A Study of Caloric Restriction and Cardiovascular Aging in Cynomolgus Monkeys (Macaca fascicularis): A Potential Model for Aging Research

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Caloric restriction has been demonstrated to retard aging processes and extend maximal life span in rodents, and is currently being evaluated in several nonhuman primate trials. We initiated a study in 32 adult cynomolgus monkeys to evaluate the effect of caloric restriction on parameters contributing to atherosclerosis extent. Following pretrial determinations, at which time a baseline measure of ad libitum (ad lib) dietary intake was assessed, animals were randomized to an ad lib fed group (control) or a caloric restriction group (30% reduction from baseline intake). The animals are being evaluated for glycated proteins, insulin, glucose, insulin sensitivity measures, and specific measures of body fat composition by CT scans (e.g., intra-abdominal fat) over specified intervals. The results from the first year of observation demonstrate a significant diet effect on body weight, and specifically intra-abdominal fat. Further, insulin sensitivity has been significantly increased after 1 year of caloric restriction compared to the ad lib fed group. These studies indicate that caloric restriction has a marked effect on a pathologic fat depot, and this change is associated significantly with an improvement in peripheral tissue insulin sensitivity.

It has been shown that caloric restriction retards aging processes in rodents (Weindruch and Walford, 1988; Snyder, 1989). 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. The mechanisms by which caloric restriction (CR) exerts its effects are unknown. However, if these findings are to be extrapolated to human beings, it will first be necessary to test varying dietary regimens in some higher species and evaluate the effect on several aging processes and age-related diseases, particularly as they relate to human health. Age-related disease such as atherosclerosis would be a valuable end point to study, but this goal has been severely hampered by lack of a suitable animal model. Cynomolgus monkeys have been shown in multiple studies by our group to be an excellent model for studying an aging adult nonhuman primate model of athero-sclerosis using dietary intervention in the form of caloric restriction compared to ad libitum food intake. The diets have been designed so that the animals have identical cholesterol intake per body weight in order to evaluate the effect of caloric restriction on atherosclerosis while maintaining similar plasma cholesterol levels. In this article, we describe the study design for this ongoing nonhuman primate trial and report the results from the first year of study.

METHODS

Animals
Thirty-two feral adult male monkeys (Macaca fascicularis) were acquired directly from Institut Pertanian (Bogar, Indonesia). After arrival at our Center, age was assessed by dentition, the animals were quarantined for 3 months, treated for internal and external parasites, and tested for tuberculosis. Routine tuberculin testing continues twice yearly. All animals are housed socially in pairs except when separated at meal time. Each monkey pair occupies a cage constructed of stainless steel with dimensions of 61 cm wide, 71 cm deep, and 86.4 cm high. Each cage allows for the placement of a sliding partition to separate the animals at mealtime. They are fitted with containers for food and have valves extending from the cage to allow water intake ad libitum. All 32 pair-caged monkeys are housed in a single
windowless room, 6 × 3.7 m in size. All rooms in the animal building utilize 100% outside air when outside air temperatures are above 40 °F. Below 40 °F, the system will mix recirculated air and outside air to maintain the temperature at approx. 72 °F. During normal operations, all animal housing areas maintain 10–15 air changes per hour and utilize 40% filters for air filtration.

Design of the Trial

The trial is a randomized one in which the independent effect of caloric restriction and its interaction with insulin resistance, intra-abdominal fat, blood and tissue glycation, and the relationship to changes in atherosclerotic lesion extent and composition are being evaluated. The design of the trial is shown in 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 (.25 mg cholesterol/Cal) containing 30% of calories from fat. While on the ad lib 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 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 also measured at baseline employing CT scans and anthropometric measurements. Following the pretrial evaluations, all of the animals were subjected to a baseline biopsy of the right femoral artery in order to quantitate the levels of both early and advanced glycated products. After the 6-month pretrial phase, animals were randomized to continue the ad libitum diet or to consume a caloric-restricted diet (30% reduction from baseline). The diets have been formulated so that the caloric restriction diet so the ratio of calcium:phosphorus would be the same in the two diets. Less calcium carbonate was added to the caloric restriction diet so that the same amount of these components was fed whether the animals were fed 100 calories/kg body weight (ad libitum) or 70 calories/kg body weight (caloric restriction). Less dextrin and sucrose were added to the caloric restriction 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 caloric restriction diet so the ratio of calcium:phosphorus would be the same in the two diets. Less alpha-cyclodextrin (non-nutritive bulk) was added to the caloric restriction diet to accommodate the additional amounts of other ingredients.

Randomization into caloric-restricted or ad libitum-fed groups. — After the 6-month pretrial evaluations, the animals were assigned to diet groups using stratified randomization, based on TPC/HDL-C ratio, age, and body weight measurements obtained during the pretrial. The randomization was designed to balance the pretrial values of total plasma cholesterol to high-density lipoprotein cholesterol concentration ratio (TPC/HDL-C), previously shown to be a major prognostic factor with the degree of atherosclerosis. As animals were pair-caged, each pair was fed either a control or CR diet.

Diet Composition

The potential for error exists in determining the ad libitum food intake of nonhuman primates due to their untidy feeding behaviors in captivity, as bits of food may be spread outside of each cage. The following procedures are being implemented to alleviate this problem. Diet is kept frozen and thawed right before each animal is fed. Food is offered in a stainless steel tray mounted on the outside of each cage. Each animal is separated from its cage mate by a sliding partition which prevents animals from transferring food to each other or retrieving dropped food from cage mate. At 1000 hr, each animal is fed its daily calculated allotment weighed for each animal. At 1500 hr, all un consumed food is removed, the amount of uneaten food is determined, the partitions are removed, and the animals are allowed to interact. The amount consumed by each monkey is then determined.

Measurement of insulin resistance, blood and tissue (i.e., skin collagen) glycation/advanced glycation, computed to-
Table 1. Composition of the Ad Libitum (Control) and Caloric Restriction Diets

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<tr>
<th>Ingredient</th>
<th>Ad Libitum Diet</th>
<th></th>
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<th></th>
<th></th>
<th>Caloric Restriction</th>
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<tr>
<td></td>
<td>Gm Per 100 gm</td>
<td>Grams Lipid</td>
<td>Mg Chol</td>
<td>Gm Per 100 gm</td>
<td>Grams Lipid</td>
<td>Mg Chol</td>
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<td>Casein, USP</td>
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<td>Dextrin</td>
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<td>Sucrose</td>
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<td>Wheat Flour, self-rising</td>
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<td>Lard</td>
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<td>Complete Vitamin Mix</td>
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<td>Ausman-Hayes Min. Mix</td>
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<td>Beta-sitosterol</td>
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<td>Protein (%) of Calories</td>
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<td>Lipid (%) of Calories</td>
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<td>Carbo (%) of Calories</td>
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<td>1.23</td>
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<td>% of fat</td>
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<tr>
<td>% of cal</td>
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<td>Saturated (%)</td>
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<td>Monounsaturated (%)</td>
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<tr>
<td>Polyunsaturated (%)</td>
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mography (CT) assessment of abdominal fat and lipid levels are being measured at multiple intervals over a 4-year period. The data collected are summarized in Table 2. Those data will be related to coronary artery atherosclerotic plaque size and complications after the 4-year period.

**Physiologic Evaluations**

**Insulin sensitivity.** — Insulin sensitivity was determined by the frequently sampled intravenous glucose tolerance test (FSIVGTT; Cobelli and Thomaseth, 1987) using a third-phase insulin infusion (Modified Minimal Model) (Finegood et al., 1990; Cefalu et al., 1995a). 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 body weight). One catheter was used for blood sampling and the other for glucose and insulin injections. Catheters were kept patent by flushing with 1–2 ml of heparinized saline between blood draws, and prior to the sampling, approx. 1–2 ml of blood was withdrawn to remove any residualized heparinized saline. One milliliter blood samples were collected at -10 and -1 minute, after which 0.5 g/kg glucose was injected over 30 seconds, beginning at Time 0. After glucose injection, blood samples (one ml) were taken at 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 28, 32, 40, 45, 50, 60, 70, 80, 90, 100, 110, 120, 140, 160, 180, 210 and 240 minutes post-glucose injection. After the 20-minute blood draw, insulin (.015 units/kg BW) was injected. At each time...
Clinical Evaluations

Adipose tissue distribution. — Computerized tomographic assessment of total abdominal, intra-abdominal, and subcutaneous abdominal fat and body measures was made in month -6 of the pretrial phase, month 0, and at 6-month intervals throughout the study.

CT scan. — The monkeys were anesthetized with ketamine HCl (10 mg/kg administered intramuscularly [i.m.]) and acepromazine (0.1 mg/kg i.m.) 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 was used for the procedure, and 120 kVp extended and arms placed adjacent to their trunks. A GE T9800 scanner was used for the procedure, and 120 kVp extended and arms placed adjacent to their trunks. A GE T analyzer 2 (Beckman Instruments, Brea, CA). Insulin was calculated against a mercury manometer according to the manufacturer’s recommendation.

Electrocardiograms. — Electrocardiograms were obtained during month 6 of the pretrial phase and will be obtained at study completion. A Hewlett-Packard electrocardiograph (Model 1500B) is used for cardiologic evaluation. Electrocardiograms are recorded between 8 and 18 minutes after ketamine HCl injection (15 mg ketamine HCl/kg body weight, administered i.m.). The animals are placed in dorsal recumbency and the standard six limb leads (I,II,III,AVR, AVL, AVF) and three chest leads (V-1,V-4,V-6) recorded.

Biochemical Parameters

Total plasma cholesterol, triglycerides, and high density lipoprotein cholesterol 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 ad libitum diets.

Cholesterol/triglyceride. — All specimens are analyzed in the Lipid Analytic Laboratory. The laboratory is in compliance with the Cooperative Lipid Standardization Program. Cholesterol and triglyceride analyses are performed using enzymatic methods on the Technicon RA-1000 analyzer. The methods for cholesterol (SM4-0139A85) and for triglyceride (SM4-0169K87, CPO Blank Method) are as described by Technicon in their technical manual. The only exception is the substitution in the cholesterol assay of the BMD236691 reagent (Boehringer Mannheim) for the Technicon reagent. Our center is fully standardized with this method and is in the continuing surveillance phase with respect to the CDC Lipid Standardization Program.

HDL cholesterol. — For determination of HDL cholesterol concentration, we use the heparin-manganese precipitation procedure described in detail in the Manual of Laboratory Operations of the Lipid Research Clinics Program. The only deviation from this procedure is that we utilize 2M MnCl2 rather than 1M MnCl2, originally suggested for the Lipid Research Clinics. The reason for this change is that we have found that small amounts of LDL cholesterol fail to precipitate in certain hyperlipoproteinemic monkeys and human beings when 1M MnCl2 is used. This is completely resolved with 2M MnCl2 without loss of HDL. For total and HDL cholesterol determinations, the RA-1000 enzymatic method is used with the Boehringer-Mannheim (BMD) high-performance cholesterol reagent. The BMD’s cholesterol reagent is used since it is made with Tris buffer which,
unlike the phosphate-buffered reagents used by many other manufacturers, does not cause positive interference in the assay of HDL cholesterol when using heparin-manganese precipitation.

**Glycation Parameters**

**Soluble blood, skin, and arterial collagen.** — Glycated hemoglobin and fructosamine were determined at month 5 and 6 of the pretrial phase and every 3 months during the trial phase. A femoral artery biopsy to determine levels of arterial collagen glycation/advanced glycation was obtained in month 6 of the pretrial period. A biopsy for skin collagen glycation was obtained at month 6 of the pretrial period and at 6-month intervals during the trial. The tissue samples (skin and artery) are currently being stored at -70°C and will be batch analyzed at study completion.

**Glycated hemoglobin.** — Total glycated hemoglobin was analyzed with automated affinity HPLC methodology (interassay c.v. = 1.2%, intraassay c.v. = 2.1%) performed on a Primus CLC-330 HPLC (Primus Corporation, Kansas City, MO) as previously reported (Cefalu et al., 1993, 1994). This method relies on boronate affinity columns to non-enzymatically trap cis-diol groups of glycated proteins. By virtue of measuring total glycation, the assay is free from interference from abnormal hemoglobin as seen in hemoglobinopathies.

**Total serum glycated proteins.** — Total serum glycated proteins were assessed with use of nitroblue colorimetric methodoligy (2nd generation fructosamine assay) as determined on a Cobas Mira Chemistry Analyzer using Roche reagents as described (Cefalu et al., 1991).

**General Chemistries**

Hematocrit, hemoglobin, white blood count, and indices were determined on a model M430 Instrument (Coulter Electrolysis, Hialeah, FL). Total protein/albumin were determined for both the caloric-restricted and ad lib fed groups during the pretrial assessments. As shown, there were no significant differences noted in the baseline measures between groups. As randomization was based on lipids, age, and body weight, the animals randomized to the CR diet appeared to have less abdominal fat prior to initiating restriction. However, these differences in abdominal fat were not statistically significant. All animals had normal EKGs at baseline.

**Caloric Intake**

Ad lib caloric intake was assessed for 3 consecutive months in the pretrial phase to get a baseline level for each individual monkey. The amount of caloric restriction for

| Table 3. Baseline Characteristics of Monkeys, Mean ± SEM |
|----------------|----------------|----------------|
| **Ad Libum-fed** (n = 16) | **Caloric Restricted** (n = 16) |
| **Age (years)** | 8 ± 1.1 | 8.4 ± 1.2 |
| **Weight (kg)** | 5.6 ± 2 | 5.5 ± 2.2 |
| **Lipids (mg/dl)** | | |
| • Total cholesterol | 283 ± 17 | 284 ± 14 |
| • HDL cholesterol | 33 ± 3 | 35 ± 2 |
| • Triglycerides | 21 ± 2 | 20 ± 1 |
| **Inulin Sensitivity (× 10^-4)** | 2.9 ± 0.4 | 3.2 ± 0.4 |
| **Abdominal Fat (cm²)** | | |
| • Total abdominal | 3328 ± 435 | 2853 ± 401 |
| • Intraperitoneal | 1775 ± 218 | 1502 ± 220 |
| • Subcutaneous | 1161 ± 170 | 1030 ± 146 |
| • Paraspin | 372 ± 64 | 320 ± 54 |
| **Blood Pressure (mmHg)** | | |
| • Systolic | 93 ± 3.6 | 93 ± 3.6 |
| • Diastolic | 53 ± 2.9 | 52 ± 2.7 |
| • Mean arterial pressure | 66 ± 3.1 | 66 ± 2.9 |

*Average of 3 values during pretrial.
each animal randomized to the CR group was therefore based on the individual animal’s ad lib intake during the pretrial. After randomization, the caloric-restricted diet was introduced over a 3-month period, with 90% of ad lib diet given in the first month, 80% in the second month, and 70% in the third month and thereafter. As demonstrated in Figure 2A, compared to the ad lib-fed animals, there has been a significant decrease in caloric intake for animals randomized to the CR group during the first year of study \((p < .001)\). At the end of the first year, animals in the CR group have averaged an \(-34\%\) decrease in dietary intake compared to the ad lib-fed animals and an \(-30\%\) decrease when compared to their ad lib intake assessed during the pretrial.

**Body Measures**

*Body weight.* — As demonstrated in Figure 2B, body weight was noted to decrease in the CR animals at the onset of caloric restriction. There has been a significant diet effect on body weight in the CR group when compared to the ad lib-fed group \((p < .05)\). CR animals have lost an average 0.3 kg of body weight since randomization, and at the end of the first year there is an 8% difference in body weight between animals.

**Lipid Measurements**

Figures 4A and 4B demonstrate the total plasma cholesterol and HDL-C levels observed during the first year of study. No diet effect was noted for these lipid parameters. Further, there was no diet effect noted for TPC:HDL-C ratio or triglyceride levels.

**Carbohydrate Metabolism**

Table 4 demonstrates parameters assessing carbohydrate metabolism during the study. There has been no significant change in levels for glycated hemoglobin or fructosamine in...
either dietary group. Table 4 also demonstrates the measurements of fasting glucose and insulin in both dietary groups. Although an increase in insulin for the ad lib-fed animals and a decrease in fasting glucose in the CR animals was noted at the end of 12 months, these changes were not statistically significant. Figure 5 demonstrates the insulin sensitivity parameter obtained with the modified minimal model technique. There has been a significant diet effect to improve insulin sensitivity in the CR animals. Compared to baseline, an increase in insulin sensitivity of 50% has occurred. No significant diet effect was noted for glucose effectiveness (data not shown).

**General Health/Cardiovascular Measures**

Despite a significant reduction in calories, the CR animals are maintaining hematologic values (hemoglobin, hematocrit, white blood count), total protein, albumin levels, BUN, and creatinine levels that are no different from the ad lib group (data not shown). No difference in blood pressure or mean arterial pressure has been noted.

**DISCUSSION**

The long-term goal of this study is to assess the independent effect of chronic caloric restriction on a specific measure of atherosclerosis, i.e., coronary artery plaque extent, in a nonhuman primate model. Caloric restriction is postulated to alter pathogenic mechanisms contributing to atherosclerosis. Therefore, in this ongoing trial, we are evaluating the role of chronic caloric restriction in modifying cardiovascular risk factors (e.g., insulin resistance, adipose tissue distribution) and glycation/advanced glycation (for both blood and tissue proteins), all of which have been strongly implicated in contributing to cardiovascular disease. In addition, we propose to evaluate the relationship of these changes to changes observed in arterial plaque/size complications determined at study completion.

The focus of this article is to present the study design, methodology, and results from the first full year of caloric restriction for this ongoing trial. The results from the first year suggest that after one year of a significant 30% reduction in total caloric intake for the calorie-restricted animals

### Table 4. Carbohydrate Measures, Mean ± SD

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<th>Baseline*</th>
<th>Restricted</th>
<th>12 months</th>
<th>Restricted</th>
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<td>Glycated hemoglobin (%)</td>
<td>3.5 ± .6</td>
<td>3.5 ± .7</td>
<td>3.4 ± .6</td>
<td>3.3 ± .6</td>
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<tr>
<td>Fructosamine (µMol/L)</td>
<td>153 ± 14</td>
<td>161 ± 17</td>
<td>145 ± 20</td>
<td>151 ± 19</td>
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<td>Glucose (mg/dl)</td>
<td>64 ± 5.2</td>
<td>66 ± 8</td>
<td>64 ± 12</td>
<td>59 ± 10</td>
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<td>Insulin (µU/ml)</td>
<td>36 ± 18</td>
<td>34 ± 13</td>
<td>54 ± 28</td>
<td>35 ± 16</td>
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*Average of 3 monthly values during pretrial.
compared to the ad lib fed group: (a) a significant decrease in weight in the caloric restriction group has occurred accompanied by a significant decrease in central adiposity as assessed by specific determination of abdominal fat depots with CT scans and by anthropometric measurements; (b) successful maintenance of total plasma cholesterol/HDL cholesterol concentrations has occurred despite a reduction in calories of 30% in the CR group; (c) no detrimental effects of CR on general chemistry profiles or cardiovascular measurements have been observed; and (d) a significant improvement in insulin sensitivity has been observed, while no significant change has been observed for glycated proteins.

We are aware of two other ongoing trials of caloric restriction in aging nonhuman primates (Ingram et al., 1990; Kemnitz et al., 1993), plus additional studies that have evaluated weight maintenance in delaying the onset to diabetes in rhesus monkeys (Hansen and Bodkin, 1993). These trials have provided significant knowledge regarding the effects and validity of caloric restriction in primates, and as they are ongoing, will continue to provide important information regarding long-term maintenance of this nutritional regimen. Our study shares similarities with the other trials, as reported observations from the other nonhuman primate trials have greatly influenced our study design. Since 1987, investigators at the National Institute on Aging (NIA) have evaluated the effect of caloric restriction in nonhuman primates at several ages across the life span. This study contains four major cohorts of animals, two male rhesus groups, one cohort of male squirrel monkeys, and a group of female rhesus monkeys. Findings from this cohort have been published in several reports (Ingram et al., 1990, 1993; Lane et al., 1995a, 1995b). A second study at the University of Wisconsin (UW) was initiated in 1989 and is designed to evaluate the effect of adult-onset CR in male rhesus monkeys (Kemnitz et al., 1993). In a third study, Hansen and Bodkin (1993) at the University of Maryland (UM) have compared calorically restricted weight-stabilized adult rhesus monkeys to ad-lib fed monkeys and evaluated factors related to the development of diabetes.

Our ongoing trial differs from the above studies primarily as we are using cynomolgus monkeys (Macaca fascicularis), whereas the other three sites are evaluating either rhesus (Macaca mulatta) (NIA, UW, UM sites) or squirrel monkeys (Saimiri sciureus) (NIA site). Our choice for cynomolgus monkeys derives from the extensive literature demonstrating their validity as an excellent model for human atherosclerosis (Rudel and Pitts, 1978; Adams et al., 1983; Hamm et al., 1983). Further, there appear to be differences in susceptibility to atherosclerosis related to physiological aspects of aging [i.e., development (puberty) and surgical menopause] in these primates (Adams et al., 1983; Weingand et al., 1986). As such, this study is unique in that it is the first study evaluating chronic caloric restriction in a higher species on atherosclerosis. In order to independently evaluate the effect of caloric restriction on atherosclerosis, it is imperative that the study be designed to significantly reduce calories, yet not to alter the cholesterol content of the diet, as that will alter a major prognostic factor for atherosclerosis, i.e., total plasma cholesterol:HDL-C ratio. This was achieved by supplementing the calorically-restricted diet with crystalline cholesterol (see diet composition). As such, cholesterol intake was similar between groups and has resulted in similar lipid profiles (Figure 4A and 4B). Therefore, this particular study design will allow the independent effect of caloric restriction on atherosclerosis to be evaluated as we have achieved similar plasma cholesterol levels between groups.

Another major difference from the other CR trials is the age of dietary initiation. In the NIA study, caloric restriction was initiated in juvenile rhesus (age .6–1 yr) and squirrel monkeys (age 1–4 yr), young adult rhesus (age 3–5 yr) and adult squirrel monkeys (age 5–10 yr), and old rhesus (>18–25 yr) and old squirrel monkeys (>10 yr). In the UW study, fully adult rhesus monkeys were studied as the age range at initiation was 8–14 years. The University of Maryland study reported results in older rhesus monkeys with an average age of 17.9 for the weight-stabilized group and 18.1 for the control group after 5–9 years of diet titration. However, our study endpoint of atherosclerosis has influenced the age at which we initiated caloric restriction. The age range of 6–9 years was chosen based on evidence from our institution that demonstrated that juvenile (2.5–3.5 years) and adult (6–12 years) monkeys that had consumed comparable atherogenic diets resulting in similar plasma lipid concentrations showed marked differences in coronary artery lesion characteristics and extent (Weingand et al., 1986). Adult animals (n = 16) developed more extensive lesions characterized predominantly as proliferative atherosclerotic plaques, while juveniles (n = 10) had minimal lesions that consisted primarily of fatty streaks. These differences in lesion characteristics and extent were not explained by differences in blood pressure. It was uncertain if these quantitative and qualitative differences in susceptibility to diet-induced atherosclerosis were due to intrinsic age-related changes in the arterial wall and/or related to the endocrine and metabolic changes associated with puberty. This observation has been referred to as "juvenile protection" and has influenced our decision about the age range at study initiation.

The diet and feeding strategy has also varied considerably between studies and resulted in different observations for body weight for the first year of study. For example, in the NIA study, the amount of food given was based on body weight and National Research Council (NRC)-estimated energy requirements. It was reported that food intake assessments revealed an actual reduction of 22–24% (Ingram et al., 1990) rather than the 30% sought on the basis of energy requirements. Nevertheless, the DR animals had a substantial reduction in body weight gain. In contrast, investigators at the University of Wisconsin (Kemnitz et al., 1993) determined ad libitum intakes for each animal during the baseline period, and caloric-restricted intakes were individually determined. They reported that actual intakes in the control animals over the first year decreased, and they assessed that the original baseline intakes were overestimated because of a transient increase in intake due to the greater palatability of the defined diet. As such, the average intake of DR animals represented a reduction of 38% from their average baseline intake, but a reduction of only 18% from the average intake of controls. Despite this, and in contrast to the NIA study, body weights in the UW study actually declined over the first
12 months. On an average, the ad lib-fed monkeys gained a kg of weight (an increase of approx. 9% in BW) from baseline, while the DR animals lost approx. 0.5 kg (approx. 4.5% loss in body weight). In the UM study (Hansen and Bodkin, 1993), animals were weighed weekly and caloric intake was adjusted based on any prior change in body weight to maintain a stable weight in each animal. Our study is similar to the UW study in that the amount of caloric intake calculated was based on ad libitum intake for each animal determined in the pretrial phase. The 30% reduction in calories for animals randomized to the CR diet was therefore based on the ad lib diet determined for each individual animal. The CR animals had daily caloric intake at consistently 30% less than the calculated ad lib intake for each animal, and averaged 34% less than the control group. Therefore, the level of dietary restriction achieved for the first full year of our study is greater than that reported for the other primate trials.

The NIA study uses both anthropometric measures and DEXA scans to monitor body fat content as does the Wisconsin group. Our trial differs in that we assessed abdominal obesity with CT scans allowing separation into intra-abdominal, subcutaneous, and paraspinous fat. Our data suggest not only a decrease in total abdominal fat mass, but a reduction in the fat depot that has been most significantly linked to cardiovascular disease and cardiovascular risk factors in both human and nonhuman primates, i.e., intra-abdominal fat mass (Kissebath et al., 1988; Shively and Clarkson, 1988; Després et al., 1989). It is of interest in our study that the intra-abdominal fat mass showed the most significant change secondary to caloric restriction, \( p < .001 \), whereas changes in subcutaneous abdominal fat mass approached significance \( (p = .07) \) in the CR animals and the paraspinous fat mass did not appear to be affected by diet. Further, this finding is of great interest from a gerontologic perspective as we have recently reported that intra-abdominal fat mass accumulates with age and it is the abdominal fat depot that can most readily explain the variance in insulin resistance with age (Cefalu et al., 1995a).

The safety of caloric restriction was demonstrated in our study as has been demonstrated in the previous trials. Although we were successful in significantly reducing calories by over 30% in the CR group, there has been no detrimental effect on hematocrit, hemoglobin, total protein, albumin, or renal function. The results from the first full year also failed to demonstrate any changes in blood pressure between groups.

Our results failed to show a significant improvement in glycated proteins between groups. These data agree with the results obtained from both the NIA study (Cutler et al., 1992) and University of Wisconsin study (Kemnitz et al., 1994), although both trials have demonstrated improvements in carbohydrate metabolism. If lessons can be learned from the rodent model, we would expect decreases in early glycated products as measured on short-lived blood proteins to be observed first (i.e., glycated hemoglobin and serum fructosamine), and would ultimately lead to reduced substrate available for advanced glycation as measured in long-lived tissue proteins. Such was the pattern we recently reported in a CR rodent (Cefalu et al., 1995b). However, a more recent article by Lane et al. (1995a) reported no change in glycated hemoglobin after 7 years of CR. The reasons as to why no change in glycated proteins has been seen in the reported long-term trials while an improvement in insulin sensitivity has occurred are not currently known.

Finally, our most significant finding is that insulin sensitivity was increased in our animals after 1 year of caloric restriction. Although there was no significant difference at baseline between dietary groups, there was a significant diet effect on insulin sensitivity. Our finding of improved insulin sensitivity in chronic caloric restriction states confirms the observations first reported by the University of Wisconsin's investigators (Kemnitz et al., 1994), and indeed our methods for assessing insulin sensitivity were similar. As such, although the UW investigators did not show a significant improvement in insulin sensitivity after the first year, it may have been that the percent of restriction was milder then intended. After the decision was made to adjust the allotment provided to the restricted animals at 18 months, the investigators demonstrated a significant increase in insulin sensitivity at 6 and 12 months later (i.e., 24 and 30 months of trial). Therefore, as demonstrated first by the University of Wisconsin group and confirmed by investigators at the NIA (Lane et al., 1995a), insulin sensitivity can be significantly improved in nonhuman primates secondary to chronic CR. The mechanism by which this occurs is currently not known but will be the focus of future investigations.

In conclusion, the results from the first year of study have demonstrated that a 30% reduction in calories can be safely achieved while maintaining plasma lipid levels. Further, a significant decrease in body weight was observed in caloric-restricted animals associated with a significant decrease in intra-abdominal fat. We observed a significant improvement in insulin sensitivity at one year, but no significant change in glycated proteins. As observed with other nonhuman primate trials, a more prolonged period of observation will be necessary to determine if significant improvement in glycated products and maintenance of improved insulin sensitivity can be demonstrated, as well as if these changes are associated with diminished progression of atherosclerosis.

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References


