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13-*cis*-RETINOIC ACID AND HEPATIC STEATOSIS IN RATS

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Abstract—The effect of administration of 13-*cis*-retinoic acid (100 mg/kg diet) on lipid metabolism was examined in male rats fed either a 20% casein + 0.3% methionine diet, a 20% casein diet, a 10% casein + 0.3% methionine diet, or a 10% casein + 0.6% methionine diet for 10 days. Hepatic triglyceride concentrations of rats fed either 10% casein diet were 3-fold greater than animals receiving diets containing 20% casein. The addition of 13-*cis*-retinoic acid to the diet further increased the total hepatic lipid (43–56%) and triglyceride (~2-fold) concentrations in rats fed the 10% casein diets. 13-*cis*-Retinoic acid supplementation did not alter the total liver lipid or triglyceride concentrations in rats fed either of the 20% casein diets. Thus, under specific dietary conditions, the administration of 13-*cis*-retinoic acid resulted in a marked accumulation of hepatic lipids which did not appear to be related to the total methionine content of the diet nor to the hepatic concentrations of *S*-adenosylmethionine and glutathione. In addition, all four groups of 13-*cis*-retinoic acid-fed rats exhibited elevations in the concentration of serum triglycerides, and 10–20% reductions in serum cholesterol concentrations.

The synthetic retinoid derivative, 13-*cis*-retinoic acid (CRA†; Isotretinoin), has been shown to be effective clinically in the treatment of severe acne [1–3] as well as in the prevention and treatment of various neoplasms in humans and animals [4–10]. Unfortunately, numerous side effects are known to be associated with CRA use [2, 3, 6, 11] such as the pronounced teratogenic potential of the drug [12, 13].

Another adverse consequence of CRA administration is its ability to increase plasma lipid concentrations in humans [3, 8, 14–18] and animals [19, 20]; however, no changes in the hepatic concentrations of lipids were demonstrated in the latter rat studies using diets containing 20–22% casein [19, 20]. Likewise, we have found that hepatic lipid concentrations were not changed in rats fed a 20% casein + 0.3% methionine diet supplemented with CRA (Schalinske KL and Steele RD, unpublished observation). In contrast, ethionine administration results in a significant accumulation of hepatic lipids and a decrease in the serum triglyceride concentration [21–25]. These alterations in hepatic and serum lipid concentrations are due to an ethionine-mediated inhibition of protein synthesis and subsequent inability to secrete triglyceride-rich lipoproteins from the liver into the bloodstream [25–29]. Thus, CRA and ethionine are two compounds which appear to differ with respect to the mechanism of altering lipid metabolism. However, a decrease in the hepatic concentration of *S*-adenosylmethionine (SAM) is charactertistic of both ethionine [30–35] and 13-*cis*-retinoic acid [36] administration. In turn, SAM concentrations reflect the supply of methionine [37], and thus the availability of methyl groups may play a role in the development of hepatic steatosis and subsequent carcinogenesis [38]. Hence, the work presented here examined the relationship of dietary protein and methionine concentration on the ability of CRA to induce hepatic steatosis in the rat.

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† Abbreviations: CRA, 13-*cis*-retinoic acid; SAM, *S*-adenosylmethionine; and VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Animals and diets. In an initial experiment, fifteen male Sprague–Dawley (Harlan Sprague–Dawley, Indianapolis, IN) rats weighing approximately 80 g, were housed in suspended wire-mesh cages in a room with a 12-hr light–dark cycle. Rats were adapted to a control diet [36] containing 10% casein + 0.3% methionine for 5 days. This moderate level of casein, with the methionine supplement, will support adequate growth in the rat [39]. After adaptation, they were fed one of three treatment diets: the control diet alone, the control diet supplemented with CRA at a level of 100 mg CRA/kg diet, or the control diet containing 0.25% DL-ethionine. CRA was added to the respective diets as 10% gelatin beadlets (Hoffmann-La Roche, Nutley, NJ); control animals received an equal amount of placebo beadlets. All diets, fed as 1% agar gels as described previously [36], and water were provided *ad lib.* throughout the study. At the end of the 10-day treatment period, rats were randomly killed by decapitation and liver samples were frozen at −70° for subsequent assessment of total liver lipid concentration.

In a second experiment, forty male Sprague–Dawley rats with initial body weights of approximately 97 g were housed in suspended wire-mesh cages in a room with a 12-hr light–dark cycle and were fed one of eight diets: either a 20% casein + 0.3% methionine diet, a 20% casein diet, a 10% casein + 0.3% methionine diet, a 10% casein +

Table 1. Effect of 13-*cis*-retinoic acid (CRA) and ethionine administration on growth, relative liver size, and total liver lipid concentration*

Treatment	Ten-day weight gain (g)	Relative liver size (% body weight)	Total liver lipids (mg/g liver)
Control	62 ± 2†	5.30 ± 0.14†	38.0 ± 2.0†
CRA	56 ± 2†	5.83 ± 0.14‡	62.0 ± 6.1‡
Ethionine	32 ± 3‡	5.92 ± 0.18‡	74.9 ± 9.0‡

* Rats were fed either a control diet (10% casein + 0.3% methionine), a control diet supplemented with 100 mg CRA/kg diet, or a control diet containing 0.25% DL-ethionine for 10 days. Data are means ± SEM, N = 5.

†‡ Mean values within a column with different symbols are significantly different ($P < 0.05$).

0.6% methionine diet, or one of the above diets containing 100 mg CRA/kg diet. The total methionine content of the first four diets was 0.86, 0.56, 0.58, and 0.88%, respectively. Changes in the dietary level of casein and/or methionine were made at the expense of cerelose. Water and diets, fed as 1% agar gels, were provided *ad lib.* throughout the study. Rats were fed the 20% casein + 0.3% methionine diet (without CRA) for a 5-day adaptation period after which animals were fed one of the eight treatment diets for 10 days. At the end of the 10-day treatment period, rats were anesthetized with ether and blood samples were obtained by cardiac puncture. Rats were killed by decapitation and livers were rapidly removed and weighed, and portions were frozen at −70° for subsequent lipid analysis. Additional liver samples were homogenized in 2 vol. of 0.4 mol/L perchloric acid for assessment of SAM and total glutathione concentration. Although rats were not fasted overnight, they were killed 3–5 hr into the light cycle, and the concentrations of serum triglycerides were similar to previously reported values in rats fasted 4–20 hr [40, 41]. In addition, all rats were killed within 90 min in order to minimize any diurnal variation. This is especially significant with respect to the determination of SAM [42].

Lipid analysis. In both experiments, the total lipid content of frozen liver samples was extracted according to the method of Folch *et al.* [43] and measured gravimetrically. In experiment 2, additional samples of the extract were utilized for the colorimetric measurement of triglycerides [44], the enzymatic determination of total cholesterol [45], and the colorimetric measurement of lipid phosphorus concentrations [46]. Blood samples were centrifuged at 16,000 *g* and aliquots of the serum were used to assess triglyceride [44] and cholesterol [45] concentrations.

SAM and glutathione analysis. Perchloric acid-homogenates were centrifuged at 9000 *g* and a sample of the resulting supernatant was applied to a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA) to obtain SAM. Quantification of SAM was accomplished using a reversed-phase HPLC system consisting of a C_{18} μBondapak column (Waters Associates) and a 5% methanol/5 mM octanesulfonic acid (pH 4.0) mobile phase in conjunction with UV detection at 254 nm [47]. An additional sample of the supernatant was utilized to measure total glutathione concentrations according to the spectrophotometric method described by Tietze [48].

Table 2. Effect of CRA on growth and relative liver size in rats fed various dietary levels of protein and methionine*

Treatment	Ten-day weight gain (g)	Relative liver size (% body weight)
20% Casein/0.3% Met	71 + 2†	5.07 ± 0.11†
+ CRA	69 ± 1†	4.98 ± 0.11†
20% Casein	62 ± 1†‡§	4.87 ± 0.15†
+ CRA	65 ± 2†‡	4.93 ± 0.06†
10% Casein/0.3% Met	57 + 5‡§	5.05 ± 0.17†
+ CRA	56 ± 4‡§	5.46 ± 0.08‡
10% Casein/0.6% Met	54 ± 2§	5.15 ± 0.11†‡
+ CRA	63 ± 4†‡§	5.49 ± 0.18‡

* The total methionine (Met) content of the 20% casein + 0.3% methionine diet, the 20% casein diet, the 10% casein + 0.3% methionine diet, and the 10% casein + 0.6% methionine diet was 0.86, 0.56, 0.58, and 0.88%, respectively. Data are means ± SEM, N = 5.

†–§ Mean values with different symbols within a column are significantly different ($P < 0.05$).

Statistical analysis. The means of each treatment group were subjected to a one-way ANOVA ($P < 0.05$) in experiment 1 and a two-way ANOVA in experiment 2. One- and two-tailed comparisons were done using the least significant difference procedure at a significance level of 5% [49].

RESULTS

The effects of CRA and ethionine feeding (experiment 1) on the weight gain, relative liver size, and total liver lipid concentrations are shown in Table 1. CRA supplementation did not effect the weight gain, whereas ethionine consumption resulted in a 48% decrease in growth compared to control animals. The relative liver size was elevated significantly by 10 and 12% in CRA- and ethionine-

Table 3. Effect of CRA on hepatic lipids in rats fed various levels of dietary protein and methionine*

Treatment	Total lipid	Triglyceride	Cholesterol	Phospholipid
	(mg/g liver)			
20% Casein/0.3% Met	31.3 ± 1.2†	5.53 ± 0.48†	1.33 ± 0.11†	15.16 ± 1.16
+ CRA	34.7 ± 1.3†‡	8.16 ± 0.56†‡	1.24 ± 0.12†	16.51 ± 0.87
20% Casein	36.3 ± 3.4†‡	5.52 ± 1.02†	1.17 ± 0.23†	16.07 ± 2.18
+ CRA	37.4 ± 1.5†‡	8.45 ± 1.37†‡	1.15 ± 0.06†	16.00 ± 0.90
10% Casein/0.3% Met	40.6 ± 1.5‡	16.31 ± 1.58§	1.27 ± 0.12†	13.94 ± 0.64
+ CRA	57.9 ± 2.4§	36.26 ± 1.79‖	1.61 ± 0.22†‡	17.77 ± 4.79
10% Casein/0.6% Met	38.9 ± 2.7‡	15.38 ± 2.18‡§	1.46 ± 0.33†	16.14 ± 2.27
+ CRA	60.8 ± 4.1§	40.04 ± 6.31‖	2.03 ± 0.17‡	20.94 ± 4.18

* The total protein and methionine (Met) content of each diet is described in Table 2. Data are means ± SEM, N = 5.

†–‖ Mean values with different symbols within a column are significantly different ($P < 0.05$).

Table 4. Effect of CRA on serum triglyceride and cholesterol concentrations in rats fed various levels of dietary protein and methionine*

Treatment	Triglycerides	Cholesterol
	(mg/dL)	
20% Casein/0.3% Met	151 ± 35†‡§	92.6 ± 0.9†
+ CRA	235 ± 20‖	81.3 ± 2.0‡
20% Casein	134 ± 13†‡	91.7 ± 2.7†
+ CRA	193 ± 16‡§‖	83.7 ± 2.5†‡
10% Casein/0.3% Met	135 ± 14†‡	91.8 ± 5.1†
+ CRA	207 ± 26§‖	70.5 ± 2.4§
10% Casein/0.6% Met	103 ± 12†	84.6 ± 3.7†‡
+ CRA	328 ± 46¶	68.0 ± 3.8§

* The total protein and methionine (Met) content of each diet is described in Table 2. Data are means ± SEM, N = 5.

†–¶ Mean values with different symbols within a column are significantly different ($P < 0.05$).

treated rats, respectively. Similarly, total liver lipid concentrations were increased markedly in both treatment groups; the hepatic lipid concentrations in rats receiving CRA in their diet were almost as high as those fed ethionine.

The ability of CRA to induce hepatic steatosis as a function of dietary protein and methionine was examined in more detail in experiment 2: specific components (e.g. triglycerides) of total liver and serum lipids were measured. The weight gain and relative liver size of rats from experiment 2 are shown in Table 2. The relative liver size was not altered due to dietary casein content. Although the addition of CRA did not result in differences in growth rate, CRA supplementation increased the relative liver size of rats fed the 10% casein + 0.3% methionine diet. A similar trend was also seen in the 10% casein + 0.6% methionine diet, but was not statistically significant.

Table 3 presents the hepatic lipid concentrations as a function of diet and CRA consumption in experiment 2. CRA significantly increased the total lipid content by 43 and 56% in rats fed 10% casein + 0.3% methionine and 10% casein + 0.6% methionine diets, respectively, whereas it had no effect on rats fed the higher protein (20% casein) diets. Hepatic triglyceride concentrations were increased markedly by low dietary protein and exacerbated by the addition of CRA. Rats fed a 10% casein diet exhibited a 2.9-fold increase in triglyceride concentration compared with animals fed a 20% casein diet. The addition of CRA further elevated the concentration of triglycerides 2.2- and 2.6-fold in rats fed 10% casein + 0.3% methionine and 10% casein + 0.6% methionine diets, respectively.

In contrast, there was little effect of diet or CRA on hepatic cholesterol and phospholipid concentrations. Only rats fed the 10% casein + 0.6% methionine diet had an increase in cholesterol levels due to CRA, whereas phospholipid concentrations remained unchanged across all treatment groups.

The serum triglyceride and cholesterol concentrations from rats in experiment 2 are shown in Table 4. All rats, except for those fed the 20% casein diet, had significant increases in serum triglyceride concentrations due to CRA consumption. The degree of hypertriglyceridemia was most pronounced (greater than 3-fold) in rats fed a 10% casein + 0.6% methionine diet. Except for animals fed the 20% casein diet, cholesterol concentrations were reduced significantly as a result of CRA. Rats fed the 10% casein diets exhibited the largest decrease (approximately 21%) in cholesterol levels.

The hepatic concentration of SAM was not changed as a result of dietary protein concentration: the mean values in rats fed a 20% casein diet were similar compared to those of animals fed a diet containing 10% casein (61.0 ± 7.6 and 63.4 ± 3.0 nmol/g liver, respectively). However, when the data are presented as a function of total dietary methionine (±CRA), a more distinct relationship among SAM concentrations, diet, and

Table 5. Effect of CRA and various levels of dietary methionine on hepatic *S*-adenosylmethionine concentrations*

	S-Adenosylmethionine (nmol/g liver)	
	− CRA	+ CRA
0.87% Methionine	70.8 ± 6.0†	54.5 ± 4.6‡
0.57% Methionine	53.7 ± 3.7‡	42.1 ± 3.4‡

* The average total methionine content of the 20% casein + 0.3% methionine diet and the 10% casein + 0.6% methionine diet was 0.87%. The average total methionine content of the 20% casein diet and the 10% casein + 0.3% methionine diet was 0.57%. Data are means ± SEM, N = 10.

†‡ Mean values within and across columns with different symbols are significantly different using a one-tailed comparison ($P < 0.05$).

Table 6. Effect of CRA and various levels of dietary methionine on total glutathione concentration in rat liver*

	Total glutathione (μmol/g liver)	
	− CRA	+ CRA
0.87% Methionine	2.0 ± 0.2†	1.6 ± 0.1‡
0.57% Methionine	2.3 ± 0.2†	1.6 ± 0.2‡

* The average total methionine content of the 20% casein ± 0.3% methionine diet and the 10% casein + 0.6% methionine diet was 0.87%. The average total methionine content of the 20% casein diet and the 10% casein + 0.3% methionine diet was 0.57%. Data are means ± SEM, N = 10.

†‡ Mean values within and across columns with different symbols are significantly different ($P < 0.05$).

CRA is apparent (Table 5). Compared with rats fed 0.87% total methionine, the concentration of SAM was lower (22–24%) in animals supplemented with CRA and in rats fed 0.57% total methionine. In addition, CRA supplementation of a 0.57% total methionine diet also reduced the concentration of SAM.

A situation similar to the hepatic SAM results was found with respect to the total glutathione concentration (Table 6). When the data were examined as a function of total dietary methionine, the total glutathione concentration was decreased significantly (20–30%) due to CRA supplementation, regardless of dietary methionine content. No difference was seen as a result of changes in the dietary methionine concentration alone, with or without CRA.

DISCUSSION

Hyperlipidemia is an often seen consequence of retinoid usage: all-*trans*-retinoic acid [20, 40, 50], etretinate [18, 51], and retinol [41] have been shown to elevate serum triglyceride concentrations. However, accumulation of lipid in the liver has only been reported in the case of excessive retinol (hypervitaminosis A) intake [41, 52, 53]. Previous CRA studies with rats which assessed hepatic lipids and serum/plasma lipid concentrations and utilized diets containing 20–22% casein found no changes in hepatic total lipid, triglyceride, cholesterol, or phospholipid concentrations with doses of CRA as high as 300 mg/kg diet [19, 20]. Earlier work in our laboratory found that rats fed a 20% casein + 0.3% methionine diet did not exhibit any signs of hepatic steatosis as well (Schalinske KL and Steel RD, unpublished observation). In our initial experiment with rats fed a 10% casein + 0.3% methionine diet (Table 1), we found that CRA supplementation did result in hepatic lipid accumulation and was as effective as ethionine in producing a fatty liver. In contrast to ethionine-treated rats, weight gain was not affected in animals supplemented with CRA; thus, dietary CRA supplementation may represent a better animal model for the study of hepatic steatosis. A noticeable difference in our initial studies compared to previous work [19, 20] was the protein and methionine content of the diets, two factors which were examined in the second set of experiments. As seen in Table 3, CRA did induce hepatic steatosis as a result of alterations in dietary protein, but not methionine, and the fatty infiltration was due predominantly to an accumulation of triglycerides. Thus, the CRA-induced accumulation of hepatic triglycerides is consistent with increased release of triglycerides into the plasma as a result of CRA administration.

The hyperlipidemic effect of CRA has been well documented in both humans and animals [3, 8, 14–20]. It has been shown to be dose-dependent [3, 16, 19, 20] and reversible [14–18]. In addition, we found that dietary CRA significantly increased serum triglyceride concentrations, regardless of the protein (10 and 20% casein) or total methionine (0.57 and 0.87%) content of the diet, whereas the serum cholesterol concentrations were consistently reduced 10–20% by feeding CRA (Table 4). This latter finding is in contrast to previous reports in humans [3, 14–18] which found total serum cholesterol concentrations were elevated due to CRA feeding: low density lipoprotein (LDL)-cholesterol concentrations were increased, whereas a decrease in the concentration of high density lipoprotein (HDL)-cholesterol was consistently reported.

Lipotrope (methionine and/or choline)-deficient diets are known to result in a fatty liver and subsequent hepatocarcinogenesis [38]; thus, we originally hypothesized the CRA-induced increase in total liver lipids due to a decrease in dietary protein may be related to the total methionine content of the diet. This was based in part on the observation that dietary CRA has been shown to decrease hepatic SAM concentrations [36], a consequence also characteristic of both dietary methyl group deficiency and ethionine feeding [32]. Likewise, the SAM concentrations measured in these studies were reduced as a result of dietary

methionine content and CRA supplementation (Table 5). However, we were unable to demonstrate that the increase in hepatic lipids due to CRA was related to the total methionine content of the diet, but rather was due specifically to the dietary protein level. In support of this, additional work in our laboratory has found that heptic SAM concentrations were decreased by over 35% due to CRA supplementation in rats fed a 20% casein + 0.3% methionine diet even though no changes were seen in the concentration of hepatic lipids (Schalinske KL and Steele RD, unpublished observation).

The tripeptide glutathione is an important compound in cellular detoxification [54]; however, the role of glutathione in the induction of hepatic steatosis is not clear. Feo *et al.* [55] reported a marked decrease in glutathione concentration in conjunction with an alcohol-induced accumulation of lipid; the change in both the hepatic concentration of glutathione and lipid was prevented by pretreatment with SAM. In contrast, it was reported [35] that hepatic glutathione concentration was increased due to ethionine, and no evidence for a relationship between glutathione and *S*-adenosyl derivatives could be demonstrated. In agreement with Glaser and Mager [56], who reported a methionine-reversible decrease in hepatic glutathione concentrations due to acute ethionine exposure, we also found that the concentration of glutathione was diminished as a result of CRA supplementation, although in our studies glutathione did not change as a function of dietary methionine content, with or without the feeding of CRA. The CRA-induced decrease found in our studies is consistent with an earlier report [36] which demonstrated that dietary CRA supplementation markedly elevated the hepatic concentration of taurine and decreased the urinary excretion of inorganic sulfate, two end products of the transsulfuration pathway, in rats fed a diet containing 1.6% total methionine. Thus, CRA appears to alter the metabolism of methionine by partitioning the degradation of cysteine towards the formation of taurine at the expense of sulfate and glutathione.

To date, this is the first report to demonstrate that CRA has the ability to markedly perturb lipid metabolism in the liver and thus may provide some insight into the well-known hyperlipidemic action of CRA. Numerous mechanisms may be responsible for CRA-induced hyperlipidemia, such as an increase in the hepatic production of very low density lipoptotein (VLDL) and/or triglycerides, a decrease in the extrahepatic tissue uptake of lipids from the circulation, or a combination of these possiblities. Bershad and co-workers [14] found that patients with CRA-induced hyperlipidemia did not exhibit alterations in the activity of lipoprotein lipase or trigylceride lipase. Marsden [17] has reported indirect evidence suggesting that CRA (and etretinate)-induced hyperlipidemia is the result of increased triglyceride synthesis, a mechanism reported to be involved in the hyperlipidemia induced by all-*trans*-retinoic acid [40, 57, 58] and vitamin A [41]. Recent studies have documented an increase in the hepatic synthesis of apo B and VLDL, an elevation in the plasma VLDL level, and a reduced uptake and degradation of plasma VLDL by the liver as a result of CRA treatment [18, 59]. In support of this hypothesis, our experiments demonstrate that under certain dietary conditions, the liver does exhibit excessive lipid accumulation in conjunction with high circulating lipid concentrations. How CRA potentially enhances hepatic triglyceride synthesis is unknown: it remains to be examined whether a specific amino acid(s) becomes limiting as dietary protein content is diminished and subsequently augments the hyperlipidemic action of CRA in the liver and the blood.

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