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Linoleic Acid and Butyrate Synergize to Increase Bcl-2 Levels in Colonocytes

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Abstract

The biological properties of polyunsaturated fatty acid (PUFA) classes have been the source of much contention. For example, *n*-3 PUFA are chemoprotective, while *n*-6 PUFA may promote tumor development. Since dietary components can have combinatorial effects, we further examined the apoptotic properties of *n*-3 or *n*-6 fatty acids when combined with different fiber sources. Mice were fed diets supplemented with either fish oil (enriched in *n*-3 PUFA) or corn oil (enriched in *n*-6 PUFA) and non-fermentable (cellulose) or fermentable (pectin) fiber sources. In complementary experiments, immortalized young adult mouse colonic (YAMC) cells were treated with docosahexaenoic acid (DHA, 22:6*n*-3) or linoleic acid (LA, 18:2*n*-6) with or without butyrate. Mice fed a fish oil and pectin diet had significantly ($p < 0.05$) increased levels of apoptosis in colonocytes compared to all other diets. Similarly, apoptosis was highly induced in DHA and butyrate co-treated YAMC cells. In contrast, in both YAMC and mouse models, LA/corn oil with butyrate/pectin treatment reduced apoptosis and enhanced expression of bcl-2. The LA and butyrate induced anti-apoptotic phenotype was reversed by knocking down bcl-2 using targeted siRNA. In comparison, overexpression of bcl-2 blocked the pro-apoptotic effect of DHA and butyrate. These data provide new mechanistic insights into the regulation of apoptosis by dietary PUFA and fiber.

Keywords

Fatty acids; Fiber; Bcl-2; Apoptosis

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Novelty: The current study documents the combinatorial effects of dietary fiber and polyunsaturated fatty acids (PUFA) on colorectal cancer risk. We demonstrate for the first time that the combination of an *n*-6 polyunsaturated fatty acid (linoleic acid) and fermentable fiber/butyrate suppress apoptosis, in part, by increasing bcl-2 expression in colonocytes.

Impact: This study will encourage cancer researchers to examine interactions between dietary fat and fiber. Our data indicate that the consumption of *n*-6 PUFA, currently being encouraged by the Heart Healthy diet, may increase colon cancer risk by suppressing apoptosis.

Introduction

Colon cancer is among the leading causes of cancer-related deaths in the world's developed countries.¹ It is now well established that colon cancer can be influenced by numerous environmental factors, and it is estimated that up to 70% of colorectal cancers can be prevented by incorporating moderate changes in lifestyle and eating habits.² Possible mechanisms of action could result from the ability of key dietary components to influence cellular dynamics in the colonic epithelium. This is noteworthy because the balance between apoptosis and proliferation is critical to the maintenance of steady-state cell populations in the intestine. For example, if cellular homeostasis between cell growth and cell death is disrupted, clonal expansion could ensue to result in the overproduction of transformed cells.³ This is attributed in part to the fact that a clearly established link exists between transformation of colonic epithelium and the progressive inhibition of apoptosis.^{3–5} Hence, agents with the potential to restore normal apoptotic signaling will reduce colon cancer risk. Importantly, measuring apoptosis can be a more accurate prognostic tool than analysis of cell proliferation to detect dietary effects on colon tumor incidence.^{4,6}

From a dietary perspective, an increasing number of published reports indicate a protective effect of fish oil and its bioactive components, *n*-3 polyunsaturated fatty acids (PUFA), against colon cancer.^{4,7–10} Eicosapentaenoic acid (20:5 $\Delta^{5,8,11,14,17}$) and docosahexaenoic acid (DHA; 22:6 $\Delta^{4,7,10,13,16,19}$) are two typical *n*-3 PUFA found in fish oil. In contrast, dietary lipids rich in *n*-6 PUFA [found in vegetable oils; e.g., linoleic acid (LA; 18:2 $\Delta^{9,12}$) and arachidonic acid (20:4 $\Delta^{5,8,11,14}$)] have been linked to an increase in colon tumor development.^{4,11,12} Furthermore, there is evidence from observational and epidemiologic studies suggesting that elevated short chain fatty acid, e.g., butyrate, levels in the lumen of the colon derived from the consumption of fermentable fiber are chemoprotective.^{13,14} In contrast, several studies have disputed the assertion that dietary fiber and fish oil reduce colon cancer risk, fueling a debate regarding the chemopreventative role of dietary *n*-3 PUFA and fermentable fiber.^{15–18} We have addressed this conundrum by examining the combined effects of dietary long-chain fatty acids with various fiber sources. Feeding animals diets containing *n*-3 PUFA (fish oil) with pectin (which ferments to butyrate) as a fiber source promotes apoptosis in the colon compared with diets high in other dietary lipids, like *n*-6 (corn oil) and *n*-9 (olive oil).^{19–21} In a follow-up study, the administration of butyrate-containing pellets for targeted release in the colon was used to demonstrate that butyrate and fish oil work coordinately in the colon to trigger apoptosis. Subsequently, DHA and butyrate was shown to work synergistically to enhance apoptosis in colonocyte cultures compared with butyrate alone.^{22,23}

Bcl-2 is a 26-kDa protein largely localized to the mitochondrial membrane, where it has the ability to inhibit apoptosis by conserving the integrity of the mitochondrial matrix to prevent escape of pro-apoptotic molecules from the mitochondria.^{24,25} Elevated levels of bcl-2 prevent cell death after tumorigenic mutations have been incurred, and a link has been established between the overexpression of bcl-2 and many types of cancer, including colorectal cancer.²⁵ Interestingly, diet has recently been shown to modulate bcl-2 expression. Specifically, elevated levels of bcl-2 were observed in rats fed corn oil and pectin as compared to a fish oil and pectin diet.²⁶ In the same experiment, bcl-2 levels were negatively correlated to apoptosis. These results suggest that the ability of PUFA and butyrate co-treatment to modulate colonocyte apoptosis may be mediated in part by changes in bcl-2 expression. In this study, we further probed the effects of fat and fiber on bcl-2 expression and colonocyte apoptosis.

Materials and Methods

Animals

C57BL/6 wild type mice were used for this experiment. All procedures followed the guidelines approved by PHS and the Institutional Animal Care and Use Committee at Texas A&M University. Mice were maintained under barrier conditions and initially consumed a Teklad commercial mouse nonpurified diet (Harlan, Houston, TX) ad libitum. Mice enrolled into the study were maintained for 4 weeks on a semi-purified defined diet that was adequate in all nutrients,²⁷ containing 320 (g/kg diet) sucrose, 200 casein, 220 corn starch, 3 DL-methionine, 35 AIN 76 salt mix, 10 AIN 76 mineral mix, 2 choline chloride, 60 fiber (cellulose, Bio-Serv, Frenchtown, NJ or high methoxyl citrus pectin, Gum Technology, Tuscon, AZ), 150 corn oil (Dyets, Bethlehem, PA) or 115 vacuum-deodorized Menhaden fish oil (Omega Protein, Houston, TX) plus 35 corn oil. There were 15 mice in the corn oil (CO) and fish oil (FO)-fed groups. Five mice from each diet group received cellulose as a fiber source and ten mice were administered pectin. The fibers were chosen because of their very different degrees of fermentability, with pectin being highly fermentable in the colon and cellulose poorly fermented.^{28,29}

Cell culture

YAMC cells, conditionally immortalized mouse colonocytes, were originally obtained from R. H. Whitehead, Ludwig Cancer Institute (Melbourne, Australia). YAMC cells (passage 12–17) were cultured under permissive conditions, 33°C and 5% CO₂ with interferon- γ , as previously described.^{30,31} Bovine serum albumin (BSA) complexed fatty acid was added to select cultures after cell plating as previously described.³¹ Select cultures were treated with 50 μ M BSA-complexed DHA or LA (Nu Chek, Elysian, MN) for 72 h, and select cultures were co-treated with 5 mM butyrate (Thermo Fisher Scientific, Inc. Waltham, MA) for the final 24 h as previously described.²² For apoptosis assays, cells were plated onto 6-well plates. For immunoblotting, cells were plated onto 150 mm dishes. For fluorescence microscopy, cells were plated onto Lab-Tek II 2-well glass chamber slides (Nalge Nunc, Inc. Rochester, NY).

TUNEL assay

Apoptotic cells were enumerated on paraformaldehyde-fixed tissues from the distal colon using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) labeling kit (Trevigen, Gaithersburg, MD) as we have previously described.³² The number of apoptotic cells was recorded in at least 100 well-oriented crypts per mouse.

Immunoblotting

For mouse colonic tissue protein extraction, the mucosa was scraped from the underlying smooth muscle and homogenized in ice-cold homogenization buffer [50 mM Tris-HCl pH 7.2, 250 mM sucrose, 2 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 100 mM sodium orthovanadate, 1% Triton X-100, 100 μ M activated sodium orthovanadate, 40 μ L/mL protease cocktail, 10 mM β -mercaptoethanol] as previously described.³³ Samples were passed through a 27-gauge needle and then centrifuged at 16,000 *g* for 20 min, following which the supernatant was collected. Protein concentration of the extract was determined using Coomassie Plus Protein assay (Pierce, Rockford, IL). Extracts were treated with pyronin sample buffer and subjected to polyacrylamide gel electrophoresis in precast 4–20% Tris-glycine mini gels (Invitrogen, Carlsbad, CA). After electrophoresis, proteins were electroblotted onto a polyvinylidene fluoride membrane with the use of a Hoefer Mighty Small Transphor Unit (San Francisco, CA) at 400 mA for 90 min. Following transfer, the membrane was incubated in 4% nonfat dry milk and 0.1% Tween 20 in PBS at room

temperature for 1 h with shaking, followed by incubation with shaking overnight at 4°C with primary rabbit anti-mouse bcl-2 antibody (Pharmingen, San Jose, CA) diluted in PBS containing 4% milk and 0.1% Tween 20. Membranes were washed with PBS containing 0.1% Tween 20 and incubated with secondary peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry, Gaithersburg, MD) per manufacturer's instructions. Bands were developed using Pierce SuperSignal West Femto maximum sensitivity substrate. Blots were scanned with a Fluor-S Max MultiImager system (Bio-Rad, Hercules, CA). For cell culture, both adherent and floating cells were harvested using homogenization buffer. The sample was passed through a 27 gauge needle and incubated on ice for 30 min, followed by centrifugation at 16,000 g for 20 min. The protein concentration of the samples was determined by Coomassie Plus Protein assay. The total cell lysate was then treated with pyronin sample buffer and processed using the same methodology as the mouse samples. For bcl-2 overexpression blots, rabbit anti-human bcl-2 polyclonal antibody from Stressgen (Ann Arbor, MI) was used.

YAMC apoptosis assay

Apoptosis was determined using nucleosomal fragmentation enzyme linked immuno-sorbent assay (ELISA) (Roche, Mannheim, Germany). Floating cells were harvested, washed, lysed, and centrifuged to sediment nuclei. The supernatant containing mono and oligonucleosomes was incubated with substrate and subsequently analyzed by ELISA. Absorbance values were measured at 405 nm and subsequently normalized to the number of adherent cells per dish to determine apoptotic index as previously described.³¹

Quantitative PCR

Real-time PCR (ABI 7900) was used to quantify mRNA expression levels of bcl-2 as previously described.³⁴ cDNA was synthesized from 2 µg total RNA using random hexamers and oligo(dT) primers with Superscript II RT (Invitrogen). PCR was performed (primer sequences available online) using predeveloped Taqman assays (Applied Biosystems, Foster City, CA). Expression levels were normalized to ribosomal 18S expression using a predeveloped assay kit from Applied Biosystems.

siRNA transfection

Twenty-four h after the initiation of fatty acid treatment, YAMC cells were seeded into either 6-well plates (1.2×10^5 cells per well) for apoptosis analysis or 150-mm dishes (1.0×10^6 cells per dish) for immunoblotting and transfected using a Qiagen (Hilden, Germany) HiPerFect kit according to the manufacturer's instructions. Cells (control, DHA, or LA treated) were not transfected or transfected with either 1 nM nontargeting siRNA or 1 nM bcl-2 siRNA (Dharmacon, Lafayette, CO). After transfection, cells were fatty acid treated for an additional 48 h. Select cultures were co-treated with butyrate (5 mM) for the final 24 h. Subsequently, cultures were harvested for either immunoblotting or ELISA apoptosis analyses.

Bcl-2 transfection

Twenty-four h after initiation of fatty acid treatment, YAMC cells were transfected with full length human bcl-2 (from Maria Hoyer-Hansen, Danish Cancer Institute) using an Amaxa (Cologne, Germany) nucleofector device, nucleofection kit L, program A-020. Additional cultures were transfected with 2 µg of pMaxGFP as a control. After transfection, cells were seeded onto 2-chambered cover glass slides at a density of 2.0×10^5 for fluorescence measurements. For immunoblotting, cells were seeded at a density of 1.0×10^6 onto 150-mm dishes. For apoptosis assays, cells were seeded into 6-well plates at a density of $1.2 \times$

10⁵. Cultures were treated with fatty acids for an additional 48 h, and select cultures co-treated with butyrate (5 mM) for the final 24 h.

Fluorescent imaging

Overexpression of bcl-2 was measured by immunocytochemistry. After transfection, cells were plated onto 2-chambered cover glass slides. After 48 h, cells were fixed with paraformaldehyde for 20 min and incubated overnight in blocking solution (1% BSA/0.1% NaN₃ in PBS). Cells were incubated with rabbit anti-bcl-2 (20 µg/mL) for 1 h, followed by incubation with Alexa 568 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 1 h. Slides were sealed using the ProLong Antifade Kit (Molecular Probes). Fluorescence was viewed with TE 300 Nikon Eclipse fluorescence microscope. Cells were excited at 595–605 nm and fluorescence emission was collected at 620 nm. Images were taken with a Photometrics Cool snap EZ digital CCD camera.

Statistical analysis

The effect of independent variables (treatment effects) was assessed using the one-way ANOVA test in SuperANOVA and SPSS. Differences among means were evaluated using the least-significant difference test of contrast. *P* values <0.05 were considered to be statistically significant.

Results

Dietary fish oil combined with fermentable fiber induces maximal levels of colonocyte apoptosis

To examine the effects of dietary lipid and fiber combination, mice were fed diets enriched with fish oil or corn oil. Each of these lipid sources was combined with either cellulose, a nonfermentable fiber source, or pectin, a fermentable fiber source. After 4 weeks of feeding, mice were sacrificed and colonic tissue was collected. A main goal was to determine if diet has a significant effect on apoptosis, since apoptosis is often suppressed during colonic transformation.^{3–5} A TUNEL assay was performed to measure the level of apoptosis in the colonic mucosa. Dietary fish oil when combined with pectin induced significantly (*p*<0.05) higher levels of apoptosis compared to all other dietary combinations (Fig. 1a). In addition, the combination of pectin with corn oil resulted in significantly (*p*<0.05) higher apoptosis compared to cellulose and corn oil.

Treatment of DHA with butyrate enhances apoptosis in YAMC cells

We next determined whether observations made *in vivo* could be reproduced using a colonocyte cell culture model. For this purpose, YAMC cells were treated with BSA-conjugated DHA or LA with or without butyrate, while control cells were untreated. After a 72 h incubation period, a nucleosomal fragmentation assay was performed to measure apoptosis. Similar to the mouse dietary model, the combination of DHA and butyrate, which correlates to a diet of fish oil and pectin, resulted in the highest apoptotic index (Fig. 1b). Neither of the long-chain fatty acid treatments alone exhibited an increase in apoptosis compared to untreated cells. In contrast, butyrate either alone or combined with LA was able to induce apoptosis compared to control; however, this induction was not as high as that observed in DHA and butyrate treated cultures.

Dietary corn oil combined with butyrate promotes expression of bcl-2 in colonic mucosa

In order to determine whether fatty acid-induced changes were mediated by bcl-2, a critical anti-apoptotic mediator,^{24,25,35} mouse colonic mucosa were analyzed by immunoblotting to measure bcl-2 expression *in situ*. Interestingly, the highest expression of bcl-2 was observed

in mice fed diets enriched in corn oil combined with pectin (Fig. 2a). In contrast, mice exhibiting the highest level of colonic apoptosis, i.e., fish oil and pectin treatment, had significantly ($p<0.05$) lower bcl-2 expression compared to corn oil and pectin treatment.

LA with butyrate treatment promotes bcl-2 expression in YAMC cells

YAMC cells were harvested and the levels of bcl-2 quantified by immunoblotting. Similar to corn oil and pectin feeding, YAMC cells treated with LA and butyrate, displayed significantly increased levels of bcl-2 compared to all other groups (Fig. 2b). LA only treatment resulted in a 2-fold increase in bcl-2 expression over control, but LA combined with butyrate induced a 3-fold increase in expression. Furthermore, mRNA analysis using quantitative PCR revealed that LA and butyrate treatment induced bcl-2 transcription (Fig. 2c). These data indicate that the YAMC cell line mimics *in vivo* observations.

Knockdown of bcl-2 enhances apoptosis in YAMC cells treated with LA and butyrate

To further probe the contribution of bcl-2 to the effects of DHA or LA when combined with butyrate, we assessed the impact of bcl-2 knockdown on YAMC apoptosis. We hypothesized that decreasing bcl-2 expression in LA and butyrate treated cells could increase the level of apoptosis up to that exhibited by DHA and butyrate treatment. YAMC cells were transfected with bcl-2 siRNA, and cultures were harvested 48 h later. Nontargeting siRNA was used as a control to ensure that any observed changes in apoptosis were not due to off-target effects. Bcl-2 siRNA treatment resulted in a 2-fold decrease in bcl-2 expression compared to untreated control cultures (Fig. 3a). All groups transfected with bcl-2 siRNA displayed at least a 2-fold decrease in bcl-2 expression (Fig. 3b). In contrast, nontargeting siRNA did not significantly alter bcl-2 expression. LA and butyrate treated cells following siRNA treatment had a comparable level of bcl-2 expression relative to DHA and butyrate treated cells without siRNA. In subsequent experiments, apoptosis was measured 48 h post transfection. Following knockdown of bcl-2, apoptosis in LA and butyrate treated cells was increased to the levels seen in DHA and butyrate cultures (Fig. 4). The pro-apoptotic effect of DHA and butyrate combination was not influenced by bcl-2 silencing.

Overexpression of bcl-2 suppresses apoptosis in DHA and butyrate treated cultures

Since silencing of bcl-2 in LA and butyrate treated cells elevates apoptosis, we next determined the effects of bcl-2 overexpression. YAMC cells were transfected with full-length human bcl-2 and examined 48 h later. Transient overexpression subsequently increased bcl-2 levels significantly (Figs. 5a and 5b). Cells treated with either DHA and butyrate or LA and butyrate demonstrated significantly ($p<0.05$) inhibited apoptosis when transfected with bcl-2 (Fig. 6). Apoptosis was only inhibited by approximately 30% in LA and butyrate treated cells following overexpression of bcl-2. In contrast, overexpression of bcl-2 suppressed apoptosis in DHA and butyrate treated cells by 2-fold to levels observed in untransfected LA and butyrate treated cells.

Discussion

The progressive inhibition of apoptosis is closely linked to the transformation of colonic epithelium.^{3–5} For this reason, the promotion of apoptosis is an important aspect of chemoprevention. In order to develop toxicologically innocuous colon cancer chemoprevention measures, we have previously shown that fermentable fiber, which generates butyrate in the colonic lumen, when combined with *n*-3 PUFA as the lipid source can promote programmed cell death.^{12,22,23,36–38} This critical observation emphasizes the need to evaluate both the lipid and fiber composition of diets. The failure to address an interaction between fat and fiber may explain why the chemoprotective effects of dietary

fiber are obscured in prospective cohort studies.^{15,39,40} In this study, we observed a 3-fold increase in apoptosis in YAMC cells treated with DHA and butyrate compared to control (Fig. 1b). This was mirrored by a 3-fold increase in apoptosis in mice fed diets enriched in fish oil and pectin compared to a corn oil and cellulose diet (Fig. 1a). We have previously demonstrated that the combination of DHA and butyrate induces apoptosis by recruiting distinct signaling pathways. Specifically, DHA and butyrate potentiate reactive oxygen species and mitochondrial calcium to induce p53-independent apoptosis.^{22,23,37} Both of these events function to increase apoptosis via the intrinsic mitochondrial apoptotic pathway. We have also demonstrated that butyrate alone induces apoptosis through a Fas receptor-mediated extrinsic pathway.³¹ This pathway induces apoptosis in a manner largely independent of the mitochondrial signaling axis.⁴¹

In contrast to the pro-apoptotic, coordinate effects of DHA and butyrate, LA and butyrate co-treatment did not induce high levels of apoptosis *in vivo* and *in vitro* (Figs. 1a and 1b). This is consistent with dietary experiments demonstrating that highly fermentable fiber or butyrate-containing pellets induce apoptosis only when fish oil is the lipid source.^{4,12,21,36} Interestingly, we observed high colonocyte levels of bcl-2 in both mice and YAMC cells administered corn oil/LA and pectin/butyrate compared to all other treatments (Figs. 2a and 2b). Although there was not an overall correlation between changes in apoptosis and bcl-2, the levels of bcl-2 and apoptosis were inversely correlated when fish oil/DHA or corn oil/LA was combined with pectin (*in vivo*) or butyrate (*in vitro*).

Since bcl-2 is an anti-apoptotic mediator,²⁵ we hypothesized that its induction could explain why LA and butyrate treated cells exhibit decreased levels of apoptosis compared to DHA and butyrate treated cells. Increased expression of bcl-2 is potentially harmful because apoptotic pathway defects may confer cells with a survival advantage, promoting a malignant phenotype.⁴¹ Bcl-2 inhibits mitochondrial-mediated apoptosis by maintaining the integrity of the outer mitochondrial membrane, most likely by inhibiting the activity of pro-apoptotic bcl-2 family members.^{42,43} Since the extrinsic apoptotic pathway operates largely independently of mitochondria, apoptosis triggered by death receptor signaling will not be directly influenced by changes in bcl-2 expression.⁴⁴ This may explain why LA and butyrate treated cells have similar levels of apoptosis compared to butyrate-only treated cells (Fig. 1b). In this scenario, the extrinsic pathway is still being induced by butyrate, which is not highly susceptible to inhibition by increased expression of bcl-2.

Silencing of bcl-2 using siRNA did not have an effect on DHA and butyrate treated cells (Fig. 4), indicating that native bcl-2 does not inhibit DHA and butyrate induced apoptosis. However, knockdown of bcl-2 in LA and butyrate treated cultures significantly enhanced apoptosis to levels comparable to DHA and butyrate treated cells (Fig. 4). These data indicate that the distinct effects on apoptosis resulting from treatment with butyrate combined with LA versus DHA can be attributed to differences in bcl-2 expression in the two treatments. The overexpression of bcl-2 in DHA and butyrate treated cells resulted in a decrease apoptosis to the level seen in LA and butyrate treated cells (Fig. 6). Since the combination of DHA and butyrate induces apoptosis through oxidation-sensitive, mitochondrial Ca²⁺-dependent (intrinsic) pathways,^{22,23} it is noteworthy that bcl-2 possesses antioxidant function and protects cells against mitochondrial oxidative stress.⁴⁵ This activity would prevent apoptosis induced by DHA and butyrate. Furthermore, bcl-2 could potentially inhibit apoptosis by blocking the release of Ca²⁺ from the endoplasmic reticulum.⁴⁶ Further studies are needed in order to determine the bcl-2 locus of action resulting from dietary fat/fiber interaction.

Currently, it is not known how dietary fat (linoleic acid) and fermentable fiber (butyrate) modulate expression of bcl-2. Bcl-2 expression has been shown to be regulated by several

mechanisms. Its levels are affected post-transcriptionally by noncoding microRNAs (miRNA), including miR-15a, miR-15b, miR-16-1, miR-21, miR-34a, and let-7a.^{47–51} Interestingly, dietary modulation of miRNA levels in the colon has recently been demonstrated.⁵² In addition, butyrate has been shown to function as a histone deacetylase inhibitor to promote gene transcription.⁵³ This activity could potentially function in concert with linoleic acid to increase expression of bcl-2. LA has been shown to activate Akt and Erk signaling in colon cancer cells.⁵⁴ Both Akt and Erk signaling pathways can act in concert to up-regulate bcl-2 expression.^{55–58} Therefore, it is likely that LA and butyrate are acting synergistically through a number of mechanisms to increase expression of bcl-2.

With respect to the relevance of the mouse diets used in this study, the fish oil-diets contained approximately 1.4% and 1.0% of energy as EPA and DHA, respectively. As a point of reference, most Japanese consume n-3 PUFA at 1–2% of energy in their diet.⁵⁹ With regard to cell culture experiments, colonocytes were harvested following fatty acid (50 μ M) and butyrate (5 mM) co-treatment. These concentrations are considered physiologically relevant because they fall well within the ranges of human/mouse blood and intestinal luminal levels.^{60–62}

In summary, we have demonstrated that colonocyte bcl-2 expression is induced by the combination of LA and butyrate. As expected, the increase in bcl-2 was associated with a decrease in apoptosis. These findings provide new insights into the mechanism of apoptotic regulation by dietary PUFA and fermentable fiber. The data suggest significant differences in the bioactivity of butyrate when combined with different long-chain fatty acids. Since LA is a common n-6 PUFA found in the Western diet (~7% energy LA; ~14 g in a 2000 kcal/day), its promotion as component of a Heart Healthy diet may be a concern.^{63,64}

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Abbreviations

CO	corn oil
DHA	docosahexaenoic acid
ELISA	enzyme linked immune-sorbent assay
FO	fish oil
LA	linoleic acid
PUFA	polyunsaturated fatty acids
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
YAMC	young adult mouse colonocytes

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FIGURE 1.

Colonocyte apoptosis *in vivo* and *in vitro*. For *in vivo* apoptosis experiments, colonic mucosa from mice fed diets enriched in fish oil or corn oil combined with cellulose or pectin was analyzed by TUNEL assay. For *in vitro* experiments, YAMC cells treated with fatty acid, butyrate, or co-treatment were analyzed by nucleosomal fragmentation assay for cell death. (a) Apoptosis levels in colonic mucosa of mice grouped by diet, $n = 5-10$ mice per diet. (b) Apoptosis levels (% of control) in YAMC cells grouped by treatment, $n = 6$ samples per treatment. *In vitro* results are representative of at least 3 independent experiments. All data are expressed as means $\pm$ S.E. Mean values not sharing common letters are significantly different, $p < 0.05$.

FIGURE 2.

Expression of bcl-2 *in vivo* and *in vitro*. For *in vivo* experiments, bcl-2 expression in colonic mucosa from mice fed diets enriched in fish oil or corn oil combined with cellulose or pectin was analyzed by immunoblotting. For *in vitro* experiments, total cell lysate from YAMC cells treated with fatty acid, butyrate, or co-treatment was analyzed by immunoblotting for bcl-2 protein expression and qPCR to determine mRNA levels. (a) Quantification of bcl-2 levels in mouse colonic mucosa grouped by treatment. *Inset*, Representative immunoblot, n = 5–10 mice per diet. (b) Quantification of bcl-2 expression (% of control) in YAMC cells grouped by treatment. *Inset*, Representative immunoblot. (c) Quantification of bcl-2 mRNA (% of control) in YAMC cells grouped by treatment and normalized to ribosomal 18S gene expression, n = 5–8 samples per treatment. *In vitro* results are representative of at least 3

independent experiments. Data are expressed as means $\pm$ S.E. Mean values not sharing common letters are significantly different, $p < 0.05$. FC, fish oil + cellulose; CC, corn oil + cellulose; FP, fish oil + pectin; CP, corn oil + pectin; C, control (no fatty acid treatment); B, butyrate; D, docosahexaenoic acid (DHA); L, linoleic acid (LA); DB, DHA + butyrate; LB, LA + butyrate.

FIGURE 3.

Knockdown of *bcl-2* in YAMC cells using siRNA. YAMC cells were either untransfected, transfected with nontargeting siRNA (1 nM), or transfected with *bcl-2* siRNA (1 nM). Cells were cultured for 48 h after transfection and subsequently harvested. Total cell lysate was analyzed by immunoblotting for *bcl-2* expression. (a) Quantification of *bcl-2* (% of control) in untreated YAMC cells grouped by transfection condition. *Inset*, Representative immunoblot. (b) Quantification of *bcl-2* levels (% of control) in YAMC cells grouped by treatment and transfection condition. Data are expressed as means $\pm$ S.E from $n=3$ samples per treatment. Results are representative from 3 independent experiments. Mean values not sharing common letters are significantly different, $p<0.05$. C, Control; NT siRNA, transfected with nontargeting siRNA; Bcl-2 siRNA, transfected with siRNA against *bcl-2*.

FIGURE 4.

Effect of bcl-2 siRNA on apoptosis in YAMC cells. Apoptosis was measured by nucleosomal fragmentation assay for cell death 48 h following siRNA transfection. Quantification of apoptotic index of YAMC cells (% of control) grouped by treatment and transfection condition. Data are expressed as means $\pm$ S.E. from $n=6$ samples per treatment. Results are representative from 3 independent experiments. Mean values not sharing common letters are significantly different, $p<0.05$. NT siRNA, transfected with nontargeting siRNA; Bcl-2 siRNA, transfected with siRNA against bcl-2.

FIGURE 5.

Transient overexpression of full-length human bcl-2 in YAMC cells. Bcl-2 expression was measured by immunofluorescence and immunoblotting 48 h post-transfection. (a) Representative fluorescence microscopy image of bcl-2 in untreated YAMC cells mock transfected or transfected with bcl-2. (b) Quantification of bcl-2 (% of control) overexpression in YAMC cells grouped by treatment and transfection condition. *Inset*, Representative immunoblot. Data are expressed as means $\pm$ S.E. from $n = 3$ samples per treatment. Results are representative from 3 separate experiments. Bcl-2, transfected with full-length human bcl-2.

FIGURE 6.

Effect of overexpression of bcl-2 on apoptosis in YAMC cells. YAMC cells were transfected with bcl-2. Apoptosis was analyzed 48 h post-transfection by nucleosomal fragmentation assay for cell death. Quantification of apoptotic index of YAMC cells (% of control) grouped by treatment and transfection condition. Data are expressed as means $\pm$ S.E. from $n = 5-6$ samples per treatment. Results are representative from 3 separate experiments. Mean values not sharing common letters are significantly different, $p < 0.05$. Bcl-2, transfected with full-length human bcl-2.