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Sodium butyrate inhibits rat insulinoma cell proliferation without affecting the cellular insulin content or insulin release

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The short-chain carboxylic acid sodium butyrate is known to induce differentiation in several cell systems. In this paper we have investigated the effect of sodium butyrate on the growth rate and hormone content and secretion in clonal rat insulinoma cells (RINm5F). Special attention was paid to the role of polyamines for these processes, since these compounds have been implicated in the regulation of growth and function of insulin-producing cells. For this purpose RINm5F cells were maintained in culture for 4 days in the presence or absence of 1–2 mM sodium butyrate. It was found that sodium butyrate dose-dependently inhibited the proliferative activity of the RINm5F cells along with a decreased cellular polyamine content. The cellular content of insulin and secretion of the hormone into the culture medium were, however, not altered by sodium butyrate treatment, and neither did this treatment impose a glucose-sensitive insulin release. The decreased polyamine content was restored by the concomitant addition of exogenous putrescine; however, despite this, the reduced cell proliferation persisted. From these findings we conclude that the decreased growth rate evoked by sodium butyrate results from other events than specific polyamine synthesis inhibition. It is furthermore demonstrated that the processes of cell proliferation and regulation of cellular insulin content and secretion can be dissociated in the RINm5F cell line.

Key words: Insulin, insulinoma, RINm5F cells, sodium butyrate.

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Introduction

The establishment of the insulin-producing cell line RINm5F has created a source of production of large numbers of clonal insulin-secreting cells needed for studies concerning insulin production at the molecular level.[1,2] The cell line has, however, a major drawback insofar as it does not respond normally to glucose with an increased insulin release, although other secretagogs have been reported to be effective in this respect.[3] Other obvious shortcomings include the neoplastic proliferation and the low hormone content of the cells.[4] An agent capable of inducing differentiation of these cells would be expected not only to induce glucose sensitivity, but also to decrease the rapid growth rate and to increase the cellular insulin content. Sodium butyrate is a short-chain carboxylic acid which has been reported to exert an inhibitory effect on tumor cell growth and to induce differentiation of RIN 1056 cells by increasing the transcription of the insulin gene.[5,6] The aim of this study was to investigate the putative differentiative action of sodium butyrate on RINm5F cell proliferation and insulin content and secretion. Special attention was paid to the role of polyamines for these processes, since these compounds have been implicated in the regulation of growth and function of insulin-producing cells.

Materials and methods

Materials

Culture medium RPMI 1640, calcium- and magnesium-free Hanks' balanced salt solution, L-glutamine, penicillin, streptomycin and fetal calf serum were obtained from Flow Laboratories

(Irvine, UK). Antibovine insulin serum was from Miles-Yeda (Rehovot, Israel), whereas crystalline mouse insulin and [^{125}I]insulin were supplied by Novo (Denmark). Sodium butyrate, putrescine, spermidine, spermine, L-proline, dansyl chloride and Hepes were from Sigma (St Louis, MO). Trypsin (0.05%)/EDTA (0.02%) solution was obtained from Gibco (Paisley, UK). Bovine serum albumin (fraction V) was from US Biochemical Corporation (Cleveland, OH) and tissue culture dishes were supplied by Nunc (Roskilde, Denmark).

Cell culture

RINm5F cells were cultured at 37°C in medium RPMI 1640 containing 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 10% fetal calf serum. Cells were dislodged from culture dishes by a brief incubation with trypsin/EDTA in calcium- and magnesium-free Hanks' solution.

Polyamine content

The cellular polyamine content was determined by one-dimensional thin layer chromatography.[7,8] For this purpose cells were washed in ice-cold phosphate-buffered saline and homogenized by sonication in 20 µl 0.3 M perchloric acid at 4°C. After centrifugation (5 min, 12 000 *g*), the supernatant was alkalinized by addition of 10 µl 1 M Na_2CO_3 followed by 75 µl of 10 mg/ml dansyl chloride in acetone. After incubation at room temperature overnight in the dark, excess reagent was reacted with 5 µl L-proline (250 mg/ml) followed by sonication for 2 min. Dansyl-polyamines were extracted in 100 µl toluene. The toluene was evaporated and the residue redissolved in 5 µl toluene for application on thin layer chromatography plates (HPTLC Fertigplatten, Kieselgel 60 F_{254}; E. Merck). The dansylated polyamines were separated by one run with ethyl acetate:cyclohexane (1:1, v/v) followed by two runs with diethyl ether:cyclohexane (2:3, v/v). The spots were scraped off the plates and the fluorescence intensities of the supernatants measured in a model LS-5 Perkin-Elmer luminescence spectrometer connected to a plate reader at an excitation wavelength of 360 nm and an emission wavelength of 510 nm. This method has, in our hands, an intra-assay variability (SEM/mean) of 6%, an interassay variability of 3% and a sensitivity of about 15 pmol (i.e. an amount of polyamine resulting in a fluorescence intensity 2 SD above the blank reading). Standard curves were linear up to at least 1000 pmol and showed correlation coefficients of 0.99 or greater.

DNA content, insulin content, insulin release and cell viability

The DNA content of the cell cultures after 4 days was determined fluorometrically as described previously.[9,10] The cellular insulin content in aqueous homogenates extracted overnight in acid ethanol[8] and accumulation of insulin in the culture media during the last 24 h of culture were determined radioimmunologically.[11] In some experiments cell cultures were subjected to short-term studies of insulin release. For this purpose cells were exposed to 2.8 or 28 mM glucose in a bicarbonate buffer[12] supplemented with 10 mM Hepes and 2 mg/ml albumin in the presence or absence of sodium butyrate. Following incubation for 30 min at 37°C, aliquots of the incubation buffer were removed and stored at −20°C pending analysis for insulin by radioimmunoassay.[11] Cell viability was estimated by Trypan blue exclusion.

Results

Polyamine content

Sodium butyrate (1 mM) failed to affect the cellular putrescine content but decreased the contents of spermidine by 35% and spermine by 55% (Table 1). At 2 mM butyrate there was also a decrease in the putrescine content to 60% of that in untreated cells, and the decreases in spermidine and spermine contents were more pronounced than at 1 mM butyrate. The concomitant addition of exogenous putrescine to cultures treated with 1 mM sodium butyrate resulted in an accumulation of this diamine, and restored the contents of spermidine and spermine.

DNA content and cell viability

Exposure of the RINm5F cells to 1 mM sodium butyrate reduced the DNA content of the cultures to about 25% of that in untreated cultures (Table 2). At 2 mM sodium butyrate this effect was even more pronounced, resulting in a 90% reduction of

Table 1. Effects of sodium butyrate on RINm5F cell polyamine content

Cell culture	Polyamine content (pmol/μg DNA)		
	putrescine	spermidine	spermine
Control	8.2 ± 1.0 (8)	302 ± 11 (8)	283 ± 13 (8)
1 mM sodium butyrate	5.6 ± 1.2 (9)	201 ± 21 (9)***	131 ± 16 (9)***
2 mM sodium butyrate	4.6 ± 0.9 (8)*	166 ± 15 (8)***	121 ± 18 (8)***
1 mM sodium butyrate + 100 μM putrescine	55 ± 7 (8)***	310 ± 14 (8)	277 ± 19 (8)

Cells were cultured for 4 days in media supplemented as indicated. Polyamines were extracted with perchloric acid, dansylated, separated by thin layer chromatography and quantified fluorometrically. Values are given as means of control cells ± SEM for the number of observations in parentheses. *$p < 0.05$ and ***$p < 0.001$ for chance differences versus untreated cells using Student's unpaired *t*-test.

Table 2. Effects of sodium butyrate on RINm5F cell viability and DNA content

Cell culture	Viability (%)	DNA content (μg/dish)
Control	94 ± 5 (8)	101 ± 12 (10)
1 mM sodium butyrate	46 ± 6 (8)***	26 ± 4 (10)***
2 mM sodium butyrate	33 ± 6 (8)***	11 ± 2 (10)***
1 mM sodium butyrate + 100 μM putrescine	51 ± 4 (4)***	23 ± 2 (4)***

Cells were cultured for 4 days in media supplemented as indicated. Cell viability was estimated by Trypan blue exclusion and the DNA content of the cultures was measured fluorometrically. Values are means ± SEM for the number of observations in parentheses. ***$p < 0.001$ for chance differences versus control cells using Student's unpaired *t*-test.

the DNA content. The viability of cells treated with 1 mM sodium butyrate was decreased to 50% of that in control cells, as assessed by Trypan blue exclusion, whereas 2 mM of the carboxylic acid elicited a 65% decrease in viability. The concomitant addition of small amounts of exogenous putrescine failed to influence the decrease in growth rate and cell viability evoked by sodium butyrate.

Insulin content and insulin release

The cellular content of insulin was not affected by sodium butyrate treatment, neither at 1 nor at 2 mM (Table 3). Similarly, no effects of sodium butyrate on the accumulation of insulin into the culture medium were detected. Neither did sodium butyrate treatment render the cells sensitive to acute glucose stimulation of insulin release (data not shown).

Discussion

The present findings confirm previous observations that sodium butyrate decreases the proliferative activity of RINm5F cells[13] and RIN 1056 cells.[6] At variance with these reports are, however, the current observations of an unaltered cellular insulin content and insulin release following sodium

Table 3. Effects of sodium butyrate on RINm5F cell insulin content and medium insulin accumulation

Cell culture	Insulin content	Insulin accumulation
Control	100	100
1 mM sodium butyrate	115 ± 12 (10)	81 ± 21 (10)
2 mM sodium butyrate	102 ± 19 (8)	83 ± 18 (8)
1 mM sodium butyrate + 100 μM putrescine	94 ± 17 (4)	90 ± 19 (4)

Cells were cultured for 4 days in media supplemented as indicated. The cellular insulin content in homogenates extracted overnight in acid ethanol and medium insulin accumulation during the last 24 h of culture were determined radioimmunologically. Values are given as percent ± SEM of control cells for the number of observations in parentheses. Control values were: insulin content: 6.4 ± 0.8 ng/μg DNA ($n = 10$) and insulin accumulation 2.0 ± 0.3 ng/μg DNA/24 h ($n = 10$).

butyrate treatment. The reasons underlying this discrepancy remain elusive but can conceivably be accounted for by different features between subclones used. In addition, in the investigation of the RIN 1056 line,[6] only the mRNA levels of the different hormones were studied. It is thus feasible that the reported increase in insulin gene transcription by sodium butyrate is not accompanied by the subsequent stimulation at the translational level.

Our finding of an increased fraction of cells taking up Trypan blue following sodium butyrate exposure suggests that the carboxylic acid exerted a cytotoxic action towards the RINm5F cells. It should be noted, however, that the Trypan blue exclusion test was carried out on the total number of cells present in culture dishes, i.e. also free-floating cells, whereas hormone assays were performed on cells attached to the culture dish surface. This readily explains the lack of decreased cellular insulin content that one would expect as a result of a decreased viability. It may also be speculated that the cells remaining after washing of the cultures represent a fraction of cells less sensitive to the toxic action of sodium butyrate.

The molecular mechanisms by which sodium butyrate exerts its effects in this cell line remain to be clarified but it has previously been demonstrated that butyrate can influence chromatin structure by inhibiting histone deacetylase, resulting in hyperacetylation of high-mobility group proteins,[14] an event which is considered to alter the transcriptional activity of the chromatin. Other mechanisms include DNA hypomethylation,[15] altered genomic nuclease sensitivity,[16] and selective interference with histone H-1 and H-2 phosphorylation.[17] Of notable interest in this context is the butyrate-induced decrease in cellular polyamine contents observed in the current investigation. Since sufficient levels of polyamines appear to be a prerequisite for maintenance of rapid tumor cell proliferation,[18] we tested the possibility that the decreased growth rate in butyrate-treated cultures resulted specifically from the observed polyamine depletion. For this purpose small amounts of putrescine, which is the metabolic precursor to the polyamines spermidine and spermine, were added concomitantly with sodium butyrate. It was found, however, that although this procedure restored the intracellular polyamine content, the decreased proliferation rate persisted. This finding rules out the possibility that the decreased polyamine content accounts for the antiproliferative activity of sodium butyrate. It also suggests that the changes in polyamine content do not reflect a specific inhibition of polyamine biosynthesis but rather are secondary to other metabolic effects by butyrate.

Our results moreover demonstrate an interesting dissociation between growth inhibition and regulation of the cellular insulin content and secretion of the hormone. More specifically, we have previously demonstrated that specific polyamine depletion decreases RINm5F cell proliferation along with an elevated insulin content and increased activity of the secretory machinery.[19] The current study shows that inhibition of cell proliferation does not necessarily result in increased insulin content and secretion.

In conclusion, sodium butyrate exerts an antiproliferative effect on RINm5F cells but fails to increase the cellular insulin content and to induce glucose-sensitive insulin release, effects causally unrelated to the decreased polyamine content. There was therefore no evidence for a differentiating effect of sodium butyrate on this subclone of the RINm5F cell line.

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