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Original Contribution

Characterization of the rat oral microbiome and the effects of dietary nitrate

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ABSTRACT

The nitrate–nitrite–NO pathway to nitric oxide (NO) production is a symbiotic pathway in mammals that is dependent on nitrate reducing oral commensal bacteria. Studies suggest that by contributing NO to the mammalian host, the oral microbiome helps maintain cardiovascular health. To begin to understand how changes in oral microbiota affect physiological functions such as blood pressure, we have characterized the Wistar rat nitrate reducing oral microbiome. Using 16S rRNA gene sequencing and analysis we compare the native Wistar rat tongue microbiome to that of healthy humans and to that of rats with sodium nitrate and chlorhexidine mouthwash treatments. We demonstrate that the rat tongue microbiome is less diverse than the human tongue microbiome, but that the physiological activity is comparable, as sodium nitrate supplementation significantly lowered diastolic blood pressure in Wistar rats and also lowers blood pressure (diastolic and systolic) in humans. We also show for the first time that sodium nitrate supplementation alters the abundance of specific bacterial species on the tongue. Our results suggest that the changes in oral nitrate reducing bacteria may affect nitric oxide availability and physiological functions such as blood pressure. Understanding individual changes in human oral microbiome may offer novel dietary approaches to restore NO availability and blood pressure.

Introduction

Since the Human Microbiome Project began in 2007, an explosion of research has led to the publication of hundreds of studies. A common theme is the role of the microbiome in disease pathology, as much work has been aimed at identifying dysbioses associated with specific disease. In contrast, less emphasis has been placed on identifying and characterizing microbiome states and activities associated with health since the study of the original HMP original cohort of 300 healthy people. Because "healthy" microbiomes can be exploited to maintain or improve health (or return dysbiotic states to healthy states), it is essential that we continue to characterize and define microbiomes of health and harness the therapeutic potential of commensal bacteria.

In the mid-1990 s, researchers began characterizing an oxygen- and NOS-independent alternative pathway to NO production, called the nitrate–nitrite–NO pathway. Nitrate (NO_3^-) and nitrite (NO_2^-), previously thought to be inert end products of NO oxidation [1], can be reduced to bioactive NO through this pathway. Systemic nitrate and nitrite in blood and tissues are now considered a pool for bioactive NO [2,3]. The reduction of nitrate to nitrite, the first step of the nitrate–nitrite–NO pathway, is dependent on the oral commensal microbiota [4]. By contributing nitrite and NO to the mammalian host via the two-electron nitrate reduction, the oral microbiome is critical for mammalian physiology. NO, a gaseous free radical, is a critical cell signaling molecule involved in host defense, mitochondrial function, inhibition of oxidative stress, nerve transmission, endothelial function, antiaggregation by platelets, antiadhesion of leukocytes, vasodilation, and regulation of blood pressure [5]. In humans, NO is also

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endogenously produced by nitric oxide synthases (NOSs), which convert L-arginine and molecular oxygen to NO and L-citrulline. Nitrite also has cell-signaling properties [6] and acts as a reservoir of NO activity [7,8]. Therefore any strategy or treatment regimen that enhances production or availability of nitrite and/or NO will have positive benefits on mammalian physiology.

One of the most important physiological roles for NO is maintenance of cardiovascular system health through blood pressure regulation, vasodilation, and inhibition of platelet aggregation and leukocyte adhesion. In fact, NO insufficiency is one of the early hallmark signs of endothelial dysfunction [9]. Numerous animal and human studies have demonstrated that nitrate and nitrite supplementation is associated with increased cardiovascular health [10]. Sodium nitrate decreases diastolic blood pressure in humans and rats [11]. Beetroot juice, a dietary source of nitrate, decreased diastolic and systolic blood pressure [11–13]. Infusion of nitrite into the blood stream was also associated with reduced blood pressure in humans via oxyhemoglobin-mediated nitrite reduction to NO [14]. In eNOS-deficient mice, dietary nitrite restored NO homeostasis and was cardioprotective [15]. Dietary nitrate supplementation protected against ischemia–reperfusion damage in mice and also increased vascular regeneration after chronic ischemia in mice [16]. In a study of elderly people with an increased risk for cardiovascular disease, dietary nitrate supplementation reversed vascular dysfunction [17]. Research suggests that the nitrate supplementation-associated benefits are due to nitrate reduction by the oral microbiome since the benefits of nitrate supplementation were lost when subjects spat out their saliva prior to ingestion or were administered an antiseptic mouthwash [12,13,18,19]. Additionally, salivary nitrate reduction is absent in germ-free animals [20,21].

The potential to exploit the symbiotic nitrate–nitrite–NO pathway to NO production is profound, particularly because adequate and sustained control of blood pressure is achieved in only about 50% of treated hypertensive patients, including all classes of antihypertensives [22]. As cardiovascular disease remains the top killer in the United States, accounting for more deaths each year than cancer, designing new diagnostics, treatments, and preventives for diseased and at-risk individuals is essential. Additionally, because NO is an important signaling molecule in various body systems, exploiting the oral microbiome to contribute to NO production and maintain NO homeostasis has the potential to affect human health beyond the cardiovascular system. Based on these studies, rats may be a suitable organism for studying the effect that the oral microbiome has on the effects of sodium nitrate supplementation and cardiovascular health. In order to determine if use of oral antiseptic mouthwash and exposure to dietary nitrate changes the oral microbiome, we compared the Wistar rats' native tongue microbiome with that of animals treated with sodium nitrate supplementation and chlorhexidine mouthwash. Our results will aid future studies aimed at using the oral microbiome to increase nitrite and nitric oxide availability to the host, but also any studies in which the oral microbiome plays a key role. Our recently published data on identifying oral nitrate reducing bacteria in humans [23] demonstrate that we have the capabilities and expertise to interrogate the rat microbiome and determine how select interventions affect NO homeostasis.

Methods

Animals

Seven-week-old male Wistar rats were purchased from Charles River Laboratories (Wilmington, MA), housed individually in the Taub Animal Facility at Baylor College of Medicine (BCM), and provided with food and water ad libitum. Animals were trained through daily handling and restraint to reduce stress during experimental manipulations. One to 2 weeks after arrival at the Taub Animal Facility, animals were surgically implanted with a telemetric blood pressure measurement device (described below). Animals were weighed regularly throughout the study and gained an average of 76.6 g by the end of the study. The daily average intake of water was 42 ml. All experimental procedures (Fig. 1) were approved by the BCM Institutional Animal Care and Use Committee.

Surgical implantation of DSI PA-C40 telemetric blood pressure probes

PA-C40 telemetric devices (DSI, St. Paul, MN) were surgically implanted into the animals. One day prior to surgery, animals received an oral dose of Carprofen (6 mg), and 15 min prior to surgery, animals received an injection of Buprenorphine (0.2 mg/kg). Animals were anesthetized with isoflurane and kept warm during surgery through use of a heated board. Tissue hydration was maintained with sterile saline solution and the eyes were protected from drying by use of a sterile, bland ophthalmic ointment. The animals were placed in dorsal recumbency and an incision was made through the skin of the abdomen and then through the abdominal wall. The catheter portion of the PA-C40 telemetric device was placed into the abdominal aorta, and the transmitter portion was placed inside the intraperitoneal cavity, with the transmitter suture rib incorporated into the abdominal wall closure. Carprofen (6 mg) was administered to the animals daily for 48 h after surgery and animals were monitored for pain and discomfort. Additional dosages of Buprenorphine were administered if needed. Animals were allowed to recover from surgery for 1–2 weeks before experimental measurements began. Baseline blood pressure measurements were obtained for 5 days before additional experimental manipulations were performed.

Sodium nitrate supplementation

After 5 days of assessing animals at baseline, animals were supplemented with sodium nitrate ($NaNO_3$, Sigma-Aldrich, St. Louis, MO) in their drinking water (1 g/L) for the remainder of the study

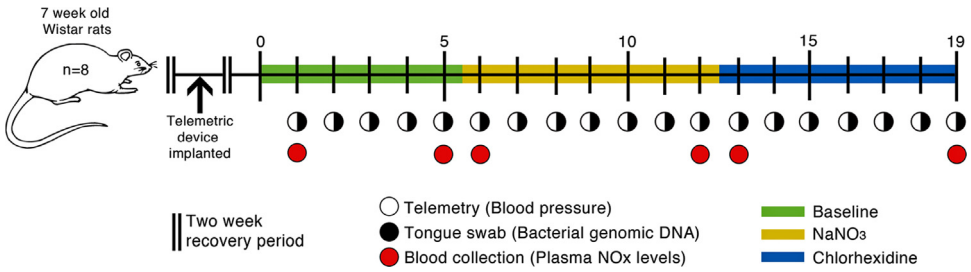

Fig. 1. Flow of experimental methods. Eight male Wistar rats were surgically implanted with the PA-C40 telemetric device, rested up to 14 days, and then blood pressure measurements, tongue swabs, and blood were collected during 5 baseline days followed by 7 days of $NaNO_3$ supplementation and an additional 5 days of $NaNO_3$ supplementation plus chlorhexidine mouthwash.