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Original Contribution

Pepsin is nitrated in the rat stomach, acquiring antiulcerogenic activity: A novel interaction between dietary nitrate and gut proteins

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ABSTRACT

Dietary nitrate is reduced to nitrite and nitric oxide (•NO) in the gut, producing reactive species able to nitrate proteins and lipids. We investigated intragastric production of •NO and nitrating agents in vivo by examining selective nitration of pepsinogen and pepsin. We further addressed the functional impact of nitration on peptic activity by evaluating the progression of secretagogue-induced ulcers. Pepsinogen nitration was assessed in healthy and diclofenac-induced ulcerated rat stomachs. Both groups were fed nitrite or water by oral gavage. Protein nitration was studied by immunofluorescence and immunoprecipitation. In parallel experiments, pentagastrin was administered to rats and nitrite was then instilled intragastrically. •NO levels were measured before and after nitrite administration by chemiluminescence. Macroscopic damage was assessed and nitrated pepsin was examined in the margin of ulcers. Protein nitration was detected physiologically in the stomach of healthy animals. Nitrite had a dual effect on intragastric nitration: overall nitration was decreased under physiological conditions but enhanced by acute inflammation. Pepsin and pepsinogen were also nitrated via a nitrite-dependent pathway. Nitration of both pepsin and its zymogen led to decreased peptic activity in response to classical substrates (e.g., collagen). Under conditions of acute ulceration, nitrite-dependent pepsin nitration prevented the development of gastric ulcers. Dietary nitrite generates nitrating agents in the stomach in vivo, markedly decreasing peptic activity. Under inflammatory and ulcerogenic conditions pepsin nitration attenuates the progression of gastric ulceration. These results suggest that dietary nitrite-dependent nitration of pepsin may have a novel antiulcerogenic effect in vivo.

Introduction

Pepsin, the most abundant gastric protease, is an enzyme involved in the digestion of proteins, from both dietary and endogenous sources. Although pepsin has been assigned a physiological role in digestion, a pathological role in peptic ulcer disease is also suggested, making it a double-edged sword [1–4]. In fact, in the gastrointestinal tract, pepsin is responsible for only 15% of protein digestion [5] and therefore, much focus has been on its pathophysiological role in gastric ulceration.

After a meal, pepsin is secreted as an inactive zymogen, pepsinogen, by chief cells located at the base of gastric glands [6,7] and becomes available in the gastric lumen [5]. Here, a dynamic interaction between pepsin and the ingested nutrients is likely to occur. Pepsin will not only cleave peptide bonds on dietary proteins but can also be simultaneously targeted for biochemical modifications induced by dietary products. In this context, dietary nitrate (mainly found in green leafy vegetables but also present in human saliva under fasting owing to nitric oxide (•NO) oxidation) has been shown to be an intragastric donor of •NO in the human stomach [8,9]. Nitrate, after an enterosalivary circulation, is concentrated and reduced to nitrite in the oral cavity [10]. In the gastric compartment, nitrite is reduced to •NO, in a process accelerated by the presence of dietary polyphenols and other reductants [11–13]. Nitric oxide plays a key role in gastroprotective mechanisms by increasing mucosal blood flow, thickening the mucus layer, and killing gut pathogens [14]. However, in addition to •NO, other bioactive nitrogen oxides (e.g., $^{\bullet}NO_2$, N_2O_3, N_2O_4) are produced at the acidic gastric pH [15,16] and their potential physiological impact has

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been largely overlooked. The nitrogen dioxide radical ($^{\bullet}NO_2$) is known to nitrate proteins under these conditions [17]. Protein tyrosine nitration is a posttranslational modification that may have a profound impact on protein function and in this context has been implicated in Alzheimer and cardiovascular diseases [18]. Nevertheless, nitrite-dependent nitration of mediators of the gastrointestinal tract has remained largely unexplored.

We propose that dietary nitrate, through the intermediate $^{\bullet}NO$, yields nitrating agents in the stomach and that pepsin is nitrated in vivo. Moreover, whereas pepsin nitration takes place at a luminal acidic pH, locally produced $^{\bullet}NO$ is able to diffuse large distances within the gastric mucosa [19,20] triggering nitration reactions in deep regions of the gastric mucosa. Accordingly, it has been shown that nitrite-derived $^{\bullet}NO$ induces pepsinogen nitration in the gastric mucosa under acute inflammation but, noteworthily, has the opposite effect in the healthy stomach, suggesting that nitrite has a dual role in the modulation of nitrating pathways in the stomach. Furthermore, the induction of gastric juice secretion with pentagastrin results in the development of gastric ulcers, which, in most cases, are accompanied by intense bleeding. However, when nitrite is instilled intragastrically, pepsin becomes nitrated and the progression of gastric ulcers is prevented. This finding, which is in accordance with in vitro experiments, confers not only functional but also pathophysiological roles on pepsin nitration.

Overall, our findings support the hypothesis that the ingestion of nitrate-enriched food induces pepsin nitration in the stomach, decreasing its ability to erode the gastric mucosa. Nitrated pepsin can thus be regarded as an antiulcerogenic molecule with the peculiarities of being produced endogenously and diet-dependent.

Materials and methods

Experimental protocol for the detection of overall and pepsinogen nitration in the gastric mucosa

Animals

All experiments were performed in accordance with European and Portuguese guidelines for animal handling in scientific research. Male Wistar rats weighing between 230 and 250 g (Charles River, Barcelona, Spain) were used. Rats were kept under standard conditions of temperature and illumination. Twenty hours before the experiments the animals were deprived of food but had access to water ad libitum.

Experimental procedure

Rats were anesthetized with urethane (1.25 g kg^{-1}, intraperitoneal (ip) injection), and gastric ulcers were induced by the administration of 30 mg kg^{-1} diclofenac (Voltaren; Novartis) by oral gavage. The rats were left in their cages lying on a heating pad for 4 h [21]. Next, sodium nitrite (1.3 mg kg^{-1}) was given again by oral gavage. After 1 h the stomach was isolated and processed for further studies. The experimental groups and the protocol used are elucidated in Fig. 1.

Determination of overall protein nitration by immunohistochemistry

The presence of nitrated proteins in the gastric mucosa was investigated by immunohistochemistry. The body region of the stomach was fixed in 4% buffered paraformaldehyde, cut, and permeabilized with Triton 0.5% (in phosphate-buffered saline (PBS)). Nonspecific binding was blocked for 2 h with 5% bovine serum albumin and 0.6% Tween (in Tris-buffered saline). Then, the sections were incubated with rabbit polyclonal antibody against nitrotyrosine [22], overnight in a humidity chamber (4 °C). After being washed with PBS, the sections were incubated with a secondary antibody against rabbit (Alexa Fluor 488; Santa Cruz Biotechnology) for 1 h. Finally the sections were washed again with PBS and the nuclei were stained with Hoechst for 5 min. The slides were then observed under a fluorescence microscope (Zeiss Axiovert 200; Carl Zeiss MicroImaging, Germany). Nitrotyrosine expression score was assessed by the judgment of two researchers blinded to the protocol using a four-level scale from 0 to 3 (0, no stain; 1, light stain; 2, moderate stain; 3, intense stain).

Tissue morphology was evaluated by hematoxylin and eosin staining under a light microscope.

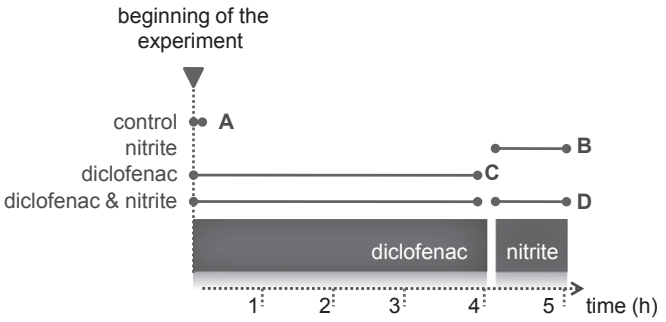

Fig. 1. Experimental procedure used to study the effect of nitrite as a nitrating agent in the acidic rat stomach in vivo. Wistar rats were divided in four groups depicted as A, B, C, and D, and the following treatments were performed: (A) no drugs were given to the rats, (B) rats received nitrite 1.3 mg kg^{-1} for 1 h, (C) rats received diclofenac 30 mg kg^{-1} for 4 h, and (D) rats were exposed to diclofenac for 4 h and nitrite for 1 h. All drugs were administered by oral gavage. Each group contained five or six animals.

Detection of nitrated pepsinogen

Gastric sample preparation

The stomachs were isolated and immediately immersed in ice-cold lysis buffer (1 mM $NaVO_4$, 10 mM β-glycine, 5 mM NaF, 1 mM phenylmethanesulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma–Aldrich) in 50 mM Hepes buffer solution: 150 mM NaCl, 2 mM EDTA, 10% glycerol, and1% Triton X-100). The tissue was roughly minced and then homogenized using a polytron. All crude homogenates showed minimal viscosity and were mixed for 30 min (4 °C), followed by a 10-min centrifugation at 15,000 g (4 °C). The supernatant was collected and frozen at −80 °C until further analysis. Protein concentration was determined using the Bradford method (Bio-Rad).

Immunoprecipitation

Solubilized proteins (800 μg) were incubated with 2 μg of a polyclonal pepsinogen antibody (Santa Cruz Biotechnology) for 3 h at 4 °C. The immune complexes were then precipitated (1 h at 4 °C) with 10 μl of protein A/G Ultralink resin (Thermo Scientific) previously washed with lysis buffer. The complexes were then washed with PBS (2000 g × 1 min, five times) and nitrated pepsinogen was eluted from the beads by adding loading buffer and further heating at 95 °C for 5 min. The samples were then applied on SDS–12% polyacrylamide gels. A Western blot against nitrotyrosine was then performed.

Western blot

After electrophoresis, proteins were transferred electrophoretically to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked with 5% defatted dry milk. Membranes were then incubated with rabbit polyclonal antibody against nitrotyrosine [22] overnight (4 °C). Membranes were washed