Sodium butyrate inhibits expression of urokinase and its receptor mRNAs at both transcription and post-transcription levels in colon cancer cells

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Abstract **The effects of butyrate on the modulation of urokinase plasminogen activator (uPA) and its receptor (uPAR) mRNAs were studied. While both mRNA levels were increased after stimulation by tumor necrosis factor alpha (TNFα), phorbol ester (PMA) and cycloheximide, they were inhibited by butyrate at 2.5 to 25 mM. Nuclear run-on transcription assays indicated that uPA mRNA was modulated by butyrate at the transcriptional level but the uPAR gene was regulated at both transcriptional and post-transcriptional levels in the presence or absence of TNFα. In the presence of PMA, however, butyrate acts at the post-transcriptional level on both genes.**

Key words: Butyrate; Urokinase; Urokinase receptor; Tumor necrosis factor

1. Introduction

Butyrate is a short chain fatty acid produced in the lumen of the human colon by bacterial fermentation of dietary fibre [1]. At concentrations present in the lumen it inhibits cell proliferation and stimulates cell differentiation [2]. Butyrate also induces apoptosis of colonic carcinoma cells in culture [2,3], increases c-*jun* and reduces c-*myc* expression in colon cancer cells [4,5]. Butyrate responsive elements have been found in the 5' flanking regions of c-*fos* and mouse calbindin-D_{28k} genes [1,6]. The effects of butyrate on the genes implicated in the cell surface proteases that may determine adenocarcinoma invasion and metastasis, however, are unknown.

Proteolysis mediated by urokinase plasminogen activator (uPA) may be central to the processes of cell migration and invasiveness under normal and pathological conditions that include cancer invasion and metastasis [7]. uPA plays its catalytic role on the cell surface by binding specifically to its receptor (uPAR) with high affinity. Receptor-bound uPA activates the proenzyme plasminogen to form plasmin which initiates a proteolytic cascade that contributes to the degradation of basement membranes and the extracellular matrix [8]. The activity of uPA on the cell surface is regulated by the number of uPAR and the level of its inhibitors in the tumour cell microenviroment. In cancers, receptor-bound uPA activity is focussed at the invasive front [9]. High levels of uPA activity and uPAR gene expression in human cancers correlate with risk of recurrence [10,11] and increasing evidence indicates that invasion and metastasis by adenocarcinoma cells can be prevented by inhibiting uPA and uPAR production [12,13].

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine that has cytocidal activity against a number of cancer cell lines and induces haemorrhagic necrosis of cancers in mice [14]. It stimulates a range of mRNAs, including uPA and uPAR mRNAs in human endothelial and colon cancer cells [15], but whether butyrate inhibits the increased uPA and uPAR expression induced by TNFα or PMA is unclear. Similarly, whether butyrate regulates uPA and uPAR mRNA at the levels of transcription or post-transcription has received little attention. This paper reports that sodium butyrate inhibits uPA and uPAR mRNA expression in a dose-dependent manner in colon cancer cell lines regardless of whether they have been stimulated with TNFα, PMA or cycloheximide. In the presence or absence of TNFα, butyrate acts, at least in part, at the transcriptional level on uPA gene expression, but acts at both the transcription and post-transcription levels on the expression of the uPAR gene. In the presence of PMA, however, butyrate acts at the post-transcriptional level on the expression of both genes.

2. Materials and Methods

2.1. Materials

Recombinant human TNFα (activity: 1 unit ~0.0455 ng) was provided by the Asahi Chemical Company, Tokyo, Japan. PMA and cycloheximide (CHX) were from Sigma Chem. Co., St. Louis, USA, and [α-^{32}P]dCTP from Amersham Ltd., UK.

2.2. Cell culture

The HCT116 [16] (American Type Tissue Collection) and LIM1215 cells [17] (Dr. R.T. Whitehead, Ludwig Institute for Cancer Research, Melbourne, Australia) were found to be free of *Mycoplasma* by Hoechst stain #33258 and of virus contamination by transmission electron microscopy of cell cultures throughout this study. Cells were maintained in RPMI-1640 medium and washed with PBS and incubated overnight or for 4 h in RPMI serum-free media before stimulation. Immediately before harvest, cell viability was consistently found to be >90%.

2.3. cDNA probes

The human uPA cDNA used was a 0.93 kb *Bgl*II–*Bam*HI fragment from the plasmid pcUKLTR6 provided by Dr. R. Miskin, the Weizmann Institute of Science, Israel [18]. The 1.144 kb human uPAR cDNA [19] used was a gift from Dr. E.K.O. Kruithof, Lausanne, Switzerland. Human 18 S ribosomal DNA (18 S rDNA) was provided by Dr. B.E.H. Maden, University of Liverpool, UK [20]. Human β-actin cDNA [21] was a gift from Dr. H.D. Campbell, The Australian National University. Human ubiquitin cDNA [22] was a gift from Dr. R.T. Baker, The Australian National University. cDNA probes were labelled with [α-^{32}P]dCTP by the random priming method [23].

2.4. RNA preparation and Northern blot analysis

RNA was purified from cells using the guanidinium isothiocyanate method, electrophoresed on 1% formaldehyde-containing agarose gels,

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Abbreviations: NaB, sodium butyrate; CHX, cycloheximide; TNF, tumor necrosis factor; PMA, phorbol 12-myristate 13-acetate; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor.

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transferred to a nitrocellulose membrane and further processed according to the method of Ausubel et al. [23]. The membranes were exposed to Kodak XAR film and multiple film exposure times were used to ensure linearity of band intensities. The intensities of mRNA bands in the autoradiographs were scanned and quantitated by a video densitometer (Model 620; Bio-Rad). mRNA intensities are calculated relative to the intensity of the 18S rRNA internal control. The RNA molecular markers used were purchased from Promega corporation.

2.5. Nuclear run-on transcription assay

Nuclei were isolated from HCT116 cells treated with TNFα, PMA or butyrate, according to the method previously described [24]. In brief, about 5×10^7 cells were washed twice with cold PBS and lysed in 4 ml NP-40 lysis buffer (10 mM Tris, pH 7.4/10 mM NaCl/3 mM $MgCl_2$/0.5% NP-40). Nuclei were then pelleted by centrifugation at $600 \times g$ for 3 min. For the transcription elongation reaction, freshly prepared nuclei were incubated at 30°C for 30 min with [α-^{32}P]UTP, and run-on analysis performed. In a given experiment, each filter was hybridized to DNA immobilized on nitrocellulose with the same amount of ^{32}P-labelled RNA. The filters were then exposed at −70°C for 7–14 days using intensifying screens. The intensities of mRNA bands in the autoradiographs were scanned and quantitated by a video densitometer (Model 620; Bio-Rad). mRNA intensities are calculated relative to the intensity of the β-actin internal control.

3. Results and discussion

3.1. Inhibition of uPA and uPAR mRNAs by sodium butyrate (NaB)

To study whether expression of uPA and uPAR mRNAs is inhibited by butyrate in the presence or absence of TNFα (440 units/ml), PMA (30 ng/ml) or CHX (20 μg/ml) in colon cancer cells, RNA was isolated from cells treated with stimuli and analyzed by Northern-blotting. Unstimulated HCT116 cells expressed low levels of 2.5 kb uPA and 1.4 kb uPAR transcripts but both mRNAs were increased in the presence of TNFα, PMA or CHX (Fig. 1 and 2).

To investigate the role of butyrate in the regulation of uPA or uPAR mRNAs, HCT116 cells were exposed to butyrate concentrations varying from 0.25–25 mM for 4 h. Northern blot analyses show inhibition of uPA and uPAR mRNAs at 2.5 mM to 25 mM (Fig. 1A), in the range of concentrations found in the human colonic lumen. At 25 mM butyrate, uPA mRNA expression was completely inhibited and uPAR mRNA levels were reduced by 60% as determined by scanning densitometry. Similar butyrate effects on uPA and uPAR mRNAs were found in another colon cancer cell lines, LIM1215 (data not shown).

To determine the effects of butyrate on TNFα or PMA-stimulated increases in uPA and uPAR mRNA levels, TNFα or PMA plus various concentrations of butyrate (0.25–25 mM) were added to the HCT116 cells for 4 h. Northern blot analyses showed a marked inhibition of uPA and uPAR mRNA expression in cells. At 25 mM, butyrate inhibited about 90% of the uPA mRNA induced by TNFα or PMA, whereas uPAR mRNA was inhibited by about 50% in the same experiment, suggesting that uPA mRNA was more sensitive to butyrate than that of uPAR (Fig. 1B,C).

In our experiments, butyrate had no effects on the total RNA yield or on cell viability. When the uPAR probe was eluted from the filters and the same RNA blot was then hybridized using ^{32}P-labelled human β-actin [21], ubiquitin cDNA [22] or 18S rDNA, no significant changes in the β-actin and ubiquitin mRNAs (data not shown) or 18S rRNA were detected regardless of whether the cells had been exposed to butyrate indicating that butyrate is selective in inhibiting gene expression.

Butyrate is a naturally occurring short chain fatty acid that decreases cell growth, increases differentiation and induces apoptosis in colon cancer cell lines [2], suggesting that locally produced butyrate may help protect colon epithelial cells from becoming neoplastic. Elevated uPA and uPAR expression has been reported for a number of human malignancies including colon, breast, bladder, and prostate cancer [10]. uPA-mediated proteolysis appears to be central to invasion by adenocarcinoma cells and inhibition of uPA activity has become a target of anti-metastatic therapy [10]. The results presented in this paper show that, in colon cancer cells, there is a strong correlation between the degree of inhibition of uPA and uPAR gene expression and the concentration of sodium butyrate. Butyrate produced by bacterial fermentation in the colon may therefore contribute to the prevention of uPA-mediated tumor cell invasion and metastasis.

Furthermore, PMA induced increases of uPA and uPAR mRNAs that were markedly inhibited by sodium butyrate sug-

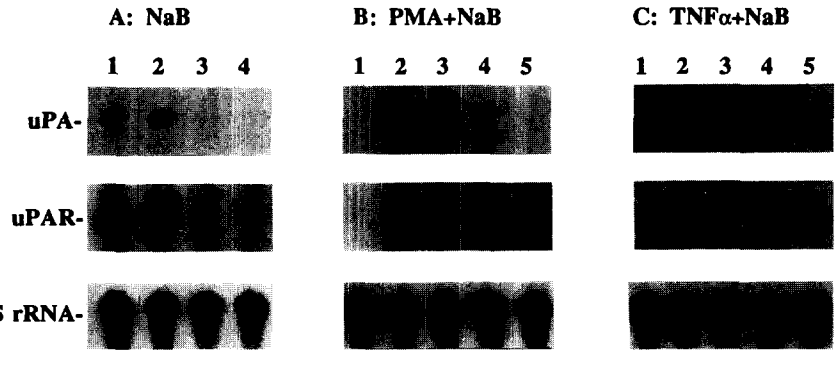

Fig. 1. Inhibition of uPA/uPAR mRNAs by sodium butyrate (NaB) in HCT116 cells. Cells were untreated or treated with PMA (30 ng/ml) or TNFα (440 units/ml) for 4 h in the presence or absence of NaB. The same Northern blot was hybridized to uPA cDNA, uPAR cDNA and 18 S rDNA probes as indicated. (A) Dose-dependent inhibition of mRNAs accumulation by NaB. Autoradiographic exposure times were 5 days (for uPA and uPAR cDNA as probes) and 1.5 h (for 18 S rDNA as probe). Lane 1, untreated; lanes 2–4, NaB at 0.25, 2.5 and 25 mM, respectively. (B) Dose-dependent inhibition of mRNAs accumulation by PMA plus NaB. Autoradiographic exposure times were 48 h (for uPA cDNA as probe), 24 h (for uPAR cDNA as probe) and 1.5 h (for 18 S rDNA as probe). Lane 1, untreated; Lane 2, PMA; lanes 3–5, PMA plus NaB. NaB at 0.25, 2.5 and 25 mM, respectively. (C) Dose-dependent inhibition of mRNAs accumulation by TNFα plus NaB. Autoradiographic exposure times were 48 h (for uPA cDNA as probe), 72 h (for uPAR cDNA as probe) and 1 h (for 18 S rDNA as probe). Lane 1, untreated; Lane 2, TNFα; lanes 3–5, TNFα plus NaB. NaB at 0.25, 2.5 and 25 mM, respectively. Each experiment was repeated at least three times and representative data shown in the figures.

gesting lumenal butyrate may also protect colon epithelial cells exposed to tumour promoters by down-regulating uPA system gene expression. TNFα is produced by many cell types including activated monocytes, macrophages and lymphocytes [14]. Tumor-infiltrating macrophages, but not colon cancer cells, may also produce TNFα mRNA and protein in colon cancer tissue [25]. The inhibitory effect of butyrate on uPA and uPAR mRNAs stimulated by TNFα suggests that the tumor microenvironment may contribute to the regulation of uPA and uPAR gene expression in colonic cancers thereby influencing tumor invasiveness. Butyrate may also induce abnormal or transformed cell lines to convert to more normal phenotype and function [2]. These data combined with the inhibitory effects of butyrate on uPA and uPAR mRNAs expression reported here, may offer the prospect of developing new therapeutic approaches to prevent invasion and metastasis by adenocarcinomas of the colon.

To investigate whether the inhibition of uPA or uPAR gene expression by butyrate depends on new protein synthesis, HCT116 cells were preincubated with CHX (20 μg/ml) for 30 min to ensure inhibition of protein synthesis at an early time point and then butyrate was added for 4 h. Northern blot analysis showed that in HCT116 cells, CHX alone induced uPA and uPAR mRNA expression suggesting that both genes are regulated by a labile repressor protein [15]. uPA and uPAR mRNAs were also induced by CHX in HCT116 cells stimulated by TNFα or PMA (Fig. 2), suggesting that stimulation of uPA and uPAR do not need *de novo* protein synthesis. uPA and uPAR mRNAs induced by CHX in the absence or presence of TNFα or PMA were inhibited by sodium butyrate (Fig. 2) indicating that the effects of butyrate on uPA and uPAR gene expression do not require synthesis of new regulatory proteins in colon cancer cells. Similar effects of butyrate inhibition were found in LIM1215 cells (data not shown). No difference was

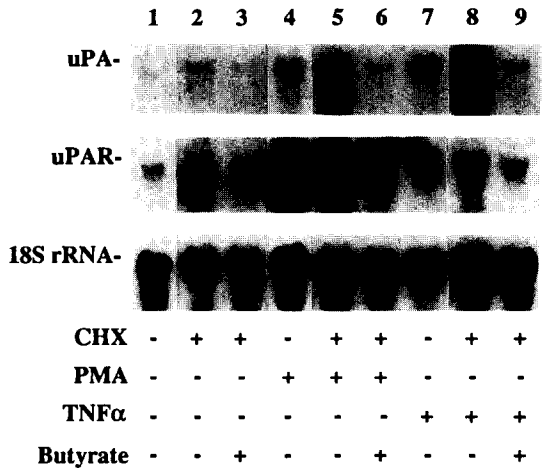

Fig. 2. Inhibition of uPA/uPAR mRNAs by sodium butyrate (NaB) in the presence of CHX in HCT116 cells. Cells were untreated or treated with CHX (20 μg/ml) for 4 h in the presence or absence of NaB (2.5 mM), PMA (30 ng/ml) and TNFα (440 units/ml). The same Northern blot was hybridized to uPA cDNA, uPAR cDNA and 18S rDNA probes as indicated. Autoradiographic exposure times were 24 h (for uPA and uPAR as probes) and 4 h (for 18 S rDNA as probe). The symbol '−' and '+' indicate untreated and treated cells respectively. Each experiment was repeated at least three times and representative data shown in the figures.

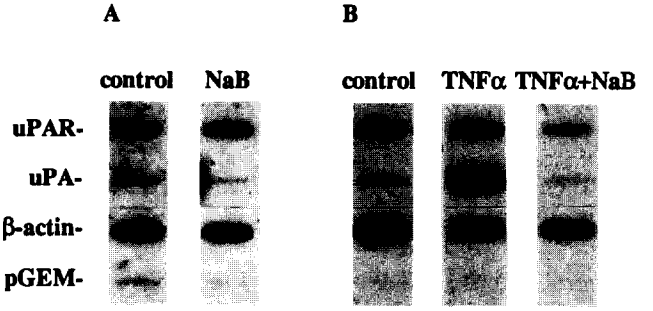

Fig. 3. Nuclear run-on transcription analysis of effects of sodium butyrate (NaB) on uPA and uPAR gene transcription in HCT116 cells. Nuclei were isolated from HCT116 cells treated with NaB (2.5 mM) (A) or TNFα (2,000 units/ml) plus NaB (2.5 mM) (B). Following in vitro transcription, RNA was purified from the nuclei and hybridized with uPAR cDNA, uPA cDNA, β-actin cDNA or vector DNA (pGEM) immobilized on nitrocellulose filters. Hybridized ^{32}P-labelled nuclear RNA was detected by autoradiography. Autoradiographic exposure time was 7 days for experiment (A), and 5 days for experiment (B). Each experiment was repeated at least twice and representative data shown in the figures.

observed in the constitutively expressed 18 S ribosomal RNA (18 S rRNA), which served as a control.

3.2. *Effects of butyrate on uPA and uPAR transcription in isolated nuclei*

To determine whether the effects of butyrate on the levels of uPA or uPAR mRNA act at the transcriptional level, nuclear run-on transcription assays were performed in HCT116 cells treated or untreated for 4 h with 2.5 mM butyrate in the presence or absence of PMA and TNFα. As shown in Fig. 3, uPA and uPAR transcripts were expressed in nuclei from unstimulated HCT116 cells. While butyrate markedly inhibited uPA mRNA expression, it has no effect on uPAR mRNA indicating that the inhibitory effect of butyrate alone on uPA expression was mediated, at least in part, at the transcriptional level. By contrast, the inhibition of uPAR mRNA by butyrate alone was not mediated at the transcriptional level, but may act via post-transcriptional mechanisms including control points of mRNA processing, transport, translation or degradation. uPA and uPAR transcripts were increased in the cells exposed to TNFα (Fig. 3), but not PMA ([15] and data not shown). Both transcripts were also markedly reduced in the cells treated with butyrate plus TNFα (Fig. 3), but not butyrate plus PMA ([15] and data not shown). These assays indicate that the inhibitory effect of butyrate in the presence of TNFα was mediated, at least in part, at the transcriptional level, whereas the inhibitory effect of butyrate in the presence of PMA may act via a post-transcriptional mechanism [15 and data not shown].

Previous reports showed that butyrate affects expression of genes including the c-*fos* and c-*myc* at both the transcription and post-transcription levels [1, 26]. Butyrate induces the c-*fos* proto-oncogene expression very rapidly at the post-trancriptional level but up-regulated the c-*fos* gene at the transcriptional level at later time in a colon carcinoma cell line, Caco-2 [1]. c-*myc* gene expression, however, was down-regulated at the post-transcriptional level in the same cell line [26], indicating that complex mechanisms may be involved in the regulation of c-*fos* and c-*myc* gene expression by butyrate. In the case of the uPA system, butyrate modulated gene expression at both the

transcription (uPA) and post-transcription (uPAR) levels suggesting that different mechanisms may be involved in the regulation of uPA and uPAR gene expression.

Human TNFα regulates gene expression in eukaryotic cells by binding to the TNFα receptors [27]. In endothelial cells, TNF receptor signal transduction pathways may involve cAMP, G-proteins, protein kinase C and other kinases [28]. The effects of TNFα may be mediated through the transcriptional factor NF-κB which is activated by the sphingomyelin pathway in HL-60 cells [27]. The nuclear run-on experiment results reported here show that butyrate inhibits TNFα-induced uPAR mRNA at the transcriptional level suggesting that butyrate may affect uPAR gene expression by interacting with TNFα signalling pathways. Therefore, the inhibitory effects of butyrate on TNFα- and PMA-induced changes in uPA and uPAR mRNA levels may reflect inhibition of TNFα signalling pathways, inhibition of uPA gene transcription and degradation of both mRNAs at the level of post-transcription.

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