

## Dietary Fish Oil Promotes Colonic Apoptosis and Mitochondrial Proton Leak in Oxidatively Stressed Mice

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### Abstract

An alteration of mitochondrial function can result in disruption of redox homeostasis and is associated with abnormal cancer cell growth. Manganese superoxide dismutase (SOD2) and glutathione peroxidase 4 (Gpx4) are two of the most important antioxidant defense enzymes that protect cells against oxidative stress. We had previously shown that n-3 polyunsaturated fatty acids (PUFA) promote colonocyte apoptosis, a marker of colon cancer risk, in part by enhancing phospholipid oxidation. To elucidate the mechanisms regulating oxidative stress-induced apoptosis *in vivo*, we fed heterozygous SOD2<sup>Het</sup>, Gpx4<sup>Het</sup>, and transgenic Gpx4<sup>Tg</sup> mice diets containing either 15% corn oil by weight (CO, enriched in n-6 PUFA) or 3.5% CO + 11.5% fish oil (FO, enriched in n-3 PUFA) for 4 weeks. Our data showed that (i) genetic predisposition to oxidative stress facilitates apoptosis in the mouse colon (Gpx4<sup>Het</sup> > SOD2<sup>Het</sup> > Wt > Gpx4<sup>Tg</sup>), (ii) dietary n-3 PUFA have an additive effect on the induction of apoptosis in Gpx4<sup>Het</sup> and SOD2<sup>Het</sup> mice; and (iii) dietary n-3 PUFA reverse the phenotype in oxidatively protected Gpx4<sup>Tg</sup> mice by elevating apoptosis to a level observed in wild-type (Wt; control) animals. Complimentary experiments examining colonic mitochondrial bioenergetic profiles indicate that FO-fed mice exhibit a significantly ( $P < 0.05$ ) increased respiration-induced proton leak relative to control CO treatment. This finding was consistent with a loss of membrane potential in response to chronic oxidative stress and supports the contention that n-3 PUFA alter mitochondrial metabolic activity, thereby enhancing apoptosis and reducing colon cancer risk. *Cancer Prev Res*; 4(8); 1267–74. ©2011 AACR.

### Introduction

Colorectal cancer is the third most common cause of cancer death in men and women with an estimated incidence in the United States of 142,570 and a mortality rate of 51,370 in 2010 (1). Due to the fact that successful treatment modalities for this malignancy are still limited, significant attention is currently being directed to preventive dietary programs which may interfere with the process of colon carcinogenesis at all stages, thereby improving patient survival.

Colonic epithelial cell homeostasis is maintained by the balance between cell proliferation and apoptosis. Because apoptosis is considered to be a useful marker of colorectal cancer risk (2, 3), higher levels are consistent with the enhanced removal of damaged cells, which is thought to prevent clonal expansion and reduce colorectal cancer risk in both animal models (2, 4) and humans (5, 6). We have shown that animals fed fish oil (FO), enriched in n-3 polyunsaturated fatty acids (n-3 PUFA), for example, docosahexaenoic acid (DHA), 22:6<sup>Δ4,7,10,13,16,19</sup> and eicosapentaenoic acid (EPA), 20:5<sup>Δ5,8,11,14,17</sup>, trigger the induction of apoptosis in the mouse/rat colon (2, 4, 7, 8). The proapoptotic effect of n-3 PUFA has also been observed in humans (9, 10).

With respect to molecular targets of n-3 PUFA, mitochondria are key organelles capable of regulating the intrinsic apoptotic pathway and mediating cell death in pathologic and stress conditions. We have recently shown that n-3 PUFA promote apoptosis in part via the generation of reactive oxygen species (ROS) such as superoxide/hydrogen peroxide, and in particular, membrane phospholipid hydroperoxides (PLOOH), which disrupt the mitochondrial permeability transition pore, enhance mitochondrial Ca<sup>2+</sup> accumulation and trigger the release of soluble intermembrane proteins (11–13). The production of ROS/PLOOH in mitochondria is strictly regulated by multiple

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antioxidant systems, that is, mitochondrial phospholipid hydroperoxide glutathione peroxidase (Gpx4), classical glutathione peroxidase, and manganese-dependent superoxide dismutase (SOD2). Among these mitochondrial antioxidant enzymes, Gpx4 and SOD2 are 2 of the essential enzymatic defense systems against oxidative damage to membrane constituents (14). Specifically, SOD2 transforms toxic superoxide, a byproduct of the mitochondrial electron transport chain, into hydrogen peroxide and diatomic oxygen. Gpx4 catalyzes the reduction of hydrogen peroxide, organic hydroperoxides, and most specifically, lipid hydroperoxides.

Due to the fact that oxidative stress is a critical regulator capable of promoting apoptosis in cancer cells (15), we hypothesized that the genetic modification of antioxidant enzymes (SOD2, Gpx4) would alter cell death-mediated responses in the colon. Since the potential for diet to modulate oxidative stress-induced apoptosis in the colon has not been clearly defined *in vivo*, in this study, haploinsufficient heterozygous Gpx4<sup>Het</sup> (16) and SOD2<sup>Het</sup> (17, 18) mice which exhibit increased mitochondrial oxidative stress, along with Gpx4<sup>Tg</sup> mice which exhibit decreased mitochondrial oxidative stress (19), were utilized to further elucidate the mechanisms of oxidative stress-mediated apoptosis induced by n-3 PUFA.

## Materials and Methods

### Animals and diets

SOD2<sup>Het</sup> (B6.129S7-Sod2<sup>tm11Eb</sup>/J) mice were originally purchased from Jackson Labs and backcrossed to C57BL/6 mice for 10 generations (17). Gpx4<sup>Het</sup> and Gpx4<sup>Tg</sup> mice were originally generated by Q. Ran at the University of Texas Health Science Center San Antonio and backcrossed to C57BL/6 mice for 10 generations (16, 19). All procedures followed the guidelines approved by Public Health Service and the Institutional Animal Care and Use Committee at Texas A&M University. Mice were genotyped at 3 to 4 weeks of age for SOD2 and Gpx4 status by PCR. Animals enrolled into the study were maintained for 4 weeks on a semipurified defined diet containing 320 (g/kg diet) sucrose, 200 casein, 220 corn starch, 3 DL-methionine, 35 AIN 76 salt mix, 10 AIN 76 mineral mix, 2 choline chloride, 60 cellulose, 150 corn oil (CO; Dyets) or 115 vacuum-deodorized menhaden FO (Omega Protein) plus 35 CO. The major differences regarding the fatty acid compositions of the 2 lipid sources were the concentrations of EPA (11%) and DHA (8%) in the FO diet and the concentration of linoleic acid (18:2<sup>Δ9,12</sup>) in the CO diet (57%). At the end of feeding period, animals were euthanized and the colon was collected. One centimeter of the distal colon was removed, fixed in 4% paraformaldehyde for 4 hour, followed by a series of ethanol washes, then embedded in paraffin. In complementary studies, mucosa was scraped from the colon and mitochondria were isolated and immediately used for metabolic analyses.

### Genotyping

Genomic DNA was extracted from 0.5 cm of mouse tail by using a Qiagen DNA Tissue Kit (Qiagen, Inc.). PCR was done by using a Platinum Taq Polymerase Kit (Gibco BRL). SOD2 mouse genotyping was described previously (8). Gpx4<sup>Tg</sup> genotype was confirmed by the 299 bp PCR product of human Gpx4 transgene (primers: 5'-GAACCTCACCAAGGTAAGGGGGCTGTG-3'; 5'-CCTTCTCTATCACCTGTCGGGGAGG AA-3'). Gpx4<sup>Het</sup> genotype was confirmed by amplifying the 880 bp PCR product of hypoxanthine-guanine phosphoribosyltransferase in addition to the 310 bp PCR product (internal standard; primers: 5'-CTACGGTGAGTAGGTAGA-3', 5'-GGCCCTGGTTTC TATGTA-3', 5'-GTAGGATATGCCCTTGAC T-3'). All primers were synthesized by the Gene Technologies Laboratory at Texas A&M University (College Station, TX).

### Phenotyping by Western blot

Colonic mucosa was homogenized in ice-cold lysis buffer containing 0.1% SDS and subjected to polyacrylamide gel electrophoresis in 4% to 20% precast mini gels as per the method of Laemmli (20). After electrophoresis, proteins were electroblotted onto a polyvinylidene difluoride membrane by using a Hoefer Mighty Small Transphor Unit (Pharmacia) at 400 mA for 1.5 hours. Following transfer, the membrane was processed and blocked in 4% nonfat dry milk and 0.1% Tween 20 in PBS at room temperature for 1 hour with shaking, followed by incubation with shaking overnight at 4°C with primary antibody (rabbit anti-Gpx4 antibody, generated by using a 17 amino acid peptide corresponding to the C terminus of Gpx4 protein as antigen) diluted in PBS containing 4% milk and 0.1% Tween 20. Membranes were washed with PBS containing 0.1% Tween 20 and incubated with secondary antibody (peroxidase-conjugated goat anti-rabbit IgG, Kirkegaard & Perry Laboratories, Inc.) as per manufacturer's instructions. Blots were scanned by using a Fluor-S Max MultiImager System (Bio-Rad).

### Measurement of apoptosis

Apoptotic cells were enumerated in paraformaldehyde-fixed sections from the distal colon by using a terminal deoxynucleotidyl transferase (TUNEL) labeling kit (Trevigen) as we have previously described (8). The number of apoptotic cells was recorded in at least 100 well-oriented crypts per mouse. Data were calculated as percentage of apoptosis index relative to the control (wild-type mice fed the control CO diet) in the respective transgenic animal models.

### Immunohistochemistry

Immunohistochemical staining for 8-hydroxy-2'-deoxyguanosine (8-OHdG) was done by using the avidin-biotin-peroxidase complex (ABC) method as described previously (21). In brief, the specimens embedded in paraffin were cut at 3 μm thickness, stained with hematoxylin and eosin or used for immunohistochemistry analysis. Representative

areas were chosen and cores of 3 mm diameter were punched out from the blocks with a precision instrument (Tissue Microprocessor; Azumaya). Cores of 24 (6 × 4 array) in a group were embedded in a paraffin block to avoid interspecimen immunostaining condition. Endogenous peroxidase activity was quenched in paraffin-embedded tissue sections with 1% H<sub>2</sub>O<sub>2</sub>. Antigen was retrieved by pretreatment with citrate buffer (antigen unmasking solution, Vector Laboratories) in a microwave oven at 37°C for 1 hour. Nonspecific protein-protein interactions were blocked with diluted rabbit serum, the slides were incubated with primary antibody (10 µg/mL for anti-8-OHdG N45.1 antibody), followed by biotinylated rabbit secondary antibody and the ABC complex for 45 minutes. 3,3'-diaminobenzidine (DAB) was used as the chromagen. Slides were washed thoroughly between incubations with PBS. Negative controls were established by replacing the primary antibody with PBS and serum. Positive staining was indicated by the presence of brown-colored precipitate. The colonic epithelial cells in immunostained specimens were evaluated by 2 registered pathologists (S.T. and Y.O.) as negative, weak, moderate, or intense (0, 1, 2, or 3, respectively). The means of the evaluation of the 2 pathologists were used for the semi-quantitative analysis.

#### Mitochondrial bioenergetic analysis

For these studies, mice were euthanized at the end of a 4-week feeding period, colon mucosa removed, and mitochondria immediately isolated by using a Mitochondrial Fractionation Kit (Active Motif). Mitochondrial bioenergetic profiles were immediately measured by using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience) as described by Wu and colleagues (22) with some modifications. Briefly, mitochondria were resuspended in mitochondrial assay buffer (MAS-1 buffer) containing 70 mmol/L sucrose, 220 mmol/L mannitol, 10 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 5 mmol/L MgCl<sub>2</sub>, 2 mmol/L HEPES, 1 mmol/L EGTA, 0.2% FA-free bovine serum albumin, and pH to 7.4 by using KOH. Mitochondria were aliquoted into Seahorse XF 24-well cell culture plates (10 µg/50 µL/well), centrifuged at 2,000 × g at 4°C for 10 minutes to facilitate mitochondria attachment to the plates. A total of 450 µL of 1.1× initiation assay buffer (MAS-1 buffer supplemented with 5.5 mmol/L succinate and 2.2 µmol/L rotenone) was added to each well immediately following centrifugation. Culture plates (containing attached mitochondria) were subsequently incubated in a non-CO<sub>2</sub> environment at 37°C for 8 minutes. Following incubation, XF 24-well culture plates were transferred to the XF24 Extracellular Flux Analyzer. Hydrated cartridges containing mitochondrial mediators, ADP (1 mmol/L), Oligomycin (2 µmol/L), FCCP (carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone; 4 µmol/L), and antimycin A (4 µmol/L) were layered over the 24-well plates. The compounds were injected at timed intervals into the samples, and the oxygen consumption rates (OCR) were monitored continuously.

#### Cardiolipin peroxidation

10-*N*-nonyl-acridine orange (NAO; Molecular Probes), binding to mitochondria, was used to estimate cardiolipin (CL) peroxidation as previously described (23), with some modifications. Freshly isolated mitochondria from colon mucosa was resuspended in a mixture (1:9, v/v) of MAS-1 buffer and buffer A (125 mmol/L KCl, 10 mmol/L HEPES, 5 mmol/L MgCl<sub>2</sub>, and 2 mmol/L K<sub>2</sub>HPO<sub>4</sub>, pH 7.4). A total of 150 µL of 20 µmol/L NAO was then added to each mitochondrial sample (10 µg/50 µL), gently mixed and incubated for 10 minutes at 25°C, followed by centrifugation at 20,000 × g for 10 minutes at 4°C. Free dye in the supernatant was determined by measuring absorbance at 495 nm, and the NAO bound to mitochondria was calculated as the total minus-free NAO.

#### Statistics

The Brown-Forsythe's test of homogeneity of variance was conducted to ensure that the variances between the treatment groups were equal. A 2-way ANOVA was used for analysis, followed by the least-squares means test for multiple comparisons. Data are expressed as means ± SE. Statistical significance was set at  $P < 0.05$ .

#### Results

##### Genotyping and functional status of Gpx4 in transgenic and heterozygous mice

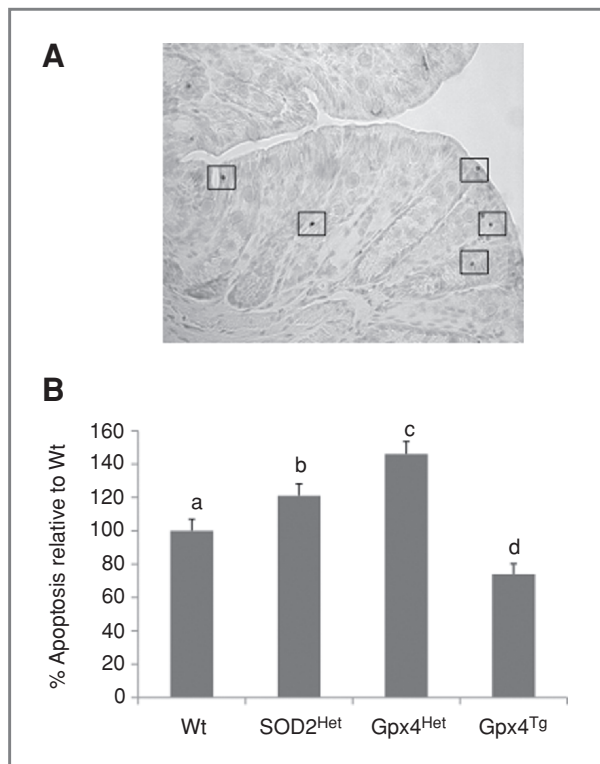
To confirm that Gpx4-targeted deletion resulted in the anticipated reduction in Gpx4 protein expression, lysates from colonic mucosa were probed by using antibodies recognizing Gpx4. As shown in Supplementary Figure S1, Gpx4<sup>Tg</sup> mice expressed higher, whereas Gpx4<sup>Het</sup> mice exhibited lower (almost nondetectable), Gpx4 expression in both the colon and liver compared with their respective wild-type siblings. The effect of SOD2 haploinsufficiency on colonic mucosa in SOD2<sup>Het</sup> mice has been previously described (8). There was a significant main effect of diet on animal body weights, with FO mice weighing significantly ( $P = 0.01$ ) less than CO-fed mice (5.8 vs. 10.4 g,  $n = 20$ ).

##### Genetic modification of antioxidant enzymes alter the induction of apoptosis in mouse colon

Regardless of dietary treatment, genetic modification alone modulated the levels of colonic apoptosis. As shown in Figure 1, both SOD2<sup>Het</sup> and Gpx4<sup>Het</sup> mice exhibited significantly ( $P < 0.05$ ) higher levels of apoptosis relative to control wild-type mice. In contrast, Gpx4<sup>Tg</sup> mice had significantly ( $P < 0.05$ ) lower levels of apoptosis compared with their wild-type siblings.

##### The proapoptotic effect of SOD2 or Gpx4 deletion is enhanced by dietary n-3 PUFA

Both allelic ablation of SOD2 ( $P = 0.04$ ) and dietary n-3 PUFA ( $P = 0.021$ ) enhanced apoptosis in the colon (Fig. 2A). Specifically, FO-fed mice exhibited a 22% increase in apoptosis relative to CO-fed mice. Similarly, apoptosis was increased by 19% in heterozygous compared



**Figure 1.** Effect of genetic modification of antioxidant enzymes on the induction of apoptosis in the colon. SOD2<sup>Het</sup>, Gpx4<sup>Het</sup>, Gpx4<sup>Tg</sup>, and respective Wt siblings were fed CO diet (control, enriched in n-6 PUFA) or FO diet (enriched in n-3 PUFA) for 4 weeks. Apoptosis in the distal colon was measured by using the TUNEL assay. A, representative TUNEL micrograph. DAB stained spots indicate apoptotic cells. B, apoptotic index was calculated as the total number of apoptotic cells per 100 crypts. Data are presented as % apoptotic index relative to respective Wt siblings (mean  $\pm$  SE, all diet groups pooled,  $n = 28-42$ ). Bars not sharing the same letters are significantly different,  $P < 0.05$ .

with wild-type mice. Overall, the effects of genotype and diet were additive, with SOD2<sup>Het</sup>/FO mice exhibiting the highest level of apoptosis, 46% higher than wild-type (Wt)/CO mice. A similar overall profile with more pronounced effects were observed in Gpx4<sup>Het</sup> mice, with both allelic ablation of Gpx4 ( $P = 0.0001$ ) and dietary n-3 PUFA ( $P = 0.001$ ) increasing apoptosis in the colon (Fig. 2B). Specifically, FO-fed mice exhibited a 29% increase in the number of apoptotic cells relative to CO-fed mice; whereas apoptosis was increased by 48% in heterozygous compared with wild-type mice. The overall effects of genotype and diet were additive, with Gpx4<sup>Het</sup>/FO mice exhibiting the highest level of apoptosis, 89% higher than Wt/CO mice.

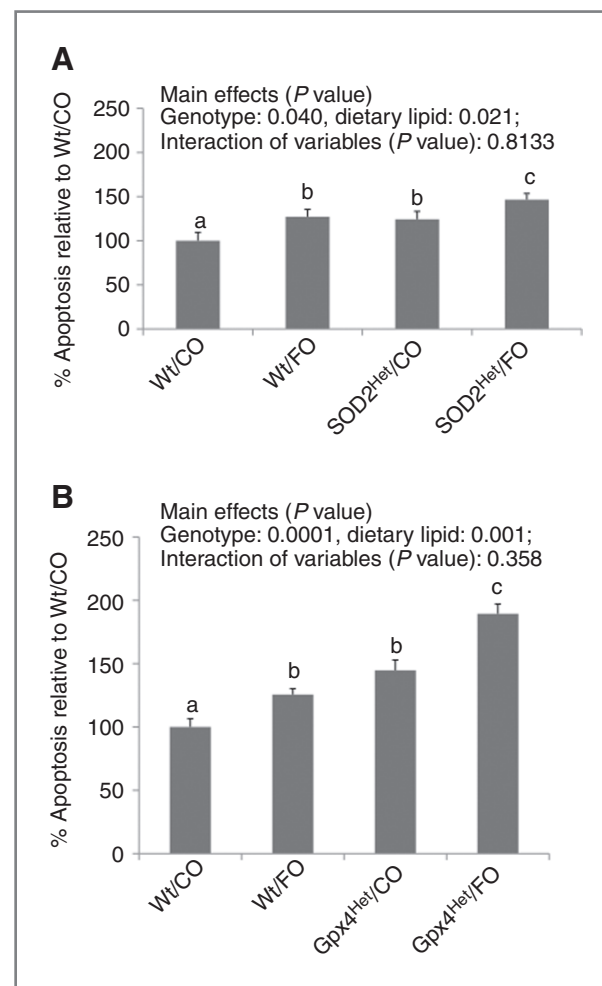
#### The antiapoptotic effect of Gpx4 overexpression is abolished by dietary n-3 PUFA

Overexpression of Gpx4 reduced apoptosis ( $P = 0.0002$ ), whereas dietary n-3 PUFA enhanced ( $P = 0.002$ ) apoptosis in the colon (Fig. 3). Specifically, Wt/FO-fed mice exhibited a 26% increase in apoptosis relative to Wt/CO-fed mice.

However, apoptosis was decreased by 25% following Gpx4<sup>Tg</sup> overexpression compared with wild-type mice. Overall, the effects of genotype and diet were antagonistic, with Gpx4<sup>Tg</sup>/FO mice exhibiting a similar level of apoptosis compared with Wt/CO mice.

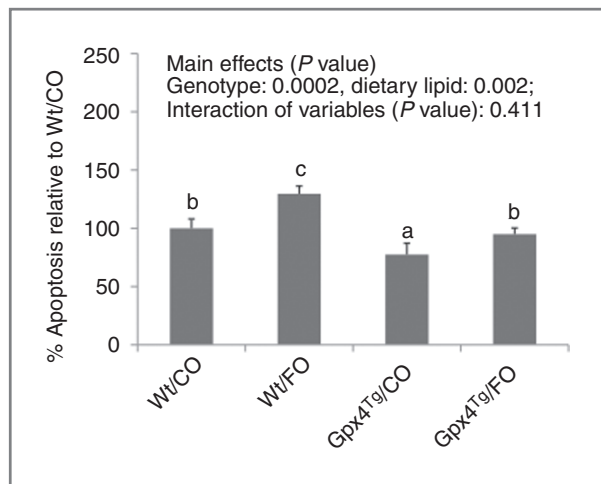
#### Gpx4 overexpression suppresses dietary n-3 PUFA induced oxidative DNA damage

Since persistent oxidative stress can damage DNA (24), we examined the effect of diet and Gpx4 status on colonic 8-OHdG levels. Dietary n-3 PUFA increased 8-OHdG DNA adduct levels in Wt/FO mice by 64% compared with the control Wt/CO (n-6 PUFA enriched) group (Fig. 4). However, the promotive effect of n-3 PUFA was diminished in Gpx4<sup>Tg</sup> mice, with both CO- and FO-fed Gpx4<sup>Tg</sup> mice exhibiting comparable levels of 8-OHdG.



**Figure 2.** The proapoptotic effect of SOD2 or Gpx4 deletion is enhanced by dietary n-3 PUFA. Data are presented as % apoptotic index relative to control Wt mice fed CO diet (mean  $\pm$  SE,  $n = 14-16$ ). A, SOD2<sup>Het</sup> mice; B, Gpx4<sup>Het</sup> mice. Values not sharing the same letter indicate a significant difference ( $P < 0.05$ ). Refer to Figure 1 for legend details.

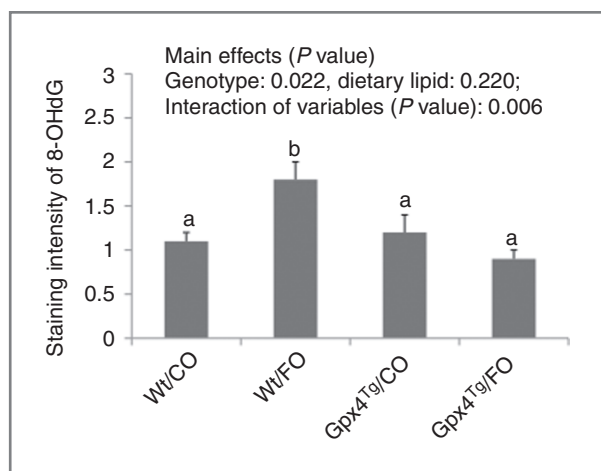




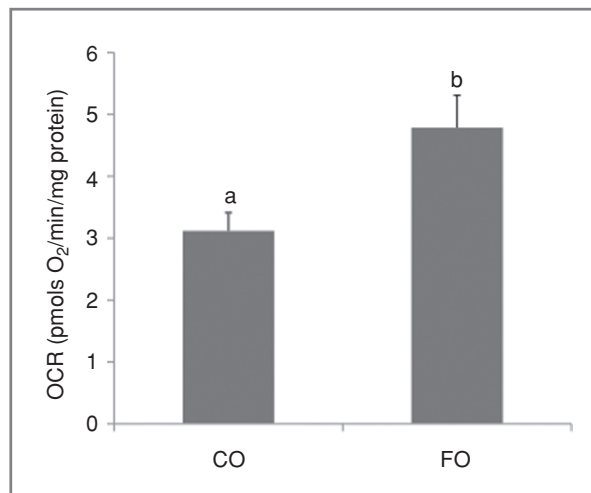
**Figure 3.** Gpx4 overexpression suppresses apoptosis in the colon. Data are presented as % apoptotic index relative to control Wt mice fed CO diet (mean  $\pm$  SE,  $n = 19$ –23). Values not sharing the same letter indicate a significant difference ( $P < 0.05$ ). Refer to Figure 1 for legend details.

### n-3 PUFA enhances colonocyte mitochondrial proton leak

A representative quantitative measure of colonocyte mitochondrial function by using the Seahorse Bioscience Extracellular Flux Analyzer is shown in Supplementary Figure S2. In this analytical system, the proton leak curve is defined as the OCR attributed to all processes contributing to ion movement across the mitochondrial inner membrane (25). Hence, the proton-leak rate represents proton "escape" into the mitochondrial matrix contribut-



**Figure 4.** Gpx4 overexpression suppresses oxidative DNA damage induced by dietary n-3 PUFA. Immunohistochemical staining of 8-OHdG was done by using 4% paraformaldehyde-fixed colonic tissue as described in the Materials and Methods. Data are presented as mean staining intensity of 8-OHdG (mean  $\pm$  SE,  $n = 5$  mice per treatment) in Gpx4<sup>Tg</sup> and Wt mice fed CO or FO diets. Values not sharing the same letter indicate a significant difference ( $P < 0.05$ ).



**Figure 5.** Mitochondrial respiration associated proton leak is enhanced by dietary n-3 PUFA. The colonic mitochondrial bioenergetic profile was measured by using a Seahorse XF24 Analyzer as described in the Materials and Methods. The proton leak curve is defined as the OCR attributed to all processes which allow ion movement across the mitochondrial inner membrane. Proton-leak data represent the difference in OCR following oligomycin and antimycin A challenge in mice fed CO or FO diet (mean  $\pm$  SE, all transgenic and Wt groups pooled,  $n = 26$ –28). Values not sharing the same letter indicate a significant difference ( $P < 0.05$ ).

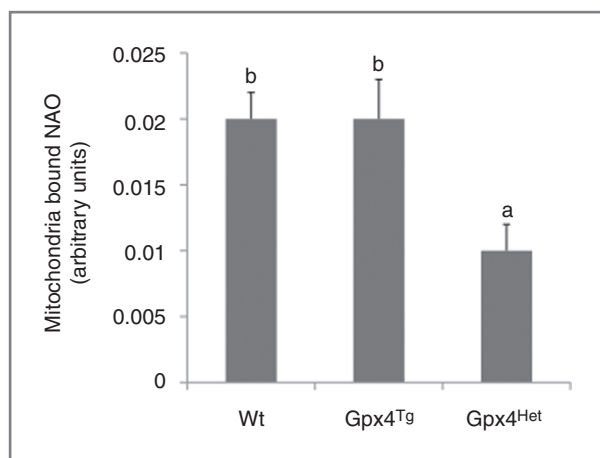
ing to the dissipation of mitochondrial membrane potential in state 4 (without ADP). Compared with the CO control (wild-type animals only) group, FO treatment significantly ( $P = 0.019$ ) enhanced the proton leak-related OCR by 54% (Fig. 5). In contrast, there was no effect of Gpx4 haploinsufficiency or overexpression on the proton leak level (Supplementary Table S1). Additional respiration parameters (state II, state III respiration, and respiratory control ratio) related to mitochondria function are shown in Supplementary Table S1.

### Gpx4 haploinsufficiency enhances mitochondrial CL peroxidation

NAO binds to CL with high affinity, and the fluorochrome loses its affinity for peroxidized CL (26). Assuming that the total CL mass in the colon is not altered by diet (27) or genetic modification (23), a reduction in bound NAO indirectly indicates an elevation in CL peroxidation. Figure 6 summarizes the significant ( $P = 0.035$ ) effect of genotype, with Gpx4<sup>Het</sup> mice exhibiting the lowest level of NAO binding, indicating an increased level of CL peroxidation relative to wild-type and Gpx4<sup>Tg</sup> animals. The separate effects of diet and Gpx4 status are shown in Supplementary Table S2.

## Discussion

Oxidative stress and gene–environment interactions play a significant role in the development of colon cancer (28). Oxidative stress results from an imbalance in the



**Figure 6.** Colonic mitochondrial CL peroxidation is enhanced in Gpx4 haploinsufficient mice. CL peroxidation was determined by measuring bound NAO in freshly isolated colonic mitochondria from Gpx4<sup>Tg</sup>, Gpx4<sup>Het</sup>, and Wt mice as described in the Materials and Methods (mean  $\pm$  SE, all diet groups pooled,  $n = 8-16$ ). Values not sharing the same letter indicate a significant difference ( $P < 0.05$ ).

production of ROS and antioxidant defenses of the cell. Although oxidative stress has been traditionally considered as a toxic byproduct of cell metabolism, cooperating with inflammatory/oncogenic signaling in cellular transformation (29), under certain circumstances it can also trigger apoptosis and play an important inhibitory role in tumor initiation (30, 31). From a mechanistic perspective, oxidative stress regulates a broad array of signal transduction pathways that link the endoplasmic reticulum, mitochondrial function and apoptosis (32, 33).

Colorectal cancer continues to pose a serious health problem in the United States. In the view of safety concerns, surrounding the use of pharmaceutical agents, such as nonsteroidal antiinflammatory drugs and tamoxifen as cancer chemopreventive agents, potentially innocuous dietary chemopreventive agents are now considered attractive alternatives (34). In this respect, there is substantial experimental, epidemiologic and clinical evidence indicating that FO containing diets rich in n-3 PUFA are protective against colon tumorigenesis (2, 9, 10, 35-38). In a major recent finding, it was shown that EPA reduced rectal polyp number and size in patients with familial adenomatous polyposis (FAP; ref. 39). Most impressive was the fact that FO derived n-3 PUFA suppressed FAP to a degree similar to the selective COX-2 inhibitor celecoxib. Collectively, these data indicate that n-3 PUFA hold promise as a chemoprevention agent for FAP and sporadic colon cancer. From a mechanistic standpoint, we have shown that n-3 PUFA promote colonic mitochondrial oxidative stress and apoptosis (a critical marker of colorectal cancer risk) in both animal and cell culture models (4, 8, 12, 13, 40). To further elucidate the mechanisms regulating oxidative stress-induced apoptosis *in vivo*, we fed SOD2<sup>Het</sup>, Gpx4<sup>Het</sup>, Gpx4<sup>Tg</sup>, and their respective wild-type siblings, diets containing either n-3 or n-6 PUFA (control)-enriched diets.

Overall, the data support our hypothesis linking the genetic predisposition to oxidative stress (e.g., SOD2 and Gpx4 haploinsufficiency) to the enhancement of apoptosis in the colon. Consistent with this outcome, the genetic enhancement of a critical mitochondrial antioxidant defense system (e.g., Gpx4 overexpression), decreased apoptosis. It is noteworthy that the level of apoptosis was higher in Gpx4<sup>Het</sup> relative to SOD2<sup>Het</sup> mice, suggesting a primary role of GPx4 in mediating oxidative stress-induced apoptosis in the colon. Interestingly, among the mitochondrial antioxidant enzymes, Gpx4 is unique because it directly reduces peroxidized phospholipids in membranes (41, 42). Therefore, Gpx4 is considered to be the primary enzymatic defense system against oxidative damage to cellular membranes (43).

Since EPA and DHA are oxidatively susceptible lipids due to their high degree of unsaturation (44), we anticipated that FO feeding would magnify the proapoptotic phenotype observed in SOD2<sup>+/-</sup> and Gpx4<sup>+/-</sup> mice. As predicted, an additive (diet + genetic deletion) proapoptotic effect was observed. The combinatorial effect of dietary n-3 PUFA and genetic prooxidative modification (SOD2 or Gpx4 haploinsufficiency) on the induction of colonic apoptosis, and the antagonist effect of dietary n-3 PUFA and genetic antioxidant modification (n-3 PUFA plus Gpx4 overexpression), further indicate the important role of oxidative stress in modulating colonic apoptosis.

As aforementioned, when the cellular level of oxidative species exceeds the capacity of the antioxidant defense system(s), this scenario can generate oxidative stress, ultimately translating into DNA damage (24). Because 8-OHdG is the most abundant oxidative DNA adduct, we determined the combined effects of dietary n-3 PUFA and antioxidant modification (Gpx4 overexpression) on the level of oxidative DNA damage within the colonic crypt. As expected, Gpx4<sup>Tg</sup> mice endowed with an enhanced mitochondrial specific antioxidant capability (19), exhibited a reduction ( $P < 0.05$ ) in the levels of DNA damage compared with wild-type siblings. In contrast, the inclusion of FO in the diet enhanced 8-OHdG levels in wild-type mice. Interestingly, we have previously reported that FO feeding lowers intestinal 8-OHdG following the initiation of an acute wounding/inflammatory episode by using dextran sodium sulfate (4, 45). This apparent discrepancy may be explained by the level of oxidative stress generated in the target tissue, that is, acute "pathologic" inflammatory agent versus chronic "physiologic" dietary effect. Because oxidative stress cannot be defined in universal terms, we propose in future studies to meticulously document the colonocyte intracellular environment following diet manipulation. This will help clarify the role of products of oxidative stress in terms of essential signals for the execution of the apoptotic program. Our overall goal is to delineate the impact of the source and severity of oxidative stress on colonocyte biology.

It has been reported that oxidative stress in mitochondria triggers an increase in proton-leak rates resulting in the depletion of membrane potential (18, 46). This is

noteworthy, because mitochondrial proton leak can contribute directly to the induction of apoptosis (47, 48). Hence, we were interested in examining colonocyte mitochondrial bioenergetic profiles in Gpx4<sup>Het</sup>, Gpx4<sup>Tg</sup>, and wild-type sibling mice. Interestingly, although there was no effect of Gpx4 haploinsufficiency or overexpression per se on proton-leak levels (Supplementary Table S1), FO feeding significantly ( $P < 0.05$ ) increased proton leak across the inner mitochondrial membrane in the colon (Fig. 5). The higher level of proton leak in all FO groups is consistent with the loss of membrane potential, which corroborates previous data indicating that colonocytes from FO-fed animals exhibit a decreased mitochondrial membrane potential, resulting in higher caspase 3 activity and the induction of apoptosis (49).

A critical function of Gpx4 is to repair oxidative damage in biological membranes, which are enriched in phospholipids. We have previously reported that n-3 PUFA promote colonocyte apoptosis in part via the generation of membrane PLOOH (11). When the level of ROS/PLOOH exceeds the detoxification capacity of the mitochondria, the resulting chronic oxidative stress, for example, oxidized CL, can directly trigger the release of proapoptotic factors from mitochondria into the cytosol (50). Since there is strong evidence that incorporation of EPA and DHA into mitochondrial membranes increases susceptibility to oxidative stress (11, 27), we used the NAO-binding method to indirectly measure the level of CL peroxidation in Gpx4 genetically modified mice fed CO or FO diets. To our surprise, no dietary effect was observed in any of the mouse models (Wt, Gpx4<sup>Tg</sup>, and Gpx4<sup>Het</sup>; Supplementary Table S2), which suggests that the effect of n-3 PUFA on phospholipid peroxidation does not directly target intestinal CL. However, we did observe elevated CL peroxidation

(lower NAO bound) in Gpx4<sup>Het</sup> mice, which is consistent with the antioxidant function of Gpx4 on membrane phospholipids. These data support our observation that genetic predisposition to oxidative stress promotes colonocyte apoptosis in a CL-dependent manner.

In summary, we have addressed for the first time the *in vivo* apoptogenic effect of dietary fat composition in oxidatively stressed animals. We show that oxidative stress promotes apoptosis in the colon. In addition, FO derived n-3 PUFA promote an oxidation–reduction imbalance in the intestine, increasing proton leak across the mitochondrial inner membrane, contributing to a permissive environment for apoptosis. We conclude that both genetic and dietary-induced oxidative stress enhance apoptosis in the mouse colon via complementary, overlapping mechanisms. Given the critical nature of apoptosis in colon cancer prevention, and the fact that inhibition of apoptosis is an integral component in the genesis of colon cancer, it is imperative to elucidate the precise mechanisms by which n-3 PUFA promote apoptosis in the colon.

#### Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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