An Increase in Reactive Oxygen Species by Dietary Fish Oil Coupled with the Attenuation of Antioxidant Defenses by Dietary Pectin Enhances Rat Colonocyte Apoptosis[1,2]

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ABSTRACT We showed previously that the dietary combination of fish oil, rich in (n-3) fatty acids, and the fermentable fiber pectin enhances colonocyte apoptosis in a rat model of experimentally induced colon cancer. In this study, we propose that the mechanism by which this dietary combination heightens apoptosis is via modulation of the colonocyte redox environment. Male Sprague-Dawley rats ($n = 60$) were fed 1 of 2 fats (corn oil or fish oil) and 1 of 2 fibers (cellulose or pectin) for 2 wk before determination of reactive oxygen species (ROS), oxidative DNA damage, antioxidant enzyme activity [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)] and apoptosis in isolated colonocytes. Fish oil enhanced ROS, whereas the combination of fish oil and pectin suppressed SOD and CAT and enhanced the SOD/CAT ratio compared with a corn oil and cellulose diet. Despite this modulation to a seemingly prooxidant environment, oxidative DNA damage was inversely related to ROS in the fish oil and pectin diet, and apoptosis was enhanced relative to other diets. Furthermore, apoptosis increased exponentially as ROS increased. These results suggest that the enhancement of apoptosis associated with fish oil and pectin feeding may be due to a modulation of the redox environment that promotes ROS-mediated apoptosis. J. Nutr. 134: 3233–3238, 2004.

KEY WORDS: • *apoptosis* • *reactive oxygen species* • *antioxidant enzymes* • *oxidative damage*

Colorectal cancer is anticipated to be the third most frequently diagnosed cancer in the United States this year, and it is predicted that almost half of individuals diagnosed will die from the disease within 5 y (1). Yet many of these cases could be prevented by appropriate diet and lifestyle modifications. Dietary fat and fiber are 2 of the most widely investigated dietary components with respect to colon cancer prevention (2,3). There is substantial evidence that diets rich in (n-3) PUFA, such as those found in fish oil (eicosapentaenoic acid and docosahexaenoic acid), protect against colon carcinogenesis (4,5), whereas diets rich in (n-6) PUFA, such as those found in corn oil, appear to promote cancer development in the colon. However, the chemopreventive abilities of fiber, fermentable and nonfermentable, have been the subject of much debate and have shown varied results in intervention trials and epidemiologic studies (6,7). Poorly fermented fibers, such as cellulose, have been considered protective in their ability to dilute putative carcinogens that may be present in the fecal stream. Yet, the products of highly fermentable fibers (i.e., butyrate) have been shown, at least in vitro, to possess chemopreventive qualities (8). We propose that the inconsistent effect seen with fiber may be attributable to the composition of the fat in the diet (9). Specifically, we have shown that the fermentable fiber, pectin, in combination with fish oil, has a protective effect in multiple stages of colon cancer (4,10).

The protective effect of this diet was shown to be primarily through enhancement of apoptosis, a form of programmed cell death (4,11). However, the mechanism by which the fish oil and pectin diet induces apoptosis has not been clearly elucidated. Recent evidence suggests that reactive oxygen species (ROS)[4] comprise an important mediator of apoptosis [reviewed in (12)]. Considering the degree of unsaturation of (n-3) PUFA in combination with the rapid colonocyte oxidation of butyrate, this protective diet may alter cellular ROS in a manner sufficient to induce apoptosis in the colonocyte. Yet

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[4] Abbreviations used: BSA, bovine serum albumin; CAT, catalase; DTT, dithiothreitol; FLARE, fragment length analysis using repair enzymes; fpg, formamidopyrimidine-DNA glycosylase; GPx, glutathione peroxidase; 8-OHdG, 8-hydroxydeoxyguanosine; ROS, reactive oxygen species; SOD, superoxide dismutase; TUNEL, dUTP nick end-labeling.

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ROS can also damage and potentially mutate DNA (13); therefore, cells employ several defenses against ROS including antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Although these enzymes are key players in preventing cellular damage caused by endogenous ROS (14,15), overexpression or addition of these enzymes to tissue systems has been shown to enhance tumorigenesis and block the action of several chemotherapeutic drugs by suppressing ROS-induced apoptosis (16,17). The ability of diet to modulate antioxidant enzyme expression and activity has been documented (18,19). However the ability of diet to simultaneously influence additional redox factors, such as ROS generation, in the colon has not been characterized. This investigation evaluates the ability of dietary lipid and fiber to alter the oxidative status of rat colonocytes, via ROS generation and modulation of antioxidant enzyme activity, thus creating an environment permissive for apoptosis.

MATERIALS AND METHODS

Animals and diets. Animal use was approved by the University Animal Care Committee of Texas A&M University and conforms to NIH guidelines. Male weanling (28-d old) Sprague-Dawley rats (n = 60; Harlan Sprague Dawley) were housed individually in raised wire cages to diminish coprophagy and maintained in a temperature- and humidity-controlled animal facility with a daily photoperiod of 12 h light and 12 h dark. The rats were stratified by body weight and assigned to 1 of 4 experimental diets (n = 15 rats/diet) as previously described (20). Experimental diets were consumed for 2 wk. This study represents a 2 × 2 factorial design with 2 types of fat (corn oil or fish oil) and 2 types of fiber (cellulose or pectin). Dietary composition included 15% dietary fat by weight (30% energy from fat) and 6% dietary fiber by weight (equivalent to 30 g fiber/d in a human diet). The fish oil diet contained 3.5 g of corn oil/100 g diet to prevent essential fatty acid deficiency (21). The types of fiber were chosen on the basis of their fermentability, with cellulose being poorly fermentable and pectin being highly fermentable (22). Corn oil and fish oil were analyzed for peroxide value (3.3 mEq/kg corn oil and 3.4 mEq/kg fish oil, respectively), fatty acid composition, and antioxidant composition. To ensure equal antioxidant content in all diets, fish oil was supplemented with α-tocopherol, γ-tocopherol, and *tert*-butyl hydroquinone equal to the levels found in corn oil. Food and water were freely available. To minimize fatty acid oxidation, diets were stored at −80°C and fresh food was provided every 24 h. Food intake and body weights were measured weekly.

Tissue collection and cell isolation. After the rats were killed by CO_2 asphyxiation and cervical dislocation, the colon was removed and flushed with warm Ca^{2+}- and Mg^{2+}-free PBS (GibcoBRL). For each rat, the last half of the colon was taken as the distal colon. The last centimeter of the distal colon was taken for histology and fixed in 4% paraformaldehyde for 4 h, followed by washing in 50 and 70% ethanol. The remaining colon segment was cut longitudinally to expose the lumen and placed in warm Ca^{2+}- and Mg^{2+}-free HBSS, 30 mmol/L EDTA, 5 mmol/L dithiothreitol (DTT), 0.1% fatty acid–free bovine serum albumin (BSA; wt/v), 1 mmol/L glutamine, and 1 mmol/L butyrate (pH 7.4). After a 15-min shaking incubation, the mucosal side was gently scraped with a rubber policeman. This procedure is designed to remove intact crypts and surface cells leaving behind the lamina propria (23). Removal of crypts and surface cells was confirmed by histological examination of the remaining intestinal tissue after the scraping procedure. The isolated crypts were then centrifuged at 100 × g and washed twice in warm HBSS containing Ca^{2+}, Mg^{2+}, 0.1% BSA (wt/v), 1 mmol/L glutamine, and 1 mmol/L butyrate. An aliquot of cells from the distal colon was taken for antioxidant enzyme analysis and fragment length analysis using repair enzymes (FLARE).

Measurement of apoptosis using the dUTP nick end-labeling (TUNEL) assay. Paraffin sections of the 4% paraformaldehyde fixed tissues were utilized for in situ measurement of apoptosis using ApopTag kits (Intergen) as previously described (24). This technology is based on the terminal deoxynucleotidyl transferase-mediated TUNEL technique. Intestinal crypts were scored according to positive staining by DAB and morphological criteria previously described by Kerr et al. (25). The apoptotic index for each crypt was determined by dividing the number of apoptotic cells in a crypt column by the crypt column height (number of cells). The mean apoptotic index of 25 crypts within a tissue was used as the apoptotic index for that tissue.

Measurement of antioxidant enzyme activity. Activities of CAT, GPx, and SOD in isolated colonocytes were measured spectrophotometrically using commercial assay kits (Calbiochem). Cell lysates were prepared by homogenization of cells in 50 mmol/L potassium phosphate buffer [250 mmol/L sucrose, 1 mmol/L EDTA, 1 mmol/L DTT, 0.1% Triton X-100 (v/v)] followed by centrifugation for 3 min at 10,000 × g. The supernatant was used for enzyme assays following protocols provided in each kit. Briefly, SOD activity was determined by measuring the rate of generation of a chromophore at 525 nm. CAT activity was determined by measuring the absorbance of quinoneimene dye at 520 nm. GPx was determined indirectly by oxidation of NADPH to $NADP^+$ measured at 340 nm using H_2O_2 as the preferred substrate. Sodium azide (NaN_3) was used to inhibit catalase competition for H_2O_2. Samples were analyzed in triplicate in 96-well microplates with standards provided in kits or purchased separately from Calbiochem. Microplates were read on a Spectra Max 250 microtiter plate reader using SoftMax Pro v.1.2 software (Molecular Devices). Activity was normalized to protein concentration as determined by Coomassie Blue assay (Pierce).

Detection of reactive oxygen species. Samples of isolated colonocytes (maintained at 37°C) were prepared in duplicate and incubated for 15 min with chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes), a fluorescence probe sensitive to such cellular oxidants as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^\bullet$), and peroxyl radicals ($OOH^\bullet$). This probe passively diffuses into cells and upon oxidation by ROS forms a fluorescent adduct that remains trapped in the cell. Fluorescence was monitored on a Meridian Ultima confocal microscope (Meridian Instruments) with a 530-nm barrier filter and laser excitation at 488 nm, as previously described (26). Fluorescence intensity was used as an indirect measure of prevalence of ROS. Data for each sample were collected from 15 fields/treatment for each rat. Viability of the cells used for analysis was determined after each treatment by staining with ethidium homodimer-1 (Molecular Probes). Mean viability was 81 ± 4.5% (n = 60).

Measurement of oxidative DNA damage using the FLARE assay. This assay is a modification of single-cell gel electrophoresis, which uses *Escherichia Coli* formamidopyrimidine-DNA glycosylase (fpg) to introduce DNA strand breaks specifically at 8-hydroxydeoxyguanosine (8-OHdG) adducts (27,28), a prevalent and potentially mutagenic oxidative DNA adduct (29). This process measures the levels of 8-OHdG on a single-cell level in intact nuclei (30). 8-OHdG adducts were quantified using a comet assay kit (Trevigen). After isolation, crypts were kept at 4°C for the entire procedure to minimize DNA repair and were dispersed into single cells by repeated aspiration through a 27-gauge needle and plated with agarose in duplicate on comet slides. Slides were exposed to lysis buffer (1% sodium lauryl sarcosinate (v/v), 2.5 mol/L NaCl, 100 mmol/L EDTA, 1% Triton X-100 (v/v), 10 mmol/L Tris base, pH 10) to remove the outer cell membrane leaving only intact nuclei. Slides were then immersed in 1X FLARE buffer (10 mmol/L HEPES-KOH, 100 mmol/L KCl, pH 7.4) followed by the addition of the fpg enzyme (Trevigen) diluted 1:50 with reaction buffer (25X FLARE buffer, 100X BSA). Control slides received only reaction buffer without the fpg enzyme. After treatment with alkali solution (1 mmol/L EDTA, pH 12.5) to denature DNA strands, slides were exposed to electrophoresis (1 V/cm, 20 min) and immersed briefly in 70% ethanol (v/v). Nuclei were viewed by epifluorescence microscopy using SYBR green staining (Molecular Probes). Quantitation of the relative tail moment [tail moment/(tail moment + head moment)] (31) was measured using Metamorph software (Nikon, Garden City, NY). One hundred randomly selected cells were analyzed per treatment group for each rat.

Statistical analysis. Analyses of ROS, antioxidant enzyme activity, and oxidative DNA damage were performed by mixed model ANOVA using SAS 8.0 (SAS Institute). Apoptotic indices were analyzed with Poisson regression using Proc Genmod. The relation between antioxidant enzymes and ROS was determined by regression analysis in SPSS. The relation between ROS and oxidative DNA damage was examined by regression analysis using the generalized estimation equation approach with an identity link (32). The covariates considered in the model included the combinations of oil and fiber as well as oxidative DNA damage nested within these treatment combinations. To explain the relation of ROS and apoptotic index, the linear relation between the log-transformed apoptotic index and ROS levels was modeled. To reduce the influence from potential outlying observations, a robust regression using Huber's weight function was performed (33). Differences were considered significant at $P < 0.05$.

RESULTS

Food intake and body weight gain. There were no significant differences in food intake or body weight gain among the experimental groups (results not shown).

Apoptosis. The combination of dietary fish oil and pectin yielded a greater apoptotic index compared with the other experimental diets ($P < 0.008$) (**Fig. 1**). These results are in agreement with previous findings from our laboratory in which a diet of fish oil and pectin enhanced apoptosis during experimentally induced colon cancer (4,11).

Antioxidant enzyme activity. The activity of CAT in colonocytes was lower ($P < 0.006$) in diets containing pectin as the fiber source compared with cellulose (results not shown). Furthermore, the combination of fish oil with pectin resulted in 75% less CAT activity ($P < 0.003$) and 35% less SOD activity ($P < 0.05$) than a diet containing corn oil and cellulose (**Fig. 2**A). There was no diet effect on GPx. Because SOD and CAT act sequentially in a pathway of ROS elimination, we expressed the data as enzyme activity ratios. In diets containing pectin as the fiber source, the mean ratio of SOD/CAT was greater relative to the cellulose-containing diets (SOD/CAT = 1.7 and 0.7, respectively) ($P < 0.02$). A similar trend ($P = 0.06$) was seen with respect to dietary fish oil (SOD/CAT = 1.6) and corn oil (SOD/CAT = 1.0). When the combination of lipid and fiber was considered, as expected, the combination of fish oil and pectin yielded a greater SOD/CAT ratio (2.2) than the combination of corn oil and cellulose (0.6) and fish oil and cellulose (0.9) ($P < 0.02$) (Fig. 2*B*).

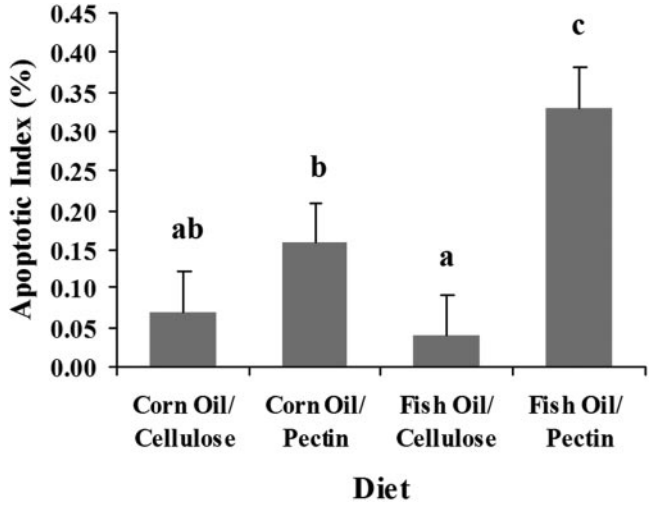

FIGURE 1 Rats fed fish oil and pectin displayed a greater apoptotic index compared with those fed corn oil/cellulose, corn oil/pectin, and fish oil/cellulose. Data are means ± SEM from 25 crypts for $n = 15$ rats/diet. The apoptotic index represents the total number of apoptotic cells in a crypt column/total number of cells in the crypt column. Bars not sharing a letter differ, $P < 0.008$.

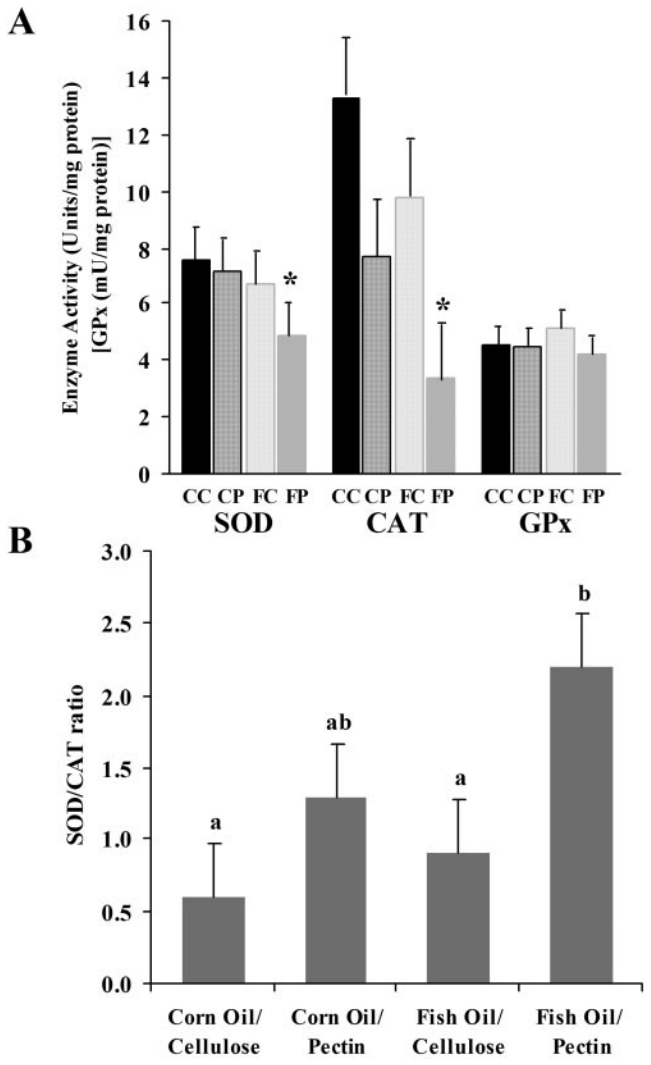

FIGURE 2 CAT and SOD activity are diminished and SOD/CAT is elevated in rat colonocytes by dietary fish oil in combination with pectin. Data are means ± SEM from $n = 10$ rats/diet with samples read in triplicate. *Different from CC (*panel A*): SOD, $P < 0.05$; CAT, $P < 0.003$. Bars not sharing a letter in *panel B* differ, $P < 0.02$. Abbreviations: CC = Corn oil/Cellulose, CP = Corn oil/Pectin, FC = Fish oil/Cellulose, FP = Fish oil/Pectin.

Reactive oxygen species. ROS levels in colonocytes were greater in rats consuming fish oil compared with corn oil ($P < 0.02$, **Fig. 3**). Dietary fiber did not significantly alter ROS levels, nor did the combination of oil and fiber.

Relation between ROS, apoptosis, and oxidative DNA damage. Regardless of diet, there was a strong exponential relation between ROS and apoptosis (**Fig. 4**). As the levels of ROS rose in rat colonocytes, the apoptotic index rose exponentially ($P < 0.005$). Across all diets, there was no difference in overall levels of oxidative DNA damage as determined by quantification of 8-OHdG adducts. However, upon examination of the relation of oxidative DNA damage to ROS levels within a rat, there were distinct diet differences (**Fig. 5**). In rats consuming the fish oil and pectin diet, oxidative DNA

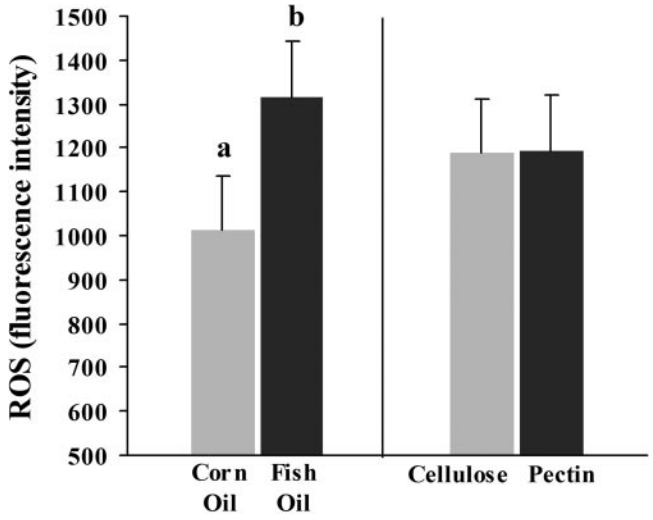

FIGURE 3 Dietary fish oil elevates ROS generation in rat colonocytes. All samples were prepared in duplicate. Data are main effect means ± SEM, $n = 15$ rats/oil or fiber group with 15 fluorescent images captured/rat. Bars not sharing a letter differ within that panel, $P < 0.05$.

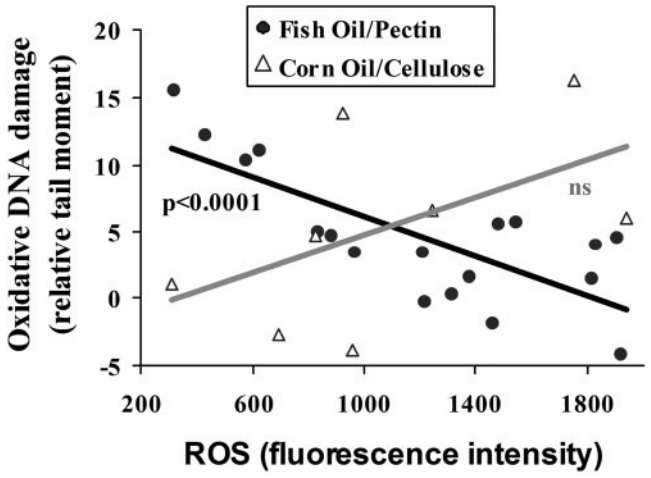

FIGURE 5 Oxidative DNA damage was inversely associated with ROS production in colonocytes from rats fed the fish oil pectin diet. Data are from $n = 26$ rats with 100 cells/rat analyzed for oxidative DNA damage and 25 crypts/rat for apoptosis. The apoptotic index was calculated as the total number of apoptotic cells in a crypt column/total number of cells in the crypt column. The fish oil pectin correlation was significant ($P < 0.0001$, $r = 0.75$). The corn oil cellulose correlation was not significant ($r = 0.53$). The slopes of the 2 lines differed, $P < 0.0019$.

damage was inversely related to ROS level ($P < 0.0001$), indicating that as the level of ROS increased, there was a decrease in the level of oxidative DNA damage. In contrast, rats consuming the other experimental diets did not exhibit this inverse relation. In fact, the relation of ROS and oxidative DNA damage in the corn oil and cellulose diet was significantly different from the fish oil and pectin diet ($P < 0.002$).

DISCUSSION

Apoptosis has been shown to be one of the most critical control processes in cancer prevention and treatment (34). In fact, induction of apoptosis is the primary mode of action for most chemotherapeutic drugs and radiation. Therefore, the ability of dietary fish oil in combination with pectin to enhance apoptosis, as shown here and in previous studies (4,5), may be a critical mechanism by which this diet is able to prevent colon cancer. The specific proapoptotic actions of dietary fish oil and pectin are not well characterized; however, the initiation and regulation of apoptosis appears to be intimately associated with modifications in the oxidative environment (12,35). The balance of ROS generation and antioxidant capacity within the cell determines the oxidative environment. When ROS exceed the antioxidant capacity of the cell, oxidative stress results. Oxidative stress can initiate and/or mediate a number of signaling cascades, including apoptosis. Thus, alterations in the cellular redox balance may comprise a potential mechanism whereby fish oil and pectin initiate apoptosis. We showed previously that dietary fish oil enhances ROS generation in colonocytes (36); however, we did not consider the antioxidant response. The intent of this study was to use primary cultures from an in vivo rat model to determine the ability of dietary fish oil and pectin to modulate cellular ROS and antioxidant capacity to promote apoptosis.

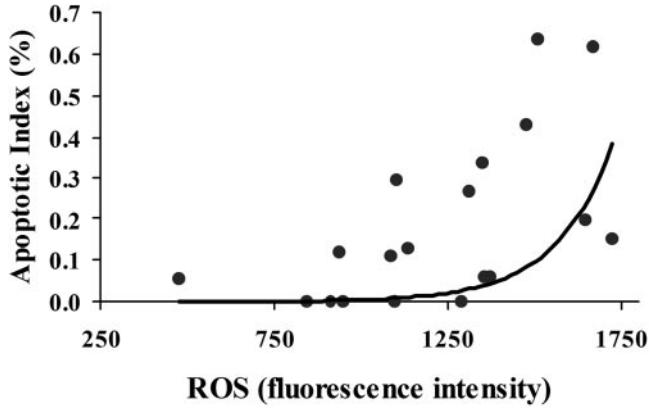

FIGURE 4 Elevation in ROS corresponds to the exponential increase in apoptotic index in rat colonocytes. Data are from $n = 20$ rats (10 fish oil/pectin, 10 corn oil/cellulose) with 15 fluorescent images captured/rat for ROS and 25 crypts/rat for apoptosis. The apoptotic index was calculated as the total number of apoptotic cells in a crypt column/total number of cells in the crypt column. Equation for the exponential relationship is $y = \exp(-1.9611 + 0.0011 \times \text{ROS}) - 0.25$.

Important determinants of cellular antioxidant capacity are the enzymes SOD, CAT, and GPx, which are responsible for the elimination of ROS. Because these enzymes act sequentially to remove ROS, the balance of the activity of these enzymes may be as critical in the defense against ROS as the activity of the enzymes alone (37). Data from this experiment showed that the activity of these enzymes, especially CAT, is influenced by dietary fiber. Specifically, antioxidant enzyme activity in colonocytes from rats fed a pectin diet was less than that observed in those fed the cellulose diets. Furthermore, the combination of fish oil with pectin in the diet resulted in even lower activity for CAT and SOD. Interestingly, the difference in CAT activity was greater than the change seen with SOD, suggesting a possible enzyme activity imbalance. SOD converts superoxide ($O_2^{\bullet -}$) to H_2O_2, which is then converted to water and/or O_2 by CAT and GPx. Thus, dramatically diminished CAT activity coupled to only a subtle reduction in SOD (an increase in SOD/CAT) may yield a system that can no longer eliminate H_2O_2 at the rate it is formed. Indeed, dietary pectin and to a lesser extent dietary fish oil elevate the SOD/CAT ratio compared with cellulose and corn oil, respectively. Furthermore, the greatest enhancement in SOD/CAT

was seen when fish oil and pectin were combined in the diet. Similar experiments in rat colonocytes found that dietary fish oil reduces antioxidant enzyme activity (18), and a recent dietary intervention trial showed that diets high in fiber and (n-3) PUFA are capable of reducing antioxidant enzyme activity in humans (19). Although the mechanism by which these dietary constituents achieve a reduction in enzyme activity is unclear, these results suggest that dietary lipid and dietary fiber, specifically fish oil and pectin, work coordinately to alter antioxidant enzyme activity and balance in a manner that may create a prooxidant environment in the colonocytes.

ROS measurements further suggest that dietary fish oil may create a more oxidative environment in the colonocytes compared with a corn oil diet. Diets with fish oil as the lipid source enhanced ROS generation in the colonocytes. This is not unexpected considering the high degree of unsaturation found in the long-chain (n-3) PUFA in fish oil. The primary (n-3) fatty acids in fish oil, eicosapentaenoic acid [20:5(n-3)] and docosahexaenoic acid [22:6(n-3)] have up to 3 times as many double bonds per molecule than the (n-6) fatty acids found in corn oil, such as linoleic acid [18:2(n-6)]. This increases the opportunity for oxidant attack and can contribute to the propagation of ROS. Furthermore, we have shown dietary (n-3) PUFA to be readily incorporated into the mitochondrial membrane (38), predisposing the mitochondria to enhanced lipid peroxidation and membrane damage and contributing to the propagation of ROS generated by the mitochondrial electron transport system (36). It is likely that many of the fatty acids consumed in these experimental diets maintained a high degree of unsaturation because the diets were kept at −80°C to prevent fatty acid oxidation. However, these conditions may not be practical outside of the laboratory setting, and less than optimal storage may enhance the degree of fatty acid oxidation before consumption. This may have a substantial impact on the physiologic effects of dietary lipid and definitely warrants further attention.

Although dietary fish oil and pectin alter the antioxidant capacity and ROS generation of colonocytes to favor a prooxidant environment, the outcome of these diet-induced cellular modifications is critical. An oxidative environment may favor apoptosis, or alternatively, may increase the potentially mutagenic event of damaging DNA. Therefore, it was important to determine apoptosis and oxidative DNA damage in the same rats in which the diet-induced changes in ROS and antioxidant enzyme activity were measured. In the fish oil/pectin diet, apoptosis was enhanced and the oxidative environment created by this diet was associated with a decline in oxidative DNA damage. Alternatively, in the corn oil/cellulose diet, apoptosis was less than in the fish oil/pectin diet and as ROS increased, oxidative damage did not decrease as in the fish oil/pectin diet. Thus, the suppression of oxidant protection systems and enhancement of ROS generation by dietary fish oil and pectin appears to protect the colon against oxidative DNA damage by promoting ROS-mediated apoptosis. However, continued investigation is required to further elucidate the multifaceted relation between cellular redox status and apoptosis. Additional investigation is also warranted to determine whether these dietary modifications of the redox environment and the resulting increase in apoptosis continue during the initiation and progression stages of colon carcinogenesis. Although we showed previously that apoptosis was enhanced by dietary fish oil and pectin during the initiation (11) and progression (4) of colon carcinogenesis, whether the mechanism is associated with alteration of the redox environment remains to be determined.

In summary, dietary fish oil and pectin work coordinately to enhance colonocyte apoptosis by modulation of the cellular redox environment. In this ex vivo model, we showed that dietary fish oil enhanced ROS in colonocytes, whereas the effects of dietary pectin were more clearly seen in the attenuation of antioxidant enzyme activity. Therefore, it is not surprising that the combination of these dietary constituents worked in concert to create an environment permissive for apoptosis, thereby protecting cells from severe and possibly mutagenic DNA damage. This study reinforces the importance of diet for the prevention of cancer and strengthens the growing realization that the effects of individual diet components may not be as important as the combination of foods consumed in the diet. Further investigations should evaluate the influence of other components of the diet matrix as well as the effect of alterations to diet components (e.g., fatty acid oxidation before consumption).

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