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Fish oil increases mitochondrial phospholipid unsaturation, upregulating reactive oxygen species and apoptosis in rat colonocytes

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We have shown that a combination of fish oil (high in n-3 fatty acids) with the butyrate-producing fiber pectin, upregulates apoptosis in colon cells exposed to the carcinogen azoxymethane, protecting against colon tumor development. We now hypothesize that n-3 fatty acids prime the colonocytes such that butyrate can initiate apoptosis. To test this, 30 Sprague–Dawley rats were provided with diets differing in the fatty acid composition (corn oil, fish oil or a purified fatty acid ethyl ester diet). Intact colon crypts were exposed *ex vivo* to butyrate, and analyzed for reactive oxygen species (ROS), mitochondrial membrane potential (MMP), translocation of cytochrome C to the cytosol, and caspase-3 activity (early events in apoptosis). The fatty acid composition of the three major mitochondrial phospholipids was also determined, and an unsaturation index calculated. The unsaturation index in cardiolipin was correlated with ROS levels ($R = 0.99$; $P = 0.02$). When colon crypts from fish oil and FAEE-fed rats were exposed to butyrate, MMP decreased ($P = 0.041$); and translocation of cytochrome C to the cytosol ($P = 0.037$) and caspase-3 activation increased ($P = 0.032$). The data suggest that fish oil may prime the colonocytes for butyrate-induced apoptosis by enhancing the unsaturation of mitochondrial phospholipids, especially cardiolipin, resulting in an increase in ROS and initiating apoptotic cascade.

Introduction

Colon cancer is the second leading cause of death from cancer in the US today, and its development is highly responsive to diet (1). The two diet components thought to have the most significant effect on colon tumor development are dietary fat and fiber. Specifically, n-3 polyunsaturated fatty acids (high in fish oils) appear protective against colon cancer in epidemiological, clinical and experimental studies (2–7). The type of dietary fiber that is the most protective against colon tumor development remains the subject of debate. One hypothesis is that fermentable fibers should be the most protective since butyrate, a short chain fatty acid derived in the colon from microbial fermentation, promotes differentiation and apoptosis in a variety of colon tumor cell lines (reviewed in ref. 8). However, there is some discrepancy between *in vitro* and *in vivo* studies regarding the efficacy of butyrate (9).

In previous studies we showed that the more fermentable fibers (pectin, guar and oat bran), provided as part of the standard American Institute of Nutrition corn oil-based diet used by most investigators, upregulated colonic cell proliferation (10,11) and actually enhanced experimental tumorigenesis, rather than being protective (12). Others reported similar findings (13,14). However, more recently when we provided the highly fermentable fiber pectin with a fish oil rather than corn oil-based diet we found a synergistic protective effect of this combination (15,16). Further experiments with the fish oil/pectin diet confirmed that the protective effect was due to an enhancement of apoptosis, rather than a decrease in cell proliferation at all stages of tumor development, i.e. initiation, promotion/progression and once tumors had formed (15–18).

The purpose of the present study was to determine why a highly fermentable fiber combined with a fish oil diet, rich in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), is better able to initiate apoptosis than the same diet combined with corn oil, rich in linoleic acid (18:2n-6). Our hypothesis was that the lipid component of the diet (fish oil or corn oil) increases the unsaturation of colonic mitochondrial cell membranes making them more susceptible to damage by reactive oxygen species (ROS). This, in turn, should lower mitochondrial membrane potential (MMP), allowing cytochrome C to translocate to the cytosol, a key event in the apoptotic cascade. According to this hypothesis, butyrate can initiate apoptosis through a ROS-dependent mechanism, only if the mitochondrial membranes are susceptible due to a high degree of unsaturation. This could explain why a diet containing a highly fermentable fiber is only protective when fish oil is the lipid source.

To test this hypothesis we provided rats one of three diets differing only in the type of fat (corn oil, fish oil or an ethyl ester diet designed to mimic the fatty acid composition of fish oil, but in a purified form). Rats were provided the semi-purified diets, intact colonic crypts were isolated and exposed to butyrate or no butyrate and measurements were made of mitochondrial levels of ROS, MMP, translocation of cytochrome C and caspase-3 activity. In addition, we evaluated the fatty acid composition of the three primary mitochondrial phospholipids as a function of diet and determined an unsaturation index for each mitochondrial phospholipid.

Abbreviations: CMH_2-DCFDA, 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAEE, fatty acid ethyl ester diet; HBSS, Hank's balanced salt solution; MMP, mitochondrial membrane potential; PE, phosphatidylethanolamine; ROS, reactive oxygen species.

Materials and methods

Animals and study design

The animal use protocol was approved by the University Laboratory Animal Care Committee of Texas A&M University and conformed with NIH guide-

Table I. Composition of the experimental diets

Ingredient	g/100 g
Dextrose	51.06
Casein	22.35
D,L-Methionine	0.34
Salt mix, AIN-76	3.91
Vitamin mix, AIN-76	1.12
Choline bitartrate	0.22
Cellulose[a]	6.00
Fat	
Corn oil diet	
Corn oil	15.00
Fish oil diet	
Menhaden fish oil[b]	11.50
Corn oil	3.50
FAEE diet	
Fatty acid ethyl esters[c]	11.50
Corn oil	3.50

[a]Cellulose (microcrystalline) was purchased from Bioserv (Frenchtown, NJ).
[b]Vacuum-deodorized Menhaden fish oil (NIH fish oil test material program, Southeast Fisheries Center, Charleston, SC).
[c]16:0 (3.45 g); 18:1(n-9)(2.74 g); 18:2(n-6)(0.54 g); 20:5(n-3)(2.50 g); 22:6(n-3) (1.60 g) from Nu Chek Prep (Elysian, MN) and glycerol (0.73 g). All three oils contained α-tocopherol, 1.0 mg/g; γ-tocopherol, 1.5 mg/g; t-butylhydroquinone, 0.03 % (w/w) as antioxidants.

lines. Thirty male 180 g Sprague–Dawley rats (Harlan Sprague–Dawley, Houston, TX) were individually housed and maintained in a temperature and humidity controlled animal facility. The rats were acclimated for 1 week prior to receiving the defined diets for 2 weeks, and then stratified by body weight so that mean initial body weights did not differ between groups.

Diets

The three defined diets (Table I) differed only in the type of fat [corn oil, fish oil or fatty acid ethyl esters (FAEE)]. The major differences among the fatty acid composition of the three dietary lipid sources were significantly higher amounts of EPA (20:5n-3) and DHA (22:6n-3) in the fish oil compared to corn oil diet, and higher amounts of linoleic acid (18:2n-6) in the corn oil diet. The FAEE diet was formulated to contain similar amounts of saturated, monoenoic and dienoic fatty acids and a similar EPA:DHA ratio as the fish oil diet. The purpose of the FAEE diet, using highly purified individual fatty acids, was to test if the lipid effects were due to the fatty acid composition of the fish oil or to some other bioactive substance found in fish oil, which would not be present in the highly purified FAEE diet. The fish oil diet and FAEE contained 3.5 g corn oil/100 g diet to ensure that essential fatty acid requirements were met. Fish oil and FAEE were supplemented to attain an equivalent level of antioxidants to the corn oil: 1 mg/g α-tocopherol and 1.5 mg/g γ-tocopherol (Archer-Daniels Midland, Decatur, IL) and 0.03% tertiary butylhydroquinone (Eastman Chemical Company, Kingsport, TN). Rats were provided with fresh diet every day and the feeders were removed and washed daily. Animals had free access to food and water at all times. No differences in 48 h food intakes were found as measured 1 week after receiving the diets. Body weights were recorded each week and no differences were detected among diet groups.

Isolation of colonic crypts and mitochondria

Rats were killed by CO_2 gas overdose, followed by cervical dislocation. The colon was removed and colonic crypts were isolated as described previously with modifications (19). Briefly, after removal of the rectum, and 1 cm of intestine for histology, the next 9 cm from the distal end of the colon was flushed with phosphate-buffered saline (PBS) to remove feces. The segment was incubated in warm calcium and magnesium-free Hank's balanced salt solution (HBSS), 5 mM dithiothreitol, 0.1% fatty acid-free BSA, 1 mM glutamine and 30 mM EDTA containing buffer (pH 7.4) for 15 min with shaking to dislodge crypt units from their attachments to the extracellular matrix. After incubation, the intact crypts were isolated by gently scraping the mucosa with a rubber policeman. As shown in Figure 1, whole crypts were selectively isolated, and the lamina propria and muscular layers were still intact after isolation of the crypts. The crypts/cell suspension was then centrifuged at 300 *g* for 3 min, the supernatant discarded and the pellet suspended in HBSS containing calcium and magnesium (pH 7.4). This centrifugation step was repeated.

Mitochondria from colonic crypts were isolated by the method of Johnson and Lardy (20). All steps and centrifugations for mitochondrial isolation were performed on ice or at 4°C. Briefly, colonic crypts were resuspended in homogenizing buffer containing 300 mM sucrose, 1 mM EDTA, 5 mM HEPES (pH 7.4), 50 μM sodium fluoride, 100 μM sodium orthovanadate, 25 μg/ml each leupeptin, pepstatin and aprotinin, and 150 μM 4-(2-aminoethyl) benzene-sulfonyl fluoride using six strokes of a Potter–Elvehjam homogenizer. The homogenate was centrifuged at 800 *g* for 10 min to pellet nuclei and unbroken cells. The supernatant was centrifuged at 15 000 *g* for 10 min and the pellet was washed with 1 ml of homogenizing buffer and re-centrifuged at 8000 *g* for 5 min. This final mitochondrial pellet was resuspended in 50 mM Tris–HCl, aliquoted and stored at –80°C until analysis.

Analysis of mitochondrial phospholipid fatty acid composition and determination of unsaturation index

Cellular lipids were extracted using chloroform/methanol (2:1, v/v) and individual phospholipid classes separated by thin-layer chromatography using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, v/v) as described previously (21). Total phospholipid was assayed as described by Duck-Chong (22). A known amount of monopentadecanoin was added to the isolated phospholipid bands which were scraped into tubes containing 6% HCl and incubated at 75°C for 16 h. Following transmethylation, fatty acid methyl esters from the cardiolipin, phosphatidylcholine and phosphatidylethanolamine (PE) fractions were further purified on silica gel plates using toluene prior to quantification by capillary gas chromatography. The unsaturation index for each fatty acid within a phospholipid was calculated by multiplying the number of double bonds in each fatty acid by the mol percentage of that fatty acid and dividing by 100. The unsaturation index for each phospholipid was generated by summing the individual fatty acid unsaturation indices.

Measurement of ROS and MMP

Isolated colonic crypts were incubated with or without 5 mM butyrate in HBSS + calcium and magnesisum (pH 7.4) for 30 min at 37°C prior to the measurement of ROS and MMP. The ROS generation was measured using 5-(and -6)-chloromethyl-2′,7'-dichlorodihydrofluorescein diacetate (CMH_2-DCFDA) (Molecular Probes, Eugene, OR) as described previously (23). Cleavage of acetate moieties by esterases inside the cell causes the 2,7-dichlorofluorescein to be trapped inside the cell and available for oxidation by peroxides or hydroperoxides to the fluorescent 2,7-DCF (23,24). Briefly, isolated crypts were incubated with 5 μM CMH_2-DCFDA in HBSS containing calcium and magnesium for 15 min at 37°C, washed with HBSS + calcium and magnesium and pelleted by centrifugation. Fluorescence intensity was measured using an Ultima confocal microscope (Meridian Instruments, Okemos, MI). Laser excitation was performed at 488 nm and emitted fluorescence was monitored with a barrier filter (530 nm). A total of 15 areas were measured for each sample.

To measure MMP, isolated colonic crypts were incubated with 10 μM rhodamine 123 (Molecular Probes) for 15 min at 37°C, washed with HBSS + calcium and magnesium and pelleted by centrifugation (25). Mitochondrial fluorescence intensity was monitored at 488 and 530 nm excitation and emission, respectively, with a confocal microscope (Meridian Instruments). A reduction of fluorescence intensity reflects the decrease of MMP. Data from a total of at least 15 areas were measured for each sample. Cell viability was tested in each sample after analysis, using ethidium homodimer-1 (Molecular Probe). Ethidium homodimer-1 is excluded by the intact plasma membrane of live cells but enters cells with damaged membranes, producing red fluorescence in dead cells. In this study, the average cell viability throughout the experiments was 87.1 ± 0.42% (n = 159).

Translocation of cytochrome C to the cytosol

Isolated crypts were incubated with or without 5 mM butyrate for 1 h, then protein was extracted as described previously (26) and protein concentrations were determined using the Coomassie Plus protein assay (Pierce, Rockford, IL). Colonic cytosol and mitochondrial fraction extracts (4 μg) were subjected to polyacrylamide gel electrophoresis in 4–20% pre-cast mini gels (Invitrogen, Carlsbad, CA), and the proteins were electroblotted onto PVDF membranes (Millipore, Bedford, MA). Following blocking, membranes were incubated in primary antibody (mouse cytochrome C monoclonal antibody, PharMingen, San Diego, CA), diluted in PBS containing 4% non-fat milk and 0.1% Tween-20, with shaking at 4°C overnight. Membranes were washed in PBS containing 0.1% Tween-20 and the target protein was detected with SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce). The membrane was scanned and intensities quantified using a Fluor-S Imaging system (BioRad Laboratories, Hercules, CA).

Measurement of caspase-3 activity

After incubation with or without 5 mM butyrate for 30 min, isolated crypts were disrupted in lysis buffer [25% lysis buffer supplied with the kit (EnzChek Caspase-3-Assay Kit, Molecular Probes) and 75% CyQuant lysis buffer

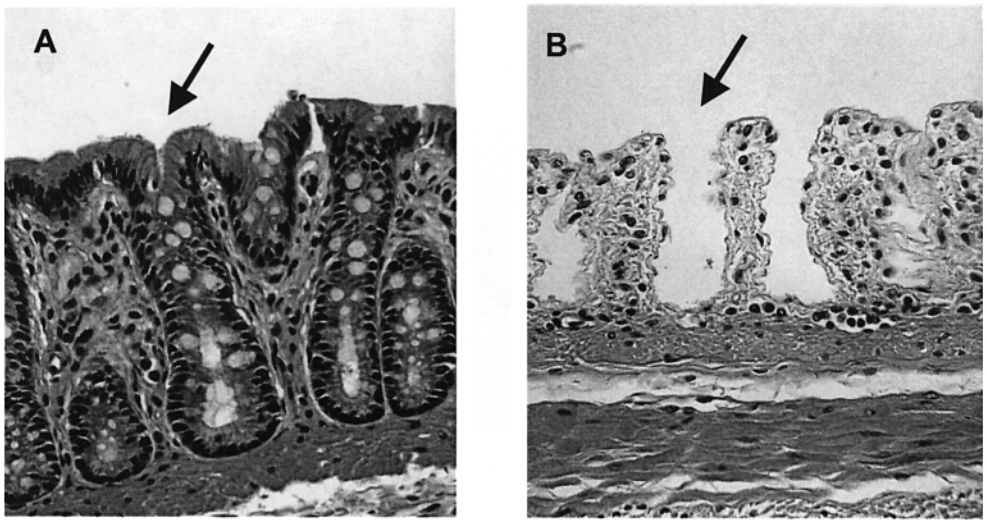

Fig. 1. Isolation of colonic crypts. (**A**) The mucosa prior to crypt isolation, the arrow identifies a colonic crypt. (**B**) The mucosa post isolation, the arrow identifies a void left by removal of a crypt.

(Molecular Probes) in order to dilute the sample, with addition of 0.1% Triton X-100]. The cell lysate was passed through a 27 gauge needle, followed by a 30 min incubation on ice. Cellular debris was removed by centrifugation at 15 000 *g* for 20 min at 4°C and the supernatant was incubated with a synthetic tetrapeptide (DEVD) substrate which is specifically cleaved by members of the caspase-3 subfamilies (EnzChek Caspase-3-Assay Kit, Molecular Probes). Standards containing 0–25 μM of R110 (proprietary, supplied with the kit) were used to determine the amount of fluorescence released. Fluorescence was measured at A496/A520 using a Wallac Victor 1420 multilabel counter (Perkin Elmer, Boston, MA) starting 30 min after the addition of substrate and continuing until the signal for all samples was within the linear portion of the standard curve. Omission of cell lysate was used as a background control. Lysate protein concentration was determined by the BioRad protein assay (BioRad).

Statistical analyses

The fatty acid composition of colonic mitochondrial phospholipids was analyzed using a one-way ANOVA to determine the effect of dietary lipid. ROS, MMP, cytochorome C translocation and caspase-3 activity were analyzed using ANOVA to determine the effect of dietary lipid and butyrate. The effect of the difference between butyrate treatment and control on ROS, MMP, cytochrome C translocation and caspase-3 activity was analyzed using one-way ANOVA to test the effect of dietary lipid. When the dietary lipid effect was significant (P value <0.05), the Student–Newman–Keul's multiple range test was used to determine which dietary lipid means were significantly different from one another. MMP and caspase-3 activity were analyzed using contrast comparisons in SAS to test the difference between corn oil versus the combined effects of the fish oil and FAEE diets. The unsaturation index of a phospholipid was regressed against the basal ROS value, the butyrate-induced ROS value and the difference (change in ROS). The Pearson's correlation coefficient for each relationship was computed from the model R^2.

Results

The fish oil and fish oil FAEE diets produced nearly identical physiological effects

There was no difference between the fish oil and FAEE diets with respect to any of the functional measurements (ROS, MMP, cytochrome C translocation and caspase-3 activity). The single difference between the fish oil and FAEE diet effects on mitochondrial phospholipid fatty acid composition was found in cardiolipin, in that the fish oil diet resulted in higher levels of linoleic acid than the FAEE diet (Table II).

Fish oil and FAEE diets increased levels of unsaturation in fatty acids of mitochondrial membrane phospholipids

The three major phospholipid classes found in the mitochondria are phosphatidylcholine, PE and cardiolipin (27). In each of these phospholipid classes, fish oil and FAEE diets increased the relative amounts of both C20:5n-3 and C22:6n-3. With one exception (i.e. in cardiolipin) the increase in C20:5n-3 and C22:6n-3 was at the expense of C18:2n-6 (Table II). When an unsaturation index was calculated for each phospholipid as a function of diet, rats fed fish oil or FAEE diets had higher unsaturation indices in each phospholipid class than did rats fed corn oil (data not shown) although this was only statistically significant for glycerophosphoethanolamine [unsaturation index = 1.65 (corn oil); 2.06 (fish oil); 2.04 (FAEE)($P < 0.05$)].

Table II. Effect of dietary lipid on fatty acid composition of the major mitochondrial phospholipids[a]

Diet	Corn oil	Fish oil	FAEE
Cardiolipin			
18:2n-6	14.70 ± 3.19*†	17.90 ± 2.41†	8.38 ± 0.86*
20:5n-3	Tr*	2.19 ± 0.88†	5.07 ± 0.73‡
22:6n-3	Tr*	1.64 ± 0.67†	1.87 ± 0.38†
Glycerophosphocholine			
18:2n-6	17.14 ± 0.42*	13.25 ± 0.25†	13.29 ± 0.22†
20:5n-3	Tr*	3.16 ± 0.15†	3.33 ± 0.28†
22:6n-3	Tr*	1.27 ± 0.10†	1.12 ± 0.29†
Glycerophosphoethanolamine			
18:2n-6	12.07 ± 0.40*	8.86 ± 0.42†	9.15 ± 0.12†
20:5n-3	Tr*	12.13 ± 0.58 †	14.67 ± 1.89†

[a]Fatty acid values are shown as mean ± standard error of the mean.
Tr: Trace amount. Within a row, means not sharing a common symbols (*, †, ‡) are significantly different ($P < 0.05$).

ROS in response to butyrate administration were highly correlated with the unsaturation index of cardiolipin

Intact colonic crypts, isolated from rats provided with diets differing in their lipid composition, were analyzed using the vital dye CMH_2-DCFDA which detects the sum of ROS generated by singlet oxygen, hydroxyl radical, superoxide, hydroperoxide and peroxide (28). Although the amount of ROS generated in rat colonocytes was higher in response to butyrate administration with the fish oil and FAEE diets compared with corn oil, this was not significant (results not shown). When ROS was regressed with the unsaturation index of mitochondrial cardiolipin—0.988 (corn oil), 1.050 (fish oil),

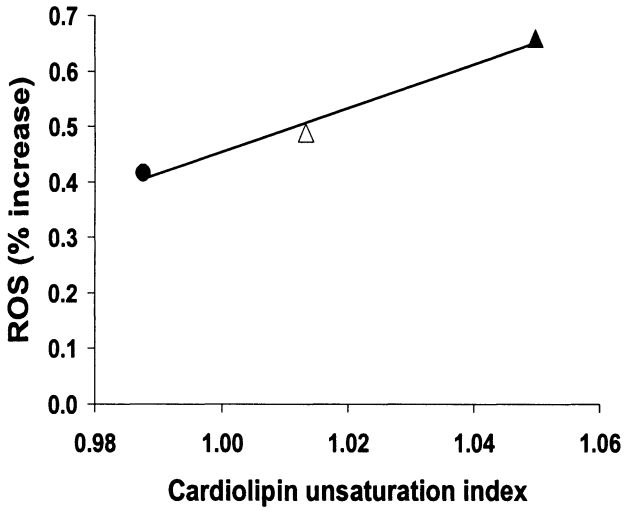

Fig. 2. Correlation of the unsaturation index of cardiolipin with ROS. The unsaturation index of mitochondrial cardiolipin was positively correlated with ROS level ($R^2 = 0.99$, $P = 0.02$). The closed circle is corn oil, the open triangle is FAEE and the closed triangle is fish oil.

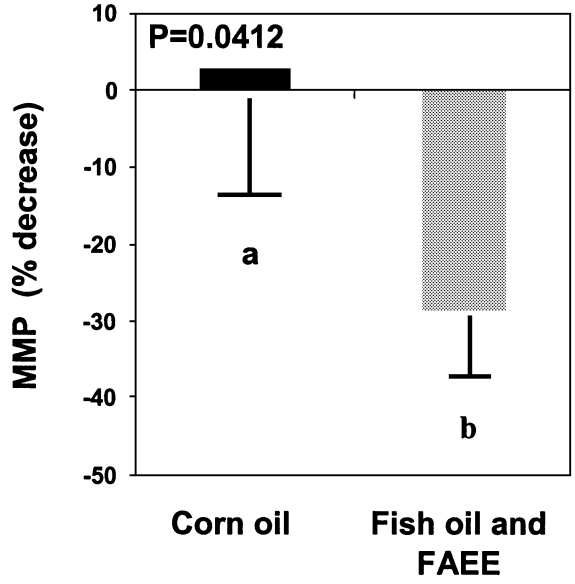

Fig. 3. Effect of diet on MMP production. The MMP decreased with fish oil and FAEE diets compared with corn oil diet ($P = 0.0412$) with 5 mM butyrate incubation. There was no significant difference on MMP between fish oil and FAEE diets.

1.013 (FAEE)—the correlation coefficient was 0.99 ($P = 0.02$) (Figure 2). In contrast, there was no significant correlation between ROS and the unsaturation index of mitochondrial phosphatidylcholine (0.98; $P = 0.11$) nor with PE (0.82; $P = 0.38$).

MMP decreased in rats fed fish oil and FAEE diets

MMP membrane potential was analyzed using the vital dye rhodamine 123. When colonic crypts were incubated for 30 min with 5 mM butyrate, MMP decreased in cells isolated from rats fed fish oil and FAEE. No such effect was observed with colon cells isolated from rats fed corn oil (Figure 3).

Cytochrome C translocated from the mitochondria to the cytosol and caspase-3 was activated in colonic crypts from fish oil and FAEE-fed rats exposed to butyrate

Figure 4 shows a representative immunoblot of cytochrome C in the cytosol and mitochondria of colonocytes, with and without butyrate administration. Prior to butyrate administration, most of the cytochrome C was located in the mitochondria (lane 3) as compared with the cytosol (lane 1). In contrast, after a 1 h incubation with butyrate, there was a translocation of cytochrome C from the mitochondria (lane 4) to the cytosol (lane 2). Both fish oil and the FAEE diets enhanced the translocation of cytochrome C to the cytosol as compared with corn oil ($P = 0.0367$) (Figure 5). Caspase-3 activity increased in response to butyrate incubation in colonic crypts isolated from rats fed fish oil and FAEE ($P = 0.0324$), but did not increase in crypts from corn oil-fed rats (Figure 6).

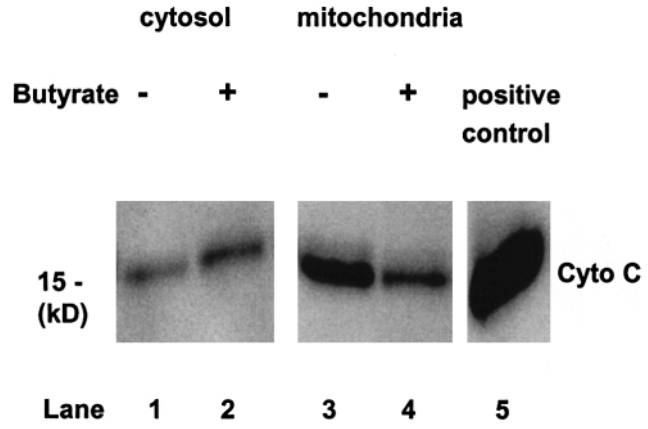

Fig. 4. A representative immunoblot of cytochrome C in the cytosol and mitochondria with and without butyrate administration. Prior to butyrate administration, most of the cytochrome C was located in the mitochondria (lane 3) as compared with the cytosol (lane 1). In contrast, after a 1 h incubation with butyrate, there was a translocation of cytochrome C from the mitochondria (lane 4) to the cytosol (lane 2). Lane 5 contains recombinant cytochrome C standard.

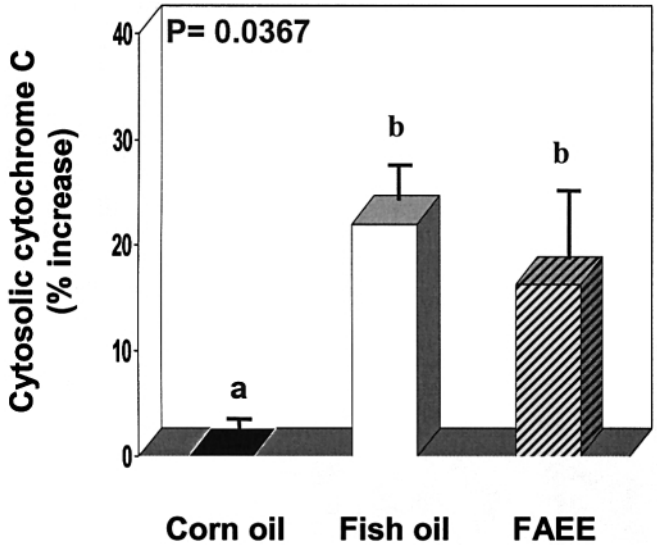

Fig. 5. Translocation of cytochrome C to the cytosol. Both fish oil and the FAEE diet enhanced the translocation of cytochrome C to the cytosol as compared with corn oil ($P = 0.0367$) after incubation with butyrate.

Discussion

A functional relationship among butyrate, mitochondria and ROS was shown previously in other research (16,17) using *in vitro* colonic carcinoma cells. Here we showed the relationship among butyrate administration, mitochondrial function, ROS, MMP and apoptosis with dietary intervention using

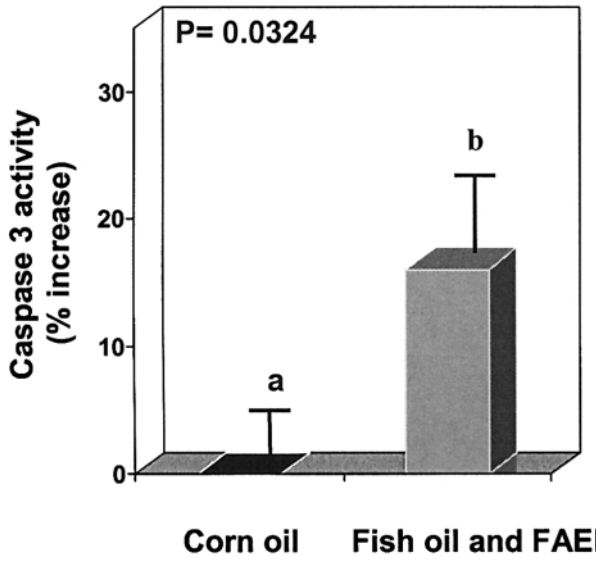

Fig. 6. Caspase-3 activity. Caspase-3 activity increased in response to butyrate incubation in colonic crypts isolated from rats fed fish oil and FAEE, but did not increase in crypts from corn oil-fed rats ($P = 0.0324$). There was no significant difference between fish oil and FAEE diet on caspase-3 activity.

normal rat colonocytes. Our results show that diet treatment alone did not induce apoptosis in rat colonocytes. However, when colon cells are isolated from rats, whether or not they respond to butyrate depends on dietary lipid pretreatment. As nearly identical results were achieved with both fish oil and FAEE diets, we conclude that the observed effects are due to the fatty acids themselves rather than some other component found in fish oil. Collectively, the data support our hypothesis that the fatty acids found in fish oil prime the cell in such a way that it is responsive to butyrate-induced apoptosis.

One specific mechanism by which fish oil or FAEE feeding primes the cell making it responsive to butyrate-induced apoptosis may be by alteration of mitochondrial membrane phospholipid fatty acids to those with higher degrees of unsaturation, as shown in the present study. Although this is the first report of alterations to colonic mitochondrial phospholipids with fish oil feeding, two other papers have reported similar effects of fish oil feeding on mitochondrial phospholipids, from rat heart and liver (29), and rat renal cortical cells (30). An *in vitro* study reports enrichment with DHA to 48% of the fatty acids in cardiolipin when human colonic adenocarcinoma (HT-29) cells are incubated with this fatty acid (31). The significance of an increase in fatty acid unsaturation is the extensive literature relating degree of fatty acid unsaturation to degree of lipid peroxidation (32). In this study, only the unsaturation index for PE was significantly different between the corn oil diet and either the fish oil or FAEE diets. However, the relationship of unsaturation index with ROS as a function of diet was significant only in the cardiolipin fraction. Cardiolipin is located exclusively in the inner mitochondrial membrane and is required for cytochrome C oxidase activity (33). The primary phospholipid of plasmalogen, which is a glycerophospholipid containing plasmenyl phospholipid, is PE (34). We have shown that fish oil feeding increases the n-3 PUFA and lowers n-6 PUFA in the sn-2 position of macrophage plasmalogen (35). Recently, it has been reported that plasmalogen phospholipids delay oxidative degradation in animal cells (36) and *in vitro* (34), suggesting that phospholipids esterified with PUFA at the sn-2 position of plasmalogen are less susceptible to oxidative damage (37). Incorporation of n-3 PUFA at the sn-2 position of plasmalogen PE would not lead to elevated ROS, as it does when incorporated into mitochondrial cardiolipin, and therefore did not result in a relationship between ROS level and unsaturation index. However, in cardiolipin, ROS level was highly correlated ($r = 0.99$, $P = 0.02$) with the unsaturation index because of the exposure of more susceptible n-3 PUFA in cardiolipin to high levels of oxygen radicals.

Although there was a numerically greater production of ROS in colon cells from rats fed fish oil and FAEE diets compared to those from rats fed corn oil diets after 30 min of incubation with buytyrate, this did not achieve statistical significance. In a follow-up study (38) we incubated colon crypts with butyrate for an additional 30 min and did detect higher levels of ROS with fish oil feeding compared to corn oil ($P = 0.06$). We conclude from this that as ROS production is a downstream event from lipid peroxidation, more time is required before the effects of butyrate on this variable are detected in our system.

Peroxidation of fatty acids in mitochondrial membrane phospholipids can have important physiological consequences in addition to down stream generation of ROS. For example, Malis *et al*. (30) provided rats with fish oil or beef tallow-enriched diets and isolated renal cortical mitochondrial membranes to determine the fatty acid composition of mitochondrial phospholipids. They demonstrated that fatty acids from fish oil were readily incorporated into renal cortical mitochondria, and that when exposed to ROS, the mitochondria enriched in n-3 fatty acids had significantly greater uncoupled respirations than those from beef tallow-fed rats. This suggests that ROS damage mitochondrial membrane phospholipids causing functional defects in the electron transport system, which could both depress MMP and cause a greater production of ROS at the sites of uncoupling. This correlates well with our finding of a decrease in MMP for fish oil but not corn oil-fed rats.

Although there are a number of correlative studies describing a relationship between lipid peroxidation and apoptosis, the specific pathway(s) by which peroxidation leads to apoptosis remains to be elucidated. In one study a positive correlation was established between the number of double bonds in fatty acids, their increased susceptibility to lipid peroxidation and their potential for inducing apoptosis (39). It has been reported that high levels of dietary fish oil suppress human carcinoma cell growth in athymic nude mice, presumably by increased accumulation of lipid peroxidation products in tumor tissue (40), which may contribute to increased apoptosis. Damage to mitochondrial membranes by lipid peroxidation may result in a decrease in MMP as we have observed in the present study. Efficient transfer of electrons in the electron transport system is required for maintenance of MMP. Cardiolipin is known to have a specific and tight association with cytochrome C oxidase, the terminal enzyme complex of the electron transport chain, and is functionally important for activity of this enzyme complex (41). Oxidative damage to cardiolipin has been shown to result in a depression of mitochondrial function (42) and release of cytochrome C into the cytosol (43) which initiates apoptosis. Also, Paradies *et al*. (44) exposed mitochondrial membranes from rat heart to a free radical generating system that resulted in mitochondrial lipid peroxidation and a marked loss of cytochrome C oxidase activity. Exogenous addition of

cardiolipin (but not peroxidized cardiolipin) was able to prevent the loss of cytochrome C oxidase activity. Shidoji *et al*. (43) suggest that peroxidation of cardiolipin in the mitochondrial inner membrane may disrupt a molecular interaction between the membrane phospholipid and the protein. These studies are consistent with the findings of our present study in which fish oil and n-3 PUFA feeding enhanced the translocation of cytochrome C into the cytosol and activated caspase-3, an early event in the apoptotic cascade.

Although production of ROS and increased susceptibility to damage by ROS are generally considered undesirable, our data suggest that the opposite may be true during colon cancer development. This finding may have practical consequences as there is increasing emphasis today on consuming antioxidants as protectants against aging and cancer, which may be misguided advice. A number of studies have now reported that administration of antioxidants can block apoptosis (45). The concept that antioxidant administration may block apoptosis and enhance tumorigenesis was recently tested in an *in vivo* system using a defined transgenic brain tumor model with known tumor apoptosis rates (46). Feeding the mice an antioxidant-poor diet for 4 months significantly reduced the mean tumor diameter by 65%. Reduced tumor burden correlated with an increased rate of tumor cell apoptosis (a 5-fold increase in the percentage of apoptotic cells in tumors of antioxidant-poor mice compared with controls). There was also evidence of increased oxidative stress in brain tumors of mice fed an antioxidant-poor diet relative to controls (46).

Although one should be careful when extrapolating results from rats to humans, of note are the major clinical trials with antioxidant supplements of beta carotene and alpha tocopherol which were halted when the antioxidant intervention arms were shown to significantly enhance the risk of lung cancer in heavy smokers (47,48). It is possible that in healthy humans, antioxidants may protect against a number of diseases. In contrast, once cancer has been initiated, ROS-induced apoptosis may be a protective mechanism to delete transformed cells. In summary, we have shown previously that a combination of fish oil and the butyrate-producing fiber pectin are protective against experimentally induced colon cancer by upregulating apoptosis (15,16). We now propose a mechanism for the upregulation of apoptosis: incorporation of n-3 fatty acids into mitochondrial phospholipids increasing their susceptibility to peroxidation resulting in decreased MMP, release of cytochrome C into the cytosol and activation of caspase-3.

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