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Normoxic Cyclic GMP-independent Oxidative Signaling by Nitrite Enhances Airway Epithelial Cell Proliferation and Wound Healing

Ling Wang[a], **Sheila A. Frizzell**[a], **Xuejun Zhao**[a,b], and **Mark T. Gladwin**[a,b,*]

[a]Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA 15213, USA

[b]Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh, Pittsburgh, PA 15213, USA

Abstract

The airway epithelium provides important barrier and host defense functions. Recent studies reveal that nitrite is an endocrine reservoir of nitric oxide (NO) bioactivity that is converted to NO by enzymatic reductases along the physiological oxygen gradient. Nitrite signaling has been described as NO dependent activation mediated by reactions with deoxygenated redox active hemoproteins, such as hemoglobin, myoglobin, neuroglobin, xanthine oxidoreductase (XO) and NO synthase at low pH and oxygen tension. However, nitrite can also be readily oxidized to nitrogen dioxide ($NO_2^{\bullet}$) via heme peroxidase reactions, suggesting the existence of alternative oxidative signaling pathways for nitrite under normoxic conditions. In the present study we examined normoxic signaling effects of sodium nitrite on airway epithelial cell wound healing. In an *in vitro* scratch injury model under normoxia, we exposed cultured monolayers of human airway epithelial cells to various concentrations of sodium nitrite and compared responses to NO donor. We found sodium nitrite potently enhanced airway epithelium wound healing at physiological concentrations (from 1uM). The effect of nitrite was blocked by the NO and $NO_2^{\bullet}$ scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (c-PTIO). Interestingly, nitrite treatment did not increase cyclic guanosine monophosphate (cGMP) levels under these normoxic conditions, even in the presence of a phosphodiesterase 5 inhibitor, suggesting cGMP independent signaling. Consistent with an oxidative signaling pathway requiring hydrogen peroxide (H_2O_2)/heme peroxidase/$NO_2^{\bullet}$ signaling, the effects of nitrite were potentiated by superoxide dismutase (SOD) and low concentration H_2O_2, whereas inhibited completely by catalase, followed by downstream extracellular-signal-regulated kinase (ERK) 1/2 activation. Our data represent the first description of normoxic nitrite signaling on lung epithelial cell proliferation and wound healing and suggest novel oxidative signaling pathways involving nitrite-H_2O_2 reactions, possibly via the intermediary, $NO_2^{\bullet}$.

Keywords

sodium nitrite; nitric oxide; cyclic GMP; cell proliferation; airway epithelium

*Corresponding author. Address: Pulmonary, Allergy and Critical Care Medicine, University of Pittsburgh, PA 15213, USA. Tel: +1 412 692 221. Fax: +1 412 692 2260. gladwinmt@upmc.edu..

1.1 Introduction

Airway epithelium provides a physical border between host and environment that protects from injurious and infectious stimuli that gain access to the respiratory tract through inspiration or aspiration [1]. Epithelial injury occurs in various lung diseases and repair of the injured epithelium is important to restore its barrier integrity. Immediately after injury, the airway epithelium initiates a repair process in order to restore the barrier integrity. Although wound repair in such *in vitro* injury models is a complex process involving both cell migration and proliferation, the initial phase of wound repair involves primarily cell migration. Indeed, after migrating cells have covered the denuded and wounded area, the barrier integrity of the bronchial epithelium is restored [1-3]. New therapies that can enhance cell migration and restore barrier integrity hold promise for the treatment of acute lung injury syndromes.

It is now appreciated that nitrite, previously viewed as a physiologically inert metabolite of nitric oxide (NO), serves as an important source of NO in vasculature and tissues [4]. Nitrite is detected in the airway epithelial lining fluid as an NO metabolite in several studies [5], but the role of this anion in epithelial function is uncertain. Based on a study showing beneficial effects of NO on epithelial growth and wound repair [6], we hypothesized that sodium nitrite may similarly enhance airway epithelial cells wound healing. However, to date, most of the signaling effects of nitrite have been characterized under hypoxic or ischemic conditions which require the reductive formation of NO by deoxygenated redox active hemoproteins [7], while in the present study the signaling effects of nitrite on airway epithelial cells wound healing occur during normoxia. Consistent with alternative normoxic oxidative signaling pathways, the effects of nitrite appear to be independent of cGMP formation and require the intermediary, $NO_2^{\bullet}$.

1.2 Material and methods

1.2.1 Cell culture and treatments

The immortalized human bronchial epithelial cell line, BEAS-2B (ATCC, Rockville, MD) was grown in bronchial epithelial growth media (BEGM, LONZA, Walkersville, MD). A papilloma virus–immortalized human bronchial epithelial cell line (HBE1), kindly provided by Dr. J. Yankaskas (University of North Carolina, Chapel Hill, NC) and primary normal human bronchial epithelial cells (NHBE) (LONZA, Walkersville, MD) were also grown in BEGM. Experiments were performed in 6- or 12- well collagen I coated plates. After changing the medium or after cell wounding (see next section), cells were treated with sodium nitrite (1-100uM, Sigma, St. Louis, MO) or the NO donor, diethylenetriamine NONOate (DETA-NONOate, Cayman Chemical, Ann Arbor, MI; t1/2 = 20 h at 37°C) for up to 24 h. Cells were pretreated, where indicated, for 15 minutes with the NO or $NO_2^{\bullet}$ scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide [8] (c-PTIO, Alexis Biochemicals), nonspecific nitric oxide synthase (NOS) inhibitor, G -nitro-L-Arginine-Methyl Ester (L-NAME, Sigma), polyethylene glycol-superoxide dismutase (PEG-SOD, Sigma) and polyethylene glycol-catalase (PEG-catalase, Sigma). None of the agents used significantly affected cell morphology or viability under these conditions.

1.2.2 In vitro wound repair assay

To investigate the effects of sodium nitrite on epithelial wound repair, we used a common *in vitro* wound assay, in which confluent cell monolayers are mechanically wounded by creating a linear scratch. BEAS-2B and HBE1 cells were grown to confluence in 12-well tissue culture plates. After introduction of a linear wound of ~0.5 mm width using a sterile P200 pipette tip, cell monolayers were rinsed with BEGM to remove all cellular debris,

fresh media was added to each well, and appropriate reagents were administered. Wound closure was photographed serially for 24 hours, using an inverted microscope (Olympus, Center Valley, PA). Wound closure was expressed as a percentage of the initial wound area, quantitated using NIH ImageJ software ((initial wound area - 24h wound area)/initial wound area × 100%).

1.2.3 Measurement of nitrite levels in conditioned medium and cell lysates

The nitrite levels in the conditioned medium and cell lysates were measured by tri-iodide based reductive chemiluminescence using a Model-280 Nitric Oxide Analyzer (NOA) from Sievers Instruments. Confluent BEAS-2B cells grown in 10cm culture dishes were lysed with nitrite preservation solution (0.8 M potassium ferricyanide, 0.1M N-ethylmaleimide, 10% NP-40). 10 μl conditioned medium or 200ul cell lysate samples were injected into the NOA purge vessel containing acidic tri-iodide reagent (prepared freshly: 2.0 g potassium iodide and 1.3 g iodine, 40 ml distilled H_2O and 140 ml glacial acetic acid), as previously described [9]. Freshly made sodium nitrite solutions of known concentrations, prepared in phosphate buffered saline, were used as standards. The area under the curve of the samples was divided by the slope from the standard curve (area/pmol) and then divided by the volume of the sample injected (μl). This calculation provides the concentration of nitrite in the samples (μM).

1.2.4 Determination of cellular cGMP

Intracellular cGMP concentration was determined in the cell lysate using a competitive enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI). Cells were pretreated with a phosphodiesterase 5 inhibitor, sildenafil (5uM, Sigma) for 15min followed by sodium nitrite (50uM) or DETA-NONOate (25uM) treatment in the presence of sildenafil for 3 hours. After treatments, cells were extracted in cold 0.1 N HCL for 30 min at 4°C, and the protein precipitate was removed by centrifugation at 1,000 × g for 10 min. The cGMP levels were measured in the supernatant, expressed as fmol/mg protein.

1.2.5 Cellular ROS detection

Intracellular superoxide production was detected using the superoxide-sensitive fluorophore dihydroethidine (DHE, Sigma). DHE is oxidized to oxoethidium by superoxide. Oxoethidium binds to DNA, becoming highly fluorescent. After treatment, cells were loaded with 5 μM DHE in fresh media at 37°C in the incubator for 30 min. The cells were then rinsed with fresh media and were imaged immediately. Images were taken on an inverted fluorescent microscope.

1.2.6 PicoGreen Cell Proliferation Assay

Cell proliferation was determined by measuring the DNA content of cell lysates using a picoGreen® dsDNA Quantification Kit (Invitrogen). BEAS-2B or NHBE Cells were grown in 24 well plates with or without nitrite treatment. At 24 hour the culture medium was replaced with 0.1%v/v Triton X-100 in PBS and frozen at −80°C. Cells were then lysed by three freeze/thaw cycles and the assay performed as per manufacturer's instructions. Samples were analysed at wavelengths of 480nm (excitation) and 520nm (emission).

1.2.7 Cell viability assay

BEAS-2B or HBE1 cells (2×10^4 per well) were loaded in 96-well plates and maintained in BEGM. Cell viability was determined with the CellTiter 96 AQueous One Solution cell proliferation assay (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. Absorbances at 490 nm (test wavelength) and at 650 nm (reference wavelength)

were measured using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Bio-TEK Instruments Inc., Winooski, VT, USA).

1.2.7 Statistical analysis

All data are expressed as the mean ± SD. Student's t-test or one-way analysis of variance (ANOVA) were used as appropriate and indicated. A p value of ≤ 0.05 was considered significant.

1.3 Results

1.3.1 Sodium nitrite promotes airway epithelial wound repair

To evaluate the effect of sodium nitrite on airway epithelial wound repair *in vitro*, confluent monolayers of BEAS-2B cells were mechanically damaged and then exposed to various concentrations (1-100uM) of sodium nitrite. The cells treated with epithelial growth factor (EGF), known to potently increase wound healing, served as positive control. As illustrated in Figure 1, sodium nitrite significantly and potently (from 1 uM) enhanced wound repair in a linear wound assay in BEAS-2B cells at 24h compared to untreated control cells. The effect of sodium nitrite on wound closure was dose-dependent in the concentration range lower than 50uM, but inhibited at higher nitrite concentrations (100μM, Figure 1A). In addition, the effect on wound repair induced by nitrite was similar to the effect with an equivalent concentration of authentic NO, delivered using the NO donor DETA-NONOate, which produces an expected 2 moles of NO per mole (Figure 1B). To confirm the results of studies with BEAS-2B cells, the experiments were also performed with another human bronchial epithelial cell line, HBE1. Similar effects were observed with nitrite in HBE1 cell wound healing (Figure 1D). Treatment with sodium nitrite for 24 hours did not affect BEAS-2B cell viability (see Supplemental Figure 1).

1.3.2 NOS independent effects of nitrite

Since respiratory epithelium constitutively expresses nitric oxide synthase (NOS), which may account for the endogenous NO production [10], we treated cells with nitrite (50uM) in the presence of the nonspecific NOS inhibitor L-NAME (1mM) to rule out this possibility. We found exposure to L-NAME did not attenuate the wound repair induced by nitrite, suggesting a NOS-independent effect (Figure 2).

1.3.3 Nitrite levels in conditioned medium and cell lysates after sodium nitrite and NO donor treatments

In order to compare nitrite levels with both treatments, we measured nitrite concentration in BEAS-2B cell media at 24h after treatment (Figure 3A). The nitrite levels measured in conditioned medium were lower than the added concentration, at approximately 70% of the added nitrite, indicating consumption of extracellular nitrite by the cells. However, only approximately 30% of the added NO was measured as nitrite after DETA-NONOate treatment. These data suggest that despite similar effects on epithelial wound healing, the actual nitrite exposure levels were different in sodium nitrite and equivalent NO treated cells. These studies indicate to us that nitrite might be mediating NO-independent signaling effects on airway epithelial wound healing. With increased extracellular nitrite treatment, the nitrite levels in cell lysates went up (Figure 3B). However, the intracellular nitrite levels were substantially lower than the extracellular levels, suggesting nitrite serves as a signaling molecule at very low concentrations, and the movement of nitrite across cell membrane is a highly controlled process, which to date has not been characterized.

1.3.4 c-PTIO blocks sodium nitrite-induced wound repair

To further characterize mechanisms of nitrite-induced wound closure, cells were pretreated with c-PTIO. The protective effects of sodium nitrite and NO donor on wound repair were markedly diminished in the presence of 100uM c-PTIO, whereas c-PTIO alone did not affect wound repair in control cells (Figure 4). It should be noted that the NO scavenger c-PTIO also scavenges $NO_2^{\bullet}$ rapidly. In fact, the rate constants for the reaction of $NO_2^{\bullet}$ with c-PTIO to form the corresponding oxoammonium cations ($PTIO^+$s) and nitrite have been measured at 1.5 - 2 × 10^7 M^{-1} sec^{-1} [8].

1.3.5 Non-cGMP signaling induced by nitrite

In order to distinguish NO signaling via soluble guanylyl cyclase (sGC) versus alternative signaling pathways via $NO_2^{\bullet}$, we directly measured intracellular cGMP levels since one cellular target involved in NO-mediated signaling is the heme center of sGC, activation of which induces the production of cGMP [11]. As illustrated in Figure 5, we found that cellular cGMP production was not increased by nitrite treatment under normoxic condition (21% oxygen) while the NO donor produced robust cGMP formation, which suggested that nitrite enhanced wound healing via a non-cGMP signaling pathway.

1.3.6 ROS production is involved in sodium nitrite-induced wound repair

To investigate whether the stimulatory effect of sodium nitrite on airway epithelial wound closure involves reactive oxidative species (ROS) signaling, we detected superoxide formation during wounding using DHE staining. As shown in Figure 6A, mechanical wounding induced significant superoxide generation at the wound edges. We next exposed wounded BEAS-2B monolayers to cell permeable antioxidants PEG-SOD and PEG-catalase. PEG-SOD (400U/ml) pretreatment significantly increased BEAS-2B wound closure, while PEG-catalase (1kU/ml) inhibited the effects of nitrite on wound closure (Figure 6B), suggesting that H_2O_2 formation may be needed in the nitrite mediated signaling pathway. To further confirm this we evaluated the effect of low concentrations (5-25uM) of H_2O_2 on nitrite mediated wound healing, revealing enhanced basal wound repair and trends towards enhanced nitrite-induced wound repair by H_2O_2 (Figure 6C). We performed a number of inhibitor studies showing that the ROS is not produced by XO, mitochondria, or uncoupled eNOS (data not shown). This supports a possible role for dual oxidase (Duox), which has been previously described [12]. We did Western Blot analysis for 3-nitrotyrosine formation in the cells but did not see any difference with or without nitrite treatment (Supplemental Figure 2). However this is not unexpected as the formation of 3-nitrotyrosine requires 2 nitrogen dioxide equivalents and our nitrite concentrations are very low. It is more likely that $NO_2^{\bullet}$ radical formed in these reactions would oxidize glutathione and protein thiol targets.

1.3.7 Sodium nitrite promotes airway epithelial cell proliferation

To identify the effects of nitrite on airway epithelial cell proliferation, we used Picogreen assay to measure DNA content after nitrite treatment. As shown in Figure 7, sodium nitrite (10-50uM) promoted BEAS-2B and primary NHBE cell proliferation in a dose dependent manner, while catalase inhibited the effect. This result demonstrates the proliferative signaling effects of nitrite on airway epithelial cells and the role of H_2O_2 in bronchial epithelial cell proliferation as well.

1.3.8 ERK1/2 activation is involved in nitrite-induced wound repair

To gain insight into the possible downstream intracellular signaling which regulates cell migration and proliferation, a specific inhibitor of the ERK1/2 activation, PD098059 was used. Prior studies of *in vitro* epithelial cell migration and wound repair suggest that the

ERK1/2 pathway plays a critical role in cell migration and proliferation [13, 14], which can be activated by ROS and reactive nitrogen species (RNS) [15]. We found pretreatment with PD098059 (10uM) attenuated the stimulatory effect of nitrite after wounding while PD098059 by itself had no significant effect on the wound closure, suggesting downstream ERK1/2 activation is involved in nitrite-induced wound healing (Figure 8).

1.4 Discussion

The airway epithelium serves a primary role as a protective barrier against inhaled environmental toxins and microorganisms. In response to airway epithelial injury, repair processes are initiated and early events in the repair process include cell spreading, migration and proliferation [3, 16]. The main finding of our present study is that sodium nitrite enhances cell proliferation and wound healing of human airway epithelial cells in normoxic physiological concentrations. Moreover, our studies reveal that intracellular signaling after stimulation with nitrite under normoxia is independent of NO and cGMP generation, but involves oxidative reactions with hydrogen peroxide and downstream ERK1/2 activation.

In our study, the initiation of wound closure by sodium nitrite occurs at relatively low levels that are well within the range of the physiological conditions found in the human body. It has been reported that the nitrite levels in human plasma range from 100 nM to 1 uM [17-20]. The salivary nitrite levels can approach 1-2 mM after a dietary nitrate load [7, 21]. Nitrite is also detected in exhaled breath condensate [22], suggesting higher levels in airway lining fluid. However, the exact concentrations of nitrite to which pulmonary epithelial cells are physiologically exposed are not known. Our studies suggest that extracellular physiological concentrations of nitrite potently promote airway epithelial cell proliferation and repair in response to mechanical injury *in vitro*. However, very high non-physiologic levels of extracellular nitrite slowed the wound repair.

Prior studies suggest nitrite protects against tissue injury in a variety of ischemic diseases (liver and heart ischemia-reperfusion injury, hypoxic pulmonary vasoconstriction, and hemorrhagic stroke) via NO-dependent mechanisms [23, 24]. Nitrite is converted to bioactive NO at low pH and oxygen tension by reduction by deoxyhemoglobin, deoxymyoglobin, xanthine oxidase and other hemoproteins [25-29]. Virtually all studies showing authentic NO generation from nitrite are conducted under anaerobic conditions. However, it is not clear how cells could convert nitrite to NO under normoxic conditions. In the present study a number of findings suggest that nitrite is signaling independent of NO. The first is that all the experiments were performed under normoxic conditions. Providing more direct evidence, we found no formation of the NO-downstream second messenger cGMP after exposure to nitrite in these studies. While c-PTIO inhibited the wound healing effect of both NO and nitrite, c-PTIO can also rapidly scavenge $NO_2^{\bullet}$ [8]. Based on lack of cGMP stimulation but potent inhibition by c-PTIO, we considered the possibility that nitrite oxidation to $NO_2^{\bullet}$ could mediate signaling. Nitrite can be oxidized to $NO_2^{\bullet}$ via heme catalzyzed peroxidase chemistry. In this reaction H_2O_2 is required which reacts with a peroxidase or redox active heme (hemoglobin, myoglobin, cytochrome C, etc.) to form an oxoiron intermediate analogous to peroxidase compound I and II (FeIV= O) which can oxidize nitrite to $NO_2^{\bullet}$ [30, 31]. For example, myoglobin reacts according to a peroxidase-like cycle forming two active intermediates, which can induce one-electron oxidation of the substrates. The MbFe(IV)==O intermediate oxidizes nitrite to NO2•. In the second mechanism, H_2O_2 reacts with iron-bound nitrite to produce an active nitrating species, which has been assumed to be a protein-bound peroxynitrite species, MbFe(III)--N(O)OO.

We therefore explored the role of H_2O_2, based on the known chemistry of heme catalyzed peroxidase oxidation of nitrite to $NO_2^{\bullet}$. The inhibition of nitrite effects on wound healing by catalase, and augmentation with SOD and H_2O_2, suggest an important role of H_2O_2 in this signaling process, strongly indicating that nitrite oxidation to $NO_2^{\bullet}$ may mediate these effects. H_2O_2 is now known to serve as a cell signaling messenger in numerous contexts, such as cell survival, wound healing etc. The signaling role of H_2O_2 is effective at very low levels and excessive H_2O_2 poses the clear risk of oxidative stress [32]. In zebra fish wound margin tissue, the H_2O_2 signal peaked 20 minutes after wounding [33] and low levels of H_2O_2 generated by NADPH oxidases (NOX) enable leukocyte recruitment and wound healing [34]. In airway epithelial cells it is reported that ROS is generated by a core component homolog dual oxidase 1 (Duox1) [12, 35, 36] and the wound edge is a major source of reactive oxygen species generated via Duox [12]. With increased extracellular nitrite and H_2O_2 levels, nitrogen dioxide ($NO_2^{\bullet}$), the one-electron oxidation product of nitrite may be generated, which could drive a number of post-translational protein modifications, including nitration of critical tyrosine residues, oxidation of thiols to form sulfenic and sulfinic groups, and S-nitrosation via thiyl radical formation or N_2O_3 formation. All of these modifications could alter downstream protein activities. In the present study, our results are consistent with previous reports, and demonstrate the importance of downstream ERK1/2 activation in epithelial cell proliferation and wound healing, supported by the result that pretreatment with PD098059 attenuated the stimulatory effect of nitrite. ERK1/2 can be activated by ROS and RNS via phosphorylation [37-41] or tyrosine nitration [42]. In addition, a variety of potential targets of ROS and RNS could contribute to ERK1/2 activation, including cell surface receptors, G proteins, upstream kinases, protein phosphatases and proteasome components, each of which could be direct or indirect targets of reactive oxygen or nitrogen species, thereby modulating the duration and magnitude of ERK1/2 activation [15].

Here we propose a new hypothesis for nitrite signaling under normoxic and hypoxic conditions based on the unique redox properties of nitrite. Nitrite is very stable *in vivo* with a half-life in humans of approximately 30-minutes [43, 44]. Its unique redox properties provide an opportunity for a one-electron oxidation to $NO_2^{\bullet}$ and a one electron reduction to NO under different oxygen conditions. The one proton adduct, nitrous acid, is readily reduced to NO and also can directly nitrosate thiol targets. Additional reactions with NO and $NO_2^{\bullet}$, or with nitrite, NO and ferric hemoglobin can generate N_2O_3 [45, 46]. Finally, the peroxidase reactions with hemoproteins and the autocatalytic reaction of nitrite with oxyhemoglobin can produce $NO_2^{\bullet}$ [47]. In Figure 9 we propose a model for nitrite signaling via oxidative and reductive pathways in biology.

There are some potential limitations to this study. First, the simulative effect of nitrite on wound healing was detected mostly in immortalized cell lines. We did not see nitrite enhanced the wound closure of primary NHBEs (data not shown). However, we found nitrite increased NHBE cell proliferation. Since the wound repair process includes cell spreading, migration and cell proliferation, the effects of nitrite on wound healing may vary in different cell types. Second, in the present study, airway epithelial cells were grown in isolated monolayers, rather than in contact with extracellular matrix and endothelial/ mesenchymal cells. It is possible that the wound repair is different from the conditions *in vivo*. Finally, we have not directly measured $NO_2^{\bullet}$ levels, as these are very low in cells. Further biochemical and *in vivo* studies are needed to confirm the validity of these findings and our hypothesis that nitrite signals via oxidative, and specifically $NO_2^{\bullet}$ dependent pathways.

In conclusion, our findings demonstrate that low, physiologically relevant concentrations of nitrite promote airway epithelial cell proliferation and wound repair via cGMP independent

pathway. Interaction with H_2O_2 and downstream activation of the ERK1/2 pathway plays a central role in this process. This study provides new information about the mechanisms involved in nitrite mediated signaling under normoxia. The role of nitrite in promoting wound healing raises the possibility of therapeutic application of this previously considered inert anion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

[1]. Zahm JM, Kaplan H, Herard AL, Doriot F, Pierrot D, Somelette P, Puchelle E. Cell migration and proliferation during the in vitro wound repair of the respiratory epithelium. Cell Motil Cytoskeleton. 1997; 37:33–43. [PubMed: 9142437]

[2]. Herard AL, Zahm JM, Pierrot D, Hinnrasky J, Fuchey C, Puchelle E. Epithelial barrier integrity during in vitro wound repair of the airway epithelium. Am J Respir Cell Mol Biol. 1996; 15:624–632. [PubMed: 8918369]

[3]. Zahm JM, Chevillard M, Puchelle E. Wound repair of human surface respiratory epithelium. Am J Respir Cell Mol Biol. 1991; 5:242–248. [PubMed: 1910810]

[4]. Gladwin MT, Crawford JH, Patel RP. The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation. Free Radic Biol Med. 2004; 36:707–717. [PubMed: 14990351]

[5]. Zhu S, Ware LB, Geiser T, Matthay MA, Matalon S. Increased levels of nitrate and surfactant protein a nitration in the pulmonary edema fluid of patients with acute lung injury. Am J Respir Crit Care Med. 2001; 163:166–172. [PubMed: 11208643]

[6]. Bove PF, Wesley UV, Greul AK, Hristova M, Dostmann WR, van der Vliet A. Nitric oxide promotes airway epithelial wound repair through enhanced activation of MMP-9. Am J Respir Cell Mol Biol. 2007; 36:138–146. [PubMed: 16980554]

[7]. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov. 2008; 7:156–167. [PubMed: 18167491]

[8]. Goldstein S, Russo A, Samuni A. Reactions of PTIO and carboxy-PTIO with *NO, *NO2, and O2-*. J Biol Chem. 2003; 278:50949–50955. [PubMed: 12954619]

[9]. MacArthur PH, Shiva S, Gladwin MT. Measurement of circulating nitrite and S-nitrosothiols by reductive chemiluminescence. J Chromatogr B Analyt Technol Biomed Life Sci. 2007; 851:93–105.

[10]. Robbins RA, Springall DR, Warren JB, Kwon OJ, Buttery LD, Wilson AJ, Adcock IM, Riveros-Moreno V, Moncada S, Polak J, et al. Inducible nitric oxide synthase is increased in murine lung epithelial cells by cytokine stimulation. Biochem Biophys Res Commun. 1994; 198:835–843. [PubMed: 7509602]

[11]. Giordano D, Magaletti DM, Clark EA. Nitric oxide and cGMP protein kinase (cGK) regulate dendritic-cell migration toward the lymph-node-directing chemokine CCL19. Blood. 2006; 107:1537–1545. [PubMed: 16249377]

[12]. Wesley UV, Bove PF, Hristova M, McCarthy S, van der Vliet A. Airway epithelial cell migration and wound repair by ATP-mediated activation of dual oxidase 1. J Biol Chem. 2007; 282:3213–3220. [PubMed: 17135261]

[13]. Sharma GD, He J, Bazan HE. p38 and ERK1/2 coordinate cellular migration and proliferation in epithelial wound healing: evidence of cross-talk activation between MAP kinase cascades. J Biol Chem. 2003; 278:21989–21997. [PubMed: 12663671]

[14]. Chen WL, Lin CT, Li JW, Hu FR, Chen CC. ERK1/2 activation regulates the wound healing process of rabbit corneal endothelial cells. Curr Eye Res. 2009; 34:103–111. [PubMed: 19219681]

[15]. Chu CT, Levinthal DJ, Kulich SM, Chalovich EM, DeFranco DB. Oxidative neuronal injury. The dark side of ERK1/2. Eur J Biochem. 2004; 271:2060–2066. [PubMed: 15153095]

[16]. Keenan KP, Combs JW, McDowell EM. Regeneration of hamster tracheal epithelium after mechanical injury. I. Focal lesions: quantitative morphologic study of cell proliferation. Virchows Arch B Cell Pathol Incl Mol Pathol. 1982; 41:193–214. [PubMed: 6191435]

[17]. Gorenflo M, Zheng C, Poge A, Bettendorf M, Werle E, Fiehn W, Ulmer HE. Metabolites of the L-arginine-NO pathway in patients with left-to-right shunt. Clin Lab. 2001; 47:441–447. [PubMed: 11596905]

[18]. Meulemans A, Delsenne F. Measurement of nitrite and nitrate levels in biological samples by capillary electrophoresis. J Chromatogr B Biomed Appl. 1994; 660:401–404. [PubMed: 7866533]

[19]. Gladwin MT, Shelhamer JH, Schechter AN, Pease-Fye ME, Waclawiw MA, Panza JA, Ognibene FP, Cannon RO 3rd. Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. Proc Natl Acad Sci U S A. 2000; 97:11482–11487. [PubMed: 11027349]

[20]. Gladwin MT, Schechter AN, Kim-Shapiro DB, Patel RP, Hogg N, Shiva S, Cannon RO 3rd, Kelm M, Wink DA, Espey MG, Oldfield EH, Pluta RM, Freeman BA, Lancaster JR Jr. Feelisch M, Lundberg JO. The emerging biology of the nitrite anion. Nat Chem Biol. 2005; 1:308–314. [PubMed: 16408064]

[21]. Lundberg JO, Gladwin MT, Ahluwalia A, Benjamin N, Bryan NS, Butler A, Cabrales P, Fago A, Feelisch M, Ford PC, Freeman BA, Frenneaux M, Friedman J, Kelm M, Kevil CG, Kim-Shapiro DB, Kozlov AV, Lancaster JR Jr. Lefer DJ, McColl K, McCurry K, Patel RP, Petersson J, Rassaf T, Reutov VP, Richter-Addo GB, Schechter A, Shiva S, Tsuchiya K, van Faassen EE, Webb AJ, Zuckerbraun BS, Zweier JL, Weitzberg E. Nitrate and nitrite in biology, nutrition and therapeutics. Nat Chem Biol. 2009; 5:865–869. [PubMed: 19915529]

[22]. Chladkova J, Krcmova I, Chladek J, Cap P, Micuda S, Hanzalkova Y. Validation of nitrite and nitrate measurements in exhaled breath condensate. Respiration. 2006; 73:173–179. [PubMed: 16549945]

[23]. Dezfulian C, Shiva S, Alekseyenko A, Pendyal A, Beiser DG, Munasinghe JP, Anderson SA, Chesley CF, Vanden Hoek TL, Gladwin MT. Nitrite therapy after cardiac arrest reduces reactive oxygen species generation, improves cardiac and neurological function, and enhances survival via reversible inhibition of mitochondrial complex I. Circulation. 2009; 120:897–905. [PubMed: 19704094]

[24]. Shiva S, Gladwin MT. Nitrite mediates cytoprotection after ischemia/reperfusion by modulating mitochondrial function. Basic Res Cardiol. 2009; 104:113–119. [PubMed: 19242636]

[25]. Gladwin MT, Grubina R, Doyle MP. The new chemical biology of nitrite reactions with hemoglobin: R-state catalysis, oxidative denitrosylation, and nitrite reductase/anhydrase. Acc Chem Res. 2009; 42:157–167. [PubMed: 18783254]

[26]. Gladwin MT, Kim-Shapiro DB. The functional nitrite reductase activity of the heme-globins. Blood. 2008; 112:2636–2647. [PubMed: 18596228]

[27]. Tiso M, Tejero J, Basu S, Azarov I, Wang X, Simplaceanu V, Frizzell S, Jayaraman T, Geary L, Shapiro C, Ho C, Shiva S, Kim-Shapiro DB, Gladwin MT. Human neuroglobin functions as a redox-regulated nitrite reductase. J Biol Chem. 286:18277–18289. [PubMed: 21296891]

[28]. Minneci PC, Deans KJ, Shiva S, Zhi H, Banks SM, Kern S, Natanson C, Solomon SB, Gladwin MT. Nitrite reductase activity of hemoglobin as a systemic nitric oxide generator mechanism to detoxify plasma hemoglobin produced during hemolysis. Am J Physiol Heart Circ Physiol. 2008; 295:H743–754. [PubMed: 18552166]

[29]. Zuckerbraun BS, Shiva S, Ifedigbo E, Mathier MA, Mollen KP, Rao J, Bauer PM, Choi JJ, Curtis E, Choi AM, Gladwin MT. Nitrite potently inhibits hypoxic and inflammatory pulmonary arterial hypertension and smooth muscle proliferation via xanthine oxidoreductase-dependent nitric oxide generation. Circulation. 121:98–109. [PubMed: 20026772]

[30]. Bian K, Gao Z, Weisbrodt N, Murad F. The nature of heme/iron-induced protein tyrosine nitration. Proc Natl Acad Sci U S A. 2003; 100:5712–5717. [PubMed: 12709594]

[31]. Brennan ML, Wu W, Fu X, Shen Z, Song W, Frost H, Vadseth C, Narine L, Lenkiewicz E, Borchers MT, Lusis AJ, Lee JJ, Lee NA, Abu-Soud HM, Ischiropoulos H, Hazen SL. A tale of two controversies: defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. J Biol Chem. 2002; 277:17415–17427. [PubMed: 11877405]

[32]. Halliwell B, Clement MV, Long LH. Hydrogen peroxide in the human body. FEBS Lett. 2000; 486:10–13. [PubMed: 11108833]

[33]. Martin P, Feng Y. Inflammation: Wound healing in zebrafish. Nature. 2009; 459:921–923. [PubMed: 19536251]

[34]. Niethammer P, Grabher C, Look AT, Mitchison TJ. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. Nature. 2009; 459:996–999. [PubMed: 19494811]

[35]. De Deken X, Wang D, Many MC, Costagliola S, Libert F, Vassart G, Dumont JE, Miot F. Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. J Biol Chem. 2000; 275:23227–23233. [PubMed: 10806195]

[36]. Geiszt M, Witta J, Baffi J, Lekstrom K, Leto TL. Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. FASEB J. 2003; 17:1502–1504. [PubMed: 12824283]

[37]. Lee YS, Bak EJ, Kim M, Park W, Seo JT, Yoo YJ. Induction of IL-8 in periodontal ligament cells by H(2)O (2). J Microbiol. 2008; 46:579–584. [PubMed: 18974961]

[38]. Rygiel TP, Mertens AE, Strumane K, van der Kammen R, Collard JG. The Rac activator Tiam1 prevents keratinocyte apoptosis by controlling ROS-mediated ERK phosphorylation. J Cell Sci. 2008; 121:1183–1192. [PubMed: 18349077]

[39]. Loo AE, Ho R, Halliwell B. Mechanism of hydrogen peroxide-induced keratinocyte migration in a scratch-wound model. Free Radic Biol Med. 51:884–892. [PubMed: 21699973]

[40]. Chen JX, Zeng H, Tuo QH, Yu H, Meyrick B, Aschner JL. NADPH oxidase modulates myocardial Akt, ERK1/2 activation, and angiogenesis after hypoxia-reoxygenation. Am J Physiol Heart Circ Physiol. 2007; 292:H1664–1674. [PubMed: 17220182]

[41]. Ruffels J, Griffin M, Dickenson JM. Activation of ERK1/2, JNK and PKB by hydrogen peroxide in human SH-SY5Y neuroblastoma cells: role of ERK1/2 in H2O2-induced cell death. Eur J Pharmacol. 2004; 483:163–173. [PubMed: 14729104]

[42]. Iwagaki A, Choe N, Li Y, Hemenway DR, Kagan E. Asbestos inhalation induces tyrosine nitration associated with extracellular signal-regulated kinase 1/2 activation in the rat lung. Am J Respir Cell Mol Biol. 2003; 28:51–60. [PubMed: 12495932]

[43]. Hon YY, Sun H, Dejam A, Gladwin MT. Characterization of erythrocytic uptake and release and disposition pathways of nitrite, nitrate, methemoglobin, and iron-nitrosyl hemoglobin in the human circulation. Drug Metab Dispos. 38:1707–1713. [PubMed: 20634335]

[44]. Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, Partovi K, Pelletier MM, Oldfield EH, Cannon RO 3rd, Schechter AN, Gladwin MT. Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation. Circulation. 2007; 116:1821–1831. [PubMed: 17893272]

[45]. Fernandez BO, Lorkovic IM, Ford PC. Mechanisms of ferriheme reduction by nitric oxide: nitrite and general base catalysis. Inorg Chem. 2004; 43:5393–5402. [PubMed: 15310219]

[46]. Basu S, Grubina R, Huang J, Conradie J, Huang Z, Jeffers A, Jiang A, He X, Azarov I, Seibert R, Mehta A, Patel R, King SB, Hogg N, Ghosh A, Gladwin MT, Kim-Shapiro DB. Catalytic generation of N2O3 by the concerted nitrite reductase and anhydrase activity of hemoglobin. Nat Chem Biol. 2007; 3:785–794. [PubMed: 17982448]

[47]. Grubina R, Huang Z, Shiva S, Joshi MS, Azarov I, Basu S, Ringwood LA, Jiang A, Hogg N, Kim-Shapiro DB, Gladwin MT. Concerted nitric oxide formation and release from the simultaneous reactions of nitrite with deoxy- and oxyhemoglobin. J Biol Chem. 2007; 282:12916–12927. [PubMed: 17322300]

Highlights

> Sodium nitrite potently enhanced airway epithelium wound healing. > Nitrite does not increase cGMP levels under normoxic condition. > Oxidative reactions with H_2O_2 and downstream ERK1/2 activation are involved.

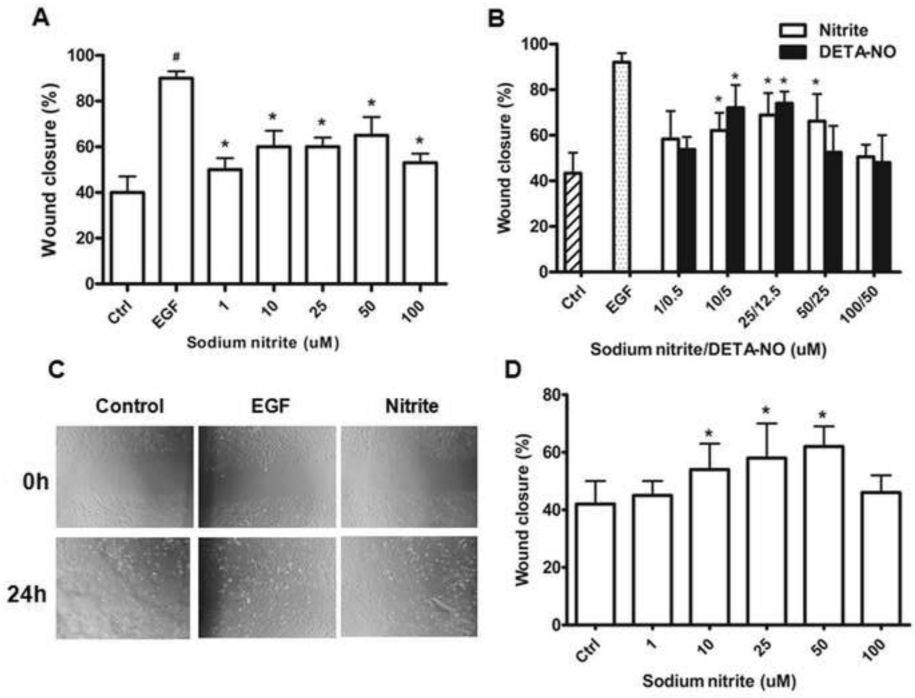

Fig. 1.

BEAS-2B and HBE1 cell wound healing were enhanced by sodium nitrite. (A) BEAS-2B cells were treated with varying doses of sodium nitrite (1-100uM) after wounding. Wound closure was significantly improved by sodium nitrite at 24 hours. (B) BEAS-2B wound repair was enhanced by both sodium nitrite (1-100uM) and by the NO donor, DETA-NONOate (0.5-50uM). (C) Confluent BEAS-2B cells were wounded by a P200 pipette tip and wound closure was monitored at 24h using a phase contrast microscope. (D) HBE1 cells were treated with varying doses of sodium nitrite (1-100uM) after wounding. Wound closure was significantly improved by sodium nitrite at 24 hours. Data represent mean ± SD. $^{*}p < 0.05$, compared with control. $^{\#}p < 0.01$, compared with control.

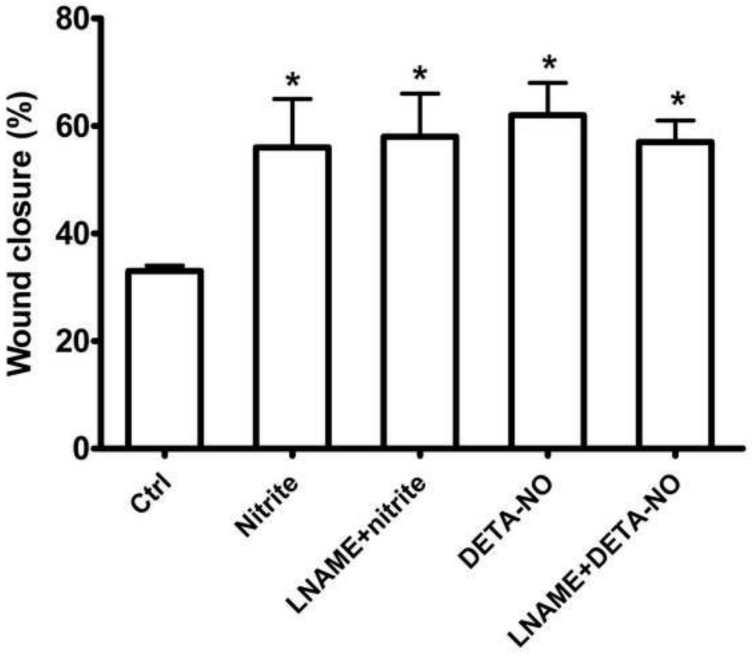

Fig. 2.

L-NAME did not attenuate the wound healing induced by nitrite or DETA-NONOate. BEAS-2B cells were pretreated with nonspecific NOS inhibitor L-NAME (1mM) for 15min, followed by treatment with nitrite (50uM) or DETA-NONOate (25uM) in the presence of L-NAME for 24h. Data represent mean ± SD. $^{*}p < 0.05$, compared with control.

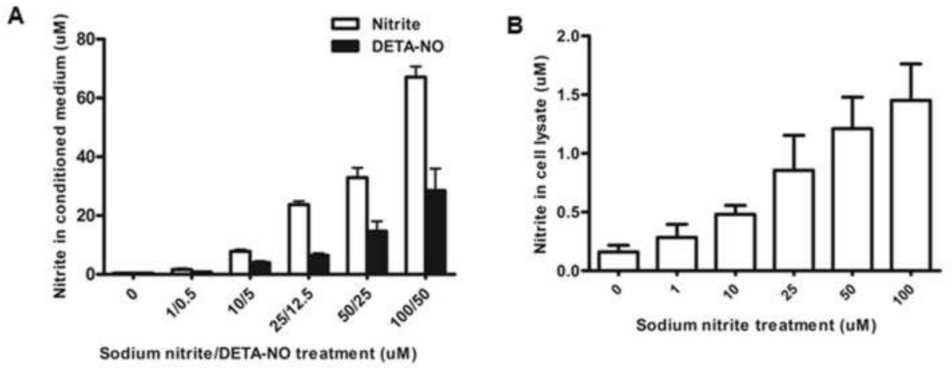

Fig. 3.

Nitrite levels in conditioned medium and cell lysates of cultured BEAS-2B cells following treatment with sodium nitrite or DETA-NONOate. (A) Conditioned medium was collected after treatment with sodium nitrite (1-100uM) or DETA-NONOate (0.5-50uM) for 24 hours. (B) Cell lysates were collected after treatment with sodium nitrite (1-100uM) for 24 hours. Nitrite levels were measured by using Nitric Oxide Analyzer. Data represent mean ± SD.

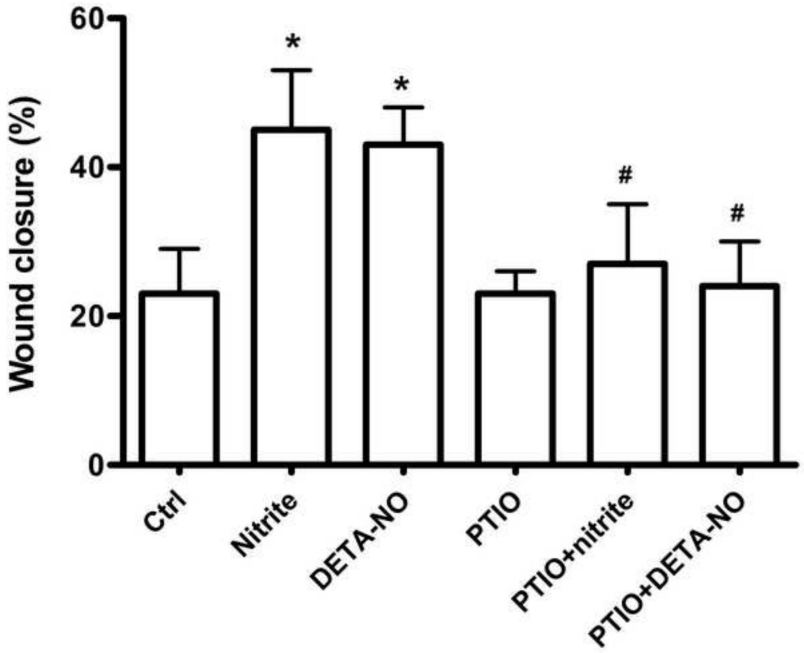

Fig. 4.

c-PTIO diminishes the improvement effects of sodium nitrite and DETA-NONOate on wound repair of BEAS-2B. BEAS-2B cells were pretreated with c-PTIO (100uM) for 15min, followed by treatment with sodium nitrite (50uM) or DETA-NONOate (25uM) for 24h. Data represent mean ± SD. $^{*}p < 0.05$, compared with control. $^{\#}p < 0.05$, compared with nitrite or DETA-NONOate.

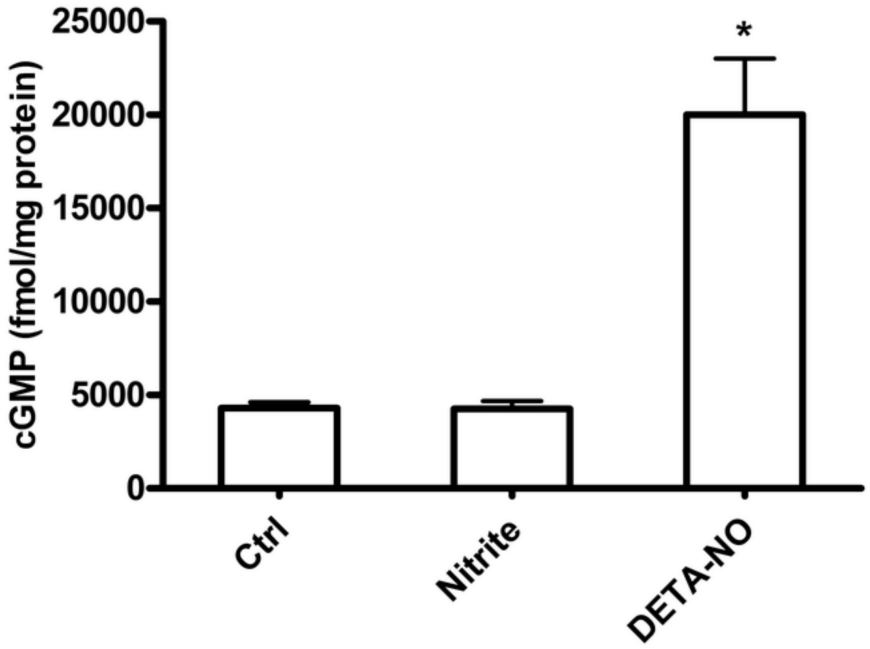

Fig. 5.

Cellular cGMP production was not increased by nitrite treatment while NO donor produced robust cGMP formation. Confluent BEAS-2B cells were pretreated with sildenafil (5uM), followed by treatment with sodium nitrite (50uM) or DETA-NONOate (25uM) for 3 hours. Cyclic GMP levels were determined in BEAS-2B cell lysates. Data represent mean ± SD. $^{*}p < 0.01$, compared with control.

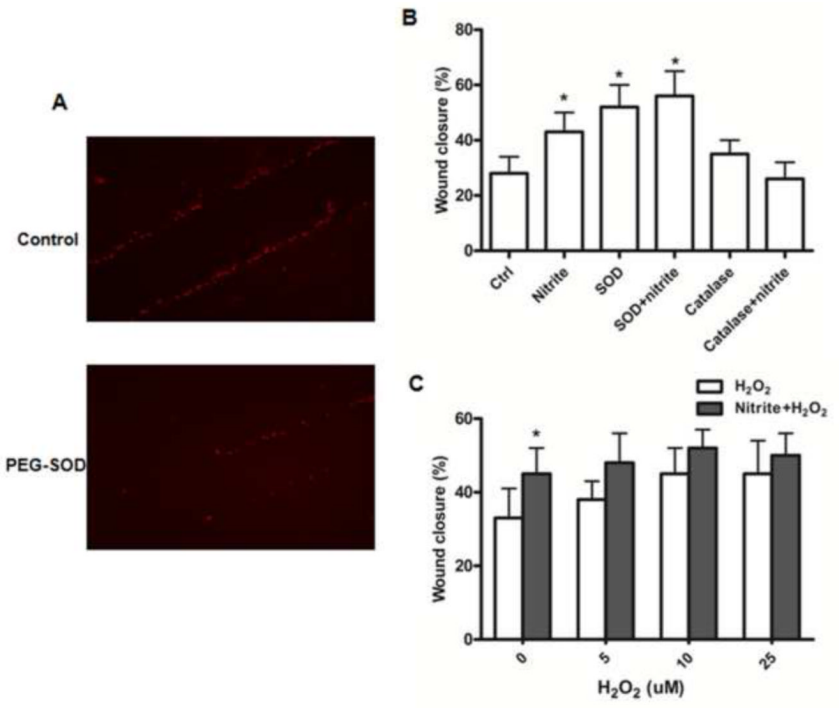

Fig. 6.

ROS production is involved in nitrite-induced airway epithelial wound repair. (A) Mechanical wounding induced significant superoxide generation at the wound edges which was inhibited by PEGSOD. (B) PEG-SOD improved the protective effect of nitrite while PEG-catalase inhibited the protective effect of nitrite. BEAS-2B Cells were pretreated with PEG-SOD (400U/ml) or PEG-catalase (1kU/ml) for 15min, followed by treatment with sodium nitrite (50uM) for 24h. (C) Low concentration of hydrogen peroxide (H_2O_2) tended to improve the protective effect of nitrite. BEAS-2B cells were treated with sodium nitrite (50uM) with or without varying concentrations of H_2O_2 (5-25uM). Data represent mean ± SD. *$p < 0.05$, compared with control.

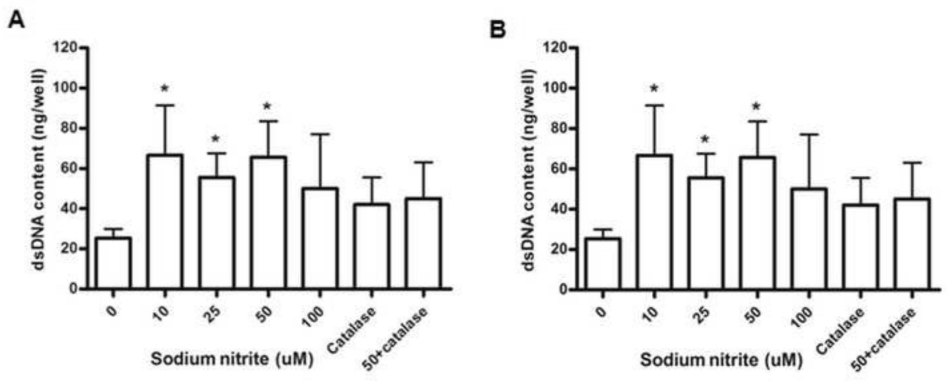

Fig. 7.

Sodium nitrite promoted BEAS-2B and NHBE cell proliferation. (A) Confluent BEAS-2B cells were treated with varying doses of sodium nitrite (10-100uM) or catalase (1kU/ml) after wounding. (B) Confluent NHBE cells were treated with varying doses of sodium nitrite (10-100uM) or catalase (1kU/ml) after wounding. Data represent mean ± SD. *$p < 0.05$, compared with control.

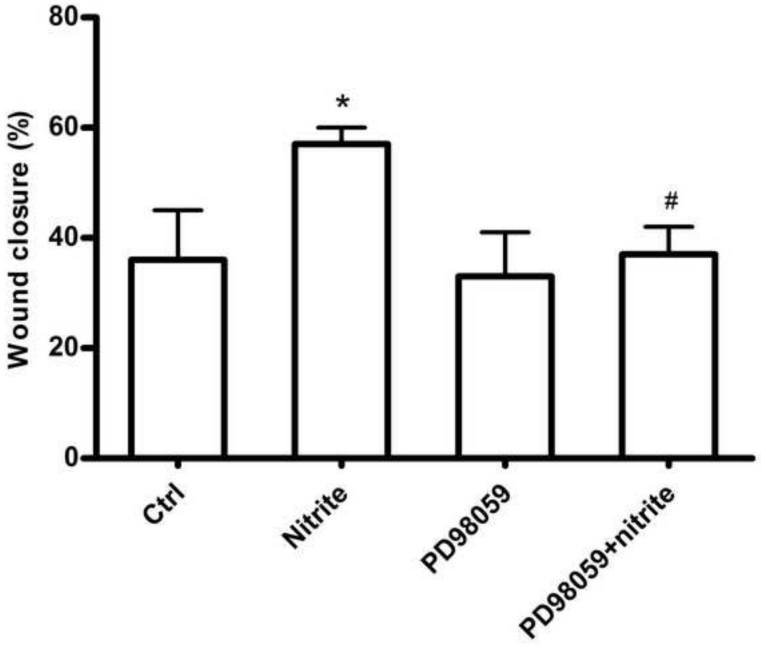

Fig. 8.

ERK1/2 activation is involved in nitrite-induced airway epithelial wound repair. BEAS-2B cells were pretreated with PD98059 (10uM) for 15min, followed by treatment with sodium nitrite (50uM) for 24h. Data represent mean ± SD. $^{*}p < 0.05$, compared with control. $^{\#}p < 0.05$, compared with nitrite treated group.

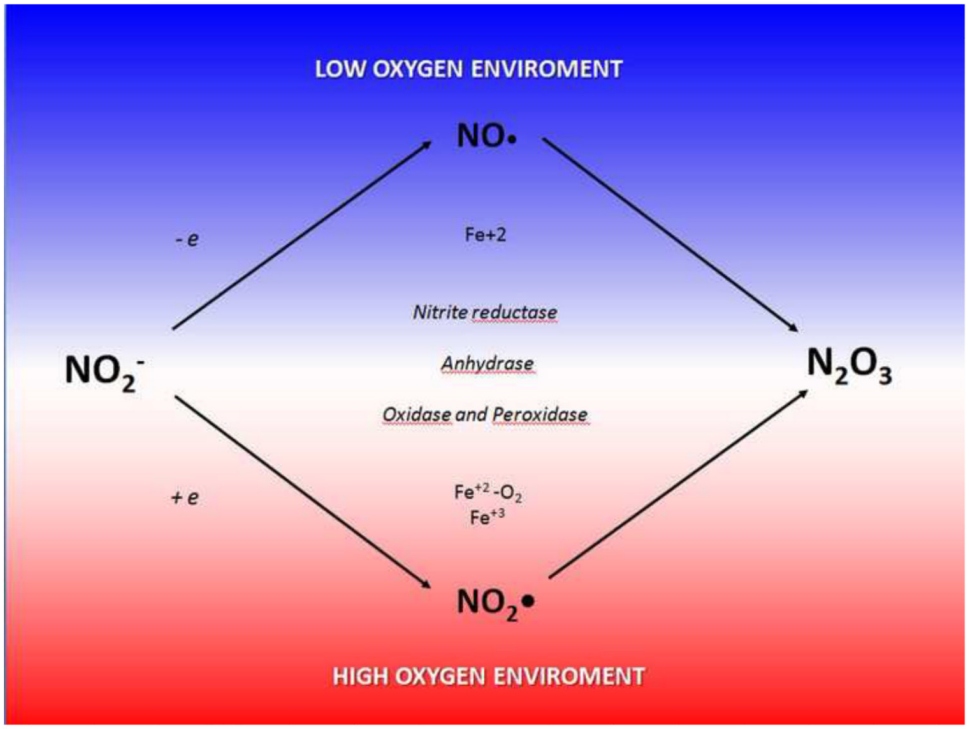

Fig. 9.

A proposed model for nitrite signaling via oxidative and reductive pathways in biology. Nitrite can be one-electron oxidized to $NO_2^{\bullet}$ and one electron reduced to NO under different oxygen conditions, mediating biological signaling cascades.