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Original Contribution

Active secretion and protective effect of salivary nitrate against stress in human volunteers and rats

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ABSTRACT

Up to 25% of the circulating nitrate in blood is actively taken up, concentrated, and secreted into saliva by the salivary glands. Salivary nitrate can be reduced to nitrite by the commensal bacteria in the oral cavity or stomach and then further converted to nitric oxide (NO) in vivo, which may play a role in gastric protection. However, whether salivary nitrate is actively secreted in human beings has not yet been determined. This study was designed to determine whether salivary nitrate is actively secreted in human beings as an acute stress response and what role salivary nitrate plays in stress-induced gastric injury. To observe salivary nitrate function under stress conditions, alteration of salivary nitrate and nitrite was analyzed among 22 healthy volunteers before and after a strong stress activity, jumping down from a platform at the height of 68 m. A series of stress indexes was analyzed to monitor the stress situation. We found that both the concentration and the total amount of nitrate in mixed saliva were significantly increased in the human volunteers immediately after the jump, with an additional increase 1 h later ($p < 0.01$). Saliva nitrite reached a maximum immediately after the jump and was maintained 1 h later. To study the biological functions of salivary nitrate and nitrite in stress protection, we further carried out a water-immersion-restraint stress (WIRS) assay in male adult rats with bilateral parotid and submandibular duct ligature (BPSDL). Intragastric nitrate, nitrite, and NO; gastric mucosal blood flow; and gastric ulcer index (UI) were monitored and nitrate was administrated in drinking water to compensate for nitrate secretion in BPSDL animals. Significantly decreased levels of intragastric nitrate, nitrite, and NO and gastric mucosal blood flow were measured in BPSDL rats during the WIRS assay compared to sham control rats ($p < 0.05$). Recovery was observed in the BPSDL rats upon nitrate administration. The WIRS-induced UI was significantly higher in the BPSDL animals compared to controls, and nitrate administration rescued the WIRS-induced gastric injury in BPSDL rats. In conclusion, this study suggests that stress promotes salivary nitrate secretion and nitrite formation, which may play important roles in gastric protection against stress-induced injury via the nitrate-dependent NO pathway.

For half a century, dietary nitrate and nitrite have been considered as the precursors of carcinogenic *N*-nitroso compounds [1–3]. However, the nitrate and nitrite anions (NO_3^- and NO_2^-, respectively) can be generated endogenously in the human body in the case of a shortage of dietary nitrate intake [4]. Thus, these anions may be essential components of the body, though their exact physiological functions are unknown. The salivary glands are important organs that mediate metabolism and dynamic nitrate balance through the enterosalivary circulation of nitrate. Dysfunction in the salivary glands has been linked to decreased nitrate secretion and increased nitrate levels in the serum and urine [5,6].

Up to 25% of circulating nitrate is actively taken up by the salivary glands, concentrated approximately 10-fold, and secreted into the saliva [7,8]. Salivary nitrate can be reduced to nitrite by the commensal bacteria in the mouth and/or stomach and then

Abbreviations: WIRS, water-immersion-restraint stress; BPSDL, bilateral parotid and submandibular duct ligature; UI, ulcer index; HPLC, high-performance liquid chromatography; LDF, laser Doppler flowmetry

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metabolized to nitric oxide (NO) in the acidic stomach [9–11]. Though most nitrate enters the systemic circulation, some salivary nitrate can be converted to nitrite and NO in other tissues [9,12]. Thus, nitrate uptake from the blood to the salivary glands seems to be a critical step in the enterosalivary circulation of nitrate. Sialin, which localizes in the lysosomes and cytoplasmic membrane of salivary glandular epithelial cells, has been shown to mediate the transport of NO_3^-, which may play an important role in the physiological regulation of systemic nitrate–nitrite–NO homeostatic balance [13,14].

The recycled salivary nitrate and nitrite absorbed into the blood can significantly influence the nitrate–nitrite–NO balance in the blood, which is important in conditions such as high blood pressure, platelet aggregation, and vascular damage [8–10]. High concentration of salivary nitrate or nitrite might be involved in host defense against oral and gastric pathogens [8,15], and dietary and recycled salivary nitrate or nitrite may also play important roles in gastric protection [16]. Salivary nitrate and nitrite that enter the stomach may enhance the bactericidal effects of gastric juice [17], increase gastric mucus blood flow and mucus thickness, and relieve chemically induced gastric ulcers [18].

However, previous studies were mainly focused on the protective effects of exogenous administration of nitrate and nitrite. In what circumstances salivary nitrate is actively secreted has not yet been determined in human beings. In this study, we investigated the effects of acute stress conditions on nitrate secretion and nitrite formation in human volunteers and the role of salivary nitrate/nitrite on stress-induced gastric injury in rats. Here, we report for the first time that the levels of salivary nitrate and nitrite are increased significantly under stress conditions and may play an important role in protection against stress-induced gastric injury via the nitrate-dependent NO pathway.

Methods

Human bungee jump protocol

Twenty-two healthy volunteers naive to bungee jumping or skydiving (14 males and 8 females, age range 21–45 years, mean age 27 years) were involved in the human stress trial. All volunteers had been living in Beijing, China, for more than 1 year and shared similar dietary habits. The trial was reviewed and approved by the ethics committees of Capital Medical University of China. Written informed consent was obtained from all subjects.

All subjects participated in a bungee jump from a height of 68 m, from a trestle platform fixed at the rock shore of the Qinlongxia Water Reservoir, Huairou District, Beijing, under the supervision and guidance of an experienced commercial bungee jump crew. The participants consumed the same food and water the day before the jump. On the morning of the study day, at 3 h before the jump, the first mixed saliva samples were collected. Subsequent saliva samples were collected immediately after the jump and 1 h after the jump. Unstimulated mixed saliva was collected in a quiet room in a germ-free centrifugal tube for 10 min; the static salivary flow rate represents the average amount of saliva per minute [19]. Saliva samples were 10,000 MW filtered and diluted before assay. The concentrations of nitrate and nitrite in the saliva were detected by high-performance liquid chromatography (HPLC) as described previously [5,6].

Before the bungee jump, the primary physical indexes (including respiratory rate, pulse rate, blood pressure) were measured just before saliva collection. These physical indexes were measured again directly after the jump. Blood samples were taken just after saliva collection before and after the bungee jump. Blood was drawn into Vacutainer tubes containing reduced glutathione–EGTA buffer for measurement of plasma noradrenalin and adrenalin. Plasma was separated and stored at $-80\,^{\circ}C$ until assays were performed. Plasma noradrenalin and adrenalin were detected by HPLC [20].

Experimental animals and study design

All animal experiments were approved by the ethics committees of Capital Medical University of China. Male adult Sprague–Dawley rats (5–6 weeks of age; body weight (bw) 180–200 g) were kept under standardized conditions at 21–22 °C with 12-h light/dark cycles. The rats were allowed to adapt to this environment in mesh-bottom cages with free access to distilled drinking water and regular pellet food for at least 7 days before the experiment. The rats were randomly divided into four groups as shown in Supplementary Fig. 1. The rats were anesthetized for each procedure with 0.4 ml/100 g bw of 10% chloral hydrate (Sigma, St. Louis, MO, USA) via intraperitoneal injection.

Bilateral parotid and submandibular gland duct ligature (BPSDL)

After the facial skin was incised, the parotid and submandibular gland ducts were surgically exposed. Bilaterally, the parotid and submandibular gland ducts were ligated to block secretion of saliva. Control rats were involved in a sham operation (sham) without any duct ligature.

Nitrate pretreatment

Half of the rats with and without BPSDL were randomly selected for nitrate supplementation (BPSDL+nitrate; sham+nitrate) and administered sodium nitrate ($NaNO_3$) dissolved in distilled water (5 mmol/L) for 7 days before the stress experiment. The daily dose of nitrate was approximately 1 mmol/kg bw as described previously [21]. The remaining rats with or without BPSDL were used as control groups (BPSDL; sham) and administered distilled water containing sodium chloride (NaCl) of the same dose. The baseline concentration of nitrate in the distilled water was less than 1 μM.

Measurement of gastric luminal NO

The concentration of gastric luminal NO gas in anesthetized fasting rats ($n=6$ for each group) was analyzed before the stress experiment as described previously [22]. Briefly, the stomach was directly inflated with NO-free air (NO <3 ppb) using a 5-ml syringe with a thin needle. External clamps were placed over the lower esophagus and duodenum to prevent leakage of the gas into these compartments during the sampling. After the air was incubated for 15 s, the gas was aspirated and immediately injected into a chemiluminescence analyzer (Interscan, USA) and the peak NO concentration recorded. The instrument's detection limit for NO was 10 ppb. Calibration of the instrument was performed with cylinder gas (10 ppm NO in nitrogen; AGA AB, Lidingö, Sweden).

Measurements of nitrate and nitrite in fasting gastric juice

Additional rats ($n=6$ for each group, the grouping is described in Supplementary Fig. 1) were fasted overnight, anesthetized, and subjected to pyloric ligature. Four hours after the ligature, the animals were sacrificed, the abdomen was opened, and another ligature was placed around the distal esophagus close to the diaphragm. The stomach was removed and its contents drained into a graduated centrifuge tube, which was centrifuged at 2000 g for 15 min. NaOH (0.1 M) was added to the sample at a v/v ratio of