Caloric Restriction and Cardiovascular Aging in Cynomolgus Monkeys (Macaca fascicularis): Metabolic, Physiologic, and Atherosclerotic Measures From a 4-Year Intervention Trial

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Caloric restriction (CR) retards aging processes, extends maximal life span, and consistently improves insulin resistance in lower species. Insulin resistance is associated with cardiovascular disease, but data is lacking demonstrating that increased insulin sensitivity reduces atherosclerosis progression. We initiated a study in 32 adult cynomolgus monkeys to evaluate increased insulin sensitivity secondary to CR on atherosclerosis extent. Following pretrial determinations, animals were randomized to a moderately atherogenic (0.25 mg cholesterol/Cal containing 30% of calories from fat)-fed control group or CR group (30% reduction) with equivalent dietary cholesterol intake. CR significantly improved insulin sensitivity and reduced intraabdominal fat over the 4-year intervention, while no significant differences were seen for the lipid profile between groups. Despite improved insulin sensitivity with CR, atherosclerosis extent did not differ between the ad libitum-fed or CR groups. These studies demonstrate that CR significantly improves insulin sensitivity, but when elevated plasma cholesterol concentrations were held similar, there was no effect on atherosclerosis extent. However, the composition of these lesions and changes in endothelial function may have been improved but were not evaluated in this study. Thus, further studies are needed to determine if improved insulin sensitivity might decrease arterial inflammation and improve endothelial function, despite no changes in atherosclerosis extent.

IT has been firmly established that caloric restriction (CR) retards aging processes particularly in lower species (1). This observation is supported by evidence that food restriction increases life span, retards age-associated physiological changes, and delays or prevents most age-associated disease (1). The mechanisms by which CR exerts its effects are unknown, but one of the most consistent physiological effects of chronic CR is an improvement in insulin resistance. The improvement of insulin resistance with CR is an important finding when considering mechanisms applicable to human health, as insulin resistance is clearly linked to the development of cardiovascular (CV) risk factors and significantly associated with CV (2,3). It also has been demonstrated that improvement in insulin resistance with either nonpharmacologic or pharmacologic approaches will improve specific CV disease risk factors in humans (4,5). Further, it has been postulated that improved insulin sensitivity on a clinical level will ultimately result in attenuation in atherosclerosis progression and a reduction in cardiovascular events either through direct effects or secondary to beneficial effects on other CV risk factors. Clinical trials in humans are currently ongoing to test that hypothesis. In the ongoing clinical trials, pharmacologic agents, that is, glitazones, are being used to increase insulin sensitivity, but nonpharmacologic intervention, that is, CR, has also been shown to be very effective in improving insulin resistance.

Given the robust findings of CR as noted in lower species, ongoing human studies of chronic CR are now under way. However, as a first step in extrapolating the findings to human beings, varying dietary regimens have been and continue to be tested in higher species such as nonhuman primates, and the effect on several aging processes and age-related diseases, particularly as they relate to human health, are being evaluated. One very important age-related disease, that is, atherosclerosis, is a valuable end point to study, particularly as it relates to the improvement in insulin sensitivity with chronic CR—but this goal has been severely hampered by the lack of a suitable animal model. However, cynomolgus monkeys have been shown in multiple studies to be an excellent model for the study of atherosclerosis and pathogenic mechanisms involved in the development of atherosclerosis (6–8). Furthermore, it has been shown that CR can be initiated safely and maintained without de-
trimental effects in nonhuman primates (9,10). Therefore, with evidence demonstrating safety of CR in nonhuman primates, our study evaluated the effect of increased clinical insulin sensitivity on the extent of atherosclerosis in a nonhuman primate.

Our specific hypothesis was that an increase in insulin sensitivity, secondary to CR over a sustained period when compared with a control diet, and with equivalent cholesterol intake, may reduce the progression of atherosclerotic lesions. To evaluate our hypothesis, we studied cynomolgus monkeys using dietary intervention in the form of CR compared to control. Due to the well-studied effect of lipids on atherosclerosis in this model, the diets were designed so that the animals had identical cholesterol intake per body weight (BW) in order to evaluate independent effects of insulin action on atherosclerosis while maintaining similar plasma cholesterol levels. Previously, we described the study design for this nonhuman primate trial and reported the results from the first year of study (11).

METHODS

Animals

Thirty-two feral adult male monkeys (Macaca fascicularis) were acquired directly from the Institute Pertanian (Bogar, Indonesia) and quarantined for 3 months. Age was assessed by dentition. All animals were housed socially in pairs except when separated at mealtime by sliding a partition to separate them. Cages were fitted with containers for food and water bottles to allow water intake ad libitum. All 32 pair-caged monkeys were housed in a single windowless room, 6 × 3.7 m in size. All rooms in the animal building utilized 100% outside air when outside air temperatures were above 40°F. Below 40°F, the system mixed recirculated air and outside air to maintain the temperature at approximately 72°F. During normal operations, all animal housing areas maintained 10–15 air changes per hour. Guidelines for the use and care of laboratory animals of the Wake Forest University School of Medicine were followed.

Design of the Trial

The trial was a randomized trial in which the independent effect of CR and its interaction with insulin resistance and the relationship to changes in atherosclerotic lesion extent and composition were evaluated (Figure 1).

Beginning in the fourth month and throughout the remainder of the pretrial (baseline, months 4–6), all of the animals were fed a moderately atherogenic diet (0.25 mg cholesterol/Cal) containing 30% of calories from fat. While on the control diet, caloric intake for each individual animal was assessed by feeding a known allotment and weighing the uneaten food. During the pretrial phase, measurements were taken monthly for plasma total cholesterol and high-density lipoprotein-cholesterol (HDL-C) concentrations. In addition, frequently sampled intravenous glucose tolerance tests (FSIVGTT-Modified Minimal Model) were done during the pretrial (baseline) to assess insulin sensitivity. Body composition was measured at baseline using computed tomography (CT) scans and anthropometric measurements. After the 6-month pretrial phase, animals were randomized to continue the control diet or to consume a caloric-restricted diet (30% reduction from baseline). The diets have been formulated so that cholesterol intake and vitamin and mineral intake per kilogram BW were similar between groups (see Diet Composition, Table 1).

Randomization into caloric-restricted or control groups.—After the 6-month pretrial evaluations, the animals were assigned to diet groups using a stratified randomization based on target plasma concentration (TPC)/HDL-C ratio, age, and BW obtained during the pretrial. The randomization was designed to balance the pretrial values of TPC/HDL-C previously shown to be a major prognostic factor with the degree of atherosclerosis. Each pair was fed either a control or CR diet.

Diet Composition

The nutritional objective of the study was to provide sufficient food for control groups to meet their estimated energy requirements based on their age and individual mean BW. The caloric-restricted diet was introduced over a 3-month transition period (90% of control intake during the first month, 80% during the second month, 70% during the third month and thereafter). The dietary restriction was based on each animal’s calculated daily ad libitum intake during the pretrial. Additional vitamin mixture, mineral mixture, beta-sitosterol, and crystalline cholesterol were added to the CR diet so that the same amount of these components was fed, whether the animals were fed 100 calories/kg BW (control) or 70 calories/kg BW (CR). Less dextrin and sucrose were added to the CR diet so the amount of calories provided from carbohydrate, and as a result the caloric density of protein and fat, would be the same in the two diets. Less calcium carbonate was added to the CR diet so that the ratio of calcium phosphorus would be the same in the two diets. Less alphacel (nonnutritive bulk) was added to the CR diet to accommodate the additional amounts of other ingredients. The composition of each diet is outlined in Table 1.

The potential for error exists in determining the daily food intake of nonhuman primates due to their untidy feeding behaviors, as bits of food may be spread throughout the cage. The following procedures were implemented to alleviate this problem. Diet was kept frozen and thawed right before each animal was fed. Food was offered as a cake in a stainless steel tray mounted on the outside of each cage. Each animal was separated from its cage mate by a sliding partition, which prevented animals from transferring food to one another or retrieving dropped food from a cage mate. At 10:00 AM, each animal was fed its daily calculated allotment. At 3:00 PM, all unconsumed food was...
removed, the amount of uneaten food was determined, the partitions were removed, and the animals were allowed to interact. The amount consumed by each monkey was then determined.

**Physiologic Evaluations**

**Insulin Sensitivity.**—Insulin sensitivity was determined by the frequently sampled FSIVGTT using a third-phase insulin infusion (Modified Minimal Model) (12) as described previously (13). Determinations were made during the pretrial and at 6-month intervals thereafter.

Monkeys were studied after an overnight fast. Two indwelling saphenous venous catheters were inserted after the animals were anesthetized with ketamine HCl (10 mg/kg BW). One catheter was used for blood sampling and the other for glucose and insulin injections. Catheters were kept patent by flushing with heparinized saline between blood draws, and, prior to the sampling, approximately 1 ml of blood was withdrawn to remove any residualized heparinized saline. One milliliter blood samples were collected at 0 and 1 minute, after which 0.5 g/kg glucose was injected over 30 seconds, beginning at Time 0. After glucose injection, blood samples (1 ml) were taken at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 28, 32, 40, 45, 50, 55, 60, 70, 80, 90, 100, 110, 120, 140, 160, 180, 210, and 240 minutes postglucose injection. After the 20-minute blood draw, insulin (0.015 units/kg BW) was injected. At each time point, glucose and insulin measurements were made. At the end of study, in addition to the minimal model analysis, we assessed insulin sensitivity with a hyperinsulinemic, euglycemic clamp after 48 months of CR. After placement of catheters, blood was taken at 0, 5, and 10 time points (baseline). A one-step euglycemic hyperinsulinemic clamp was then done on each animal. A bolus injection of insulin was administered for 4 minutes, and hyperinsulinemia maintained by means of a continuous insulin infusion. Blood samples were drawn at 5-minute intervals and glucose levels maintained in the 4.9–5.5 mmol/L range over 120 minutes by a 20% dextrose infusion. The steady-state plasma glucose and insulin levels were 5.4 ± 0.55 mmol/L and 17.8 ± 7.8 pmol/L, respectively. Glucose was assayed by the glucose oxidase method on a glucose Analyzer 2 (Beckman Instruments, Brea, CA). Insulin was assayed by radioimmunoassay (Incstar Corp., Stillwater, MN).

**Clinical Evaluations**

**Adipose tissue distribution.**—Computerized tomographic assessment of total abdominal, intraabdominal, and sub-
cutaneous abdominal fat and body measures was done in month -6 of the pretrial phase, month 0, and 6-month intervals throughout the study. The monkeys were anesthetized with ketamine HCl (10 mg/kg administered intramuscularly [IM]) and acepromazine (0.1 mg/kg IM) prior to the CT scan. The monkeys were positioned on their backs with legs extended and arms placed adjacent to their trunks. A GE CT9800 scanner (GE, Milwaukee, WI) was used for the procedure, and 120 kVp and 100 mutant allele-specific amplification (MASA) with a 2-second scan time were used as the radiographic parameters. The CT density score used to identify fat was previously determined and was found to be -140 to -40 Hounsfield units, with the reference range from -1000 (air) to +1000 (dense bone) with zero being the density of water (14). Intraabdominal, total, and subcutaneous fat was determined for a 1 cm scan taken at the umbilicus, as described previously (11).

**Blood pressure/heart rate.** Blood pressure and heart rate were measured during the pretrial phase and at 6-month intervals thereafter. Monkeys were sedated with 15 mg ketamine HCl/kg BW IM. Between 8 and 18 minutes after sedation, at least three measurements of systolic blood pressure, diastolic blood pressure, and heart rate were taken. The average of these measurements was used to determine the animal’s blood pressure and heart rate. Blood pressure measurements were made with a Dinamap Portable Adult/Pediatric and Neonatal Vital Signs Monitor (Model 8100; Critikon, Inc., Tampa, FL), as described (11).

**Biochemical Parameters**

**Plasma lipids and lipoproteins.** Total plasma cholesterol, triglycerides, and HDL-C for all animals were measured during months 4, 5, and 6 of the pretrial phase, and then every 3 months after starting the caloric-restriction or control diets. All samples were analyzed in the Clinical Chemistry Laboratory, which was in compliance with the Cooperative Lipid Standardization Program. Cholesterol and triglyceride analyses were performed using enzymatic methods on the Technicon RA-1000 analyzer (Bayer Corp., Indianapolis, IN) high-performance cholesterol reagent. Glycated hemoglobin was analyzed with automated affinity high-pressure liquid chromatography (HPLC) methodology (interassay coefficient of variation = 1.2%, intraassay coefficient of variation = 2.1%) performed on a Primus CLC-330 HPLC (Primus Corporation, Kansas City, MO) as previously reported (15). Total serum glycated proteins were assessed using nitroblue colorimetric methodology (second generation fructosamine assay) as determined on a Cobas Mira Chemistry Analyzer (Roche Diagnostics, Nutley, NJ) using Roche reagents (Roche Diagnostics) as previously described (16).

**General Chemistries**

Hematocrit, hemoglobin, white blood count, and indices were determined on a model M430 Instrument (Coulter, Electrolysis, Hialeah, FL). Total protein and albumin were determined by the Cobas Mira Chemistry Analyzer using Roche Reagents.

**Atherosclerosis Measures**

After 4 years of study, the monkeys were anesthetized deeply with sodium pentobarbital (100 mg/kg, IV) and exsanguinated. The heart was removed and the coronary arteries were perfused for 1 hour at 100 mmHg pressure using 10% neutral buffered formalin. Fifteen blocks were removed (5 serial blocks from each of the left circumflex, left anterior descending, and right coronary artery). Each block was embedded in paraffin and sections (5 μm) were taken and stained with Verhoeff-van Gieson. The sections were projected and the intimal or plaque area was recorded using a hand-held stylus with a computer-assisted digitizer. The extent of coronary artery atherosclerosis was expressed as the cross-sectional area of plaque as described previously (17). The mean of 15 sections was calculated for each animal.

**Statistical Methods**

A randomized single-blind design was used to avoid selection bias in the allocation of subjects to intervention groups. Although a double-blind design is preferred, as it reduces any bias in reporting of subjective improvements and/or occurrences of adverse effects as well as any investigator bias in the collection of outcome measures, all investigators who were responsible for the evaluations of end-point measures were blinded to group assignments. All biochemical assessments were made by technicians with no knowledge of group assignments.

The effects of the CR diet on the trial evaluations measured at the specified intervals postrandomization were estimated using repeated measures analysis of covariance (ANCOVA). Analysis of group differences was adjusted for the prerandomization levels of the outcome measure being tested in order to reduce the variance explained by prerandomization predictors. All tests of hypotheses and reported p values are two-sided. Whenever a baseline value was used as a covariate in an ANCOVA model, an interaction term between the group and covariate was initially included to check the parallelism assumption. If, and it was always the case, the interaction was not significant at the .10 level of significance, the interaction term was omitted. Histograms and summary statistics were evaluated for each outcome measure. If moderate skewness was indicated, a logarithmic transformation was used for analysis of test hypotheses.

Estimates of intervention effects were obtained at each follow-up observation. Tests of time of follow-up by
intervention effects were conducted to test for consistency of effects over the follow-up period. Average intervention effects over the follow-up period were estimated and tested for significance.

**RESULTS**

The baseline characteristics of the animals are demonstrated in Table 2. As shown, there were no significant differences in age, weight, lipids, or blood pressure between groups. Furthermore, abdominal CT scans and insulin sensitivity measures obtained in the pretrial phase demonstrated no differences in body composition or insulin sensitivity between groups.

**Caloric Intake**

Figure 2 demonstrates caloric intake for both CR and control animals in the pretrial phase and over the duration of study. As demonstrated, after 10% monthly reduction in intake for 2 months, a consistent 30% decrease in caloric intake in the CR group was maintained ($p < .001$).

**Body Weight/Fat Distribution**

There was a progressive increase in BW in the control group over the duration of study. In contrast, CR caused a significant reduction in BW (Figure 3A). The effect of CR on various abdominal fat depots measured by CT was also assessed. The control group had a gradual increase in both total fat and intraabdominal fat over the entire duration of study, whereas there was a significant effect of CR to reduce both total abdominal fat and intraabdominal fat mass (Figure 3B).

**Insulin Sensitivity**

There was no significant difference at baseline (prior to CR initiation) between groups. There was a significant diet effect, however, as the CR group was significantly more insulin sensitive than the control group beginning at month 6 of the intervention and maintaining the effect over the entire duration of the study (Figure 4A). In addition to the minimal model analysis, we assessed insulin sensitivity with the hyperinsulinemic euglycemic clamp after 48 months of CR. The whole-body insulin-stimulated glucose disposal was significantly higher in animals randomized to CR when compared with controls (Figure 4B).

**Lipid/Lipoprotein and Atherosclerosis Measures**

The mean values for plasma lipid and lipoprotein measures are summarized in Table 3. There were no significant differences in plasma lipids and lipoprotein concentrations as expected due to the increased cholesterol in the diets of the CR group. In fact, there was a tendency for increased total and very low-density lipoprotein (VLDL) cholesterol. There was no statistical difference in glycated hemoglobin or fructosamine levels (data not shown), nor a significant difference noted for blood pressure between groups. Coronary artery atherosclerosis extent (intimal area) was not significantly different between groups (Figure 5). There was a significant correlation between both total and VLDL cholesterol and atherosclerosis extent ($r = 0.43$ and $0.45$, $p < .05$).

**DISCUSSION**

This study demonstrates that CR has a profound effect to improve insulin sensitivity and reduce intraabdominal fat with aging. The effect of CR was noted during the first year of observation and was maintained over the 4 years of observation as demonstrated not only from periodic measures of insulin sensitivity with minimal model assessment, but determination of hyperinsulinemic, euglycemic clamps at the end of the study. However, despite the improvement in sensitivity, atherosclerosis extent did not appear to be altered with the effect of CR.

The improvement of insulin sensitivity reported in the study confirmed the observations that had been reported by numerous investigators assessing CR in higher species.
Specifically, Kemnitz and colleagues (18) first reported improved sensitivity by similar models in rhesus monkeys. This finding has also been confirmed by investigators at the National Institute on Aging (NIA) and the University of Maryland when evaluating CR and insulin action in non-human primates (19,20). It is now clear that an improvement in insulin sensitivity appears to be one of the most consistent features of chronic CR, as observed in both rodent and nonhuman primate models. Indeed, ongoing human studies are currently evaluating CR on parameters assessing carbohydrate metabolism.

Despite the improved BW and insulin sensitivity with CR, there was no difference in plasma lipid and lipoprotein concentrations between groups. However, this is not a surprising finding, as this was expected from the design of the study. As hyperlipidemia has a profound effect on atherosclerosis extent in this model, we sought to maintain similar plasma lipid levels between groups, and this was achieved by supplementing the CR diet with crystalline cholesterol (Table 1). Despite no significant difference in lipid levels in this study, we have reported that lipid levels may be favorably affected by CR in other species when dietary cholesterol is not held constant. Specifically, Edwards and colleagues demonstrated that triglyceride levels were significantly lowered by CR, compared with controls, in a cohort of rhesus monkeys in the University of Wisconsin CR project (21). Verdery and colleagues also reported decreased triglyceride concentrations in adult animals and increased levels of HDL2B, the fraction associated with cardioprotection, in a cohort of rhesus monkeys in the NIA trials (22). However, in the studies of Edwards (21) and Verdery (22), no additional cholesterol was fed to the CR monkeys, which was in contrast to the present study.

In addition to the lipoprotein concentration, LDL composition has also been assessed with chronic CR. We had previously reported that CR did not alter the LDL chemical composition in our cohort of cynomolgus monkeys, whereas in the rhesus, significant increases in cholesterol esters and significant decreases in triglyceride and phospholipid content in the LDL particle were found (23). The reason for these changes between the species are not clear, but it appears that dietary cholesterol may override the effects of CR on the LDL cholesterol concentration and composition. The effect of CR on additional properties of the LDL particle have been evaluated; LDL proteoglycan binding was shown not to be affected in the cynomolgus cohort, but was improved in the University of Wisconsin cohort (21,24). Once again, it is likely that the dietary manipulations affected these results. Finally, LDL oxidation...
secondary to CR was not shown to be altered in either the
cynomolgus or rhesus model (23).

Although our study did not demonstrate an effect of CR
to reduce lipid levels as we sought to maintain plasma
cholesterol with our study design, the effect of CR to
modulate lipids and lipoproteins may also be related to the
species studied, as different observations have been noted in
rodents. Specifically, Liepa and colleagues (25) and Masoro
and colleagues (26) found that food restriction in rats fed
a cholesterol-free semisynthetic diet markedly affected
plasma lipids. Specifically, it was demonstrated in Fisher
344 rats fed either ad libitum or 60% of ad libitum intake
that postabsorptive serum cholesterol and phospholipid
concentrations increase in the ad libitum-fed rats with
increasing age. However, food restriction did not influence
the serum levels of these lipids in young rats but delayed
the age-related increase in concentrations (25). Other studies in
rodents suggest that, although restriction of components of
the diet other than calories can affect both longevity and
age-associated pathology, CR in all cases had the major
effect (27–30). Thus, the failure of CR to affect plasma
lipids for the monkeys in this study may relate not only
to dietary cholesterol supplementation but to species
differences in the response to CR.

The results from all ongoing nonhuman primate CR trials
have demonstrated that CV risk factors, such as BW and
intraabdominal fat and insulin sensitivity, are significantly
improved by CR. It would be hypothesized that these
beneficial effects on CV risk factors would ultimately
reduce atherosclerosis extent. However, our data here
confirm our pilot findings in the aorta (24) and suggest that
atherosclerosis extent in the coronary arteries is not reduced
with improved insulin sensitivity with comparable hyperlipidemia. As there was a significant correlation between
atherosclerosis extent and plasma lipids, hyperlipidemia
may overwhelm other potential beneficial effects of an
enhanced insulin sensitivity and reduced fat content. Clearly, hyperlipidemia was present and equal in both
groups as the total cholesterol averaged well over 9.1 mmol/L
(350 mg/dl) for both groups. However, there does not
appear to be an independent effect of insulin sensitivity and
body fat to retard atherosclerosis progression in this study.
The observation that there does not appear to be an
independent effect of insulin sensitivity to reduce athero-
sclerosis extent in the presence of hyperlipidemia was also
observed in a rodent study. Specifically, we randomized
three groups of apolipoprotein E (APOE) knockout mice to
placebo treatment or treatment with the pharmacologic
agents troglitazone and rosiglitazone. The insulin sensitivity
was significantly improved in both groups of animals
randomized to pharmacologic therapy when compared with
controls. However, similar levels of hyperlipidemia were
maintained for all three groups (total cholesterol
\( > 15 \text{ mmol/L} \)) and, as a result, no significant changes were
observed for lesion extent at the end of the study (31).

Table 3. Plasma Lipid and Lipoproteins and Coronary Artery
Atherosclerosis Extent

<table>
<thead>
<tr>
<th>Lipids (mmol/L)</th>
<th>Control</th>
<th>Caloric Restricted</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>9.23 ± 0.39</td>
<td>9.8 ± 0.31</td>
<td>.13</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>62 ± 0.05</td>
<td>59 ± 0.03</td>
<td>.69</td>
</tr>
<tr>
<td>V+LDL cholesterol</td>
<td>8.61 ± 0.36</td>
<td>9.38 ± 0.31</td>
<td>.12</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>28 ± 0.04</td>
<td>23 ± 0.03</td>
<td>.39</td>
</tr>
</tbody>
</table>

Notes: Mean ± standard error of mean.
HDL = high-density lipoprotein; V+LDL = very low-density lipoprotein.
Therefore, the finding from the present study, and from our rodent data, suggested that improved parameters such as insulin sensitivity might not independently improve atherosclerosis extent in the presence of marked hyperlipidemia.

Summary
Chronic CR is effective in reducing body fat and improving insulin sensitivity in aging. However, when plasma cholesterol levels are kept constant and elevated, an increase in insulin sensitivity was not associated with a decrease in atherosclerosis extent. As LDL may be argued to be the major risk factor for atherosclerosis, the favorable effect of insulin sensitivity and body fat distribution may be negated in the face of elevated cholesterol. However, the composition of these lesions and changes in inflammation and endothelial function may have been improved but were not evaluated in this study.

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