# Regulation of apolipoprotein E synthesis and mRNA by diet and hormones

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**Abstract** Rats were fasted or fasted and refed simple purified diets so the effects of individual carbohydrates or fats could be studied. Freshly isolated hepatocytes from these animals were used to measure both apoE synthesis and mRNA levels so any changes in apoE synthesis that might occur without changes in its mRNA could be detected. Some of these experiments were done with both sexes. Both fasting and fasting and refeeding a 60% glucose fat-free diet significantly increased spoE synthesis. However, cyclic AMP is not likely to rapidly mediate the effect of fasting since dibutyryl cAMP slightly lowered (rather than increased) apoE synthesis and mRNA when injected into rats for 4.5 h. Dietary fat had no effect either in the absence of carbohydrate or when consumption of carbohydrate was constant in pair-fed rats. ApoE mRNA levels remained normal for 4 days in primary hepatocytes cultured in medium that had only amino acids as an energy source. Added hormones or fructose had no significant effect. Thus, only fasting and fasting and refeeding glucose were able to significantly change apoE synthesis or mRNA levels. Synthesis of apoE may be regulated to increase when apoE is secreted with very low density lipoprotein or when apoE in secreted high density lipoprotein is needed to acquire cholesteryl esters for the synthesis of bile salts and acids by liver.—**Kim, M.H., R. Nakayama, P. Manos, J. E. Tomlinson, E. Choi, J. D. Ng, and D. Holten.** Regulation of apolipoprotein E synthesis and mRNA by diet and hormones. *J. Lipid Res.* 1989. **30:** 663 -671.

**Supplementary key words** cultured primary hepatocytes • cAMP • insulin • dexamethasone

Apolipoprotein E (apoE) is a 35,000 dalton glycoprotein found in several classes of mammalian lipoproteins including VLDL, chylomicrons, chylomicron remnants, and HDL with apoE (for review, see reference 1). The major site of apoE synthesis is the liver (2–4). Recently, however, apoE synthesis has been demonstrated in mouse macrophages (5, 6) and in extrahepatic tissues, such as adrenal, testis, ovary, spleen, kidney, and lung (7–10).

ApoE plays important roles in the metabolism of triglycerides and cholesterol by mediating the interaction of specific lipoproteins with extrahepatic and hepatic LDL receptors (1) and hepatic apoE receptors (HDL with apoE and chylomicron remnant receptors) (11–16). Thus, apoE may play a role in reverse cholesterol transport where cholesterol, acquired by HDL from plasma membranes, is esterified and transferred to apoE-containing chylomicron or VLDL remnants which are rapidly removed by receptors on the liver and oxidized to bile salts and acids in that organ (for a review, see 17). The observation that apoE variants are observed in patients with familial type III hyperlipidemia (18, 19) illustrates the importance of understanding more about how diet effects apoE metabolism.

Most studies indicate that although dietary cholesterol and fat change serum concentrations of apoE, there is little effect on apoE synthesis and/or mRNA levels in liver (7, 20, 21). However, there are conflicting reports about whether these high cholesterol and fat diets increase apoE synthesis in rats given propylthiouracil to make them more susceptible to atherosclerosis (21, 22) and few studies have been done on the effect of fatty acids in the absence of cholesterol. In cultured hepatocyte systems, added fatty acids or glucose stimulates triglyceride synthesis but does not change apoE synthesis or mRNA levels (23, 24). However, feeding sucrose to rats increases both lipogenesis and apoE synthesis (25). This led to the proposal that coinduction of both lipogenesis and apolipoprotein synthesis by dietary carbohydrate could increase the capacity of liver cells to both assemble and secrete triglyceride-rich lipoproteins (25). In the present study we used rats to determine the extent to which dietary carbohydrate and saturated or polyunsaturated fat coordinately

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TYA, eicosa-5, 8, 11, 14-tetraynoic acid; TSM, 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$; TSE, 10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA; HEPES, 2 M HEPES, pH 6.6; PBS, 10 mM sodium phosphate, pH 7.2, 0.15 M NaCl; TDPBS, PBS with 1% each Triton X-100 and sodium deoxycholate; MOPS, 0.2 M morphalinopropanesulfonic acid, pH 7.0, 50 mM sodium acetate, 5 mM EDTA; pApoE, apoE cDNA in pUC 9; SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0; SSPE, 0.3 M NaCl, 20 mM $NaH_2PO_4$, 2 mM EDTA, pH 7.4; PUFA, polyunsaturated fatty acid.

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regulate the induction of lipogenic enzymes and apoE. These studies were extended to include a comparison to female rats. Conflicting reports on the effect of fasting on apoE synthesis (24, 26) also led us to reexamine this question and to test the possibility that cyclic AMP, increased in response to fasting, induces apoE synthesis.

## EXPERIMENTAL PROCEDURES

### Materials

Nembutal was purchased from Abbot Laboratory and collagenase from Cooper Biomedical. Acrylamide and N, N-methylene-bis-acrylamide were from Bio-Rad Laboratories. Protosol was obtained from New England Nuclear Biolabs and Betafluor was from National Diagnostics. L-[4,5-$^3$H]leucine (45 Ci/mmol or 120 Ci/mmol) was from Amersham. [$^{32}$P-$\gamma$]dATP and [$^{32}$P-$\alpha$]dCTP (3000 Ci/mmol) were from ICN Radiochemicals. IgSorb was from the Enzyme Center Inc. and dibutyryl cAMP was from Sigma. Oligo(dT)12-18 was obtained from Collaborative Research. Nitrocellulose filters were obtained from Schleicher and Schuell. Restriction enzymes were purchased from Bio-Rad Laboratories. Eicosa-5, 8, 11, 14-tetraynoic acid (TYA) was from Dr. J. Hamilton (Hoffman-LaRoche, Inc., Nutley, NJ).

### Treatment of Animals

Young adult Sprague-Dawley rats were purchased from Bantin and Kingman, Inc. (Fremont, CA), housed in individual cages, and maintained on a Purina chow diet until use. Chow-fed animals were maintained on the same Purina chow diet until they were killed. Rats were fasted for 2 days or fasted for 2 days and then refed a diet containing 60% glucose, 30% casein, 5% salt mixture, 2% vitamin mixture, and 3% cellulose for 3–4 days as previously described (27). Rats fed the high fat diet were fasted for 2 days and then refed a diet containing 52.2% lard, 33.7% casein, 8.3% corn oil, 5.7% minerals, and vitamins as described (28). For the cAMP treatment, rats were fasted for 2 days, refed the 60% fructose fat-free diet for 3–4 days, and then injected intraperitoneally with a solution containing dibutyryl cAMP and theophylline at doses of 50 mg and 20 mg/kg body weight, respectively. Control animals were injected with saline instead of cAMP. Four and one-half h later, the livers were used to determine effects on apoE synthesis and mRNA.

The procedure used to determine the effect of polyunsaturated fatty acids and the inhibition of arachonidate metabolism was described previously (29). Briefly, rats weighing about 130 g were put on a 3-h meal feeding conditioning phase for 10–30 days. Two groups of rats were fed 15 g of the following diet (g/100 g): 2–3 safflower oil, 67–68 glucose, 20 casein, 3 cellulose, 5 salt mix, 2 vitamin mix, 0.3 D,L-methionine, and 0.1 butylated hydroxytoluene. All meals were fed in nonspill metabolism feeders. After the rats were conditioned to eat all of the diet during the 3-h meal period, they were switched to the high carbohydrate diets used by Clarke and Clarke (30) for a 7-day induction phase. The fat-free diet contained (g/100 g) 70 glucose, 20 casein, 3 cellulose, 5 salt mix, 2 vitamin mix, 0.3 D,L-methionine, and 0.1 butylated hydroxytoluene. After the induction phase, rats were divided into three dietary restriction phase groups and fed the following for 4 days: group 1, the fat-free diet; group 2, the fat-free diet plus 5% safflower oil; group 3, the fat-free diet plus 5% safflower oil and 0.05% TYA. Rat weight and food consumption were monitored daily. All rats were killed 1 h after cessation of meal feeding. There were seven to eight rats in each group.

### Isolation of hepatocytes

Rat hepatocytes were prepared by the collagenase perfusion method as described by Procsal, Winberry, and Holten (27). Rats were anesthetized and the liver was cannulated and perfused with collagenase in Kreb's bicarbonate buffer at 37°C for 30 min. The liver cells were suspended in Hams F-12/SA medium with modifications as described by Winbery et al. (31). The Hams F-12/SA medium was supplemented with arginine but lacked leucine and ornithine. One ml of cell suspension was saved for RNA isolation. The cells were pulsed with [$^3$H]leucine (one-tenth the volume of packed cells, 45 Ci/mmol for fasted-refed rats, 120 Ci/mmol for fasted or chow-fed rats) for 60 min at 37°C with continuous oxygenation (95% $O_2$, 5% $CO_2$) and shaking. The cells were collected and suspended in an equal volume of 0.15 M NaCl, 1 mM phenylmethylsulfonylfluoride and frozen at −70°C. The postmicrosomal fraction was prepared from the frozen cell suspension by thawing and pulsing twice for 20 sec, on ice, with a sonicator (model W-375 from Ultrasonics Inc.) set at 60% duty cycle at an output of 3. One ml of sonicated cell suspension to be used for immunoprecipitation of apoE was added to an equal volume of 4% Triton X-100, 4% deoxycholate, and PBS at pH 7.2 and mixed well. The cell suspension was centrifuged at 100,000 *g* for 60 min to give the postmicrosomal fraction used for the immunoprecipitation. The radioactivity incorporated into total soluble proteins was determined by spotting 10 $\mu$l of postmicrosomal fraction on Whatman 3MM filter paper discs (2.4 cm in diameter) as previously described (29) except that two ethanol washes were used after two 5-min 5% TCA rinses at room temperature. The discs were dried under a sun lamp and then extracted with 300 $\mu$l of Protosol at 55°C for 3 h. After cooling to room temperature, 5 ml of Betafluor was added and radioactivity was determined by scintillation counting.

### Isolation of RNA from hepatocytes

Two 500-$\mu$l aliquots of hepatocyte suspension were placed in two Eppendorf plastic tubes. Total cytoplasmic RNA was isolated as described by Maniatis, Fritsch, and Sambrook (32) with modifications (P. Zehnder, personal communication). Cells were briefly pelleted and resuspended in 350 $\mu$l of 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$ (TSM) containing 0.5% NP-40 and 18.42 $\mu$l of RNasin (20 U/$\mu$l). The mixtrue was vortexed occasionally for 3 min at 4°C, centrifuged to pellet nuclei, and the supernatant was transferred to a new Eppendorf tube containing 350 $\mu$l of 10 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA (TSE) plus 1% SDS. The supernatant was extracted eight to ten times with 0.7 ml of phenol–chloroform–isoamyl alcohol 24:24:1. The phenol had been preequilibrated with TSE, 0.1% 8-hydroxyquinoline, and 0.2% 2-mercaptoethanol. RNA was precipitated from the aqueous layer with two volumes of ethanol and 1/20 volume of 3 M sodium acetate at −20°C. Total cytoplasmic RNA was dissolved in 100 $\mu$l of 10 mM Tris, pH 7.5, 1 mM EDTA, 0.1% SDS, and stored at −70°C. The RNA concentration was calculated by determining the $A_{260}$ units and using the conversion factor of 25 $A_{260}$ units/mg RNA. In one experiment, vanadyl-ribonucleoside (rather than RNasin) was added to inhibit RNase. RNA from cultured hepatocytes was isolated by using guanidinium thiocyanate (33).

### Primary culture of hepatocytes

Hepatocytes from 2-day fasted rats were plated and cultured in serum-free medium lacking hormones, carbohydrate, or fat as described previously (34). Hepatocytes were plated onto collagen-coated nylon discs in L15 medium which lacked galactose and pyruvate, and arginine was replaced with ornithine. The amino acid composition was as described by Oliver, Edwards, and Pitot (35); HEPES (25 mM), $NaHCO_3$ (1.189 g/l), fraction V-fatty acid-free bovine serum albumin (2 mg/ml), penicillin and streptomycin (50 $\mu$g/ml each) were also present. This was the basal medium. Three million cells were added to each 60-mm plate and the medium was changed 4 h after plating and every 24 h thereafter.

### Preparation of the rabbit anti-apoE serum

Rats were fed an atherogenic diet containing propylthiouracil (22) and the apolipoproteins were isolated from $\beta$-VLDL by ultracentrifugation and delipidation as previously described (36). ApoE was purified by SDS PAGE electrophoresis and electroelution. This procedure was repeated to insure a pure preparation of apoE, and rabbits were then immunized. To test antiserum specificity, Western blots were run on rat serum and a rat liver supernatant fraction prepared as described above. In each case, the anti-apoE serum reacted with a single protein which migrated with pure rat apoE.

### Quantitation of apoE synthesis

The immunoprecipitation and SDS PAGE were performed as previously described (37). The immunoprecipitation reaction contained 100 $\mu$l of the postmicrosomal fraction, 15 $\mu$l of rabbit anti-rat apoE serum, 85 $\mu$l of PBS (10 mM sodium phosphate, 0.15 M NaCl) at pH 7.2 and 1% Triton X-100 and deoxycholate. The reaction mixture was incubated at 4°C for 24 h with continuous mixing and then 200 $\mu$l of 10% (w/v) IgSorb suspended in 1% Triton X-100, 1% deoxycholate in PBS at pH 7.2 (TDPBS buffer) was added and mixed at room temperature for 1 h. The immunoprecipitate was collected by centrifugation, washed with 400 $\mu$l of TDPBS buffer, and resuspended in 600 $\mu$l of this buffer. The suspension was layered on a 1 M sucrose pad and centrifuged at 12,000 *g* for 5 min at 4°C. The pellet was washed twice with 0.01 M sodium phosphate buffer, pH 7.5, and the apoE-IgG complex was dissociated by extracting the pellet twice with 2% SDS and 2% 2-mercaptoethanol at 100°C for 5 to 10 min. The two extractions were combined and subjected to SDS PAGE. The radioactivity in the apoE band was determined by scintillation counting of the sliced gel. The relative rate of apoE synthesis was calculated as the percent of cpm in immunoprecipitable apoE per cpm in total soluble proteins.

### Preparation of apoE cDNA

An apparently full-length apoE cDNA was obtained by screening our rat liver cDNA library with a 28-base synthetic oligodeoxydnucleotide probe derived from the published sequence of rat apoE mRNA (38). Authenticity of the apoE cDNA was established by extensive restriction mapping which, in every case, matched results predicted by the published sequence (39). The plasmid (papoE) contained the apoE cDNA in pUC9.

### Northern blot analysis

Total RNA was electrophoresed on a 1% agarose gel-containing 2.2 M formaldehyde in buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA). RNA was denatured at 50°C for 15 min in gel buffer containing 50% formamide and 2.2 M formaldehyde prior to loading onto the gel. After electrophoresis, RNA was transferred to a nitrocellulose filter in 3.0 M NaCl, 0.3 M sodium citrate (SSC), baked, prehybridized, and hybridized overnight to nick-translated [$^{32}$P]papoE ($7.0 \times 10^7$ cpm) in 50% formamide, 5 × Denhardts, 5 × SSPE (10 × SSPE = 3 M NaCl, 0.2 M $NaH_2PO_4$, 20 mM EDTA, pH 7.4), 0.1% SDS, 100 $\mu$g/ml heat-denatured salmon sperm DNA at 42°C. The filter was washed four times at room

temperature in 2 × SSC, 0.1% SDS followed by washing twice at 65°C for 45 min in 1 × SSC, 0.1% SDS. The filter was blotted dry and autoradiography was performed with intensifying screens at −70°C.

### Quantitation of poly $A^+$ RNA

The poly $A^+$ RNA content present in total RNA was determined by RNA dot-blot hybridization using a [$^{32}$P]-oligo(dT)$_{12-18}$probe (40). Twofold serial dilutions from 4 to 0.125 μg of total RNA were denatured in 6 × SSC and 7.4% formaldehyde and bound to the nitrocellulose filter. Two μg of *E. coli* tRNA was included as a negative control. Prehybridization and hybridization were carried out in buffers as described for Northern blot analysis. The filter was washed four times (5 min each) at room temperature in 6 × SSPE/0.05% sodium pyrophosphate, then at 32°C for 15 min in 6 × SSPE/0.05% sodium pyrophosphate. The autoradiogram was scanned and densities were integrated with the LKB Ultrascan-XL-laser densitometer. The integrated density versus μg RNA per dot was plotted and the slope was measured.

### Quantitation of apoE mRNA

The cDNA insert (about 1100 bp) from papoE was labeled with [$^{32}$P-α]dCTP by random priming with hexadeoxynucleotides (41). RNA dot blot hybridization to [$^{32}$P]apoE cDNA was used to quantitate apoE mRNA. Twofold serial dilutions from 4 to 0.125 μg of total RNA denatured in 6 × SSC and 7.4% formaldehyde were bound to a nitrocellulose filter. The dot blot was prehybridized and hybridized to the [$^{32}$P]apoE cDNA insert (about 5 × $10^7$ cpm) at 42°C in buffer as described for Northern blot analysis. The filter was washed four times at room temperature in 6 × SSPE/0.1% SDS for 5 min each, then washed twice in 0.1 × SSPE/0.1% SDS at room temperature for 30 min and subjected to autoradiography. The autoradiogram was scanned as described for the quantitation of poly $A^+$ RNA. The integrated densities were plotted against μg of RNA applied and the slope was measured. The relative content of apoE mRNA was calculated as the slope for apoE mRNA relative to the slope for poly $A^+$ RNA. In the experiment using PUFA and TYA, limiting amounts of poly $A^+$ RNA required us to pool individual samples from each group (seven or eight rats/group) before quantitating dot blots for apoE mRNA.

## RESULTS

Identification of nutrients and hormones that regulate apolipoprotein synthesis is essential if one is to understand the structure–function relationships involved in regulating promoters on the genes coding for these important proteins. Identification of promoter sequences on the apoE gene are ongoing in a number of laboratories but specific signals regulating apoE synthesis have yet to be elucidated. Considerable progress has been made, particularly by Davis et al. (23–25), but very few studies have been done using purified, simple diets where the effect of individual carbohydrates or fats could be studied.

The initial report by Boogaerts et al. (25) that dietary sucrose increased the synthesis of apolipoproteins secreted with VLDL led to the proposal that coinduction of both lipogenesis and apolipoprotein synthesis by dietary carbohydrate could increase the capacity of liver cells to both assemble and secrete triglyceride-rich lipoproteins. In that experiment, hepatocytes were prepared from rats with or without sucrose in the drinking water. These were cultured with radioactive amino acids for up to 18 h and the radioactivity in secreted apolipoproteins was determined. Under these conditions, the synthesis of apoB, apoA-I and apoE was approximately doubled by dietary sucrose.

Our objective was to use defined diets to determine the extent to which dietary manipulation of individual fats or carbohydrates might regulate apoE synthesis. We chose to determine the rate of apoE synthesis by immunoprecipitating apoE from pulse-labeled liver cells rather than by measuring the radioactivity in secreted apolipoproteins. We also measured the relative level of apoE mRNA so we could detect any regulation of apoE synthesis that might be independent of changes in apoE mRNA.

The conditions used here have been shown to produce large changes in lipogenesis (42) and in the synthesis of several lipogenic enzymes (43, 44). Control rats were fed the chow diet. Experimental rats were fasted for 2 days or fasted 2 days and refed a high glucose or fructose, no-fat diet, or a high fat, no-carbohydrate diet. In one experiment, the rats fed the high fructose, fat-free diet were injected with dibutyryl cAMP under conditions that decrease the synthesis of the lipogenic enzyme glucose-6-P dehydrogenase (G6PD) (45).

Hepatocytes, freshly isolated from these rats, were labeled with [$^3$H]leucine and postmicrosomal supernatants were obtained. The radiolabeled apoE was quantitatively immunoprecipitated with rabbit anti-rat apoE serum and immunoprecipitates were analyzed by SDS PAGE. **Fig. 1A** shows the entire gel profile of radiolabeled apoE immunoprecipitated from hepatocytes isolated from a rat fasted for 2 days and refed a high glucose fat-free diet for 4 days. It had a major peak with a molecular weight of 35,000 which comigrated with pure apoE. In order to ascertain whether the first immunoprecipitation brought down all of the apoE present in the postmicrosomal fraction, a second immunoprecipitation was carried out using the supernatant remaining after the first immunoprecipitation. There was no peak corresponding to apoE in the

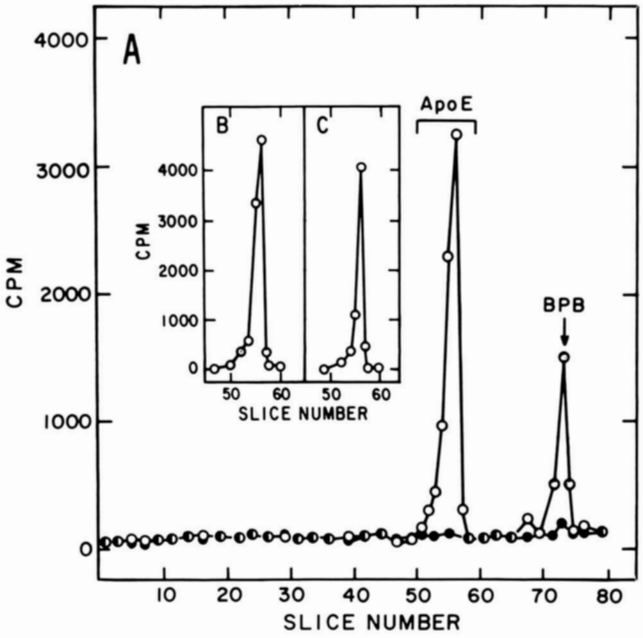


**Fig. 1.** Analysis by SDS polyacrylamide gel electrophoresis of apoE immunoprecipitated from $10^6$ cpm of radioactivity in total soluble protein. Hepatocytes were pulsed with [$^3$H]leucine for 60 min and the apoE was immunoprecipitated with rabbit anti-rat apoE serum (○) and IgSorb. Nonimmune rabbit serum (●) was used for the immunoprecipitation as a negative control (Fig. 1A). Under these conditions, the radioactivity in each peak is directly proportional to the relative rate of apoE synthesis. Fig. 1A shows the radioactivity in the entire gel when the solubilized immunoprecipitate from a fasted rat refed the 60% glucose fat-free diet was subjected to SDS PAGE. The inserts show the radioactivity in the apoE peak immunoprecipitated from a fasted rat (B) or from a rat fed the chow diet (C). The radioactivity in each apoE peak is 7785 cpm (A), 10,070 cpm (B) and 6560 cpm (C). BPB, bromphenol blue.

second immunoprecipitation (data not shown) indicating that all of the apoE was removed by the first immunoprecipitation. As a control, nonimmune serum was incubated with the postmicrosomal fraction and, as expected, was unable to react with any protein. The relative rate of apoE synthesis was obtained from the radioactivity present in the apoE peak by normalizing to the radioactivity in the total TCA-precipitable proteins.

Since apoE is a secreted protein in vivo, it was essential to determine whether any apoE was released into the medium during pulse-labeling of hepatocytes under the conditions used. Therefore, medium was also used for immunoprecipitation. The amount of apoE released into the medium was insignificant (less than 5% of that in hepatocytes, data not shown). Thus, the radioactivity in apoE immunoprecipitated from the hepatocyte supernatant fraction can be used to calculate the relative rate of apoE synthesis.

The peak of radioactivity associated with the bromphenyl blue dye marker was usually smaller than in Fig. 1A. We do not believe it represents a liver protein that reacts with the anti-apoE serum or a proteolytic fragment of apoE since the antiserum did not react with any protein smaller than apoE on a Western blot of a rat liver supernatant fraction, and the peak also appeared when antiserum versus other proteins was used (data not shown). Its uniform size and the use of the protease inhibitor phenylmethylsulfonylfluoride in the preparation of the supernatant fraction also make it unlikely that it was a proteolytic fragment of apoE; thus the radioactivity in this peak was excluded in calculating apoE synthesis. The insert in Fig. 1 shows representative apoE peaks immunoprecipitated from rats starved for 2 days (B) or rats fed the standard chow diet ad libitum (C). All peaks in Fig. 1 have been normalized to the same amount of radioactivity in total protein so the radioactivity in each peak is directly proportional to the relative rate of apoE synthesis.

ApoE mRNA was quantitated by probing dot blots of total RNA with [$^{32}$P]apoE cDNA in an assay similar to that described by Reardon et al. (46). **Fig. 2** shows a Northern gel probed with [$^{32}$P]papoE. A plot of the migration of the 18 and 28S rRNA indicated that the probe reacted with a single mRNA of about 1100 bases. This demonstrates the specificity of the apoE cDNA. Fig. 2 also shows representative dot blots of total RNA hybridized to the [$^{32}$P]apoE cDNA. These and additional data were scanned and normalized for poly A$^+$ RNA content as described in the Methods section in order to quantitate the amount of apoE mRNA.

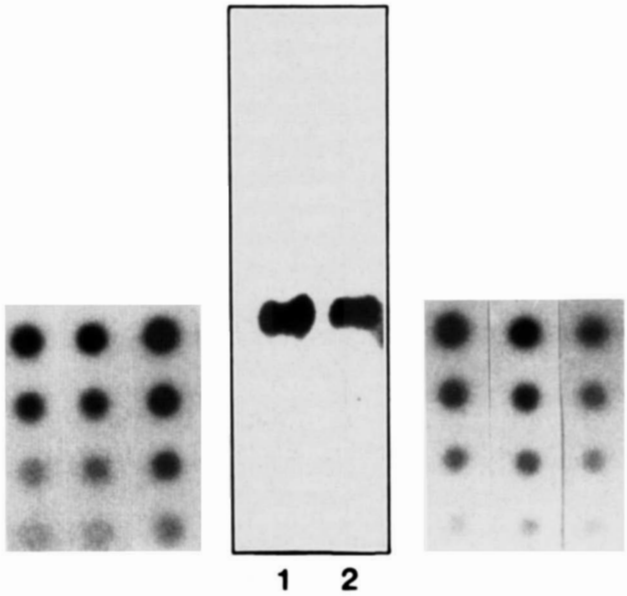


**Fig. 2.** Northern and dot blot analysis of total RNA isolated from rat hepatocytes. Total cytoplasmic RNA was isolated from rat hepatocytes by phenol–chloroform extraction using RNasin to inhibit RNase. The Northern blot, in the center, had 10 μg (lane 1) and 5 μg (lane 2) of total RNA hybridized to nick-translated papoE. Representative dot blots, using from 4 to 0.5 μg of total RNA isolated from livers of rats subjected to the six conditions reported in Table 1, are shown flanking the Northern blot. From left to right, the dietary conditions are 1) Purina chow; 2) fat, carbohydrate-free; 3) fasted; 4) glucose, fat-free; 5) fructose, fat-free without cAMP; 6) fructose, fat-free + cAMP.

TABLE 1. Effect of a high carbohydrate, fat-free diet and cAMP or a high fat, carbohydrate-free diet on the relative rate of synthesis and mRNA levels for liver apoE in male rats

| Diet | Relative Rate of ApoE Synthesis | Relative Level of ApoE mRNA |
|---|---|---|
| Purina chow | 0.66 ± 0.05[a] | 1.0 ± 0.076[a'] |
| Glucose, fat-free | 0.72 ± 0.07 | 1.2 ± 0.12[c] |
| Fat, carbohydrate-free | 0.71 ± 0.06 | 0.88 ± 0.06 |
| Fasted, 2 days | 1.1 ± 0.08[b] | 1.4 ± 0.06[b'] |
| Fructose, fat-free | 0.60 ± 0.04 | 1.1 ± 0.13[d] |
| Fructose, fat-free + dibutyryl cAMP | 0.56 ± 0.03 | 0.82 ± 0.11[e] |

Results are expressed as the mean ± standard error with four rats in each group (except there were three rats in the group fed the high glucose, fat-free diet, and five rats in the high fructose, fat-free diet group given cAMP). The Purina Chow was fed ad libitum. All others were fasted for 2 days prior to being refed the various diets.

Results designated [a] and [b] are significantly different ($P < 0.005$) as are [a'] and [b'] ($P < 0.01$); [a'] and [c], and [d] and [e] differ by $0.05 < P < 0.1$.

The results of the dietary treatments are shown in **Table 1.** The only dramatic effect was the significant increase in apoE synthesis and mRNA in fasted rats. This confirms previous results by Davis et al. (24) who showed slightly larger changes when rats were fasted for an additional day and disagrees with a recent report by Wilcox and Heimberg (26). Neither of the high carbohydrate diets nor the high fat diet caused a significant change in apoE synthesis or mRNA. The increase in apoE mRNA by dietary glucose was of marginal significance and dietary fructose had no significant effect. G6PD synthesis measured in the same animals showed the normal 45- to 60-fold changes we had reported previously (47).

Since fasting increases apoE synthesis and cAMP levels in liver (48), we used cAMP to see whether it could be the mediator of apoE induction in livers of fasted rats. The experimental design was one we had used previously to show that rat liver G6PD synthesis and mRNA were rapidly repressed by cAMP (46, 47). The results show no significant effect of cAMP on apoE synthesis or mRNA although both decreased somewhat.

**Table 2** shows the response of female rats to fasting and refeeding a 60% glucose fat-free diet. Since there had been a tendency for apoE synthesis and mRNA levels to increase in male rats under these conditions, we increased the number of animals in the two groups so small changes could be detected with greater confidence. Also, since apoE synthesis and mRNA levels always changed similarly in male rats (Table 1), only apoE mRNA levels were measured in the female rats. Experiment 1 illustrates that there was no significant difference in the level of apoE mRNA between the male and female rats. Experiment 2 illustrates that fasting females for 2 days increased apoE mRNA somewhat more than it had in males. Refeeding fasted females the 60% glucose, fat-free diet significantly increased apoE mRNA. Additional experiments will be necessary to determine whether the larger increase in apoE mRNA in females refed the glucose diet (1.7-fold) than in males refed the same diet (1.2-fold) is due to chance or to a real difference between how males and females respond to these diets. In any event, our results with the females support results by Boogaerts et al. (25) that a high carbohydrate diet causes an increase in apoE synthesis in liver.

With one exception (22), dietary lipids have had little effect on apoE synthesis in the rat; these diets included complex mixtures of lipids and cholesterol and contained carbohydrate (7, 20–22). As shown in Table 1, a high fat (mostly saturated) diet lacking carbohydrate had no effect on apoE synthesis or mRNA in liver. However, since polyunsaturated fatty acids (PUFA) are much more effective in decreasing lipogenesis, we tested the effect of PUFA alone under conditions where the amount of carbohydrate consumed was held constant by pair feeding. This insured that any changes observed would be due to the fatty acids and not the effect of the high glucose diet seen in Tables 1 and 2. In addition, an inhibitor of arachidonic

TABLE 2. Effect of a high glucose, fat-free diet or a 2-day fast on the levels of apoE mRNA in female rats

| Diet | Number of Animals | Relative Level of ApoE mRNA |
|---|---|---|
| Experiment 1 | | |
| Purina chow | 3 females | 1.0 ± 0.13 |
| Purina chow | 3 males | 0.79 ± 0.15 |
| Experiment 2 | | |
| Purina chow | 3 females | 1.0 ± 0.16[a] |
| Fasted 2 days | 6 females | 1.7 ± 0.10[b] |
| Glucose, fat-free | 5 females | 1.6 ± 0.10[c] |

Other conditions are as described in Table 1. Differences in the radioactivity of the apoE cDNA probe account for differences between experiments 1 and 2.

Results designated [a] and [b] are significantly different ($P < 0.005$); [c] and are significantly different ($P < 0.025$).

acid metabolism (TYA) was included to see whether inhibition of eicosanoid production would effect apoE synthesis (**Table 3**). Previously, the mRNA of two lipogenic enzymes was measured in these animals (29). Although there were no changes in apoE mRNA, there were significant decreases (1.5- to 3-fold) in 6-phosphogluconate dehydrogenase and G6PD mRNA in rats fed PUFA and this was prevented by TYA. Despite the ability of PUFA to regulate lipogenesis and lipogenic enzyme synthesis in liver, PUFA does not regulate apoE mRNA levels and inhibition of eicosanoid formation also has little effect on apoE mRNA.

In 1985 Elshourbagy et al. (49) reported that insulin and dexamethasone were able to increase apoA-I and apoA-IV mRNA levels in cultured hepatocytes but that apoE mRNA did not change. More recently, Lin (50) reported that insulin increased cellular and secreted apoE in cultured hepatocytes. We therefore reexamined the effect of these hormones on apoE mRNA levels in a primary hepatocyte culture system we recently optimized for induction of the lipogenic enzyme G6PD (34). We anticipated that our optimized culture conditions might be better able to detect subtle effects of hormones on apoE mRNA levels. The conditions we chose have been shown to change G6PD synthesis and activity 70-fold (ref. 34 and P. Manos, unpublished results). The results, presented in **Table 4,** show that insulin, dexamethasone, and fructose, at concentrations we have shown to be optimum for G6PD induction, had no significant effect on apoE mRNA levels. Dexamethasone, however, consistently caused a slight decrease in apoE mRNA.

## DISCUSSION

We have presented a comprehensive series of experiments to resolve some of the controversy in the literature regarding the regulation of apoE synthesis by diet and by fasting. Defined purified diets were used so the effect of individual components could be identified.

TABLE 3. Effect of polyunsaturated fatty acids (PUFA) and TYA on the level of apoE mRNA[a]

| Diet | Relative Levels of ApoE mRNA |
|---|---|
| Glucose | 1.0 |
| Glucose + PUFA | 1.0 |
| Glucose + PUFA + TYA | 1.1 |

[a]Under conditions where the dietary carbohydrate consumed was constant.

TABLE 4. Effect of insulin, dexamethasone, and fructose on apoE mRNA levels in cultured hepatocytes

| Conditions | Relative Levels of ApoE mRNA |
|---|---|
| Basal medium | 1 |
| Insulin | $1.1 \pm 0.3^a$ |
| Dexamethasone | $0.54 \pm 0.04^b$ |
| Insulin + dexamethasone | $0.68 \pm 0.1$ |
| Insulin + dexamethasone + fructose | $0.81 \pm 0.15$ |

Hepatocytes prepared from fasted rats were cultured for 4 days in the basal medium which lacked serum, hormones, carbohydrate, or fat, or in this medium supplemented with dexamethasone ($10^{-6}$ M), insulin ($10^{-7}$ M), or fructose (5 mM). ApoE mRNA on Northern or dot blots was quantitated in four separate experiments and corrected for the amount of polyA$^+$ RNA on each blot. Results are normalized to the apoE mRNA present in the basal medium in order to correct for any differences in probe radioactivity.

For [a] and [b], $0.05 < P < 0.1$.

The finding that carbohydrate in the diet increased apoE synthesis led us to reevaluate the effect of lipids on apoE synthesis because dietary fat can decrease the consumption of synthetic diets and carbohydrate (51). Thus, any effect of dietary fat might have been masked by changes in the amount of carbohydrate consumed. We used PUFA at a concentration known to decrease lipogenic enzyme mRNA and synthesis in pair-fed rats to insure they consumed the same amount of carbohydrate (see Table 1 in reference 29). Under these carefully controlled conditions there was no effect of safflower oil on apoE mRNA. Our results in Tables 1 and 3 are consistent with those of several others who used more complex diets with cholesterol and concluded that dietary lipids do not regulate apoE synthesis in liver (20, 21, 23, 24).

In Table 2, we show that there is no significant difference in apoE mRNA levels in male and female rats. Fasting or fasting and refeeding the 60% glucose diet both increased apoE mRNA levels significantly. To our knowledge, this is the first comparison of the sexes in the regulation of apoE synthesis in rat liver.

The primary hepatocyte culture system we used in our studies completely avoids exposure of the cells to serum, hormones, carbohydrate, or fat. The energy source in this medium is amino acids. Hepatocytes cultured in this basal medium for 4 days maintain apoE mRNA at levels equal to those in intact liver. This was determined by running a standard liver poly A$^+$ RNA preparation, isolated from a fasted rat refed a high carbohydrate diet, on each Northern or dot blot (data not presented). Thus, maintenance of apoE mRNA levels does not require hormones, carbohydrate, or fat in the medium (at least for 4 days). These results are consistent with the inability of carbohydrates, hormones, or fats to alter apoE synthesis or mRNA levels in cultured hepatocytes (Table 4; references 23, 24, 48). The decrease in apoE mRNA by $10^{-6}$ M dex-

amethasone was not statistically significant but was consistently observed. However, glucocorticoids are not likely to regulate apoE synthesis in vivo since a lower concentration in cultured hepatocytes or injection into rats did not alter apoE mRNA levels (49).

Since many of the effects of fasting on protein synthesis are mediated by cAMP, we used the procedure in Table 1 to see whether cAMP could induce apoE synthesis in vivo. Although these conditions had been used previously to demonstrate the cAMP can rapidly decrease G6PD synthesis and mRNA (46, 47), there was no effect on apoE synthesis. The small decrease in apoE mRNA was not significant. Thus, cAMP is not able to mediate a rapid induction of apoE synthesis. If cAMP is involved, it must act over a longer time period.

At the present time, only fasting or a high carbohydrate, lipogenic diet are clearly identified as conditions that regulate apoE synthesis in rat liver. The synthesis of apoE is not regulated like the synthesis of the enzymes that produce fatty acids in liver (lipogenic enzymes) since the synthesis of these enzymes is decreased by cAMP and fasting, and regulated by insulin, glucocorticoids, thyroxine, and PUFA (for a recent review, see reference 52). However, an intermediate in carbohydrate metabolism has been proposed to regulate the induction of lipogenic enzymes (53) and a similar mechanism could be responsible for the induction of apoE by dietary carbohydrate.

It might seem unusual that a high carbohydrate diet and fasting both increase apoE synthesis in liver since these conditions have opposite effects on the regulation of carbohydrate and lipid metabolism and on the synthesis of many enzymes involved in lipogenesis (52). In the liver of the fed rat, apoE is secreted with VLDL and the increase in apoE synthesis by dietary carbohydrate may be linked to this process. In the fasting rat, little apoE is secreted with VLDL; rather it seems to be associated with HDL (24, 26). Recently Mackinnon et al. (54) have shown that HDL with apoE is an important source of cholesteryl esters for bile synthesis. It may be important to increase apoE synthesis in the fasting rat as a means of continuing to provide cholesterol for bile synthesis so fats can be absorbed when feeding is resumed. Thus, the increase in apoE synthesis by dietary carbohydrate and by fasting may simply reflect the multiple complex functions of apoE in lipid metabolism.

This research was supported in part by a grant from the American Heart Association (California affiliate).

*Manuscript received 18 July 1988 and in revised form 18 October 1988.*

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