Caloric restriction increases HDL₂ levels in rhesus monkeys (Macaca mulatta)

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Caloric restriction increases HDL₂ 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 compared with 30 controls. 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 HDL₂b, 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.

aging; nutrition; primates; atherosclerosis; high-density lipoprotein

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 CR monkeys in group O were studied. The animals were housed in conditions that have been described previously (27).

Cohort I. The young cohort (10 control and 10 CR) consisted of 20 monkeys. The study in this report began in 1987, and these animals entered the CR protocol at 6–7 yr of age. The CR animals were fed the restricted diet ad libitum to maintain body weight 70% of ad libitum weight. The control animals were fed the same diet, and both groups were maintained in the same environment throughout the study.

Cohort II. The young cohort (10 control and 10 CR) consisted of 20 monkeys. The study in this report began in 1988, and these animals entered the CR protocol at 3–5 yr of age. The CR animals were fed the restricted diet ad libitum to maintain body weight 70% of ad libitum weight. The control animals were fed the same diet, and both groups were maintained in the same environment throughout the study.

RESULTS

Plasma Concentrations of Lipids, Lipoproteins, and Other Factors

Plasma lipids, lipoproteins, and other factors were measured in cohort I and cohort II. The results are presented as means ± SE. The control and CR groups were compared using analysis of variance (ANOVA) and Student’s t tests. The results are presented in Table 1.

Table 1. Plasma Lipids, Lipoproteins, and Other Factors in Study Monkeys

<table>
<thead>
<tr>
<th>Factor</th>
<th>Control</th>
<th>CR</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>210 ± 5</td>
<td>195</td>
<td>0.05</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>60 ± 3</td>
<td>65</td>
<td>0.01</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>90 ± 5</td>
<td>55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>30 ± 2</td>
<td>35</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apolipoprotein A-II</td>
<td>25 ± 2</td>
<td>20</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

HDL Apolipoprotein Subfractions

The HDL subfractions corresponding to human HDL₂b were also measured. 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.
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.

Diets. 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 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.

Table 1. Characteristics of experimental and control groups

<table>
<thead>
<tr>
<th>Group J</th>
<th>Group A</th>
<th>Group O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>CR</td>
<td>Control</td>
</tr>
<tr>
<td>n</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Age, yr</td>
<td>7.5 ± 0.4*</td>
<td>7.3 ± 0.3</td>
</tr>
<tr>
<td>Weight, kg†</td>
<td>8.8 ± 0.4*</td>
<td>7.1 ± 0.4*</td>
</tr>
<tr>
<td>BMI, kg/m²†</td>
<td>2.7 ± 0.1*</td>
<td>2.2 ± 0.1*</td>
</tr>
<tr>
<td>Lean mass, kg†</td>
<td>8.1 ± 0.3*</td>
<td>6.6 ± 0.3*</td>
</tr>
<tr>
<td>Fat, %†</td>
<td>9.7 ± 2.3</td>
<td>5.6 ± 1.0</td>
</tr>
</tbody>
</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).

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 microtiter plates. Triglyceride measurements were corrected for plasma glycerol content. Analyses were calibrated against standards certified by the National Bureau of Standards. HDL and LDL 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, NJ) 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 were obtained by subtracting the average scan for one population from the average scan of another 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 for significance 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.
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 HDL$_2$ 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, apo A-I or apo A-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 HDL$_{2b}$ protein by an average of 15.8 mg/dl $[F(1, 53) = 4.45, P < 0.04]$. This increase in HDL$_{2b}$ is nearly the same as the increase in the sum of HDL$_{2b}$ protein and HDL$_1$ 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, apo A-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 HDL$_{2b}$, HDL$_{2a}$, and HDL$_{1}$ 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
development of atherosclerosis in nonhuman primates eating very low fat diets. CR increased serum levels of the larger HDL subfractions, HDL2b and HDL1-2b. 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 HDL2b-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, HDL2 and HDL3 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 HDL2b 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

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, HDL2b, 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

Table 3. Multivariate analysis of lipid and lipoprotein measurements

<table>
<thead>
<tr>
<th>Dependent Variables (mg/dl)</th>
<th>Independent Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglyceride</td>
<td>P &lt; 0.1</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>HDL2 cholesterol</td>
<td>P &lt; 0.02</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>P &lt; 0.15</td>
</tr>
<tr>
<td>Apolipoprotein A-II</td>
<td>P &lt; 0.15</td>
</tr>
<tr>
<td>HDL2 protein</td>
<td>P &lt; 0.15</td>
</tr>
<tr>
<td>HDL1-2b protein</td>
<td>P &lt; 0.15</td>
</tr>
</tbody>
</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.

Fig. 2. HDL apolipoprotein normalized difference between HDL subfractions from calorically restricted and control rhesus monkeys. Gradient gel electrophoresis results were normalized on the basis of HDL apolipoprotein levels, and densitometric scans from controls were subtracted electronically from scans from calorically restricted animals. Significant density at Stokes’ radius >5 nm shows that caloric restriction (CR) specifically increased HDL protein in HDL2a, HDL2b, and HDL1 size range. Absence of residual density at Stokes’ <5 nm shows that smaller HDL subfractions were unaffected by CR. Quantitative analysis by Gaussian deconvolution of scans showed that CR increased HDL protein in this size range by ~10 mg/dl.
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.

We gratefully acknowledge the assistance of Howard Baldwin and the support of Dr. Reubin Andres and the Laboratory of Clinical Physiology at the Gerontology Research Center, National Institute on Aging (NIA), Baltimore, MD. We also acknowledge the excellent animal care and technical support provided by Edward Tilmont, Lauren J. Thonson, and Stephen Jay and the continuing support of the veterinary resources program of the National Center for Research Resources (Dr. Ruth Woodward, veterinarian, Dr. Joseph Knapka, and Dennis Barnard, nutritionist).

Dr. Larry Rudel and his staff at Bowman Gray School of Medicine, Winston-Salem, NC, were instrumental in providing the enzyme-linked immunosorbent assay measurements of high-density lipoprotein apolipoproteins.

R. B. Verderi was supported in part by Bowman Gray School of Medicine, The Arizona Center on Aging, The University of Arizona, and NIA Special Emphasis Research Career Award Grant K01-AG-00414.

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Received 16 December 1996; accepted in final form 20 June 1997.

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of rhesus monkeys subjected to long-term diet restriction.


