Rutoside as Mucosal Protective in Acetic Acid-Induced Rat Colitis

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Abstract: The effect of the flavonoid rutoside on acetic acid-induced rat colitis was studied. Rats were pretreated orally with different doses of the flavonoid (10, 25, and 100 mg/kg) 48, 24, and 1 hour prior to colitis induction and examined for colonic damage 24 hours later. Colonic inflammation was characterized by gross and microscopical injury, bowel wall thickening, abolition of fluid absorption, glutathione depletion, enhanced leukotriene B4 synthesis, and increased levels of myeloperoxidase and alkaline phosphatase activities. Rutoside treatment (25 and 100 mg/kg) reduced histologic injury and prevented the increase in alkaline phosphatase activity, but it had no effect on myeloperoxidase levels or leukotriene B4 synthesis. In addition, glutathione depletion was effectively counteracted at the dose of 25 mg/kg, whereas fluid absorption was achieved at the highest dose assayed. It is concluded that rutoside has an acute anti-inflammatory activity in this model which may be related to a putative direct protective effect on intestinal cells, mainly enterocytes, in which the antioxidative properties of the flavonoid may play a role.

Key words: Rutoside, acetic acid rat colitis, intestinal anti-inflammatory activity, antioxidative activity.

Introduction

Flavonoids comprise a large group of polyphenolic compounds of low molecular weight that are widespread in the plant world (1–3). Flavonoids are known inhibitors of a number of enzymes involved in intracellular signal transduction, such as hydro- lases, transferases, oxidoreductases, and kinases (1), many of which are activated in inflammation. Furthermore, a number of cells of the immune system are downregulated by certain flavonoids in vitro (2). On the other hand, most flavonoids are potent antioxidative/radical scavenging agents. Therefore, flavonoids may exert significant anti-inflammatory effects in vivo, as has been suggested elsewhere (2). One of the most common flavonoids in nature is quercetin (3,3',4',5,7-penta- hydroxyflavone), which is normally found as its glycosidic forms, such as rutoside (3-rhamnosyl-glucosylquercetin) and quercitrin (3-rhamnosylquercetin). In previous studies we have demonstrated that quercetin can affect the oxidative status of the rat intestine, protecting it from lipoperoxidative insult (4). On the other hand, quercitrin is helpful in mucosal recovery from lactose-induced chronic diarrhea in rats (5).

The chronic inflammatory diseases of the gut (ulcerative colitis and Crohn's disease) have been the subject of intense investigation in the last few years. Despite this effort, their treatment remains empirical and there is a clear demand for new anti-inflammatory agents that may be helpful in these conditions (6). Flavonoids may be useful in inflammatory bowel disease because of their multilevel anti-inflammatory action, radical scavenger activity, and low toxicity. We have previously shown that quercitrin is helpful in trinitrobenzene-sulfonic acid rat colitis (7). The aim of the present study is to evaluate the effect of the administration of rutoside, one of the major flavonoids in nature, on acetic acid-induced experimental colitis in the rat, a well established model of colonic inflammation with some resemblance to human acute intestinal inflammation (8, 9).

Materials and Methods

Chemicals

Glutathione reductase was obtained from Boehringer Mannheim (Barcelona, Spain), p-nitrophenyl phosphate was obtained from Merck (Madrid, Spain) and acetic acid was purchased from Merck-Schuchardt (Hohenbrunn, Germany). All other reagents, including rutoside, were obtained from Sigma (Madrid, Spain).

Induction of acetic acid colitis

Female Wistar rats (230–260 g), obtained from the Laboratory Animal Service of the University of Granada, were randomly distributed in several experimental groups. Animals were housed in makrolon cages (3–4 rats per cage) and maintained in our laboratory in air-conditioned animal quarters with a 12-h light-dark cycle. Animals were provided with free access to tap water and food (Panlab A.04) for 1 week before the experiments began. The induction of acetic acid colitis was adapted from that originally described by Macpherson and Pfeiffer (8). Animals were fasted for 24 h and slightly anaethesized with diethyl ether. Under anaesthesia, colitis was induced with an enema of 2 ml of 4% (v/v) acetic acid, which was neutralized after 10 sec with 2 ml of phosphate-buffered saline (PBS, pH = 7.4), administered while holding the animals.
in a supine position. These solutions were delivered by means of a Teflon cannula (o.d. 2 mm) inserted 7 cm through the anus. After administration was completed, animals were kept in a head-down position until they recovered from anaesthesia, and then were returned to their cage. Rats from the non-colitic (normal) group received 2 ml of PBS administered rectally.

Animals were divided into 5 groups and 3 of them (n = 7) were administered rutoside (dissolved in 1 ml of distilled water) 48, 24, and 1 h before colitis induction. Each group received one different dose: 10, 25, or 100 mg/kg, p.o., by means of an esophageal catheter. An acetic acid control group (n = 14) and a phosphate buffer (PBS) control group (n = 14) were also included for reference. Animals from both groups were daily given 1 ml of distilled water orally. All animals were studied for in vivo colonic fluid absorption 24 h after colitis induction and afterwards they were sacrificed and examined for colonic damage.

In vivo colonic fluid absorption

The tied-off rat colon technique proposed by Beubler and Juan (10) was used and modified to the experimental needs. The animals were anaesthetized with urethane (1.2 g/kg i.p.) and the entire colon rinsed carefully with 20 + 20 ml of warm saline to remove fecal contents. The lumen was then dried out by injecting 20 + 20 ml of air and further gentle manual expression. Immediately after cleaning, the colon was filled with 1.0 ml of warm Tyrode solution, and ligated at both ends and the animals were left on a warmed pad (37°C) for exactly 1 h. At the end of this period the abdominal cavity was re-opened and the tied-off colon carefully removed. The remaining fluid was collected in a tube and weighed. A positive difference between the final and the initial volume (as determined gravimetrically) was considered as secretion and vice versa. The results were expressed as µl/g · h wet tissue for normalization. The composition of the Tyrode solution was (mM): 136.7 NaCl, 5.4 KCl, 1.8 CaCl2, 1.05 MgCl2, 0.42 NaH2PO4, 11.9 NaHCO3, and 5.5 glucose. Preliminary experiments showed that performing the tied-off technique had no significant effect on the biochemical parameters measured in this study.

Assessment of colonic damage

The colonic segments were placed on an ice-cold plate, cleaned of fat and mesentery, and blotted on filter paper. Each specimen was weighed and its length measured under a constant load (2 g). The colon was longitudinally opened and scored for macroscopically visible damage on a 0 to 10 scale by two observers unaware of the treatment, according to the criterium described by Bell et al. (11), which takes into account the extent as well as the severity of colonic damage.

One rat in each group was randomly selected for histological determination. The samples (~1 cm long) were collected from the most severely damaged area and fixed by immersion in neutral phosphate buffered 10% (v/v) formalin. The tissue was dehydrated in ethanol and embedded in paraffin. Sections (3–4 µm) were cut on a rotary microtome, mounted on glass slides and stained with hematoxylin/eosin for light microscopic examination.

Biochemical analysis

Preparation of tissues: The colon was subsequently divided longitudinally in 4 pieces for biochemical determinations. Two fragments were frozen at −30°C for myeloperoxidase (MPO) and alkaline phosphatase (AP) plus protein determination, and another sample was weighed and frozen in 1 ml of 5% (w/v) trichloroacetic acid for total glutathione content determination. The remaining sample was processed for measurement of leukotriene B4 (LTB4) synthesis. All biochemical measurements were completed within 1 wk from the time of sample collection and were performed in duplicate.

Analytical methods: Myeloperoxidase activity was measured according to the technique described by Krawisz et al. (12); the results are expressed as MPO units/g wet tissue, and one unit of MPO activity was defined as that degrading 1 mmol/min of hydrogen peroxide at 25°C. Alkaline phosphatase activity was evaluated following the method of Bessey et al. (13); the results are expressed as µU/mg protein. Protein content was quantitated using the Bradford (14) method, using bovine serum albumin (fraction V) as a standard. Total glutathione content was determined by the recycling assay described by Anderson (15), the results are expressed as nmol/g wet tissue. LTB4 synthesis was evaluated by enzyme-linked immunosorbent assay (Amersham, Madrid, Spain), and the results expressed as ng/g wet tissue (16).

Statistical analysis

All results are expressed as mean ± SEM. Differences among means were tested for statistical significance using one-way analysis of variance (ANOVA) and a posteriori least significance tests. Nonparametric data (score) are expressed as median (range) and were analyzed with the Mann-Whitney U test. All statistical analyses were carried out with the Statgraphics 5.0 software package (STSC, Maryland), with statistical significance set at p < 0.05.

Results

Effect of acetic acid administration

A single intracolonic administration of 4% acetic acid produced a syndrome characterized by diarrhea, anorexia, loss of weight, and severe colonic damage. Average food intake in the acetic acid control group was 47.5% of that of the PBS control group (21.2 ± 1.3 g/rat·day). Body weight was reduced by 3.7 ± 0.7% in the acetic acid-treated animals, whereas normal rats increased 6.2 ± 0.6%. The intrarectal instillation of acetic acid to the rats led to the development of diffuse changes in the colon consisting of erythema, inflammation, and confluent areas of extensive erosions and hemorrhage extending approximately 2 cm in length in average. They were assigned a median score of 6.5 (5.0). The histological sections showed hyperemia, edema of the submucosa, epithelial disruption mucosal erosions with goblet cell depletion, and a mixed inflammatory infiltrate. The caecum was spared from inflammation. None of the rats died throughout the experiment.

In vivo colonic fluid transport was dramatically affected by inflammation, changing from net absorption to net secretion (Table 1). Colonic inflammation was also characterized by a 4-fold increase in MPO, a 2-fold increase in AP activity, a 4-fold...
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Table 1  Effects of rutoside treatment (10, 25 and 100 mg/kg) on damage score and changes in colonic weight as well as in colonic fluid absorption 24 h after acetic acid administration.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Damage scorea (0–10)</th>
<th>Colonic weightb (mg/cm)</th>
<th>Fluid absorptionc (μl/g · h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS Control</td>
<td>14</td>
<td>0</td>
<td>82.9 ± 1.9</td>
<td>−413 ± 110</td>
</tr>
<tr>
<td>AA Control</td>
<td>14</td>
<td>6.5 (5)**</td>
<td>131.0 ± 8.2**</td>
<td>+153 ± 53**</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>7</td>
<td>4 (6)**</td>
<td>130.3 ± 12.9**</td>
<td>+30 ± 103**</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>7</td>
<td>2.5 (4)<strong>;</strong></td>
<td>109.0 ± 7.6**</td>
<td>+59 ± 42**</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>7</td>
<td>3 (5)**</td>
<td>112.7 ± 9.4*</td>
<td>−38 ± 95**;**</td>
</tr>
</tbody>
</table>

a Score data are expressed as median (range).
b Colonic weight data are expressed as mean ± SEM.
c Colonic fluid absorption data are expressed as mean ± SEM. A negative value denotes absorption, whereas a positive value denotes secretion.

Table 2  Effects of rutoside treatment (10, 25 and 100 mg/kg) on colonic glutathione (GSH) content, myeloperoxidase (MPO) activity, alkaline phosphatase (AP) activity and LTB4 synthesis 24 h after acetic acid administration.

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH (nmol/g tissue)</th>
<th>MPO (U/g tissue)</th>
<th>AP (μU/mg protein)</th>
<th>LTB4 (ng/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS Control</td>
<td>1030 ± 69</td>
<td>17.2 ± 2.7</td>
<td>6.2 ± 1.1</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>AA Control</td>
<td>560 ± 56**</td>
<td>65.5 ± 12.8*</td>
<td>14.7 ± 2.7**</td>
<td>4.2 ± 2.1**</td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>567 ± 106**</td>
<td>53.9 ± 14.6**</td>
<td>15.6 ± 1.5**</td>
<td>3.0 ± 1.2**</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>908 ± 122**</td>
<td>76.4 ± 11.7%</td>
<td>6.7 ± 1.1**</td>
<td>6.1 ± 1.5**</td>
</tr>
<tr>
<td>100 mg/kg</td>
<td>596 ± 77**</td>
<td>104.7 ± 29.6**</td>
<td>7.6 ± 1.6*</td>
<td>5.8 ± 2.4**</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM.

* p < 0.05, ** p < 0.01 vs. PBS control group.

Effect of rutoside treatment on experimental colitis

Treatment of colitic animals with rutoside had no effect on body weight change or food intake (data not shown). The colonic weight of animals treated with 25 mg/kg of rutoside was significantly lower compared to the saline group (p < 0.05) (Table 1). Treatment with either 25 mg/kg or 100 mg/kg of rutoside significantly reduced macroscopical colonic damage (p < 0.01 and p < 0.05, respectively), whereas the dose of 10 mg/kg was without effect. Thus, 5/7 of the rats treated with 25 mg/kg of rutoside only showed hyperemia and one linear ulcer surrounded by inflamed tissue. On the other hand, the majority (4/7) of the rats treated with 100 mg/kg of rutoside presented a site of gross ulceration and inflammation, not extending more than one centimeter along the colon. Histological sections obtained from treated rats displayed grossly similar changes to those observed in the acetic acid control group, i.e., the colonic wall appeared thickened and inflammatory cells were present, specially in the submucosa. However, there were no signs of epithelial sloughing and goblet cell depletion seemed to be less severe (Fig. 1).

Treatment with 100 mg/kg of rutoside improved colonic fluid transport, as shown in Table 1, reversing fluid secretion into absorption, although the absorption rate was still significantly reduced compared to the saline group.

The results from the biochemical analysis are shown in Table 2. Rutoside treatment did not reduce colonic tissue MPO activity. However, treatment with either 25 or 100 mg/kg of rutoside decreased AP activity by 54% (p < 0.01) and 48% (p < 0.05), respectively, compared to the acetic acid control group. There was no modification in the colonic levels of LTB4 after treatment of colitic rats with rutoside in comparison with non-treated animals. Total glutathione levels were increased by 61% in those rats treated with 25 mg/kg of rutoside (p < 0.01), but not with other doses.

Discussion

Instillation of 4% acetic acid in the rat colon elicited an intense acute inflammatory response, which was evidenced by macroscopical and microscopical damage of the colonic wall, elevation of inflammatory markers, impairment of fluid absorption, and loss of body weight. These features are in agreement with those reported elsewhere (17, 18).

Administration of rutoside prior to colitis induction partially prevented colonic inflammation at the doses of 25 and 100 mg/kg, whereas the dose of 10 mg/kg was without effect. Thus, rutoside treatment ameliorated the severity of the inflammatory lesions and reduced the damaged area. A reduction in colonic weight was also observed in the group treated with the dose of 25 mg/kg compared to the acetic acid control group (p < 0.05), confirming the anti-inflammatory effect of the flavonoid (Table 1).
Myeloperoxidase is an enzyme predominantly located in the azurophilic granules of neutrophils, which justifies its widespread use as a marker for inflammation (11, 18). In the present experiment, MPO levels were increased 4-fold as a result of colonic inflammation. Rutoside treatment failed to reduce colonic MPO activity at all the doses assayed. This is somewhat surprising, since most of the pharmacological agents reported to exert an anti-inflammatory effect in experimental colitis show a concomitant reduction in MPO activity (7, 16, 18). Neutrophil infiltration is not an early event in acetic acid colitis, since these phagocytes are recruited to the site of inflammation through the chemotactic action of a number of mediators, most notably interleukin 1 (16, 18), which are released in response to tissue injury. Therefore, the lack of effect of the flavonoid on MPO levels (i.e., neutrophil infiltration) suggests that it does not interfere with the release of such mediators. On the other hand, the ability of mammals to hydrolyze flavonoid glycosides to the corresponding aglycone has been previously reported (19); thus, when rutoside gets to the intestine, it can be metabolized by microbial glycosidases present in the rat intestine to release its aglycone, quercetin (20). Quercetin is a known inhibitor of neutrophil function and MPO (21), an effect which could justify the beneficial effect of the flavonoid even though MPO levels were unchanged. Nevertheless, it has been demonstrated that neutrophil depletion does not attenuate the acute colonic injury induced in rats by the intracolonic instillation of acetic acid (17), and so it is unlikely that this mechanism can solely account for the beneficial effect of rutoside.

Fig. 1 Histological sections of colonic mucosa obtained from (A) the PBS control group; (B) acetic acid control group; (C, D) groups treated with 25 or 100 mg/kg of rutoside, respectively. Original magnification: ×400. (B) shows epithelial sloughing and goblet cell depletion compared with (A). Epithelium appears preserved in (C) and (D).
There is considerable evidence involving LTB₄ as a key proinflammatory mediator in colonic inflammation (12). Arachidonic acid metabolism in acetic acid experimental colitis has been shown to closely resemble its human counterpart. As it has been previously described, the present study shows that LTB₄ synthesis is stimulated in colonic specimens obtained from colitic rats. Rutoside treatment failed to inhibit LTB₄ synthesis at all doses, even though quercetin, the aglycone of rutoside, is an inhibitor of lipoxygenase (22). These data parallel those obtained in the MPO assay, since neutrophils are generally considered as the major site of arachidonic acid metabolism (and, accordingly, LTB₄ production) in inflammatory bowel disease, both in humans and in rats (11, 17), and further suggest that rutoside treatment does not directly interfere with neutrophil function.

Alkaline phosphatase is a phenotypic marker of differentiation which is upregulated in experimental chronic diarrhea (5). We have also demonstrated that this enzymatic activity constitutes a sensitive and reliable marker of experimental colonic inflammation in the rat (7). Interestingly, treatment with 25 or 100 mg/kg of rutoside significantly reduced AP activity in the rat colon to normal values, showing a positive correlation with the anti-inflammatory effect of the flavonoid. This effect suggests that rutoside treatment somehow preserved the enterocytes from insult. This is consistent with the observation of grossly normal epithelium in histological sections obtained from rats treated with either dose of rutoside.

Oxygen (and nitrogen) free radicals have been proposed to play a role in the pathophysiology of intestinal inflammation, and antioxidative therapy (glutathione, desferrioxamine, superoxide dismutase, sulfasalazine) has been successfully tried in human and experimental colitis (7, 23). Decreased glutathione levels, which are indicative of oxidant stress, have been detected both in human (24) and experimental colonic inflammation (7). Such oxidative stress has also been detected in our study, since colitis produced a ~50% drop in total glutathione levels. Rutoside treatment effectively counteracted glutathione depletion at the dose of 25 mg/kg, but this effect disappeared as the dose was increased. This is in agreement with the dose-response relationship observed with the related flavonoid quercitin in trinitrobenzene-sulfonic acid rat colitis (7). These data demonstrate that rutoside has a glutathione sparing effect in rat colitis which is attributable to its antioxidative properties, and this may be a mechanism of action involved in the effect of the flavonoid.

One of the classic symptoms of colonic inflammation is diarrhea. Several authors have directly examined the impact of experimental colitis on hydroelectrolytic transport. Thus, Federak et al. (9, 25) have demonstrated that acetic acid-induced colitis is accompanied by a significant decrease in colonic basal net sodium and chloride absorption, as a consequence of colonic barrier disruption and increased permeability. Disturbances in permeability have been suggested to be involved in the pathogenesis of inflammatory bowel disease. Therefore, impairment of fluid absorption, as found in our study (Table 1), primarily reflects the loss of viable epithelial surface in the early stages of colitis. Only the highest dose of rutoside assayed (100 mg/kg), was able to reverse secretion into net absorption, although to a limited degree, compared with normal fluid absorption (saline treated animals). Considering the aforementioned reduction in damaged area by rutoside at these doses, amelioration of colonic transport may be partly ascribed to mucosal protection from inflammation. Nevertheless, there must be other mechanisms involved, since treatment with 25 mg/kg of rutoside did not have such effect on fluid absorption while reducing the injured area.

In summary, our data show that treatment of experimentally induced colitis with rutoside, one of the most common flavonoids in nature, provides a colonic mucosal protective effect. The anti-inflammatory activity of rutoside seems to be unrelated to impairment of neutrophil function or lipooxygenase inhibition, and may be ascribed to preservation of the mucosa in which protection against oxidative insult (at lower doses) or amelioration of colonic fluid absorption (at higher doses) may play a role.

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