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Research Paper

Role of nitrite, urate and pepsin in the gastroprotective effects of saliva

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ABSTRACT

Dietary nitrate is now recognized as an alternative substrate for nitric oxide (•NO) production in the gut. This novel pathway implies the sequential reduction of nitrate to nitrite, •NO and other bioactive nitrogen oxides but the physiological relevance of these oxidants has remained elusive. We have previously shown that dietary nitrite fuels an hitherto unrecognized nitrating pathway at acidic gastric pH, through which pepsinogen is nitrated in the gastric mucosa, yielding a less active form of pepsin *in vitro*. Here, we demonstrate that pepsin is nitrated *in vivo* and explore the functional impact of protein nitration by means of peptic ulcer development. Upon administration of pentagastrin and human nitrite-rich saliva or sodium nitrite to rats, nitrated pepsin was detected in the animal's stomach by immunoprecipitation. •NO was measured in the gastric headspace before and after nitrite instillation by chemiluminescence. At the end of each procedure, the stomach's lesions, ranging from gastric erosions to haemorrhagic ulcers, were scored. Nitrite increased gastric •NO by 200-fold ($p < 0.05$) and nitrated pepsin was detected both in the gastric juice and the mucosa ($p < 0.05$). Exogenous urate, a scavenger of nitrogen dioxide radical, blunted •NO detection and inhibited pepsin nitration, suggesting an underlining free radical-dependent mechanism for nitration. Functionally, pepsin nitration prevented the development of gastric ulcers, as the lesions were only apparent when pepsin nitration was inhibited by urate. In sum, this work unravels a novel dietary-dependent nitrating pathway in which pepsin is nitrated and inactivated in the stomach, preventing the progression of gastric ulcers.

1. Introduction

Nitrate, from green leaf vegetables, is involved in a plethora of physiological mechanisms not only in the gut but also systemically [1]. The implications of nitrate for human health rely on its ability to trigger an NO-synthase independent reductive pathway leading to the formation of nitric oxide (•NO), the *nitrate-nitrite-nitric oxide pathway* [2]. Nitrate reduction to nitrite and •NO is translated into increases of gastric mucosal blood flow and mucus production, inhibition of inflammatory pathways and prevention of microbial infections [3–5]. Nitrate consumed in green leafy vegetables is absorbed in the small intestine and mixes in blood with the nitrate derived from endogenous •NO generation. Then, c.a. 25% is taken up by the salivary glands and secreted into the oral cavity [6]. Here, metagenomic approaches have recently characterized symbionts that reduce nitrate to nitrite [7]. At the acidic gastro-oesophageal junction, nitrite is non-enzymatically metabolized to different nitrogen oxides, including •NO [8, 9]. Although most of the biological effects of nitrate have been attributed to •NO, it is clear that a complex network of chemical reactions culminates in the production of higher nitrogen oxides, some with the capability to modify both endogenous and exogenous macromolecules [10–12]. Some of these oxides (such as nitrogen dioxide radical, $•NO_2$) induce nitration, in which a nitro group ($-NO_2$) is inserted into a tyrosine residue within proteins or a fatty acid chain, yielding nitrated proteins or lipids, as recently shown [10,13]. This observation is of note as nitrated lipids can, in turn, signal to induce the expression of anti-inflammatory genes [14] and, therefore, a meal containing foods with both nitrate and oleic or linoleic acids, may fuel the production of anti-inflammatory molecules in the stomach that might also be absorbed into the circulation.

In this regard, we have also recently demonstrated that pepsinogen, the precursor of pepsin, is nitrated through a nitrate-dependent pathway in the stomach [13]. Pepsin is a gastric protease responsible for the breakdown of 15% of dietary proteins but, importantly, it is also known to erode the gastric mucosa,

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pinpointing its involvement in the development of peptic ulcer disease [15]. In this context, *in vitro* studies have shown that pepsin derived from nitrated pepsinogen has a lower proteolytic activity than pepsin derived from the non-nitrated zymogen [13], anticipating a potential gastroprotective effect of nitrated pepsin. In the present work it is shown that dietary nitrite induces pepsin nitration *in vivo* through a mechanism likely involving the generation of $\bullet NO_2$. Nitration decreases the proteolytic function of pepsin, preventing the development of acute gastric ulcers.

2. Materials and methods

2.1. Ethical approval

All experiments were performed according to European Community Council Directive for the Care and Use of Laboratory Animals (86/609/ECC) and approved by the local institutional animal care committee (ORBEA committee). Adult male Wistar rats (260–300 g) were purchased from Charles River, Barcelona and kept under 12 h cycles of light/dark for 7 days. During the period of acclimatization they were fed a standard chow and had access to water *ad libitum*. Before the experiments rats were fasted for 20 h (to minimize gastric contents) but had free access to water.

2.2. Surgical procedure

Rats were anaesthetized by the administration of a mixture of ketamine and xylazine (4:1, intraperitoneally, ip) and laid supine under a heating pad. Pentagastrin 20 μg kg^{-1} was administered ip to stimulate gastric secretion 15 min before the surgery. Animals were under anaesthesia at the moment of pentagastrin administration in order to prevent discomfort or pain. A laparotomy was then performed and the hepatogastric ligament was cut to facilitate handling of the stomach. External clamps were applied in the lower oesophagus and pylorus to avoid the passage of air and juices to the adjacent compartments and luminal levels of •NO were measured by chemiluminescence (see bellow). Sodium nitrite (1.3 mg kg^{-1}) was then directly instilled into the gastric lumen through a thin needle and 5 min later, gastric •NO was again measured. Rats were sacrificed by cardiac arrest 30 min after nitrite instillation. Samples of gastric juice were collected, the stomach was dissected out and gastric lesions were evaluated. Gastric tissue and juice were then snap frozen until further analysis (see Fig. 1). In another set of experiments, the same procedure was performed but immediately before nitrite instillation, 400 μM urate was injected into the gastric lumen. Nitrite-enriched human saliva (collected after the ingestion of 90 g of lettuce) was also used instead of sodium nitrite (n=4). Typically, the volumes of the solutions added to the stomach was 1 mL except for sodium nitrite for which small adjustments were made to ensure the administration of the same dose to all animals. In order to add the same amount of salivary nitrite, the volume of saliva administered was c. a. 4 mL were added.

3. Measurement of gastric •NO

Gastric •NO concentrations were determined by using a high sensitive and specific chemiluminescence methodology, as previously described [5,16]. Briefly, after a laparotomy, 4 mL of •NO-free air (typically less than 4 parts per billion) was injected in the stomach lumen through a thin needle, avoiding major gastric arteries. External clamps were used to prevent the spreading of injected gas to other gastrointestinal compartments. After 15 s, 4 mL of air was aspirated and immediately injected into a chemiluminescence analyser (CLD88 Exhalyzer, EcoMedics) to determine •NO concentration. The same procedure was performed 5 min after instillation of nitrite.

4. Collection of human saliva and determination of nitrite concentration

A sample of c.a. 10 mL of saliva was collected from a human volunteer who underwent an overnight fasting. Then, 90 g of iceberg lettuce (nitrate load) was ingested and saliva was again collected one hour later. After centrifugation (12,000 *g*x10 min), nitrite content was determined by chemiluminescence (CLD88 Exhalyzer, EcoMedics). Briefly, 100 μL of supernatant was injected into a closed chamber connected to the chemiluminescence analyser containing a reducing mixture of 45 mM potassium iodide and 10 mM iodine in glacial acetic acid continuously bubbled with nitrogen at 56 °C. Under these conditions, nitrite is reduced to •NO, which is quantified by the analyser following a calibration curve obtained from standard nitrite solutions.

4.1. Preparation of stomach homogenates

The glandular mucosa surrounding the gastric lesions was minced in ice-cold lysis buffer (50 mM Tris pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% NP 40, 1 mM PMSF and protease inhibitor cocktail, Sigma Aldrich; typically 100 mg of tissue was homogenized in 1 mL of buffer) with a pair of small scissors. The suspension was further triturated with a bullet blender (Labmark) and centrifuged at 12,000 *g* x 10 min (4 °C). The supernatant was collected and total protein was quantified by the Bradford method (Bio-Rad). Care was taken to avoid artifactual nitration due to media acidification.

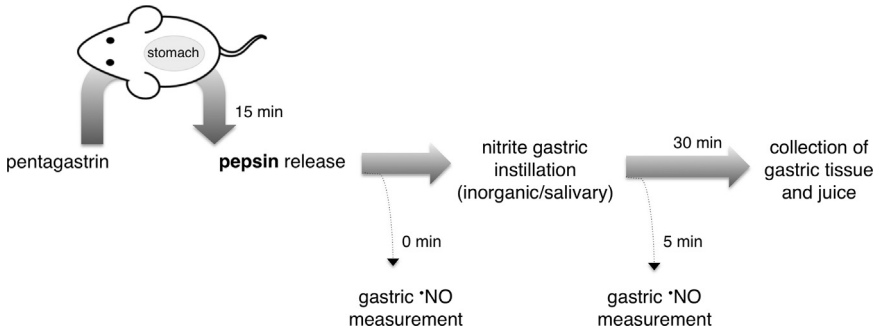

Fig. 1. Experimental design. Briefly, Wistar rats were anesthetized and pentagastrin was administered intraperitoneally to stimulate pepsin release. A laparotomy was performed 15 min later and gastric •NO was measured in the gastric headspace. Then, nitrite or human saliva was instilled into the stomach and, 5 min later •NO was again measured. After 30 min, animals were sacrificed and both gastric juice and tissue were collected.