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LMP2 Knock-Out Mice Have Reduced Proteasome Activities and Increased Levels of Oxidatively Damaged Proteins

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ABSTRACT

The proteasome is a large intracellular protease, composed of multiple subunits, that is present in all eukaryotic cells. Proteasome inhibition is known to occur during normal aging, and is believed to contribute towards an age-related increase in oxidative stress, although at present the mechanisms responsible for mediating age-related changes in proteasome activity have not been elucidated. At present the relationship between proteasome subunit expression, proteasome activity, and protein oxidation during normal aging has not been elucidated. In the present study we observed that the absence of LMP2, a specific proteasome subunit, decreases proteasome activities in both the brain and liver, with increased levels of protein oxidation occurring in both tissues. Results from this study demonstrate for the first time that individual proteasome subunits are important for the regulation of age-related changes in both proteasome activity and protein oxidation. *Antioxid. Redox Signal.* 8, 130–135.

INTRODUCTION

As part of normal aging, as well as in a wide variety of age-related disorders, there is a well-documented increase in the level of protein oxidation (2, 19). Although the mechanisms responsible for mediating elevated levels of protein oxidation have not been elucidated, increasing evidence suggests that increased protein oxidation levels may play a causal role in promoting a variety of deleterious physiological disturbances observed in both normal aging and age-related disease (2, 19). The proteasome is a large intracellular protease that is composed of multiple subunits, possesses several proteolytic activities, and is responsible for mediating the degradation of a wide variety of intracellular protein substrates (3, 4). Numerous reports suggest that proteasome inhibition occurs in a variety of age-related disorders, as well as normal aging, with proteasome inhibition possibly playing a particularly important role in mediating increased levels of protein oxidation (5, 6, 12, 13, 14, 17, 20, 22). At present the mechanism responsible for mediating proteasome inhibition in each of these conditions has not been elucidated.

In order to identify the contribution of individual proteasome subunits towards overall proteasome function, as well as general physiological and cellular homeostasis, a number of transgenic mice have been established and well characterized. One of the first mice generated for this purpose was the low molecular weight protein 2 knock-out mouse (LMP2 KO) (21). LMP2 is regarded as an inducible β subunit, and is thought to play an important role in the inducible- and immunology-related activities of proteasome (3, 4). Nearly all studies using transgenic mice deficient for individual proteasome subunits have focused on elucidating the role of individual proteasome subunits towards the regulation of immunological function, with no previous studies examining whether the absence of a single proteasome subunit has any effect on age-related impairment of proteasome function. Additionally, no previous study

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has examined whether absence of a single proteasome subunit alters the level of protein oxidation in young or aging mice.

The focus of the present study was to determine if proteasome subunit expression becomes altered during normal aging, to determine the relationship between altered proteasome subunit expression and age-related declines in proteasome activity, to determine the relationship between age-related increases in protein oxidation and age-related proteasome inhibition, and to elucidate if the absence of an individual proteasome subunit (LMP2 subunit) alters any of these processes.

MATERIALS AND METHODS

Transgenic mice

Breeding pairs of LMP2 KO mice were a generous gift of Dr. L. Van Kaer (Vanderbilt University), and have been described extensively in previous studies (15, 16, 21). These mice were developed on a C57BL/6 background, with mice of this same background used as controls in all studies. All animals were maintained on a 12-hour light/dark cycle, and were maintained in a sterile pathogen-free environment, which was confirmed by the routine serological examination of sentinel mice in each room. A total of 8 wild-type mice and 8 LMP2 KO mice were utilized for each of the experimental conditions, in each of the biochemical assays reported. Tissues from each animal were harvested separately, and independent samples were used in all analyses. Only the brain and liver tissue were used in this study. Previous studies have documented that LMP2 KO mice appear normal, and possess no obvious health deficits up to 1 year of age (21).

Analysis of proteasome activity and proteasome expression

Proteasome activity was determined as described previously (7–9, 18). Briefly, cell lysates were collected and protein aliquots (1 μg/μl) generated in proteasome activity buffer (10 mmol/L Tris-HCl, pH 7.8, 1 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 5 mmol/L $MgCl_2$). Substrates specific for each of the proteasome proteolytic activities were added to proteasome activity buffer, and the cleavage of each of these peptides utilized to quantify the amount of proteasome activity. For analysis of 26S proteasome activity, 2 m*M* ATP was added to proteasome activity buffer. A standard curve of free 7-amido-4-methylcoumarin (MCA) was utilized for quantification of proteasome activity. Fluorescence was monitored at 340 nm excitation and 440 nm emission. Proteasome-mediated degradation of oxidized BSA was conducted using BODIPY labeled BSA (Molecular Probes, Carlsbad, CA), exactly as was reported previously for proteasome-mediated degradation casein (18), with the exception that BSA was mildly oxidized with $FeSO_4$ prior to analysis.

Proteasome subunit expression was quantified using a custom gene array, which utilizes membrane-bound gene-specific PCR products, ranging from 200 to 500 base pairs. The ^{32}P-labeled cDNA, derived from the retro-transcription of total RNA, was utilized as probe. A serial dilution of β-actin and 18S rRNA fragments was utilized for normalization and quantification. Data was collected using a phosphoimager (Fuji, Stamford, CT) and quantified using NIH software (Scion Image, Frederick, MD). Each gene was analyzed in duplicate and normalized to β-actin expression. For each experimental time point and condition, data were obtained from eight separate mice. Data obtained from array studies was confirmed by conducting real time RT PCR for several of the proteasome subunit genes, as described previously from our laboratory (9).

Analysis of protein oxidation

Protein oxidation was determined by analysis of protein carbonyl content as described previously (1, 10, 11), using the 2,4-dinitrophenylhydrazine (DNPH) procedure, which has been demonstrated previously to be a reliable and sensitive measure of protein oxidation. A 5% (weight/volume) tissue homogenate was prepared in TBS containing protease inhibitor cocktail (Sigma Chemical, St. Louis, MO) homogenized, and centrifuged at 700 × *g*. The resulting supernatant was treated with 10 m*M* DNPH dissolved in 2 *N* HCl, or with 2 *N* HCl alone in the controls. Samples were then incubated for 1 hour at room temperature, precipitated with 10% trichloroacetic acid, centrifuged for 3 minutes at 16,000 × *g,* and the resulting pellet washed three times with ethanol/ethyl acetate. The pellet was then redissolved in guanidine containing 10 m*M* phosphate buffer-trifluoroacetic acid (pH 2.3). The difference in absorbance between the DNPH-treated and the HCl-treated samples was determined at 366 nm, and the results expressed as nmol carbonyl groups/mg of protein using the extinction coefficient of 22.0 $mmol^{-1}cm^{-1}$ for aliphatic hydrazones.

Statistical analysis

Statistical significance was determined using a Student's *t*-test to do pairwise comparisons between two individual groups. Analysis was conducted using SigmaPlot software and p value of < 0.05 set for statistical significance.

RESULTS

Proteasome activity

In order to determine if proteasome subunit expression is altered during normal aging, we conducted studies in young (4-month-old) and aging (12-month-old) wild-type mice (C57BL/6). These studies revealed that the majority of proteasome subunits examined demonstrated a greater than 10% increase in expression with increased age, in the brain (15/21), with a smaller percentage of subunits increased in the liver (6/21) (Table 1). The alterations in proteasome subunit expression in the liver were markedly different from what is observed in the brain, suggesting that the alterations in proteasome expression observed during aging is tissue specific. Studies in transgenic mice that lacked functional LMP2 expression, LMP2 KO mice (11), revealed a nearly identical ratio of increased proteasome subunit expression during normal aging of both the brain and liver (Table 1), as compared to wild-type mice.

We next examined proteasome activity in the brain and liver of aging wild-type and LMP2 KO mice. Analysis of 20S proteasome function in the brain and liver of young mice revealed

Table 1. Alterations in Proteasome Subunit Expression in Brains and Liver of Aging Control and Mutant Mice

		BRAIN		*LIVER*	
		Wild type (ratio 12 mos/4 mos)	*LMP2$^{-/-}$ (ratio 12 mos/4 mos)*	*Wild type (ratio 12 mos/4 mos)*	*LMP2$^{-/-}$ (ratio 12 mos/4 mos)*
b1	(20Sα)	1.56	1.55	0.89	1.10
b2	(20Sα)	1.07	1.12	1.33	1.30
C2	(20Sα)	1.13 (2.29)	1.14 (1.01)	1.20 (2.48)	0.60 (0.70)
C8	(20Sα)	1.36	1.37	1.18	1.09
C9	(20Sα)	1.29	1.03	1.22	1.40
C10	(20Sα)	1.26	1.09	1.24	0.82
PRE1	(20Sα)	1.74	1.32	0.89	0.94
C5	(20Sβ)	1.12	1.06	1.03	0.96
MB1	(20Sβ)	0.76	1.09	0.91	0.76
LMP2	(20Sβ)	1.12	NA	1.01	NA
LMP7	(20Sβ)	1.01	1.33	0.92	1.24
LMP10	(20Sβ)	1.09 (1.06)	0.99 (0.98)	0.95 (0.69)	1.58 (1.20)
PRE4	(20Sβ)	1.17	1.18	1.06	1.97
S1	(19S)	1.31	1.14	0.94	1.17
S4	(19S)	1.20	1.14	1.07	1.36
S9	(19S)	1.15	1.04	1.01	0.90
S11	(19S)	1.08	1.20	1.39	0.54
S12	(19S)	1.23	1.20	0.99	1.20
p28	(19S)	1.22	1.15	1.02	1.60
PA28α	(11S)	1.19 (1.10)	1.11 (1.02)	0.98 (0.88)	1.14 (1.06)
PA28β	(11S)	0.99	1.66	1.04	0.52

Expression of individual proteasome subunits was determined using a custom proteasome subunit expression array designed to quantify the expression for the indicated proteasome subunit at the level of RNA. The expression of each gene was normalized to β-actin expression, and the mean value of expression for each individual gene calculated from 8 separate experiments. Data are expressed as the ratio of the means for the indicated experimental conditions. Real time RT PCR analysis was utilized to confirm data obtained from custom proteasome subunit expression array, and is presented in parenthesis.

that LMP2 KO mice had significantly lower chymotrypsin-like activity in the brain, as compared to young wild type mice (Fig. 1A). Within the liver of young LMP2 KO mice there was a significant decrease in all 20S proteolytic activities of the proteasome (Fig. 1B), as compared to young wild-type mice. In both the brain and liver, young LMP2 KO mice demonstrated a significant decrease in chymotrypsin-like and post-glutamyl peptidase activities of the 26S proteasome (Figs. 1C and 1D) as compared to young wild-type mice. In aging wild-type mice, the only significant decreases in any individual proteasome activity consisted of a impairment in 26S chymotrypsin-like activity in the brain and 26S post-glutamyl peptidase activity in the liver (Figs. 1C and 1D). In LMP2 KO mice there were significant age-related impairment in multiple activities of the 20S and 26S proteasome, which were evident in both the liver and the brain (Figs. 1A–D). The ability of proteasome to degrade mildly oxidized protein was impaired in the liver, but not the brain, of aging wild-type mice (Figs. 1E and 1F). In LMP2 KO mice there was a significant age-related decrease in proteasome mediated degradation of mildly oxidized protein in both the liver and brain (Figs. 1E and 1F). In most cells both the 20S and 26S proteasome complexes are present, and with each enzyme mediating different functions. In general, the activities of 26S proteasome are highly ATP dependent, primarily degrade ubiquitinated proteins, and are therefore under the regulation of all proteins involved in regulating ubiquitin-mediated proteolysis. On the other hand, the 20S proteasome is ATP independent, and primarily degrades proteins independent of ubiquitination. In light of this it is quite expected that the 20S and 26S should show a different response to aging and the stress associated with the loss of LMP2 expression.

Protein oxidation

Analysis of protein oxidation, via quantification of protein carbonyl levels, revealed that young LMP2 KO mice exhibited higher levels of protein oxidation in both the brain and liver (Figs. 2A and 2B), as compared to wild-type mice. Aging wild-type mice exhibited increased levels of oxidized protein in both the brain and liver (Figs. 2A and 2B), as compared to 4-month-old mice. LMP2 KO mice demonstrated an age-related increase in oxidized protein levels in both the brain and liver (Figs. 2A and 2B), with the brain exhibiting higher levels of oxidized protein than age-matched wild-type mice (Fig. 2A).

DISCUSSION

In the present study both the brain and liver exhibited age-related increases in proteasome subunit expression. Within

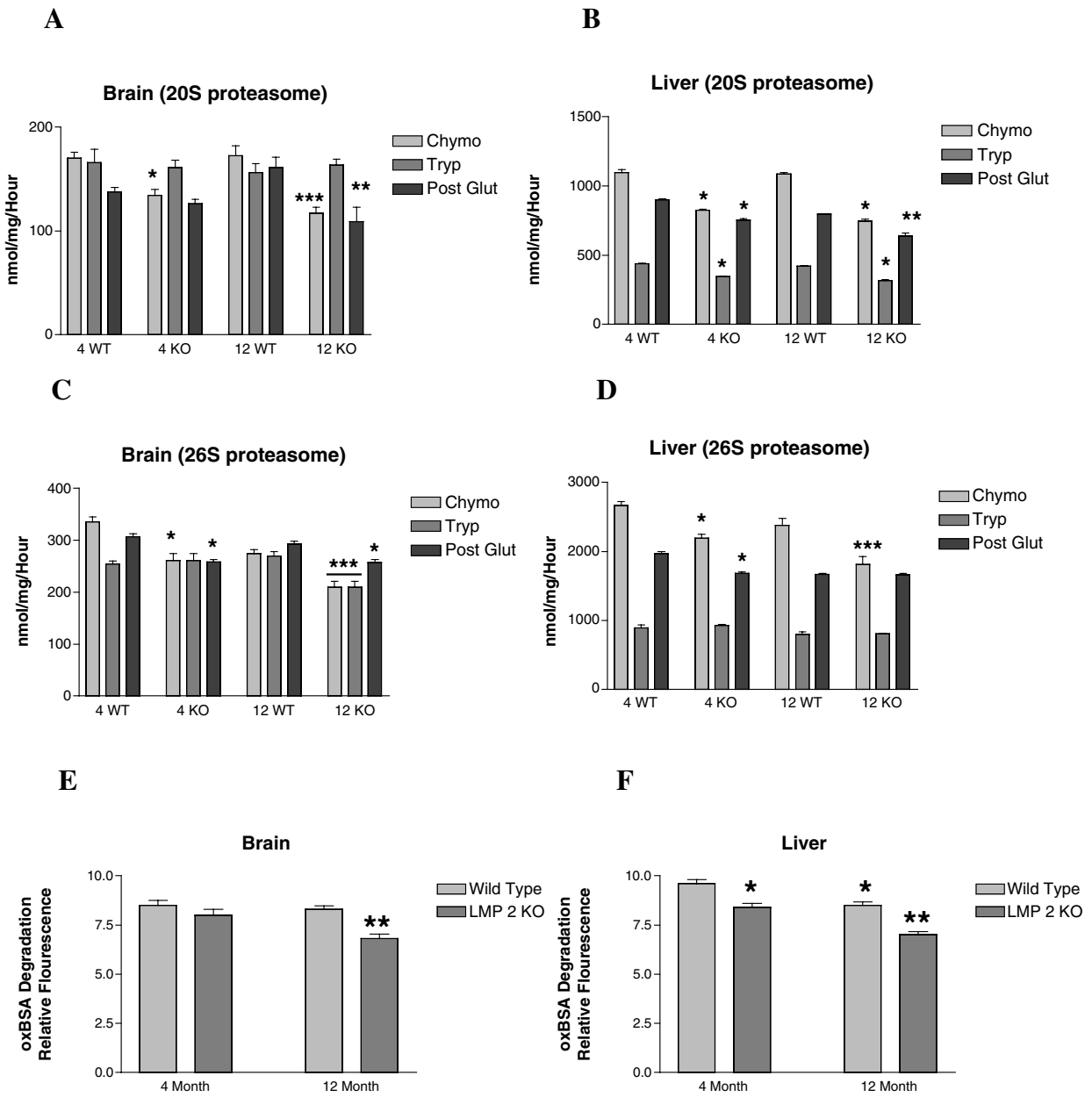

FIG. 1. Role of LMP2 in age-related alterations in proteasome activity. The chymotrypsin-like, trypsin-like, and post-glutamyl peptidase activities of the 20S and 26S proteasome were determined in the brain (**A, C**) and liver (**B, D**). Proteasome activity was analyzed in the brain and liver of young (4-month-old) and aging (12-month-old) LMP2 KO mice (KO), and nontransgenic wild-type mice (WT). The ability of the proteasome to degrade mildly oxidized bovine serum albumin (oxBSA) was determined in the brain (**E**) and liver (**F**) of young and aging KO or WT mice. All data are expressed as the mean and SEM of results from 16 young (8 WT/8 KO) and 16 aging mice (8 WT/8 KO). *$p < 0.01$ compared to age-matched animal of same background; **$p < 0.01$ compared to 4-month-old mice; ***$p < 0.03$ compared to 4-month-old animal and age-matched animal of different background.

the brain, a higher percentage of proteasome subunits exhibited increased expression with age, as compared to the liver. These data suggest a potentially important role for proteasome subunit plasticity in maintaining proteasome function during normal aging, and may indicate that a plasticity in proteasome subunit expression is particularly important in the brain.

No significant decreases in 20S proteasome activity was observed in either the brain or liver of aging wild-type mice, even though slight impairments in individual 26S proteasome activities were observed in both the brain and liver. A significant age-related impairment in proteasome-mediated degradation of oxidized protein was observed in the liver of wild-

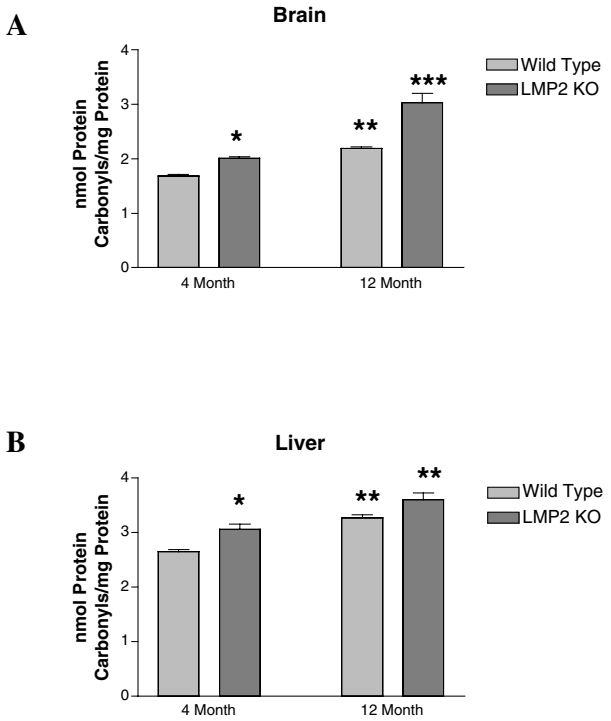

FIG. 2. Role of LMP2 in age-related alterations in protein oxidation. The amount of protein oxidation (protein carbonyls) was quantified in the brain (**E**) and liver (**F**) of young (4-month-old) and aging (12-month-old) LMP2 KO mice (KO), and nontransgenic wild type mice (WT). All data are expressed as the mean and SEM of results from 16 young (8 WT/8 KO) and 16 aging mice (8 WT/8 KO). *$p < 0.01$ compared to age-matched animal of same background; **$p < 0.01$ compared to 4-month-old mice; ***$p < 0.03$ compared to 4-month-old animal and age-matched animal of different background.

type mice, with both the brain and liver exhibiting an age-related increase in oxidized protein levels. Taken together, these data may indicate that the 26S proteasome is more vulnerable to age-related inhibition, and suggest that the ability of the proteasome to degrade oxidized protein may be regulated by factors other than the maintaining individual proteasome proteolytic activities. Data also indicate that the initial age-related elevations in oxidized protein levels are mediated by factors other than gross proteasome inhibition.

LMP2 KO mice demonstrated a more robust age-related inhibition of individual proteasome proteolytic activities, as well as impairments in proteasome-mediated degradation of oxidized protein. Interestingly, age-related impairments in both 20S and 26S proteasome function were observed, with both evident in both the brain and liver of LMP2 KO mice. In the brain, but not the liver, this inhibition was associated with a significant exacerbation of age-related increases in oxidized protein levels. These data suggest that losing the expression of a single proteasome subunit can result in gross changes in proteasome function. Additionally, our data indicate that these changes in proteasome function are exacerbated during normal aging, and may result in an elevated level of protein oxidation.

Cumulatively, our data suggest that proteasome subunit expression is altered during normal aging, with increased expression of multiple proteasome subunits possibly playing an important role in preserving proteasome function during normal aging. In the present study, the absence of a single proteasome subunit resulted in a more robust age-related impairment in proteasome activity, and age-related elevation in oxidized protein levels. Such observations highlight the potential adaptability of the proteasome, and suggest that the proteasome attempts to respond to age-related stressors by increasing proteasome subunit expression in order to maintain proteasome function. The maintenance of proteasome activity appears to be particularly important for preventing the secondary, but not initial, age-related increase in oxidized protein levels. Many factors are involved in initiating the increased oxidative damage observed in aging tissues, with increased free radical generation being an important mediator of oxidative damage. Consistent with these observations, recent studies have demonstrated the ability of low-level oxidative stress to increase proteasome subunit expression in neural cells, which was associated with a preservation of proteasome activity and only slight increases in oxidized protein levels (9, 12, 22).

In future studies it will be critical to elucidate fully the subtle nuances of proteasome subunit expression, developing a firm understanding of what causes age-related alterations in proteasome subunit expression, and elucidating mechanisms which serve to maintain proteasome activity throughout the aging process. Such interventions may lead to more successful aging and thus provide a therapeutic benefit to a host of age-related diseases.

ABBREVIATIONS

ATP, adenosine triphosphate; BSA, bovine serum albumin; DNPH, 2,4-dinitrophenylhydrazine; EDTA, ethylenediaminetetraacetic acid; HCl, hydrochloric acid; LMP, latent membrane protein.

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