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Effect of aging and late onset dietary restriction on antioxidant enzymes and proteasome activities, and protein carbonylation of rat skeletal muscle and tendon

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Abstract

Many studies have shown that lifelong dietary restriction (DR) can retard aging processes. Very few reports, however, are found that examined the effect of late onset DR on biochemical parameters in aging animals [Goto, S., Takahashi, R., Araki, S., Nakamoto, H., 2002b. Dietary restriction initiated in late adulthood can reverse age-related alterations of protein and protein metabolism. *Ann. NY Acad. Sci.* 959, 50–56]. We studied the effect of every-other-day feeding, initiated at the age of 26.5 months and continued for 3.5 months, on antioxidant enzymes, protein carbonyls, and proteasomes of the gastrocnemius muscle and tendon in rats. Age-related increase in the activity and content of Cu, Zn-SOD and the content of Mn-SOD was attenuated by the DR in both tissues. The same was true for glutathione peroxidase and catalase activities. Significant increase with age in protein reactive carbonyl derivatives (RCD) in the tendon was noted that was partially reversed by the DR. No significant change of RCD, however, was observed in the skeletal muscle. The age-related and DR-induced changes of the RCD in the tendon appeared to be associated with proteasome activity that decreases with age and increases by the DR. It is suggested that the late onset DR can have beneficial effects on the locomotive functions by reducing age-associated potentially detrimental oxidative protein damage in the tendon. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Dietary restriction; Late onset; Aging; Protein carbonylation; Proteasome; Antioxidant enzyme; Skeletal muscle; Tendon

1. Introduction

The free radical theory of aging proposes that aging is primarily caused by radicals derived from cellular metabolic processes and/or exogenous sources such as chemicals and irradiations (Harman, 1956, 1981). Age-associated decline of physiological

functions can be the result of significantly increased production of reactive oxygen species (ROS), including highly reactive hydroxy radicals which overwhelm the capability of antioxidant systems and scavenging activities of oxidatively modified molecules. The decline might also be due to decreased efficiency of repair activities that lead to the accumulation of oxidative damage together with the processes mentioned earlier. ROS can modify lipids, proteins and DNA. Accumulation of oxidized proteins

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appears to occur at a much higher extent (i.e. on the order of 5–10% of total cellular proteins on average, Stadtman, 1992) than that of oxidatively modified lipid (Miyazawa et al., 1993) or DNA (Richter et al., 1988; Kaneko et al., 1996), which is far below 0.1% as a steady state level. Moreover, oxidative modifications of amino acid residues in antioxidant and repair enzymes would curb their biological activities resulting in further accumulation of oxidative damage (Levine et al., 1981; Stadtman, 1992). Therefore, the degree of oxidative modifications of these enzymes is considered to have significant deteriorating consequences on cell function.

It has been reported by several groups of investigators that reactive carbonyl derivatives (RCD) of proteins which are believed to be formed by ROS-induced modification of side chains of arginyl, aspartyl, glutamyl, lysyl, prolyl, and/or thronyl residues accumulate with age (Stadtman, 1992; Goto et al., 2002a). Accumulation of RCD in the brain proteins appears to be closely related to impaired cognitive function with age, suggesting that accumulation of carbonylated proteins is not just a result but likely to be a causative factor of age-associated decline of physiological functions in the brain (Carney et al., 1991; Forster et al., 1996; Butterfield et al., 1997; Radák et al., 2001). It should also be mentioned, however, that the increase in oxidative damage appears to occur in a limited number of species of proteins rather than proteins in general (Goto et al., 1999), and the issue of age-related general changes of protein carbonyl contents is controversial (Goto and Nakamura, 1997).

Aging of mammalian species including human and rat is associated with decline in muscle mass and force generation (Lexell et al., 1983; Holloszy et al., 1991). Force generated in the sarcomere in the muscle is transmitted to bones to result in locomotion in which process the tendon, a connective tissue, plays a curricular role. Age-related changes in the antioxidant system and oxidative damage to proteins of the skeletal muscle have been reported (Ji et al., 1990; Ji, 1993; Leeuwenburgh et al., 1994; Aspnes et al., 1997). The tendon, however, has remained unexplored in this respect.

Dietary restriction (DR) can increase average as well as maximum life span of a variety of mammalian species as well as non-mammalian species such as

fish, nematodes and insects, serving as an important tool to study mechanisms of normal and decelerated aging (Yu, 1996; Sohal and Weindruch, 1996). A lifelong DR is known to attenuate age-related increase in the accumulation of oxidative damage in different organs (Sohal et al., 1993; Chen and Yu, 1994; Kaneko et al., 1997; Radák and Goto, 1998). It has been shown that proteins in hepatocytes from dietarily restricted old mice have significantly shorter half-lives than those of their ad libitum fed counterparts (Ishigami and Goto, 1990). These results suggest possible beneficial effects of DR on protein functions that decrease with age. Limited information is available, however, on whether a shorter period of DR initiated late in life has beneficial effects on the oxidative status in proteins. If proved, the late onset DR would provide a biological basis of potential intervention for human aging after middle age (Goto et al., 2002b).

In the present study, we investigated the activity and content of antioxidant enzymes, proteasome activities and RCD in the gastrocnemius muscle and the connected Achilles tendon of young control (10 month-old) and aged rats subjected to either ad libitum feeding or DR initiated at the age of 26.5 months and continued for 3.5 months.

2. Materials and methods

2.1. Animals

Male rats (F344/DuCrj) were purchased from Charles River, Japan at the age of 4 weeks and maintained under specific pathogen-free conditions in our animal facility (Toho University) with free access to laboratory chow CE-7 (Clea Japan, Tokyo) and water. The composition of the diet was as follows: protein, 17.2%; fat, 3.5%; fiber, 5.5%; ash, 5.9%; nitrogen-free extract, 59.9%; water, 8.0% (data obtained from the supplier). Under these conditions the rats had a mean life span of 29 months (Takahashi and Goto, 1987a). In the present study, rats 10 months old (young) and 30 months old (ad libitum fed old or dietarily restricted old, see below) were used.

Table 1
Effect of age and DR on antioxidant enzyme activity and content in rat gastrocnemius muscle

	AD10	AD30	EOD30
Cu, Zn-SOD content ($\mu\text{g}/\text{mg}$ protein)	1.42 \pm 0.31	1.88 \pm 0.41*	1.61 \pm 0.28
Cu, Zn-SOD activity (units/mg protein)	12.3 \pm 1.5	19.8 \pm 2.3*	12.8 \pm 2.0 [#]
Mn-SOD content ($\mu\text{g}/\text{mg}$ protein)	0.60 \pm 0.03	1.79 \pm 0.28*	0.59 \pm 0.04 [#]
Mn-SOD activity (units/mg protein)	6.52 \pm 0.84	8.41 \pm 0.96*	5.80 \pm 0.60 [#]
GPX (units/mg protein)	12.6 \pm 1.1	18.8 \pm 1.5*	14.3 \pm 1.3 [#]
CAT ($k \times 10^{-4}/\text{mg}$ protein)	0.52 \pm 0.05	1.15 \pm 0.08*	0.57 \pm 0.02 [#]
DT-diaphorase (nmol/mg protein)	0.21 \pm 0.02	0.18 \pm 0.01	0.19 \pm 0.01

AD10, 10 month-old rats fed ad libitum; AD30, 30 month-old rats fed ad libitum; EOD30, rats dietary restricted from 26.5 months of age for 3.5 months. Values are mean \pm SD of six animals per group. * $p < 0.05$ (AD10 versus AD30); [#] $p < 0.05$ (AD30 versus EOD30).

2.2. Dietary restriction

DR of rats by weekday every-other-day (EOD) feeding was initiated at 26.5 months of age and continued for 3.5 months. In the EOD group, the diet (CE-7) was provided on Mon, Wed, and Fri at the noon hour and removed from the food hoppers the following noon. Control animals were maintained similarly but fed ad libitum. Details of the effects of this regimen on various parameters of the aged animals will be reported elsewhere (Takahashi et al., in preparation).

2.3. Assays

The gastrocnemius muscle and Achilles tendon were excised and stored at -80°C until used for the experiments. The tissue extracts were prepared as described (Nakamura and Goto, 1996; Hayashi and Goto, 1998). Measurements of immunoreactive Cu, Zn-SOD and Mn-SOD were made by an enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies against SOD isoenzymes (Radák et al., 1995). The total SOD activity was determined by the method of Crapo et al. (1978). The unit of enzyme activity was defined as the amount of the enzyme that inhibited the rate of cytochrome *c* reduction by 50%. The mitochondrial Mn-SOD activity was measured in the presence of potassium cyanide because the cyanide inhibits the Cu, Zn-SOD activity but not the Mn-SOD. Cu, Zn-SOD activity was calculated by subtracting Mn-SOD activity from total SOD activity. Total activity of glutathione peroxidase (GPX) was assayed with cumene hydroperoxide as a substrate according to Tappel (1978).

The activity of catalase (CAT) was measured by the method of Aebi (1984). The oxidative carbonyl modification of proteins was evaluated by spectrophotometric assay as we described earlier (Nakamura and Goto, 1996; Radák et al., 1997, 1998).

The proteasome degrades oxidatively or otherwise modified proteins and has at least five distinct peptidase activities (Orlowski et al., 1993). In the present study, two types of activities among these were measured after separation of the enzyme from lower molecular weight proteases on glycerol gradient centrifugation as described previously (Hough et al., 1987; Hayashi and Goto, 1998). The peptidase activities were determined fluorometrically by measuring the release of 4-methyl-7-amino-coumarin (MCA) from fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-MCA (SUC-LLVY-MCA) and butyloxycarbonyl-Leu-Arg-Arg-MCA (BOC-LRR-MCA) for chymotrypsin-like and trypsin-like activities, respectively.

The method reported by Erstner (1967) was used to determine DT-diaphorase activity. The assay mixture contained 25 mM Tris-HCl (pH 7.4), 0.3 mM NADH, 0.04 mM 2,6-dichloroindophenol and 0.2% Tween-20. The reaction was started by the addition of the enzyme fractions, and the reduction of the substrate was followed at 600 nm.

2.4. Statistical analysis

The biochemical assay data were assessed by ANOVA, followed by Scheffe's post-hoc test. When applicable, an unpaired Student's *t*-test was used. The significance was set at $p < 0.05$.

Table 2
Effect of age and DR on antioxidant enzyme activity and content in rat Achilles tendon

	AD10	AD30	EOD30
Cu, Zn-SOD content ($\mu\text{g}/\text{mg}$ protein)	0.12 ± 0.01	$0.17 \pm 0.01^*$	$0.13 \pm 0.01^\#$
Cu, Zn-SOD activity (units/mg protein)	1.42 ± 0.02	$1.83 \pm 0.04^*$	$1.36 \pm 0.02^\#$
Mn-SOD content ($\mu\text{g}/\text{mg}$ protein)	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.01
Mn-SOD activity (units/mg protein)	0.75 ± 0.02	$0.88 \pm 0.04^*$	$0.72 \pm 0.03^\#$
GPX (units/mg protein)	2.31 ± 0.23	$3.87 \pm 0.58^*$	2.90 ± 0.34
CAT ($k \times 10^{-4}/\text{mg}$ protein)	0.117 ± 0.010	$0.169 \pm 0.020^*$	$0.123 \pm 0.010^\#$
DT-diaphorase (nmol/mg protein)	UD	UD	UD

Animals used are the same as in Table 1. Values are mean \pm SD of six animals per group. * $p < 0.05$ (AD10 versus AD30), # $p < 0.05$ (AD30 versus EOD30).

3. Results

3.1. Body weight

The EOD feeding resulted in about 30% loss of body weight in the first 80 days, but no significant change was observed thereafter (details will be reported elsewhere; Takahashi et al.). The control rats fed ad libitum maintained a steady body weight throughout the experimental period.

3.2. Antioxidant enzyme activities (Tables 1 and 2)

The activities of antioxidant enzymes were many-fold lower in the tendon than in the skeletal muscle. The Cu, Zn-SOD activity and its content increased significantly with age but the increase was attenuated or reversed in dietarily restricted old animals in both the skeletal muscle and tendon. On the other hand, the

Mn-SOD activity and its content increased as a function of age in the skeletal muscle but no significant alteration was observed in the tendon. The DR reduced the age-related increase in the Mn-SOD content in the skeletal muscle. No significant change was observed in the content in tendon by DR, however. The activities of GPX and CAT increased with age significantly and this change disappeared with DR in both tissues. The activity of DT-diaphorase did not change with age or DR.

3.3. Reactive carbonyl derivatives in proteins (Fig. 1)

The RCD content did not change significantly with age or with DR in the skeletal muscle (Fig. 1(a)). In contrast, a marked increase of 200% was observed in the tendon in the aged group but this was reduced to about 150% of the level of the young animals in DR groups (Fig. 1(b)).

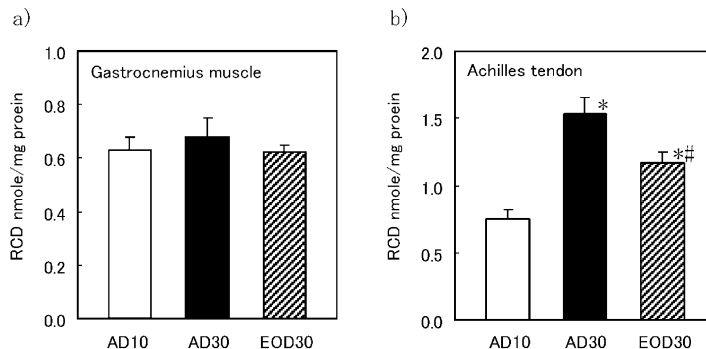


Fig. 1. Effect of age and DR on RCD in proteins of the gastrocnemius muscle (a) and Achilles tendon (b). AD10, 10 month-old rats; AD30, 30 month-old rats; DR30, rats dietary restricted from 26.5 months of age for 3.5 months. Values are mean \pm SD of six animals per group. * $p < 0.05$ (AD10 versus AD30), # $p < 0.05$ (AD30 versus EOD30).

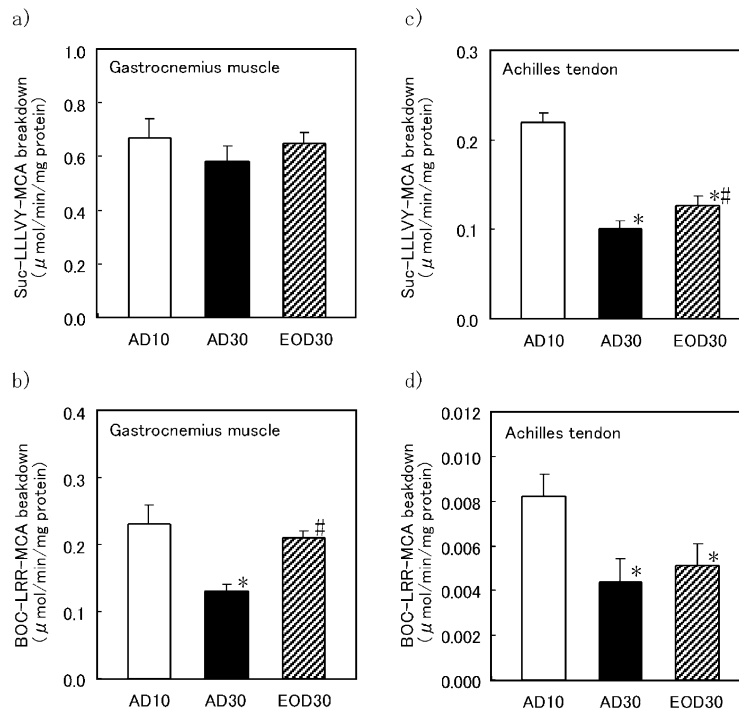


Fig. 2. Effect of age and DR on the chymotrypsin-like activity (breakdown of SUC-LLVY-MCA) (a,c) and trypsin-like activity (breakdown of BOC-LRR-MCA) (b,d) in the gastrocnemius muscle (a,b) and Achilles tendon (c,d). Animals used are the same as in Fig. 1. Values are mean \pm SD of six animals per group. * $p < 0.05$ (AD10 versus AD30), # $p < 0.05$ (AD30 versus EOD30).

3.4. Proteasome activity (Fig. 2)

The trypsin-like activity of proteasome declined remarkably ($\sim 45\%$) with age in the skeletal muscle, and DR restored higher trypsin-like activity, comparable to the level of young animals (Fig. 2(a)). A similar tendency was observed in the chymotrypsin-like activity in the tissue (Fig. 2(b)). However, the effects of age and DR on the chymotrypsin-like activity in the tissue were not significant.

In the tendon, both chymotrypsin- and trypsin-like activities of the proteasome declined markedly (50–60%) with age (Fig. 2(c) and (d)). These activities tended to increase in dietarily restricted aged-animals but the change was marginally significant in chymotrypsin-like activity (Fig. 2(c)) and not significant in trypsin-like activity (Fig. 2(d)).

4. Discussion

The present findings suggest that the tendon accumulates a significant amount of RCD with aging, and this could be due to the age-associated decline in proteasome activity and/or to increase in the generation of ROS. The accelerated age-related change in the tendon might also be related to low proteasome activity (Fig. 2) and very low antioxidant enzyme activities (Tables 1 and 2) in this tissue as compared with the skeletal muscle. This might explain part of the clinical consequences since it could lead to marked increase in the healing period of tendon injury, which often occurred in aged individuals (Smith et al., 1999).

The lifelong DR has been shown to delay accumulation of oxidatively modified DNA with age (Kaneko et al., 1997) and age-associated abnormalities in the skeletal muscle (Aspnes et al., 1997). The

RCD is reported to increase with age in different organs (Stadtman, 1992; Sohal et al., 1993) including the skeletal muscle (Mecocci et al., 1999; Sato et al., 1998). However, a recent study by Bejma and Ji (1999) shows that aging is not associated with increase in RCD content in the skeletal muscle. In the present study, we did not observe a significant effect of DR on the RCD accumulation in this muscle. Therefore, 3.5 months of DR initiated at old age does not seem to be an effective means of reducing the accumulation of RCD in this tissue.

Age-related decline of proteasome activity has been reported for the liver (Shibatani and Ward, 1996; Hayashi and Goto, 1998; Anselmi et al., 1998). The present data revealed, for the first time to our knowledge, that aging is associated with significant decline of proteasome activity in the tendon and to some extent in the skeletal muscle. This age-related decline in the proteasome activity may at least partly account for the increase in oxidatively modified proteins in the connective tissue. In the skeletal muscle, the effects of lowered proteasome activity may be compensated by high antioxidant enzyme activities, thus keeping the RCD level low.

It has been proposed that the accumulation of oxidatively modified proteins, such as those detected by RCD (Grune et al., 1997; Goto et al., 2001) is related to the half-life of proteins: namely, proteins with short half-lives have a small amount of RCD while those with long half-lives accumulate more RCD. Hence, the activity of proteolytic systems appears to play a crucial role in the accumulation of RCD. In general, the half-lives of proteins such as collagens and elastin in the tendon are longer than intracellular proteins and this could be one reason for greater accumulation of RCD in proteins in this tissue compared with those in the skeletal muscle.

Despite intensive investigation, the mechanisms by which DR effects ROS generation and extends life span are still largely unknown (Masoro, 2000). One hypothesis is that DR lowers the ROS generation in mitochondria (Weindruch et al., 1980; Sohal and Weindruch, 1996). The fact that the activity and content of mitochondrial Mn-SOD increased with aging in the skeletal muscle is in agreement with the finding that the RCD content does not change with age. The Mn-SOD activity would be down regulated by DR, suggesting reduced ROS generation. How-

ever, the mitochondrial SOD activity and content did not change with age significantly in the tendon, probably indicating that mitochondrial superoxide formation does not increase with aging in this tissue.

Age-related increase in antioxidant enzyme activity in the skeletal muscle suggests that the increased scavenging activity was not efficient enough to cope with the deleterious effects of ROS to avoid oxidative damage of macromolecules (Ji et al., 1990; Oh-Ishi et al., 1995). On the other hand, the age-associated increase in antioxidant enzyme activities was attenuated in dietary restricted animals, indicating that the DR might depress the rate of ROS generation. Attenuation of antioxidant enzymes in the skeletal muscle by our regimen of DR is in accordance with the finding of Luhtala et al. (1994) in their lifelong DR experiment. The very notable difference in the activity of antioxidant enzymes between the two tissues is most probably due to the difference in the metabolic activity, that in the skeletal muscle being higher than that in the tendon. It should be mentioned that the observed changes are also possibly due to increased activity of DR animals compared to sedentary ones since DR animals tended to move around more perhaps searching food.

Taken together, we found in this study that the tendon accumulates significantly larger amount of RCD than the connecting skeletal muscle with age. The activities and contents of SOD isoenzymes are many-fold lower in the tendon than the skeletal muscle and there is an increase in antioxidant enzyme activity in both tissues with age. The activity of proteasome decreases in both tissues and this decrease is more prominent in the tendon. DR attenuates and reverses the age-associated increase in antioxidant enzyme activity, but the low degree of RCD accumulation in the tendon suggests that the accumulation is due to decrease in the turnover of proteins rather than an increase in ROS formation. Finally, it should be stressed that potentially beneficial outcomes of DR may be attained even when initiated late in life, the finding in keeping with our previous reports (Takahashi and Goto, 1987b; Ishigami and Goto, 1990).

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