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Protein oxidation associated with aging is reduced by dietary restriction of protein or calories

(protein restriction/caloric restriction/protein carbonyls/free radicals)

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ABSTRACT **The accumulation of unrepaired oxidative damage products may be a major factor in cellular aging. Both oxidative lesions in DNA and oxidatively damaged proteins have been shown to accumulate during aging. The accumulation of oxidized proteins in Fischer 344 rats was compared for animals consuming protein-restricted and calorically restricted diets—both of which have been shown to extend lifespan. Rats were fed diets restricted in either protein (5% or 10% of the diet as compared with the normal 20% casein), or calories (25% or 40% less than normal), or total diet (40% less than normal). In addition, some of the rats fed a diet providing 5% or 20% protein were irradiated twice weekly (125 rads per exposure; 1 rad = 0.01 Gy). The level of oxidative damage to proteins (protein carbonyls) was determined in rats sacrificed at various times. The oxidative damage to proteins increased with aging and with radiation. Either protein or calorie restriction markedly inhibited the accumulation of oxidatively damaged proteins. Protein restriction reduced the accumulation of oxidatively damaged proteins during the oxidative stress of chronic irradiation.**

Oxidants generated during normal metabolism appear to play a significant role in the processes of aging and carcinogenesis (1, 2). They produce an array of oxidative-damage products including DNA damage lesions such as 8-hydroxy-2′-deoxyguanosine (3), peroxidative damage to lipids within mitochondrial and cell membranes (4, 5), inactivation by metal-catalyzed oxidation reactions of metabolic enzymes such as glutamine synthetase and glucose-6-phosphate dehydrogenase (6), and oxidative damage to proteins resulting in protein carbonyl derivatives (7).

Numerous studies suggest that calorie restriction (CR) markedly extends lifespan and inhibits carcinogenesis (8–12). Protein restriction (PR) produces these same effects (12–16). Only a few reports have examined both CR and PR for their resultant effects upon lifespan extension within the same study design (11, 17–20). All but one (20) of these studies (11, 17–19) demonstrated that CR alone, PR alone, and both, i.e., total dietary restriction (TDR), resulted in significant increases in lifespan. While some investigators have concluded that PR does not markedly increase lifespan (20, 21), the levels of protein fed in these studies may not have been low enough to cause PR comparable in degree to the levels of CR used in most studies.

There are many similarities between CR and PR in their effects on various physiological factors that can affect lifespan. For example, CR leads to improved antioxidant defenses (22, 23) and reduced levels of oxidative damage products (22, 24, 25). PR studies have led to similar observations (refs. 26 and 27; L.D.Y., unpublished observation). Another variable important to lifespan that is similarly affected by CR or PR is the rate of body weight gain, which is a strong correlate to risk of premature death and cancer (with smaller body size being a protective factor for both) (28). In general, a smaller body size correlates with reduced proliferation, and since cell division rates are a key factor in mutagenesis (29, 30), it is plausible that reduced cell division rates play a role in avoiding premature aging and cancer (28–30).

The decrease in the rate of aging and cancer by CR is often accompanied by a loss or decrease in the ability to reproduce. This has been interpreted by evolutionary biologists as part of the fundamental trade-off between reproduction and maintenance (31, 32). It has been speculated that a hormonal switch in starving animals decreases reproductive capacity and increases the maintenance functions that prolong life (31). PR may, in part, act in much the same way. Animal studies have shown that low protein feeding delays onset of puberty and decreases growth rate (and significantly inhibits mammary tumorigenesis) (33). A recent study in the People's Republic of China also has suggested that age at menarche is significantly prolonged by low protein intake (34).

Specific metabolic rate in various species is strongly inversely correlated with lifespan and with oxidative damage to DNA (35). Both CR and PR have been shown to affect metabolic rate (10, 22, 36, 37), but whether the restriction results in an increase or decrease of metabolic rate seems to largely depend upon the level of the restriction (37). Mild CR or PR tends to increase metabolic rate whereas severe restrictions appear to decrease the rate. Also, both CR and PR (i.e., mild restriction—not deficiency) tend to boost cell-mediated immunity (stimulation of natural killer cell activity) with only mild or no suppression of humoral immunity (38–40).

We estimate that the DNA hits per cell per day from endogenous oxidants are normally 10^5 in the rat and 10^4 in the human (3, 41, 42). These oxidative lesions are effectively but not perfectly repaired; the normal steady-state level of oxidative DNA lesions is about 10^6 per cell in the young rat but rises to about twice this level in the old rat (3, 43). Oxidants are produced as by-products of mitochondrial electron transport, various oxygen-utilizing enzyme systems, peroxisomes, and other processes associated with normal aerobic metabolism, as well as by lipid peroxidation. Oxidants that escape the body's numerous antioxidant defenses can damage critical cellular macromolecules, including DNA and proteins, and this damage can promote early aging, mutations, and cancer.

Since aging is associated with the accumulation of damage products over time, it is likely that treatments shown to minimize production of oxygen radical damage products could increase functional lifespan (44). Stadtman and others

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Abbreviations: CR, calorie restriction; PR, protein restriction; TDR, total dietary restriction.

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have studied accumulation of oxidatively damaged proteins and inactivation of metabolic enzymes during aging. They found that, during aging, many critical enzymes inactivated by mixed-function oxidation (MFO) systems accumulated as inactive forms (45). Subsequent studies indicated that modifications of histidine residues of malic enzyme accumulated with age (46). Oliver *et al.* (7) showed that oxidative modification of enzymes by MFO systems led to oxidative damage to proteins which accumulated during aging. Stadtman and Oliver (6) showed that aging resulted in inactivation of glyceraldehyde-3-phosphate dehydrogenase, aspartate aminotransferase, phosphoglycerate kinase, and glucose-6-phosphate dehydrogenase, as well as increased protein oxidation. These oxidatively damaged proteins, or protein carbonyls, are also generated profusely during ischemia–reperfusion (47) and during oxidative stress induced by hyperoxia (48). A reversal of the age-related increase in oxidative damage to brain proteins and improvement in memory after administration of a free radical-trapping compound have also been reported (49). Collectively, these observations suggest that the accumulation of these oxidized proteins play an important role in the disruption of normal cellular functioning that can promote early aging and, further, that treatments with the capacity to reduce or reverse accumulation of these oxidative damage products will help to restore normal functioning and perhaps extend lifespan.

We have evaluated the effects of administration of diets known to extend lifespan on accumulation of oxidized proteins in the rat. This study was undertaken to determine (*i*) the combined and separate effects of PR and CR on the accumulation of oxidized proteins and (*ii*) the effect of PR on the accumulation of oxidized proteins during chronic oxidative stress (irradiation), which may be a mimic of early aging. The effect of age on the accumulation of oxidized proteins at a constant level of protein intake was also examined. In addition, we compare and discuss PR and CR for their similar effects on physiological changes associated with aging. The effects of these protein and calorie restrictions on oxidative DNA damage will be discussed in a separate paper.

MATERIALS AND METHODS

Animals and Diets. Weanling male Fischer 344 (F344) rats (Charles River Breeding Laboratories) with average body weights of 40–60 g were randomized into treatment groups. Animal care and protocols were in accordance with institutional guidelines and were approved. Rats were fed AIN 76-A diet (50) (Dyets, Bethlehem, PA) providing either 5%, 10%, or 20% casein (equivalent to 4.35%, 8.7%, or 17.4% protein, since casein is 87% protein) with sucrose and corn starch being substituted proportionately and isoenergetically for casein. The 25% CR and 40% CR groups were fed AIN 76-A diets containing 26.66% or 33.33% casein, respectively (with other nutrients similarly increased), so that when restricted amounts of the diet were fed, the CR animals were getting all other nutrients at a level equivalent to the normal 20% casein group. The TDR group received AIN 76-A diet at 40% restriction, resulting in 40% restriction of all nutrients (including protein) as compared with the 20% casein group. Table 1 summarizes the compositions of these diets. Rats were individually caged and received food and water ad libitum with the exceptions of the 25% CR and 40% CR groups and the 40% TDR group, which were fed daily. A temperature of 23°C and a 12-hr light/dark cycle were maintained throughout the study. Signs of ill health were monitored daily throughout the 15-week study period. An additional group of rats was fed the 20% casein diet and then killed at 60 weeks. Two-day food intakes and body weights were recorded at regular intervals.

Experimental Protocol. After acclimatization to the animal facility (during which all animals were fed AIN 76-A diet providing 20% casein), rats were provided diets of various compositions (Table 1) for 6 or 12 weeks. Since the animals were about 3 weeks old upon dietary intervention, their age at the end of these periods was 9 or 15 weeks. Rats receiving radiation were exposed twice weekly (≈125 rads per exposure) throughout the study to cesium-137 in a whole-body irradiator. This exposure level, although high, was chosen because it was considered sublethal yet would generate an array of oxygen radicals. More importantly, rats could be chronically exposed to these radicals. Selected groups of rats at 6, 12, and 60 weeks following dietary intervention were anaesthetized and decapitated. Multiple slices of the median liver lobe were immediately frozen on dry ice and subsequently stored at −85°C.

Protein Oxidation Determination. Protein carbonyl content was determined as described by Levine *et al.* (51), with slight modifications (A. Reznick and L. Packer, personal communication). Several extra tissue washes were carried out, which resulted in slightly lower total protein carbonyl values than are sometimes reported. Weighed liver tissue samples (200–300 mg) were minced in 3 ml of potassium phosphate buffer (100 mM, pH 7.4) containing 0.1% (wt/vol) digitonin (Sigma) and 1 mM EDTA along with the protease inhibitors leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), and aprotinin (0.5 μg/ml) (all from Sigma) to prevent proteolysis of oxidized proteins during sample preparation. Minced tissue was incubated for 15 min at room temperature. The supernatant was removed and centrifuged (3000 × *g* for 10 min) and the resulting supernatant was divided into two 1-ml portions. Protein concentration of the soluble protein fraction was determined on one portion by using a standard albumin (dissolved in 6 M guanidine hydrochloride) curve (52). Protein carbonyl content was determined spectrophotometrically on the other portion by the 2,4-dinitrophenylhydrazine (DNPH) method (7). Both portions were sequentially precipitated with 4 ml of 20% (wt/vol) trichloroacetic acid and 4 ml of 10% trichloroacetic acid and then washed three times with 4 ml of 1:1 (vol/vol) ethanol/ethyl acetate. Precipitates were dissolved in 2 ml of 6 M guanidine hydrochloride. DNPH supernatants (for protein carbonyl determination) were scanned over the range 320–410 nm with a Shimadzu

Table 1. Diet composition (g/kg)

Component	5% casein	10% casein	20% casein	25% CR	40% CR
Casein	50	100	200	266.67	333.33
DL-Methionine	0.75	1.5	3	4.00	5.00
Corn starch	185.00	173.0	150	123.10	96.17
Sucrose	617.25	578.5	500	410.23	320.52
Cellulose	50	50	50	66.67	83.33
Corn oil	50	50	50	66.67	83.33
Mineral mix*	35	35	35	46.67	58.33
Vitamin mix*	10	10	10	13.33	16.66
Choline bitartrate	2	2	2	2.66	3.33

*AIN 76-A vitamin and mineral mixes (50).

UV160U spectrophotometer recording the peak (at about 360 nm). Protein concentration samples were read separately at 280 nm. Results are expressed as nmol of DNPH incorporated per milligram of protein to give nmol of protein carbonyl per milligram of protein. The data on protein carbonyls in Table 3 was obtained at a different time and with newer reagents than the data in Table 2. The variability between the 15-week 20% casein data in the two tables may be due to this. The results within each experiment showed less variability, as can be seen by the SEM.

Statistical Analysis. Body weights and oxidized protein content data were compared by Student's t test, using Minitab (53).

RESULTS

As seen in Fig. 1, animals fed the 5% casein diet were significantly smaller ($P < 0.05$) throughout the study period than animals fed the 10% or 20% casein diets. Rats fed the 10% casein diet grew at essentially the same rate as rats fed the 20% casein diet. Rats fed at 25% restriction of calories (25% CR) were significantly smaller ($P < 0.05$) than rats fed the 5% casein diet ad libitum. Similarly, rats fed at 40% restriction of calories (40% CR) and at 40% restriction of total diet (40% TDR) were significantly smaller ($P < 0.05$) than rats in any other group. The 40% TDR rats were slightly smaller than the 40% CR rats.

At the end of the study, the 25% CR, 40% CR, and 40% TDR groups were smaller than the 20% casein ad libitum fed group by 18.4%, 29.4%, and 32.1%, respectively. CR studies often report that animals are smaller in size than ad libitum controls by an amount proportional to the degree of CR. Thus, it might be expected that these groups of animals would be smaller by approximately 25%, 40%, and 40%, respectively. It is possible that, had the animals been fed these diets for a longer period of time, these differences in size might have been reached.

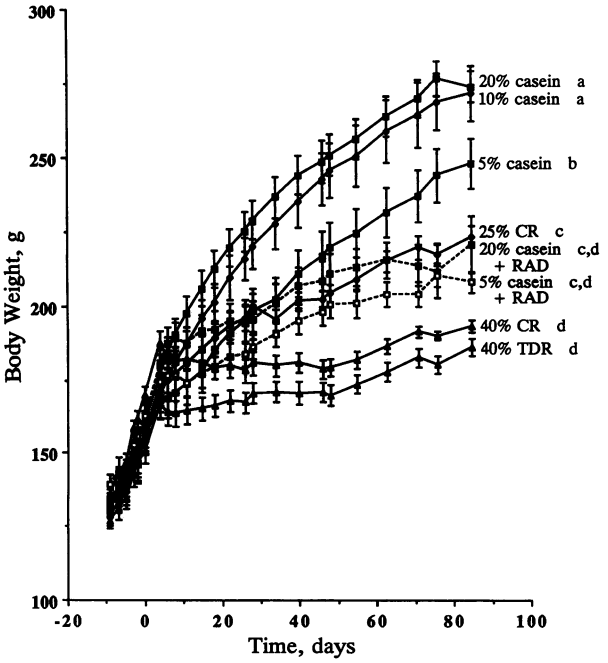

FIG. 1. Body weights (mean ± SEM) for rats fed AIN 76-A diet providing 20%, 10%, or 5% casein or at 25% or 40% CR, or at 40% TDR as compared with ad libitum consumption for rats fed the 20% casein diet and for rats that were fed the 5% or 20% casein diet and were irradiated (+RAD). Different letters (a–d) indicate means that are significantly different at $P < 0.05$.

Rats that were irradiated were significantly smaller ($P < 0.05$) throughout the study period than those that were not irradiated. Irradiated rats fed the 5% casein diet were only slightly smaller than irradiated rats fed the 20% casein diet.

Food consumption results are not shown. However, the 5% casein-fed rats consumed *more* food throughout the study period than the 20% casein-fed rats (about 12–15% by weight) despite the facts that the 5% casein-fed rats were smaller and the diets were isoenergetic as was seen in earlier studies (15, 16, 37).

The degree of oxidative damage to protein as it is affected by PR, CR, and TDR is shown in Table 2. The data suggest that oxidative damage to protein is markedly reduced by both PR and CR. An additional group of rats was killed at 60 weeks. The level of protein carbonyls in these animals was 3.36 ± 0.06 nmol of protein carbonyl per mg of liver protein. Collectively, the results from this study also suggest that the level of oxidative damage to protein increases with increasing age (at constant protein intake), as has been reported by Stadtman and others (6, 7, 48). Starke-Reed and Oliver (48) showed that these increases were most significant after 100 weeks of age. While our total values of protein carbonyls are lower than have been reported by these workers (perhaps because we used a method employing extra washes), our findings and conclusions are in agreement with those of Starke-Reed, Stadtman, and Oliver.

Table 3 shows data from the portion of the study designed to examine whether PR confers protection against oxidative damage to proteins in animals receiving chronic irradiation. Irradiation markedly increased the accumulation of oxidative damage to protein. This finding was expected because irradiation is known to generate an array of oxygen radicals. Of interest is the observation that the level of oxidative damage to proteins was reduced by PR in irradiated animals. Proportionately, the irradiation-induced increase in protein carbonyls for 5% casein-fed animals was 24.7%, while for the 20% casein-fed animals this increase was 66.8% (when comparisons were made to their respective dietary treatment groups). These results suggest that PR confers protection against oxidative damage to proteins during the oxidative stress of chronic irradiation.

DISCUSSION

Results from this study lend further support to the idea that oxidative damage to macromolecules may play a major role in aging and the degenerative diseases associated with it. These data should not be taken to suggest that CR and PR necessarily act to extend lifespan via the mechanism of reduced oxidative damage to proteins. Rather, these data

Table 2. Oxidative damage to protein as influenced by PR or CR

Rat age	Diet	n	Protein carbonyls, nmol/mg
9 weeks	5% casein	5	$1.85 \pm 0.06^{a,b}$
	20% casein	5	1.95 ± 0.10^{a}
	40% TDR (12% casein)	5	1.73 ± 0.06^{b}
15 weeks	5% casein	7	1.67 ± 0.10^{a}
	10% casein	7	2.03 ± 0.03^{b}
	20% casein	9	2.94 ± 0.18^{c}
	25% CR (20% casein)	6	$2.33 \pm 0.12^{b,d}$
	40% CR (20% casein)	7	2.41 ± 0.10^{d}
	40% TDR (12% casein)	8	$1.96 \pm 0.13^{a,b}$

Means ± SEM (at the same time point) with different superscripts are significantly different at $P < 0.05$.

Table 3. Oxidative damage to protein as influenced by irradiation and PR

Diet	n	Protein carbonyls, nmol/mg	
		No irradiation	Irradiation
5% casein	7	1.90 ± 0.02[a]	2.37 ± 0.01[a]
20% casein	9	2.20 ± 0.01[b]	3.67 ± 0.08[b]

Rats were 15 weeks old when sacrificed. Within-column means (± SEM) with different superscripts are significantly different at $P < 0.05$.

show that both CR and PR significantly inhibit oxidative damage to proteins and that PR confers protection against oxidative damage to proteins during the oxidative stress of chronic irradiation. Moreover, these data suggest that PR inhibits oxidative damage to proteins as significantly as CR does (at the levels of restriction examined).

The final observation is critical, since studies have often been taken to conclude that CR has a greater effect than PR on inhibiting age-related decline and on lifespan extension (11, 17, 18). However, CR studies are often done by simply feeding restricted amounts of the same diet given to ad libitum fed controls. Thus, restricted animals also receive less protein (as well as proportionately less of all other dietary constituents). Clearly, with such a design, the portion of lifespan extension attributable to PR is totaled along with that attributable to CR. Only a few studies have examined both PR and CR for their combined and separate effects upon lifespan extension within the same study design (11, 17–20). These studies have suggested that both CR and PR significantly extend lifespan, with CR having the greatest impact. However, in some of these studies, the protein levels chosen may not have been low enough to represent a level of PR comparable to the level of CR. It is possible that, had different levels of PR been chosen, the increases in lifespan attributable to PR might have been much closer to those attributable to CR.

PR is a more feasible option for humans than CR. CR studies often deprive calories by 25–40%. Human feeding studies have shown that this level of caloric deprivation is extremely difficult (if not impossible) for most humans to adopt over a lifetime. In contrast, animals fed low protein diets are able to eat more total calories but gain less weight than animals fed high protein diets (15, 16). Thus, the starving associated with CR is not similarly associated with PR. In addition, protein-restricted animals have additional health benefits including lower total serum cholesterol (15), enhanced immune surveillance (40), fewer tumors (15), more energy (54), and longer lifespan (11, 13, 14, 17, 18).

Chronic exposure to ionizing radiation is often used as a model of accelerated aging (55), and it is known that high doses of ionizing radiation shorten lifespan (56). Our data suggest that PR confers protection against oxidative damage to proteins during the oxidative stress of chronic irradiation. This finding is consistent with reports suggesting that low protein intake boosts antioxidant defenses including increased levels of superoxide dismutase and catalase in muscle and liver (refs. 26 and 27; L.D.Y., unpublished observation). In addition, De *et al.* (27) have reported significantly decreased lipid peroxidation and decreased lipofuscin accumulation in animals fed low protein diets. To our knowledge, however, ours is the first report showing that diets known to extend lifespan (both CR and PR) can reduce the accumulation of oxidized proteins.

In this study, rats on CR regimens grew at a slower rate than rats consuming low protein diets. Since body size is a strong correlate to premature death and cancer (28), these data suggest that calorically restricted rats might live longer than protein-restricted rats (at least at these chosen levels of intake) simply because they are smaller. In addition, cell division rates are depressed in calorically restricted animals (57) and, possibly, in protein-restricted animals (58) [although some studies have suggested the opposite conclusion with extremely low protein intake (59)]. One possibility for the observed reductions in oxidative damage to proteins for both the CR and PR groups is that cell division and protein synthesis are decreased.

Metabolic rate is inversely correlated with lifespan (60). It has been suggested that animals with high metabolic rates (and short lifespans) produce more oxygen radicals, causing greater rates of damage to macromolecules (35). CR and PR produce alterations in various aspects of energy metabolism, including decreased body temperature (61, 62) and increased physical activity with PR (54, 62), but the association of CR and PR (37, 61, 63) or CR (62–64) with oxygen consumption is puzzling since most studies suggest that some levels of PR and CR actually *increase* oxygen consumption (37, 61, 64). Increased oxygen consumption would seem to lead to greater oxidative damage.

While oxygen consumption and metabolic rate may play a role in determining lifespan (60) and oxidative damage rates (35), perhaps the more relevant variable, in terms of extending functional lifespan, is the capacity to deal with oxygen and/or oxygen radicals. Since the rate of production of oxygen radicals is enormous, even appreciable changes in oxygen consumption does little to alter total oxidative hits per day. However, dietary restrictions may markedly increase the capacity to quench free radicals, which may markedly decrease the accumulation of oxidative damage (regardless of initial oxygen consumption and metabolic rate). Both CR and PR have been shown to boost antioxidant defenses (refs. 22, 23, 26, and 27; L.D.Y., unpublished observation), resulting in reduced accumulation of various oxidative damage products (22, 24, 25). This view is in accord with the suggestion by Masoro *et al.* (10) that food restriction does not slow aging by decreasing metabolic rate.

The conclusions that can be drawn from this study are as follows: (*i*) PR inhibits oxidative damage to proteins, (*ii*) CR inhibits oxidative damage to proteins, (*iii*) PR reduces oxidative damage to proteins during the oxidative stress of chronic irradiation, and (*iv*) irradiation increases oxidative damage to proteins. Results of Stadtman and colleagues (7, 48, 49) indicating that age increases oxidative damage to protein have also been further supported.

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