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Mitochondrial lipid peroxidation is influenced by dietary factors in early colon carcinogenesis

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Oxidative damage to mitochondrial proteins, lipids, and DNA seem to influence the promotion and progression of tumors. High-fat diets and diets high in iron decrease manganese superoxide dismutase activity, a mitochondrial antioxidant, in colon mucosa. Lipid peroxidation products are low in microsomal preparations from colonic mucosa even under peroxide-inducing conditions. However, damage specific to mitochondrial membranes is unknown. This study was designed to investigate dietary lipid and iron effects on fatty acid incorporation and lipid peroxide formation in mitochondrial membranes of colonic mucosa. Male Fischer rats were fed high-fat diets containing either corn oil or menhaden oil with an iron level of either 35 or 535 mg/kg diet. Animals were given two injections of the colon carcinogen, azoxymethane, or saline. Colon tissue was collected 1 and 6 weeks after injections. Mitochondrial and microsomal fractions were prepared for fatty acid analysis and quantitation of lipid peroxidation products. Results showed that lipid composition of both subcellular fractions were influenced by diet. Fatty acid composition of mitochondria differed from microsomes, but overall saturation remained constant. Peroxidation products in mitochondrial membranes were significantly greater than in microsomal membranes. Dietary treatment significantly affected mitochondrial peroxidation in carcinogen-treated animals. Therefore, mitochondria from colon mucosa are more susceptible to peroxidation than are microsomes, dietary factors influence the degree of peroxidation, and the resulting damage may be important in early colon carcinogenesis. (J. Nutr. Biochem. 8:696–701, 1997) © *Elsevier Science Inc. 1997*

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Introduction

Mitochondrial integrity and function are important in carcinogenesis.[1] Tumors of various sites, as well as tumor cell lines, exhibit abnormalities in mitochondrial morphology, enzymatic activity, and transport mechanisms. Changes in the mitochondrial DNA of cancer cells seem to influence the promotion and progression of tumors.[1] Mitochondria are sites of oxygen radical production, and without adequate antioxidant protection, oxygen radicals cause damage to target proteins, membrane lipids, and DNA of this subcellular organelle.[2]

Evidence from literature suggests that dietary antioxidants contribute to overall protection against the development and progression of colon tumors.[3] The risk for development of colon cancer increases in models involving chronic inflammation, ulcerative colitis, or parasitic infection. Damage resulting from reactive oxygen species is the possible link between these conditions and the development of cancer.[3] Measurement of lipid peroxidation products in colonic mucosa has been of unknown value, however. Lipid peroxidation products are consistently low in total membrane and microsomal preparations from colonic mucosa in spite of inducing conditions both in vivo and in vitro.[4–6] The lipid composition of mucosal membranes is affected by the diet. However, the incorporation of polyunsaturated fatty acids in this tissue does not increase membrane peroxidation to an extent seen in other tissues. The antioxidant protection of colonic mucosa is significant even under peroxide-inducing conditions.[4]

Manganese superoxide dismutase (MnSOD) is an antioxidant enzyme, found within the mitochondria, which catalyzes the conversion of superoxide radicals to hydrogen

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Table 1 Composition of experimental diets (g/100 g)

Component	CO[a]	MO[a]
Casein	22.60	22.60
Cornstarch	28.05	28.05
Dextrinized cornstarch	10.00	10.00
Sucrose	8.00	8.00
Corn oil[b]	20.00	1.00
Menhaden oil[b]		19.00
Fiber	5.65	5.65
AIN-93G mineral mix	3.95	3.95
AIN-93 vitamin mix	1.13	1.13
L-Cystine	0.34	0.34
Choline bitartrate	0.28	0.28

[a]Iron content is 35 mg/kg diet. Two additional diets, COFe and MOFe were supplemented with ferric citrate to provide 535 mg of iron/kg diet. [b]Contains 0.02% TBHQ.

peroxide.[7] The activity of MnSOD is an important step in the protection of mitochondria from oxidative damage. MnSOD activity appears to be important during carcinogenesis as its activity is greatly reduced, or absent, in transformed cells.[7–9]

This laboratory reported that diets high in fat or iron decrease MnSOD activity in colon mucosa.[10] It is not known if this decrease in antioxidant protection results in peroxidation and damage specific to mitochondrial membranes of the colon. This study was designed, therefore, to investigate fatty acid incorporation and lipid peroxide formation in mitochondrial membranes of colonic mucosa. Diets rich in corn oil or fish oil were used to compare fatty acid composition of mitochondrial and microsomal membrane preparations, and measure lipid peroxidation products after treatment with saline or the colon carcinogen, azoxymethane.

Methods and materials

Animals

Weanling male Fischer-344 rats were purchased from Charles River (Kingston, NY USA) and fed one of the four experimental diets described below for 1 week. After this adjustment period, animals from each diet group were given two weekly injections of either azoxymethane (AOM) or saline in the protocol described below. Animals remained on the diets throughout the experiment and tissue was collected 1 and 6 weeks after carcinogen treatment. Animals were housed individually in the institutional Laboratory Animal Research Facility. Institutional and National Research Council Guidelines were followed for their care and maintenance. Body weights and food consumption data were recorded.

Carcinogen administration

AOM was dissolved in sterile saline immediately prior to use and given to animals in two weekly intraperitoneal injections at a dose of 12 mg AOM/kg body weight.[11] Animals used for control were given injections using an equal volume of sterile saline.

Diet

Four high-fat diets, based on the AIN-93G formulation,[12] were used for the study (*Table 1*). The diets were purchased from Dyets (Bethlehem, PA USA). The CO diet contained 20% corn oil; the MO diet contained 19% menhaden oil plus 1% corn oil to supply sufficient essential fatty acids. The AIN mineral formulation provided 35 mg/kg iron as ferric citrate. Two corresponding diets, COFe and MOFe, contained additional ferric citrate to provide 535 mg/kg iron. Recommended procedures were followed to prevent peroxidation of the diets.[13] This included addition of tert-butylhydroxyquinone (TBHQ) to the lipid sources; storage of the diets below 4°C; and feeding of the animals twice weekly, discarding any uneaten diet.

Tissue preparation

Rats were killed with CO_2, colons removed, slit longitudinally, rinsed, immediately frozen in liquid nitrogen and stored at −85°C. At the time of assay, colons were thawed on ice and mucosal tissue scraped. Mucosal tissue was then homogenized for 20 sec in approximately 1:5 weight:volume buffer containing 0.2 mol/L mannitol, 50 mmol/L sucrose, 1 mmol/L EDTA in 10 mmol/L HEPES-NaOH, pH 7.4.[14] A nuclear pellet was removed by centrifugation at 1000 × g for 10 min. The heavy mitochondrial fraction was then obtained by centrifugation of the supernatant at 3000 × g for 10 min. The resulting pellet was washed twice by resuspending in one half the original volume of buffer followed by centrifugation at the same speed for the same amount of time. The microsomal fraction was then obtained by centrifugation of the supernatant at 105,000 × g for 60 min.

Purity of cell fractions

Mitochondrial and microsomal fractions were assayed for mitochondrial monoamine oxidase (MAO) activity as a measure of cell fraction purity. MAO was measured spectrophotometrically by the method of Salach and Weyler using Kynuramine-HBr as a substrate.[15] In that procedure, 0.05 mol/L sodium phosphate buffer, pH 7.2, 0.2% Triton X-100, with 1 mmol/L Kynuramine-HBr were incubated at 30°C for 2 min. The solution was then measured at 314 nm for 2 min after addition of sample. The units are defined in this procedure as 1 μmol of product min^{-1} mg $protein^{-1}$.

Fatty acid analysis

Fatty acid analysis was performed on both mitochondrial and microsomal pellets prepared as described above. Total lipids were extracted from the cell fractions using methanol and chloroform.[16] Transmethylation of the fatty acids was accomplished using methanolic HCl and 2,2-dimethoxypropane. Composition analysis of fatty acid methyl esters was then determined on a Shimadzu GC 14A using flame inonization and a Shimadzu Chromatopac integrator. Peaks were identified using authentic standards and results expressed as percent composition.

Table 2 Monoamine oxidase activity (μmol/min/mg)

Diet	Mitochondria[a,b]	Microsome[a,b,c]
CO	4.55 ± 0.86	0.85 ± 0.29
MO	4.73 ± 0.91	0.67 ± 0.35
COFe	4.31 ± 0.72	1.05 ± 0.54
MOFe	3.80 ± 0.74	0.86 ± 0.44

[a]Mean ± SEM of 3 rats.
[b]No differences according to diet ($P > 0.05$).
[c]Each diet differs from mitochondrial fraction ($P < 0.05$).

Table 3 Fatty acid composition of microsome fraction (% composition)

Fatty acid	CO	MO	COFe	MOFe
14:0	1.6 ± 0.1—b	4.0 ± 0.4—a	2.1 ± 0.4—b	4.5 ± 0.3—a
16:0	13.3 ± 5.9—a	14.9 ± 6.6—a	13.5 ± 6.5—a	13.8 ± 7.1—a
16:1	2.1 ± 0.8—b	7.1 ± 0.8—a	1.9 ± 0.3—b	7.1 ± 0.3—a
18:0	14.3 ± 2.1—a	14.8 ± 1.8—a	16.4 ± 1.4—a*	16.4 ± 1.5—a
18:1	27.4 ± 7.8—a	23.1 ± 2.0—a	21.4 ± 2.7—a	24.4 ± 2.2—a
18:2 n-6	21.3 ± 1.3—a*	11.7 ± 2.1—b*	22.8 ± 2.0—a*	10.7 ± 1.7—b*
18:3 n-6	6.0 ± 1.1—a*	7.2 ± 1.5—a*	6.1 ± 1.8—a*	6.6 ± 1.3—a*
18:3 n-3	1.1 ± 0.8—a	0.8 ± 0.5—a	1.2 ± 0.6—a	1.1 ± 0.2—a
20:4 n-6	11.4 ± 0.5—a*	5.4 ± 0.7—b*	13.0 ± 2.0—a*	5.6 ± 0.1—b*
20:5 n-3	ND—b	6.1 ± 1.0—a	ND—b	5.5 ± 0.5—a
22:4 n-6	1.0 ± 0.6—a	tr—b	0.6 ± 0.1—a	tr—a
22:5 n-3	tr—b	1.7 ± 0.2—a	tr—b	1.4 ± 0.3—a
22:6 n-3	tr—b	3.1 ± 0.2—a	tr—b	2.6 ± 0.5—a*
DBI	1.5 ± 0.1—a	1.6 ± 0.1—a	1.5 ± 0.1—a	1.5 ± 0.1—a

Mean ± SEM 5 rats; ND, none detected; tr, trace.
Means within each row with different letters are different ($P < 0.05$).
Double bond index (DBI) is calculated as sum of percent composition of each fatty acid × number of double bonds/100.
*Indicates difference from mitochondrial fraction ($P < 0.05$).

Thiobarbituric acid-reacting substances

Thiobarbituric acid reacting substances (TBARS) were measured as an estimate of lipid peroxidation products using previously published methods.[4] Briefly, 200 μL microsomal or mitochondrial suspension was heated with 400 μl 50 mmol/L sodium phosphate, 0.1 mmol/L EDTA, pH 7.0 buffer, 600 μL 10% trichloroacetic acid, and 1.0 mL 0.5% 2-thiobarbituric acid for 45 min. Tubes were cooled and then centrifuged. Absorbance of the supernatant was read at 535 nm against a standard curve of 1,1,3,3-tetramethoxypropane. Results are expressed as nmol malondialdehyde per mg protein.

Statistical analysis

The SAS statistical software[17] was used for data analysis. Differences in assay parameters were determined according to diet, carcinogen treatment, and subcellular fraction. Significance for analysis of variance, and *t* test procedures was defined as $P < 0.05$. Student-Newman-Keuls' test was used to separate treatment means when the initial analysis of variance indicated a difference.

Results

Monoamine oxidase activity

Table 2 shows MAO activity as an indicator of purity of the two subcellular fractions. MAO activity was greater in the mitochondria-enriched fraction than in the microsomes. There were no differences in either cell preparation according to diet.

Fatty acid composition

Tables 3 and *4* show the fatty acid composition of microsome and mitochondrial subcellular fractions of colon mucosa from animals fed the different test diets. The corn oil containing diets, CO and COFe, resulted in higher composition of linoleic acid (18:2 n-6) and arachidonic acid (20:4 n-6) in both the mitochondrial and microsomal fractions. The menhaden oil containing diets, MO, and MOFe, resulted in higher concentrations of the n-3 fatty acids

Table 4 Fatty acid composition of mitochondrial fraction (% composition)

Fatty acid	CO	MO	COFe	MOFe
14:0	2.6 ± 1.0—a	4.1 ± 0.9—a	1.9 ± 1.1—a	4.8 ± 0.7—a
16:0	19.0 ± 0.8—a	15.2 ± 5.6—ab	23.3 ± 1.9—a	5.5 ± 0.9—b
16:1	1.8 ± 0.2—c	5.2 ± 0.8—b	1.5 ± 0.2—c	8.1 ± 0.9—a
18:0	7.3 ± 0.6—b	13.0 ± 2.1—a	8.2 ± 0.7—b	15.0 ± 1.0—a
18:1	23.9 ± 1.1—bc	26.2 ± 1.5—ab	22.1 ± 0.7—c	28.8 ± 0.2—a
18:2 n-6	36.9 ± 2.2—a	20.8 ± 2.3—b	33.7 ± 3.3—a	18.5 ± 0.5—b
18:3 n-6	2.6 ± 0.5—a	0.6 ± 0.3—b	2.5 ± 0.6—a	0.6 ± 0.1—b
18:3 n-3	0.6 ± 0.3—a	1.9 ± 0.7—a	0.3 ± 0.1—a	2.0 ± 0.2—a
20:4 n-6	4.4 ± 0.3—ab	1.7 ± 0.7—b	5.4 ± 0.5—a	3.3 ± 0.5—ab
20:5 n-3	ND—c	4.7 ± 1.1—b	ND—c	6.8 ± 1.2—a
22:4 n-6	0.4 ± 0.3—a	tr—a	0.5 ± 0.3—a	tr—a
22:5 n-3	tr—a	2.9 ± 1.3—a	tr—a	1.9 ± 0.1—a
22:6 n-3	tr—c	3.6 ± 0.5—b	tr—c	4.8 ± 0.2—a
DBI	1.3 ± 0.0—a	1.5 ± 0.1—a	1.3 ± 0.1—a	1.7 ± 0.1—a

Mean ± SEM 5 rats.
Means within each row with different letters are different ($P < 0.05$).
Double bond index (DBI) is calculated as sum of percent composition of each fatty acid × number of double bonds/100.

Table 5 n-3 and n-6 fatty acids in subcellular fractions of colon mucosa (% composition)

Diet	Microsome	Mitochondria
	n-3	
CO	1.7 ± 0.8—b	1.2 ± 0.4—b
MO	11.6 ± 1.2—a	13.1 ± 2.1—a
COFe	2.2 ± 0.9—b	1.0 ± 0.3—b
MOFe	10.7 ± 1.1—a	15.5 ± 1.2—a
	n-6	
CO	39.7 ± 2.2—a	44.3 ± 2.2—a
MO	24.5 ± 3.2—b	23.2 ± 2.2—b
COFe	42.6 ± 2.2—a	42.1 ± 2.6—a
MOFe	23.1 ± 3.0—b	22.5 ± 2.6—b

Means in each column with different letters are different ($P < 0.05$). Total n-6 and n-3 fatty acid composition of mitochondria did not differ from that of microsomes.

including: eicosapentaenoic acid (20:5 n-3), docosapentaenoic acid (22:5 n-3), and docosahexaenoic acid (22:6 n-3). Adjustments in incorporation of unsaturated and monounsaturated fatty acids resulted in double bond indexes (DBI) that were not different by either diet or subcellular fraction.

There were differences in fatty acid composition according to subcellular fraction. Mitochondrial membranes contained higher levels of linoleic acid and lower levels of δ-linolenic and arachidonic acids in all diet groups. The total n-6 and n-3 fatty acid composition of mitochondria did not differ from that of microsomes (*Table 5*).

Lipid peroxidation products

Figures 1 and *2* show TBARS levels of colonic microsomal and mitochondrial fractions 1 and 6 weeks after AOM injections in animals fed the test diets. Microsomal peroxidation products were low and showed no differences by either diet or AOM treatment at 1 week. At the 6-week time point, microsomal peroxidation was low in saline-treated animals and increased in animals treated with AOM. There were no differences in TBARS values according to diet in the microsomal fractions.

Peroxidation products in mitochondrial membranes were significantly greater than in microsomal membranes in all groups measured. Dietary treatment significantly affected mitochondrial peroxidation in carcinogen-treated animals. MO-fed animals had the lowest mitochondrial TBARS values. Iron supplementation increased lipid peroxidation of MO-fed animals significantly but only to a level similar to the CO-fed group.

Discussion

The mitochondrial and microsome subcellular fractions used in this study were prepared by methods of centrifugation. MAO, a flavin containing mitochondrial aromatic

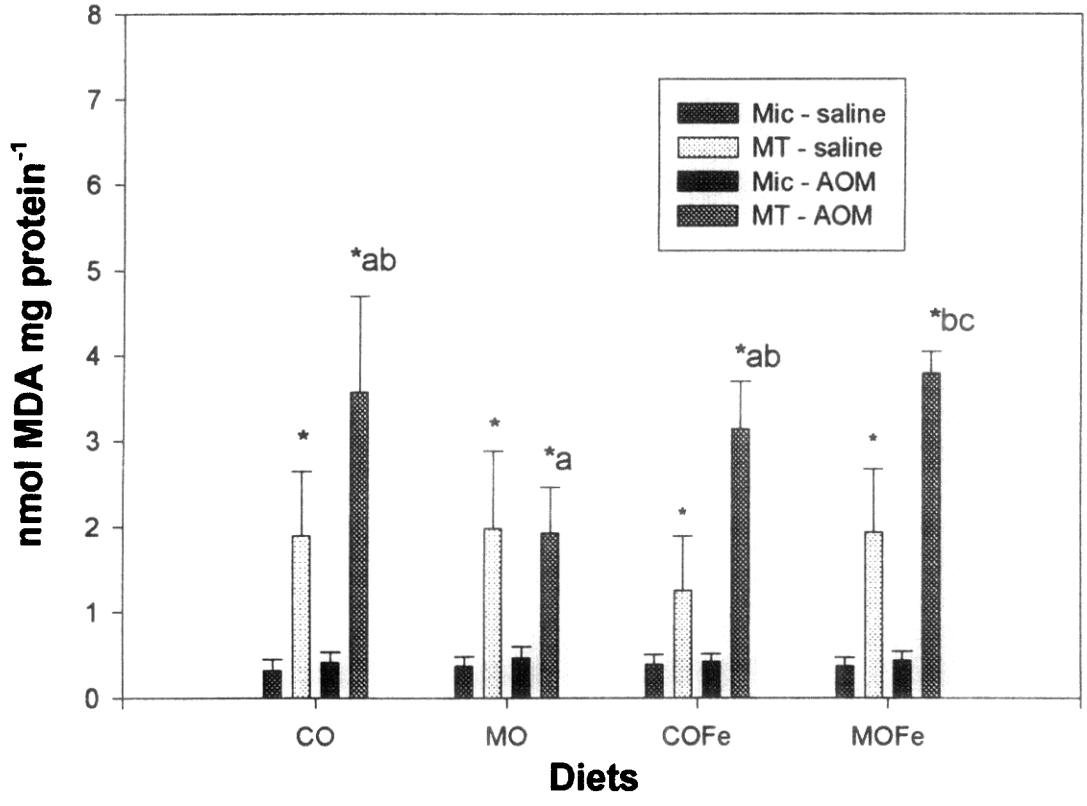

Figure 1 Thiobarbituric acid-reacting substances in mitochondrial (MT) and microsomal (MIC) fractions of colon mucosa from rats fed different diets 1 week after AOM injection. Values represent mean ± SEM of 5 rats. An * indicates mean for MT differs from MIC ($P < 0.05$). Letters indicate differences according to diet in MT of animals injected with AOM.

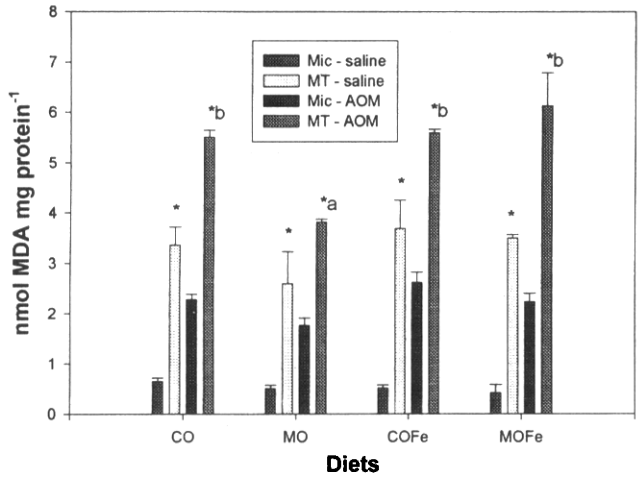

Figure 2 Thiobarbituric acid-reacting substances in mitochondrial (MT) and microsomal (MIC) fractions of colon mucosa from rats fed different diets 6 weeks after AOM injection. Values represent mean ± SEM of 5 rats. An * indicates mean for MT differs from MIC ($P < 0.05$). Letters indicate differences according to diet in MT of animals injected with AOM.

amine oxidase, activity was measured in cell fractions as an indication of purity.[15] There are two isoenzymes of MAO. This study measured isoenzyme A using Kynuramine-HBr as substrate, the primary form found in rat mucosa.[15] *Table 2* shows that the method of centrifugation used in this study was an effective method of obtaining a mitochondria-enriched fraction.

The results from this study show that fatty acid composition of dietary lipids influenced the lipid composition of both the microsomal and mitochondrial subcellular compartments in colonic mucosa. *Table 5* shows that both microsomes and mitochondria contained high levels of n-3 fatty acids from animals fed a fish oil diet or high levels of n-6 fatty acids when fed a corn oil-based diet. Using DBI as a rough estimate of the percent of unsaturated fatty acids in each of the membranes, revealed that ultimate membrane saturation was tightly controlled, regardless of dietary factors. Unsaturated and monounsaturated fatty acids increased or decreased proportionately to maintain membrane fluidity. Interestingly, the distribution of n-6 fatty acids was different in mitochondrial enriched fraction as compared with microsomes. Microsomes contained high levels of linoleic acid (18:2 n-6) and low levels of arachidonic acid (20:4 n-6), whereas the opposite was true for mitochondrial fractions.

In spite of similarities in apparent membrane saturation, there were significant differences in quantity of lipid peroxidation products measured in the subcellular fractions. Mitochondrial fractions exhibited greater TBARS values than microsomal fractions at all points. The high level of antioxidant protection of colonic mucosa has been noted by this and other laboratories.[4,5] The inability to detect, or even induce, lipid peroxidation in microsomal fractions of this tissue made correlation of oxidant damage and carcinogenesis difficult. The current results indicate, however, that mitochondria from this tissue are susceptible to peroxidation, that dietary factors may influence this, and that the resulting damage may be important in colon carcinogenesis.

Results from *Figures 1* and *2* show that animals fed a menhaden oil-based diet had lower lipid peroxide levels than those fed a CO-based diet. Previous studies of cancer risk in this tissue, have indicated that fish oil-based diets result in a lower risk for tumor development than corn oil-based diets.[18] A reduced level of mitochondrial damage may explain part of that effect. It should be noted that iron supplementation increased the level of peroxidation in the MO group to the same level as the CO-fed animals, however.

The relationship of mitochondrial lipid peroxidation and MnSOD activity is also of interest. This laboratory has reported that diets high in fat or high in iron have resulted in a decrease in MnSOD activity.[10] This decrease in MnSOD activity is a potential mechanism for the increased peroxide levels seen in the mitochondria of AOM-treated animals. A correlation between specific changes in diet and changes in enzyme activity is not easy because mitochondrial membranes from MO-fed animals exhibited both low levels of MnSOD activity and low TBARS levels.

Recent literature suggests mitochondria play a central role in programmed cell death.[19] Mitochondrial permeability transition involves opening of inner mitochondrial membrane pores. With this opening, there is a loss of membrane potential, uncoupling of oxidative phosphorylation, oxygen radical generation, and induction of a poorly described death signaling complex. Mitochondrial permeability transition may cause oxidative stress due to increased oxygen radical production and it may also be considered a consequence of oxidative stress.[19] Our results show that dietary lipid composition modifies mitochondrial lipid composition and susceptibility to lipid peroxidation in early stages of colon carcinogenesis. Implications are that this may be a potential mechanism for dietary fat involvement in colon cancer risk.

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