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Oxidative damage to the promoter region of SQSTM1/p62 is common to neurodegenerative disease

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Abstract

Recently we reported that declined SQSTM1/p62 expression in Alzheimer disease brain was age-correlated with oxidative damage to the p62 promoter. The objective of this study was to examine whether oxidative damage to the p62 promoter is common to DNA recovered from brain of individuals with neurodegenerative disease. Increased 8-OHdG staining was observed in brain sections from Alzheimer's disease (AD), Parkinson disease (PD), Huntington disease (HD), Frontotemporal dementia (FTD), and Pick's disease compared to control subjects. In parallel, the p62 promoter exhibited elevated oxidative damage in samples from various diseases compared to normal brain, and damage was negatively correlated with p62 expression in FTD samples. Oxidative damage to the p62 promoter induced by H₂O₂ treatment decreased its transcriptional activity. In keeping with this observation, the transcriptional activity of a Sp-1 element deletion mutant displayed reduced stimulus-induced activity. These findings reveal that oxidative damage to the p62 promoter decreased its transcriptional activity and might therefore account for decreased expression of p62. Altogether these results suggest that pharmacological means to increase p62 expression may be beneficial in delaying the onset of neurodegeneration.

Keywords

Oxidative stress; neurodegenerative disease; p62 promoter

Introduction

Oxidative stress induced by reactive oxygen species (ROS) results in damage to lipid, protein and DNA. Oxidative damage to DNA includes: oxidatively modified bases, abasic (AP) sites, single-strand and double-strand breaks (Friedberg et al., 2004). All of these DNA oxidative lesions are particularly harmful since they may cause not only mutations which can be inherited by the next generation, leading to genome instability, but may also regulate gene expression (Shibutani et al., 1991; Ghosh and Mitchell, 1999). Among the five nucleobases, guanine is the most susceptible to oxidation because of its high electron density (Steenken, 1989). 8-hydroxydeoxyguanosine (8-OHdG) is the major type of DNA oxidative adduct, which serves as a common biomarker of DNA oxidative damage (Steenken, 1989; Kasai, 1997). Hydroxyl

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radical, singlet oxygen and peroxynitrite may produce 8-OHdG. In addition this modification may be a mutagen converting G:C to T:A. DNA-base excision repair (BER) is the primary DNA repair pathway for base oxidative modifications, as well as single strand breaks (Krokan et al., 1997; 2000). The major enzyme in BER is oxoguanine DNA glycosylase 1 (OGG1), which repairs 8-OHdG in both nuclear and mitochondrial DNA in human cells (Boiteux and Radicella, 2000). The 8-OHdG glycosylase of *E. coli*, Fpg, has both N-glycosylase and AP-lyase activities (Boiteux et al., 1992; Tchou and Grollman, 1995).

Progressive and irreversible accumulation of DNA oxidative damage has been implicated in several age-associated diseases, including neurodegenerative diseases (Filipcik et al., 2006; Nakabeppu et al., 2007; Yang et al., 2008). Alzheimer's disease (AD), the most common form of neurodegenerative disorder, is characterized by memory loss and behavioral abnormalities. The pathological hallmarks of AD are extracellular β -amyloid plaques and intracellular neurofibrillary tangles. Oxidative modifications to both nuclear DNA and mitochondrial DNA are increased in AD brains (Gabbita et al., 1998; Wang et al., 2005; Migliore et al., 2005). Elevated levels of oxidative DNA damage were also observed in lymphocytes from AD patients (Mórocz et al., 2002). Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder, and the pathological characteristics of PD are the nigral dopaminergic cell loss and cytoplasmic inclusions called Lewy bodies that are composed of aggregated α -synuclein (Mouradian, 2002). Oxidative DNA damage leads to the degeneration of dopaminergic neurons associated with PD (Alam et al., 1997; Zhang et al., 1999; Nakabeppu, 2007). Huntington's disease (HD) is an autosomal-dominant neurological disease characterized by abnormal body movement and lack of coordination. HD is caused by intranuclear inclusions of mutant huntingtin with an expansion of the trinucleotide repeat (CAG) in the *huntingtin* gene leading to neuronal loss in the striatum and cortex (The Huntington's Disease Collaborative Research Group, 1993; Vonsattel and DiFiglia, 1998). Increased 8-OHdG levels have been observed in the caudate region of HD brain and in the brain of HD transgenic mice (Browne et al., 1997; Bogdanov et al., 2001). 8-OHdG level and deleted mitochondrial DNA molecules are also elevated in the peripheral blood of HD individuals (Chen et al., 2007). FTD and Pick's disease share some characteristics such as atrophy of the frontal and anterior temporal cortex, associated with neuronal loss and gliosis (Hauw et al., 1996; Brun, 1993). To date, however, there is little evidence revealing elevated 8-OHdG levels in either FTD or Pick's disease, but mitochondrial DNA damage has been reported in these two neurodegenerative diseases (Su et al., 2000; Mawrin et al., 2004).

Sequestosome 1/p62, also known as A170 and ZIP (PKC-zeta-interacting protein), was originally cloned as a phosphotyrosine-independent ligand of the p56^{lck} Src homology (SH2) domain (GenBank accession no. **BC019111.1**) (Park et al., 1995), and identified as a ubiquitin binding protein (Vadlamudi et al., 1996). Sequestosome 1/p62 contains multiple domains including PB1, ZZ, TRAF6, PEST and UBA that enable the protein to serve as a scaffold for regulation of ubiquitination and phosphorylation (Wooten et al., 2006; Moscat et al., 2007). The UBA domain of p62 has been implicated in shuttling ubiquitinated substrates to the proteasome for protein degradation (Seibenhener et al., 2004; Geetha et al., 2008). p62 is oxidatively-induced in cells from both humans and mice (Ishii et al., 1996), and p62 has been localized to aggresomes of various neurodegenerative diseases (Zatloukal et al., 2002). Also, an absence of p62 leads to the loss of aggresomes and neuronal cell death (Nakaso et al., 2004; Babu et al., 2008). Moreover, p62 has been reported to activate the antioxidant response element (ARE) and protect cells from oxidative stress (Liu et al., 2007). p62 expression is regulated at the transcriptional level (Nakaso et al., 2004) and the p62 promoter is enriched in CpG (Vadlamudi and Shin, 1998), which may be targeted by oxidative stress damage. Interestingly, oxidative damage to a subset of genes in the human genome has been correlated with increased 8-OHdG within these genes' promoter regions and decreased transcriptional activity (Lu et al., 2004). In this regard, we recently reported that increased oxidative

modification within the p62 promoter correlated with declined p62 expression in AD brain and in a mouse model of AD (Du et al., 2009). This study was undertaken to further examine the correlation between oxidative damage to the p62 promoter in relation to other neurodegenerative diseases such as: FTD, HD, Pick's and PD.

Materials and methods

Human brain samples

FTD, HD, Pick's and PD adult human brain frontal cortex samples were obtained from the Harvard Brain Tissue Resource Center, McLean Hospital, Boston, MA., in accordance with the Institutional Review Board-approved guidelines. AD and age-matched control (normal human brain) samples (frontal cortex) were obtained from Emory University Alzheimer's Disease Research Center, Emory University, Atlanta, GA. AD cases met CERAD and NIA-Reagan Institute for the neuropathologic diagnosis of AD (Mirra et al., 1991). The samples were as closely aged-matched as possible. The information about all samples is summarized in Table 1.

Western blotting

Brain tissue was homogenized in 1 mg/ml of ice-cold buffer (1 M sucrose in 0.1 M MES, 1 mM EDTA, 0.5 mM MgSO₄, pH 7), and centrifuged at 50,000 X g for 20 minutes at 4°C. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin (BSA) as a standard. The lysate was subjected to SDS-PAGE in 10% acrylamide gels. Samples were transferred from the gel to a nitrocellulose membrane. The blot was blocked with 7% milk in TBS-Tween (20 mM Tris, 8g /L NaCl, 0.1% Tween 20, pH 7.5) and incubated with primary antibody followed by secondary antibody. The blot was then processed with ECL reagent (Amersham Pharmacia Biotech, Pittsburgh, PA) for 2 minutes and exposed to ECL film. Gel and Graph Digital software (Silk Scientific Corporation, Orem, Utah) was used to scan and quantify the signal.

DNA isolation

Total DNA was isolated as described previously (Lu et al., 2004). Genomic DNA was isolated from brain tissues by DNeasy Tissue Kit (Qiagen, Valencia, CA) with following modifications to minimize *ex vivo* oxidation artifacts. All buffers were purged with nitrogen and supplemented with 50 µM phenyl-tert-butyl nitron (PBN) (Sigma, St. Louis, MO). The high temperature incubation was replaced by 4 hours incubation at 37°C. Following elution with ddH₂O, purified DNA was stored at -80°C.

Oxidative DNA damage assay

Quantitative real time PCR was employed to determine the level of damaged DNA within the human p62 promoter (Lu et al., 2004; Du et al., 2009). The primers designed for each amplicon are shown in Table 2. In brief, the formamidopyrimidine glycosylase (fpg) (New England Biolabs, Ipswich, MA) cleavage reaction was performed by incubating 250 ng of total genomic DNA with 8 units of fpg and 100 µg/ml of BSA in a total volume of 50 µl at 37°C for 12 hours, followed by incubation at 60°C for 10 minutes to inactivate fpg. An aliquot of the reaction mixture was used for quantitative PCR assay. Real time quantitative PCR was carried out on an ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master mix. All reactions were performed in a 25 µl mixture containing 1X SYBR Green master mix, 0.2 µM primer mix (forward and reverse), and template DNA for QPCR, respectively. A standard curve derived from 5-fold serial dilutions of genomic DNA was used to determine the absolute concentrations of intact DNA in the template. Oxidative damage was calculated as (intact DNA in non-treated aliquot - intact DNA in fpg-treated

aliquot) / (intact DNA in non-treated aliquot). Negative controls (absence of template for RT-PCR) were used to monitor nonspecific amplification. PCR products were verified by melting curves. Fluorescence was converted into DNA concentration using a standard curve. All DNA samples were analyzed three independent times.

8-OHdG immunohistochemistry

AD blocks were obtained from Emory University Alzheimer's Disease Research Center, Emory University, Atlanta, GA. Normal and other disease blocks were obtained from Harvard Brain Tissue Resource Center, McLean Hospital, Boston, MA. Goat anti-8-oxo-deoxyguanine polyclonal antibody (Chemicon, Temecula, CA) was used to detect 8-OHdG in sections. After deparaffinization with Xylene, brain sections (5 microns) were hydrated through graded ethanol. Endogenous peroxidase activity in the tissue was eliminated by incubation with 3% H₂O₂ in methanol for 15 minutes. The sections were treated with 20 µg/ml proteinase-K for 45 minutes at 37°C and nonspecific binding sites were blocked by 10% normal goat serum in PBS for 4 hours. The sections were incubated with goat 8-OHdG antibody (1:100) in 5% normal goat serum overnight at 4°C followed by addition of rabbit anti-goat secondary antibody (1:400) in PBS for 45 minutes. An antibody control was also performed, where the section was incubated with all reagents but the primary antibody. Next, ABC reagent was used to enhance the signal and immunostaining was developed by DAB for 3–5 minutes. Immunostaining was developed by DAB for 3–5 minutes.

DNA Immunoprecipitation

Incorporation of 8-OxodG into genomic DNA was assayed by DNA immunoprecipitation as described (Akatsuka et al., 2006), employing a goat anti-8-oxo-deoxyguanine polyclonal antibody (Chemicon, Temecula, CA). Genomic DNA was extracted from brain tissue (50 mg) and concentration was measured. 5 µg genomic DNA was digested with TSP45I (New England Biolabs, Ipswich, MA) for 16h at 65°C. Digested DNA was incubated with 5 µl 8-oxo-dG antibody, 10% BSA, and 1X PBS in a total volume of 400 µl reaction at 4°C for 3 hours. Anti-goat IgG agarose was added and mixing resumed for another 3 hours at 4°C. The beads were centrifuged and washed once with a low salt immune complex buffer (0.1% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, 50 mM HEPES-KOH pH 7.5, 140 mM NaCl) with rotating 3 minutes, once with a high salt wash buffer (0.1% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, 50 mM HEPES-KOH pH 7.5, 500 mM NaCl) with rotating 5 minutes, once with a LiCl wash buffer (0.1% sodium deoxycholate, 1 mM EDTA, 0.5% NP-40, 250 mM LiCl, 10 mM Tris-HCl pH 8.0) with rotating 3 minutes, and twice in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with rotating 3 minutes each time. The washed agarose beads were eluted with 100 µl freshly prepared elution buffer (50 mM Tris-HCL and 10 mM EDTA, pH 8.0) twice. DNA was extracted from combined elute by phenol-chloroform and ethanol precipitation, and dissolved in 10 µl ddH₂O. DNA concentration was determined by 260 nm adsorption. The same volume of immunoprecipitated DNA was used for PCR with primers specific for amplicons in the p62 promoter. Gel and Graph Digital software (Silk Scientific Corporation, Orem, Utah) was used to quantify the signal of PCR product and relative intensity was analyzed statistically.

Cell transfection and Dual-Luciferase assay

Luciferase reporter plasmid derived from the promoter of p62 gene (p62 promoter-pGL3) was transfected into HEK 293 cells along with a pRL-TK-Renilla control plasmid (pGL3: pRL = 7:1) and prostate-derived Ets factor (PDEF) construct (pGL3: PDEF = 5:1) (Thompson et al., 2003). Forty-eight hours after transfection, cells were lysed and analyzed by Dual-Luciferase Reporter Assay (Promega). Reporter luciferase activity (pGL3) was normalized to Renilla-luciferase activity (pRL) as control for transfection efficiency. Relative promoter activity was

expressed as the ratio of the luminescence of pGL3 to the luminescence reading of pRL. The duplicate wells were transfected and treated or not, and each well was assayed in triplicate. The experiment was replicated three independent times.

Sp-1 deletion mutation

The Sp-1 deleted form of the p62 promoter was made by Quickchange II XL Site-directed mutagenesis kit (Stratagene, La Jolla, CA 92037). The primers for Sp-1 deleted mutation were follows: 5'GCTTCCTTCTCCCCTCCCCCAGTCTCTTC 3' (Forward) and 5'GAAGAGACTGGGGGAGGGGAGAAGGAAGC 3' (Reverse). Wild type p62 promoter-pGL3 construct was used as the template. PCR and transformation were performed following the kit manual. Four clones were picked to prepare plasmids and sent for sequencing. The entire promoter was further sequenced to validate an absence of any PCR induced mutations.

Statistical analyses

Possible differences between group means and statistical relationships between the level of p62 expression and p62 promoter damage were analyzed using one-tailed t tests, ANOVA and correlation analyses (SAS v 9.1, SAS Institute Inc., Cary, NC, U.S.A.). For significant differences, alpha was set at 0.05. Group-wise alpha values were adjusted for multiple comparisons using the Stepdown Sidak algorithm. One-tailed probabilities were selected in comparisons where *a priori* evidence (Du et al. 2009) indicated that responses relative to control could be expected to be unidirectional (increased or decreased).

Results

Oxidative modification to the p62 promoter in DNA from various disease samples

Oxidative stress is a major risk factor associated with neurodegenerative disease (Lin and Beal, 2006; Butterfield, 2006). We recently reported that reduced p62 expression occurred in both AD brains and transgenic AD mice compared to controls, which correlated with oxidative damage to the p62 promoter (Du et al., 2009). In order to investigate whether this observation is common to other neurodegenerative diseases, DNA oxidative damage in normal and various neurodegenerative disease brains was examined. Since 8-OHdG has been described as one of the best biomarkers of DNA oxidative damage (Steenken, 1989; Kasai, 1997), the staining of brain sections with antibody to 8-OHdG was undertaken (Fig. 1). No immunoreactivity was present in samples treated with DNase I or in sections incubated without primary antibody (Fig. 1). Although, RNA has been reported to immunoreact with 8-OHdG antibody (Nunomura et al., 1999), the majority of the immunoreactivity was specific for DNA since pretreatment of the section with DNase completely eliminated immunoreactivity. In brain sections taken from individuals with various neurodegenerative diseases such as AD, FTD, HD, Pick's, or PD, 8-OHdG immunoreactivity was apparent. Close examination revealed a significant degree of cytoplasmic and some nuclear staining (Fig. 1, inset-arrow). These results reveal oxidative DNA damage is a feature common to the pathogenesis of these various neurodegenerative diseases.

P62 promoter is oxidatively modified

We have recently shown that the human p62 promoter contains a CpG island which is a target for oxidative stress modification (Du et al., 2009). Since higher oxidative DNA damage was found in samples from various neurodegenerative diseases, we next evaluated oxidative damage to the p62 promoter in DNA isolated from normal tissue compared to that obtained from diseased individuals. Three amplicons were designed to test the damage index of the p62 promoter based upon their degree of GC richness (> 60%) and the position of putative transcription factor binding sites (Fig. 2A). These amplicons were then employed in a DNA

damage assay to assess the degree of oxidative damage within the p62 promoter (Lu et al., 2004; Du et al., 2009). Brain tissue from AD, FTD, Pick's, HD and PD individuals were assessed for the degree of promoter damage. DNA damage was significantly higher in the samples from the various diseases compared to those of normal brain (Fig. 2B). On average, the degree of damage to amplicon 3 in AD, Pick's, FTD, and PD are higher than damage to either amplicon 1 or 2, possibly because DNA repair occurs more slowly at sites further from the transcription start site (Tu et al., 1996). In order to validate the results obtained with the DNA damage assay, DNA immunoprecipitation with an anti-8-OHdG antibody was employed to confirm the level of the modified base (Akatsuka et al., 2006). Two samples each were chosen from normal and each neurodegenerative disease. From the relative intensity of specific PCR products, the observed levels of oxidative modification in amplicon 2 for all five disease samples were significantly higher than controls (range = 28X to 258X greater response; Fig. 2C). These findings were congruent with the damage assay and immunohistochemical analysis, revealing increased 8-OHdG levels in DNA isolated from diseased samples compared to normal samples.

Absence of a genetic mutation in the p62 promoter

Oxidative modification of guanine in some transcription factor binding sites has been reported to inhibit transcription factor binding resulting in reduced protein expression (Ghosh and Mitchell, 1999). In samples from AD individuals, we recently observed that p62 expression was negatively correlated with damage to the promoter (Du et al., 2009). Because FTD samples showed enhanced DNA oxidative damage to all three amplicons (Fig. 2B), we examined if the same correlation existed between the level of p62 expression and oxidative damage in FTD diseased individuals (Fig. 3A). Expression of p62 and tubulin, as control, in five FTD samples was examined. A correlation analysis was undertaken between the relative expression of p62, normalized to the expression of tubulin, and the average oxidative damage that occurred to the promoter (Fig. 3B). The relative expression of p62 was strongly negatively correlated with p62 promoter damage ($r = -0.85$; $p = 0.067$). These findings are congruent with our earlier study (Du et al., 2009).

8-OHdG has the mutagenic potential to make G $\rightarrow$ T transversion (Hatahet et al., 1998). Since p62 expression is regulated at the transcriptional level (Thompson et al., 2003; Nakaso et al., 2004; Du et al., 2009) and nucleotide alternations in p62's promoter region might affect transcription factor binding, we examined whether there is possible genetic variation caused by DNA oxidative damage to the p62 promoter. Four normal, six AD, and two FTD brain samples were selected for further study. The p62 promoter (2 Kb of 5'-flanking region) was amplified by two specific primers, forward and reverse (Table 2A). Five sequencing primers, PWF1-S, PWF2-S, PWF3-S, PWF4-S, and PWR1-S were used to sequence the p62 promoter from various samples (Table 3; Fig. 4). The sequencing results revealed no genetic variants in the p62 promoter in samples from either AD or FTD brain, compared with normal samples. Altogether, these results suggest that declined p62 levels are not likely caused by base mutation in the promoter region.

Oxidative damage to p62 promoter decreases p62 promoter activity

In order to further understand the mechanism whereby oxidative modification to the p62 promoter might affect its transcriptional activity, a p62 promoter construct was treated with 100 μ M H₂O₂ *in vitro*, transfected into HEK cells, followed by assay to examine p62 basal transcriptional activity (Fig. 5A). Promoter activity was decreased 40% compared to cells transfected with the non-treated p62 promoter construct (Fig. 5A). In parallel, oxidative damage to either amplicon 1, 2 or 3 of the H₂O₂-treated p62 promoter was also evaluated. The degree of damage was significantly higher for amplicon 2 and 3 (Fig. 5B). Collectively, these results demonstrate that oxidative damage to the p62 promoter resulted in decreased p62

promoter transcriptional activity. Likewise, these findings are concordant with previous results showing that long-term H₂O₂-treatment of HEK cells led to reduced p62 protein expression (Du et al., 2009).

Deletion of an Sp-1 inhibited induction of p62 promoter activity by oxidative stress

Close examination of the 3 amplicons which span the p62 promoter reveal that amplicon 3 contains a transcription factor binding site for Sp-1 (Fig. 2A), with a guanine assessable for oxidative modification. It has also been reported that oxidative modification of an Sp-1 binding site can inhibit transcription factor binding (Ghosh and Mitchell, 1999). A p62 promoter mutant which lacked an Sp-1 binding site was generated by PCR. Since p62 is induced in response to stress (Ishii et al., 1996), the mutant promoter construct and the wild type (WT) construct were transfected into HEK cells and induction of promoter activity was assessed by H₂O₂ treatment of cells (Fig. 6). The induction of the WT p62 promoter by H₂O₂-treatment was 1.8 times greater compared to that of non-treatment, while cells transfected with the Sp-1 deleted p62 promoter construct failed to induce promoter activity. An unpaired t-test revealed a significant difference between induction of WT compared to the mutant (Fig. 6), suggesting deletion of Sp-1 transcription factor binding site abolished the induction of the p62 promoter by oxidative stress.

Discussion

Neurons are particularly vulnerable to the attack of oxidative stress due to high O₂ consumption in brain leading to accumulation of ROS (Schulz et al., 2000; Droge and Schipper, 2007). Growing evidence indicates that DNA oxidative damage may play a common role in the pathogenesis of several neurodegenerative diseases, such as AD, PD, HD, and ALS (Lovell and Markesbery, 2007a and 2007b; Migliore et al., 2005; Nakabeppu et al., 2007; Bogdanov et al., 2001; Warita et al., 2001). Alternatively, lesions may serve as a consequence of oxidative stress and have been suggested to function as a primary line of antioxidant defense (Smith et al., 2002; Castellani et al., 2006; Hayashi et al., 2007; Nakamura et al., 2007). However, two limitations to our study were the lack of region-specific samples associated with pathological lesions and the small sample size utilized in for mutation analysis. The robust immunostaining obtained with all of the samples suggest that oxidative damage may be wide-spread in the brain and may be a common disturbance. The small number of samples employed for the DNA sequencing aspect does not rule out that more rare mutations might exist in the population at large. Thus, this should not be considered a definitive study in that regard.

Since neurons are highly differentiated, long-lived, and irreplaceable, antioxidant and DNA repair systems are critical for longevity. A defective DNA damage response might cause neurodegeneration. BER dysfunction was observed in brain from individuals with both AD and mild cognitive impairment (MCI) due to reduced OGG1 activity (Weissman et al., 2007). Therefore, neuronal loss in AD might result from the combined effects of increased DNA oxidative damage and impaired DNA repair. Oxidative damage to nuclear and mitochondrial DNA has also been observed in PD brains and brain cells of α -synuclein mutant mice (Nakabeppu et al., 2007; Yasuhara et al., 2007). Increased 8-OHdG levels has been observed in HD transgenic mice, post-mortem HD caudate, and peripheral blood of human HD patients (Bogdanov et al., 2001; Browne et al., 1997; Chen et al., 2007). Also, the activities of some antioxidant enzymes such as superoxide dismutase (SOD1), glutathione peroxidase (Gpx) in erythrocytes (Chen et al., 2007), and catalase in skin fibroblasts of patients with HD (del Hoyo et al., 2006) were decreased. Although few investigations regarding 8-OHdG levels in FTD and Pick's disease have been made, DNA fragmentation was noted in both diseases (Su et al., 2000; Gleckman, et al., 1999). DNA fragmentation has also been observed in PD (Bender et al., 2006), and suggested to result from oxidative DNA damage (Yang et al., 2008). In a study

by Lu et al., (2004), they examined a large array of genes whose promoters were oxidatively modified, the common element being the high GC content. Our findings are consistent with promoters of this type as being a target for oxidative modification.

The role of p62 in neurodegenerative disease is still not fully understood. However, various studies support a role for p62 in the formation of inclusion bodies and trafficking of proteins for degradation. p62 was found as an oxidative stress-induced protein (Ishii et al., 1996), suggesting a protective function under oxidative stress. This protein is a common component of ubiquitin positive inclusions, found in various neurodegenerative diseases such as NFT in AD, and Lewy bodies in PD (Kuusisto et al., 2001; Zatloukal et al., 2002). In HD, p62 has been shown to protect neuronal cells from toxicity of misfolded proteins by enhancing aggregate formation (Bjørkøy et al., 2005). Protein oligomers, such as A β oligomers, which generate ROS toxic to cells, can be stored in aggresomes to protect cells from oxidative stress. p62 also interacts with LC3, a marker of the autophagosome, to facilitate degradation of ubiquitinated protein aggregates by autophagy (Pankiv et al., 2007). An absence of p62 in brain of mice deficient in p62 leads to accumulation of insoluble polyubiquitin aggregates (Wooten et al., 2008), as well as an absence of aggresomes in response to proteasome inhibition (Wooten et al., 2006). Moreover, p62 has been reported to activate the antioxidant response element (ARE) and protect cells from oxidative stress (Liu et al., 2007). p62 can stimulate NF-E2-related factor 2 (Nrf2) nuclear translocation to activate the expression of many antioxidant enzymes. Oxidative damage to DNA, especially to some age-related gene promoter can down-regulate gene expression (Lu et al., 2004). Furthermore, decreased p62 expression is correlated with increased oxidative damage to the human p62 promoter in AD brain (Du et al., 2009). Because p62 expression is regulated at the transcriptional level (Thompson et al., 2003; Nakaso et al., 2004), the effects of oxidative damage within the p62 promoter were examined. Decreased transcriptional activity was observed along with corresponding oxidative DNA damage within the p62 promoter upon *in vitro* H₂O₂ treatment. It has been reported that the oxidative modification to Sp-1 element can abolish the transcription factor binding (Ghosh and Mitchell, 1999). We found that the Sp-1 element deleted mutation in the human p62 promoter displayed reduced stress-induced activation compared to the WT p62 promoter. Altogether, these results reveal that oxidative modification to the p62 promoter decreases its transcriptional activity, and could therefore contribute to down-regulated p62 expression. In contrast, increased p62 expression has been associated with deficiency in autophagy and may be used as a marker of the autophagic activity of the cell (Yue, 2007).

We propose that p62 plays a central role in regulation of neurodegeneration by its ability to activate survival signaling, given its function as a scaffold (Moscat et al., 2007), as well as, an ability to traffic polyubiquitinated substrates for proteasomal degradation (Seibenhener et al., 2004; Geetha et al., 2008), and to sequester toxic misfolded ubiquitinated proteins for autophagy (Nakaso et al., 2004; Wooten et al., 2006; Komatsu et al., 2007). Our findings suggest that elevation of p62 levels in brain might serve as a potential therapeutic target for treatment of various neurodegenerative diseases. Further studies in appropriate models are needed to test this idea.

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Fig. 1.

Representative immunohistochemical staining for 8-OHdG in brain. Sections were treated with DNase I or incubated without primary antibody did not display any immunoreactivity for 8-OHdG. Little or no immunoreactivity was observed in sections taken from normal brain. DNA damage is present in neurodegenerative diseased brains than in normal brains. Variable but reproducible staining to 8-OHdG was observed in sections of AD, FTD, HD, Pick's and PD. The staining was mostly cytoplasmic with some nuclear deposits (Inset-arrow).

C

Fig. 2.

Profile of oxidative damage in 3 amplicons within p62 promoter of normal and various samples from neurodegenerative diseased brains. **A.** Three amplicons, amplicon 1 (−1085 ~ −954), amplicon 2 (−407 ~ −321) and amplicon 3 (−1608 ~ −1484), were designed within the human p62 promoter. Amplicon 2 is the closest amplicon to the transcription initiation site and amplicon 3 is the furthest one. GC content of each amplicon and transcription factor binding sites within the p62 promoter were shown. **B.** Oxidative damage to each amplicon in control and various neurodegenerative diseased brains was assessed. Neurodegenerative diseased samples (N shown in the bar) include Alzheimer's Disease (AD), Pick's, Frontotemporal Dementia (FTD), Huntington Disease (HD) and Parkinson Disease (PD). Oxidative damage is expressed as Mean % ± S.D. One-way ANOVAs indicated significant overall differences among the 6 groups for each amplicon (amplicon 1, $F = 6.21$, $p < 0.001$; amplicon 2, $F = 4.03$, $p < 0.01$; amplicon 3, $F = 4.98$, $p < 0.01$). Individual comparisons between control and disease associated damage levels were conducted using one-tailed t tests with the group-wise p -value adjusted for multiple tests by the Stepdown Sidak approach. Damage to each amplicon from all 5 disease groups was significantly higher than control subjects (* indicates adjusted $p < 0.05$). **C.** DNA immunoprecipitation of amplicon 2 within the p62 promoter with a polyclonal antibody to 8-OHdG (Chemicon) in normal and various neurodegenerative diseased brain samples. Input DNA for each sample is shown. Relative density of each band was quantified. The PCR products of amplicon 2 from each of the neurodegenerative diseased samples were

higher than control samples. ANOVA indicated overall statistical significance among the 8-OHdG levels in the 5 diseased and 1 control groups ($F = 5.69$, $p = 0.028$). Significant differences between individual diseases and the control are indicated by ** (adjusted $p < 0.001$).

A **B** **Fig. 3.**

Correlation between p62 expression and oxidative damage in the p62 promoter in FTD. **A.** p62 expression in 5 human FTD brain tissues. An equal concentration of whole lysate protein (50 μ g) from 5 human FTD brains were Western blotted with p62 and tubulin. P62 relative expression was expressed as the ratio of the relative intensity of p62 to that of tubulin. **B.** Correlation analysis between p62 expression and the average oxidative DNA damage of 3 amplicons. p62 relative expression is negatively correlated with DNA damage within the p62 promoter ($r = -0.85$; $p = 0.067$).

Amplification and Sequencing primers for p62 promoter

Fig. 4.

Diagram of primers used for p62 promoter amplification and sequencing. Two specific primers, PWF and PWR were used to amplify p62 promoter from genomic DNA. The location of five sequencing primers, PWF1-S, PWF2-S, PWF3-S, PWF4S, and PWR1S were shown.

A **B** **Fig. 5.**

Oxidative stress reduces p62 promoter activity. **A.** p62 promoter activity decreased upon *in vitro* H₂O₂ treatment. p62 promoter-pGL3 was incubated in the absence or presence of 100 μ M H₂O₂ for one hour at 4°C *in vitro*. Non-treated and H₂O₂-treated p62 promoter-pGL3 were transfected into HEK cells along with pRL-TK and PDEF. Cells were lysed and analyzed by Dual-Luciferase Reporter Assay after 48 h. Relative promoter activity was expressed as Mean $\pm$ S.E.M. (N = 3). **B.** Oxidative DNA damage to 3 amplicons within the p62 promoter upon *in vitro* H₂O₂ treatment. Non-treated p62 promoter-pGL3 construct and *in vitro* 100 μ M H₂O₂-treated p62 promoter-pGL3 were subjected to oxidative DNA damage assay for three amplicons. The 10 X dilution of the p62 promoter-pGL3 construct was used to generate standard curve. * $p < 0.05$, significant difference.

Fig. 6.

Induction of the wild type and Sp-1 deleted p62 promoter upon H₂O₂ treatment. WT p62 promoter-pGL3 and Sp-1 deletion mutant were transfected into HEK cells along with pRL-TK and PDEF. The cells were treated with 600 μM H₂O₂ for 16 h, post-transfection, lysed and subjected to dual-luciferase assay. The p62 promoter-pGL3 activity was normalized by pRL-TK activity. The induction was expressed as the ratio of the relative promoter activity of 600-μM-treatment to that of non-treatment (Mean ± S.E.M. (N = 6); ** $p < 0.001$).

Table 1

Features of Subject Group

Brain Number	Distributive Dx	Age	Race/Sex	PMI	Other disease	Source
OS00-06	Normal	60	b/f	8	-	ADRC, Emory
OS00-23	Normal	68	w/f	11	-	ADRC, Emory
OS01-112	Normal	65	w/f	6	-	ADRC, Emory
OS02-35	Normal	75	w/f	6	-	ADRC, Emory
OS03-299	Normal	69	w/m	2.5	-	ADRC, Emory
OS00-05	Mild AD	74	w/m	<6	-	ADRC, Emory
OS01-73	Mild AD	76	w/f	15	-	ADRC, Emory
OS01-126	Mild AD	87	w/f	32	-	ADRC, Emory
OS01-129	Mild AD	92	w/f	NA	-	ADRC, Emory
OS00-38	Severe AD	92	w/f	6	-	ADRC, Emory
OS01-02	Severe AD	69	w/f	5.5	-	ADRC, Emory
OS01-11	Severe AD	80	b/f	5	-	ADRC, Emory
5746	FTD	75	m	14.83	-	HBTRC
5882	FTD	88	f	8.67		HBTRC
B6482	FTD	69	m	17.33		HBTRC
B6605	FTD	73	m	11.8	Pneumonia	HBTRC
B6806	FTD	73	f	22.83	Respiratory failure	HBTRC
4413	Pick's	70	m	24		HBTRC
4943	Pick's	95	f	27.5	Sepsis	HBTRC
b5025	Pick's	76	f	11.08		HBTRC
b5035	Pick's	75	f	20	Pneumonia	HBTRC
5507	HD	48	f	26.72		HBTRC
5567	HD	50	m	14.78		HBTRC
B6385	HD	75	f	23.2		HBTRC
B6606	HD	69	f	12.08		HBTRC
5657	PD	72	m	26.25		HBTRC
5731	PD	85	m	15.75		HBTRC
b6647	PD	84	f	12.75		HBTRC
b6653	PD	73	f	20.97		HBTRC

Brain Number	Distributive Dx	Age	Race/Sex	PMI	Other disease	Source
b6812	PD	75	m	19.42		HBTRC

Table 2

Amplicons and Primer Pairs used for Real-time PCR

P62 promoter	Amplicon	Primer sequence	T _m (°C)	
Human	Amplicon 1	5' - CAT TCA CAC CTG TGG ACC AGC -3'	58.5	Sense
		5' -CTT GCA GGA GCT GGA GAA ACC-3'	58.3	Antisense
	Amplicon 2	5' -GAC CTA GCA GCC TCC TGA TAT GG-3'	58.7	Sense
		5' -TGG CCA TGA CTC AGC AAT ATC CTC-3'	58.8	Antisense
	Amplicon 3	5' -AGC TTC CCA AAG ACT CCC TCT TCT-3'	59.6	Sense
		5' -TCC CAT GAC TTT GAC TCA GCA GGT-3'	60.3	Antisense

Table 3

Samples for sequencing of the human p62 promoter

A

Sample	1	2	3	4	1	2	3	4	6	1	2
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B

Primers for amplification and sequencing of the human p62 promoter

Primer	Sequence	Tm(°C)
PWF	5'-TTA CAA CAT GCT GGG CAG CCA CAG-3'	60.3
PWR	5'-AGC AGA AGC TGA AGC GGC GAA TCT-3'	63.0
PWF1-S	5'-AGG TAA GAG GTC ACT GAG ATG GGT-3'	58.9
PWF2-S	5'-GCC AAA TGG CGA GAA AGC AAA GGA-3'	60.6
PWF3-S	5'-CAT TCA CAC CTG TGG ACC AGC-3'	58.5
PWF4-S	5'-TAC CTG CTG AAT TGA GGA GCC CAT-3'	60.2
PWF1-S	5'-AGA AGC TGA AGC	59.2

A												
Samples for sequencing of the human p62 promoter												
Sample	1	2	3	4	1	2	3	4	5	6	1	2
GGC GAA TCT-3'												