Colonic epithelial cell activation and the paradoxical effects of butyrate

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Butyrate may have paradoxical effects on epithelial cells of similar origin. This study aimed to examine the hypothesis that one mechanism that dictates a cell’s response to butyrate is its state of activation. First, the responses to 24 h exposure to butyrate (1–2 mM) of normal and neoplastic human colonic epithelial cells activated by their isolation and primary culture, and of colon cancer cell lines, LIM1215 and Caco-2, were examined. In primary cultures of normal and cancer cells, butyrate had no effect on alkaline phosphatase activities but significantly suppressed urokinase receptor expression by a mean ± SEM of 30 ± 12% and 36 ± 9%, respectively. Interleukin-8 secretion was suppressed by 44 ± 7% in normal cells (P < 0.05) but was unchanged in cancer cells. In contrast, the cell lines significantly increased alkaline phosphatase activities by >50%, urokinase receptor expression >2-fold and interleukin-8 secretion >3-fold in response to butyrate. Secondly, the effect of butyrate on Caco-2 cells was examined with or without prior exposure to a specific activating stimulus [tumour necrosis factor alpha (TNFα)]. Interleukin-8 secretion increased by 145 ± 23% and 132 ± 17% on 24 h exposure to 2 mM butyrate or 0.1 µM TNFα alone, respectively. However, in cells pre-treated with TNFα, butyrate significantly inhibited secretion by 34 ± 7% below unstimulated levels. The response to butyrate of urokinase receptor, whose expression was not stimulated by TNFα, was unchanged. These effects were mimicked by trichostatin A, an inhibitor of histone deacetylase, suggesting that butyrate’s paradoxical effects may have been operating by the same mechanism. In conclusion, some of the paradoxical effects of butyrate do not appear to represent inherent differences between normal and transformed cells. Rather, the response may be determined by the state of activation of the cells.

Introduction

Butyrate is a short-chain fatty acid that is physiologically relevant to the colonic epithelium. It is a product of fermentation of luminal carbohydrates, is found in millimolar concentrations in the lumen, and is readily taken up by epithelial cells (1). Butyrate is the principal energy source for the colonic epithelium (2). It has been implicated in colorectal tumorigenesis since it exerts a multitude of anti-tumour effects in transformed cells in vitro, such as modulation of cell proliferation, differentiation and apoptosis, and the expression of multiple proteins (3,4). Butyrate has been found to induce hyperacetylation of histones by the inhibition of histone deacetylase (5) and the promoters of several genes contain a highly conserved sequence, the butyrate response element (6–8). In vivo, luminal butyrate concentrations are inversely correlated with tumour size in experimental colorectal tumorigenesis (9) and direct rectal or caecal instillation of butyrate reduced the size and number of tumours in experimental carcinogenesis (10,11). It is, therefore, no surprise that considerable experimental effort is being expended in order to define the effects of butyrate and the mechanism(s) by which it acts.

The cellular effects of butyrate are complex, especially since those in one cell system may be the complete antithesis of those in a different but related cell systems. This so-called ‘butyrate paradox’ has been observed in relation to cell proliferation, differentiation and apoptosis (3,4,12). The biological basis for these contrasting effects has, however, not been ascertained but there are three main explanations. First, they may reflect inherent differences in the cells, based on genetic differences. For example, Velazquez et al. (13) proposed that mutated G-proteins might have altered specific affinity for butyrate, which leads to inhibition of mevalonate-associated G-protein activation. Secondly, the ability of cells to β-oxidize butyrate may influence their response to butyrate (14). The rate of removal of butyrate from the cytoplasm should influence the availability of butyrate to exert its effects. Thus, cells that oxidize butyrate poorly may have higher intracellular concentrations of butyrate leading to direct butyrate-mediated effects, such as inhibition of histone deacetylase (5) with subsequent suppression of proliferation (15) and induction of apoptosis (16). Evidence to directly support such a hypothesis has recently been presented (17).

We hypothesize a third possibility, that the responses of cells to butyrate may depend on the cells’ state of activation, independently of β-oxidation. For example, butyrate may have opposite effects on the secretion of a certain protein in cells activated to secrete that protein than it would if that cell were in an unstimulated state. Paradoxical effects of butyrate on markers of proliferation and differentiation have been described in colonic epithelial cells freshly isolated from resected colon compared with those in colon cancer cell lines (12). A major difference between the cells studied, however, is that, for normal cells, the relatively harsh extraction process used and the subsequent culture activate the cells to, for example, synthesize several proteins, such as urokinase (u-PA) and its receptor (u-PAR), and interleukin-8 (IL-8) (18–20). The possibility that such an effect might influence the response to butyrate of the synthesis of those proteins has not previously been directly addressed.

The present study aimed to help resolve the issue of the

**Abbreviations:** DMEM, Dulbecco’s minimal essential medium; HBSS, Hank’s balanced salt solution; IL-8, interleukin-8; TNFα, tumour necrosis factor-α; TSA, trichostatin A; u-PA, urokinase; u-PAR, urokinase receptor.
mechanism by which the paradox might work by addressing the potential influence of cell activation. Two approaches were taken. First, we sought to determine whether normal and neoplastic colonic epithelial cells do indeed behave differently in response to butyrate when the potential variable of activating the cells via isolation/culture techniques was similar for each cell type. Secondly, in a more controlled experimental setting, the response to butyrate of a colon cancer cell line, Caco-2, following its specific activation by tumour necrosis factor α (TNFα) was compared with that of unstimulated cells.

Expression and secretion of specific proteins

Cells were cultured in medium (outlined above) with or without sodium butyrate at the highest concentrations that were reliably non-toxic to the cells. Previous experience indicated that, for freshly isolated cells, a concentration of 1 mM was best tolerated. In cell lines, 2 mM was well tolerated and achieved maximal responses. For the end points used, preliminary experiments showed that 1 mM produced similar effects, but to a lesser degree. In some experiments, the cells were cultured with trichostatin A (TSA; ICN Pharmaceuticals, Costa Mesa, CA), a specific inhibitor of histone deacetylase (15), at a concentration of 1 μM, which was not toxic and had maximal effects in preliminary experiments. In other experiments, cells were pre-incubated for 24 h with TNFα (a gift of Boehringer Mannheim, Mannheim, Germany) at a concentration of 10^{-7} M, and then washed. Cells were cultured at 37°C and 95% air/5% CO₂ and harvested at varying times over a 24 h period. Harvesting was performed by aspiration of freshly isolated cells from the culture wells or, for cell lines, by washing the cells and then scraping them from the substratum using a plastic policeman. The cellular material was placed into 1 ml Tris–mannitol buffer (50 mM tris-mannitol and 2 mM trizma base in dH₂O, pH 7.4) and mechanically homogenized at 4°C. Culture supernatants were also collected. Cell homogenates and supernatants were stored at −20°C until assayed.

Urokinase content of cell homogenates and culture supernatants was measured by commercial ELISA (#393 American Diagnostica) (22). Urokinase receptor concentrations were measured by ELISA (#394 American Diagnostica) in cell homogenates (23). An in-house ELISA was used to measure the content of IL-8 in culture supernatants. The sample (100 μl/well) was added to microtitre plates pre-coated with anti-IL-8 polyclonal antibodies (R&D Systems, Minneapolis, MN) and incubated at 25°C for 2 h. The plate was washed and 100 μl of mouse immunoglobulin–biotinylated conjugate (Dako (Australia) Pty Ltd, Botany, NSW, Australia), diluted 1:20 000, added to each well. After incubation at 25°C for 1.5 h, the plates were washed, 100 μl monoclonal anti-IL-8 was added, incubated for 2 h at 25°C and then 100 μl streptavidin–peroxidase, diluted 1:10 000, added to each well. After a further 30 min incubation, the wells were washed and tetramethylbenzidine added. The colour reaction was stopped by addition of 1 M phosphoric acid and the absorbances were read at 450 nm using standards calibrated against WHO reference material. All values were expressed relative to the protein content of the relevant cell homogenate. This was measured using bovine γ-globulin as standard (24).

Statistical analyses

Data were expressed as mean ± SEM and a P-value <0.05 was considered statistically significant. All analyses were performed using Microsoft Excel (1995 release). Paired data were compared using a paired t-test and unpaired data with a two-tailed Student’s t-test.

Results

Characterization of isolated cancer cells

Examination of cell pellets using light and transmission electron microscopy showed, for all pellets, >96% of cells to be cancer cells, either as single cells or in clumps. Non-epithelial cells had the morphological features of lymphocytes and occasional erythrocytes were also observed. The cancer cells were large and variable in size, with some showing surface microvilli and others containing apoptotic bodies. The cancer cells at both time zero and after 4 h in culture medium looked healthy and viable, although occasional necrotic cells were present. Mitotic cancer cells were present at both time zero and after 4 h in culture medium.

The metabolic stability of cancer cells in primary culture was indicated by the similar rates of uptake of [3H]leucine uptake or of [3H]thymidine in the first 4 h of culture and in the 18–24 h period in four experiments (Figure 1). The G/P ratio was also similar over those two time periods (Figure 1).

The phenotypic characteristics of cancer cells were examined at the end of 24 h culture and this was compared with those of normal epithelial cells and colon cancer cell lines (Figure 2). As previously demonstrated with normal cells (18,20), cancer cells secreted u-PA and IL-8, supernatant levels of u-PA were >3-fold that of the corresponding cell-associated
levels at the end of 24 h culture. Cancer cells secreted more u-PA and had more u-PA and u-PAR associated with them than normal cells. The secretion of IL-8 from cancer cells was 3-fold greater from normal cells (Figure 2). Both cancer cell lines exhibited phenotypic profiles that differed markedly from the other cell types. Of particular note were the relatively low levels of secretion of both u-PA and IL-8 by Caco-2 cells and of u-PA by LIM1215 cells.

**Effect of butyrate on cell characteristics**

The effect of 24 h exposure of the cell types to 1 or 2 mM butyrate is shown in Table I. As previously reported (18–20) in normal colonic epithelial cells, butyrate significantly reduced cell-associated u-PA and u-PAR expression, and the secretion of u-PA and IL-8, but had no significant effect on alkaline phosphatase activity. Cancer cells responded similarly to their normal counterparts in direction and in the magnitude of the response for alkaline phosphatase and cell-associated u-PA and u-PAR expression. However, the secretion of u-PA was suppressed to a lesser degree by butyrate ($P = 0.009$) and IL-8 was unaffected by butyrate ($P = 0.0015$ compared with change in normal cells). These patterns of responses differed markedly from those in the cancer cell lines, in which butyrate induced significant increases in alkaline phosphatase activities, u-PAR expression and IL-8 secretion. Urokinase expression and secretion by the cells was suppressed by butyrate in LIM1215 cells but stimulated in Caco-2 cells.

**Effect of pre-incubation with TNFα on butyrate-mediated responses in Caco-2 cells**

Pre-incubation of Caco-2 cells for 24 h with TNFα more than doubled IL-8 secretion over the subsequent 24 h compared with cells not exposed to TNFα (Figure 3). A similar degree of stimulation of IL-8 secretion was observed when the cells were exposed to 2 mM butyrate for 24 h. However, in cells pre-treated with TNFα for 24 h and then washed, 24 h exposure to butyrate suppressed IL-8 secretion to a mean of 34% below basal levels of secretion ($P = 0.03$; Figure 3). Exposure of Caco-2 cells to TSA, a specific inhibitor of histone deacetylase, significantly enhanced secretion of IL-8 >3-fold. However, this marked stimulation was abrogated when cells pre-incubated with TNFα were subsequently exposed to TSA (Figure 3).

Exposure of Caco-2 cells for 24 h to TNFα had no effect on u-PAR expression (Figure 3). TSA increased u-PAR expression to a similar degree as for butyrate. Pre-incubation with TNFα did not change the direction or magnitude of the response of u-PAR to butyrate or to TSA (Figure 3).

**Discussion**

Butyrate paradoxically affects colonic epithelial cells isolated from histologically normal large bowel when compared with
responses in colon cancer cell lines. This is not restricted to proliferation and differentiation (12), but also concerns the expression of u-PAR and secretion of IL-8. Butyrate suppresses both in normal cells and stimulates both in cell lines. Cell lines, however, are not the ideal comparator for isolated normal cells, since they represent a selected population of colon cancer lines, however, are not the ideal comparator for isolated normal cells, since they represent a selected population of colon cancer cell lines. This is not restricted to colon cancer cell lines (unpublished data).

The most obvious difference between cancer cell lines and normal cells that have adapted to conditions in vitro and have not recently undergone a disruptive isolation process. For these reasons, a technique, modified from that successfully used to isolate viable normal colonic epithelial cells (21), was developed to obtain unselected populations of neoplastic colonic epithelial cells in high purity. The cells were uniformly viable following their isolation and, under the conditions of study, remain viable for the experimental period as shown ultrastructurally, by constant rates of protein and DNA synthesis, and by their synthesis and secretion of the proteins, u-PA and IL-8. As might be expected, the cancer cells had a different phenotype to autologous normal cells. This was exemplified by the presence of abnormal ultrastructural features, by the elevated expression of components of the u-PA system and by increased secretion of IL-8. Urokinase and its receptor have been implicated in the ability of cancer cells to invade tissue (25) or to metastasize (26), and of normal epithelial cells to migrate (27). They may also play a role in the control of colonic epithelial turnover (28). Interleukin-8 promotes the migration of keratinocytes (29) and of at least some colon cancer cell lines (unpublished data).

Responses to butyrate reflect specific effects and not global changes in the rate of protein synthesis (12,30). They were qualitatively similar for primary cultures of normal and cancer cells. The major exception was the lack of response in cancer cells in the secretion of IL-8. This observation may relate to inherent differences between the cell types. Alternatively, the marked stimulation of the secretion of IL-8 observed with freshly isolated cancer cells may have rendered them less amenable to inhibition by butyrate. Indeed, butyrate was significantly less potent in reducing the expression of u-PA and u-PAR than in normal cells. In contrast, the pattern of responses to butyrate in both cell lines was qualitatively different to those in the isolated cells. Butyrate markedly stimulated alkaline phosphatase activity, as previously reported (12,31,32) and IL-8, an effect not previously observed. It also stimulated expression of u-PAR, contrary to recent findings in other cell lines where butyrate reduced u-PAR mRNA levels (33,34). However, in both of these studies, concentrations of butyrate used were higher (>2.5 mM) and in the range at which we have observed evidence of cell toxicity in the same or other cell lines (12,31,35). Butyrate’s effects on secretion and cell-associated expression of u-PA differed between the two cell lines. Urokinase secretion was suppressed by butyrate in LIM1215 cells, as also reported (at albeit higher concentrations) by Dang et al. (33), but was stimulated in Caco-2 cells. Spontaneous secretion and cell expression of u-PA by Caco-2 cells was the lowest across all cell types (<10% of that for freshly isolated cancer cells), and this may have influenced butyrate’s effects (see below).

The paradox in many of the responses to butyrate appears, therefore, to be less a reflection of effects in neoplastic as opposed to normal cells, but more a manifestation of responses in freshly isolated as opposed to in vitro-adapted cell lines. In other words, at least some of the paradoxical effects of butyrate previously reported might reflect factors other than intrinsic changes in the epithelial cells after tumorigenic transformation. The most obvious difference between cancer cell lines and freshly isolated cancer cell populations used in the present study is that the fresh cancer cells were subjected to the stresses of the extraction technique and the unaccustomed culture conditions away from their usual substratum. The effects of isolation and culture in vitro on normal colonic crypt cells include the induction of synthesis of u-PA (36), u-PAR (19) and IL-8 (20), a progressive increase in alkaline phosphatase activity of the cell population (30), and the induction of apoptosis (37). The cells are, therefore, in an ‘activated’ state, in which multiple intracellular pathways are stimulated. The stimulus for such cell activation is likely to be cell injury due to, for example, the trauma of the isolation process. These pathways are not stimulated in the cell lines, as shown by, for example, their low rates of secretion of IL-8 and u-PA.

Table I. The effect of 24 h of exposure to butyrate on phenotypic and secretory characteristics of isolated colonic epithelial cells from histologically normal mucosa and from cancer, and of LIM1215 and Caco-2 cell lines

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Cancer</th>
<th>LIM1215</th>
<th>Caco-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>n(^a)</td>
<td>9–10</td>
<td>9–11</td>
<td>6–8</td>
<td>4–8</td>
</tr>
<tr>
<td>Alkaline phosphatase(^b)</td>
<td>–4 (10)</td>
<td>–13 (7)</td>
<td>+107 (43)(^c)</td>
<td>+56 (27)(^c)</td>
</tr>
<tr>
<td>Cell-associated urokinase</td>
<td>–51 (18)(^c)</td>
<td>–31 (13)(^c)</td>
<td>+27 (8)(^c)</td>
<td>+172 (38)(^c)</td>
</tr>
<tr>
<td>Secreted urokinase</td>
<td>–51 (8)(^c)</td>
<td>–19 (7)(^c)</td>
<td>–49 (7)(^c)</td>
<td>+93 (25)(^c)</td>
</tr>
<tr>
<td>Cell-associated urokinase receptor</td>
<td>–30 (12)(^c)</td>
<td>–36 (9)(^c)</td>
<td>+314 (89)(^c)</td>
<td>+137 (17)(^c)</td>
</tr>
<tr>
<td>Secreted interleukin-8</td>
<td>–44 (7)(^c)</td>
<td>–7 (7)</td>
<td>+489 (175)(^c)</td>
<td>+197 (18)(^c)</td>
</tr>
</tbody>
</table>

\(^a\)Number of replicate experiments (not all indices were measured in all cell populations studied).
\(^b\)Percentage (SEM) of the change in each index (calculated as units or weight per mg cell protein) induced by butyrate compared with control.
\(^c\)P < 0.05 (paired t-test).

Fig. 3. The effect of 24 h exposure to butyrate (But) and TSA on IL-8 secretion and u-PAR expression by Caco-2 cells with and without pre-incubation for 24 h with TNFα. The results are shown as mean ± SEM of 3–5 replicate experiments. Stars represent effects that were significantly different to control values (P < 0.05; Student’s t-test).
These observations suggest that the modulatory effect of butyrate on the expression of a specific factor may depend on the state of stimulation of specific pathways that lead to the expression of that factor. One way of addressing this hypothesis is to determine whether stimulation of a cell line to produce a protein could alter butyrate’s effect on the expression of that protein. A suitable stimulus was pre-exposure to TNFα, since it stimulated IL-8 but not u-PAR expression by Caco-2 cells (though it has been reported to stimulate u-PAR mRNA levels in other cell lines (33)). Indeed, butyrate inhibited IL-8 secretion in cells cultured with TNFα. Its stimulatory effect on u-PAR expression, however, was not altered, indicating that the conditions being applied were not toxic to the cells. Thus, a ‘butyrate paradox’ involving IL-8 was created by specific stimulation of cells to express IL-8. A similar inhibitory effect of butyrate on IL-8 secretion was recently reported in Caco-2 cells stimulated by exposure to interleukin-1β but the effect of butyrate on unstimulated cells was not reported (38).

It might be anticipated that the underlying mechanism for diametrically opposite effects of butyrate on the same cell involves different pathways of action by butyrate. However, both effects may have involved inhibition of histone deacetylase, an intracellular action of butyrate (5) that has been implicated as a major mechanism for several of its end effects, including induction of apoptosis (39), inhibition of proliferation (39,40), stimulation of p21WAF1/C1P1 expression (40), and inhibition of interleukin-1β-induced secretion of IL-8 secretion (38). Trichostatin A is a potent and specific inhibitor of that enzyme and its exposure to the cells mimicked butyrate’s effects in both situations for both end points. Furthermore, TSA also potentely stimulates the secretion of IL-8 from unstimulated LIM1215 cells (unpublished data). While these observations do not directly prove that butyrate is indeed acting by inhibition of histone deacetylation in its effect on IL-8 secretion in Caco-2 cells, they proved strong circumstantial evidence. TNFα activates a surface receptor that leads to a cascade of signalling events that result in synthesis of transcription factors, which, in turn, lead to transcription of messenger RNA for IL-8. Hyperacetylation of histones, the result of inhibition of histone deacetylase, alters the accessibility of DNA to the binding of transcription factors (41). Butyrate may be acting, therefore, on ‘downstream’ nuclear events that permit increased or decreased transcription of specific factors depending upon the spectrum of transcription factors to which the DNA is exposed, the nature of which is dependent on ‘upstream’ signalling events.

In conclusion, the results of the present study demonstrate that some of the effects of butyrate previously reported in colon cancer cell lines do not occur in freshly isolated colon cancer cells, and where the responses more closely mimic those observed in normal colonic epithelial cells. For the indices examined, the ‘butyrate paradox’ does not, therefore, represent intrinsic differences between normal and neoplastic cells from the colonic epithelium but more likely reflects the effects on the cells of the extraction techniques employed and the culture conditions used. The creation of a ‘butyrate paradox’ in Caco-2 cells with regard to IL-8 secretion and the possible key role that inhibition of histone deacetylation may play for both effects of butyrate indicate the importance of the state of cell activation in predicting the response of cells to butyrate. Whether the state of activation plays a role in determining the proliferative or apoptotic response of colonic epithelial cells to butyrate requires investigation. Nevertheless, these issues must be carefully considered in interpretation of any studies examining the modulatory effects of butyrate and in the extrapolation of findings in any experimental system to those in a specific situation in vivo.

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References

P.R. Gibson et al.


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