FEBS Letters 546 (2003) 228–232 FEBS 27369

Role of the proteasome in protein oxidation and neural viability following low-level oxidative stress

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Received 13 March 2003; revised 2 May 2003; accepted 12 May 2003

First published online 27 May 2003

Edited by Ulrike Kutay

Abstract Numerous studies suggest that proteasome inhibition may play a causal role in mediating the increased levels of protein oxidation and neuron death observed in conditions associated with oxidative stress. In the present study we demonstrate that administration of non-toxic levels of oxidative stress does not result in impairment of 20S/26S proteasome activity, and actually increases the expression of specific proteasome subunits. Non-toxic levels of oxidative stress were observed to elevate the amount of protein oxidation in the presence of preserved proteasomal function, suggesting that proteasome inhibition may not mediate increases in protein oxidation following low-level oxidative stress. Preserving basal proteasome function appears to be critical to preventing the neurotoxicity of low-level oxidative stress, based on the ability of proteasome inhibitor treatment to exacerbate oxidative stress toxicity. Taken together, these data indicate that maintaining neural proteasome function may be critical to preventing neurotoxicity, but not the increase in protein oxidation, following low-level oxidative stress.

Key words: Neuron; Neurotoxicity; Oxidative stress; Proteasome; Protein oxidation

1. Introduction

The proteasome is a large intracellular multicatalytic protease that is responsible for mediating the degradation of a vast array of oxidized, misfolded, and aggregated proteins [1–4]. The proteolytic core of the proteasome, referred to as the 20S proteasome, is composed of multiple α- and β-subunits [5–8]. Additional cap-like protein complexes, referred to as the 11S and 19S proteasomes, can bind to the 20S proteasome resulting in the formation of the 26S proteasome [5–8].

Increasing evidence suggests that the activity and composition of the proteasome can be altered in immune cells in response to inflammatory stimuli [9,10]. The functional importance of such changes is highlighted by studies in transgenic mice that have demonstrated that the ability to alter proteasome homeostasis in these immune cells is essential to maintaining proper immune function [11–14]. Despite the tremendous progress that has been made in understanding proteasome biology in the immune system, the role of the proteasome in other systems, such as the nervous system, is only beginning to be elucidated.

Numerous studies indicate that oxidative stress occurs in a number of neurodegenerative conditions, and may play a causal role in the neurodegeneration observed in those conditions [15–25]. Recent studies have also indicated a possible causal role for proteasome inhibition in the neurotoxicity associated with oxidative stress [22]. Pharmacological inhibition of the proteasome is sufficient to induce neural death [26–31], consistent with proteasome inhibition playing a causal role in the neurodegeneration observed in conditions such as Alzheimer's disease (AD) and Parkinson's disease (PD). However, at present the role of the proteasome in preventing or promoting the elevations in protein oxidation and neuron death following low-level oxidative stress has not been elucidated.

2. Materials and methods

2.1. Materials

Proteasome substrates were purchased from Bachem, the protein oxidation kit from Intergen, and all reverse transcription polymerase chain reaction (RT-PCR) supplies from Invitrogen. All cell culture media and serum were obtained from Gibco Life Sciences. The proteasome antibodies were purchased from Affinity Bioreagents Incorporated. All remaining chemicals and reagents were purchased from Sigma Chemical.

2.2. Establishment of primary rat cortical neurons and maintenance of neural cell lines

Primary cortical neurons were established and maintained from E18 Sprague–Dawley rats as described previously [27]. Neural SH-SY5Y cells were purchased from the American Tissue Culture Collection, and grown in minimum essential medium containing 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. Cells were maintained in 5% CO_2 in a 37°C incubator, with cells of fewer than 20 passages utilized for all studies.

2.3. Analysis of cell survival

Cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) conversion as described previously [26,32–34]. At least eight cultures were utilized for each time point.

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0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00582-9

Results from MTT assays were confirmed using Hoechst 33258 staining as described previously [32–34]. Cell survival in primary neurons was determined as described previously [27], utilizing repeated visual cell counts in the same microscopic field, with cell counts made before and after treatment utilized for determining the percentage of neuron death.

2.4. Analysis of proteasome activity

Proteasome activity was determined as described previously [26,33]. 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 $MgCl_2$). The activity of the 20S and 26S proteasome was distinguished by the absence or inclusion of 2 mmol/l ATP, as described previously [2,35]. Chymotrypsin-like and post-glutamyl peptidase activities of the proteasome were determined by measuring the rate of Suc-Leu-Leu-Val-Tyr-MCA and Suc-Leu-Leu-Glu-MCA, respectively. Fluorescence was monitored at 340 nm excitation and 440 nm emission.

2.5. Analysis of protein oxidation

The levels of protein oxidation were determined by dot blot analysis using an oxidized protein detection kit as described previously [36,37]. This assay is based on the immunochemical detection of protein carbonyl groups derivatized with 2,4-dinitrophenyl hydrazine, according to the manufacturer's instructions. All samples were performed in duplicate.

2.6. Analysis of proteasome expression

For real-time RT-PCR analysis of proteasome subunit expression total RNA was collected from cells at the times indicated as described previously [33,38]. For each reaction a total of 150 ng of total RNA was amplified according to the manufacturer's instructions using SYBR® Green PCR Master Mix reagent, as described previously [39]. The following parameters were utilized: LMP2: 30 min at 48°C, 5 min at 95°C, 30 s at 94°C, 30 s at 56°C, 50 s at 72°C; LMP7: 30 min at 48°C, 5 min at 95°C, 30 s at 94°C, 30 s at 52°C, 50 s at 72°C; LMP10: 30 min at 48°C, 5 min at 95°C, 30 s at 94°C, 30 s at 57°C, 50 s at 72°C; PRE4: 30 min at 48°C, 5 min at 95°C, 30 s at 94°C, 30 s at 56°C, 50 s at 72°C; 18S: 30 min at 48°C, 5 min at 95°C, 30 s at 94°C, 30 s at 48°C, 50 s at 72°C. LMP2, LMP7, LMP10, PRE4, and 18S were amplified for 26, 26, 18, 12, and 9 cycles respectively. Primers used for amplification were as follows: LMP2: 5′-GAT GCT GCG GGC GGG AGC ACC ACC-3′, 3′-CAG TTC ATT GCC CAA GAT GAC TC-5′ (640 bp); LMP7: 5′-CTG CTT GGC ACC ATG TCT GGC TG-3′, 3′-TCT ACT TTC ACC CAA CCA TCT TC-5′ (443 bp); LMP10: 5′-ATC CTG GGC GGC GTA GAC CTG AC-3′, 3′-TTA CTC CAC CTC CAT AGC CTG CAC-5′ (411 bp); PRE4, 5′-TGG CTC GTT TCC GCA ACA TCT CTC-3′, 3′-GGT CTG TGT AGA CAA TGG TCC CTC-5′ (524 bp); 18S, 5′-GAC AGG ATT GAC AGA TTG ATA G-3′, 3′-CAC TTG TCC CTC TAA GAA GTT G-5′ (174 bp). Following amplification one major band was detected by gel electrophoresis, with all amplifications adhering to the SYBR® Green dissociation protocol. Data were collected and analyzed using ABI Prism 2000®. Proteasome subunit expression at the protein level was conducted by Western blot analysis, as described previously from our laboratory [26,27]. Data are representative of results from at least four separate experiments.

2.7. Statistical analysis

Statistical significance was determined using Student's *t*-test, with a *P* value of < 0.05 required for significance.

3. Results

3.1. Relationship between neural proteasome activity, protein oxidation, and neural viability

In order to determine the relationship between oxidative stress-induced alterations in neural viability, proteasome function, and protein oxidation we conducted studies in human neural SH-SY5Y cells. Following exposure to increasing concentrations of H_2O_2 cells were analyzed for cellular viability by quantification of MTT reduction and analysis of nuclear morphology. Administration of H_2O_2 caused a dose- and time-dependent induction of neural death, with addition of 50 μM H_2O_2 significantly increasing neural death at both 8 h and 24 h post administration (Fig. 1A,B). Analysis of protein oxidation in this experimental paradigm revealed that each of the concentrations of H_2O_2 utilized for survival studies caused an elevation in the levels of protein oxidation as early as 3 h following treatment (Fig. 1C). Analysis of 20S and 26S proteasome activity revealed that no significant alteration in either 20S or 26S proteasome activity was evident 3 h following oxidative stress treatment (Fig. 1D), with 1–10 μM H_2O_2 inducing small elevations in 20S proteasome activity in several experiments (data not shown). Addition of toxic concentrations of H_2O_2 (50 μM) was observed to inhibit both 20S and 26S chymotrypsin-like proteasome activity following a 24 h incubation (Fig. 1E). Identical results were obtained with analysis of 20S and 26S post-glutamyl peptidase activity of the proteasome (data not shown).

3.2. Oxidative stress increases the expression of multiple proteasome subunits

In order to elucidate the effect of oxidative stress on proteasome subunit expression, we analyzed the expression of multiple proteasome subunits following the addition of 10 μM H_2O_2. At the protein level increased levels of the inducible proteasome subunits LMP2, LMP7, and LMP10 were observed following H_2O_2 treatment (Fig. 2). No elevation in β-tubulin expression was observed following administration of oxidative stress (Fig. 2), confirming that elevations in proteasome subunit expression were not an artifact of oxidative stress treatment. Real-time RT-PCR revealed that elevated levels of LMP2 and LMP10 mRNA were evident following treatment with 10 μM H_2O_2 (Fig. 3A). Additionally, expression of the proteasome subunit PRE4, a subunit responsible for post-glutamyl peptidase activity, was elevated following treatment with 10 μM H_2O_2 (Fig. 3A). No alteration in 18S rRNA was observed following oxidative stress treatment (Fig. 3A). Electrophoresis of RT-PCR products revealed that only one major product was generated by RT-PCR reaction (Fig. 3B).

3.3. Proteasome activity is necessary to prevent the toxicity of low-level oxidative stress

Analysis of proteasome activity and proteasome expression following low-level oxidative injury suggested that neural proteasome biology was dramatically altered, and may play a role in preventing neural death following exposure to oxidative injury. In order to directly determine if maintenance of proteasome activity played a causal role in preventing oxidative stress-induced neurotoxicity, we conducted studies in which neural SH-SY5Y cells were co-administered amounts of H_2O_2 and proteasome inhibitor that did not induce neurotoxicity when administered individually. Co-administration of sub-lethal levels of H_2O_2 and proteasome inhibitor was sufficient to induce significant levels of neural cell death in SH-SY5Y cells (Fig. 4A). Similar results were obtained in primary rat cortical neurons co-administered sub-lethal levels of H_2O_2 and MG115 (Fig. 4B).

4. Discussion

Data from the present study indicate that neural 20S proteasome and neural 26S proteasome activity is not inhibited

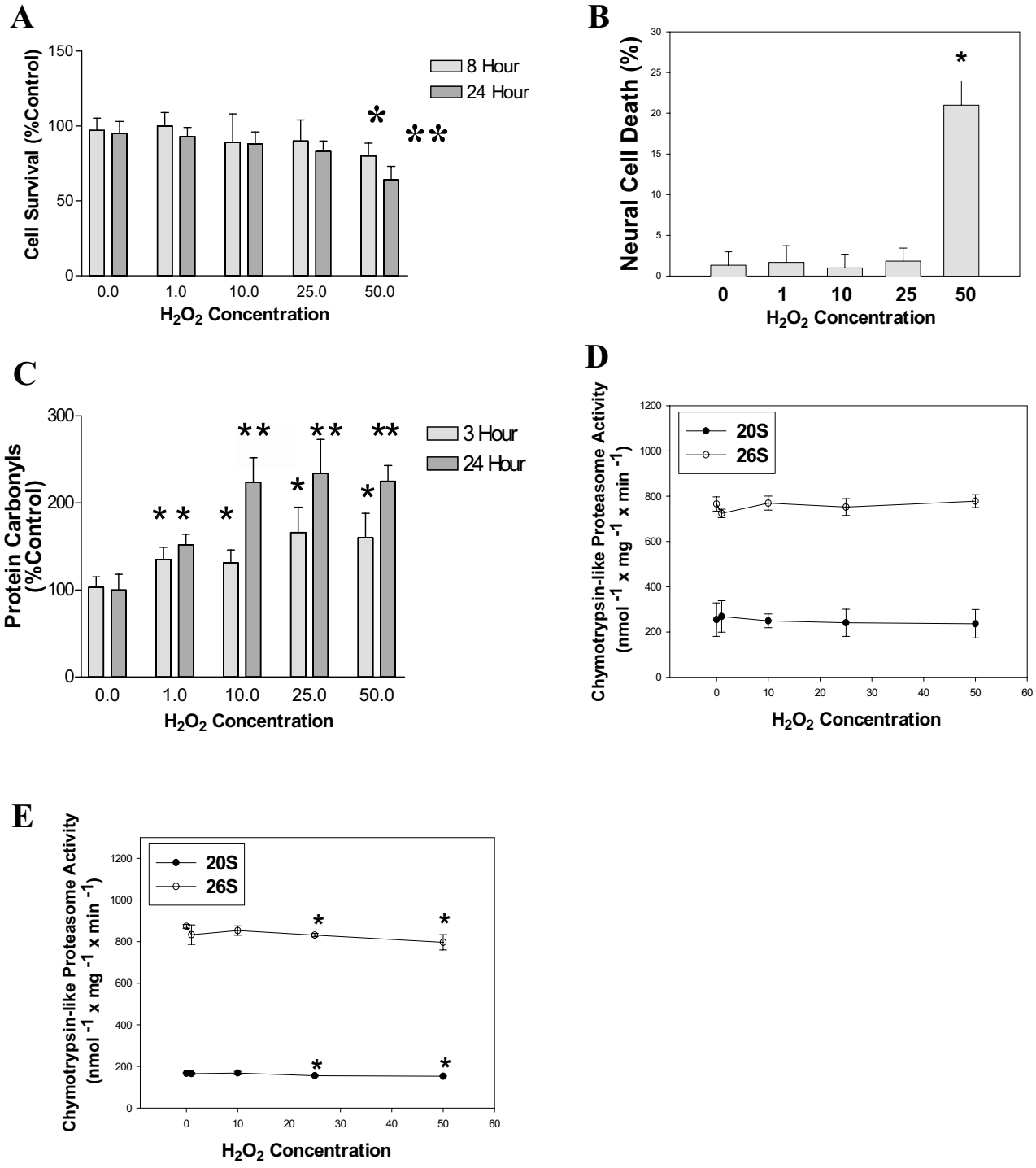

Fig. 1. Oxidative stress alters neural viability, protein oxidation, and proteasome function. Neural SH-SY5Y cells were exposed to increasing concentrations of H_2O_2 and analyzed for cell death using either MTT reduction (A) or nuclear morphology (B) as an index of neural survival. MTT reduction was analyzed 8 and 24 h post treatment, with nuclear morphology analyzed 24 h post treatment. Protein carbonyl formation was analyzed in neural SH-SY5Y cells exposed to increasing concentrations of H_2O_2 (C), at 3 or 24 h post administration. Neural SH-SY5Y cells were analyzed for 20S (−ATP) or 26S (+ATP) proteasome activity 3 h (D) or 24 h (E) following the administration of increasing concentrations of H_2O_2. Data presented are the mean and S.E.M. of at least six samples from at least two separate experiments. $^*P < 0.05$ compared to control cultures; $^{**}P < 0.05$ compared to cultures treated for 3 h.

following exposure to low-level oxidative stress. Such data indicate that neural proteasome activity may not necessarily be as vulnerable to oxidative stress-induced inhibition as previously anticipated. In future studies it will be important to determine if aging affects the ability of neural cells to maintain proteasome activity following low-level oxidative stress. At present it is unclear whether proteasome inhibition is responsible for causing elevations in oxidative stress within the brain, or whether oxidative stress may be responsible for mediating proteasome inhibition in the brain. In the present study, ad-

ministration of mild oxidative stress (1 and 10 μM H_2O_2) was observed to elevate the amount of protein oxidation within neural cells in the absence of detectable levels of proteasome inhibition. These data suggest that proteasome inhibition may not necessarily be a mediator of increased protein oxidation following low-level oxidative stress.

Studies in this paper demonstrate for the first time the ability of the neural proteasome to undergo dramatic and selective alterations in proteasome subunit expression following the administration of oxidative stress. Previous studies from our laboratory have demonstrated that the expression of aggregate-prone proteins (polyglutamine-containing proteins) chronically increases the expression of LMP2 expression in neural cells [33], suggesting that increases in LMP2 expression may be a mechanism that neural cells utilize to survive in the presence of aggregate-prone proteins. In that same study, neural cells expressing elevated levels of LMP2 under basal conditions were unable to elevate proteasome activity in response to heat shock stress [33], suggesting that chronic expression of inducible proteasome subunits may adversely affect the ability of the neural proteasome to respond to subsequent stress. Such data strongly suggest that the expression of individual proteasome subunits is essential to the preservation of neural proteasome function and neuronal viability. It is likely that aging affects the ability of neural cells to increase proteasome subunit expression following a variety of stressors [40], as such an attenuation in proteasome plasticity may directly contribute to age-related inhibition of proteasome activity.

In the present study, neural 20S and 26S proteasome activity were decreased to a similar degree by administration of lethal oxidative injury. Because the observed decrease in proteasome activity did not occur within the first 3 h of oxidative injury, and occurred to a similar degree as the amount of neural death, these data suggest that the observed decreases in proteasome activity following oxidative injury may be due in a large part to a decrease in the number of viable neural cells. Such an observation may be particularly important in neurodegenerative conditions that are associated with the presence of decreased proteasome activity and elevated levels of oxidative stress. For example, at present it is unclear whether the loss of proteasome activity in neurodegenerative disorders such as AD and PD are due to the inhibition of proteasome activity in neural cells, or whether loss of proteasome activity is an artifact of increased levels of neural death observed in those conditions. It is interesting to point out that the studies describing impairments of proteasome activity in AD and PD report only seeing a loss of proteasome activity in the brain regions exhibiting the largest amount of neuron death, and report impairments in multiple proteasome proteo-

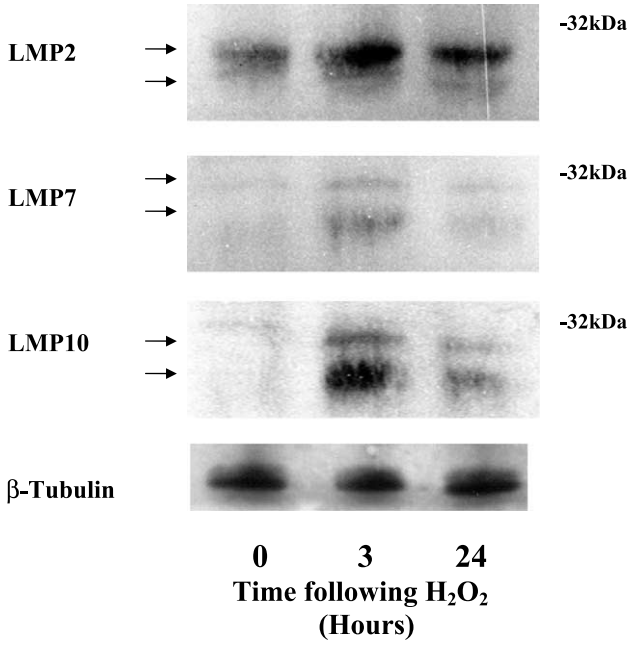

Fig. 2. Oxidative stress alters the expression of proteasome subunits at the protein level. At the times indicated, proteins were collected from neural SH-SY5Y cells treated with 10 μM H_2O_2. Equal amounts of cellular lysates (50 μg) were then separated by electrophoresis, transferred to a nitrocellulose membrane, and immunoreacted with the indicated antibody. Data are representative of results from at least two experiments.

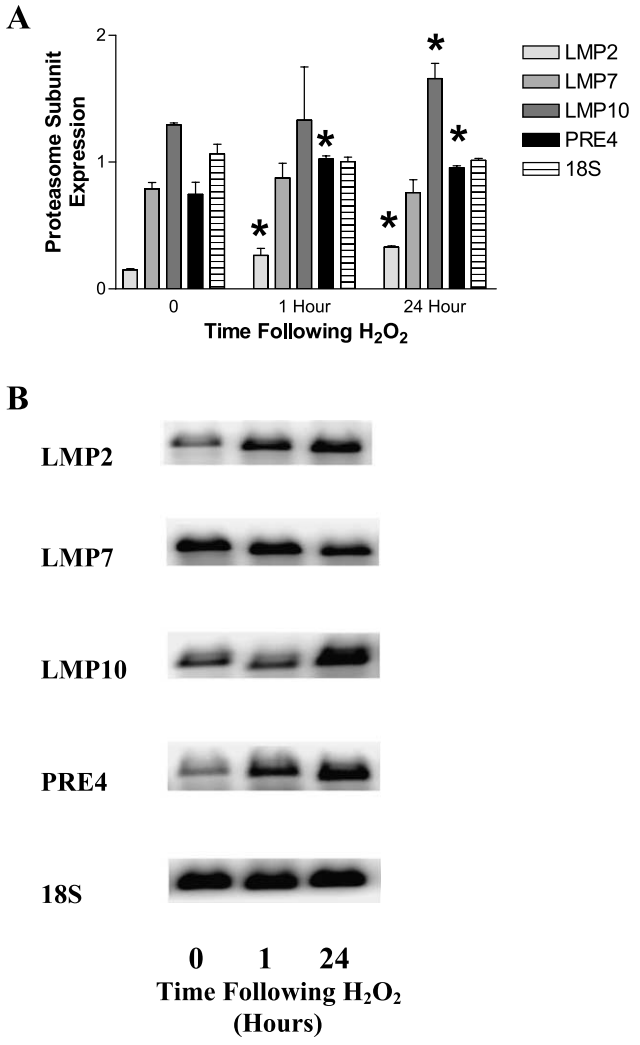

Fig. 3. Oxidative stress alters the expression of proteasome subunits at the mRNA level. A: Neural SH-SY5Y cells were analyzed via real-time RT-PCR analysis for the amount of LMP2, LMP7, LMP10, PRE4, or 18S rRNA expression following the administration of 10 μM H_2O_2. B: Analysis of RT-PCR products revealed that only one major band was generated following the RT-PCR procedure. Samples were collected at the times indicated and equal amounts of mRNA subject to real-time RT-PCR. Data are expressed as the mean and S.E.M. of results from two separate experiments.

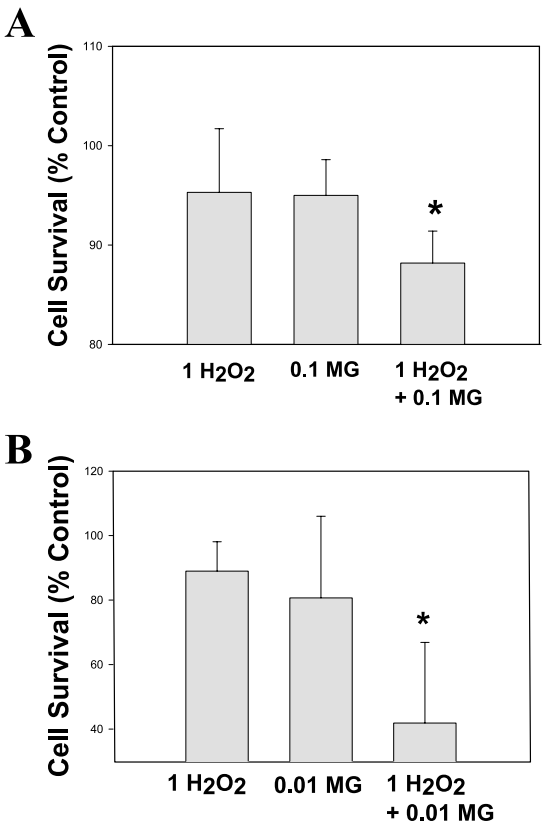

Fig. 4. Pharmacological inhibition of proteasome activity exacerbates the toxicity of oxidative stress. Neural SH-SY5Y cells (A) or primary rat cortical neurons (B) were co-treated with non-toxic concentrations of H_2O_2 (1 μM) and non-toxic concentrations of MG115 (0.1 or 0.01 μM) and analyzed for cellular viability 24 h following treatment. Data presented are the mean and S.E.M. of at least six samples from at least two separate experiments. $^*P < 0.05$ compared to control cultures.

lytic activities, with both the 20S and 26S proteasome activities inhibited [23–25].

Cumulatively, data from the present study indicate the importance of identifying proteasome inhibition in AD and PD, but also developing an understanding of how proteasome biology changes in each of these disorders. In particular it is critical to develop a better understanding of the effects of normal aging on the dynamics of neural proteasome activity, proteasome expression, and proteasome composition within the brain.

Acknowledgements: This work was supported by the American Heart Association (J.N.K.), and grants from the National Institutes of Health (AG018437, AG005119, J.N.K.). The authors wish to thank Dr. William R. Markesbery for his continual support.

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