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FREE RADICAL-INDUCED LIVER INJURY. II. EFFECTS OF INTRAPERITONEALLY ADMINISTERED 2,2′-AZOBIS(2-AMIDINOPROPANE) DIHYDROCHLORIDE ON THE FATTY ACID PROFILES OF HEPATIC TRIACYLGLYCEROL AND PHOSPHOLIPIDS

HIROYUKI SHIMASAKI*, WAT-HAN SAYPIL and NOBUO UETA

Department of Biochemistry, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi, Tokyo 173, Japan

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Liver injury induced by the radical initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and carbon tetrachloride (CCl_4) was examined by the analysis of lipids in the liver of rats. Increased triacylglycerol (TAG) was found in the liver within 24 hr following injection of these drugs. In AAPH-treated and CCl_4-treated rats, it was 2.1 and 1.8 times that in the controls, respectively. TAG-palmitate and -oleate were found at particularly increased levels, while polyunsaturated fatty acid profiles of hepatic phospholipids were essentially the same for the treated and untreated rats. It is evident from these findings that radical initiators cause no decrease in polyunsaturated fatty acids in hepatic lipids, but accumulate TAG in the liver. Such a condition is the equivalent of liver injury in the rats in whose diets vitamin E has long been deficient.

KEY WORDS: Free radical, vitamin E, 2,2′-azobis(2-amidinopropane) dihydrochloride, carbon tetrachloride, liver injury.

INTRODUCTION

The administration of CCl_4 to experimental animals is known to cause severe liver injury, such as centrilobular necrosis and fatty liver.[1–4] As the mechanism by which this compound exerts hepatotoxic action, it is generally considered that CCl_4 is metabolized by hepatic microsomal cytochrome P-450 to the trichloromethyl free radical, which in turn reacts with molecular oxygen to form the highly toxic peroxy radical adduct, trichloromethylperoxy radical.[5] Terao and Niki[6] recently found an azo compound, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), to damage biological tissue when administered intraperitoneally to mice. AAPH is a well-known radical intiator both *in vitro* and *in vivo*, and generates free radicals at a constant rate, the first-order rate constant k_d being 1.1×10^6/s at 37° C.[6] AAPH radicals react with molecular oxygen to form peroxy radicals[7] which react rapidly with polyunsaturated fatty acids to produce lipid hydroperoxides.[7,8] To date, however, no lipid analysis of tissues from AAPH-treated animals has been conducted. The present study was thus

*To whom correspondence should be addressed: Dr. Hiroyuki Shimasaki, Department of Biochemistry, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi, Tokyo 173, Japan.

conducted to examine the influence of free radicals on the polyunsaturated fatty acids of tissue lipids in AAPH-treated animals. A means was also sought to study the mechanism of the hepatic injurious action of free radicals in vitamin E-deficient animals.[9]

MATERIALS AND METHODS

Materials

2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and carbon tetrachloride (CCl_4) were obtained from Wako Pure Chemical Ind., (Tokyo, JPN). Fatty acid standards for GLC analysis were purchased from Nu-Chek-Prep., Inc. (Elysian, MN). Other reagents were obtained from Kanto Chemical Co., Inc. (Tokyo, JPN), and organic solvents were furnished by Wako Pure Chemical Ind. (Tokyo, JPN).

Animals

Male Sprague-Dawley rats, 8 weeks old, were purchased from the Tokyo Animal Experimental Laboratory (Tokyo, JPN), and fed a control diet[9] for 4 weeks prior to AAPH- or CCl_4-treatment.

AAPH and CCl_4 administration

Three groups of 6 rats each (12 weeks old) were injected intraperitoneally with 0, 45 and 100 mg/kg of AAPH in saline solution as described by Terao and Niki.[6] One group was orally administered 0.66 ml/kg of CCl_4 dissolved in 0.3 ml olive oil. All the rats were made to fast 24 hr after treatment.

Lipid extraction

At intervals of 24 hr following the administration of AAPH or CCl_4, ca 5 ml of blood from the animals were collected in heparinized tubes before sacrifice by decapitation. Total lipids were extracted from the plasma and tissue with chloroform/methanol as described previously.[9]

Fatty acid analysis

The lipids were transesterified under a nitrogen atmosphere with 5% anhydrous HCl-methanol at 85° C for 2 hours. Pentadecanoic acid was used as the internal standard and fatty acid analysis was conducted by gas liquid chromatography.[10]

Statistical analysis

All data were expressed as means ± S.D. Statistical analysis was performed by the Student t-test with $p < 0.05$ being considered significant.

RESULTS

Effects of AAPH administration on hepatic lipids and fatty acid profiles

The animals which had received 100 mg/kg of AAPH exhibited dyspnea and cyanosis, and died within 10 min. Those which had received 45 mg/kg of AAPH, and five rats injected intraperitoneally with 0.2 ml of physiological saline as controls were sacrificed for lipid analysis. AAPH caused increase in hepatic lipids within 24 h after injection. The amount of total lipids was 92.7 ± 18.9 mg/wet weight of liver, this being about 2.1 times that in the controls (Table I). The amount of triacylglycerol (TAG) increased in the liver, and the levels of TAG-palmitate and -oleate increased more than 2.8-fold and 3.4-fold as compared to those in controls, respectively. TAG-linoleate also increased in the liver of AAPH-injected rats (Figure 1). The fatty acid levels of hepatic phospholipids were essentially the same for the AAPH-injected rats and the controls (data not shown). Total fatty acids in the plasma were noted to decrease significantly in the AAPH-treated rats ($p < 0.01$), while this drug had no effect on red blood cell lipids under the experimental conditions used (Table II).

Effects of CCl_4 administration on hepatic lipids and fatty acid profiles

The oral administration of CCl_4 (0.66 ml/kg body weight) led to an increase in hepatic lipids within 24 h following treatment (Table I). The fatty acid profile of triacylglycerol was similar to that of the AAPH-injected rats (Figure 1). Total fatty acids in plasma decreased significantly in CCl_4-treated rats ($p < 0.02$), while the red blood cells of the CCl_4-treated and control rats showed no significant differences (Table II). It should be noted that changes in the fatty acid levels of hepatic phospholipids, such as phosphatidylcholine and phosphatidylethanolamine, were not significantly different between the treated and untreated groups under the experimental conditions (data not shown).

DISCUSSION

Peroxy radicals, produced by reaction of AAPH radicals with molecular oxygen, have been shown to be capable of altering membrane phospholipids due to lipid peroxidation of polyunsaturated fatty acids in acyl-residues of the lipids *in vitro*.[7,8] Terao and

TABLE I
Changes in liver weights and total lipids of rats treated with AAPH and CCl_4

	Control (n = 5)	AAPH[1] (n = 6)	CCl_4[2] (n = 6)
Liver (g)	17.2 ± 0.8	19.2 ± 1.5	17.2 ± 1.9
(mg/g wet weight of liver) Total lipids[3]	43.4 ± 3.8	92.7 ± 18.9[a]	78.6 ± 20.3[b]

Mean ± S.D.
[1]Rats were sacrificed at 24 hr after intraperitoneal administration of 20 mg 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH).
[2]Rats were sacrificed at 24 hr after oral administration of 0.66 ml CCl_4/kg body weight.
[3]AAPH or CCl_4 groups versus control group were significantly different ([a]$p < 0.001$ and [b]$p < 0.01$).

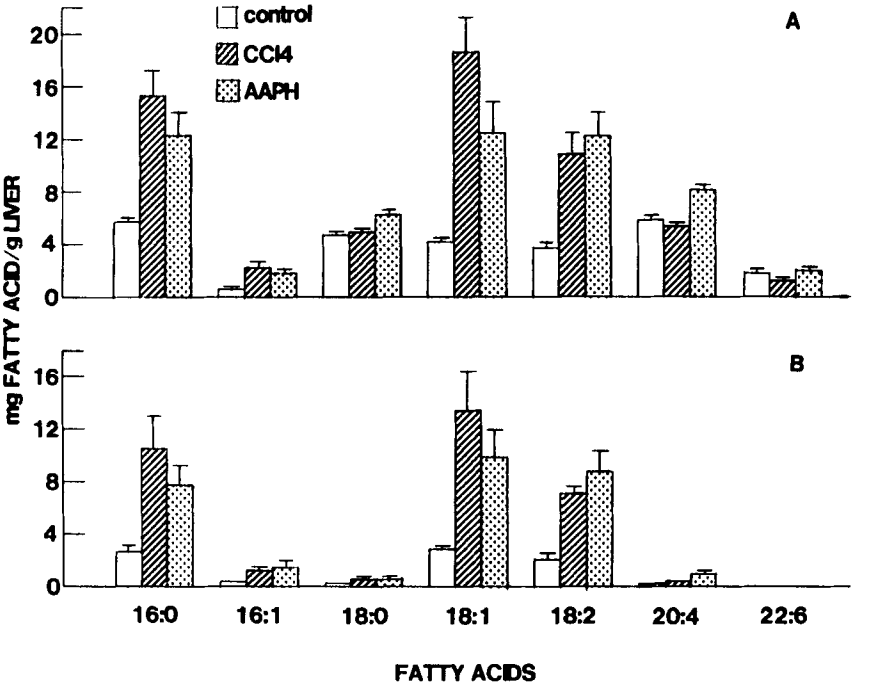

FIGURE 1 Effects of administered AAPH and CCl_4 on the fatty acid levels of hepatic total lipids (A) and triacylglycerol (B) in rats. Extraction and analysis of lipids were performed as described under Methods. Values are means ± S.D. from 5 or 6 animals in each group.

TABLE II
Levels of major fatty acids in plasma and red blood cell total lipids of rats treated with AAPH and CCl_4

	Plasma (mg/μl Plasma)			Red Blood Cells (μg/μmol Phosphorus)		
	Control (n = 5)	AAPH[1] (n = 5)	CCl_4[2] (n = 6)	Control (n = 5)	AAPH[1] (n = 5)	CCl_4[2] (n = 6)
16:0	0.34 ± 0.10	0.18 ± 0.03	0.19 ± 0.05	22.55 ± 0.53	24.99 ± 2.16	23.97 ± 5.61
16:1	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	1.12 ± 0.17	1.05 ± 0.81	0.15 ± 0.07
18:0	0.15 ± 0.02	0.07 ± 0.02	0.12 ± 0.03	9.65 ± 0.91	9.70 ± 0.99	10.10 ± 2.66
18:1	0.25 ± 0.09	0.13 ± 0.03	0.13 ±0.04	7.21 ± 0.46	7.85 ± 0.78	7.58 ± 1.20
18:2	0.24 ± 0.08	0.14 ± 0.03	0.12 ± 0.03	7.23 ± 0.43	8.66 ± 1.18	7.61 ± 1.13
20:4	0.38 ± 0.04	0.13 ± 0.09	0.24 ± 0.07	14.71 ± 1.39	18.50 ± 1.52	15.53 ± 3.48
Total[3]	1.40 ± 0.31	0.69 ± 0.18[a]	0.81 ± 0.21[b]	62.24 ± 3.38	70.75 ± 8.53	64.77 ± 15.6

Mean ± S.D.
[1)]Rats were sacrificed at 24 hr after intraperitoneal administration of 20 mg AAPH.
[2)]Rats were sacrificed at 24 hr after oral adoministration of 0.66 ml CCl_4/kg body weight.
[3)]AAPH and CCl_4 groups versus control group were significantly different ([a]$p < 0.01$ and [b]$p < 0.02$).

Niki[6] recently found that experimental animals administered AAPH exhibited numerous fat droplet formations and damage to certain biological membranes in the liver. However, they did not conduct an analysis of the lipids in the livers from these animals. The results of the present study clearly indicate that the administration of AAPH and CCl_4 causes TAG accumulation in the liver of rats, and that the levels of TAG-palmitate and -oleate increase particularly in liver lipids. The cause of this TAG accumulation is not known, but may possible arise from free radical-induced liver cell injury. Essentially the same results were noted for rats fed a vitamin E-deficient diet for 4, 6, and 9 months.[9]

The PUFA profiles of hepatic phospholipids of animals administered either AAPH CCl_4 and control rats were essentially the same. It may not be possible to detect the loss of PUFA in AAPH- or CCl_4-treated animals for most unchanged membrane phospholipids *in vivo*, possibly due to *in vivo* homeostatic mechanisms that protect tissue lipids from free radical-mediated lipid peroxidation.[11,12] Similar results have been reported for hepatic phospholipids of rats fed a vitamin E-deficient diet.[9]

It is important to bear in mind that although there were no significant differences in plasma total lipids of the vitamin E-deficient and control rats,[9] their levels decreased significantly in the AAPH- and CCl_4-treated rats. Early studies[13,14] showed that CCl_4 administered to experimental animals inhibits protein synthesis in liver cells, causing a decrease in plasma lipoproteins. Thus possibly, AAPH or its radicals may inhibit protein synthesis in liver cells, so that both plasma lipoproteins and lipids decrease. The liver cell injury resulting from dietary vitamin E deficiency may be less severe than that in AAPH- and CCl_4-treated animals. The present findings, however, do support the possibility that vitamin E deficiency leads to free radical-induced liver cell injury *in vivo*.[9]

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