Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 8845–8849, November 1990
Medical Sciences

Incorporation of marine lipids into mitochondrial membranes increases susceptibility to damage by calcium and reactive oxygen species: Evidence for enhanced activation of phospholipase A_2 in mitochondria enriched with $n-3$ fatty acids

(fish oils/ischemia/oxygen free radicals)

CHARLES D. MALIS*, PETER C. WEBER†, ALEXANDER LEAF*, AND JOSEPH V. BONVENTRE*‡

*Medical Services, Massachusetts General Hospital, and Departments of Medicine and Preventive Medicine, Harvard Medical School, Boston, MA 02114; and †Institut fur Prophylaxe und Epidemiologie der Kreislaufkrankheiten an der Universitat Munchen, Munich, Federal Republic of Germany

Contributed by Alexander Leaf, July 12, 1990

ABSTRACT **Experiments were designed to evaluate the susceptibility of mitochondrial membranes enriched with $n-3$ fatty acids to damage by Ca^{2+} and reactive oxygen species. Fatty acid content and respiratory function were assessed in renal cortical mitochondria isolated from fish-oil- and beef-tallow-fed rats. Dietary fish oils were readily incorporated into mitochondrial membranes. After exposure to Ca^{2+} and reactive oxygen species, mitochondria enriched in $n-3$ fatty acids, and using pyruvate and malate as substrates, had significantly greater changes in state 3 and uncoupled respirations, when compared with mitochondria from rats fed beef tallow. Mitochondrial site 1 (NADH coenzyme Q reductase) activity was reduced to 45 and 85% of control values in fish-oil- and beef-tallow-fed groups, respectively. Exposure to Ca^{2+} and reactive oxygen species enhanced the release of polyunsaturated fatty acids enriched at the *sn*-2 position of phospholipids from mitochondria of fish-oil-fed rats when compared with similarly treated mitochondria of beef-tallow-fed rats. This release of fatty acids was partially inhibited by dibucaine, the phospholipase A_2 inhibitor, which we have previously shown to protect mitochondria against damage associated with Ca^{2+} and reactive oxygen species. The results indicate that phospholipase A_2 is activated in mitochondria exposed to Ca^{2+} and reactive oxygen species and is responsible, at least in part, for the impairment of respiratory function. Phospholipase A_2 activity and mitochondrial damage are enhanced when mitochondrial membranes are enriched with $n-3$ fatty acids.**

Dietary fish oils, enriched in long chain polyunsaturated fatty acids, eicosapentaenoic acid [20:5 ($n-3$); EPA] and docosahexaenoic acid [22:6 ($n-3$); DHA], may be important in the prevention of atherosclerotic vascular diseases (1, 2). Little is known, however, regarding their potential adverse effects.

Since phospholipase A_2 (PLA_2) is activated in the presence of peroxidized fatty acids and membranes enriched with $n-3$ fatty acids will have more unsaturated bonds and likely be more susceptible to peroxidation, these membranes might be more susceptible to PLA_2-induced degradation. Lorenz *et al.* (3) and Von Schacky *et al.* (4), however, reported that phospholipid enrichment with $n-3$ fatty acids resulted in decreased eicosanoid production and suggested that $n-3$ fatty acids serve as poor substrates for PLA_2 action.

We and others have implicated PLA_2 as a mediator of cellular injury associated with Ca^{2+} and reactive oxygen species in ischemia, toxin exposure, and trauma for the following reasons. (*i*) Ca^{2+} activates PLA_2 (5). (*ii*) Ca^{2+} enhances lipid peroxidation (6) and, even in the absence of increased $[Ca^{2+}]$, PLA_2 activation can be potentiated by the presence of peroxidized fatty acids in phospholipids (7). (*iii*) Reactive oxygen species enhance vasoactive eicosanoid synthesis in various tissues (6, 8, 9) and PLA_2 is the rate-limiting enzyme for eicosanoid production. (*iv*) Free fatty acids (FFAs) (10–12) and lysophospholipids (13–15), released by PLA_2, can damage cell membranes and increase membrane permeability to Ca^{2+} (16), thereby increasing cellular $[Ca^{2+}]$ and enhancing the effects listed above.

Mitochondria play a central role in cell injury associated with many pathophysiological states. PLA_2 has been implicated as a mediator of mitochondrial damage (17). We have reported (18) that Ca^{2+} and reactive oxygen species produced functional mitochondrial defects that mimicked those produced with ischemia *in vivo* (19, 20).

In the present study, we correlated functional abnormalities with changes in mitochondrial phospholipid fatty acid composition as well as the amount and composition of released FFAs after exposure to Ca^{2+} and reactive oxygen species. The results indicate that mitochondrial membranes enriched with EPA and DHA are more susceptible to PLA_2-mediated injury produced by Ca^{2+} and reactive oxygen species, as reflected by reductions in respiratory control, uncoupled respiration, and site I [NADH coenzyme Q (CoQ) reductase] activity of the electron transport chain. These functional disturbances were associated with enhanced release of polyunsaturated fatty acids from mitochondrial membranes.

METHODS

Feeding Protocol. Male Sprague–Dawley rats were fed a diet containing either 2% (wt/wt) corn oil and 20% (wt/vol) beef tallow or fish oils (menhaden oil, lot 2370; Zapata Haynie, Reedville, VA), with vitamin E supplementation at 80 international units/day, for at least 4 weeks.

Mitochondrial Function. Renal cortical mitochondria from rats weighing 250–325 g were prepared at 4°C and respiration was monitored polarographically at 24°C, as reported (18). Mitochondrial protein (1 mg) was added to 1 ml of respiration medium consisting of 140 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgCl_2$, 30 μM EGTA, and 5 mM Tris·HCl (pH 7.2). Respiratory substrate was either sodium pyruvate (5 mM) and

Abbreviations: FO, fish oil; BT, beef tallow; PLA_2, phospholipase A_2; Δ_4Ach, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; HX/XO, reactive oxygen species generated by hypoxanthine and xanthine oxidase, respectively; FFA, free fatty acid; CoQ, coenzyme Q.

‡To whom reprint requests should be addressed at: Renal Unit, Jackson 8, Massachusetts General Hospital, 32 Fruit Street, Boston, MA 02114.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

sodium malate (5 mM) or sodium succinate (10 mM). When succinate was used, rotenone (4 μM) was included in the medium. Mitochondrial respiration was studied 3 min subsequent to the addition of (*i*) 25 μM hypoxanthine (HX) and 0.3 unit of xanthine oxidase (XO), which generate reactive oxygen species, or (*ii*) $CaCl_2$ (30 nmol/mg), followed by HX and XO. No exogenous iron was added. State 4 respiration was measured for 2 min. ADP (300 nmol) was then added and state 3 respiration was determined. After all ADP was consumed and state 4 respiration was reestablished, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; 100 pg/mg of mitochondrial protein) was added to assess uncoupled respiration. NADH CoQ reductase activity was assayed by the method of Di Virgilio and Azzone (21) with modifications (18).

Mitochondrial Phospholipid Extraction. Phospholipids were extracted and fatty acid composition was determined, as described (3, 4, 22) with some modifications. Butylated hydroxytoluene (final concentration, 0.2%) was added as an antioxidant to the tissue. 17:0 and 20:3 ($n-6$) FFAs were added to the phospholipid extract, serving as internal standards for extraction efficiency and degradation, respectively. Analysis was carried out with a Hewlett–Packard (model 5890A) gas chromatograph equipped with an integrator (model 3393A), and a Durabond-225 fused-silica capillary column consisting of 50% cyanopropylmethyl/50% methylphenylpolysiloxan, 30 mm long, 0.25 mm internal diameter, and a 0.15-μm coated phase. Injection and detector port temperatures were 200 and 250°C, respectively. The oven temperature was 90°C for 0.5 min, increased 7°C/min to 200°C, and maintained at 200°C for 30–35 min.

FFA Release. Mitochondrial fatty acid release into buffer containing 0.5% bovine serum albumin was determined as an index of mitochondrial PLA_2 activity. Fatty acids were extracted from the medium with $CHCl_3$/*n*-heptane/methanol, 56:42:2 (vol/vol), with butylated hydroxytoluene. To form the FFA methyl esters, diazomethane was added to the residue and evaporated with N_2. Fatty acid methyl esters were analyzed by gas chromatography, as described above.

Statistics. Statistical analysis was performed using analysis of variance, with Scheffe analysis for comparisons between groups. Data are presented as mean ± SEM.

RESULTS

Phospholipid Fatty Acid Content. Phospholipids in mitochondria from fish-oil-fed rats (FO mitochondria) had reduced amounts of oleic acid [18:1 ($n-9$)], linoleic acid [18:2 ($n-6$)], and arachidonic acid [20:4 ($n-6$); Δ_4Ach], and 15- and 3-fold increases in EPA and DHA content, respectively, when compared to phospholipids from mitochondria from beef-tallow-fed rats (BT mitochondria) (Fig. 1).

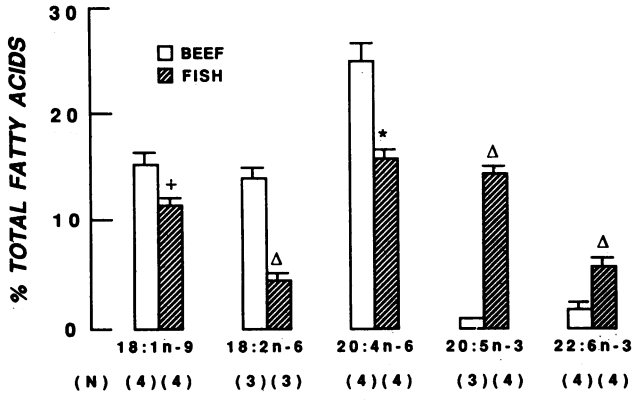

FIG. 1. Incorporation of fish oils into mitochondrial membranes with the feeding protocol employed. Data are expressed as percent of total fatty acids. A diet high in fish oils resulted in reduced content of oleic acid [18:1 ($n-9$)], linoleic acid [18:2 ($n-6$)], and Δ_4Ach [20:4 ($n-6$)] (75, 30, and 65% of BT mitochondria, respectively), while resulting in significant (15- and 3-fold, respectively) increases in EPA [20:5 ($n-3$)] and DHA [22:6 ($n-3$)] when compared with mitochondria from beef-tallow-fed rats. +, $P < 0.05$; *, $P < 0.005$; Δ, $P < 0.0005$ compared with BT mitochondria.

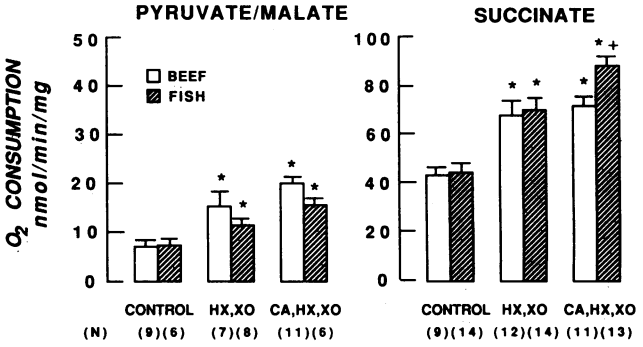

FIG. 2. Effect of calcium (CA) and reactive oxygen species (HX/XO) on basal (state 4) respiration of mitochondria isolated from beef-tallow- or fish-oil-fed rats. Either pyruvate and malate (site I substrates) (*Left*) or succinate (a site II substrate) (*Right*) were used as substrates. *, $P < 0.01$ compared to control; +, $P < 0.01$ compared with BT mitochondria treated with Ca^{2+} and HX/XO.

Mitochondrial Respiration. By using pyruvate and malate as (site I) substrates, FO and BT mitochondria exposed to reactive oxygen species (HX/XO) or Ca^{2+} and reactive oxygen species (Fig. 2 *Left*) developed significant increases in basal (state 4) respiration over controls. Similarly, by using succinate as (a site II) substrate, state 4 respiration also increased with exposure to reactive oxygen species alone or plus Ca^{2+} (Fig. 2 *Right*). When Ca^{2+} was added with HX and XO, FO mitochondria had a significantly higher succinate-supported (basal) respiration than did BT mitochondria. Increases in state 4 respiration indicate enhanced mitochondrial membrane permeability.

FO mitochondria exposed to Ca^{2+} and reactive oxygen species, with pyruvate and malate as substrates, had a significant marked decrease of ADP-stimulated (state 3) respiration to 55% of that measured in BT mitochondria (Fig. 3 *Left*). In contrast, mitochondria from both feeding groups had equivalent decreases in ADP-stimulated respiration with succinate as substrate (Fig. 3 *Right*). An accentuated reduction in ADP-stimulated respiration with site I but not site II substrates in FO mitochondria might be explained by a defect in the electron transport chain.

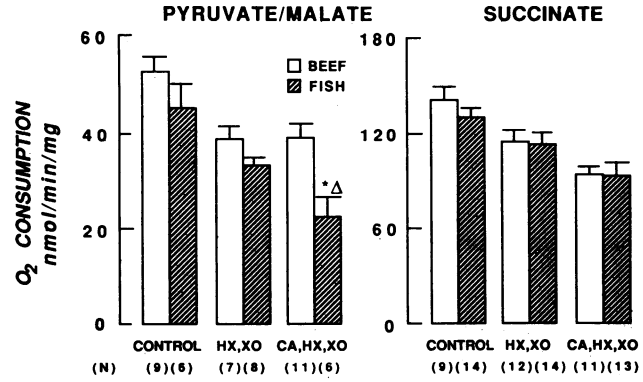

FIG. 3. Effect of calcium (CA) and reactive oxygen species (HX/XO) on ADP-stimulated (state 3) respiration in mitochondria isolated from beef-tallow- and fish-oil-fed rats. Experiments were performed with either pyruvate and malate (*Left*) or succinate (*Right*) as substrates. * Δ, $P < 0.01$ compared to control FO mitochondria and Ca^{2+}/HX/XO BT mitochondria.

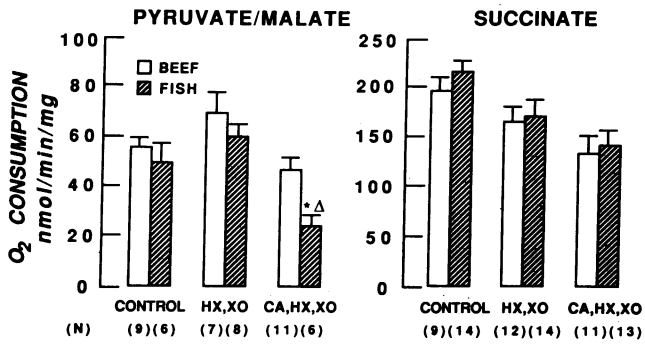

FIG. 4. Effect of calcium (CA) and reactive oxygen species (HX/XO) on uncoupled respiration in mitochondria isolated from rats fed beef tallow or fish oil. Pyruvate and malate (*Left*) or succinate (*Right*) were employed as substrates. * Δ, $P < 0.01$ compared to control FO and Ca^{2+}/HX/XO BT mitochondria.

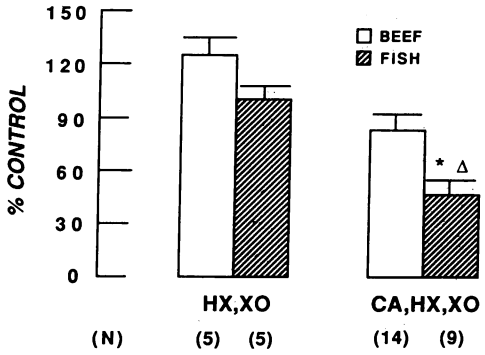

FIG. 5. Effect of calcium (CA) and reactive oxygen species (HX/XO) on rotenone-suppressible NADH CoQ reductase in mitochondria isolated from rats fed beef tallow or fish oil. Data are presented as percent control rotenone-suppressible activity. * Δ, $P < 0.01$ compared to HX/XO exposure of FO mitochondria and Ca^{2+}/HX/XO exposure of BT mitochondria.

The integrity of the electron transport chain was evaluated by assessing uncoupled respiration. With pyruvate and malate as substrates, FO mitochondria sustained a much larger reduction in uncoupled respiration upon exposure to Ca^{2+} and reactive oxygen species as compared with BT mitochondria (Fig. 4 *Left*). With succinate as substrate, there were graded reductions in uncoupled respiration in mitochondria exposed to HX/XO alone and to Ca^{2+}/HX/XO. However, there were no differences in uncoupled respiration comparing FO and BT mitochondria (Fig. 4 *Right*). These results suggest that FO mitochondria could have a defect in the electron transport chain localized to site I.

NADH CoQ Reductase Activity. The function of site I of the electron transport chain was determined by assaying NADH CoQ reductase. Rotenone, a specific inhibitor of this enzyme, suppressed activity by 65% in FO and BT mitochondria. With exposure to Ca^{2+} and reactive oxygen species, BT mitochondria had a modest reduction in site I activity to 85% of control rotenone-suppressible activity. In contrast, site I activity in FO mitochondria fell to 45% of control, indicating a marked defect at site I of the electron transport chain (Fig. 5).

PLA_2 and Mitochondrial Damage. To assess the role of PLA_2 in the production of mitochondrial damage, mitochondrial phospholipid fatty acid content and FFAs released from mitochondria were analyzed after exposure to Ca^{2+}, reactive oxygen species (HX/XO), Ca^{2+}/HX/XO, or Ca^{2+}/HX/XO/dibucaine, an inhibitor of PLA_2 activity (Tables 1, 2, and 3).

In FO mitochondria, phospholipid fatty acid content did not change upon exposure to Ca^{2+} alone. After exposure to reactive oxygen species (HX/XO), Δ_4Ach [20:4 ($n-6$)], EPA [20:5 ($n-3$)], and DHA [22:6 ($n-3$)] content decreased significantly.

Ca^{2+} alone caused no detectable change in the FFAs released. FFAs were released from FO mitochondria after exposure to RAD alone, and FFA release was further enhanced with exposure to both Ca^{2+} and HX/XO. The increased release of 20:4 and 20:5 FFAs exceeded the increases observed in any of the other FFAs. Whereas the increases in these two FFAs were 8- and 5-fold above control, none of the others exceeded a 3-fold increase. Significant increases were also measured in medium 22:6 ($n-3$) with Ca^{2+} and HX/XO.

Since polyunsaturated fatty acids occupy the *sn*-2 position of phospholipids and these are the fatty acids that were most affected by the treatments, it may be concluded that PLA_2 activity was increased by exposure to Ca^{2+} and HX/XO. We have reported (18) that dibucaine, a PLA_2 inhibitor (23), protected mitochondria against functional defects induced by Ca^{2+} and reactive oxygen species. Dibucaine (*i*) protected the electron transport chain, presumably at the level of NADH, CoQ reductase; (*ii*) preserved F_1-ATPase and ADP translocase activity; and (*iii*) prevented complete uncoupling of mitochondrial respiration (18). When dibucaine was added to FO mitochondria prior to Ca^{2+} and HX/XO exposure,

Table 1. Fatty acid content of mitochondrial phospholipids and characterization of released fatty acids from FO mitochondria after each experimental treatment

Treatment	*n*	Fatty acids, %						
		16:0	18:0	18:1	18:2	20:4	20:5	22:6
FO mitochondrial phospholipid fatty acid content								
Control	6	11.4 ± 0.4	15.2 ± 0.9	3.6 ± 0.2	8.0 ± 0.4	14.4 ± 0.5	6.8 ± 0.1	3.2 ± 0.2
Ca^{2+}	6	10.3 ± 0.5	13.1 ± 0.6	3.5 ± 0.1	7.8 ± 0.3	13.6 ± 0.7	6.5 ± 0.2	3.2 ± 0.2
HX/XO	6	9.8 ± 0.7	13.8 ± 0.5	3.1 ± 0.2	7.4 ± 0.1	8.0 ± 0.3‡	3.8 ± 0.2‡	2.6 ± 0.2*
Ca^{2+}/HX/XO	6	10.4 ± 0.6	14.4 ± 0.4	3.1 ± 0.2	7.5 ± 0.2	8.1 ± 0.4‡	3.8 ± 0.1‡	2.7 ± 0.2
Ca^{2+}/HX/XO/Dibu	4	12.0 ± 0.2	15.6 ± 0.9	3.7 ± 0.2	8.2 ± 0.3	7.9 ± 0.4‡	3.9 ± 0.4‡	2.8 ± 0.2
FO mitochondrial FFAs released								
Control	6	2.9 ± 0.8	2.6 ± 0.5	1.6 ± 0.3	3.2 ± 1.2	1.2 ± 0.2	1.0 ± 0.4	0.5 ± 0.1
Ca^{2+}	6	2.6 ± 0.2	2.5 ± 0.3	1.1 ± 0.5	4.6 ± 1.7	1.1 ± 0.2	0.8 ± 0.2	0.6 ± 0.1
HX/XO	5	4.6 ± 0.4	3.9 ± 0.1	1.8 ± 0.2	6.2 ± 0.9	6.7 ± 0.8‡	3.7 ± 0.4‡	1.2 ± 0.1‡
Ca^{2+}/HX/XO	6	5.7 ± 0.2†	4.8 ± 0.3†	2.8 ± 0.2†	8.0 ± 0.4†	9.3 ± 0.6‡	5.1 ± 0.4‡	1.5 ± 0.2‡
Ca^{2+}/HX/XO/Dibu	4	5.8 ± 0.7*	5.5 ± 0.8*	2.9 ± 0.2†	3.4 ± 0.3¶	5.6 ± 0.9§	3.6 ± 0.6†	1.2 ± 0.2*

Individual fatty acid content is expressed as percent of total phospholipid fatty acids or total FFAs (mean ± SEM) where the total includes the added standards. The fatty acid content in the phospholipids of mitochondria exposed to 30 μM Ca^{2+} alone, reactive oxygen species alone (HX/XO), the two combined (Ca^{2+}/HX/XO), and Ca^{2+}/HX/XO combined with dibucaine (Dibu) is shown as is the FFA composition of the medium in the same experiments. Compared with controls: *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$. Compared with Ca^{2+}/HX/XO group: §, $P < 0.01$; ¶, $P < 0.001$.

Table 2. Fatty acid content of mitochondrial phospholipids and characterization of released fatty acids from BT mitochondria after each experimental treatment

Treatment	*n*	Fatty acids, % 16:0	18:0	18:1	18:2	20:4	20:5	22:6
		BT mitochondrial phospholipid fatty acid content						
Control	6	11.8 ± 0.6	14.8 ± 0.5	5.2 ± 0.1	11.0 ± 1.2	20.2 ± 1.1	1.4 ± 0.2	1.3 ± 0.1
Ca^{2+}	5	10.3 ± 0.6	12.8 ± 0.5	4.7 ± 0.3	9.6 ± 1.4	18.5 ± 1.5	1.2 ± 0.2	1.2 ± 0.1
HX/XO	4	10.3 ± 0.7	13.9 ± 0.3	4.8 ± 0.4	10.0 ± 1.1	14.3 ± 0.6†	1.2 ± 0.2	1.3 ± 0.2
Ca^{2+}/HX/XO	6	10.6 ± 0.5	14.5 ± 0.6	4.8 ± 0.2	10.1 ± 0.9	15.0 ± 0.8†	1.4 ± 0.2	1.2 ± 0.5
Ca^{2+}/HX/XO/Dibu	6	11.4 ± 0.6	14.5 ± 0.7	5.1 ± 0.3	10.1 ± 1.1	13.1 ± 1.0‡	0.9 ± 0.2	0.9 ± 0.1
		BT mitochondrial FFAs released						
Control	9	3.8 ± 0.2	3.1 ± 0.2	3.8 ± 0.5	5.3 ± 0.7	1.4 ± 0.3	1.4 ± 0.3	0.4 (2)
Ca^{2+}	3	2.8 ± 0.3	2.8 ± 0.2	5.0 ± 0.3	5.4 ± 1.1	0.6 ± 0.2	ND	ND
HX/XO	3	3.1 ± 0.5	3.2 ± 0.2	5.7 ± 0.5	6.2 ± 1.4	3.0 ± 0.4*	0.3 (2)	0.2 ± 0.0
Ca^{2+}/HX/XO	7	3.9 ± 0.3	3.1 ± 0.2	4.6 ± 0.7	6.8 ± 0.8	6.5 ± 1.4†	0.5 ± 0.3	0.5 ± 0.2
Ca^{2+}/HX/XO/Dibu	7	4.5 ± 0.3	4.0 ± 0.2	4.4 ± 0.6	5.8 ± 0.7	4.5 ± 1.1*	0.6 ± 0.1	0.5 ± 0.2

Individual fatty acid content is expressed as percent of total phospholipid or FFAs (mean ± SEM) where the total includes the added standards. The experiments are analogous to those described for fish-oil-fed rats in Table 1. ND indicates that measurements were not made. Compared with controls: *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$. Dibu, dibucaine.

there were reductions in media levels of 18:2, 20:4, and 20:5 FFAs, as compared with Ca^{2+} and HX/XO exposure alone. Dibucaine did not modify the levels of saturated FFAs that largely occupy the *sn*-1 position of phospholipids. The small release of saturated fatty acids with Ca^{2+} and HX/XO, not inhibited by dibucaine, indicates the presence of an acylhydrolase activity other than PLA_2.

When compared with FO mitochondria, BT mitochondria showed fewer changes in phospholipid fatty acid composition after the same level of exposure to Ca^{2+} and reactive oxygen species (Table 2). As expected, the level of 20:4 ($n-6$) was much higher and that of 20:5 ($n-3$) much lower in BT compared with FO mitochondria. The phospholipids in the BT mitochondria showed no significant changes in fatty acid content with any of the treatments, with the exception that 20:4 ($n-6$) content fell with exposure to HX/XO but was not further reduced by the combination of Ca^{2+} and HX/XO. Control levels of the 20:5 and 22:6 fatty acids were very low and did not show significant changes. Dibucaine failed to reverse the decrease in the content of the 20:4 fatty acid.

With exposure to HX/XO or to Ca^{2+}/HX/XO, release of Δ_4Ach increased and this increase was diminished, but not significantly, by dibucaine. As with the FO mitochondria the effects of Ca^{2+} and HX/XO exposure were limited to changes in the major fatty acids occupying the *sn*-2 position of the phospholipids.

Table 3 shows the absolute quantities of Δ_4Ach and EPA released from the FO and BT mitochondria in response to the experimental treatments. As in Tables 1 and 2, there is an increased release of Δ_4Ach and EPA with exposure to HX/XO or to Ca^{2+}/HX/XO, and a reduction in fatty acid release upon addition of dibucaine. With exposure to Ca^{2+} and HX/XO, the total amount of medium Δ_4Ach plus EPA was much larger in the FO mitochondria than in BT mitochondria. This is consistent with a greater PLA_2 activity in the FO mitochondrial membranes.

DISCUSSION

PLA_2 activation is believed to contribute to tissue injury in a number of pathophysiological processes in kidney (17, 24), brain (25), heart (26, 27), and liver (28). We have suggested (18) that PLA_2 activation played an important role in Ca^{2+}- and reactive oxygen species-induced injury to mitochondria. The current experiments supported this hypothesis, since Ca^{2+} and reactive oxygen species stimulate the selective release of unsaturated fatty acids that is inhibitable by dibucaine, a PLA_2 inhibitor.

Membranes enriched with highly unsaturated $n-3$ fatty acids would be more susceptible to peroxidation by reactive oxygen species, with generation of toxic lipid hydroperoxides (29), propagating further damage. Peroxidized fatty acids may also potentiate activation of PLA_2 (7), which would amplify the injury. The increased levels of FFAs would in turn likely increase membrane permeability to Ca^{2+} (16), which might further activate PLA_2. In addition, elevated levels of lysophospholipids, other products of PLA_2 activation, reduce mitochondrial membrane potential, and alter mitochondrial Ca^{2+} uptake and release (30).

The larger change in unsaturated fatty acid content of membrane phospholipids found in FO mitochondria is consistent with the greater functional impairment in respiratory activity, as compared with BT mitochondria. The increased functional impairment was due, in part, to an increased permeability of the FO mitochondrial membranes to protons

Table 3. Release of Δ_4Ach and EPA from FO and BT mitochondria

Treatment	*n*	Fatty acid, nmol/mg of mitochondrial protein: FO mitochondria Δ_4Ach	EPA	Sum	*n*	BT mitochondria Δ_4Ach	EPA	Sum
Control	6	2.5 ± 0.3	1.4 ± 0.6	3.9	8	3.1 ± 0.6	1.8 ± 1.0	4.9
Ca^{2+}	6	2.5 ± 0.4	1.3 ± 0.5	3.8	7	1.6 ± 0.4	0 ± 0	1.6
HX/HO	6	18.0 ± 2.3‡	9.9 ± 1.1‡	27.9	8	7.8 ± 1.4*	0.2 ± 0.1	8.0
Ca^{2+}/HX/XO	6	29.7 ± 2.0‡	16.2 ± 1.6‡	45.9	14	17.6 ± 3.7†	1.1 ± 0.6	18.7
Ca^{2+}/HX/XO/Dibu	4	14.6 ± 2.5‡¶	9.5 ± 1.9‡§	24.1	15	11.4 ± 2.5*	0.6 ± 0.3	12.0

Quantities of Δ_4Ach, EPA, and the sum of Δ_4Ach + EPA are expressed as nmol per mg of mitochondrial protein and were calculated from the respective areas of the gas chromatograms corresponding to each fatty acid compared with the area occupied on the chromatograms by the internal standard fatty acids and the amounts of the latter applied to the chromatogram. Compared with controls: *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$. Compared to Ca^{2+}/HX/XO group: §, $P < 0.05$; ¶, $P < 0.01$. Dibu, dibucaine.

and to a defect in the electron transport chain at site I, NADH CoQ reductase.

By using an isolated mitochondrial preparation, we are able to define susceptibility to a well-defined set of pathophysiological stimuli (i.e., Ca^{2+} and reactive oxygen species). Although these observations are important for the understanding of toxic and ischemic injury "*in vivo*," we cannot conclude that fish-oil-fed animals will suffer enhanced tissue damage. Other effects of $n-3$ fatty acids may also modify the degree of tissue damage resulting from ischemia or toxic influences *in vivo*. Blood flow is enhanced during reperfusion of ischemic hearts in $n-3$ fatty acid-fed rats (31), consistent with the enhanced vascular relaxation we found in postanoxic isolated aortic rings from fish-oil-fed rats (32). Less leukotriene B_4, which promotes neutrophil chemotaxis and aggregation, is produced by human monocytes after diets containing $n-3$ fatty acid supplements (33). $n-3$ fatty acids directly impair thromboxane A_2 production (22). Furthermore, neutrophils from humans ingesting fish oil supplements have a reduced ability to produce reactive oxygen species (34). Thus, predicting tissue damage depends upon an understanding of the complex interactions of all these different factors that are influenced by $n-3$ fatty acids.

In conclusion, these studies further support a primary role for PLA_2 in mitochondrial damage associated with Ca^{2+} and reactive oxygen species. Activation of PLA_2 is further enhanced when mitochondria are enriched in $n-3$ fatty acids. These mitochondrial defects could reduce ATP production and contribute to cell death secondary to various toxic or ischemic influences *in vivo*. In the organism, these changes may be partially offset by other effects of fish oil supplementation, such as increased postischemic blood flow and reduced chemotaxis with reduced free-radical generation by neutrophils. Nevertheless, it is important to appreciate adverse as well as beneficial effects of $n-3$ fatty acids to adequately evaluate the consequences of membrane enrichment with these lipids.

Our appreciation is extended to Zapata Haynie. (Reedville, VA) for providing menhaden oil. This work was supported by National Institutes of Health Grants DK 39773, DK 38452, DK 38165, and NS 10828 as well as Deutsche Forschungsgemeinshaft Grant WE 681. C.D.M. is supported by Clinical Investigator Award K08 DK 01320. J.V.B. is an Established Investigator of the American Heart Association.

1. Leaf, A. & Weber, P. C. (1988) *N. Engl. J. Med.* **318,** 549–557.
2. Kinsella, J. E., Lokesh, B. & Stone, R. A. (1990) *Am. J. Clin. Nutr.* **52,** 1–28.
3. Lorenz, R., Sprengler, U., Fischer, S., Duhm, J. & Weber, P. C. (1983) *Circulation* **67,** 504–511.
4. Von Schacky, C., Siess, W., Fischer, S. & Weber, P. C. (1985) *J. Lipid Res.* **26,** 457–464.
5. Bonventre, J. V. & Swidler, M. (1988) *J. Clin. Invest.* **82,** 168–176.
6. Braughler, J. M. (1987) in *Oxygen Radicals and Tissue Injury*, Proceedings of a Brook Lodge Symposium, ed. Halliwell, B. (Fed. Am. Soc. Exp. Biol., Bethesda, MD) (Augusta, MI), pp. 99–104.
7. Sevanian, A. & Kim, E. (1985) *J. Free Radicals Biol. Med.* **1,** 263–271.
8. Baud, L., Nivez, M. P., Chansel, D. & Ardaillou, R. (1981) *Kidney Int.* **20,** 332–339.
9. Au, A. M., Chan, P. H. & Fishman, R. A. (1985) *J. Cell. Biochem.* **27,** 449–453.
10. Chan, S. H. & Higgins, E., Jr. (1978) *Can. J. Biochem.* **58,** 111–116.
11. Chua, B. H. & Shrago, E. (1977) *J. Biol. Chem.* **252,** 6711–6714.
12. Lamers, J. M. J. & Hulsmann, W. C. (1977) *J. Mol. Cell. Cardiol.* **9,** 343–346.
13. Das, D. K., Engelman, R. M., Rousou, J. A., Breyer, R. H., Otani, H. & Lemeshow, S. (1986) *Am. J. Physiol.* **251,** H71–H79.
14. Corr, P. B., Gross, R. W. & Sobel, B. E. (1984) *Circ. Res.* **55,** 135–154.
15. Okayasu, T., Curtis, M. T. & Farber, J. L. (1985) *Arch. Biochem. Biophys.* **236,** 638–645.
16. Philipson, K. D. & Ward, R. (1985) *J. Biol. Chem.* **260,** 9666–9671.
17. Smith, M. W., Collan, Y., Kahnyg, M. W. & Trump, B. F. (1980) *Biochim. Biophys. Acta* **618,** 192–201.
18. Malis, C. D. & Bonventre, J. V. (1986) *J. Biol. Chem.* **261,** 14201–14208.
19. Rouslin, W. & Millard, R. W. (1981) *Am. J. Physiol.* **240,** H308–H313.
20. Nishida, T., Shibata, H., Koseki, M., Nakao, K., Kawashima, Y., Yoshida, Y. & Tagawa, K. (1987) *Biochim. Biophys. Acta* **890,** 82–88.
21. Di Virgilio, F. & Azzone, G. F. (1982) *J. Biol. Chem.* **257,** 4106–4113.
22. Von Schacky, C., Fischer, S. & Weber, P. C. (1985) *J. Clin. Invest.* **76,** 1626–1631.
23. Reinhart, P. H., Van De Pol, E., Taylor, W. M. & Bygrave, F. L. (1984) *Biochem. J.* **218,** 415–420.
24. Patel, Y., Stewart, J., Matthys, E. & Venkatachalam, M. A. (1982) *Clin. Res.* **30,** 541 (abstr.).
25. Bazan, N. G. (1970) *Biochim. Biophys. Acta* **218,** 1–14.
26. Chien, K. R., Han, A., Sen, A., Buja, M. & Willerson, J. T. (1984) *Circ. Res.* **54,** 313–322.
27. Chien, K. R., Sen, A., Reynolds, R., Chang, A., Kim, Y., Gunn, M. D., Buja, L. M. & Willerson, J. T. (1985) *J. Clin. Invest.* **75,** 1770–1780.
28. Chien, K. R., Abrams, J., Serroni, A., Martin, J. T. & Farber, J. L. (1978) *J. Biol. Chem.* **253,** 4809–4817.
29. Barber, A. A. & Bernheim, F. (1967) *Adv. Gerontol. Res.* **2,** 355–403.
30. Lenzen, S., Gorlich, J.-K. & Rustenbeck, I. (1989) *Biochim. Biophys. Acta* **982,** 140–146.
31. Force, T., Malis, C. D., Guerrero, L., Varadarajan, G. S., Bonventre, J. V., Weber, P. & Leaf, A. (1989) *Am. J. Physiol.* **257,** H1204–H1210.
32. Malis, C. D., Varadarajan, G. S., Force, T., Weber, P. C., Leaf, A. & Bonventre, J. V. (1988) *Circulation* **78,** II-216 (abstr.).
33. Lee, T. H., Hoover, R. L., Williams, J. D., Sperling, R. I., Ravalese, J., III, Spur, B. W., Robinson, D. R., Corey, E. J., Lewis, R. A. & Austen, K. F. (1985) *N. Engl. J. Med.* **312,** 1217–1224.
34. Fisher, M., Upchurch, K. S., Levine, P. H., Johnson, M. H., Vaudreuil, C. H., Natale, A. & Hoogasian, J. J. (1986) *Inflammation* **10,** 387–392.