SERT and TPH-1 mRNA expression are reduced in irritable bowel syndrome patients regardless of visceral sensitivity state in large intestine

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VISCERAL HYPERSENSITIVITY is thought to play a role in irritable bowel syndrome (IBS) symptom generation (46). A subset of IBS patients is hypersensitive to colorectal distension, indicating that physiologic stimuli are perceived with increased intensity and even cause pain (3, 29, 33). This may result from altered expression of receptors, transporters, and enzymes that determine the availability of their agonists and thereby increasing sensitivity of peripheral afferent nerve endings. Both serine protease and serotonergic signaling modulate colorectal sensitivity (5, 7–9, 22, 41, 42, 45).

IBS patients have increased serine protease activity in colonic mucosa, which activates mucrine extrinsic primary afferent neurons in culture and induces hyperalgesia and allodynia in mice upon colorectal distension (7, 42). These responses are diminished after preincubation with serine protease inhibitor.

Mast cell tryptase and nonpancreatic trypsin might be responsible for increased serine protease activity in colonic mucosa. Expression of mast cell tryptase and trypsinogens, zymogens of active trypsins, are elevated in colonic mucosa from IBS patients (7). Origin of enhanced trypsinogen expression has not been elucidated. Trypsin IV, produced by enterocytes, is a likely candidate. The nociceptive effects of colonic mucosal supernatant are protease-activated receptor (PAR)-2 dependent (7). Both mast cell tryptase and trypsin IV can activate PAR-2 (7, 35).

Activation of PAR-2 on epithelial cells leads to increased paracellular permeability, which might increase passage of luminal antigens and toxins with consequent mucosal immune activation contributing to visceral hypersensitivity (8, 9, 41). Activation of PAR-2 on nerve terminals can provoke the release of neuropeptides such as substance P (44). Substance P triggers the degradation of mast cells thereby releasing inflammatory mediators that may stimulate primary afferents (2, 4, 13, 20).

Serotonin (5-HT) also modulates visceral sensitivity of the gastrointestinal tract (5, 45). 5-HT is synthesized in enterochromaffin (EC) cells by the rate-limiting enzyme tryptophan hydroxylase (TPH)-1 and in the brainstem and myenteric plexus neurons by TPH-2 (14, 30, 36, 50). Uptake of 5-HT into enterocytes is mediated by serotonin reuptake transporter (SERT), a serotonin-selective transport protein (11, 49). Contradictory results on EC cell counts, 5-HT content and expression of TPH-1, and SERT in mucosal rectal biopsies of IBS patients have been reported (6, 12, 18, 34, 43). Increased EC cell numbers, higher 5-HT content, enhanced release, and impaired uptake of 5-HT have been shown in conditions characterized by overt mucosal inflammation, such as animal models of colitis and inflammatory bowel disease (12, 16, 21, 31, 32, 52). These findings indicate that alterations in 5-HT signaling may be due to active inflammation. Notably, low-grade inflammation has been shown in IBS patients (39, 43, 51). Recently, in patients with IBS, increased colonic 5-HT release was correlated with mucosal mast cell infiltration (15).

Another recent study in pediatric patients with IBS demonstrated that children showing mild increases in rectal mucosal immune cell counts had higher 5-HT content and lower SERT mRNA expression (20). Since only a subgroup of IBS patients is visceral hypersensitive to colorectal distension, it is conceivable that differences in the portion of IBS patients exhibiting colorectal hypersensitivity underline discrepancies in serotonergic signaling pathway components in IBS patients. Also, for serine protease signaling supporting evidence that alterations in pathway components correlate with visceral hypersensitivity to colorectal distension is lacking. In this study we determined whether or not altered colorectal mucosal serine protease and
serotonergic signaling pathways components are related to rectal visceral hypersensitivity of IBS patients. As changes in serine protease and 5-HT signaling are associated with immune activation, the inflammatory status of the biopsy specimens was carefully assessed.

MATERIALS AND METHODS

Subjects

Twenty-three patients with IBS according to Rome II criteria participated in the study. Using the Rome II criteria four patients were classified as constipation-predominant IBS (IBS-C), 11 diarrhea-predominant IBS (IBS-D) patients, and eight as IBS patients with alternating bowel habits. Organic gastrointestinal disorders were ruled out by colonoscopy or sigmoidoscopy. Exclusion criteria also included celiac disease, diabetes mellitus, major abdominal surgery, endocrine, central nervous system, or severe psychiatric disorders as assessed by history taking, physical examination, laboratory tests, and, when considered appropriate, duodenal biopsy, lactose tolerance test, fecal culturing, and abdominal imaging. Fifteen subjects who underwent a negative screening colonoscopy or sigmoidoscopy because of family history of colon cancer, unexplained anemia, hemorrhoids, or previous colonic polyps, and who had no gastrointestinal symptoms, were used as control subjects. A series of questions about the medical history and the Rome II criteria served to check the health status of the controls. During endoscopy no signs of abnormality, including inflammation in the descending colon, sigmoid, or rectum were seen in any of the subjects. All participants were not permitted to use erythromycin, octreotide, serotonin antagonists or agonists, antidepressives, and prokinetics 1 wk before the endoscopy and rectal barostat test. Furthermore, laxatives, anti-diarrheals, antacids containing magnesium or aluminum salts, antispasmodic agents, calcium antagonists, or nitrates and opioids or other narcotic analgesics were not permitted 48 h before the endoscopy and rectal barostat test. The study was approved by the medical ethics committee of the University Medical Centre Utrecht, and written informed consent was obtained from all participants.

Study Protocol

After an overnight fast, the subjects underwent a sigmoidoscopy or colonoscopy. Bowel preparation for the sigmoidoscopy consisted of sennoside containing laxatives (Prunacol) and phosphosoda enema (Colex) at the morning of the sigmoidoscopy. Bowel preparation for colonoscopy consisted of macrogol containing laxatives (Colofort). Four mucosal biopsy specimens of the rectum and sigmoid per subject were obtained. Biopsies for mRNA expression analysis and ELISA measurement were immediately snap frozen in liquid nitrogen and subsequently stored at −80°C. Biopsy for histologic examination was stored in paraformaldehyde 2% at 4°C. Within a week from the endoscopy, sensitivity to rectal distension was measured in IBS patients.

mRNA Expression Analysis

Total RNA isolation, cDNA synthesis, and monitoring of mRNA levels of PAR-2, trypsinogen IV, and TPH-1 were performed as described previously (26).

TagMan MGB probe (oligonucleotides 5’-3’: ccagcacagtctcagaa)-based detection was used for the quantification of SERT mRNA levels. Polymerase chain reaction of SERT was carried out using 5 μl of diluted cDNA, 12.5 μl 2× iQ Supermix (Bio-Rad, Hercules, CA), and 300 nM of the forward and reverse primer each, and 100 nM TagMan MGB probe 5’-labeled with FAM and a nonfluorescent quencher at the 3’-end. MgCl₂ was added to obtain a final concentration of 5 mM in a total volume of 25 μl. Thermal cycling conditions consisted of a 3 min 95°C initial denaturation step, followed by 50 cycles of 15 s denaturation at 95°C and 1 min annealing and extension at 60°C.

For normalization purposes three endogenous reference genes were measured; porphobilinogen deaminase (PBGD), β-actin (ACTB), and GAPDH. Expression levels in the various biopsy specimens were quantified by calculating initial target concentrations using the obtained threshold cycle values and the relative standard curve (26). Subsequently, for each sample the level of a gene of interest was divided by that of the geometric mean of the three reference genes to obtain the normalized mRNA expression (26, 48).

Measurement of 5-HT and Substance P Content of Mucosal Biopsies

The biopsy specimens were homogenized in 1 ml ice-cold buffer [1× PBS, 40 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin-A, 1 mM PMSF] using the Omni M H homogenizer. Suspending were centrifuged at 25,000 rpm for 20 min at 4°C. In the supernatants, the 5-HT content (RE 59121; IBL, Hamburg, Germany), substance P content (583751; Cayman Chemical, Ann Arbor, MI), and protein content (QuantitPro BCA assay; Sigma, Saint Louis, MO) were quantified using an enzymatic immunoassay kit according to manufacturer’s instructions. C18-SPE extraction of the supernatants prior to substance P content measurement was performed (20). After the extraction, the samples were reconstituted in enzyme immunoassay buffer. Substance P content values out of the linear range of the standard curve (<20%) were assigned the content value of 20% for statistical purposes. The contents of 5-HT and substance P were expressed per milligram of protein (±SE).

Histopathology and Immunohistochemistry

Fixed biopsies were embedded in paraffin wax with orientation optimized using a dissecting microscope to ensure that sections (5 μ) were perpendicular to the mucosa. The sections were stained with hematoxylin and eosin (H & E) and immunohistochemistry staining with monoclonal antibodies for CD3 (detects T lymphocytes, 1/100; DAKO, Glostrup, Denmark), mast cell tryptase (1/800; DAKO), and 5-HT (rabbit anti-human, PSE 1/200; Eurodiagnostica, Malmö, Sweden). A single expert pathologist did a conventional histologic assessment on H & E-stained sections of the biopsies. The mean numbers of mast cells were expressed per millimeter squared of the total mucosal area. The mean number of intraepithelial T lymphocytes were expressed per 100 enterocytes. The mean number of 5-HT-immunoreactive cells were evaluated per colonic gland. Acceptable crypts were defined as being midaxial, with U-shaped sections extending from the muscularis to the lumen with an intact structure.

Rectal Barostat Test

A barostat (Distender Series II Barostat, Protocol Plus; G&J Electronics, Willowdale, Ontario, Canada) was used to inflate a disposable 500-ml polyethylene bag tightly wrapped on the distal end of a polyvinyl tube in the rectum for physiological measurements (53). The barostat allows recording of rectal volume at a fixed pressure level, which is an indirect measure of rectal tone. Rectal tone represents the volume of the rectum after 15 min at a pressure of minimal distending pressure (MDP) ± 2 mmHg. In addition, by inflating the intrarectal barostat bag, sensitivity to rectal distension can be assessed in a controlled fashion. The rectum was evacuated with an enema of tap water. The tightly folded bag was inserted into the rectum. During the measurement, the patient was in a prone position to reduce the gravitational effects of the abdominal organs. After an adaptation period of 10 min, MDP was determined. MDP was defined as the minimum pressure required to overcome mechanical forces and at which the intrabag volume was > 30 ml. To assess basal rectal tone, operating pressure was set at MDP + 2 mmHg, and intrabag volume
was measured for 15 min. Rectal distensions were performed according to a rapid phasic, isobaric distension protocol. The pressure increment was 4 mmHg above MDP, each step lasting 1 min and separated by 1-min intervals at MDP level. The inflation rate was set at 32 ml. After 45-s distension, distension-evoked sensations of urgency, discomfort, and pain were graded from none to an extreme amount on a visual analog scale. At the threshold for urgency, discomfort or pain, or when the maximum pressure of 50 mmHg was reached, the bag was deflated, and this completed the distension session.

The discomfort and pain thresholds were defined as the amount of pressure above MDP at which the subject reported a score > 80% on the VAS. The threshold for discomfort or pain lower than MDP + 32 mmHg was used in this study to define visceral hypersensitivity (3, 17). If the subject requested to stop the distensions before 80% on any of the VAS scores was reached, the thresholds were not determined. Wall tension was calculated assuming a cylindrical shape of the balloon in the rectum. Laplace’s law was applied to a cylinder for calculation of wall tension.

Statistics

To obtain normally distributed data, the normalized mRNA levels were first transformed by taking the natural logarithm. The relative differences between the groups are expressed as a fold change. An independent-samples t-test for evaluation of difference in mRNA expression, 5-HT, and substance P content and EC cell, intraepithelial lymphocytes (IEL), and mast cell counts between controls and IBS patients was performed. Regional differences of mRNA expression, 5-HT, and substance P content and EC cell, IEL, and mast cell counts between sigmoid and rectum were evaluated by applying an univariate one-way ANOVA and a post hoc test (Bonferroni correction).

A Mann-Whitney U-test was performed for evaluation of difference in mRNA expression, 5-HT, and substance P content and EC cell, IEL, and mast cell counts between controls and rectal hypersensitive (+) and (-) IBS patients. A P < 0.05 was considered significant. All statistical analysis was performed using commercially available software (SPSS 12.0.1; SPSS Science, Chicago, IL for Microsoft Windows).

RESULTS

Subjects

Twenty-three IBS patients (14 female) and 15 controls (6 female) were included in the study. The mean age (range) of the IBS patients was 39.5 (22–65) yr vs. 42.1 (22–62) yr of the controls. There were no statistical differences between patients and controls with regard to gender distribution and age.

Rectal Barostat Test

Rectal sensitivity was measured in 19 IBS patients (Table 1). Of the 19 IBS patients who underwent rectal sensitivity measurement, eight were IBS-A, 7 IBS-D, and 4 IBS-C patients.

Table 1. Rectal barostat parameters in the irritable bowel syndrome (IBS)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hyper (-)</th>
<th>Hyper (+)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDP, mmHg</td>
<td>8.0 ± 1.1</td>
<td>9.5 ± 0.92</td>
<td>0.316</td>
</tr>
<tr>
<td>Discomfort, mmHg</td>
<td>38.0 ± 1.7</td>
<td>21.3 ± 1.9</td>
<td>0.001</td>
</tr>
<tr>
<td>Pain, mmHg</td>
<td>38.9 ± 1.1</td>
<td>26.7 ± 2.2</td>
<td>0.002</td>
</tr>
<tr>
<td>Rectal tone, ml</td>
<td>90.1 ± 15.6</td>
<td>128.1 ± 9.7</td>
<td>0.063</td>
</tr>
<tr>
<td>Rectal wall tension, mmHg/cm</td>
<td>26.9 ± 3.3</td>
<td>37.2 ± 4.3</td>
<td>0.094</td>
</tr>
</tbody>
</table>

Values are means ± SE. Patients were divided into a hypersensitivity (+) group (n = 12) on the basis of a discomfort/pain threshold ≤ minimal distending pressure (MDP) + 32 mmHg and hypersensitivity (-) group (n = 7).

Table 2. mRNA expression of PAR-2, trypsinogen IV, SERT, and TPH-1 normalized against geometric mean of reference genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sigmoid</th>
<th>Rectum</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-2</td>
<td>2.2 ± 0.13</td>
<td>4.6 ± 0.48</td>
</tr>
<tr>
<td>Controls</td>
<td>2.3 ± 0.11</td>
<td>4.3 ± 0.38</td>
</tr>
<tr>
<td>Trypsinogen IV</td>
<td>0.51 ± 0.06</td>
<td>0.94 ± 0.13</td>
</tr>
<tr>
<td>Controls</td>
<td>0.73 ± 0.11</td>
<td>1.0 ± 0.21</td>
</tr>
<tr>
<td>SERT</td>
<td>5.5 ± 1.5</td>
<td>2.6 ± 1.2*</td>
</tr>
<tr>
<td>Controls</td>
<td>14.3 ± 5.9</td>
<td>2.8 ± 0.54</td>
</tr>
<tr>
<td>TPH-1</td>
<td>0.63 ± 0.13</td>
<td>1.5 ± 0.23*</td>
</tr>
<tr>
<td>Controls</td>
<td>0.90 ± 0.14</td>
<td>3.0 ± 0.59</td>
</tr>
</tbody>
</table>

Values are means ± SE of normalized mRNA level. The mRNA expression cannot be compared between the different genes since different standard curves were used. PAR, protease-activated receptor; SERT, serotonin reuptake transporter; TPH, tryptophan hydroxylase. *P < 0.05 compared with controls.

mRNA Expression

For all SYBR Green-based assays, amplification yielded a single product, which size was equivalent to that predicted from the relevant sequence. In sigmoid and rectum the level of mRNA expression of the reference genes (ACTB, GAPDH, and PBGD) was comparable between controls and IBS patients. Between hypersensitivity (+) and (-) IBS patients the level of mRNA expression of the reference genes was comparable in sigmoid and rectum.

The mRNA expression results for PAR-2, trypsinogen IV, SERT, and TPH-1 are shown in Table 2.

PAR-2. In sigmoid and rectum no differences were observed in PAR-2 expression normalized against the geometric mean of the three reference genes between controls and IBS patients.

Trypsinogen IV. In sigmoid and rectum no differences in trypsinogen IV expression between controls and IBS patients were observed. No significant difference in trypsinogen IV expression between rectal hypersensitivity (+) and (-) IBS patients in sigmoid and rectum was observed.

TPH-1. In IBS patients, the expression of TPH-1 normalized against the geometric mean of the three reference genes was lower (P = 0.005) in rectum compared with controls. The relative decrease of TPH-1 expression was 2.2 in the rectum. In, respectively, the sigmoid and rectum of the hyper-sensitivity (+) IBS patients, TPH-1 expression was 1.8-
2.2-fold lower \((P = 0.015\) and \(P = 0.051\)) compared with controls (Fig. 1). In rectum of the rectal hypersensitivity \((-)\) IBS patients, TPH-1 expression was 2.4-fold lower \((P = 0.012)\) compared with controls. In both regions no significant difference in TPH-1 expression between rectal hypersensitivity \((+)\) and \((-)\) IBS patients was observed.

**SERT.** In IBS patients, the expression of SERT normalized against the geometric mean of the three reference genes was 2.4-fold lower in rectum compared with controls \((P = 0.013)\). The mRNA expression of SERT was not significantly different in sigmoid mucosa of controls and IBS patients. However, in rectal hypersensitivity \((+)\) patients, SERT transcript level was 2.1-fold lower in the rectum compared with controls \((P = 0.028)\). SERT transcript level was not significantly different in hypersensitivity \((+)\) and \((-)\) patients (Fig. 1C). No significant difference in SERT mRNA expression in sigmoid mucosa of controls and hypersensitivity \((+)\) or \((-)\) IBS patients was observed. Finally, in sigmoid no significant difference in SERT expression between rectal hypersensitivity \((+)\) and \((-)\) IBS patients was observed.

**Mucosal 5-HT and Substance P Content**

No significant differences in mucosal serotonin content of IBS patients and controls in sigmoid \((56.1 \pm 7.4\) vs. \(48.7 \pm 7.7\) pg·ml\(^{-1}\)/mg protein) and rectum \((75.9 \pm 8.5\) vs. \(61.9 \pm 11.4\) pg·ml\(^{-1}\)/mg protein) were observed.

No significant differences in mucosal 5-HT content in sigmoid and rectum of rectal hypersensitivity \((+)\) and \((-)\) IBS patients and controls were observed.

Rectal substance \(P\) content was higher in IBS patients compared with controls \((5.31 \pm 0.61\) vs. \(3.40 \pm 0.49\) pg·ml\(^{-1}\)/mg protein; \(P = 0.045)\). No significant differences in mucosal substance \(P\) content between IBS patients and controls sigmoid \((5.38 \pm 0.55\) vs. \(3.99 \pm 0.54\) pg·ml\(^{-1}\)/mg protein) were observed. In both regions no significant differences in substance \(P\) content between rectal hypersensitivity \((+)\) and \((-)\) IBS patients were observed.

**Histopathology and EC, IEL, and Mast Cell Counts**

In all H \& E-stained biopsies a normal colonic mucous membrane was present. The lamina propria displayed a slight
amount of small lymphocytes and clusters of histiocytes. Eosinophils, fibrosis, and plasma cells were not seen. Some macrophages displayed some minimal brownish granula in the cytoplasm, not yet concomitant with pseudomelanosis. No signs of microscopic colitis, collagenous colitis, or other inflammatory conditions were seen. There were no differences found in histopathological findings between IBS patients and controls.

In Table 3, EC cell, IEL, and mast cell counts in IBS patients and controls are presented. The number of EC cells was comparable between patients and controls (P > 0.05) in both regions. No significant difference in the number of EC cells was observed between hypersensitivity (+) and (−) patients.

The number of EC cells was larger in the rectum compared with the sigmoid in controls (P ≤ 0.001). The number of mast cells per millimeter squared in the sigmoid and rectum was comparable between IBS patients and controls (P > 0.05). No significant difference in the number of mast cells was observed between hypersensitivity (+) and (−) patients.

No significant differences were found in the number of IEL per 100 enterocytes in the sigmoid and rectum of IBS patients and controls. Furthermore, no significant difference in IEL number was observed between hypersensitivity (+) and (−) patients was observed. Regional differences in IEL number were also not observed in either IBS patients or controls.

DISCUSSION

This study reveals reduced mRNA expression of TPH-1 and SERT in the rectum of both rectal hypersensitivity (+) and hypersensitivity (−) IBS patients compared with controls. Therefore, we conclude that differences in the portion of IBS patients exhibiting colorectal hypersensitivity are unlikely to explain discrepancies in studies on serotonergic pathway components in IBS patients. Our data confirm the observations of Coates et al. (12) who also reported that TPH-1 and SERT mRNA expression are reduced in the bowel of patients with IBS. Camilleri et al. (6) did not look at TPH-1, but did not find a significant difference in SERT mRNA expression. Given that IBS is a heterogeneous disorder, these discrepant results may be explained by studying different populations although subjects meet Rome criteria. More importantly, we believe that the confounding effect of inflammation causes the disparity (12, 18, 23, 31, 52). Similar to the present work, in the study of Coates et al. analysis was confined to subjects without underlying inflammation, whereas Camilleri included subjects with increased numbers of inflammatory cells.

Decreased SERT expression may be expected to increase 5-HT available for receptor activation. Although lower TPH-1 expression corresponds with reduced 5-HT synthesis and may therefore (partially) compensate for the decreased inactivation of 5-HT. Altered 5-HT availability may predispose to the manifestation of altered bowel habits or changes in stool frequency and/or consistency. In addition, our finding of reduced TPH-1 and SERT expression both in rectal hypersensitivity (+) and (−) patients does not exclude the involvement of serotoninergic dysfunction in visceral hypersensitivity. Although reduced SERT transcript levels indicate a decreased capacity to remove 5-HT from the interstitial space once it is released, SERT mRNA expression is not a measure of SERT function. Reduced SERT activity could contribute to visceral hypersensitivity through increased 5-HT availability. Thus, it is possible that SERT activity discriminates between rectal hypersensitivity (+) and (−) patients. Whatever may be the consequences for 5-HT availability, differences in sensitivity of 5-HT3 receptors on rectal extrinsic primary afferents may determine visceral sensitivity. The 5-HT3 receptor is a pentameric complex, and receptor subtypes comprising different combinations of subunits may differ in their response to 5-HT (24, 37). Recent findings support a role of mucosal 5-HT in eliciting visceral pain sensation and hypersensitivity in IBS through 5-HT3 receptors. Release of 5-HT from colonic mucosa correlated with the severity of abdominal pain/discomfort in patients with IBS (15). Furthermore, in vitro studies demonstrated that colonic mucosal supernatants of patients with IBS increased the firing rate of rat mesenteric sensory neurons and that the stimulating effects was blunted by the 5-HT3 receptor antagonist granisetron (15).

Rectal substance P content was higher in IBS patients compared with controls. However, no significant differences in substance P content between rectal hypersensitivity (+) and (−) IBS patients were observed. Substance P is released from extrinsic primary afferents upon activation and subsequently triggers the degranulation of mast cells, of which the contents may in turn stimulate the extrinsic primary afferents. Therefore, it is conceivable that elevated substance P correlates with visceral hypersensitivity. Evidence for a clear-cut relationship with visceral sensitivity is also not supported by a recent study showing substance P immunoreactive fibers to be increased in recto-sigmoid mucosa of IBS patients compared with controls although not related to abdominal pain (1). Besides from nerve endings, substance P is also secreted by enteroendocrine cells and inflammatory cells (38). No significant differences in the 5-HT synthesizing subtype of enteroendocrine cells, mast cells, and IEL were observed in the colorectal mucosal biopsies between IBS patients and controls in our study. Notably, it has been demonstrated in IBS-D that patients exhibiting rectal hypersensitivity had significantly higher total enteroendocrine cell densities in their rectal mucosa (40).

The number of EC cells was larger in the rectum compared with the sigmoid in controls (P < 0.05). No significant difference in the number of EC cells was observed between hypersensitivity (+) and (−) patients.

No significant differences were found in the number of IEL per 100 enterocytes in the sigmoid and rectum of IBS patients and controls. Furthermore, no significant difference in IEL number was observed between hypersensitivity (+) and (−) patients was observed. Regional differences in IEL number were also not observed in either IBS patients or controls.

### Table 3. Enterochromaffin (EC) cells, intraepithelial lymphocytes (IEL), and mast cell counts in IBS patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Sigmoid</th>
<th>Rectum</th>
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</thead>
<tbody>
<tr>
<td>EC cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBS</td>
<td>2.2 ± 0.31</td>
<td>2.8 ± 0.41</td>
</tr>
<tr>
<td>VH (+)</td>
<td>2.4 ± 0.38</td>
<td>2.3 ± 0.51</td>
</tr>
<tr>
<td>VH (−)</td>
<td>2.0 ± 0.54</td>
<td>3.6 ± 0.90</td>
</tr>
<tr>
<td>Controls</td>
<td>1.8 ± 0.25</td>
<td>3.8 ± 0.55</td>
</tr>
<tr>
<td>IEL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBS</td>
<td>0.03 ± 0.005</td>
<td>0.02 ± 0.003</td>
</tr>
<tr>
<td>VH (+)</td>
<td>0.04 ± 0.013</td>
<td>0.02 ± 0.006</td>
</tr>
<tr>
<td>VH (−)</td>
<td>0.04 ± 0.007</td>
<td>0.03 ± 0.012</td>
</tr>
<tr>
<td>Controls</td>
<td>0.03 ± 0.004</td>
<td>0.02 ± 0.003</td>
</tr>
<tr>
<td>Mast cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IBS</td>
<td>111.9 ± 8.5</td>
<td>97.1 ± 8.2</td>
</tr>
<tr>
<td>VH (+)</td>
<td>103.6 ± 11.4</td>
<td>94.4 ± 13.8</td>
</tr>
<tr>
<td>VH (−)</td>
<td>124.9 ± 17.6</td>
<td>111.9 ± 11.9</td>
</tr>
<tr>
<td>Controls</td>
<td>94.8 ± 7.2</td>
<td>84.6 ± 5.0</td>
</tr>
</tbody>
</table>

Values are mean ± SE. Mast cells are expressed per millimeter squared, IEL per 100 enterocytes, and EC cells per colonic gland. VH (+), rectal hypersensitivity on the basis of a discomfort/pain threshold ≤ MDP + 32 mmHg; VH (−), no rectal hypersensitivity.

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We acknowledge that our study has some limitations. In rectal hypersensitivity (−) patients the reduction in rectal SERT mRNA did not reach statistical significance due to an outlier. Before one can be certain that SERT mRNA expression is reduced in the rectum of IBS patients without rectal hypersensitivity larger studies are needed. Furthermore, a threshold for discomfort or pain lower than MDP + 32 mmHg (i.e., ∼40 mmHg) was used in this study to identify rectal hypersensitivity (+) IBS patients. Previously, it was demonstrated by Bouin et al. (3) that a pain threshold of 40 mmHg best discriminated IBS patients from healthy volunteers. Although 90.7% of the IBS patients studied by Bouin were hypersensitive in the rectum, the 40 mmHg pain threshold does not necessarily indicate hypersensitivity as would a 95% reference range obtained from a group of healthy volunteers. Considering this, it has to be noted that our hypersensitivity (+) group might contain normosensitive IBS patients. On the other hand, the use of a VAS score of ≥ 80% for the discomfort or pain threshold instead of 30% as applied by Bouin probably lowers the number of false-positive hypersensitivity (+) IBS patients. We cannot rule out that our choice for defining hypersensitivity might have masked significant differences between hypersensitive (+) and (−) IBS patients. In addition, combining the three IBS subgroups may have influenced the results. There is evidence that IBS-C patients have increased mucosal 5-HT concentration and EC cell numbers, whereas IBS-D patients were reported to have reduced mucosal 5-HT concentration (8, 25). Although the number of IBS-C and IBS-A patients does not differ between the hypersensitivity (+) and (−) groups, six out of seven IBS-D patients who underwent the rectal barostat test were hypersensitive. The higher number of IBS-D patients in the hypersensitivity (+) group might have led to reduced mucosal 5-HT concentration and may have precluded the detection of a significant difference between hypersensitivity (+) and (−) patients. Comparing a group of patients with possibly increased as well as reduced mucosal 5-HT concentrations to controls may have leveled out differences. Future studies should test relationship of alterations in serine protease and serotonergic signaling components with visceral hypersensitivity by comparing hypersensitivity (+) and (−) patients for each IBS subgroup individually.

With regard to data interpretation we want to draw attention to the following. First, as preparation for colonoscopy macrogol-containing laxatives were used, whereas sennoside-containing laxatives and sodium phosphate enemas were used for sigmoidoscopy. Sigmoidoscopy and consequently sennoside-containing laxatives were used more in IBS patients (17/23) than in controls (3/15) due to clinical preference of the physician. It has been reported that a single dose of sennoside-containing laxatives increases the proliferation rate of colonic epithelial cells and reduces crypt height (47). During cell proliferation, mRNA expression of housekeeping genes, such as GAPDH and ACTB, is enhanced (16). Similar reference gene expression in rectum and sigmoid of IBS patients compared with controls indicates that different bowel preparation for colonoscopy and sigmoidoscopy has not affected the results. Second, no significant differences were observed in PAR-2 expression between controls and IBS patients. Since we used mucosal biopsies, information on PAR-2 expressed by sensory nerves and the myenteric plexus located in the submucosal area is not included. Finally, in rectal hypersensitivity (+) IBS patients, TPH-1 mRNA expression was also significantly reduced in the sigmoid. In rectal hypersensitivity (−) patients, no significant difference in sigmoidal TPH-1 transcript level was observed probably due to a smaller difference in expression level and/or the smaller sample size.

In conclusion, in biopsies carefully evaluated for inflammation, abnormalities in colorectal components of serotonergic signaling showed no differences between rectal hypersensitivity (+) and (−) IBS patients. Although a clear pattern of alterations has not yet emerged, and their relationship with regard to motility, secretion, and visceral sensitivity has not been resolved, our results support a role for disordered enteric serotonergic signaling in the pathophysiology of IBS.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


