Regulation by nutritional status of lipids and apolipoproteins A-I, A-II, and A-IV in inbred mice

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Abstract This study illustrates that genetic strain and feeding status can markedly influence tissue lipid concentrations and mRNA levels of apolipoprotein genes. C57BL/6 and BALB/c mice were maintained for 2 weeks on four test diets differing in amount of cholesterol and type of fat, and fasted for 4 h or 16 h prior to collection of tissues. For both strains, the primary effect of fasting from 4 h to 16 h was to paradoxically elevate triglyceride levels in plasma and liver, and to elevate hepatic apoA-IV mRNA levels. Triglyceride secretion rates, estimated after the injection of Triton WR-1339, suggested that elevations in plasma triglyceride levels were due to reduced clearance of very low density lipoproteins. Although plasma glucose levels decreased with fasting time for both strains, insulin levels decreased for BALB/c but not C57BL/6 mice regardless of diet. This suggests that factors thought to be mediated by insulin, (e.g., plasma free fatty acid concentrations; hepatic apoA-IV mRNA levels) may be influenced by local changes in insulin sensitivity, which are controlled genetically and are not reflected by plasma insulin levels. In summary, nutritional status influences a constellation of factors involved in lipid transport that also show strong genetic components and may influence subsequent analyses of gene expression in the mouse system.—**LeBoeuf, R. C., M. Caldwell, and E. Kirk.** Regulation by nutritional status of lipids and apolipoproteins A-I, A-II, and A-IV in inbred mice. *J. Lipid Res.* 1994. **35:** 121–133.

Supplementary key words mouse • nutrition • apolipoprotein regulation • genetics

Inbred mice are proving to be valuable models for identifying genetic factors in mammalian plasma lipid transport. For instance, hundreds of inbred strains are commercially available and many have been shown to exhibit substantial differences in plasma lipoprotein quantity, size, and chemical composition (1–3), apolipoprotein regulation (4), and activity of key enzymes in mammalian lipid transport (5, 6). In addition, the use of recombinant inbred strain and backcross analyses have demonstrated the importance of dietary–genetic interactions in the control of lipid transport (7) and associated diseases (8, 9). The structural genes for nearly all of the major apolipoproteins and many enzymes determining plasma lipoprotein structure and concentration have been mapped to mouse chromosomes (reviewed in ref. 9). Finally, genetically engineered mice with transgenes controlling the overexpression and disruption of lipid transport genes have been developed (reviewed in ref. 9). How nutritional status influences the expression of lipoprotein transport genes in normal and genetically modified strains is not entirely clear. In particular, the physiological consequences of food restriction on the expression of lipid transport proteins has not been well characterized and is the subject of this report.

A common practice in metabolic and molecular genetic studies is to collect tissues from mice after an overnight (12–16 h) fasting period to avoid variations in lipid parameters due to postprandial fluctuations. However, food deprivation in mammals alters a constellation of physiological parameters as tissues convert from utilizing glucose to fats and eventually amino acids as fuel (10). Fuel conversion occurs rapidly (3–6 h) in small mammals such as mice and rats as evidenced by marked decreases in tissue glycogen levels (10, 11). Other changes associated with semi-starvation conditions include decreases in plasma insulin and glucose levels and increases in plasma FFA and triglyceride concentrations.

Several reports show that modulation of hepatic lipogenesis by food restriction alters the transcriptional and/or synthetic regulation of apolipoprotein genes. In hepatocytes taken from rats fasted for 3 days (12), a decreased rate of triglyceride secretion was accompanied by a 50% decrease in apoB-48 synthesis, although synthesis of apoB-100 remained unchanged. In contrast, apoE synthesis was elevated 2- to 4-fold. The effect of fasting rats for 48 h on the mRNA levels of several plasma proteins showed marked decreases in mRNA for apoA-II, more moderate decreases in mRNA levels of apoE, A-IV, albumin, transferrin, and transthyretin, and a 33% in-

Abbreviations: apo, apolipoprotein; FFA, free fatty acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high density lipoprotein; LDL, low density lipoprotein; TC, total cholesterol; Triton, Triton WR-1339; VLDL, very low density lipoprotein.

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crease in apoA-I mRNA (13). Poullain et al. (14) measured plasma apolipoprotein levels of rats fasted for 3 days. While changes in hepatic apoB mRNA levels were dependent upon diet, apoA-I and A-IV mRNAs showed marked decreases and apoE mRNA showed, a marked increase compared to fed animals. Baum, Teng, and Davidson (15) showed that refeeding rats fasted for 48 h with a high carbohydrate diet resulted in a 30-fold increase in hepatic triglyceride content and a shift in apoB editing from production of both B-100 and B-48 to produce primarily apoB-48. The refed rats also exhibited moderate increases in apoA-I, A-IV, and E mRNA levels. Thus, interpretation of gene regulation must include consideration of nutritional status.

Our laboratory is undertaking a systematic study in mice to identify and characterize genes susceptible to manipulation by dietary cholesterol and fatty acids. C57BL/6 and BALB/c mice are used to model dietary responses in mice known to differ in atherosclerosis susceptibility (8) and other lipoprotein parameters (1, 4). During the course of these studies, we observed marked deviations in plasma lipid and hepatic apolipoprotein mRNA levels within each individual mouse strain. In preliminary studies, differences in fasting times appeared to account for much of the intrastrain variation. Thus in this report, we examine several parameters of lipid transport in the plasma and liver as a function of two fasting times commonly used in the literature for mouse studies. We have found that fasting time dramatically alters the plasma and hepatic concentrations of triglyceride and the mRNA levels of apolipoprotein A-IV in the liver.

MATERIALS AND METHODS

Animals

Female BALB/cByJ (BALB/c) and C57BL/6J (C57BL/6) mice from 6 to 8 weeks of age were obtained from the Jackson Laboratory, Bar Harbor, ME. Mice were fed a pelleted rodent chow diet (Wayne Rodent BLOX 8604, Teklad, Madison, WI) for 2 weeks prior to initiation of diet studies. Mice were maintained in a temperature-controlled (25°C) facility with a strict 12-h light/dark cycle. Mice were given free access to food and water. Food was removed from the mice 4 h or 16 h prior to the collection of blood from the retroorbital sinus into tubes containing anticoagulant and antimicrobial agents (1 mM EDTA, 50 μg/ml gentamicin sulfate, 0.05% sodium azide). Plasma was stored for 1 week or less at 4°C prior to analysis. Mice were killed by cervical dislocation and tissues obtained for lipid analyses were placed directly into liquid nitrogen and stored at −70°C. This project was approved by the Animal Care and Use Committee of the University of Washington (Protocol #2140-01).

Diets and feeding

Four diets were used in this study. Pelleted rodent chow and an "atherogenic" diet containing rodent chow known to elicit fatty streak lesions in strain C57BL/6 mice (8) were obtained from Teklad Test Diets (Madison, WI). The rodent chow diet contained approximately 4% fat, 24% protein, and 4.5% crude fiber. The atherogenic diet, described in detail by Paigen et al. (16), provided 30% kcal from fat (cocoa butter) and contained 1.25% cholesterol and 0.5% sodium cholate. Two high fat diets containing safflower oil and 0.5% sodium cholate with (saff/high) and without (saff/low) added cholesterol (0.5%) were prepared in our diet kitchen as described (17). The two safflower oil diets were calorically balanced and differed only in cholesterol content. These diets provided approximately 24% kcal from protein, 43% kcal from carbohydrate, and 33% kcal from fat. A detailed description of dietary contents has been given (17). Diets were stored in air-tight containers at −20°C and fresh diet was provided daily. Animal acceptance of the semi-synthetic diets was improved by gradual introduction of diet over a 4-day period: 2 days of 50:50 chow to semi-synthetic diet, 2 days of 25:75 chow to semi-synthetic diet, followed by 2 weeks of 100% semi-synthetic diet (17).

Mice were fed test diets during three separate study periods owing to the availability of mice. In the first, mice were fed the atherogenic diet for 2 weeks and plasma and tissues were collected as described above. HDL cholesterol values were not obtained for these atherogenic diet-fed mice due to insufficient volumes of plasma. The second feeding period consisted of mice fed rodent chow and two safflower oil-containing diets which were fed concurrently. HDL were separated from other lipoproteins prior to the freezing of plasma samples for these mice. Plasma and tissue analyses as described below were determined for animals of both sets of feeding studies simultaneously. The third feeding study consisted of mice maintained on rodent chow for the Triton WR-1339 experiments as described below.

Tissue lipid, glucose, and insulin determinations

Lipids were extracted from mouse liver using the method of Folch, Lees, and Sloane Stanley (18), then modified to contain Triton X-100 as described by Carr, Andresen, and Rudel (19). The hepatic and plasma total and HDL cholesterol concentrations were determined using a colorimetric kit (Diagnostic Kit, No. 236691, Boehringer Mannheim, Indianapolis, IN) with cholesterol standards (Preciset #125512, Boehringer Mannheim). HDL cholesterol values were measured after the selective precipitation of VLDL/LDL by phosphotungstate (20). Triglyceride concentrations in liver and plasma were determined after removal of free glycerol (Diagnostic Kit, No. 450032, Boehringer Mannheim). Hepatic phos-

pholipids were determined according to Turner and Rouser (21). Plasma free fatty acid quantities were determined using a colorimetric kit (No. 990-75401, Wako Chemicals USA, Inc., Dallas, TX). Plasma glucose concentrations were determined on a Beckman Glucose Analyzer II utilizing a glucose oxidase system. Plasma insulin quantification was by radioimmunoassay (22) using an antibody to guinea pig insulin and human insulin standards.

Apolipoprotein quantitation

Plasma apolipoprotein concentrations were determined by slot-blotting. All antibodies were determined to be monospecific by Western blot analysis of mouse plasma. Rabbit antiserum to apoA-I was prepared from isolated mouse apoA-I. ApoA-I was first separated from other HDL proteins by Sephadex column chromatography as described (23) and further isolated from a Coomassie-stained band from 15% SDS-polyacrylamide gels. Antiserum to apoA-II was prepared as described (23) and results using this antiserum also described (20). ApoA-IV was detected using antiserum developed in rabbit to an apoA-IV fusion protein (24) which was generously provided by Deborah Purcell-Huynh and Aldons J. Lusis (University of California, Los Angeles, CA). Plasma aliquots (25 μl for apoA-II, 50 μl for apoA-IV, and 5 μl for apoA-I of plasma diluted 1:500) were applied to nitrocellulose filters (0.45 μm pore size, Schleicher and Schuell, Keene, NH) using a Minifold II slot-blotter (Schleicher and Schuell) according to manufacturers' instructions. Filters were then removed from the blotter, air-dried for 30 min, rinsed briefly in water, and soaked for 15 min in 15% hydrogen peroxide. After a brief rinse in distilled water, filters were baked under vacuum for 1 h. Apolipoproteins were detected after incubation of filters with monospecific antisera followed by incubation with iodinated Protein A (20). Antisera dilutions of 1:1000 were used. Signal intensities for apoA-II and apoA-IV were linear for volumes ranging from 2.5 to 100 μl of plasma diluted by 1:500 with water. For apoA-I, linearity was achieved in the range of 2.5 to 25 μl of plasma diluted by 1:500. This method routinely detected 5 ng of apolipoprotein.

Triton WR-1339 experiments

Plasma triglyceride accumulation with time was assessed following injection of Triton WR-1339 (designated in text as Triton; Tyloxapol, Sigma Chemical Co., St. Louis, MO) via the tail vein of mice. Triton was dissolved in 0.9% NaCl (15% Triton w/v) and injected at a dose of approximately 300 mg/kg body weight between 10–11:30 AM after fasting mice for either 4 h or 16 h. Blood samples for measurement of triglyceride and cholesterol were obtained at 30, 60, and 120 min after injection of Triton. Blood samples were also taken at each time point from mice injected with normal saline (sham injected). An aliquot of plasma was used to determine the concentration of Triton following precipitation of plasma proteins by 1:10 isopropanol and spectroscopic reading at 278 nm as described (25). Triglyceride accumulation rates (**Table 1**) were determined similarly to Kasim et al. (26) as: rate (mg/min) = 1/3 $[(TG_{30}\text{-}TG_0)/30 + (TG_{60}\text{-}TG_0)/60 + (TG_{120}\text{-}TG_0)/120] \times$ plasma volume, where TG_{30}, TG_{60}, and TG_{120} are triglyceride concentrations at 30, 60, and 120 min, respectively, and TG_0 is triglyceride concentration for sham-injected mice (n = 3–6) used at each time point. The plasma volume used was established for mice at 5.77% of body weight (27).

Messenger RNA quantitation

Mouse cDNA probes for apoA-I and apoA-II were as described (20). A partial mouse apoA-IV cDNA probe (7) was generously provided by Deborah A. Purcell-Huynh and Aldons J. Lusis (University of California, Los Angeles, CA). RNA was isolated from mouse livers and quantitated by Northern blotting as previously described

TABLE 1. Statistical parameters describing effects of fasting time, diet, and strain on plasma and liver measurements[a]

	Plasma									Liver					
							Apolipoprotein			mRNA					
ANOVA Factors	TC	HDL-C	TG	Gluc	Ins	FFA	A-I	A-II	A-IV	A-I	A-II	A-IV	C	TG	PL
Time (T)	<0.001	0.353	<0.001	<0.001	<0.001	<0.001	0.045	<0.001	<0.001	0.019	0.339	<0.001	0.469	<0.001	0.229
Diet (D)	<0.001	<0.001	<0.001	<0.001	0.167	0.101	<0.001	<0.001	<0.001	0.003	0.001	<0.001	<0.001	<0.001	0.246
Strain (S)	0.115	<0.001	<0.001	0.181	<0.001	<0.001	0.803	<0.001	0.003	0.595	0.294	<0.001	<0.001	<0.001	0.008
T × D	<0.001	0.170	0.006	0.391	0.300	0.009	0.004	0.008	0.064	0.697	0.075	0.001	0.688	0.008	0.535
T × S	0.114	0.211	0.131	0.007	0.001	0.016	0.058	0.852	0.983	0.483	0.002	0.010	0.253	0.067	0.093
D × S	<0.001	<0.001	<0.001	<0.001	0.124	<0.001	0.006	<0.001	<0.001	0.011	0.064	<0.001	<0.001	0.072	<0.001
T × D × S	0.012	0.003	<0.001	0.579	0.219	0.001	0.032	0.109	0.685	0.659	0.819	<0.001	0.103	0.061	0.776

Table abbreviations are time (T), diet (D), strain (S), total cholesterol (TC), HDL cholesterol (HDL-C), triglyceride (TG), glucose (Gluc), insulin (Ins), free fatty acids (FFA), cholesterol (C), and phospholipid (PL).

[a]ANOVA, analysis of variance for probability > F. A 3-factor model was used for initial analysis of plasma and liver parameters. Further 2-factor analyses were performed in some cases. *P* values for differences between individual samples are given in the text.

(1, 5) using a cDNA probe (Gibco BRL, Gaithersburg, MD) for glycerol-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard.

Statistical analysis

Results are reported as means ± SEM. Statistical differences were determined by multi-factorial ANOVA using SYSTAT for the Macintosh (SYSTAT, Inc., Evanston, IL). Post-hoc analyses of significance was made by Fisher's protected least squares difference test. $P < 0.05$ was accepted as statistically significant.

RESULTS

Body weights were determined three times during this study for rodent chow- and saffiower oil diet-fed mice. Initial body weights of BALB/c and C57BL/6 mice were 19.6 ± 1.3 g and 18.2 ± 1.2 g, respectively (mean ± standard deviation) for the 30 mice of each strain which were then randomly distributed between diet groups. Within each strain, final body weights were nearly identical for all diet groups, suggesting that the animals were healthy and ate comparable calories. Final body weights were 22.1 ± 1.4 g for BALB/c mice and 19.0 ± 1.0 g for C57BL/6 mice. Body weights for mice fed the atherogenic diet were not determined in this study, but mice have been shown in many preliminary experiments to either maintain or gain body weight (1–5% of initial body weight) over 2 weeks of feeding (R. LeBoeuf, unpublished data). Final body weights of mice used in the Triton studies are presented in **Table 2.**

Plasma lipid and lipoprotein concentrations

Results of plasma lipid parameters were examined by three-way ANOVA in terms of fasting time, mouse strain, and diet. As shown in Table 1, significant 3-way interactions were observed for plasma total cholesterol (TC), HDL cholesterol, and plasma triglyceride concentrations. A main effect on plasma total cholesterol (TC) levels was dietary cholesterol (**Fig. 1**). Significant increases in TC values, regardless of fasting time, were observed between mice of the same strain when fed the atherogenic diet (cocoa butter with cholesterol) as compared to rodent chow ($P < 0.001$), and when fed the saff/high (safflower oil with cholesterol) as compared to saff/low (safflower oil without cholesterol) diets ($P < 0.001$). Interaction between fasting time and diet was reflected in decreased TC levels at 16 h for C57BL/6 and BALB/c mice fed the atherogenic diet ($P < 0.001$) and for C57BL/6 mice fed saff/high ($P < 0.001$). Significant strain differences were seen for mice fed the atherogenic diet ($P < 0.001$ at 4 h; $P < 0.01$ at 16 h), saff/low at 4 h ($P < 0.001$), and saff/high at 4 h ($P < 0.05$) and 16 h ($P < 0.001$).

TABLE 2. Triglyceride (TG) secretion rates following injection of Triton WR-1339[a]

Group	Strain	Time of Fasting	Body Weight	TG Secretion Rate[b]
		h	*g*	*mg/min*
A	C57BL/6	4	19.1 ± 0.3	1.74 ± 0.03
B	C57BL/6	16	19.4 ± 0.4	1.73 ± 0.35
C	BALB/c	4	21.3 ± 0.2	5.22 ± 0.92
D	BALB/c	16	21.2 ± 0.8	3.42 ± 0.55

[a] Data presented as mean ± SEM for 3 to 10 mice.
[b] Calculated as described in Materials and Methods. A two-way ANOVA (strain versus fasting time) showed significance only due to strain ($F = 20.79$, $P < 0.002$). Fisher's least-significant-difference test: A, C: $P < 0.002$; B, D; $P < 0.055$; A, B and C, D: not significant.

HDL cholesterol levels were mainly effected by diet and strain (Table 1). For mice fed safflower oil diets, HDL cholesterol values (Fig. 1) were significantly lower upon addition of dietary cholesterol at both fasting times for C57BL/6 ($P < 0.001$) and BALB/c ($P < 0.05$ for 4 h; $P < 0.001$ for 16 h). BALB/c mice exhibited significantly higher HDL cholesterol levels for all diets as compared to C57BL/6 mice ($P < 0.005$). The amount of plasma obtained from mice fed the atherogenic diet was not sufficient, due to technical difficulties, to allow determination of HDL cholesterol. In previous studies, HDL cholesterol values were 30 and 60 mg/dl for atherogenic diet-fed C57BL/6 and BALB/c mice fasted overnight (20).

Plasma triglyceride concentrations were sensitive to all effects except the interaction of time and strain (Table 1). Thus, time effected similar changes in plasma triglyceride levels for both strains. In particular, the 16 h fasting time was associated with significant increases in triglyceride levels ($P < 0.04$–0.001) for all but two cases (C57BL/6 fed atherogenic; BALB/c fed saff/low). Diet also provided a main influence on plasma triglyceride levels which were also strain- and time-dependent. For instance, triglyceride concentrations for C57BL/6 mice were not significantly different at 4 h. At 16 h, an effect of diets containing cholesterol was seen, as plasma triglyceride was lower for mice fed the atherogenic diet compared to rodent chow ($P < 0.001$) and saff/high compared to saff/low ($P < 0.001$). In contrast, BALB/c mice fed high fat diets (atherogenic and safflower oil) showed significantly lower triglyceride levels compared to BALB/c mice fed rodent chow at 4 h of fasting ($P < 0.01$). At 16 h, BALB/c mice showed an increase ($P < 0.001$) or no change in triglyceride levels with the addition of dietary cholesterol. Thus, triglyceride responses were complex and dependent upon a combination of nutritional and genetic factors.

Plasma glucose, insulin, and free fatty acid (FFA) concentrations

A loss of tissue glucose stores, a decrease in plasma insulin, and an increase in plasma FFA are characteristics of semi-starvation conditions (10, 11). Regardless of diet or

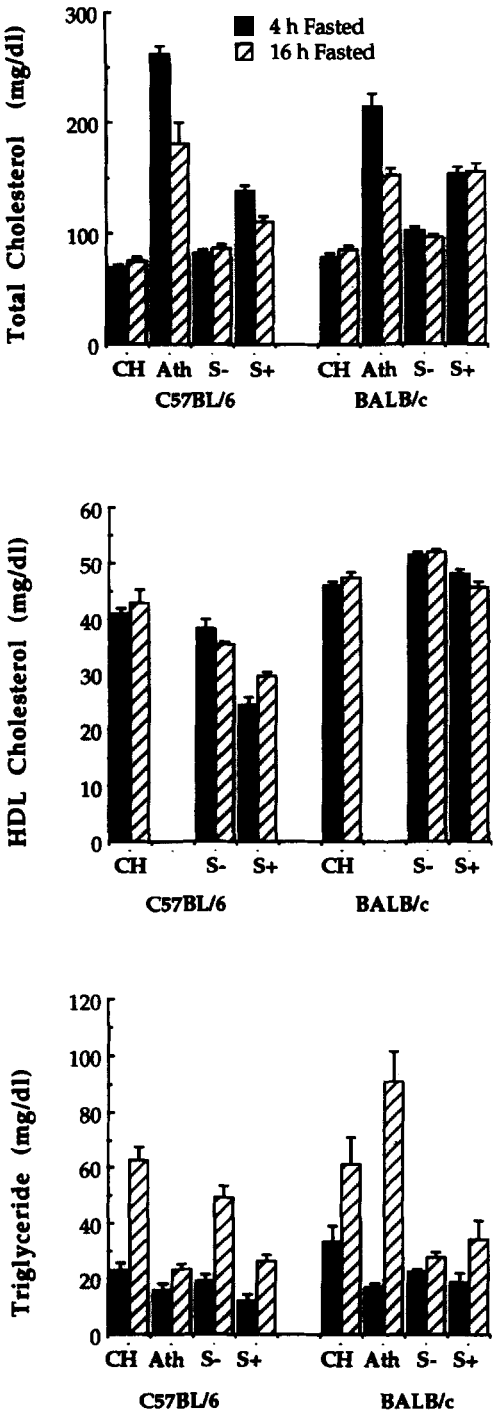


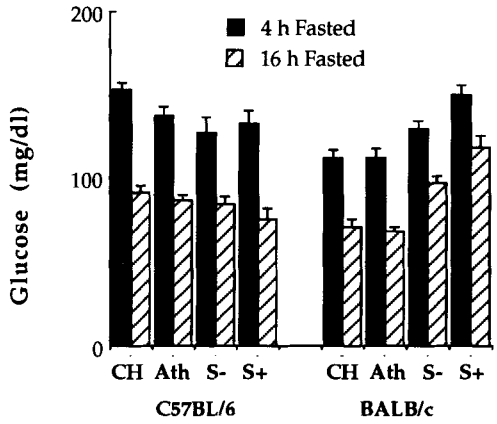
Fig. 1. Plasma lipid concentrations in response to diet and fasting time for C57BL/6 and BALB/c mice. Mice were maintained on either rodent chow (CH), a diet containing rodent chow, 150 g/kg cocoa butter with 12.5 g/kg cholesterol, and 5 g/kg sodium cholate (Ath), or semi-synthetic diets containing 138 g/kg safflower oil and 5 g/kg sodium cholate without (S−) or with (S+) 5 g/kg cholesterol for 2 weeks. Animals were fasted for either 4 h (solid bar) or 16 h (hatched bar) and bled from the orbital sinus between 11 AM and 1 PM. Plasma total cholesterol, HDL cholesterol, and triglyceride were determined as described under Materials and Methods. Each bar represents the mean value ± SEM obtained for 10 mice. Significance levels are given in the text.

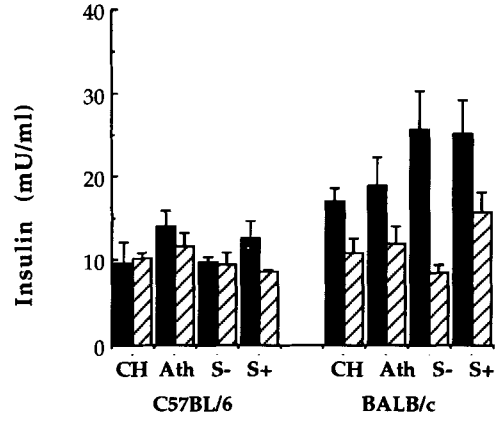
strain, plasma glucose concentrations were approximately 30% lower at 16 h as compared to 4 h of fasting (**Fig. 2**) ($P < 0.001$). Concomitant changes in mouse plasma insulin and FFA concentrations did not necessarily accompany the glucose changes and were dependent upon strain and diet. For instance, significant decreases in plasma insulin levels with fasting time were seen for BALB/c ($P < 0.05$–0.001) but not C57BL/6 mice. Plasma insulin levels varied modestly (9–14 mU/ml) for C57BL/6 mice fed all diets at both 4 and 16 h fasting times. FFA levels increased significantly for C57BL/6 mice fed all diets upon fasting for 16 h as compared to 4 h ($P < 0.005$). BALB/c mice exhibited inconsistent changes in FFAs with time and diet. FFA levels were significantly higher at 16 h only for atherogenic diet-fed BALB/c mice ($P < 0.001$), decreased for saff/low-fed mice ($P < 0.001$), and were unchanged for rodent chow- and saff/high-fed mice. Differences between strains included significantly higher levels of insulin for BALB/c compared to C57BL/6 mice at 4 h ($P < 0.001$). In addition, FFA levels were higher for BALB/c versus C57BL/6 mice ($P < 0.001$–0.02) for all but safflower oil-fed mice fasted 16 h. Thus, glucose, insulin, and FFA levels showed differential effects due to fasting time, genetic, and dietary factors.

Plasma apolipoprotein (apo) concentrations

Overall, plasma apoA-I, A-II, and A-IV concentrations tended to remain the same or decrease from 4 h to 16 h of fasting (**Fig. 3**). ApoA-I concentrations showed effects from multiple interactions (Table 1). Thus, diet and time effects were strain-dependent. For instance, at 4 h of fasting, apoA-I levels for safflower oil-fed C57BL/6 mice were higher than observed for mice fed the rodent chow-based diets ($P < 0.001$). The apoA-I levels for these safflower oil-fed mice decreased significantly with increased fasting time ($P < 0.003$). In contrast, apoA-I levels for BALB/c mice remained relatively constant for all conditions at 120–140 mg/dl. Significant differences between strains were seen for 4 h-fasted mice fed rodent chow and safflower oil diets ($P < 0.04$).

There were four main observations concerning changes in apoA-II concentration. First, the primary effect of fasting time occurred for mice fed rodent chow, for which decreases were seen in both strains at 16 h ($P < 0.004$). Second, within each fasting time group, mice fed rodent chow had higher levels of apoA-II than mice fed the high fat diets for strains BALB/c ($P < 0.001$) and C57BL/6 ($P < 0.03$). Third, BALB/c mice showed greater concentrations of apoA-II than C57BL/6 mice ($P < 0.002$–0.05) for all diets except saff/low at 4 h. Finally, apoA-II levels were significantly decreased for C57BL/6 mice fed saff/high as compared to saff/low diets ($P < 0.001$) and the atherogenic versus rodent chow diets ($P < 0.03$), suggesting that dietary cholesterol may influence plasma apoA-II concentrations in this strain.





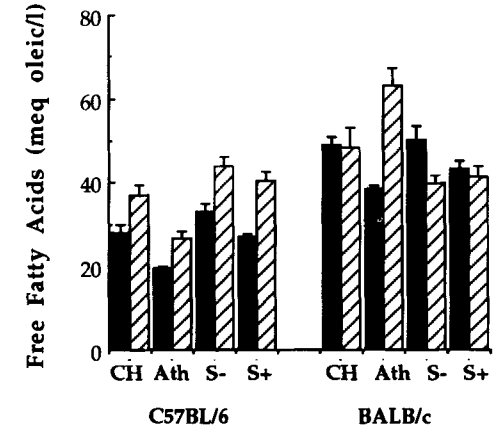
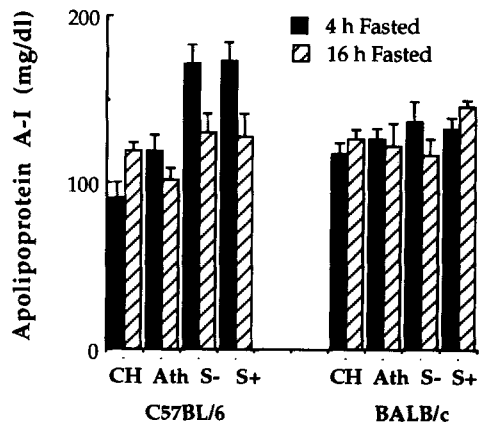
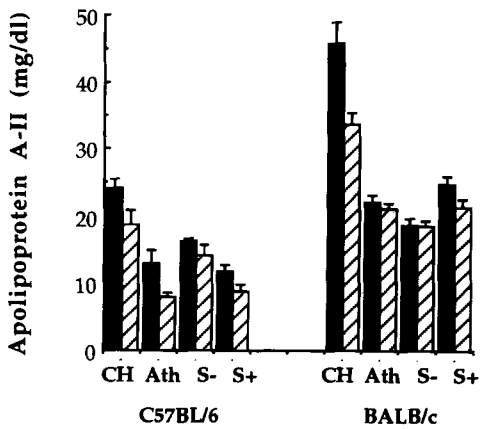


Fig. 2. Plasma glucose, insulin, and free fatty acid concentrations in response to diet and fasting time for C57BL/6 and BALB/c mice. Experimental conditions are as described in the legend to Fig. 1. Diets are rodent chow (CH), cocoa butter and cholesterol (Ath), and safflower oil without (S−) or with (S+) cholesterol. Glucose and free fatty acid determinations were done colorimetrically and insulin levels were determined by radioimmunoassay as described under Materials and Methods. Each bar represents the mean value ± SEM obtained for 3–10 animals. Significance levels are given in the text.





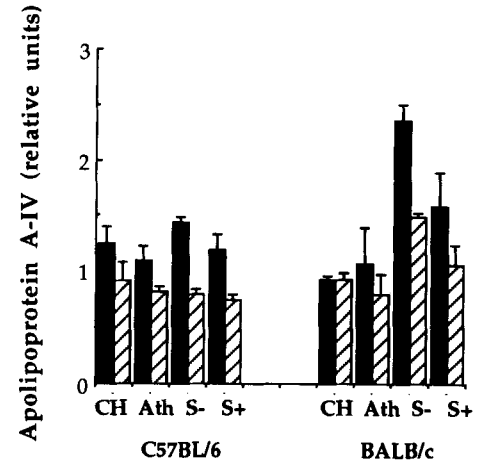


Fig. 3. Plasma apolipoprotein A-I, A-II, and A-IV concentrations in response to diet and fasting time for C57BL/6 and BALB/c mice. Apolipoprotein levels were determined by quantitative immunoblotting as described under Materials and Methods. Experimental conditions are as described in the legend to Fig. 1. Diets are rodent chow (CH), cocoa butter and cholesterol (Ath), and safflower oil without (S−) or with (S+) cholesterol. Each bar represents the mean value ± SEM obtained for 3–5 mice. Significance levels are given in the text.

ApoA-IV concentrations tended to be lower at 16 h versus 4 h, but reached significance only for mice fed safflower oil diets ($P < 0.008$). Within each fasting time group, apoA-IV concentrations were the same for C57BL/6 mice fed all diets. In contrast, BALB/c mice fed the saff/low diet had significantly higher apoA-IV concentration than BALB/c mice fed the other diets ($P < 0.004$). This diet was the only one to result in a significant difference between strains at both fasting times ($P < 0.001$).

Hepatic mRNA levels

GAPDH mRNA was used as our internal standard and it was not influenced by fasting time, diet, or mouse strains used here. This conclusion was based on the signal intensity of GAPDH mRNA bands on autoradiograms which reflected the ethidium bromide stain intensity of 18S and 28S bands seen in gels prior to blotting. In addition, many of our samples were run repeatedly in different combinations on separate agarose gels showing, in side-by-side comparisons, that no trends in GAPDH mRNA levels were observed.

Although hepatic apoA-I mRNA levels tended to increase with fasting time, only one of these values (BALB/c fed rodent chow; $P < 0.05$) reached statistical significance due to large variations (**Fig. 4**). Diet effects consisted of lower apoA-I mRNA values for BALB/c mice fed safflower oil as compared to the chow and atherogenic diets ($P < 0.001$–0.05). C57BL/6 apoA-I mRNA levels were not significantly different among all conditions. A diet–strain effect was seen for mice fed the atherogenic diet in which apoA-I mRNA levels were higher for BALB/c than C57BL/6 mice ($P < 0.006$).

ApoA-II mRNA levels (Fig. 4) were influenced by time and strain interactions. Based on 2-way ANOVA analyses, BALB/c showed significant increases in apoA-II mRNA levels with time of fasting ($P < 0.004$). This accounted for the higher levels of apoA-II mRNA levels seen for BALB/c versus C57BL/6 mice at 16 h ($P < 0.003$). Moderate diet effects were also observed. At 16 h, apoA-II mRNA levels were reduced for C57BL/6 mice fed the high fat diets (atherogenic and safflower oil diets) versus rodent chow, but statistical significance was obtained only for the saff/high diet ($P < 0.006$). ApoA-II mRNA levels for BALB/c mice fed safflower oil were lower than for mice fed rodent chow and atherogenic diets. In fact, for BALB/c mice, changes in apoA-II mRNA levels correlated to those of apoA-I ($r^2 = 0.845$; $P < 0.001$).

ApoA-IV mRNA levels (Fig. 4) were markedly responsive to fasting time ($P < 0.001$) with one exception (BALB/c mice fed the saff/low diet). ApoA-IV mRNA levels increased from 2- to 24-fold with fasting overnight as compared to 4 h. C57BL/6 mice showed no significant differences in apoA-IV mRNA levels as a function of diet at either fasting time. At 16 h, BALB/c mice fed the

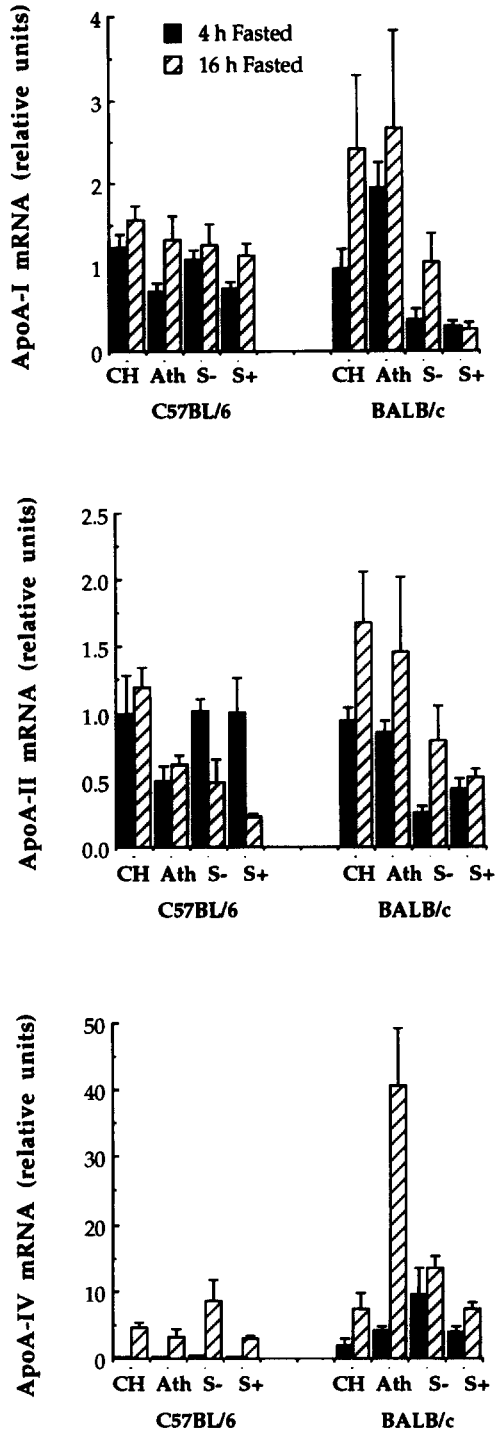


Fig. 4. Hepatic levels of mRNA specific for apolipoprotein A-I, A-II, and A-IV for C57BL/6 and BALB/c mice. Experimental conditions are as described in the legend to Fig. 1. Diets are rodent chow (CH), cocoa butter and cholesterol (Ath), and safflower oil without (S−) or with (S+) cholesterol. Total hepatic RNA was isolated and 20 μg of each preparation was subjected to Northern analysis using ^{32}P-labeled cDNA probes for the apolipoproteins and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described under Materials and Methods. Autoradiograms were examined by scanning densitometry to quantitate the relative levels of the apolipoprotein signals. Values shown are the mean densitometric units for apolipoprotein mRNA normalized to levels of GAPDH ± SEM for 3–5 mice. Significance levels are given in the text.

atherogenic diet displayed significantly elevated apoA-IV mRNA levels as compared to mice fed the remaining diets ($P < 0.001$–0.017).

Hepatic lipid concentrations

The primary determinant of hepatic cholesterol concentration was dietary cholesterol (**Fig. 5**). Thus, the atherogenic and saff/high diets resulted in over 5-fold increases in hepatic total cholesterol levels compared to rodent chow and saff/low diets, respectively, for both strains ($P < 0.001$). There was no effect of fasting time on hepatic cholesterol levels within strains, and strain effects were limited to the two diets containing added cholesterol. C57BL/6 exhibited significantly higher levels of hepatic cholesterol than BALB/c mice ($P < 0.001$).

Hepatic triglyceride levels were markedly sensitive to fasting time and were higher for 16 h versus 4 h fasted mice of both strains fed all diets ($P < 0.001$–0.011). Thus, elevations in both hepatic and plasma triglyceride levels were observed with overnight fasting as compared to short-term fasting. Several main effects were also seen. For instance, saff/high diets resulted in higher triglyceride levels as compared to saff/low diets for all cases ($P < 0.001$). When contrasting rodent chow-to atherogenic diet-fed mice, a significant decrease was seen for C57BL/6 mice fasted for 16 h ($P < 0.004$), a change not seen for BALB/c mice.

Fasting time did not show a main effect on hepatic phospholipid levels. Phospholipid values were relatively constant (12–18 mg/g tissue) for C57BL/6 and BALB/c mice fed all diets except the atherogenic diet. Strain type was a main effect but yielded significant differences between strains only for the atherogenic diet ($P < 0.001$).

VLDL production

To assess the contribution of triglyceride-rich lipoprotein production to the increased plasma triglyceride levels at 16 h of fasting as compared to 4 h, plasma triglyceride levels were determined with time after injection of mice with Triton WR-1339. Since plasma triglyceride levels increased with fasting time regardless of diet (Fig. 1), mice fed rodent chow were used.

C57BL/6 and BALB/c mice fasted for 4 and 16 h exhibited steady increases in plasma triglyceride levels over the entire 120 min (regression analysis values ranged from r^2 = 0.81–0.99). Plasma triglyceride values were significantly elevated above those of control saline-injected mice at each time point (data not shown). The values at each time point were used to estimate rates of triglyceride secretion as described in Materials and Methods. As shown in Table 2, triglyceride secretion rates for C57BL/6 mice were the same at 4 and 16 h and significantly lower than values calculated for BALB/c mice at each time point. Triglyceride secretion rates at 4 and

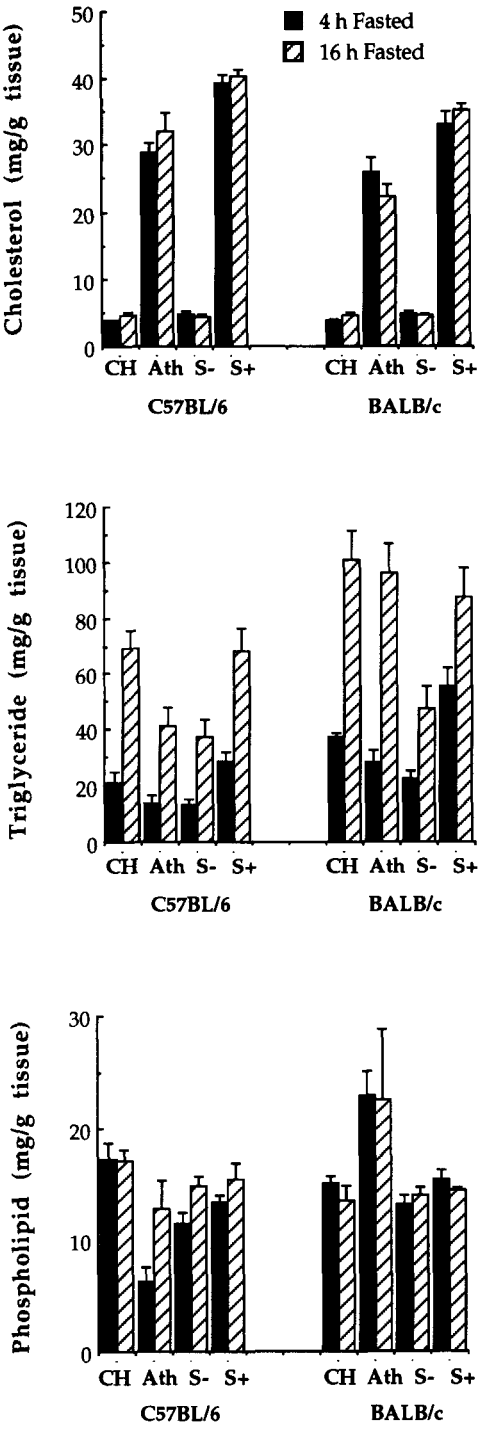


Fig. 5. Hepatic total cholesterol, triglyceride, and phospholipid concentrations in response to diet and fasting time for C57BL/6 and BALB/c mice. Experimental conditions are as described in the legend to Fig. 1. Diets are rodent chow (CH), cocoa butter and cholesterol (Ath), and safflower oil without (S−) or with (S+) cholesterol. Lipids were extracted according to Folch et al. (18) and lipid determinations were done using spectrophotometric analyses as described under Materials and Methods. Each bar represents the mean value ± SEM obtained for 4–5 mice. Significance levels are given in the text.

16 h observed for BALB/c were not significantly different. Thus, genetic factors appeared to be the main influence on rates of plasma triglyceride secretion in these mice.

DISCUSSION

This report demonstrates that aspects of lipoprotein transport are altered by nutritional status in mice. For nutritional studies, it is helpful to fast animals prior to collection of blood and/or tissues to avoid fluctuations due to postprandial events. Our results show that duration of fasting has a strong influence on many metabolic parameters in mice. The primary effect of fasting for 16 h as compared to 4 h was to decrease plasma glucose levels, elevate triglyceride levels in the plasma and liver, and increase the amount of hepatic apoA-IV mRNA. These changes were generalized phenomena as they occurred regardless of strain and diet. Other features of increased fasting were modest changes in levels of plasma total and HDL cholesterol, and apolipoproteins, and in liver apoA-I and apoA-II mRNA. Variations in these parameters were also seen due to diet and genetic background. Finally, changes in plasma insulin and FFA concentrations were differentially sensitive to fasting time, strain, and diet.

In this study, two categories of diets were used. The first category included two diets previously shown to differentiate atherosclerosis susceptibility among mouse strains (8). These diets consisted of either chow fed alone or mixed with cocoa butter, cholesterol and bile acid. The second category included two semi-synthetic diets developed to systematically identify and characterize genes susceptible to manipulation by dietary cholesterol and polyunsaturated fatty acids (17). These diets represent two categories used by us (8, 17) and others (3, 28) for studies of mouse lipid transport and differ markedly in amount and type of many components, including protein, fiber, bile acid, and fats. Yet, major changes in plasma lipids and expression of at least one apolipoprotein were observed regardless of diet, suggesting that extent of food restriction needs to be taken into consideration in describing mouse phenotypes.

A major finding was that fasting for 16 h resulted in elevated plasma triglyceride levels compared to fasting for 4 h. In humans (29 and refs. therein) and rats, overnight fasting leads to decreased plasma triglyceride levels. However, reports conflict as to the effect of fasting on plasma triglyceride concentrations in rodents. Triglycerides have been reported either to increase (10, 30, 31) or decrease (14, 32, 33) during fasting. The contradictory reports may be partially explained by the extent of fasting, as decreased plasma triglyceride levels were found in studies using severe starvation (> 24 h) conditions.

The Triton WR-1339 studies showed that VLDL production was not altered by fasting. Thus, the increased triglyceride levels in plasma at 16 h must be due to decreased VLDL clearance. The reduced clearance of VLDL at 16 h was likely due to decreased lipolysis by LPL (5). Lipolysis of triglyceride is a necessary step for efficient catabolism of VLDL (34) and chylomicrons (35). Consistent with this is the observation that, in rats, LPL activity changes rapidly in response to changes in nutritional status (36). Interestingly, a main determinant of triglyceride secretion rate was strain type with BALB/c mice exhibiting significantly greater triglyceride secretion rates than C57BL/6 mice.

Hepatic triglyceride levels were also elevated at 16 h versus 4 h of fasting. During starvation, de novo fatty acid synthesis is decreased (37, 38) and hepatic triglyceride is derived primarily from esterification of glycerol with preformed exogenous fatty acids (39). In fact, in vivo studies suggest that increased availability of circulating FFAs and glycerol stimulate triglyceride synthesis in the liver (39). This may have occurred in our mice, as elevations in plasma free fatty acids with increased fasting time were observed, at least for C57BL/6 mice, and fasted mice are known to exhibit high levels of free glycerol (30–40 mg/dl) (10, 40). Thus, compared to the 4 h-fasted mice, our 16 h-fasted mice were probably synthesizing more triglycerides from peripherally derived glycerol and FFAs, and more of these triglycerides were retained rather than secreted from the liver. Several reports suggest that VLDL secretion is dependent upon the availability of apoB (12, 39) as well as lipid components. Thus, one explanation for lack of VLDL production in our mice at 16 h was low apoB protein synthesis upon overnight fasting, which is currently being tested.

Another major effect of fasting was on hepatic apoA-IV mRNA levels, which were markedly elevated at 16 h compared to 4 h. It is tempting to speculate that the coincidental increases in hepatic apoA-IV mRNA and triglyceride levels suggest a role for apoA-IV in intracellular triglyceride storage, packaging into lipoproteins, and/or secretion. Such roles for apoA-IV in the intestine have been suggested based on elevations in both apoA-IV mRNA and protein levels in response to fat feeding (7, 41). Expression of apoA-IV in the liver, but not intestine, has repeatedly been shown to be sensitive to hormonal as well as dietary controls (42, 43). For instance, treatment of rats with thyroid hormone (42, 44), corticosteroids (43, 45), or dietary sucrose (46) elevates hepatic apoA-IV mRNA levels, while hypothyroidism induced by PTU (42, 44, 47), estrogen (42, 48, 49), and clofibrate (42) reduces hepatic apoA-IV mRNA. Comparing these and other studies of hepatic apoA-IV expression to measurements of hepatic triglyceride accumulation and secretion (**Table 3**) suggests that conditions that increase liver

TABLE 3. Relationships between hepatic apoA-IV mRNA, and hepatic triglyceride (TG) concentration and VLDL secretion as a function of fasting, hormone, and dietary treatment[a]

	ApoA-IV mRNA		TG Accumulation		VLDL Secretion	
Treatment	Effects	Ref.	Effects	Ref.	Effects	Ref.
Fasting < 48 h	↑	here, 15	↑	48	---, ↑, ↓	30, 31, 37
Hyperthyroid	↑	15, 42, 44, 47	---	44	↑	44, 50
Dietary sucrose	↑	46	↑	55	↑	55
Corticosteroids						
Dexamethazone	↑	43, 45, 56	↑	56–58	↑	45, 56, 58–60
Hydrocortizone	↑	42, 45	↑	61	↑	45
Insulin	↑	43	↑	57, 62	↓	30, 57, 59
Fasting > 48 h	↓	13, 14			↓	32, 33, 63
PTU	↓	42, 44, 47	↓	39, 44	↓	44, 50
Estrogens	↓	42, 49	↓	59	↑	60, 64
Fibrates	↓	42	↓	65	↓	66, 67

[a]Effects of treatments compared to control values is noted as an increase (↑), decrease (↓), or no change (---), as described within the listed references.

triglyceride accumulation also increase apoA-IV mRNA. However, accumulation but not secretion was seen for estrogen treatment and short-term fasting (< 48 h) (50), as also shown in this study, suggesting an uncoupling between triglyceride accumulation and secretion. Thus, we hypothesize that in the liver of rodent and perhaps avian species, apoA-IV is needed for storage of triglyceride and/or packaging into VLDL, but that increased expression of apoA-IV does not lead to enhanced secretion of VLDL.

Recently, Reue et al. (51) showed that both transcription and turnover contribute to steady-state hepatic apoA-IV mRNA concentration. Thus, elevations in apoA-IV mRNA levels with fasting time were likely due to a combination of these events. Reue et al. (51) also showed that the expression of apoA-IV in liver contributes to plasma concentrations of apoA-IV protein in mice. In contrast to hepatic mRNA levels, apoA-IV plasma protein levels were lower for mice fasted 16 h versus 4 h of fasting. Since plasma apoA-IV is derived from both hepatic and intestinal sources (43), the decrease in plasma apoA-IV levels for 16 h-fasted mice probably reflected a balance between catabolism of liver-derived plasma apoA-IV and diminished contributions from intestinal synthesis.

Although our relative mRNA values for apoA-I and A-II for 16 h-fasted C57BL/6 and BALB/c fed chow and atherogenic diets were comparable to those of Williams et al. (7), the apoA-IV mRNA values were quite different. For instance, Williams et al. (7) observed nearly a 4-fold increase in apoA-IV mRNA upon feeding C57BL/6 mice the atherogenic diet compared to mice fed rodent chow. Chow fed BALB/c mice exhibited nearly 3-fold higher apoA-IV mRNA levels as compared to C57BL/6 mice and 2-fold reduced levels upon feeding atherogenic diet. Our work showed a marked increase in hepatic apoA-IV mRNA levels at 16 h versus 4 h of fasting for both strains fed all diets. In contrast, apoA-I and A-II mRNA levels were less affected by fasting time. Our results imply that fasting time affects the regulation of apoA-IV in a generalized way (not dependent upon diet or genetic background). Discrepancies with mRNA levels reported by Williams et al. (7) were also described by Reue et al. (51) who attributed the differences to non-genetic factors. We suggest that such non-genetic factors influencing hepatic levels of apoA-IV mRNA are differences in the exact times of fasting and perhaps time of day (diurnal cycle) during which tissues were collected.

Cholesterol levels in plasma were higher for mice fed diets with added cholesterol compared to the companion diets without added cholesterol, as observed previously (17, 20). Plasma HDL cholesterol concentrations were also influenced by the diets, particularly for the C57BL/6 mice. Several reports have shown that C57BL/6 mice fed diets rich in cholesterol, saturated fats, and bile acids exhibit marked decreases in HDL cholesterol levels and that this effect is subdued in other strains such as BALB/c and C3H/He (2, 8). The decrease in C57BL/6 HDL levels is mediated by increased HDL catabolism controlled, at least in part, by the gene *Ath-1* (8). The dietary component responsible for this effect is unknown. Since C57BL/6 mice have shown the decrease in HDL cholesterol with saturated fat-(2, 8, 28, 52), polyunsaturated fat- (Fig. 1), and low fat- (52) based diets, the primary dietary component responsible for decreased HDL levels is likely to be cholesterol. In support of this idea is the observation that high levels of dietary fat fed to C57BL/6 mice in the absence of cholesterol result in the maintenance (Fig. 1) or elevation (52) of plasma HDL cholesterol levels in comparison to mice fed low-fat diets.

Decreases have been observed in HDL levels for C57BL/6 mice after cholesterol feeding that are accompanied by decreases in apolipoprotein components. In particular, apoA-I and apoA-II levels decrease by approximately 30% and 50%, respectively, compared to levels seen for mice fed control diets (2, 8). Our C57BL/6 mice

did not show the characteristic drop in plasma apoA-I levels upon consuming the atherogenic diet. Perhaps longer feeding times (> 2 wks) are required to cause a more extensive loss of plasma apoA-I. In contrast to apoA-I, apoA-II plasma levels decreased as much as 2-fold for both C57BL/6 and BALB/c mice fed any of the high-fat diets compared to levels for mice fed chow within each time point. At 16 h of fasting, the decreases were greatest for C57BL/6 mice fed the atherogenic and saff/high diets suggesting that both the high fat and cholesterol dietary components contributed to loss of apoA-II.

C57BL/6 and BALB/c mice varied in the response of insulin levels to fasting times. Insulin levels contribute to the regulation of at least two lipolytic enzymes, lipoprotein lipase and hormone-sensitive lipase (53). In adipose tissue, lipoprotein lipase and hormone sensitive lipase show reciprocal regulation by insulin. Thus, under conditions of starvation, lipolysis of plasma triglycerides decreases and lipolysis of triglycerides stored in adipose tissue accelerates. Consistent with these expectations, C57BL/6 mice exhibited diet-independent decreases in glucose and elevations in plasma concentrations of triglyceride and FFA. Surprisingly, concomitant decreases in insulin levels were not seen, suggesting that the role of insulin in lipase activities may be locally mediated or that lipase activities are altered by factors other than or in addition to insulin in this strain. In contrast, BALB/c mice exhibited decreases in both glucose and insulin levels. Although levels of plasma FFAs tended to be higher in BALB/c than C57BL/6 mice fed the same diets, overt changes in FFA levels due to fasting time were irregular and dependent upon diet in BALB/c mice. This indicates a different balance between tissue lipolysis and hepatic uptake of plasma FFA than seen for C57BL/6 mice. That genetic differences exist among strains with respect to insulin sensitivity has been demonstrated by Surwit et al. (54) who disassociated fasting glucose levels from insulin sensitivity among progeny of C57BL/6 and A/J mouse strains. Thus, increased fasting resulted in elevations in plasma triglyceride concentrations, but concomitant changes in plasma insulin and FFA concentrations were strain-dependent.

In summary, duration of fasting alters many metabolic parameters used to characterize the lipid transport system in mice. In addition, the magnitude of these alterations is influenced by dietary and genetic factors. Mice are particularly sensitive to food restriction-induced depletion of their lipid reserves because of their high overall metabolic rate even as compared to rats (11). Significant changes in patterns of fuel utilization occur within 3 h (10) after feeding that are reflected in changes in lipid metabolism. Thus, we suggest that 4 h as opposed to overnight fasting is preferable prior to studies of the lipid transport system in mice.

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