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Significance of Impairment of Antioxidants in Colonic Epithelial Cells Isolated From TNBS-Iuduced Colitis Rats

Walter-Leopoldo Zea-IRIARTE1, Kazuya MAKIYAMA1, Shinji GOTO6, Kunihiko MURASE1, Yoshishige URATA6, Ichiro SEKINE3, Kohei HARA6, Takahito KONDO6

1) Second Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto Nagasaki 852, Japan.
2) Department of Pathological Biochemistry, Nagasaki University School of Medicine, 1-12-4 Sakamoto Nagasaki 852, Japan.
3) Department of Pathology, Atomic Disease Institute, Nagasaki University School of Medicine, 1-12-4 Sakamoto Nagasaki 852, Japan.

The functional status of glutathione (GSH), its related enzymes and Cu, Zn-superoxide dismutase (SOD) in colonocytes isolated from trinitrobenzene sulphonic (TNBS)-induced colitis rats was studied. Colitis (T group) was induced in Wistar rats with 42 mg TNBS dissolved in 0.35 ml of 40% (v/v) ethanol instilled into the colon. The animals were sacrificed on day 14 and compared with saline-instilled rats (S group). The GSH concentration and the enzymatic activities of glutathione peroxidase (GPx), glutathione S-transferase (GST), and SOD were spectrophotometrically estimated. The severity of colitis was assessed histologically and by myeloperoxidase activity (MPO) in whole colonic tissue. The body weight loss of the rats in the T group was marked. In colonocytes isolated from rats in the T group, the concentration of GSH (7.9 ±1.4 vs. 11.3 ± 0.4 nmol/mg protein, p < 0.05) and the activities of GST (104.4 ± 10.3 vs. 146.2 ± 18.5 mU/mg protein, p < 0.05) and SOD (74.4 ± 8.9 vs. 99.8 ± 7.5U/mg protein, p < 0.05) were lower, but the activity of GPx (430.0 ± 14.1 vs. 283.9 ± 10.0 mU/mg protein, p < 0.05) was higher than in the S group. As expected, the activity of MPO in the T group was higher than in the S group (371.2 ± 14.7 vs. 158.9 ± 8.4 mU/mg tissue, p<0.05) and histologically, colitis was only observed in rats in the T group. In conclusion, the functional status of antioxidants in the colonic epithelial cells of rats challenged with TNBS solution is impaired. This impairment may make them more susceptible to oxidative damage that may contribute to the development of the lesions observed in this model. Further studies at the molecular level are necessary to investigate these novel findings in this model and their potential application for testing new therapeutic approaches in inflammatory diseases of the intestinal tract.

Key Words : glutathione, glutathione peroxidase, glutathione S-transferase, superoxide dismutase, trinitrobenzene sulphonnic acid, colitis, colon.

Introduction

The gastrointestinal (GI) tract is well supplied with antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and its related enzymes1. The importance of these defense mechanisms against reactive oxygen metabolites (ROMs) is well established2. For instance, GSH alone has ROMs-scavenging ability, but it is also critical for the function of glutathione peroxidase (GPx) and glutathione S-transferase (GST). The former metabolizes a variety of potentially important organic peroxides (i. e., DNA and lipid peroxides) and works in concert with CAT to remove H2O2 from the cells, and the latter forms conjugates with a broad spectrum of electrophilic toxins to GSH (xenobiotic metabolism) and has selenium-independent peroxidase activity4. Furthermore, SOD (a family of metalloproteins) catalyzes the dismutation of O2 to H2O2 that virtually eliminated reactions leading to the formation of ·OH7.

On the other hand, a pathophysiological role of ROMs has been proposed in a variety of diseases of the GI tract4 and in inflammatory bowel disease (IBD) such as ulcerative colitis (UC) and Crohn's disease (CD), since inflammatory cells (i. e., neutrophils, eosinophils, and macrophages) can produce large amounts of ROMs8 and the therapeutic effect of sulfasalazine and 5-aminosalicylic acid in the treatment of UC may be partially based on their scavenging abilities9,10. Regardless of the role that ROMs may play11, the above mentioned antioxidants have received little attention in IBD. Understanding the functional status of antioxidants will provide insight into the pathophysiology of IBD and/or allow new therapies and drugs to be tested. In this context, the rat model of colitis based on the intracolonic administration of an ethanolic solution of trinitrobenzene sulphonnic acid (TNBS) is particularly useful12. The inflammatory lesions induced by TNBS resemble IBD in both histologic and morphologic
Induction of chronic colitis

Yokohama, Japan) weighing 240-280 g were used. The animals were housed in rack-mounted wire cages with a 12-hour light/dark cycle, and acclimated for at least one week prior to the experiment. Standard laboratory pelleted formula (Oriental Yeast Co., Ltd., Fukuoka, Japan) was provided. The animals were fasted for 48 h prior the experiment with free access to tap water. The body weight of each rat was measured three times a week.

Animals

Eight-week-old male Wistar rats (Charles River Japan, Yokohama, Japan) weighing 240-280 g were used. The animals were housed in rack-mounted wire cages with a maximum of five animals per cage. The rats were placed in a room with a constant temperature of 21°C-23°C and a 12-hour light/dark cycle, and acclimated for at least one week prior to the experiment. Standard laboratory pelleted formula (Oriental Yeast Co., Ltd., Fukuoka, Japan) was provided. The animals were fasted for 48 h prior the experiment with free access to tap water. The body weight of each rat was measured three times a week.

Induction of chronic colitis

The animals were weighed and lightly anesthetized with intraperitoneal (IP) injection of 30 mg/kg body weight of pentobarbital sodium. Colitis was induced by intracolonic administration of 42 mg of TNBS dissolved in 0.35 ml of 40% (v/v) ethanol. Before administration of the solution, a colonoscopy was performed in each rat using an Olympus-BF type 2 J bronchofiberscope (Olympus Co., Tokyo, Japan) to ensure that at least a 6-cm segment, starting from the anus, was stool-free. Colonic instillation of the solution was performed using 1-ml syringe fitted to a Swan-Ganz catheter inserted into the colon as the tip was placed in about 6 cm proximal to the anus. Placing the catheter properly, its tip-balloon was inflated with 2.5 ml of air, followed by instillation of 0.35 ml of TNBS solution. One milliliter of air was injected into the catheter to flush it. The catheter remained in place for about 2 min keeping the rat in the supine position. Finally, the catheter was withdrawn and the rats were kept in their cages until they were sacrificed on day 14.

The severity of colitis was assessed histologically and by measurement of the activity of myeloperoxidase (MPO), a biochemical marker of inflammation.

Materials and Methods

Chemicals

Phenylmethylsulfonyl fluoride (PMSF), o-dianisidine, GSH, glutathione reductase, xanthine oxidase, NADPH and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Dithiothreitol (DTT) from Boehringer Mannheim GmbH (Mannheim, Germany). Eagle’s minimal essential medium and fetal bovine serum (MEM-FBS) were from GIBCO (Grand Island, NY, USA). 2, 4, 6-Trinitrobenzene sulfonic acid sodium salt was from Nacalai Tesque, Inc. (Kyoto, Japan). Pentobarbital sodium from Abbot Laboratories (Nenbutal, North Chicago, IL, USA). Bicinchoninic acid from Pierce Chemical Co. (Rockfort, IL, USA). Trimethylammonium bromide (HTAB), 1-chloro-2,4-dinitrobenzene, 5,5'-dithiobis-2-nitrobenzoate, and all other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Experimental Design

The 30 rats included in this study were randomly divided into two experimental groups as follows: (1) saline group (S group, n = 10), intracolonic instillation of isotonic saline solution, and (2) TNBS (T group, n = 20), intracolonic instillation of TNBS-ethanol solution. The T group was further divided into two subgroups of 10 rats each for biochemical and histological studies, respectively.

Histologic Studies

Ten rats from each group were weighed and sacrificed with a pentobarbital overdose. Laparotomy was performed and the entire colon of each rat was removed. The colons were immediately rinsed with ice-cold isotonic saline solution and opened by longitudinal incision on a piece of glass on dry ice. The specimens were then quickly examined and macroscopic damage was scored on a scale of 0 (none) to 6 (most severe), in a blind fashion as previously described with some modifications. Ten specimens taken from each rat of the S group were used as controls (Table 1).

Three fragments from each colon for microscopic assessment were taken from the most representative damaged areas and pinned on a cork block. The remnant colonic tissue was stored at -80°C. A code number was assigned to each fragment. The specimens were fixed in 10% formalin for 24 h, embedded in paraffin and then processed routinely. The assessment was performed in a blind fashion by light microscopy after staining with hematoxylin and eosin (H & E) and periodic acid-Schiff (PAS). The damage was scored on a scale of 0 (none) to 6 (most severe), as previously described with some
Table 1  Criteria for Assessment of Colonic Damage

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
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<tbody>
<tr>
<td>Macroscopic</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Normal colonic tissue</td>
</tr>
<tr>
<td>1</td>
<td>Localized hyperemia, but no ulcer or scar</td>
</tr>
<tr>
<td>2</td>
<td>Linear ulcer or ulcer scar with no significant inflammation</td>
</tr>
<tr>
<td>3</td>
<td>Linear ulcer or ulcer scar with inflammation at one site</td>
</tr>
<tr>
<td>4</td>
<td>Two or more sites of ulceration and inflammation extending ≤ 1 cm</td>
</tr>
<tr>
<td>5</td>
<td>Two or more sites of ulceration and inflammation &gt; 1 cm or one major site of inflammation and ulceration extending &gt; 1 cm along the length of the colon with or without strictures</td>
</tr>
<tr>
<td>6</td>
<td>Score 5 plus perforation and/or adhesions</td>
</tr>
<tr>
<td>Microscopic</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Normal colonic tissue</td>
</tr>
<tr>
<td>1</td>
<td>Inflammation or focal ulceration (&lt; 1 cm) limited to the mucosa</td>
</tr>
<tr>
<td>2</td>
<td>Focal or extensive ulceration (≥ 1 cm) and inflammation limited to the submucosa</td>
</tr>
<tr>
<td>3</td>
<td>Focal or extensive ulceration and transmural inflammation limited to the muscularis propia</td>
</tr>
<tr>
<td>4</td>
<td>Focal (&lt; 1 cm) ulceration limited to the serosa and transmural inflammation</td>
</tr>
<tr>
<td>5</td>
<td>Extensive (≥ 1 cm) ulceration limited to the serosa and transmural inflammation</td>
</tr>
<tr>
<td>6</td>
<td>Focal or extensive transmural ulceration and inflammation, and perforation</td>
</tr>
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Modified from references 3, 15, 23, 24

Preparation of Colonic Tissue and Colonic Epithelial Cells

To prepare colonic tissue for measurement of the activity of MPO, a 1-cm segment proximal to the point of instillation of the TNBS solution was immediately excised and divided into two samples of approximately 100 mg each. It was then weighed, frozen on dry ice, stored at −20°C and used within one week.

The isolation of epithelial cells was performed according to distended intestinal sac method with some modifications. This method was selected to avoid contamination with inflammatory or mesenchymal cells that produce antioxidants as well that can bias the results. Briefly, 10 rats from each group were sacrificed and their colons were removed excluding the ceca. The colon was placed on a piece of glass on ice and the fecal content was gently milked out. Immediately, a 6-cm catheter (9-French) was inserted approximately 5 mm into the lumen at each end. It was fixed in place by a tight suture of silk to avoid leakage from the lumen. The length of the colon was rinsed thoroughly with an intestinal wash solution (0.15 M NaCl, 1 mM DTT and 0.23 μM PMSF) using two 10-cm syringes fixed to the catheters. The large intestine was then filled (approximately 10 ml) with preoxygenated (100% O₂ and warmed to 37°C) preoxygenated (100% O₂ and warmed to 37°C) buffer A (96 mM NaCl, 27 mM sodium citrate, 1.6 mM KCl, 8 mM KH₂PO₄, 5.6 mM Na₂HPO₄, and 0.23 μM PMSF) and submerged in oxygenated 0.15 M NaCl solution, and then incubated at 37°C for 15 min. Buffer A was then drained and discarded. Immediately, the colon was filled with preoxygenated (100% O₂ and warmed to 37°C) buffer B (109 mM NaCl, 2.4 mM KH₂PO₄, 4.3 mM NaHPO₄, 1.5 mM EDTA, 10 mM D-glucose, 5 mM glutamine, 0.55 mM DTT, and 0.23 μM PMSF), incubated at 37°C for 15 min, and then the solution was drained and pooled. The cells were collected in MEM-FBS and pelleting

Table 2  Effect of TNBS Solution on the Concentration of GSH and the Enzymatic Activities of GPx, GST and SOD in Colonic Epithelial Cells Isolated from TNBS-Induced Colitis Rats

<table>
<thead>
<tr>
<th>Assays</th>
<th>Sham-Instilled (S) (n=10)</th>
<th>TNBS (T) (n=10)</th>
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<tbody>
<tr>
<td>GSH* nmol/mg protein</td>
<td>11.3 ± 0.4</td>
<td>7.90 ± 1.4</td>
</tr>
<tr>
<td>a GPx₇ mU/mg protein</td>
<td>283.9 ± 10.0</td>
<td>430.0 ± 14.1</td>
</tr>
<tr>
<td>a GST mU/mg protein</td>
<td>146.2 ± 18.5</td>
<td>104.4 ± 10.3</td>
</tr>
<tr>
<td>a SOD mU/mg protein</td>
<td>99.8 ± 7.5</td>
<td>74.4 ± 8.9</td>
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Colonocytes isolated from rats 14 days after intracolonic administration of TNBS solution (T group) and sham-instilled rats (S group). GSH: glutathione, GPx: glutathione peroxidase, GST: glutathione S-transferase, SOD: Cu, Zn-superoxide dismutase. Values are expressed as mean ± SD. *p < 0.05 vs. T group vs. T group p < 0.05.
at 250 rpm for 5 min at 4°C and washed three times in PBS (9 parts 0.154 M NaCl and 1 part 0.1 M NaH$_2$PO$_4$/Na$_2$HPO$_4$, pH 7.4). The wet weight of each cell pellet was determined and then the pellet was stored at −80°C. Homogenization of the cell pellets was performed within 24 h.

Assessment of Cell Yield and Viability

Qualitative assessment of purity and morphology of cell preparations was made by light microscopy using Papanicolau’s and Giemsa stainings. Samples from all colonic remnants were also taken and stained with H & E and PAS stainings to ensure the effectiveness of the isolation technique. Cell viability was evaluated by the conventional trypan blue exclusion method. Any dye penetration was interpreted as non-viability. Cell viability was considered acceptable if greater than 80%, otherwise the sample was discarded.

Preparation of Homogenate

To measure the activity of MPO, the colonic tissues were minced in a beaker on ice containing 0.5% HTAB buffer, transferred to a test tube and homogenized with a Polytron generator (PT 1200, Kinamatica AG, Lucerne, Switzerland). The homogenates were centrifuged at 15,000 rpm for 15 min at 4°C and then the supernatant was used for the measurement of MPO activity.

To determine the concentration of GSH and the activity of GPx, GST, and SOD, the cell pellets were kept on ice and homogenized using an ultrasonic cell disrupter (Sonifier W-200p, Branson Sonic Power Co., Dambury, CT, USA). The solution used for homogenization contained 1 mM EDTA and 0.5 mM PMSF in PBS. The homogenates were centrifuged at 15,000 rpm at 4°C for 30 min and aliquots of the supernatant were stored at −80°C. Enzymatic studies and protein concentrations were then performed within one week.

Estimation of Protein and Glutathione Concentrations

The protein concentration in each homogenate of isolated colonic epithelial cells from each rat was determined by the method of Redinbaugh and Turley with BSA as the standard. The GSH concentration, expressed as nmol/mg protein, was determined enzymatically by the method of Beutler.

Estimation of Enzymatic Activities

The activity of MPO was measured spectrophotometrically (UV-2200 spectrophotometer, Shimadzu, Tokyo, Japan) according to Bradley et al. One unit of MPO activity was defined as that converting 1 µmol of hydrogen peroxide to water per minute at 25°C and expressed in mU/g tissue. The activities of GPx and GST, expressed as mU/mg protein, were also estimated spectrophotometrically as described by Beutler and 1 U of enzymatic activity was defined as 1 µmol substrate changed per minute at 37°C and 24°C, respectively. SOD activity was determined by the method of the inhibition of the reduction of nitroblue tetrazolium by SOD at 25°C, as described by Beauchamp and Fridovich. Fifty percent inhibition was defined as 1 U of activity and expressed as U/mg protein.

Previous preliminary studies in our laboratory showed that TNBS did not interfere with the results of the enzymatic assays. All enzymatic studies were performed in duplicate.

Statistical Analysis

All data are expressed as mean ± SD. Comparisons of parametric data (body weight, GSH concentration and enzymatic studies) were performed using Student’s t-test for unpaired observations. With all statistical analyses, an associated probability (p value) of ≤5% was considered significant.

Results

Body Weight

All rats in the T group lost body weight (Fig.1) in comparison with the S group. The rats in the T group lost body weight from 261.9 ± 9.2 g at the beginning of the experiment to 225.4 ± 9.2 g and 210.1 ± 8.6 g on days 7 and 14, respectively. In contrast, the rats in the S group gained body weight normally 266.3 ± 8.9 g at the beginning, to 311.8 ± 6.9 g on days 7, and to 328.0 ± 14.0 g at the end of the experiment (p < 0.05 between both groups on days 7 and 14).

Pathologic Studies

Macroscopically, the colons from the rats in the S group were normal. All rats challenged with TNBS-solution developed chronic colitis and the average macroscopic damage score was 4.4 ± 0.7. The lesions were skip and irregularly located from the point of instillation to before the cecum; no lesions were located in the cecum itself or before the point of instillation. Strictures were developed in 30% (3 of 10) of the rats. Macroscopically, all rats challenged with TNBS solution developed colitis. The inflammatory-cell infiltration around the ulcers was mainly composed of neutrophils, eosinophils and mononuclear cells. Fibrosis was observed in scars and around ulcers at the healing stage. Although the inflammatory-
cell infiltration was not quantified, these cells were more abundant in colons with much higher damage in a score-dependent fashion. The average microscopic damage score was 4.2 ± 0.9. The colons of the rats in the S group were histologically normal.

Glutathione Concentration

TNBS-ethanol solution decreased the concentration of GSH (Fig. 2). The mean concentration of GSH in the colonic epithelial cells from rats in the T group was 30% lower than those isolated from rats in the S group (7.9 ± 1.4 vs. 11.3 ± 0.4 nmol/mg protein, p < 0.05).

Enzymatic Activities

As a response to the inflammatory reaction, the activity of MPO in all rats challenged with TNBS solution increased (Fig. 3). The activity of MPO in the colon of the rats of the T group was 133.6% higher than that of rats in the T group (371.2 ± 14.7 vs. 158.9 ± 8.4 mU/g tissue, p < 0.05).
Fig. 4 Effect of TNBS-solution on the activities of GPx, GST and SOD in colonic epithelial cells isolated from TNBS-induced colitis rats. Activities of GPx, GST and SOD in colonic epithelial cells isolated from rats after intracolonic administration of TNBS solution (T group) and sham-instilled rats (S group). Values are mean ± SD and n = 10 for each group. The symbols denote the p value of each enzymatic assay, as for S group vs. T group: * p < 0.05.

In the colonic epithelium of rats challenged with TNBS solution, the activity of GST and SOD decreased (Fig. 4). As for GST activity, it decreased 28.5% in the rats in the T group in comparison with that of the S group (104.4 ± 10.3 vs. 146.2 ± 18.5 mU/mg protein, p < 0.05). As for SOD activity, it decreased in 25.5% rats in the T group in comparison with that of the rats in the S group (74.4 ± 8.9 vs. 99.8 ± 7.5 mU/mg protein, p < 0.05). On the other hand, the activity of GPx was increased in the colonocytes of all rats challenged with TNBS solution. It increased in 14.4% in rats of the T group in comparison with that of the rats of the S group (430.0 ± 14.1 vs 283.9 ± 10.0 mU/mg protein, p < 0.05).

Discussion

Experimental studies in vivo concerning the antioxidant defense mechanisms of colonic epithelial cells have been difficult to perform because of several problems such as contamination of samples with mesenchymal and inflammatory cells or other elements that may bias the results. As the distended intestinal sac method yielded viable colonocytes with negligible contamination with inflammatory cells or other kinds of tissues, this is the first study to investigate the functional status of antioxidants in isolated colonic epithelial cells in the TNBS-induced colitis model. We have reconfirmed that TNBS-ethanol solution induced colitis and demonstrated that the enzymatic activities of SOD and GST are impaired, that of GPx is enhanced and that the concentration of GSH is decreased. As antioxidants are impaired and these are sine qua non protecting the cells against ROMs, rats challenged with TNBS solution may be more susceptible to oxidative damage. This impairment can be explained on the basis of the deleterious effect of ROMs on antioxidants. These ROMs in TNBS-induced colitis may originate from the colonic epithelium that can metabolize TNBS and generate ROMs and inflammatory cells (neutrophils, eosinophils and macrophage) that are characteristic of the inflammation in this type of colitis. It can be speculated that TNBS-ethanol may initiate a vicious cycle that starts when the mucosal barrier is broken by ethanol allowing TNBS to bind substances of high molecular weight (i.e., cell-surface proteins) in colonic tissue causing more inflammation and other immunological reactions, i.e., macrophage-mediated recognition and lysis of TNBS-modified autologous cells within mucosa, that may lead to the generation of ROMs (and other mediators, like cytokines and prostaglandins) by inflammatory and colonic epithelial cells as well as other sorts of cells, i.e., microvascular endothelium. These ROMs would impair cellular antioxidants favoring cell damage or death and leading to the development of more inflammation and the ulcerative lesions observed in this model. This would amplify the inflammatory reaction, causing more lesions to develop, and finally the chronic stage would be reached. If this hypothetical progression of events indeed takes place in this experimental model, it may be more complicated and other mechanisms may also be involved, i.e., inflammatory cell-mediated damage and other cell-derived products such as proteinases and cationic proteins to name but a few. The idea that ROMs have a deleterious effect on antioxidant enzymes is not new. It has been demonstrated in our laboratory that the activity of γ-glutamylcysteine synthetase (γ-GCS), an enzyme that catalyzes the rate-limiting step of GSH synthesis, as well as the activities of GST, GPx and SOD are impaired by ROMs. Direct inhibition by ROMs of SOD, CAT and other peroxidases has also been pointed out. In concert with this, in alveolar macrophages of elderly cigarette smokers who are exposed to increased oxidative stress, a decrease in the activity of GSH synthesis was also observed as well as the activities of GST, GPx and SOD are impaired by ROMs. In other experimental studies, we have found that in rat embryos exposed to oxidative stress induced by a high concentration of glucose in the culture medium, the expression of γ-GCS mRNA is very sensitive to such oxidant stimulus, this causes changes in the activity of γ-GCS that modulate the metabolism of GSH. In this regard, it is possible that in the current study ROMs generated in TNBS-induced colitis may alter the expression of γ-GCS mRNA and thereby decrease the activity of γ-GCS that, in turn, alters the metabolism of GSH as well.
as its related enzymes in colonic epithelial cells. Similar mechanisms at the mRNA level may also take place concerning in the activity of SOD. In contrast, the activity of GPx behaved in a different manner. One potential explanation is that the oxidative stress induced by TNBS may enhance the expression of GPx mRNA, thus increasing the availability of GPx. We have also found high activity of GPx in abdominal X-ray irradiation-induced colitis in rats (unpublished observations). These mechanisms are far from clear and further studies at the molecular level are currently underway in our laboratory to clarify the relationship among TNBS-induced colitis, production of ROMs, and their effect on antioxidants.

As the therapy for IBD remains unsatisfactory to date and corticoids in high doses are frequently required, new therapeutic strategies are needed. In this regard, as antioxidants can be pharmacologically manipulated with scavengers, it seems possible that this model of chronic colitis may be used for testing new drugs with scavenging abilities. Indeed, studies in our laboratory (unpublished observations) treating TNBS-induced colitis rats with rebamipide, 2-(4-chlorobenzoylamino)-3-[2-(1H-quino- non-4-yl)]-propionic acid, a drug that has scavenging ability and has been used to treat gastric ulcer, have demonstrated that it preserves antioxidants and attenuates the severity of colitis. We found that rebamipide preserves the activities of GST and SOD and prevents the drop in the GSH concentration. The attenuation of colitis was observed clinically, histologically, and biochemically, as assessed by macroscopic and microscopic criteria, by improvement of body weight and the lack of development of colonic strictures as well as by lower the activity of MPO in rebamipide-treated rats than in the controls.

In conclusion, the functional status of antioxidants in the colonic epithelial cells of rats challenged with TNBS solution are impaired. This impairment may make them more susceptible to oxidative damage that may contribute to the development of the lesions observed in this model. Further studies at the molecular level are warranted to investigate these novel findings in this model and their potential application for testing new therapeutic approaches in inflammatory diseases of the intestinal tract.

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References