Calcium butyrate: Anti-inflammatory effect on experimental colitis in rats and antitumor properties

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Abstract. Butyric acid is a physiological component of the colonic environment that possesses anti-inflammatory and antitumor properties, among others. However, little is known regarding its effects following direct application on the colonic surface. This study was conducted to investigate the topical anti-inflammatory effect of calcium butyrate in chemically-induced colitis in rats and to evaluate its antitumor properties in vivo and in vitro. The anti-inflammatory activity of calcium butyrate was evaluated in dinitrobenzene sulfonic acid-induced colitis in rats, following intracolonic instillation for 6 consecutive days and its in vivo antitumor activity was evaluated in F344 rats with the azoxymethane (AOM)-induced aberrant crypt foci (AFC) test, following intracolonic instillation for 4 weeks. The in vitro antiproliferative activity was assessed by incubation for 48 h with the HT29, SW620 and HCT116 intestinal tumour cell lines to evaluate the rate of ⁴H-thymidine uptake. In dinitrobenzene-induced colitis, the intracolonic instillation of calcium butyrate completely prevented body weight reduction in the animals and counteracted the local noxious effects of the irritant by reducing colon edema (-22.7%, P=0.048) and the area of mucosal damage (-48%, P=0.045). In the AOM-induced AFC test, the intracolonic instillation of calcium butyrate significantly reduced the number of AFC in the entire colon (-22.7%, P<0.05). Calcium butyrate, following incubation with the HT29, SW620 and HCT116 tumour cell lines, induced a significant antiproliferative, dose-dependent effect (P=0.046 to P=0.002) in all three strains, as measured by the reduction in ⁴H-thymidine uptake. Calcium butyrate directly applied to the mucosa of the rat colon was able to ameliorate colonic inflammation, suggesting a possible beneficial role in the treatment of inflammatory colon diseases. Moreover, calcium butyrate exhibited notable antitumor effects in vivo and in vitro; however, their clinical relevance requires confirmation by additional clinical investigations.

Introduction

Butyric acid is a short-chain fatty acid produced in the colon by microflora fermenting dietary fibres, starch and lactate (1,2). The rationale supporting the use of butyric acid in intestinal disorders is associated with its well recognized ability to supply energy to the colocytes in order to regulate their metabolism, control their motility and water/ electrolyte absorption and protect against infections and the onset of inflammatory processes (1,3,4). Moreover, butyric acid is actively involved in the control of the pathological growth of the colocytes (5-8), directing their replication through mechanisms involving inhibition of DNA synthesis and restoration of apoptosis (6,9-17). These mechanisms are closely associated with the properties of butyric acid acting as a potent histone deacetylase inhibitor and as a p21 and p53 gene activator (18-25). The preventive effects of butyric acid on colorectal cancer may also be mediated by enhancing TGF-β signalling and its suppressor function in the gut epithelial cells (26). Butyric acid is also a potent activator of acid sphingomyelinase (27), leading to the production of well known antiproliferative molecules, such as ceramide and sphingosine (28-30). The multiple effects of butyric acid on the biology of colocytes prompted the investigation of the anti-inflammatory properties of calcium butyrate directly applied on the colon surface in a chemically induced model of experimental colitis, in order to evaluate its possible role in colon cancer development in vivo and in vitro.

Materials and methods

Ethical and general considerations. All the in vivo experiments were approved by the local Institutional Ethics Committee and were performed in agreement with the Italian Legislative Decree 116/27.01.1992 regarding the protection of animals used for experimental or other scientific purposes. All the experiments used animals kept in temperature- and light-controlled rooms, maintained at a constant temperature of ~24°C and a 12-h light/dark cycle. The animals were fed...
standard animal pellets (Mucedola s.r.l., Milan, Italy) and tap water ad libitum.

**Effect of calcium butyrate on dinitrobenzene (DNB)-induced colitis in rats.** Groups of 8-10 male Wistar rats (Charles River, Lecco, Italy) for a total of 27 animals weighing ~200-250 g were used. The test was performed as previously described (31), with the exception of a longer treatment duration. Colitis was induced in animals fasted overnight and under light ether anaesthesia, by instillation into the colonic lumen of 30 mg DNB dissolved in 0.25 ml ethanol 50%. The administration of the irritant was performed through a rectal catheter (outer diameter 0.3 mm) placed ~7 cm proximally to the anus. Following DNB instillation and insufflation with 0.5 ml air, the catheter was removed. Calcium butyrate (CAS 5743-36-2; Garuda Chemicals, Mumbai, India) was suspended in SV17874, composed of 0.4% Tween-80, 0.5% carboxymethylcellulose and 0.9% benzyl alcohol in sterile saline, and was administered into the colon by the same procedure as described for the irritant, at concentrations of 10 and 30 mg in 0.25 ml. The administration lasted for 6 days, including 3 days prior to and 3 days after colitis induction. Twenty-four hours after the last administration, the animals were weighed and sacrificed by CO₂ inhalation. Immediately after the sacrifice the intestine was exteriorized. The colon was isolated, cleaned from the mesenterium, vessels and fat and was placed unstretched on a ruler. Starting from the anal verge, a 10-cm segment of the distal colon was cut and was weighed after rinsing the lumen with saline. Subsequently, the isolated portion of the colon was longitudinally opened and pinned down on a cork table with the mucosal surface facing the observer. The mucosal damage area (MDA) was measured with the Image-Pro Plus image analysis system, version 3.0 for Windows 95/NT (Media Cybernetics 1993-97, www.mediacy.com). The image acquisition was performed with a PC camera (Chicony iCam 330; Chicony Electronics Co., Ltd., New Taipei, Taiwan) and the MDA was expressed in mm².

**Effect of calcium butyrate on induction of aberrant crypts (ACs) and aberrant crypt foci (ACF) in rat colon.** Groups of 10-12 female F344 rats (Charles River), for a total of 33 animals, weighing ~150 g, were used. The animals received two subcutaneous injections of 15 mg/kg body weight azoxymethane (AOM; Sigma-Aldrich, Milan, Italy), with an interval of 7 days between the injections, to induce the formation of ACs in the colon. Calcium butyrate was suspended in SV17874 and administered once a day, at concentrations of 0.7 and 3.5 mg in 0.25 ml, for 4 consecutive weeks, beginning on the day prior to the first AOM injection. The administration of calcium butyrate was performed through a rectal catheter (outer diameter 0.3 mm) placed ~7 cm proximally to the anus. Twenty-four hours after the last intracolonic administration, the animals were euthanized by CO₂ inhalation. Immediately after the sacrifice, the entire colon was isolated, cleaned from the mesenterium, vessels and fat and was longitudinally opened. The opened colon was transversally cut at the splenic flexure level to separate the proximal from the distal colon. The segments were rinsed with buffer saline and were fixed flat in 10% phosphate-buffered formalin (pH 6.9-7.1) for 16 h. The segments were stained with methylene blue (0.01% in phosphate-buffered neutral formalin) for 30 min, briefly washed with deionised water and placed on microscope slides with the mucosal side facing up. The mucosal surface was then investigated under a microscope (magnification, x40) for the detection of ACs and ACF. The ACs were distinguished from the surrounding normal crypts by their easier staining, increased size, thicker epithelial lining and increased pericryptal zones (32). The number of single ACs and the number of foci containing ACs were counted and recorded separately for the proximal and distal colon.

**Effect of calcium butyrate on proliferation of colon cancer cells.** HT29, SW620 and HCT116 human colon cancer cells were obtained from the National Research Institute on Cancer (Genova, Italy). All the cell lines were grown in complete Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Milan, Italy), supplemented with 1% L-glutamine 200 mM, 1% penicillin-streptomycin 10,000 U/ml and 10% foetal bovine serum (FBS; Invitrogen Life Technologies) and were maintained at 37°C in an humidified incubator with 50 ml/l CO₂. The cells were then dispensed in 96-well Nuncion Surface plates (Thermo Scientific, Milan, Italy) at a density of 3x10⁵ cells/ml and were incubated for 48 h at 37°C with calcium butyrate. Calcium butyrate was suspended in DMEM without FBS and was tested at concentrations of 1.25, 2.5 and 5 mM. Complete DMEM was used as negative control. During the last 18 h of incubation the cells were labelled with methyl-³H-thymidine (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). The effect of calcium butyrate on the proliferation of HT29, SW620 and HCT116 cells was assessed by the ³H-thymidine uptake test, which was performed at the end of the incubation period. The radioactivity was read with a Beckman LS6000SC Beta Counter (Beckman Coulter Inc., Fullerton, CA, USA) and the proliferation activity was expressed as mean counts per minute.

**Statistical analysis.** Significant differences among the experimental groups were evaluated with one-way analysis of variance, in which pairwise multiple comparisons were performed with the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

In DNB-induced colitis (Table I), the intracolonic instillation of 30 mg calcium butyrate completely prevented body weight reduction in the animals (P=0.002) and counteracted the local noxious effects of the irritant, reducing colon edema (-23%, P=0.048) and the area of mucosal damage (-48%, P=0.045).

In the AOM-induced ACF test, the occurrence of ACs and ACF in the distal colon of the control group was ~2.5 times higher compared to that in the proximal colon (Table II), resembling the different incidence of colorectal cancer detected in humans. The intracolonic instillation of 3.5 mg calcium butyrate reduced the global occurrence of ACs and ACF in both colonic districts, with a more evident activity in the distal colon. The inhibitory effect reached a statistical significance for both AC and ACF in the distal colon (-24.7 and -23.5%, respectively; P<0.05) and for ACF in the proximal colon (-20.2%, P<0.05).
Table I. Effect of calcium butyrate on DNB-induced colitis in rats.

<table>
<thead>
<tr>
<th>Local treatment (dose)</th>
<th>Body weight (g)</th>
<th>Colon weight (g)</th>
<th>MDA (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial ± SD</td>
<td>Final ± SD</td>
<td>Δ%</td>
</tr>
<tr>
<td>Vehicle</td>
<td>286±35</td>
<td>27±35</td>
<td>-13</td>
</tr>
<tr>
<td>CB (10 mg)</td>
<td>275±42</td>
<td>262±38</td>
<td>-13</td>
</tr>
<tr>
<td>CB (30 mg)</td>
<td>291±46</td>
<td>295±50</td>
<td>3.8</td>
</tr>
</tbody>
</table>

DNB, dinitrobenzene; MDA, mucosal damage area; CB, calcium butyrate; SD, standard deviation.

Table II. Effect of calcium butyrate on AOM-induced aberrant crypt foci in rats.

<table>
<thead>
<tr>
<th>Local treatment (dose)</th>
<th>Distal colon</th>
<th>Proximal colon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AC number</td>
<td>ACF number</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Δ%</td>
</tr>
<tr>
<td>Vehicle</td>
<td>138.3±36.8</td>
<td>-</td>
</tr>
<tr>
<td>CB (0.7 mg)</td>
<td>138.7±22.7</td>
<td>0.3</td>
</tr>
<tr>
<td>CB (3.5 mg)</td>
<td>104.1±16.2</td>
<td>-24.7</td>
</tr>
</tbody>
</table>

AOM, azoxymethane; AC, aberrant crypts; ACF, aberrant crypt foci; CB, calcium butyrate; NS, not significant; SD, standard deviation.
The results of $^3$H-thymidine uptake after a 48-h incubation of HT29, SW620 and HCT116 cells with calcium butyrate are presented in Table III. A significant, dose-dependent inhibitory effect on cell proliferation was observed in all three cell clones at any tested dose ($P=0.046$ to $P=0.002$). These cells differed regarding sensitivity to calcium butyrate and, as a general rule, SW620 and HCT116 cells appear to be more sensitive compared to HT29 cells.

**Discussion**

The anti-inflammatory activity of butyric acid has been already described in the literature (1,3,8); however, to the best of our knowledge, there is no currently available report regarding the effects of local administration to the colon. Our experiments demonstrated that calcium butyrate exerted a notable anti-inflammatory effect when directly instilled on the mucosal surface of the colon in rats, suggesting that oral formulations engineered to deliver butyric acid to the colon may be useful in the treatment of inflammatory bowel diseases. Our results reinforce previous, although sparse, clinical observations suggesting an efficacy of oral and rectal preparations of butyric acid in the management of inflammatory (33-36) and non-inflammatory (37) bowel disorders. In our experiments, we also demonstrated that calcium butyrate exhibited a notable antitumor activity in vivo, inducing a significant reduction in the occurrence of ACs and ACF in the colon of rats challenged with AOM. These findings may be of value, since ACF are considered to be dysplastic lesions, potentially evolving to colon cancer (32,38). Despite the fact that previous in vivo studies did not provide conclusive results regarding the beneficial effect of oral butyrate on the prevention of precancerous lesions in the gut (39,40), our results demonstrated that these effects were achieved when calcium butyrate was delivered directly into the colon. The apparent higher susceptibility of the distal colon to calcium butyrate represents further evidence of the local activity of this substance. The discrepancy between our results and those of previous studies lies with the fact that, in our study, calcium butyrate was administered topicaly, whereas following oral administration of plain formulations, the rapid and complete absorption of butyric acid in the upper part of the gastrointestinal tract may have prevented its adequate availability to the colon. This suggests that, for calcium butyrate to be active by the oral route, it should be administered in formulations delivering the substance directly to the colon, in order to provide and maintain effective concentrations in the mucosa to confer anticarcinogenic benefits. Finally, we demonstrated that calcium butyrate inhibited the proliferation of human HT29, SW620 and HCT116 colon cancer cell lines in vitro. The inhibitory effect of calcium butyrate on cancer cell proliferation was dose-dependent and did not appear to be attributable to a direct cytotoxic mechanism, since butyric acid, when incubated with normal cells, such as human lymphocytes, did not affect their viability (41). The complex of in vivo and in vitro antitumor activities of calcium butyrate opens new perspectives in the long-term strategies of colorectal cancer prevention in humans, considering that butyric acid is a substance physiologically produced in the colon, cost-effective and very well tolerated. When compared to other pharmacological approaches.
recently suggested for the prevention of colon cancer (i.e., COX-2 inhibitors and other NSAIDs), calcium butyrate offers the additional advantage of lack of interference with complex biological systems, such as prostaglandins, leukotrienes and thromboxanes, whose long-term disruption may be associated with unwanted and unpredictable effects. The data obtained from our experiments suggest a possible beneficial role of topical calcium butyrate administration in the treatment of inflammatory colon diseases. Moreover, calcium butyrate exhibited notable antitumor activity in vivo and in vitro; however, their clinical relevance requires confirmation by further well-designed clinical investigations.

References