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Influence of Caloric Restriction on the Development of Atherosclerosis in Nonhuman Primates: Progress to Date

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Caloric restriction (CR) has been observed to retard aging processes and extend the maximum life span in rodents. In an effort to evaluate the effect of this nutritional intervention on physiologic variables in higher species, several nonhuman primate trials are ongoing. In particular, a study evaluating the independent effect of CR on the extent of atherosclerosis was initiated in 1993 in 32 adult cynomolgus monkeys. Therefore, the trial was designed to achieve identical cholesterol intake after animals were randomized to a control group or a calorie-restricted group (30% reduction from baseline caloric intake). The animals were routinely evaluated for glycated proteins, plasma insulin and glucose levels, insulin sensitivity, and specific measures for abdominal fat distribution by CT scans over a 4-year interval. The results from 4 years of intervention demonstrate that CR improves cardiovascular risk factors (such as visceral fat accumulation) and improves insulin sensitivity. In contrast to other primate studies with normolipidemic animals, CR had no independent effects on plasma lipid levels and composition in the presence of equivalent amounts of dietary cholesterol intake. Preliminary analysis of atherosclerotic lesion extent in the abdominal aorta has failed to demonstrate differences between control animals and CR animals. Follow-up studies are being conducted to determine the effect of CR on atherosclerosis extent in coronary and carotid arteries.

Key Words: **atherosclerosis; insulin; glucose; obesity; lipids.**

INTRODUCTION

Atherosclerosis is a major public health problem and a well described, age-related process. The clinical sequelae of atherosclerosis, e.g., coronary heart disease and cerebrovascular disease, are reported to be responsible for half of all deaths occurring annually and 70–80% of deaths in individuals over 65 years of age (American Heart Association, 1994; Thom, *et al*., 1992). In addition, 50% of all patients hospitalized for acute myocardial infarction are reported to be over 65 years of age (Gurwitz, *et al*., 1991). The projected increase in the number of individuals who will be over 65 by the year 2030 is estimated to be 20% of the U.S. population or ~35 million people. The well-observed increase in morbidity and mortality for these older persons due to cardiovascular disease is of great interest in gaining understanding of the role of aging in the progression of atherosclerosis (Duncan, *et al*., 1996; Wei and Gersh, 1987).

Atherosclerosis is a multifactorial process in which hypertension, smoking, gender, and lipids, in addition to age, are considered major risk factors (McGill, 1978; NCEP Expert Panel, 1993; Schaefer, *et al*., 1995). However, the major risk factors, in combination, explain only about half of the individual variability in the extent and severity of atherosclerosis and the incidence of coronary heart disease. Genetics also plays a major role, but a hypothesis of great gerontological interest is that age-related conditions (e.g., abdominal obesity, insulin resistance, and glycation/advanced glycation) may contribute to the progression of atherosclerosis (Cerami, 1985; Cerami, *et al*., 1985; Folsom, *et al*., 1989; Gillum, 1987; Haffner, *et al*., 1988; Horiuchi, *et al*., 1996).

Caloric restriction (CR) retards aging processes in a number of lower species; evidence that CR increases life span, retards age-associated physiologic changes, and delays or prevents most age-associated diseases has been substantiated (Snyder, 1989; Weindruch and Walford, 1988). Yet, the mechanism by which CR exerts its effects is unknown. However, if the findings are to be extrapolated to human beings, it will first be necessary to study CR in higher species to evaluate its effect on several aging processes and age-related diseases, particularly as they relate to human health. In this regard, an age-related disease such as atherosclerosis, with its considerable morbidity and mortality in older persons, would be a valuable endpoint to study. Unfortunately, this goal has been hampered by lack of a suitable animal model, as rodents do not generally develop atherosclerosis. Further, a trial of many years' duration would be required in order to evaluate the specific role of aging, *per se*, on the development of atherosclerosis in humans. Therefore, an animal model that is representative for both aging processes and atherosclerosis would serve a valuable role by allowing observations and conclusions drawn from a much shorter clinical trial period. As such, cynomolgus monkeys have been well described in multiple studies as an excellent

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model for both atherosclerosis and mechanisms involved in the development of atherosclerosis (Adams, *et al*., 1983; Cefalu and Wagner, 1997; Clarkson, *et al*., 1984, 1987; Weingand, 1989). Once prior studies demonstrated the safety of CR in nonhuman primates (Ingram, *et al*., 1990; Kemnitz, *et al*., 1993), a clinical trial to evaluate the effect of CR on the pathogenesis and extent of atherosclerosis in cynomolgus monkeys was initiated.

This report summarizes clinical observations from the trial regarding cardiovascular risk factors and presents preliminary findings of the effect of CR on atherosclerosis extent. The observations regarding the lipid findings are compared to the ongoing CR project in rhesus monkeys conducted at the University of Wisconsin (UW).

MATERIALS AND METHODS

Animals. A clinical trial to evaluate the effect of CR on the pathogenesis and extent of atherosclerosis was initiated in l993 at the Wake Forest University School of Medicine, Comparative Medicine Clinical Research Center in Winston-Salem, North Carolina. We hypothesized that CR over a sustained period, when compared with an *ad libitum* (AL) diet with equivalent cholesterol intake, would retard the progression of atherosclerotic lesions by attenuating age-associated increases in vascular tissue and blood levels of early and advanced glycated products (e.g., glycated protein and protein cross-links), improving peripheral insulin sensitivity and reducing intra-abdominal fat. The hypothesis was tested in an aging cohort of cynomolgus monkeys (*Macaca fascicularis*) using dietary intervention in the form of CR compared to ad libitum food intake. The diets were designed so that the animals randomized to CR had identical dietary cholesterol intake with the control animals, despite the reduced calories, in order to evaluate the effect of CR on atherosclerosis, independent of plasma cholesterol levels. The study design, diet composition, specific methods, and results of the first year of study have been previously reported (Cefalu, *et al*., 1997b).

Briefly, beginning in l993, 32 feral adult male monkeys were initially fed a moderately atherogenic diet (0.25 mg cholesterol/Cal) containing 30% of calories from fat. After a 6-month pretrial phase, animals were randomized to continue the control diet or to consume a CR diet (30% reduction from each animal's AL baseline dietary intake). To ensure that cholesterol intake per kg body weight was identical between the groups, the CR diet was supplemented with crystalline cholesterol. The dietary intervention continued for 4 years. Insulin resistance, blood and tissue glycation/advanced glycation, abdominal fat content, and lipid levels were measured at specified intervals over 4 years as previously outlined (Cefalu, *et al*., 1997b). The trial evaluations are outlined in Table 1.

In an effort to provide a comparative basis for assessing CR, findings from the Wake Forest cohort were compared to those observed for rhesus monkeys (*Macaca mulatta*) as part of the UW CR project. The diets and experimental design of the UW CR trial have been previously described (Kemnitz, *et al*., 1993). Briefly, after obtaining baseline measures, animals from the UW cohort were assigned to 1 of 2 treatment regimens: control (fed AL 6–8 h a day) or CR (fed 70% of their baseline intake). The rhesus monkeys were fed a purified diet with corn oil (10% of diet weight) as the major source of fat. Results for body weight, physical activity, metabolic rate, carbohydrate metabolism, and immunologic function have been previously reported (Kemnitz, *et al*., 1993; Kim, *et al*., 1997; Ramsey, *et al*., 1997; Roecker, *et al*., 1996). Animals from the UW study were randomized in 2 phases: Study of Group 1 (30 males) was initiated in l989 and study of Groups 2 (30 females) and 3 (16 males) was initiated in l994. The Group 1 ages ranged from 17–23 years, and Groups 2 and 3 had age ranges of 12–18 years at the time of the present studies. The methods used for the UW cohort (rhesus monkeys) and the Wake Forest cohort (cynomolgus monkeys) for lipid composition and low density lipoprotein (LDL)-proteoglycan (PG) interaction were similar, as described previously (Edwards, *et al*., 1998).

TABLE 1
Trial Evaluations

Types of measurements
Descriptive
Age
Clinical
Body weight, blood pressure, EKG, CT assessment of abdominal fat, and body measures
Biochemical
Lipids (TPC, TG, LDL-C, HDL-C)
Physiologic
Frequent sampling intravenous glucose tolerance testing (modified minimal model)
Glycemic indices
Tissue (iliac artery glycation and advanced glycation)
Skin collagen (glycation and advanced glycation)
Blood (glycated hemoglobin, fasting glucose, serum fructosamine)

Atherosclerosis measurements. Atherosclerosis extent for the Wake Forest cohort was assessed in the abdominal aorta. Sections of aorta for histologic studies were fixed in paraformaldehyde, then dehydrated, embedded in paraffin, and stained with Verhoeff-van Gieson's stain. Sections were projected onto a screen and the intimal area was measured with a digitizer (Clarkson, *et al*., 1980). The extent of atherosclerosis was expressed both as the mean intimal area and as the mean intimal area divided by the internal elastic lamina.

Summary of Progress to Date

The clinical characteristics, study design, and diet composition of the CR trial in cynomolgus monkeys were previously reported (Cefalu, *et al*., 1997b). The monkeys randomized to CR had a 30% reduction of caloric intake compared to the control group, which resulted in lower body weight. The effects of CR to improve insulin resistance and abdominal obesity were observed after less than one year of study. Insulin sensitivity, for example, was improved by 6 months of intervention and was maintained through month 30 of the study (Cefalu, *et al*., 1997a) (Fig. 1). In addition, both total and intra-abdominal fat masses were reduced in the monkeys randomized to CR (Fig. 2).

As discussed above, additional crystalline cholesterol was added to the diets fed to CR monkeys to maintain a similar cholesterol intake to controls. Total plasma cholesterol (TPC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), or TPC/HDL-C ratios did not differ between CR and control groups (Fig. 3). In contrast are the observations from the UW monkey cohort, where CR significantly reduced plasma triglyceride (TG) concentration with no significant effect on TPC, HDL-C or LDL-C (Edwards, *et al*., 1998) (Table 2). Also, CR in the UW cohort significantly reduced LDL phospholipid and TG content, while significantly increasing cholesterol ester content (Edwards, *et al*., 1998).

The compositional changes in LDL induced by CR in the rhesus monkeys (UW cohort) appeared to alter the functional properties of the particle. Specifically, *in vitro* binding studies that assessed LDL interaction with isolated arterial PG demonstrated a 35% decrease in the amount of LDL complexed to PG for LDL from CR vs. control rhesus monkeys (Fig. 4A). A similar analysis was performed for LDL from CR and control cynomolgus monkeys from the Wake Forest study. As demonstrated in Figure 4B, there was no difference in LDL complexed to PG for this animal cohort. In addition to the LDL-PG interaction studies, LDL oxidation studies were determined for both cohorts of animals. Employing 2 methods of assessing oxidation of LDL, no difference

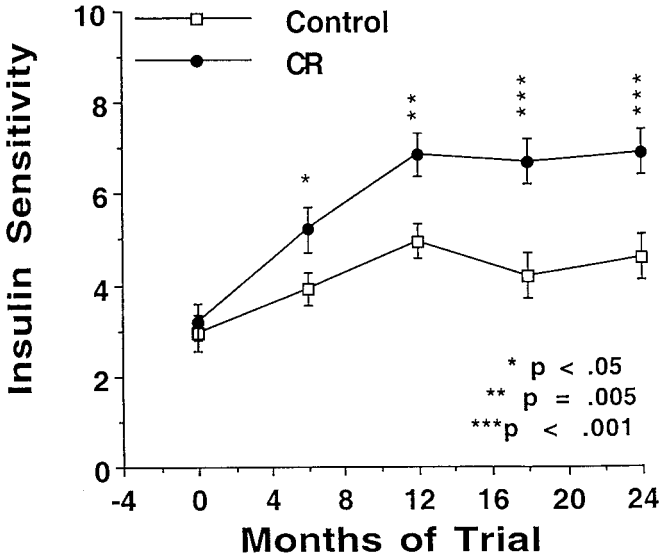

FIG. 1. Insulin sensitivity measures in control and CR animals as part of the Wake Forest University cohort.

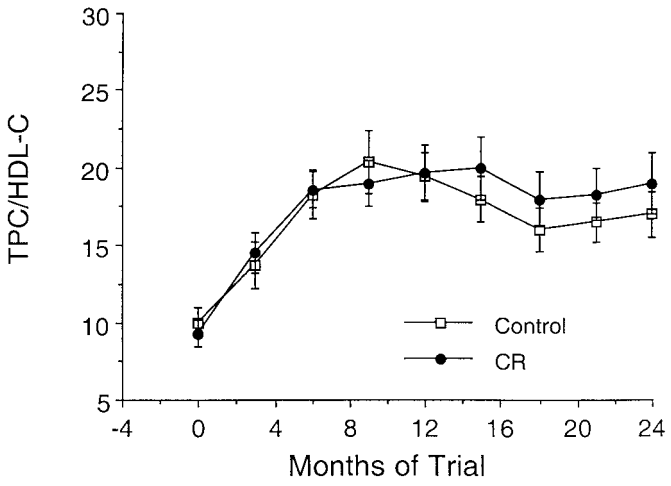

FIG. 3. Total plasma cholesterol/HDL-cholesterol ratio in cynomolgus monkeys.

between CR and control groups was found in either trial (authors′ unpublished observations).

To evaluate the effect of CR on atherosclerosis, intimal area was measured in the abdominal aorta. As shown, there was no difference in lesion size for the aorta in monkeys randomized to CR when compared to control (Fig. 5).

DISCUSSION

We have presented findings to date on the influence of CR in altering cardiovascular risk factors and atherosclerosis extent in a nonhuman primate model of atherosclerosis. In addition, we have compared lipid findings from two species of monkeys subjected to similar degrees of CR, but with different nutritional composition. The data indicate that CR favorably improves risk factors such as body weight, abdominal obesity, and insulin resistance in both rhesus and cynomolgus monkeys. With specific regard to lipids, no effect of CR was observed in lipid measurements of the cholesterol-fed cynomolgus monkeys, whereas CR favorably improved lipid profile, LDL chemical composition, and LDL-PG interaction in rhesus monkeys. Further, the atherosclerosis extent for the abdominal aorta was not found to be different, in preliminary analyses between the cholesterol-fed cynomolgus monkeys randomized to CR, from that of the controls.

The observation that CR improves body weight and reduces body fat in nonhuman primates has been previously reported in three cohorts of nonhuman primates. Lane, *et al*. (1992) have demonstrated from the NIA study that chronic CR was effective in reducing body fat in the CR rhesus monkeys, compared to controls. These observations were also demonstrated in

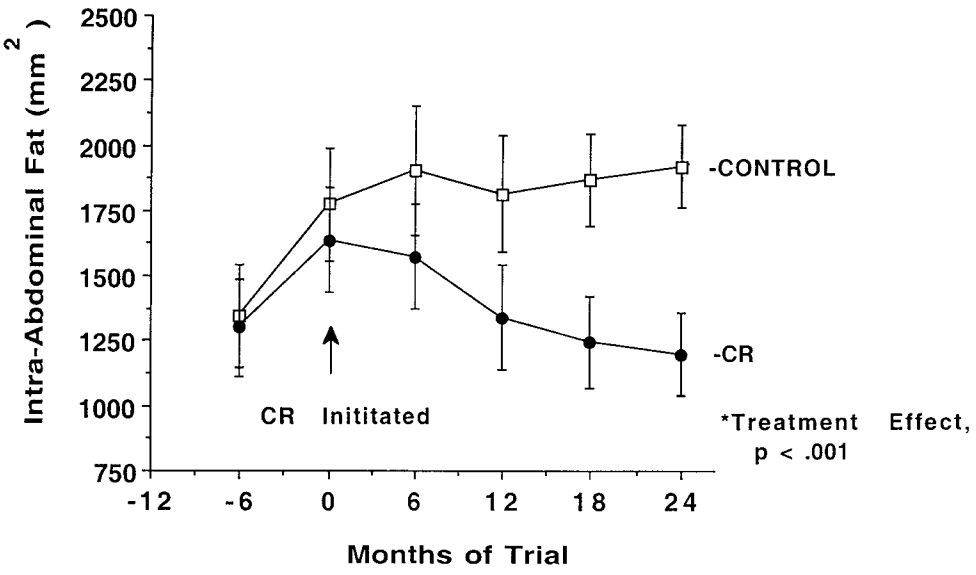

FIG. 2. Demonstrates the effect of CR vs. control on intra-abdominal fat mass in cynomolgus monkeys.

TABLE 2
Plasma Lipids

	TPC	TG	HDL-C	LDL-C
Rhesus				
Control ($n = 34$)	182 ± 6 (170,193)	139 ± 24 (91,187)	88 ± 4 (79,97)	69 ± 4 (60,77)
CR ($n = 32$)	175 ± 6 (162,188)	66 ± 8 (48,83)	92 ± 4 (84,99)	70 ± 3 (64,76)
p value[a]	0.45	0.0001	0.49	0.77

Note. From Edwards, *et al.*, 1998.
[a] t test of CR versus control. TG log-transformed prior to analysis.

rhesus monkeys studied by Kemnitz, *et al*. (1993) from the University of Wisconsin. In addition, Hansen and Bodkin (1993) have reported weight maintenance studies in rhesus monkeys in their cohort. These studies have used both anthropometric measures and DEXA scans to monitor body fat content, and there appears to be little doubt that chronic CR favorably improves body fat content. However, the cynomolgus monkey trial differs from the other primate studies in that abdominal fat distribution was assessed with CT scans, allowing separation into intra-abdominal, subcutaneous, and paraspinous fat. Our data show not only a decrease in total abdominal fat mass, but also a reduction in the intra-abdominal fat depot that has been most significantly linked to cardiovascular disease in both human and nonhuman primates (Després, *et al*., 1989; Fujioka, *et al*., 1987; Kissebah, *et al*., 1988; Matsuzawa, *et al*., 1995; Shively, *et al*., 1987). It is of interest in our study that the intra-abdominal fat mass showed the most significant change secondary to CR in the first year of study when compared to both the subcutaneous and paraspinous fat (Cefalu, *et al*., 1997b). Further, this finding is of interest from a gerontologic perspective as it has been recently reported that central adiposity accumulates with age and it is the intra-abdominal fat depot that can most readily explain the variance in insulin resistance with age (Cefalu, *et al*., 1995; Coon, *et al*., 1992; Kohrt, *et al*., 1993).

This finding of improved insulin sensitivity in chronic CR states confirms the observations first reported by the UW investigators (Kemnitz, *et al*., 1994), and indeed the methods for assessing insulin sensitivity (viz., minimal model technique) were similar. This finding has been confirmed by investigators at the NIA (Lane, *et al*., 1995) and the University of Maryland (Hansen and Bodkin, 1993) when evaluating CR and insulin action in nonhuman primates. It is now clear that an improvement in insulin sensitivity is one of the most consistent features of sustained CR as observed in both rodent and nonhuman primate studies.

In addition to body weight and insulin levels, detailed lipid evaluations were assessed. In cynomolgus monkeys randomized to CR yet fed the identical dietary cholesterol as the control group, CR did not alter the lipid levels (Fig. 3). It must be pointed out, however, that this was expected by the design of the study. Indeed, we sought to maintain similar plasma lipid levels between groups, and this was achieved by supplementing the CR diet with crystalline cholesterol. However, when

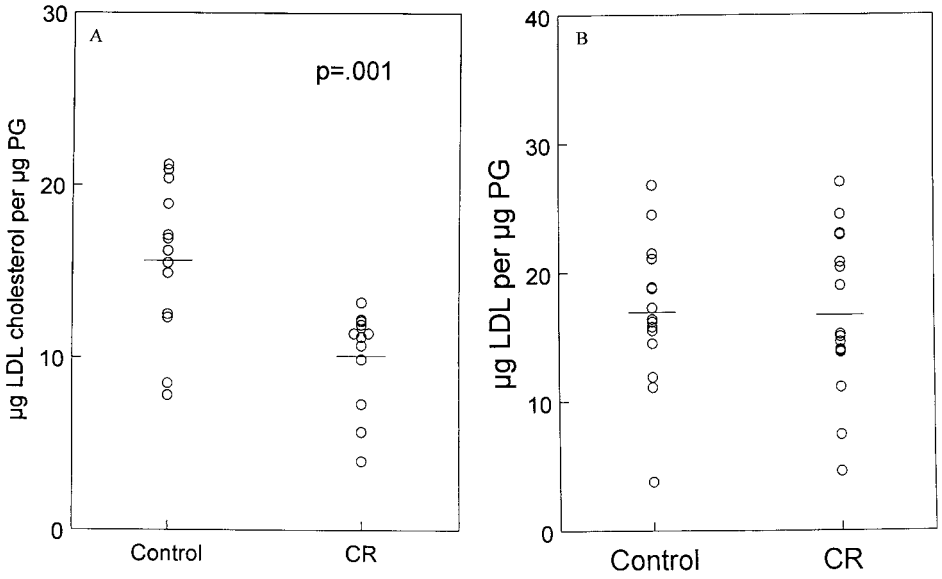

FIG. 4. Demonstrates plasma LDL-proteoglycan binding in rhesus monkeys as part of the UW study (A), and for the cynomolgus monkeys as part of the Wake Forest University study (B).

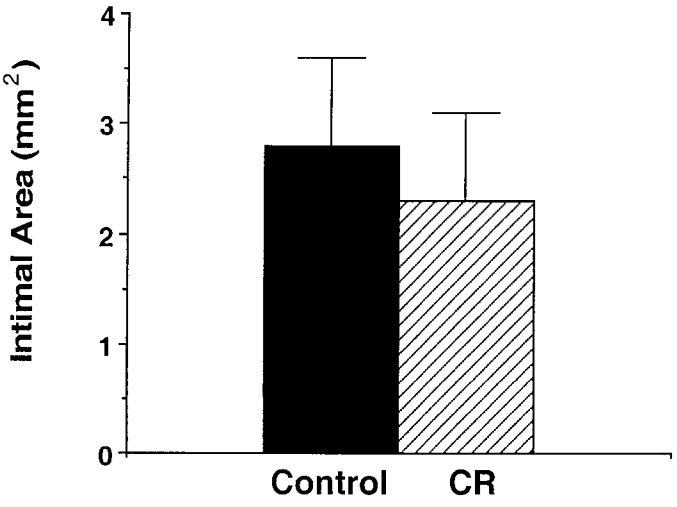

FIG. 5. Demonstrates intimal area observed for cynomolgus monkeys.

comparing the effect of CR *per se* on lipid levels in monkeys as part of the UW study, we found that lipid levels are favorably affected by CR in rhesus monkeys (Edwards, *et al*., 1998). In particular, TG levels were significantly lowered by CR compared to controls in the UW CR project. It was also observed that specific lipid and lipoprotein profiles may be favorably affected by CR, as demonstrated by Verdery, *et al*. (1997) for rhesus monkeys in the NIA cohort. Specifically, CR resulted in decreased TG levels in adult animals and increased levels of HDL2b, the HDL fraction associated with cardioprotection. Differences in HDL size were associated with glucose, insulin, and lean body mass.

In addition to the lipid levels, there has been significant interest in the LDL chemical composition. It is now clear that all LDL are not equally atherogenic and that even in the absence of high levels of LDL, certain characteristics of LDL particle composition and structure increase the risk for atherosclerosis. For the monkeys in the Wake Forest University trial, there appeared to be no effect of CR to alter the LDL chemical composition (authors′ unpublished observations). In contrast, in rhesus monkeys subjected to CR at the UW, significant increases in cholesterol esters and significant decreases in TG and phospholipid content in the LDL particle were found (Edwards, *et al*., 1998). It appears that dietary cholesterol may override the effects of CR on LDL chemical composition. However, species differences in sensitivity of lipid metabolism to CR cannot be ruled out.

Mechanisms underlying the atherogenicity of particular LDL particles are poorly understood. One process that is thought to mediate the entrapment and retention of LDL in arterial tissue is its interaction with arterial PG (Hurt-Camejo, *et al*., 1997). *In vitro* PG-LDL binding studies have shown that human LDL with high affinity for PG is associated with coronary heart disease (Camejo, *et al*., 1976; Linden, *et al*., 1989). Moreover, experimental changes in the dietary fat intake of nonhuman primates modulated the PG binding properties of LDL in a direction consistent with the effects of the diet on atherosclerosis (Edwards, *et al*., 1991).

LDL binding to arterial PG was measured as part of the cynomolgus monkey trial. The results failed to demonstrate an effect of CR on LDL-PG binding. In contrast, however, we have previously reported a 35% decrease in the amount of LDL complexed to PG from CR vs. control rhesus monkeys as part of the UW cohort. In that study, a strong positive correlation was observed between PG binding and numbers of TG molecules per particle (Edwards, *et al*., 1998). Phospholipid molecules per particle were also significantly associated with PG binding when the groups were pooled, but not when control and CR groups were analyzed separately. It was reported that among LDL particle composition variables, the number of molecules of TG per LDL particle remained significantly correlated with LDL-PG binding, following control for treatment allocation and duration of treatment (Edwards, *et al*., 1998). It appeared that no other LDL particle composition was significantly related to binding. The explanation for the lack of effect on LDL-PG binding in animals randomized to CR in the Wake Forest cohort, as compared with the UW cohort, is most likely due to differences in diet composition. In addition, the dietary cholesterol intake was similar for the CR and control animals in the Wake Forest study, resulting in no difference in plasma lipid composition. Also, the rhesus monkeys were primarily fed a purified diet with corn oil representing 10% of diet weight as the major source of fat, while the cynomolgus monkeys consumed high fat and high cholesterol diets. Indeed, as seen from the results, cynomolgus monkeys achieved lipid levels of approximately 350 mg/dl of total cholesterol compared to roughly half that in the rhesus cohort.

The results from all of the ongoing nonhuman primate CR trials have demonstrated that cardiovascular risk factors such as insulin sensitivity, body weight, and body fat distribution are improved by CR. Beneficial effects on these risk factors should ultimately reduce atherosclerosis extent. As part of the Wake Forest trial, arteries were obtained to assess lesion extent and composition. At the time of writing this manuscript, we have completed the analysis for the abdominal aorta and have found no difference in atherosclerosis extent. These findings may not be all that surprising, given the lack of change in major risk factors in the CR group, most notably lipid levels, LDL chemical composition, and PG binding. One of the major aims of the Wake Forest trial was to maintain plasma lipid levels and to observe the effect of CR *per se*. It is, thus, fair to say that this was indeed a hyperlipidemic model, and that the experimental design demonstrated that CR improves cardiovascular risk factors, such as insulin sensitivity and body fat distribution. However, these appeared to be negated in the face of markedly elevated cholesterol. In contrast, the UW rhesus cohort, whose lipid intake was relatively lower when compared to the cynomolgus monkeys and whose serum levels were more moderate, showed compositional and quantitative changes in the profile.

Therefore, these early results demonstrate that CR may have profound and favorable effects on cardiovascular risk factors such as insulin sensitivity and visceral fat accumulation. However, favorable atherosclerosis outcomes to be expected by these improved parameters may be negated in hypercholesterolemic states.

In conclusion, it is apparent that CR is effective in reducing body fat and body fat distribution and in improving insulin sensitivity. Our particular study demonstrates that in the presence of hyperlipidemia, CR may not favorably improve atherosclerosis extent, nor functional characteristics of the LDL. Further studies on the ongoing UW and NIA trial are required to fully evaluate the influence of CR on atherogenesis in nonhuman primates.

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