Effects of Butyrate on the Expression of Insulin-Like Growth Factor Binding Proteins in Bovine Kidney Epithelial Cells

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Abstract: Sodium butyrate induces cell cycle arrest and apoptosis in bovine kidney epithelial cells primarily *via* down-regulating cell cycle-related gene expression and enhancing expression of pro-apoptotic genes. The insulin-like growth factor (IGF) system plays an essential role in these processes. Understanding of regulation of insulin-like growth factor binding proteins (IGFBPs) by butyrate helps reveal the mechanisms by which butyrate induces many physiological processes. In this study, we investigated effects of butyrate on the expression of insulin-like growth factors (IGF1 and IGF2), as well as their receptors (IGF1R and IGF2R) and binding proteins (IGFBPs), in Madin Darby bovine kidney (MDBK) epithelial cells. Our results demonstrated that IGF1 expression was below detection using real-time RT-PCR whereas expression of IGF2 was significantly up-regulated by butyrate in both mRNA and protein levels. Unlike IGF1R, whose expression remained unchanged, IGF2R was also up-regulated by butyrate. Butyrate significantly enhanced expression of IGFBP3 and IGFBP5, while the expression of IGFBP4 and IGFBP6 was down-regulated. Our results suggested that IGF2, not IGF1, may play a critical role in regulating cell cycle progression and programmed cell death by apoptosis in MDBK cells.

Keywords: Bovine, IGF, IGFBP, butyrate.

INTRODUCTION

As one of the natural microbial fermentation products in the gastrointestinal tract, butyrate plays a significant role in energy metabolism in ruminants [1]. Butyrate also has a multitude of cellular regulatory effects that modulate cell differentiation, proliferation, and motility primarily by acting as a histone deacetylase (HDAC) inhibitor. Our previous studies established that butyrate plays a role in inducing cell cycle arrest and apoptosis [2, 3, 4, 5]. The biological endpoint of a 24h treatment by 10mM sodium butyrate was resultant death of approximately 38% of total cells, mainly due to apoptosis [2]. In bovine kidney epithelial cells, up to 8% of all genes tested had an altered gene expression pattern resulting from butyrate treatment [4]. The butyrate-induced genes related to multiple signal pathways such as cell cycle control, extracellular matrix remodeling, and apoptosis, were discussed in details [4, 5].

The insulin-like growth factor (IGF) system plays an essential role in cell growth, proliferation, differentiation, transformation, and apoptosis [6]. Insulin-like growth factor binding proteins (IGFBPs) act as carrier/transport proteins in biologic fluids to control IGF efflux. In addition to their roles in prolonging IGF half-lives, IGFBPs themselves possess biological functions independent of their ability to modulate IGF activities [7]. For example, IGFBP3 has been shown to act as a ligand for nuclear receptor RXR-α, involving in apoptosis regulation [8]. Our previous study in cattle determined that IGF2, which regulates a broad range of gene networks, is significantly up-regulated by butyrate [5].

Although butyrate exerts its major physiological functions in the gastrointestinal tract of ruminants, we chose bovine kidney epithelial cells (MDBK) in this study for several reasons. First, our previous studies demonstrated that MDBK cells expressed butyrate receptors and were responsive to exogenous butyrate treatment. MDBK as an established bovine cell line with inducible apoptosis and cell cycle regulatory events was also readily available. In addition, the data shown that functional type I IGF receptors were present and the cells were indeed responsive to exogenous IGF1 treatment as evidenced by resultant increase of DNA synthesis [9]. These same authors shown that MDBK cells virtually excluded any passage of IGF1 across epithelial cell monolayers; and in term of high trans-epithelial electrical resistance values, which indicated great impediment of electrolyte and macromolecular movement, MDBK cells displayed characteristics more comparable to colon carcinomas cells. In this study, we investigated how butyrate regulates expression of IGFs, IGF receptors and six IGFBPs under three cell cycle conditions: normal cell cycle where cells undergo exponential growth, cells under 72h of serum deprivation (~79% of cells are arrested in G_1/ G_0 phases), and cells after 10h of release from serum deprivation (~81% of cells are in S phase). Our results, obtained using real-time RT-PCR and Western blot analysis, provided insights into the mechanism by which butyrate regulates cell cycle arrest and apoptosis.

MATERIALS AND METHODOLOGY

Cell Culture

The Madin-Darby bovine kidney epithelial cells (MDBK, American Type Culture Collection, Manassas, VA., Catalog No. CCL-22) were cultured in Eagle's minimal essential medium supplemented with 5% fetal bovine serum (Invitrogen, Carlsbad, CA) in 25 cm^2 flasks with medium renewal twice per week. Cell cultures were maintained in a water-

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jacked incubator with 5% CO_2 at 37ºC. Sub-cultivations were performed when cells attained 80 to 90% confluence, according to the product information supplied by American Type Culture Collection. For normal cell cycle experiment, the cells were treated with 10mM of sodium butyrate (Calbiochem, San Diego, CA) for 24h at approximately 50% confluence (during the exponential growth phase). For the serum deprivation experiment, cells were rinsed with serum-free media and then replaced with media containing 0.5% fetal bovine serum and cultured for 72h. 10mM of sodium butyrate was then added to the media and the cells were cultured for an additional 24h. For the synchronization experiment, cells were cultured for 72h in the media containing 0.5% fetal bovine serum, then released into fresh media containing 5% fetal bovine serum for 10h; the released cells were then cultured at 10mM of sodium butyrate for additional 24h. Under each of the three conditions, the control was time-matched PBS. The treatment and control groups each had 3 replicates. The harvested cells were snap frozen in liquid N2 and stored at -80ºC until RNA and protein extraction.

Flow Cytometry

The detailed procedure was previously described [2, 3, 4]. Briefly, cells collected by trypsinization were washed and re-suspended in PBS. Two volumes of ice-cold 100% ethanol were added drop-wise into tubes and mixed with cells in suspension by slow vortexing. After incubation with RNase I, cells were then stained with propidium iodide (PI). Measuring the fluorescence by flow cytometry provided a measure of the amount of PI taken up by the cells and, indirectly, the amount of DNA content, which reflects the status of cell cycle progression. Cells in G_1/G_0 phases have 2C DNA contents and do not undergo any DNA synthesis. Cells in S phase undergo rapid DNA synthesis and have DNA contents ranging from 2C to 4C whereas cells in M/G_2 phases have 4C DNA content again without any DNA synthesis activity. The DNA content was analyzed using a flow cytometer (FC500, Beckman Coulter, Palatine, IL) and collected data were analyzed using Cytomics RXP software (Beckman Coulter). >10,000 cells per sample were analyzed using flow cytometry.

Preparation of Cell Extracts and Western Blot Analysis

Preparations of cells and protein extracts, SDS-PAGE and Western Blot analysis were described previously [2, 4]. Briefly, the protein from different samples was separated by SDS PAGE on two identical 4 to 20% polyacrylamide gradient gels. One gel was stained with SimpleBlue (Invitrogen) and one was transferred to a membrane and probed with polyclonal anti-IGF2R (Novus Biologicals, Littleon, CO, USA) and anti-IGFBP2 (Abcam, Inc., Cambridge, MA, US, which also cross-reacts with IGFBP1) antibodies. The relative densities of the target bands on the Western Blots from three experiments were quantified with image software UN-SCAN-IT (Silk Scientific, Orem, Utah, USA). The data were then statistically analyzed using one-way analysis of variance (ANOVA).

Real-Time RT-PCR

Total RNA was extracted as previously described [4]. Briefly, total RNA was first extracted using Trizol (Invitrogen) followed by DNase digestion and purification using an RNeasy Mini kit (Qiagen, Valenica, CA). RNA integrity was verified using a Bioanalyzer 1000 (Agilent, Palo Alto, CA).

Real-time RT-PCR analysis was carried out using the iQ SYBR Green Supermix kit (Biorad) with 200 nM of each amplification primer (Table **1**) and the 1st-strand cDNA (100 ng of the input total RNA equivalents) in a 25 µl reaction volume. The amplification was carried out on an iCycler iQ™ Real Time PCR Detection System (BioRad) with the following profile: 95ºC – 60s; 40 cycles of 94ºC-15s, 60ºC –30s, and 72ºC –30s. The melting curve analysis was performed for each primer pair. Expression levels of β-actin remained constant and were used as endogenous controls. Relative gene expression data were calculated using the $2^{-\Delta\Delta CT}$ method [10]. The data were then analyzed using unpaired Student's t-test with $P<0.05$ being taken as significant.

RESULTS

Butyrate Induces Cell Cycle Arrest and Apoptosis Independent of Cell Cycle Status

In our previous study [2, 4], we monitored butyrate induced cell death and cell cycle arrest in MDBK cells in a time/dose-dependent manner using flow cytometry and

Table 1. **Primers Used in Real-Time RT-PCR**

Gene	GenBank Accession#	Forward Primer	Reverse Primer
IGF1	NM_001077828	ACAGGAATCGTGGATGAG	ACTCCCTCTACTTGTGTTC
IGF2	NM_174087	CGCTCAGAGAGGCCAAGAGT	GGCTCACTTCTAATCGCTGGAT
IGF1R	XM_606794	TGACGAGAGACATCTATGAGAC	GACCCCGAAGGACCAGAC
IGF2R	NM_174352	TGCCTCCCGAGTCAGTAAC	AATCTTTGGGTGGGTTGTTTG
IGFBP1	NM_174554	TTTTTCAAGTGGTTCAATGGAG	CAACATTTAAGTTTCCCTGCC
IGFBP2	NM_174555	AGCACCTCTACTCCCTACAC	GTTCACACACCAGCACTCC
IGFBP3	NM_174556	AATCGCCCTTGCTTGGTG	GGTCGTGGCTGTGTTTATATTC
IGFBP4	NM_174557	CTGAGACCCAATCCCAACAC	TCTCCATCAGGCACATACATTC
IGFBP5	XM_600908	CACACTGACAACCCCAAATCTG	GCCTGAAGGTCCAGCATCC
IGFBP6	NM_001040495	AAGGAGAGTAAGCCCCAAGCA	CGGGAAGGAGTGGTAGAGGTC

Western blotting. The cell population profiles were investigated under three experimental conditions at a single selected dose that was proven to be capable of generating maximum biological impact. First, under the normal cell cycle condition, the exponentially growing cells consist of approximately 41% of G_1/G_0 cells and 49% of S phase cells (Table **2**). As previously reported, butyrate induced cell cycle arrest and drastic changes in cell population profiles in MDBK cells [4]. A significant increase in the number of cells in G_1 phase (with 2C DNA contents) and decrease in S phase cells (falling between the 2C and 4C DNA contents) was observed (Fig. **1**), suggesting the cells were indeed arrested at the G_1/S boundary and that DNA replication was blocked by butyrate treatment. Under serum deprivation condition, the cell population was predominantly at the G_1/G_0 phases (~79%). However, when released from serum deprivation for 10h, synchronization occurred and ~81% of the cell population was shifted to S phase (Table **2**). Under all three conditions, sodium butyrate induced cell cycle arrest and cell death as identified by TUNEL assay [2], suggesting the anti-proliferative and pro-apoptotic effects of butyrate were independent of cell cycle status. Accumulation of acetylated histone 3 (H3) was also observed under all three conditions (data not shown), indicating that the biological effects of butyrate in these cells were due to its histone deacetylase inhibitory activity.

Table 2.   Flow Cytometric Assessment of the Cell Population*

	% G_1/G_0	% S	% M/G_2
Normal cell cycle (N)	41.4 ± 1.5	48.7 ± 1.8	9.9 ± 0.4
Serum Deprivation (G)	79.4 ± 1.0**	12.3 ± 0.8**	8.3 ± 0.6
Release (S)	15.8 ± 1.9**	80.6 ± 3.4**	3.6 ± 3.5

*Data represent Mean ± SEM of 4 separate experiments.
**P <0.01 based on one way ANOVA.
N = Cells in normal cell cycle.
G = Cells after serum deprivation (predominantly in G_1/G_0 phase).
S = Cells after serum deprivation and release (predominantly in S phase).

Real-Time RT-PCR

Gene expression of IGFs, IGF receptors, and six IGF binding proteins regulated by butyrate was monitored under three experimental conditions using real-time RT-PCR. The expression of IGF1 under all three conditions was below detection after 40 cycles of amplification, regardless of butyrate treatment (Table **3**). The butyrate treatment induced significant (P<0.05) up-regulation of IGF2 under these conditions (143 to 363 fold). In the control group, serum deprivation (S) and release (G) alone induced IGF2 mRNA up-regulation ~ 5 fold compared to control cells under normal cell cycle.

Insulin-like growth factor 1 receptor (IGF1R) expression was detectable and remained constant under all three conditions. Butyrate induced slightly down-regulation of IGF1R. However, the changes were not significant. In contrast to IGF1R, IGF2R expression was enhanced by butyrate under all three conditions 4-10 fold. Butyrate induced upregulation of IGF2R more in the G_1/G_0 phase dominated cell population than in the cell populations of predominantly S phases (Table **3**).
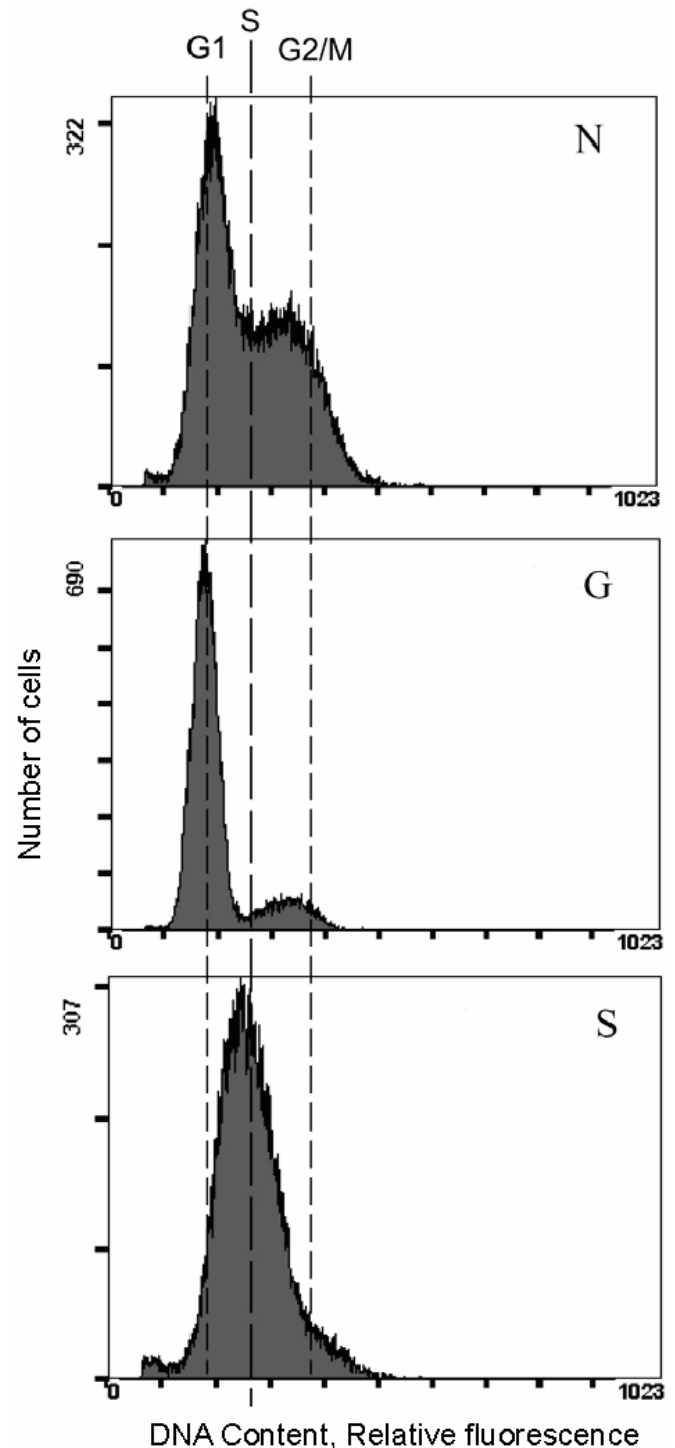

Fig. (1). Cell population profiles determined by flow cytometry. Histogram plots of flow cytometry analysis of MDBK cells under three experimental conditions were displayed: N: cells with normal cell cycle; G: cells after 72h serum deprivation; and S: cells released for 10h after 72h of serum deprivation. Deconvolution of the DNA histogram using Cylchred software. Data represent means from three replicates. G_1: cells in G_1/G_0 phases; S: cells in S phase; G_2/M: cells in M/G2 cell cycle phases.

Butyrate regulated all insulin-like growth factor binding proteins (IGFBPs) except IGFBP2 in bovine kidney epithelial cells. Butyrate up-regulated IGFBP3 and IGFBP5 whereas IGFBP4 and IGFBP6 were down-regulated slightly

(Table **3**). IGFBP3 was significantly up-regulated by butyrate under all three conditions (14-24 fold).

Table 3.   Gene Expression Induced by Sodium Butyrate Under Three Experimental Conditions Measured Using Real-Time RT-PCR

	Treatment (Mean Fold; n=3)					
	N+CT	**S+CT**	**G+CT**	**N+SB**	**S+SB**	**G+SB**
IGF1	BD	BD	BD	BD	BD	BD
IGF2	1.0	4.6	4.7*	143.4*	298.5*	363.0*
IGF1R	1.0	1.0	1.2	0.9	0.8	0.9
IGF2R	1.0	0.9	1.4	4.7**	4.5*	10.7
IGFBP1	1.0	0.6	1.6	5.1	1.9	5.9
IGFBP2	1.0	0.9	0.9	1.8	0.6	0.9
IGFBP3	1.0	1.2	1.4	21.2*	14.6**	23.9*
IGFBP4	1.0	1.0	1.0	0.3**	0.3*	0.2**
IGFBP5	1.0	0.9	1.2	2.3	2.3*	3.5*
IGFBP6	1.0	1.1	1.3	0.7	0.5**	0.4*

N = Cells in normal cell cycle; S = Cells after serum deprivation and release (predominantly in S phase); G = Cells after serum deprivation (predominantly in G_1/G_0 phase). CT = control; SB = treated with sodium butyrate at 10mM for 24h. BD = below detection after 40 cycles. In the control group (CT), the two cell-cycle stages (S and G) were compared to normal (N), respectively. In the treatment group, SB treatment was compared to the untreated control in their respective cell cycle stages (for example, G+SB *vs* G+CT). * = $P<0.05$; ** = $P<0.01$.

Western Blot Analysis

Due to availability of antibodies that recognize bovine proteins, only a limited number of proteins were analyzed using Western blot analysis. Overall trends indicated that there was a good correlation between mRNA expression and protein expression. The parallel expression was evident for all proteins analyzed in this system. As Fig. (**2**) shows, SB induced IGF2R expression in protein level under all three conditions. However, a greater induction of IGF2R was achieved when the population was predominantly G_1/G_0 -phase cells (G), in an agreement with the mRNA results measured by real-time RT-PCR (Table **3**). Also consistent with the mRNA results, IGFBP2 protein was not significantly altered by butyrate.

DISCUSSION

Insulin-like growth factors (IGF) play a pivotal role in regulating cell proliferation and differentiation as a potent mitogen. They are also a critical modulator of cellular growth and transformation in many tissues [6]. IGF1 acts in a paracrine and endocrine manner to affect bovine mammary gland development [11]. In addition, IGFs are capable of inhibiting programmed cell death by apoptosis in various cell types [12]. Although IGFs exert their biological functions through their receptors for signal transduction, the biological activities of IGFs are regulated by their binding proteins. The four major functions of IGFBPs have been documented [7]: acting as carrier proteins in biological fluids with very high affinity binding to both IGF1 and IGF2 to ensure virtually all circulating IGFs are bound to IGFBPs; prolonging IGF half-
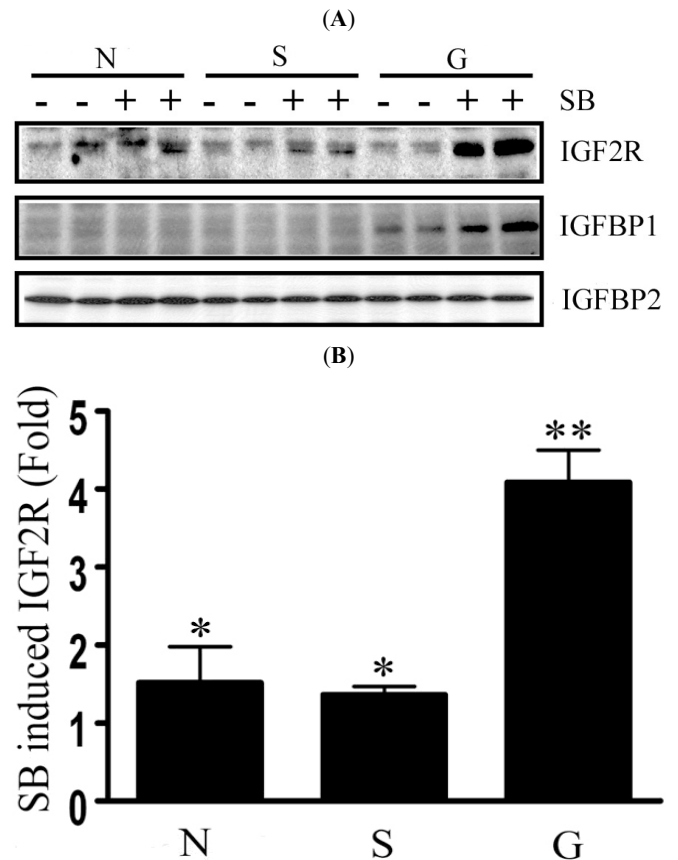
Fig. (2). Western blot analysis of IGF2R, IGFBP1, and IGFBP2 under three experimental conditions. N: cells with normal cell cycle; G: cells after 72h serum deprivation; and S: cells released for 10h after serum deprivation. SB: sodium butyrate treatment at 10mM for 24h. (**A**) Protein from different samples was separated by SDS PAGE on 4 to 20% polyacrylamide gradient gels. The gel was transferred to a membrane for Western blotting with polyclonal anti-IGF2R and anti-IGFBP2 antibodies. The samples were loaded in duplicate. (**B**) Butyrate-induced fold change of IGF2R protein. Western Blots from three experiments were quantified using UN-SCAN-IT image software. Polyclonal anti-IGFBP2 antibody recognizes both IGFBP1 and IGFBP2. * = $P<0.05$; ** = $P<0.01$. Data are presented as means ± SEM.

lives; providing a mean of tissue- and cell type-specific localization; and modulating interaction of IGFs with their receptors. Most recently, IGFBPs have been found to have direct biological functions that are independent of IGFs [13, 14]

In this study, we systematically investigated the expression regulation of the IGF system by butyrate in bovine cells. Our results indicated that IGF1 was below detection and IGF1R remained non-responsive to butyrate treatment. The observation of strong up-regulation of both IGF2 and IGF2R by butyrate suggested that IGF2 may play a more important role in bovine kidney epithelial cells *in vitro*, even though in many systems, most of the actions of both IGF1 and IGF2 are mediated by the IGF1R [7]. This is also in sharp contrast to murine mammary glands where IGF1 was always expressed at significantly higher levels than either IGF2 or IGFR and plays a more important role during mammary

morphogenesis [15, 16]. Indeed, mRNA expression of IGF2 and IGF2R was significantly induced by butyrate under all three conditions, resulting in a parallel increase in IGF2R protein level (Fig. **2A**). As an imprinted gene in cattle and canines, IGF2R recognizes multiple ligands such as IGF2, retinoic acid [17], and TGFβ, having a broad range of physiological functions including its association with tumorigenesis [18] and apoptosis. IGF2R is generally regarded as a negative regulator of IGF2 by acting as a sink. However, it has been shown that over-expression of soluble IGF2R leads to an increase in IGF2 levels *in vivo* [19]. It is possible that over-expression of IGF2R induced by butyrate in MDBK cells is involved in pro-apoptotic processes. Therefore, understanding actions of IGF2R in IGF signaling pathway in MDBK cells becomes intriguing.

Among six IGFBPs, it seemed that as the predominant IGFBP secreted [20], IGFBP2 was not regulated by butyrate in both mRNA and protein levels in MDBK cells. Both IGFBP4 and IGFBP6 were down-regulated by butyrate. Down-regulation of IGFBP6 observed in this study also confirmed our previous findings using microarrays [4]. IGFBP6 has a 100-fold higher affinity for IGF2 than for IGF1 [21]. This seemingly paradoxical observation, that while IGF2 expression is strongly up-regulated by SB, its highest affinity binding protein is down-regulated, suggested that different functions of various IGFBP members modulated IGF signaling transduction. Butyrate up-regulated IGFBP3, and IGFBP5; and, specifically, IGFBP3 expression was increased 14 to 24 fold by butyrate in our observation. It has been demonstrated that IGFBP3 induces apoptosis in an IGF-independent manner through the activation of caspases involving a death receptor-mediated pathway in human breast cancer cells [22]. Besides increased cleavage of caspases 7 and 8, IGFBP3 is also capable of increasing the ratio of pro-apoptotic Bax and Bad to anti-apoptotic Bcl-2 and Bcl-X_L proteins [23]. Our observation also suggested that butyrate induced up-regulation of CDKN1A (p21) was mediated by IGFBP3 to achieved its inhibitory effect on cell proliferation and cell cycle. It has been documented that butyrate and other HDAC inhibitors increase IGFBP3 expression by activating the IGFBP3 promoter *via* an Sp1/Sp3 multiprotein complex formed on the butyrate responsive element in the promoter region [13]. IGFBP5 is a negative regulator of IGF1 [24]. In mouse mammary epithelial cells, IGFBP5 expression is increased during apoptosis induced by TGFβ-3 treatment along with a parallel increase of cleaved apoptotic marker caspase 3 [25]. The causal relationship between IGFBP5 and apoptosis was documented in transgenic mice expressing IGFBP5 [26, 27] and the effects of IGFBP5 may be mediated in part by IGF-independent effects involving potential interactions with the extracellular matrix system (ECM). The biological significance of IGFBP5-ECM association has been carefully examined [28]. Indeed, our previous network analysis identified a complex network of genes involving IGF2, matrix metallo-proteinases (MMP1 & MMP13), TIMP3, connective tissue growth factor and IGFBPs that are regulated by butyrate in MDBK cells [5]. Although butyrate up-regulated both IGFBP3 and IGFBP5 in this study, our *in silico* analysis suggested the promoter regions of these two bovine genes shared few structural similarities. There exist multiple Sp1 sites within a 70bp region upstream from the transcription start site (TSS) in bovine IGFBP3. However, unlike its human counterpart [13], the stretch of 11 p53 consensus binding sites is absent in bovine IGFBP3. The TATA box does not appear in the IGFBP5 promoter region. While Sp1 and AP-1 sites exist, a single p53 binding site is located 11 bp downstream of TSS in bovine IGFBP5. Therefore, different kinds of transcriptional machinery may be involved in transcriptional regulation of both genes by butyrate. Together, these studies suggested that IGFBP3 and IGFBP5 may play an essential role in regulating butyrate-inducing cell death. Our future work will include comparing the expression profiles induced by both butyrate and exogenous IGFBPs and siRNA to IGFBPs in apoptotic pathway regulation in MDBK cells. Dissecting the mechanism of butyrate in regulating genes associated with cell death by apoptosis, cell proliferation and differentiation will not only facilitate designing more potent HDAC inhibitors for cancer but also help to understand ruminant physiology, which in turn could lead to improvement in energy utilization efficiency of cattle.

CONCLUSION

In this study, we systematically investigated the expression regulation of the IGF system by sodium butyrate using both real-time RT-PCR and Western blot analysis in bovine kidney epithelial cells under three experimental conditions: cells under normal cell cycle progression, cells after serum deprivation, and cells after release from serum deprivation. Our results suggested that IGF2, not IGF1, may play an essential role in regulating cell cycle progression and programmed cell death by apoptosis; and over-expression of IGF2R induced by butyrate may be involved in pro-apoptotic processes in MDBK cells. While the functions of IGFBPs seemed diverse, IGFBP3 and IGFBP5 were shown to be a primary regulator of butyrate-induced cell growth arrest and apoptosis. Because butyrate functions as both a nutrient and signaling molecule in ruminants, our findings enable better understanding of biological functions of short-chain fatty acids such as butyrate, during cattle energy metabolism, cell growth and proliferation.

ACKNOWLEDGEMENTS

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