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Butyrate Cytoprotection of Colonic Epithelial Cells May Be Mediated Through Inhibition of Heat Shock Protein 70

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Abstract: Butyrate, normally found in the colon, is used in therapy to reduce epithelial cell damage and mucosal inflammation in conditions like ulcerative colitis and radiation colitis. Heat shock proteins (Hsp), particularly Hsp70, are also known to be important in cellular responses to injury. We hypothesized that butyrate may act to protect epithelial cells through induction of Hsp70. HT29 intestinal epithelial cell monolayers were exposed to two forms of non-lethal stress; heat or chemical in presence or absence of butyrate. Cells were radiolabeled with ^{35}S-methionine and incubated at $37°C$ for 2 h. Cells were lysed, subjected to 1D SDS-PAGE, followed by autoradiography. In additional studies, the effect of initial exposure to sub-lethal heat on subsequent exposure to lethal heat was examined. Expression of the inducible form of Hsp70 and cell viability was assessed. Non-lethal stress induced Hsp70 expression in HT29 cells and this was inhibited by butyrate, but not by acetate or propionate. This effect of butyrate was seen only at lower concentration. Inhibition of Hsp70 synthesis was accompanied by an increased ability of the cells to tolerate exposure to a second lethal heat stress. Butyrate increased colonic epithelial cell survival in the face of lethal heat stress and significantly reduced Hsp70 expression.

Key words: Butyrate, heat shock protein 70, thermotolerance, colonic; nuclear factor kappa B

Introduction

Butyrate, a short chain fatty acid produced by the bacterial fermentation of carbohydrate, plays an important physiological role in the maintenance of the colonic epithelial integrity. Apart from being the major energy source for the colonic epithelium, it regulates colonic mucosal growth, barrier function and epithelial proliferation (Cook and Sellin, 1998). Evidence from clinical studies and experimental animals has shown that butyrate deficiency is associated with chronic mucosal atrophy (Harig *et al.*, 1989; Roediger, 1980a). Butyrate has also been linked to the pathogenesis of Ulcerative Colitis (UC), an inflammatory bowel disease of unknown etiology (Roediger, 1980a). Butyrate enemas have been used to reduce colonic mucosal inflammation in inflammatory conditions like UC and radiation colitis (Breuer *et al.*, 1991; Vernia *et al.*, 2000). Recently we have demonstrated that butyrate ameliorates experimental colitis induced in rats by dextran sulfate sodium (Venkatraman *et al.*, 2003).

The heat shock proteins (Hsp) or stress proteins are important in the cellular response to stress and in cellular homeostatic functions such as protein synthesis and protein transport across membranes. However, in addition to these basic functions, Hsp are now increasingly recognized to play a role in immune activation and recognition and thus can be cytotoxic (Srivastava *et al.*, 1998;

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Wallin *et al.*, 2002). Emerging evidence also supports a role for Hsp in the inflammatory response, suggesting that Hsp participate in cytokine signal transduction and in the control of cytokine gene expression (Chen *et al.*, 1999; Asea *et al.*, 2000a; Kol *et al.*, 2000). For example, it has been demonstrated that Hsp70 can exert a deleterious effect by mediating release of inflammatory cytokines through a CD14-dependent mechanism (Asea *et al.*, 2000b). Under these conditions, suppression of Hsp activation may be beneficial in ameliorating colonic epithelial cell damage and consequent inflammation. These studies were undertaken to examine the hypothesis that cytoprotection of colonic epithelial cells by butyrate could be mediated through modulation of heat shock protein expression. Here we report that butyrate protected HT-29 cells in response to lethal stress. This effect of butyrate maybe mediated by inhibition of Hsp70.

Materials and Methods

Stress Protein Response in HT-29 Cells

The stress response was induced in HT-29 cells, a colon carcinoma cell line. HT-29 cells were grown to confluence for 4 days in RPMI-1640 medium, supplemented with 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 7.5% NaHCO$_3$ and 10% fetal calf serum. The cells were trypsinized, centrifuged (5000 rpm×5 min) and suspended in HEPES buffer (130 mM NaCl, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 5.0 mM HEPES and 0.1 mM phenyl methyl sulfonyl fluoride).

Five hundreds microliter of cell suspension (1×10^5 cells mL^{-1}) were maintained at 37°C for 30 min in a shaking water bath. Sodium butyrate (10 mM final concentration) or NaCl (10 mM; as control) were added to the suspensions. The cells were heat stressed by incubating for 30 min at 43°C. Control incubations were done, maintaining the cells at 37°C throughout. The cells were then labeled with 60 µCi mL^{-1} ^{35}S methionine (BRIT, India) and incubated for 2 h at 37°C. In other experiments, the effect of adding 10 mM butyrate after heat shock was also investigated and compared to the effect when added before heat shock.

The Hsp response to chemical injury was also investigated. Five hundred microliter of cell suspension (1×10^5 cells mL^{-1}) was incubated with 0.5 mM deoxycholic acid (Sigma Chemical Co, USA) at 37°C for 15 min in a shaking water bath. The cells were centrifuged at 5000 rpm for 5 min and the cell suspension was washed and re-suspended in 250 µL^{-1} of HEPES buffer. ^{35}S-methionine (60 µCi mL^{-1}) was added to the suspension and the cells incubated at 37°C for 2 h after addition of sodium butyrate or NaCl (10 mM final concentration). Cell viability was not appreciably affected between the control and test after thermal or detergent injury (data not shown). This degree of heat or detergent injury was therefore adjudged to be sufficient to induce a stress protein response, but inadequate to cause cell death.

Thermotolerance

Five hundres microliter of the HT-29 cell suspension (1×10^5 cells mL^{-1}) was maintained at 37°C for 30 min in a shaking water bath. Sodium butyrate (10 mM final concentration) or NaCl (10 mM as control) were added to the suspensions. The cells were heat stressed by incubating at 43°C for 30 min and then further incubated at 37°C for 2 h. Control incubations were done, maintaining the cells at 37°C throughout. All cells except control were then subjected to a second, more severe, heat stress at 46°C for 15 min, followed by incubation at 37°C for 60 min. Cell viability was assessed at 0, 15, 30 and 60 min after the second heat shock by quantitating release of lactate dehydrogenase (LDH) into the medium (Venkatraman *et al.*, 2003).

SDS-PAGE and Autoradiography

HT-29 cells were resuspended in HEPES buffer. To 250 μL^{-1} of suspension, 60 $\mu Ci\ mL^{-1}$ of ^{35}S-methionine was added and cells were incubated at 37°C for 45 min. The cells were then centrifuged at 3000 rpm for 3 min and the cell pellet resuspended in 150 μL^{-1} HEPES buffer. The resuspended cells were lysed by sonication of 2 μ amplitude for 2 min (MSE Soniprep 150, UK). The protein concentration of the sonicated suspensions was assayed by Lowry's method. Samples were initially boiled for 5 min in protein dissociation buffer (9% SDS, 16% β-mercaptoethanol, 15% glycerol, 1M Tris, pH 6.7, containing 0.02% of bromophenol). Equal amounts of protein were then loaded on to 10% SDS-PAGE gel. Ten percent separating gel contained 10% acrylamide, 0.1% SDS, 0.075% ammonium persulphate, 0.05% TEMED and 0.373 M Tris, pH 8.8). Stacking gel containing 5% acrylamide, 0.1% bis acrylamide, 0.1% SDS, 0.2% ammonium persulphate, 0.069% TEMED and 0.138M Tris, pH 6.8. Electrophoresis of the gels was carried out for 6 h at 120V in buffer containing 0.02 M Tris, 0.192 M glycine and 0.1% SDS, of pH 8.6. They were then stained overnight in Coomassie blue stain (methanol: acetic acid: water 50:10:40 V/V/V containing 0.1% Coomassie brilliant blue R). Gels were destained, dried and exposed to X-ray film at -80°C for 14 days. The films were developed and scanned using a HP scanner and density of the protein bands quantitated using Scion Image for Windows (Scion Corporation, 1998).

Western Blot

Western blot analysis was carried out as described earlier (Towbin *et al.*, 1979). After electrophoresis, gels were immersed in transfer buffer (25 mm Tris, 192 mM glycine, 20% methanol) and blotted on to 0.45 μm nitrocellulose membranes (Millipore, MA, USA) using a Biorad power supply. The membranes were covered with mouse anti-HSP70 monoclonal antibody (SPA-810, Stressgen, Victoria, BC, Canada) diluted 1:5000 with blocking solution and incubated for 2 h at 23°C. After washing thrice, the blot was incubated with goat anti-mouse antibody conjugated with alkaline phosphatase (Genei, Bangalore, India) diluted 1:1000 with blocking solution for 2 h at 23°C. After further washing, alkaline phosphatase was developed using NBT/BCIP (Genei, Bangalore, India) as substrate. All incubation procedures were performed under gentle agitation at 23°C.

Statistics

All numerical values were expressed as mean±standard error. The Mann-Whitney two-tailed test was used to assess significance of differences between means of groups. p-values less than 0.05 were considered statistically significant.

Results

Inhibition of Hsp70 Synthesis by Butyrate in HT-29 Cells in Response to Non-lethal Thermal Stress

To assess the *in vitro* increase in protein synthesis in response to non-lethal thermal stress, intestinal epithelial cells were loaded with ^{35}S-methionine and then subjected to stress. Cellular proteins were then separated using 1D-SDS PAGE. Control cells without heat stress showed faint protein bands of different molecular weights, reflecting incorporation of the added ^{35}S-methionine into basally produced protein. Incubation of cells at 42°C for 30 min followed by 2 h incubation at room temperature led to several prominent protein bands indicating incorporation of ^{35}S-methionine into
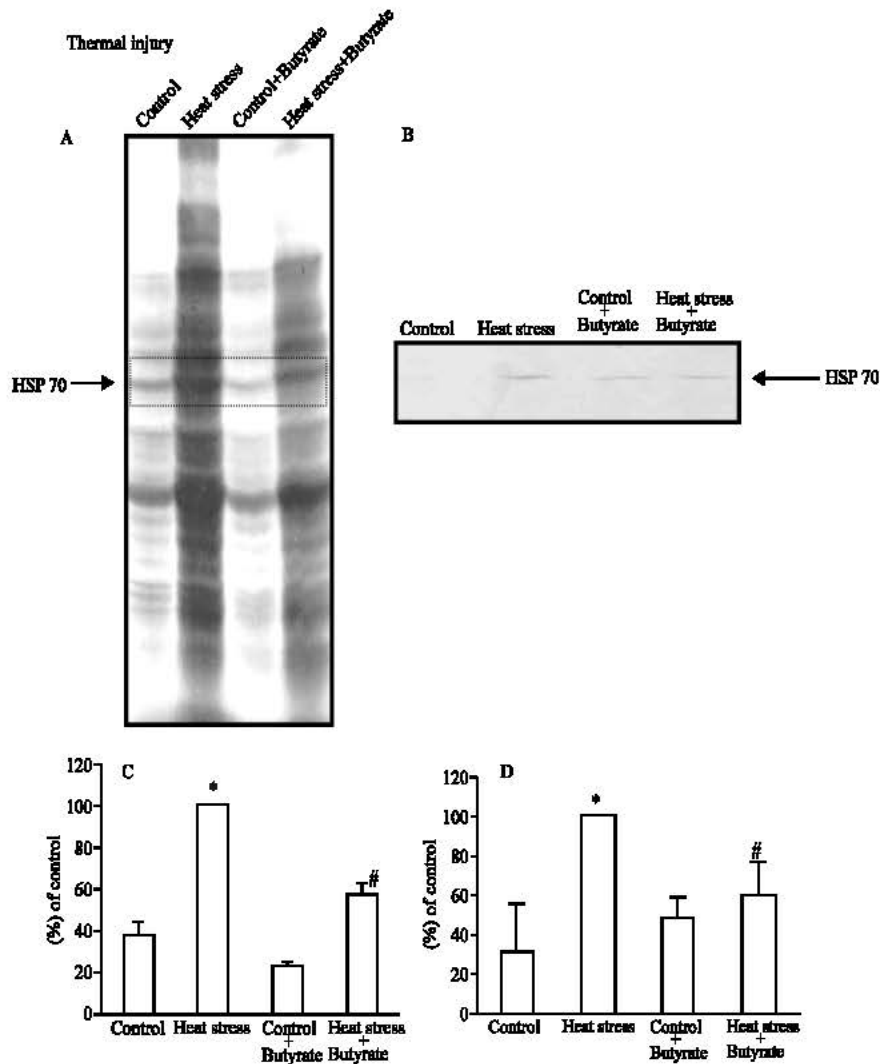
Fig. 1: Inhibition of Hsp70 by butyrate in cells subjected to non-lethal thermal stress. (A) HT-29 cells in culture were exposed to 43°C for 30 min in the presence or absence of 10 mM butyrate. Cells were incubated for 2 h at 37°C after addition of ^{35}S methionine. Equal amounts of protein loaded in each lane were separated on a 10% SDS-PAGE gel and the synthetic pattern was determined by autoradiography. Stressed cells showed increased neo-protein formation at the 72 kDa position (indicated by arrow). (B) The identity of Hsp70 protein was confirmed by immunoblotting. Protein separated electrophoretically was transferred to nitrocellulose and then probed with antibody specific for inducible form of the Hsp70. (C) Percentage inhibition of Hsp 70 production by butyrate, based on densitometry of the bands from the gel (A). (D) Percentage inhibition of Hsp70 production by butyrate, based on densitometry of Western blot bands. Butyrate inhibited Hsp70 production by 43% after thermal injury. *p<0.0001 compared to non-stressed control. Non-lethal thermal stress vs non-lethal thermal stress with butyrate -#p<0.001
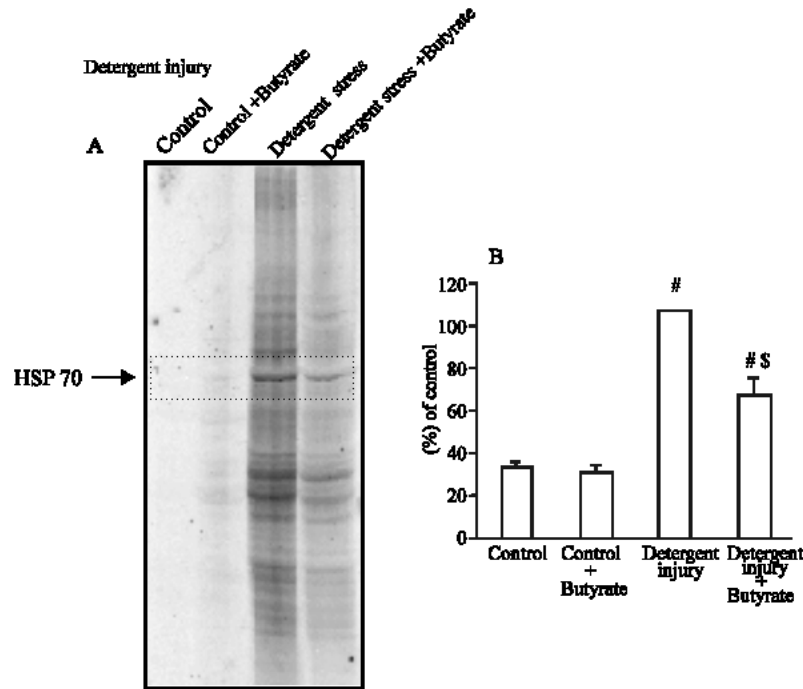
Fig. 2:   Inhibition of Hsp70 by butyrate in cells subjected to non-lethal detergent stress HT-29 cells in culture were exposed to 0.5 mM deoxycholic acid for 15 min in the presence or absence of 10 mM butyrate. Cells were incubated for 2 h at 37°C after addition of ^{35}S methionine and incorporation of the isotope into new protein determined by autoradiography following SDS-PAGE. Butyrate significantly inhibited Hsp70 synthesis (A). Percentage inhibition of HSP 70 production by butyrate was quantitated by densitometry of the bands from the gel. Butyrate inhibited HSP70 production by 32% after detergent injury (B) Detergent injury vs detergent injury with butyrate $^{\$}$p<0.005, *p<0.0001 compared to non-stressed control

stress-related neoproteins (Fig. 1a). A band at approximately 70 kDa (indicative of Hsp72, the inducible form of Hsp70) was among the most prominent following stress. Interestingly, thermally stressed cells incubated in presence of butyrate showed significant inhibition of ^{35}S-methionine incorporation into protein at the 70 kDa position (Fig. 1c). The 70 kDa protein was confirmed to be Hsp72 by immunoblotting using a specific antibody against the inducible form of Hsp70. Analogous to the metabolic labeling data, increased expression of Hsp70 was seen in thermally stressed cells and presence of butyrate resulted in significant inhibition of this induction (Fig. 1b and d). Control cells incubated in presence and absence of butyrate showed production of Hsp70 in the basal state, using antibody to either the constitutive or inducible forms of the protein.

Inhibition of Hsp70 Synthesis by Butyrate in HT-29 Cells in Response to Non-lethal Detergent Stress

To determine whether the induction of Hsp70 and its inhibition by butyrate is common to other injuries, HT-29 cells were subjected to non-lethal detergent injury and induction of protein synthesis examined. Cells were exposed to 0.5 mM deoxycholic acid for 15 min, the medium replaced and the cells further incubated with ^{35}S-methionine for 2 h. Figure 2 show that increased ^{35}S-methionine incorporation into many proteins was noted in detergent injured cells compared to the non-stressed
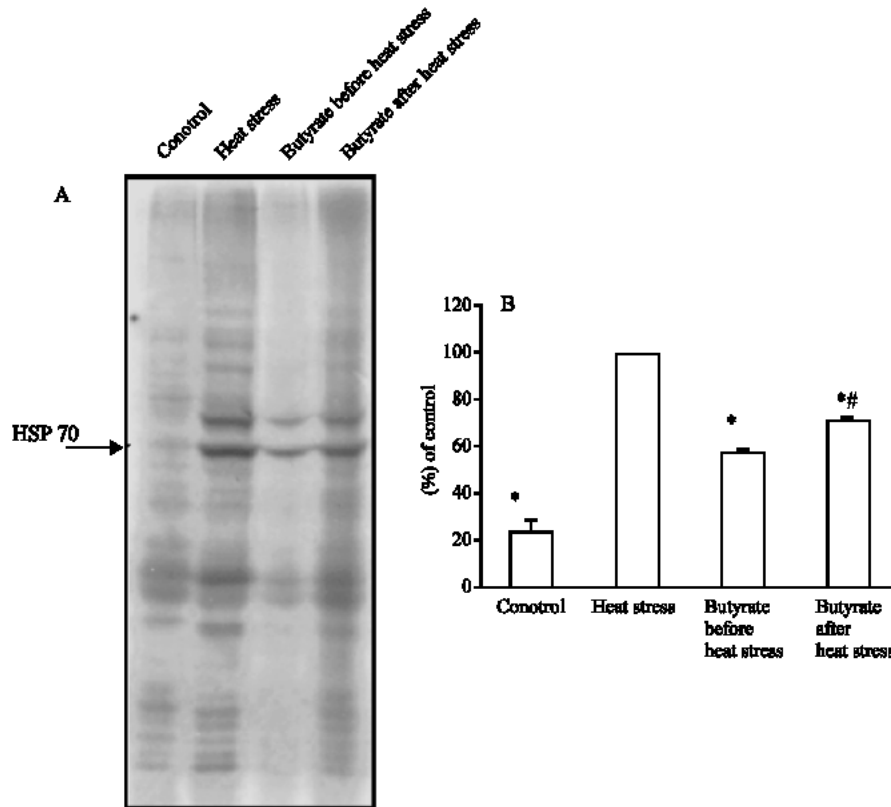
Fig. 3: Effect of timing of butyrate exposure on inducible Hsp70 synthesis (A) HT-29 cells were heat stressed at 43°C for 30 min and further processed as described in Fig. 1. Inhibition of HSP70 synthesis was significantly greater when 10 mM butyrate was added prior to heat shock compared to addition after heat shock. (B) Percentage inhibition of HSP 70 production by butyrate, based on densitometry of the bands from the gel (A). * $p < 0.001$ compared with heat stressed cells. # $p < 0.001$ compared with heat stressed cells with butyrate added before stress

controls. As in heat-treated cells, this was very prominent in the 70 kDa range, indicative of Hsp70 synthesis. This increase in ^{35}S-methionine incorporation into new proteins and specifically into proteins at 70 kDa position, was also inhibited by butyrate (Fig. 2).

Timing of Butyrate Exposure in Relation to Heat Shock and its Effect on Hsp70 Levels

The decrease in Hsp70 levels due to decreased protein synthesis could be further amplified if butyrate also had an effect on protein turnover. To determine if butyrate could also function to increase Hsp70 levels by decreasing protein degradation after induction due to heat stress, further studies were carried out with butyrate addition both prior to heat shock as well as subsequent to heat treatment. Figure 3a and b, inhibition of the stress protein response was more marked if butyrate was added to the medium prior to heat shock compared to addition of butyrate after heat shock, indicating that the primary effect of the molecule was an early event, influencing neo protein synthesis and its presence after induction did not have a significant effect.
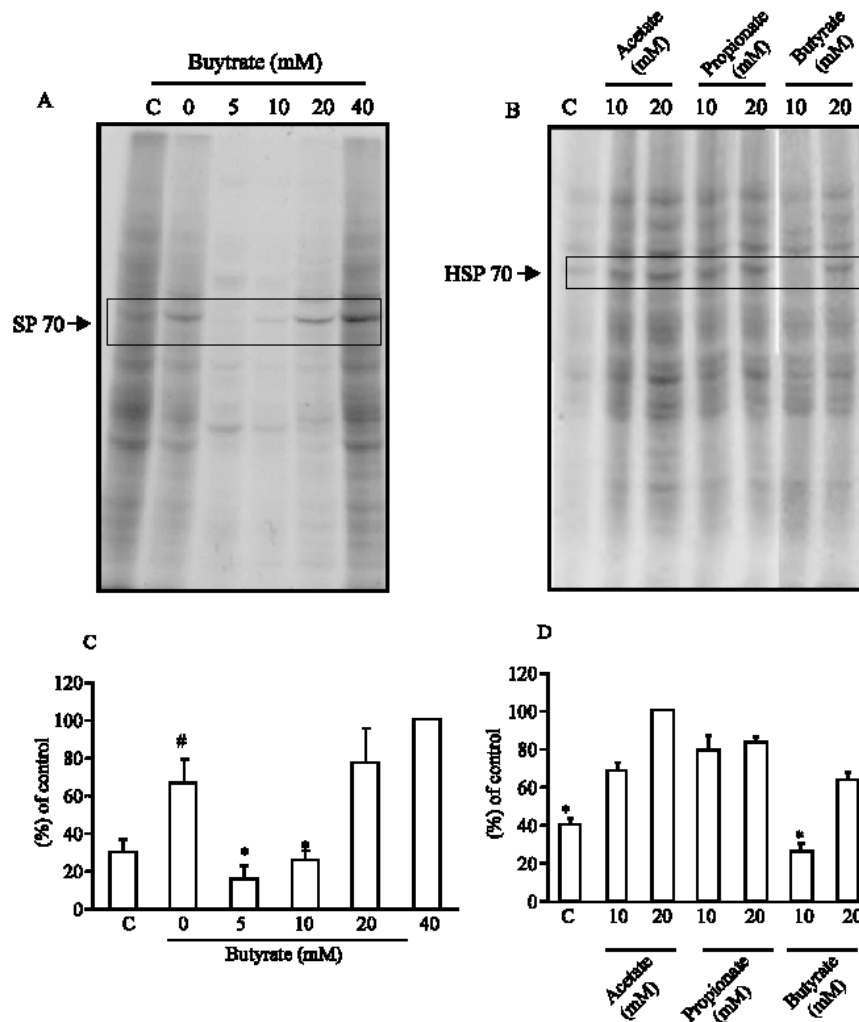
Fig. 4: Biphasic effect of butyrate on Hsp70 induction HT-29 cells subjected to non-lethal thermal stress, then lysed and subjected to 1D-SDS-PAGE, followed by autoradiography. (A). Effect of butyrate concentration on Hsp70 synthesis. Concentration below 10 mM inhibited Hsp70 synthesis and concentration above 10 mM increased Hsp70 formation. (B) Effect of other short chain fatty acids on Hsp70 synthesis. Acetate and propionate did not alter the levels of Hsp70 formation in response to non-lethal thermal stress. (C) Densitometric relative values of Hsp70 synthesis from stressed cells incubated in presence of different concentrations of butyrate. Thermally stressed cells vs control -*p<0.002. Thermally stressed cells vs thermally stressed cells incubated with different concentrations of butyrate-*p<0.001. (D) Densitometric relative values of Hsp70 synthesis in cells incubated in presence of different short chain fatty acids. Thermally stressed cells vs thermally stressed cells incubated with butyrate-*p<0.001
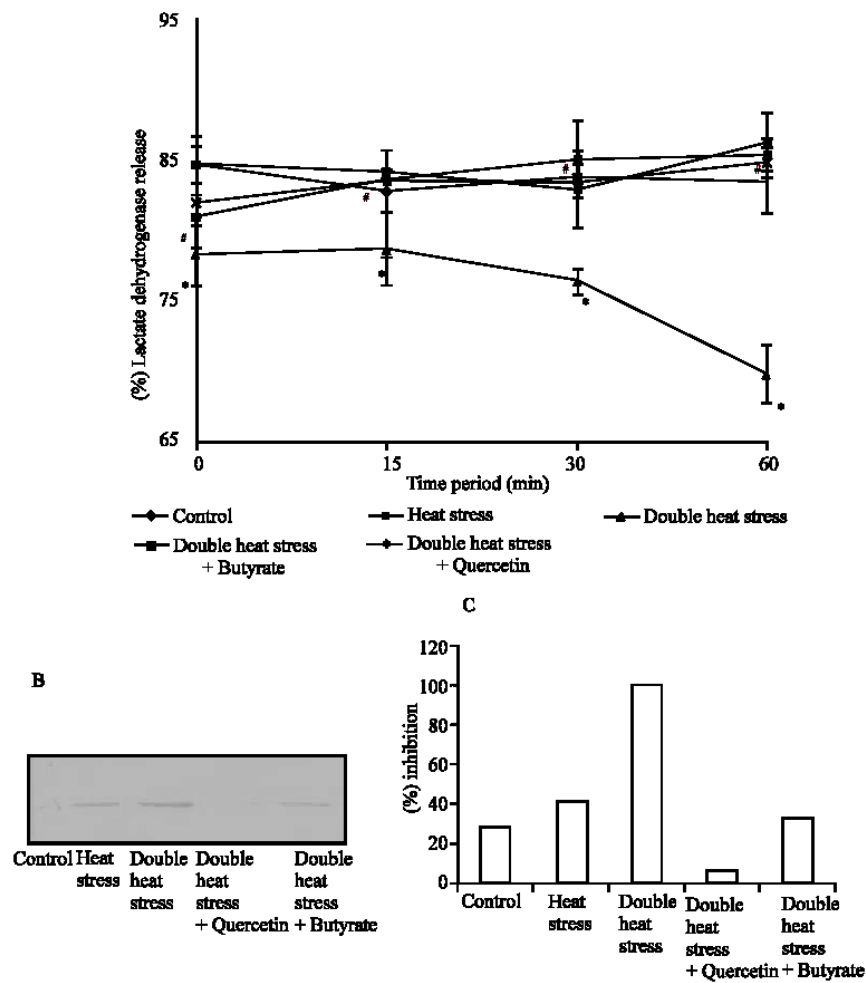
Fig. 5: Butyrate increased thermotolerance of HT-29 cells subjected to lethal heat stress. For experiments involving double heat stress, HT-29 cells were exposed to initial non-lethal heat stress, 43 °C for 30 min and then further incubated at 37 °C for 2 h in presence and absence of 10 mM butyrate or 0.2 mm quercetin. All cells were then subjected to a second, more severe, heat stress at 46 °C for 15 min. Control incubations were done, maintaining the cells at 37 °C throughout. Cell viability was assessed by LDH release into the medium. Exposure to the second thermal injury significantly reduced viability of HT-29 cells compared to control cells, whereas addition of butyrate significantly improved the thermotolerance of these cells (A). *p<0.05 compared with the same time period value for control. #p<0.05 compared with the same time period value for double heat-stressed cells incubated with butyrate. (B) Western blot for inducible Hsp70. (C) Quantitative densitometry of bands obtained by protein blot

Effect of Butyrate Concentration on Hsp70 Production in Response to Stress

Butyrate is known to have a biphasic effect on cell viability. At lower concentrations it increases cell viability and at higher concentrations it is known to be cytotoxic. In the next series of experiments, the effect of butyrate concentration on Hsp70 induction was examined. Thermally stressed HT-29 cells

were metabolically labeled in presence of different concentrations of butyrate (0, 5, 10, 20 and 40 mM). At lower concentrations (up to 10 mM) there was significant inhibition of Hsp70 induction. However, at higher concentrations (20 mM and above) there was significant induction of Hsp72, suggesting a biphasic role of butyrate on Hsp72 induction (Fig. 4a and c). To understand if this effect was a common response to all short chain fatty acids, cells were incubated in presence of various concentrations of acetate and propionate. Reduction of Hsp70 production in response to stress appeared to be a property unique to butyrate, not being found with 10 and 20 mM of acetate and propionate, which are other short chain fatty acids found in the colon (Fig. 4b and d).

Prevention of Hsp70 Induction by Butyrate Is Associated with Thermotolerance

Cells exposed to nonlethal heat stress are known to become transiently resistant to a subsequent heat shock. It is traditionally considered that this development of thermotolerance is an effect of induction of Hsp70 in response to the first heat stress. Figure 5a shows that a decrease in cell viability was noted in cells subjected to lethal heat stress alone, but prior non-lethal thermal stress in the presence of 10 mM butyrate provided significant protection against this. An increase in expression of Hsp70 was seen in cells subjected to non-lethal stress, which further increased after the subsequent exposure to lethal heat stress (Fig. 5b and c). Experiments were also carried out using quercetin, which is an inhibitor of Hsp70. Treatment of cells with (0.2 mM) quercetin prevented induction of Hsp70. Exposure to butyrate reduced the expression of Hsp70 but at the same time increased cell viability after exposure to lethal stress. Quercetin treatment was similarly accompanied by increased cell viability after exposure to lethal stress.

Discussion

Butyrate a four carbon molecule formed from bacterial fermentation of carbohydrate has a variety of effects with particular reference to colonic epithelial physiology and disease. It has been assumed that the protective effect of butyrate in this context results predominantly from influencing energy availability in the colonocyte, since it is the primary source of energy in this cell type (Cook and Sellin, 1998; Roediger, 1980b; Roediger, 1982). Butyrate, alone or in combination with other short chain fatty acids, has been used in the therapy of a number of diseases characterized by colonic mucosal inflammation including ulcerative colitis. However, the cellular events mediating the cytoprotective effect of butyrate are not well understood and formed the focus of this study. Butyrate protected HT29 epithelial cells from heat and detergent stress, an effect that was accompanied by inhibition of Hsp70. The induction of stress proteins in response to variety of injurious events is a highly conserved cellular response. A number of stressful conditions including environmental, physiological or pathological stimuli induce a marked increase in heat shock protein synthesis, referred to as the stress response (Lindquist, 1986). In concordance with earlier studies, thermal stress in colonic epithelial cells resulted in an induction of the heat stress response (Yokota *et al.*, 2000; Chow and Zhang, 1998; Musch *et al.*, 1996b). Until recently, heat shock proteins (also known as heat stress proteins) have mostly been regarded as intracellular molecules that mediate a range of essential housekeeping and cytoprotective functions. Butyrate has been considered cytoprotective to the colonic epithelium, especially because it has effects on colonocyte oxidative metabolism, colonocyte regeneration and colonic mucosal blood flow. The cytoprotective effect of butyrate has primarily been studied in intact animals. The present study shows that butyrate protected epithelial cells in monolayer culture against deleterious influences such as heat injury or detergent injury. Intuitively, we expected that butyrate would protect cells probably by amplifying the heat shock response to stress.

Contrary to expectation, butyrate treatment resulted in a decrease in the heat stress mediated induction of Hsp70. This effect on heat shock protein induction was biphasic, with the effect seen only at concentrations of butyrate below 10 mM. At higher concentrations, butyrate amplified the induction of Hsp70 synthesis. The inhibitory effect was most prominent when butyrate was added before the heat treatment.

Interest in the role of Hsp's as intercellular signaling molecules has been fuelled by the observations that these molecules can be released and are present in the extracellular environment under physiological conditions as well as pathological conditions. Even though the mechanism and physiological significance of Hsp release is not very clear, emerging evidence suggests that Hsp may have additional roles as an immune regulator. For example, a death-promoting effect of Hsp70 itself has been described after CD95 activation in Hsp70 transfected Jurkat cells (Liossis *et al.*, 1997); while in acute myeloid leukemic cells, apoptosis correlated with the intracellular Hsp7070 level (Chant *et al.*, 1996).

Butyrate treatment provided thermo-tolerance to the intestinal epithelial cells, preventing the loss of cell viability on exposure to lethal heat stress. There have been studies suggesting that heat shock can increase susceptibility to cell lysis (Dressel *et al.*, 2000; Roigas *et al.*, 1998) and it has been reported that Hsp70 is capable of inducing proinflammatory cytokines via the CD14/Toll like receptor complex mediated signal transduction pathway (Asea *et al.*, 2000a b; Dybdahl *et al.*, 2002). The reported activation of the innate immune system by Hsp's has been hailed as an important new function of Hsp with broad biological significance. Earlier studies have demonstrated that induction of proinflammatory cytokines by Hsp70 may actually contribute to the pathogenesis of certain acute and chronic inflammatory diseases (Moseley, 1998; Pockley *et al.*, 2002). Furthermore, the observed Hsp70 cytokine effect is mediated by activation of nuclear factor kappa B (Asea *et al.*, 2000b; Venkatraman *et al.*, 2003). We have recently demonstrated in an animal model of inflammatory bowel disease that inhibition of Hsp70 by butyrate decreased the activation of NFκB and was cytoprotective (Venkatraman *et al.*, 2003). Recently there has been another study which has shown butyrate decrease Hsp70 synthesis in colonic cell line (Gehrmann *et al.*, 2005).The present study clearly demonstrate that decrease in Hsp70 levels are not necessarily associated with cell death in response to lethal stress and that butyrate did not increase cell viability through increase in Hsp70.

The present studies demonstrate that induction of Hsp70 did not confer protection to colonic epithelial cells against lethal heat. These cells already express very high levels of Hsp70 constitutively (Beck *et al.*, 1995; Musch *et al.*, 1999). In cells already expressing relatively high levels of Hsp70, further induction of the protein may not provide any additional protection for the cell. In the HT-29 cell line butyrate and quercetin, both of which inhibited Hsp70 production, conferred increased survival ability on cells exposed to lethal heat. This suggests that the effects on cell survival may have been secondary to inhibition of Hsp70. Even though earlier studies have suggested that Hsp70 provides cytoprotection in the IEC-18 intestinal epithelial cell line, it should not be overlooked that those particular cells have very low basal levels of Hsp70 (Musch *et al.*, 1996; Urayama *et al.*, 1998). Hence the effect observed could be cell type specific. It is known that Hsp70 induces chemokine and cytokine release in response to certain stimuli and this effect could have been attenuated by butyrate. Butyrate reduced mucosal inflammation in dextran sulfate colitis in rats, an effect accompanied again by reduced levels of Hsp70 expression in the mucosa. Butyrate is a molecule found in abundance in the human and mammalian colon and has many important effects on colonic physiology, including roles in trophy, growth, differentiation and repair of the colonic mucosa.

The present study provide another explanation for the beneficial effect of butyrate on survival of colonic epithelial cells, an effect that may be of clinical importance in a variety of conditions involving the colonic mucosa.

Acknowledgement

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