The Effect of Caloric Restriction on Glycation and Glycoxidation in Skin Collagen of Nonhuman Primates

David R. Sell, Mark A. Lane, Mark E. Obrenovich, Julie A. Mattison, April Handy, Donald K. Ingram, Richard G. Cutler, George S. Roth, and Vincent M. Monnier

The accumulation of Maillard reaction products increases with age in long-lived proteins and can be retarded by caloric restriction. Here we determined whether caloric restriction inhibits formation of glycation and glycoxidation products in skin collagen of squirrel and rhesus monkeys between 1990–1997. Restricted monkeys (n = 11, n = 30, respectively) were maintained at 70% of caloric intake of controls (n = 25, n = 32, respectively). Glycation was assessed by furosine and glycoxidation by pentosidine and carboxymethyl-lysine. With age, the rate of furosine formation moderately but nonsignificantly (p > .05) increased in both control monkey groups. It significantly (p = .011) decreased in the caloric-restricted rhesus, but not squirrel monkeys. Caloric restriction did not significantly decrease the pentosidine or carboxymethyl-lysine rates in either species of monkeys. These results suggest that caloric restriction, when maintained long-term in nonhuman primates, tends to decrease glycation, but not glycoxidation.

One of the primary goals of aging research is to improve quality of life in old age by delaying or preventing age-related disease and disabilities. Because of this objective, there is a pragmatic interest in the development and study of interventions that retard aging and its deleterious processes. The most efficacious intervention evaluated to date is caloric restriction (CR). This nutritional intervention is able to retard the deleterious effects of aging and extend both average and maximum life spans in rodents and other short-lived species (1–3).

Since its initial report in the mid-1930s, experimentation on CR has been made using these short-lived animals. Beginning in the late 1980s, various studies were initiated in longer-lived nonhuman primates (4–6) to assess the relevance of CR to aging in humans (7,8). Currently, 3 major trials at the National Institute on Aging (NIA), the University of Wisconsin (UW), and the University of Maryland (UMD) are under way to evaluate CR in monkeys (2). The goal of the 3 studies is to determine whether CR will exert beneficial antiaging and antidisease effects in monkeys, a species much more closely related to humans than previously investigated (9).

Several hypotheses have been proposed regarding mechanisms by which CR retards aging and disease (3). One that bears interest and importance to the present research has its biological plausibility in equilibrates with ambient glucose concentration (20), and there is a progressive decline in collagen turnover with age (21).
Collagen Glycation and Caloric Restriction in Monkeys

However, these factors are likely accelerated by the decline in both glucose tolerance and insulin responsiveness with age in rodents (22,23), monkeys (24–26), and humans (27). Importantly, CR, an important modulator of aging rate, is able to ameliorate glucose tolerance when maintained long term in rodents (22,28) and rhesus monkeys (24–26). Even in a short-term study using 18-year-old rhesus monkeys maintained on CR (30%) for 1 year, insulin response to a glucose challenge was improved over that observed in ad libitum-fed control monkeys (29).

It has previously been hypothesized that the age-related increase in levels of glycation and glycoxidation may be associated with the deterioration in glucose tolerance and insulin responsiveness with age (3,10,30,31). Indeed, CR was able to inhibit the age-related formation of these products in rodent skin and tendon (10,19,32). Thus, consistent with this hypothesis, the objective of the present study was to determine whether chronic CR in squirrel and rhesus monkeys, 2 species susceptible to the effects of glucose intolerance at late age, would also inhibit glycation and glycoxidation of skin collagen as reflected in measures of pentosidine, furosine, and carboxymethyl-lysine.

Methods

Animals and Diets

The cohorts of squirrel and rhesus monkeys biopsied for this study and its design of the dietary restriction trial, including animal husbandry, care, and dietary regimens, have been described elsewhere (2,4,26) and are briefly recapitulated here. Animals were maintained at the Primate Unit, National Institutes of Health (NIH) Animal Center, Poolesville, Maryland. Experimental animals were restricted to 70% of intakes for controls of comparable age and body weight; i.e., 30% CR, as previously detailed (26). The diet had been supplemented with higher than recommended levels of essential vitamins and minerals to ensure that the restricted monkeys were not deficient in essential nutrients.

Monkeys entered the study at juvenile, adult, or old age and were followed up to 7 years from 1990 to 1997. The age stratification to these age groupings have been previously published (2,4,26) and summarized in Table 1.

Procurement of Skin Biopsies

Skin biopsies were collected from a total of 98 individual monkeys at five different time periods consisting of years 1990, 1991, 1993, 1995, and 1997. At each sampling, a 4-mm skin biopsy was punched from the region of the proximal dorsal/lateral left forearm for squirrel monkeys, or from the dorsal/lateral region above the left fifth rib for rhesus monkeys, by the attending veterinarian at the NIH Animal Center. All biopsies were performed under ketamine anesthesia (7–10 mg/kg intramuscular). The biopsies were immediately rinsed twice in 0.9% saline and frozen at −80°C until shipped on dry ice to Case Western Reserve University (Cleveland, OH). Upon arrival, the specimens were immediately stored frozen at −80°C until processing.

Tissue Processing

Biopsies were coded and received without knowledge of the species, age, diet, or group. As previously described (16), each biopsy was frozen in a dry-ice methanol bath followed by removal of excess hair and any noticeable fat with a scalpel blade. The remaining portion of the biopsy was coarsely minced and suspended in 5 ml of 2:1 chloroform-methanol for delipidation on a rotary shaker at 4°C for 18 hours. After centrifugation at 1710 × g (Sorvall RT 6000B; Du Pont, Wilmington, DE), the solvent was aspirated. Tissues were homogenized (Polytron PT 10/35; Brinkmann, Westbury, NY) in pH 7.4 phosphate-buffered saline at 4°C for 1 minute and centrifuged at 14,123 × g (Sorvall RC5C; Du Pont) for 20 minutes. The supernatant was discarded. Samples were extracted sequentially by rotary-shaking for 18 hours at 4°C in salt, acetic acid, and pepsin as previously described (16). Upon centrifugation, the pellet of insoluble collagen was lyophilized.

Table 1. Summary of Body Weight, Serum Glucose, and Insulin Levels of Monkeys Represented in the Studya

<table>
<thead>
<tr>
<th>Data</th>
<th>Parameter</th>
<th>Age Grouping</th>
<th>Control</th>
<th>Caloric restricted</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhesus 1997</td>
<td>Body weight (kg)</td>
<td>JA</td>
<td>11.8 ± 0.72 (20)</td>
<td>9.0 ± 1.79 (16)</td>
<td>.004</td>
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<tr>
<td></td>
<td></td>
<td>O</td>
<td>10.1 ± 0.91 (7)</td>
<td>7.8 ± 0.48 (8)</td>
<td>.035</td>
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<td></td>
<td>Glucose (mg/dl)</td>
<td>JA</td>
<td>65.7 ± 2.01 (20)</td>
<td>66.1 ± 2.40 (15)</td>
<td>.878</td>
</tr>
<tr>
<td></td>
<td></td>
<td>O</td>
<td>64.4 ± 1.99 (7)</td>
<td>63.4 ± 1.21 (8)</td>
<td>.649</td>
</tr>
<tr>
<td></td>
<td>Insulin (IRI µIU/ml)</td>
<td>JA</td>
<td>17.8 ± 4.66 (20)</td>
<td>9.6 ± 1.63 (15)</td>
<td>.143</td>
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<td></td>
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<td>O</td>
<td>11.5 ± 2.34 (6)</td>
<td>10.9 ± 2.25 (7)</td>
<td>.847</td>
</tr>
<tr>
<td>Glucose tolerance test (GTT Rhesus)</td>
<td>Glucose (mg/dl)</td>
<td>JA</td>
<td>58.2 ± 1.95 (17)</td>
<td>55.3 ± 1.62 (18)</td>
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<td>O</td>
<td>65.4 ± 8.35 (8)</td>
<td>53.9 ± 3.06 (8)</td>
<td>.220</td>
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<tr>
<td></td>
<td>Insulin (IRI µIU/ml)</td>
<td>JA</td>
<td>12.3 ± 1.08 (16)</td>
<td>7.5 ± 1.18 (18)</td>
<td>.003</td>
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<td>O</td>
<td>13.3 ± 2.03 (8)</td>
<td>12.0 ± 3.85 (8)</td>
<td>.775</td>
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<td>Squirrel 1997</td>
<td>Body weight (kg)</td>
<td>JA</td>
<td>0.87 ± 0.09 (14)</td>
<td>0.85 ± 0.12 (10)</td>
<td>.395</td>
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<tr>
<td></td>
<td>Glucose (mg/dl)</td>
<td>JA</td>
<td>74.9 ± 3.35 (13)</td>
<td>77.1 ± 3.09 (9)</td>
<td>.652</td>
</tr>
</tbody>
</table>

Notes: aParameter ± SEM with the total number of monkeys represented shown in parentheses.

1 JA = juvenile; A = adult; O = old. Age groups were initiated at the beginning of caloric restriction during the year 1987 as follows (age in years): squirrel monkeys, J < 5; A < 10; O < 16; rhesus monkeys, J < 2; A < 5; O = 13–24.
Acid Hydrolysis and Quantitation of Collagen

Up to 5 mg of tissue was weighed and hydrolyzed in 13 × 100 mm Kimax screw-top tubes (Kimble/Kontes, Vineland, NJ) with 1 ml of de-aerated 6 N HCl (Fisher Optima Brand, Fisher Scientific, Pittsburgh, PA). Each tube was purged for 1 minute with N₂ before sealing the tube with a PTFE [polytetrafluoroethylene]-faced, rubber-lined screw cap. The samples were placed in a heated oven at 110°C for 18 hours. The HCl was evaporated with a SpeedVac Concentrator (Model AS160; Thermo-Savant, Holbrook, NY). After drying, each residue was resuspended in 1 ml of distilled water and filtered with a Spin-X Centrifuge Tube Filter (Costar, Corning Inc., Corning, NY) containing a 0.45-μm cellulose acetate membrane. The filtrate was collected in a 2-ml polypropylene tube after centrifugation at 7000 × g using a microcentrifuge TOMY (MRX-151; Peninsula Laboratories, Belmont, CA). The amount of collagen in each filtered sample was determined by the hydroxyproline assay as previously described (16). This assumes that collagen is composed of 14% hydroxyproline by weight (33).

Analytical Assays

Furosine was assayed using a modification of the method of Resmini and colleagues (34). An aliquot of acid-hydrolyzed collagen sample equivalent to 90 μg hydroxyproline was dried-down by the SpeedVac (Thermo-Savant) in a 12 × 75 mm glass tube. The residue was reconstituted with 200 μl of water followed by the injection of a 50-μl aliquot, the equivalent to 22.5 μg hydroxyproline, onto a 4.6 × 250 mm furosine-dedicated high-pressure liquid chromatography (HPLC) column (Spec Furo; Alltech, Deerfield, IL) using a Waters HPLC system (Waters, Milford, MA) as previously described (10). Buffer A contained 0.4% acetic acid (v/v). Buffer B was the same as buffer A except for the addition of 0.27% KCl (w/v). Initially, buffer A was run isocratically for 12.5 minutes, then a gradient from 0%–10% B for 7 minutes, and finally isocratic at 10% B for a further 15.5 minutes. The elution of furosine at 33 minutes was monitored at 280 nm using a model 486 Waters absorbance detector interfaced to a computer loaded with Azur chromatography software (Datalys, Saint Martin D’Heres, France).

Pentosidine was assayed using the same HPLC system as previously described for furosine with pyridoxamine (Sigma, St. Louis, MO) used as the internal standard. The equivalent of 45 μg hydroxyproline contained in 100 μl of water-reconstituted sample and spiked with 50 ng pyridoxamine was injected onto a 4.6 × 250 mm Vydac 218TP54 HPLC column (The Separations Group, Hesperia, CA). Solvent A was water and 0.01 M heptafluorobutyric acid (HFBA), Solvent B contained 60% acetonitrile and 0.01 M HFBA. Initially, 2% B was run isocratically for 9 minutes, and linearly increased to 19% B at 10 minutes, 28% B at 35 minutes, 31% B at 44 minutes, and 100% B at 45 minutes. The latter was held for 10 minutes before column reequilibration. Pyridoxamine and pentosidine eluted at 23 and 37 minutes, respectively, as monitored by a Jasco 821-FP spectrofluorometer (Jasco, Easton, MD) at excitation/emission 335/385 nm.

Carboxymethyl-lysine was determined by a dual-column procedure previously described by this laboratory (35). The first step involved the collection of the peak using the HPLC gradient and solvent system described for pentosidine. With this system, carboxymethyl-lysine eluted at 9 minutes as identified by postcolumn derivatization described below using a standard. The underivatized peak from each sample was collected into 13 × 75 mm borosilicate tubes in a volume of 5 ml using a FRAC-100 fraction collector (Amersham Pharmacia Biotech, Piscataway, NJ). The contents were dried down in a SpeedVac concentrator (Thermo-Savant) and reconstituted with 200 μl of solvent A from the second injection system. A volume of 50 μl from each sample was reinjected onto a reverse-phase column as described for pentosidine using the following solvent system: solvent A contained 5% 1-propanol (Mallinckrodt ChromAR HPLC; Mallinckrodt Baker Inc., Paris, KY), 3 grams/liter sodium dodecyl sulfate (Fluka, Buchs, Switzerland), and 1 gram/liter monobasic sodium phosphate (Mallinckrodt) adjusted to pH 2.8 with ultra-pure phosphoric acid (Aldrich, Milwaukee, WI); solvent B, same as solvent A except for 1-propanol concentration increased to 60%. Carboxymethyl-lysine was separated by applying a linear gradient of 15% to 22% B from 0 to 30 minutes at a flow rate of 1 milliliter/minute. The elution of carboxymethyl-lysine at 28 minutes was detected by postcolumn derivatization with o-phthalaldehyde in the presence of 2-mercaptoethanol (Aldrich) as described (35) at excitation/emission 340/455 nm with the Jasco spectrofluorometer.

Experimental Measurements of Body Weight, Glucose, and Insulin, and Administration of the Glucose Tolerance Test

Body weight and endocrine data are presented in Table 1 for the year 1997. These data represent a snapshot of data relevant for the comparison of glycation and glycoxidation markers. Procedures for measurements have been detailed previously (26) and hence are only summarized here. All skin samples were obtained while the monkeys were under ketamine anesthesia after an overnight fast. Blood samples were obtained by a single puncture of the femoral vein within 10–12 minutes of ketamine anesthesia. Serum was prepared by centrifugation and stored at −20°C before analysis. Glucose levels were measured by the glucose oxidase method using a Beckman Glucose Analyzer (Beckman Instruments, Fullerton, CA), and insulin by radioimmunoassay (Diagnostic Products, Los Angeles, CA) as described (26). The method for administrating the glucose tolerance test to these monkeys has previously been detailed by Lane and colleagues (29).

Statistical Analyses

Pearson correlations, regression equations, and analyses of variance (ANOVA) were by SPSS (Graduate Version 9, SPSS, Inc., Chicago, IL). Other statistical procedures including tests for homogeneity and normality of data and the comparison of slopes between regression lines are described elsewhere (32,36). Statistical significance was accepted as p < .05.
RESULTS

The Effect of Age and Dietary Regimen on Body Weight

Analyses were performed following stratification of both squirrel and rhesus monkeys into juvenile (J), adult (A), and old (O) age groups as defined in Table 1. Data consisting of 1997 body weights along with serum glucose and insulin levels at fasting, and a glucose tolerance test administered to rhesus monkeys, are summarized in Table 1. For most analyses, the J + A groups were combined since they differed little in terms of age at the time of analyses (i.e., 1–5 year range).

For rhesus monkeys, the results show the following: (a) Body weights were significantly reduced by CR in both J + A (p = .004) and O (p = .035) monkey groups; (b) CR induced no reduction in glucose levels in any of these groups nor was there a significant age-related increase in serum glucose; and (c) there was a nonsignificant (p = .143) trend toward reduced insulin levels in the CR J + A groups, but not in the older group. Subsequent analysis of glucose tolerance data test with more samples and tolerance analyzed under more controlled conditions showed a trend toward lower glucose in the CR groups of control versus CR (Table 2). Results showed that slopes (i.e., rate of change in furosine...
levels) were significantly different between control and CR for rhesus ($p < .005$), but not squirrel monkeys ($p > .05$).

Second, and as expected, pentosidine significantly increased with age in both control squirrel ($p = .004$) and rhesus monkeys ($p = .02$) as modeled by nonlinear regression (Figure 2, Table 2). For CR monkeys, the increase was significant for squirrel ($p = .007$), but not rhesus ($p = .49$). Subsequently, the rate of pentosidine formation was modeled by exponential functions as shown in Table 2: $y = a(10^{bx})$, where $y =$ pentosidine level and $x =$ age, of which the acceleration was assessed by coefficient “b,” or the slope of the age function given in Table 2. Rates were not affected by control versus CR feeding in squirrel monkeys ($b = 0.025$ vs. 0.028). In rhesus monkeys, the rate was decreased about 3-fold by CR ($b = 0.007$ vs. 0.002). However, the difference in slopes for the latter comparison between the control and CR rhesus monkeys did not reach statistical significance ($p = .40$). In further investigation, pentosidine-formation rates in these graphs (Figure 2) were compared for squirrel versus rhesus monkeys. In all cases, these rates were found significantly greater in squirrel monkeys compared with rhesus monkeys for both controls (i.e., 0.025 vs. 0.007, $p = .036$) and diet-restricted monkeys (i.e., 0.028 vs. 0.002, $p < .005$), as previously described (16).

Thirdly, carboxymethyl-lysine levels showed no significant ($p > .05$) change with age, species, or diets (Figure 3); thus, no further statistical analyses were made.

Analysis according to age groupings.—When levels were stratified into age groups as defined in Table 1, there were no significant ($p > .05$) differences within a species for age group and dietary regimen as tested by ANOVA for all three parameters (data not shown). However, the analysis of furosine levels versus age groups and diets showed several interesting trends in older monkeys of both species.

First, this analysis failed to show CR as a significant factor when furosine levels were pooled across all age groupings of rhesus monkeys ($p > .80$), although the age grouping $\times$ diet interaction was significant ($p = .023$). This interaction can be observed in the data clusters for rhesus monkeys presented in Figure 1, where levels are reduced in the cluster for CR rhesus versus the cluster for control rhesus monkeys at ages >16 years, but not in these clusters at ages <16 years. A plot of this interaction as a function of age groupings (i.e., juvenile, adult, and old, coded as 1, 2, and 3, respectively) showed that levels nonsignificantly ($p = .18$) increased with age grouping in control rhesus monkeys and significantly ($p = .043$) declined with age grouping in CR rhesus monkeys.

Second, and interestingly, furosine levels were consistently though not significantly ($p > .05$) lower in CR rhesus monkeys compared with the controls, but only in the old age grouping at each assay period when furosine levels were determined (i.e., 1993, 1995, and 1997: 288 ± 31, 239 ± 18, 280 ± 36 versus 375 ± 118, 313 ± 72, 354 ± 34 pmoles/mg collagen, respectively). In this analysis, the overall difference in mean levels (i.e., 264 ± 17 versus 339 ± 38) approached statistical significance ($p = .064$). Likewise, there was a nonsignificant ($p = .079$) trend for furosine levels measured in 1997 to be reduced by CR in the adult group of squirrel monkeys versus the controls (i.e., 242 ± 27 vs. 341 ± 45 pmol/mg collagen).

Interrelations Between Parameters of Glycoxidation and Glycation

Levels for furosine, pentosidine, and carboxymethyl-lysine were found significantly related to each other as summarized for all data points from individual monkeys, diets, and species as shown in Figure 4. Furosine was most significantly ($p < .0001$) correlated ($r = .37$) with
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whole without regard to individual monkeys did differences
measurements were pooled and collectively analyzed as a
7 years, a great deal of variability was still noted. Only when
parameters were followed individually in each monkey over
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monkeys correlated significantly with their respective
Likewise, 1997 skin furosine levels of individual rhesus
formation rates between 1990–1997 were significantly (p < .05) difference between control and CR groups, although
levels of insulin in squirrel monkeys, were not significantly (p > .05) different between control and CR groups, although
toward reduced insulin in CR groups was apparent in all
group (Table 1). Insulin levels during the glucose
tolerance test administered to rhesus monkeys were
significantly lower in CR J + A groups versus the controls; however, no differences were noted in the O groups of
monkeys (Table 1). Although generally consistent with previous results from the NIA study (26), variability in
response among monkeys to the effects of CR may account for the lack of statistical significance in certain analyses within the current study. Nonetheless, there appeared to be beneficial effects of CR
in reducing collagen glycation especially in old rhesus
monkeys as indicated by slope analyses of furosine levels
Figure 1. A trend toward lower glycation of skin collagen
was also observed in adult squirrel monkeys that had advanced to a biologically older age, where glycation markers
are supposedly more sensitive to intervention, compared with rhesus monkeys of similar age if one considers the
differences in life spans (25 vs. 40 years) between these 2
monkey species (16).

Most experimental trials with nonhuman primates have
shown that CR increases insulin sensitivity compared with
control animals (24,26,37,38). However, data as to whether
CR reduces plasma glucose and glycohemoglobin levels are
conflicting. The nutritional composition of diets fed to
monkeys as well as their body composition (particularly fat),
the extent and method of implementing the reduction in
-calories, and the required length of time after implementa-
tion of the CR regimen for observable effects to occur are all
in question (25,26,38). A further challenge is that primates

Correlations Among Body Weight, Serum Glucose,
and Insulin Levels, and Parameters of Glycoxidation
and Glycation of Skin in Old Monkeys

Parameters presented in Table 1 and Figures 1–3 were
evaluated for correlations by bivariate analysis within
individual monkeys, as explained in Methods. Significant
results of this analysis are presented in Figure 5A–C. The
results showed that serum glucose measured in individual
rhesus monkeys correlated significantly with their respective
skin furosine levels (r = .75, p = .001) and body weight
(r = .61, p = .016), all measured in 1997 (Figure 5A and B).
Likewise, 1997 skin furosine levels of individual rhesus
monkeys correlated significantly (r = .56, p = .029) with the
respective body weight (Figure 5C). In addition, pentosidine
formation rates between 1990–1997 were significantly (r = .49, p = .034) greater in control versus CR rhesus monkeys
(data not shown).

DISCUSSION

The objective of this study was to determine whether CR
is effective in retarding the age-related increase in glycation
and glycoxidation levels in monkey skin as previously
observed in skin of rodents (10,19,32). The results show
that, with the exception of carboxymethyl-lysine, levels
increased with age in monkey skin as previously noted with
rodents. However, a surprisingly large variability existed
among monkeys and species for levels of furosine, pentosi-
dine, and carboxymethyl-lysine in skin collagen. When
parameters were followed individually in each monkey over
7 years, a great deal of variability was still noted. Only when
measurements were pooled and collectively analyzed as a
whole without regard to individual monkeys did differences
emerge as shown in Figures 1–3. Overall, slope analyses
indicated that the age-related increase of furosine was
significantly (p = .011) inhibited by CR feeding in rhesus
monkeys versus the controls. Secondly, these same analyses
indicated that CR did not significantly (p > .05) affect the
age-related increase of glycoxidation as measured by
pentosidine in skin.

In the present study, fasting levels of serum glucose and
insulin in rhesus monkeys, as well as fasting levels of
glucose in squirrel monkeys, were not significantly (p > .05) different between control and CR groups, although
a trend toward reduced insulin in CR groups was apparent in
all age groups (Table 1). Insulin levels during the glucose
tolerance test administered to rhesus monkeys were
significantly lower in CR J + A groups versus the controls;
however, no differences were noted in the O groups of
rhesus monkeys (Table 1). Although generally consistent with previous results from the NIA study (26), variability in
the degree of individual responses among monkeys to the
effects of CR may account for the lack of statistical significance in certain analyses within the current study.
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tion of the CR regimen for observable effects to occur are all
in question (25,26,38). A further challenge is that primates

Figure 4. Correlations between measured levels of furosine, pentosidine, and carboxymethyl-lysine (CML) in monkey skin collagen. Equations for regression lines:
A, pentosidine = 3.36 + 0.013 (furosine); n = 165, r = .37, p < .0001. B, CML = 94 + 0.133 (furosine); n = 154, r = .23, p = .005. C, CML = 4.3 + 0.025 (pentosidine); n = 155, r = .32, p < .0001. □ = control squirrel monkeys; ■ = caloric-restricted squirrel monkeys; ○ = control rhesus monkeys; ● = caloric-restricted rhesus monkeys.
raised in captivity have a predisposition for the development of obesity and diabetes (6). In short, the rhesus trial study at UMD (24) and the study with Cynomolgus monkeys at Wake Forest University (37) reported no reduction in plasma glucose levels even after long-term CR (i.e., 9 years). In the UW study of CR in rhesus monkeys (25,38), reductions in plasma glucose were reported to occur at 18 months after initiation. In the NIA trial, reductions were observed at 4 years after implementation (26), but not at 3 years (39). To avoid the problems associated with variations in glucose levels in blood, resulting from stress handling and anesthesia (40–42), glycohemoglobin levels are frequently measured as a useful index of cumulative glycemia over a period of 5–8 weeks measured in humans (43). Although not studied here, glycohemoglobin levels have not been significantly reduced by CR in any of the studies (26,37,39). However, the reductions in glycohemoglobin levels in the UW study (25,38) approached statistical significance ($p = .073$).

In the present study, pentosidine and carboxymethyl-lysine levels were measured in skin collagen as markers of glycoxidation. For squirrel monkeys, levels were not affected by CR. For rhesus monkeys, there was a non-significant trend ($p > .20$) for CR to lower pentosidine levels in the old monkey group for the last two assay periods occurring in 1995 and 1997. Furthermore, the overall pentosidine formation rate measured between 1990–1997 was found significantly ($p = .034$) retarded by CR within individual monkeys of the old age group at the end of the study (i.e., 1997). A, Serum glucose versus skin furosine; $y = 54 + 0.032x$, $n = 14$, $r = .75$, $p = .001$; diet $p = .38$ (NS). B, Body weight versus serum glucose; $y = 54 + 1.1x$, $n = 14$, $r = .61$, $p = .016$; diet $p = .49$ (NS). C, Body weight versus skin furosine; $y = 94 + 24x$, $n = 14$, $r = .56$, $p = .029$; diet $p = .57$ (NS). ○ = control rhesus monkeys; ● = calorie-restricted rhesus monkeys.

Both the UW and the present study use rhesus monkeys. However, the comparison of present results with those of UW study may not be prudent without consideration of differences in experimental designs between the two. First, in the UW study (38), the control monkeys, but not restricted monkeys, are approximately 33% heavier in body weight compared with those in the NIA study described in Table 1 (e.g., at age 18 years, 15 vs. 10 kg). Second, the control monkeys used in the UW study (38) are reported to have a greater percentage of body fat in comparison with the ones used here, i.e., 30% vs. 14% (26). Third, the semipurified diet fed to monkeys in the UW study is formulated with a mixture of 28.5% sucrose and 30% cornstarch. Regarding this dietary factor, spontaneous obesity occurs in maturing squirrel monkeys fed semipurified diets containing 25%–35% sucrose (47). Furthermore, previous studies with rodents had shown that high levels of sucrose in diets increases insulin resistance (48) and decreases life span (49). However, levels used in these two rodent studies were much higher (i.e., 65%–67%) than that presently in use in monkey studies. Interestingly, in the study by Kim and colleagues (48), Wistar rats fed a semipurified diet formulated with either 65% sucrose or 50% cornstarch were reported to have a greater visceral fat accumulation, higher fasting plasma insulin levels (but not glucose), and insulin resistance of the skeletal muscle glucose transporter in comparison with those fed a standard laboratory chow diet. When considering these results with rodents and the diet issue, in the UW study of rhesus monkeys by Zainal and colleagues (46), the type of diet fed to monkeys, whether a standard laboratory chow or a semipurified diet containing a mixture of sucrose and cornstarch, had a significant influence on oxidation levels measured in tissue sections of skeletal muscle. More specifically, measurements using electron microscopy showed that levels for control monkeys fed the semipurified diet were significantly elevated over those from the CR group and controls fed the laboratory chow diet (46).

In this research, we have compared rhesus (Macaca mulatta) with squirrel (Saimiri sciurius) monkeys representing...
Old and New World primates, respectively. As observed in captivity, rhesus monkeys have a maximum life span of approximately 40 years (50). In comparison, the smaller squirrel monkey (Table 1) can live up to 27 years, although few live beyond their early 20s (51). Both species show neuropathological changes at old age consistent with β-amyloidosis in the brain starting at approximately 25 years for rhesus and 15 years for squirrel monkeys (51). Furthermore, mortality of both species results, in part, from incidences of cardiovascular disease and diabetes (2.50), both of which are associated with obesity (9). In this factor, both species have a propensity for the development of spontaneous obesity under the right conditions of diet and husbandry. However, obesity in squirrel monkeys occurs at an earlier age of 3–5 years compared with 8–12 years for rhesus monkeys, an observation that may be related to the difference in their life spans and maturation rates (47). From these observations, one may conclude that the shorter-lived squirrel monkey ages at a faster rate than the longer-lived rhesus monkey. Consistent with this conclusion is the finding that the rate of pentosidine formation in skin collagen is significantly greater for squirrel versus rhesus monkeys for both control and CR cohorts (Figure 2, Table 2) as previously reported (16).

In the present study, potential sources of variability in furosine, pentosidine, and carboxymethyl-lysine levels measured in skin collagen are noted (Figures 1–3). For one, monkeys used in this study represent a genetically heterogeneous population of animals in contrast to rodent studies where inbred strains have generally been used (10,19,32). However, the amount of variation presently observed here for pentosidine with age is no greater than that previously published for healthy humans and other animals (16) where heterogeneity also existed without regard to genetic susceptibility of certain individuals to age-related diseases, such as diabetes, which affect pentosidine levels in skin (17,52,53). Second, individual differences in collagen turnover could affect levels measured in skin. This factor is important due to the findings that protein turnover is a primary determinant in the accumulation of these products in collagen (54). In that regard, the half-life of skin collagen is reported to be 15 years compared with 117 years for cartilage in humans (54). In the present investigation, skin was chosen as the sample tissue because it could easily be obtained by biopsy at multiple times from the same animal longitudinally over the course of study without adversely affecting the health of the animal. Third, some of the variability likely derives from changes in procedures, both collection and assay, that can occur over the 7-year period. For example, different laboratory personnel have been involved in measurements. To compensate for differences among assays, an internal standard was used in the pentosidine assay (i.e., pyridoxamine), and biopsies from the last 2 sampling periods (1995 and 1997) were frozen and assayed at the same time.

Finally, comments should be made as to what this study means to the mechanism by which CR acts. The ability of CR to decrease collagen glycation, but not glycoxidation, suggests that it might have decreased cumulative glycemia especially in older monkeys. The other possibility, i.e., that CR would have increased collagen turnover, would appear less likely since neither carboxymethyl-lysine nor pentosidine were decreased. However, the origin of these advanced glycation end products is still unclear since they can form not only from glucose but also from lipids and ascorbic acid, respectively (13,14,18).

**Conclusion**

Significant differences in glycation rates were observed in this study between control and CR monkeys, and there was a trend toward lower pentosidine accumulation in CR rhesus monkeys. These differences are essentially manifested in the old group of rhesus monkeys, ages >16 years (Figures 1, 2, and 5). These data are best explained by the fact that CR tends to decrease glycemia, or perhaps inhibits the age-related decrease in collagen turnover. Significant correlations were observed in the older group of individual rhesus monkeys consisting of serum glucose with skin furosine levels, and serum glucose with body weight (Figure 5). However, these correlations were statistically significant only when data from control and CR monkeys were both pooled together, and not significant when analyzed separately (Figure 5).

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