Caloric Restriction in Rhesus Monkeys Reduces Low Density Lipoprotein Interaction with Arterial Proteoglycans

Iris J. Edwards, 1 Lawrence L. Rudel, 1 James G. Terry, 2 Joseph W. Kemnitz, 3,4 Richard Weindruch, 3,4,5 and William T. Cefalu 2

Departments of 1 Pathology and 1 Internal Medicine of Wake Forest University School of Medicine, Winston-Salem, North Carolina.
3 Department of Medicine, University of Wisconsin–Madison, Madison.
4 Wisconsin Regional Primate Center, Madison.
5 GRECC, William S. Middleton VA Medical Center, Madison, Wisconsin.

Caloric restriction (CR) has been shown to retard aging processes in many species. We investigated effects of CR on plasma low density lipoproteins (LDL), a major risk factor for the age-associated process of atherosclerosis. Studies emphasized effects of CR on LDL composition and their interaction with arterial proteoglycans (PG). Rhesus monkeys were fed a control diet (n = 13) or subjected to CR (n = 12 fed 30% less calories) for >5 years. Plasma LDL cholesterol concentrations were similar for control and CR groups (82 ± 8 vs 72 ± 6 mg/dL, mean ± SEM). LDL was isolated by ultracentrifugation and HPLC. LDL particles from CR animals had a lower molecular weight (2.9 ± 0.1 vs 3.2 ± 0.1 g/mol, p < .05) and were depleted in triglyceride (249 ± 16 vs 433 ± 49 mol/particle, p < .005) and phospholipid (686 ± 20 vs 837 ± 33 mol/particle, p < .007). Lower PG binding was measured for LDL from CR animals (10.1 ± 0.8 vs 15.6 ± 1.1 fig LDL cholesterol/ftg PG, p < .005). This was associated with the lower triglycerides (r = .76, p < .0005) and phospholipids (r = .48, p < .01). Thus, a dietary intervention which may retard aging inhibits a proposed mechanism of atherogenesis.

CALORIC restriction (CR) has been shown to counteract the biological effects of aging in species ranging from protozoans to rodents (1,2). Three interrelated outcomes have now been reported in studies of mice and rats: increased lifespan, retardation of age-associated physiologic decline, delay or prevention of age-related diseases. Trials are now in progress in nonhuman primates to determine influences of CR on aging in species more closely related to man.

Atherosclerosis is an age-related process which is the leading cause of human mortality in western society. In atherogenesis, lipid derived from apolipoprotein B (apoB)-containing lipoproteins is deposited in the arterial wall (3,4) and elevated plasma low density lipoproteins (LDL) represent a widely accepted risk factor for atherosclerosis. However, it is now clear that all LDL are not equally atherogenic and that even in the absence of high levels of LDL, certain characteristics of LDL particle composition and structure increase the risk for atherosclerosis.

Mechanisms underlying the atherogenicity of particular LDL are poorly understood. One process which is thought to mediate the entrapment and retention of LDL in arterial tissue is its interaction with arterial proteoglycans (PG) (5). In vitro PG-LDL binding studies have shown that human LDL with high affinity for PG was associated with coronary heart disease (6,7). Moreover, experimental changes in the dietary fat intake of nonhuman primates modulated the PG binding properties of LDL in a direction consistent with the effects of the diet on atherosclerosis, that is, an atherogenic diet enhanced and an atherosclerosis-protective diet decreased the PG-binding capacity of LDL (8–10). The present studies were undertaken to examine effects of CR in the nonhuman primate model on the composition and properties of plasma LDL which may affect their PG-binding potential and thus their atherogenicity.

RESEARCH DESIGN AND METHODS

Animals

The animals used for these studies were rhesus monkeys (Macaca mulatta), housed at the Wisconsin Regional Primate Research Center. Their care, diets, and the experimental design of the parent trial to which they are assigned have been previously described (11). Briefly, following baseline measurements, animals were assigned to one of two groups: control, which were fed ad libitum for 6–8 hours per day; or caloric restricted (CR), which were fed at 70% of their baseline intake for the same number of hours. The monkeys were fed purified diet with corn oil (10% of the diet weight) as the major source of fat. The animals’ body size and composition, physical activity, metabolic rate, glucose tolerance, insulin sensitivity as well as indices of immunologic function have been previously reported (11–13). Sixty-six animals were included in the parent trial (34 controls, 32 CR). These animals, when 8–14 years of age, were randomized into the trial in two phases: Group 1
LDL-PG binding studies and LDL composition analysis were conducted using a randomly chosen subset of 25 of these animals (13 control and 12 CR). Seventeen males (42% of all male animals) and 8 females (31% of all females) were included in the studies. The first (n = 9) and third (n = 8) groups of males were equally represented. Animals in the parent trial and those included in the LDL-PG studies were similar in body weight (10.3 kg vs 10.8 kg, respectively, \( p = .50 \)). The substudy animals were also comparable to the parent trial animals in plasma glucose (\( p = .79 \)), plasma insulin (\( p = .63 \)), and insulin sensitivity (\( p = .55 \)).

**Measurement of Plasma Lipids**

After an 18-hour fast, animals were sedated with ketamine hydrochloride (10 mg/kg), and blood was drawn from the femoral vein into tubes containing 0.1% EDTA (pH 7.4, final concentration). Plasma was prepared by centrifugation at 1000g, 30 minutes, 4°C, and placed under nitrogen and on ice for overnight shipment to Wake Forest University School of Medicine. Standardized methods were used for lipid determinations: total cholesterol (TPC), triglycerides (TG), and high density lipoprotein (HDL) cholesterol were quantified in the Centers for Disease Control and Prevention (CDC)-standardized Bowman Gray Lipid Research Laboratory. LDL cholesterol was estimated by the Friedewald formula (14).

**Lipoprotein Preparation**

Plasma of 25 animals (13 control and 12 CR) were selected at random for isolation of LDL by sequential density gradient ultracentrifugation. Plasma (4–5 mL) was first ultracentrifuged for 20 hours at 40,000 rpm at 4°C. The infranate (density > 1.006) was density adjusted with potassium bromide and ultracentrifugation was repeated for 43 hours at 4000 rpm at 4°C. The supernatant (density < 1.063) containing LDL was collected and stored under argon in the dark for up to 8 weeks prior to PG binding and composition analysis. LDL molecular weight was determined by column chromatography on Superose 6B and chemical compositions of LDL were measured by standard colorimetric and enzymatic procedures (9).

**Proteoglycan Preparation**

The thoracic aortas of two male, adult cynomolgous macaques fed a monkey chow diet were used to prepare a PG pool for binding assays. At necropsy the arteries were excised, stripped of adventitia, and quick-frozen in liquid nitrogen for storage at –70°C. For PG extraction, tissues were thawed, minced into 2–3 cm² sections, and incubated for 36 hours at 4°C in 4.0 M guanidine HCl, 0.05 M sodium acetate (pH 4.5, 30 mL/g wet tissue) containing protease inhibitors (15). Extracted tissues were removed by filtration through Whatman No. 1 paper, and the extract was concentrated using CF 25 cones (Amicon, Inc., Beverly, MA). Following exchange dialysis into 7.0 M urea, 0.05 M Tris, 0.15 M NaCl, pH 7.2, the solution containing the PG was chromatographed on DEAE Sephadex (24 mL column) by washing with three bed volumes of loading buffer and eluting with three bed volumes of 7.0 M urea, 0.05 M Tris, 1.0 M NaCl, pH 7.2. Dialysis and chromatography was repeated once to improve purification. Recoveries were monitored by dimethylene blue assay (16), and the proteoglycan content of the final preparation was measured as hexuronic acid (17). Agarose electrophoresis of papain-liberated GAG (18) demonstrated that the PG preparation was predominantly chondroitin sulfate PG. Aliquots of the PG preparation were stored at –20°C until used in LDL binding assays.

**Proteoglycan-Low Density Lipoprotein Interactions**

The assay used has been fully described in previous publications (8,9,19). Briefly, 120 µg LDL (as cholesterol) was incubated with 1 µg PG (as hexuronic acid) in 1.1 mL of buffer containing 5 mM Tris, 6 mM KCl, 15 mM CaCl₂, 1 mM MgSO₄, pH 7.2, at room temperature for 30 minutes. Insoluble PG-LDL complexes that formed were separated by centrifugation at 1000g for 30 minutes, and LDL in the pellet was measured using an enzymatic assay for cholesterol. Data are presented as amount of LDL cholesterol present in insoluble complexes with PG.

PG binding studies were conducted between 7 and 61 days after LDL isolation (29 ± 4 days). There was no correlation between LDL storage time and level of PG-LDL interaction (\( r = .09, p = .68 \)). LDL storage time was 28 ± 5 days for control and 30 ± 6 days for CR animals (\( p = .74 \)).

**Data Analysis**

Values presented are mean ± SEM (95% confidence levels). Differences between groups were evaluated by t test. Pearson univariate and partial correlation coefficients are presented. Plasma TG, LDL TG, and LDL cholesterol ester were log-transformed prior to analysis because of skewed distributions. General linear models were used for multivariable analyses controlling for treatment and group effects. Analyses were performed using SAS version 6.1 (SAS Institute, Inc., Cary, NC).

**RESULTS**

**Plasma Lipids**

Plasma lipids and lipoprotein concentrations for all animals in the parent trial are shown in Table 1. There was no difference in total cholesterol, LDL cholesterol, or HDL cholesterol, and LDL/HDL ratios were similar between the groups. Significantly lower plasma triglycerides were present in the CR group with their value being only 49% of control. In the subset of animals used to provide LDL for composition analyses and measurement of PG binding, differences in plasma TG did not reach statistical significance due to restricted sample size (Table 2). However, total cholesterol (\( p = .15 \)), LDL cholesterol (\( p = .13 \)), HDL cholesterol (\( p = .50 \)), and TG (\( p = .75 \)) in the substudy animals were representative of the larger parent trial.
LDL Composition

LDL was isolated from plasma and its chemical composition was determined. The percentage composition of LDL is given in Table 3. LDL from CR animals were significantly depleted in TG and phospholipids and were relatively richer in cholesteryl ester and protein. The percentage of free cholesterol was similar between the groups. LDL particles from CR versus control animals were smaller as evidenced by a lower molecular weight (2.9 ± 0.1 vs 3.2 ± 0.1 g/umol [mean ± SEM], p < .05) for CR and 2.6 ± 0.1 for control (n = 12). In order to understand further which of the compositional variables were related to the particle size and percentage differences, these data were converted to numbers of LDL particles, whereas the number of TG and phospholipid molecules per LDL particle were not different between the groups. As shown in Table 3, the number of protein, cholesteryl ester, and free cholesterol molecules per LDL particle were not different between the groups, whereas the number of TG and phospholipid molecules were significantly lower in LDL from CR animals (249 ± 16 vs 433 ± 49 [TG, p < .005] and 686 ± 20 vs 837 ± 33 [phospholipid, p < .001]) for CR and control animals, respectively. The overall charge of the LDL as assessed by agarose electrophoresis was similar between groups (data not shown).

LDL Interaction with Arterial PG

To determine whether the compositional changes in LDL induced by CR affected functional properties of the particles, an in vitro binding assay was used to measure LDL interaction with isolated arterial PG. The results are shown in Figure 2. There was a 35% decrease in the amount of LDL complexed to PG for LDL from CR versus control animals (10.1 ± 0.8 vs 15.6 ± 1.1 [p = .001]). Based on mass measurements and LDL molecular weights, the mean number of LDL particles bound per μg PG was 4.2 ± 0.6 (×1012) for LDL from CR animals and 7.1 ± 0.7 (×1012) for LDL from control animals, a 41% reduction.

To examine more closely the influence of CR-induced changes in LDL particle composition on the reduced PG binding, regression analyses were performed. A strong positive correlation was observed between PG binding and number of TG molecules per particle (r = .76, p < .0005) (Figure 3A). The correlations within the diet groups were r = .56 (p < .05) for CR and r = .67 (p < .01) for control. Phospholipid molecules per particle were significantly associated with PG binding when groups were pooled (r = .48, p < .01) (Figure 3B), but not when control and CR groups were analyzed separately. There were no statistically significant relationships between PG binding and LDL particle content of cholesteryl ester, free cholesterol, or protein (not shown). Among LDL particle composition variables, number of molecules of TG per LDL particle (partial r = .64, p = .002) remained significantly correlated with LDL-PG binding after control for treatment allocation (control or CR) and duration of treatment (animal group). No other LDL particle composition variables were significantly related to LDL-PG binding after control for treatment and duration, and none of these variables contributed significantly to models that included molecules of TG in LDL.

### Table 1. Plasma Lipids of All Animals in Parent Trial

<table>
<thead>
<tr>
<th></th>
<th>TPC</th>
<th>TG</th>
<th>HDLC</th>
<th>LDLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>182 ± 6</td>
<td>139 ± 24</td>
<td>88 ± 4</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>(n = 34)</td>
<td>(170,193)</td>
<td>(91,187)</td>
<td>(79,97)</td>
<td>(60,77)</td>
</tr>
<tr>
<td>CR</td>
<td>175 ± 6</td>
<td>66 ± 8</td>
<td>92 ± 4</td>
<td>70 ± 3</td>
</tr>
<tr>
<td>(n = 32)</td>
<td>(162,188)</td>
<td>(48,83)</td>
<td>(84,99)</td>
<td>(64,76)</td>
</tr>
</tbody>
</table>

*p* test of CR versus control. TG log-transformed prior to analysis.

### Table 2. Plasma Lipids of Animals in PG-LDL Binding Studies

<table>
<thead>
<tr>
<th></th>
<th>TPC</th>
<th>TG</th>
<th>HDLC</th>
<th>LDLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>202 ± 9</td>
<td>142 ± 55</td>
<td>97 ± 5</td>
<td>82 ± 8</td>
</tr>
<tr>
<td>(n = 13)</td>
<td>(183,221)</td>
<td>(22,261)</td>
<td>(86,108)</td>
<td>(66,98)</td>
</tr>
<tr>
<td>CR</td>
<td>178 ± 12</td>
<td>84 ± 19</td>
<td>89 ± 4</td>
<td>72 ± 6</td>
</tr>
<tr>
<td>(n = 12)</td>
<td>(152,204)</td>
<td>(43,126)</td>
<td>(79,99)</td>
<td>(59,85)</td>
</tr>
</tbody>
</table>

*p* test of CR versus control. TG log-transformed prior to analysis.

### Table 3. LDL Chemical Composition

<table>
<thead>
<tr>
<th></th>
<th>LDL MW (g/μmol)</th>
<th>LDL Percent Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.2 ± 0.1</td>
<td>35.6 ± 1.2 CE TG FC PL蛋白</td>
</tr>
<tr>
<td>(n = 13)</td>
<td>(3.0,3.4)</td>
<td>(32.9,3.8) TG FC PL蛋白</td>
</tr>
<tr>
<td>CR</td>
<td>2.9 ± 0.1</td>
<td>39.1 ± 0.4 CE TG FC PL蛋白</td>
</tr>
<tr>
<td>(n = 12)</td>
<td>(2.6,3.1)</td>
<td>(38.1,4.0) TG FC PL蛋白</td>
</tr>
</tbody>
</table>

*p* test of CR versus control. TG log-transformed prior to analysis.
Figure 1. Chemical composition of LDL particles from caloric-restricted (CR) and control animals. Values are molecules per particle and represent means ± SEM. Values were calculated using the following molecular weight values: PL (phospholipid) 775; TG (triglyceride) 900; FC (free cholesterol) 384; CE (cholesteryl ester) 675; Pro (protein, based on apoB 100) 512,000. *p < .05 by Student t test.

Figure 2. The formation of particulate complexes with PG of LDL from caloric-restricted (CR) and control animals. LDL (120 µg as cholesterol) was incubated with PG (1 µg as hexuronate) in 5mM Tris, 6mM KCl, 15mM CaCl₂, 1.5 mM MgSO₄, pH 7.2, for 30 minutes at room temperature. The formation of particulate complexes was measured as cholesterol in a 1500g pellet. Data are presented as µg of LDL cholesterol present in insoluble complexes with 1 µg PG. Values are shown for individual animals.

DISCUSSION

Ongoing trials of CR in aging nonhuman primates have already provided valuable information on beneficial effects of this nutritional intervention in higher species. Improved peripheral insulin sensitivity associated with the chronic CR state has now been confirmed as a consistent metabolic feature for all nonhuman primate trials (20–23). Because insulin resistance has been identified as an independent correlate of human atherosclerosis (24), this is one potentially antiatherogenic effect of CR. Another, as suggested by the present studies, is by effects on lipoproteins. These studies introduce a new mechanism whereby CR may have a beneficial effect on risk factors for the development of atherosclerosis in primates. They demonstrate that CR results in plasma LDL with reduced binding potential for arterial PG. In vivo, this may lead to lower LDL entrapment and retention in the arterial intima of restricted compared to ad-libitum fed animals and may be a mechanism whereby the age-associated progression of atherosclerosis may be retarded.

The lipid profiles of the animals in the present study were not different between CR and ad-libitum fed animals, with the exception of plasma triglyceride levels which were markedly decreased in CR animals. The absence of an increase in HDL to accompany the decrease in TG was sur-
These are collectively termed the "atherogenic lipoprotein phenotype" (33). Because each of these abnormalities may be atherogenic and all are metabolically interdependent, it is difficult to establish the relative importance of LDL particle size to atherogenesis. In addition, this dyslipidemic state frequently coexists with insulin resistance, hypertension, and a procoagulant state, that is, "Syndrome X," a condition associated with high risk of cardiovascular disease (30,34). In monkeys, dietary manipulation has shown that LDL particle size per se is not the atherogenic variable of LDL. Dietary polyunsaturated fat induced an increase in LDL particle size in cynomolgous macaques and a reduced LDL size in African green monkeys, and yet in both species, it was associated with a decrease in atherosclerosis (35). Rather, it was suggested that some other variable, often but not always, associated with particle size may be the more important determinant of LDL atherogenicity.

The relationship between LDL size and its PG-binding properties is also unclear. In the present study, although the mean particle size was smaller and PG binding was lower for LDL from CR animals, the relationship between LDL molecular weight and its PG-binding ability did not reach statistical significance (r = .3, p = .15). This is consistent with results of previous studies with nonhuman primate LDL. In cynomolgous macaques, LDL particle size was smaller and its avidity for arterial PG was lower when diets containing fish oil compared to lard were fed (8). When LDL from those animals were subfractionated to examine more homogeneous populations of particles, LDL binding to arterial PG increased with increasing molecular size of the subfractions for lard-fed but not fish oil–fed animals (9). This suggests that some variables, often but not always, associated with particle size may be the more important determinant of LDL binding to PG. When unfraccionated LDL from individual humans were allowed to interact with arterial PG, there was selective binding of a subpopulation of particles which were more dense, had a higher pi, a lower core-to-surface ratio, and higher apoB content than the remaining population (36). Although the relationship of LDL particle size to atherosclerosis in monkeys may be species- and diet-dependent, the present study in rhesus monkeys is consistent with reports in humans that lower plasma TG are associated with less atherogenic LDL (28–30,32).

The interaction between LDL and PG occurs between negatively charged sulfate groups on glycosaminoglycan chains of PG and positively charged amino acids (primarily lysines) of LDL apoB (37). The charge density of an LDL population appears to be a major factor in its PG-binding capacity (for review, see 36), and it has been shown that different PG-binding sequences of apoB may act cooperatively to enhance the interaction with PG (38). This suggests that a conformation of LDL, which brings positively charged recognition sequences of apoB in close proximity to each other, will increase the avidity of the particle for PG. The correlation of LDL particle TG and phospholipid content with PG binding (Figure 3) may also be an indication that particle structure, and the associated conformation of apoB, are important in the interaction between LDL and PG. Models for the manner by which apoB intercalates into the lipid of the LDL particle indicate regions of protein association with core lipids as well as with surface phospholipids. It is possible that certain degrees of lipidation of an LDL particle result in conformations of apoB where positively charged PG-recognition sequences of apoB are positioned in close proximity to increase the avidity of PG binding. Overall charge differences in LDL were not apparent between groups in the present study. Clearly more information about LDL particle structure may help to provide reasons for the association identified here.

A recent publication has shown that oxidation of LDL decreases its ability to bind to PG (39). Oxidation is unlikely to be a factor in the differences in binding between CR and control animals in the present studies, because two different methods of assessing oxidation of LDL indicated no difference between the two groups (authors, unpublished data). Another factor which may influence LDL–PG interaction is the apoE content of LDL, but this association can only be demonstrated for specific subspecies of LDL (9,10). Although apoE content of plasma was similar between control and CR animals in the present study (data not shown), the apoE content specifically of LDL has not been determined. Isolation of subfractions of LDL will be required to assess whether apoE plays a role in the PG-binding differences observed in this study.

In conclusion, this study demonstrates that chronic CR in nonhuman primates results in reduced interaction of LDL with arterial PG. These data suggest a mechanism by which CR could reduce retention of atherogenic lipoproteins in the artery wall, thereby reducing their atherogenic potential. It is not known if CR reduces the risk of atherosclerosis in rhesus monkeys and atherosclerosis extent was not evaluated in the present studies. Therefore, the definitive end point and its relation to LDL–PG interaction is still to be evaluated.

Acknowledgments

This work was supported by NIA AG14190 (IJE), AG00578 and AG10816 (WTC), AG11915 (RW), and NIH Grant HL49373 (LLR).
The authors wish to acknowledge the technical contributions of Ramesh Shah and Martha Wilson, PhD, and secretarial assistance of Shelton Phillips. An abstract of these studies was presented at the 50th Annual Scientific Meeting of The Gerontological Society of America, Cincinnati, Ohio, November, 1997.

Address correspondence to Dr. Iris J. Edwards, Department of Pathology—Comparative Medicine, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157. E-mail: iedwards@wfubmc.edu

REFERENCES


Received January 21, 1998
Accepted May 11, 1998