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Normoxic cyclic GMP-independent oxidative signaling by nitrite enhances airway epithelial cell proliferation and wound healing

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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 ($NO_2^{\cdot}$) 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 1 μM). The effect of nitrite was blocked by the NO and $NO_2^{\cdot}$ 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/$NO_2^{\cdot}$ 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, $NO_2^{\cdot}$.

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 cell 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 cell 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, $NO_2^{\cdot}$.

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Material and methods

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 (1–100 μM, 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 min with the NO or NO_2^- 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.

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 h, 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 − 24 h wound area)/initial wound area × 100%).

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 10 cm culture dishes were lysed with nitrite preservation solution (0.8 M potassium ferricyanide, 0.1 M *N*-ethylmaleimide, 10% NP-40). 10 μl conditioned medium or 200 μl 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).

Determination of cellular cGMP

Intracellular cGMP concentration was determined in the cell lysates using a competitive enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI). Cells were pretreated with a phosphodiesterase 5 inhibitor, sildenafil (5 μM, Sigma) for 15 min followed by sodium nitrite (50 μM) or DETA-NONOate (25 μM) treatment in the presence of sildenafil for 3 h. 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 1000 × g for 10 min. The cGMP levels were measured in the supernatant, expressed as fmol/mg protein.

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.

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 h 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 480 nm (excitation) and 520 nm (emission).

Cell viability assay

BEAS-2B or HBE1 cells (1×10^4 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).

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 $\leqslant 0.05$ was considered significant.

Results

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 (1–100 μM) of sodium nitrite. The cells treated with epithelial growth factor (EGF), known to potently increase wound healing, served as positive control. As illustrated in Fig. 1, sodium nitrite significantly and potently (from 1 μM) enhanced wound repair in a linear wound assay in BEAS-2B cells at 24 h compared to untreated control cells. The effect of sodium nitrite on wound closure was dose-dependent in the concentration range lower than 50 μM, but inhibited at higher nitrite concentration (100 μM, Fig. 1A). In addition, the effect on wound repair induced by nitrite was similar to the effect with an equivalent concentration of authentic NO,