Proapoptotic Effects of Dietary (n-3) Fatty Acids Are Enhanced in Colonocytes of Manganese-Dependent Superoxide Dismutase Knockout Mice1–3

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Abstract
We recently demonstrated that (n-3) PUFA trigger the induction of apoptosis in the colon by enhancing phospholipid oxidation and mitochondrial Ca2+ accumulation. To further elucidate the mechanisms regulating oxidative stress-induced apoptosis in vivo, a 2 × 2 experiment was designed using both wild type (control) and manganese-dependent superoxide dismutase (SOD2) heterozygous knockout mice (SOD2+/−), which exhibit increased mitochondrial oxidative stress. Mice were fed diets differing only in the type of fat [corn oil or fish oil containing (n-3) PUFA] at 15% by weight for 4 wk. Dietary (n-3) PUFA treatment enhanced (22%) apoptosis in colonic crypts. In addition, SOD2 haploinsufficiency enhanced (20%) apoptosis, which was further increased (36%) by (n-3) PUFA feeding. Dietary lipid source and genotype interactively modulated nitrotyrosine levels (P = 0.027) and inflammation (P = 0.032). These findings demonstrate that the proapoptotic effects of (n-3) PUFA are enhanced in oxidatively stressed SOD2+/− mice. Thus, (n-3) PUFA appear to promote an oxidation-reduction imbalance in the intestine, which may directly or indirectly trigger apoptosis and thereby reduce colon cancer risk. J. Nutr. 139: 1328–1332, 2009.

Introduction
The balance between proliferation and apoptosis is critical to the maintenance of steady-state number for cell populations in the colon. In general, dysregulation of this mechanism can disrupt homeostasis, resulting in clonal expansion, with the resultant overproduction of affected cells (1). It has now been clearly established that the transformation of colonic epithelium to carcinoma is in part associated with a progressive inhibition of apoptosis (1–3). Hence, chemotherapeutic agents that restore normal apoptotic pathways have the potential to effectively treat cancers that depend on aberrations of the apoptotic pathway to develop (4). Importantly, we have demonstrated that measurements of apoptosis have greater prognostic value to detect dietary effects on colon tumor incidence than do measurements of cell proliferation (2,5).

A very exciting outcome of recent studies was the demonstration that rats fed fish oil containing (n-3) PUFA, such as docosahexaenoic acid [(DHA),11 22:6(n-3)] and eicosapentaenoic acid [(EPA), 20:5(n-3)], have higher levels of colonic apoptosis, conferring resistance to alkylation- and oxidation-induced DNA damage (5–9). The protective effect of (n-3) PUFA was evident at both the initiation and postinitiation stages of carcinogenesis (2,8). This important observation identifies a clear mechanism by which dietary (n-3) PUFA exert a significant and protective modulatory effect on colonocyte deletion in the whole animal. In addition, these data are consistent with recent observations indicating that (n-3) PUFA promote apoptosis of colonic mucosa in humans (10,11).

The intrinsic apoptotic pathway channels cell death signals via the mitochondrion, which serves as a damage/oxidative stress sensor and monitor of metabolic status (12). Consistent with previous reports linking oxidative stress and apoptosis (13–15),
we have recently demonstrated that cis-unsaturated fatty acids, particularly from the (n-3) PUFA family, induce this pathway 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\(^{2+}\) accumulation, and trigger the release of soluble intermembrane proteins (9,16–19). Oxidative stress regulates a broad array of signal transduction pathways that regulate mitochondrial function and apoptosis (20). The production of ROS/PLOOH in mitochondria is strictly regulated by mitochondrial PLOOH glutathione peroxidase, classical glutathione peroxidase, and manganese (Mn)-dependent superoxide dismutase (SOD2). Among these mitochondrial antioxidant enzymes, SOD2 is considered to be a primary enzymatic defense system against oxidative damage to cellular membranes (20). However, the potential for diet to modulate oxidative stress-induced apoptosis in the colon has not been clearly defined in vivo.

In this study, SOD2 heterozygous knockout mice (SOD2\(^{+/-}\)), which exhibit increased mitochondrial oxidative stress (13,21), were used to further elucidate the regulatory mechanisms of oxidative stress-mediated apoptosis induced by (n-3) PUFA in the colon. We hypothesized that heterozygotes, compared with wild type (wt) mice, would exhibit elevated levels of colonic apoptosis and that (n-3) PUFA would magnify this phenotype.

**Materials and Methods**

**SOD2\(^{+/-}\) mice and diets.** SOD2\(^{+/-}\) (B6.129S7-Sod2\(^{+/-}\);J) mice were originally purchased from Jackson Labs and backcrossed to C57BL/6 mice for 10 generations (22). All procedures followed the guidelines approved by Public Health Service and the Institutional Animal Care and Use Committee at Texas A&M University. The colony of SOD2\(^{+/-}\) mice used for this study was generated by breeding SOD2\(^{+/-}\) mice to wt C57BL/6 mice. Mice were screened at 3–4 wk of age for the SOD2 deletion by PCR analysis of DNA extracted from 0.5 cm of mouse tail using a Qiagen DNA tissue kit. PCR was performed (primer sequences available online) on an ABI 7900 instrument. cDNA was synthesized from 2 mg total RNA using random hexamers and oligo(dT) primers with Superscript II RT (Invitrogen). PCR was performed (primer sequences available online) using predeveloped Taqman assays (Applied Biosystems). Expression levels were normalized to ribosomal 18S expression using a predeveloped assay kit from Applied Biosystems.

**Measurement of apoptosis.** Apoptotic cells were enumerated on paraformaldehyde-fixed tissues in the distal colon using a terminal deoxynucleotidyl transferase 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.

**Determination of isoprostane levels.** Total (free and esterified) F\(_2\)-isoprostane (8-iso-PGF\(_{2\alpha}\)), F\(_1\)-isoprostanes, and F\(_3\)-neuroprostanes were measured in hydrolyzed lipid extracts of snap-frozen colonic mucosa samples to quantify noncyclooxygenase free radical-catalyzed peroxidation of arachidonic acid, EPA, and DHA, respectively (26). 8-iso-PGF\(_{2\alpha}\) masscharge ratio (m/z 569), F\(_1\)-isoprostanes (m/z 567), and F\(_3\)-neuroprostanes (m/z 593) were measured by stable isotope dilution, negative ion chemical ionization GC-MS as pentafluorobenzyl ester, trimethylsilyl ether derivatives. [\(^{2}\)H\(_4\) ]-8-isoPGF\(_{2\alpha}\) was used as an internal standard (m/z 573).

**Analysis of histopathology.** Large intestinal segments were rolled, cassetted (“Swiss roll”), fixed in 70% ethanol, and paraffin-embedded as previously described (2). Colonic samples were examined by a board-certified pathologist who was unaware of the treatment groups. Because intestinal lesions were multifocal and of variable severity, the grades given to any section of intestine took into consideration the number of lesions as well as their severity. As previously described (27), a grade of 0 to 4 was based on the following criteria: 0, no unexpected inflammatory cell populations; 1, a few small mucosal lymphocytic aggregates; 2, extension of lymphatic cells beyond discrete segregates, and/or large discrete aggregates; 3, more severe than 2, and involving the submucosa; 4, essentially a trans-mural inflammatory lesion.

**Immunohistochemistry.** Following antigen retrieval with 10 mmol/L citrate, pH 6.0 at 100°C for 5 min, formalin-fixed, paraffin-embedded sections from distal colon were processed using rabbit polyclonal anti-nitrotyrosine (Chemicon International, AB5411) to quantify in situ nitrotyrosine staining as previously described (28). The degree of immunopositive nitrotyrosine staining in the distal colon was expressed as square root of integrated pixel intensities (29).

**Statistics.** The Brown-Forsythe’s test of homogeneity of variance was conducted and indicated that the variances between the treatment groups were equal. Subsequently, we used a 2-way ANOVA model to fit the data. T statistics were used to test the differences between treatment effects. Results are shown as means ± SE. Differences with P-values <0.05 were considered significant.

**Results**

**Genotyping and functional status of SOD2 in heterozygous mice.** To establish that the SOD2-targeted mutation resulted in a reduction of MnSOD (SOD2) protein in heterozygous mice, lysates from colonic mucosa were probed using antibodies recognizing Cu/ZnSOD (SOD1) or SOD2. Germ line transmission of HPRT was verified and SOD2 was reduced by ~50% in SOD2 heterozygous (+/-) mice (Supplemental Fig. 1). In contrast, SOD1 expression did not change. Because SOD2 null mice are embryonic lethal (22), only wt and heterozygous mice were used in this study.

**Western blot.** Colonic mucosa was homogenized in ice-cold lysis buffer containing 0.1% SDS and subjected to PAGE in 4–20% precast mini gels. After electrophoresis, proteins were electroblotted onto a polyvinylidene fluoride membrane with the use of a Hoefer Mighty Small Transphor Unit (Pharmacia) at 400 mA for 1.5 h. After transfer, the membrane was processed in 4% nonfat dry milk and 0.1% Tween 20 in PBS at room temperature for 1 h with shaking, followed by incubation with shaking overnight at 4°C with primary antibody (rabbit anti-SOD1 antibody, Chemicon; rabbit anti-SOD2 antibody, Upstate) 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) per the manufacturer’s instructions. Blots were scanned with a Fluor-S Max Multimager system (Bio-Rad).

**Real-time PCR.** To quantify mRNA expression levels of inducible nitric oxide synthase, tumor necrosis factor-α, intracellular adhesion molecule-1, prostaglandin synthase-2, and interleukin-1β, we performed real-time PCR on an ABI 7900 instrument. cDNA was synthesized from 2 μg total RNA using random hexamers and oligo(dT) primers with Superscript II RT (Invitrogen). PCR was performed (primer sequences available online) using predescribed Taqman assays (Applied Biosystems). Expression levels were normalized to ribosomal 18S expression using a predeveloped assay kit from Applied Biosystems.

Diet-induced colonocyte apoptosis in SOD2\(^{+/-}\) mice
The proapoptotic effect of (n-3) PUFA feeding is enhanced in SOD2\(^{1/-}\) mice. Both dietary (n-3) PUFA (\(P = 0.002\)) and allelic ablation of SOD2 (\(P = 0.007\)) enhanced apoptosis in the distal colon (data not shown). Specifically, mice fed FO exhibited a 22\% increase in apoptosis compared with CO-fed mice. A representative terminal deoxynucleotidyl transferase labeling micrograph is shown (Supplemental Fig. 2). Similarly, apoptosis was increased by 20\% in heterozygous compared with wt mice. Overall, the effects of genotype and diet were additive, with SOD2\(^{1/-}\)/CO-PUFA-fed mice exhibiting the highest level of apoptosis, 36\% higher than wt-/(n-6) PUFA-fed mice (Fig. 1).

Because SOD2\(^{1/-}\) mice exhibit increased mitochondrial oxidative stress (21), the level of arachidonic acid 20:4(n-6) derived F2-isoprostane, a proinflammatory marker of oxidant injury was quantified in colonic mucosal samples. SOD2\(^{1/-}\) and wt mice did not differ (data not shown). Interestingly, (n-3) PUFA supplementation suppressed total F2-isoprostane levels, regardless of genotype (Fig. 2). In contrast, the interaction between dietary lipid source and genotype was close to being significant for the EPA-derived F3 (\(P = 0.063\)) and DHA-derived F4 (\(P = 0.059\)) isoprostane levels.

Interactive effect of dietary lipid and SOD2 heterozygous deficiency modulate inflammation in the colon. Enhanced mitochondrial oxidative stress and resultant dysfunction associated with SOD2 haploinsufficiency can trigger tissue-specific inflammatory responses. Therefore, intestinal levels of nitrotyrosine, a biomarker of inflammation (28), were quantified. Dietary lipid source and genotype interactively (\(P = 0.027\)) influenced colonic nitrotyrosine levels (Supplemental Fig. 3; Table 1). Specifically, (n-3) PUFA treatment tended to increase nitrotyrosine scores in wt mice (FO-fed wt > CO-fed wt; \(P = 0.063\)), whereas levels were decreased relative to (n-6) PUFA-treated SOD2\(^{1/-}\) mice (FO-fed SOD2\(^{1/-}\) < CO-fed SOD2\(^{1/-}\); \(P = 0.049\)). In general, results from nitrotyrosine immunohistochemistry were consistent with inflammation scores (Table 1). Dietary lipid source and genotype interactively (\(P = 0.032\)) modulated the inflammatory score in the colon. Levels of genes typically upregulated in inflammatory bowel disease, including inducible nitric oxide synthase, tumor necrosis factor-\(\alpha\), intracellular adhesion molecule-1, prostaglandin synthase, and interleukin-1\(\beta\), did not differ among the groups (\(P = 0.21–0.95\)) (Supplemental Table 1), suggesting that (n-3) PUFA ameliorate a mild form of intestinal inflammation associated with mitochondrial oxidative stress.

**Discussion**

A growing body of literature supports the contention that bioactive food components containing (n-3) PUFA reduce colon cancer risk, in part by increasing colonocyte apoptosis (5,8,10,11,27). At present, the molecular and cellular mechanisms by which the primary pleiotropic molecules, EPA and DHA (16–18), upregulate apoptosis effector mechanisms in colonocytes have not been clearly defined. Interestingly, in previous studies it was noted that (n-3) PUFA actually promote an oxidation-reduction imbalance in the intestine, which was associated with increased apoptosis and reduced colonic tumors (9,16,30). Recently, we have demonstrated that DHA promotes mitochondrial oxidative stress and increases mitochondrial Ca\(^{2+}\) levels, which directly contribute to the induction of apoptosis in the colon (9,18,19). Therefore, although oxidative stress has been traditionally considered as a toxic by-product of cell metabolism, cooperating with inflammatory/oncogenic signaling in cellular transformation (31,32), under certain circumstances it can also trigger apoptosis and play an important inhibitory role in tumor initiation (15).
In this study, genetically manipulated mice were utilized to take advantage of the fact that SOD2+/− haploinsufficiency increases mitochondrial oxidative stress (13,21). Although previous investigators have linked apoptosis in this model to tissue decline and early senescence, we approached the SOD2+/− mouse from a chemoprevention standpoint and hypothesized that mitochondrial oxidative stress will promote colonic apoptosis and that (n-3) PUFA will magnify this phenotype. The current experiments support this hypothesis, because the combination of SOD2 haploinsufficiency and (n-3) PUFA culminated in increased apoptosis (Fig. 1). These results are consistent with evidence indicating that the incorporation of EPA and DHA into mitochondrial membranes increases oxidative stress, which induces apoptosis (14,16,18,33). This observation is further supported by the fact that a mitochondrial antioxidant, MitoQ, can block the effects of DHA on promotion of apoptosis in colonocytes (18,34). From a colon cancer risk perspective, it is also worth emphasizing that the localization of alkylation and oxidation-induced DNA damage and apoptosis in colonic crypts is different between these 2 forms of DNA damage (5,6,29). As opposed to methylation-induced DNA adducts, oxidative adducts are primarily localized to the upper part of the crypt. Hence, an increase in apoptosis in the upper part of the crypt will remove oxidative DNA damage. This is consistent with previous observations indicating that fish oil feeding protects intestinal cells against oxidative DNA damage, in part via deletion mechanisms (7). In regard to the relevance of apoptosis at the top of crypts, there is evidence that the development of adenomatous polyps proceeds through a “top-down” mechanism (35). Therefore, even though stem cells are located in the base of the crypt, targeted apoptosis in the superficial portions of the mucosa would reduce the propagation of damaged cells.

Persistent oxidative stress associated with SOD2+/− mice produced a mild form of intestinal inflammation as exhibited by the modest elevation in nitrotyrosine and inflammation score data and the failure to induce mucosal proinflammatory gene expression. Nitrotyrosine serves as a biochemical marker for inflammation and increases in protein tyrosine nitration occur in human inflammatory bowel disease and colorectal carcinomas (36). Paradoxically, (n-3) PUFA feeding modestly increased inflammatory indices in wt mice. This was unexpected based on the putative antiinflammatory properties of (n-3) PUFA (37). We propose that (n-3) PUFA may have influenced both mucosal inflammation and repair. Hence, it is likely that the stimulatory environment, e.g. SOD2+/− background, determines whether the effect of EPA/DHA will be protective or deleterious to the host.

F3-isoprostanes are generally considered to be proinflammatory mediators and have been implicated in the pathogenesis of a variety of diseases, including cancer (38). Because SOD2+/− mice exhibit increased mitochondrial oxidative stress (13,21), it was anticipated that isoprostanes formed nonenzymatically from the free radical-induced oxidation of arachidonic acid would be enhanced by SOD2 deletion. However, no increase was observed, suggesting that the mitochondrial oxidative stress associated with haploinsufficiency does not trigger overt lipid peroxidation in the colon. This may in part be attributed to the fact that peroxidized lipids in SOD2+/− mouse tissues accumulate in an age-dependent manner (13). Therefore, this process may occur in SOD2+/− mouse colon but at a later time. Another interesting finding of our study relates to the observation that dietary (n-3) PUFA reduced the levels of F3-isoprostanes in colonic mucosa. Although these observations are consistent with previous reports (39), the interpretation is complicated by the fact that EPA and DHA supplant arachidonic acid and can therefore dramatically alter both the spectrum and biological properties of isoprostane metabolites produced by cells located in the intestinal mucosa (40). Examination of EPA and DHA-derived nonenzymatic peroxidation, i.e. F3 and F4 isoprostanes, revealed a complex genotype × diet interaction. Further studies are needed to elucidate these effects.

In conclusion, SOD2 haploinsufficiency enhanced apoptosis in the colon, which was further increased by (n-3) PUFA feeding. These results are consistent with a growing body of evidence indicating that mitochondrial oxidative stress promotes apoptosis in the colon. For example, when the level of ROS/PLOOH exceeds the capacity of the mitochondria to detoxify them, the resulting chronic oxidative stress can directly trigger the release of proapoptotic factors from mitochondria into the cytosol (41). Collectively, these observations may explain a growing number of reports suggesting that antioxidant supplements have unwanted consequences to our health (42). This is based on the concept that antioxidant administration may block apoptosis and thereby enhance colon tumorigenesis. Therefore, it is important to determine whether antioxidant administration in vivo can suppress apoptosis and enhance colon cancer risk.

**Literature Cited**


**TABLE 1** Histological evaluation of mucosal nitrotyrosine levels and inflammation clinical score from wt and SOD2+/− mice fed CO and FO diets for 4 wk

<table>
<thead>
<tr>
<th></th>
<th>CO</th>
<th>FO</th>
<th>CO</th>
<th>FO</th>
<th>Genotype (G)</th>
<th>Diet (D)</th>
<th>G × D</th>
<th>Main effects, P-values</th>
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</thead>
<tbody>
<tr>
<td>Nitrotyrosine</td>
<td>0.83 ± 0.31a</td>
<td>1.83 ± 0.26b</td>
<td>1.57 ± 0.43b</td>
<td>0.83 ± 0.31a</td>
<td>0.937</td>
<td>0.937</td>
<td>0.032</td>
<td></td>
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<tr>
<td>Inflammation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.047</td>
<td>0.030</td>
<td>0.027</td>
<td></td>
</tr>
</tbody>
</table>

1 Values are means ± SE, n = 9–10 (nitrotyrosine) or 6–8 (inflammation). Means in a row without a common letter differ, P < 0.05.
2 Values are the square root of integration intensity of nitrotyrosine staining in the distal colon.
3 Values are the mean clinical score of the entire colon.