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Oxidative stress alters neuronal RNA- and protein-synthesis: Implications for neural viability

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Abstract
Recent studies have demonstrated that impaired protein synthesis occurs in several neurodegenerative conditions associated with oxidative stress. Studies have also demonstrated that administration of oxidative stressors is sufficient to impair different and discrete regulatory aspects of protein synthesis in neural cells, with the majority of these studies focused on the effects of oxidative stressors towards initiation factors. Currently, little is known with regards to oxidative stress effects on the rates of RNA- and protein-synthesis, or the relationship between oxidant-induced impairments in RNA-/protein-synthesis to subsequent neuron death. In the present study, we demonstrate that administration of an oxidative stressor (hydrogen peroxide) induces a significant and time-dependent decrease in both RNA- and protein-synthesis in primary neurons and neural SH-SY5Y cells. Increases in RNA oxidation and disruption of ribosome complexes were selectively observed following the longer durations of oxidant exposure. Significant correlations between the loss of RNA- and protein-synthesis and the amount of oxidant-induced neuron death were also observed. Interestingly, the addition of a protein synthesis inhibitor (cycloheximide) did not significantly alter the amount of neuron death induced by the oxidative stressor. These data demonstrate that oxidant exposure promotes a time-dependent decrease in both RNA- and protein-synthesis in neurons, and demonstrate a role for elevations in RNA oxidation and ribosome dysfunction as potential mediators of impaired protein synthesis. These data also suggest that there is a complex relationship between the ability of oxidative stressors to modulate RNA- and protein-synthesis, and the ability of oxidative stressors to ultimately induce neuron death.

Keywords: *Aging, Alzheimer's disease, neuron, ribosome, stroke, transcription*

Introduction

Cells in the central nervous system (CNS) exhibit a wide array of oxidative modifications, with the levels of oxidative damage in the CNS known to be significantly modulated by a variety of intracellular and extracellular stressors. Oxidative stress is a condition in which cellular stressors generate sufficient levels of oxidative damage that cellular homeostasis is adversely affected. Understanding the basis for oxidative stress in the CNS, and the contribution of oxidative stress to neurodegeneration in the CNS, is an intense area of investigation. For example, oxidative stress is believed to contribute to neuron death and the development of neuropathology in a variety of clinical settings including Alzheimer's disease (AD), Parkinson's disease (PD), stroke, as well as normal aging [1–3]. This concept is based primarily on the fact that increased levels of oxidative damage are observed to occur in each of these conditions, with the addition of oxidative stressors sufficient to mimic many neuropathological and neurophysiological features present in each of these conditions [1–3]. Additionally, several lines of evidence suggest that decreasing the levels of oxidative stress may be beneficial in delaying or preventing

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pathogenesis in a variety of neurodegenerative settings [1–5]. Despite such progress, the basis whereby oxidative damage promotes neuronal toxicity and dysfunction remains to be firmly elucidated.

In order to successfully respond to the constant barrage of stressors cells encounter, all cells must continually synthesize the different proteins that are essential for maintaining cellular homeostasis. The processes of transcription and translation are exquisitely regulated in order to allow for the controlled genesis of RNA and protein molecules. Alterations in any of the multiple steps of RNA or protein synthesis can dramatically alter the rates at which new macromolecules are generated, and thereby compromise the ability of cells to successfully respond to cellular stressors. Increasing evidence suggests that declines in protein synthesis occurs during aging, stroke and age related diseases of the nervous systems such as AD [6–11] In many of these conditions, it is known that there is also an increase in RNA oxidation and disruption of ribosome function and ribosome assembly [9,12–15]. Additionally, studies have demonstrated that exposure to oxidants is sufficient to decrease the expression and activation of factors that regulate translation (initiation and elongation factors) [16–19]. These data suggest that interplay between oxidative stress and the synthesis of RNA and protein molecules may be an important in promoting neuronal dysfunction and neuropathology in the CNS [20,21].

The purpose of this study was to begin the process of understanding the relationship between oxidative stress-induced increases in RNA oxidation, ribosome dysfunction, and neurotoxicity to the ability of oxidative stressors to impair RNA- and protein-synthesis. Using primary rat neuron cultures and human neural SH-SY5Y cells we observed that there is a significant and complex interplay between each of these factors. Cumulatively, these data have important implications for understanding the mechanism(s) by which oxidative stress disrupts RNA- and protein-synthesis and promotes subsequent neurotoxicity.

Materials and methods

Establishment of primary neuron cultures

Neuron cultures were established as described previously [22–24]. Briefly, the cerebral cortex of 18-day-old Sprague–Dawley rat embryos were dissected and manually dissociated in Hanks' balanced salt solution. Cells were plated onto polyethyleniminecoated 35 mm dishes containing neurobasal medium supplemented with B-27 and maintained in a CO_2 incubator at 37°C. Seven days after initial plating, cultures were used for experimentation. SH-SY5Y cells were plated and maintained as described in previous publications from our laboratory [25].

Analysis of neural viability

Neuron survival was determined by quantification of neuronal morphology and nuclear morphology as described previously [22–24]. Briefly, viable neurons were counted in premarked microscope fields ($\times 10$ objective) before experimental treatment and 24 h after treatment, with the viability of neurons assessed by morphological criteria. Neurons with intact neurites of uniform diameter and a soma with a smooth appearance were considered viable. Neurons with fragmented neurites and a vacuolated and/or swollen soma were considered nonviable. The MTT assay was used as an additional means of assessing viability in both primary neurons and SH-SY5Y cells as reported previously by our laboratory [22–25].

Analysis of RNA and protein synthesis

For analysis of RNA synthesis the cultures were incubated with ^{32}P uridine triphosphate (10 μCi) for 30 min as described previously by our laboratory [24]. Total RNA was isolated from treated neurons according to the manufacturer's instruction (TriReagent©, Sigma). Total RNA was precipitated with ethanol overnight and the amount of incorporated ^{32}P uridine measured using a scintillation counter. For analysis of protein synthesis the cultures were incubated with ^{35}S methionine (10 μCi) for 30 min (in methionine-free medium) and proteins precipitated using TCA, and counted using scintillation counter as described previously by our laboratory [24]. For electrophoresis analysis, an aliquot of the lysate (10 μg) was collected following the initial ^{35}S methionine pulse, and separated on a 7.5% SDS-PAGE gel.

Analysis of RNA oxidation

The amount of RNA oxidation was measured as described previously from our laboratory [9,26]. For this analysis, we utilized slot–blot studies with 2.5 μg of total RNA loaded onto each well. The amount of RNA was calculated using the optical density (OD) at 260 nm ($OD_{260\,nm}$). The purity of RNA was determined by calculating the 260/280 nm ratio, and ensuring that the ratio was between 1.7 and 2.0 for all samples. The RNA was isolated from cell cultures using standard procedures (TriReagent) described previously from our laboratory [9,26].

Results

Oxidant exposure results in increased neural death

In order to establish a model to study the effects of oxidative stress on neuronal RNA- and protein-synthesis, we conducted studies using hydrogen peroxide as an oxidative stressor. In this model,

we exposed primary neurons to increasing durations (0–60 min) of hydrogen peroxide (0.1 mM), followed by a washout and recovery period. We observed that 30 and 60 min treatments with hydrogen peroxide resulted in significant levels of neuron death, detectible by both neuron counts (Figure 1(A)) as well as MTT assay (Figure 1(B)), 24 h following initial oxidant exposure. A small increase in neuron death was observed following 10 min oxidant exposure (accompanied by a 24 h washout period) when using neuron counts as a determinant of viability (Figure 1(A)), but not when using the MTT assay (Figure 1(B)). Conducting the same form of experimentation with human neural SH-SY5Y cells, using MTT as a measure of neural viability, resulted in a nearly identical pattern of neural death (Figure 1(C)). No increase in neural death, using either the primary neuron counts or MTT assay, was observed within the first 4 h of oxidant exposure (data not shown). Taken together, these results indicate that increasing levels of hydrogen peroxide exposure induces a reproducible time-dependent increase in neural death in both primary neurons and SH-SY5Y cells.

Oxidant exposure impairs RNA- and protein-synthesis in neural cells

In our next set of studies, we measured the rates of RNA- and protein-synthesis in neural cells using our aforementioned oxidative stress model. For these studies RNA- and protein-synthesis were analyzed 2 h following oxidant exposure. Significant impairments in RNA-synthesis were observed in both neurons and SH-SY5Y cells following 10 min oxidant exposure (Figure 2), with 60 min oxidant exposure impairing RNA-synthesis to a greater degree than 10 min exposure in both cell types. A time-dependent decrease in protein-synthesis was also observed in both cell types following oxidant exposure (Figure 3), although the impairment in protein-synthesis following 10 min oxidant exposure was much more severe in SH-SY5Y cells as compared to primary neuron cultures (Figure 3). Electrophoresis analysis of the radiolabeled proteins, and unlabeled protein levels using coomassie staining, revealed a timedependent decreases in all proteins in both cell types (Figure 4). It is important to note that all RNA and protein analyses were conducted 2 h following oxidant exposure, which is prior to any detectible increases in neural death, and are therefore not an artifact of increased neural death.

Relationship between impairment of RNA- and protein-synthesis with oxidative stress-induced neuron death

In order to determine if there was a correlation between the neuron death induced by hydrogen peroxide exposure, and the declines in RNA- and protein-synthesis, we conducted linear regression analysis. In this analysis, we used the mean neural survival (MTT assay) and the levels of RNA- and protein-synthesis inhibition, to determine the statistical relationship between each of these values. In these studies, we observed that there was a significant and

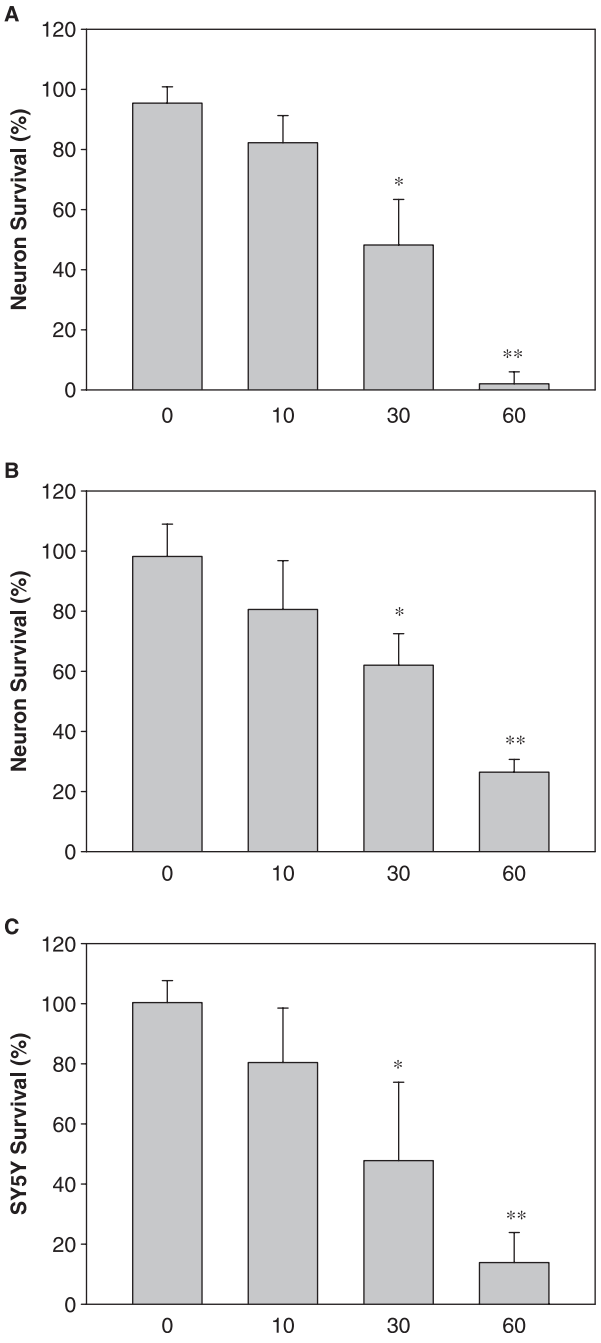

Figure 1. Administration of oxidative stressor induces a time-dependent increase in neural death. Primary rat neurons were treated with hydrogen peroxide (0.1 mM) for increasing lengths of time (0–60 min) followed by awashout of the hydrogen peroxide, and analysis of neural viability 24 h following initial oxidant treatment. Neurons were analyzed for neural toxicity using repeated counts of neural fields (A) or MTT assay (B) as measures of viability 24 h following oxidant exposure. Identical experiments in SH-SY5Y cells were conducted using MTT as a measure of neural viability (C). Data represent the mean and SEM from 10 individual cultures from two separate experiments. $*p < 0.05$ as compared to control cultures; $**p < 0.05$ as compared to cultures receiving 10 min hydrogen peroxide treatments.

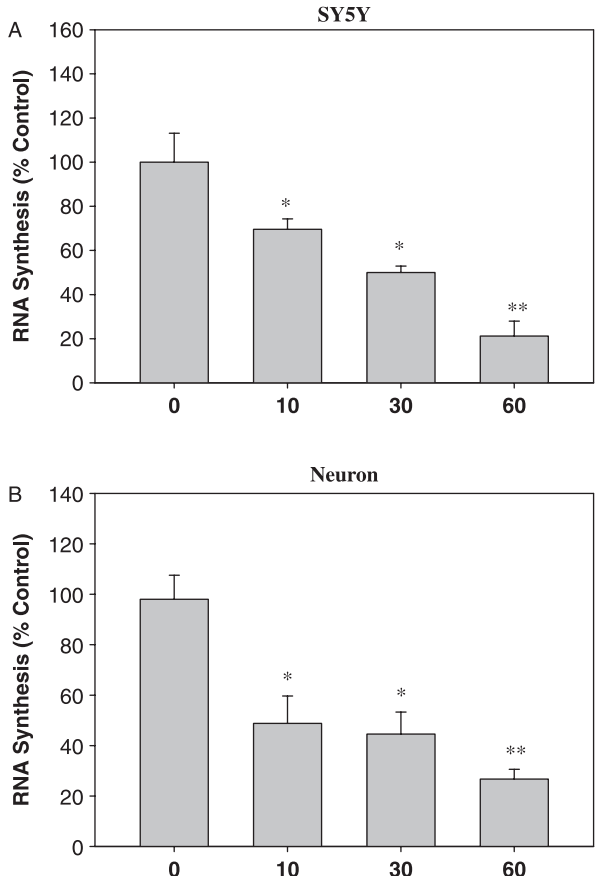

Figure 2. Administration of oxidative stressor induces a time-dependent decrease in RNA synthesis. SH-SY5Y cells (A) and primary rat neurons (B) were treated with hydrogen peroxide (0.1 mM) for increasing lengths of time (0–60 min) followed by a washout of the hydrogen peroxide, and analyzed for alterations in the rate of RNA synthesis 2 h following initial oxidant treatment. Data represent the mean and SEM from 10 individual cultures from two separate experiments. $\star p < 0.05$ as compared to control cultures; $\star\star p < 0.05$ as compared to cultures receiving 10 min hydrogen peroxide treatments.

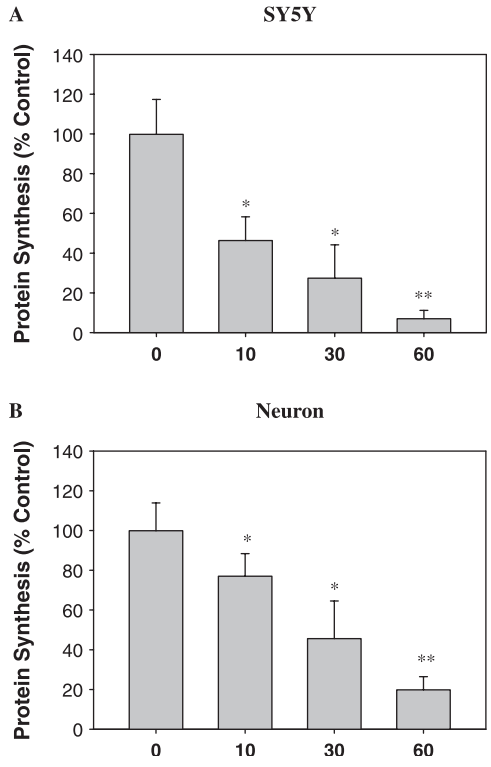

Figure 3. Administration of oxidative stressor induces a time-dependent decrease in protein synthesis. SH-SY5Y cells (A) and primary rat neurons (B) were treated with hydrogen peroxide (0.1 mM) for increasing lengths of time (0–60 min) followed by a washout of the hydrogen peroxide, and analyzed for alterations in the rate of protein synthesis 2 h following initial oxidant treatment. Data represent the mean and SEM from 10 individual cultures from two separate experiments. $\star p < 0.05$ as compared to control cultures; $\star\star p <$ 0.05 as compared to cultures receiving 10 min hydrogen peroxide treatments.

positive correlation between the loss of neuron viability and impairments in RNA-synthesis ($R^2 = 0.98$), and the loss of neuronal viability with the impairments in protein-synthesis ($R^2 = 0.90$) in SH-SY5Y cultures. Similarly, there was a significant and positive correlation between the loss of neuron viability and impairments in RNA synthesis ($R^2 = 0.86$) and impairments in protein synthesis ($R^2 = 0.98$) in primary neuron cultures. Because of the significant correlation between declines in protein-synthesis induced by oxidants, and the oxidant-induced increase in neuronal death, we next conducted studies to determine if the addition of a protein synthesis inhibitor (cycloheximide) could promote an exacerbation of oxidant-induced neuron death. In these studies, we observed that cycloheximide did not exacerbate the toxicity of hydrogen peroxide in either SH-SY5Y cells or primary neurons (Figure 5). Interestingly, cycloheximide treatment was actually observed to trend towards increased neural viability with the 30 min hydrogen peroxide treatment in both SH-SY5Y cells and primary neurons (Figure 5).

Oxidant exposure increases RNA oxidation and alters ribosome homeostasis

We next examined the levels of RNA oxidation present in our oxidative stress model, measuring the levels of RNA oxidation (8-OhG) as described previously by our laboratory [9,26]. In these studies, we observed elevated levels of RNA oxidation following 30 min, and to a lesser extent following 60 min oxidant exposure, for SH-SY5Y cells and primary neurons (Figure 6). In contrast, significant levels of RNA oxidation were not observed for either cell type following 10 min oxidant exposure (Figure 6). Analysis of polyribosomes revealed a similar pattern of oxidant-induced alterations, whereby 10 min exposure to oxidants did not induce significant alterations in polyribosomes, and 30 and 60 min oxidant exposures significantly altered ribosome

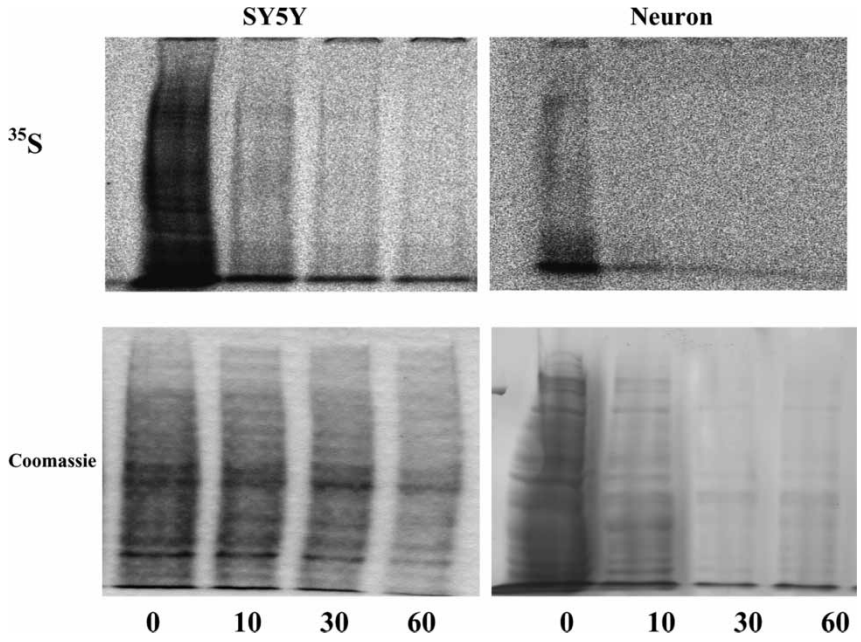

Figure 4. Administration of oxidative stressor reduces levels of most proteins. SH-SY5Y cells and primary rat neurons were treated with hydrogen peroxide (0.1 mM) for increasing lengths of time (0–60 min) followed by a washout of the hydrogen peroxide. Following 2 h washout period an aliquot of the protein lysate was collected and separated by electrophoresis on a 7.5% SDS-PAGE gel. Radiolabeled proteins were detected by overnight autoradiography, and total proteins detected by coomassie staining. Data are representative of results from three separate experiments.

homeostasis (Figure 7). Although 10 min exposure did not significantly alter polyribosome profile, it was observed to significantly decrease the amount of ribosome precursors (Figure 7). The 60 min oxidant exposure caused a near ablation of both ribosome precursors and polyribosome complexes (Figure 7).

Discussion

The current studies indicate that increasing the duration neural cells are exposed to an oxidative stressor promotes increasingly more severe impairments to both RNA- and protein-synthesis. Interestingly, the 10 min oxidant exposure appears to impair RNA- and protein-synthesis in a manner that is biochemically distinct from the RNA- and protein-synthesis observed following longer oxidant exposures. Specifically, the 10 min oxidant exposure impaired RNA- and protein-synthesis in a manner devoid of detectible elevations in RNA oxidation, or the presence of pronounced disruptions in polyribosome homeostasis. These data highlight the importance of considering that the amount and duration of oxidant exposure could promote declines in RNA-/protein-synthesis via multiple and distinct mechanisms within the CNS, and therefore the basis for declines in RNA- and protein-synthesis in the different neurodegenerative conditions is almost certainly multifactoral. For example, most studies to date have focused on a role for oxidative stressors decreasing protein synthesis in neural cells in an eIF2α dependent manner [16–19]. In these studies, acute and chronic exposure to hydrogen peroxide resulted in an increase in eIF2α phosphorylation which is known to be sufficient to inhibit translation [6,7]. Our data indicate that a 10 min exposure to hydrogen peroxide is sufficient to significantly impair RNA-synthesis, which will certainly contribute to impaired rates of translation independent of any effects on eIF2α. Additionally, longer exposures to hydrogen peroxide were observed to increase RNA oxidation and promote ribosome homeostasis, which are also sure to alter the rates of translation independent of effects on eIF2α. Recent studies have demonstrated that addition of the protein synthesis inhibitor cycloheximide increases phosphorylation in eIF2α [27], and impairs protein synthesis independent of effects on eIF2α, raising the possibility that increased levels of eIF2α phosphorylation in different neurodegenerative conditions may actually be a consequence and not a cause for oxidative stress-induced impairments in protein synthesis. Studies are currently underway to understand the relationship between oxidative stress-induced effects on RNA- and protein-synthesis with other related events such as eIF2α phosphorylation and endoplasmic reticulum stress.

Recent studies have demonstrated an important interplay between the proteasome and proteolytic pathway with the processes of transcription and translation. For example, impairment in proteasome function is sufficient to impair protein synthesis in neural cells [20,24], impair ribosome biogenesis [28]

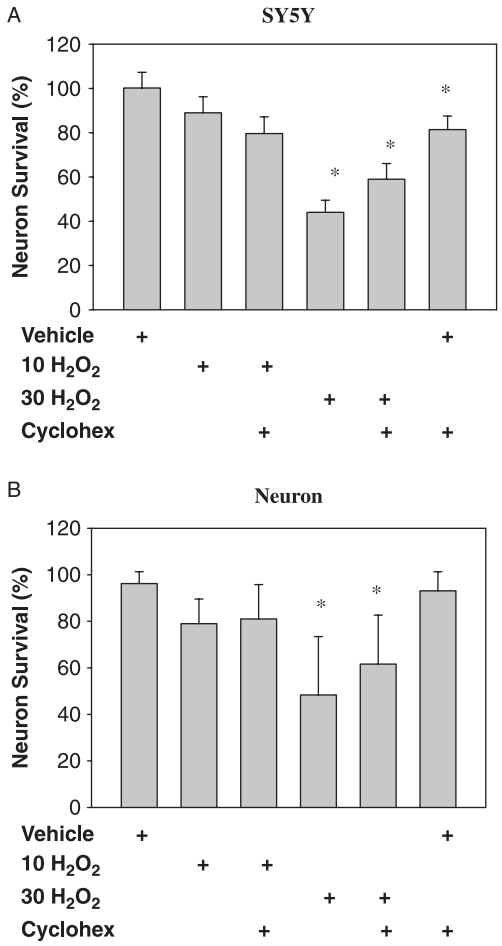

Figure 5. Administration of protein synthesis inhibitor does not exacerbate toxicity of hydrogen peroxide administration. Neural SH-SY5Y cells (A) and primary neuron cultures (B) were treated with hydrogen peroxide for 10 or 30 min followed by a washout period, and ultimately analyzed for neural viability using the MTT assay. Some cultures received the protein synthesis inhibitor cycloheximide (1 μM) following hydrogen peroxide treatments, or received cycloheximide treatments alone. Data represent the mean and SEM from 10 individual cultures from two separate experiments. $\star p < 0.05$ as compared to control cultures.

and impair the regulation of essential transcription factors [20]. Additionally, it is well established that oxidative stressors are capable of inhibiting proteasome function [29,30]. These data raise the possibility that oxidative stress-induced impairments in proteasome function may contribute to the declines in transcription and translation observed in the present study, as well as in a variety of neurodegenerative settings. In the short term impairing, the levels of RNA- and protein-synthesis in the face of impaired proteolytic capabilities would be expected to be beneficial. For example, in such settings cells would not be expected to be burdened with generating macromolecules in a pro-oxidative stress environment, and thus avoid being confronted with a decreased capacity for degrading and removing the different oxidized cellular components as they are synthesized. Prolonged impairments in macromolecule synthesis would be expected to be extremely deleterious to neural viability. In particular, decreased RNA- and protein-synthesis would be expected to impair the ability of neural cells to generate molecules to replace those which have been oxidatively damaged, or to synthesize molecules that impair the downstream effects of macromolecule oxidation. In this feed forward pathway, repeated episodes of oxidative stress could contribute to neural dysfunction and neuropathology via the promotion of long lasting impairments in the replacement of oxidized proteins, and an impaired genesis of proteins which protect against the effects of oxidatively damaged molecules.

Two important biochemical events were selectively observed following longer exposures to oxidative stressors, and were not evident following the shortest duration of oxidant exposure. The first of these events was an elevation in the levels of RNA oxidation. A number of studies have now documented that increased levels of RNA oxidation are evident in multiple neurodegenerative settings [9,12–15]. Our data suggest that exposure to levels of oxidative stress sufficient to impair RNA- and protein-synthesis in neural cells do not necessarily contribute to increases in the levels of RNA oxidation. These data do not exclude the possibility that selective RNA's exhibit elevated levels of RNA oxidation, as has been reported in AD [15,31]. We hypothesize that the increase levels of RNA oxidation in response to longer durations of oxidant exposure are mediated by overriding the ability of neural cells to degrade oxidized RNA, and surpassing the capacity for neural cells to protect RNA from oxidation through interactions with ribosomes and RNA binding proteins. Because of the widespread increase in RNA oxidation in a variety of disorders [9,12–15], we believe that RNA oxidation is extremely stable in many clinical and experimental settings. Such stability may be useful in using RNA oxidation as a selective and early marker for increases in oxidative stress, and may have important implications for ultimately developing pathology, with oxidized mRNA demonstrated recently to increase the generation of translational errors [32], which may be an essential component for mediating oxidative stress-induced neuron death. The second modification selectively observed with longer durations of oxidant exposure was a pronounced loss of ribosome homeostasis. Recent studies from our laboratory have demonstrated that even the earliest stages of AD there is a significant reduction in ribosome content, impaired function of ribosomes, and pronounced elevations in oxidative damage to ribosome complexes [9,12]. Our studies suggest that these presumably irreversible and significant effects require longer durations of oxidant exposure, and are not a guaranteed consequence of being exposed to an oxidative stressor. In the present study, we observed that SH-SY5Y cells exhibited more pronounced

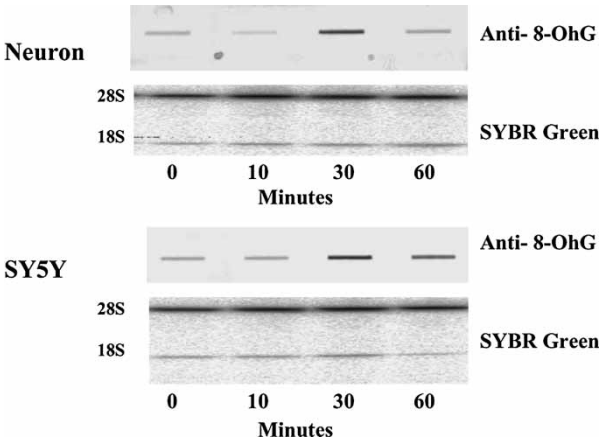

Figure 6. Administration of oxidative stressor induces a time-dependent increase in RNA oxidation. Equal amounts of purified RNA (2.5 μg) from the different experimental conditions were subjected to slot blot analysis of RNA oxidation using anti-8OhG as a measure of RNA oxidation. An aliquot of the RNA from each condition was separated on an agarose gel and subjected to SYBR green staining to ensure equal loading and integrity of RNA (based on presence of 28 and 18S bands). Data is representative of results from three separate experiments.

sensitivity to oxidant-induced impairments in protein synthesis. These data suggest that the capacity of oxidative stressors to impair different components of transcription and translation may be cell type specific, and that this variability may ultimately contribute to the differential vulnerability between different cell types to undergo oxidative stress following exposure to an oxidative stressor.

The data in the present study indicate that decreasing protein synthesis does not cause an exacerbation in the toxicity of oxidative stressors in either primary neurons or neural SH-SY5Y cells. The findings demonstrate an important complexity for the contribution declines in RNA- and protein-synthesis play in the toxicity of oxidative stressors. Clearly, in the present study we observed significant correlations between the loss of RNA- and protein-synthesis with eventual toxicity. Such data strongly link observed toxicity with the decrease synthesis of each of these molecules. The inability of cycloheximide to exacerbate toxicity in this acute model may represent the maximial toxicity that is achieved via oxidative stress-induced impairments in protein synthesis. Cumulatively, these data indicate a complex role

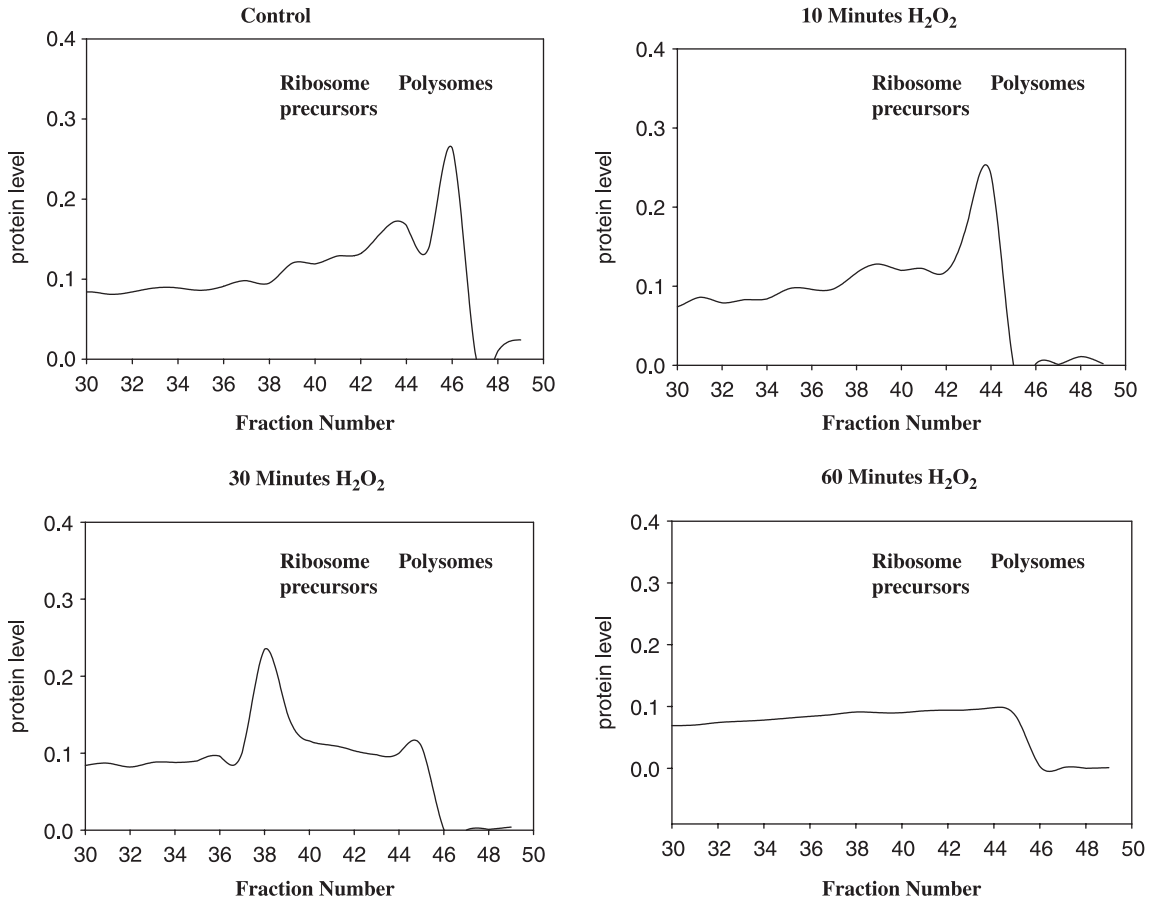

Figure 7. Administration of oxidative stressor induces a time-dependent increase in ribosome disturbances. The amount of ribosome precursors and polyribosomes were detected in neural SH-SY5Y cells following treatment with hydrogen peroxide as described previously by our laboratory [9,12]. Data are representative of results from three separate experiments. Nearly identical results were obtained in primary neuron cultures.

for impairments in RNA- and protein-synthesis contributing to neuronal oxidative stress, and highlight the need for conducting further experimentation in elucidating the importance of this emerging research area for AD, PD, stroke and aging research.

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