Docosahexaenoic acid accumulates in cardiolipin and enhances HT-29 cell oxidant production

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Abstract **The objective of this study was to investigate membrane fatty acids for their effects on mitochondrial function in live cells. Mitochondrial potential and oxidant production were measured in human colonic adenocarcinoma (HT-29) cells with membranes enhanced in either oleic acid, linoleic acid, arachidonic acid, eicosapentaenoic acid, or docosahexaenoic acid. Docosahexaenoic acid-enriched cells had increased mitochondrial potential and produced 5-fold more cellular oxidants than did cells enriched with any other fatty acid. Oxidant production in fatty acid-enriched HT-29 cells did not correlate with the degree of unsaturation for total membrane fatty acids. However, there was a strong correlation between the degree of fatty acid unsaturation of cardiolipin, a critical inner-mitochondrial membrane phospholipid, and oxidant production. Cardiolipin acyl composition is known to influence the activity of electron transport complexes, an effect that can increase mitochondrial oxidant production. Docosahexaenoic acid was enriched to 48 mol% of the fatty acids present in HT-29 cell cardiolipin. These results demonstrate the importance of membrane acyl composition to mitochondrial potential and oxidant production in live cells. Additionally, results suggest that docosahexaenoic acid increases cell oxidant production by accumulating in cardiolipin, where its presence alters electron transport efficiency.—**Watkins, S. M., L. C. Carter, and J. B. German. **Docosahexaenoic acid accumulates in cardiolipin and enhances HT-29 cell oxidant production. *J. Lipid Res.* 1998.** 39: **1583–1588.**

Supplementary key words cardiolipin • docosahexaenoic acid • mitochondria • fatty acid metabolism • HT-29 cells • oxidation • mitochondrial potential • flow cytometry • dihydrorhodamine 123

Cardiolipin (CL) is a diphospholipid (diphosphatidyl glycerol) required for the structural integrity of the mitochondria and for the proper function of the electron transport chain. In tissues with high respiration rates, such as heart, CL can account for 25% of the phospholipids in the inner-mitochondrial membrane (IMM) (1). Enzyme complexes involved in electron transport and ATP synthesis, including cytochrome *c* oxidase (2, 3), NADH reductase (4, 5), cytochrome b_1c_1 complex (5, 6), and ATP synthase (6, 7), require the close association of several CL molecules for activity. The structural properties of CL that favor these associations result from a unique four-fatty acid structure. In mammals, CL acyl composition is unusually sensitive to diet, and in humans it is rich in the essential dietary fatty acid linoleic acid (LA, 18:2 n–6) (8). Lysocardiolipins or CL with a low LA content are not effective activators of electron transport complexes in vitro (2, 9). Despite the importance of CL acyl composition to efficient electron transport, CL has the most diet-responsive and changeable fatty acid composition among phospholipids. In mammals, CL has been modified to contain 85–90 mol% LA (10, 11), 50 mol% docosahexaenoic acid (DHA, 22:6 n–3) (12), or 50 mol% oleic acid (OA, 18:1 n–9) (12) by changing the acyl composition of the diet.

The highly unsaturated acyl composition of CL is curious given the presence of CL in the IMM. The IMM is exposed to a high concentration of reactive oxygen species (ROS) in the form of superoxide radicals (13, 14). Not surprisingly, mitochondria from animals that have endured oxidative stress, including older animals (14) and those that have experienced ischemia-reperfusion (14–16), are specifically depleted of CL. It is not known whether CL plays a direct role in the production of ROS or is simply susceptible to degradation in their presence. This distinction is important as intracellular ROS can induce mitochondrial damage (17), and possibly cell death (18–20). Despite the known effects of CL and CL acyl composition on the in vitro activities of electron transport complexes, membrane fatty acid composition has not been investigated for its role in changing mitochondrial potential or ROS production. In this study, mitochondrial potential and cellular ROS production were measured in live HT-29 cells that had been enriched with specific fatty acids. The extensive incorporation of DHA into HT-29 cell CL and the increased mitochondrial potential and oxidant production in DHA-enriched cells suggest a

Abbreviations: AA, arachidonic acid; CL, cardiolipin; DHR123, dihydrorhodamine 123; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IMM, inner-mitochondrial membrane; LA, linoleic acid; OA, oleic acid; PUFA, polyunsaturated fatty acid; R123, rhodamine 123; ROS, reactive oxygen species.

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specific action for this fatty acid in modulating mitochondrial functions.

MATERIALS AND METHODS

Materials

Unless otherwise noted, all cell culture reagents were obtained from Sigma Chemical, Co., St. Louis, MO. Fatty acids were obtained from Nu Chek Prep, Inc., Elysian, MN and fluorescent probes were from Molecular Probes, Inc., Eugene, OR.

HT-29 cell culture

Human colonic adenocarcinoma cells (HT-29), obtained from American Type Culture Collection (Rockville, MD) at passage 128, were maintained at less than six passages after receipt. All cultures were maintained at 37°C and 5% CO_2 in 150-cm^2 culture flasks (Fisher Scientific, Pittsburgh, PA). The growth medium, consisting of McCoy's 5A medium (with 10% fetal bovine serum, 100 units penicillin/mL, 100 μg streptomycin/mL and 0.219 mg l-glutamine/mL added) was changed every 48 h.

Fatty acid enrichments

Upon reaching 80–90% confluence, HT-29 cells were split onto 78-cm^2 Optilux petri dishes (Fisher Scientific, Pittsburgh, PA) at a final concentration of approximately 10 million cells per dish. The cells were incubated for 24 h prior to enrichment with fatty acids. Fatty acid enrichments were administered by culturing the cells in growth medium supplemented with 50 μm OA, LA, arachidonic acid (AA, 20:4 n–6), eicosapentaenoic acid (EPA, 20:5 n–3), or DHA for 36 h. Fatty acids were added to the growth media in ethanol, with a final ethanol concentration of 0.03% (vol/vol). Control cells were grown in medium containing 0.03% ethanol (vol/vol), but without added fatty acid. To harvest, cells were washed twice in Dulbecco's phosphate-buffered saline and scraped into 2 mL methanol containing 0.1% (w/v) butylated hydroxytoluene as an antioxidant. HT-29 cell lipids were extracted by homogenization in chloroform–methanol–water 2:1:0.9 (vol/vol/vol) (21). After homogenization, the chloroform phase containing the lipid extract was collected and concentrated under a stream of nitrogen. Total HT-29 cell phospholipids were isolated from the lipid extract by thin-layer chromatography using a solvent mixture of petroleum ether–ethyl ether–acetic acid 80:20:1 (vol/vol/vol) (22). Cardiolipin was isolated from the lipid extract (including other phospholipids) by thin-layer chromatography using a solvent system of chloroform–methanol–acetic acid–water 50:37.5:3.5:2 (vol/vol/vol) (23). Phospholipid and CL bands were identified using authentic standards obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Isolated phospholipids or CL were scraped from the thin-layer chromatography plates and methylated by incubation with 3 N methanolic-HCl in sealed vials under a nitrogen atmosphere for 18 h. Fatty acid methyl esters were extracted from the methylation mixture with hexane and quantified by gas chromatography. Results shown with error bars are expressed as means ± SD for a minimum of three samples.

Flow cytometry

Mitochondrial potential and cellular oxidant production were assessed by flow cytometry using a FACScan (Becton-Dickenson, San Jose, CA) equipped with an argon laser. Mitochondrial potential was determined by measuring the rhodamine fluorescence of 2 million fatty acid-enriched HT-29 cells that had been incubated with 0.01 mg rhodamine 123 (R123)/mL for 30 min at 37°C (24). Changes in the mitochondrial potential were calculated from the mean R123 fluorescence of each fatty acid-enriched cell population and expressed as a percentage difference from control cells. Cellular oxidant production was determined by measuring the rhodamine fluorescence of 2 million fatty acid-enriched HT-29 cells that had been incubated in growth medium containing 1 μm dihydrorhodamine 123 (DHR123) for 30 min at 37°C (25). DHR123 is a non-fluorescent reduced R123 derivative that is freely permeable to cell membranes. Intracellular oxidation in the presence of H_2O_2 and cytochromes converts DHR123 to the fluorescent R123, which is retained intracellularly by the mitochondrial potential (26). Independently treated quadruplicate samples were pooled prior to flow cytometry, and 10,000 cells were counted for each analysis. Values for mitochondrial potential and cellular oxidant production were derived from single-parameter R123 and DHR123 fluorescence histograms, respectively. As fatty acid enrichments altered mitochondrial potential, and therefore the maximal ability of HT-29 cells to retain oxidized R123, a normalized cellular oxidant production value was calculated. The normalized ROS production was determined as the ratio of DHR123 fluorescence to R123 fluorescence for each cell population. The normalized ROS production is expressed as a difference between the fatty acid-enriched cells and controls (see Fig. 2B).

Assessment of membrane unsaturation

As a general assessment of the structure of CL, the average number of double bonds per CL fatty acid was calculated using the fatty acid compositional data presented in Table 1. The average number of double bonds per fatty acid was also determined for the total phospholipid fraction. Plots of normalized ROS production and the average number of double bonds per fatty acid for both CL and total phospholipids were constructed. Linear regressions were performed and the degree of correlation was assessed using the coefficient of determination (r^2-value).

RESULTS

Incorporation of fatty acids

Total HT-29 cell phospholipids contained 38, 18, 25, 13, or 11 mol% OA, LA, AA, EPA, or DHA, respectively, in cells enriched with each of those fatty acids (**Table 1**). Cardiolipin from fatty acid-enriched HT-29 cells contained 26, 41, 6, 9, or 48 mol% OA, LA, AA, EPA, or DHA, respectively, in cells enriched with those fatty acids (Table 1). HT-29 cells significantly incorporated each of the exogenously added fatty acids; however, only DHA and LA preferentially accumulated in CL. Cardiolipin also accumulated substantial amounts of OA but not EPA or AA. The selective incorporation of DHA and LA, but not AA or EPA, into HT-29 cell CL suggested that the enzymatic activities responsible for the unique acyl composition of CL in vivo were intact and similarly expressed in cultured HT-29 cells.

Mitochondrial potential

The change in mitochondrial potential for cells enriched with each of the fatty acids and control cells is shown in **Fig. 1**. The mitochondrial potential of cells enriched with OA, LA, and AA was diminished 11, 18, and 5%, respectively, relative to control cells. The mito-

TABLE 1. Fatty acid composition of total cell phospholipids (PL) and cardiolipin (CL) from HT-29 cells enriched with various fatty acids

Fatty Acid		Fatty Acid Enriched in HT-29 Cells					
		Control	OA	LA	AA	EPA	DHA
14:0	PL	4.5 ± 0.4	2.7 ± 0.1	3.4 ± 0.4	4.1 ± 0.6	3.3 ± 0.5	5.1 ± 1.0
	CL	3.2 ± 0.5	12.9 ± 8.7	6.0 ± 3.3	2.8 ± 0.8	6.0 ± 4.3	5.6 ± 4.1
16:0	PL	24.9 ± 0.3	23.5 ± 1.5	25.3 ± 3.0	18.2 ± 15.3	22.3 ± 1.0	27.6 ± 0.4
	CL	18.6 ± 2.9	10.9 ± 2.5	10.8 ± 1.9	11.7 ± 0.8	19.6 ± 4.5	6.4 ± 0.6
16:1	PL	14.1 ± 1.5	10.3 ± 0.3	12.0 ± 2.5	7.1 ± 1.1	12.1 ± 4.0	9.1 ± 1.0
	CL	25.5 ± 1.4	23.3 ± 2.8	9.1 ± 0.8	28.6 ± 5.4	14.9 ± 5.4	11.8 ± 3.9
18:0	PL	9.1 ± 0.3	8.9 ± 0.3	9.5 ± 0.3	15.8 ± 2.4	10.5 ± 1.8	12.2 ± 0.7
	CL	6.0 ± 1.0	3.9 ± 1.0	4.8 ± 1.8	4.0 ± 0.4	7.2 ± 2.0	2.7 ± 0.1
18:1	PL	28.1 ± 0.3	38.1 ± 0.8	18.8 ± 0.6	17.8 ± 2.8	24.9 ± 2.1	17.9 ± 2.2
	CL	15.5 ± 0.6	25.9 ± 3.0	8.3 ± 0.5	17.2 ± 0.6	13.0 ± 2.7	9.7 ± 2.3
18:1n–7	PL	3.2 ± 0.3	2.6 ± 0.1	2.4 ± 0.0	2.8 ± 0.5	2.2 ± 0.8	2.4 ± 0.2
	CL	10.3 ± 1.0	11.6 ± 1.4	12.0 ± 0.9	16.2 ± 1.0	7.7 ± 2.4	6.6 ± 1.7
18:2	PL	2.2 ± 0.2	1.9 ± 0.1	18.1 ± 0.9	1.6 ± 0.3	1.6 ± 0.4	1.5 ± 0.1
	CL	8.3 ± 0.5	6.4 ± 0.8	41.1 ± 5.2	10.4 ± 0.8	9.7 ± 3.7	4.0 ± 1.0
20:4	PL	5.9 ± 0.7	5.4 ± 0.1	4.1 ± 0.1	24.9 ± 3.9	4.5 ± 0.9	4.1 ± 0.1
	CL	0.7 ± 0.2	0.6 ± 0.1	1.5 ± 0.8	6.0 ± 0.8	2.0 ± 0.4	0.7 ± 0.0
20:5	PL	0.7 ± 0.6	1.0 ± 0.0	0.7 ± 0.0	0.6 ± 0.2	13.0 ± 0.1	5.2 ± 0.8
	CL	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.9	0.0 ± 0.0	8.7 ± 0.4	0.5 ± 0.0
22:6	PL	2.3 ± 0.2	1.6 ± 0.1	1.4 ± 0.1	1.6 ± 0.3	1.8 ± 0.4	11.6 ± 1.3
	CL	3.2 ± 0.5	0.2 ± 0.0	1.4 ± 0.8	0.3 ± 0.6	2.3 ± 0.3	47.7 ± 5.0
Other	PL	5.0	3.9	4.3	5.5	3.8	3.3
	CL	8.7	4.3	4.6	2.8	8.9	4.3

Data are expressed as the mol% fatty acid in the sample and are averages of three independently treated samples ± SD. OA, LA, AA, EPA, and DHA represent HT-29 cells enriched with oleic acid, linoleic acid, arachidonic acid, eicospentaenoic acid, and docosahexaenoic acid, respectively.

chondrial potential of cells enriched with EPA was not different from that of control cells. Mitochondrial potential in DHA-enriched cells was increased 11% relative to control cells.

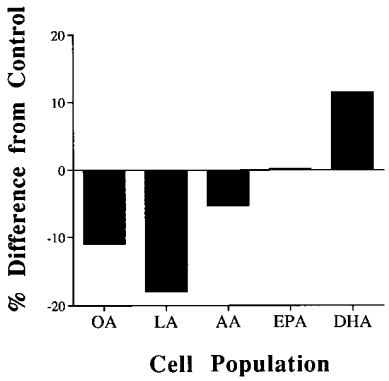


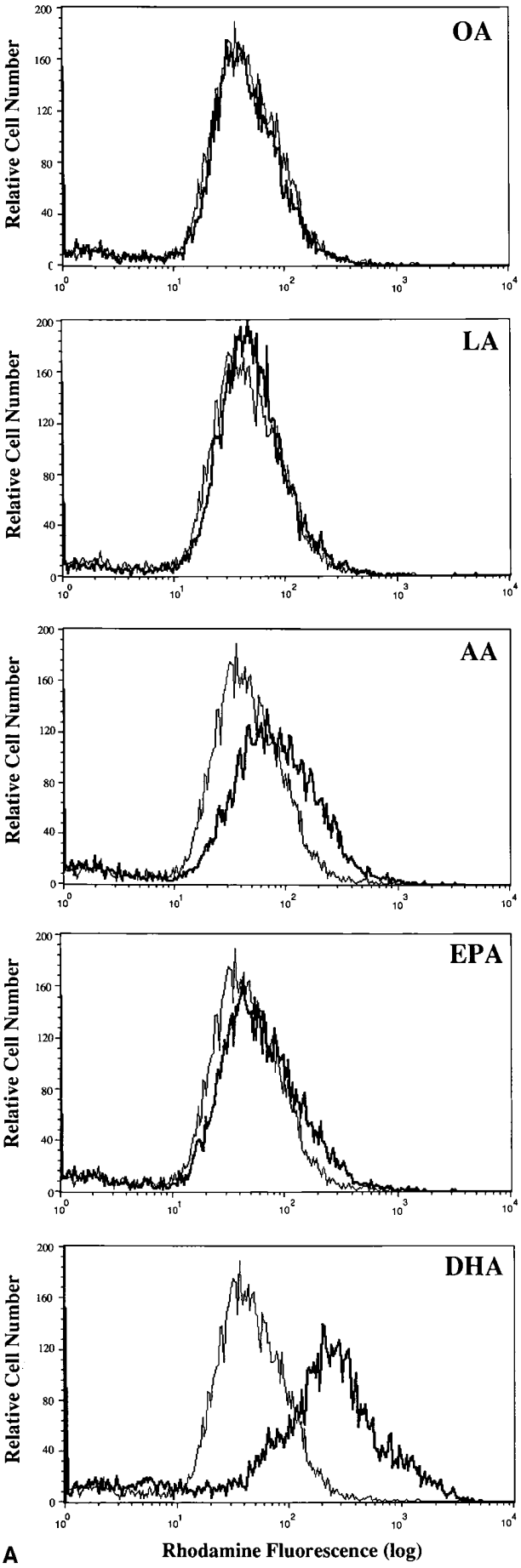
Fig. 1. Mitochondrial potential in HT-29 cells enriched with oleic acid (OA), linoleic acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA), or docosahexaenoic acid (DHA). Fatty acid-enriched cells (two million per replicate) were incubated with 0.01 mg R123/mL for 30 min at 37°C and analyzed for rhodamine fluorescence by flow cytometry. Four independently treated and stained samples were pooled for each analysis. Values for mitochondrial potential were obtained from the mean rhodamine fluorescence of each population. Data are expressed as a percentage difference in the mean rhodamine fluorescence for fatty acid-enriched cell populations and for that of control cell populations.

Cellular oxidant production

One-parameter DHR123 fluorescence histograms for HT-29 cells enriched with each fatty acid are shown in **Fig. 2A**. The mean DHR123 fluorescence of cells enriched with DHA was 490% greater than that in control cells. Cells enriched with OA, LA, AA, or EPA had mean DHR123 fluorescences that were changed −6, 11, 96, or 41% over that of controls, respectively. Because changes in mitochondrial potential indicate a differential ability to retain oxidized R123, cell oxidant production data were also normalized for mitochondrial potential (Fig. 2B). Normalized ROS production by cells enriched with OA, LA, AA, EPA, or DHA was, respectively, 6, 35, 94, 40, or 429% greater than that produced by control cells. These values were not substantially different from the mean DHR123 fluorescence values obtained from one-parameter DHR123 fluorescence histograms (Fig. 2A).

Degree of phospholipid unsaturation

As a general assessment of phospholipid structure, the average number of double bonds per fatty acid present in CL or total phospholipids was determined. Cardiolipin from cells enriched with OA, LA, AA, EPA, or DHA contained an average of 0.81, 1.31, 1.16, 1.27, or 3.37 double bonds per fatty acid, respectively. Total phospholipids of HT-29 cells enriched with OA, LA, AA, EPA, or DHA contained an average of 0.99, 1.07, 1.56, 1.45, or 1.52 double bonds per fatty acid, respectively. Linear regressions for the normalized ROS production (reported as a percent-



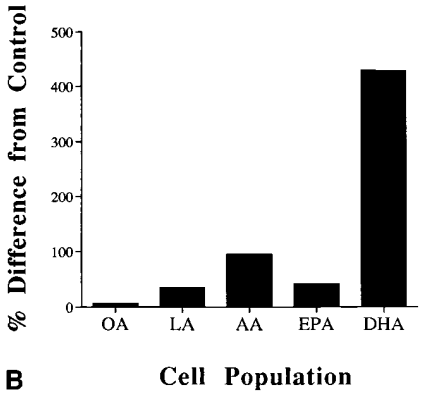


Fig. 2. A: One-parameter rhodamine fluorescence histograms for fatty acid-enriched HT-29 cells treated with dihydrorhodamine 123 (DHR123). Light lines represent control cell populations and dark lines represent oleic acid (OA), linoleic acid (LA), arachidonic acid (AA), eicosapentaenoic acid (EPA), or docosahexaenoic acid (DHA)-enriched cell populations. Cells (two million per replicate) were incubated with 1 μm DHR123 for 30 min at 37°C and analyzed for rhodamine fluorescence by flow cytometry. DHR123 reacts with intracellular ROS to form fluorescent R123, which is then retained by the mitochondria, enabling a flow cytometric assessment of cellular oxidant production. Each histogram is the result of four independently treated and stained samples that were pooled prior to analysis. B: Normalized cellular oxidant production in fatty acid-enriched HT-29 cells. As the retention of oxidized R123 varied between cell populations, a normalized DHR123 fluorescence was calculated. The ratio of rhodamine fluorescences between cells treated with DHR123 and R123 was determined and the values obtained for each fatty acid-enriched cell population were plotted as a percentage difference from controls. Normalized ROS production values were not substantially different from those obtained directly from DHR123 fluorescence histogram analyses. This shows that changes in rhodamine fluorescence from cells treated with DHR123 were not the result of differences in the capacity of mitochondria to retain oxidized R123. These data demonstrate a large and specific amplification of cellular ROS production in DHA-enriched HT-29 cells.

age difference from control cells) and the average numbers of double bonds per fatty acid for both CL and total phospholipids are shown in **Fig. 3**. The coefficient of determination (r^2) for ROS production and total phospholipid unsaturation was 0.308, indicating that the degree of unsaturation of total phospholipids was not correlated with the amount of ROS produced by HT-29 cells. However, the r^2-value for ROS production and CL fatty acid unsaturation was 0.962, demonstrating the strong correlation between CL unsaturation and the production of ROS.

DISCUSSION

The present study investigated the contribution of individual membrane fatty acids to cellular ROS production and mitochondrial potential in whole cells. Changes in these functions were consistent neither with how well the enriched fatty acid changed total HT-29 cell acyl composition nor with the degree of unsaturation of total HT-29

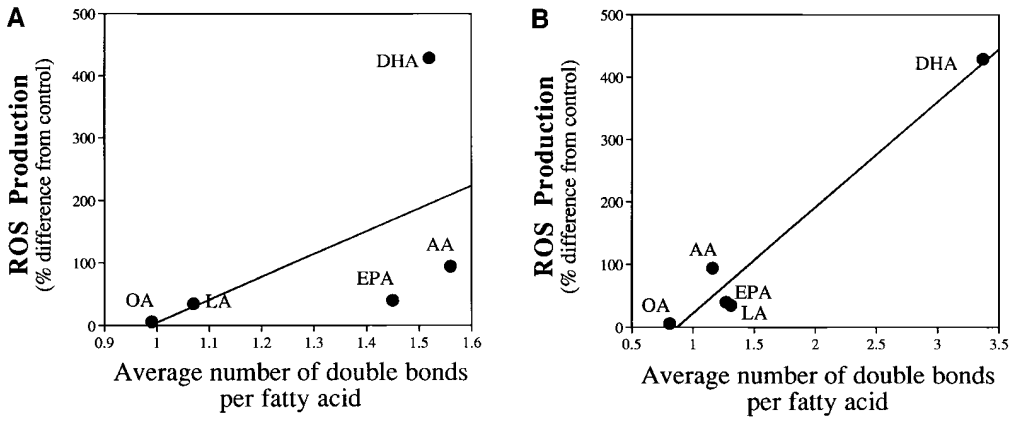


Fig. 3. A: Relationship between ROS production and total HT-29 cell membrane unsaturation for cells enriched with various fatty acids. Values for normalized ROS production are taken from Fig. 2B. The number of double bonds is expressed as an average per membrane fatty acid, and was calculated from fatty acid analysis data. OA, LA, AA, EPA, and DHA represent the values obtained from cells enriched with oleic acid, linoleic acid, arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid, respectively. A linear regression was performed to determine whether a relationship existed between these variables, and the coefficient of determination (r^2) was 0.308. Therefore, the degree of unsaturation of total cell membranes did not correlate with cellular oxidant production. B: Relationship between ROS production and cardiolipin (CL) unsaturation for cells enriched with various fatty acids. Values for normalized ROS production are taken from Fig. 2B. The number of double bonds is expressed as an average per CL fatty acid, and was calculated from fatty acid analysis data. A linear regression was performed to determine whether a relationship existed between these variables, and the coefficient of determination (r^2) was 0.962, suggesting a role for CL acyl composition in the production of cellular ROS.

cell membranes. This suggested that membrane PUFA were capable of changing cell functions, including ROS production, through mechanisms that did not involve a general membrane peroxidation reaction. These mechanisms may explain why individual fatty acids have unique effects on mitochondrial function.

Although free fatty acids are known to uncouple energy production in isolated mitochondria, the intracellular concentration of free fatty acids is exceptionally small, and this phenomenon is not likely to be relevant to live cells (27). HT-29 cell mitochondrial potential was influenced most drastically by LA and DHA. Linoleic acid and DHA were also the fatty acids that incorporated most extensively into CL, suggesting a possible role for CL acyl composition in mitochondrial potential. The presence of CL is required for the activity of mitochondrial enzymes involved in the maintenance of membrane potential, including cytochrome *c* oxidase, cytochrome b_1c_1 complex, ATPase, ADP/ATP carrier protein, and NADH reductase (8). Changes in CL–enzyme complex interactions induced by altering CL acyl composition are known to modulate complex activity (8, 9). The effects of membrane acyl composition on mitochondrial potential are at least partially mediated by CL acyl composition.

Large changes in the production of cellular ROS were observed in HT-29 cells enriched with unsaturated fatty acids. A general peroxidation of membrane unsaturated fatty acids was not responsible for these changes, as ROS production did not correlate with the degree of membrane unsaturation (Fig. 3A). In fact, DHA was the fatty acid least incorporated into total HT-29 cell phospholipids, yet DHA-enriched cells produced 5-fold more ROS than any other fatty acid-enriched cell population. The specific amplification of ROS by DHA suggested that DHA accumulated in a critical subcellular pool. This critical pool is CL, as DHA accounted for 48 mol% of CL fatty acids, a striking contrast to the 11 mol% present in total HT-29 cell phospholipids.

Because CL structure influences electron transport efficiency (2, 5, 6, 9, 28), which in turn is proposed to modulate mitochondrial superoxide radical production (14, 29), it was logical to investigate the degree of CL unsaturation for its relationship with cellular ROS production. Indeed, there was a strong correlation between the number of unsaturated bonds per CL fatty acid and ROS production (Fig. 3B). The unique effect of DHA on ROS production is thus explained by the selective accumulation of DHA in CL. The changes in CL structure that accompany a large increase in DHA content would be substantial as DHA has a highly kinked and rigid structure as the result of its six unsaturated bonds.

As the result of its accumulation in CL, DHA is proposed to have amplified ROS production by modulating electron transport efficiency and increasing the production of superoxide radicals. It has previously been shown that cells treated with DHA necrose as the result of intracellular ROS production. However, consistent with the proposal stated above, this effect is eliminated in cells depleted of mitochondrial DNA, and that therefore do not synthesize electron transport complexes (30). It is also possible that CL fatty acids participate directly in peroxidation reactions. This would explain the decrease in the CL content of tissues from older animals or in cells subjected to oxidant stresses (14, 16). A direct participation of DHA in peroxidation reactions would not be independent of changes in electron transport efficiency, however, as damaged CL would further impair CL–complex interactions. Changes in CL structure also explain the failure

of the polyunsaturated AA or EPA to produce results similar to those produced by DHA. While enriched in total HT-29 cell phospholipids to a greater extent than DHA, AA or EPA were not substantially incorporated into CL. Cardiolipin rich in LA has been presumed to provide the optimal structure for interacting with electron transport complexes; however, LA was not superior to OA in preventing the production of cellular ROS. The present data provide evidence for an important effect of CL acyl composition in the production of cellular ROS. Cardiolipin is a dynamic and important phospholipid pool that is critical to the normal activity of the mitochondria, and it is clear that CL fatty acid composition should be considered when interpreting the cellular effects of dietary fats and oils.

The similarities between the acyl composition of HT-29 cell CL and CL isolated from animal tissues show that cultured HT-29 cells are appropriate for testing the effects of fatty acids on mitochondrial functions. The present study demonstrates that mitochondrial potential and ROS production are affected by membrane acyl composition. However, these effects are likely to be mediated by specific mechanisms rather than non-specific PUFA peroxidation. The influence of CL acyl composition on mitochondrial potential and cellular ROS production provides evidence for a novel means to modulate mitochondrial function through diet. These results suggest that CL acyl composition can modulate the increased ROS production observed in aging and cell death processes.

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