonization of new hosts. Drugs that inhibit Mtb's defenses against RNS may therefore be useful even in settings where immunodeficiency impairs the expression of host iNOS or hypoxia limits its action.

Methods

Isolation and differentiation of primary human monocytes

Heparinized peripheral blood was collected by venepuncture from healthy human donors who provided informed consent under an IRB approved protocol. Monocytes were isolated and differentiated as described (29, 34). Briefly, mononuclear cells isolated by centrifugation over Ficoll-Paque (GE Healthcare) were subject to positive selection using magnetic beads coupled to anti-CD14 antibodies (Milteyi Biotec) and cultured in 60% RPMI, supplemented with 1% glutamax, 40% human plasma and GM-CSF and TNFα (0.5 ng ml^{-1} each) at $5x10^5$ cells ml^{-1}. 30% of the total culture volume was replaced with fresh medium and cytokines twice a week. For Cytodex1 bead culture, Cytodex 1 beads were prepared according to the instructions provided by the manufacturer (GE Healthcare). The cells were plated at a density of $2x10^6$ cells ml^{-1} per well of a 24 well plate and 50% of the medium was replaced three times a week. After 2 weeks at 10% or 21% O_2 and 5% CO_2 at 37°C in a humidified atmosphere, the cells were activated with IFNγ (5 ng ml^{-1}) and infected with Mtb the following day. No antibiotics were used at any point.

To remove most nitrate from the medium, 1 day prior to infection the cells were washed 3 times with PBS and the medium was replaced with DMEM with 10% human plasma, GM-CSF, 0.5 ng mL^{-1} TNFα and 5 ng mL^{-1} IFNγ.

Measurement of nitrite

Nitrite levels in the supernatants of macrophage cultures were measured by the Griess assay (41, 56) using nitrite standards prepared in the same medium used to culture the macrophages.

Preparation of Mtb

M. tuberculosis H37Rv was grown in Middlebrook 7H9 broth supplemented with 0.2% glycerol, 0.5% BSA, 0.2% dextrose and 0.085% NaCl (ADNaCl) with 0.05% Tween 80. The *narG, nirB* and complemented strains (36, 42) were generously provided by Dr. F. Bange. Single-cell suspensions were collected in the supernatant after centrifugation at 120 G for 10 minutes. For macrophage infection, ~$2x10^7$ bacteria were pelleted by centrifugation and resuspended in PBS. CFUs were determined by serial dilution in 0.1% TritonX-100, and plating on Middlebrook 7H11 agar with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco, BD) supplemented with 0.5% glycerol for 2-3 weeks at 37°C, 5% CO_2.

Pimonidazole staining

Uninfected or Mtb-infected (MOI: 5) macrophages were treated with or without 200 μM pimonidazole for 24 hours, washed and stained anti-pimonidazole antibody coupled to allophycocyanin (APC) (Hypoxyprobe RedAPC Kit) as recommended by the manufacturer and counterstained with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI). APC and DAPI signals were quantified at the Cell Screening Core Facility, Weill Cornell Medical College and analyzed using MetaXpress High Content

Image Acquisition & Analysis Software (Molecular Devices). The APC signal was normalized to the number of nuclei per image.

Intrabacterial ATP

Single-cell suspensions of Mtb ($7x10^8$ per condition) were incubated in 7H9/ADNaCl with 0.05% Tween 80 at an OD_{580} of 0.07 in 1% O_2, 5% CO_2 at 37 °C in a humidified atmosphere, pelleted by centrifugation, washed once in PBS, pelleted and processed immediately for ATP measurement or frozen at -80°C for later use. For a single endpoint measurement, the pellet was resuspended in 220 µl of standard lysis buffer: 100 mM Tris-OH and 1 mM PMSF, pH 7.4, with 1x protease inhibitor (Roche: cOmplete ULTRA Tablets, Mini, EDTA-free, EASY pack catalogue number: 05892791001) added immediately prior to bacterial lysis in a bead-beating homogenizer with silica beads for 2 cycles with incubation on ice between them. After brief centrifugation to pellet silica beads, 1:1:2 (v/v/v) ratio of cell lysate (50 µL), lysis buffer and ATP reagent were added in triplicate to a black 96-well opaque plate for the BacTiter-Glo Microbial Cell Viability Assay (Promega). Luminescence was read immediately using a SpectraMax L Luminescence Microplate Reader (Molecular Devices). For rate measurements, the lysate was filter-sterilized (0.22 µm Corning Costar Spin-X Plastic Centrifuge Tube Filters at 10,000G for 20-30 minutes). ATP was measured in an aliquot and reagent ATP was added to the remaining lysate from which triplicate 10 µL aliquots were immediately dispensed to the assay plate. At the indicated times, 90 µL of lysis buffer and 100 µL of ATP reagent were added and luminescence measured. ATP

standards diluted in lysis buffer were prepared fresh for each experiment. The intrabacterial ATP for each sample was normalized to the protein concentration of each sample (Bio-Rad Protein Assay).

Quantitative RT-PCR

Macrophages ($5x10^6$ per T25 flask) were cultured in 10% or 21% oxygen and infected with Mtb (MOI: 40) for 10 hours. The monolayer was washed twice with PBS. Trizol (2 mL) was added to each flask and the cells detached and using a rubber scraper. Alternatively, $\geq 2x10^9$ bacteria were incubated per condition in 1% or 21% oxygen for 3 days in standard medium, in the absence of nitrate. An equal volume of buffer containing 5 M guanidinium thiocyanate, 25 mM sodium citrate, 20 mM N-lauryl-sarcosine, 0.7% v/v β-mercaptoethanol was added. The bacteria were pelleted by centrifugation, 1 mL of Trizol added per sample and the suspension beaten with silica beads. RNA extraction was conducted using an RNeasy kit (Qiagen) in accordance with the manufacturer's instructions, except that off-column DNAse digestion was performed for 2 hrs at 37 °C. One mg of total RNA was reverse-transcribed using the GeneAmp RNA PCR Kit (Applied Biosystems). Quantitative RT-PCR was performed using gene specific primers (TaqMan Gene Expression Assay, Life Technologies) and the SuperScript III Platinum Two Step qRT-PCR Kit (Life Technologies) with a 7900HT Fast Real Time PCR System (Applied Biosystems). Each experiment was performed in triplicate. Values within the linear range of the primers were normalized to values for 16S rRNA.

RNA Sequencing

At least $5x10^9$ bacteria per sample were incubated in 1% oxygen and left untreated or treated with 5 mM nitrate or 2.5 mM nitrite. After 3 days RNA was collected from one flask of untreated Mtb. The remaining flasks (untreated or nitrate- or nitrite- treated) were transferred to 21% oxygen for an additional overnight incubation before RNA collection. RNA samples with an RNA Integrity Number (RIN) (Bioanalyzer (Agilent Technologies 2100) value > 8 were processed further. Ribosomal RNA was removed by hybridization with magnetic bead-coupled oligonucleotides (MicrobExpress Kit, Life Technologies). Libraries were prepared according to the manufacturer's instructions and resultant RNA sequenced (HiSeq2000/1000, Illumina). Single-ended sequencing reads were aligned to the reference genome of Mtb H37Rv using the Burrows-Wheeler Alignment tool (20, 50) (Accession NC_000962.3). Cufflinks (53) was used to measure transcript abundances in reads per kilobase of exon per million mapped reads (RPKM) as well as to find differentially expressed genes. Regulation was considered significant for changes with a p value corrected for multiple comparisons (p_{corr}) < 0.05 (Supplementary Tables 1 and 2, see Appendix 1). The samples labeled "nitrate" and "untreated," were prepared in 3 independent experiments. Only one sample was available for the "nitrite" and "untreated 1% oxygen" conditions. These samples were only used to evaluate the expression all 47 genes of the *DOS* regulon as a unit, with the expression of each gene serving as a control for the expression of the others.

Statistical analysis

Statistical analysis was performed as indicated in the figure legends using Prism 5.0f for Macintosh (GraphPad Software, San Diego, CA).

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CHAPTER 3

NITRITE IMPACTS THE SURVIVAL OF *MYCOBACTERIUM TUBERCULOSIS* IN RESPONSE TO ISONIAZID AND HYDROGEN PEROXIDE [*]

Summary

When access to molecular oxygen is restricted, *Mycobacterium tuberculosis* (Mtb) can respire an alternative electron acceptor, nitrate. We found that Mtb within infected primary human macrophages *in vitro* at physiologic tissue oxygen tensions respired nitrate, generating copious nitrite. A strain of Mtb lacking a functioning nitrate reductase was more susceptible than wild type Mtb to treatment with isoniazid during infection of macrophages. Likewise, nitrate reductase-deficient Mtb was more susceptible to isoniazid than wild type Mtb in axenic culture, and more resistant to hydrogen peroxide. These phenotypes were reversed by the addition of exogenous nitrite. Further investigation suggested that nitrite might inhibit the bacterial catalase. To the extent that Mtb itself is the most relevant source of nitrite acting within Mtb, these findings suggest that inhibitors of Mtb's nitrate transporter or nitrate reductase could enhance the efficacy of isoniazid.

Introduction

Nitrate (NO_3^-) and nitrite (NO_2^-) arise in the mammalian host from three major sources—dietary, bacterial and host. Dietary nitrate is absorbed into the blood. Some is reduced by oral and gastrointestinal

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commensals, producing nitrite, which is also absorbed. Nitric oxide synthases (NOSs), including those that are constitutively expressed, produce nitric oxide, which autoxidizes to nitrate and nitrite. Even in healthy individuals, in whom relatively little of the high-output NOS isoform (iNOS) is expressed (1), nitrate is present in plasma at levels of 20-40 μM (2). In patients with tuberculosis (TB), iNOS and other NOSs are expressed in granulomas (1, 3).

At oxygen tensions of 1% or lower, Mtb itself can produce nitrite as it maintains ATP synthesis and redox homeostasis by reducing an alternative electron acceptor, nitrate. Mtb constitutively expresses the *narGHJI* operon, which encodes Mtb's only functional nitrate reductase (4, 5). However, transport of nitrate into the bacterium depends on transcription of a nitrate transporter encoded by *narK2*, a member of the dormancy survival (*DOS*) regulon (6-8). The *narK2* gene is expressed in axenically cultured Mtb only when oxygen levels fall below 1%. Induction of *narK2* markedly enhances mycobacterial nitrate respiration (4). RNA transcripts from *narG,* which encodes a subunit of the nitrate reductase, and *narX,* an inactive fused nitrate reductase downstream of *narK2* in a shared operon, have been detected in lungs of patients with TB (4, 9, 10), and RNA transcripts encoding the nitrate transporter, *narK2*, were elevated in the lungs of Mtb infected mice (11). Thus, mycobacterial nitrate reduction likely occurs in the infected host.

Our laboratory recently converted our culture system for primary human macrophages from conventional gas-phase conditions of 21% O_2, 5% CO_2 to 10% O_2, 5% CO_2 on the grounds that 10% O_2 is physiologic for tissue macrophages (12). To our surprise, infection of the

macrophages *in vitro* with wild type Mtb under 10% oxygen resulted in extensive accumulation of nitrite in the supernatant. Evidence will be presented elsewhere that macrophages were not the source of nitrite in this system. In contrast, as shown below, Mtb's NarG was the source, despite the non-hypoxic level of oxygen in the gas phase.

Wayne has proposed that nitrate respiration supports the establishment of a non-replicating persistent state (NRP) in response to hypoxia (13). We hypothesized that Mtb lacking the capacity for nitrate respiration might demonstrate an impaired entrance into NRP and therefore remain more susceptible to drugs that target replicating Mtb. The goal of the present study was to assess the contribution of nitrate respiration to Mtb's sensitivity to the drugs isoniazid (INH), ethambutol, streptomycin and rifampicin. Compared to wild type Mtb, *narG*-deficient Mtb, which is unable to produce nitrite, was selectively hyper-susceptible to INH, as well as hyper-resistant to hydrogen peroxide. INH requires oxidative activation by the mycobacterial catalase/peroxidase KatG in order become cidal. The resultant isonicotinoyl radical adducts with cellular pyridine nucleotides and potently inhibits InhA, a member of type II fatty acid synthesis pathway involved in cell wall mycolic acid biosynthesis (14, 15). These findings imply that the action of INH on Mtb may be blunted by nitrite contributed by the host, the pathogen or both.

Results

Nitrate respiration enhanced mycobacterial resistance to isoniazid within primary human macrophages.

We observed no difference in survival of wild type (Wt Mtb) and

narG-deficient Mtb (NarG Mtb) during infection of primary human macrophages under 10% O_2 and 5% CO_2 (Figure 1a). During the course of infection, large quantities of nitrite arose in the supernatant of macrophages infected with wild type Mtb, which was not observed in the supernatants of macrophages infected with *narG*-deficient Mtb (Figure 1b). To test the role of nitrate reduction on the susceptibility of Mtb to antibiotics, we infected macrophages with wild type, *narG*-deficient and complemented strains (NarGc Mtb) and treated the cultures with drugs. Mtb deficient in *narG* was hyper-susceptible to INH, with a 10-fold decrease in the MBC99 compared to wild type (Figure 1c-d). In contrast, *narG* made no observable difference to the antibacterial actions of streptomycin, rifampicin or ethambutol on Mtb within macrophages (Supplementary Figure 1a-f, see Appendix 2).

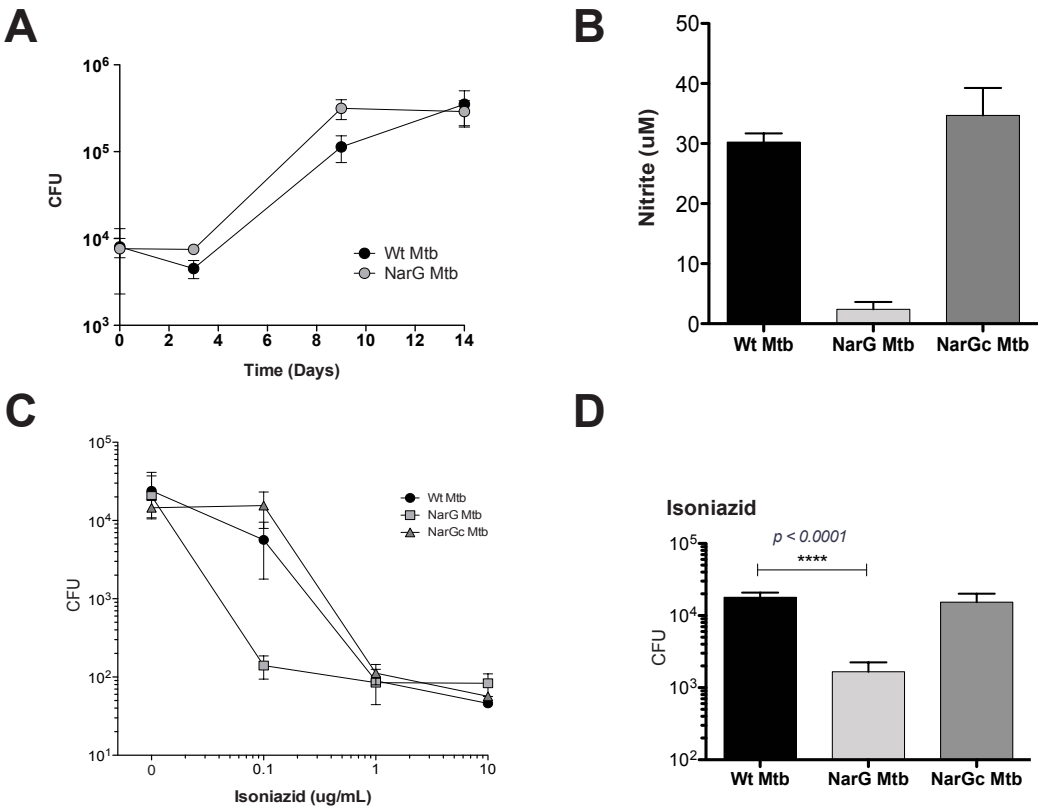


Figure 1. Effect of nitrate respiration on mycobacterial resistance to isoniazid within primary human macrophages. (A) Survival of wild type and *narG*-deficient (NarG) Mtb within macrophages (MOI: 0.1, corresponding to 10,000 bacteria). (B) Nitrite levels arising in the supernatant of macrophages 3 days after infection with wild type Mtb (Wt Mtb), NarG Mtb and the complemented strain (NarGc Mtb) (MOI: 5). (C) Susceptibility of Mtb to INH within infected macrophages (MOI: 0.1) over 3 days. Means ±SEM from one experiment representative of 3 independent experiments using cells from different donors. (D) As in (C), pooling results from the 3 donors (means ± SEM) at a single INH concentration administered to the culture for 3 days (0.1 μg mL^{-1}). The p value was determined by an unpaired t test.

Addition of nitrite reversed the hyper-susceptibility of narG-deficient Mtb to isoniazid.

The hyper-susceptibility of *narG*-deficient Mtb to INH may have resulted from loss of the metabolic energy generated by nitrate reduction or from failure to produce nitrite. To distinguish between these possibilities, we added exogenous nitrite to the medium of infected macrophage cultures treated with INH. Because the standard culture medium consisted of 40% human plasma and 60% RPMI 1640, it contained high concentrations of nitrate. Therefore, to limit the accumulation of nitrite produced by wild type Mtb, we replaced the medium immediately prior to infection with a low-nitrate medium (to minimize perturbation we refrained from washing the cells thoroughly; thus, medium exchange was not complete). Addition of exogenous nitrite enhanced bacterial survival in the presence of INH in a dose-dependent manner (Figure 2a-c). That this effect was less striking in the case of wild type Mtb was likely due to residual nitrate in the medium, allowing for low-level nitrite production (Figure 2a).

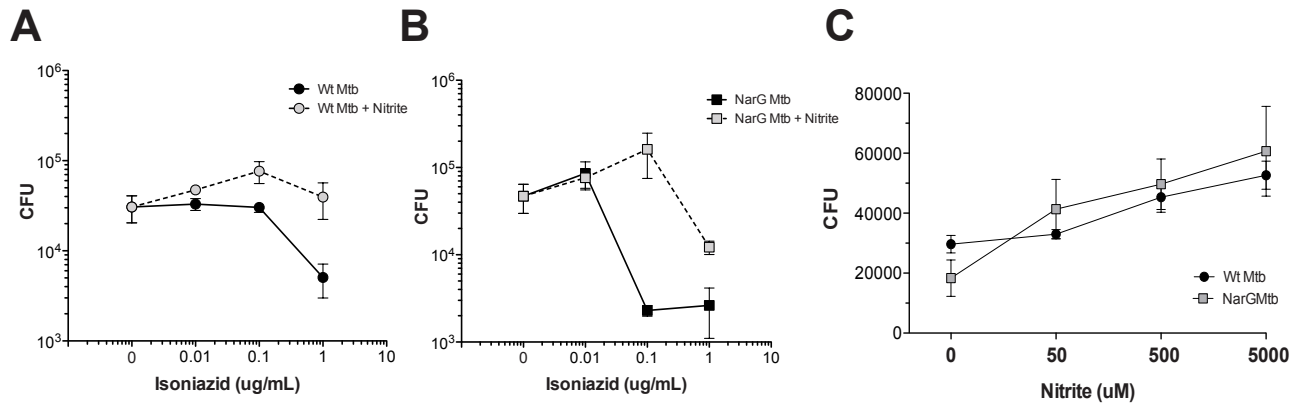


Figure 2. Impact of exogenous nitrite on the hyper-susceptiblity of narG-deficient Mtb to isoniazid. (A, B) The effect of nitrite on the survival of (A) wild type or (B) *narG*-deficient (NarG) Mtb within INH-treated infected macrophages (MOI: 0.1). The standard culture medium was replaced with low-nitrate medium. Where indicated by the dashed lines, exogenous sodium nitrite (final concentration 1 mM) was added to the culture medium along with the indicated concentrations of INH. CFU were harvested 3 days later. (C) Survival of wild type and *narG*-deficient (NarG) Mtb within macrophages treated with increasing concentrations of nitrite in the presence of a single concentration of INH (0.1 μg mL^{-1}). The standard culture medium was replaced with low-nitrate medium and exogenous sodium nitrite was added at the indicated final concentrations. Each result is representative of at least two independent experiments (means ± SEM).

Mtb deficient in narG Mtb was hyper-susceptible to isoniazid in axenic culture.

Mtb respires nitrate when infecting human macrophages under 10% oxygen, but in axenic culture, as noted, nitrate respiration is barely detectable until oxygen is lowered to 1% or less. This implied that within macrophage phagosomes, the functional level of oxygen perceived by

Mtb was far lower than the level present in the gas phase. The nitrate reductase assay is routinely used in clinical laboratories to identify drug resistant Mtb (16). Although the assay is carried out under room air (21% oxygen), nitrate reductase activity is measured at the end of a prolonged incubation period, which may indicate that the medium in which Mtb is incubated becomes hypoxic. Alternatively, while aerobic cultures of Mtb do not express the nitrate transporter, *narK2*, nitrate may gradually diffuse across the plasma membrane and be reduced to nitrite. In order to test the role of nitrate respiration in Mtb's susceptibility to INH in the absence of macrophages, we cultured wild type and *narG*-deficient Mtb in 21% oxygen or 1% oxygen. No measurable survival differences were observed for the two strains in room air in response to isoniazid, streptomycin, or rifampicin (Figure 3a-c). Under 1% oxygen, however, the survival of *narG*-deficient INH-treated Mtb dropped by 2-3 $\log_{10}$ as compared to wild type and complemented strains (Figure 3d), which was due to the induction of the nitrate reductase system. Next, we examined survival as a function of time rather than concentration. A single application of INH led to a drop of 2 $\log_{10}$ in CFU of the *narG*-deficient strain over 9 days, while the CFU of the wild type and complemented strains remained constant (Figure 3e). Exogenous nitrite enhanced the survival of the *narG*-deficient and wild type strains cultured in the absence of nitrate and treated with INH (Figure 3f).

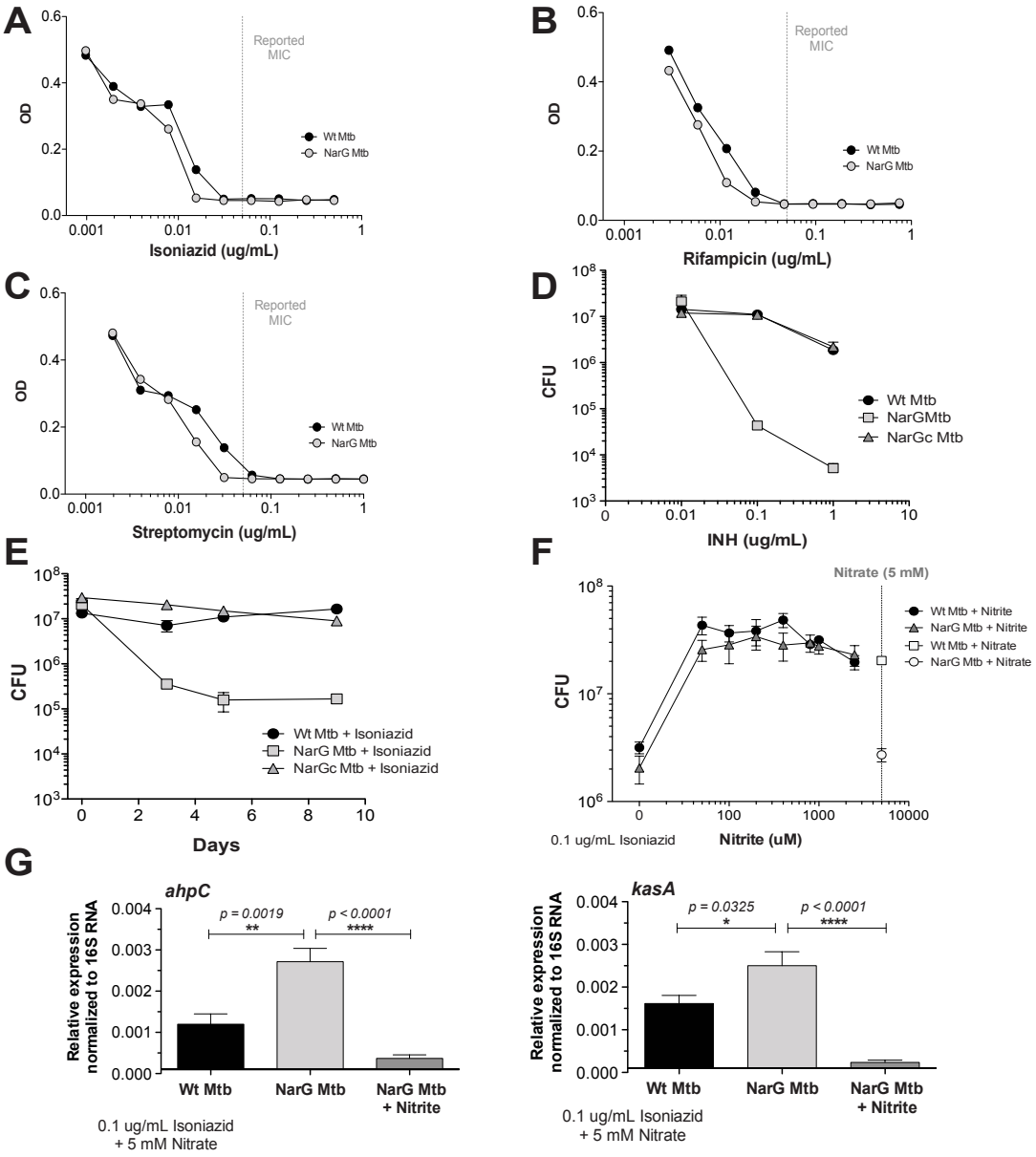
Following INH treatment and its subsequent activation by KatG, Mtb induces the expression of *kasA* and *ahpC* (17). The *narG*-deficient strain demonstrated greater induction of *kasA* and *ahpC* RNA transcripts than the wild type strain in response to 0.1 μg mL^{-1} INH, and induction

was attenuated following treatment with nitrite (Figure 3g). INH requires oxidative activation by the mycobacterial catalase/peroxidase KatG in order to become bioactive. These results suggest that when presented with the same concentration of INH under conditions that could sustain nitrate respiration by wild type Mtb, *narG*-deficient Mtb converted a larger proportion of the INH prodrug to its active form than wild type Mtb. This implied that the production of nitrite during respiration of nitrate by wild type Mtb might partially inhibit KatG.

Figure 3. Impact of narG-deficiency on susceptibility of Mtb to isoniazid in axenic culture. (A-C) Survival of wild type and *narG*-deficient Mtb in the presence of the indicated concentrations of (A) isoniazid, (B) rifampicin or (C) streptomycin in 21% oxygen over 5 days, initial (OD 0.1). Published MICs are provided for reference (46). (D, E) Survival of wild type, *narG*-deficient (NarG) and complemented (NarGc) strains cultured in 1% oxygen (D) for 3 days with the indicated concentrations of INH or (E) as a function of time over 9 days with 0.1 μg mL^{-1} INH. (F) Survival of wild type and *narG*-deficient Mtb incubated with increasing concentrations of exogenous nitrite or one fixed dose of nitrate, as indicated, over 3 days in 1% oxygen with 0.1 μg mL^{-1} INH. Results for (A-F) are means ± SEM representative of 2 independent experiments. (G) Expression of transcripts of *ahpC* and *kasA* by wild type and *narG*-deficient Mtb treated with 0.1 μg mL^{-1} INH and 5 mM nitrate for 16 hrs at 1% oxygen relative to 16S RNA. Where indicated, 2.5 mM nitrite was also added. Bars indicate means ± SEM from 3 independent experiments analyzed by unpaired t tests.

Figure 3 (continued)

BROTH CULTURE OF MTB IN THE ABSENCE OF HUMAN MACROPHAGES



narG-deficient Mtb was not hyper-susceptible to treatment with ethionamide.

To further investigate the role of KatG in mediating the hyper-susceptibility of *narG*-deficient Mtb to INH, we infected human macrophages with Mtb and treated the cultures with ethionamide (ETH). ETH and INH are close chemical analogues. Both are prodrugs that when activated inhibit the same target, InhA. However, ETH is activated by the monoxygenase EtaA rather than by KatG (18, 19). Survival of wild type and *narG*-deficient Mtb treated with ETH within infected human macrophages did not differ (Figure 4a). Therefore, the INH susceptibility of *narG*-deficient Mtb is unlikely to be due to differential dependence on InhA and may instead be due to differences in the activation of INH by KatG.

Nitrate reduction enhanced the lethality of hydrogen peroxide.

KatG is the mycobacterial catalase peroxidase that is also responsible for the cellular activation of INH. Levels of KatG mRNA determined by qRT-PCR and KatG protein detected by western blot did not differ among wild type, *narG*-deficient and complemented strains of Mtb (Supplementary figure 2b-c, see Appendix 2). Moreover, we were unable to measure differences in the extent of KatG dependent oxidation of INH via nitroblue tetrazolium staining of polyacrylamide gel electrophoregrams of lysates obtained from the three strains (data not shown). Nonetheless, to help assess whether the catalytic activity of KatG might differ between wild type and *narG*-deficient Mtb, the strains were incubated for several hours with nitrite or nitrate at 1% oxygen to allow

for nitrate respiration and then treated with hydrogen peroxide, the natural substrate of KatG. We reasoned that if the activity of KatG were attenuated by nitrite, the wild type and complemented strains might be more susceptible to hydrogen peroxide than *narG*-deficient Mtb. In accord with this hypothesis, *narG*-deficient Mtb was more resistant to hydrogen peroxide than the other two strains (Figure 4b). The enhanced mycobactericidal effect of hydrogen peroxide on wild type Mtb incubated with nitrate was also observed following the addition of increasing concentrations of exogenous nitrite in the absence of nitrate (Figure 4c and Supplementary Figure 2b, see Appendix 2).

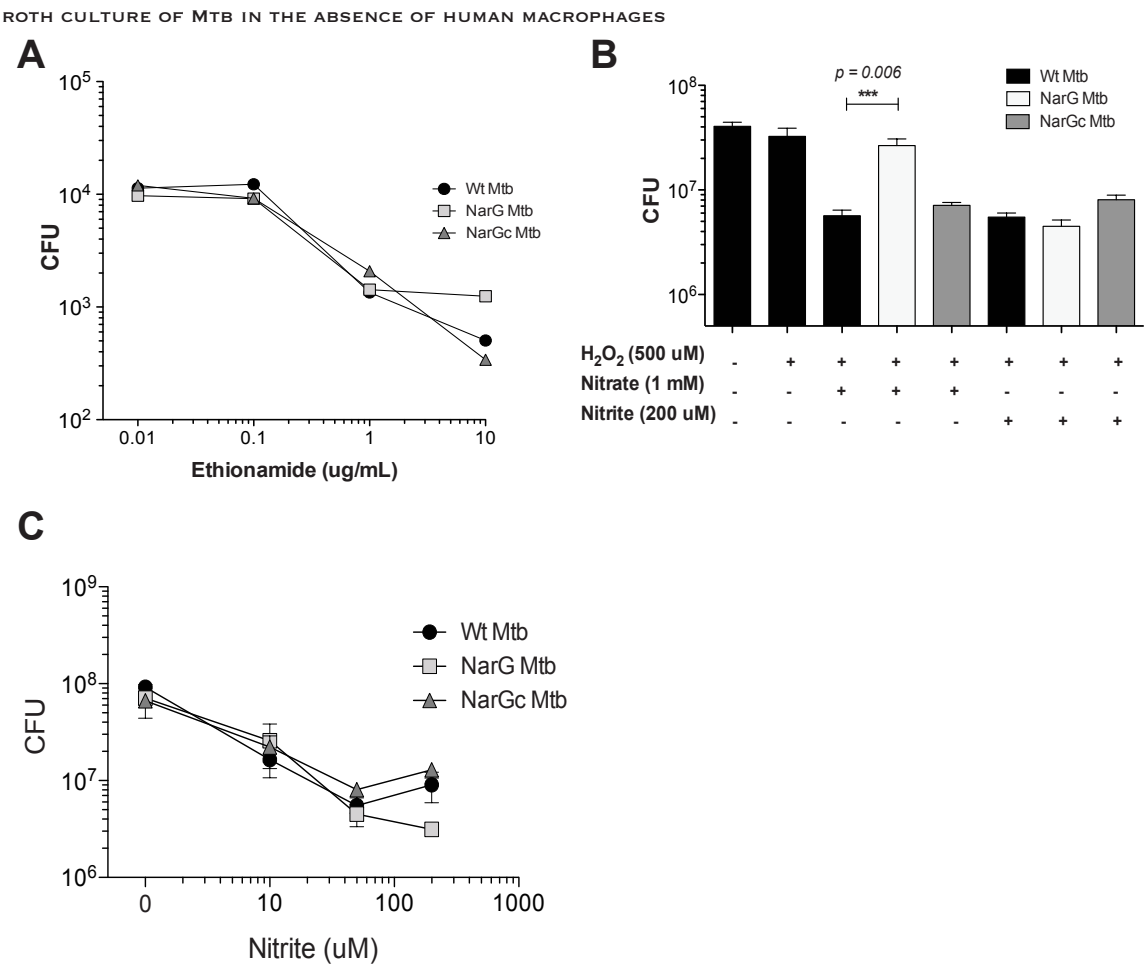


Figure 4. Impact of narG-deficiency on susceptibility of Mtb to ethionamide and hydrogen peroxide. (A) Survival of wild type and *narG*-deficient Mtb within infected primary human macrophages (MOI: 0.1) treated with the indicated concentrations of ethionamide for 3 days at 10% oxygen. (B) Survival of Mtb in axenic culture treated with nitrate or nitrite for 8 hours in 1% oxygen, to allow for nitrate respiration to occur, and then treated with hydrogen peroxide (500 μM) and incubated in 1% oxygen overnight (initial OD_{580}: 0.1). These are pooled data from 2 independent experiments and were analyzed by an unpaired t test. (C) Survival of wild type, *narG*-deficient (NarG Mtb) and complemented strains (NarGc Mtb) (initial OD580: 0.1) following treatment with both hydrogen peroxide (500 μM) and increasing concentrations of nitrite as indicated. Individual experiments representative of at least 2 independent experiments. Bars indicate means ± SEM.

Discussion

Herein we report that deficiency of *narG* increases the susceptibility of Mtb to INH and decreases its susceptibility to hydrogen peroxide. Prior to this work, the knockout of only one gene, *cydC*, had been reported to increase the susceptibility of Mtb to INH (20). The mechanism in the present study most likely involves nitrite-mediated impairment of the activity of KatG that takes place when wild type Mtb reduces nitrate to nitrite, which is absent in *narG*-deficient Mtb. Although nitrate is usually omitted from mycobacterial culture media, it is a physiologic constituent of human body fluids. Although Mtb does not reduce nitrate to nitrite in axenic culture unless the culture is hypoxic, we found that Mtb does reduce nitrate to nitrite when cultured within macrophages at physiologic oxygen tensions. Thus, phenotypic INH resistance arising from Mtb's nitrate respiration is likely to occur in the Mtb-infected host.

Interactions of catalase and other peroxidases with nitrite are complex (21-24). When we incubated Mtb with nitrite, lysed the cells, separated the proteins by non-reducing polyacrylamide gel electrophoresis and incubated the gel with INH and a tetrazolium dye as described (25), we did not see impairment in the activity of catalase compared to lysates from Mtb not exposed to nitrite (not shown). However, the biochemical environment within intact mycobacteria differs markedly from that of gels in room air, and tetrazolium is not a physiologic oxidant for INH. Moreover, *katG* mutations that cause INH resistance do not necessarily impact the rate of INH-NAD adduct formation by KatG relative to wild type Mtb, pointing to the potential for dissociation between the functional

effects of KatG mutations and the results of biochemical assays for KatG activity (26-29).

The following evidence supports the hypothesis that nitrite interfered with the INH-activating function of catalase in the intact mycobacterium: Mtb deficient in *narG* was both more susceptible to INH and more resistant to hydrogen peroxide than wild type Mtb. Second, *narG*-deficient Mtb was not hyper-susceptible to treatment with ETH. Third, compared to wild type and complemented strains, *narG*-deficient Mtb demonstrated higher expression of *ahpC* and *kasA*, two genes induced by treatment with INH, and their expression was reduced by the addition of nitrite to the cell culture. Taken together, these results suggest that Mtb lacking *narG* achieves greater KatG-mediated activation of INH than wild type Mtb. Lastly, in agreement with this hypothesis, *narG*-deficient Mtb better resisted treatment with hydrogen peroxide. The mutation of genes other than *katG* can also cause INH resistance. For example, mutations that impair the catalytic activities of Ndh, a type II NADH dehydrogenase, and MshA and MshC, which contribute to mycothiol biosynthesis, can cause INH resistance. However, inactivating mutations in any one of these genes also cause ETH resistance (30-32), which was not observed in this study.

Nitric oxide or nitrite at low pH, which generates nitric oxide (33), can potentiate the bactericidal activity of hydrogen peroxide towards *E. coli* (2, 21, 34, 35). However, to our knowledge, this is the first report that nitrite at neutral pH can synergistically enhance the bactericidal action of hydrogen peroxide. Production of hydrogen peroxide by phagocytic cells is an important component of host antibacterial defense

(36). Mycobacterial nitrate respiration may enhance the lethality of host reactive oxygen species *in vivo*. This may result from inhibition of KatG by nitrite or from inhibition of a variety of antioxidant defense proteins, including KatG, by products more reactive than nitrite to which nitrite can give rise. In *E. coli*, nitrate reductase is an important source of species reactive enough to nitrosylate proteins, as evidenced by an 80% reduction in protein S-nitrosylation in *narG*-deficient as compared to wild type bacteria (37-39). Rhee *et al*. identified 29 mycobacterial proteins that were S-nitrosylated when Mtb was treated with nitrite at low pH, including KatG (40). In addition, heme peroxidases such as KatG oxidize nitrite to more reactive species, such as nitrogen dioxide ($^{\bullet}NO_2$) and nitryl chloride (NO_2Cl) (21-24).

INH is widely reported to lose mycobactericidal activity when Mtb is cultured in conditions that prevent it from replicating. However, when the oxygen in an axenic, nitrate-containing culture of Mtb was reduced below 1%, *narG*–deficient Mtb, which was not replicating, became highly INH sensitive. We confirmed that effect and demonstrated its reversal with exogenous nitrite. To the extent that a relevant source of nitrite for Mtb residing in a macrophage in the human host may be Mtb itself, the question arises whether the cidality of INH for non-replicating *narG*-deficient Mtb could be phenocopied in non-replicating wild type Mtb by an inhibitor of Mtb's nitrate transporter or nitrate reductase. If so, such agents might significantly reduce the time required to cure latent TB with INH or active TB with INH-containing regimens, reducing the incidence of emergent drug resistance associated with incomplete treatment (41, 42). InhA is essential to aerobically and perhaps also hypoxically cultured

mycobacteria as two compounds that targeted both InhA as well as fatty acid synthase type 1 were cidal to non-replicating Mtb (43). Nevertheless, it remains unclear whether the reduced survival of *narG*-deficient Mtb treated with INH resulted only from the inactivation of InhA or by inhibition of additional mycobacterial target(s). A mechanistic understanding of the hyper-susceptibility of *narG*-deficient Mtb to INH may reveal new INH target(s) amenable to inhibition by other compounds.

Methods

Isolation and differentiation of primary human monocytes

Isolation of human monocytes and their differentiation into macrophages was as described (12). In brief, heparinized peripheral blood was collected by venipuncture from healthy human donors who provided informed consent under an IRB approved protocol. Peripheral blood mononuclear cells were first isolated by centrifugation of whole blood over Ficoll-Paque (GE Healthcare). The buffy coats were collected and monocytes were isolated by positive selection using magnetic beads conjugated to anti-CD14 antibodies (Milteyi Biotec). Following isolation, the human monocytes were plated at a density of 500,000 cells ml^{-1} per well of a 96 well plate. The culture medium consisted of 60% RPMI 1640, supplemented with 1% glutamax, 40% human plasma and GMCF and TNFα (0.5 ng ml^{-1} each). 30% of the total culture volume was replaced with fresh medium and cytokines every 3 to 4 days. Replacing fresh medium was conducted as rapidly as possible in room air before the cells were placed back in the low oxygen incubator. Differentiation of the

monocytes was conducted over 2 weeks in our standard medium at 10% O_2 and 5% CO_2 at 37°C in a humidified atmosphere in a chamber flushed with N_2 under the control of a PRoOX sensor and ProCO_2 regulator (BioSpherix). They were then activated with IFNγ (5 ng ml^{-1}) before infection with *M. tuberculosis* the following day at the desired multiplicity of infection (MOI).

Preparation of Mtb and infection of human macrophages with Mycobacterium tuberculosis

M. tuberculosis H37Rv was grown in Middlebrook 7H9 broth supplemented with 0.2% glycerol, 0.5% BSA, 0.2% dextrose and 0.085% NaCl with 0.05% Tween 80. The *narG*–deficient and complemented strains were generated as described (44). Cultures were started with 1 mL stock originally frozen at -80°C in log phase and then grown over 4 to 5 days to optical densities (OD) of 0.5-1.25 before the start of an experiment. A single-cell suspension was generated by centrifugation at 120 g for 10 minutes. For macrophages infection, roughly $200x10^6$ bacteria were centrifuged, in order to be able to observe a pellet. The 7H9 was completely removed and the cells were washed with PBS. The bacteria were then resuspended in culture medium and the desired number of Mtb was added to the macrophage culture.

Certain experiments required that we remove the nitrate-containing medium from the cell culture. In this case, 1 day prior to infection the cells were washed with room temperature PBS 3 times and a low nitrate formulation of DMEM with 10% human plasma was added along with 0.5 ng mL^{-1} GMCSF and TNFα and 5 ng mL^{-1} IFNγ.

Measurement of nitrite

Nitrite levels produced in the co-culture supernatants of infected macrophages were measured by the Griess assay. Briefly, 100 µl of supernatant was removed and to this were added 50 µl of 2% sulfanilamide with 5% phosphoric acid and 50 µl of 0.2% N-1-napthylethylenediamine dihydrochloride. Nitrite standards were prepared in the same medium used to culture the macrophages. Absorbance was measured at 550 nm immediately after addition of the reagents to the supernatant samples.

Treatment of infected macrophages

Macrophages were incubated with Mtb at the indicated MOI for 4-5 hours under 10% oxygen. The supernatant was removed and the macrophages were washed 3 times with room temperature PBS to remove extracellular bacteria. Fresh medium was added to the cells, followed by the addition of nitrite, nitrate and/or an antimycobacterial drug such as INH. The cultures were then returned to the low oxygen incubator and incubated for a subsequent 3 days with the desired compound. Drugs were freshly dissolved before each experiment in DMSO, except for streptomycin, which was dissolved in water.

CFU determination from infected macrophages

Following incubation with Mtb for 3 days, the macrophages were inspected by microscopy to ensure continued confluence and adherence to the well. The supernatant was collected and the cells were gently washed

3 times with room temperature PBS to wash away remaining compounds. Following the wash steps, the cells were again visualized to confirm that the monolayer was intact and then lysed by incubation in 0.5% Triton X-100 for 10 minutes at 37°C. The lysate was serially diluted in 0.1% Triton X-100 and plated on Middlebrook 7H11 agar with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco) supplemented with 0.5% glycerol. Colonies were enumerated following 3 weeks of culture at 37 °C, in 21% O_2, 5% CO_2.

Treatment of Mtb in axenic culture

Mtb was added at the indicated OD to 96 well plates in 7H9 broth with or without 5 mM nitrate or the indicated concentration of nitrite, supplemented with 10 ADNaCl (0.2% glycerol, 0.5% BSA, 0.2% dextrose, 0.085% NaCl) and 0.05% Tween 80. The indicated antibiotic was added immediately and the plate was incubated in 1% oxygen for 3 days or at 21% oxygen for 5 days, as indicated. The Mtb was resuspended, serially diluted in 0.1% Triton-X 100 and plated for CFU as above. Alternatively, for OD measurements, the Mtb was resuspended in the culture medium and the absorbance was read at 580 nm on the fifth day of incubation with the indicated compound. In the case of exposure to hydrogen peroxide, the plate was incubated in 1% oxygen for 8 hours with or without 1 mM nitrate or the indicated concentration of nitrite. At this point, the indicated concentration of hydrogen peroxide was added and the cells were incubated overnight at 1% oxygen before plating for CFU.

KatG western blot and measurement of KatG dependent oxidation of INH

A minimum of $4x10^9$ bacteria per condition were suspended in 7H9, 10% ADNaCl at an OD of 0.2 in the presence or absence of 2.5 mM nitrite. Cultures were incubated for 1 day in 1% oxygen. The cultures were collected by centrifugation and resuspended in 200 µl of lysis buffer containing 50 mM $NaPO_4$, 1 mM PMSF and 4x complete protease inhibitor cocktail (Roche). The cells were lysed in a bead-beating homogenizer with silica beads 3 times with intermittent incubation on ice. The lysate was collected and filter sterilized and concentrated at 4°C to ~100 µl using Amicon Ultra-0.5 mL centrifugal filters from Millipore with a molecular weight cut-off of 3 kDa. Protein concentration was measured using the Bio-Rad assay and equal amounts of protein from each sample were loaded into 7.5% reducing gels. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane, blocked with Odyssey blocking buffer from LI-COR Biosciences and stained with anti-KatG antibody (obtained through BEI Resources, NIAID, NIH: Monoclonal Anti-*Mycobacterium tuberculosis* KatG, gene Rv1908c, clone IT-57 (CDA4) (culture supernatant), NR-13793) and anti-DlaT antibody (1:1000) and then visualized with fluorophore-coupled secondary antibodies (45). To test the degree of KatG dependent oxidation of INH, 50 µg of total cell lysate was loaded into 7.5% non-reducing gels. After electrophoresis, the gel was incubated at room temperature for 15 - 30 minutes in 50 µLs of $NaPO_4$ buffer containing 62 mg INH, and 2 mM nitroblue tetrazolium (NBT) at pH 7 to which hydrogen peroxide was added to a final concentration of 800 µM (25).

Quantitative PCR of RNA from Mtb growing in macrophages or broth culture

Five million human macrophages were cultured per T25 flask in 10% oxygen and infected with wild type, *narG*-deficient or the complemented strains of Mtb at an MOI of 40 for 10 hours. Supernatant was removed and the monolayer was washed 2 times with room temperature PBS. Trizol (2 mL) was added to each flask and the cells were detached using a rubber policeman. For broth cultures, at least $2.5x10^9$ bacteria per condition were incubated in 1% oxygen for 3 days with or without 2.5 mM nitrite and 0.1 μg mL^{-1} INH. Then an equal volume of 5M GTC buffer containing 5M guanidine thiocyanate, 25 mM sodium citrate, 20 mM N-lauryl-sarcosine, 0.7% v/v β- mercaptoethanol, was added. The bacteria were pelleted by centrifugation and 1 mL of Trizol was added per sample. The suspension was beaten with silica beads, 3 times. RNA was extracted using an RNeasy kit (Qiagen) in accordance with the manufacturer's instructions with the exception that off column DNAse digestion was performed for 2 hrs at 37 °C. To generate cDNA, total RNA (900 ng) was reverse transcribed using the GeneAmp RNA PCR Kit (Applied Biosystems). Quantitative RT-PCR was performed using gene specific primers (Life Technologies) and the SuperScript III Platinum Two Step qRT-PCR Kit (Life Technologies) with a 7900HT Fast Real Time PCR System (Applied Biosystems). Each experiment contained experimental triplicates. All reported values were within the linear range of the primers and the experimental results were normalized to 16S rRNA values.

Statistical analysis

Statistical analysis was performed as indicated in the figure legends using the standard statistical software Prism version 5.0f for Macintosh, GraphPad Software, San Diego California USA

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CHAPTER 4

CONCLUSIONS

Within Mtb-infected humans, nitrite levels rise to such substantial levels as to be measured within the plasma (1, 2). This nitrite is likely a product of the host's immune response as well as Mtb's hypoxic respiratory pathway. However, these enzymatic processes are inversely regulated by oxygen tension (3, 4). Therefore, in the host whether Mtb faces normoxia or hypoxia it will encounter nitrite and the RNS that generate or arise from it. That Mtb resides within hypoxic tissue in the human host is inferred from experiments with infected model hosts, which develop hypoxic lesions containing Mtb (5-7). In addition, lung tissue resected from patients with TB contains RNA transcripts that encode proteins essential to nitrate respiration (8, 9). Therefore, Mtb likely respires nitrate within the human host.

The majority of infected individuals will never eradicate Mtb, though most successfully sequester the pathogen. Subpopulations of mycobacteria residing within the human host are thought to enter into a dormant, non-replicating state and to survive for the lifetime of the host (10). Persisting mycobacteria will serve as a nidus for disease reactivation and infection of new hosts (10, 11). The extent to which nitrate respiration contributes to Mtb's persistence *in vivo* remains unknown. However, the host is comprised of heterogeneous microenvironments in which Mtb may transiently be exposed to concentrations of oxygen sufficient to support active replication (12). While renewed growth may be advantageous and

contribute to the colonization of new hosts, ill-timed growth may disrupt the dynamic equilibrium that exists between the pathogen and host and compromise bacterial dissemination. Once a granulomatous lesion containing Mtb erodes into a bronchus, droplet nuclei containing Mtb are expelled from the diseased individual to enter and infect a naïve host (13). Therefore, in the setting of sustained normoxia, Mtb-derived nitrite may limit untimely growth and provide a store of metabolic energy to provision the colonization of naïve hosts.

Nitrate respiration regulated the expression profile of genes that are also regulated by exposure of Mtb to pH 5.5, hydrogen peroxide, nitric oxide, hypoxia, iron-depleted medium and residence within mouse macrophages. Therefore, nitrate respiration may support the survival of Mtb faced with hostile host stresses. In fact, this has already been demonstrated for Mtb exposed to low pH, RNS (14) and sudden anaerobiosis (15). We have reported that the combination of Mtb-derived nitrite and hydrogen peroxide is more lethal to Mtb than treatment with hydrogen peroxide alone (Cunningham-Bussel, manuscript in preparation, see chapter 2). However, while short incubations with nitrite synergized with hydrogen peroxide to kill Mtb, longer incubations seemed to promote the survival of hydrogen peroxide treated Mtb, although this remains to be confirmed (data not shown). Therefore in regards to hydrogen peroxide, nitrite may exert opposing effects depending both upon the concentration and length of exposure of Mtb to nitrite prior to treatment with hydrogen peroxide.

We also observed that in the absence of nitrate respiration, as compared to bacteria that continued to respire nitrate, Mtb became hyper-

susceptible to INH. Therefore, inhibitors of the nitrate reductase or nitrate/proton symporter *narK2* may synergize with INH to significantly reduce the length of time required to treat TB. That INH remained bactericidal even under non-replicating conditions suggested that the hyper-susceptibility of *narG*-deficient Mtb to INH might have been due to the inhibition of a cellular protein(s) other than the recognized protein target of INH, InhA. Furthermore, regardless of any synergy with INH, nitrate respiration may prove to more broadly support the survival of Mtb *in vivo*. Therefore, compounds that prevent nitrate respiration may prove to be valuable additions to the arsenal of antitubercular antibiotics.

Future directions

Future experiments will need to first address whether nitrate respiration benefits the survival of Mtb in a model organism. Similarly, the observation that nitrate respiration allows Mtb to resist INH should also be tested *in vivo*. High-throughput screening of chemical libraries against axenic cultures of Mtb may identify novel compounds that inhibit the mycobacterial nitrate reductase and nitrate/proton symporter *narK2*. In addition, it may be possible to identify the protein target(s) of INH in non-replicating Mtb lacking a functional nitrate reductase by screening for spontaneous revertants of *narG*-deficient Mtb that lose their hyper-susceptibility to INH. Alternatively, this may be accomplished by screening a library of transposon insertion mutants generated in *narG*-deficient Mtb for variants that have altered susceptibility to INH. The mutants that demonstrate either increased or reduced sensitivity may

contain transposon insertions in genes that regulate the susceptibility of non-replicating Mtb to INH.

In vivo experiments to determine the impact of nitrate respiration on Mtb within the host will require a model organism that develops hypoxic, TB-associated lesions, such as the guinea pig, rabbit or macaque (5). These organisms better mimic human tissue pathology as compared to the standard mouse model, which is not hypoxic (5-7). Recently, an alternative mouse model was reported to develop hypoxic lesions in response to infection with Mtb (16). This mouse strain may therefore be a more suitable model system in which to investigate the contribution of nitrate respiration to the persistence of Mtb *in vivo*.

Both the host and pathogen are potential sources of nitrite *in vivo*. Therefore, it will be important to determine the amount of nitrite individually produced by the host and pathogen and to selectively abolish these catalytic activities during the course of infection. The production of host-nitrite can be limited by the use of selective or non-selective NOS inhibitors (17, 18) or greatly repressed by genetic deletion of iNOS (19). Pathogen derived nitrite production is abolished by the genetic deletion of *narG* (4). In addition to the selective abrogation of nitrite generated by the host or pathogen, nitrite can be supplemented by adding nitrate or nitrite to the diet of infected model hosts (20, 21). Dietary nitrate and nitrite supplementation can greatly increase the concentration of plasma and tissue nitrite as well as the concentration of tissue S-nitrosylation (20). Therefore, an additional experiment might involve infection of iNOS deficient host organisms with *narG*-deficient Mtb followed by the supplemental administration of exogenous nitrate or nitrite, which may

contribute to determining the impact of nitrite on Mtb *in vivo*.

Mycobacterial nitrate respiration caused a non-heritable resistance to INH, which remains mechanistically unexplained. Nitrite and the RNS that arise from it are known to post-translationally modify cellular proteins and interfere with their function (22-25). Drug resistance is often attributed to the acquisition of heritable genetic mutations that protect bacterial populations from cidal compounds. However, non-heritable survival has also been described to account for the persistence of genetically susceptible bacteria treated with bactericidal antibiotics (26, 27). Mtb persists in vivo, despite treatment with antimycobacterial antibiotics such as INH (28). Therefore, despite an underlying genetic sensitivity to INH, the survival of subpopulations of Mtb treated with INH may be due to the resistance afforded by mycobacterial nitrate respiration. Nitrite is highly diffusible and therefore could non-heritably manipulate array of cellular targets across a population of bacteria. Therefore, INH resistance is but one functional consequence of mycobacterial nitrate respiration; others likely remain to be characterized.

Nitrite has previously been demonstrated to regulate mammalian gene expression (23, 29). However, we did not observe any impact of mycobacterial nitrate respiration on the transcriptome of Mtb infected human macrophages. However, unlike the transcriptional profiles of groups of genetically identical mice or bacteria, the transcriptional profile of Mtb infected macrophages was analyzed using RNA collected from three genetically dissimilar individuals. We propose that the lack of an observable impact of nitrate respiration on the transcriptome of infected primary human macrophages may be due the reportedly variable gene

expression that exists between unrelated humans (30).

In summary, we have demonstrated that mycobacterial nitrite reduction impacts a variety of previously described mycobacterial cellular processes. However, we anticipate that Mtb-derived nitrite broadly impacts not only the cellular processes of Mtb itself, but also the host. Nitrate respiration may therefore play a central role in the interaction between the host and mycobacterial pathogen, the full extent of which remains to be characterized.

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PUBLICATIONS

1. Cunningham-Bussel A, Zhang T, Nathan C *Mycobacterium tuberculosis* generates nitrite in human macrophages at physiologic oxygen tensions: impact on mycobacterial ATP consumption and gene expression. *(manuscript submitted)*.

2. Cunningham-Bussel A, Bange F-C, Nathan C Nitrite impacts the survival of *Mycobacterium tuberculosis* in response to isoniazid and hydrogen peroxide. *Microbiology Open, in press*.

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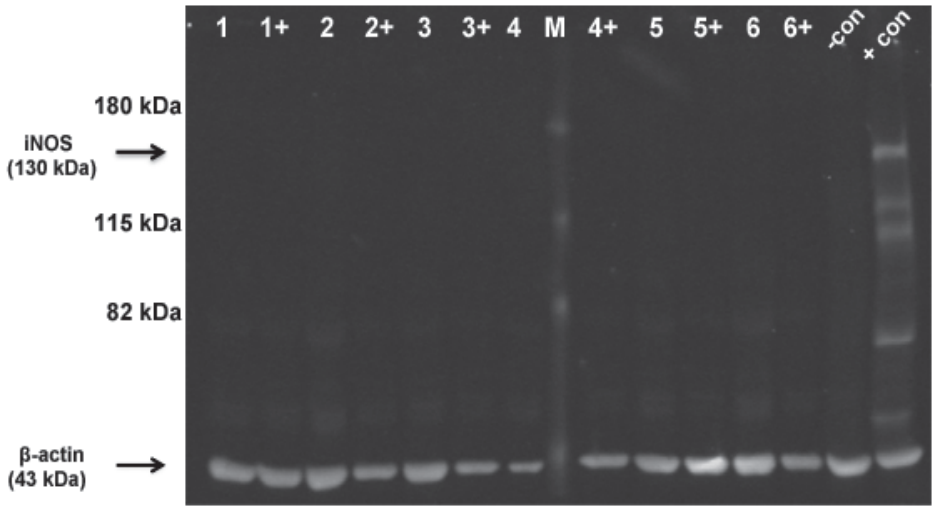
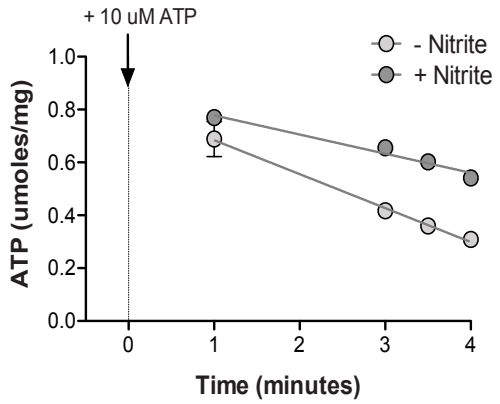
APPENDIX 1

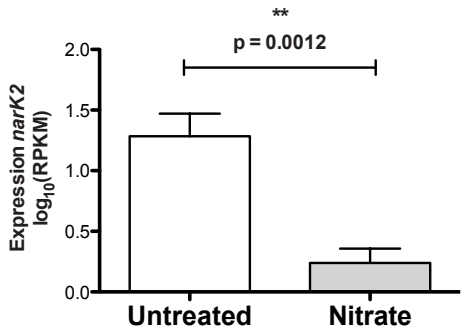


Supplementary Figure 1. iNOS was not detected within Mtb-infected primary human macrophages. Macrophages were either infected with Mtb (MOI: 5, indicated by a "+" sign) or remained uninfected for 3 days at 10% oxygen. Results are shown for 6 individual donors. Lysates were separated by reducing SDS polyacrylamide gel electrophoresis and probed with anti-iNOS antibody (Santa Cruz Biotechnology sc-8310) and anti-β-actin (abcam 8226). The positive control (+ con) was generated by lipofectamine-assisted transfection of HEK293 cells with a pcDNA3.1 plasmid construct containing human iNOS. To generate the negative control, HEK293 cells were transfected with an empty plasmid.



	- Nitrite	+ Nitrite
Slope	-0.1285 ± 0.01356	-0.07246 ± 0.005091

Supplementary Figure 2. Consumption of exogenous ATP added to cell lysates from nitrite treated or untreated bacterial cultures. Wt Mtb was incubated in the presence or absence of 2.5 mM nitrite for 3 days in 1% oxygen; cell lysates were incubated for 20 minutes at room temperature to allow for the complete metabolism of endogenous ATP. Reagent ATP (10 μM) was then added to the cell lysates and ATP content measured at intervals. Results are from 1 experiment representative of 3.



Supplementary Figure S3. Average expression of *narK2* by untreated and nitrate treated wild type Mtb. Means ± SD, n=3 independent experiments. The p value was determined by an unpaired t test.

Supplementary Table 1. Genes significantly induced by nitrate. (p_{corr} <0.05), (n=3)

Gene ID	Gene	RATIO (Nitrate/Untreated)	p corr <0.05
Rv2780	*ald*	9.38	2.58E-03
Rv1994c	*cmtR*	4.22	2.39E-02
Rv1464	*csd*	28.91	2.72E-04
Rv3743c	*ctpJ*	5.82	4.23E-03
Rv3019c	*esxR*	6.10	3.49E-03
Rv3020c	*esxS*	6.86	1.28E-03
Rv2590	*fadD9*	5.04	3.42E-02
Rv0752c	*fadE9*	4.23	3.34E-02
Rv1909c	*furA*	7.74	2.41E-03
Rv2384	*mbtA*	5.65	4.61E-03
Rv2383c	*mbtB*	30.00	5.59E-04
Rv2380c	*mbtE*	13.40	1.02E-02
Rv2386c	*mbtI*	12.52	3.40E-05
Rv2385	*mbtJ*	4.85	5.07E-03
Rv3206c	*moeB1*	6.42	4.96E-02
Rv0747	*PE_PGRS10*	4.56	7.39E-03
Rv0834c	*PE_PGRS14*	7.11	1.94E-02
Rv0978c	*PE_PGRS17*	11.21	1.19E-05
Rv2615c	*PE_PGRS45*	6.30	1.07E-02
Rv3746c	*PE34*	7.84	3.40E-05
Rv1402	*priA*	6.01	1.22E-03
Rv0142	*Rv0142*	6.16	1.80E-03
Rv0398c	*Rv0398c*	3.77	2.51E-02
Rv0465c	*Rv0465c*	4.86	3.26E-02
Rv0575c	*Rv0575c*	10.3	5.67E-06
Rv0841	*Rv0841*	3.91	4.91E-02
Rv1049	*Rv1049*	6.40	1.09E-03
Rv1050	*Rv1050*	3.90	4.84E-02
Rv1057	*Rv1057*	4.46	2.16E-02
Rv1375	*Rv1375*	9.53	2.35E-03
Rv1403c	*Rv1403c*	15.91	2.12E-07
Rv1405c	*Rv1405c*	14.53	4.71E-05
Rv1461	*Rv1461*	34.70	1.71E-03
Rv1578c	*Rv1578c*	3.83	4.89E-02
Rv1670	*Rv1670*	4.18	3.09E-02
Rv1671	*Rv1671*	4.40	2.39E-02
Rv1767	*Rv1767*	4.05	3.51E-02
Rv1907c	*Rv1907c*	4.14	2.08E-02
Rv1986	*Rv1986*	3.93	1.32E-02
Rv2324	*Rv2324*	4.03	3.58E-02
Rv2616	*Rv2616*	100.96	1.00E-11
Rv2617c	*Rv2617c*	137.41	4.23E-10
Rv2618	*Rv2618*	26.9	4.08E-04
Rv2619c	*Rv2619c*	20.75	3.11E-05
Rv2621c	*Rv2621c*	21.63	1.78E-06
Rv2622	*Rv2622*	10.26	1.21E-06
Rv2650c	*Rv2650c*	5.21	6.03E-03
Rv2651c	*Rv2651c*	4.00	2.39E-02
Rv2729c	*Rv2729c*	4.28	1.14E-02
Rv2781c	*Rv2781c*	6.21	7.53E-04
Rv3289c	*Rv3289c*	8.99	3.29E-04
Rv3402c	*Rv3402c*	6.94	2.36E-04
Rv3403c	*Rv3403c*	4.70	2.51E-02
Rv3832c	*Rv3832c*	6.02	2.94E-02
Rv3839	*Rv3839*	28.77	4.23E-10
Rv3840	*Rv3840*	4.57	1.05E-02
Rv3288c	*usfY*	6.32	7.30E-03

Supplementary Table 2. Genes significantly repressed by nitrate. (p_{corr} <0.05), (n=3) Genes labeled with an asterix are members of the *DOS* regulon as defined by (20).

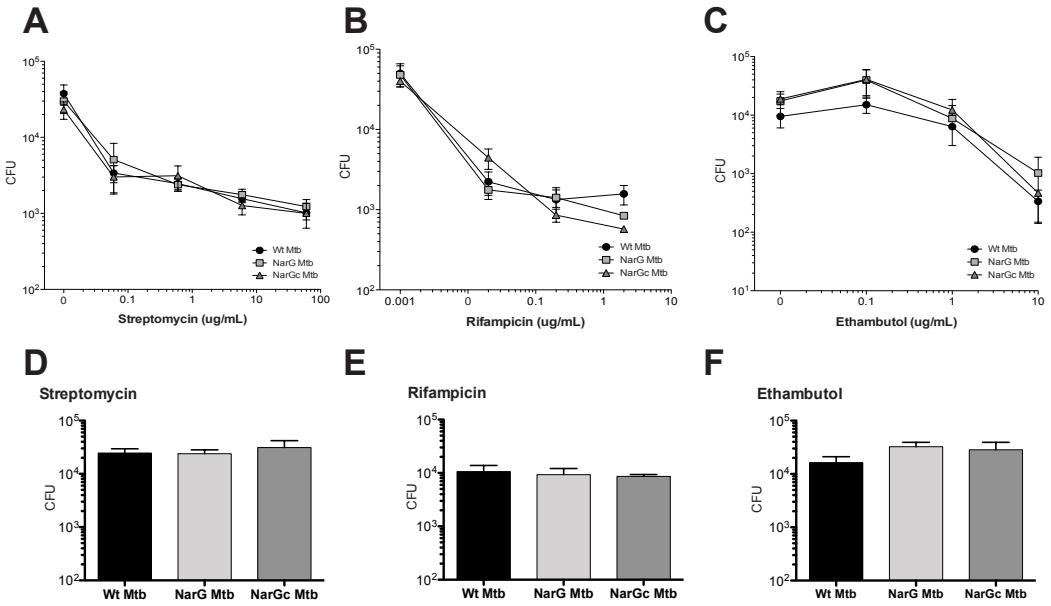
	Gene ID	Gene	RATIO (Nitrate/Untreated)	p corr <0.05
*	Rv0569	*Rv0569*	0.06	4.01E-10
*	Rv2007c	*fdxA*	0.05	4.67E-10
*	Rv2627c	*Rv2627c*	0.06	7.49E-10
*	Rv2626c	*hrp1*	0.06	1.08E-09
*	Rv3841	*bfrB*	0.05	4.29E-09
*	Rv0570	*nrdZ*	0.08	2.76E-08
*	Rv1733c	*Rv1733c*	0.09	0.000000036
*	Rv2031c	*hspX*	0.07	0.000000192
*	Rv2625c	*Rv2625c*	0.09	0.000000408
*	Rv1739c	*Rv1739c*	0.10	0.000000643
*	Rv2032	*acg*	0.10	0.00000105
*	Rv2628	*Rv2628*	0.11	0.00000134
*	Rv1738	*Rv1738*	0.10	0.0000014
*	Rv0572c	*Rv0572c*	0.12	0.00000188
*	Rv2623	*TB31.7*	0.09	0.00000203
*	Rv3134c	*Rv3134c*	0.10	0.00000203
*	Rv1997	*ctpF*	0.11	0.00000272
*	Rv2030c	*Rv2030c*	0.09	0.00000429
*	Rv3130c	*tgs1*	0.09	0.0000295
*	Rv1998c	*Rv1998c*	0.16	0.0000559
*	Rv3127	*Rv3127*	0.08	0.0000559
*	Rv2624c	*Rv2624c*	0.15	0.000211
*	Rv2029c	*pfkB*	0.09	0.00027
*	Rv3131	*Rv3131*	0.04	0.0018
*	Rv3126c	*Rv3126c*	0.18	0.00482
*	Rv2006	*otsB1*	0.21	0.00698
*	Rv1996	*Rv1996*	0.21	0.0076
*	Rv1736c	*narX*	0.16	0.00991
*	Rv1737c	*narK2*	0.09	0.0358
*	Rv2028c	*Rv2028c*	0.15	0.0488
	Rv1195	PE13	0.10	0.00000123
	Rv0700	rpsJ	0.16	0.000454
	Rv2190c	Rv2190c	0.17	0.000626
	Rv0315	Rv0315	0.18	0.000646
	Rv1196	PPE18	0.17	0.00278
	Rv0227c	Rv0227c	0.20	0.00306
	Rv0892	Rv0892	0.19	0.00508
	Rv0714	rplN	0.22	0.0052
	Rv0237	lpqI	0.22	0.0063
	Rv0718	rpsH	0.23	0.00635
	Rv2952	Rv2952	0.21	0.00789
	Rv2033c	Rv2033c	0.22	0.0102
	Rv2563	Rv2563	0.23	0.0108
	Rv0717	rpsN1	0.25	0.0109
	Rv0009	ppiA	0.23	0.0131
	Rv1884c	rpfC	0.23	0.0148
	Rv2564	glnQ	0.25	0.015
	Rv2450c	rpfE	0.19	0.0209
	Rv1886c	fbpB	0.18	0.0239
	Rv0933	pstB	0.27	0.025
	Rv2816c	Rv2816c	0.28	0.0269
	Rv0719	rplF	0.27	0.0278
	Rv1515c	Rv1515c	0.30	0.0342
	Rv2945c	lppX	0.29	0.0359
	Rv2959c	Rv2959c	0.27	0.0419
	Rv2416c	eis	0.31	0.0431

Gene ID	Gene	RATIO (Nitrate/Untreated)	p corr <0.05
Rv2917	Rv2917	0.31	0.0431
Rv0934	pstS1	0.27	0.0475
Rv3628	ppa	0.30	0.0475
Rv0935	pstC1	0.30	0.0476
Rv0165c	mce1R	0.28	0.0492
Rv0867c	rpfA	0.28	0.0494
Rv2431c	PE25	0.27	0.0494

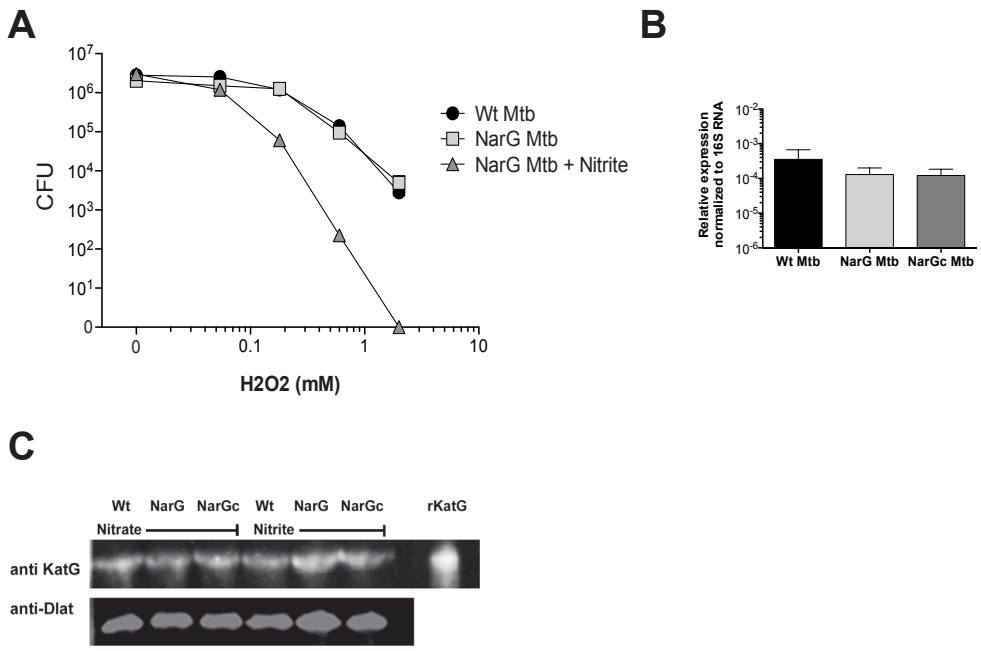
Supplementary Table 3. Genes that are significantly regulated by nitrate and no other tested stimuli. *(p_{corr} <0.05)* The expression of genes regulated only by nitrate and no other stimuli reported in Table 1.

Gene	Gene ID	Entrez Gene ID	Untreated (RPKM)	Nitrate (RPKM)	RATIO (Nitrate/Untreated)	p_{corr} <0.05	Function	Functional category
Rv0978c	*PE_PGRS17*	885077	0.38	4.25	11.21	0.000012	Function unknown	PE/PPE
Rv2781c	*Rv2781c*	888488	4.24	26.37	6.21	0.0008	Function unknown	Intermediary metabolism and respiration
Rv1402	*priA*	886716	0.31	1.88	6.01	0.0012	Functions as a helicase within the primosome	Information pathways
Rv3743c	*ctpJ*	885106	0.50	2.89	5.82	0.0042	Cation-transporting ATPase	Cell wall and cell processes
Rv0237	*lpqI*	886693	3.11	0.69	0.22	0.0063	Probable conserved lipoprotein	Cell wall and cell processes
Rv0747	*PE_PGRS10*	888662	1.61	7.33	4.56	0.0074	Function unknown	PE/PPE, lipid metabolism
Rv2615c	*PE_PGRS45*	888215	1.35	8.50	6.30	0.0107	Function unknown	PE/PPE
Rv2563	*Rv2563*	887516	3.78	0.85	0.23	0.0108	Active transport of glutamine	Cell wall and cell processes
Rv0752c	*fadE9*	888684	2.85	12.08	4.23	0.0334	Function unknown	Lipid metabolism
Rv1515c	*Rv1515c*	886459	1.94	0.57	0.30	0.0342	Function unknown	Conserved hypothetical
Rv2917	*Rv2917*	887758	1.34	0.42	0.31	0.0431	Function unknown, but involved in lipid degradation	Conserved hypothetical, cell wall and cell processes
Rv0841	*Rv0841*	3205068	9.10	35.58	3.91	0.0491	Function unknown	Cell wall and cell processes
Rv0165c	*mce1R*	886818	4.50	1.27	0.28	0.0492	Probable transcriptional regulatory protein Mce1R (probably GntR-family)	Regulatory proteins

APPENDIX 2



Supplementary Figure 1. Effect of nitrate respiration on mycobacterial resistance to streptomycin, rifampicin and ethambutol within primary human macrophages. (A-C) Survival of wild type, *narG*-deficient (NarG) and complemented strains (NarGc) within macrophages (MOI: 0.1, corresponding to 10,000 bacteria) treated with the indicated concentrations of (A) rifampicin (B) streptomycin or (C) ethambutol over 3 days in 10% oxygen. The results shown in A-C are single experiments that are representative of at least two independent experiments conducted using cells isolated from at least two distinct human donors. (D-F) contain the pooled CFU data from at least two independent experiments conducted with cells from at least 2 individual human donors shown for one drug concentration as follows: (D) rifampicin (0.2 μg mL^{-1}) (E) streptomycin (0.6 μg mL^{-1}) or (F) ethambutol (0.1 μg mL^{-1}). Bars indicate means ± SEM.



Supplementary Figure 2. Nitrite treatment hyper-sensitized Mtb to hydrogen peroxide treatment without an observed difference in the expression of *katG* or a measurably reduced degree of its peroxidase activity. (A) The survival of axenic cultures of Mtb (OD 0.1) following treatment with the indicated concentration of hydrogen peroxide at 1% oxygen incubated overnight with 1 mM nitrite. This is representative of 2 independent experiments. (B) The mRNA expression of *katG* by wild type, *narG* -deficient (NarG) and the complemented (NarGc) strains within infected human macrophages following incubation for three days at 10% oxygen. The given experiment is the pooled result of 2 independent experiments. (C) Axenic cultures of wild type, *narG*-deficient and the complemented strains of Mtb were incubated over one day at 1% oxygen and lysed to collect total cell protein. Individual proteins from the total cell lysate were separated by native gel electrophoresis and KatG and the housekeeping gene DLAT were visualized by fluorophore coupled antibody staining. This is representative of three independent experiments. Bars indicate means ± SEM.