Effect of *SOD1* Overexpression on the 20S Proteasome during Aging

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**Abstract**

Metabolism of oxygen derivatives has been shown to be altered in Down syndrome (DS) due to the overexpression of the Cu/Zn superoxide dismutase gene (*SOD1*) on chromosome 21. Transgenic mice for the human *SOD1* gene (*hSOD1*) exhibit some features of the syndrome. Oxidation of proteins and oxidative stress are involved in normal and pathological aging. The proteasome is an adaptative system to eliminate the modified proteins which can be deleterious. As *SOD1* overexpression has been shown to be either deleterious or protective according to tissues and paradigms, we have measured in function of age the 20S proteasome activities in neural tissues (cerebral hemisphere, cerebellum and cortex) and in the thymus and the heart from control and transgenic mice. Indeed, although *SOD1* overexpression is very deleterious in thymus and heart, it has little effect in cerebral hemisphere and cortex depending on the proteolytic activity measured. Conversely in the cerebellum the three proteolytic activities decrease dramatically in transgenic old mice while it was not modified in control mice during aging. The results of this study suggest that some phenotypes of DS present in thymus, heart and neural tissues of *hSOD1* transgenic mice might be partially due to the modulation of the 20S proteasome expression during aging.

**Keywords**

*SOD1*, Transgenic Mice, Proteasome, Aging, Thymus, Heart, Neural Tissues, Cerebellum

1. Introduction

Metabolism of oxygen derivatives has been shown to be altered in Down syndrome [1]-[5] due to the overexpression of the Cu/Zn superoxide dismutase *SOD1* gene on chromosome 21 [5] [6]. Transgenic mice for the

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human SOD1 gene (hSOD) were the first model for studying Down syndrome [7] and since then two other transgenic lines have been developed [8] [9]. hSOD1 transgenic mice exhibit some features of the syndrome such as premature thymic involution and early T lymphocytes development [10] [11], deficiency in spatial memory and LTP which can be restored by antioxidants [12], abnormal sleep pattern [13], abnormal neuropathic pain [14] and cardiac abnormalities [15]. SOD1 overexpression has been shown to be either deleterious or protective according to tissues tested and physiological situations studied [16] and indeed SOD-1 overexpression was shown to be protective against in vivo insults as glutamate and MPTP toxicity but deleterious for aging processes [17]-[20].

Protein oxidation in cells is a consequence of radical oxygen species production as they can oxidize specific amino acids in proteins that will be rapidly degraded by proteases [21]. Organisms have developed a complex and adaptative proteolytic system, the proteasome, to degrade selectively oxidized and damaged proteins [22]-[24] thus preventing their accumulation which can be deleterious in many diseases (review in: [25] [26]). The 26S proteasome consists of a 20S catalytic core and one or two regulatory complexes. The 20S core is a cylindrical unit consisting of 28 subunits, arranged as four heptameric rings whose activity is to degrade oxidized proteins in an ATP- and ubiquitin-independent manner (Adams et al., 2003) [27]. Under basal conditions, the 26S proteasome is used in an ATP- and ubiquitin-dependent manner to eliminate damaged proteins but under stress conditions, probably due to its higher resistance to degradation, the 20S proteasome becomes the major degradation machinery for damaged proteins thus preventing the pathogenicity caused by protein aggregates [28] [29]. The levels of ROS have been suggested to increase during aging and to contribute to aging associated alterations in many different tissues [30]. Modifications in the proteasome activities have been hypothesized to be involved in normal and pathological aging [29] [31] and it has been suggested that proteasome activation could be a novel anti-aging and anti-neurodegenerative strategy [32]-[34].

It is rather well known that persons with Down syndrome are at risk for aging [35] [36]. This aging concerns various systems: the dermic (skin dryness), the immune (early thymic involution), the ocular (early cataract, keratoconus), the musculoskeletal system, the neural system (Alzheimer-like neuro-anatomopathology).

As SOD1 is an important scavenging factor against oxidative stress mediated by ROS and was shown to be either protective or deleterious in various paradigms, we checked if “in vivo” SOD1 overexpression modifies during aging the three “in vitro” proteolytic activities of the 20S proteasome in various neural organs (cerebral hemisphere, cerebellum and cortex) and in two other tissues affected by SOD1 overexpression (thymus and heart). We showed that the evolution during aging of the SOD1 activity was different in thymus, cerebral hemisphere, cortex and cerebellum from transgenic mice. Although the 20S proteasome content did not differ in function of age in the various tissues, the proteasome activities were completely different in the thymus or the heart compared to neural tissues for both controls and transgenic mice. SOD1 overexpression has a deleterious effect in the thymus and the heart as well as in the cerebellum but with a much less extent in the cerebral hemisphere and the cortex.

2. Material and Methods
2.1. Animals
hSOD-1 transgenicmice (KT line) have been obtained by microinjecting in FVB/N blastocysts a 11.5 genomic fragment of the human SOD-1 gene including its own promotor [37]. Homozygous hSOD-1 mice (SS) and control mice were generated from crosses between hemizygous (S+) transgenic mice. Transgenic and control mice used in the present study were from 15 days until 24 months-old depending on the tissue tested and were maintained on a 12 hr:12 hr dark cycle, with food and water ad libitum. We used tissues of at least four animals of each type. All experiments were conducted in accordance with the ethical standards of French and European regulations (European Communities Council Directive, 86/609/EEC). All procedures with experimental animal were approved by University Animals Care Committee, according to the Guide for the Care and Use of Laboratory Animals.

2.2. Genotyping
The presence of transgene in the off springs (S/+ or SS) was confirmed by polymerase chain reaction using DNA extracted from the tail as previously [9].
2.3. Tissues Extraction

Mice were killed by decapitation and the various tissues were rapidly removed, frozen in liquid nitrogen at −80°C prior to utilization. Tissues were immersed in ice-cold homogenization buffer (20 mM HEPES, 0.1 mM EDTA, 1 mM β-mercapto-ethanol, pH 7.8) and homogenized (1 ml buffer/100 mg tissue) utilizing an ultraturrax. The homogenates were then centrifuged at 12,000 g for 20 minutes (4°C). Supernatants were collected and protein concentration was determined using the Bradford reagent (Bio-Rad, Bethesda, USA) and bovine serum albumin (Sigma, St. Quentin-Fallavier, France) as the standard protein prior to measurements of SOD-1 and proteasome activities.

2.4. Measurement of SOD Activities

SOD activity was performed using the commercially available Superoxide Dismutase Assay Kit from R & D systems. This method employs xanthine and xanthine oxidase to generate superoxide radicals which convert Nitro Blue Tetrazolium (NBT) into NBT-diformazan which absorbance is measured at 560 nm. SOD activity is quantified by the degree of inhibition of this reaction.

2.5. Proteasome Activities Measurements

Three activities of the 20S proteasome were measured in protein extracts for the various tissues of transgenic mice (S/+ or SS) and controls in absence or presence of the proteasome-specific inhibitor MG132 (N-Chz-Leu-Leu-Leucinal) to obtain the real proteasome activity. Chymotrypsine-like, trypsin-like and caspase-like activities were assayed with specific fluorogenic peptides LLVY-MCA (chymotrypsine-like), LSTR-MCA (trypsin-like) and LLE-NA (caspase-like) as previously described (Le Pecheur 2005). Excitation/Emission wavelengths were measured at 350/440 nm for LLVY-MCA and LSTR-MCA and at 333/410 nm for LLE-NA.

2.6. Western Blotting for Proteasome

Extracted proteins (20 µg) were suspended in Laemmli buffer containing 0.5% mercaptoethanol and denatured at 95°C for 10 min. Proteins were separated on 12% SDS-polyacrylamide gel and transferred to a Hybond nitrocellulose membrane (Amersham Life Science, USA) for 1 h at room temperature. Specific sites on the membrane were blocked 1 h at room temperature in a 5% non-fat dry milk/0.2% Tween20-PBS solution. After blocking, membranes were incubated with primary antibody (polyclonal rabbit anti-20S proteasome, 1:2000 or monoclonal mouse anti-β-actin, 1:2000 (Sigma, St. Quentin-Fallavier, France) overnight at 4°C in 5% non-fat dry milk/0.2% Tween20 in PBS. A secondary anti-mouse or rabbit horseradish peroxidase-conjugated antibody (Sigma, St. Quentin-Fallavier, France) was used followed by ECL detection (Amersham Life Sciences, USA) according to the supplier’s instructions. Images of protein band intensities were obtained with the LAS-3000 imaging system (Fuji Photo Film Co., Ltd.). Densitometry analysis was performed using an image analyzer (Multi Gauge software, Fuji Photo Film Co., Ltd.) and densitometry measurements of the 20S proteasome levels were normalized to β-actin. All experiments were performed using two biological replicates for each sample and three different samples for each age. Scans analysis of western blots were performed using Multi Gauge® V2.02 software.

2.7. Statistical Analysis

One test ANOVA Newman-Keuls Multiple Comparison Test was calculated using Prism Graph Pad® Software; p ≤ 0.05 was assessed as significant. All data are represented as mean ± SEM. ***, °°° and ####p ≤ 0.001; **, °° and ##p ≤ 0.01; *, ° and #p ≤ 0.05.

3. Results

3.1. SOD-1 Activities

3.1.1. In Thymus

SOD1 activity was measured for controls and S/+ mice aged 0.5, 1, 3 and 12 months. The SOD1 activity (Figure 1(a)) was increased until one month in transgenic and control mice (36 U/mg and 4 U/mg respectively)
and then decreased in the 3 and 12 month animals for both S+ and control mice (15 and 3 U/mg). When the activities were compared between controls and S+ tissues the overexpression was highest for the 1 month old animals (7 fold) and was around 3 - 4 fold for the other ages. Unfortunately we did not have access to SS samples for measurements of SOD1 activities in the thymus.

3.1.2. In Cerebral Hemisphere
SOD1 activity was measured in S+, SS and control animals aged 1, 2, 12 and 24 months (Figure 1(b)). In control tissues there was a small increase in SOD1 activity (2 to 5 U/mg) for young to old animals. In the tissues from both S+ and SS animals there is a slight increase in SOD1 activity from 1 to 2 months (25 to 32 U/mg and 30 to 40 U/mg respectively), the activity being stable in older animals. The overexpression was between 6 and 9 fold in S/+ and SS animals respectively when compared to control ones. The activities in SS tissues were slightly higher than in S/+ (13% around).

3.1.3. In Cerebellum
SOD1 activity was measured in S+, SS and control animals aged 1, 2, 12 and 24 months (Figure 1(c)). The SOD1 activity profile was similar to that in cerebral hemisphere for both S+ and SS and control tissues. In controls there was an increase in SOD1 activity (2 to 4 U/mg) for young to old animals. In the tissues from both S+ and SS animals there is a slight increase in SOD1 activity from 1 to 2 months (15 to 25 U/mg and 18 to 35 U/mg respectively), the activity being stable in older animals. The overexpression was between 6 and 8 fold in S/+ and SS animals when compared to control ones. The activities in SS tissues were slightly higher than in S/+ (12% around).

3.2. Proteasome Activities in Thymus, Heart, Cerebral Hemisphere, Cerebellum and Cortex

3.2.1. In Thymus
The proteasome activities were measured in control and S/+ animals aged 15, 80 days and 8 months and in SS animals aged 80 days and 8 months (Figures 2(a)-(c)). In tissues for control animals, chymotrypsine- and trypsin-like activities were dramatically decreased in function of age whereas the caspase-like activity was decreased from 15 to 80 days only. In S/+ mice there was a decrease only at 8 months for chymotrypsine- and trypsin-like activities and no modification in the caspase-like activities. When the three activities for S/+ tissues were compared to control ones at 15 days there was a decrease of 40% around whereas chymotrypsine- and caspase-like activities in tissues from S/+ and SS at 80 days and 9 months were similar. A slight decrease in the trypsin-like activity was measured in S/+ and SS tissues when compared to controls aged 8 months.

3.2.2. In Heart
The proteasome activities were measured in control and S/+ and SS animals aged 1, 4 and 11 months
Figure 2. Proteasome activities in thymus (for 15, 80 days and 8 months-old mice) and in heart (for 1, 4, 11 months-old mice). The homogenates for chymotrypsin—((a) and (d)), trypsin—((b) and (e)) and caspase-like ((c) and (f)) activities for thymus and heart respectively were measured as described in materials and methods. Results for S/+ and SS mice are in yellow and red boxes respectively and control mice in white boxes. The results are expressed as percentage of the 100% for each proteasome activity in control mice (100% for the 15 days thymus and 100% for the 1 month heart). In the thymus the effect of age, expressed as mean ± SEM was obtained for each group (control, S/+ and SS) using a Student t-test for unpaired samples: *p < 0.05, **p < 0.01 and ***p < 0.001 for control mice and °p < 0.05, °°p < 0.01, °°°p < 0.001 for S/+ and SS mice. Comparison of the values for S/+ and SS mice in yellow and red boxes respectively versus control mice in white boxes using a student t-test for unpaired samples is marked ###p ≤ 0.001. In the heart, all the comparisons within a group or between groups are given with the same symbols *p < 0.05, **p < 0.01 and ***p < 0.001 and we could not add other comparisons for technical reasons.

(Figures 2(d)-(f)). In tissues from control mice there was a decrease for the three activities in function of age which a more dramatic decrease for the chymotrypsine activity than for the two others. In control animals aged between 11 and 1 month old comparison in the three activities shows that the chymotrypsine-like activity was decreased by 60%, the trypsin-like by 45% and the caspase like by 35% only. In S/+ tissues there was a dramatic decrease in the chymotrypsine-like and caspase-like activities in function of age, a decrease in trypsin-like activity was present for 11 months tissues only. But in tissues from SS animals there was a slight decrease in function of age and in the three activities. It should be pointed out that tissues from one month old animals exhibit already reduced proteasomes activities similar to those of the control ones at 11 months.

3.2.3. In Neural Tissues
The proteasome activities were measured in control and SS animals aged 3, 8 and 16 months in the cerebral hemisphere (Figures 3(a)-(c)), the cerebellum (Figures 3(d)-(f)) and in 7, 15 and 24 months in the cortex (Figures 3(g)-(i)). In the three tissues from control mice the proteasome activities were not modified in function of age. In the tissues from SS animals there was almost no decrease for the three activities in cerebral hemisphere and a 25% decrease for the chymotrypsine- and caspase-like activity in the cortex of 24 months mice when compared to those of 7 months. Conversely in the cerebellum there was already a slight decrease for 8 month animals when compared to 3 months and a dramatic decrease at 16 months. When SS tissues were compared to control ones there was a slight decrease (12% around) in the three activities in the cerebral hemisphere but for 8 and 16 months tissues only. The same profile was obtained for the cerebellum but with a more drastic decrease in 16 month tissues for the three activities (30% to 13% according to the proteasome activities).

3.3. Expression of the 20S Proteasome in Thymus, Cerebral Hemisphere, Cerebellum and Cortex
To determine if the proteasome activities decrease measured in thymus and neural tissues of hSOD-1 transgenic
mice could be due to a decrease of 20S proteasome levels, we assayed by western blot analysis the amount of 20S proteasome as described in material and methods. The thymus samples were from S/+ , SS and control mice aged 1 months old, the cerebellum samples were from 3, 8, 16 months old SS and control mice, the cerebral hemisphere samples were from 3, 8, 16 months old SS and control mice and cortex samples were from 7 and 15 months old SS and control mice. As shown in Figure 4 there was no modification of the proteasome content in the tissues tested according to age or status (S/+ , SS or control mice).

4. Discussion

Previous results on the same mice have shown that oxidation might explain the alteration of proteasome activities in the cortex of SOD transgenic mice [38]. Proteasome inactivation during aging is probably a general phenomenon, but is variable among selective cell types and tissues some of which being more vulnerable to age-related alterations. Thus we have measured proteasome activities in function of age in neural tissues and for comparison in two other tissues thymus and heart for which SOD1 overexpression has been shown to induce some abnormalities. We measured the proteasome activities in the thymus and the heart of both hemizygous (S/) and homozygous (SS) transgenic mice and in the neural tissues for homozygous (SS) transgenic mice only.

As shown in the results there was in thymus a decrease in function of age for the three activities in both control and transgenic animals but in transgenic animal it was mostly in old animals and for the chymotrypsin- and trypsin-like activities only. These differences between thymus of transgenic and controls animals could be explained by the fact that there was already a decrease by 40% of the three activities in transgenic young animals. The decrease is enhanced in older transgenic animals (8 months) for two of three proteasome activities Thus in thymus, SOD1 overexpression, which is enhanced by a 7 fold in activity, is deleterious in very young animals but not in older ones. Besides the early thymic involution and the early T cell differentiation previously shown [10] [11], our present results confirm the deleterious effect of SOD1 overexpression in the thymus physiology. Interestingly, it was recently shown a decrease in the beta 5t subunit of the proteasome in the thymus of...
individuals with T21 which might explain at least partly the thymic and immunological abnormalities in these patients [39].

In the heart, previous results obtained in the rat species have shown decline in 20S proteasome activities during aging between 3 and 26 months old animals [40] [41]. In the present study using tissues of S/+, SS and controls 1 - 11 months old mice, the three proteasome activities were decreased in function of age for the three types of animals. SOD1 overexpression, induces a much earlier decrease but with a similar level as in old controls. Thus during aging the proteasome activities were decreased in heart for control and transgenic mice and SOD1 overexpression increases this deleterious effect of aging.

In the aging CNS, multicatalytic proteasome activity has been shown to be decreased in the cortex and hippocampus of very old rats but not in the cerebellum and brain stem [30]. In another study, when 15-months old rats and mice were compared with 5-months old animals [42], the chymotrypsin-like and the trypsin-like activities were decreased in the cortex and the cerebellum respectively. In the mouse (6 weeks and 15 months animals) from the same study, the chymotrypsin-like and the trypsin-like activities were decreased in the cortex and the cerebellum respectively. In the murine model to study trisomy 21, the Ts65Dn mice, chymotrypsin-like activity in the cerebellum was reduced by 35% [43].

In the present study we measured the three 20S proteasome activities in tissues from cerebral hemisphere and cerebellum for SS and controls 3, 8 and 16 months old animals and in the cortex 7, 15 and 24 months old animals. As shown in the results, no significant modification in function of age was shown in control mice for the three activities in the various tissues. This result can be correlated to the fact that fibroblasts from centenarians have a normal proteasome [44]. Conversely, when tissues of SS mice were compared to controls, the three proteasome activities were almost similar in the 3 months old animals and were decreased in tissues from older animals especially in the cerebellum in which the decrease was between 50 and 30% according to the activity measured. The present result is in accordance with that obtained in the Ts65Dn mice [43]. Such decreases were also measured in the cortex but to a lesser extent. These present results can be correlated to the results of a recent study showing that in the frontal cortex of DS brain (average age 24.7 for control brains and for DS brain 23.1), the three proteasome activities were decreased [44]. Our results might also help to understand better how oxidative stress is involved in the proteostasis network involved in Down syndrome before Alzheimer pathology will be present [45].
5. Conclusions

The present study was performed to investigate if “in vivo” SOD1 overexpression induces modifications in the proteolytic activities of the 20S proteasome in various organs (thymus, heart, cerebral hemisphere, cortex and cerebellum) during aging. The results obtained show that although the SOD1 overexpression measured by the enzymatic activity is very high in most tissues tested there are significant difference among tissues during aging:
- In tissues of control mice, the SOD1 activities remain very high in neural tissues but not in thymus
- SOD overexpression induces a reduction in the proteasome activities by 20% - 30% around in all tissues from young mice.
- During aging in control mice, the proteasome activities are almost not modified in neural tissues but are decreased dramatically in thymus and heart.
- During aging in transgenic mice, the proteasome activities decrease earlier than in controls but quantitatively no more than in the oldest control mice.
- During aging in transgenic mice, the proteasome activities decrease dramatically in heart and cerebellum.

Thus these results demonstrate that some tissues are more protected than others and it is particularly interesting that our results showed that cortex and cerebral hemisphere are protected during aging while cerebellum is not.

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