Quantification of Neurotensin Binding Sites at Different Locations in Inflammatory Bowel Disease and Control Human Intestine

W.P. ter Beek
I. Biemond
E.S.M. Muller
C.B.H.W. Lamers

Department of Gastroenterology-Hepatology, Leiden University Medical Centre, The Netherlands
Abstract

**Background:** Recently, interest in gastro-intestinal neuropeptides and their receptors has greatly increased because of the possibility of using agonist or antagonists in patient care. Neurotensin is involved in intestinal processes such as inflammation, secretion and motility by interacting with specific cell-surface receptors. The knowledge of intestinal neurotensin receptor expression is growing, but it is still incomplete, especially with regard to the human situation. **Aim:** To further explore the localization and number of neurotensin binding sites in mucosa and muscle of control human colon and ileum and in tissue of patients with inflammatory bowel disease (IBD). **Methods:** Full thickness intestinal tissue samples were collected from 23 control patients and 28 patients with IBD (11 Crohn’s disease (CD) and 17 ulcerative colitis (UC)). The tissue of patients with IBD was categorized on the basis of macroscopic appearance and myeloperoxidase (MPO) expression in inflamed and noninflamed samples. For detection of neurotensin binding sites a quantitative autoradiographic method was used. **Results:** Neurotensin binding to ileal muscle of control patients (22±6 fmol/g) was significantly lower than the binding to control colonic muscle (120±16 fmol/g), whereas mucosal binding was even less (7±4 and 13±4 fmol/g for ileum and colon, respectively). There was a significant inverse correlation (r = -0.68) between inflammation as quantified by the MPO method and neurotensin binding to colonic muscle in patients with IBD. In ileum the number of binding sites decreased significantly in muscle of inflamed tissue samples as assessed macroscopically by the pathologist compared to muscle in noninflamed samples of patients with CD (6±2 vs.18±4 fmol/g). **Conclusions:** Neurotensin binding was strongly present in colonic muscle of controls and significantly less in the ileal muscle. In patients with IBD a decrease in neurotensin binding sites was seen in intestinal smooth muscle, which is correlated with the degree of inflammation. The control intestinal mucosa expressed a small number of neurotensin binding sites, while IBD patients even had a slightly lower neurotensin binding.
Introduction

The tridecapeptide neurotensin was first isolated from bovine hypothalamus [1] and later from bovine intestine [2]. In the intestine, neuroendocrine cells (N cells) in the mucosa and enteric neurons release neurotensin [3]. The highest concentration of neurotensin-like immunoreactivity is found in the ileum [4]. Within the gastrointestinal tract neurotensin has many different functions; this peptide acts on pancreatic and gastric acid secretion, it is involved in the initiation of hormone release, and it plays a role in motility and chloride secretion [3,5,6].

In animals several studies have been carried out on the distribution of neurotensin receptors in the control intestine, showing neurotensin binding to the smooth muscle and to the plexuses of the small intestine [4,7-9] and colon [10]. In contrast, the knowledge of neurotensin receptor distribution in humans is still incomplete. Reubi et al. showed neurotensin binding sites in colonic muscle and nerves surrounding adenocarcinoma [11]. Other studies have also shown binding sites in the colonic muscle [12,13]. To our knowledge there are no data on the distribution of neurotensin receptors in human ileum and the reports on neurotensin receptors in the mucosa are contradictory. Using immunohistochemistry Riegler et al. have detected neurotensin receptors at the bottom of the crypts and in the lamina propria of human colonic mucosa [14], but other studies did not show mucosal binding [12,15,16].

It recently became clear that neurotensin also plays a role in the inflammatory response. In vitro studies have shown that inflammatory cells express the neurotensin receptor and that neurotensin increases vascular permeability, stimulates mast cell degranulation, phagocytosis, and histamine and chloride secretion [14,17-19]. Castagliuolo et al. have shown that pre-treatment with a neurotensin antagonist reduces the acute symptoms of inflammation in the Clostridium difficile toxin-A inflammation model in rats. They concluded that neurotensin and its receptor are important in the acute inflammatory response in the colon [17]. However, little is known about the role of neurotensin and its receptors in the inflammatory process in the human intestine. Ulcerative colitis (UC) and Crohn’s disease (CD), both forms of inflammatory bowel disease (IBD), are characterized by chronic inflammation accompanied by changes in motility and
diarrhoea. But to the best of our knowledge, no studies have examined the neurotensin receptor expression in IBD.

The aim of this study was to describe the distribution and number of neurotensin binding sites in mucosa and muscle of control human intestine. In addition, neurotensin binding sites in tissue (both inflamed and noninflamed) of patients with IBD were studied and compared with control tissue. To address these issues storage phosphor autoradiography was used, a technique to quantify and locate peptide binding to frozen tissue sections.

**Materials and methods**

**Tissue sampling**

Full thickness intestinal tissue specimens were obtained within 30 minutes after surgery from 11 patients with CD (mean age 38 years; range 18-73 years) and 17 patients with UC (mean age 38 years; range 19-72 years), both from macroscopically inflamed and/or noninflamed areas as assessed by the pathologist. Samples of patients with CD included colonic and ileal tissue, whereas only colon specimens were taken from the patients with UC. For controls, tissue was taken at least 10 cm from the affected site from 23 patients with non-inflammatory diseases (mean age 56 years; range 34-74 years). The tissue was embedded in Tissue-Tek® O.C.T. compound, rapidly frozen on dry ice and stored until use at 80°C.

**Storage phosphor autoradiography**

Cryostat tissue sections (14 μm) were cut at -20°C, mounted on gelatine-coated glass slides and stored overnight at -80°C. Several methods [20] of 125I-neurotensin binding to tissue sections were tested and optimised resulting in the following protocol. Slides were air dried for 30 min and pre-incubated in 50 mM Tris-HCl (pH 7.0) containing 0.5% BSA for 20 min. For total binding, slides were incubated with 50 mM Tris-HCl, 0.25 mg/ml bacitracin, 4 μg/ml leupeptin, 2 μg/ml chymostatin, 130 mM NaCl, 7.7 mM KCl, 5 mM MgCl₂, 1 mM ethylene glycol-bis(β-
aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), and 75 pM $^{125}$I-neurotensin (Perkin Elmer Life Science, Boston, Massachusetts, USA) at pH 7.0 for 180 min at room temperature. Alternate serial sections were incubated with addition of 1 μM non-radioactive neurotensin (Bachem AG, Switzerland) to determine non-specific binding. After incubation, sections were washed five times for 5 min with 50 mM Tris-HCl pH 7.0 containing 0.5% BSA at 4°C. Washed slides were rapidly dried with a stream of cold air. Slides were placed in a storage phosphor cassette for 40 h at room temperature. Laser scanning the screen in the Phosphor Imager® (Molecular Dynamics, Sunnyvale, California, USA) visualized the latent image stored on the storage phosphor screen. The data of the digitised image were processed with ImageQuant® software (Molecular Dynamics, Sunnyvale, California, USA). Slides with 10 μl drops of different concentrations of radiolabeled ligand were used for standardization. Rat brain sections acted as positive control. Binding was expressed in fmol/g tissue. To determine the specific binding, the non-specific binding was subtracted from the total binding. Serial sections were stained with hematoxylin and eosin to distinguish between smooth muscle and mucosa.

**MPO-assay**

The extent of neutrophil infiltration was quantified by measuring myeloperoxidase activity (MPO) to confirm the macroscopic classification of inflammation and to grade the extent of inflammation. Tissue was homogenized and 25 μl of the homogenate was used in an assay described by Krawisz *et al.* [21] to detect MPO activity. The reactions were followed kinetically for 30 minutes and a sample of human polymorphonuclear neutrophils was used for standardization. MPO activity was expressed in arbitrary units.

**Data-analysis**

Data were expressed as mean ± SEM (standard error of mean). Unpaired Student’s t-tests were used to infer significant differences between groups. Pearson correlation was calculated between ligand binding and MPO activity. Values of p < 0.05 were considered significant.
Results

Neurotensin binding to control intestine

The distribution of neurotensin binding in control human colon and ileum is shown in figure 1. Strong neurotensin binding is observed to colonic smooth muscle of control patients (figure 1A-C), while binding to ileal muscle is lower (figure 1D-F). In mucosa of control patients, only weak binding of neurotensin is observed to both colon and ileum. Using the ImageQuant® software neurotensin binding sites were quantified and neurotensin binding is expressed in fmol per gram tissue. In control tissue neurotensin binding is significantly higher to colonic muscle than to ileal muscle (120±16 vs 22±6; figure 2). Neurotensin binding to mucosa is low (13±4 and 7±4 fmol/g for colon and ileum, respectively) and this is significantly different from the binding to muscle (figure 2).

Figure 1. {superscript}125I-neurotensin Binding to Control Human Colon (A-C) and Ileum (D-F)
A, D: Specific binding of {superscript}125I-neurotensin, the intensity of the greyscale is proportional to the number of binding sites. B, E: Hematoxylin/eosin staining of the serial sections, the pink coloured tissue is smooth muscle and the purple tissue is mucosa. C, F: The precise location of neurotensin-binding is shown by merging the binding image with the hematoxylin/eosin staining, which gives a qualitative result.
Figure 2. **125I-neurotensin Binding in Human Intestine** Binding in 19 colon and 7 ileum samples is measured with autoradiography and expressed in fmol/g tissue and measured. * p<0.05 versus smooth muscle, # p<0.05 versus colon.

**Neurotensin binding to inflamed intestine**

The MPO-assay showed that the classification in macroscopically inflamed and noninflamed areas is correlated with the degree of neutrophil infiltration (r=0.42, p=0.004). MPO values in the inflamed IBD group were significantly higher than those in the noninflamed IBD group (9.7±0.8 vs. 5.5±1.3 U/mg tissue; p<0.02). Neutrophil infiltration in control tissue was comparable to the infiltration in the noninflamed IBD group (5.1±0.7 vs. 5.5±1.3 U/mg tissue). In tissue samples of patients with IBD, the same distribution pattern of neurotensin binding is seen as in controls. Most binding is found to colonic smooth muscle and mucosal neurotensin binding is low (figure 3).

Comparing the quantity of neurotensin binding in patients with IBD and controls showed a decrease of the binding in both mucosal and muscular inflamed and noninflamed IBD tissue, but the difference was not statistically significant (table 1). Within the IBD group there was no significant difference between the neurotensin binding in CD and UC patients, although there was a tendency of lower neurotensin binding to colonic muscle of inflamed CD samples compared to UC samples (42±11 vs. 88±21 fmol/g). Neurotensin binding to ileal muscle, all from CD
Figure 3 $^{125\text{i}}$-neurotensin Binding to Macroscopic Inflamed UC Colonic Tissue (A-C) and CD Ileal Tissue (D-F). A, D: Specific binding of $^{125\text{i}}$-neurotensin, the intensity of the greyscale is proportional to the number of binding sites. B, E: Hematoxylin/eosin staining of the serial sections, the pink coloured tissue is smooth muscle and the purple tissue is mucosa. C, F: The precise location of neurotensin-binding is shown by merging the binding image with the hematoxylin/eosin staining, which gives a qualitative result.

patients, significantly decrease in the muscle of inflamed tissue samples ($p=0.02$ vs. noninflamed muscle; table 1). If the degree of inflammation (MPO-activity) in patients with IBD is correlated with neurotensin binding, a significant negative correlation ($r=-0.68; p=0.03$) is seen in the colonic muscle. In the mucosa, no
differences are found between inflamed and noninflamed areas (table 1) of patients with IBD and there is no correlation with MPO activity.

Table 1. Quantity of $^{125}$I-neurotensin Binding (fmol/g tissue) in Human Colon and Ileum as Measured by Autoradiography

<table>
<thead>
<tr>
<th></th>
<th>colon</th>
<th></th>
<th>ileum</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mucosa</td>
<td>muscle</td>
<td>mucosa</td>
<td>muscle</td>
</tr>
<tr>
<td>Mean (SEM) n</td>
<td></td>
<td>Mean (SEM) n</td>
<td></td>
<td>Mean (SEM) n</td>
</tr>
<tr>
<td>control</td>
<td>13 (4) † 18</td>
<td>120 (16) 19</td>
<td>7 (4) 5</td>
<td>22 (6) † 7</td>
</tr>
<tr>
<td>noninflamed IBD</td>
<td>6 (2) † 4</td>
<td>93 (25) 8</td>
<td>2 (1) † 4</td>
<td>18 (4) † 4</td>
</tr>
<tr>
<td>inflamed IBD</td>
<td>6 (3) † 15</td>
<td>82 (16) 19</td>
<td>7 (3) 5</td>
<td>6 (2) † 5</td>
</tr>
</tbody>
</table>

† p<0.05 vs. muscle; † p<0.05 vs. colon; * p<0.05 vs. noninflamed IBD

Discussion

This study describes the distribution of neurotensin binding sites in human colon and ileum of patients with IBD and control samples.

Neurotensin binding in control intestine

Previous studies on the distribution of neurotensin receptors in human control colon by autoradiography have only shown neurotensin receptor expression in the muscular and neuronal compartments of the colon [11,12]. However, Riegler et al. have detected neurotensin receptors in human colonic mucosa using immunohistochemistry [14]. In agreement with that study [14], our study has shown neurotensin binding to the mucosa of control human colon and the binding was subsequently quantified. The binding in the mucosa appeared to be lower than in the muscle. As it is generally accepted that neurotensin is involved in the secretion of chloride and fluid [14], both of which are regulated in the mucosa, the finding of neurotensin receptors in the mucosa was not totally unexpected. A possible
explanation for the fact that previous studies did not find neurotensin binding to the mucosa could be that neurotensin receptor expression in tissue surrounding adenocarcinomas as studied by Reubi et al. [11] is different from tissue further away from the affected area. Furthermore, it should be noted that Azriel and Burcher [12], who were unable to demonstrate mucosal neurotensin binding, applied a photographic emulsion technique which is not as sensitive as the storage phosphor autoradiography used in our study [20].

To the best of our knowledge, no studies have described the neurotensin receptor expression in human ileum. From animal studies [4,7-9], it is known that the ileum expresses neurotensin receptors, however, the level of expression is less than that found in the colon [10]. Our results on neurotensin binding sites in human ileum are in agreement with these earlier animal studies.

**Neurotensin binding to inflamed intestine**

Castagliuolo et al. have shown that the mRNA content of the neurotensin receptor in rats increases in the mucosa immediately after *Clostridium difficile* toxin A injection [17]. This suggests that neurotensin and its receptors play a role in the process of acute inflammation. No data are available on the role of the neurotensin receptor in inflammatory processes in humans. There are studies that have shown that the human peripheral lymphocytes express the neurotensin receptor and that human neutrophils react *in vitro* with locomotion and phagocytosis upon neurotensin stimulation [18,19]. We investigated the neurotensin binding in patients with a chronic inflammatory intestinal disease, inflammatory bowel disease (IBD). The mucosal increase in neurotensin receptor mRNA seen in rats [17] does not correspond with our findings in humans with chronic intestinal inflammation. This could be due to various factors, such as species-related differences, differences between acute and chronic inflammation and/or differences between mRNA and protein expression. In the smooth muscle of patients with IBD a decrease in neurotensin binding was found when compared to controls, although the difference was not statistically significant. However, correlation analysis showed that in colonic IBD muscle samples the degree of inflammation is inversely correlated with the number of binding sites. Depending on the degree of inflammation a decrease
was seen in the amount of neurotensin binding. It is known that neurotensin is a peptide involved in the regulation of intestinal motility. Therefore a decrease of binding sites for neurotensin could affect this motility, possibly resulting in a disturbed motility, as often seen in patients with IBD. When comparing inflamed with noninflamed areas of the intestine of patients with IBD, a significant decrease in neurotensin binding in patients with CD was seen in macroscopic inflamed ileum compared to noninflamed ileum. In the inflamed colon of patients with IBD the decrease in neurotensin binding compared to controls was rather small (82±16 vs. 93±25 fmol/g) and did not reach statistical significance. However, the decrease in the three CD samples was larger than in the UC samples (42±11 vs. 88±21 fmol/g) suggesting that changes in muscular neurotensin binding are more pronounced in CD than in UC patients. This is in agreement with the fact that the inflammatory process affects the smooth muscle in CD, but not in UC patients.

In summary, in control human intestinal mucosa neurotensin binding is detectable, however it is less than in the intestinal muscle. Within the intestine the expression of binding sites for neurotensin in the colon is significantly higher than in the ileum. In patients with IBD a small decrease was seen in neurotensin binding compared to controls sites. This was more pronounced in the inflamed samples than in the noninflamed IBD samples. There was an inverse correlation between the degree of inflammation and neurotensin binding. These findings may be related to the disturbed motility seen in patients with IBD. The present findings warrant further studies on the (patho)physiological significance of neurotensin receptors in both muscle and mucosa of the bowel.

References


