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Protein oxidation and enzyme activity decline in old brown Norway rats are reduced by dietary restriction

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Abstract

The effect of aging and diet restriction (DR) on the activity of creatine kinase (CK), glutamine synthetase (GS) and protein carbonyl formation in the cerebellum, hippocampus and cortex of male and female brown Norway (BN) rats has been investigated. It was demonstrated that CK activity in three different regions of the rat brain declines with age by 30%. Age-related decrease of GS activity was only 10–13% and did not reach statistical significance. Consistent with previously published studies, age-related increase of protein carbonyl content in each brain area studied has been observed. Preventive effects of a caloric restricted diet on the age-associated protein oxidation and changes of the activity of CK and GS in the brain was observed for both aging male and female BN rats. DR delayed the accumulation of protein carbonyls. Age-related changes of CK activity in rat brain were abrogated by DR. The activity of GS in the brain of old rats subjected to the caloric restricted diet was higher than that in the brain of young animals fed ad libitum. The results are consistent with the notion that DR may relieve age-associated level of oxidative stress and lessen protein damage. © 1998 Elsevier Science Ireland Ltd.

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1. Introduction

Although the basic causes of aging are still unknown, the free radical theory of aging (Harman, 1956, 1981, 1992) proposes that oxygen-derived free radicals result in a cumulative damage to cellular components and this may be a casual factor in aging and in some age-associated neurodegenerative diseases (Ames et al., 1993). There is a considerable body of evidence that the mechanism by which oxidative stress affects the aging process is through the infliction of molecular damage which leads to the accumulation of oxidative damage to DNA, lipids and proteins (Sohal et al., 1994a; Ames, 1989; Beal, 1995; Butterfield et al., 1997a; Butterfield and Stadtman, 1997). Changes in the antioxidant defenses and indicators of oxidative stress have been shown, such as decreased activities of superoxide dismutase, catalase and glutathione reductase (Sohal et al., 1990; Mo et al., 1995).

There are numerous reports in the literature indicating that oxidative damage to proteins results in the modification of amino acid residues leading to the formation of carbonyl derivatives (Butterfield and Stadtman, 1997; Stadtman and Oliver, 1991; Davies, 1987). While a relationship between protein oxidation and aging have been suggested (Stadtman, 1992; Stadtman et al., 1992; Oliver et al., 1987; Starke-Reed and Oliver, 1989; Smith et al., 1992), it has also been reported that oxidative modification of proteins can modulate biochemical characteristics of protein such as enzymatic activity (Oliver, 1997; Levine, 1983a). Oxidative modification of proteins by oxygen-free radicals and as a consequence of that, carbonyl groups formation which increase with age, has been well described in the literature (Beal, 1995; Butterfield et al., 1997a; Butterfield and Stadtman, 1997; Stadtman, 1992; Sohal et al., 1994b). Previous studies have demonstrated that oxidative processes often result in decreased activity of key enzymes, including glutamine synthetase (GS), creatine kinase (CK) and tyrosine hydroxylase (Floyd, 1990; Howard et al., 1996; Hensley et al., 1995). In aging rats and humans there is a decrease in the levels of cardiac CK activity (Schuyler and Yarbrough, 1990; Chesky et al., 1980). Age-related declines in GS and CK activities have been observed in gerbil and human brain tissues (Carney et al., 1991; Smith et al., 1991).

It is well known that a restriction of caloric intake can extend the life span of rodents when compared to controls fed ad libitum (for review, see Cohen (1979)). Reduced caloric intake has been shown to retard the decline of many physiological functions and delay the onset of some age-related pathologies (Holehan and Merry, 1986; Mascro, 1988; Gabbita et al., 1997). Dietary restricted rats showed higher activities of some liver lysosomal enzymes (Ferland et al., 1990) and maintain less oxidatively damaged DNA (Sohal et al., 1994a) compared to rats fed ad libitum.

The specific aim of the present study was to determine the effects of age and diet restriction (DR) on protein carbonyl content and on the activity of GS and CK enzymes in different rat brain areas.

2. Materials and methods

2.1. Animals and diet

Brown Norway (BN) male and female rats (3 or 30 months old) were used in this study. The rats were maintained at the National Institute on Aging/National Center for Toxicological Research Project on Caloric Restriction Colonies (Jefferson, AR) until one month prior to target ages, whereupon they were shipped by air to the Sanders-Brown Center on Aging at the University of Kentucky, Lexington, KY. The absence of a significant pathology was confirmed by medical examination of each animal used in the study. Each rat had an individual 'health certificate' and was examined by a vet prior to shipping. An additional examination of the animals was carried out by our Animal Facility vet upon arrival to verify their healthy status. The rats were housed individually in $21 \times 10 \times 8$ solid bottom polycarbonate cages with wire tops for placement of food and water. The rats maintained on DR had daily access to 60% of the intake of a companion group of rats given ad libitum access (AL) to the diet (NIH-31 pellets, Purina Feeds). The DR was implemented after the animals were 16 weeks old. The DR rats were fed a special NIH-31 formulation providing a correction for intake of essential nutrients (Witt et al., 1989). The rats were maintained on a 12-h light, 12-h dark cycle with light portion beginning at 06:00 h. Following a two- to three-week adaptation period, the rats were used for experiments.

The three-month old group included 10 males and eight females which were fed ad libitum. The 30-month old male group included eight rats fed ad libitum and six rats maintained on a calorically restricted diet, while the 30-month old female group included eight rats fed ad libitum and seven rats maintained on a diet.

2.2. Tissue use

After decapitation the whole brain was removed, dissected on a cold stage and the cortex, cerebellum and hippocampus were removed and submerged in liquid nitrogen. All tissue was stored at -80°C until analyzed.

Frozen brain areas were thawed, minced and resuspended in 10 mM HEPES buffer (pH 7.4), containing 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$ and proteinase inhibitors leupeptin (0.5 μg/ml), pepstatin (0.7 μg/ml), aprotinin (0.5 μg/ml), type IIS soybean trypsin inhibitor (0.5 μg/ml) and PMSF (40 μg/ml). The homogenate was centrifuged at $16\,000 \times g$ for 10 min and the supernatant fluid was recovered and used for the determination of oxidized proteins and GS and CK activity.

2.3. Enzyme assays

CK activity was determined colorimetrically by using a commercial kit (Sigma). One unit (U) of total CK activity was determined as the amount of the enzyme that will convert 1.0 μmol of creatine to creatine phosphate/min at 37°C, pH 9.0.

GS activity was determined by the method of Rowe et al. (1970) as modified by Miller et al. (1978) and corrected for nonspecific glutaminase activity by comparison in the presence and absence of ADP and arsenate. The specific GS activity was determined as units per mg of protein, where 1 U will form 1 μmol of γ-glutamyl hydroxamate/min at 37°C. All results are represented as mean values of triplicates $\pm$ S.E.M.

2.4. Determination of protein carbonyl content

The protein carbonyl content was determined spectrophotometrically using the 2,4-dinitrophenylhydrazine (DNPH)-labeling procedure as described (Butterfield et al., 1997a; Butterfield and Stadtman, 1997; Howard et al., 1996; Levine et al., 1990). The aliquots of water-soluble proteins containing 0.2 mg of total protein were treated with 10 mM DNPH dissolved in 2N HCl, or with 2N HCl alone as blanks. Samples were then incubated for 1 h at room temperature, stirred every 10 min and protein was reprecipitated with 10% trichloroacetic acid. The precipitates were centrifuged for 3 min at $16\,000 \times g$, washed three times with ethanol/ethyl acetate (1:1, vol/vol) and dissolved in 6 M guanidine hydrochloride in 10 mM phosphate buffer-trifluoroacetic acid (pH 2.3). The absorbance at 380 nm of the samples treated with DNPH/HCl was determined versus the samples treated with HCl alone. Results were expressed as nmol of carbonyl groups incorporated per mg of protein calculated from the extinction coefficient of 21.0 mM^{-1} cm^{-1} for aliphatic hydrazones. For all assays the results are reported as the mean of duplicates $\pm$ S.E.M.

2.5. Statistical analysis

Statistical analysis was made using ANOVA followed by Dunnett's test for multiple comparisons.

3. Results

3.1. CK activity

CK activity was measured in water-soluble protein extracts of the cerebellum, cortex and hippocampus isolated from young and old BN male and female rats. No gender difference was found in total CK activity of young male and female rats, so all data were combined together. The results show (Fig. 1A,B) that CK activity decreased in brain areas of aged BN rats, both males and females, which were fed ad libitum. The decrease was $\approx 30\%$ and statistically significant in all three brain areas of aged female rats ($P < 0.05$ or $P < 0.01$) and in the cerebellum and cortex of aged male rats ($P < 0.01$), while in the hippocampus of old BN males the decrease was less pronounced ($\approx 87\%$ of the CK level in young controls, $P > 0.1$). In contrast, CK activity in all brain areas of DR male and female rats was not decreased, reaching ≈ 90–105% of the CK level of young animals.

3.2. GS activity

No gender difference was found in GS activity of young male and female BN rats, so, as with CK activity, all data were combined together. Fig. 2A,B demonstrate the mild decline of GS activity in brain areas of aged BN rats fed ad libitum. The mean GS activity was $\approx 87–95\%$ of the three-month old animals, although this difference was not statistically significant ($P > 0.1$).

In DR animals the activity of GS was higher, reaching or even exceeding the young animals level. In DR 30-month male rats, the GS activity was the highest

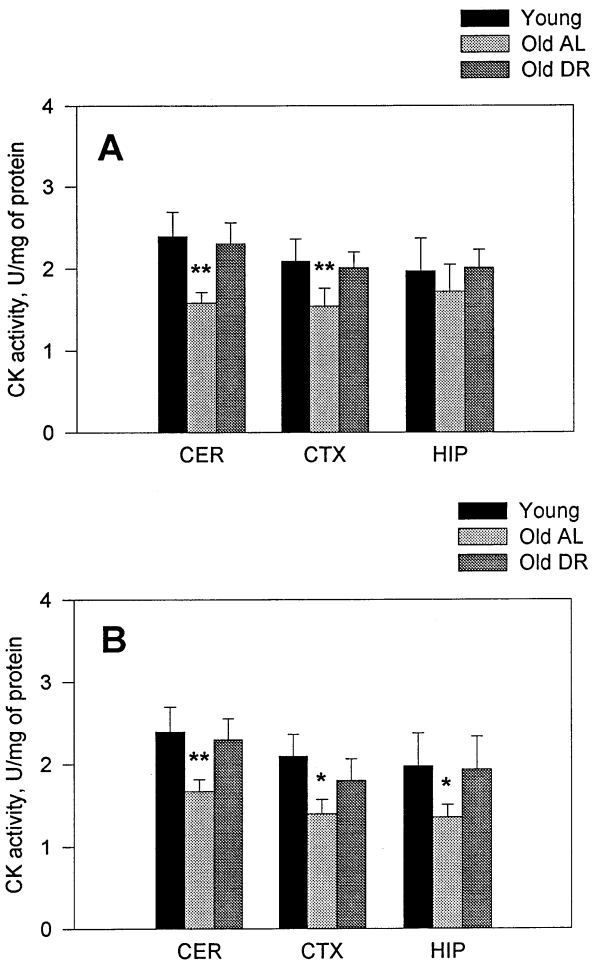

Fig. 1. Activity of creatine kinase (CK) in the homogenates of the cerebellum, cortex and hippocampus from male (A) and female (B) brown Norway rats at the age of three months (young) and at 30 months fed ad libitum (old AL) and dietary restricted (old DR). Bars represent mean percentage CK activity ($\pm$ S.E.M.); * $P < 0.05$ and ** $P < 0.01$, relative to young control group.

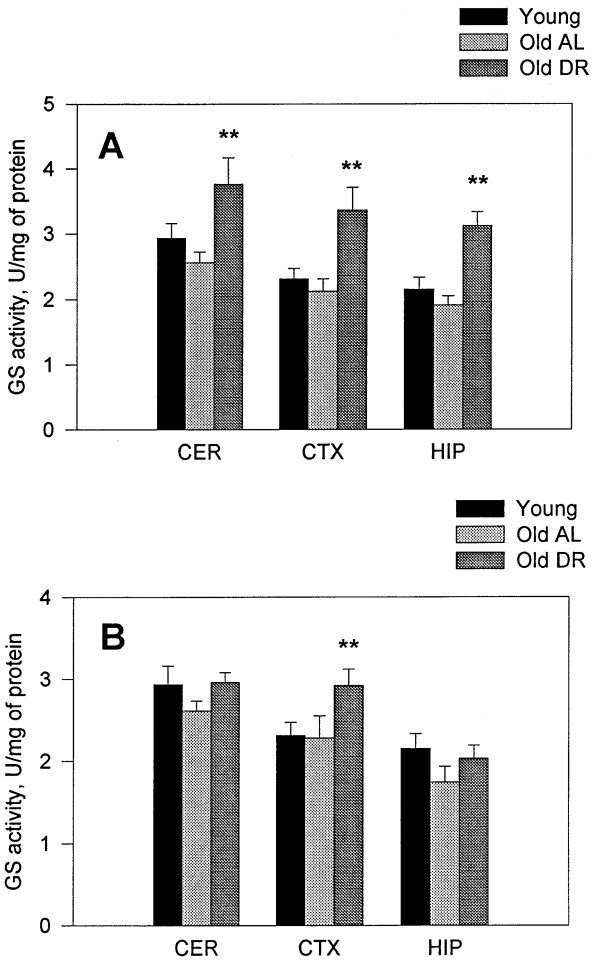

Fig. 2. Activity of glutamine synthetase (GS) in the homogenates of the cerebellum, cortex and hippocampus from male (A) and female (B) brown Norway rats at the age of three months (young) and at 30 months fed ad libitum (old AL) and dietary restricted (old DR). Bars represent mean percentage GS activity ($\pm$ S.E.M.); * $P < 0.01$ and ** $P < 0.001$, relative to young control group.

(Fig. 2A), with 28% increase in cerebellum and 45% increase in cortex and hippocampus when compared to young three-month old rats ($P < 0.001$). DR 30-month female rats demonstrated exactly the same level of GS activity as three-month old animals (Fig. 2B), with 25% increase of GS activity in the cortex ($P < 0.01$).

3.3. Protein carbonyl content

Fig. 3 shows very little amounts of protein carbonyls (≈ 1 nmol/mg protein) present in different brain areas of young animals (combined data for males and females). As expected, aged 30-month old animals, fed ad libitum, demonstrated the increase in protein carbonyl content, which was 5–7 times that of young three-month old animals ($P < 0.0001$). Aged DR rats had less protein carbonyls in each brain area studied, although this level was about two times higher in old female DR rats and 2.5–3.3 times higher in old male DR rats than in young animals ($P < 0.0001$).

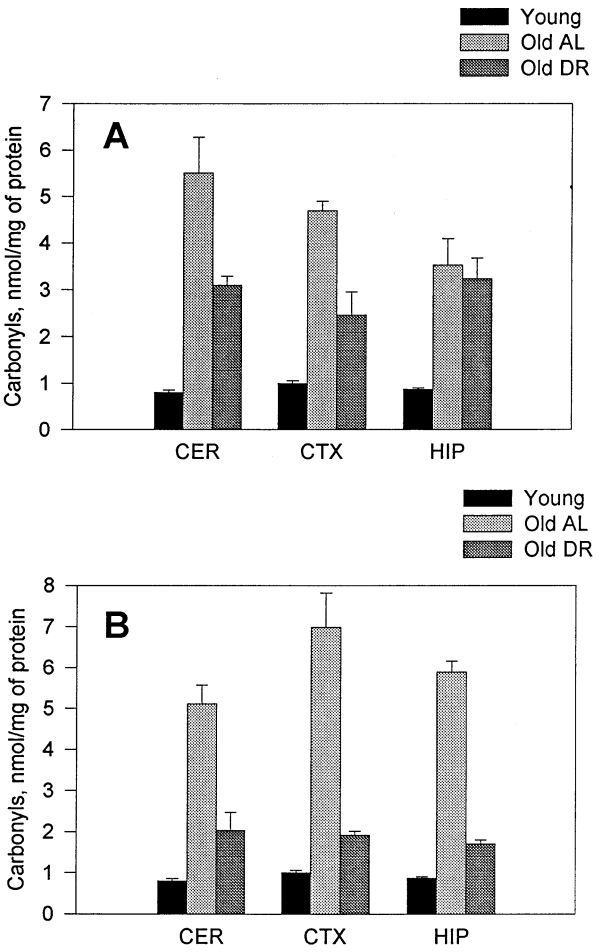

Fig. 3. Protein carbonyl content in the homogenates of the cerebellum, cortex and hippocampus of male (A) and female (B) brown Norway rats at the age of three months (young) and at 30 months fed ad libitum (old AL) and dietary restricted (old DR). Bars represent mean percentage carbonyl content ($\pm$ S.E.M.); $P < 0.0001$ in all groups and brain areas studied, relative to young control group.

4. Discussion

Free radical-mediated protein oxidation occurs in aging and may have important functional consequences. Oxidative damage, detectable by the protein carbonyl modifications, is associated with aging and it is postulated that oxidative damage to cellular components is a causal factor in aging (Harman, 1956, 1981, 1992; Butterfield et al., 1997a; Butterfield and Stadtman, 1997; Stadtman and Oliver, 1991; Sohal and Allen, 1986; Stadtman, 1986). It is well-documented now that the level of oxidatively modified proteins is increased in the aging brain (Butterfield et al., 1997a; Smith et al., 1992; Sohal et al., 1994b; Smith et al., 1991; Stohal et al., 1995). Many enzymes were shown to be sensitive to oxidation (Stadtman and Oliver, 1991; Stadtman, 1986; Fucci et al., 1983). Enzyme activity loss with age may be early indicators of tissue damage because oxidative inactivation of enzymes may lead to a disruption of cellular metabolism and may impair the capacity of the cell to repair the damage, especially if DNA undergoes a significant degree of oxidative damage (Sohal et al., 1994a; Stohal et al., 1995; Taylor et al., 1995). Antioxidant enzymes, which normally protect brain tissue against free radicals, may themselves be inactivated (Pigeolet et al., 1990) and this may contribute to an increase of free radical damage.

The activity of CK declines with age in the heart tissue of rodents and humans by $\approx 30\%$ from young controls (Schuyler and Yarbrough, 1990; Chesky et al., 1980; Steinhagen-Thiessen and Hilz, 1976) and in brain tissue of human patients by $\approx 40\%$ (Smith et al., 1991). We observed the decrease of CK activity in both male and female old rats fed ad libitum, which was $\approx 65-70\%$ of the level observed in young animals, the results consistent with those observed by others.

CK is considered to be one of the most vulnerable enzymes to oxidative stress (Thomas et al., 1994; Banerjee et al., 1991). Previous studies have demonstrated the decreased cardiac capacity of senescent rats which some authors link to the decreased CK activity (Schuyler and Yarbrough, 1990). It has been shown also that Alzheimer's disease patients have decreases in phosphocreatine and ADP levels and AD brain undergoes severe energetic stress (Pettegrew et al., 1994). This may be due in part to the dramatic decrease of CK activity in AD brain (Hensley et al., 1995; Burbaeva et al., 1992; Aksenov et al., 1997) while it has been demonstrated that oxidative stress is very important in the pathogenesis of AD (Harman, 1995; Butterfield, 1997; Markesbery, 1997; Bowling and Beal, 1995; Smith et al., 1996, 1995).

Glutamine synthetase undergoes oxidative inactivation and is very sensitive to oxidative stress (Levine et al., 1981; Levine, 1983b; Butterfield et al., 1997b). It has been shown that GS activity decreases with age in the human and gerbil cortex by $\approx 50\%$ (Hensley et al., 1995; Carney et al., 1991; Smith et al., 1991), which may lead to the accumulation of L-glutamate with resulting glutamate neurotoxicity. We did not see that much difference in GS activity: in both male and female old rats, GS was only 10–13% lower than in young animals. However, our data are consistent with those obtained by Mo et al. (1995), who demonstrated that GS activity was 8% lower in 24- versus 12-month old mice.

It is well known that the life span of diet-restricted rodents is longer than ad libitum fed animals (Ross, 1961). Although it is still unclear if the effects of low caloric intake on life span are primarily due to a slowdown of the basic mechanism of aging, it have been reported that some age-related biochemical and physiological changes can be delayed by DR (Taylor et al., 1995; Weindruch et al., 1986; Masoro et al., 1991). Delayed accumulation of carbonyls in DR Fisher 344 rats was demonstrated by Youngman et al. (1992). It was also shown that the increase of protein carbonyls with age can be retarded, but not eliminated, by DR. Sohal et al. (1994b) reported ≈ 2.5 and 3.5 nmoles carbonyls/mg protein in the brain of nine- and 23-month old mice fed ad libitum, which is close to our observations. DR mice had $\approx 20\%$ less carbonyls than AL animals, somewhat less pronounced than we had observed. The mechanism underlying the protective effect of dietary restriction on the age-related accumulation of oxidized proteins is unclear. It is possible that the decrease of caloric intake improves mitochondrial respiration and, therefore, decreases free radical production (Sohal et al., 1994b). Alternatively, DR may decrease the accumulation of damaged proteins by increasing the rate of their proteolytical degradation (Taylor et al., 1995). In addition, calorie restricted diet may improve the rate of protein synthesis and, therefore, may help to restore the level of active enzyme.

Restoration by DR of the activity of CK and GS enzymes to the young level has not been demonstrated. It has been shown that DR Sprague Dawley rats had higher activity of beta-galactosidase, arylsulfatase B and catepsin D than AL rats, but the activities never reached the young animals level (Ferland et al., 1990). Some improvements in the liver enzymes activity of old mice were reported by Koizumi et al. (1987).

Since DR animals exhibit lower level of protein carbonyls, lower level of DNA damage, lower rates of $O_2^{\cdot}$ and H_2O_2 generation, lower ROS production, less altered lipid fluidity and higher activity of some key enzymes, including antioxidant defense enzymes (Sohal et al., 1994a,b; Gabbita et al., 1997; Stohal et al., 1995; Taylor et al., 1995; Chung et al., 1992; Yu et al., 1990), DR rats seem to have milder consequences of age-related oxidative stress. We suggest that DR not only prolongs life span, but also modulates age-associated increased level of oxidative stress and lessens protein damage in aging.

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