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Colon cancer cell apoptosis is induced by combined exposure to the n-3 fatty acid docosahexaenoic acid and butyrate through promoter methylation

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Abstract

DNA methylation and histone acetylation contribute to the transcriptional regulation of genes involved in apoptosis. We have demonstrated that docosahexaenoic acid (DHA, 22:6 n-3) and butyrate enhance colonocyte apoptosis. To determine if DHA and/or butyrate elevate apoptosis through epigenetic mechanisms thereby restoring the transcription of apoptosis-related genes, we examined global methylation; gene-specific promoter methylation of 24 apoptosis-related genes; transcription levels of *Cideb*, *Dapk1*, and *Tnfrsf25*; and global histone acetylation in the HCT-116 colon cancer cell line. Cells were treated with combinations of (50 μM) DHA or linoleic acid (18:2 n-6), (5 mM) butyrate or an inhibitor of DNA methyltransferases, and 5-aza-2′-deoxycytidine (5-Aza-dC, 2 μM). Among highly methylated genes, the combination of DHA and butyrate significantly reduced methylation of the proapoptotic *Bcl2l11*, *Cideb*, *Dapk1*, *Ltbr*, and *Tnfrsf25* genes compared to untreated control cells. DHA treatment reduced the methylation of *Cideb*, *Dapk1*, and *Tnfrsf25*. These data suggest that the induction of apoptosis by DHA and butyrate is mediated, in part, through changes in the methylation state of apoptosis-related genes.

Keywords

docosahexaenoic acid; butyrate; apoptosis; DNA methylation; epigenetics

Introduction

During carcinogenesis, gene transcription is aberrantly regulated via epigenetic changes. These changes include DNA methylation and histone modification and occur primarily in the promoter region of select genes.[1,2] Clinical studies have demonstrated a correlation

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between DNA methylation and the pathological and epidemiological features of colon cancer.[3–5] Recent data indicate that promoter methylation of proapoptotic genes suppresses transcription, resulting in the survival of cancer cells.[6] For instance, *Dapk1* is a positive mediator of apoptosis, a tumor suppressor candidate, and is known to be heavily methylated in colon and bladder cancer.[7,8] Restoring transcription of tumor suppressor genes by the reversal of these epigenetic processes is considered a latent target of cancer prevention and treatment.

We have previously shown that a combination of fish oil (high in docosahexaenoic acid [DHA], an n-3 polyunsaturated fatty acid [PUFA]) and the fiber pectin (fermented to short-chain fatty acids including butyrate by the colonic microflora) is protective against colon carcinogenesis in part by up-regulating the apoptotic removal of DNA damaged cells.[9] In contrast, diets highly enriched in corn oil (enriched in linoleic acid [LA], an n-6 PUFA) suppress apoptosis and promote colon cancer.[10] We have demonstrated that apoptosis induction by the combination of fish oil and pectin is in part contingent upon DHA incorporation into mitochondrial phospholipids,[11] while butyrate functions as a energy substrate and histone deacetylase inhibitor.[12] Another potential mechanism involved in tumor suppression by fish oil/pectin is through the transcriptional regulation of key tumor suppressors or oncogenes. In a previous study, we demonstrated the effect of fish oil/pectin on global changes in gene expression profiles in carcinogen-injected Sprague–Dawley rats. One result from the pathway analyses of those data was the observation that the expression of genes that promote apoptosis was up-regulated in rats consuming the fish oil/pectin diet at the tumor endpoint.[13] Moreover, we have reported that cells isolated from carcinogen-induced colon tumors contain DNA that is highly methylated in the promoter region of *Bcl2*, an antiapoptotic mediator, which was associated with induction of apoptosis in colonocytes from rats consuming a fish oil/pectin diet.[14]

However, it has not been determined if DHA and/or butyrate can directly affect the aberrant promoter methylation of cancer-promoting genes. Therefore, the aim of this study was to investigate the epigenetic regulation of apoptosis-related genes in a colon cancer cell line exposed to DHA and/or butyrate. For this purpose, we examined global DNA methylation and histone acetylation in combination with gene-specific promoter methylation of 24 apoptosis-related genes, in HCT-116 human colon cancer cells. Bender *et al.* demonstrated that 5-Aza-dC regulated p16 DNA methylation and gene expression in seven cancer cell lines including HCT-116 cells.[15] The study by Schneider-Stock *et al.* showed that 5-aza-cytidine, the analog of 5-Aza-dC, induced demethylation and significant down-regulation of DNMT1 and DNMT3a gene transcription in HCT-116 cells.[16] Therefore, we chose to use the well-characterized HCT-116 cell line to test the effects of DHA/butyrate on DNA promoter methylation, using 5-Aza-dC as a positive control.

Materials and methods

Cell culture

HCT-116 cells were cultured in McCoy's 5 A media (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and 2 mM GlutaMAX (Gibco) at 37°C in 5% CO_2. Cells (passages

11–13) were seeded onto 100-mm cell culture dishes or six-well plates at a density of 3.0×10^4 cells and allowed to attach for 24 h.

Experiment I—Fifty μM bovine serum albumin (BSA)-complexed DHA or LA (Nu Chek Prep. Inc.) was used to treat cells for 72 h.[17] Cells were co-incubated with 5 mM sodium butyrate (Sigma) during the final 12 h of the fatty acid treatment period.[18] 5-Aza-dC (2 μM), a potent inhibitor of DNA methylation (Santa Cruz Biotechnology) served as a positive control, and cells were treated for 48 h. Negative control cells were incubated in media without BSA-complexed fatty acids or butyrate.

Experiment II—The goal of this experiment was to develop a more thorough time course for gene expression relative to treatment with 5-Aza-dC, fatty acids, and/or butyrate. For this purpose, all cells received the assigned treatments at time 0, and gene expression was monitored in cells harvested at 48, 72, and 96 h post-treatment initiation. The treatments included 50 μM BSA–DHA or BSA–LA, 5 mM butyrate, or 2 μM 5-Aza-dC (Figure 1).

Global DNA methylation assay

Genomic DNA was isolated from HCT-116 cells using QIAamp DNA mini kit (Qiagen). Global methylation was quantified using a MethylFlash methylated DNA quantification kit (Epigentek). Briefly, 100 ng of genomic DNA was pipetted into multiwell plates coated with a 5-methylcytosine antibody and incubated at 37°C for 90 min. DNA was subsequently washed and a capture antibody added prior to incubation at room temperature for 30 min. At the end of the incubation period, the capture antibody solution was removed and replaced with an enhancer solution followed by another 30-min incubation at room temperature. The extent of global methylation was measured by reading absorbance at 450 nm. The relative methylation status of each sample was determined using positive and negative controls provided in the kit.

Gene specific DNA methylation

DNA methylation of apoptosis-related genes was determined using a Methyl-Profiler DNA methylation PCR array (SABiosciences, MeAH-121 C-2). Genes included on the array are listed in Table 1. Briefly, 1 μM of genomic DNA from HCT-116 cells was treated with a mock enzyme, a DNA methylation sensitive enzyme, a DNA methylation-dependent enzyme, or both the DNA methylation sensitive and dependent enzymes at 37°C overnight. The relative DNA methylation status was determined using Ct values from quantitative real time PCR (qRT-PCR) as previously described.[19] Data are presented as a percentage of cellular DNA containing methylated gene promoters, which included a combination of both the intermediate level of methylation and hypermethylated DNA.

Gene expression using qRT-PCR

Total RNA was extracted from HCT-116 cells using an RNAqueous Kit (Ambion) and DNase treated. The cDNA was synthesized from 2 μg total RNA using random hexamers and oligo dT primers with SuperScript II reverse transcriptase (Invitrogen). Transcript levels were determined using Taqman gene expression assays (Applied Biosystems; *Cideb* Hs00205339_m1, *Dapk1* Hs00234489_m1, and *Tnfrsf25* Hs00980365_g1) with an ABI

Prism 7900HT PCR sequence detector. Expression levels were normalized to Eukaryotic *18S* rRNA expression (Hs99999901_s1). Linearity of each assay was assessed prior to analysis of the samples. Negative controls that were prepared during the reverse transcription reactions by eliminating the RT enzyme were analyzed for each gene.

Histone H3 and H4 acetylation

The acetylation levels of histone H3 and H4 were assessed using the EpiQuik total histone H3/H4 acetylation detection fast kit (Epigentek). Histone proteins were extracted as per kit instructions and the sample concentrations were measured using a BCA Protein Assay Kit (Pierce). Separate assays were performed to determine acetylation status for the H3 and H4 histones. These assays used 2 μg of nuclear proteins incubated with antiacetylated histone H3-specific or H4-specific antibodies. In each assay a detection antibody was added along with a color development reagent, and the absorbance was measured at 450 nm.

Statistical analysis

Data were analyzed using one-way ANOVA and Duncan's multiple range tests for global methylation, transcription level of *Dapk1* and *Tnfrsf2*, and global H3 or H4 histone acetylation. To determine if gene-specific methylation was different between each treatment group and the negative control group, the statistical analysis was carried out using a Wilcoxon rank test. Data are presented as means ± SEM, and means were considered different when the resulting *P* value was less than 0.05.

Results

Global methylation

In Experiment I, we determined the effects of the PUFA and/or butyrate treatments on the level of global DNA methylation in HCT-116 cells exposed to the PUFA for 72 h and to butyrate for 12 h prior to sample collection. Both DHA and butyrate independently decreased global methylation whereas the combination of DHA and butyrate did not produce a significant demethylating effect compared to the negative control (media only). In comparison, the positive control, 5-Aza-dC (2 μM), significantly suppressed global methylation, as expected (Figure 2).

Gene-specific promoter methylation of apoptosis-related genes

We examined DNA methylation status at promoter CpG islands of 24 apoptosis-related genes using samples generated in Experiment I (Table 2). Of the 24 apoptosis-related genes on the assay card, the extent of promoter methylation in six genes (*Bcl2*, *Bcl2l11*, *Cideb*, *Dapk1*, *Tnfrsf25*, and *Ltbr*) exceeded 60% in the negative control group. The positive control treatment, 5-Aza-dC, induced demethylation only when the CpG-rich region of a gene promoter was heavily methylated (>60%).

Treatment with 5-Aza-dC resulted in a 28% decrease in the level of promoter methylation in *Dapk1*, compared to the negative control (media only). The promoter methylation level of *Bcl2l11* was 97% inhibited by 5-Aza-dC compared with the negative control. Butyrate had demethylating effects on the *Bcl2l11* promoter, and DHA treatment significantly decreased

the levels of promoter methylation of *Cideb*, *Dapk1*, and *Tnfrsf25* compared to the negative control group. Although the combination of DHA and butyrate did not affect global methylation, it did result in gene-specific demethylation of *Bcl2l11, Cideb, Dapk1, Ltbr*, and *Tnfrsf25*.

The transcription levels of Tnfrsf25 and Dapk1

To further develop the time course of gene expression, in Experiment II HCT-116 cells were incubated with experimental agents for 48, 72, and 96 h and the transcription levels of *Cideb*, *Dapk1*, and *Tnfrsf25* were quantified by qRT-PCR. These genes were chosen because of their hyper-methylated status and response to 5-Aza-dC, DHA, and the combination of DHA and butyrate noted in Experiment I. The 5-Aza-dC treatment induced the expression of *Dapk1* and *Tnfrsf25* after 72 and 96 h incubation (Figure 3). The transcript level of *Cideb* was only detectable following 5-Aza-dC treatment, suggesting that 5-Aza-dC restored the expression of this gene (data not shown). *Dapk1* expression was elevated by more than 100 and 200% at 72 and 96 h of 5-Aza-dC treatment, respectively, as compared with the negative control cells. Butyrate dramatically up-regulated *Tnfrsf25* and *Dapk1* transcript levels in 48-h samples, but declined thereafter. Similar to the data obtained with butyrate alone, the combination of butyrate with DHA or LA resulted in a rapid elevation in transcript levels, followed by a subsequent decline in expression.

Global histone H3/H4 acetylation

The increase in transcript levels of *Tnfrsf25* and *Dapk1* by butyrate treatment led us to examine whether gene expression was being influenced by its affect on histone acetylation status. Therefore, we assessed the global histone acetylation levels of H3 and H4 in HCT-116 cells from Experiment I. Cells treated with 5-Aza-dC demonstrated no significant changes in histone acetylation state. Global H3 histone acetylation levels were elevated in butyrate treated cells compared to the negative controls, but there were no interactions between butyrate and the PUFA treatments that influenced H3 acetylation beyond that obtained with butyrate alone. The global H4 histone acetylation level increased in cells treated with LA or LA combined with butyrate (Figure 4).

Discussion

The transcriptional regulation of apoptosis-associated genes by DNA methylation is one of the mechanisms by which cancerous cells avoid apoptosis. The coordinated hypermethylation of proapoptotic gene promoters and hypomethylation of antiapoptotic gene promoters suppress normal regulation of apoptosis.[6] Studies have been conducted on DNA methylation status of apoptosis-related genes in prostate cancer,[20] glioblastoma multiforme,[21] and bladder cancer.[7] In general, the methylation status of apoptosis-related gene promoter regions correlates with tumor phenotype, suggesting that DNA methylation of apoptosis-related genes could be a biomarker for cancer diagnosis.

In this study, we investigated the promoter methylation status of 24 apoptosis-related genes in human colon cancer cells exposed to DHA, LA, butyrate, or a combination of PUFA and butyrate. Methylation of apoptosis-related genes was targeted because we previously

demonstrated that the combination of fish oil (n-3 PUFA) and pectin (butyrate) enhanced colonocyte apoptosis, which in part, was associated with the expression of genes involved in apoptosis.[13,18,22] The goal of this paper, therefore, was to determine if DHA and/or butyrate modulate the expression level of apoptosis-related genes by suppressing promoter methylation in human colon cancer cells. Hypermethylation was detected for six apoptosis-related genes, including *Bcl2, Bcl2l11, Cideb, Dapk1, Ltbr,* and *Tnfrsf25*, which are also methylated in colon cancer or other types of cancers.[8,23–26] Friedrich *et al*. reported similar results in bladder tumor samples, wherein *Dapk1* and *Tnfrsf25* were hypermethylated compared with non-malignant adjacent tissue.[7]

Kolar *et al*. demonstrated that DHA or LA independently did not increase colonocyte apoptosis in HCT-116 cells.[18] In the present study, DHA and LA treatments decreased methylation of *Cideb, Dapk1*, and *Tnfrsf25* promoters, which are all linked to a proapoptotic phenotype. Cell death-inducing DFFA-like effector b (Cideb) induces apoptosis in a caspase-dependent manner via cytochrome c release from mitochondria.[27] *Dapk1* is a member of the serine/threonine protein kinase family and a positive regulator of apoptosis.[28] TNF receptor superfamily 25 (Tnfrsf25), also known as DR3, is a membrane-bound death receptor capable of inducing apoptosis.[29] The combination of LA with butyrate did not result in significant changes in the methylation status of these three genes. However, LA decreased methylation of Bcl-2, an antiapoptotic mediator.

DHA combined with butyrate also reduced promoter methylation of five proapoptotic genes *Cideb, Dapk1, Tnfrsf25, Bcl2l11,* and *Ltbr*, but did not affect methylation of the Bcl-2 promoter. We have previously demonstrated that combination chemotherapy using DHA and butyrate most effectively enhanced colonocyte apoptosis in HCT-116 cells.[18] Induction of apoptosis by the combination of DHA and butyrate may therefore result from their effects on methylation, as well as other effects induced by this combination.[30] We demonstrate for the first time that part of the enhanced apoptosis induced by the combination of DHA and butyrate may be attributable to the demethylation of proapoptotic genes in colon cancer cells.

Butyrate alone decreased promoter methylation of *Bcl2l11* as well as global methylation. In addition, butyrate induced a fourfold increase in the transcription levels of *Tnfrsf25* and *Dapk1* at 48 h and increased their expression when combined with LA and DHA. These increases in gene expression may be attributed to an effect of butyrate on histone deacetylation [29]. It is known that histone acetylation mainly occurs at the promoter region of genes in the process of transcription whereas histone deacetylation occurs in the promoter region leading to gene silencing.[31] The promoter region of *Dapk1* and *Tnfrsf25* is proximate to sites where histone modifications occur.[32] Therefore, the increased histone acetylation by butyrate might contribute to the induction of gene expression of these genes. We have previously reported that butyrate enhances global histone H3 methylation [14] and this result is comparable to that of Kobori *et al.*, who noted that 10 mM butyrate enhanced 25% more histone H3 acetylation than a control group in human colon cancer cells.[33] However, it will be necessary to study site-specific histone acetylation to fully appreciate the influence of histone modifications on increases of *Dapk1* and *Tnfrsf25* expression. Currently, we are

globally assessing histone acetylation using chromatin immunoprecipitation sequencing to fully determine the impact of diet on histone modifications in the colonic mucosa.

Bcl2l11, also known as Bim, antagonizes Bcl-2 and promotes apoptosis.[34] In agreement with the increased apoptosis levels previously reported,[18] promoter methylation of *Bcl2l11* decreased significantly with 5-Aza-dC, butyrate, and the combination of butyrate with DHA or LA relative to the negative control group. Considering that with the exception of the positive control the demethylation of the Bcl2l11 promoter required butyrate, our findings suggest that butyrate is primarily responsible for demethylation of the Bcl2l11 promoter, and by doing so contributes to the induction of apoptosis.

With respect to the time-dependent increases in gene expression due to the 5-Aza-dC treatment, our results are comparable to those of others showing that 5-aza-cytidine, the analog of 5-Aza-dC, increased the expression level of p16 as incubation time progressed.[16] Indeed, a 72-h exposure to 2 μM 5-Aza-dC suppressed *Dapk1* promoter methylation by 28% and induced a 1.5-fold increase in *Dapk1* transcription (Figure 3). Recently, Shu *et al.* reported that curcumin resulted in a similar reduction in promoter methylation of *Neurog1* in prostate cancer cells and that this reduction led to the twofold increase of *Neurog1* mRNA expression.[35] However, the decreases in promoter methylation caused by DHA, compared to the negative control in this study were less than 50% of the decrease obtained with the positive control (5-Aza-dC, 18–27%).

The 5-Aza-dC treatment, a DNMT inhibitor, has been extensively studied and shown to inhibit DNA methylation and restores the expression level of tumor suppressor genes.[36] This compound is incorporated into DNA and acts as a substrate for DNMT. However, it adheres to DNMT and remains as a covalent protein–DNA adduct, thereby suppressing DNA methylation. Dietary compounds, such as epigallocatechin-3-gallate (green tea compound) interrupt DNMT activity by blocking the active site of the enzyme.[37] Therefore, it is possible that the demethylating effect of DHA might be exerted through mechanisms other than that employed by 5-Aza-dC. DHA is primarily incorporated into phosphatidylethanolamine (PE), which is converted to phosphatidylcholine through the addition of methyl groups from S-adenosyl methionine.[38] Recently, Kale *et al.* found that when DHA was lacking, there was less PE-containing DHA, and the resulting excess in available methyl groups could be used for DNA methylation by DNMT.[39] This finding suggests that DHA may be capable of suppressing DNA methylation by consuming methyl groups. Therefore, DHA may indirectly suppress DNMT activity whereas 5-Aza-dC directly inactivates DNMT. In our current study, DHA inhibited global methylation and promoter methylation of proapoptotic genes, yet the demethylating effect of DHA was not sufficient to induce gene expression. Previously, it has been reported that reduced promoter methylation by butyrate was not sufficient to allow re-expression of genes.[40]

In summary, we have shown that the combination of DHA and butyrate promotes apoptosis in part by suppressing promoter methylation of the proapoptotic genes *Bcl2l11*, *Cideb*, *Dapk1*, *Ltbr*, and *Tnfrsf25* in colon cancer cells. We demonstrated that DHA has global and modest gene-specific demethylating activity, which may have implications for a synergistic effect with the inhibitors of DNA methylation, such as 5-Aza-dC. Although these results

were obtained with a single human colon cancer cell line, they replicate, and thus further validate previous *in vivo* observations [14].

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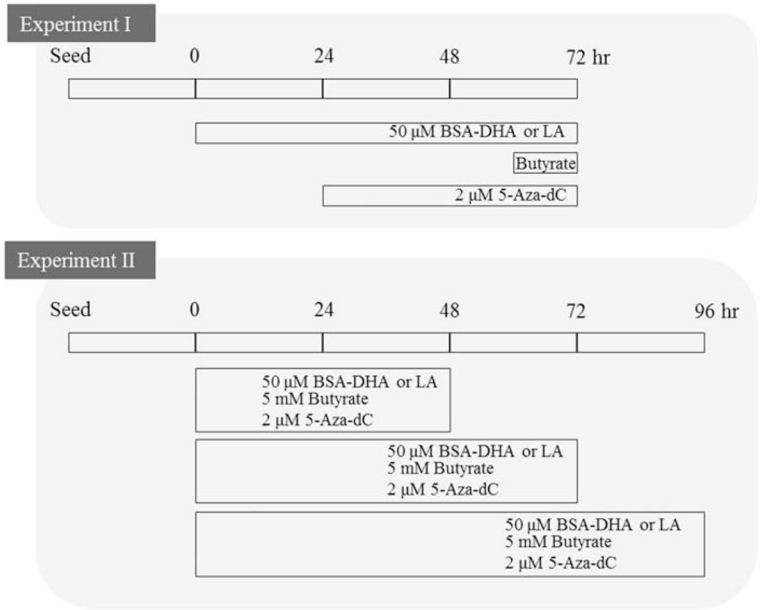

Figure 1.
Design of cell culture experiments

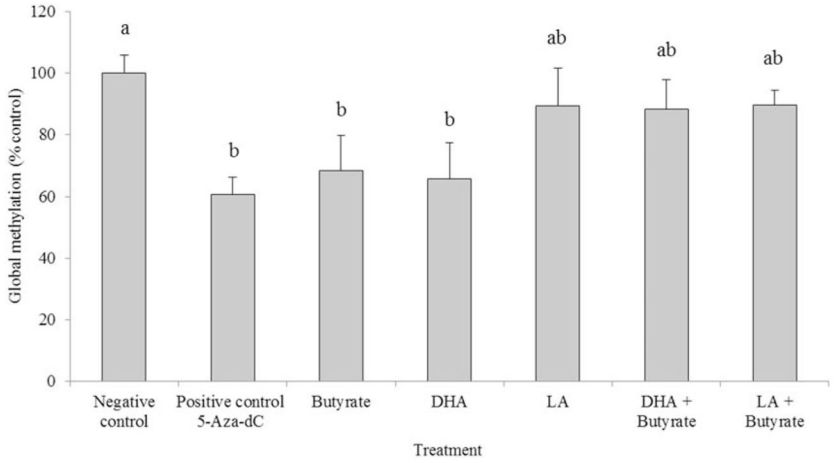

Figure 2.

Global DNA methylation status (percent of the negative control) in HCT-116 cells cultured with 5-Aza-dC (48 h), butyrate (12 h), DHA (72 h), LA (72 h), or co-treatment (see "Experiment I" section for details of Experiment I). Values are means ± SEM. Values not sharing common letters are significantly different, $P < 0.05$, $n = 6$ per treatment. Negative controls represent cells incubated in media only. DHA: docosahexaenoic acid; LA: linoleic acid

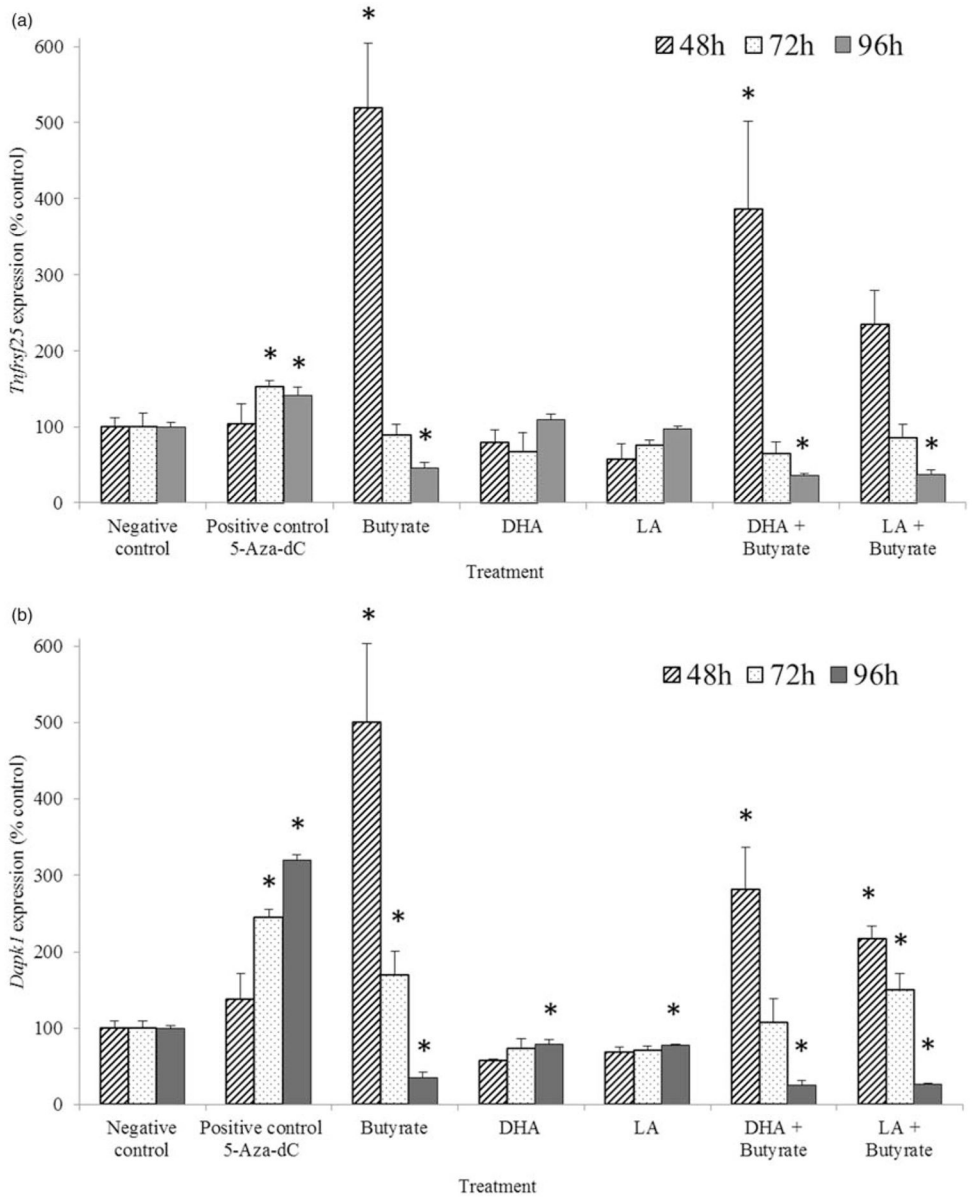

Figure 3.

Time-course analysis of *Tnfrsf25* (Panel a) and *Dapk1* (Panel b) transcript levels (expressed as percentage of the negative control) in HCT-116 cells cultured with 5-Aza-dC, butyrate, DHA, LA, or co-treatment for 48, 72, or 96 h (see "Experiment II" section for details of Experiment II). Values are means ± SEM. *Different from the negative control sample measured at the same time point, $P < 0.05$, $n = 3$ per treatment. The negative controls were cells incubated in media only

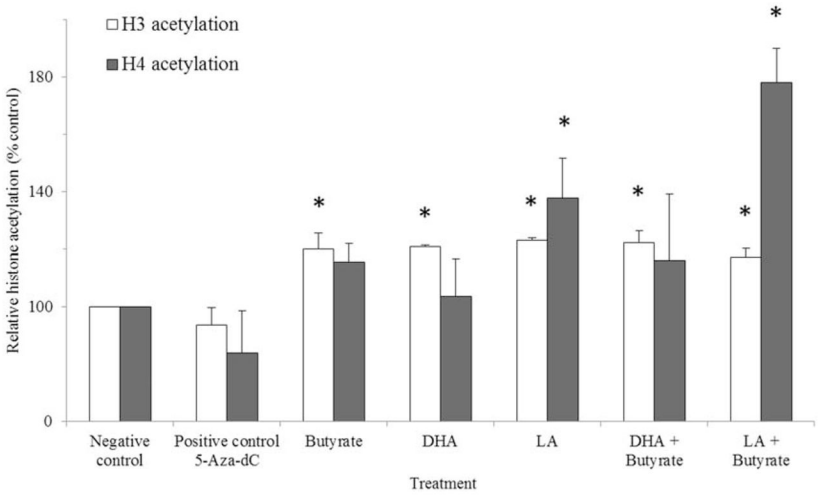

Figure 4.

H3 and H4 histone acetylation (expressed as percentage of the negative control) in HCT-116 cells incubated with 5-Aza-dC (48 h), butyrate (12 h), DHA (72 h), LA (72 h), or co-treatment (see "Experiment I" section for details of Experiment I). Values are means ± SEM. *Different from the negative control, $P < 0.05$, $n = 3$ per treatment. The negative controls were cells incubated in media only

Table 1

Gene specific DNA methylation targets included in the PCR array

RefSeq accession number	Symbol	Description
NM_009684	*Apaf1*	Apoptotic peptidase activating factor 1
NM_007522	*Bad*	BCL2-associated agonist of cell death
NM_009736	*Bag1*	Bcl2-associated athanogene 1
NM_007527	*Bax*	Bcl2-associated X protein
NM_009741	*Bcl2*	B-cell leukemia/lymphoma 2
NM_009754	*Bcl2l11*	BCL2-like 11 (apoptosis facilitator)
NM_153787	*Bclaf1*	BCL2-associated transcription factor 1
NM_007544	*Bid*	BH3 interacting domain death agonist
NM_007546	*Bik*	Bcl2-interacting killer
NM_007465	*Birc2*	Baculoviral IAP repeat-containing 2
NM_009761	*Bnip3l*	BCL2/adenovirus E1B interacting protein 3-like
NM_009810	*Casp3*	Caspase 3
NM_015733	*Casp9*	Caspase 9
NM_009894	*Cideb*	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector B
NM_009950	*Cradd*	CASP2 and RIPK1 domain containing adaptor with death domain
NM_029653	*Dapk1*	Death associated protein kinase 1
NM_010044	*Dffa*	DNA fragmentation factor, alpha subunit
NM_010175	*Fadd*	Fas (TNFRSF6)-associated via death domain
NM_007836	*Gadd45a*	Growth arrest and DNA-damage-inducible 45 alpha
NM_007545	*Hrk*	Harakiri, BCL2 interacting protein (contains only BH3 domain)
NM_010736	*Ltbr*	Lymphotoxin B receptor
NM_178589	*Tnfrsf21*	Tumor necrosis factor receptor superfamily, member 21
NM_033042	*Tnfrsf25*	Tumor necrosis factor receptor superfamily, member 25
NM_011640	*Trp53*	Transformation related protein 53

Table 2

Extent of apoptosis-related gene promoter methylation (%)[a] in HCT-116 cells cultured with 5-Aza-dC, butyrate, DHA, LA, or co-treatment

Gene symbol	Apoptosis	Negative control	Positive control 5-Aza-dC	Butyrate	DHA	LA	DHA + butyrate	LA + butyrate
Highly methylated genes								
Bcl2	Anti	98.83 ± 0.27	80.62 ± 4.02*	98.26 ± 0.36	94.55 ± 3.31	97.78 ± 0.25*	97.87 ± 0.48	98.35 ± 0.35
Bcl2l11	Pro	68.86 ± 1.74	2.10 ± 0.63*	52.58 ± 10.24*	53.79 ± 10.84	62.22 ± 2.66	52.11 ± 9.82*	60.48 ± 3.02*
Cideb	Pro	94.63 ± 1.52	77.01 ± 2.81*	88.83 ± 2.58	84.69 ± 3.27*	85.02 ± 0.94*	86.89 ± 2.00*	89.62 ± 2.37
Dapk1	Pro	96.35 ± 0.28	69.84 ± 2.90*	94.86 ± 0.67	93.45 ± 1.32*	94.88 ± 0.16*	94.10 ± 0.31*	94.88 ± 0.55
Ltbr	Pro	64.55 ± 1.29	29.39 ± 4.87*	61.64 ± 1.21	59.68 ± 2.70	59.99 ± 1.15*	57.94 ± 1.54*	61.11 ± 1.10
Tnfrsf25	Pro	97.20 ± 0.53	76.79 ± 2.30*	95.43 ± 0.90	92.80 ± 1.85*	93.26 ± 0.53*	94.07 ± 0.99*	95.41 ± 0.93
Not highly methylated genes								
Apaf1	Pro	0.76 ± 0.10	0.45 ± 0.13	0.40 ± 0.13	0.67 ± 0.23	0.53 ± 0.12	0.54 ± 0.22	0.32 ± 0.07*
Bad	Pro	1.08 ± 0.09	2.16 ± 0.89	2.25 ± 0.58	2.32 ± 0.61	2.13 ± 0.52*	1.90 ± 0.38*	1.55 ± 0.56
Bag1	Anti	6.81 ± 0.58	14.84 ± 5.43*	15.88 ± 3.66	15.89 ± 3.80	15.67 ± 1.93*	14.13 ± 2.93*	12.85 ± 4.22
Bax	Pro	0.69 ± 0.07	0.65 ± 0.11	0.58 ± 0.20	0.81 ± 0.18	0.65 ± 0.14	0.75 ± 0.26	0.41 ± 0.08*
Bclaf1	Pro	0.96 ± 0.11	1.15 ± 0.39	1.40 ± 0.34	1.68 ± 0.38	1.49 ± 0.31	1.49 ± 0.19*	1.30 ± 0.40
Bid	Pro	6.56 ± 0.24	7.52 ± 2.74	12.78 ± 2.89	11.23 ± 2.53	13.03 ± 1.20*	11.60 ± 1.39*	12.04 ± 2.4*
Bik	Pro	2.84 ± 0.20	10.67 ± 5.25*	9.84 ± 2.91	24.80 ± 9.70*	30.42 ± 9.85*	8.92 ± 2.35*	7.48 ± 3.30
Birc2	Anti	2.00 ± 0.17	3.67 ± 1.47	4.62 ± 1.06	4.92 ± 1.36	5.16 ± 1.05*	4.16 ± 0.93*	3.17 ± 1.15
Bnip3l	Pro	0.81 ± 0.10	0.69 ± 0.15	1.03 ± 0.39	1.12 ± 0.21	1.07 ± 0.11	1.03 ± 0.24	0.61 ± 0.19
Casp3	Pro	1.21 ± 0.05	2.06 ± 0.67	10.74 ± 8.13*	2.38 ± 0.61	2.31 ± 0.40*	2.00 ± 0.35*	1.71 ± 0.54
Casp9	Pro	3.58 ± 0.34	5.11 ± 0.31*	3.22 ± 0.19	3.44 ± 0.24	3.15 ± 0.26	3.74 ± 0.62	2.80 ± 0.10
Cradd	Pro	1.54 ± 0.56	1.33 ± 0.23	1.48 ± 0.32	1.59 ± 0.37	1.35 ± 0.18	1.69 ± 0.34	1.00 ± 0.20
Dffa	Pro	0.80 ± 0.05	0.65 ± 0.14	0.68 ± 0.25	0.77 ± 0.16	0.74 ± 0.19	0.79 ± 0.19	0.45 ± 0.09*
Fadd	Pro	0.50 ± 0.09	0.50 ± 0.10	0.49 ± 0.19	0.71 ± 0.18	0.67 ± 0.17	0.59 ± 0.12	0.28 ± 0.08
Gadd45a	Pro	10.85 ± 1.26	17.73 ± 6.69	27.02 ± 7.71	32.17 ± 10.33	31.76 ± 5.09*	26.42 ± 6.85	22.36 ± 7.30
Hrk	Pro	16.14 ± 1.23	32.76 ± 4.88*	32.95 ± 6.15	37.31 ± 6.85*	46.33 ± 4.10*	33.85 ± 7.14	30.83 ± 6.23*
Tnfrsf21	Pro	0.67 ± 0.08	0.58 ± 0.19	0.67 ± 0.24	0.79 ± 0.23	0.48 ± 0.10	0.62 ± 0.22	0.37 ± 0.08*

Gene symbol	Apoptosis	Negative control	Positive control 5-Aza-dC	Butyrate	DHA	LA	DHA + butyrate	LA + butyrate
Trp53	Pro	1.20 ± 0.50	1.75 ± 0.67	1.92 ± 0.47	2.11 ± 0.47	2.01 ± 0.39*	1.88 ± 0.33	1.49 ± 0.54

[a] Data are presented as a percentage of cellular DNA containing methylated gene promoters, which included a combination of both the intermediate level of methylation and hypermethylated DNA.

* Different from the negative control group (P <0.05). Data are expressed as means ± SEM from five to six samples per treatment. The negative controls represent cells incubated in media only.