Effect of dietary lipid and vitamin E on mitochondrial lipid peroxidation and hepatic injury in the bile duct-ligated rat[1]

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Abstract To assess whether lipid peroxidation of hepatic mitochondria is asosciated with cholestatic hepatic injury we examined the effect of bile duct ligation (BDL) versus sham surgery on mitochondrial lipids of rats maintained on one of seven diets. Diets included vitamin E-deficient (E−) and vitamin E-sufficient (E+) combined with normal lipid (11.9% calories as stripped corn oil), high lipid (35% calories as stripped corn oil), or n-3 fatty acid (fish oil) supplementation. Rats were killed 17 days after surgery, mitochondria were isolated by differential centrifugation, and lipid-conjugated dienes and thiobarbituric acid-reacting substances (TBARS) were measured in mitochondrial lipids as indices of lipid peroxidation. BDL resulted in significant increases in lipid peroxidation in all dietary groups. The E- high lipid diets (with either corn oil or fish oil) were associated with higher lipid peroxide and serum bilirubin values in BDL rats compared to the normal lipid diets. Fish oil supplementation did not ameliorate cholestatic or oxidative injury. Serum alanine aminotransferase, bilirubin, alkaline phosphatase, and cholylglycine levels correlated significantly with levels of mitochondrial conjugated dienes and TBARS. These data suggest that free radical stress occurs during BDL in the rat and may result in mitochondrial lipid peroxidation, and that diets high in lipid may increase free radical damage to hepatic mitochondria. The role of free radicals in cholestatic hepatic injury requires further investigation. —**Sokol, R. J., M. Devereaux, and R. A. Khandwala.** Effect of dietary lipid and vitamin E on mitochondrial lipid peroxidation and hepatic injury in the bile duct-ligated rat. *J. Lipid Res.* 1991. **32:** 1349-1357.

Supplementary key words cholestasis • free radicals • α-tocopherol • bile acids • n-3 fatty acids • n-6 fatty acids • fish oil

The pathogenesis of hepatic injury during cholestasis is poorly understood. Recent observations suggest that oxidant or free radical stress may play a role in cholestatic hepatic injury. A preliminary report showed that incubation of bile acids with isolated rat hepatocytes promoted oxidative modification of lipids (lipid peroxidation) concurrent with loss of hepatocyte viability and that α-tocopherol or superoxide dismutase inhibited these effects (1). It has also been shown that bile acids enhance the release of oxygen free radicals from activated rat polymorphonuclear leukocytes (2), inflammatory cells that are present in both experimental (3, 4) and human cholestatic liver lesions (5). Cu, Zn-superoxide dismutase and catalase, two of the free radical scavenging enzymes in liver, have been reported to be low in the diseased human liver, thereby potentially increasing the susceptibility of the liver to injury by oxygen-derived free radicals (6). Furthermore, the severity of cholestatic liver injury in adults with primary biliary cirrhosis and in bile duct-ligated rats was related to increasing levels of dietary fat intake (7), a situation that may increase the substrate for lipid peroxidation (polyunsaturated fatty acids), thereby making the liver more susceptible to oxidant stress (8). In addition, elevated plasma concentrations of lipid peroxides have been reported in children with chronic cholestatic liver disease (9). Thus, there is a growing body of evidence suggesting that free radicals may be generated during cholestasis, that the diseased liver may have a diminished capacity to scavenge free radicals, and that lipid peroxidation may be associated with cholestatic liver injury.

The aim of this study was to directly assess whether peroxidation of hepatic mitochondrial lipids is associated with cholestatic liver injury in the bile duct-ligated rat and

Abbreviations: ALT, alanine aminotransferase; BDL, bile duct ligation; E+, vitamin E-sufficient; E−, vitamin E-deficient; CO, corn oil; FO, fish oil; TBARS, thiobarbituric acid-reacting substances.

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to determine whether modifications in dietary lipid and vitamin E intake could alter this injury. We investigated mitochondria in this model because of previous demonstration of lipid peroxidation in mitochondrial membranes in other hepatic injury models wherein free radical generation has been implicated in the pathogenesis of tissue injury (10, 11). The rationale for testing dietary lipid intake in this model was that ingested fatty acids are incorporated into cell membrane phospholipids, and that polyunsaturated fatty acids of both the n-6 and n-3 families are highly susceptible to lipid peroxidation (12). Vitamin E intake was also modified to change susceptibility to lipid peroxidation inasmuch as vitamin E is the primary membrane-bound, lipid-soluble antioxidant that protects membrane fatty acids from oxidant stress (13). In human cholestatic states, malabsorption (14–16) and subsequent deficiency of vitamin E are common (17–21), rendering patients more susceptible to oxidant injury to the central nervous system (22, 23) and possibly the liver. An additional reason for studying n-3 fatty acids in this model is because use of n-3 fatty acid (fish oil) supplements has been suggested as a means of reducing liver cell injury and disease progression in patients with chronic liver disease (24) through inhibition of prostanoid and leukotriene pathways (25) and inhibition of cytokine synthesis by peripheral blood monocytes (26) and Kupffer cells (27). However, inasmuch as the n-3 fatty acids in fish oil are polyunsaturated, supplementation with fish oil may increase susceptibility to peroxidation of membrane lipids (12). Therefore, the effect of n-3 fatty acid supplementation on cholestatic injury and lipid peroxidation was also assessed in this animal model. Previous studies have demonstrated that manipulating dietary lipid intake alters mitochondrial membrane content of n-6 and n-3 fatty acids (28). Thus, the strategy for our study was to achieve the desired vitamin E and fatty acid status in hepatic subcellular membranes by dietary means.

METHODS

Animals and diets

Weanling male Sprague-Dawley rats obtained from Sasco, Inc., (Omaha, NE) were housed in polyethylene cages with stainless steel wire tops and 12-h light-dark cycles. Rats were randomly assigned to receive one of six semisynthetic liquid diets (Dyets, Inc., Bethlehem, PA) or standard Purina Lab Chow (Ralston Purina Co., Chicago, IL) including: E + 11.9%CO, vitamin E-sufficient (E + ; 50 IU all-*rac*-α-tocopheryl acetate/kg diet), normal lipid (11.9%CO; 11.9% of calories as stripped corn oil); E-11.9%CO, vitamin E-deficient (< 10 IU all-*rac*-α-tocopheryl acetate/kg diet), normal lipid; E + 35%CO, E + , high lipid (35% of calories as stripped corn oil); E-35%CO, E-, high lipid; E + 35%FO, E + , high lipid, fish oil-supplemented (11% of calories as stripped corn oil, 24% of calories as stripped menhaden oil); and E-35%FO. All rats were pair-fed for 6 weeks to equal the growth of the E-35%CO rats. The E-35%CO diet was designed to maximize susceptibility to lipid peroxidation and to mimic the approximate percentage of calories as lipid in the average Western human diet. The E-35%FO diet provided linoleic acid from the corn oil equal to the amount in the E-11.9%CO diet plus additional n-3 fatty acids at levels shown to suppress eicosanoid pathways (24). The lipid composition and fatty acid distribution of each diet is shown in **Table 1**. The liquid diets were purchased as powder which was refrigerated in the dark until reconstitution with distilled water for use within 24 h. Reconstituted liquid diets were kept refrigerated in the dark until use. E- diets contained 0.02% butylated hydroxyanisole to prevent in vitro peroxidation of diet lipid. Each rat received a new fresh supply of liquid diet each day.

Bile duct ligation procedure

After 6 weeks of diet, rats underwent bile duct ligation and transection (BDL) or sham surgery. Under pentobarbital anesthesia (65 mg/kg i.p.) and oxygen supplementation, the common hepatic duct was located through a midline abdominal incision, double ligated near the liver, and transected between ligatures. Two ml of sterile saline and 5,000 units of penicillin G were instilled into the peritoneum at the completion of surgery, and the incision was closed using stainless steel surgical staples. Sham surgery

TABLE 1. Lipid and fatty acid composition of diets

Composition	11.9% Corn Oil Diet	35% Corn Oil Diet	35% Fish Oil Diet
Stripped corn oil (g/l)	13.25	38.9	13.0
Menhaden oil (g/l)	0	0	25.9
Percent calories			
Fat	11.9	35.0	35.0
Carbohydrate	68.8	45.7	45.7
Protein	19.3	19.3	19.3
Fatty acid profile (%)			
14:0	trace	trace	6.0
16:0	9.7	9.7	14.6
16:1	trace	trace	8.3
16:2			1.1
16:3			1.1
16:4			1.2
17:0			0.6
18:0	1.9	1.9	2.5
18:1	26.0	26.0	16.3
18:2	61.7	61.7	21.6
18:3	0.7	0.7	1.3
18:4			2.3
20:0			0.1
20:1			1.1
20:4			1.5
20:5			10.3
22:5			1.6
22:6			6.1

was identical to the ligation procedure, including locating and manipulating the common hepatic duct, except that the bile duct was not ligated or divided. Rats were maintained on the respective preoperative diet after surgery and were pair-fed to the weight gain of the BDL rats on the E-35%CO diet.

Blood, liver, and mitochondrial studies

Rats were killed 17 days after BDL or sham surgery by exsanguination from the inferior vena cava through a laparotomy incision with the animal under light ether anesthesia. To assess the severity of liver injury, blood was obtained from the inferior vena cava and serum was analyzed for total bilirubin, alkaline phosphatase, and ALT by automated laboratory procedures and for cholylglycine by radioimmunoassay (29). Vitamin E status was assessed by serum α-tocopherol measured by high pressure liquid chromatography with absorbance detection (30) and the ratio of serum α-tocopherol to total serum lipid concentration (31, 32).

The liver was removed, weighed, and processed immediately for the following studies: hepatic α-tocopherol concentrations were assessed on frozen liver, stored at −70°C, by high pressure liquid chromatography with fluorescence detection (33), and expressed per mg wet weight of liver and per mg hepatic protein (34). To quantitate oxidant injury to the liver, lipid peroxidation of fresh hepatic mitochondria was assessed by thiobarbituric acid-reacting substances (TBARS) and lipid-conjugated dienes, by previously described methods (10). TBARS were expressed as nmol/mg mitochondrial protein. Lipid conjugated dienes were expressed as the difference in absorbance at 233 nm (peak absorbance for conjugated dienes), normalized to a denominator of 1 mg lipid per ml cyclohexane, between each experimental rat and a mean of values from three chow-fed adult rats analyzed simultaneously each day. Mitochondria were isolated from 6 g of liver by centrifugation through a self-generating sucrose-Percoll gradient (75%/25%:vol/vol) as previously described (10). The purity and contamination of each membrane preparation was assessed by the enrichment and percentage recovery of microsomal (reduced nicotinamide-adenine dinucleotide phosphate (NADPH) cytochrome C reductase (35), mitochondrial (succinate dehydrogenase) (36), and lysosomal (N-acetyl glucoseaminidase) (37) marker enzymes. Enrichment was calculated by dividing the specific activity of the mitochondrial fraction by that of the original liver homogenate; percentage recovery was calculated by dividing the total activity of the mitochondrial fraction by that of the homogenate.

Statistical analysis

Statistical analysis for differences among rats in the experimental groups was performed by the analysis of variance and Scheffe F test. Analysis for differences between two groups was performed by the Student's *t*-test. Linear regression analysis was used to determine relationships between variables. A P value <0.05 was considered statistically significant. All values are expressed as the mean ± standard error of the mean (SEM) unless otherwise stated.

RESULTS

Growth and vitamin E status

Rats in all experimental groups showed similar growth because of the pair-feeding study design (**Table 2**). Liver weight was increased in the BDL groups. Serum α-tocopherol concentrations and ratios of α-tocopherol:total serum lipid concentrations and ratios of α-tocopherol: total serum lipid concentrations confirmed the expected vitamin E status of each experimental group (Table 2). Hepatic α-tocopherol concentrations were significantly lower in BDL rats compared to sham-operated animals (expressed per g liver and per mg protein) in all the E+ dietary groups (Table 1); however, total hepatic α-tocopherol content did not differ between BDL and sham-operated rats (data not shown). Rats maintained on E- diets had significantly lower hepatic α-tocopherol concentrations than E+ rats.

Lipid peroxidaton in mitochondrial membranes

Enrichment and recovery of mitochondrial membrane fractions based on organelle marker enzymes were similar in the seven experimental groups (**Table 3**). Mitochondria were enriched a mean of 8.5- to 11.0-fold with mild microsomal and minimal lysosomal contamination. Recovery of mitochondria ranged from a mean of 74.7 to 93.5%.

Hepatic mitochondria showed evidence of in vivo lipid peroxidation from all BDL groups compared to sham-operated rats by analysis of both lipid-conjugated dienes and TBARS (**Table 4**). However, mitochondria from E-35%CO and E-35%FO BDL rats had significantly higher levels of lipid-conjugated dienes and TBARS compared to other BDL rats. There were no significant differences between lipid peroxidation values of E-35%CO compared to E-35%FO rats.

Assessment of hepatic injury and correlation to lipid peroxidation

Liver injury was documented in BDL rats by elevated serum alkaline phosphatase, total bilirubin, cholylglycine, and ALT levels compared to sham-operated rats. In the E-35%CO and E-35%FO rats, serum bilirubin values were significantly higher than those in other BDL rats (**Table 5**). To determine whether the severity of cholestatic liver injury correlated with the degree of lipid peroxidation, serum alkaline phosphatase, bilirubin, cho-

TABLE 2. Growth and vitamin E indices in experimental groups

Dietary Group	Surgery	N	Weight	Liver Weight	Serum α-Tocopherol	Serum α-Tocopherol: Total Lipids	Liver α-Tocopherol	
							μg/g Wet Weight	μg/mg Protein
			g	*g*	*μg/ml*	*mg/g*		
E + 11.9%CO	BDL	12	308 ± 2	16.6 ± 0.7[a]	7.3 ± 0.5	1.61 ± 0.17	6.1 ± 0.9[a]	0.18 ± 0.03[a]
	Sham	8	308 ± 2	10.4 ± 0.5	6.3 ± 0.4	1.50 ± 0.13	8.2 ± 1.1	0.31 ± 0.05
E-11.9%CO	BDL	5	298 ± 1	15.9 ± 0.5[a]	0.6 ± 0.2	0.16 ± 0.06	1.4 ± 0.2	0.09 ± 0.01
	Sham	6	301 ± 1	10.3 ± 0.1	0.6 ± 0.2	0.17 ± 0.03	1.6 ± 0.2	0.08 ± 0.01
E + 35%CO	BDL	5	308 ± 1	16.6 ± 0.6[a]	7.3 ± 0.4	2.08 ± 0.39	4.4 ± 0.6[a]	0.16 ± 0.01[a]
	Sham	7	305 ± 1	9.8 ± 0.4	6.7 ± 0.4	2.31 ± 0.50	9.9 ± 1.0	0.34 ± 0.05
E-35%CO	BDL	9	305 ± 3	16.4 ± 0.5[a]	0.9 ± 0.2	0.22 ± 0.05	2.2 ± 0.4	0.08 ± 0.01
	Sham	8	304 ± 3	11.0 ± 0.4	0.4 ± 0.1	0.14 ± 0.05	1.5 ± 0.4	0.07 ± 0.01
E + 35%FO	BDL	7	316 ± 1	17.7 ± 0.7[a]	5.4 ± 0.8	0.67 ± 0.08	4.2 ± 0.2[a]	0.20 ± 0.04[a]
	Sham	6	314 ± 2	10.4 ± 0.6	2.9 ± 0.5	1.36 ± 0.07	6.2 ± 0.7	0.33 ± 0.05
E-35%FO	BDL	6	311 ± 1	17.0 ± 0.4[a]	0.8 ± 0.3	0.15 ± 0.05	1.7 ± 0.2	0.09 ± 0.01
	Sham	6	311 ± 1	10.4 ± 0.7	0.4 ± 0.1	0.15 ± 0.02	1.5 ± 0.2	0.07 ± 0.01
Rat chow	None	11	310 ± 3	11.5 ± 0.3	4.7 ± 0.3	1.03 ± 0.11	11.6 ± 1.4	0.60 ± 0.07

All values mean ± SEM. See Methods for definition of dietary group terms; BDL, bile duct ligation; Sham, sham surgery.
[a] $P < 0.05$ vs. shams in same dietary group.

lylglycine, and ALT were compared to mitochondrial TBARS and conjugated dienes by linear regression analysis. Significant correlations were found between all variables (**Table 6**). Representative relationships are illustrated in **Figs. 1–4**.

DISCUSSION

The experiments conducted in this study demonstrate that peroxidative decomposition of mitochondrial lipids occurs in the chronically BDL rat model of cholestatic liver injury. Both lipid-conjugated dienes and TBARS were significantly elevated in mitochondrial lipids from BDL rats compared to sham-operated, pair-fed control rats. This finding was present in rats receiving low lipid or high lipid diets, predominantly n-6 or n-3 fatty acid-containing diets, and E+ or E- diets. Diets that theoretically should maximize the susceptibility to lipid peroxidation, by increasing substrate (polyunsaturated fatty acids) and decreasing protection (α-tocopherol) against free radical modification of lipids were found to

TABLE 3. Enrichment and recovery of mitochondrial membrane fractions

Dietary Group	Surgery	N	Succinate Dehydrogenase		NADPH Cytochrome C Reductase		N-Acetyl Glucoseaminidase	
			Enrichment	Recovery (%)	Enrichment	Recovery (%)	Enrichment	Recovery (%)
E + 11.9%CO	BDL	7	10.2 ± 1.3	78.1 ± 5.9	5.2 ± 1.0	18.1 ± 3.3	2.0 ± 0.6	12.4 ± 2.8
	Sham	6	9.5 ± 0.6	84.8 ± 5.7	3.7 ± 0.8	18.2 ± 3.4	1.7 ± 0.3	11.8 ± 2.4
E-11.9%CO	BDL	5	9.5 ± 0.9	74.2 ± 10.1	2.3 ± 0.5	25.4 ± 5.3	1.6 ± 0.6	11.0 ± 1.1
	Sham	6	8.9 ± 2.5	82.3 ± 5.7	3.4 ± 0.8	24.7 ± 5.8	1.5 ± 0.2	15.7 ± 3.2
E + 35%CO	BDL	5	9.4 ± 0.8	79.0 ± 9.8	2.4 ± 0.8	19.6 ± 5.1	1.8 ± 0.4	7.0 ± 2.5
	Sham	7	9.7 ± 0.7	79.3 ± 5.7	2.9 ± 0.3	23.4 ± 3.8	1.3 ± 0.2	14.4 ± 3.8
E-35%CO	BDL	5	9.9 ± 0.5	76.0 ± 5.8	3.6 ± 0.9	16.0 ± 3.0	1.6 ± 0.2	12.6 ± 1.9
	Sham	6	9.5 ± 0.7	79.2 ± 6.1	2.9 ± 0.6	20.0 ± 2.0	1.9 ± 0.7	16.0 ± 1.0
E + 35%FO	BDL	7	10.2 ± 0.5	76.4 ± 5.1	3.3 ± 0.6	16.9 ± 2.2	3.0 ± 0.5	10.1 ± 0.9
	Sham	6	10.4 ± 0.9	93.5 ± 3.2	2.9 ± 0.9	18.0 ± 3.3	1.4 ± 0.3	7.5 ± 1.3
E-35%FO	BDL	6	8.5 ± 0.8	74.7 ± 6.2	5.3 ± 1.0	16.0 ± 2.7	2.0 ± 0.7	12.5 ± 2.9
	Sham	6	11.0 ± 0.9	76.3 ± 2.9	3.7 ± 0.9	23.0 ± 5.5	1.9 ± 0.3	6.7 ± 0.8
Rat chow	None	12	8.5 ± 0.5	79.8 ± 3.6	4.5 ± 0.9	15.3 ± 2.3	2.8 ± 0.7	12.4 ± 2.8

All values mean ± SEM. There were no statistically significant differences among groups in any column by ANOVA.

TABLE 4. Lipid peroxidation of hepatic mitochondrial membranes from experimental rats

Dietary Group	Surgery	N	Absorbance at 233 nm/mg Lipid	Difference in Absorbance at 233 nm/mg Lipid	TBARS (nmol/mg Protein)
E + 11.9%CO	BDL	12	0.84 ± 0.04[a]	0.19 ± 0.05[a]	3.1 ± 0.2[a]
	Sham	6	0.67 ± 0.06	−0.03 ± 0.03	1.8 ± 0.2
E-11.9%CO	BDL	5	0.69 ± 0.14[a]	0.17 ± 0.12[a]	5.0 ± 0.3[a]
	Sham	6	0.48 ± 0.04	−0.01 ± 0.05	2.2 ± 0.2
E + 35%CO	BDL	5	0.60 ± 0.16[a]	0.11 ± 0.05[a]	4.1 ± 0.4[a]
	Sham	7	0.48 ± 0.04	0.02 ± 0.05	1.8 ± 0.3
E-35%CO	BDL	11	0.92 ± 0.07[a,b]	0.40 ± 0.05[a,b]	5.3 ± 0.2[a]
	Sham	10	0.69 ± 0.11	−0.04 ± 0.05	2.2 ± 0.4
E + 35%FO	BDL	7	0.51 ± 0.09[a]	0.19 ± 0.07[a]	4.6 ± 0.4[a]
	Sham	6	0.37 ± 0.03	0.06 ± 0.02	2.8 ± 0.4
E-35%FO	BDL	5	0.67 ± 0.04[a]	0.38 ± 0.07[a,b]	5.8 ± 0.4[a]
	Sham	6	0.36 ± 0.04	0.03 ± 0.03	3.2 ± 0.5
Rat chow	None	12	0.41 ± 0.03	−0.01 ± 0.02	1.8 ± 0.2

All values mean ± SEM.
[a] $P < 0.05$ by ANOVA vs. sham and rat chow groups.
[b] $P < 0.05$ by ANOVA vs. other BDL groups.

enhance the degree of mitochondrial lipid peroxidation and were also associated with increased liver injury (serum bilirubin). Subcellular organelle marker enzyme studies demonstrated similar purity and lack of contamination of mitochondrial preparations from all dietary and surgical groups, thus confirming that the lipid peroxidation findings were not caused by differences in membrane populations isolated from the various dietary groups or from BDL versus sham-operated rats. Contamination of mitochondrial fractions with lysosomal membranes estimated by marker enzymes (Table 3) was mild, particularly in view of the observation that the lipid content of hepatic mitochondria is approximately 100 times that of lysosomes (38). Therefore, the contribution of lysosomes to the lipid component of our mitochondrial fractions could not generate sufficient lipid peroxides to account for the observed mitochondrial lipid peroxidation.

As anticipated, serum alkaline phosphatase, total bilirubin, cholylglycine, and ALT were elevated in all

TABLE 5. Liver blood tests in experimental groups

Dietary Group	Surgery	N	Alkaline Phosphatase	Total Bilirubin	Cholylglycine	ALT
			IU/l	*mg/dl*	*μM*	*IU/l*
E + 11.9%CO	BDL	12	567 ± 45	9.5 ± 0.9	60.3 ± 10.1	184 ± 9
	Sham	8	247 ± 42	0.8 ± 0.3	2.2 ± 0.8	44 ± 7
E-11.9%CO	BDL	5	489 ± 34	8.7 ± 0.7	52.2 ± 2.5	217 ± 28
	Sham	6	206 ± 35	0.4 ± 0.1	1.3 ± 0.2	44 ± 7
E + 35%CO	BDL	5	663 ± 134	9.0 ± 0.6	55.2 ± 6.1	231 ± 18
	Sham	7	290 ± 61	0.5 ± 0.2	2.9 ± 0.8	48 ± 9
E-35%CO	BDL	11	617 ± 62	13.0 ± 1.4[a]	60.2 ± 2.7	223 ± 14
	Sham	10	256 ± 36	0.6 ± 0.1	2.8 ± 0.2	59 ± 13
E + 35%FO	BDL	7	420 ± 27	9.0 ± 0.5	65.3 ± 5.5	155 ± 12
	Sham	6	254 ± 39	2.1 ± 0.6	1.4 ± 0.3	64 ± 9
E-35%FO	BDL	6	571 ± 25	12.3 ± 0.5[a]	66.7 ± 5.5	178 ± 28
	Sham	6	196 ± 38	1.6 ± 0.2	2.1 ± 0.3	43 ± 5
Rat chow	None	13	225 ± 26	0.6 ± 0.1	1.6 ± 0.3	38 ± 4

All values mean ± SEM. Values for each variable from BDL rats were significantly ($P < 0.05$) higher compared to sham rats in same dietary group and compared to control rats.
[a] $P < 0.05$ by ANOVA vs. other BDL groups.

TABLE 6. Correlations between mitochondrial lipid peroxidation and indices of hepatic injury in experimental rats

Liver Injury Indices	Mitochondrial TBARS		Mitochondrial Lipid-Conjugated Dienes	
	r Value	*P* Value	*r* Value	*P* Value
Serum total bilirubin	0.79	0.0001	0.64	0.0001
Serum alkaline phosphatase	0.63	0.0001	0.49	0.001
Serum ALT	0.75	0.0001	0.63	0.0001
Serum cholylglycine	0.74	0.0001	0.56	0.0001

Correlations were calculated by linear regression analysis using data points from all groups of experimental animals.

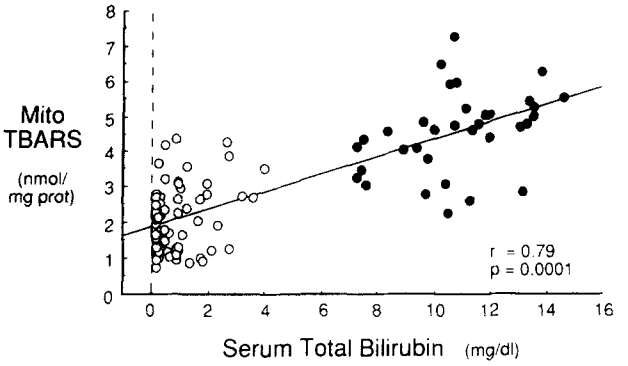

Fig. 2. Relationship between serum total bilirubin level and concentration of hepatic mitochondrial TBARS from rats on various diets 17 days after bile duct ligation (solid circles) or sham surgery (open circles).

BDL rat groups compared to sham-operated rats. Of interest were the significant correlations between the degree of lipid peroxidation and the biochemical markers of the severity of hepatic injury (Table 6), suggesting that oxidant damage paralleled the cholestatic injury to hepatocytes. This study was not designed to conclusively determine whether lipid peroxidation was involved in the pathogenesis or, alternatively, occurred as a consequence of hepatocyte injury in this animal model. However, mitochondrial lipid peroxidation in other animal models of liver injury has been associated with a wide range of functional consequences that may impair cellular energy metabolism and viability (reviewed in ref. 39). Peroxidation of phospholipid fatty acids may perturb cellular function by altering membrane permeability and changing the activity of lipid-dependent membrane enzymes (40). In addition, aldehyde products of lipid peroxidation (e.g., 4-hydroxynonenal) may exhibit cytotoxic properties of their own (41). Specific examples of mitochondrial alterations associated with lipid peroxidation caused by oxidant stresses include mitochondrial swelling (42), decreased transmembrane potential (43), decreased respiratory control ratio (44, 45), uncoupling of oxidative phosphorylation (46), and depression of cytochrome C oxidase activity (47). The functional consequences of mitochondrial lipid peroxidation in the BDL model of cholestasis are currently under investigation.

An additional important effect of lipid peroxidation during hepatic injury may be the stimulation of fibrogenesis. Chojkier et al. (48) and Houglum, Brenner, and Chojkier (49) have recently shown that iron-induced lipid peroxidation increased collagen gene transcription and collagen synthesis in cultured human fibroblasts, and that α-tocopherol prevented the generation of lipid peroxide products and collagen gene expression (49). Thus, lipid peroxide may be one of the signals from injured hepatocytes that stimulate collagen synthesis, thus linking hepatocellular injury to hepatic fibrosis. Although collagen synthesis has not been formally studied in the BDL model, periportal fibrosis and cirrhosis ultimately lead to the demise of BDL rats (3).

To establish that free radical injury and lipid peroxidation play a role in cholestatic liver injury, it will be necessary to demonstrate that inhibitors of free radical generation or free radical scavengers can ameliorate the hepatic injury as well as reduce lipid peroxidation. Pre-

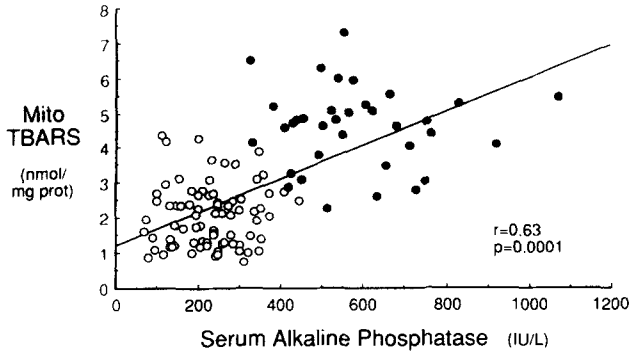

Fig. 1. Relationship between serum alkaline phosphatase level and concentration of hepatic mitochondrial TBARS from rats on various diets 17 days after bile duct ligation (solid circles) or sham surgery (open circles). Solid line represents regression line.

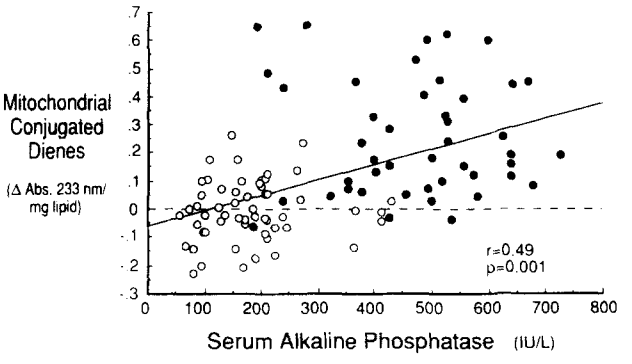

Fig. 3. Relationship between serum alkaline phosphatase level and mitochondrial lipid-conjugated dienes from rats on various diets 17 days after bile duct ligation (solid circles) or sham surgery (open circles).

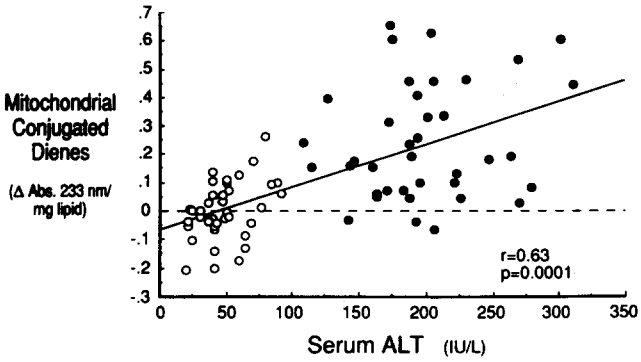

Fig. 4. Relationship between serum ALT level and mitochondrial lipid-conjugated dienes from rats on various diets 17 days after bile duct ligation (solid circles) or sham surgery (open circles).

liminary studies from our laboratory using the iron-chelating agent deferoxamine as a free radical inhibitor have demonstrated reduced hepatic injury, diminished lipid peroxidation, and improvement of the histological lesion in BDL rats (50). Further studies in this area of investigation are needed to characterize the mechanism by which free radicals are generated during cholestasis and to determine whether therapy to inhibit these processes may offer long-term beneficial effects.

A second objective of our study was to determine the effect of n-3 fatty acid supplementation on the hepatic injury and lipid peroxidation of mitochondria in the BDL rat. Our data demonstrate that n-3 fatty acid supplementation does not ameliorate the cholestatic injury caused by BDL in either E+ or E- rats, and that n-3 fatty acids promote lipid peroxidation in E- BDL rats as effectively as n-6 fatty acids. If lipid peroxidation of mitochondria is indeed deleterious to the hepatocyte, then n-3 fatty acid supplementation may exacerbate cholestatic injury. Thus, caution should be exercised in recommending fish oil supplements to humans with cholestatic liver disease until studies clearly demonstrate the safety and efficacy of this treatment.

Our findings of increased hepatic and mitochondrial injury in rats consuming a high lipid diet are similar to observations made in animal models of alcoholic liver injury, another pathologic state wherein involvement of free radicals has been postulated (51). Studies have demonstrated a direct relationship between the amount of dietary corn oil (up to 35% of ingested calories) in ethanol-treated rats and the severity of hepatic steatosis (52-54), zonal necrosis (53, 54), and centrilobular fibrosis (53, 54). In addition, a diet containing 41% lipid versus 10% lipid calories in chronic ethanol-treated rats resulted in abnormalities in mitochondrial respiratory function (55). Moreover, Nanji and French (56) found that the mortality rates due to alcoholic cirrhosis in 17 countries correlated with the per capita consumption of polyunsaturated fats. Thus, intake of polyunsaturated fatty acids may be an essential factor in the pathogenesis of alcoholic liver disease; however, its relation to mitochondrial lipid peroxidation in that condition has not been evaluated.

In our study, there were no significant increases in lipid-conjugated dienes or TBARS in sham-operated rats receiving the E-35%CO or E-35%FO diets in contrast to the BDL rats receiving these diets. Thus, lipid peroxidation of hepatic mitochondria is not promoted merely by alteration of fatty acid substrate and antioxidant levels, but rather requires a stimulus for oxidant stress that exceeds the free radical scavenging capability of the hepatocyte. Our study suggests that cholestasis produced by BDL leads to free radical generation and the resulting lipid peroxidation parallels the extent of hepatocyte cholestatic injury. Characterization of the mechanisms by which cholestasis produces an oxidant stress and the role of oxidative damage to hepatic mitochondria in the pathogenesis of cholestatic injury require further investigation.

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