Caloric restriction increases HDL$_2$ levels in rhesus monkeys (Macaca mulatta)

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Verdery, Roy B., Donald K. Ingram, George S. Roth, and Mark A. Lane. Caloric restriction increases HDL$_2$ levels in rhesus monkeys (Macaca mulatta). Am. J. Physiol. 273 (Endocrinol. Metab. 36): E714–E719, 1997.—Caloric restriction (CR) prolongs the life of rodents and other small animals, but the benefits of CR for primates and people are as yet unknown, and mechanisms by which CR may slow aging remain unidentified. A study of rhesus monkeys, Macaca mulatta, is underway to determine if CR might prolong life span in primates and to evaluate potential mechanisms for life prolongation. Thirty rhesus monkeys in three age cohorts, restricted to 70% of ad libitum calorie intake for 6–7 yr, were obtained in three age groups and started CR in two cohorts. Plasma lipid, lipoprotein, and high-density lipoprotein (HDL) apolipoproteins and subfractions were measured and compared with weight, percent fat, glucose, and insulin level. CR caused decreased triglyceride levels in adult monkeys and increased levels of HDL$_2b$, the HDL subfraction associated with protection from atherosclerosis. Multivariate statistical analyses showed that differences in lipid and lipoprotein levels occurring with CR could be accounted for, at least in part, by decreased body mass and improved glucose regulation. These studies have used a novel dietary modification paradigm in nonhuman primates focused on calorie reduction. Results suggest that CR, as mediated by its beneficial effect on body composition and glucose metabolism, could prolong human life by decreasing the incidence of atherosclerosis.

VERDERY, Roy B., Donald K. Ingram, George S. Roth, and Mark A. Lane. Caloric restriction increases HDL$_2$ levels in rhesus monkeys (Macaca mulatta). Am. J. Physiol. 273 (Endocrinol. Metab. 36): E714–E719, 1997.—Caloric restriction (CR) to 60–80% of ad libitum energy intake increases the life span of laboratory rodents and other small animals (29). CR maintains certain physiological functions in a youthful state, and, concomitant with life span extension, CR delays development of age-associated diseases. Although affected processes and diseases are specific to species and strain of experimental animal, they include glucose homeostasis, malignancies, renal failure, and cardiomyopathy (29).

A study of effects of CR in nonhuman primates was begun several years ago (11). It has been hypothesized that CR will delay the onset of age-associated diseases in these animals. It has also been hypothesized that CR will positively impact risk factors for these diseases and that careful study of the mechanisms of the effects of CR on risk factors for the development of age-associated diseases will give insight into basic mechanisms underlying aging.

One age-associated disease of great interest in this study of nonhuman primates is atherosclerosis, since atherosclerosis remains the most important age-associated disease in humans. Macaca species have been used for over 25 years as models for the study of effects of dietary manipulations on risk factors (10), particularly lipoprotein risk factors (21), for the development of atherosclerosis (20).

The dietary model employed in CR is different from that previously used in studies of modification of risk factors for atherosclerosis. Most studies using nonhuman primates to examine the effects of diet on lipoprotein risk factors employ isocaloric diets with modification of cholesterol (21), fatty acid distribution (26), or the relative proportion of fat, carbohydrate, and protein energy (10). These studies have been instrumental in furthering our understanding of the role of cholesterol, saturated fat, and total dietary fat in the development and potential regression (5) of atherosclerosis. In contrast, in the CR experiment in this report, the diet for both controls and restricted animals contained 5% fat and only 4.5 mg cholesterol/100 g, and CR monkeys received and consumed the same diet restricted to 30% less per kilogram body weight than age-matched control animals (11).

Studies of risk factors for atherosclerosis in long-term CR have been limited. In rats, CR lowers serum cholesterol and triglycerides relative to controls (4, 19, 23). However, high-density lipoprotein (HDL) cholesterol levels in rodents have been reported to be both lower (4) and higher (23) in CR than in controls.

In this report, we describe the effects of long-term CR in nonhuman primates in the study mentioned above (11) on plasma lipids, lipoproteins, and HDL apolipoproteins. With the diet employed, total fat intake was very low (5% of total dietary weight). We report that CR, in nonhuman primates, restricting animals to 70% of control energy intake causes significant increases in levels of apolipoprotein A-I (apo A-I) in specific HDL subfractions corresponding to human HDL$_2b$. These increases in HDL accompanying CR are statistically predicted by relatively low plasma glucose and insulin levels and the low lean body mass that accompanied CR (17).

METHODS

Animals. Male rhesus monkeys (Macaca mulatta) were obtained in three age groups and started CR in two cohorts. Cohort I entered the study in 1987, and cohort II entered the study in 1988. The young group consisted of 20 juvenile monkeys (group J, 10 control and 10 CR; 0.6–1.0 yr old at entry). The adult group consisted of 20 animals (group A, 10 control and 10 CR; 3–5 yr old at entry). The old group consisted of 17 animals (group O, 10 control and 7 CR; >18 yr old at entry). All monkeys in groups J and A and all but one of
the monkeys in group O were born in captivity and had known birth dates. One group O monkey was caught wild, but detailed records from the time of captivity were available to accurately determine minimum age. Before this study, no monkey had been used for invasive experimentation.

Housing, feeding, blood sampling, and husbandry practices were as previously described (11). The primate unit is fully accredited by the American Association for Accreditation of Laboratory Animal Care. All procedures relevant to the study were approved by the Animal Care and Use Committee of the Gerontology Research Center, National Institute on Aging.

Diet. The diet consisted of 71.6% crude carbohydrate, 15.4% crude protein, 5% crude fat, and 8% fiber by weight. Caloric density was 3.8 kcal/g. Dietary fat was ~55% polyunsaturated, ~25% monounsaturated, and ~20% saturated and included ~4.5 mg cholesterol/100 g. All monkeys received two meals each day, at 0700 and 1400. Control monkeys were fed a specified amount based on National Research Council guidelines, as previously described (11, 17). CR monkeys received 30% less food per kilogram body weight than what the age-matched control animals received, as described previously (11). Quarterly measurements of food consumption over the course of the study have shown that the average intake in CR monkeys was 27% less than in controls (Lane, unpublished data). Because all monkeys had reached a stable body weight, there was no adjustment in food allotments during the time period over which the present data were collected. To avoid nutrient deficiency, the diet was supplemented with vitamins, minerals, and trace elements at a level 40% higher than that normally provided to the age-matched control animals received, as described previously. To avoid nutrient deficiency, the diet was supplemented with vitamins, minerals, and trace elements at a level 40% higher than that normally provided to the age-matched control animals. Because all monkeys had reached a stable body weight, there was no adjustment in food allotments during the time period over which the present data were collected. To avoid nutrient deficiency, the diet was supplemented with vitamins, minerals, and trace elements at a level 40% higher than the recommended daily allowance for rhesus monkeys. Because all monkeys were fed the same diet, the experimental manipulation was a reduction in total calories and not an alteration of a specific dietary component.

Body composition. All measurements and samples were obtained under ketamine anesthesia (7–10 mg/kg im) after an overnight fast, unless otherwise specified. Body weight was determined by weighing anesthetized monkeys on an electronic scale (Sartorius Model F135S), and body mass was obtained with the monkey in left lateral recumbency, using a custom-made sliding ruler. Estimates of fat and lean mass were obtained with the monkey in a supine position on the scan table (mode P4–12, elapsed time ~10 min). Lunar pediatric software (version 1.03 E) was used for all scans and data analyses.

Measurement of lipids and lipoproteins. A blood sample, obtained while animals were under ketamine anesthesia, after an overnight fast, was used for all analyses of lipids and lipoproteins. Plasma was separated immediately from blood cells and kept at 4°C until analysis. Total cholesterol and triglyceride levels were determined using standard enzymatic techniques (1, 24) adapted for implementation in microtitre plates. Triglyceride measurements were corrected for plasma glycerol content. Analyses were calibrated against standards certified by the National Bureau of Standards. HDL and HDL2 levels were measured using the dextran-sulfate double precipitation method previously described (7). An aliquot of plasma was kept frozen at ~70°C for analysis of apolipoproteins. HDL apo A-I and apo A-II were measured in batches in the laboratory of Dr. Larry Rudel (Bowman Gray School of Medicine, Winston-Salem, NC) using apolipoprotein standards purified from Macaca fascicularis (15).

HDL subfraction distributions were measured as previously described (28). Plasma was adjusted to a density of 1.25 g/ml with KBr, and total lipoprotein fraction was obtained by ultracentrifugation (50,000 rpm, 24 h, Ti 50.4 rotor, Beckman, Palo Alto, CA). The lipoprotein fraction was subjected to gradient gel electrophoresis on 4–30% polyacrylamide gradient gels obtained commercially (Pharmacia, Piscataway, N J) or prepared using the technique of Margolis and Kendrick (22). Gels were fixed, stained with Coomassie blue and destained under standard conditions, and scanned to obtain a digitized scan for further analysis. Particle sizes were determined by calibration of each gel, using standard proteins. Intensities were calibrated by normalizing the total HDL area to the measured HDL apolipoprotein concentration. Population-averaged scans were obtained as previously described (9) and represent the apolipoprotein concentration per particle averaged over the population. Differences between populations represent both the differences in total HDL apolipoprotein concentration and the differences in HDL subfraction distribution.

Other tests. Other laboratory tests, including total blood count, chemistry profile, and insulin levels were obtained on samples sent to commercial laboratories.

Data analysis. Data were entered into a computerized database and analyzed with standard statistical packages (SAS, Cary, NC). Differences between groups were analyzed using Student's t-test for continuous variables. Differences among categorical variables were analyzed using analysis of variance. Multivariate linear models were calculated with stepwise addition of independent variables. Effects with P < 0.05 were considered significant. Averages are presented ± 1 SE.

Table 1. Characteristics of experimental and control groups

<table>
<thead>
<tr>
<th>Table 1. Characteristics of experimental and control groups</th>
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<tbody>
<tr>
<td><strong>Group J</strong></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>n</td>
</tr>
<tr>
<td>Age, yr</td>
</tr>
<tr>
<td>Weight, kg</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
</tr>
<tr>
<td>Lean mass, kg</td>
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<tr>
<td>Fat, %</td>
</tr>
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</table>

Values are means ± SE. CR, calorie restriction; BMI, body mass index. See METHODS for description of groups J, A, and O. *Significant differences due to CR (P < 0.05). †Significant differences due to age group (P < 0.05).
though there were consistent trends toward lower BMI (1,54) when animals were considered together, the effects of CR on 

in CR animals. When the young group was considered alone, the effect of CR on reducing BMI was significant (F(1,19) = 9.21, P < 0.01), although the effect on reducing percent body fat, while indicating a trend [F(1,19) = 2.80, P < 0.15], was not significant. When all animals were considered together, the effects of CR on BMI [F(1,54) = 3.15, P < 0.1] and percent body fat [F(1,54) = 1.49, P < 0.25] were not significant, although there were consistent trends toward lower BMI and body fat in restricted monkeys.

Lipid and lipoprotein levels. Plasma lipid and lipoprotein levels by age group are shown in Fig. 1 and presented in Table 2. Total HDL cholesterol [F(2,54) = 4.11, P < 0.03] and HDL2 cholesterol [F(2,54) = 7.02, P < 0.002] levels were higher in the younger animals. Total triglyceride level increased with age [F(2,154) = 3.93, P < 0.03]. There were no significant effects of age group on total or LDL cholesterol levels, apoA-I or apoA-II levels, or levels of other HDL subfractions.

HDL subfraction levels. Effects of CR on HDL subfraction levels by age group are shown in detail in Table 2. CR increased levels of HDL2a protein by an average of 15.8 mg/dl [F(1,53) = 4.45, P < 0.04]. This increase in HDL2a is nearly the same as the increase in the sum of HDL2a and HDL1 protein (15.6 mg/dl) and the increase in apo A-I (15.0 mg/dl) associated with CR, although these other differences were not statistically significant at the P < 0.05 level. Lower triglyceride levels occurred in CR in young and middle age groups [F(1,33) = 4.52, P < 0.05], but triglyceride levels in older monkeys were not affected by CR. CR did not significantly change levels of total or LDL cholesterol, apoA-II levels, or levels of the other HDL subfractions.

Gradient gel electrophoresis of HDL. Because CR raised apo A-I levels by 15.8 mg/dl, ~19%, by increasing concentrations of the larger HDL subfractions, we calculated the average gradient gel electrophoresis results by subtracting protein-normalized control scans from protein-normalized CR scans using the method used previously for analyzing differences in HDL seen in transgenic mice (9). Protein-normalized differences between these two groups are presented in Fig. 2. These results clearly demonstrate that all of the increase in apo A-I occurred in subfractions of the size of HDL2a, HDL2b, and HDL1 and that there were no significant effects of CR on smaller HDL subfractions. Additionally, this figure demonstrates that the younger monkeys may have been more responsive to CR than the older animals, although this result was not statistically significant.

Multivariate analysis. To develop hypotheses for possible mechanisms connecting age and CR with

Table 2. Lipid and lipoprotein measurements in dietary restriction

<table>
<thead>
<tr>
<th></th>
<th>Group J</th>
<th>Group A</th>
<th>Group O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 9)</td>
<td>CR (n = 10)</td>
<td>Control (n = 9)</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>27 ± 3*†</td>
<td>24 ± 5*†</td>
<td>46 ± 3*†</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>124 ± 10</td>
<td>114 ± 7</td>
<td>105 ± 7</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>73 ± 8†</td>
<td>70 ± 6†</td>
<td>48 ± 4†</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>46 ± 6</td>
<td>40 ± 4</td>
<td>48 ± 5</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>81 ± 14</td>
<td>110 ± 23</td>
<td>86 ± 11</td>
</tr>
<tr>
<td>Apolipoprotein A-II</td>
<td>15 ± 2</td>
<td>15 ± 2</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>HDL₃ₐ protein</td>
<td>8 ± 2</td>
<td>10 ± 1</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>HDL₃b protein</td>
<td>13 ± 2</td>
<td>15 ± 1</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>HDL₃₃ protein</td>
<td>24 ± 5</td>
<td>22 ± 3</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>HDL₃₄ protein</td>
<td>17 ± 4</td>
<td>19 ± 8</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>HDL₃₅ protein</td>
<td>30 ± 9*</td>
<td>55 ± 14*</td>
<td>22 ± 3*</td>
</tr>
<tr>
<td>HDL₃₆ protein</td>
<td>34 ± 9*</td>
<td>59 ± 15*</td>
<td>25 ± 4*</td>
</tr>
</tbody>
</table>

Values are means ± SE in mg/dl. HDL, high-density lipoprotein; LDL, low-density lipoprotein. *Significant differences due to CR, P < 0.05. †Significant differences due to age group (P < 0.05).
changes in lipid and lipoprotein levels, multivariate linear regression analyses were conducted using BMI, percent body fat, lean body mass, plasma glucose levels, and basal plasma insulin levels as independent variables and lipid and lipoprotein levels as dependent variables. Table 3 shows that variation in triglyceride levels is associated with variations in BMI and percent fat. Variations in HDL, HDL$_{2b}$, apo A-I, and HDL subfraction levels are associated with variations in glucose, lean body mass, and, to a lesser extent, insulin levels.

**DISCUSSION**

Our findings demonstrate that long-term CR without malnutrition markedly improved risk factors for the development of atherosclerosis in nonhuman primates eating very low fat diets. CR increased serum levels of the larger HDL subfractions, HDL$_{2b}$ and HDL$_{1-2b}$. Body weight, BMI, and lean body mass were also reduced by long-term CR. The HDL subfraction result is not a chance occurrence, because it occurred independently in the three separate age cohorts and because the increase in HDL$_{2b-1}$ was inversely related to the decrease in triglyceride levels caused by CR, as expected from other data on HDL metabolism (9). In data not presented, from two other analysis cycles of the same cohorts, CR had similar effects on triglyceride levels and HDL subfractions.

Our study is unique for primate studies of lipoprotein response to diet because total food intake has been manipulated and the proportion of carbohydrates, fat, and protein has been kept constant. Nonetheless, our findings are consistent with findings in other studies that changed the relative composition of the diet and studied effects of low total fat, low saturated fat, and low cholesterol diets on nonhuman primates. All of these diets cause low plasma cholesterol levels (3–6). In the present study, triglyceride and cholesterol levels were generally low in both CR and control monkeys, consistent with the low-cholesterol, low-fat diet. There have been no long-term studies of CR in nonhuman primates before now. In a study of 7–12 mo of diet and/or exercise in overweight men, HDL$_2$ and HDL$_3$ increased in response to exercise and, to a lesser extent, to a weight-reducing diet (22, 23). In the present study, salutary effects of low-calorie diets were demonstrated in lean, rather than overweight, monkeys, suggesting that CR may benefit normal-weight people.

Our findings agree in general with those reported in rodents on long-term CR. Rats on CR weigh less, and CR results in reduced adiposity (for review see Ref. 29). Several studies have reported that CR reduces serum triglycerides (4, 19, 23). Although Choi et al. (4) reported that CR reduced HDL levels in young rats, Masoro et al. (23) showed that CR reduced HDL in young rats but not in old rats (30 mo of age).

The mechanisms underlying changes in HDL subfraction levels occurring with CR are unknown. Presumably, the changes are caused by changes in production and/or turnover of specific HDL components or subfractions, and the changes in production and turnover may be mediated by changes in glucose homeostasis and body composition caused by CR.

This study of long-term effects of CR in nonhuman primates is continuing, and the effects of CR on the development of atherosclerosis are not yet known. With the low-fat diet employed, even the controls may have no atherosclerosis, even though the association of obesity, impaired glucose tolerance, high cholesterol levels, and low HDL levels on the development of experimental atherosclerosis in Macaca species is well known (8). In humans, an inverse association between HDL$_{2b}$ levels and prevalence and incidence of atherosclerosis has been well established (3, 6, 13, 27). Our findings, therefore, predict that the effects of CR on body weight, BMI, glucose homeostasis, and lipid and lipoprotein

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**Table 3. Multivariate analysis of lipid and lipoprotein measurements**

<table>
<thead>
<tr>
<th>Dependent Variables (mg/dl)</th>
<th>Independent Variables</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>BMI</td>
</tr>
<tr>
<td>Triglyceride P &lt; 0.1 P &lt; 0.0001</td>
<td>P &lt; 0.02</td>
</tr>
<tr>
<td>HDL cholesterol P &lt; 0.05</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>HDL$_2$ cholesterol P &lt; 0.15</td>
<td>P &lt; 0.15</td>
</tr>
<tr>
<td>HDL$_{2b}$ protein P &lt; 0.15</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>HDL$_{2b}$ protein P &lt; 0.05</td>
<td>P &lt; 0.05</td>
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</table>

Results of stepwise multivariate analysis. Only independent variables that entered the model with P < 0.15 were included. Independent variables significant at the P < 0.15 level, when considered singly in univariate regression analysis, were significant predictors of dependent variables with univariate P < 0.05.
levels will demonstrate that CR, when added to a low-fat diet, will reduce the incidence of atherosclerosis. This study demonstrates for the first time that the CR dietary paradigm, known for extending the life span of rodents and other short-lived species, improves the cardiovascular risk profile in nonhuman primates. These findings add to a growing body of evidence that CR induces a wide range of physiological changes in nonhuman primates that will delay the onset of age-related diseases, slow aging processes, and perhaps extend the life span. Other studies have shown that 30% CR-induced changes in glucose regulation may delay the onset of diabetes or reduce its complication rate (2, 14, 17). Combined with other physiological data suggesting that nonhuman primates respond to CR in a way that is similar to rodents (16–18, 25), the present results strengthen the possibility that CR will have effects on aging and life span in long-lived mammals that are similar to those reported in rodents. Even if CR does not extend the life span in long-lived species, the emerging picture is that CR will delay the onset of some age-associated diseases.

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