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Differential changes in Substance P, VIP as well as neprilysin levels in patients with gastritis or ulcer

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ABSTRACT

The protective effect of capsaicin-sensitive sensory nerve (CSSN) activation was recently demonstrated in human gastric mucosa. We here examined changes in neuropeptides, specifically Substance P (SP), calcitonin-gene related peptide (CGRP) and vasoactive intestinal peptide (VIP) in patients with chronic gastritis or ulcer. Furthermore changes in neprilysin levels, which hydrolyse these neuropeptides, were determined. Gastric biopsies were obtained from both lesion- and normal-appearing mucosa of 57 patients. The presence of H. pylori infection was verified with rapid urease assay. Neuronal and non-neuronal levels of SP, VIP, CGRP and neprilysin activity were determined in freshly frozen biopsies. Immunohistochemical localization of neprilysin was performed in 30 paraffin embedded specimens. We here found that neuronal SP levels decreased significantly in normally appearing mucosa of patients with gastritis while levels of non-neuronal SP increased in diseased areas of gastritis and ulcer. The presence of H. pylori led to further decreases of SP levels. The content of VIP in both disease-involved and uninvolved mucosa, and expression of neprilysin, markedly decreased in patients with gastritis or ulcer. Since VIP, as well as SP fragments, formed following hydrolysis with neprilysin is recognized to have gastroprotective effects, decreased levels of VIP, SP and neprilysin may predispose to cellular damage.

1. Introduction

Chronic gastritis, as well as peptic and duodenal ulcers is common and important health problem that may predispose to malignancy. Most widely used therapies for these conditions target reduction in gastric acid secretion. Recent studies, however, demonstrate that hypochlorhydria and gastric atrophy actually predispose patients to gastric cancer [13]. Furthermore, the chronic use of proton pump inhibitors associates with an increased risk of infectious complications and nutritional deficiencies [2]. Hence a more effective treatment strategy would not alter the normal physiology of the gastric mucosa while providing mucosal protection is required.

Previous studies have demonstrated that capsaicin-sensitive sensory nerves (CSSN) protect the gastric mucosa from damage by varied stimuli such as stress, ethanol ingestion and aspirin [15,26,30,47,61]. The gastrointestinal system is rich in CSSN that contain neuropeptides such as Substance P and calcitonin-gene related peptide (CGRP) [10]. Activation of CSSN surrounding blood vessels in the submucosa of the gastric wall appears to play a major role activating vasodilation when the mucosa is challenged by acid [25]. Mucosal innervation by CSSN also activates protective secretion of mucus in response to enhanced gastric acidity, thereby providing a locally activated defense system [1]. Sensory neuropeptides are also involved in gastric perception and increased neuropeptide levels were reported in patients with helicobacter pylori-positive functional dyspepsia [41].

Capsaicin, a pungent ingredient of chili pepper, activates cellular responses via TRPV1/VR1 capsaicin (vanilloid) receptors [6]. A controlled randomized prospective study demonstrated that capsaicin ingestion protects against gastric microbleeding induced by indomethacin or ethanol [44]. Protective effect of CSSN was also shown in patients with chronic gastritis with H. pylori infection and extent of protection does not depend on the presence or absence of H. pylori [9]. Similar gastroprotective effects of capsaicin analogs were also reported in patients [43]. Hence, better understanding peptide-mediators involved in gastroprotective effects of CSSN is important for the discovery and development of new, more effective and less toxic treatments of gastrointestinal disorders.

There are only a few human studies that focus on quantifying changes in individual neuropeptides of CSSN in different gastrointestinal pathologies. Results of these studies appear to contradict the findings of studies with capsaicin. For example SP, one

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of the principle neuromediators of CSSN involved in vasodilatation, angiogenesis, inflammation and healing [32,56,59], was found increased in the mucosa of patients with chronic gastritis [31]. Similar increases were also detected in immunoreactive vasoactive intestinal peptide (VIP) containing nerve fibers [53]. In another study, expression of capsaicin receptors, CGRP and SP was found to be higher in the mucosa of patients with chronic gastritis compared with that of normal controls [9].

These discrepancies may relate to multiple factors. Firstly, levels of sensory neuropeptides were localized using immunohistochemical methods which are semi-quantitative. Secondly, peptide levels were primarily measured at the lesion site. Sensory neuromediators are also found in immune cells and immune cells infiltrating the lesion site which would confound interpretation of increased neuronal peptide levels [53]. Thirdly, each neuropeptide may have multiple properties with some gastroprotective, and others not. For example, animal studies have demonstrated that CGRP is involved in gastroprotection provided by the activation of CSSN but not by SP [45]. Lastly, factors which modify the action of sensory neuropeptides are also likely to have important regulatory roles. Neprilysin might be one such factor. Neprilysin (also identified as neutral endopeptidase or CD10) is an enzyme involved in hydrolysis of SP, VIP and CGRP [7,33,55]. To the best of our knowledge, there is no study examining the changes in neprilysin levels and activity in patients with chronic gastritis or ulcer.

The goal of our study is to clarify these possibilities by measuring levels of sensory neuropeptides (SP, CGRP) as well as VIP which is primarily localized in intrinsic gastric myenteric neurons [52] from both lesion-free and diseased areas of patients with gastritis or ulcers using a two-step acetic acid extraction method. This protocol developed by us [16], differentiates neuronal peptides from non-neuronal ones. Secondly, we also quantified changes in neprilysin activity and correlated these with its tissue distribution using immunohistochemistry. Furthermore possible sex-dependent changes were also evaluated.

2. Materials and methods

This study was performed on 57 patients who had upper gastrointestinal endoscopy for symptoms of dyspepsia from January 2008 to May 2009 in the Department of Gastroenterology. The Control Group consisted of patients who had symptoms without any pathological changes by endoscopic examination (functional dyspepsia). Exclusion criteria included the presence of chronic rheumatologic diseases, malignancy, Diabetes Mellitus or chronic lung disease. The age of the patients ranged from 23 to 50 years (mean ± SD; 42.7 ± 7.5 years) with a male/female ratio of 1.53:1. The study protocol was approved by the Committee of Ethics at the Faculty of Medicine, Akdeniz University, Antalya, Turkey. Procedures were conducted according to the principles expressed in the Declaration of Helsinki. Patients meeting these criteria were interviewed by one of us and asked to give consent for obtaining biopsies during the endoscopy. Thirty-two of the patients included in this study had gastritis, eleven had duodenal ulcer, one patient had peptic ulcer and 10 of them had no pathological findings. None of the patients were under specific treatment for gastritis or ulcer. Ten patients reported to take a proton pump inhibitor irregularly (one or two times in a week). Endoscopic appearance of the lesions was used for the diagnosis of gastric and duodenal ulcers. Biopsy specimens from the pathological areas were evaluated microscopically in patients with gastritis. Biopsies were obtained from the lesion, from neighboring normal looking gastric mucosa at the farthest possible distance from the lesion in case of gastritis and a 3–4 cm distant from the ulcer. These biopsies were snap-frozen in liquid nitrogen and kept at −80 °C until the assays. Presence of H. pylori infection verified using the rapid urease assay (CLO test, Pylori Tek, Hpfast) which was performed at the time of endoscopy.

For immunohistochemical studies, 30 paraffin-embedded gastric specimens resected between 2000 and 2006 were selected from the database of the Department of Pathology, Akdeniz University Medical School. Clinical data and pathologic slides were reviewed in each case. The age of these patients ranged from 25 to 68 years (mean ± SD, 43.0 ± 12.8 years) with a male/female ratio of 2:1. Tissue specimens were divided into four groups: Group I (Control Group): Gastric mucosa from 6 previously healthy patients who had an urgent surgery for gastric perforation due to penetrating injuries. Group II: Normal appearing gastric mucosa from 6 patients who underwent partial gastrectomy for bleeding peptic ulcer. Group III: Gastric mucosa adjacent to ulcers from 7 patients operated for gastric ulcer. Group IV: Gastric mucosa from 11 patients diagnosed with gastritis.

2.1. Measurement of peptide levels

We previously established a method to differentially measure SP levels present in the nerve endings as well as in non-neuronal tissue [14,16]. Briefly, biopsy specimens were cut into small pieces and kept in 1 ml of 2% acetic acid at 95 °C for 10 min. Sequential collections of supernatants were performed in which the first 10 min extraction included predominately neuronal SP, while the second extraction re-incubated in 1 ml of 2% acetic acid at 95 °C for 50 min yielded the non-neuronal compartment of the peptide.

Supernatants were dried completely then reconstituted in 150–300 μl of sample buffer from the SP EIA kit (Cayman Chem., Catalog No. 583751). From each sample, 25 and 50 μl were used for immunoassay, which gave results within confidence interval (%95). Tissue extractions were also used for quantifying VIP (Bachem-Pennisula Laboratories, cat. no.: S1183) and CGRP (Phoenix Pharmaceuticals, cat. no.: EK-015-02) by immunoassay. Detection limit for SP was 4–500 pg/ml, and we have tested multiple dilutions of the samples in order to avoid very low and very high concentrations, since the assay is most sensitive at 20–80 percentiles (approximately 10–250 pg/ml). Under these conditions both intra- and interassay variabilities were less than 20%. IC_{50} for VIP measurements was 200 pg/ml and same precautions were taken to keep intra- and interassay variabilities at less than 20%.

2.2. Measuring NEP like activity

Dansyl-D-Ala-Gly-p-nitro-Phe-Gly, dansyl-D-Ala-Gly and fosforamidon were purchased from Sigma (St. Louis, MO). Measurement of NEP activity was performed as described previously with some modifications [5]. Briefly snap-frozen tissues were weighed and sonicated 5 times on ice for 15 s in ice-cold 50 mM Tris–HCl, (pH 7.4) buffer which included 1% Triton-X100. The homogenates were centrifuged at 10,000 × *g* for 3 min to remove cellular debris and nuclei and then stored at −80 °C until used.

Samples (10 μl) were pre-incubated with enalapril, an angiotensin-converting enzyme (ACE) inhibitor, to prevent cleavage of the fluorogenic substrate (*N*-dansyl-D-Ala-Gly-*p*-nitro-Phe-Gly) by ACE, in the presence or absence of phosphoramidon, a specific NEP inhibitor. Following this pre-incubation, the fluorogenic substrate was added, and samples were incubated for an additional 2 h at 37 °C. The final concentrations were 16.5 μM for enalapril, 16.5 μM for phosphoramidon, and 200 μM for the substrate, in a reaction volume of 160 μl. After 2 h of incubation, fluorescence absorbance was recorded using a BIOTEC FX800 Reader. The amount of product was estimated by measuring its fluorescence intensity at 562 nm with excitation at 342 nm. Arbitrary fluorescence units for each sample were compared with a standard curve prepared using Dansyl-D-Ala-Gly (cleavage