A Diet-Induced Hypercholesterolemic Murine Model to Study Atherogenesis Without Obesity and Metabolic Syndrome

Karsten Hartvigsen, Christoph J. Binder, Lotte F. Hansen, Apaïs Rafia, Joseph Juliano, Solvi Hörkkö, Daniel Steinberg, Wulf Palinski, Joseph L. Witztum, Andrew C. Li

Objective—Western-type high-fat/high-cholesterol diets used to induce atherogenesis in low-density lipoprotein (LDL) receptor-deficient mice also lead to obesity with concomitant metabolic complications, eg, hypertriglyceridemia, hyperinsulinemia, and insulin resistance. Our aim was to design a diet inducing atherosclerosis through moderate hypercholesterolemia without associated parameters of the metabolic syndrome.

Methods and Results—Male LDL receptor-deficient mice were fed regular chow (RC; 0.01% cholesterol/4.4% fat), cholesterolemia-enriched regular chow (HC; 1% cholesterol/4.4% fat), or Western diet (WD 0.06% cholesterol/21% milk fat) for 28 weeks. HC-feeding led to elevated plasma (≈20.7 mmol/L [800 mg/dL]) and LDL cholesterol and accelerated atherosclerosis. Plasma triglycerides were unaffected. Compared with RC-fed controls, HC-fed mice had normal body weight gain and normal fasting levels of glucose, free fatty acids, and insulin. In contrast, WD-fed mice were extremely hypercholesterolemic (>41.4 mmol/L), obese, hypertriglyceridemic, hyperinsulinemic, insulin resistant, and showed adverse health such as skin/fur abnormalities and hepatic steatosis. Although atherosclerotic surface areas in the entire aorta were similar in HC-fed and WD-fed mice, lesions in aortic origin cross sections were significantly larger in WD-fed mice. However, morphology was similar in lesions of equal size.

Conclusions—The HC diet induced moderate hypercholesterolemia and extensive atherosclerosis and should be useful to study specific aspects of atherogenesis in the absence of confounding effects of the metabolic syndrome. (Arterioscler Thromb Vasc Biol. 2007;27:878-885.)

Key Words: atherosclerosis ■ insulin resistance ■ lipoproteins ■ metabolic syndrome ■ mouse models

Atherosclerosis is a complex disorder that displays many of the characteristics of a chronic inflammatory process.1 To accelerate atherogenesis in mice, plasma cholesterol levels must be raised extensively.2 With extreme degrees of hypercholesterolemia, multiple pathways that can independently sustain lesion formation appear to be activated.3 In this setting, for instance induced by the administration of a so-called Western diet (WD), the extreme hypercholesterolemia and associated features of the metabolic syndrome such as obesity, hypertriglyceridemia, and hyperinsulinemia can overcome the modulating impact of immune functions that can be often demonstrated at more moderate levels of hypercholesterolemia.4,7 Thus, a diet that increases low-density lipoprotein (LDL) cholesterol levels modestly, without associated parameters of the metabolic syndrome, would be preferable for many studies of atherogenesis.

The LDL receptor-deficient (LDLR⁻/⁻) mouse on the C57BL/6 background fed a high-fat/high-cholesterol WD is a commonly used murine model of atherosclerosis.2 Young LDLR⁻/⁻ mice on regular chow (RC) have twice the plasma cholesterol levels of C57BL/6 mice, ie, ≈5.2 mmol/L (200 mg/dL),8 which slowly increases to ≈9.0 mmol/L (350 mg/dL) with age.9,10 The increased plasma cholesterol is found primarily in atherogenic LDL with only small amounts in very low–density lipoprotein (VLDL).9 This lipoprotein profile is more comparable to the human profile, for instance, than that of RC-fed apolipoprotein E-deficient (ApoE⁻/⁻) mice, which have elevated cholesterol-enriched VLDL-like particles as the major lipoprotein.11

Because RC-fed LDLR⁻/⁻ mice develop little atherosclerosis,2,11 various high-fat/high-cholesterol WDs have been used. However, they not only induce extreme hypercholesterolemia but also lead to marked weight gain with concomitant components of the metabolic syndrome, eg, hypertriglyceridemia caused by elevated VLDL levels, decreased high-density lipoprotein (HDL) levels, hyperinsulinemia, and insulin resistance.12-15 In addition, the WD generates pathologies independent of atherosclerosis, eg, changes in fur and skin/fur abnormalities and hepatic steatosis.
skin integrity that lead to ulceration and infection and decreases in body weight and plasma lipids, caused in part by hepatic steatosis and gastritis. The associated infections and metabolic disturbances may independently impact atherogenesis and complicate interpretation of atherosclerosis data. Thus, alternative diets that produce atherosclerosis without these confounding effects would be helpful.

For these reasons, we developed a simple cholesterol-enriched regular chow diet (high-cholesterol [HC] diet, 1% cholesterol plus 4.4% fat) that induces more moderate hypercholesterolemia (=20.7 mmol/L [800 mg/dL]), predominately increases LDL cholesterol, and leads to atherosclerosis without concomitant obesity and other health and metabolic consequences.

Methods

Animals and Diets

All mice were tenth-generation male LDLR−/− mice on the C57BL/6j background. Mice were weaned at 21 days of age and fed RC consisting of 0.01% cholesterol and 4.4% fat (TD8604; Harlan-Teklad, Madison, Wis) until initiation of the diet interventions. In 3 separate studies, groups of mice were matched for age, litter, body weight, and plasma cholesterol and triglycerides, and housed in cages equipped with rodent enrichments (igloo and gnawing bone; Bio-Serv, Frenchtown, NJ) in a facility with a 12-hour light cycle. Animals had ad libitum access to water and food. All animal experiments were performed according to NIH guidelines and were approved by the University of California, San Diego Animal Subjects Committee.

In addition to the RC diet, 2 experimental diets were used. One diet was a widely used WD consisting of 0.06% cholesterol and 21% milk fat (TD98383; Harlan-Teklad). The second diet consisted of regular chow that was only enriched with cholesterol and designated as HC diet. This HC diet was prepared as follows: 1.0% (wt/wt) chow (Sigma-Aldrich, St. Louis, Mo) was dissolved in diethyl ether and sprayed over RC pellets in a fume hood. The diet was stored at −20°C after the diethyl ether had evaporated. The HC diet was changed twice per week, whereas the milk fat-containing WD was changed 3 times per week because of the visual discoloration (white to yellow), suggestive of accelerated lipid peroxidation. The compositions of the diets are listed in Table 1 (see http://atvb.ahajournals.org).

Intervention Design

Study 1 included 2 age groups of LDLR−/− mice, designated “younger” (age 14.1±0.2 weeks; n=23) and “older” (age 44.1±0.2 weeks; n=36), and each was divided into 3 diet intervention groups and fed either RC, HC diet, or WD for 28 weeks. Mice were bled after 0 and 14 weeks, subjected to an oral glucose tolerance test (OGTT) after 19 to 22 weeks, and euthanized after 28 weeks of feeding for atherosclerosis analysis.

Study 2 included LDLR−/− mice (age 16.5±0.2 weeks; n=44) that were divided into 3 diet intervention groups and fed commercial pelleted custom-made diets (Harlan-Teklad) that had been enriched with 0.00% (TD8604; n=12), 0.15% (TD44049; n=8), 0.25% (TD99260; n=8), 0.50% (TD97234; n=8), and 1.00% (TD97131; n=8) cholesterol, using the TD8604 RC as the base. Plasma was obtained after 0 and 4 weeks of cholesterol feeding and analyzed for lipid levels. Study 3 included LDLR−/− mice (age 34.1±0.4 weeks; n=25) that were fed the HC diet. Mice were bled after 0, 4, and 15 weeks of HC feeding to monitor lipid levels and euthanized in groups of 4 to 7 mice after 8, 12, 18, 24, 28, and 40 weeks of HC feeding for atherosclerosis analysis.

Results

Impact of Diets on Body and Organ Weights and Hepatic Lipid Content

The consequences of HC feeding were compared with those of RC and WD feeding in both younger and older male LDLR−/− mice (starting age 14 and 44 weeks, respectively). Figure 1 shows the body weight gain over 28 weeks of RC, HC, and WD feeding of the younger and older LDLR−/− mice. Independently of age, HC-fed mice gained weight equal to RC-fed mice, whereas WD-fed mice gained 25% to 35% more body weight (P<0.01; Figure 1). All HC-fed and RC-fed mice appeared healthy and active throughout the 28-week intervention period (even though the “older” mice were 17 months old). Some WD-fed mice exhibited signs of adverse health, including a sudden decrease in plasma lipid levels and body weights (>10 grams over 7 days), and/or abnormal changes in fur and skin integrity (including ulcerations). In fact, 3 of the 12 older WD-fed mice had to be euthanized before 12 weeks of feeding and a fourth mouse after 18 weeks. All other mice were euthanized after 28 weeks of feeding and hearts, spleens, livers, and pancreases were weighed (supplemental Figure IA to ID). No statistical differences in organ weights were observed between the HC-fed and RC-fed mice, whereas WD-fed mice, independently of age, had 45% to 65% heavier livers (P<0.01). Livers from HC-fed and RC-fed mice appeared normal, in

Atherosclerosis, Hepatic, and Plasma Analyses

Atherosclerosis lesion areas were measured in cross-sections of the aortic origin and as surface area in the en face preparation of the entire aorta. Trichrome-stained cross-sections were used to assess lesion morphology. Plasma and blood collected after a 3-hour fast or an OGTT were analyzed for levels of cholesterol, triglycerides, glucose, insulin, and free fatty acids. Cholesterol and triglyceride distribution in plasma lipoproteins separated by fast protein liquid chromatography (FPLC) were determined. Hepatic lipid content was measured. All experimental data are expressed as mean±SEM. See http://atvb.ahajournals.org for full description of methods and statistics.
Lipoprotein Levels

In addition, the older WD-fed mice also had heavier yellow with patchy coloration indicative of hepatic steatosis. In contrast to the livers from WD-fed mice, which appeared yellow with patchy coloration indicative of hepatic steatosis,

Figure 2. HC-fed LDLRI/− mice have isolated elevation of LDL cholesterol. Fasting plasma cholesterol (A) and triglyceride (B) levels in younger and older RC-fed, HC-fed, and WD-fed LDLRI/− mice. Representative cholesterol (C) and triglyceride (D) profiles of fast protein liquid chromatography separated lipoproteins from intra-grouped plasma of 28-week RC-fed, HC-fed, and WD-fed younger mice. Statistically significant differences between age-matched HC-fed mice and RC-fed or WD-fed mice at the same time point are indicated with **P < 0.01 and ***P < 0.001. Data are given as mean ± SEM.

Livers were analyzed for cholesterol and triglyceride content. Compared with RC-fed and HC-fed mice, WD-fed mice were found to have highly significant and massive hepatic accumulation of both free and esterified cholesterol as well as triglycerides (supplemental Figure IIA, IIB). In fact, the levels of lipid accumulation are comparable to that seen in leptin-deficient C57BL/6 mice consistent with hepatic steatosis. In contrast, HC-fed mice had only mild, although significant, elevations of hepatic free and esterified cholesterol as well as triglycerides compared with RC-fed mice.

Impact of Diets on Plasma Lipid and Lipoprotein Levels

Figure 2 shows 3-hour fasting plasma lipid levels and lipoprotein distributions in younger and older LDLRI/− mice after RC, HC, and WD feeding. Independently of age, HC-fed mice had significantly elevated plasma cholesterol compared with RC-fed mice (≈20.7 versus ≈7.8 mmol/L [800 versus 300 mg/dL]; Figure 2A) without concomitant changes in plasma triglyceride levels (≈2.0 mmol/L [175 mg/dL]; Figure 2B). This is in marked contrast to the effect of the WD, which induced extreme hypercholesterolemia (>41.4 mmol/L [1600 mg/dL]) and hypertriglyceridemia (>6.8 mmol/L [600 mg/dL]). Cholesterol and triglyceride lipoprotein distribution in male mice (Figure 2C, 2D, respectively) by FPLC after 28 weeks of feeding showed that the HC diet induced an almost exclusive elevation in LDL cholesterol with minimal changes in HDL levels and only trace increases in VLDL levels. The same lipoprotein profile was also observed in HC-fed male LDLRI/− mice after 14, 40, and 80 weeks of feeding. Together, this shows a reproducible cholesterol distribution in LDL, HDL, and VLDL of ≈83%, 14%, and 3%, respectively. In contrast, WD-fed mice had markedly different lipid profiles, as both LDL and VLDL particles were greatly enriched in cholesterol and triglyceride (Figure 2C, 2D), suggesting different phenotypes and perhaps atherogenicity. The lipoprotein profiles were confirmed by agarose gel electrophoresis (supplemental Figure III). Female LDLRI/− mice fed the same HC-diet had plasma cholesterol and triglyceride levels of ≈18.1 mmol/L (700 mg/dL) and ≈0.85 mmol/L (75 mg/dL), respectively, and a lipoprotein cholesterol distribution similar to HC-fed male mice (data not shown).

To investigate if the increase in plasma cholesterol levels could be titrated according to the content of dietary cholesterol, we fed male LDLRI/− mice for 4 weeks with commercial custom-made TD8604 RC-based diets that had been enriched with 0.00% (RC), 0.15%, 0.25%, 0.50%, and 1.00% cholesterol (study 2). All cholesterol-enriched diet groups had significantly higher plasma cholesterol after 4 weeks of feeding (P < 0.001). Statistically significant differences between diet groups at the same time point are indicated with *P < 0.05 and ***P < 0.001. Data are expressed as mean ± SEM.

Figure 3. Titration of plasma cholesterol levels in male LDLRI/− mice fed 0% (RC), 0.15%, 0.25%, 0.50%, and 1.00% cholesterol-enriched commercial pelleted custom-made regular chow (study 2). All cholesterol-enriched diet groups had significantly higher plasma cholesterol after 4 weeks of feeding (P < 0.001). Statistically significant differences between diet groups at the same time point are indicated with *P < 0.05 and ***P < 0.001. Data are expressed as mean ± SEM.

After 4 weeks of feeding, all 5 diet groups had significantly different mean plasma cholesterol levels of 8.2, 11.7, 17.8, 20.7, and 25.9 mmol/L (317, 453, 687, 800, and 1003 mg/dL),
respectively). Body weights and plasma triglycerides were unaffected by the cholesterol feeding (data not shown).

Impact of Diets on Glucose and Insulin Metabolism
To assess the effect of these diets on nonlipid metabolic parameters, blood was collected at 0, 14, and 20 weeks of feeding after a 3-hour fast. HC-fed and RC-fed mice had nearly identical fasting levels of glucose (Figure 4A, 4B), as well as normal insulin-to-glucose ratios (E, F) as compared with RC-fed control mice (all data points; \( P > 0.05 \)). In contrast, WD-fed mice are relatively hyperinsulinemic and at least for the older mice have lower glucose levels. Statistically significant differences between age-matched RC-fed and HC-fed mice versus WD-fed mice at the same time point are indicated with \( **P < 0.01 \) and \( ***P < 0.001 \) and differences between RC-fed mice versus WD-fed mice are indicated with \( ^{P}P < 0.05 \) and \( ^{P}P < 0.01 \). Data are given as mean±SEM.

Figure 4. HC-fed LDLR/−/− mice have normal fasting levels of glucose (A, B) and insulin (C, D), as well as normal insulin-to-glucose ratios (E, F) as compared with RC-fed control mice (all data points; \( P > 0.05 \)). In contrast, WD-fed mice are relatively hyperinsulinemic and at least for the older mice have lower glucose levels. Statistically significant differences between age-matched RC-fed mice and HC-fed mice versus WD-fed mice at the same time point are indicated with \( **P < 0.01 \) and \( ***P < 0.001 \) and differences between age-matched RC-fed mice and WD-fed mice at the same time point are indicated with \( ^{P}P < 0.05 \) and \( ^{P}P < 0.01 \). Data are expressed as mean±SEM.

Mice were subjected to an OGTT after 19 to 22 weeks of feeding. Figure 5A to 5D illustrates that HC-fed and RC-fed mice had similar responses to the glucose challenge (all time points: \( P > 0.05 \)). The blood glucose profiles (Figure 5A, 5B) show that despite higher glucose levels at 7.5 and 15 minutes after glucose administration for the WD-fed mice, the glucose dynamics were similar among all groups. In contrast, in both age groups, the WD-fed mice had very abnormal and highly variable insulin responses to the administered glucose, ie, \( \approx 3 \)-fold insulin elevations (WD, 0.94 to 1.3 nmol/L [5.4 to 7.3 ng/mL] versus HC and RC, 0.23 to 0.40 nmol/L [1.3 to 2.3 ng/mL]), \( \approx 5 \)-minute delayed insulin peak (WD, 7.5 to 15 minutes versus HC and RC, 7.5 minutes), and \( \approx 30 \)-minute delayed return to fasting insulin levels in addition to fasting hyperinsulinemia (WD versus HC and RC, both \( P < 0.01 \)). Direct comparison of insulin levels between diets at the different time points revealed significantly higher insulin levels in the WD-fed mice (Figure 5C, 5D). Thus, in contrast to RC-fed and HC-fed mice, WD-fed mice were hyperinsulinemic, and the glucose and insulin responses after OGTT suggested a degree of insulin resistance.

Impact of Diets on Atherosclerosis
Figure 6 reports the extent of atherosclerosis in the entire aorta of WD-fed and HC-fed mice, as evaluated by the en face method and at the aortic origin by cross-sectional analysis. Independently of age and diet, no significant differences in en face aortic lesion areas were obtained (Figure 6A, 6C). There were no quantitative differences in the extent of lesion formation in arch, thoracic, and abdominal subsections.
of the aorta (data not shown). The en face method yields a 2-dimensional measurement and does not take into account lesion volume. To address this, we weighed the carefully cleaned aortas, as we have previously shown that aorta weight correlates well with extent of atherosclerosis, but again we found no differences (results not shown). In contrast, cross-sectional analysis of the aortic origin showed that WD-fed mice had 35% to 45% larger lesions than HC-fed mice (younger mice, $0.377\pm 0.048$ versus $0.547\pm 0.039 \text{ mm}^2/\text{section}$; $P=0.022$; Figure 6B; older mice, $0.423\pm 0.037$ versus $0.565\pm 0.041 \text{ mm}^2/\text{section}$; $P=0.021$; Figure 6D).

Lesion composition assessed in trichrome-stained cross-sections of the aortic origin showed that the majority of lesions in both groups of mice were quite advanced and contained areas of collagen-rich fibrous caps, necrotic cores, cholesterol clefts, and cellular enrichment adjacent to the lumen. To determine whether diets resulted in compositional differences, we compared similar-sized small, intermediate, and large cross-sectional lesions, as described in Methods. In general, no overall qualitative differences in cellular composition were noted, but the degree of core necrosis appeared to be increased in some lesions from the WD-fed mice. An example is shown in supplemental Figure V, which shows a series of cross-sections over a distance of 333 \( \mu \text{m} \) from one lesion under one valve leaflet from 2 representative HC-fed and WD-fed mice with equal lesion burden.

To delineate the temporal lesion progression of atherosclerosis in HC-fed LDLR mice, a separate study was designed (study 3). Twenty-five 34-week-old male LDLR mice were fed the HC diet and euthanized in 6 cohorts of 3 to 5 mice after 8, 12, 18, 24, 28, and 40 weeks of feeding. Again, these HC-fed mice appeared healthy despite their old age and the long diet intervention, ie, obesity and changes in fur and skin integrity were not observed. Steady plasma lipid levels were achieved within 4 weeks of initiation of diet (supplemental Figure VIA) and lipoprotein distributions (supplemental Figure VIB) were identical to those shown for HC-fed mice in Figure 2C and 2D. The en face analysis of the aorta demonstrated progressive lesion formation throughout the entire aortic tree (Figure 6E).

**Discussion**

The present work demonstrates that male LDLR mice fed 1% cholesterol-enriched regular chow, without other added fat or cholate, had predominant elevation of LDL cholesterol and extensive atherosclerosis, without concomitant alterations characteristic of the metabolic syndrome, typically seen with use of WDs, eg, increases in weight gain, VLDL triglycerides, VLDL cholesterol, glucose, insulin, and alterations in glucose/insulin dynamics. Moreover, HC-fed mice appeared as healthy as RC-fed mice and did not display features typically seen with WD-fed mice such as fatty infiltration of livers and/or changes in skin and fur integrity that often lead to ulceration. Thus, the HC diet minimized conditions that might independently impact atherogenesis. Also, compared with WD-fed mice, the degree of plasma cholesterol elevation with HC feeding was less. Because severe hypercholesterolemia may override effects that may otherwise impact atherogenesis, the development of atherosclerosis at more moderate levels is a distinct advantage for many studies, for example, those of the impact of immune deficiency. Thus, we propose that the simple HC diet described here is advantageous for many atherosclerosis studies in mice.

The metabolic syndrome is defined as a cluster of factors that leads to a substantial increase in cardiovascular disease. Some of the known components are: (1) obesity; (2) dyslip-
idemia, ie, elevated VLDL with associated hypertriglyceridemia and low HDL; (3) hyperinsulinemia, insulin resistance, and glucose intolerance; (4) hypertension; (5) proinflammatory state, eg, high C-reactive protein in humans or corresponding SAA in mice; and (6) prothrombotic state.15 Our work has demonstrated that atherosclerotic male LDLR−/− mice fed the high-cholesterol diet do not have the first 3 components, which are central features of the metabolic syndrome. Recently, Teupser et al,20 feeding male LDLR−/− mice a semi-synthetic low-fat HC diet, demonstrated that dietary cholesterol did not induce hypertension in this setting. Moreover, Chait et al21 recently reported >5-fold elevated plasma SAA levels in WD-fed male LDLR−/− mice compared with RC-fed controls. In collaboration with Chait et al, we determined SAA levels in the mice from study 1 and confirmed the strong SAA-elevating effect of WD, which could not be demonstrated with HC feeding (unpublished data, 2005). Thus, male LDLR−/− mice fed low-fat, cholesterol-enriched chow do not have confounding effects of the metabolic syndrome.

The concept of a low-fat, cholesterol-enriched RC is not novel.2 However, this is the first report to our knowledge that directly compares such a diet with RC and a WD. Teupser et al,20 feeding male LDLR−/− mice low-fat, cholesterol-enriched RC diets, using the American Institute of Nutrition’s cholesterol-free, casein-containing, semi-synthetic AIN-76A diet (Table I), LDLR−/− mice fed 0% or 0.02% cholesterol-enriched diets had plasma cholesterol levels of ≈12.9 mmol/L (500 mg/dL), whereas mice fed 0.15%, 0.3%, or 0.5% cholesterol-enriched AIN-76A had cholesterol levels of ≈36.2 mmol/L (1400 mg/dL).20 On the AIN-76A diet, only mice with cholesterol levels of 36.2 mmol/L developed atherosclerosis in the aorta, and in addition these mice developed a complex lipoprotein profile with high levels of unusual triglyceride-poor, cholesterol-rich VLDL.20 In contrast, in our studies with regular murine chow enriched with 1% cholesterol only, the LDLR−/− mice had plasma cholesterol levels of ≈20.7 mmol/L (800 mg/dL) and developed reproducible aortic lesions. In fact, feeding male LDLR−/− mice of different ages the HC diet for a period of 28 weeks revealed a high degree of reproducibility of total aortic en face lesions: 20.2±2.0% (beginning age 14 weeks, n=7; Figure 6A), 17.1±1.4% (beginning age 28 weeks, n=19; unpublished data from an unrelated study), 17.7±2.5% (beginning age 34 weeks, n=4; Figure 6E), and 21.3±2.2% (beginning age 44 weeks, n=12; Figure 6C).

To further investigate the uses of the cholesterol-enriched TD8604 RC, we attempted to titrate the plasma cholesterol levels in male LDLR−/− mice using commercial custom-made cholesterol-enriched diets with 0.15%, 0.25%, 0.5%, and 1% added cholesterol (study 2). We found that these diets significantly increased plasma cholesterol 1.8-, 2.5-, 2.9-, and 3.8-fold, respectively (Figure 3), which demonstrated the feasibility of manipulating plasma cholesterol levels between 11.7 to 25.9 mmol/L (450 to 1000 mg/dL) using cholesterol-enriched TD8604 RC as base. Interestingly, such dose-response curve of dietary cholesterol using the AIN-76A as base was not observed by Teupser et al, who found 2 different cholesterol levels in response to various dietary cholesterol amounts, ie, ≈12.9 mmol/L at <0.02% dietary cholesterol and ≈36.2 mmol/L at >0.15% dietary cholesterol.20 Moreover, our study 2 revealed that the cholesterol-sprayed HC diet and the commercial custom-made 0.5% cholesterol-enriched diet, TD97234, resulted in equal plasma cholesterol levels. The explanation for the dose-dependent response using the TD8604 RC as base, but not the semi-synthetic AIN-76A, is most likely caused by the content of casein and fructose in AIN-76A (Table I). Compared with plant-derived proteins, casein has been reported to elevate levels of plasma and liver cholesterol and triglycerides, as well as atherosclerosis in rodents.22 Likewise, sucrose and fructose induce hypertriglyceridemia and are more atherogenic compared with starch.22 Thus, using the TD8604 RC as base for cholesterol-enriched low-fat diets may have advantages over the AIN-76A diet with respect to titration of plasma cholesterol, lipoprotein distribution, and liver lipid levels.

WD-fed male LDLR−/− mice on the C57BL/6J background are known to be susceptible to obesity,22–24 However, we found that enriching RC with cholesterol (ie, the HC diet) did not induce the marked weight gain seen with WD feeding (Figure 1). It is interesting to note that the intervention changed the weight trajectory of the mice. After 28 weeks of intervention, the younger RC-fed and HC-fed mice were 42 weeks old and weighed ≈33.5 grams, which was less than expected, because the older RC-fed and HC-fed mice weighed ≈37.5 grams at 44 weeks of age at the beginning of the diet intervention. We have consistently made this observation and speculate that procedures such as blood drawing and OGTT are the cause. This emphasizes the importance of internally controlled mouse interventions.

This report confirms that WD-fed male LDLR−/− mice developed obesity, hyperinsulinemia, and some degree of insulin resistance, as we and others have reported.12–14 In contrast, female LDLR−/− mice respond differently to WD feeding; therefore, we focused the present study on male mice. Although insulin resistance and diabetes are known to accelerate atherosclerosis in humans, it has been difficult to show this in murine models.23 Previously, we have shown that the WD-induced metabolic complications in the male LDLR−/− mice may be dramatically improved by PPARγ ligands, and that these agents decrease the extent of atherosclerosis in these male mice.14 However, it is not clear whether the anti-atherogenic impact of these agents is caused by improvement of the insulin resistance. In fact, it appears more likely that their anti-atherogenic impact is more related to a decrease in the inflammatory properties of macrophages and/or to advantageous alterations in intracellular cholesterol metabolism.14,24,25 Thus, it is of considerable interest that in our current studies, despite the metabolic complications and the 2- to 3-fold higher cholesterol exposure in the WD-fed mice compared with HC-fed mice, the extent of aortic atherosclerosis was not significantly different (Figure 6A, 6C). In contrast, quantification of the extent of atherosclerosis at the aortic origin revealed that the WD-fed mice had 35% to 45% larger lesion burden compared with HC-fed mice (Figure 6B, 6D). Nevertheless, qualitative comparison of lesion composition in lesions of same size in the 2 diet groups did not reveal any gross differences (supplemental Figure V). It
should be noted that the relatively long diet interventions (chosen chiefly with regards to the metabolic parameters) used in this study have resulted in very large and advanced lesions, and it is conceivable that shorter interventions would have yielded a better differentiation of the atherogenic properties of the tested diets.

Because WD feeding caused significantly greater hypercholesterolemia, the larger lesion burden in the aortic origin of WD-fed mice was expected. In contrast, the finding of equal aortic en face lesion burden with HC and WD feeding was unexpected. Thus, there appears to be site-specific responses in the arterial tree to the WD and HC diets. Such site-specificity in murine atherosclerosis models in response to different diets, gender, or immunologic interventions is now well-described.26 In this regard, it is worth emphasizing that the lipoprotein cholesterol and triglyceride profiles from the HC-fed LDLR −/− mice are different from those of the WD-fed mice. Much of the elevated cholesterol found in the WD-fed mice is associated with larger VLDL-like particles (Figure 2C-D), and at least as compared with LDL particles, these are relatively less atherogenic.27,28 Duff et al29 originally commented on the failure of diabetes, which caused a marked enrichment of such large VLDL, to accelerate atherosclerosis in rabbits presumably for the same reasons. Also, the cholesterol and triglyceride profiles (Figure 2C, 2D) reveal that LDL particles from WD-fed mice, compared with HC-fed mice, have higher triglyceride content, suggesting LDL particles with different phenotype and potentially atherogenicity.29 Of course, the failure to detect differences in aortic surface atherosclerosis using the en face method may be caused by the fact that it is a 2-dimensional measure and does not take into account the thickness and depth of the lesions, which potentially becomes increasingly problematic with the severity of lesion progression.

The HC diet provided an intermediate level of plasma cholesterol of ∼20.7 mmol/L (800 mg/dL), which distributed between VLDL, LDL, and HDL as ∼0.65 (3%), ∼17.1 (83%), and ∼2.8 (14%) mmol/L, respectively (Figure 2C). This cholesterol distribution is comparable to those obtained in 2 "apoB-100 only" mouse models of atherosclerosis; ie, LDLR −/− ApoB100/100 and LDLR −/− Apobec1 −/− mice.9,28,30 RC-fed LDLR −/− ApoB0/100 and LDLR −/− ApoB1/1 mice have plasma cholesterol levels of ∼7.8 mmol/L (300 mg/dL) and ∼14.2 mmol/L (550 mg/dL), respectively, and both develop aortic atherosclerotic lesions covering ∼14% of the surface of the entire aorta at 34 to 40 weeks.9,11,28 In our study, we found that LDLR −/− mice after 28 weeks of HC feeding develop total aortic en face lesions of ∼20%, which is consistent with the higher plasma cholesterol level (∼20.7 mmol/L [800 mg/dL]). The cholesterol distribution within the lipoproteins of these 3 murine models of atherosclerosis is much more comparable to the usual human profile than those of the commonly used LDLR −/− mice on high-fat/high-cholesterol diets or ApoE −/− mice, as well as LDLR −/− mice fed the cholesterol-enriched casein-containing AIN-76A diet.

Thus, the HC diet described here and the WD and the cholesterol-enriched AIN-76A diets described by others produce different biological consequences. Overall, at the level of the aorta, the HC diet appears to be as atherogenic as the WD, despite marked differences in total cholesterol content. In contrast, at the aortic origin, the WD was more atherogenic. These data emphasize the complexity of lesion formation at different sites in the aortic tree.26

In summary, the present work describes an alternative and simple murine model of diet-induced atherogenesis, in which LDLR −/− mice are fed casein-free RC enriched with cholesterol without other added fat or cholate. We propose that this simple diet may be advantageous for many atherosclerosis studies in mice.

Addition Note

During the review process of our manuscript, Goldberg, Dansky, and colleagues published data relevant to this report (Wu et al, J Lipid Res 2006;47:2215–2222). In 2 independent diet interventions using male LDLR −/− mice, the authors tested the effects of low-fat and high-fat diets in the setting of matched levels of moderate hypercholesterolemia. Their diets were based on the casein-containing AIN-76A diet, similar to that used by Teupser et al.30 Compared with the mice fed the low-fat diet, the mice fed the high-fat diet became obese, hypertriglyceridemic, hyperglycemic, hyperinsulinemic, and insulin resistant, and thus showed a type 2 diabetic phenotype. Despite this, no differences in atherosclerotic lesion burden and composition could be demonstrated after 20 weeks of feeding. The authors did observe, however, that in one group of mice fed diets for 40 weeks, the mice fed the high-fat diet had larger lesions at the aortic origin but not in the entire aorta (en face method) and brachiocephalic artery, and also had a more atherogenic lipoprotein profile. Although, the authors did not compare their test diets to a baseline RC that does not induce hypercholesterolemia, their report supports our observations that low-fat atherogenic diets do not induce parameters of the metabolic syndrome and that the type 2 diabetic/insulin resistance phenotype has limited atherogenic potential in the male LDLR −/− mouse model.

Acknowledgments

We thank Florencia Casanada, Richard Elam, Jennifer Pattison, and Mercedes Silvestre for excellent technical assistance.

Sources of Funding

NIH-HL56989 (SCOR in Atherosclerosis and Molecular Medicine). K.H. was supported by a fellowship from the AHA (Western States Affiliates), a faculty start-up grant from The Sam and Rose Stein Institute for Research on Aging, and grants from the Novo-Nordisk/Danish-American Foundation, the Reinholdt W. Jorck & Hustrus Foundation, the Otto Mønsteds Foundation, the Arvid Nilssons Foundation, and the Villum Kann Rasmussen Foundation. C.J.B. was supported by a fellowship from the AHA (Western States Affiliates). A.C.L. was supported by a beginning grant-in-aid from the AHA (Western States Affiliates).

Disclosure

None.

References


A Diet-Induced Hypercholesterolemic Murine Model to Study Atherogenesis Without Obesity and Metabolic Syndrome
Karsten Hartvigsen, Christoph J. Binder, Lotte F. Hansen, Apaïs Rafia, Joseph Juliano, Sohvi Hörkkö, Daniel Steinberg, Wulf Palinski, Joseph L. Witztum and Andrew C. Li

Arterioscler Thromb Vasc Biol. 2007;27:878-885; originally published online January 25, 2007; doi: 10.1161/01.ATV.0000258790.35810.02
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/27/4/878

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/01/25/01.ATV.0000258790.35810.02.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplementary Methods

Supplemental Methods

Atherosclerosis Analysis

The extent of atherosclerosis was quantified by computer-assisted image morphometry in Sudan IV-stained en face preparations of the entire aorta and in cross sections through the aortic origin of paraffin-embedded hearts, as previously described.\(^1\) A trichrome stain, consisting of hematoxylin, fuchsin red/picric acid, and aniline blue, was utilized for cross sectional analysis of plaque burden and composition.\(^1,2\)

Diet-induced differences in lesion composition from the HC- and WD-fed mice were compared in aortic origin lesions of equal size. For this purpose, 10 small atherosclerotic lesions (0.054\(\pm\)0.004 and 0.055\(\pm\)0.004 mm\(^2\)), 10 intermediate lesions (0.152\(\pm\)0.003 and 0.153\(\pm\)0.002 mm\(^2\)), and 10 larger lesions (0.250\(\pm\)0.006 and 0.250\(\pm\)0.003 mm\(^2\)) were selected from both the HC and WD groups, respectively, based exclusively on size, because intimal/medial ratios are not appropriate for sections of the sinus of Valsalva.

Blood and Plasma Analyses

Following a 3-hour fast, mice were anesthetized with Isoflurane and retro-orbital blood was obtained via EDTA-coated microcapillary tubes (at 11 a.m.). Glucose was immediately measured in 1 \(\mu\)L blood using a FreeStyle glucometer (TheraSense, Alameda, CA, USA) prior to rapid isolation of plasma. Insulin levels were measured in 2 \(\mu\)L plasma using the Mercodia Ultrasensitive Mouse Insulin ELISA kit (Alpco Diagnostics, Windham, NH, USA). Unbound
free fatty acids (FFA) was determined in 1 μL plasma using the fluorescent probe ADIFAB2 (FFA Sciences LLC, San Diego, CA, USA).\(^3\) Plasma cholesterol and triglycerides levels were determined using automated enzymatic assays (Roche Diagnostics, Indianapolis, IN, USA and Equal Diagnostics, Exton, PA, USA). Fresh plasma samples were pooled according to intervention groups and lipoproteins were fractionized by size using fast protein liquid chromatography (FPLC) equipped with a Superose 6 column, and cholesterol and triglyceride levels were determined in each fraction (250 μL).\(^4\) Whole plasma was also studied by 1.0% agarose gel electrophoresis according to a standard protocol.

**Oral Glucose Tolerance Test (OGTT)**

Four weeks prior to the OGTT, mice from Study-1 were individually handled daily to accustom them to the procedure. After 19-22 weeks of feeding, mice were fasted for 3 hours and then gavaged with glucose (1 mg/g body weight) using a 10% glucose solution. Retro-orbital blood samples were taken with EDTA-coated microcapillary tubes after anesthesia with Isoflurane at 0, 7.5, 15, 30, 60, and 90 min after glucose administration. Glucose and insulin levels were measured as described above.

**Hepatic Lipid Analyses**

Livers from younger mice of Study-1 were collected immediately after perfusion with ice-cold 2 μmol/L EDTA-containing phosphate-buffered saline (PBS) at pH 7.4 and snap frozen in liquid nitrogen. Liver cholesterol was quantified as previously described.\(^5,6\) Briefly, 100 μg of 5α-cholestane (Steraloids, Newport, RI, USA) was added as an internal standard to 44±2 mg (n=21) liver tissue in duplicates and homogenized in 1.5 mL cold ddH₂O. Lipids were extracted.
using 2.5 mL cold methanol and 5 mL cold dichloromethane with vigorous vortexing after each addition. The lower organic phase was recovered and split into two equal aliquots and dried down with argon gas. Total cholesterol was measured in one aliquot after saponification by adding 2 mL of 1 M NaOH in 95% ethanol. Free cholesterol was measured in the other aliquot by adding 2 mL of 95% ethanol. Both tubes were incubated for 1 hr at 85°C followed by addition of 500 μL ddH₂O, 200 μL 5M NaCl, and 2 mL n-hexane. After vortexing, the upper organic phase was recovered, dried under argon gas, dissolved in 25 μL toluene, and injected (1 μL) onto a packed silica column in a Varian gas-liquid chromatograph. Data were normalized using the internal standard after daily calibration with a panel of external standards containing both 5α-cholestane and cholesterol (Sigma-Aldrich) in serial dilutions. Data are expressed as mg cholesterol/g wet liver weight. Cholesterol ester content was calculated as the difference between total and free cholesterol.

Liver triglyceride content was quantified in the same liver extracts as previously described. In brief, the lipid extract used for free cholesterol determination was dried under argon gas following addition of 6 mg Triton X-100 (Sigma-Aldrich) in dichloromethane. Lipids were resuspended in 100 μL 0.9% NaCl by sonication and the triglyceride content was determined as described in the Methods for plasma triglycerides. Data are expressed as mg triglycerides/g wet liver weight.

**Statistical Analyses**

All experimental data are expressed as mean±SEM. Differences in mean values were analyzed with the parametric one-way ANOVA with the Tukey-Kramer multiple comparison post-test unless otherwise noted (InStat 3.06; GraphPad Software). *p*<0.05 was considered
significant. Cholesterol distribution in VLDL, LDL, and HDL was estimated as the area under
the curve, following Gaussian multi-peak analysis of cholesterol content in each FPLC fraction
(Origin Pro 7; OriginLab Corporation).

REFERENCES

1. Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models:
correlation between lesions in the aortic origin and in the entire aorta, and differences in the
extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient

2. Calara F, Silvestre M, Casanada F, Yuan N, Napoli C, Palinski W. Spontaneous plaque
rupture and secondary thrombosis in apolipoprotein E-deficient and LDL receptor-deficient

3. Richieri GV, Ogata RT, Kleinfeld AM. The measurement of free fatty acid concentration
with the fluorescent probe ADIFAB: a practical guide for the use of the ADIFAB probe.

proliferator-activated receptor γ ligands inhibit development of atherosclerosis in LDL


Table S-1

Table S-1: Composition of diets used in this report; i.e. the standard RC diet, the cholesterol-enriched HC diet, and the semi-purified WD diet. The semi-purified AIN-76A diet used by Teupser et al.\(^6\) was included for comparison.

<table>
<thead>
<tr>
<th>Diet</th>
<th>RC</th>
<th>HC</th>
<th>WD</th>
<th>AIN-76A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TD8604(^a)</td>
<td>TD8604 + cholesterol</td>
<td>TD98338(^b)</td>
<td>D10001(^c)</td>
</tr>
<tr>
<td>Protein (%wt)</td>
<td>24.5</td>
<td>24.5</td>
<td>17.3</td>
<td>20.3</td>
</tr>
<tr>
<td>Carbohydrate (%wt)</td>
<td>46.6(^d)</td>
<td>46.6(^d)</td>
<td>49.0</td>
<td>66.0</td>
</tr>
<tr>
<td>Fat (%wt)</td>
<td>4.4</td>
<td>4.4</td>
<td>21.2</td>
<td>5.0</td>
</tr>
<tr>
<td>Fiber (%wt)</td>
<td>36.9</td>
<td>36.9</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Specific ingredients:

<table>
<thead>
<tr>
<th>Component</th>
<th>RC</th>
<th>HC</th>
<th>WD</th>
<th>AIN-76A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (g/kg)</td>
<td>-</td>
<td>-</td>
<td>195.0</td>
<td>200.0</td>
</tr>
<tr>
<td>DL-Methionine (g/kg)</td>
<td>-</td>
<td>-</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Sucrose (g/kg)</td>
<td>-</td>
<td>-</td>
<td>341.5</td>
<td>150.0</td>
</tr>
<tr>
<td>Corn starch (g/kg)</td>
<td>-</td>
<td>-</td>
<td>151.4</td>
<td>500.0</td>
</tr>
<tr>
<td>Milk fat (g/kg)</td>
<td>-</td>
<td>-</td>
<td>210.0</td>
<td>-</td>
</tr>
<tr>
<td>Corn oil (g/kg)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>50.0</td>
</tr>
<tr>
<td>Cellulose (g/kg)</td>
<td>-</td>
<td>-</td>
<td>50.0</td>
<td>50.0</td>
</tr>
</tbody>
</table>

| Added cholesterol (g/kg) | - | 10 | 0.1 | - |
| Total cholesterol (g/kg) | 0.1 | 5-10 | 0.6 | 0.0 |
| (%wt)                     | (0.01%) | (~0.5-1.0%) | (0.06%) | (0.0%) |
| Overall (kcal/g)          | 3.1 | 3.1 | 4.5 | 3.9 |

\(^a\) The TD8604 regular chow is a complete and balanced fixed formula diet designed to provide optimum nutrition to research mice. The ingredients include: vitamin and mineral mixtures, soybean meal, wheat, corn, cane molasses, brewers dried yeast, fish meal, and soybean oil (which provides 1.9%wt of the essential fatty acid, linoleic acid); \(^b\) The semi-purified TD98338 diet is a modification of the more widely used TD88137 diet in which the only change is a reduction of added cholesterol from 1.5 g/kg to 0.1 g/kg; \(^c\) Research Diets, Inc., New Brunswick, NJ, USA; \(^d\) Estimated as the nitrogen-free extract; \(^e\) Milk fat (21%) contributes 0.5 g cholesterol/kg diet.

Suppl. page 6
Figure S-1

Figure S-1: HC-fed male LDLR\(^{-/-}\) mice have equal heart, spleen, liver, and pancreas weights (panel A, B, C, and D, respectively) compared to control RC-fed mice after 28 weeks of feeding. WD-fed older mice had significantly heavier spleens than HC- and RC-fed mice. Both older and younger WD-fed mice have significantly heavier livers than HC- and RC-fed mice. No statistical differences were detected in organ weights between the HC- and RC-fed mice, although the livers were slightly heavier in the older HC-fed group (p=0.053). Statistically significant differences between age-matched RC-, HC-, and WD-fed mice are indicated in the panels; *p<0.05, **p<0.01, and ***p<0.001. Data are expressed as mean±SEM.
Figure S-2

Figure S-2: Young HC-fed male LDLR\(^{-/-}\) mice have mildly elevated liver content of free and esterified cholesterol as well as triglycerides (panel A and B, respectively) compared to control RC-fed mice after 28 weeks of feeding. In sharp contrast, livers of WD-fed mice accumulate massive amounts of lipids. Statistically significant differences between RC-fed mice and the two other diet groups (HC- and WD-fed mice) are indicated in the panels; \(*p<0.05\) and \(***p<0.001\), whereas differences between HC-fed mice and WD-fed mice are indicated in the panels; \(\$\$p<0.01\) and \(\$\$\$p<0.001\). Data are expressed as mean±SEM. The liver triglyceride content data in panel B was analyzed using the nonparametric ANOVA Kruskal-Wallis Test in combination with Dunn’s Multiple Comparison Test.
Figure S-3

Figure S-3: HC-fed male LDLR\(^{-/-}\) mice have predominant elevation of LDL cholesterol compared to RC-fed mice. Agarose gel electrophoresis of intra-group pooled 3-hour fasted whole plasma from LDLR\(^{-/-}\) mice fed indicated diets for 14 weeks. Strong lipid staining of pre-\(\beta\) particles from WD-fed mice, but only weak staining from HC- and RC-fed mice, indicated high VLDL (and IDL) levels in the WD-fed mice. The lipid staining intensity of \(\beta\) particles, representing LDL (and IDL), increased from RC- to HC- to WD-fed mice. Moreover, intensity of lipid staining of \(\alpha1\) and \(\alpha2\) particles indicates equal levels of HDL fractions between the different groups of mice.
Figure S-4

Figure S-4: Independently on RC-, HC, and WD-feeding, male LDLR−/− mice had similar fasting levels of unbound FFA (all data points; p>0.05). Data are given as mean±SEM.
Figure S-5

Figure S-5: In general, no overall qualitative differences in lesion morphology in atherosclerotic lesions of equal size were noted, but the degree of core necrosis and cholesterol cleft formation appeared to be slightly increased in some lesions from the WD-fed mice. Lesion composition was assessed by trichrome staining and showed that the majority of lesions in both groups of mice were advanced, containing areas of collagen-rich fibrous caps, necrotic cores, cholesterol clefts, and cellular enrichment adjacent to the lumen. Serial 9-μm cross sections over a distance of 333 μm from one lesion under one valve leaflet are shown from a representative HC-fed (left column) and WD-fed (right column) mouse. The area of each lesion is given in mm². Note the extent of atherosclerosis in the coronary artery with both diets (in the lower right corner of later sections). The trichrome stain was used to visualize the collagen-rich extracellular matrix (blue stain), cell cytoplasm and intercellular fibers (pink stain), and cell nuclei (black stain). Original magnification ×100.
Figure S-5
Figure S-6

Figure S-6: The HC diet elevates LDL cholesterol in male LDLR⁻/⁻ mice (Study-2 in the manuscript). Panel A; Time course of total plasma cholesterol and triglyceride levels (n=25, 25, 5, 4, 16, 4, 4, 4, 4, 3 per time point). Panel B; Representative cholesterol and triglyceride profiles of plasma lipoproteins by FPLC after 40 weeks of feeding. Similar profiles were obtained at 14, 28, and 80 weeks of HC-feeding. Data are expressed as mean±SEM.