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Measuring nitrate reductase activity from human and rodent tongues

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ABSTRACT

Reduction of salivary nitrate to nitrite by oral microbes expressing nitrate-reductase has emerged as a crucial pathway in systemic NO homeostasis in humans and other mammals. Selective depletion of oral microbes prevents dietary nitrate-dependent lowering of blood pressure, inhibition of platelet aggregation and ischemic injury. To date, most studies interrogate enterosalivary nitrate reduction by following changes in saliva or plasma nitrite and NO-signaling (functional) end points. Little is known about whether, and if so how, nitrate-reductase enzymatic activity per se (i.e. independent of nitrate levels) is a variable and may account for any individual to individual variation. Here, we describe a minimally invasive protocol that allows for NR activity determination from human, rat and mouse tongue scrapes/swabs. We validate this method using selective application of antiseptic agents to the distal tongue surface which decreased NR activity by >80% and show that bacterial number is a significant variable in measured NR activities between males and females. Also, we show that NR activity is >80% lower in smokers (humans) and after bromine gas exposure (mice), suggesting that exposure to inhaled reactive substances inhibit NR activity identifying a potentially new mechanism by which environmental toxicants promote dysfunction in NO-bioavailability. The described method will facilitate studies testing whether NR specific activity is a variable in different pathophysiologic settings, and in turn how this activity modulates enterosalivary nitrate-reduction.

1. Introduction

While the major source of nitric oxide (NO) in mammals are the nitric oxide synthases (NOS), recent studies have shown alternate sources also exist. Specifically, dietary nitrate can be reduced to nitrite, which in turn is reduced to NO and other nitrosating species at low oxygen tensions and low pH ensuring NO-homeostasis in hypoxic tissues [1,2]. Endogenously formed nitrate or dietary nitrate, is first concentrated into the saliva reaching millimolar concentrations. There, orally residing nitrate reducing bacteria, concentrated on the dorsal tongue, reduce nitrate to nitrite [3]. The nitrite is swallowed after which it can be further reduced to stimulate NO-dependent signaling processes by several putative mechanisms involving metalloproteins [4,5]. Functionality of this enterosalivary nitrate circuit has been demonstrated in human and experimental models with compelling data showing that nitrate consumption lowers blood pressure, inhibits platelet aggregation, improves exercise performance and inhibits inflammatory tissue injury [6–9]. Most importantly, these effects are abrogated with prior depletion of the oral microbiome using antiseptic mouthwash.

Key components of the enterosalivary circuit of NO formation are the oral facultative anaerobes expressing active nitrate reductases. Several studies have demonstrated that nitrate reductase activity is concentrated in the posterior dorsal region of the tongue with at least 15 species of bacteria expressing nitrate reductase identified from human samples [10,11]. To date, the majority of measures used to follow enterosalivary nitrate-reduction are changes in saliva and plasma nitrite levels after nitrate administration, together with functional end points (e.g. blood pressure, exercise performance or platelet aggregation). Few studies have directly measured nitrate reductase (NR) activity. In one protocol, human volunteers held potassium nitrate solution in the mouth

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and then nitrite formation measured after a given time. Kanady and Jones et al. swabbed tongues, then added nitrate and followed nitrite formation ex vivo [12]. However, to our knowledge no systematic and reproducible protocol has been described assessing oral NR activity. Our objective was to further develop a method for NR activity determination, with our primary goal being to measure NR specific activity, controlling for bacterial number. We show that under normal conditions, NR activity may vary from individual to individual, but this effect is mediated by varying amounts of bacteria collected in tongue scrapes. Our secondary goal was to develop a protocol that can be used with mice with a view to test if NR activity changes in different pathophysiological conditions. Using human and animal models, we demonstrate that NR activity is lower in instances of exposure to inhaled toxicants (such as cigarette smoke and bromine) and suggest that this may play a role in the development of diseases associated with environmental toxins.

2. Materials and methods

2.1. Materials

Sterile wood cotton tipped applicators (6 cm length) were purchased from Fisher (Cat. No. 23-400-115). Sterile disposable multifunction Lab spatulas were purchased from Sigma Aldrich (Z677787-100 EA). 20% Chlorhexidine digluconate solution was purchased from Sigma Aldrich (C9394-25 ML). Brain heart infusion (BHI) broth and tryptic soy blood agar (TSBA) was purchased from anaerobes systems (Morgan Hill, CA). Ketamine HCL 100 mg/ml and Xylazine 100 mg/ml solutions were purchased from VetOne (Boise, ID). Male and female C57/Bl6 mice (10–12 weeks, 20–25 g) and Sprague Dawley rats (8–10weeks, 200–300 g) were purchased from Envigo (Indianapolis, IN). All protocols involving animals were reviewed and approved by the UAB IACUC committee.

2.2. Human subjects

Healthy human subjects who were non-smokers or active smokers (>20 cigarettes per week) were enrolled. Subjects were identified after response to fliers or using the UAB Lung Health Center database. All enrolled subjects did not use antiseptic or over the counter mouthwash (at least 1 month), were not currently or recently (last 3 months) on antibiotics. All procedures were according to UAB Institutional Review Board approved procedures.

2.3. Collection of posterior tongue swab

2.3.1. Human

Scrapings from the posterior tongue were collected from male and female adults using the narrow end of a sterile disposable lab spatula. Five gentle scrapes were performed in one direction left to right, followed by a further 5 scrapes in the other direction. Scrape volumes of approximately 30 μl were collected. 70 μl of normal saline was used to rinse the spatula and total volume (100 μl) collected into eppendorf tubes. 400 μL of BHI was then added to the samples which were then vortex mixed for 30–45 s. All protocols were approved by institutional review board.

2.3.2. Mice

Male and female C57/Bl6 mice (10–12weeks) were briefly anesthetized by intramuscular injection of xylazine and ketamine (7 and 70 mg/kg body weight respectively) into the lower left femoris region. The oral cavity was exposed by suspending mice from the top jaw. Tongues were gently pulled out from the oral cavity using blunted forceps. Sterile cotton tipped applicators soaked in 200 μl of sterile saline were then used to swab the posterior dorsum of the tongue with 10 identical strokes from back to front. Care was taken to swab only the posterior tongue. Mice were then returned back to cages and typically awoke within 20–30min. Applicators were placed into 1 ml of BHI and briefly vortex mixed for 30–45 s. After mixing, applicators were removed and placed in to empty Eppendorf tubes and centrifuged at 5000 rpm for 45 s to remove excess culture liquid from the cotton fibers. Any excess culture liquid obtained was then transferred to the original 1 mL of BHI culture broth.

2.3.3. Rat

Male and female Sprague Dawley rats were anesthetized by intraperitoneal injection of xylazine and ketamine (4.5 and 45 mg/kg body weight respectively) and tongue swabs collected as described for mice.

All procedures involving animals were approved by institutional IACUC.

2.4. Human nitrate reductase activity

After mixing of tongue scrapes in BHI broth, two aliquots (100 μl each) were incubated at 37 °C for 10min. Ten μl was then taken to measure baseline nitrite levels. Water (vehicle control) was added to one aliquot, and sodium nitrate (varying concentrations) added to the other and both incubated at 37 °C. At indicated times, samples were vortex mixed (~5sec) to ensure homogenous sampling and 10 μl collected to measure time dependent changes in nitrite. Nitrite was measured by triiodide based reduction coupled with ozone chemiluminescence as previously described using a Sievers 280i Nitric Oxide analyzer (GE analytical instruments, Boulder, CO) [13]. Nitrite levels were determined by comparison with standard curves measured daily; detection limits were 1–10 pmol. In parallel, bacterial load was measured on tongue scrapes after initial dilution into BHI broth (described below). Nitrate reductase activity was calculated by normalizing initial rates of nitrite formation with corresponding colony forming units.

2.5. Mouse and rat nitrate reductase activity

Initial studies failed to show detectable changes in nitrite formation after nitrate addition to freshly collected mouse or rat tongue swabs. For this reason, swabs were split into two (each of 500 μl) and cultured in a total of 1.5 ml of BHI broth in either aerobic (21% O_2) or anaerobic (0.5% O_2) incubators at 37 °C. For aerobic conditions the broth was cultured in a rotary shaker (200 rpm, 37 °C). For anaerobic growth, medium was placed in an air sealed chamber and the inner O_2 of the chamber depleted by passing N_2 gas through the chamber till the O_2 meter reached <0.5%. The whole chamber was then placed in the 37 °C incubator. Incubation times were varied as described in results. After indicated incubation times, aliquots were taken and CFU determined, and sodium nitrate-dependent nitrite formation measured as described above.

2.6. Bacterial counts

CFU were determined using TSBA agar by the drop plate method. For human samples, 10 μl BHI containing tongues scrapes were serially diluted in sterile normal saline until 10^{-3} and 10^{-4} dilutions. For rat and mice, 10 μl were collected from BHI containing bacteria after varying culture times (0, 6, 12, 18 h) and 10^{-5} and 10^{-6} dilutions then plated on TSBA agar medium. For 0 h and 6 h, lower dilutions (10^{-1}–10^{-3}) were also tested. For each sample, a minimum of 3 drops (10 μl each) of each dilution were plated and incubated overnight at 37 °C under aerobic or anaerobic conditions.