EFFECTS OF FISH OIL AND BUTYRATE ON DIET-MEDIATED APOPTOSIS AT THE PROMOTION STAGE OF COLON CARCINOGENESIS

A Thesis

by

ANNE HENRY NEWTON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

August 2004

Major Subject: Nutrition

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Approved as to style and content by:

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ABSTRACT

Effects of Fish Oil and Butyrate on Diet-Mediated Apoptosis at the Promotion Stage of Colon Carcinogenesis. (August 2004)

Anne Henry Newton, B.S., Texas A&M University

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We have previously shown that dietary fish oil and the fiber pectin protect against colon cancer in rats by increasing apoptosis induced by reactive oxygen species (ROS) at the initiation stage of tumorigenesis. We hypothesized that fish oil would incorporate into the cardiolipin of colonic mitochondrial membranes, creating an environment in which butyrate, a fermentation product of pectin, would also increase ROS and lead to apoptosis, as evidenced by decreased mitochondrial membrane potential (MMP), enhanced caspase-3 activity and cytochrome c translocation from the mitochondria, thus protecting against colon cancer by removing DNA damaged cells at the promotion stage of carcinogenesis. Sixty rats were provided a diet containing 15% corn or fish oil for 11 wk and injected with azoxymethane (AOM) or saline at wk 3 and 4. At wk 11, colonocytes were exposed to +/- butyrate ex vivo for 30 or 60 min. ROS and MMP were measured using fluorescence microscopy, and cytochrome c concentration and caspase-3 activity were measured using ELISA assays. Cardiolipin fatty acid enrichment was measured via TLC and GC. Butyrate increased ROS ($p<0.0001$) regardless of diet or treatment group. In colonic crypts from fish oil-

consuming rats, butyrate reduced MMP ($p=0.05$). However, butyrate had no effect on MMP if the rats were consuming corn oil.

In colonocytes from rats consuming fish oil, butyrate decreased mitochondrial cytochrome c (11%; $p=0.02$) concomitant with an increase in caspase-3 activity (17%; $p=0.04$) in the distal colon. In fish oil-fed animals, the n-3 fatty acids DHA and EPA were incorporated into cardiolipin at the expense of n-6 fatty acids. Regression analysis revealed a positive relationship between DHA ($R=0.49$, $p=0.03$) and EPA ($R=0.59$, $p=0.02$) and cytosolic cytochrome c content. As the percentage of DHA and EPA in the cardiolipin increased, the level of cytochrome c in the cytosol increased. These relationships were not seen in rats consuming corn oil and suggest that these results, induced only by the combination of butyrate with fish oil, may lead to increased apoptosis at the promotion stage of colon carcinogenesis via a mitochondria-mediated mechanism.

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CHAPTER I

INTRODUCTION

Colon cancer incidence. Colon cancer is the third most commonly diagnosed type of cancer and the second leading cause of cancer death in both men and women in the United States. The American Cancer Society estimates that 150,000 people will be diagnosed with colon cancer and 57,000 will die due to the disease in 2004. A combination of genetic and environmental factors has been indicated as the primary cause of cancer and research has shown that up to one-third of all cancer deaths are related to diet (1). This is further supported by migrant studies, which suggest that lifestyle, rather than genetics, may be the more important factor in colon cancer risk. For example, the incidence of colon cancer in Japan is lower than in the United States. However, Japanese immigrants living in the U.S. have an increased risk of developing colon cancer compared to Japanese living in Japan. Furthermore, first and second generation offspring of Japanese immigrants who have adopted a Western diet have rates of colon cancer incidence equal to that of Americans from other ethnic groups (1). Epidemiological studies have shown that the level and types of dietary fat and fiber may modulate the risk of developing colon cancer (2-4).

This thesis follows the style and format of the Journal of Nutrition.

Colon cancer development. Colonic epithelial tissue in both humans and rodents is dynamic, constantly renewing itself through a cycle of proliferation and apoptosis (programmed cell death). This is normally a very tightly controlled cycle, with human colonocytes being completely replaced every four to eight days (5). However, age, hormones and dietary factors can all influence this cycle (6). Cells of the colonic epithelium are arranged in patterns called crypts, invaginations that open into the lumen. Within each crypt exists a complicated network of communicating cells, all going through the different stages of renewal, differentiation or apoptosis (7). Cell division occurs primarily in the bottom two-thirds of the crypt, where stem cells reside, as well as along the crypt axis. Cells move up the crypt toward the luminal surface, differentiating as they age, losing their ability to proliferate. Eventually the differentiated colonocytes undergo apoptosis and/or are exfoliated into the fecal stream (8,9).

Colon cancer development is the result of repeated environmental injury to the cells of the colonic epithelium and the response of cells to that injury over an extended period of time (6). Typically in colon cancer development, there is an imbalance in crypt homeostasis: a state of uncontrolled cell division, decreased differentiation and impaired apoptosis in the epithelial cells of the colon (10,11). The eventual result of this overgrowth of cells is development of colon polyps, then cancerous tumors. Therefore, it is believed that apoptosis is an important mechanism designed to remove DNA damaged cells (6,12).

Cancer development occurs in three loosely defined stages: initiation, promotion and progression, with each stage possessing a different set of morphological

characteristics. The initiation stage of colon carcinogenesis can be due to a variety of insults, one of which is oxidative DNA damage, which can result in the formation of DNA adducts. Changes in the DNA structure can lead to mutations in specific genes, including those responsible for controlling cell proliferation and apoptosis. The genetic changes that most often accompany colon cancer development are mutations in the adenomatous polyposis coli (APC) gene, K-ras, deleted in colon cancer (DCC) gene and p53, a tumor suppressor gene (13).

The promotion stage of colon carcinogenesis involves clonal expansion, in which the crypt becomes large and abnormally shaped compared to normal crypts. Groupings of these abnormal crypts are termed aberrant crypt foci (ACF). Abundant evidence exists suggesting that high multiplicity ACF, or foci that incorporate large numbers of aberrant crypts, are preneoplastic lesions of colon cancer (6,14). While the initiation and progression stages of tumorigenesis are the products of irreversible genetic changes in the cell, the promotion stage involves alterations in genetic expression, which have been shown to be reversible. This suggests that the promotion stage of tumorigenesis is an ideal target for colon cancer prevention measures (15-17).

Apoptosis. Apoptosis (programmed cell death) is a highly studied phenomenon, which plays an important role in the development of organs and later, in maintaining tissue homeostasis (18). An above normal level of apoptosis has been implicated in degenerative disorders such as Alzheimer's disease and Parkinson's disease, as well as AIDS and stroke. Alternatively, a below-normal level of apoptosis is believed to play a role in autoimmune disorders such as lupus erythematosus and in cancer (19). A decline

in apoptosis has been shown to play a prominent role in colon cancer development equal to, if not greater than, the role of unchecked proliferation (10). In a study by Hong et al. (20), colon cancer was experimentally induced in rats via injections of azoxymethane and the resulting DNA damage was measured in the colonic crypts. DNA damage was found primarily in the proliferating cells and stem cells; however, apoptosis also was seen primarily in the proliferating and stem cells. Rich et al. (21) also report that the injury threshold necessary to induce apoptosis is lower in stem cells than other cells, suggesting that apoptosis is an extremely important protective mechanism that removes DNA-damaged stem cells, thereby preventing proliferation of mutated cells.

Apoptosis is an organized, ATP-dependent process, usually initiated by physiologic factors such as DNA damage, which follows a specific order of events. Apoptosis is characterized by non-inflammatory cell shrinkage, membrane blebbing, chromatin condensation, packaging of cellular debris into apoptotic bodies, and elimination via phagocytosis (22). It has been shown in cell lines that major cellular injury (that which renders the cell unable to produce ATP) usually results in necrotic death, while less severe damage leads to apoptosis. This is because in the initial stages of apoptosis, membrane integrity is maintained in a subset of the cell's mitochondria, and the electron transport chain and ATP production remain intact (23-25).

Apoptosis is not a new discovery, but the exact regulators of the pathway are still unclear. It is apparent that more than one pathway does exist. Apoptosis can be extrinsic, mediated via death receptors such as Fas/CD95, or intrinsic, activated through the mitochondria (26,27). The method by which the mitochondria execute apoptosis is

multifaceted and its steps are interrelated. It is believed that mitochondria-mediated apoptosis may be the result of electron transport loss and uncoupling of ATP production, release of proteins that activate caspases, the proteases involved in apoptotic breakdown of the cell, and changes in the cell's redox potential (28). The mitochondria can also play a distinct role in death receptor-mediated apoptosis, acting as the point of amplification of the death signal. Once the death signaling pathway reaches the mitochondria, extrinsic apoptosis may follow the same route as intrinsic apoptosis (29).

Caspase cascade. One early step in mitochondria-mediated apoptosis is an increase in permeability of the outer and inner mitochondrial membranes (30). This increased permeability leads to a release of proapoptotic proteins from the mitochondrial intermembrane space into the cytosol of the cell. These proteins include apoptosis inducing factor (AIF), which translocates to the nucleus and condenses chromatin (31), cytochrome c, a key electron-transfer protein in oxidative respiration, and Smac/DIABLO, a pro-death protein that binds inhibitor-of-apoptosis (IAP) proteins. Upon release into the cytosol, cytochrome c binds with apoptotic protease activating factor-1 (Apaf-1). This ATP-dependent oligomerization forms what is known as the "apoptosome." The apoptosome is responsible for the recruitment of procaspase-9, which is then converted to the active caspase-9 (32,33).

Formation of the apoptosome/caspase-9 complex induces a cascade of caspase activation (33). The caspases are a family of cysteine proteases, which cleave proteins after aspartic acid residues. The caspases have been dubbed the "central executioners" of the apoptotic pathway and fall into two categories: initiator caspases (caspase-2, -8,

and –9) and effector caspases (caspase-3, -6, and –7). Caspases are synthesized as zymogens, known as pro-caspases. The pro-caspases are activated by proteolytic cleavage of the zymogen by either a pro-apoptotic protein or by autocatalytic activation. Once one caspase is activated, a "caspase cascade" ensues, culminating in the activation of the effector caspases. These effector caspases are responsible for much of the DNA cleavage and cellular degradation seen in apoptosis (29,32). The linear path from cytochrome c to Apaf-1 to caspase-9 to caspase-3 has been confirmed in gene knock out experiments (29).

Cytochrome c release. The release of cytochrome c from the mitochondria is regulated by the bcl-2 family of proteins. Bid and Bax are two examples of the pro-apoptotic bcl-2 proteins that have been shown to induce release of cytochrome c from the mitochondria. The exact method by which this occurs is unproven, but one school of thought is that the bcl-2 proteins form a protein channel in the outer mitochondrial membrane that facilitates the release of cytochrome c into the cytosol of the cell (32,34). Another hypothesis states that the bcl-2 proteins interact with the permeability transition pore complex, which spans the inner and outer mitochondrial membranes and contains a voltage-dependent anion channel (VDAC) (33,35,36). In either case, the bcl-2 proteins play a role in the increasing mitochondrial membrane permeability seen in apoptosis. The bcl-2 protein family contains both pro and anti-apoptotic members. Therefore, many of the pathways that signal cell death also signal cell survival. It is suggested that the fate of the cell is influenced by the state of the cell at that moment (22). Diet may play a role in modulating the state of the cell, thus influencing cell death or survival.

Mitochondrial membrane potential. Mitochondria act as stress sensors in the cell, as noted by reactive oxygen species (ROS)-induced increases in mitochondrial membrane permeability (37). Increased permeability of the outer mitochondrial membrane leads to a breakdown of the inner mitochondrial membrane and thus, a decrease in mitochondrial membrane potential (MMP). MMP is the electrochemical gradient maintained by the pumping of electrons across the inner mitochondrial membrane. Loss of MMP is an early event in the apoptotic cascade, occurring almost simultaneously with the release of cytochrome c (31). This property makes MMP an excellent marker for oxidative stress, both in vitro and in vivo (38). It is still unclear whether cytochrome c release or loss of MMP comes first, and it is possible that the order of events depends on the apoptotic pathway that is triggered (30).

MMP is coupled with the oxidative phosphorylation pathway and production of ATP. When MMP drops during apoptosis, the mitochondrial electron transport chain is uncoupled from ATP synthesis. A loss of MMP represents the point of no return for cells destined for apoptosis (37). Apoptosis is ATP-dependent, thus it makes sense that by uncoupling ATP synthesis, apoptosis could not continue and necrosis, which follows many of the same death pathways, but is not dependent on ATP, would take over (24). However, depolarization and release of cytochrome c does not happen to all of the mitochondria in a cell at one time. This heterogeneity of the mitochondria within a given cell may allow for production of enough ATP to complete the apoptotic breakdown of the cell (23).

Reactive oxygen species. Apoptosis usually occurs in terminally differentiated cells, but can also occur when cells experience an environmental insult, usually a minor cellular injury that does not disrupt the cell membrane (12,25). Such injury can occur in the form of oxidative DNA damage from reactive oxygen species. ROS include molecules such as superoxide, hydrogen peroxide and the hydroxyl radical and are produced endogenously in the mitochondria and during immune response. In the past, ROS and the resulting oxidative DNA damage have been considered to be deleterious. Indeed, ROS can promote cancer by interfering with signaling cascades, which may cause proliferation of DNA damaged cells (39). However, ROS have also been shown to play a necessary role as second messengers in cell signaling (36) and as inducers of apoptosis, suggesting that an increase in ROS may actually provide protection against DNA adduct formation (12,40,41). Other laboratories have found that quenching of ROS via antioxidant exposure or caspase inhibitors slows down the process of both necrosis and apoptosis (42). A clinical trial involving smokers given large doses of beta-carotene, a well-known antioxidant, had to be cut short due to an increase in lung cancer incidence (43). This study emphasizes the possibility that ROS serve a protective role under conditions in which cells may be transformed, or precancerous.

Whether ROS cause proliferation, growth arrest or cell death is dependent upon the cell type affected and the dose and duration of oxidative insult. It is believed that ROS act by affecting the activity of enzymes and transcription factors leading to altered gene expression. For example, in response to oxidative stress, the p53 tumor suppressor

gene attempts to induce apoptosis by generating more ROS. This suggests that a threshold level of ROS is necessary to carry out apoptosis (40).

Cardiolipin. The antitumor and antiproliferative effects of ROS may be due to lipid peroxidation, as lipid peroxidation may be part of the signaling pathway for ROS-induced apoptosis (44,45). Findings suggest that execution of programmed cell death is dependent upon an environment permissive to apoptosis, and this environment may be modulated by diet, through incorporation of fatty acids into the inner mitochondrial membrane (5,46). Cardiolipin (CL) is a structural phospholipid of the inner mitochondrial membrane and is highly susceptible to diet-mediated changes (47). CL is comprised mainly of unsaturated fatty acids and, as such, is at increased risk for oxidative damage (35,48). CL is directly associated with cytochrome c and is necessary for cytochrome c interaction with the inner mitochondrial membrane. The fact that mitochondrial proteins associate directly with CL and that CL is susceptible to oxidative damage due to its unsaturated fatty acid makeup and its close proximity to the electron transport chain, a primary source of ROS, suggests that CL plays a specific role in mitochondrial functions such as apoptosis (47).

Oxidative damage to CL via ROS may result in a loss of the CL-cytochrome c interaction, dissociation of cytochrome c from the inner mitochondrial membrane and release of cytochrome c into the cytosol, possibly via a voltage-dependent anion channel (VDAC) and/or the mitochondrial transition pore (35). Changes in the fatty acid composition of CL affect phospholipid packing and interaction with proteins in the bilayer. Watkins et al. (47) showed in vitro that docosahexanoic acid and

eicosapentanoic acid (DHA and EPA), the long chain polyunsaturated fatty acids (PUFA) found in fish oil, are preferentially incorporated into the CL. Incorporation of DHA and EPA into CL may increase the likelihood of lipid peroxidation signaling the release of cytochrome c, a decrease in mitochondrial membrane potential and eventually apoptotic death (46,49). Chapkin et al. (50) found that in vivo incorporation of DHA and EPA into the mitochondrial phospholipids caused decreases in mitochondrial membrane potential and increases in caspase-3 activation.

Dietary fiber and butyrate. The role of dietary fiber in colon cancer prevention has been studied in depth, with unclear results. Animal model studies showed that wheat bran, a poorly fermentable fiber, prevented ACF and subsequent tumor formation in experimentally induced colon cancer (51-53). Fermentable fibers, such as pectin, have also been shown to have chemoprotective effects (46). These fermentable fibers bypass absorption in the small intestine and are fermented in the colon by colonic microbes. The microbial fermentation of fiber results in the production of water, carbon dioxide, hydrogen, methane and short-chain fatty acids (SCFA). Ninety percent of the SCFAs produced in the colon are composed of acetate, propionate and butyrate, with butyrate providing up to 25% of the total SCFAs (54).

Butyrate is preferentially used by colonocytes as an energy source and many in vitro studies have examined the effects of butyrate on cell proliferation, differentiation and apoptosis (54). Hague et al. (55) found butyrate to be an inducer of apoptosis, specifically in transformed cells. Numerous other cell line studies found butyrate to be antiproliferative and proapoptotic (9,56-61). The method by which butyrate triggers

apoptosis has yet to be elucidated. Litvak et al. (58) propose that butyrate induces the cell cycle inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ and the proapoptotic genes Bax, Bak and Bik. An in vitro study by Ruemmele et al. (60) suggests that butyrate induces cell death via a mitochondria-mediated pathway, and Chapkin et al. (9) assert that butyrate acts through induction of a Fas death receptor pathway. One study involving two colon cancer cell lines suggests that butyrate-induced apoptosis is activated through different pathways in varying types of colon cancers (62).

It has been shown both in vitro and in vivo that uptake of butyrate by colonocytes results in enhanced mitochondrial function and increased ROS production (46,61). A study involving dimethylhydrazine-treated rats showed that both consumption of the fiber pectin and intrarectal instillation of butyrate resulted in reduced levels of antiapoptotic Bcl-2 proteins compared to rats fed a standard diet (63). It appears that the protective role of fiber depends on the type of fiber (fermentable or non-fermentable) and the type of fat present in the diet, as well as the stage of tumorigenesis of the colonocytes (64).

Dietary fish oil and n-3 PUFA. The relationship between dietary fat and colon cancer has been extensively studied. Epidemiological work has shown that fish oil consumption is inversely related to breast, prostate and colorectal cancer in some populations (3,4). It is believed that the long-chain n-3 polyunsaturated fatty acids (PUFAs) found in fish oil, docosahexanoic acid (DHA) and eicosapentanoic acid (EPA), are the primary effectors of the chemoprotective effect seen with fish consumption. DHA and EPA have been shown to reduce cancer incidence in animal feeding trials

(11,46,65-69) and have chemoprotective effects in human colorectal cancer studies (70-74). Fish oil is believed to produce this effect by increasing apoptosis in cancerous cells (11).

In vitro studies offer some insight into the anti-tumorigenic mechanisms of fish oil. Chen and Istfan (75) found that DHA induced apoptosis in HT-29 colon cancer cells via a lipid peroxidation-mediated apoptosis signaling pathway, suggesting that DHA may increase cell sensitivity to ROS. A study comparing mechanisms of apoptosis induced by n-3 versus n-6 fatty acids in HL-60 cells demonstrated that n-3 PUFAs stimulated generation of ROS, resulted in decreased MMP and the release of cytochrome c and activation of caspase-3. These effects were not seen in cells exposed to n-6 PUFAs (76). Hong et al. (46) showed that rats consuming fish oil (and fatty acid ethyl esters of DHA and EPA) incorporated the n-3 PUFAs into the mitochondrial membrane, resulting in increased ROS, decreased MMP, cytochrome c translocation to the cytosol and caspase-3 activation.

Hypothesis and specific aims. Based on the literature, we hypothesized that the observed enhancement of apoptosis with fish oil supplementation is due to alterations in mitochondrial phospholipid composition, which create a permissive environment for butyrate-induced apoptosis. Our specific aims were as follows:

- Determine, in an ex vivo system, if fish oil alters mitochondrial phospholipid composition, thus creating a permissive environment for butyrate-induced apoptosis.
- Using a 2x2x2 factorial design (corn oil or fish oil; AOM or saline; +/- butyrate), during the stage of promotion, determine the effect on known indicators of mitochondrial function, reactive oxygen species, cardiolipin fatty acid composition and butyrate-induced apoptosis.

CHAPTER II

MATERIALS AND METHODS

Experimental design. Animal work was completed in accordance with an animal use protocol approved by the University Laboratory Animal Care Committee of Texas A&M University (conforms to NIH guidelines). Sixty male weanling Sprague Dawley rats (Harlan Sprague Dawley, Houston, TX) were divided into four groups of 15 rats each in a 2x2 experimental design. Animals were fed defined diets containing cellulose as the fiber source and either corn oil or fish oil as the lipid source for a total of 11 wk. Three weeks after beginning experimental diets, rats were injected with either the colon-specific carcinogen azoxymethane (AOM; Sigma Chemical, St. Louis, MO) at 15 mg/kg rat body wt or an equivalent volume of saline. A second injection followed 1 wk later. Seven weeks after the second injection, animals were terminated **(Figure 1)**.

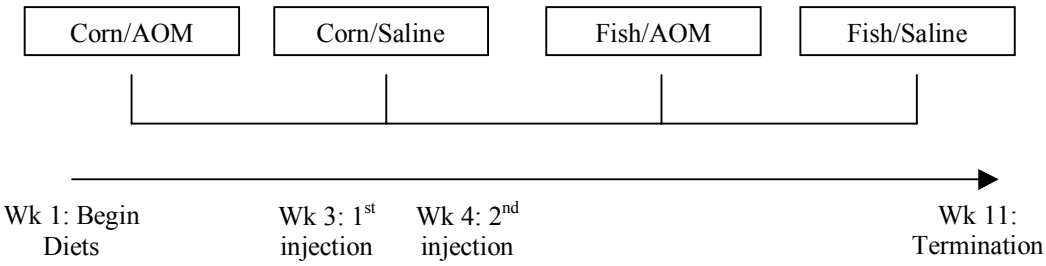

Figure 1 Experimental timeline.

Animals were housed separately in wire bottom cages in a temperature and humidity controlled facility with a light/dark cycle of 12 h and were provided free access to food and water. Animal weights and 48-h food intakes were measured 1 wk prior to the first injection, 1 wk after the second injection and 3 d prior to termination.

Experimental diets. Both defined diets contained cellulose (6 g/100 g) as a fiber source, and either corn oil, rich in n-6 fatty acids, or fish oil, rich in n-3 fatty acids, as a lipid source. The lipid was provided at 15 g/100 g of the diet by weight. The fish oil diet contained 3.5 g of corn oil/100 g diet to provide essential fatty acids. Antioxidants were added to each of the oils in order to ensure equivalent levels in both diets **(Table 1).** Rats were provided with fresh food in clean bowls daily.

TABLE 1

Composition of experimental diets

Ingredient	**g/100 g**
Dextrose	51.06
Casein	22.35
Methionine	0.34
Salt mix, AIN-76	3.91
Vitamin mix, AIN-76	1.12
Choline bitartrate	0.22
Cellulose	6.00
Corn oil[1]	15.00
or Fish oil/ Corn oil[1,2]	11.50/3.50

[1]TBHQ, [2]α, γ tocopherols- antioxidants added to oils

Crypt isolation. Seven weeks after the second injection, the rats were terminated by CO_2 asphyxiation and cervical dislocation. Colons were removed and after removal of the rectum, 1 cm was taken from both the proximal and distal ends, placed in cassettes and fixed in 4% paraformaldehyde for histology. The remaining colon was divided into proximal and distal portions **(Figure 2).**

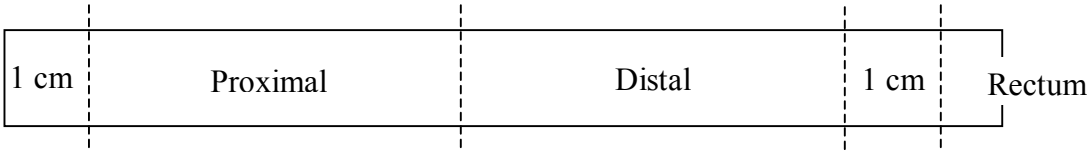

FIGURE 2 Division of the colon for analysis of intact crypts.

Colonic crypts were isolated as previously described (46,77). The tissues were opened and rinsed with warm PBS to remove feces, then placed in separate beakers with 40 ml of buffer (containing HBSS without calcium and magnesium, EDTA, DTT, Glutamine and BSA, pH 7.4) for 15 min with shaking to loosen crypts. Crypts were then separated from the colonic mucosa by scraping with a rubber policeman. Dislodged intact crypts were then transferred to conical tubes and centrifuged at 300 x g for 3 min to form a pellet. The supernatant was discarded and the pellet washed with 40 ml of buffer (containing HBSS plus calcium and magnesium, Glutamine and BSA, pH 7.4). The wash was performed once more and aliquots of the crypt solution were taken for use in subsequent assays. Crypt solutions were kept at a constant temperature of 37°C and transported to the Image Analysis Lab for ROS and MMP measurement.

Measurement of ROS and MMP. As previously described (46,77,78), proximal and distal crypt solutions were incubated with or without 5 mMol/L butyrate for 30 min. For each crypt solution, one aliquot was treated with butyrate, while two aliquots were treated only with buffer, serving as controls. One control sample was analyzed prior to the butyrate treated sample, and the other after the butyrate-treated sample. This served to establish a basal ROS level and assure that this level did not change over time. CMH_2DCFDA dye (Molecular Probes, Eugene, OR) was added to the samples and incubated for 15 min. This dye enters cells via passive diffusion where it is acted upon by intracellular esterases and thiols, which trap it inside the cell. When reactive oxygen species are present, the trapped dye is oxidized, causing it to fluoresce. The fluorescence intensity was then measured using an Ultima Confocal Microscope. Ten to 15 images of each sample were captured to generate an average intensity reading. Higher fluorescence intensity, represented by white coloring, indicates that there is a greater amount of oxidized CMH_2DCFDA dye and thus a greater amount of reactive oxygen species present in the sample **(Figure 3a).**

Rhodamine 123 (Molecular Probes, Eugene, OR) was used to determine mitochondrial membrane potential. The dye stains the mitochondria in a potential-dependent manner. The dye enters the mitochondria, and if the membrane potential is low, depolarization of the membrane occurs and the dye leaks out, resulting in lower cellular fluorescence. If the membrane is intact, the dye remains inside, resulting in a more intense fluorescent signal **(Figure 3b)**.

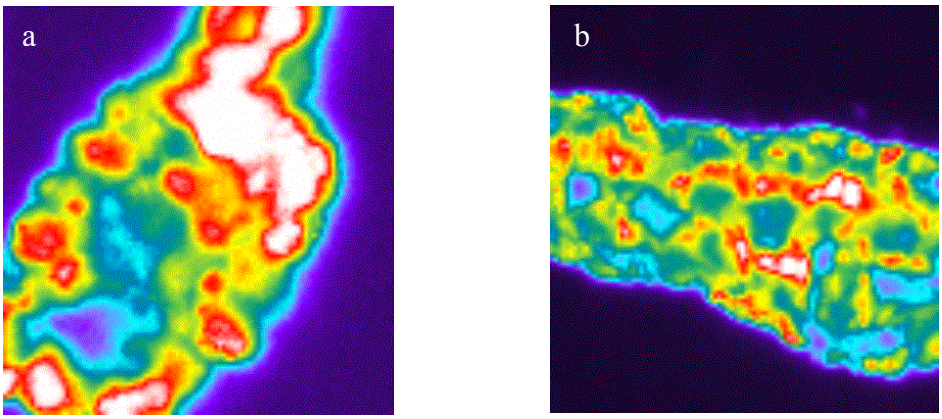

FIGURE 3 Measurement of ROS (a) and MMP (b) via fluorescence microscopy.

After the ROS and MMP fluorescence was measured, Ethidium Homodimer (Molecular Probes, Eugene, OR) was added to each sample to assess crypt viability using a Scanalytics microscope with an ultraviolet lamp. Ethidium Homodimer is impermeable to intact membranes, and thus only enters dead cells, making them appear red when viewed with a UV light.

Measurement of caspase-3. Caspase-3 activity was measured as described previously (46). Isolated, intact colonic crypts were incubated with or without 5 mMol/L butyrate. Aliquots of 0.25 ml were taken at 30, 60 and 120 min during the incubation period for caspase-3 analysis. Samples were centrifuged at 200 x g for 5 min, and pellets washed with PBS twice to remove butyrate before storing at -80°C prior to analysis. For the assay, the cells were lysed in 1X lysis buffer plus 0.1% Triton X [25% Enzcheck Caspase-3 Assay Kit Lysis Buffer (Molecular Probes, Eugene, OR) and 75% CyQuant lysis buffer (Molecular Probes, Eugene, OR)]. The suspension was passed through a 27-gauge needle and allowed to incubate on ice for 30 min. The lysed solution

was then microfuged at 2700 x g to remove cellular debris. The supernatant was placed in a clean tube and an equal amount of lysis buffer was added. The diluted supernatant was transferred to a 96-well microplate and incubated for 30 min at room temperature with Z-DEVD-R110 substrate solution (Enzcheck Caspase-3 Assay Kit, Molecular Probes, Eugene, OR). Caspase-3 has substrate specificity for the amino acid sequence Asp-Glu-Val-Asp (DEVD), which is incorporated into a bisamide derivative of Rhodamine 110 (R110), used as the substrate solution in this assay. Enzymatic cleavage of the bisamide substrate produces fluorescent R110 through two steps. The fluorescence produced over time is indicative of enzyme activity. Fluorescence was measured at A496/A520 using a Wallac Victor 1420 multilabel counter (Perkin Elmer, Boston, MA) every 30 min for 4 hr, until the readings for the samples fell within the linear portion of the standard curve. The activity (slope) was calculated and divided by the total protein content to determine the activity per μg protein. The protein concentration of the samples was determined using the microassay procedure of the Coomassie Plus protein assay (Pierce, Rockford, IL).

Isolation of mitochondria. Isolated crypts were incubated with or without 0.005 mol/L sodium butyrate for 60 min. Mitochondria were isolated as previously described (46). The following steps were all performed on ice or at 4°C. Crypts were washed with homogenization buffer containing 0.33 mol/L sucrose, 0.001 mol/L EDTA, 0.005 mol/L Hepes, and 0.003 mol/L DTT plus protease inhibitor, pH 7.4. The solution was homogenized using a Potter-Elvehjam homogenizer and then centrifuged at 600 x g for 10 min. The supernatant was saved and the nuclei pellet discarded. The supernatant was

centrifuged at 15,000 x g for 10 min, resulting in separation of the cytosol and mitochondrial fractions. The mitochondrial pellet was suspended in RIPA buffer containing 0.05 mol/L tris-HCl, 0.5% deoxycholate, 1% NP-40, 0.1% SDS, 0.005 mol/L sodium fluoride and protease inhibitor, passed through a 29-gauge needle and allowed to incubate on ice for 30 min. The solution was then centrifuged at 12,000 x g for 10 min to remove undissolved material. The cytosolic and mitochondrial fractions were aliquoted and stored at -20°C until analysis.

Cytochrome c translocation. Total protein concentrations of the cytosol and mitochondrial fractions were measured using the Coomassie Plus protein assay and the BCA protein assay (Pierce, Rockford, IL). Cytochrome c translocation was determined by the Quantikine M Rat/Mouse Cytochrome c kit (R&D Systems, Minneapolis, MN). This assay utilizes a "sandwich ELISA" method involving two antibodies. The 96-well microplate is coated with a monoclonal antibody specific for murine cytochrome c. Samples, standards and controls were added to the wells along with a second monoclonal antibody specific for cytochrome c, causing cytochrome c to be "sandwiched" between the two antibodies. Any unbound material was removed from the plate by washing with a solution of buffered surfactant, and a substrate solution containing stabilized hydrogen peroxide and stabilized chromogen was added to the wells. The second antibody is enzyme-linked to horseradish peroxidase, which reacts with the substrate solution, producing a blue color. Upon addition of the stop solution (diluted HCl), the product turns yellow. Based on sample content of DTT, a substance that interferes with this assay, samples were diluted to concentrations of 3% with

calibrator diluent (contained in kit). Fifty microliters of each sample, standard or control were added to the ELISA plate in triplicate, along with 75 µl of Cytochrome c Conjugate (the second antibody, included in the kit). The plate was incubated at room temperature for 2 h, followed by 5 washes with 400 µl of Wash Buffer (included in kit). Substrate Solution (in kit) was added to each well, the plate covered and incubated for 30 min at room temperature protected from light. Stop Solution (100 µl, included in kit) was added to each well and the optical density was read at 450 nm (correction wavelength 540 nm) on a spectrophotometer (Bio-Tek Synergy HT).

Phospholipid enrichment. Phospholipid enrichment was measured by the method of Chapkin et al. (50). Isolated crypts were incubated with or without butyrate and mitochondria were isolated and stored as described above. The lipid was extracted from the mitochondrial pellet and resuspended in MeOH/$CHCl_3$. The mass of the total phospholipids in the sample was determined using the Phosphorus Assay. Each sample of lipid extract (80 µl) was placed in a glass tube. To each sample tube and 3 blank tubes, 30 µl of 10% $Mg(NO_3)_2$ in MeOH was added and dried under N_2. Each tube was then held over the flame of a Bunsen burner for approximately 30 s. The tubes were cooled at room temperature and 400 µl of 1 M HCl was added. To generate the standard curve, 400 µl of 0, 0.24, 0.48, 0.72 and 0.96 µg of P dissolved in 1 M HCl were added to glass tubes. The samples, blanks and standards were heated in a water bath for 15 min at 95°C. The tubes were then incubated with 2 ml of a solution containing ammonium molybdate, 4 M HCl, malachite green and Tween 20 for 1 ½ hr or until a green color

developed. Each sample, blank and standard was added to a 96–well microplate (300 µl) and absorption was measured at 635 nm using the Bio-Tek Synergy HT.

Individual phospholipids (cardiolipin, phosphatidylethanolamine, and phosphatidylcholine) were separated using thin layer chromatography. Samples were spotted onto a silica gel TLC plate and placed in a glass tank containing a solvent of $CHCl_3$, MeOH, HoAc and H_2O. The plate was allowed to run until the solvent reached the top. The plate was sprayed with a solution of 8-anilino-1-napthalene-sulfonic acid and viewed with a black light. The separated bands of phospholipid were scraped into individual tubes containing 3 ml of 6% HCl/MeOH and incubated for 15 h at 76°C.

The fatty acid content of each cardiolipin sample was determined using gas chromatography. Total phospholipid mass was determined by the method of Duck-Chong (79). Fatty acid enrichment (Mol%) of cardiolipin samples was then calculated.

Statistical analysis. Food intake and body weight gain data were collected and analyzed using two-way ANOVA. ROS, MMP, caspase-3, cytochrome c and phospholipid data were analyzed using a split plot design and a mixed model procedure (PROC MIXED) in SAS. Regression and correlation analyses were performed using PROC GLM and PROC CORR in SAS. Differences were considered significant at $P<0.05$.

CHAPTER III

RESULTS

Food intake and weight gain. Food intake and weight gain values of experimental animals are shown in **Table 2**. No significant differences in weight gain or food intake were noted among the four diet/treatment groups.

TABLE 2

Animal weight gain and food intake[1]

Diet/Treatment	Weight Gain (g)	Pre-injection Food Intake (g)	Post-injection Food Intake (g)	Pre-termination Food Intake (g)
Corn Oil/AOM	335.33 ± 6.67	40.55 ± 1.51	46.29 ± 1.40	42.62 ± 1.85
Corn Oil/Saline	344.42 ± 7.63	37.83 ± 1.05	43.28 ± 1.71	43.50 ± 1.88
Fish Oil/AOM	338.96 ± 9.60	38.92 ± 1.10	43.54 ± 1.53	46.65 ± 2.41
Fish Oil/Saline	340.25 ± 5.09	38.57 ± 0.75	47.24 ± 1.69	44.38 ± 1.20

[1]Food intakes were collected at three time points over 48-hr periods. Weight gain is the difference between initial and final weights of the animals over the 12-wk experiment period. Values represent means ± SEM (n=60).

Reactive oxygen species. Butyrate caused a significant increase in reactive oxygen species in crypts isolated from the distal colon (compared to control, non-butyrate incubation), regardless of diet or carcinogen treatment (**Figure 4**). In the proximal colon, butyrate increased ROS compared to control only in animals fed fish oil (**Figure 5**).

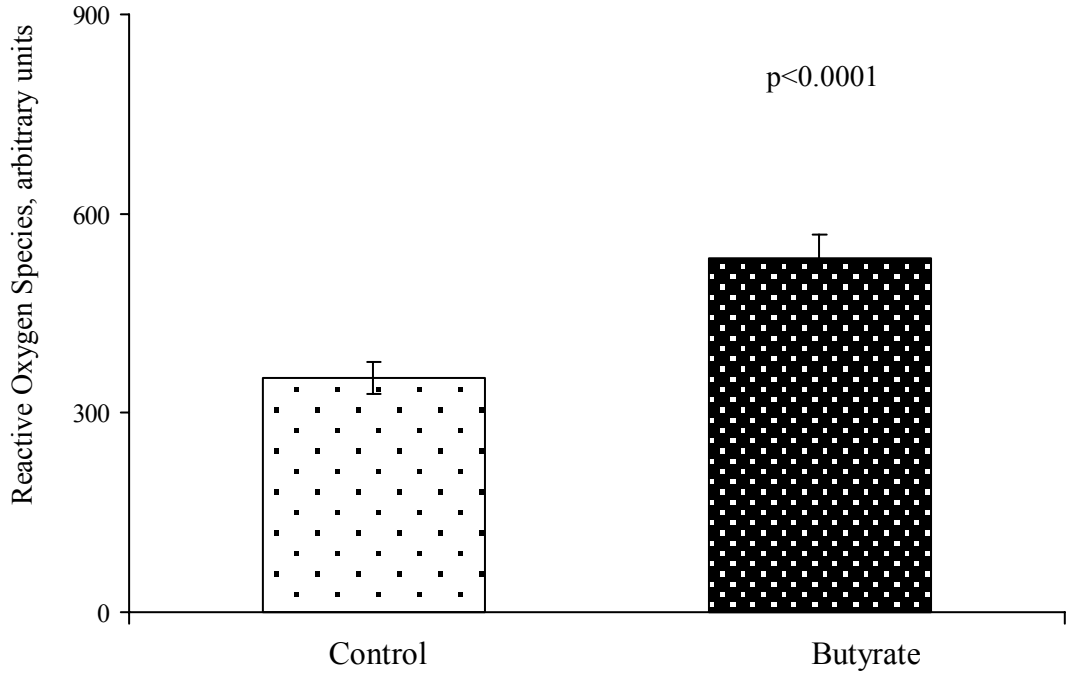

FIGURE 4 Effect of butyrate on ROS in the distal colon. ROS values are assigned arbitrary units. Values represent mean ROS ± SEM (n=60). See **Table B1** for actual values.

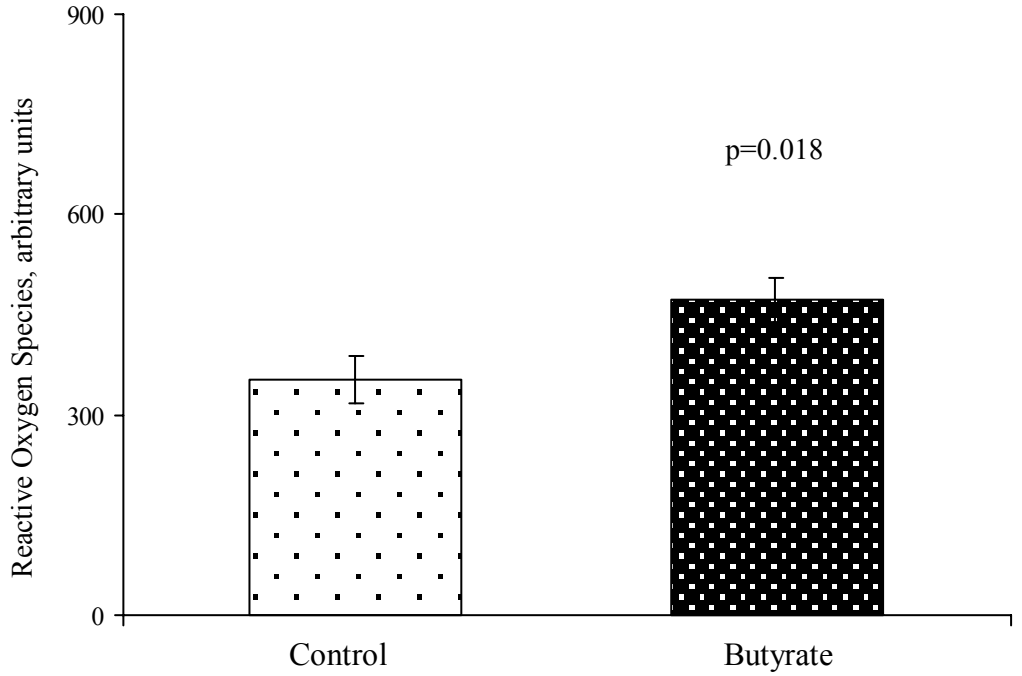

FIGURE 5 Effect of butyrate on ROS in the proximal colon of fish oil-fed animals. ROS values are assigned arbitrary units. Values represent ROS means ± SEM (n=60). See **Table B1** for actual values.

Mitochondrial membrane potential. Butyrate incubation caused an overall decrease in MMP compared to control in crypts isolated from the distal and proximal regions of the colon (**Figure 6**). However, in the distal colon, this butyrate-induced decrease in MMP did not occur in colonocytes from rats injected with AOM (**Figure 7**). Post hoc tests revealed that in the distal colons of rats injected with AOM, butyrate exposure did elicit a significant decrease in MMP in the subgroup that consumed the fish oil diet, but not the corn oil diet (**Figure 8**). However, in the proximal colon, butyrate caused a decrease in MMP compared to control in corn oil, but not fish oil-fed animals.

Also, in the proximal colon, there was no difference in butyrate-induced decreases in MMP between saline and AOM treated rats (**Table B2**).

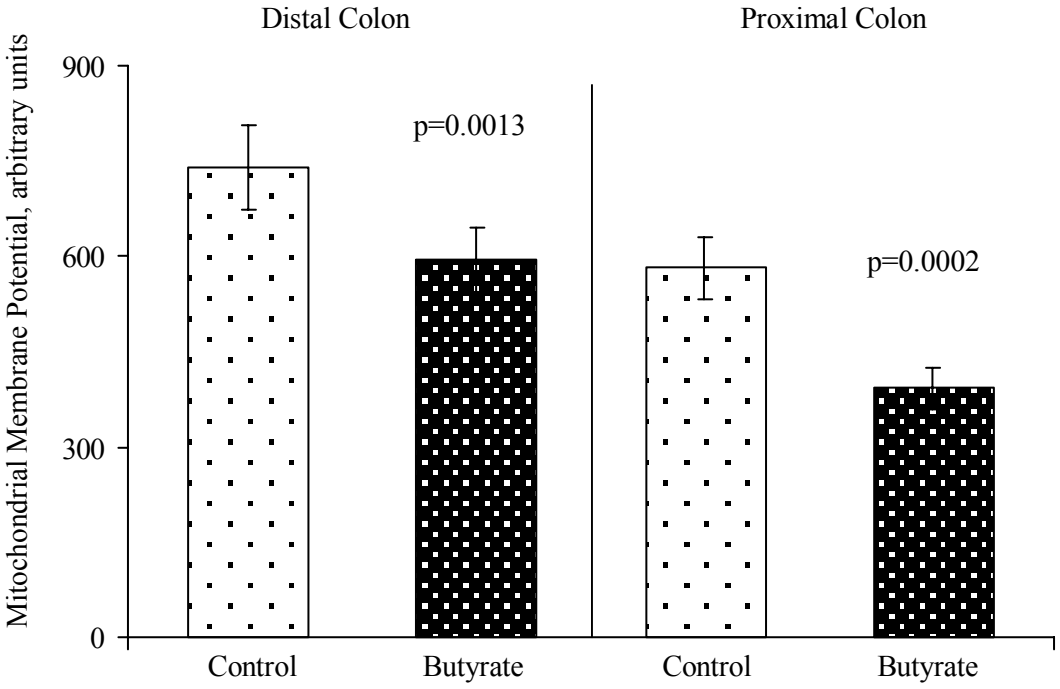

FIGURE 6 Effect of butyrate incubation on MMP. MMP values are assigned arbitrary units. Values represent MMP means ± SEM (n=60). See **Table B2** for actual values.

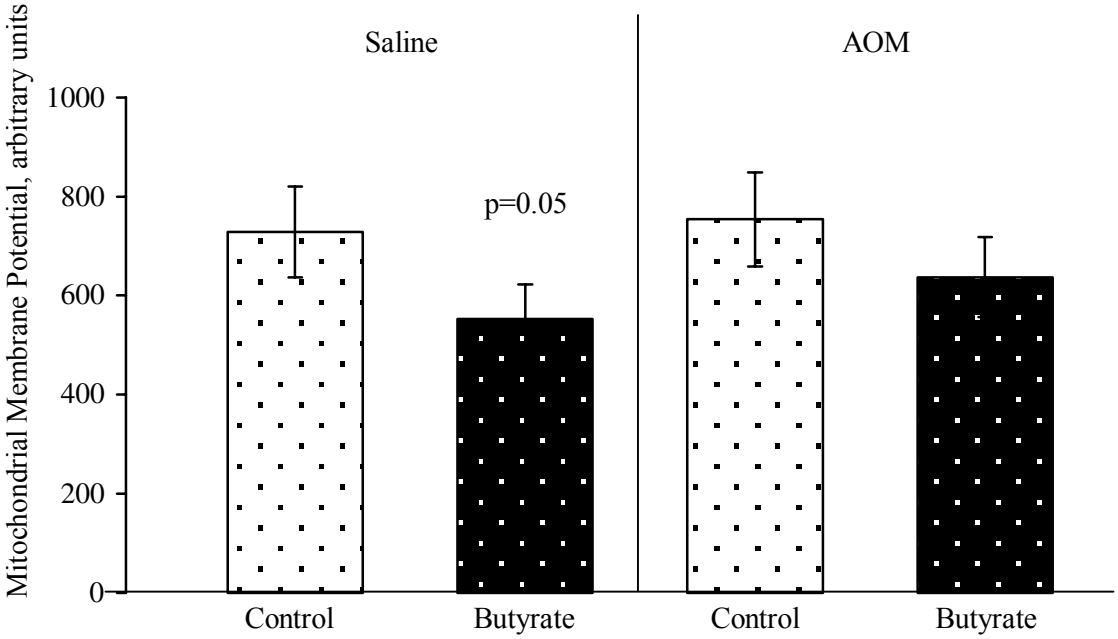

FIGURE 7 Butyrate decreased MMP in rats injected with saline, but not AOM (distal colon). MMP values are assigned arbitrary units. Values represent MMP means ± SEM (n=60). See **Table B2** for actual values.

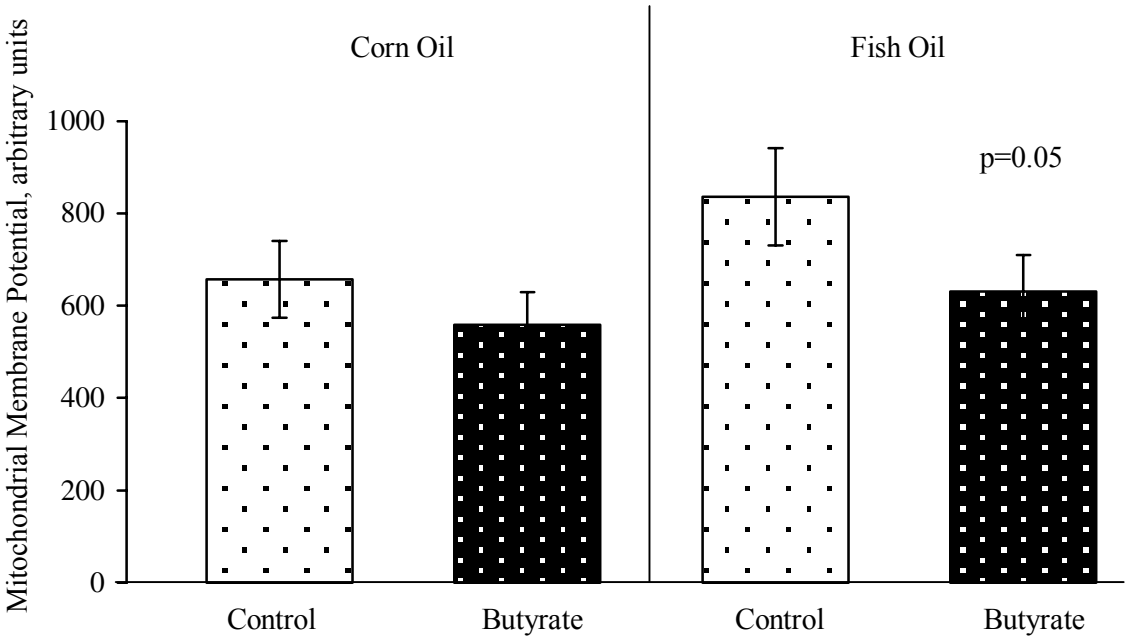

FIGURE 8 Butyrate decreased MMP in AOM-injected rats fed fish oil, but not corn oil (distal colon). MMP values are assigned arbitrary units. Values represent MMP means ± SEM (n=60). See **Table B2** for actual values.

Cell viability. Cell survival was crucial to the analysis of ROS and MMP. Therefore, cell viability was measured via incubation with Ethidium Homodimer. Six samples were evaluated, recorded and the averages determined. Viability did not differ among groups and remained above 90% throughout the data collection process. The average viability for each diet/treatment group is listed in **Table 3**.

TABLE 3
Viability of cells during ROS/MMP analysis[1]

Diet/Treatment	Cell Viability (%) Distal Colon	Cell Viability (%) Proximal Colon
Corn Oil/AOM	91.8 ± 1.9	90.93 ± 0.67
Corn Oil/Saline	93.2 ± 0.5	91.89 ± 0.63
Fish Oil/AOM	91.5 ± 0.8	91.02 ± 0.80
Fish Oil/Saline	92.5 ± 0.6	89.80 ± 1.42

[1]Viability was evaluated on each sample after the incubation period. Values represent the percentage of live cells per sample and are presented as means ± SEM (n=60).

Cardiolipin enrichment. Cardiolipin FA enrichment was determined via thin layer chromatography (TLC) and gas chromatography (GC). There was significantly greater incorporation of the n-3 polyunsaturated fatty acids DHA and EPA into the cardiolipin of the mitochondrial membrane in animals that consumed fish oil compared to corn oil. There was also a corresponding reduction in n-6 fatty acids (20:2 and 20:3) in the cardiolipin of fish oil fed rats compared to corn oil fed rats (**Table 4**).

TABLE 4
Fatty acid enrichment of cardiolipin[1]

	Distal Colon		Proximal Colon	
FA	Corn Oil	Fish Oil	Corn Oil	Fish Oil
16:0	22.13 ± 5.41	19.48 ± 5.30	19.12 ± 5.41	21.02 ± 5.30
16:1 n-7	**2.31 ± 0.46[a]**	**5.68 ± 0.48[b]**	**2.21 ± 0.46[a]**	**5.12 ± 0.45[b]**
18:1 n-9	9.02 ± 0.88	11.12 ± 0.86	8.32 ± 0.88	12.71 ± 0.86
18:1 n-7	**5.36 ± 1.33[a]**	**11.02 ± 1.3[b]**	**7.18 ± 1.3**	**9.16 ± 1.3**
18:2 n-6	31.53 ± 4.08	27.38 ± 4.00	37.34 ± 4.08	29.35 ± 4.00
18:3 n-6	3.61 ± 0.33	1.68 ± 0.33	1.73 ± 0.33	1.82 ± 0.33
20:2 n-6	**5.6 ± 0.53[a]**	**0.72 ± 0.52[b]**	**6.36 ± 0.53[a]**	**0.62 ± 0.52[b]**
20:3 n-6	**4.74 ± 0.60[a]**	**2.42 ± 0.59[b]**	**1.86 ± 0.60[a]**	**1.13 ± 0.59[b]**
20:4 n-6	3.29 ± 0.57	2.33 ± 0.56	3.07 ± 0.57	2.31 ± 0.56
20:5 n-3	**Trace[a]**	**3.46±0.96[b]**	**Trace[a]**	**2.48 ± 0.47[b]**
20:3 n-3	1.45 ± 0.31	0.96 ± 0.30	0.83 ± 0.31	0.93 ± 0.30
22:6 n-3	**Trace[a]**	**0.92 ± 0.25[b]**	**Trace[a]**	**3.22 ± 0.65[b]**

[1]Fatty acids values are presented as mean Mol % ± SEM (n=20). Only major FAs are reported. Row values without the same superscript differ ($p<0.05$). Trace <0.1%.

Cytochrome c translocation. To determine if the colonocytes were undergoing apoptosis, cytochrome c translocation and caspase-3 activity were measured by ELISA assay. Butyrate incubation caused an 11.6% decrease ($p=0.01$) in mitochondrial cytochrome c content in the distal colon and a 14.9% decrease ($p=0.02$) in the proximal colon in animals fed fish oil (**Figure 9**). Butyrate did not elicit a significant change in cytochrome c in corn oil fed animals.

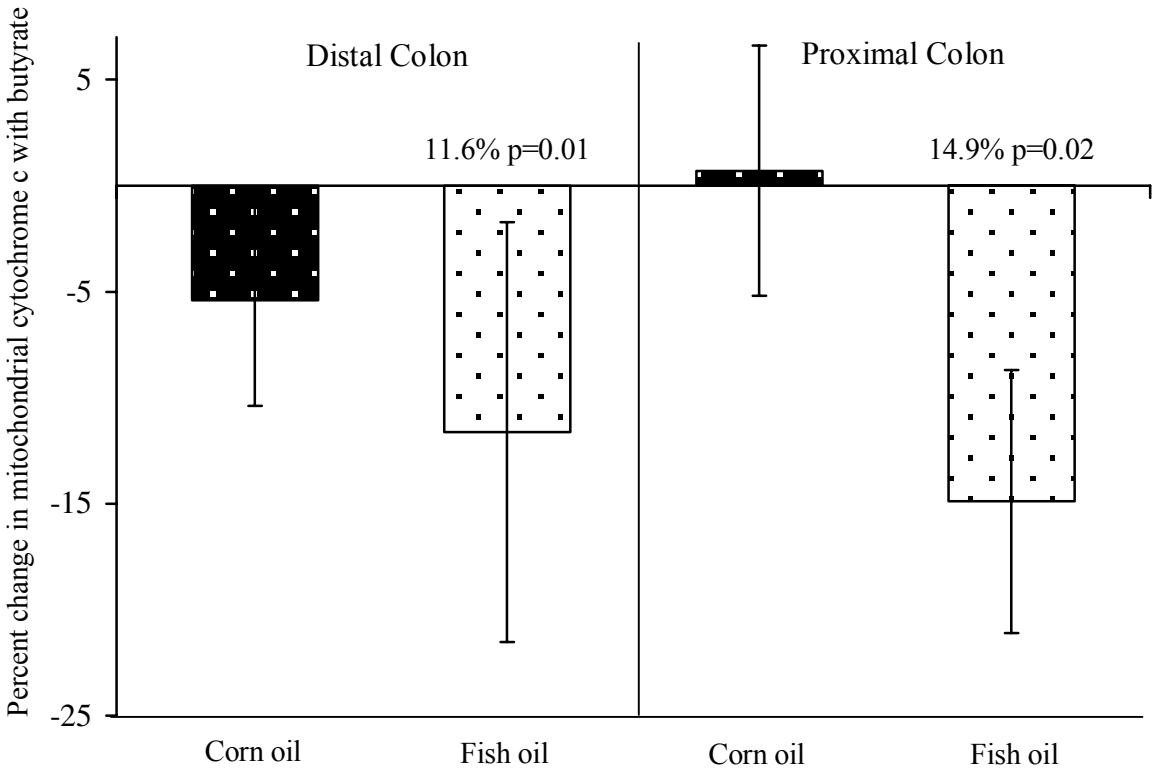

FIGURE 9 Effect of butyrate incubation on mitochondrial cytochrome c content. Values represent the % change in mitochondrial cytochrome c content with butyrate incubation ± SEM compared to control (n=60). See **Table B3** for actual values.

Caspase-3 activity. Caspase-3 activity was measured at 30 and 60 min of butyrate incubation. At the 30-min time point, butyrate induced a 17.4% increase (p=0.04) in caspase-3 activity in colonocytes from crypts in the distal colon of animals fed fish oil. No significant increase in caspase-3 activity was seen in the distal colons of animals fed corn oil. In the proximal colon, butyrate induced a 21.9% increase (p=0.05) in caspase-3 activity in animals fed fish oil and a 39.1% increase (p=0.002) in animals fed corn oil (**Figure 10**). No significant changes in caspase-3 activity were seen at the 60-min time point.

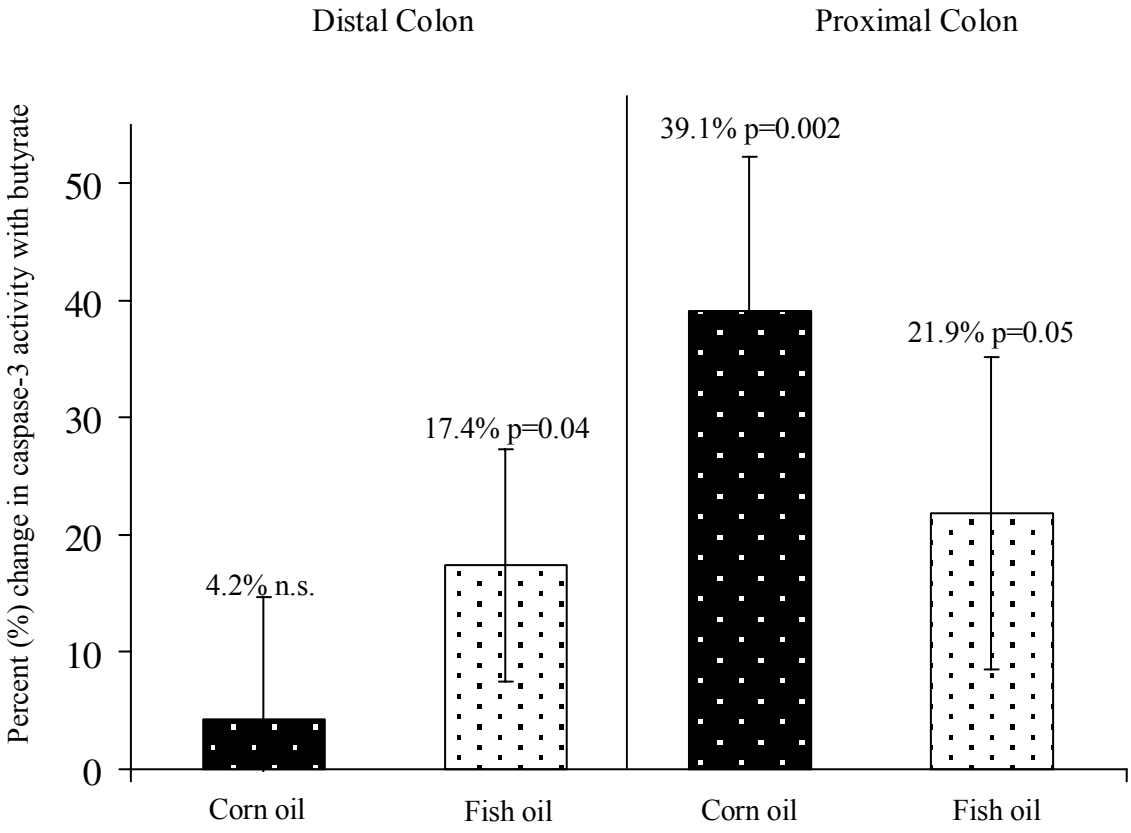

FIGURE 10 Effect of butyrate incubation on caspase-3 activity. Values represent the percent change in caspase-3 activity with 30 min butyrate incubation ± SEM compared to control (n=60). See **Table B4** for actual values.

Regression data. Regression analysis revealed a significant positive relationship between the molar percentage of DHA and EPA in the cardiolipin and cytosolic cytochrome c content (with butyrate incubation) in the distal colon (**Figure 11**). As the molar percentage of DHA and EPA in the cardiolipin increased, the amount of cytochrome c found in the cytosol also increased. Regression analysis further revealed a significant positive relationship between the molar percentage of 18:2 n-6 in the cardiolipin and MMP in the distal colon (**Figure 12**). As the amount of 18:2 n-6 in the

cardiolipin increased, the MMP (with butyrate incubation) increased. No significant relationships were found in the proximal colon between fatty acid enrichment of the cardiolipin and cytochrome c or MMP.

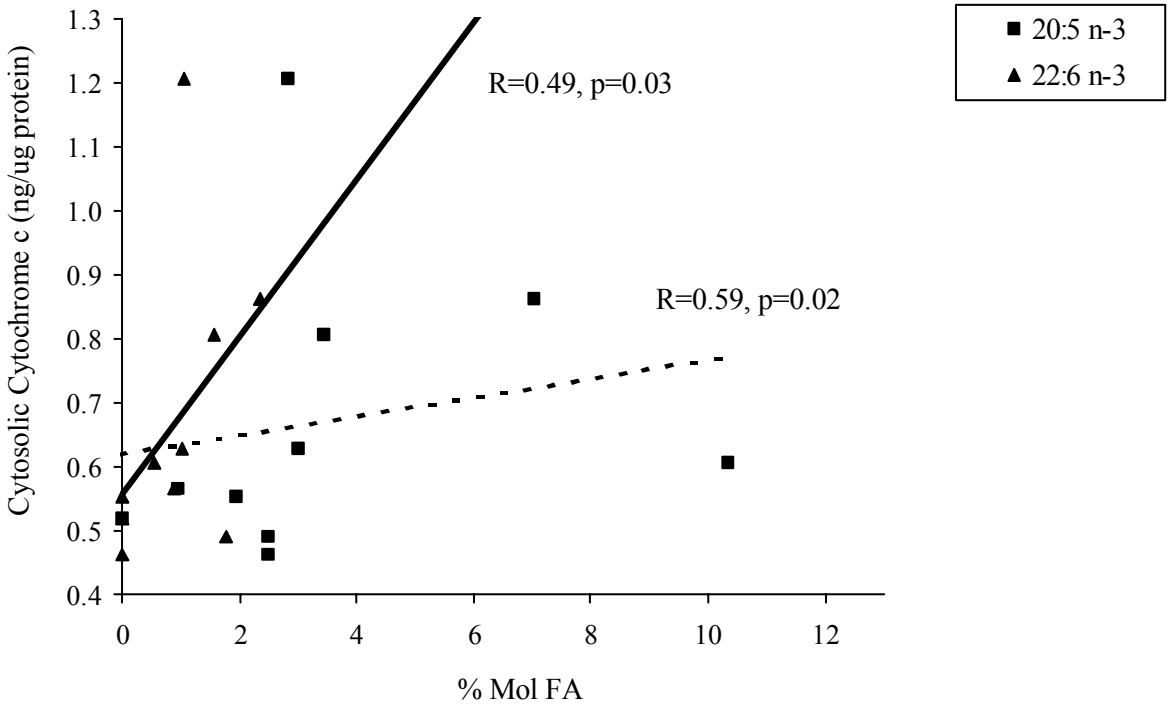

FIGURE 11 The relationship between Mol% DHA and EPA in cardiolipin and cytosolic cytochrome c content. Values represent the relationship between %mol 20:5 n-3 and 22:6 n-3 and the amount of cytosolic cytochrome c in the distal colon with butyrate incubation (n=10).

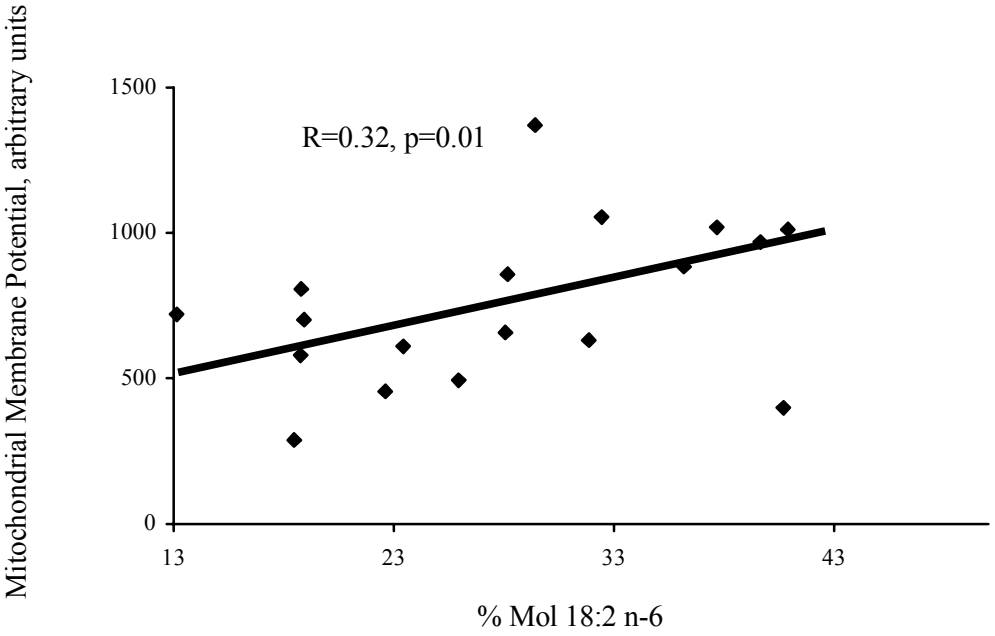

FIGURE 12 The relationship between Mol% 18:2 n-6 in cardiolipin and cytosolic cytochrome c content. Values represent the relationship between %mol 18:2 n-6 and MMP with butyrate incubation in the distal colon (n=20). MMP values are reported in arbitrary units.

CHAPTER IV

DISCUSSION

The literature suggests that a decrease in apoptosis may be an important change that occurs in transformed cells, allowing the growth of cancerous tumors (10). Earlier work has shown that the combination of dietary fish oil and the fiber pectin protects against colon cancer by increasing apoptosis (11,50). This study provides one possible mechanism by which the combination of fish oil and pectin may increase apoptosis, and thus protect against colon cancer by removing DNA damaged cells at the promotion stage of carcinogenesis.

Cardiolipin enrichment. Using thin layer chromatography (TLC) and gas chromatography (GC), we determined that consumption of fish oil leads to increased incorporation of the n-3 fatty acids DHA and EPA into cardiolipin, a phospholipid of the mitochondrial membrane at the expense of n-6 fatty acids. This finding is supported by the literature, which states that PUFAs are preferentially incorporated into the cardiolipin (47). Enrichment with DHA and EPA, long chain PUFA, has been shown to affect the structure of cardiolipin, resulting in a mitochondrial membrane that is more susceptible to damage from reactive oxygen species (35,47,49). Changes in the fatty acid composition of CL affect interaction with cytochrome c. Incorporation of DHA and EPA into CL may increase the likelihood of lipid peroxidation signaling the release of cytochrome c, a decrease in mitochondrial membrane potential and eventually apoptotic death (46,49). Hong et al. (46) found that fish oil consumption increases the unsaturation index of the cardiolipin, making the membrane more susceptible to damage

from ROS. This vulnerability is perpetuated by the location of cardiolipin in the mitochondrial membrane in close proximity to the electron transport chain, a primary source of ROS production (47). This susceptibility to ROS may increase the likelihood of ROS-induced apoptosis, resulting in the removal of DNA damaged cells.

Reactive oxygen species. This study demonstrated that exposure of the distal colon to butyrate caused an increase in ROS in colonic crypts, regardless of the diet or carcinogen treatment the animal received. Butyrate is the preferred energy substrate for colonocytes and exposure to butyrate may increase oxidative phosphorylation, thus increasing production of ROS (40,55,56). This ROS may then react with the cardiolipin of the mitochondrial membrane, causing a loss of the cardiolipin-cytochrome c association via lipid oxidation. Butyrate is a product of microbial fermentation of fermentable fibers, such as pectin (54). This finding may explain the previously found increase in ROS in colonocytes from animals fed pectin and may also be one mechanism by which the fiber pectin protects against colon cancer (10,11). In the proximal colon, butyrate caused an increase in ROS only in colonocytes from animals that consumed the fish oil diet. This finding may further support the protective synergy of fish oil and pectin (11).

Mitochondrial membrane potential. A decrease in mitochondrial membrane potential is an initiating event in apoptosis, occurring simultaneously with the release of cytochrome c (35). Therefore, MMP was measured as a biomarker of apoptosis. Analysis of MMP revealed a butyrate-induced decrease in MMP overall. However, in the distal colon, butyrate could not induce a significant decrease in MMP in colonocytes

from animals that were injected with the carcinogen AOM. A decrease in MMP is an early step in apoptosis, so the inability of butyrate to decrease MMP may indicate greater inhibition of apoptosis in colonocytes of carcinogen-injected animals. Transformed cells often lose the ability to undergo apoptosis, causing over growth of cells and the eventual development of tumors (19,80). When post hoc analyses were performed, it was noted that butyrate was able to induce a decrease in MMP in colonocytes from carcinogen-injected animals only if they received the fish oil diets. This effect was not seen in animals that consumed the corn oil diets. Furthermore, in the distal colon we found that as the molar percentage of the n-6 fatty acid 18:2 (found abundantly in corn oil) in the cardiolipin increased, mitochondrial membrane potential also increased ($R=0.32$, $p=0.01$), suggesting that colonocytes from corn oil fed animals may be resistant to apoptosis. This finding is supported by in vitro studies, which showed that n-6 fatty acids, like those prominent in corn oil, actually increase proliferation in cancer cell lines (81). In the proximal colon, butyrate was able to induce a significant decrease in MMP in animals fed corn oil but not fish oil, a result that directly opposes findings in the distal colon. Earlier work has shown the proximal and distal regions of the colon react quite differently to environmental stimuli (82). These results suggest that diet may not be as important an influence on apoptosis in the proximal colon as in the distal colon. This may be explained by epidemiological studies, which have shown that the risk of developing tumors in the proximal colon is not mediated by diet (83).

Cytochrome c. As previously stated, the structure of the mitochondrial membrane is such that there exists an intimate relationship between cardiolipin and the mitochondrial protein cytochrome c (46,49) and it is believed that loss of this association may be one stimulus for the release of cytochrome c. (35). As a second biomarker of apoptosis, changes in cytochrome c content in both the mitochondria and cytosol were measured via ELISA. During apoptosis, cytochrome c is released from the mitochondria into the cytosol, where it eventually initiates caspase-3 activity, (32,33). In this study, butyrate incubation caused an 11.6% decrease ($p=0.01$) in mitochondrial cytochrome c content in the distal colon and a 14.9% decrease ($p=0.02$) in the proximal colon of rats fed fish oil, suggesting that cytochrome c is being released from the mitochondria, as is seen during apoptosis. No significant effect of butyrate was seen in colonocytes from animals fed corn oil. Furthermore, our data show that as the molar percentages of DHA and EPA (n-3 fatty acids found in fish) in the cardiolipin of the distal colon increased, the cytosolic cytochrome c content increased ($R=0.49$, $p=0.03$; $R=0.59$, $p=0.02$). This finding strengthens the proposed relationship between butyrate, fish oil and apoptosis. In the proximal colon, no relationship was found between cytochrome c translocation and fatty acid enrichment of the cardiolipin, again supporting the epidemiological evidence (83).

Caspase-3. Caspase-3 is an enzyme activated downstream of cytochrome c and its activation is indirectly dependent upon cytochrome c translocation to the cytosol (32). Caspase-3 acts as an effector caspase, degrading DNA and leading to cellular breakdown during apoptosis. In the proximal colon, butyrate induced a 21.9% increase ($p=0.05$) in caspase-3 activity in animals fed fish oil and a 39.1% increase ($p=0.002$) in animals fed corn oil. While the percent change with butyrate was greater in corn oil than in fish oil, these changes were not significantly different from one another. In the distal colon, butyrate incubation caused a 17.4% ($p=0.04$) increase in caspase-3 activity in fish oil fed animals, but had no significant effect in animals fed corn oil. This finding provides further support for the apoptosis-inducing power of the fish oil/butyrate combination. When taken together, the butyrate-induced changes in cytochrome c translocation and caspase-3 activity suggest that colonocytes in the distal colons of animals fed fish oil may be more readily undergoing apoptosis than those from animals fed corn oil.

CHAPTER V

SUMMARY AND CONCLUSIONS

Evidence points to diet as a primary environmental factor influencing colon cancer risk in the United States. Epidemiological studies, cell line experiments and animal trials have found dietary fish oil to be a chemoprotective food component. Diet studies in animal models of colon carcinogenesis have found the combination of dietary fish oil and the fermentable fiber pectin to be protective against tumor formation (11). This study sought to define the mechanisms by which the dietary components may be protective against colon cancer at the promotion stage. The results of the study indicate that butyrate, a fermentation product of the fiber pectin, increases ROS in colonic crypts. Furthermore, the data suggest that this increase in ROS may be inducing apoptosis via a mitochondria-mediated mechanism. Colonic crypts from animals fed fish oil diets were found to be more susceptible to butyrate-induced decreases in MMP. More importantly, these changes were seen in animals injected with carcinogen, suggesting a greater propensity for butyrate-induced apoptosis and removal of DNA damaged cells in fish oil-fed animals. The results of cardiolipin analysis indicate that when animals consume fish oil, the cardiolipin fatty acid composition is altered, leading to increased levels of n-3 PUFA in the membrane, and thus, greater susceptibility to ROS-induced release of cytochrome c. These data are supported by increased caspase-3 activity with butyrate incubation in animals fed fish oil, and regression data, which show strong relationships between cardiolipin fatty acid content and butyrate-induced changes in cytochrome c.

Thus, this study may explain the previously found protective effect of dietary fish oil and pectin (11), and suggests that these dietary components increase apoptosis in transformed cells via a mitochondria-mediated mechanism.

Future research. While these data are compelling, further work must be done to justify human diet trials. One primary concern is that in order to see a diet effect in this study, animals received either corn oil or fish oil exclusively for the entire span of the project. It is unreasonable to assume that a free-living human being would choose to include only one fat source in the diet. An animal study involving different levels of fatty acids (n-6 and n-3) in various combinations at multiple time points in the cancer development process is warranted. Once results are generated in rat models that provide an estimated level of consumption at which chemoprevention is optimized, human trials should then follow.

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APPENDIX A

EXPERIMENTAL PROTOCOLS

Diet Mixing Protocol using Teklab vitamin premix
15% fat, 6% fiber (CO/cellulose, FO/cellulose)

For mixing 200 kg (8 batches) of dry premix:

1. **Take oil (fish) out of freezer 24 h prior to mixing and allow it to thaw.**
2. **Gather supplies for mixing diets and take to basement:**

   (2) Large mixing bowls for Hobart mixer (found in Lupton lab)
   (2) Beaters for Hobart mixer (found in Lupton lab)
   Pre-labeled Ziploc bags (approx. 1 kg of diet per bag)
   Small Rubbermaid weighing bins
   (3) Scoops
   70% EtOH in spray bottle for clean-up
   Box of gloves, Paper towels, Scissors, Colored labeling tape, Sharpies, dust masks
   "Lid" to mixing bowl (the trash can lid with the hole in it)

3. **Gather supplies for mixing oils and load onto large cart for transport to Chapkin lab:**

   (10) 5000 ml plastic beakers
   Pkg 25 ml sterile pipettes
   Pkg 10 ml sterile pipettes
   (2) Automatic pipettes
   Diapers
   Paper towels
   4 large plastic bins full of ice

4. **To mix the oils (Corn first):**

(Corn oil, Tenox and MTS-70 are stored in 4°C refrigerator in Dr. Chapkin's lab)

   a. Use the top-loading scale in Dr. Chapkin's lab
   b. Level the scale
   c. Place an empty 5000 ml beaker on the scale and tare
   d. Label the beaker (corn or fish oil)

e. Add the appropriate weight of corn oil to the beaker (Use the 2 extra beakers as "overflow" beakers to remove any excess oils during the measuring process)
f. Repeat steps c-e 3 times for a total of 4 beakers of corn oil
g. Next, weigh out the fish oil in the same manner as the corn oil. Then add the appropriate amount of corn oil to the fish oil. Repeat for a total of 4 beakers of fish oil.
h. To each beaker of oil, add the appropriate weights of Tenox 20A and MTS-70 using sterile pipettes After each addition, stir the oil with a sterile pipette for 1 min or until well mixed
i. Cover the beakers with foil and pack in ice until use
j. Flush original stock containers of oil with nitrogen and return them to refrigerator or freezer

5. Measuring the dry mix:

a. To the basement, bring the 8 beakers of ice-packed oil, all above listed supplies, and the top-loading scale from Dr. Chapkin's lab.
b. Make sure both doors to the mixing room are shut!
c. Re-bubble the scale
d. Make sure the bowls and mixing beakers are adequately labeled with colored tape and Sharpies to avoid confusion!
e. Place one Rubbermaid bin on the scale and tare
f. Begin to scoop the dry premix from the barrel into the bin, trying to stir up as little dust as possible (gloved hands are best throughout the mixing process)
g. Weigh out 5000 g and pour into the large mixing bowl labeled for corn oil diet. Repeat for a total of 25000 g (the barrel should be all but empty)

6. Mixing the diets:

a. Attach the bowl of dry mix
b. Attach the beater labeled for corn oil diets to the Hobart mixer and raise the bowl (use crank)
c. Thoroughly stir one of the beakers of corn oil and pour about ½ of the oil into the bowl of dry mix.
d. Cover the bowl with the "lid" and mix on setting 1 for 2 min (until the oil has blended in)
e. Add the remaining oil and continue to mix on setting 1 until blended (about 5 min)
f. Stop the mixer and lower the bowl. Thoroughly scrap the beater using gloved hands. Raise the bowl
g. Mix on setting 2 for 10 min

During the mixing period, begin measuring the dry mix as directed above and pour into the bowl labeled for fish oil diet. When the corn oil diet is finished mixing, remove the bowl and scrape the beater. Attach the fish oil beater and bowl and mix as directed above. Bag the mixed corn oil diet, wipe out the bowl and begin the measuring process once again.

7. To bag the diets:

a. Fill the pre-labeled Ziploc bags about 2/3 full using the scoop labeled for that particular diet
b. Push the air from the bags and seal, and then place the sealed bag into a freezer bag. Remove the air and seal. Pack into boxes or barrels. Store in -20°C freezer

8. Clean up:

a. Wash the bowls, beaters, scoops, bins, beakers, etc in the large sink and place on the straining rack to dry.
b. Sweep and mop the floor and wipe down all surfaces with 70% EtOH and a paper towel
c. When the bowls and beaters are dry, wipe down with 70% EtOH
d. Return all equipment

Food Intake Protocol

Food intakes were measured over a 48 h period at three time points during the feeding trial:

1 wk prior to injection #1
1 wk post injection # 2
3 d prior to termination

Day 1

Weigh out food and record weight. Place paper on top of the bedding under the double wire racks.

Day 2

Weigh the food remaining in the food cup and the food spilled on the paper and record. Urine and feces on the paper should be avoided when weighing the food.
Weigh out fresh food and record. Place fresh paper on top of the bedding under the double wire racks.

Day 3

Weigh the food remaining in the food cup and the food spilled on the paper and record. Urine and feces on the paper should be avoided when weighing the food.
Subtract the remaining and spilled food weights from the fed weights to determine the intake for each 24 h period. Add together the intake amounts to determine the 48 hr intake.

Animal Termination, Crypt Isolation, Image Analysis

I. Preparation-several days before

A. Preparation of stock solutions (Stock solutions are prepared for entire experiment)

1. EDTA (Sigma #ED4SS) 50X = 56g EDTA / 100 ml HBSS-CaMg
2. BSA (Sigma A-6003) 100X = 10g BSA / 100 ml HBSS-CaMg
3. Gln (Gibco BRL #35050-061 can be purchased 200 mM, 200X
4. Rhodamine 123 (Molecular Probes) MMP Dye stock= 10 mg/4 ml MeOH
5. Ethidium Homodimer (Molecular Probes=to one vial add 117 µl DMSO and 467 µl ddH2O.

B. Aliquot stock solutions: Place in epi or centrifuge tubes. The aliquot amount is based on the number of rats to be collected. (See chart in section III C.)

1. EDTA (50X) store at 4 °C
2. BSA (10%) store at –20 °C
3. Gln (200 mM) store at –20 °C (expires 24 mo from purchase)
4. Rhodamine 123 store at -20°C (aliquot 50 µl per tube-use 1 tube/rat)
5. Ethidium Homodimer store at -20°C

II. Preparation-day before

A. Autoclave necessary equipment (for three rats)

1. 3 1000-ml bottles (for PBS, HBSS-CaMg, HBSS+CaMg)
2. 1 250-ml bottle (for HBSS+buffer to take to image lab)
3. 2 1000-ml beakers (in which to pH solutions)
4. 6 50-ml glass flasks for shaking
5. 9 rubber policemen (put in beaker and cover with foil for autoclaving)
6. 3 magnetic stirrers (wrap in foil)
7. 2 large graduated cylinders (to measure PBS & HBSS. Measure – before +)
8. 3 sets of forceps
9. 5 pairs of scissors –3 small sharp, 1 blunt-ended, 1 large sharp (can be used for >1 rat)
10. 2 spatulas

B. Make 10X PBS

1. 10X PBS:
   a. 1 vial Dulbecco's Phosphate Buffered Saline (PBS) w/o CaMg (Gibco BRL #21600-069)
   b. Add ddH2O to bring to 1 L
   c. Dissolve, store at room temperature

C. Label supplies (for 1 rat)

1. 3 1000-ml small-top bottles (PBS, HBSS-CaMg, HBSS+CaMg)
2. 6 50-ml glass flasks for shaking (label w/ rat number, and Proximal or Distal)
3. 1 250-ml bottle (for HBSS+ buffer at imaging lab)
4. Disposable supplies in pre-labeled RAT PACKS

D. Prepare the fixation solutions

1. 70% EtOH (RT), 4% PFA (4°C)
2. 50% EtOH, 70% EtOH (4°C)

III. Preparation-day of experiment (2 h before)

A. Prepare equipment

1. Turn on shaking water bath (set dials: shaking to 6, temp to 3.2, H-M-L to M)
2. Turn on bench-top water bath (set to 37 °C)
3. Turn on warming plate (set dial to 2.75 and warm to 37 °C)

B. Prepare solutions (Must be made fresh each kill day)

1. Thaw BSA and Glutamine
2. Measure DTT
3. Make 1X PBS
   a. Mix 100 ml 10X PBS with 900 ml ddH2O
   b. Filter PBS into autoclaved container (Filter using sterile attachment and filter paper. Apply vacuum.)

4. Warm solutions (filtered PBS, HBSS-CaMg, HBSS+CaMg) in bench-top water bath at 37 °C and bubble with 95% O2 / 5% CO2 for 30 min
5. Pour some bubbled PBS into sterile squirt bottle and place in water bath

6. Make HBSS+CaMg + Gln + BSA buffer and HBSS-CaMg + EDTA + DTT + Gln + BSA buffer with stock solutions and aliquots
   a. Use sterilized stir bars and keep solutions warm (using hot plate)

C. Solution concentrations

	Hank's (ml)	50X EDTA (ml)	DTT (mg) *powder form	200X Gln (ml)	100X BSA (ml)
HBSS-CaMg	150 ml HBSS-CaMg	3 ml	115.5 mg	0.75 ml	1.5 ml
HBSS+CaMG	300 ml HBSS+CaMg			1.5 ml	3 ml

For 1 Rat:

For 2 Rats:

	Hank's (ml)	50X EDTA (ml)	DTT (mg) *powder form	200X Gln (ml)	100X BSA (ml)
HBSS-CaMg	300 ml HBSS-CaMg	6 ml	231 mg	1.5 ml	3 ml
HBSS+CaMg	500 ml HBSS+CaMg			2.5	5 ml

	Hank's (ml)	50X EDTA (ml)	DTT (mg) *powder form	200X Gln (ml)	100X BSA (ml)
HBSS-CaMg	400 ml HBSS-CaMg	8 ml	308 mg	2 ml	4 ml
HBSS+CaMg	800 ml HBSS+CaMg			4 ml	8 ml

For 3 Rats:

*Final Concentrations:

30 mM EDTA
5 mM DTT
1 mM Gln
0.1% BSA

D. Adjust the solutions to pH 7.4 at 37 °C using the pH meter

1. Calibrate the pH meter (instructions for Orion model 420A)

a. Make sure the meter has been stored with the blue plug in the probe and that the probe is full of liquid.
b. Fill 2 small plastic beakers about ½ way with calibration buffer (pH 7.0, yellow and pH 10.0, blue)
c. Remove the blue plug from the probe.
d. Press "power" button on the meter
e. Press "2nd" (green writing) and then press "cal" (for calibrate)
f. Place the meter probe into the beaker of pH buffer solution. Use the yellow, pH 7.0 first. Swirl the solution and then hold the probe steady.
g. The meter will read the pH and then beep and the word "ready" will blink. Press "yes"
h. Holding the probe over a large weigh boat, rinse well with ddH20
i. Now place the cleaned probe into the beaker with the blue pH 10.0 buffer.
j. The meter will read the pH and then beep and the word "ready" will blink. Press "yes"
k. The meter display will now say "measure"
l. The meter is now calibrated.

2. Measure the pH of your solutions
   a. Again, rinse the probe with ddH20 and place the probe in the solution to be measured.
   b. Use the arm to hold the probe. Keep the solution on the hot plate, stirring constantly with a sterilized stir bar.
   c. The meter will begin to display the pH. To adjust the pH of the solution, add 50% HCl and/or 5 M NaOH.
   d. Use the drop bottles and add the acid/base one drop at a time.
   e. When the solution reaches the desired pH (in this case, 7.4), press "yes"
   f. The solution is now ready for use and the next solution can be measured.
   g. Be sure to rinse the probe before inserting it into a new solution and before storage.
   h. Store the probe in the bottle of solution with the blue plug IN.

*Note: If the meter turns off during use, just push "power" and continue.

E. Prepare the butyrate treatment
   1. Make 200 mM butyrate stock
      a. Sodium butyrate (MW 110.09, ACROS 26419-0050, Sigma B5887)
         110.09 mg/mM x 200 mM = 22.018 g/L HBSS+CaMg
         = 22.018 mg/ml HBSS+CaMg Buffer

      b. Filter sterilize using syringe and 0.2 um filter
      c. Aliquot 750 µl and store at -80°C (750 µl is enough for 3 rats)
      d. Place butyrate on ice for use

IV. Crypt Isolation Procedure

A. Euthanize rat by CO_2 asphyxiation (3 min) and cervical dislocation
B. Open abdomen and remove colon, including rectum
C. Discard rectum and measure colon length.
   1. If colon is at least 17 cm long, remove approximately 2 cm at both proximal and distal ends.
   2. Cassette the tissue and place 1 cm of each tissue in EtOH and 1 cm of each PFA. (If colon is not quite long enough, use only about .75 cm. If the colon is 16 cm or less in total length, do not collect tissues for cassetting.)
   3. Divide colon into proximal and distal sections and place tissues in weigh boats with warm PBS.
   4. Holding the tissue with forceps, use scissors to cut down the tissue long-ways, opening up the colon.
   5. Rinse the tissue with warm PBS until free of visible fecal matter.
   6. Place tissues in 50 ml flasks containing 40 ml HBSS-CaMg + EDTA + DTT
   7. Incubate colon for 15 min with #6 shaking
   8. Pour contents of flask into sterile (labeled) petri dish.
   9. Hold the tissue with forceps and gently scrape the mucosal side of the tissue with a rubber policeman. Discard the serosal layer.
      a. The tissue will begin to curl after it is cut open. Usually, the side that curves to the outside is the mucosal side (this is the side you scrape). The outside of the colon will have many visible blood vessels (this is NOT the side you scrape!)
   10. Using a sterile pipette, transfer the contents of the petri dish into a 50 ml centrifuge tube
   11. Centrifuge at 100 xg for 3 min at room temp.

12. Remove the supernatant with a pipette and resuspend in 40 ml of HBSS+CaMg buffer to wash cells
13. Centrifuge at 100 xg for 3 min at room temp.
14. Remove the supernatant with a pipette and resuspend in 40 ml of HBSS+CaMg buffer.
15. Aliquot crypt suspension (40 ml)
16. Keep the crypt suspension at 37°C.

To the Image lab:

·Crypt suspension in warm water
·Warm HBSS+ buffer
·Chamber slides/cover slips
·Racks for 15 and 50 ml test tubes and epis
·Labeled epi tubes, 15 ml centrifuge tubes
·DMSO
·Butyrate stock (on ice)
·Pipettes and tips/ timers
·Rhodamine 123 and CMH2DCFDA dyes

IV. Image Analysis (Butyrate/no butyrate, MMP/ROS)

A. Butyrate treatment (final concentration 5mMol/L)
1. Centrifuge the crypt solution (in the 15-ml tubes) 2-3 min at 200 RPM
2. Hold in incubator at 37°C until needed
3. Aliquot 950 µl HBSS+ buffer, 25 µl of 200 mM butyrate stock, and 25µl of cell pellet into appropriate butyrate tubes
4. Incubate the solution for 30 min at 37°C

B. Control treatment (no butyrate)
1. Follow steps 1 and 2 above
2. Aliquot 975 µl HBSS+ buffer, 25 µl cell pellet into appropriate tubes
3. For control 1, add dye immediately. For control 2, incubate 30 min at 37°C

C. Measure MMP
1. Add 1µl R123 stock (mol wt 380.83; final stock concentration 6.56 mMol/L). Incubate last 15 min of cell incubation
2. Centrifuge at lowest RMP for 2 min
3. Remove supernatant and resuspend in 975 µl HBSS+ buffer
4. Centrifuge at lowest RMP for 2 min
5. Remove supernatant and resuspend in 975 µl HBSS+ buffer
6. Centrifuge at lowest RMP for 2 min

7. Transfer 10 µl of cell pellet to chamber slide
8. Give to Rola for image analysis (she will put on the cover slip).

D. Measure ROS
   1. Prepare CMH_2-DCFDA
      a. Add 20 µl DMSO to vial of CMH_2-DCFDA (stored in freezer; final stock concentration 4.32 mMol/L)
      b. Pipette up and down slowly to mix
   2. Add 1 µl 5 mM CMH_2-DCFDA for last 15 min of incubation
   3. Centrifuge at lowest RMP 2 min and remove supernatant
   4. Add 975 µl HBSS+ buffer
   5. Centrifuge at lowest RMP 2 min and remove supernatant
   6. Transfer 10 µl of cell pellet to chamber slide. Cover with cover slip, being careful not to squish the cells
   7. Give to Rola for image analysis

E. Check viability
   1. After analysis of each treatment, add 1 µl of Ethidium Homodimer to cells remaining in tube.
   2. Invert tube and incubate for 5 min at 37°C
   3. Transfer 2 µl of cell pellet to a chamber slide
   4. Cover cells with cover slip
   5. At the Scanalytics microscope, using UV lamp, select 10 different frames of cells and estimate the percentage that are viable (green=viable, red=not viable)

Isolation of Mitochondria from Colonic Crypts

1. Place 20 ml of crypt solution on ice for lipid analysis

2. With remaining 10 ml crypts, mix gently by inversion and divide into two 5 ml aliquots in 15 ml conical tubes; Add +/- butyrate
   a. To non-butyrate (-) tube, add 50 µl HBSS
   b. To butyrate (+) tube, add 50 µl of 0.5 M sodium butyrate (5 mM final conc.)
   c. Start timer immediately; mix gently by inversion
   d. Place all tubes horizontally at 37°C in Forma Shaker at 70 rpm

3. At 30, 60, 120 min, mix gently by inversion and remove 250 µl from each tube for caspase-3 processing

4. At 60 min, take remaining 4.25 ml from each tube for cytochrome c analysis prep
   a. Spin down crypts at 200 x g for 5 min at 4°C
   b. Resuspend pellet in 200 µl complete MITO homogenization buffer; transfer to epi tube
   c. Homogenize using micro-pestle with motor on ice, 6 strokes; use additional 100 µl complete MITO buffer to rinse off pestle
   d. Spin at 600 x g for 10 min at 4°C in microfuge
   e. Transfer supernatant to 1.5 ml epi tube and discard nuclei pellet
   f. Spin supernatant at 15,000 x g for 10 min at 4°C in microfuge
   g. Transfer supernatant (cytosol fraction) to new 1.5 ml epi-tube; save pellet
   h. Mix supernatant and separate into 30 µl aliquots; store at –20°C
   i. Resuspend mitochondrial pellet into 100 µl RIPA buffer with pipetting
   j. Pass through 29 gauge needle and incubate on ice 30 min
   k. Spin at 12,000 x g for 10 min; remove unsolubilized material
   l. Transfer supernatant (solubilized mitochondrial preparation) to new 1.5 ml epi-tube; mix and separate into 11 µl aliquots; store at –20 °C
   m. Save aliquot of complete MITO buffer and RIPA buffer for protein analysis

RIPA buffer:

50 mMol/L Tris-HCl
0.5% deoxycholate
1% NP-40
0.1% SDS
50 uMol/L NaF
Protease Inhibitor

MITO buffer:

300 mMol/L sucrose
1 mMol/L EDTA
5 mMol/L Hepes
3 mMol/L DTT
Protease Inhibitor

Selection of cytochrome c assay kit

Cytochrome c is a mitochondrial membrane protein, which acts as an electron carrier in the electron transport chain. It is generated in the cytosol of the cell, where it exists as an apoprotein. Apocytochrome c is transported into the intermitochondrial membrane space where the enzyme cytochrome c heme lyase covalently attaches a heme group to the N-terminus, creating holocytochrome c. The holoprotein is located within the intermitochondrial membrane space, and functions to transport electrons from Complex III to Complex IV of the oxidative phosphorylation pathway. Upon receipt of apoptotic signals, holocytochrome c is released from the mitochondria into the cytosol where it binds with Apaf-1, caspase-9 and dATP, forming the "apoptosome." Activation of caspase-9 triggers a caspase cascade, and thus, caspase-dependent apoptosis.

In the past, our lab has determined cytochrome c translocation via Western blots. However, we wished to use an ELISA assay for this study because of the greater consistency and decreased likelihood of human error during the assay procedure. Because we are interested in the release of cytochrome c into the cytosol (holoprotein) and not simply the total amount of cytochrome c present (holo and apoproteins), we used an assay sensitive to the holoprotein. Furthermore, we were working with subcellular fractions of rat colon cell lysates, so we had to use an assay kit that would work in solublized mitochondrial and cytosolic fractions from murine species. Finally, we had to employ an assay kit that was insensitive to interfering substances, most notably, dithiothreitol (DTT). Our solubilized fractions contained a 3 mM DTT concentration.

We searched through multiple kit options and considered the following:
Cytochrome C ELISA Kit, Chemicon International, Inc. #APT200 (Temecula, CA)
Specific for human cytochrome c only
Cytochrome C ELISA, Oncogene Research Products #QIA74 (San Diego, CA)
Specific for human cytochrome c only
Cytochrome C Assay Kit, Kamiya Biomedical Company #KT-002 (Seattle, WA)
Specific for human cytochrome c only
FunctionELISA Cytochrome C, Active Motif #48006 (Carlsbad, CA)
Specific for human cytochrome c only, not holo-specific
Interfering substances not a problem
Quantikine M Rat/Mouse Cytochrome C Immunoassay, R&D Systems #MCTC0 (Minneapolis, MN)
Specific for murine cytochrome c
Specific for holocytochrome c
Interfering substances: Sulfhydryl reducing agents, including DTT concentrations in the sample must be ≤0.1 mM.

Based on the given information, we utilized the Quantikine M Assay from R&D Systems. The assay sensitivity to DTT forced us to dilute the samples, however, a greater dilution was necessary to fit the samples within the standard curve generated by the standard supplied in the kit.

Quantikine Rat/Mouse Cytochrome C Assay
R&D Systems #MCTC0

Prepare Reagents:

1. Rat/Mouse Cyt. c Kit Control
   - reconstitute the Kit Control 1.0 ml ddH_2O
   - make 200 µl aliquots and store for up to 1 mo at -20°C
   - assay the control undiluted

2. Rat/Mouse Cyt. c Conjugate
   - add 0.5 ml Conjugate Concentrate to 11.0 ml Conjugate Diluent
   - use sterile container; protect from light
   - store for up to 1 wk at 4°C

3. Wash Buffer
   - if crystals have formed in concentrate, warm to room temperature and mix gently until dissolved
   - add 25 ml Wash Buffer Concentrate to 600 ml ddH_2O
   - store for up to 1 mo at 4°C

4. Substrate Solution
   - mix Color Reagents A and B together in equal portions within 15 min of use
   - protect from light

5. Rat/Mouse Cyt. c Standard
   - Reconstitute the r/m Cyt. c Standard with 2.0 ml Calibrator Diluent RD5-18
   - Allow to sit for 5 min with gentle mixing prior to dilution (makes 100ng/ml stock)
   - Store reconstituted stock for up to 1 mo at -20°C

   Perform serial dilutions to achieve proper standard curve:

   200 µl stock + 600 µl Calibrator Diluent = 25 ng/ml
   200 µl 25ng/ml + 200 µl Calibrator Diluent = 12.5 ng/ml
   200 µl 12.5 ng/ml + 200 µl Calibrator Diluent = 6.25 ng/ml
   200 µl 6.25 ng/ml + 200 µl Calibrator Diluent = 3.12 ng/ml
   200 µl 3.12 ng/ml + 200 µl Calibrator Diluent = 1.56 ng/ml
   200 µl 1.56 ng/ml + 200 µl Calibrator Diluent = 0.78 ng/ml

   Calibrator Diluent serves as the zero standard/blank (0 ng/ml)

For a higher standard curve, create 40 ng/ml high standard by mixing 320 µl of 100ng/ml stock with 480 µl Calibrator Diluent. Perform serial dilutions to reach standards of 20, 10, 5, 2.5 and 1.25 ng/ml.

Assay Procedure:

1. Prepare all reagents and standards as indicated. Thaw Cytochrome c samples (cytosolic or mitochondrial fraction); each plate can accommodate 6 rats (4 samples each) in triplicate, plus controls and standards.

2. Add 75 µl of rat/mouse Cytochrome c Conjugate to each well.

3. Add standards, samples and controls
   Standards: 48.5 µl diluted rat/mouse Cyt. c Standard + 1.5 µl buffer*
   Blank: 48.5 µl Calibrator Diluent + 1.5 µl buffer*
   Control: 48.5 µl rat/mouse Cyt. c Kit Control + 1.5 µl buffer*
   Samples: 48.5 µl Calibrator Diluent + 1.5 µl sample
   (Results in 3% sample concentration.)
   *(for cytosolic fraction, add MITO buffer; for mitochondrial fraction, add RIPA buffer.)

4. Mix by gently tapping plate frame for 1 min; cover with adhesive strip and incubate 2 hr at room temperature

5. Aspirate each well and wash; repeat process four times for a total of five washes. Wash by filling each well with 400 µl Wash Buffer using an automatic plate washer. (Complete removal of liquid at each step is crucial to good performance.) After the last wash, remove any remaining wash buffer by aspirating and then blot against clean paper towels.

6. Add 100 µl of Substrate Solution to each well. Incubate for 30 min at room temperature; protect from light.

7. Add 100 µl Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

8. Determine the optical density of each well within 30 min using the Bio-Tek Synergy HT. Set the wavelength to 450 nm and the correction wavelength to 540 nm. Consider only the values that fall within the linear portion of the standard curve.

Cytochrome c Cytosolic Fraction Protein Quantitation
Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL); microplate protocol

Preparation of Standards (BSA) diluted with ddH_2O:

Vial	Diluent volume	Volume/Source BSA	Final [BSA}
A	0	300 µl Stock	2000 µg /ml
B	125 ul	375 µl Stock	1500 µg /ml
C	325 ul	325 µl Stock	1000 µg /ml
D	175 ul	175 µl vial B	750 µg /ml
E	325 ul	325 µl vial C	500 µg /ml
F	325 ul	325 µl vial E	250 µg /ml
G	325 ul	325 µl vial F	125 µg /ml
H	400 ul	100 µl vail G	25 µg /ml
I	200 ul	200 µl vial H	12.5 µg /ml
J	400 ul	0	0 µg /ml = Blank

Store at 4°C

Assay Procedure

1. Pipette 10 µl of each standard or unknown sample into the appropriate microplate wells*
2. Add 300 µl of the Coomassie Plus Reagent to each well and mix with plate shaker for 30 s.
3. Remove plate from shaker and incubate 10 min at room temperature
4. Measure the absorbance at 595 nm with the Bio-Tek Synergy HT; subtract the reading for the blank
5. Using the standard curve, determine the protein concentration of the unknown samples.

*For the cytosolic Cytochrome c samples, the standard curve had to be adjusted to a range that included 0, 3.125, 6.25, 12.5, 25, 125, 250, 500, 750 and 1000 µg /ml. The samples were added to the wells as follows:

Standards: 5 µl BSA standard + 5 µl MITO buffer
Blank: 5 µl MITO buffer + 5 µl dH_2O
Sample: 5 µl sample + 5 µl dH_2O

Cytochrome c Mitochondrial Fraction Protein Quantitation
BCA Protein Assay Reagent Kit (Pierce, Rockford, IL); microplate protocol

Preparation of Standards (BSA) diluted with ddH_2O:

Vial	Diluent volume	Volume/Source BSA	Final [BSA}
A	0	300 µl Stock	2000 µg /ml
B	125 ul	375 µl Stock	1500 µg /ml
C	325 ul	325 µl Stock	1000 µg /ml
D	175 ul	175 µl vial B	750 µg /ml
E	325 ul	325 µl vial C	500 µg /ml
F	325 ul	325 µl vial E	250 µg /ml
G	325 ul	325 µl vial F	125 µg /ml
H	400 ul	100 µl vail G	25 µg /ml
I	400 ul	0	0 µg /ml = Blank

Store at 4°C

Preparation of Working Reagent (for entire 96-well plate):
Mix 20 ml reagent A + 0.392 ml reagent B; vortex briefly

Assay Procedure

1. Pipette 10 µl of each standard or unknown sample into the appropriate microplate wells*
2. Add 200 µl of the Working Reagent to each well and mix with plate shaker for 30 s.
3. Cover plate and incubate 30 min at 37°C
4. Cool plate to room temperature
5. Measure the absorbance at 562 nm with the Bio-Tek Synergy HT; subtract the reading for the blank
6. Using the standard curve, determine the protein concentration of the unknown samples.

*The samples were added to the wells as follows:

Standards: 10 µl BSA standard + 10 µl RIPA buffer
Blank: 10 µl RIPA buffer + 10 µl dH_2O
Sample: 10 µl sample + 10 µl dH_2O

Caspase-3 Sample Preparation

1. Take 250 µl aliquot of isolated crypts after +/- butyrate treatment
2. Spin down at 200 x g, 5 min, 4°C; discard supernatant
3. Resuspend crypt pellet in 2 ml PBS; gently pipette up and down
4. Spin at 200 x g, 5 min, 4°C
5. Carefully remove all PBS (pipette off most, pulse microfuge, pipette dry)
6. Freeze cell pellets at -80°C until ready to analyze caspase-3 activity

Caspase-3 Activity Assay Protocol

Stock Solutions: made per instructions, Caspase-3 Activity Assay Kit, Molecular Probes, Eugene, OR)
Protocol: Laurie Davidson, Nancy Turner, Molecular Probes Kit

Make Stock Solutions:

1. 5mM Z-DEVD-R110
   - Thaw kit components A and B
   - Add 264 µl of B directly to vial of A
   - Gentle heating to 50 °C to dissolve (use water bath)
   - Aliquot 60 µl/tube; Store –20 °C, desiccate, no light

2. 1M DTT Stock
   - Add 650 µl deionized water directly to vial of DTT; vortex
   - Aliquot 100 µl/tube; Store –20 °C

3. 1mM Stock AcDEVD-CHO Inhibitor
   - Thaw components B and F
   - Add 400 µl B directly to vial of F; vortex
   - Aliquot 10 µl/tube; Store –20 °C, desiccate

4. 5mM R110 Reference Standard
   - Thaw components B and G
   - Add 273 µl B directly to vial of G
   - Aliquot 10 µl/tube; Store –20 °C, no light

Protocol:

1. turn on centrifuge; fast cool to 4°C
2. Make 15 ml 1X lysis buffer (enough for one 96-well microplate)
   - 187.5 µl 20X lysis buffer, Enzcheck
   - 562.5 µl 20X lysis buffer, Cyquant
   - 150 µl Triton X
   - 14.1 ml dH_2O
3. Add 250 µl 1X lysis buffer to frozen pellet; allow cells to thaw
4. Pass suspension through 27 gauge needle; incubate on ice 30min
5. Make 7 ml 2X reaction buffer
   - 1400 µl component D
   - 70 µl 1M DTT stock
   - 5530 µl dH_20
6. Make 5 ml 2X substrate solution
   - 50 µl Z-DEVD-R100 stock
   - 4.95 ml 2X reaction buffer
7. Microfuge at 5000 rpm for 5 min. Place 100 µl supernatant into new .65 ml tube; add 100 µl lysis buffer
8. Remove 30 µl to .65 ml tube labeled for protein analysis; store at –80 °C
9. Transfer 50 µl diluted supernatant in triplicate to 96-well microplate; use 50 µl lysis buffer for blank
10. **Prepare standards:**

Make 1600 µl 1X reaction buffer

- 800 µl 2X reaction buffer
- 800 µl dH_20

Make 550 µl 25 µm R110 standard (protect from light)

- 2.75 µl 5mM R110 standard stock
- 547.3 µl 1X reaction buffer

Dilute with 1X reaction buffer to concentration: 0, 0.0625, 0.125, 0.25, 0.5, 1, 2, and 5

11. Place 100 µl R110 standard into separate wells
12. Add 50 µl substrate solution to each well, including blanks
13. Incubate 30 min at room temperature; protect from light
14. Measure fluorescence at A496/A520 every 30 min until the signal is within the linear portion of the standard curve

As a control, add material from practice animal +/- inhibitor. In 2 wells, add 1 µl AcDEVD-CHO stock (+inhibitor). In 2 wells, add 1 µl DMSO (-inhibitor). No caspase activity should be measured in wells containing AcDEVD-CHO.

Steps to calculate caspase-3 protein values

Sample Collection and Processing
5 ml aliquot of crypts taken for caspase/Cytochrome c analyses
250 µl * 3 incubations= 750 µl for caspase-3
4.25 ml for Cytochrome c

100 µl of supernatant was diluted by half and used to measure caspase-3 activity. 30 µl of diluted caspase supernatant and was taken for protein quantitation. Protein quantitation was first attempted using the Coomassie Plus Protein Assay (Pierce, Rockford, IL). Several problems arose:

1. Using the test tube protocol, the found protein concentrations were too low to fit into a reliable, linear portion of the standard curve.
2. Using the microplate protocol, which allowed samples to be assayed undiluted, the lysis buffer in which the samples were suspended interfered with the standard curve.
3. Multiple dilutions were performed to establish the optimal sample concentration, however the interfering agents were apparent even at 20% concentration. At that level, the protein in the sample fell to near immeasurable levels.
4. Dr. Dangett in the Biochemical Protein Analysis Laboratory recommended the BCA Assay, microplate procedure. While the lysis buffer interference was reduced, the reliability of the found protein values was still questionable.

We determined that the best course of action was to use the found protein values from the Cytochrome c samples to calculate the caspase protein values.

Cytochrome C total protein determination

Cytosolic protein value given by KC4 software * .240 (total ml of collected protein sample stored as aliquots) = **total cytosolic protein**
Mitochondrial protein value given by KC4 software * .088 (total ml of collected protein sample stored as aliquots) = **total mitochondrial protein**

total cytosolic protein + total mitochondrial protein= total protein in cyt. c samples

In other words…
{(total vol for all aliquots of mito fraction) * [mito prot]}+ {(total vol for all aliquots of cyto fraction) * [cyto prot]} = total quantity of prot in 4.25 ml of sample

Caspase-3 protein values were determined using the protein values found for Cytochrome c

total quantity of prot in 4.25 ml of sample/4.25 ml = **protein concentration in caspase-3 sample (ug/ml)**

Quantitate fatty acid mass of major phospholipids from rat colon mitochondria

Begin with isolated mitochondria in pellet form

1. Use 1.8 ml 0.1 M KCl to transfer the pellet from epi-tube to 25 ml screw-top glass tube; break up pellet completely

2. Add 9 ml Folch (MeOH/$CHCl_3$, 1:2, v/v) to the tube

3. Vortex for 1 min

4. Use Pasteur glass pipette to transfer the lower phase to 12 ml screw-top glass tube; flush with N_2, store at -20°C

5. Dry the extract under N_2

6. Redissolve the lipids in exactly 1 ml Folch; transfer to 4 ml glass vial. Flush with N_2, store at -80°C

7. Transfer 100 µl of the sample to a 2 ml glass vial, flush with N_2, store at -20°C (will be used for phosphorus assay, to measure the mass of total phospholipids)

8. Dry the remaining 0.9 ml sample under N_2

9. Redisssolve the sample in 25 µl Folch.

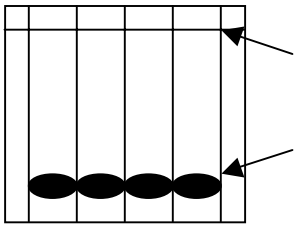
10. Activate the TLC plate (Silica Gel 60, EM Science, Gibbstown, NJ) for 1 hr at 110°C

12. Prepare the glass tank by rinsing with MeOH (Omnisolv, EM Science, Gibbstown, NJ) and allow to evaporate under hood

13. Prepare the solvent mix for the TLC system:
    - 50 ml $CHCl_3$
    - 37.5 ml MeOH
    - 3.5 ml HoAc (Tracepur Acetic Acid, EM Science, Gibbstown, NJ
    - 2 ml H_2O (HPLC grade, Fischer)

14. Place 1/3 sheet of Whatman #1 filter paper inside tank to draw solvent upward

15. Spot the sample (25 µl) to the TLC plate to separate individual phospholipids; spot samples in center of plate, avoiding outer edges. Add 25 µl Folch and spot sample again.



16. Allow plate to run until solvent almost touches top lane

17. Prepare leak-proof tubes:
    a. add 1 ml acetone to clean screw-top tubes; mark liquid line on tube with Sharpie
    b. heat in oven at 80°C for 1 hr; select tubes that have not lost acetone due to evaporation = leak proof
    c. empty acetone from tubes and allow tubes to dry
    d. rinse tube and lid with MeOH
    e. add 500 ng 17:0 free fatty acid (serves as internal standard); dry under N_2
    f. Add 3 ml 6% HCl/MeOH to the tube (light sensitive)

    18. After TLC plate is done, spray with 1% 8-anilino-1-napthalene-sulfonic acid (ANS)** under hood and view with black light. Circle cardiolipin, PE and PC bands lightly with pencil
        **Dilute dry chemical (Sigma, St. Louis, MO) with ddH_2O

    19. Scrape bands individually into leak-proof tubes containing internal standard and 6% HCl/MeOH; flush mix with N_2, Vortex for 1 min

    20. Methylate at 76°C for 15 hr

    21. Extract fatty acid methyl esters (FAME):
        a. add 2 ml hexane and 1 ml 0.1 M KCl to the leak-proof tube
        b. vortex for 1 min; centrifuge at 3000 rpm for 5 min
        c. use glass Pasteur pipette to transfer the upper phase to 4 ml glass vial
        d. add 2 ml hexane and repeat steps b and c
        -if not running GC immediately, flush vial with N_2 and store at –20°C
        e. dry down the FAME under N_2
        f. redissolve in 25 µl CH_2Cl_2
        g. inject 1 µl into GC (to calculate mass of phospholipids)

Phosphorus Assay

For determining total phospholipid quantity in lipid extract

I. Preparation of stock solutions
   a. 4 M HCL
      i. Add 242 ml concentrated HCl (12.4 M) to 500 ml ddH_2O.
   b. 1 M HCL
      i. Add 50 ml 4 M HCl to 150 ml ddH_2O.
   c. 10% $Mg(NO_3)_2$-$6H_2O$ in MeOH (w/v)
      i. Add 5 g $Mg(NO_3)_2$-$6H_2O$ to 50 ml MeOH.
   d. Solution A (4.2% ammonium molybdate in 4 M HCl)
      i. Slowly add 21 g ammonium molybdate to 500 ml 4 M HCl. Store at 4°C.
   e. Solution M (0.05% malachite green, BDH)
      i. Add 0.5 g malachite green to 1 L ddH_2O; stir for 30 min. Store at 4°C in brown bottle (light sensitive).
   f. Solution T (1.5% Tween 20 v/v)
      i. Add 1.5 ml Tween 20 to 100 ml ddH_2O.
   g. Solution AM (1:3 v/v, Solution A/Solution M)
      i. Mix 600 ml of Solution M with 200 ml of Solution A; stir for 30 min. Filter through two layers of Whatman #1 filter paper. Store at 4°C in brown bottle (light sensitive); stable for several months.
   h. Solution AMT
      i. Mix 100 ml of Solution AM with 4 ml of Solution T. Store at 4°C; stable for several days.

II. Phosphorus Assay Preparation
   a. Label 13x100 mm borosilicate disposable glass tubes with sample name, blank and standards 1-5. Assay standards in triplicate, samples in duplicate (if available).
   b. Prepare water bath under fume hood. Cover water tray with foil to preserve heat. Keep temperature close to 100°C.

III. Assay Procedure
   a. Add 90 µl lipid extract into 13x100 mm borosilicate disposable glass tube. Add directly to bottom of tube for best recovery.
   b. Add 30 µl 10% $Mg(NO_3)_2$-$6H_2O$ in MeOH (w/v) to the sample tubes and blank tubes
   c. Vortex gently
   d. Dry under N_2

* Perform following steps under vent hood: fumes are toxic!

e. Using a Bunsen burner, hold individual tubes over a flame with tongs for approximately 30 sec, or until solution has dried (a white residue will appear).
    i. Watch for bubbling and overflow of contents
    ii. Noxious brown fumes will be generated
f. Cool tubes by sitting at room temperature in non-coated metal rack
g. Add 400 µl of 1 M HCl to the sample tubes. Vortex, making sure the white residue is completely dissolved. For standard curve, add 400 µl KH_2PO_4 standards (dissolved in 1 M HCl) to standard tubes (0, 0.24, 0.48, 0.72, 0.96 µg P).
h. Heat in water bath at 90-95°C for approximately 15 min.
i. Cool tubes by sitting at room temperature
j. Add 2 ml of Solution AMT to the tubes
k. Vortex for 1 min.
l. Incubate the tubes at room temperature in the dark for 1-2 hr without stirring, until the color is developed and stabilized (a bright green color will form).
m. Transfer 300 µl of standards, blanks and samples to 96 well microplate; read at 635 nm.
n. Plot standard curve; calculate mass of samples.

References:

- Duck-Chong and Coral. A rapid sensitive method for determining phospholipid phosphorus involving digestion with magnesium nitrate. Lipids 14:492-497.
- Protocol of Dr. Yang-Yi Fan

APPENDIX B

DATA TABLES

TABLE B1
Reactive oxygen species[1]

ROS: Distal Colon			
Diet/Treatment	Butyrate	Control	P-value
	532.67 ± 37.55[a]	351.25 ± 25.15[b]	<0.0001*
Fish Oil	505.68 ± 50.41[a]	343.23 ± 34.73[b]	0.002*
Corn Oil	561.10 ± 55.93[a]	359.46 ± 36.41[b]	0.0003*
Saline	522.59 ± 52.09[a]	328.00 ± 37.69[b]	0.0001*
AOM	542.94 ± 54.12[a]	376.15 ± 38.67[b]	0.003*
ROS: Proximal Colon			
Diet/Treatment	Butyrate	Control	P-value
	428.76 ± 31.22	375.44 ± 26.71	0.12
Fish Oil	473.62 ± 49.54[a]	353.40 ± 35.27[b]	0.018*
Corn Oil	388.19 ± 39.36	398.86 ± 40.44	0.82
Saline	425.26 ± 43.84	383.14 ± 38.23	0.39
AOM	432.29 ± 94.48	367.86 ± 37.30	0.18

[1]ROS values were assigned arbitrary units. Values represent the means ± SEM.

TABLE B2
Mitochondrial membrane potential[1]

MMP: Distal Colon			
Diet/Treatment	Butyrate	Control	P-value
	593.41 ± 52.96[a]	740.26 ± 66.07[b]	0.0013*
Fish Oil	630.43 ± 79.56[a]	835.47 ± 105.44[b]	0.05*
Corn Oil	558.64 ± 70.50	656.88 ± 82.90	0.26
Saline	552.63 ± 69.74[a]	728.07 ± 91.88[,b]	0.05*
AOM	637.21 ± 80.42	753.78 ± 95.13[,]	0.24
MMP: Proximal Colon			
Diet/Treatment	Butyrate	Control	P-value
	392.92 ± 32.68[a]	580.74 ± 47.90[b]	0.0002*
Fish Oil	516.67 ± 60.24	596.39 ± 69.54	0.32
Corn Oil	298.78 ± 35.44[a]	565.49 ± 65.94[b]	<0.0001*
Saline	375.03 ± 44.48[a]	582.66 ± 67.94[,b]	0.003*
AOM	411.62 ± 47.99[a]	578.82 ± 67.49[,b]	0.02*

[1]MMP values were assigned arbitrary units. Values represent the means ± SEM.

TABLE B3

Percent change in mitochondrial cytochrome c with butyrate incubation[1]

Diet	Distal Colon	P-value
Fish Oil	-11.6 ± 5.0	0.02*
Corn Oil	-5.4 ± 5.1	0.30
Diet	**Proximal Colon**	**P-value**
Fish Oil	-14.9 ± 6.2	0.02*
Corn Oil	0.7 ± 5.9	0.91

[1]Values represent mean % changes [(butyrate-control)/control] with butyrate incubation compared to control ± SEM. Decreases signified by (-).

TABLE B4

Percent change in caspase-3 activity[1]

Diet/Treatment	Distal Colon	P-value
Fish Oil	17.4 ± 9.9	0.04
Corn Oil	4.2 ± 10.5	0.34
Diet/Treatment	**Proximal Colon**	**P-value**
Fish Oil	21.9 ± 13.3	0.05
Corn Oil	39.1 ± 13.1	0.002

[1]Values represent mean % changes [(butyrate-control)/control] with 30 min butyrate incubation compared to control ± SEM.

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