REGULATION OF THE P62 PROMOTER BY OXIDATIVE DAMAGE IN

NEURODEGENERATIVE DISEASE AND AGING

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REGULATION OF THE P62 PROMOTER BY OXIDATIVE DAMAGE IN NEURODEGENERATIVE DISEASE AND AGING

Yifeng Du

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REGULATION OF THE P62 PROMOTER BY OXIDATIVE DAMAGE IN

NEURODEGENERATIVE DISEASE AND AGING

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Yifeng Du, daughter of Guoliang Du and Guilan Xu, was born on June 17, 1972, in Zhangjiakou City, Hebei Province, People's Republic of China. She attended Lanzhou University in Gansu Province and graduated with a Bachelor of Science degree in Biology in July, 1994. She worked in Zhangjiakou-Gist-Brocades Pharmaceutical Company Limited after graduation for two years. Then, she entered Lanzhou University again and got a Master of Science degree in Biochemistry in July, 1999. After working as a lecturer in Capital University of Medical Sciences in Beijing for five years, she entered the Graduate School of Auburn University, with Cellular and Molecular Biosciences Fellowship, in August 2004.

DISSERTATION ABSTRACT

REGULATION OF THE P62 PROMOTER BY OXIDATIVE DAMAGE IN NEURODEGENERATIVE DISEASE AND AGING

Yifeng Du

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Oxidative stress is regarded as the damage to a biological system caused by reactive oxygen species (ROS) when the prooxidant overwhelms the antioxidant defense and repair ability. Growing evidence reveals that oxidative stress is a major contributor to aging and neurodegeneration. P62 is a scaffold protein that is utilized for cell signaling, receptor trafficking, and inclusion formation. P62 knockout mice exhibit Alzheimer's Disease-like phenotype. The structure and function of p62, ROS formation and oxidative damage to macromolecules, the antioxidant defense system, the relationship among p62, oxidative stress, aging, and neurodegenerative disease were reviewed in Chapter I.

Declined p62 expression levels were observed in Alzheimer's Disease (AD) brains compared to tissue from normal individuals. My hypothesis was that reduced p62 level might be caused by oxidative damage to the p62 promoter. In order to test this hypothesis, two specific objectives were undertaken: (1) examine age-associated oxidative damage to the p62 promoter in AD; (2) examine whether this damage is common in various neurodegenerative disease. In Chapter II, we showed that oxidative damage to the p62 promoter was significantly higher in DNA from AD brain than normal brain. Also, this damage was age-dependent in both WT mice and normal brains. The negative correlation between oxidative damage to the p62 promoter and the p62 expression level was demonstrated in human brains, mice, and HEK cells. In Chapter III, we showed that no genetic variance was found between the p62 promoter in normal and diseased brains. However, oxidative damage to the p62 promoter was significantly higher in various neurodegenerative diseases than normal brains. Decreased activity and induction of the p62 promoter were caused by oxidative damage to the p62 promoter and the deletion of Sp-1 binding site. Altogether, these findings revealed that p62 level was regulated by oxidative damage to the p62 promoter.

P62 regulation and future works were summarized in Chapter IV. Proteasome or autophagy inhibition up-regulates p62 protein levels. Decreased p62 level results in the reduced delivery of substrate to autophagy, leading to more damaged organelles. Since p62 knockout mice showed higher oxidative stress, the mechanism leading to this result will be further investigated and mitochondria dysfunction will be examined in WT, p62 knockout, and p62 over-expressed mice.

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LIST OF ABBREVIATION

AD	Alzheimer's Disease
AID	aPKC interaction domain
ALIS	Aggresome-like induced structure
ALS	Amyotrophic Lateral Sclerosis
AP	Apurinic
aPKC	Atypical protein kinase C
APP	Amyloid precursor protein
ARE	Antioxidant response element
BER	Base excision repair
BSA	Bovine serum albumin
CA II	Carbonic anhydrase II
CaM1	Calmodulin 1
CAT	Catalase
CBP	CREB-binding protein
CK	Casein kinase
CMA	chaperon-mediated autophagy
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid

CuZnSOD	Copper and zinc SOD
DOPA	3, 4-dihydroxyphenylalanine
DPC	DNA-protein crosslinking
DRP2	Dihydropyrimidinase-related protein-2
ECL	Enhanced chemiluminescence
EMSA	Eletrophoretic mobility shift assays
ENO1	Enolase 1
ETC	Electron transport chain
FAD	Familial Alzheimer's disease
FeSOD	Iron-containing SOD
FPD	Familial Parkinson's disease
Fpg	Formamidopyrmidine DNA glycolyase
FTD	Frontotemporal Dementi
GFAP	Glial fibrillary acidic pro
GPx	Glutathione perxidase
GS	Glutamine synthetase
GST	Glutathione-S-transferase
HD	Huntington's Disease
HEK	Human Embryonic Kidney
HNE	Hydroxynonenal
HOC1	Hypochlorous acid
Hsp90	Heat shock protein 90
hTDG	human thymine-DNA glycosylase

IB	Inclusion bodies
IKK	IκB kinase
IL-1	Interleukin-1
IRAK	Interleukin-1 receptor-associated kinase
IκB	Inhibitor of NF-κB
JNK	Jun N-terminal kinase
KO	Knock out
LAMP	Lysosomal membrane protein
LC3	Microtubule-associated protein 1 light chain 3
LIR	LC3 interacting region
MBs	Mallory bodies
MCI	Mild cognitive inpairment
MEFs	Mouse embryonic fibroblasts
MEK	Mitogen-activated protein kinase kinase
MEK1	Meiosis-specific serine/threonine protein kinase
MnSOD	Mn^{2+}superoxide dismutase
MPTP	1-methhyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
Msr	Methionine sulfoxide reductase
mtDNA	Mitochondrial DNA
MURF-2	Muscle-specific RING finger-2
nbr1	Neighbor of BRCA1 gene 1

NBR1	Next to breast cancer 1
NER	Nucleotide excision repair
NFT	Neurofibrillary tangles
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor
Nqo1	NAD(P)H dehydrogenase quinine 1
OGG1	DNA glycosylase 1
OPCA	OPR, PC, and AID
OPR	Octicosapeptide repeat
PAGE	Polyacrylamide gel electrophoresis
PB1	Phox and Bem1
PBN	Phenyl-tert-butyl nitrone
PC	Motif exiting in mammalian $p40^{phox}$ and yeast Cdc24p
PD	Parkinson's disease
PDB	Paget's disease of bone
PDEF	Prostate-derived Ets factor
PDK 1	Pyruvate dehydrogenase kinase, isozyme 1
PEG-3	Progression elevated gene-3
PGM 1	Phosphoglycerate mutase 1
PIN1	Peptidyl-prolyl cis/trans isomerase
PKC- ζ	Protein kinase C zeta
PKM2	Pyruvate kinase M2

PSI	Proteasome inhibitor
PUFAs	Phospholipid unsaturated fatty acids
RANK-L	Receptor activator for Nuclear Factor κB ligand
RING	Really interesting new gene
RIP	Receptor interacting protein
ROS	Reactive oxygen species
SATP	Signal transduction and adaptor protein
SDS	Sodium dodecyl sulfate
SH2	Src homology 2
SOD	Superoxide dismutase
SQSTM1	Sequestsome 1
TCA	Tricarboxylic acid
TFIIB	Transcription factor B related to initiation of transcription by RNA polymerase II
TNF	Tumor necrosis factor
TNF-R1	Tumor necrosis factor α receptor 1
TPI	Triose phosphate isomerase
TRADD	TNFR1 associated death domain protein
TRAF6	Tumor necrosis factor receptor-associated factor 6
TrkA	Tropomyosin receptor kinase A
UA	Uric acid

UBA	Ubiquitin associated domain
UCHL-1	Ubiquitin carboxy terminal hydrolase L-1
UPS	Ubiquitination-proteasome system
V-DAC1	Voltage dependent anion channel
WB	Western blot
WT	Wild type
ZIP	Zeta protein kinase C interacting Protein
ZZ	ZZ-type zinc finger domain
γ-SNAP	Gamma soluble NSF attachment protein

CHAPTER I. LITERATURE REVIEW

THE INVOLVEMENT OF P62 AND OXIDATIVE STRESS IN NEURODEGENERATIVE DISEASE

PART I. P62

Human p62/Sequestsome 1 (SQSTM1) (Gene bank #: BC019111.1), also known as A170, the mouse p62 (Gene bank #: BC006019.1), or zeta protein kinase C-interacting protein (ZIP), the rat p62 (Gene bank #: BC061575), is a highly conserved cytoplasmic protein which has been shown to play important roles in signaling, trafficking, and inclusion formation (Wooten et al., 2006). P62 was first identified as a phosphotyrosine-independent ligand of the Src homology 2 (SH2) domain of $p56^{lck}$, a T-cell-specific Src family tyrosine kinase in 1995 (Park et al., 1995), and the first cDNA of human p62 was cloned in 1996, also, it was found that the p62 protein is composed of 440 amino acids(Joung et al., 1996). In the same year, an oxidative stress induced gene A170 was cloned in murine peritoneal macrophages. The A170 protein is 90% identical to human p62 (Ishii et al, 1996). In 1997, ZIP, composed of 239 amino acids, was identified in rat. ZIP showed 97% similarity with mouse sequence A170, and 91% with human p62 (Puls et al., 1997). A cDNA encoding a 442-amino-acid protein named signal transduction and

adaptor protein (STAP) was cloned from the mouse osteoblastic cell line MC3T3-E1, which is identical to mouse A170 (Okazaki et al., 1999). All of these proteins share highly-conserved domains suggesting these domains have important functions. These structural motifs contain Phox and Bem1 (PB1) domain, including Src homology regions 2 (SH2) binding domain and atypical protein kinase C (aPKC) interaction motif (AID), at the N-terminal; a ZZ zinc finger acting as a potential protein binding module; a binding site for ring-finger protein tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6); two PEST sequences, and ubiquitin-associated (UBA) domain at its C-terminus (Geetha and Wooten, 2002) (Fig. 1-1).

1. Structure of p62

As a member of the src family tyrosine kinase, $p56^{lck}$ contains SH1, SH2 and SH3 domains. SH2 domain is about 100-amino-acids long and the conformation of this domain contains 2 alpha helices and 7 beta sheets. SH2 is highly conserved in several signaling molecules and mediates protein-protein interactions by binding phosphotyrosine containing proteins. However, the binding between p62 and SH2 domain is suggested to be phosphotyrosine-independent because the mutation of the only tyrosine residue (Tyr-9) in the 50 N-terminal amino acids of p62, which are critical for its binding to SH2, did not affect the binding between p62 and SH2. Moreover, phosphoamino acid analysis of immunoprecipitated p62 did not recover any phophotyrosine from growing cells (Joung et al., 1996).

ZIP was reported to interact with the regulatory domain of protein kinase C zeta (PKC-ζ) but not the classic PKCs through its AID domain. The PKC family includes

classical (α, βI, βII, γ) PKC, novel (ξ, ε, η, θ) PKC, and atypical (ζ, λ /ι) PKC. The regulatory domain of the aPKCs has only one zinc-finger, but other PKC members have two. Also, aPKC does not have a C2 domain. These structural differences make aPKCs insensitive to Ca^{2+} and diacylglycerol, which can activate other PKC isoforms. Interaction analysis of a series of ZIP deletion mutants with full-length PKC-ζ showed that amino acids 41-105 of ZIP contained the binding site for PKC-ζ. This region is between the N-terminal and ZZ finger domain and contains a short amino acid motif with the consensus sequence $YXDEDX_5SDEE/D$ (Puls et al., 1997). The homologous regions of this motif were also found in yeast Cdc24, yeast scd1, and human MEK5, and mouse SATP/A170 (Okazaki et al., 1999). Meanwhile, interaction analysis of a series of PKC-ζ deletion mutants with full-length ZIP indicated that N-terminal amino acids 79-145 of PKC-ζ containing the pseudosubstrate site, which is called phox and Bem (PB1) domain, mediates ZIP biding (Puls et al., 1997; Hirano et al., 2004). Two alternatively spliced ZIP forms, ZIP1 and ZIP2, were reported in 1999. ZIP1 is the same as ZIP cloned by Puls, and ZIP2 is a 27-amino acid alternatively spliced form. Both can bind to PKC-ζ and Kvβ2 subunits of potassium channel, and serve as a link in the PKCζ-ZIP- Kvβ2complex (Gong et al., 1999). ZIP1 and ZIP2 have different activities in stimulating phosphorylation of Kvβ2, and the ratio of these two alternative transcripts may be regulated by hormone stimulation to modulate the intracellular phosphorylation level (Gong et al., 1999). ZIP3, a new C-terminal splice variant of the ZIP family, was found to interact with the GABAc receptor and PKCζ simultaneously acting as a scaffold to be involved in the neurotransmission (Croci et al., 2003). The human homologue of ZIP, p62, can bind to the V1 domains of both PKCζ and PKC-λ /ι, although with lower affinity for

PKC-ζ (Sanchez et al., 1998). The interaction between human p62 and aPKC has been found to be a PB1-PB1domain interaction (Hirano et al., 2004). The PB1 domain is evolutionarily conserved and classified into type I and type II. Type I has a PC motif, which exits in mammalian p40^{phox} and budding yeast Cdc24p, also called OPR (octicosapeptide repeat) or AID motif containing highly conserved acidic and hydrophobic residues; it is also named the OPCA (OPR, PC, and AID) motif. The acidic residues on the OPCA motif usually is used as an interaction site by type I, whereas a conserved lysine on the opposite side of the OPCA motif is used as an interaction site by type II (Ito et al., 2001). The aPKC PB1 domain works as a type I PB1 which has a conserved acidic surface, while p62 serves as the type II PB1 domain which has a basic binding site. These two different PB1 domains in aPKC and p62 respectively interact with each other to form the PB1-PB1 complex (Hirano et al., 2004).

The ZZ finger motif was first observed in the cDNA of p62, and it is a cysteine-rich region, expected to be a metal-binding site (Joung et al., 1996). Most ZZ sequences contain six cysteine residues that are supposed to participate in Zn^{2+} binding. Three Cys-X_2-Cys motifs in ZZ finger domain are important for their folding to form a domain structure in the presence of one or two Zn^{2+} ions (Ponting et al., 1996). The homologous regions of the ZZ finger are also found in mouse STAP/A170 (Okazaki et al., 1999), yeast ADA2 (Berger et al., 1992), and some nuclear proteins such as human transcriptional adaptor protein p300 (Eckner et al., 1994), mouse cyclic AMP response element binding protein (CREB)-binding protein (CBP) (Chrivia et al., 1993), and *Drosophila* ref (2) P protein (Gay and Contamine, 1993). However, the ZZ finger in p62 is a possible binding site for second messengers. The ZZ finger was proposed as a

putative protein binding site in dystrophin, utrophin, and transcriptional adaptors because the corresponding region of CBP is known to interact with transcription factor related to initiation of transcription by RNA polymerase II (TFIIB), c-Fos, and SV40 large T antigen (Kwok et al., 1994; Janknecht and Hunter, 1996). It was proposed that the ZZ finger can bind to the receptor interacting protein (RIP), a death domain kinase that associates with the tumor necrosis factor (TNF) α receptor 1 (TNFα -R1) through its interaction with the adapter molecule TNFR1 associated death domain protein (TRADD) (Sanz et al., 2000).

TNF receptor associated factor 6 (TRAF6) is a member of TRAF protein family, which mediates signal transduction from members of the TNF receptor superfamily and the members of the Toll/IL-1 family (Wu and Arron, 2003). TRAF also interacts with various protein kinases like interleukin-1 receptor-associated kinase 1 (IRAK1) and PKCzeta, which provides a link between distinct signaling pathways. TRAF6 serves as an ubiquitin ligase (E3) in the ubiquitin-proteasome pathway (Babu, et al., 2005), and selectively synthesizes K63-polyubiquitin chains onto target substrates (Wooten, et al., 2001). TRAF6 is also a transducer in the NF-κB pathway that activates IkappaB kinase (IKK) in response to proinflammatory cytokines (Sun, et al., 2004). TRAF6 interacts with p62 through TRAF6 binding site, the region between ZZ finger motif and the PEST sequence (Sanz et al., 2000). Interaction analysis was done using a series of p62 deletion mutants and p62 isoform. $P62^{ZIP2}$, which is generated by alternative splicing and lacks amino acids 225–251, severely abolished the association of TRAF6, suggesting the binding site for TRAF6 is in the region from amino acids 225 to 251. In addition, p62 selectively interacts with C-terminal TRAF domain of TRAF6, but not with TRAF2 or

TRAF5. The TRAF domain is the most conserved domain in all TRAFs, but the TRAF domain of TRAF6 shares only 29% identity in this region, which makes it the most divergent (Cao, et al., 1996). The N-terminal effector domain of TRAF6 interacts with PKCzeta. The interaction between p62 and TRAF6 is also interleukin-1 (IL-1) dependent (Sanz et al., 2000).

There are two PEST sequences in p62 located in the regions from amino acid 266 to 294 and 345 to 377 (Geetha & Wooten, 2002). The PEST motif is rich in proline (P), glutamate (E), serine (S), and threonine (T) and has been found in many short-lived proteins and acts as a signal peptide for rapid protein degradation. The PEST sequence was reported to exist in some critical regulatory proteins related to important biological events such as c-myc, c-fos, p53 etc (Rogers et al., 1986; Rechsteiner and Roger, 1996). The phosphorylation of some serines and threonines in this region of p62 might be involved in regulation of p62 function. In addition, the PEST sequence is present in most calmodulin-binding proteins because the glutamate in the PEST sequence is negatively charged, and the phosphorylation of S and T in the PEST sequence producs a very strong negatively-charged region which can interact with calmodulin (Wang et al., 1989).

The UBA domain was initially shown to bind to ubiquitin non-covalently and was found in many other proteins (Vadlamudi et al., 1996; Hofmann and Falquet, 2001). Analysis of several deletions of p62 revealed that the C-terminal 80 amino acids are essential for binding with ubiquitin. The region of amino acids 368-434 at the C-terminal of p62 is defined as a UBA domain (Vadlamudi et al., 1996; Geetha and Wooten, 2002). The UBA domain is more likely to bind polyubiquitin chains containing four or more ubiquitins over monoubiquitin (Shin, 1998; Wilkinson et al., 2001). A UBA domain has a

compact bundle with three amphipathic α-helix where a hydrophobic surface is formed on one side that might mediate protein-protein interactions (Shin, 1998; Bertolaet et al., 2001). This conserved hydrophobic patch composed of amino acids methionine, glycine, and phenylalanine (MGF) serves as a binding site for polyubiquitin and the conserved leucine in helix 2 is also necessary for binding polyubiquitinated proteins (Mueller and Feigon, 2002) and for sequesteration-aggregate formation as well (Seibenhener et al., 2004).

2. Functional Aspects of p62

A. Signal Scaffold

As previously described, p62 contains multiple protein-protein interaction motifs, enabling it to act as a scaffold. So far, the extensively studied function of p62 as a scaffold is for the activation of the transcription factor nuclear factor kappa B (NF-κB). NF-κB is a family of transcription factors involved in cell survival and differentiation (Ghosh and Karin, 2002). Usually, NF-κB is bound by its inhibitor protein, IκB, and is inactive in the cytoplasm. When a cell is activated by triggers such as nerve growth factor (NGF) and IL-1, IκB is phosphorylated by IκB kinase (IKK) and is targeted to the ubiquitination-proteasome system (UPS) for degradation (Ghosh and Karin, 2002). Then, the released NF-κB enters the nucleus to regulate gene expression. aPKC can bind to IKKs *in vivo* and *in vitro*, and PKCζ selectively phosphorylates and activates IKKβ (Lallena et al., 1999). Upon differential stimulation, p62 interacts with TRAF6 directly, which is essential to the activation of NF-κB in cells triggered by NGF, TNFα, IL-1, or receptor activator for Nuclear Factor κB ligand (RANK-L) (Sanz et al., 2000; Wooten et

al., 2001; Duran et al., 2004). In the TNFα signaling cascade, p62 binds to PKCζ through PB1, TRAF6 through TRAF6 binding site, and adaptor protein RIP through ZZ finger respectively. N-terminal of TRAF6 interacts with PKCζ in a dimerization-dependent manner, and RIP associates the PKCζ to the TNFα receptor, finally activating the NF-κB pathway (Fig.1-1). NF-κB pathway is also triggered by IL-1 in a similar manner. Upon IL-1 stimulation, IL-1 receptor interacts with Myeloid differentiation primary response 88 (Myd88), an adaptor protein, which binds the IL-1 receptor–associated kinase (IRAK). Then, IRAK recruits TRAF6, followed by p62 recruitment. Therefore, the aPKC-p62-TRAF6-RIP/IRAK complex is necessary for activation of NF-κB upon TNFα or IL-1 stimulation (Sanz et al., 2000). P62-TRAF6 complex is also important to neurotrophin such as NGF stimulated NF-κB pathway. There are two NGF receptors in cells, TrkA and p75. In presence of NGF, p62 binds to TrkA directly, but not p75. In contrast, TRAF6 interacts with p75 but not TrkA. P62-TRAF6 cpmplex serves as a bridge between p75 and TrkA and this complex allows aPKC to phosphorylate IKK leading to activation of NF-κB (Wooten et al., 2001). NGF-induced NF-κB activation is also regulated by p62 mediated TRAF6 polyubiquitination through UBA domain of p62 (Wooten et al., 2005). aPKC-p62-TRAF6 ternary complex activated NF-κB pathway was also found in RANK-activated osteoclastogenesis suggesting p62 is an important component in the control of induced bone remodeling (Duran et al., 2004). However, p62 may work as a negative modulator during NF-κB activation in the progression of Paget's disease of bone (PDB) (Rea et al., 2006). They found that mutations in UBA domain of p62 lead to increased activation of NF-κB and osteoclast formation but wildtype p62 inhibited NF-κB activation compared with empty vector, suggesting that the UBA domain encodes

regulatory elements in RANK-mediated osteoclast formation and bone resorption involved in PDB (Rea et al., 2006). P62 also plays important roles in receptor internalization and trafficking. TrkA internalization, trafficking and sorting are regulated by Lys(63)-linked polyubiquitin chains and p62 (Geetha and Wooten, 2002; 2006). P62 directly binds the juxtamembrane region of TrkA in a phosphotyrosine-dependent manner, and this complex is internalized in the endosomal compartment. The absence of p62 prevents endosomal localization and signaling. Furthermore, TrkA is Lys63-polyubiquitinated and the inhibition of polyubiquitination interrupts signalling and internalization.

Besides the NF-κB pathway, p62 also works as a scaffold in other signaling pathways. P62 interacts with Neighbor of BRCA1 gene 1 (nbr1) through PB1 domain and with muscle-specific RING finger-2 (MURF-2) by UBA domain. The nrb1-p62-MuRF2 complex is involved in the control of muscle gene expression and protein turnover (Lange et al., 2005).

B. A Ubiquitin Binding Protein

UPS is the major system for intracellular protein degradation in eukaryotes. Ubiquitin, an 8.5 kD polypeptide, selectively binds to proteins to form polyubiquitin-protein conjugates. Different kinds of polyubiquitin chains can be formed by different lysine in ubiquitin such as K63, K48, K33, K29, K27, K11, and K6. The polyubiquitin-protein is, in most cases, a signal for protein degradation. The polyuniquitination of substrates requires three classes of enzymes: E1 (Ub-activating enzyme), E2 (Ub-conjugating enzymes), and E3 (Ub-protein ligase) (Glickman and Ciechanover, 2002).

The proteasome can bind to the polyubiquitin chain and remove it from a target protein, and degrade the protein substrate. P62 serves as a shuttling factor in the delivery of poly-ubiquitinated proteins for 26S proteasomal degradation because the UBA domain of p62 binds K63 polyubiquitin chains of ubiquitinated substrates, and PB1 domain of p62 interacts with S5a subunit of 19S proteasomal particle (Seibenhener et al., 2004). TRAF6 has been identified as a new gene (RING) finger E3 ubiquitin ligase and the interaction of p62 with TRAF6 can stimulate TRAF6 autoubiquitination and E3 ligase activity, then, transferring K63 polyubiquitin chains onto target substrates (Wooten et al., 2005) (Fig.1-1). The ubiquitination of TRAF6 is also involved in the regulation of NF-κB pathway in RANK-mediated osteoclast formation mentioned before. Due to the ubiquitin-binding characteristic, p62 might be recruited to ubiquitinated protein aggregates, into which cytoplasmic multiubiquitinated proteins segregate, and the degree of the segregation increases when the proteasome is impaired, so p62 is also named "sequestosome" (Shin, 1998; Babu et al., 2005; Kuusisto, et al., 2001a). Inclusion bodies containing p62 and ubiquitinated proteins were found in many neurodegenerative diseases, such as neurofibrillary tangles in Alzheimer's disease, Lewy bodies in Parkinsons disease, and huntingtin aggregates in Huntington's diseases (Kuusisto et al., 2001b, 2002; Nagaoka et al., 2004; Nakano, et al., 2004; Zatloukal, et al., 2002).

C. A link Between Protein Aggregates and the Autophagosome

The murine p62 homolog, A170, was first characterized as an oxidative stress-related gene from mouse macrophages (Ishii et al., 1996), and the induction of A170 is a response of macrophages to mild oxidative stress, but severe oxidative stress reduced A170 expression (Ishii et al., 1997). Research from the same lab confirmed the

transcriptional regulation of A170 upon oxidative stress (Ishii et al., 1999). Moreover, A170 was phosphorylated in murine macrophages by two proteins of 40 and 44 kDa with kinase activity, which are similar to α and α' subunits of casein kinase II (CK II), suggesting A170 protein is not an antioxidant itself, but likely a modulator of signal transduction to induce cellular responses under oxidative stress (Yanagawa et al., 1997). Moreover, p62 overexpression was also found in breast tumors and regulated by Ets factor (Thompson et al., 2003), and P62/A170 was transcriptionally activated during the formation of the aggregates in Lewy bodies from Parkinson's disease (Nakaso et al., 2004). Increased p62 is also associated with polyglutamine inclusions (Nagaoko et al., 2004). Furthermore, p62 plays an important role in NGF-related signal transduction during the differentiation of neuronal cells (Wooten et al., 2001). These findings revealed that the mRNA and protein level of p62/A170 can be induced by signals for proliferation, differentiation and oxidative stress. However, little is known about why p62/A170 can be induced and what roles it plays against oxidative stress.

Upon oxidative stress, proteins can be oxidized by ROS and these oxidized proteins can be degraded by 20S or 26S proteasome or chaperone-mediated autophagy (CMA) in mammalian cells. Autophagy is an important mechanism to remove organells and long-lived protein aggregates formed in cells because of aging, oxidative stress, mutations, misfolding etc. However, it is not clear how the autophagic machinery recognizes such aggregates. P62 is a common component in protein aggregates found in various protein aggregation diseases (Zatloukal et al., 2002). Hyperphosphorylated tau, the major component of neurofibrillary tangles (NFTs), was accumulated in neurodegeneration due to genetic inactivation of p62 (Babu et al., 2008). Also,

K63-polyubiquitinated tau protein fails to be degraded by proteasome in p62 knockout mice (Babu et al., 2005). The decline in p62 may contribute to the accumulation of insoluble aggregated polyubiquitinated proteins. However, it has been demonstrated p62 can form cytoplasmic bodies with ubiquitin-containing protein aggregates by both PB1 and UBA domains, and recruits polyubiquitinated protein aggregates to autophagy machinery. P62 overexpression protects against cell death induced by overexpression of huntingtin (Nagaoko et al., 2004). P62 co-localizes with many types of polyubiquitinated protein aggregates, the autophagy marker LC3, and late endosomes/lysosome marker CD63. Furthermore, a recent study showed that p62 interacts directly with LC3 by 22 amino acids long LC3 interacting region (LIR) and p62 is required for the formation of aggresome-like induced structure (ALIS) (Bjørkøy et al., 2005). Also, p62-positive cytoplasmic bodies were degraded by autophagy (Pankiv et al., 2007). The loss of autophagy in the central nervous system of mice has been shown to result in accumulation of polyubiquitinated proteins in inclusion bodies (Komatsu et al., 2006). All of this evidence suggests that p62 may link the recognition of polyubiquitinated protein aggregates with the autophagosome. The increased p62 levels may increase aggregate formation by the PB1 domain, then degraded by autophagy, and this may be a protective response to oxidative stress.

Figure 1-1

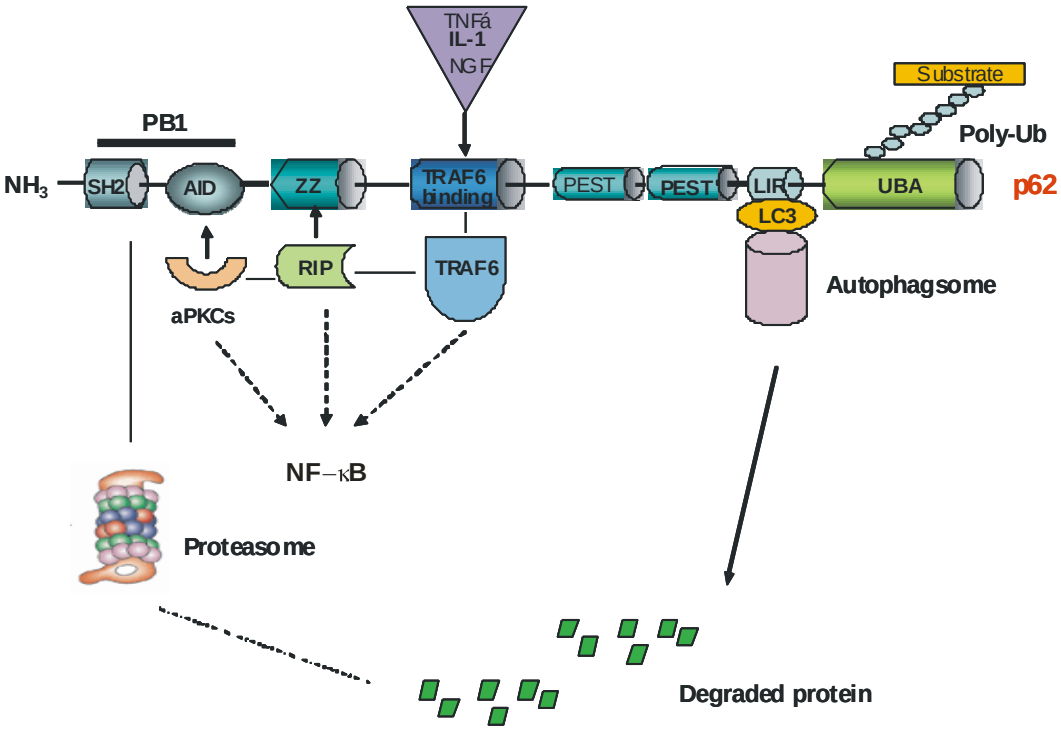


Figure 1-1. p62 structure and function. The primary structure of p62 possesses 6 motifs: PB1 domain including SH2 and AID motif at the N-teminal; a ZZ zinc finger act as a potential protein binding module; a binding site for TRAF6; two PEST sequences, and ubiquitin-associated (UBA) domain at its C-terminus. P62 serves as a scaffold with multiple motifs. Under stimulation of TNFα, IL-1 or NGF, aPKCζ-p62-TRAF6 complex activate NF-κB pathway. UBA domain of p62 can bind polyubiquitinated proteins and shuttle them to proteasome for degradation, and LIR interacts weith LC3 and mediate protein aggregates to autophagosome for drgradation.

D. Activator of the antioxidant response element

Recently, p62 was found as an activator of the antioxidant response element (ARE). It can activate the ARE and lead to up-regulation of ARE-dependent target genes with neuroprotective effects in primary mouse-derived cortical neurons (Liu et al., 2007).

3. The p62 Promoter

The genomic structure and promoter analysis of p62 was reported in 1998 (Vadlamusi and Shin, 1998). 20 kb long human *p62* gene was isolated and characterized. There are seven introns and eight exons in *p62* gene. About 1,800 nucleotides of 5'-flanking region of *p62* gene was cloned and analyzed. The 5'-flanking region of *p62* gene lacks both TATA and CAAT box, but conatins a G/C rich region immediately upstream of transcription initiation site. The 5'-flanking region from -950 to the first exon + 267, which is CpG dinucleotides rich, is defined as CpG island. The state of methylation of cytosine in 5'-CpG affects the relative activity of a promoter (Bird, 1986, 1992). Moreover, guanine (G) is sensitive to oxidative stress, but little is known about the effects of oxidative modified guanine on activity of a promoter. The luciferase assay of various lengths of the 5'-flanking region suggest that the basic region for p62 transcription locates within 357 nucleotides 5' of the transcription start site, and an enhancer region lies in the region -873 to -1408. Increased transcript and protein levels of p62 were observed during cell proliferation and differentiation, and oxidative stress (Lee, et al., 1998; Ishii, et al., 1996). A number of potential binding sites for known transcription factors are present in the p62 promoter region, revealing multiple regulatory features of the p62 promoter for responding to different signals. The basic region contains three Sp-1

sites, two AP-2 sites, one AP-1/TRE sites, and one PEA3 transcription factor binding site. The enhancer region has the binding sites for Ets-1 family transcription factors, including Ets-1a, Pu.1 and PEA3, as well as c-myc. There are binding sites for Ets-1b and PEA3 between enhancer and basic region. Upstream enhancer region includes the consensus sequences for AP-2/TRE, NF-B, PEA3/Pu.1 and Sp-1 binding. Sp-1 is a ubiquitous zinc finger containing transcription factor that binds GC/GT boxes and regulates the expression of several viral and cellular genes containing TATA-less promoters (Azizkhan et al., 1993; Kadanoga and Tijan 1986). Ets family members are nuclear targets of signal transduction (Wasylyk et al., 1998). Many Ets transcription factors have been shown linked to cancer by increased or decreased expression (Dittmer and Nordheim, 1998). Ets-1a element (-1175 to -1166) in enhancer region, not Ets1-b element (-516 to -509) within p62 promoter, binds prostate-derived Ets factor (PDEF) to activate p62 promoter in breast cancer cells in response to the proteasome inhibitor (PSI) (Thompson et al., 2003). PEA3 is induced by serum, EGF, v-src, H-ras etc. (D'Orazio et al., 1997); Pu.1 is involved in monocytic differentiation of bone marrow progenitor cells (Rosmarin et al., 1995). PEA3, Pu.1 and SP-1 all have been demonstrated to be overexpressed in cancer models. PEA3 is overexpressed in human breast cancer cells and mouse mammary tumors (Trimble et al., 1993; Benz et al., 1997), also maintains basal promoter activity of the cancer-related progression elevated gene-3 (PEG-3) (Su et al., 2000). Pu.1/SP-1 is overexpressed in erythroleukemia cell lines (Afrikanova et al., 2002) and involved in the development of myelomonocytes, B-cells, and macrophages (Nagamura-Inoue et al., 2001; Yamada et al., 2001). AP-1 and NF-κB are regulated by intracellular oxidation and reduction state (Sen and Packer, 1996). AP-1 binds to DNA sequence as homo- or

heterodimers of jun and fos (Angel and Karin, 1991). NF-κB regulates the expression of nuclear genes (Baeuerle and Baltimore, 1988). Thus, all of these cis-elements might be responsible for the regulation of the p62 gene upon signals for proliferation, differentiation, and oxidative stress.

Oxidative damage to promoter regions of many age-related genes has been observed. The mRNA and protein expression level of some vulnerable genes involved in learning, memory and neuronal survival are declined and the promoters of genes with reduced expression in aged cortex are selectively damaged by oxidative stress and show reduced base-excision DNA repair. These genes include calmodulin 1 (CaM1), Tau, Ca-ATPase, PKC, microtubule-associated protein 2 (MAP2), meiosis-specific serine/threonine protein kinase (MEK1) etc. (Lu et al., 2004). The promoter region is especially vulnerable to oxidative stress for the following reasons: 1) the promoter region usually has high G/C content that is very sensitive to oxidative DNA damage; and, 2) the damage to promoter region is not protected by transcription-coupled repair (Tu et al., 1996). Basically, a promoter containing many transcription factor binding sites located upstream -50 relative to the major transcription start site is repaired slowly, almost 50% damage are left unrepaired after 24 hours. However, 90% of damage between -40 to +100 are repaired in 4 hours, suggesting the rate of repair on both DNA strands increases dramatically near the transcription initiation site. For the transcribed DNA strand, a general gradient of repair efficiency with faster repair within the 5'-end and diminished repair towards the 3'-end of the gene was observed. Incomplete repair of lesions within transcription factor binding sites may easily lead to DNA mutations, consequently, resulting in changes in gene expression (Tu et al., 1996). It was demonstrated that the

oxidative modified DNA base, 8-OxodG, altered the interaction between *cis* element and sequence-specific transcription factor (Ghosh and Mitchell, 1999). The site-specific guanine residues in consensus binding sequences for transcription factors AP-1, Sp1 and NF-κB were replaced by 8-OxodG and eletrophoretic mobility shift assays (EMSA) were performed to examine the interaction between DNA sequence and transcription factors. Results indicated that a single 8-OxodG was sufficient to inhibit transcription factor binding to AP-1 and Sp1 sequences, but had no effect on binding to NF-κB, regardless of the position of 8-OxodG (Ghosh and Mitchell, 1999). Furthermore, the impaired transcriptional activity of mutated Parkin promoter by hydrogen peroxide was observed. The mutation of wild type T to variant G in Parkin promoter at -258 leads to the impairment of upregulation of transcriptional activity upon oxidative stress. Upon H_2O_2 treatment, the transcriptional upregulation of wild type Parkin promoter (-258T) was significantly higher than variant (-258G), suggesting guanine (G) is more sensitive to oxidative stress and individuals with -258G genotype may have a higher risk of developing Parkinson's disease (PD) (Tan et al., 2005).

4. Involvement of p62 in Neurodegenerative Diseases

Inclusion bodies (IB), which are formed from aggregating proteins, is a major pathological characteristic in various neurodegenerative diseases like Aβ plaque and tangles in AD, Lewy bodies in PD, Bunina bodies in Amyotrophic Lateral Sclerosis (ALS), and polyglutamine in Huntington's Disease (HD). Accumulating evidences indicated that p62 is a common component of various inclusion bodies (Zatloukal et al., 2002). Previous studies in our lab reveal that p62 specifically binds proteins with K63-polyubiquitin attached on its UBA domain, followed by shuttling of the ubiquitinated

protein to proteasome for degradation (Seibenhener et al., 2004). Increased accumulation of insoluble ubiquitinated proteins was detected in neurons of p62 knockout mice (Wooten et al., 2008). Early accumulation of p62 was found in neurofibrillary tangles in AD (Kuusisto et al., 2002). Depletion of p62 retards the degradation of polyubiquitinated tau protein (Babu et al., 2005) and genetic inactivation of p62 results in accumulation of hyperphosphorylated tau, suggesting the possible role of p62 in tangle formation (Babu et al., 2008). Ubiquitin, p62, and α-synuclein were detected in Lewy bodies in PD (Kuusisto et al., 2003). Also, the transcriptional activation of p62 was found during the aggregate formation after proteasome inhibitor treatment, proposing the possible mechanisms of Lewy body formation in PD (Nakaso et al., 2004). P62 was identified as a protein induced by expression of mutant huntingtin (Nagaoka et al., 2004), and was suggested to be involved in linking polyubiquitinated protein aggregates to the autophagy machinery (Bjørkøy et al., 2005). In addition, p62 was found in ubiquitin-immunoreactive intraneuronal inclusions in ALS with dementia (Nakano et al., 2004). p62 enhances aggregate formation in model systems of family ALS, and deletion of UBA domain of p62 dramatically decreased the p62-facilitated aggregate formation (Gal et al., 2007). P62 was also found in mallory bodies (MBs) in liver cells. Moreover, deficiency of p62 blocked the formation of MBs, whereas overexpression of p62 enhanced their formation, suggesting the role of p62 in MB formation may be similar to inclusion bodies in neurodegenerative disease (Nan et al., 2004). The roles of p62 in signaling, polyubiquitination, inclusion formation, and trafficking in neurodegenerative diseases have been well summrized (Wooten et al., 2006). Deletion of p62 showed significant neurodegeneration and AD-like phenotype such as increased anxiety, depression,

impaired learning and memory, and reduced serum brain-derived neurotrophic factor levels, indicating p62 plays important roles in neurodegenerative diseases (Wooten, et al., 2005; Babu et al., 2008).

PART II. OXIDATIVE STRESS

Reactive Oxygen Species (ROS)

The term of "Oxidative Stress" was named by Helmut Sies in 1985 (Sies, 1985). Oxidative stress means the damage to cells caused by free radical, which is defined as any atom or group of atoms with one or more unpaired electrons in its outer shell, which may enter into chemical-bond formation. In most molecules, there are two paired electrons within each orbital spinning in the opposite direction, which keep the molecule relatively stable and less reactive. However, when one or more electrons, especially within the outer orbital, is/are unpaired, the molecule becomes relatively unstable and more reactive with other molecules. Many of these molecular species are oxygen and sometimes nitrogen centered. The most common free radicals are formed from the reduction of molecular oxygen to water, and are typically referred to as reactive oxygen species (ROS). ROS are highly reactive and toxic molecules, and have come to be increasingly recognized for their importance to human health and disease. Under physiological conditions, low levels of ROS are present in cell and the cellular redox state is tightly regulated. Only when the generation of ROS increases and overwhelms the antioxidation defence ability, ROS cause toxicity. Oxidative damage is a major factor of injury in many common human diseases like cancer, neurodegenerative disease, aging,

and atherosclerosis. ROS includes molecules like hydrogen peroxide (H_2O_2), ions like the hypochlorite ion (OCl^-), free radicals like hydroxyl radicals ($OH^\bullet$) and superoxide anion ($O_2^{\bullet-}$). The structures of these radicals are shown in Fig. 1-2. The radicals can quickly react with other molecules or radicals to reach the stable configuration of 4 pairs of electrons in their outermost shell. This reaction changes not only the target molecule but also passes the unpaired electron along to the target to generate a second free radical or other ROS, which will react with a new target. Indeed, the high reactivity of ROS is caused by the generation of such molecular chain reactions, finally amplifying their effects many fold.

Figure 1-2

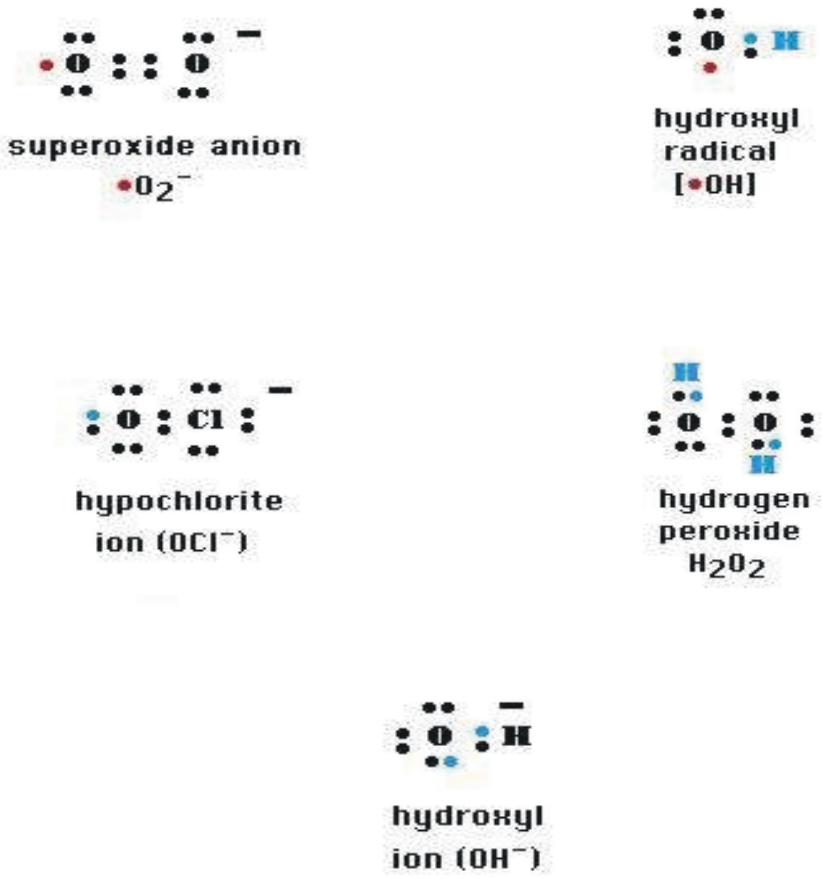


Figure 1-2. Structure of ROS. ROS include free radical, which is a molecule with an unpaired electron (shown in red) in its outer shell of electrons such as superoxide anion and hydroxyl radical; ions like hydroxyl ion and hypochlorite, and molecules like hydrogen peroxide.

ROS Formation

ROS can be generated from both external sources (sunlight, pollution, other forms of radiation, etc.) and endogenous sources (mitochondria, enzymes).

1. ROS Generated by Ionizing Radiation

Biological oxidants can come from environmental sources; for example, exposure to ultraviolet light and cigarette smoke. Radiation is an important cause of ROS. The most significant forms of radiation are light, heat, and ionizing radiation. Ionizing radiation can penetrate cells and create ions in the cell. Then, these can cause permanent alterations in DNA. Ionizing radiation includes X-rays, γ-rays, electrons ("beta" particles), neutrons, and alpha particles (helium nuclei). Actually, most radiation injury is not mediated by the radiation itself, but by the ROS generated by it. This causes not only the acute radiation injury, but also the long-term mutagenic effects, even leukemia and lymphomas years later.

2. Formation of ROS in the Mitochondria

ROS are physiological by-products of cell respiration and are generated as a result of normal metabolism. Oxidative phosphorylation is the major endogenous source of ROS. The production of mitochondrial $O_2^{\bullet-}$ is generated primarily at complex I (NADH dehydrogenase) and III (cytochrome bc_1 complex) of electron transport chain (ETC), also can be generated by components of tricarboxylic acid (TCA), pyruvate dehydrogenase, and α-ketoglutarate dehydrogenase in TCA cycle (Finkel and Holbrook, 2000, Starkov, et al 2004). The electrons accumulated in the early stages of the ETC (Complex I and CoQ) because of ETC inhibition are donated to molecular oxygen to produce superoxide anion

($O_2^{\bullet-}$). Superoxide anion is catalyzed by the mitochondria Mn^{2+}superoxide dismutase (MnSOD) to generate hydrogen peroxide (H_2O_2). Then, H_2O_2 is degraded to H_2O by glutathione peroxidase (GPx) and catalase. H_2O_2 can also be converted to the highly reactive hydroxyl radical ($OH^{\bullet}$) by the fenton reaction (Wallace, 1999). Reduced forms of iron and copper are two important transition-metal ions for the fenton reaction. They are able to cleave organic hydrogenperoxides, generating hydroxyl radicals that initiate a series of reactions, finally forming stable products such as lipid hydroxides (Dean et al, 1997). Superoxide anion can be formed on the outer mitochondrial membrane, in the matrix, and on both sides of the intermitochondrial membrane. Complex I generates $O_2^{\bullet-}$ only in the matrix of mitochondria. On the other hand, complex III releases $O_2^{\bullet-}$ toward both the intermembrane space and the matrix (Muller et al. 2004). α-Ketoglutarate dehydrogenase generates H_2O_2 in the mitochondria matrix (Starkov, et al. 2004). In addation, a large amount of H_2O_2 is generated by the outer mitochondrial membrane due to the deamination catalyzed by flavoprotein (monoamine oxidase), which is localized on the outer mitochondrial membrane (Han et al. 2003). The $O_2^{\bullet-}$ generated in the matrix is eliminated in that compartment, whereas the $O_2^{\bullet-}$ of the intermembrane space may be carried to the cytoplasm with anion channels (Han et al. 2003).

$$O_2 + e \rightarrow O_2^{\bullet-}$$

$$O_2^{\bullet-} + e + 2H^+ \rightarrow H_2O_2$$

$$H_2O_2 + e \rightarrow OH^{\bullet} + OH^-$$

$$OH^{\bullet} + OH^- + e \rightarrow H_2O + O_2$$

Another source of ROS is the modest leakage from the ETC of mitochondria, chloroplasts and endoplasmic reticulum (Dean et al., 1997). During the transfer of electrons to molecular oxygen, about 1% to 5% of electrons in the respiratory chain lose their way and participate in $O_2^{\bullet-}$ formation (Reddy and Beal, 2005). ROS exposure can inactivate the iron-sulfur (Fe-S) centers of ETC complexes I, II, III, and TCA cycle aconitase, resulting in declined mitochondrial energy production. Long term ROS exposure even leads to oxidative damage to mitochondria and cellular proteins, as well as, lipids and nucleic acids (Wallace, 1999).

3. ROS Produced Enzymatically

ROS usually can be synthesized by NADPH oxidases, which are located on the cell membrane of phagocytes-neutrophils, monocytes, and polymorphonuclear cells. Phagocytic cells can produce superoxide anion ($O_2^{\bullet-}$) by NADPH oxidase and most of the $O_2^{\bullet-}$ can form hydrogen peroxide (H_2O_2) with itself during their oxidative burst. Also, NADPH oxidase of endothelial cells is involved in signal transduction processes in angiogenesis and apoptosis (Babior, 2000b; Ray and Shan, 2005). In addition, a large number of ROS such as hypochlorous acid (HOCl), hydroxyl radical ($OH^{\bullet}$), and peroxynitrite ($ONOO^-$), are be formed by various enzymes. All of these ROS can not only kill invading microorganisms, but contribute to oxidative damage within tissues. They might be an important pathogenic factor in many diseases and chronic infections (Babior, 2000a). For example, HOCl can be produced by myeloperoxidase in neutrophils to kill micro-organisms (Aratani et al., 2006) and $O_2^{\bullet-}$ and H_2O_2 can be generated by xanthine oxidase to mediate ischemia-induced tissue injury (Yokoyama et al., 1990). The oxidation

of hypoxanthine is catalyzed by xanthine oxidase to produce xanthine and H_2O_2, and then to uric acid and H_2O_2.

Molecular Targets of ROS

Generally, the harmful effects of ROS on the cell are most often damage of DNA, oxidations of polydesaturated fatty acids in lipids, and oxidations of amino acids in proteins.

1. Protein Oxidation

Proteins are likely the most critical target of toxic damage, since protein is the most abundant, non-water component of biological system. Protein oxidative damage can be caused by radiation-induced radicals, metal-ion-catalyzed systems such as the Fenton reaction system, singlet oxygen and photochemical reactions, endogenous radicals that present on tyrosine, modified tyrosine, tryptophan, modified tryptophan, glycine or thiols, or radicals from other biological processes like malondialdehyde and 4-hydroxynonenal (4-HNE). Many radicals are electron-deficient, so they can react most rapidly with electron-rich side chains of amino acids like Trp, Tyr, His, Met, Cys, Phe, and Arg. In contrast, side chains of Ala, Asp, and Asn are least reactive. Much of the chemistry of products of protein oxidation and chemistry of protein oxidation have been illustrated by Davies and Dean (Dean, 1997). There are two kinds of oxidative modifications of proteins, reversible and irreversible modifications. Basically, the oxidative modifications to methionine and cysteine can be reversed by several antioxidant enzymes. The irreversible protein oxidative modification is the formation of carbonyls from amino acids, which is used as an index for assessing protein oxidation.

Protein oxidation might lead to rapid and severe effects. The oxidation might happen to both side chain and backbone of amino acid. Oxidation products of various amino acids and methods for measurment of these products have been elucidated by Davies (Davies, et al., 1999). The major products of aliphatic side-chain oxidation are hydroperoxides, alcohols amd carbonyl compounds in the presence of oxygen. The oxidation products of Arg are 5-hydroxy-2-aminovaleric acid and glutamic semialdehyde. The major oxidation products arising from Cys, Met are disulfides and oxyacids. Many specific products are formed from aromatic side-chain oxidation, like 3, 4-dihydroxyphenylalanine (DOPA) and di-tyrosine from tyrosine; m-tyrosine and dimers of hydroxylated species from Phe; N-formylkynurenine from Trp. Little is known about the oxidation products of Ser, Thr, Glu, Asp , Gln, and Asn, though it has been shown that alcohols and carbonyl-containing materials were formed. Three major methods have been developed to measure the oxidation products: measuring carbonyl group, HPLC and GC/MS. The advantages, disadvantages, and their applications were compared (Davies, et al., 1999). As for backbone oxidation, α–carbon radical oxidized by direct or indirect reaction is a common intermediate in most mechanisms. The reactive intermediates of protein oxidation can damage other targets like other proteins, DNA, and lipids.

Oxidative modifications of protein lead to many biochemical consequences such as formation of new species, DOPA; dimerization or aggregation, unfolding/ conformational changes, loss of structural/ functional activity, alternations in cellular handling/turnover, effects on gene regulation and expression, modulation of cell signalling, and induction of apoptosis and necrosis (Davies, 2005). Several proteins

have been identified that are oxidatively damaged in various diseases. For example, glutamine synthetase (GS) and creatine kinase (CK) are two enzymes that are particularly sensitive to oxidative modification in the neuronal system. GS and CK were oxidatively inhibited by Aβ (Aksenov et al., 1997; Yatin et al., 1999). Creatine kinase BB, β–tubulin, and β-actin were oxidatively modified in the brain in AD (Aksenov, et al., 2001). Butterfield's group indentified several proteins oxidatively damaged in mild cognitive inpairment (MCI) and AD. Enolase 1 (ENO1), Pyruvate kinase M2 (PKM2), peptidyl-prolyl cis/trans isomerase (PIN1), and GS were oxidatively damaged in hippocampus of MCI, and PIN1, dihydropyrimidinase-related protein-2 (DRP2), carbonic anhydrase II (CA II), phosphoglycerate mutase 1 (PGM 1), α-enolase, triose phosphate isomerase (TPI), gamma soluble NSF attachment protein (γ-SNAP), and ubiquitin carboxy terminal hydrolase L-1 (UCHL-1) were oxidatively modified in AD hippocampus (Butterfield et al., 2006; Sultana et al., 2006).

2. Lipid Oxidation

Lipid peroxidation is initiated with hydrogen atom abstraction by ROS such as hydroxyl ($^{\bullet}OH$), alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$) etc. from unsaturated fatty acid chains, followed by propagation, and termination stages (Catala, 2006). Major membrane bilayer component phospholipid unsaturated fatty acids (PUFAs) are particularly vulnerable to peroxidation because the double bond in the unsaturated fatty acid weakens the C-H bonds on the carbon atom close to the double bond, making the hydrogen atom more easily abstracted from methylene ($-CH_2-$) leaving an unpaired electron on the carbon ($-^{\bullet}CH-$). The lipid with $-^{\bullet}CH-$ is called lipid radical, which then reacts with molecular oxygen and abstract hydrogen atom from other fatty acid to

generate lipid hydroperoxide (LOOH) and second lipid radical (Catalá, 2006). The major compounds formed from lipid peroxidation are reactive hydroxy-alkenals like 4-hydroxy-2-nonenal (4-HNE). Hydroxy-alkenals from lipid oxidation is highly reactive although they are more stable than free radicals and exhibit some biological effects. 4-HNE can modify protein structure, regulate enzyme activity, work as a major signalling molecule, regulate mitochondrial uncoupling, and serve as subcellular messengers in gene regulatory and signal transduction pathways (Catalá, 2009). HNE is regarded as an index of oxidative stress. HNE has been shown to induce apoptosis in PC12 cells and cultured rat hippocampal neurons (Kruman et al., 1997), mediate Aβ toxicity (Uchida, 2003), induce differentiation (Friguet and Szweda, 1997), and damage proteasomal function (Ferrington and Kapphahn, 2004) suggesting that HNE is a mediator of oxidative stress-induced toxicity. Some other reactive aldehydes like acrolein, malondialdehyde, propanal, butanal, pentanal etc. were also identified (Esterbauer et al., 1991). All these reactive aldehydes can disperse from cells to other distant sites and attack other targets. Lipid peroxidation also leads to changes in the membrane structure, fluidity, and its physical properties like permeability, then affects membrane function (Catalá, 2009).

3. DNA Oxidative Damage

Oxidative damage to DNA *in vivo* is quite extensive. Hydroxyl radicals can transport across nuclear membrane to oxidatively modify DNA in the presence of metal ions. Both mitochondrial and nuclear DNA can be damaged by oxidative modification. MtDNA is more easily oxidized than nuclear DNA because high ROS production in mitochondria, deficiency of histone protection and limited repair capacities in mtDNA

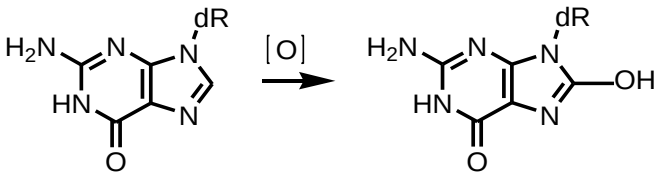
(Wallace 1992; Hashiguchi et al 2004). It has been estimated that one base is modified per 130,000 bases in nuclear DNA and per 8,000 bases in mitochondrial DNA (Richter et al., 1988). ROS can attack DNA bases, sugar, leading to strand breaks, DNA-DNA and DNA-protein cross-links (Nackerdien et al., 1991). The hot spots for free radical attack are C5, C6 in pyrimidines, the methyl in thymine, and C4, C5 and C8 in purine (Von Sonntag, 1991). So far, more than 20 oxidized base adducts have been found including 8-hydroxyl-2'-deoxyguanosine (8-OHdG), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (fapyguanine), 8-hydroxy-adenine, 4,6-diamino-5-formamidopyrimidine (fapyadenine) and 5-hydroxycytosine etc (Wang, et al. 2005). The most common oxidative lesion of thymine is the 5,6-dihydro-5,6-dihrdroxythymine (thymine glycol). The oxidative modification to DNA might cause mutations in genome. The 5-methyl oxidation products, 5-hydroxymethyluracil (5-HMU) and 5-formyluracil (5-fU) are formed by exposure to the gamma irradiation during Fenton reactions (Douki et al., 1996). The global conformation of the duplex with 5-HMU is normal, but 5-fU can cause T-C transitions and T-A transversions when analyzed in *E. coli*. (Miyabe et al., 2001). Oxidation of cytosine is a potent premutagenic lesion. Major oxidative modified product of cytosine, cytosine glycol, is highly unstable and can be deaminated readily to form uracil glycol (Wallace, 2002), which can be dehydrated to form 5-hydroxyuracil (5-OHU). Because both uracil glycol and 5-OHU pair with adenine, they might cause C-T transitions (Kreutzer & Essigmann, 1998). Two oxidative damaged products of adenine are 7, 8-dihydro-8-oxoadenie (8-oxoA) and 2-hydroxyadanine (2-OHA). Single 8-oxoA lesions have been found to cause A-G and A-C mutations in mammalian cells (Kamiya et al., 1995), and 2-OHA can cause A-G and A-T mutation when replicated in

E. coli (Kamiya and Kasai, 1997). Guanine is most senstive base to oxidative stress. The most common oxidized DNA base, 8-OHdG, is the most frequently studied lesion and serves as a marker of DNA oxidation (Kasai, et al., 1997). Hydroxyl or oxygen radicals react with deoxyguanine at C8-positions to form 8-hydroxyldeoxyguanine (8-OHdG). 8-OHdG can be formed both in absence and presence of oxygen, although the formation of 8-OHdG is favored in the presence of oxygen. This base modification occurs in about one in 10^5 deoxyguanidine residues in a normal cell (Valko et al, 2006). 8-OHdG can convert to the keto form, 7-hydroxy-8-oxo-2'-deoxyguanosine (8-OxodG), which is more stable and likely to accumulate in the genomic DNAs in nuclei and mitochondria during aging (Fig.1-3A). All these modifications might disrupt DNA replication, transcription, translation, and even cause mutations. Usually, deoxyguanosine adopts *anti* forms, but after oxidative modification, the *syn* conformation of 8-OxodG is favored energetically (Kouchakdjian, 1991). This *syn* conformation allows 8-OxodG to mispair with deoxyadenosine. Escaping from the DNA repair system, this mismatch results in G-T transversions (Pinz, 1995) (Fig.1-3B). Oxidative damage can be transferred from base to a sugar, and the 4' hydrogen on the sugar ring (ribose/deoxyribose) is very sensitive to ROS attack resulting in the collapse of the sugar, and single strand break. DNA-protein crosslinking (DPC) is produced by UV light or ROS. Usually the crosslinking takes place between base and aromatic amino acids in the protein by transferring electron and hydrogen (Peak et al., 1985). Increased DNA and RNA oxidative damage and decreased repair to damaged DNA have been found in many diseases including cancer, atherosclerosis, neurodegenerative diseases, and aging. Both DNA and RNA were found to be oxidatively damaged in

neurodegenerative diseases. 8-OHdG level in mitochondrial DNA was markly increased in spinal motorneurons of transgenic ALS mice, a neurodegenerative disease model (Warita et al., 2001). The oxidatively modified bases in both nuclear and mitochondrial DNA occur in the MCI, the earliest detectable phase of AD (Wang et al., 2006). The increased 8-OHdG level was found in the lymphocytes, ventricular cerebrospinal fluid (CSF), and brains from AD patients (Morocz et al., 2002; Lovell, et al., 1999; Gabbita, et al., 1998). RNA oxidation was also demonstrated in AD (Nunomura et al., 1999). Moreover, some evidence showed that 8-OHdG level was increased in Parkinson disease (PD) and Down's syndrome (Sato et al., 2005; Seidl et al., 1997). Besides neurodegenerative diseases, DNA oxidation was also found in breast cancer cells, young children with acute leukemia, and aging (Ruiz-Ramos, et al., 2009; Yang, et al., 2008; Kondo, et al., 2001; Wolf, et al., 2002).

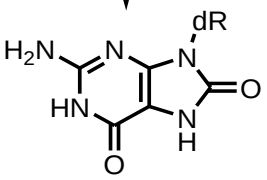
Figure 1-3

A



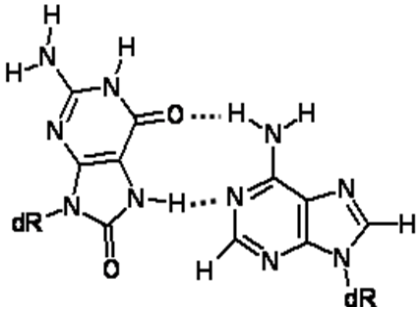
deoxyguanosine
(dG)

8-hydro-deoxyguanosine
(8-OHdG)

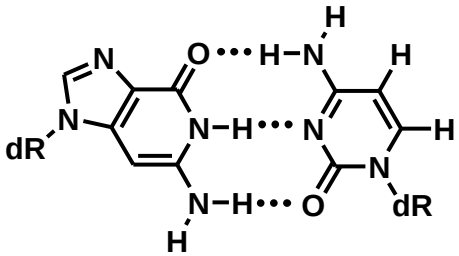


7-hydro-8-oxo-deoxyguanosine
(8-OxodG)

B



8-OxodG: dA



dG: dC

Figure 1-3. A. Formation and mispair of 8-OHdG. Deoxyguanine undergoes oxidation at 8-position to form 8-OHdG, which is the enol form. 8-OHdG can convert to the keto form, 8-OxodG, which is more stable and likely to accumulate in nuclear and mitochondrial DNAs during aging. B. In normal conditions, deoxyguanosine adopts *anti* forms and pairs with deoxycytosine, but after oxidative modification, the *syn* conformation of 8-OxodG is favored energetically. This *syn* conformation allows 8-OxodG to mispair with deoxyadenosine. Escaping from the DNA repair system, this mismatch results in G-T transversions.

Antioxidant System

Organisms are protected from oxidative stress by a series of defense mechanisms. Oxidative damage is also regarded as the imbalance between prooxidant and antioxidant in favor of the former. Davies has classified the oxidant defense into primary and secondary defense systems (Davies, 1986).

1. The Primary Defenses

A. Enzymatic antioxidant

The primary defenses include antioxidant compounds. Superoxide dismutases, glutathione peroxidase, and catalase are three major antioxidant enzymes.The best characterized antioxidant enzyme is superoxide dismutase (SOD). SODs dismutate $O_2^{\bullet-}$ into H_2O_2 and O_2. H_2O_2 is then reduced by catalases (CATs) into H_2O and O_2 to eliminate the superoxide-driven oxidative damage (Fig.1-4). Three kinds of SODs have been classified based on their amino acid sequence data. The manganese-containing SODs (MnSOD) are found in prokaryotes and in the mitochondria of eukaryotic cells. The iron-containing SODs (FeSOD) exist in prokaryotes and some plants. Mn/FeSOD all are comprised of dimers or tetramers of 21 kDa subunits with high sequence homology and conserved protein folds (Wintjens, 2004). A single manganese or iron atom bound at the active site within each subunit, serves to catalyze the disproportionation of superoxide to oxygen and hydrogen peroxide. The copper and zinc SODs (CuZnSOD) are present in the cytosol of eukaryotic cells and in chloroplasts. All these three SODs catalyze same reaction. The catalytic mechanism of CuZnSOD is the

alternate redox state of Cu (II) at the enzyme's active site during the interactions with superoxide (Tainer et al., 1983).

Glutathione peroxidases (Gpx) can reduce lipid hydroperoxides or H_2O_2 to their corresponding alcohols or water. An example reaction that glutathione peroxidase catalyzes is: $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$. There are 7 Gpx isozymes in cells, Gpx1 to Gpx7, encoded by different genes, with different celullar location and substrate specificity. They are capable of reducing a variety of organic and inorganic hydroperoxides to the hydroxy compounds using GSH or other reducing equivalents (Ursini et al, 1995). Gpx1 was found in the cytoplasm of almost all mammalian tissues and it prefers hydrogen peroxide as substrate (Muller, et al., 2007). Glutathione is the major intracellular thiol playing an important role in the maintenance of the intracellular redox state. Reduced glutathione (GSH) is monomer and oxidized glutathione (GSSG) is dimer. Glutathione-S-transferase (GST) catalyzes the conjugation of reduced glutathione with a sulfhydryl group to electrophilic centers on various substrates (Douglas, 1987). There are at least four classes of cytosolic dimeric isoenzymes in the mammalian GST super-family: Alpha, Mu , Pi and Theta (Wilce and Parker, 1994). Glutathione can directly remove free radicals or act as a substrate for Gpx and GST during the detoxification of hydrogen peroxide, lipid hydroperoxides, and phospholipid hydroperoxides.

CAT exists in almost all plants and animals and detoxifies H_2O_2 to form H_2O and O_2. The enzyme is a tetrameric molecule with four identical subunits, and each subunit is around 60 kDa (Nicholls, et al., 2001). In mammals, tissue distribution of CAT activity is in the order of liver, kidney, heart, and brain. Human serum CAT activity is markedly

increased in patients with HIV infection or adult respiratory distress syndrome (Leff er al., 1992a and 1992b), but CAT activity was decreased in an AD model carrying apolipoprotein E4 (Kharrazi, et al., 2008). Under "normal" oxidative stress, the production of intracellular H_2O_2 can be removed by Gpx. However, if the intracellular production of H_2O_2 is increased due to certain reasons, CAT is very critical to detoxify H_2O_2. Recently, several unexpected characteristices of CAT such as oxidase activity, resource of ROS exposed to ultraviolet light, and preventing substrate inactivation using unbound NADP(H) were reviewed (Kirkman, et al., 2006).

B. Nonenzymatic antioxidant

Although enzymes suppressing the formation of ROS work efficiently, free radicals are still formed *in vivo*. The radical-scavenging antioxidants can scavenge and stabilize free radicals before they attack target molecules. There are hydrophilic and lipophilic radical-scavenging antioxidants. Vitamin C, uric acid and bilirubin are hydrophilic, while Vitamin E, ubiqunol and carotenoids are lipophilic.

Vitamin E (α-tocopherol) is a fast lipid-soluble antioxidant found in cells. Vitamin E reacts with a variety of free radicals at high rates, producing the corresponding α-tocopherol radical by the donation of a hydrogen to a lipid or lipid peroxyl radicals. It was suggested that the long side chain of α-tocopherol, although necessary for incorporation in the membrane, decreases its activity in the membranes (Niki, 1999). Vitamin E deficiency happens in people unable to absorb fat properly or individuals with genetic abnormalities in the α-tocopherol transfer protein (Triantafillidis, et al., 1998; Cavalier, et al., 1998). Symptoms of vitamin E deficiency include muscle weakness,

Figure 1-4

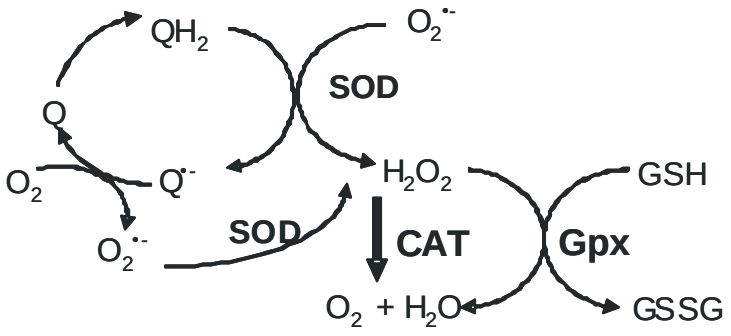


Figure 1-4. Role of antioxidant enzymes. Three major antioxidant enzymes are superoxide dismutases, glutathione peroxidase, and catalase. SODs dismutate $O_2^{\bullet-}$ into H_2O_2 and O_2. H_2O_2 is then reduced by catalases (CATs) into H_2O and O_2. Glutathione peroxidases (Gpx) can reduce H_2O_2 to water. Meanwhile, Gpx oxidize GSH to GSSG. Under "normal" oxidative stress, the production of intracellular H_2O_2 can be removed by Gpx. However, CAT is very critical to detoxify H_2O_2 when intracellular production of H_2O_2 is increased due to certain reasons.

impaired vision, unsteady gait and finally, kidney and liver function fail. Vitamin E has been shown to help protecting against a variety of chronic diseases associated with oxidative stress, including Alzheimer's disease, cardiovascular disease, cancer, stroke, cataract, diabetes, Parkinson's disease, and rheumatoid arthritis. Vitamin E can prevent or delay heart disease by limiting the oxidation of LDL-cholesterol (Jialal, 1995). Studies have shown the relationship between lower rates of heart disease with higher vitamin E intake (Knekt et al., 1994), whereas other studies showed that there is no benefits at all (Stampfer et al., 1993, Water, et al., 2002). The possible explanation for the contradictory findings is that many other nutrients besides vitamin E are necessary to promote a healthy heart because all antioxidants work in an antioxidant network. Some evidence is associated with higher intake of vitamin E with decreased incidence of prostate cancer, breast cancer (Chan et al., 1998), colon cancer (Bostick et al., 1993), bladder cancer (Jacobs et al., 2002), and lung cancer (Gackowski et al., 2005). Oxidative stress contributes to the development of AD and other neurodegenerative diseases; therefore, researchers hypothesize that vitamin E, as an antioxidant, could significantly lower the risk of developing AD. However, recent studies suggests that vitamin E does not slow progression of AD or mild cognitive impairment (MCI), and vitamin E has no significant effect on oxidative DNA damage measured by urinary 8-OHdG (Huang et al., 2000; Nocentini et al., 2001), but some evidence does reveal that high dose of vitamin E may result in some mild functional improvement. Vitamin E dietary supplementation was shown to prevent lymphocyte DNA damage following exercise or *ex vivo* H_2O_2 challenge in humans (Hartmann et al,1995; Panayiotidis et al., 1997).

Vitamin C (Ascorbic acid) is a very important and powerful hydrophilic antioxidant and is capable of scavenging aqueous radicals but can not scavenge radicals within the membranes or lipoproteins efficiently. Many studies suggest that vitamin C can reduce the radicals in aqueous solution or oxidation induced by aqueous radical such as in lungs and in the lens of the eye, but the rate of reduction decreases as the radicals go into the membrane (Gotoh et al., 1996). However, the most important feature of vitamin C involved in its antioxidant potency is its capacity of recovery of vitamin E radicals which is formed when vitamin E scavenges radicals (Kojo, 2004). Therefore, the primary antioxidant partner of vitamin C is vitamin E to protect against oxidation within the membranes. The majority of *in vivo* studies demonstrated a reduction in markers of oxidative DNA, lipid and protein damage after supplementation with vitamin C. It was reported that vitamin C protects membranes against oxidation (Retsky et al., 1999); long-term vitamin C supplementation at high dose (500 mg together with vitamin E) decreases the level of oxidative DNA damage in mononuclear blood cells of smokers (Moller et al., 2004). Some studies also suggest that vitamin C might reduce cancer risk. For example, high risk populations of gastric cancer have low serum levels of vitamin C (You et al., 2000); a consistent protective effect of vitamin C has also been found in lung and colorectal cancer (Knekt, 1991). Moreover, some studies showed that vitamin C can regulate factors that may influence gene expression, apoptosis and other cellular function. Vitamin C treatment of cells exposed to UV-B irradiation led to a 50% decrease in JNK phosphorylation, therefore inhibiting the JNK/AP-1 signalling pathways (Valko et al., 2006). However, there is no consistent efficacy for vitamin C and vitamin E in preventing

or treating AD (Boothby and Doering, 2005). Probably the combinations with other food components are needed for effective protection or they are not the key protective agents.

Carotenoids are pigments in plants and microorganisms. β-carotenoid can physically quench singlet oxygen without degradation, and react with free radicals such as $ROO^{\bullet}$, $OH^{\bullet}$, and $O2^{\bullet-}$ chemically. Studies indicated that β-carotene has an antiproliferative effect on various cancer cell lines, age-related muscular degeneration, and other diseases. Carotenoids may also regulate transcription factors (Sharoni et al., 2004). For example, treatment with β-carotene to cells exposed to oxidative stress showed suppressed activation of NF-κB and TNF-α inflammatory cytokines. Beta-carotene may also up-regulate anti-apoptotic protein Bcl-2 during the process of apoptosis in healthy cells, but reduce the expression of Bcl-2 in cancer cells, thus decreasing growth of cancer cells. Studies found that carotenoids show antioxidant ability at low oxygen or high carotenoid concentrations, but they will lose antioxidant ability, or even become pro-oxidant at high pressures of oxygen or low carotenoid concentrations (Kennedy, 1992).

Uric acid (UA) is the end product of purine metabolism in humans. UA is regarded as an efficient antioxidant due to its repair of glutathionyl radicals and reaction with $OH^{\bullet}$, and $O_2^{\bullet-}$. Monoanion urate (UH^-) can react with radicals ($R^{\bullet}$) by the general electron transfer ($UH^- + R^{\bullet} \rightarrow U^{\bullet} + RH$). Then, the radical form of uric acid ($U^{\bullet}$) decays to stable molecular products like allantoin, glyoxylate, and oxalate. UA can serve as an antioxidant in *in vitro* systems, isolated organs, and in the human lung (Becker, 1993). UA also converts metal ions, like iron and copper, to lower reactive forms which can not catalyze free-radical reactions (Miura et al., 1993). UA has shown neuroprotective effects after

experimental cerebral ischaemia (Yu et al., 1998). It was proposed that higher UA concentrations in humans help maintain normal blood pressure, thus providing a survival advantage under low dietary salt conditions (Watanabe et al., 2002). However, there are also some contradictory evdiences. For instance, Heinig argued that uric acid is a true risk factor for cardiovascular disease (Heinig & Johnson, 2006). This is probably because the free radical form of uric acid is quite reactive and has a longer life time than that of the radical initially scavenged. Vitamin C can repair the uric acid radicals to enhance the antioxidant properties of uric acid (Simic and Jovanovic, 1989).

Ubiquinone (Coenzyme Q10) is a lipid-soluble substance found in human body. Its presence is ubiquitous in nature and is an intermediate of electron transport system in the mitochondria. Ubiquinone also acts as an antioxidant by electron / hydride transfer reaction: $QH_2 + O_2^{\bullet-} \rightarrow Q^{\bullet-} + H_2O_2$ in both mitochondria and lipid membranes (Cadenas et al, 1992) (Fig.1-4). Ubiquinone is necessary for activation of mitochondrial uncoupling proteins, which can reduce mitochondrial free radical generation (Echtay et al., 2000, 2002). Increased mitochondrial uncoupling proteins protects against brain damage associated with stroke and epilepsy (Mattiasson et al., 2003). Moreover, ubiquinone can interact with vitamin E and reduce oxidized tocopherol and recover its reduced antioxidant form (Lass and Sohal, 1998). Many diseases linked to low level of ubiquinone including cardiovascular disease, PD, HD, muscular dystrophy, breast cancer, diabetes, and AIDS. Ubiquinone inhibits ischemia-induced neuronal injury in the hippocampus (Ostrowski, 2000). Declined ubiquinone level was found in PD patients and humans with aging (Shults et al., 1997; Edlund et al., 1992). All of these suggest that supplemental ubiquinone might be useful in the treatment of neurological disorders. To

date, many studies have reported the positive neuroprotection effects of ubiquinone in neurodegenerative diseases (Young, et al., 2007; Dhanasekaran, 2005). The potency of ubiquinone as a treatment to PD has been reviewed by Shults (Shults, 2005; Lewitt, 2006) and a phase II clinical trial of ubiquinone demonstrated a positive effect to decrease the development in PD (Shults et al., 2002).

2 The Secondary Defenses

The secondary defenses are repair enzymes to eliminate molecules, which are damaged by ROS and escaped the primary antioxidant defenses. These enzymes are responsible for repair of membrane phospholipids, proteins, and DNA.

A. Repair of oxidized phospholipid

Two enzymes are very critical to membrane phospholipid repair, phospholipase A2 and fatty acyl transferases. The repair reaction is as follows: fatty acyl hydroperoxides is the product of oxidation of membrane phospholipids. Fatty acyl hydroperoxides can be hydrolyzed by phospholipase A2 to produce a free fatty acid hydroperoxide and lysophospholipid. Then, the free fatty acid hydroperoxide is reduced by Gpx in the cytosol, and the lysophospholipid in the membrane is reacylated by the acyl-CoA/acyltransferase (Van Kuijk et al., 1987).

B. Repair and degradation of oxidized protein

The oxidative modified proteins tend to accumulate if they are not repaired or removed. Accumulation of oxidatively damaged proteins is one of the hallmarks in the aging process and neurodegenative diseases (Berlett and Stadtman, 1997; Friguet, 2006; Stadtman and Berlett, 1997). In eukarotic cells, oxidatively modified proteins can be

repaired by some enzymes, or degraded by the proteasome system and autophagy. Usually, the reversible oxidative modification can be reduced by several enzymes. Cysteine sulfenic acids and disulfide bridges can be reduced by thioredoxin/ thioredoxin reductase system and the glutaredoxin/glutathione/glutathione reductase system. Most oxidative modification of methionine occurs in the protein interaction domains and damage the interaction of the oxidized protein (Petropoulos & Friguet, 2006). Methionine sulfoxide reductase (Msr) repairs oxidized methionine back to methionine both *in vitro* and *in vivo* (Vogt, 1995; Sun et al., 1999). Msr activity and expression were reduced in various regions of the brains of Alzheimer's patients (Gabbita et al., 1999) and during aging (Petropoulos, 2001). This might explain the accumulation of non-repaired oxidized proteins in some age-related diseases.

Irreversible oxidative modification of protein is usually hydroxylation or carbonylation and selectively degraded by proteasome (Grune et al., 2003). Oxidized proteins often cause protein unfolding which is more proteolytic sensitive. 20S and 26S proteasomes all can degrade unfolded proteins in an ubiquitin-independent manner (Shringarpure et al., 2003). Additionally, the ubiquitin-26S proteasome pathway is also involved in the degradation of oxidized proteins from lens cells (Shang et al., 2001). Iwai proposed that lightly oxidized proteins can be ubiquitylated and degraded by the 26S proteasome, while heavily oxidized proteins causing unfolding may be degraded by the 20S or 26S proteasome without ubiquitination (Iwai, 2003). The impairment of the proteasome system in aging and neurodegenerative diseases has been documented by many studies (Diaz-hernandez et al., 2003; Grune et al., 2004). Decreased proteasome activity is sufficient to increase the levels of intracellular protein oxidation (Ding and

Keller, 2001). Consequently, the oxidized proteins lacking degradation damage the proteasomal pathway and promote further protein aggregates. Usually, lysosome was not considered to remove oxidized proteins because of its lack of selectivity. However, chaperone-mediated autophagy (CMA) was shown to be activated during oxidative stress recently (Kiffin et al., 2004), and deficiency of autophagy accumulates a higher level of oxidized proteins due to a lower degradation rate (Xiong et al., 2007). It was proposed that oxidative modifications make proteins unfold and expose hidden CMA-targeting motif, which is recognized by the cytosolic chaperone complex to activate CMA (Kaushik, 2006). Then, CMA substrates can be targeted to the lysosomal membrane where it interacts with the lysosomal membrane protein (LAMP) (Cuervo and Dice, 1996) through its targeting motif. Recent studies suggest that there is a cross-talk between proteasome and lysosome pathway. Proteasome impairment during aging and in age-related neurodegenerative causes the induction of autophagy and some genes important to regulating lysosomal activity (Keller et al., 2004; Ding et al., 2004) (Fig.1-5).

Oxidatively modified proteins can also be degraded by an ATP-stimulated Lon protease (Bota et al., 2002). It was indicated that Lon protease can degrade oxidized protein within the mitochondrial matrix to maintain mitochondrial structure and function (Bulteau, 2006). Interestingly, Lon protease is inactivated during aging (Delaval et al., 2004).

Figure 1-5

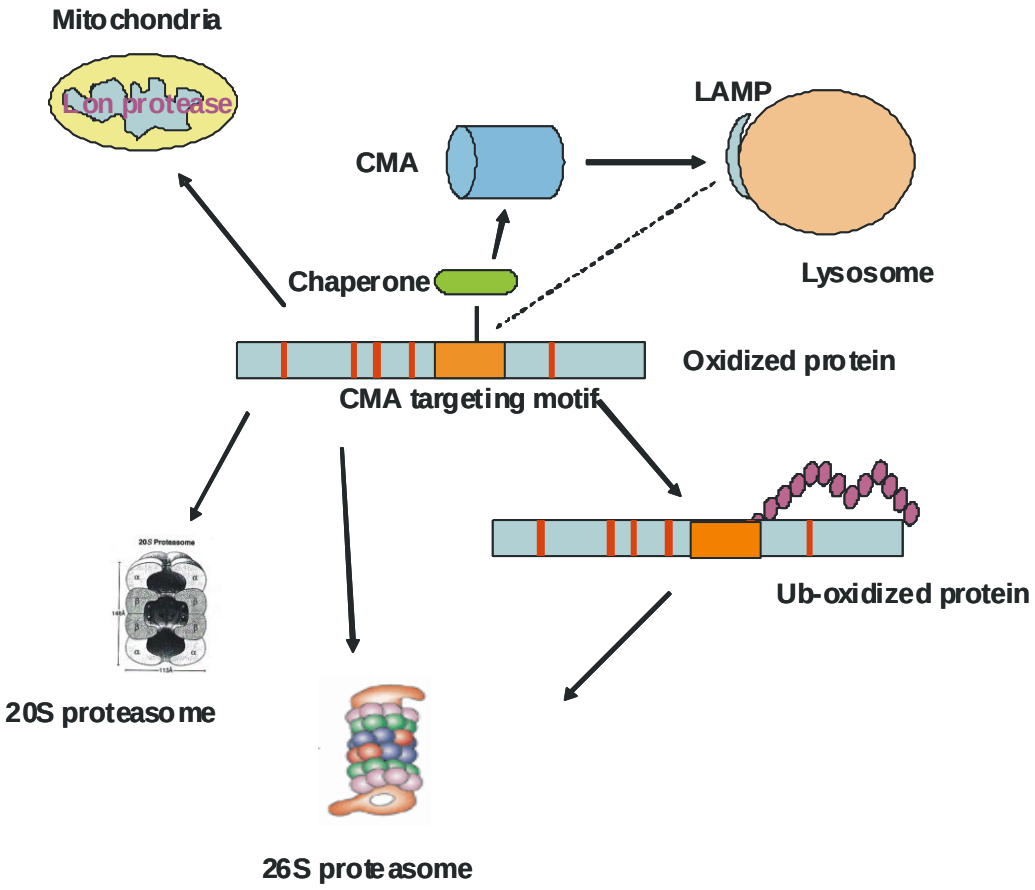


Figure 1-5. Degradation of oxidized protein. Oxidized protein can be degraded directly by 20S and 26S proteasome in an ubiquitin-independent pathway, or by 26S proteasome after ubiquitination. Exposed CMA-targeting motif in oxidized protein can be recognized by the cytosolic chaperone complex, then, activate chaperone-mediated autophagy (CMA). Oxidized protein can be targeted to the lysosomal membrane where it interacts with the lysosomal membrane protein (LAMP) by its targeting motif. Oxidative modified proteins can also be degraded by an ATP-stimulated Lon protease in mitochondria.

C. Repair of oxidatively modified DNA

DNA damage results in decreased cellular proliferation, genomic instability, and impaired gene expression. Long-term accumulation of these leads to aging, cancer, and other diseases such as neurodegenerative diseases. Organisms possess their own repair system to damaged DNA. Different types of DNA damage adopt a different repair pathway. Pyrimidine dimer caused by UV exposure and other bulky lesions are removed by the nucleotide excision repair (NER) pathway. Non-bulky lesions including oxidative modified bases, abasic sites, and strand breaks are repaired mainly by the base excision repair (BER) pathway.

8-OHdG is a major product of DNA oxidation and its keto conformation is 8-OxodG. *E. coli* has an evolved repair system to 8-OHdG consisting of three genes, mutT, mutM, and mutY (Michaels, 1992; Tajiri, 1995). Enzyme coded by mutT hydrolyzes 8-OxoGTP to 8-OxoGMP, preventing the incorporation of 8-OxoGTP into DNA during replication. mutM codes for formamidopyrmidine DNA glycolyase (Fpg), an enzyme that removes 8-OxodG from DNA when it paires with C. N-glycosylase coded by mutY, removes A mismatched with 8-OxodG. Inactivation of any of fpg and mutY genes results in G to T transversions (Cabrera et al., 1988). Fpg has both N-glycosylase and AP-lyase activities (O'connor and Laval, 1989). N-glycosylase of Fpg cleave damaged purines from DNA, genrating an apurinic (AP) site, then, the AP-lyase activity of Fpg remove AP site by β-elimination. The gap produced by hydrolysis of the phosphodiester linkage 5' to the AP site is repaired by the DNA polymerase and DNA ligase (Tchou and Grollman, 1993). Substrate specificity of Fpg protein has been studied by Tchou in 1994. They examined binding affinity of Fpg with different DNA duplex containing a single

defined oxidative modified base like 8-OxodG. Fpg protein can bind to the C8-keto group of 8-OxodG in the major groove of DNA duplex and cleave duplexes containing OxodG:dG and OxodG:dT more efficiently that duplexes conatining oxodG:dC, which is cleaved more efficiently than oxodG:dA. In *Drosophila melanogaster*, the ribsome S3 has both DNA glycosylase and AP lyase activity against 8-OxodG (Yacoub et al., 1996). In eukaryotes, the oxidative modification of deoxyguanine can be removed by the Ogg1 proteins, which is a functional analog of Fpg in eukaryotes with specific DNA glycosylases/AP lyases activities. Yeast OGG1 was isolated in 1996 (Van der Kemp et al., 1996). Mouse and human Ogg1 was cloned and characterized in 1997 (Thomas, 1997). A single Ogg1 gene in human cells (hOgg1) produces four isoforms: type Ia, Ib, Ic and II by alternative splicing (Arai, et al., 1997). Type Ia is involved in repair of 8OxodG in nuclear DNA, others in mitochondrial DNA repair. The human, mouse, and yeast OGG1 all have the helix-hairpin-helix motif that is a characteristic of DNA glycosylases family and highly conserved. A mechanism of repair was suggested that 8-oxodG can insert into the hOGG1 active site and structure of human OGG1 is an effective gate-keeping strategy for lesion discrimination (Banerjee et al, 2005). *E. coli* still possesses other enzymes that catalyze the oxidized and ring opened bases such as endonuclease VIII (Nei), 3-methyladenine DNA glycosylase II (AlkA), and endonucleases III (Nth). The oxidatively damaged thymine can be removed by a DNA glycosylase and Nei enzyme, and 5-fU paired with A is removed by AlkA. Mammalian Nth homologues have been cloned from mouse, bovine, and human. Human Nth is localized in both nucleus and mitocnohdria (Ikeda et al., 1998; Takao et al., 1998). Oxidized cytosine and uracil, 5-OHC and 5-OHU, also can be removed by Nth, Nei, and Fpg in *E. coli.* In mammalian cells, the enzyme to

remove oxidatively damaged thymine is human thymine-DNA glycosylase (hTDG). Oxidatively damaged 8-OxoA can be efficiently removed by yeast or human Ogg1 when paired with C. In contrast, the Fpg protein of *E. coli* cleaves 8-OxoA/C at a very slow rate compared with yeast Ogg1 (Girard et al., 1998).

PART III. OXIDATIVE DAMAGE, AGING, AND NEURODEGENERATUVE DISEASE

Neurons appear to be especially vulnerable to the attack by oxidative stress. Firstly, the brain processes 20% of basal O_2 consumption because of the high metabolic rate although the brain accounts for only 2% of the body weight. High O_2 consumption increases the opportunity for neurons to be attacked by ROS. A neuron needs to use ATP produced by mitochondria to maintain low gradients (high K^+, low Na^+). Therefore, inhibition of ATP synthesis can cause neuronal cell death (Beal et al., 1992). Nitric oxide ($NO^{\bullet}$) is another cause for oxidative stress in the nervous system. This highly diffusible free radical gas has been discovered as a neurotransmitter in CNS. $NO^{\bullet}$ is involved in neuron death and inhibition of NO production can block the neuron death occurring in stroke and neurodegenerative disorders such as PD (Yun et al., 1996). Although, normal NO production plays important roles in brain, but excessive NO can kill neurons. Thirdly, the important antioxidant, glutathione content is lower in neurons than in astroglial cells (Cooper, 1997) because of the limitation of cysteine uptake by neurons, which is the substrate for glutathione synthesis (Sagara et al., 1993). Deficiency of glutathione is involved in many neurodegenerative disorders associated with oxidative stress. Fourthly,

neuron membrane contains a high proportion of polyunsaturated fatty acids, catecholamines, and irons. Polyunsaturated fatty acids can react with free radicals to form peroxyl radicals resulting in damaged membrane. High level of iron leads to oxidative stress with the iron-catalysed formation of ROS. In addition, the catecholamine adrenaline can break down to free radicals (Singh et al., 2004). The last reason, but very important, is aging. Age is a key risk factor in neurodegenerative disorders because effects of attacks by free radicals can accumulate over years (Benzi and Moretti, 1995). Other identified risk factors like genetic and nongenetic reasons are also involved in aging and neurodegenerative diseases (Beal et al., 1997). Neurons are post-mitotic cells and can not be replaced as they die. Therefore, this can explain some aging and neurodegenerative diseases related to loss of function and death of neurons. Apoptosis is the major form of neuron cell death in neurodegenerative diseases. Oxidative stress is involved in the neuronal-cell death by apoptosis (Emerit et al., 2004). Also, mitochondria impairment caused by oxidative stress plays an important role in all neurodegenerative diseases (Beal, 1992).

The neurodegenerative disease is a processes based on certain genetic mutations, starting with biochemical changes, ultimately, leading to clinical characters and pathologies. Neurodegenerative diseases can be classified based on their genetic features as following: 1) single gene diseases such as HD; or 2) heterozygote gene diseases such as familial Alzheimer's disease (FAD), familial Parkinson's disease (FPD), and familial ALS. The onset of sporadic AD and PD, is later than familial forms and environmental contributions play a role. Oxidative stress is a major mechanism involved in the process

from gene to disease besides protein aggregates, proteasome dysfunction, mitochondria dysfunction and apoptosis.

AD is becoming more and more common in developed nations as the population includes more and more older persons. Some people develop AD as early as 30 or 40 years of age while others do not develop it until their late 70's or 80's. In familial AD, two mutations on the gene encoding amyloid precursor protein (APP) were identified: presenilin 1 (PSEN1) on chromosome 14 and presenilin 2 (PSEN2) on chromosome 1. In sporadic AD, the E4 variant of cholesterol transporter apolipoprotein E (apoE) increases deposition of fibrillar beta-amyloid. Mutations in the *tau* gene which codes for tau, a protein that is associated with microtubules, can be found in some AD cases. The abnormal tau accounts for helical filaments found in neurofibrillary tangles (Nussbaum et al., 2003; Tanzi and Bertram, 2001). The pathologic hallmarks of AD are neuritic plaques, which are composed of amyloid-β, and neurofibrillary tangles, which are composed of hyperphosphorylation of tau protein. Since the number of plaques increases with age, the number needed for diagnosis of AD is age-dependent. AD brain is under extensive oxidative stress, including protein oxidation, lipid peroxidation, and DNA oxidative damage (Markesbery, 1997). Proteomic identification of many oxidative modified proteins in AD brains, mild cognitive impairment (MCI), and ApoE-knockout mice model has been reported by Butterfield group and Markesbery group (Butterfield et al., 2006; Castegna et al., 2002; Sultana et al., 2005; Choi et al., 2004; Aksenov et al., 2001). All of these proteins include β-actin, creatine kinase BB (CKBB), glutamine synthase (GS), ubiquitin carboxy-terminal hydrolase-1 (UCHL-1), peptidyl prolyl *cis-trans* isomerase (Pin1), dihydropyrimidinase-related protein-2 (DRP2), carbonic

anhydrase II (CA II), phosphoglycerate mutase 1 (PGM1), enolase 1 (ENO1), pyruvate kinase M2 (PKM2), triose phosphate isomerase (TP1), gamma soluble NSF attachment protein (γ-SNAP), chaperonin subunit 5, mortalin, and glial fibrillary acidic protein. Several studies have already shown an increase in lipid peroxidation in AD brain. Aldehyde and 4-HNE produced by oxidation of PUFA in brain membrane were increased in several regions of AD brain (Subburao et al., 1990; Markesbery et al., 2005; Pamplona et al., 2005). Both nuclear and mitochondria DNA can be oxidatively damaged in MCI (Wang et al., 2006). RNA oxidation was also demonstrated in AD (Nunomura et al., 1999). Elevated levels of 8-OHdG, the marker of oxidative DNA damage, was found in the lymphocytes, ventricular cerebrospinal fluid (CSF), and brains from AD patients (Morocz et al., 2002; Lovell, et al., 1999; Gabbita, et al., 1998). Oxidative damage in AD may result from mitochondria dysfunction or peptide Aβ (Christen, 2000; Butterfield et al., 2001; Butterfield and Lauderback, 2002; Hensley et al., 1994). Aβ was demonstrated to produce H_2O_2 in cultured cells by metal ion reduction and H_2O_2 mediated Aβ toxicity (Huang et al., 1999; Behl et al., 1994). On the other hand, oxidative DNA damage in MCI and AD were also caused by impairment of the oxidized DNA repair enzyme, OGG1. Significant BER dysfunction in AD brains resulting from reduced OGG1 activity was found (Weissman et al., 2007). Recently, OGG1 activity was shown to be significantly decreased in nuclear specimens from AD and MCI, but there is no difference in mitochondria OGG1 protein. Also, this decreased OGG1 activity occurs early in the progression of AD, contributing to increased 8-OHdG level in AD and MCI (Shao et al., 2008).

Most cases of PD are sporadic. Patients usually present with movement problems like slowness of voluntary movement, rigidity, as well as cognitive deficits. Two mutations were identified to be involved in rare familial forms of PD: an autosomal dominant form with mutations in the alpha-synuclein gene and an autosomal recessive form with mutations in the ubiquitin-protein ligase (parkin) gene (Nussbaum and Ellis, 2003). Neuronal degeneration is caused by the loss of the pigmented neurons in the substantia nigra. Pathological hallmark is Lewy bodies in the cerebral cortex, as well as the substantia nigra. Inhibition of mitochondrial complex I by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) leads to ATP depletion and ROS generation (Rosenberg, 2002). A growing body of evidence supports the oxidative stress in PD. For example, accumulating protein carbonyls, which is protein oxidation product and 4-HNE, which is lipid peroxidation product, were found in PD brain (Alam et al., 1997; Yoritaka et al., 1996). The oxidative stress might be a result of defects of UPS and decreased antioxidant in PD. Impaired UPS might induce oxidative stress relevant to neuron cell death in PD (McNaught and Jenner, 2001), and decreased antioxidant enzyme activity has been reported in PD (Spina and Cohen, 1989). Oxidative damage in nucleic acid in PD had been well reviewed by Nakabeppu recently (Nakabeppu et al., 2007). They found significant increase in 8-OHdG as well as the repair enzymes may cause dopamine neuron loss.

HD is an autosomal dominant disorder that affects individuals between the ages of 20 and 50 years. Patients have abnormal body movement and lack of coordination. The abnormal gene encoding huntingtin protein is located on chromosome 4 and contains increased trinucleotide CAG repeat sequences. The large number of CAG repeats are

expressed as an elongated huntingtin protein (Martin, 1999), which lead to neuronal loss in the striatum and cortex (DiFiglia et al., 1997). Mechanisms of neurodegeneration in HD have been summarized (Gil and Rego, 2008). Mitochondrial dysfunction is the major reason for generation of ROS in HD. Elevated leukocyte 8-OHdG level and plasma malondialdehyde in peripheral tissue of HD have been observed (Chen et al., 2007). Six proteins were significantly oxidized in HD mouse model, R6/2 transgenic mice. These proteins include α-enolase, γ-enolase, creatine kinase (CK), voltage dependent anion channel (V-DAC1), heat shock protein 90 (Hsp90), and aconitase (Perluigi et al., 2005). Among them, aconitase, γ-enolase, and CK are also found oxidized in striatum from HD. In addition, the increased carbonyl levels of glial fibrillary acidic protein (GFAP), which is expressed in proliferated astrocytes, a major marker of atrophy of the striatum and cortex in HD, was identified (Sorolla et al., 2008; Eng et al., 2000). Decreased activities of SOD1, Gpx, and catalase were also reported (Chen et al., 2007; del Hoyo et al., 2006).

ALS is characterized by the loss of motor neurons caused by a mutation of Cu-Zn SOD (SOD1). Males are affected more frequently than females. Around 20% of familial ALS are caused by mutations in SOD1. The transgenic mice carrying mutant SOD1 gene can develop ALS, but $SOD^{-/-}$ mouse can not develop ALS suggesting mutant SOD is the possible cause in ALS (Boillee et al., 2006). Because mutant SOD1 is more easily denatured to release zinc ions and the pro-oxidant copper ion than normal form *in vivo*, oxidative stress in ALS may be related to impaired ability of mutant SOD1 to bind zinc (Emerit et al., 2004). Increased 8-OHdG level and impaired mitochondrial hOGG1, oxidative DNA repair enzyme, were found in the spinal motor neurons of ALS (Kikuchi et al., 2002).

Frontotemporal Dementia (FTD) is a neurodegenerative disorders with neuronal loss, gliosis in the frontal and anterior temporal cortex (Brun, 1987). Pick's disease is a subset of FTD sharing some characteristics such as atrophy of the frontal and anterior temporal cortex, associated with neuronal loss and gliosis (Hauw et al., 1996; Brun, 1993). However, pathological characteristics of FTD contain ubiquitin-positive, tau-negative inclusions (Bergmann et al., 1996), while the key marker of Pick's disease is a Pick body, which is composed of hyper-phosphorylated tau deposits (Brun, 1993). To date, there is little evidence revealing elevated 8-OHdG levels in either FTD or Picks Disease, but mitochondrial DNA damage has been identified in these two neurodegenerative diseases (Su et al., 2000; Mawrin et al., 2004). Also, like in HD, GFAP was found to be a major target of oxidation in Pick's Disease (Muntané et al., 2006).

"Free radical theory" of aging proposed by Harman more than 50 years ago suggested that endogenous free radicals generated in cells accumulate with age and could be attributed to deleterious effects on cell components (Harman, 1956). Although the concept of endogenous free radicals remained controversial at beginning, the discovery of SOD, which removes superoxide anions, gives a strong support to this theory (McCord and Fridovich, 1969). The age-related increase in products of lipid peroxidation, protein oxidation, and DNA oxidative modification in both human and experimental animals have been well-documented by Droge (Droge and Schipper, 2007). They also propose putative mechanisms accounting for the elevated age-related oxidative damage: 1) age-related decline in antioxidant enzyme activity such as Gpx, SOD; 2) oxidative mitochondrial damage leading to increased ROS production; 3) dysregulation of iron and

calcium homeostasis; and, 4) age-related decrease in plasma cysteine concentrate, which is used for GSH biosynthesis. Besides all these reasons, oxidative stress also might result from impaired removal of oxidized protein during aging (Friguet, 2006). Age-related impairment of proteasome has been found in various organs or cell types (Friguet et al., 2000). In addition, age-related declines of both Lon gene and protein, which is used to degrade oxidized protein in mitochondria, have been indicated in mouse skeletal muscle (Bulteau et al., 2006). Moreover, age-related decline of DNA repair capacity causing accumulated DNA oxidative damage with age has been documented (Chen et al., 2007). This study also suggested that DNA damage leads to cellular senescence or apoptosis, which results in disrupted tissue structure and depleted tissue stem cell, finally organ function and aging (Chen et al., 2007). It was proposed that increasing oxidative damage to mitochondrial DNA leads to impaired mitochondrial function and integrity, which in turn release more ROS resulting in more DNA damage (Finkel and Holbrook, 2000). Findings from an extensive study on genes from aged humans reveal that there exist a set of genes whose promoters are selectively damaged in an age-dependent manner resulting in reduced expression (Lu et al., 2004). This project focuses on investigating the oxidative damage to p62 promoter, the mechanism whereby damage to the promoter may regulate p62 expression, and to establish the relationship among oxidative damage within p62 promoter, aging, and neurodegenerative disease.

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*CHAPTER II. AGE –ASSOCIATED OXIDATIVE DAMAGE TO THE P62 PROMOTER: IMPLICATIONS FOR ALZHEIMER'S DISEASE

ABSTRACT

Absence of the p62 gene in mouse brain leads to biochemical and cognitive deficits that resemble Alzheimer's disease (AD). In this context, the objective of this study was to examine the age-associated oxidative damage to the p62 promoter in AD. Increased 8-OHdG staining, a marker of oxidative stress, was observed in brain sections from mice deficient in the p62 gene compared to control. Treatment of MEF cells deficient in p62 with H_2O_2 resulted in decreased cell survival and an absence of Nrf2 nuclear translocation. The mouse p62 promoter exhibited elevated oxidative damage with increasing age and the degree of p62 promoter damage was also age-correlated in human brain samples. In human subjects, the expression of p62 was decreased in AD brain relative to age-matched controls, and likewise decreased p62 expression correlated with oxidative damage to the promoter. Treatment of HEK cells with H_2O_2 resulted in decreased p62 expression concomitant with increased promoter damage. Consistent with these findings, a transgenic AD mouse model also exhibited increased p62 promoter

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damage and reduced p62 levels in brain. Altogether, our results reveal that oxidative damage to the p62 promoter correlates with decreased expression of p62 and may contribute to age-associated neurodegenerative disease such as AD and others.

INTRODUCTION

Oxidative stress is the result of imbalance between productions of reactive oxygen species (ROS) and a biological system's ability to detoxify the reactive intermediates or repair the oxidative damage. Low levels of ROS can act as signaling molecules in various intracellular processes (Scherz-Shouval and Elazar, 2007). However, when the ROS level overwhelms the antioxidant and repair system, cells might be damaged by these molecules. Progressive and irreversible accumulation of oxidative damage contributes to the aging process, and is thus implicated in development of several age-associated diseases, such as neurodegenerative diseases (Filipcik et al., 2006). Oxidative damage to DNA is particularly harmful since it may cause mutations that can be inherited by the next generation, leading to genome instability. Among the five nucleobases, guanine is the most susceptible to oxidation because of its high electron density (Steenken, 1989).

The predominant DNA oxidative adduct is 8-hydroxydeoxyguanosine (8-OHdG), which serves as a common biomarker of DNA oxidative damage (Steenken, 1989; Kasai, 1997). Most 8-OHdG lesions are repaired by a base excision repair (BER) pathway. It has been reported that when the ability of the repair system declines, cumulative oxidative DNA damage in the mitochondria and nuclear genomes of neurons may play a critical role in brain aging and neurodegenerative disorders such as AD, Parkinson’s disease and

Amyotrophic Lateral Sclerosis (Markesbery and Lovell, 2006; Nakabeppu et al., 2007; Warita et al., 2001).

Relationships have been established among increased DNA damage, defective DNA repair, aging, and age-associated neurodegenerative disease (Lovell and Markesbery, 2007; Kikuchi et al., 2002; Martien and Abbadie, 2007). However, the exact mechanism by which oxidative DNA damage lead to neurodegeneration or neuronal cell death still remains obscure. Human p62 is oxidatively-induced in human and mouse cell lines (Ishii et al., 1997), and the p62 protein has been localized to aggresomes of various neurodegenerative diseases (Zatloukal et al., 2002). This gene was first identified by Park et al., as the ligand for the $p56^{lck}$ (Park et al.,2002), and referred to as Sequestosome 1/SQSTM1. In humans, variants of p62/SQSTM1 have been linked to Pagets Disease of Bone (Layfield and Hocking, 2004). In mouse, the gene is related to A170/STAP (Signal Transduction Adapter Protein (Okazaki et al., 1999); whereas in rat, the gene is referred to as ZIP, the zeta interacting partner of the atypical protein kinase C (Puls et al., 1997). The p62 protein contains several interaction motifs that endow the protein with scaffolding abilities (Moscat et al., 2007). At its C-terminal tail p62 possesses an ubiquitin associated domain that interacts with K63 polyubiquitin chains and the N-terminus possesses a PB1 domain. A ZZ-finger recruits the atypical PKC and other proteins, whereas the TRAF6 binding site (TBS) recruits the E3 ubiquitin ligase TRAF6. Functionally, p62 serves to connect signaling pathways associated with two post-translational modifications. An absence of p62 leads to the loss of aggresomes and neuronal cell death (Nakaso et al., 2004). Moreover, p62 has been reported to activate the

antioxidant response element (ARE) and protect cells from oxidative stress (Liu et al., 2007). We reported that p62 deficiency in mice results in an AD-like phenotype (Babu et al., 2008). These mice display age-dependent neurofibrillary tangles (NFTs), memory deficits, loss of synaptic plasticity, and accumulation of polyubiquitin.

Age is one of the main risk factors for AD (Droge and Schipper, 2007). Findings from an extensive study on transcription profiling of brain from aged humans reveals that there exist a set of genes whose promoters are selectively damaged in an age-dependent fashion resulting in reduced expression (Lu et al., 2004). The majority of these genes play a role in synaptic plasticity, vesicular transport and mitochondrial function, similar to the reported functional role for p62 (Moscat et al., 2007). The aims of this study was: 1) to determine if p62 promoter was damaged in an age-dependent manner in humans and mice; 2) to examine p62 expression in human brain from late-stage AD individuals; and, 3) to examine the relationship between p62 promoter damage and p62 expression. Altogether, these findings reveal that reduced p62 may be an additional risk factor for AD and other neurodegenerative diseases.

MATERIALS AND METHODS

GenBank Information

Human p62: Sequestsome 1 (SQSTM1), Gene bank #: BC019111.1 (Park et al., 1995). Mouse p62: A170/ STAP, Gene bank #: BC006019.1 (Okazaki et al., 1999). Rat p62: ZIP, Gene bank #: BC061575 (Puls, et al, 1997).

Human brain samples and cell lines

Post mortem samples of frontal cortex derived from normal human adult without neurological disease were provided by Dr. Steven Carroll at the University of Alabama Birmingham, Department of Pathology, Birmingham, AL. 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 criteria for the neuropathologic diagnosis of AD (Mirra et al., 1991; NIA-Reagan Institute, 1997). Additional control samples were obtained from the Harvard Brain Tissue Resource Center, McClean Hospital, Boston, MA. The control samples used in this study had no clinical history of neurological disease and were neuropathologically normal (Table 2-1). WT and p62 $^{-/-}$ Mouse Embryo Fibroblast (MEF) cells were obtained from Dr. Jorge Moscat, University of Cincinnati, Cincinnati, OH. A triple transgenic mouse model of AD (3xTg-AD) was provided by Dr. Frank Laferla, UCLA, Los Angeles, CA. Knock-out mice (p62 $^{-/-}$) were generated as described previously (Rodriguez et al., 2006). All animals employed in this study were handled according to the Auburn University Institutional Animal Care and Use Committee, which abides by National Institutes of Health guidelines.

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 the following modifications to minimize *ex vivo* oxidation artifacts. All buffers were purged with nitrogen and supplemented with 50 μM phenyl-tert-butyl nitrone (PBN) (Sigma, St.

Louis, MO). The high temperature incubation was replaced by 4 hours incubation at 37°C. Following elution with ddH_2O, purified DNA was stored at -80°C.

Oxidative DNA damage assay

The DNA damage assay was developed by Lu et al. (Lu et al., 2004). Quantitative real time PCR was employed to determine the intact DNA level of specific amplicons within both the human and mouse p62 promoter. 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 min. 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

Clone N45.1 (JaICA, Tokyo, Japan), which specifically recognizes 8-OHdG was used for immunostaining (Akatsuka et al, 2006; Ihara et al., 1999). After deparaffinization with Xylene, WT and p62 $^{-/-}$ brain sections (5 microns) were hydrated through graded ethanol. Endogenous peroxidase activity in the tissue was eliminated by incubation with 3% H_2O_2 in methanol for 15 min. The sections were treated with 20 µg/ml proteinase K for 45 min at 37°C to digest proteins and nonspecific binding sites were blocked by adding the mouse Ig blocking reagent in the MOM kit (Vector Lab, Burlingame, CA) for 4 hours. The sections were incubated with 7.5 µg/ ml anti-8-OHdG monoclonal antibody, N45.1 in MOM diluent overnight at 4°C, followed by addition of biotinylated anti-mouse IgG reagent. No primary antibody control was also performed. Next, ABC reagent was used to enhance the signal and immunostaining was developed by DAB for 3-5 min. Quantification of 8-OHdG was conducted employing the Gel and Graph Digital software (Silk Scientific Corporation, Orem, Utah). Five different regions were taken from WT sections with corresponding regions examined from p62 $^{-/-}$ sections to quantify the amount of 8-OHdG per section.

Western blotting

Brain tissue was homogenized in 1 ml/gm of ice-cold 1 M sucrose in 0.1 M MES, 1 mM EDTA, 0.5 mM $MgSO_4$, pH 7, and centrifuged at 50,000 X g for 20 minutes at 4°C. Cytoplasmic fraction was collected from WT and p62 $^{-/-}$ MEF cells using Nuclear Extract Kit (Active Motif, Carlsbad, CA). 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 two min. and exposed to ECL film. Gel and Graph Digital software (Silk Scientific Corporation, Orem, Utah) was used to scan and quantify the signal.

Cell viability assay

WT and p62 $^{-/-}$ MEF cells were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum at 37^{o}C in a 5% CO_2 humidified incubator. WT and p62 $^{-/-}$ MEF cells were seed in 96-well plate at 10,000 cells per well. After 48 h, the cells were treated with various doses of H_2O_2 for 18 h (Liu et al., 2007). Each dose was tested in duplicate and the experiment replicated four independent times. Thereafter, cell viability was measured using CellTiter-Glo (Promega, Madison, WI).

Statistical analyses

Possible differences among group means and statistical relationships between the level of p62 expression and p62 promoter damage were analyzed using t-tests, ANOVAs and correlation analyses (SAS v9.1, SAS Institute Inc., Cary, NC, U.S.A.). For significant differences, global alpha was set at 0.05. Adjustments for multiple comparisons were derived using step down Sidak procedures (t-tests) or Tukey's studentized range tests (ANOVAs).

RESULTS

Absence of p62 results in oxidative stress

The predominant DNA oxidative adduct, 8-OHdG, which is induced by ROS, serves as the biomarker for oxidative DNA damage (Steenken, 1989; Kasai, 1997). The degree of oxidative damage was examined by analyzing matched brain sections from WT and p62 $^{-/-}$ mice stained with 8-OHdG at the same time. Since 6-month-old p62 $^{-/-}$ mice exhibit an AD phenotype (Okazaki et al., 1999), the levels of 8-OHdG in WT and p62 $^{-/-}$ brain sections were examined by immunostaining with a monoclonal antibody specific for 8-OHdG (N45.1) at 2-, 6-, and 12-months- of age (Ihara et al., 1999). Immunoreactivity for 8-OHdG was present in both cortical and hippocampal neurons, although hippocampal neurons in the CA1 to CA3 region of p62 $^{-/-}$ brain were more intensely stained (Figure 2-1A). Absence of primary antibody did not yield any immunoreactivity. Both WT and p62 $^{-/-}$ mice displayed elevated levels of 8-OHdG with increasing ages and sections from p62 $^{-/-}$ brain possessed more intensive 8-OHdG reactive neurons, which was more apparent by 12 mos of age. Quantitative evaluation of 8-OHdG intensity was made by scanning the same unit area from each stained section of WT and p62 $^{-/-}$ mouse brain (Figure 2-1B). These findings confirmed that both WT and p62 $^{-/-}$ mice displayed significant age-dependent elevated levels of 8-OHdG. Moreover, 8-OHdG levels in p62 $^{-/-}$ mouse brain sections were significantly higher than those in age-matched WT mouse brain sections. The 12- month-old p62 $^{-/-}$ mouse brain sections possessed a significant increase in DNA oxidative damage compared to sections from either 2- or 6-month old brain, revealing that loss of p62 enhanced oxidative stress in brain.

What is the consequence in this loss of p62? P62 activates the antioxidant response element and confers resistance to toxicity induced by oxidative stress (Liu et al., 2007). In order to investigate whether p62 has the ability to protect cells from oxidative insult, WT and p62 $^{-/-}$ MEF cells were treated with H_2O_2 for 18 h and cell viability was measured. Deficiency of p62 significantly decreased cell survival in response to H_2O_2 treatment, particularly at higher doses (Figure 2-2A). The Nrf2 transcription factor regulates expression of the antioxidant response element by translocation to the nucleus. In mice deficient in p62, Nrf2 nuclear translocation was low (Komatsu et al., 2007), furthermore, decreased nuclear Nrf2 levels have been observed in neurons from AD brain (Ramsey et al., 2007). Treatment of WT MEF cells with H_2O_2 resulted in decreased cytoplasmic Nrf2 without any alteration in tubulin levels. By comparison, Nrf2 failed to leave the cytoplasmic fraction in p62 KO cells treated with H_2O_2 (Figure 2-2B), revealing that p62 regulates the activity of Nrf2. Collectively, these results suggest that p62 protects cells from oxidative stress through a Nrf2-dependent pathway (Liu et al, 2007; Komatsu et al., 2007).

P62 promoter is oxidatively modified in an age-dependent manner

Since p62 expression is regulated at the transcriptional level (Nakaso et al.,2004) and the p62 promoter contains a CpG island (Vadlamudi et al., 1998), which is sensitive to oxidative modification (Lu et al., 2004), studies were undertaken to examine if the p62 promoter is subject to age-dependent oxidative damage. To test for DNA damage, an amplicon was chosen within the mouse p62 promoter and three amplicons were designed within the human p62 promoter (Table 2-2; Figure 2-3A). These amplicons were selected based upon their degree of GC richness (> 60%) and the position of putative transcription

factor binding sites (Vadlamudi et al., 1998). A recently developed DNA damage assay (Lu et al., 2004) was validated with these amplicons and used to examine the degree of oxidative damage to the mouse p62 promoter in DNA samples isolated from mice with increasing age. Oxidative damage to the mouse p62 promoter increased significantly in 12-month-old brains compared to either the 2-, or 6-month-old brains, but there was no significant difference between 2-, and 6-month-old samples (Figure 2-3B). This result was consistent with an increase in 8-OHdG levels with age observed by immunostaining (Figure 2-1A and B). Parallel studies were undertaken to examine whether oxidative damage to the human p62 promoter was also age-related. Samples selected for this analysis were obtained from postmortem brain of individuals with no known history of neurological disease (Table 2-1) and the damage to three amplicons were shown in Figure 2-3C. Sites further from the transcription initiation site are more difficult to repair than those which are closer (Tu et al., 1996). Therefore, amplicon 3, which is furthest from the transcription initiation site (Figure 2-3A), was selected for correlation analysis to examine if any relationship existed between the degree of oxidative damage exhibited by this amplicon and the age of the individual (Figure 2-3D). A significant positive correlation was observed ($r = 0.589$, $p < 0.05$). Likewise, damage to amplicons 1 and 2 were also significantly correlated with age (A1: $r = 0.842$, $p < 0.05$; A2: $r = 0.934$, $p < 0.01$). Collectively, these findings reveal that the p62 promoter is subject to age-related oxidative damage in both mice and humans.

Oxidative damage to the p62 promoter is associated with lower p62 expression

Because the major risk factor for AD is age and p62 $^{-/-}$ mice possess an AD-like phenotype (Babu et al., 2008), to better understand the relationship between loss of p62

and AD, studies were undertaken to examine whether there are disturbances in p62 expression in AD. Postmortem human brain samples from middle and late stage AD individuals (Braak IV and VI, respectively) and controls with no neurological disease were employed (Table 2-1). Western blot analysis of lysates employing p62 monoclonal antibody revealed significantly higher p62 expression levels in human normal brain than samples obtained from AD brain (Figure 2-4A). No alterations in actin levels were observed. DNA oxidative damage in three amplicons of the same five normal and seven AD samples were also examined. The average degree of damage exhibited by either amplicon 1, 2 or 3 of the AD samples was significantly higher than that of normal samples (Figure 2-4B). The correlation between the average degree of oxidative damage to the promoter and the relative expression of p62 in these samples was analyzed. A significant negative correlation existed between the p62 expression level and the average degree of oxidative damage to 3 amplicons in the p62 promoter ($r = - 0.6765$, $p < 0.05$, Fig. 2-4C). Thus, high DNA oxidative damage contributes to low levels of p62 expression. To further define the relationship between p62 expression and promoter damage, human embryonic kidney cells (HEK) cells were treated with $H_2O_2/FeCl_2$ as previously described (Liu et al., 2007). Treatment with increasing dose of H_2O_2 resulted in reduced p62 expression (Figure 2-5A) along with concomitant increase in damage to all three amplicons in the p62 promoter (Figure 2-5B). To extend this analysis to a relevant AD mouse model, the triple transgenic mouse model of AD (3xTg-AD), which exhibits many features of AD neuropathology (Oddo et al., 2003), were employed. The 3xTg-AD mice harbor APP_{Swe}, $Tau_{P301L,}$ and $PS1_{M146V}$ human genes. NonTg mice were used from the same strain and they process the endogenous wild-type mouse PS1 gene.

The expression of p62 and potential damage to the p62 promoter in these mice was examined. Relative p62 expression in NonTg mice was significantly higher than that in 3xTg-AD mice (Figure 2-6A); however there were no alterations in the actin levels. The same samples were analyzed for p62 promoter damage; oxidative damage to the p62 promoter in 3xTg-AD mice was significantly higher than in NonTg mice (Figure 2-6B). Altogether, the findings in the AD mouse model are congruent with the findings in human AD brain samples, further suggesting a functional relevance.

DISCUSSION

Brain aging is associated with a progressive imbalance between intracellular levels of ROS and antioxidant defenses. Clearly, multiple cellular interactions are involved simultaneously in directing the aging process. Aberrant signaling and oxidative stress associated with aging have been reviewed recently and include age-related derangement of redox-regulated signals, age-related changes in anti-oxidant enzymes, age-related decrease in plasma cysteine, and dysregulation of iron and calcium homeostasis etc. (Droge and Schipper, 2007). Extensive studies reveal that DNA damage accumulates with age. For example, age-associated increases in tissue oxidative DNA damage in Sprague-Dawley rats correlated with 8-OHdG levels (Wolf et al., 2005). Also, in a type 2 diabetes model, 8-OHdG levels are increased in an age-dependent manner (Ihara et al., 1999). Our findings are in keeping with age as a factor for accumulation of oxidative damage. This is likely due to removal of DNA adducts by an anti-oxidant repair system efficiently in young and mature animals, but the production of ROS increases due to

reduced antioxidant response and a decline in the capacity of DNA repair with age along with deficiency of p62.

Accumulating evidence demonstrates that p62 plays a yet to be fully understood role in neurodegenerative disease. For instance, p62 may protect neuronal cells from toxicity of misfolded proteins by enhancing aggregate formation under stress conditions (Bjørkøy et al., 2005), and aggresomes containing p62 have been observed in surviving cells (Nakaso et al., 2004). Because of the link between transcriptional expression of p62 (Nakaso et al., 2004; Thompson et al., 2003), and the GC content within the p62 promoter, as well as multiple transcription factor binding sites responding to diverse signals (Vadlamudi et al., 1998), we hypothesized a relationship between oxidative damage to the p62 promoter and age within this region. Damage to the mouse p62 promoter in mice increased with age, and damage to the human p62 promoter was also significantly age-correlated, revealing that age-related oxidative stress results in oxidative damage to p62 promoter. Moreover, loss of p62 enhanced oxidative stress and DNA damage. Higher 8-OHdG levels and oxidative damage were observed in p62 $^{-/-}$ mice compared to WT mice. It was recently reported that higher ROS production in p62-deficient cells is a consequence of impaired NF-κB activation, and ROS production can further increase c-Jun N-terminal kinase (JNK) activation, which can lead to cell death (Duran et al., 2008). Although the question of whether elevated DNA damage is a causative factor of aging or is only correlative with aging still can not be answered by present observations, parallel observations in mouse models of sporadic and genetic forms of AD reveal that decreased p62 expression may enhance the appearance of AD markers (Chen et al., 2007).

The promoters of some down-regulated genes with age are oxidatively damaged and the base-excision repair enzyme, human OGG1 can recover the oxidative DNA damage (Lu et al., 2004). Moreover, nuclear OGG1 levels and activity in mild cognitive impairment (MCI) and AD brains were decreased compared to normal subjects (Lovell et al., 2000). Prolyl isomerase Pin1 catalyzes the conversion between the *cis* and *trans* conformations of phosphorylated Ser/Thr-Pro motifs in peptides and is involved in AD (Yaffe et al., 1997; Lu, 2004). Reduced Pin1 levels associated with Pin1 promoter polymorphisms and oxidative inhibition of Pin1 were found in late-onset AD (Segat et al., 2007; Sultana et al., 2006). Oxidative damage to three amplicons in the human p62 promoter and the amplicon in the mouse p62 5'-flanking region were used as the index of p62 promoter damage. Age-associated oxidative damage to the p62 promoter was significantly correlated with p62 expression in AD brains, human cells treated with H_2O_2 as well as the 3XTg-AD mouse model (Figure 2-4, 2-5, and 2-6). Samples with higher p62 expression have lower promoter damage, and samples with lower expression have higher promoter damage. In the human p62 promoter, transcriptional factor binding sites for PEA3 and Sp-1 are located within the amplicon 3, a site for Pu.1 is located in the amplicon 1, and amplicon 2 includes AP-1/ TRE elements (Vadlamudi et al., 1998). Oxidative modifications of guanine within AP-1 and Sp-1 binding sites have been shown to completely inhibit the binding of AP-1 and Sp-1 transcription factors to DNA (Ghosh and Mitchell, 1999). Therefore, oxidative modification of transcription factor binding sites within the p62 promoter could account for down-regulated p62 expression. Thus, p62 is also an age-related promoter that is sensitive to oxidative modification resulting in

decreased expression (Lu et al., 2004). Further studies will be needed to pinpoint the transcription factor binding site involved in regulating p62 expression.

An absence of p62 leads to reduced nuclear localization of Nrf2 (this study and Komatsu et al., 2007), revealing that p62 regulates Nrf2 activity by a yet-to-be-defined mechanism. The transcriptional activation of the phase II detoxification enzymes is linked to a *cis*-acting element called the antioxidant response element (ARE) which regulates constitutive and inducible gene expression. Nrf2 plays a central role in gene expression of phase II detoxification enzymes and some antioxidant genes (Shih et al., 2005). In postmortem brain from AD individuals, Nrf2 levels in the nucleus are also decreased (Ramsey et al., 2007). Interestingly, there are several links between p62 and AD: 1) p62 deficient mice possess AD-tau brain pathology along with learning and memory deficits, and neurodegeneration (Babu et al., 2008); 2) p62 deficient brain exhibit a loss of Nrf2 nuclear localization (Komatsu et al., 2007) similar to AD brain (Ramsey et al., 2007); and, 3) AD brain exhibits reduced levels of p62.

In summary, we have observed that age-associated oxidative damage to the p62 promoter is negatively correlated with p62 expression and reduced Nrf 2 activity (Figure 2-7). Previously we have shown that loss of p62 results in accumulation of K63 polyubiquitin chains (Seibenhener et al., 2004), and neurodegeneration (Babu et al., 2008). Based on these results, we propose that age-associated oxidative damage to the p62 promoter leads to down-regulated p62 expression in AD brain, and thus contributes to enhanced oxidative stress through a feedforward mechanism. P62 is well studied as a scaffold for signaling pathways involved in neurotrophin signaling, ubiquitination, and inflammation (Moscat et al., 2007). At its C-terminal tail p62 possesses an ubiquitin

associated domain. A major hallmark in AD and other neurodegenerative diseases is the accumulation of ubiquitin rich aggregates (Oddo, 2008; Lim, 2007). Soluble protofibrils, monomers, and small oligomers are cytotoxic (Morrissette et al., 2009). The role of p62 is to capture these misfolded proteins and mediate their degradation by the proteasome, or autophagy when the proteasome is overwhelmed (Wooten et al., 2006; Seibenhener et al., 2007). Therefore, a decline in p62 expression as a result of oxidative damage to the promoter would have functional consequences that impair degradation of aggregated proteins such as tau and others (Figure 2-7). Altogether these findings suggest that decline in p62 expression may put one at risk for AD, and other diseases related to protein conformation and folding. In this context, increasing p62 levels might be a viable strategy to prevent a wide-spectrum of oxidative stress-related diseases.

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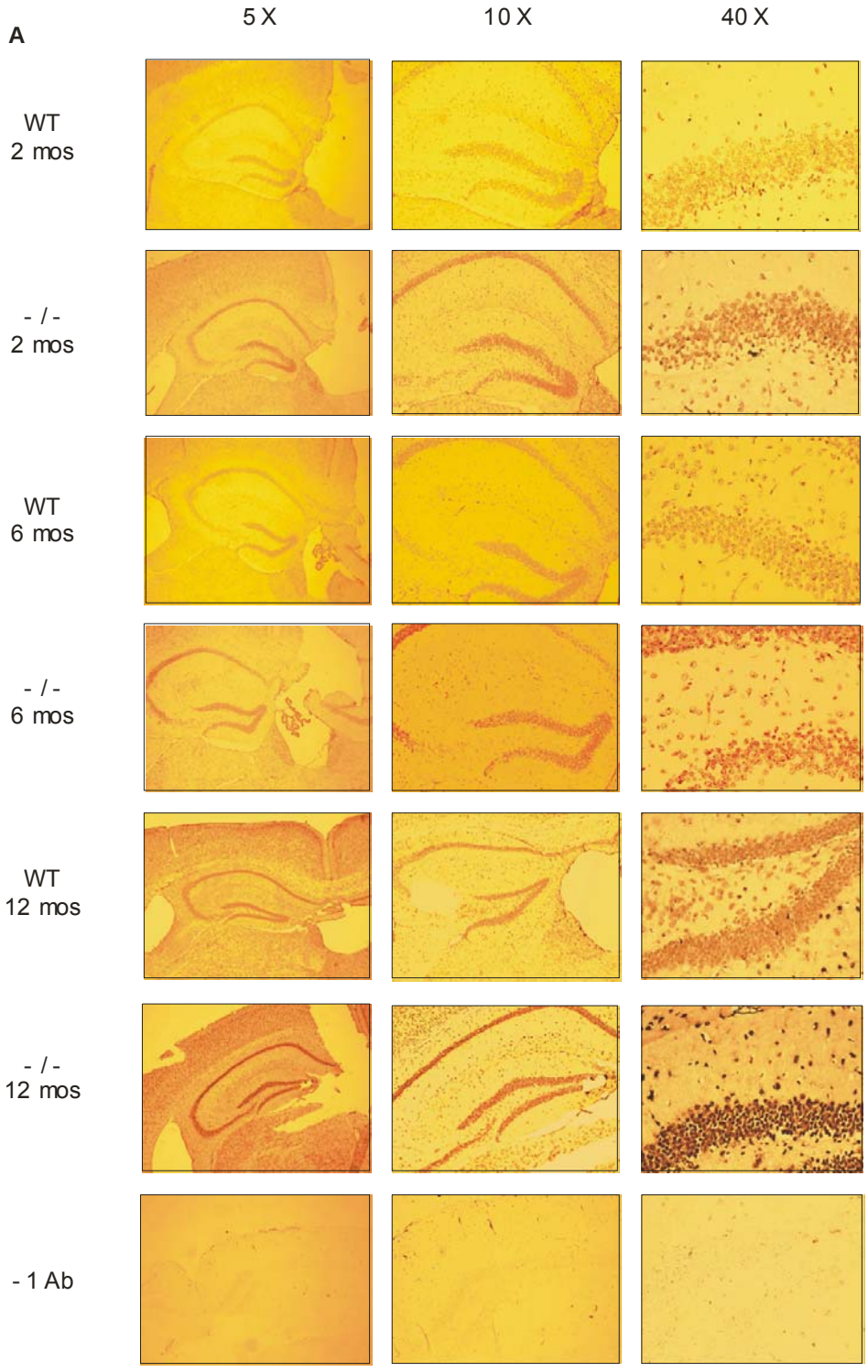
Figure 2-1



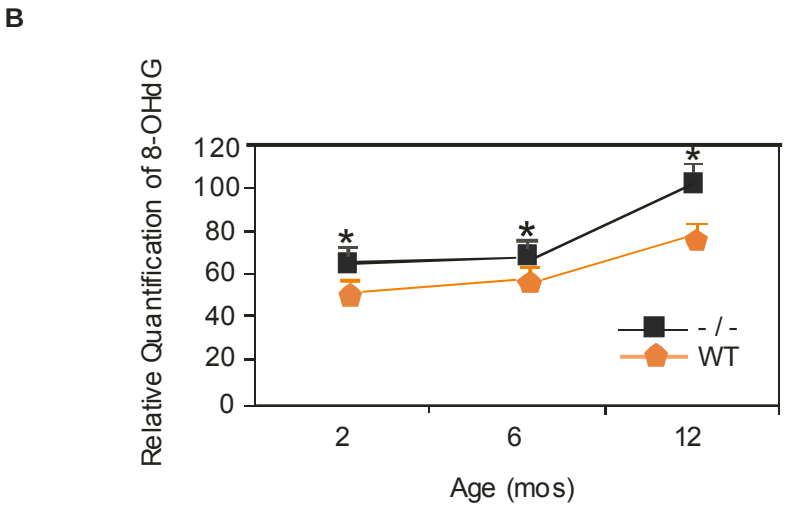
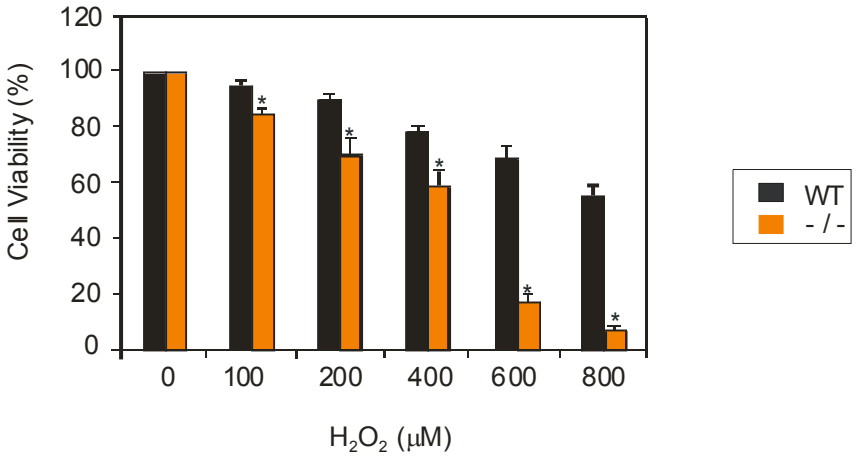


Figure 2-1. Oxidative stress increased in an age-dependent manner. **(A)** Immunostaining of 8-OHdG (N45.1) in brains of WT and p62 $^{-/-}$ mice with age. After proteinase K treatment, 2, 6, 12 months WT and p62 $^{-/-}$ mice brain sections were stained with anti-8-OHdG monoclonal antibody (7.5 µg/ ml) and processed with the MOM kit. Negative controls with no primary antibody are also shown. The results are representative of staining conducted on sections from 4 individual mice for each genotype. For each age, images were processed at 5 X, 10 X, and 40 X magnification. **(B)** Three corresponding regions from CA1, CA3, and dentate gyrus (DG) of the hippocampus from each section were scanned. The quantification of 8-OHdG for each section was expressed by the mean average pixel (Pixel total/ region size). The relative intensities were averaged and reported as Mean +/- S.D. Two-way ANOVA results were: Age, F = 46.50, $p << 0.05$; WT / p62 $^{-/-}$, F = 38.53, $p << 0.05$; interaction = NS). Asterisk (*) indicates significant (< 0.05) multiple comparison adjusted p values for individual comparisons of WT vs p62 $^{-/-}$ data for each age class.

Figure 2-2

A


120
100
80
60
40
20
0
Cell Viability (%)
*
*
*
*
*
0
100
200
400
600
800
H2O2 (μM)
WT
- / -

B

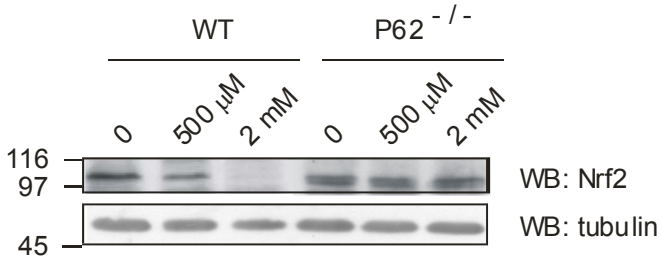
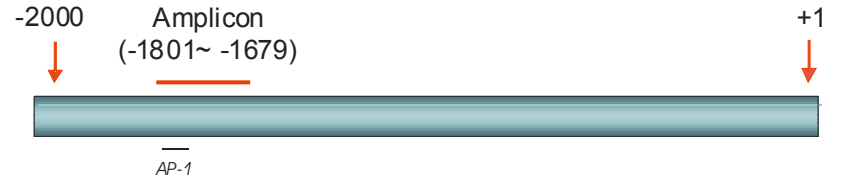

WT
P62 - / -
0
500 μM
2 mM
0
500 μM
2 mM
116
97
45
WB: Nrf2
WB: tubulin

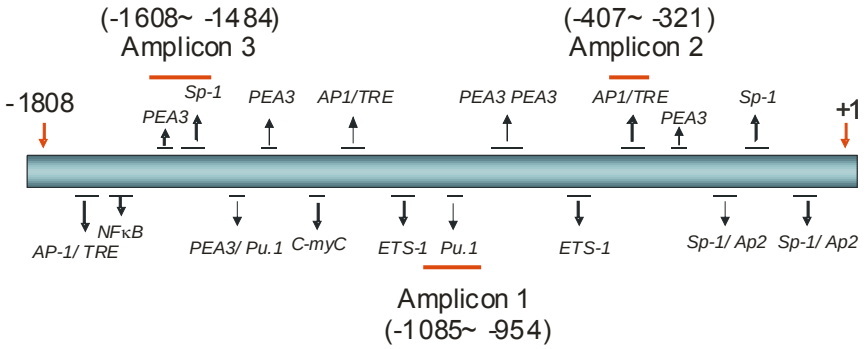
Figure 2-2. p62 mediates protection from oxidative stress *in vitro*. **(A)** WT and p62 $^{-/-}$ MEF cells were seeded in 96-well plates at 10,000 cells per well. After 48 h incubation, the cells were treated with various concentrations of H_2O_2 for 18 h. Cell viability was assessed using the CellTiter-Glo assay kit. The percentage of surviving cells is expressed as Mean +/- S.E.M (N = 4). Statistical significance of WT compared to -/- values identified by asterisk, indicating multiple comparison adjusted $p < 0.05$. **(B)** Deficiency of the p62 prevents the Nrf2 translocation from cytosol to nuclei upon oxidative stress. WT and p62 $^{-/-}$ MEF cells were pre-treated with leptomycin B (20 ng/ml) for 8h followed by treatment with or without 500 μm or 2 mM H_2O_2 for 30 minutes. Equal concentration of cytoplasmic protein (80 μg) was Western blotted with anti-Nrf2 and anti-tubulin antibodies as shown.

Figure 2-3

A

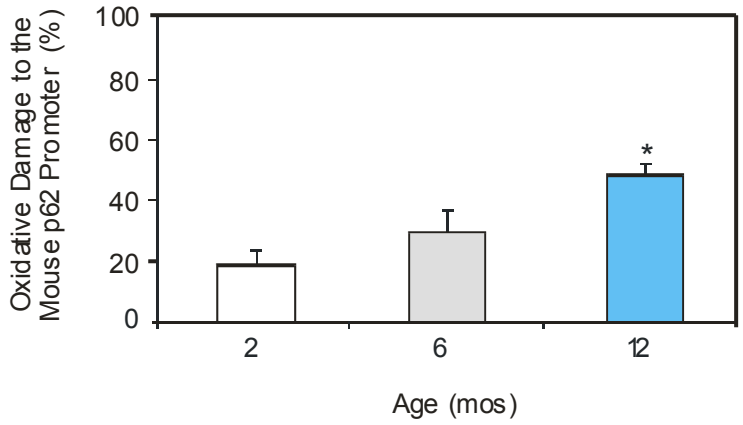


The amplicon within mouse p62 promoter

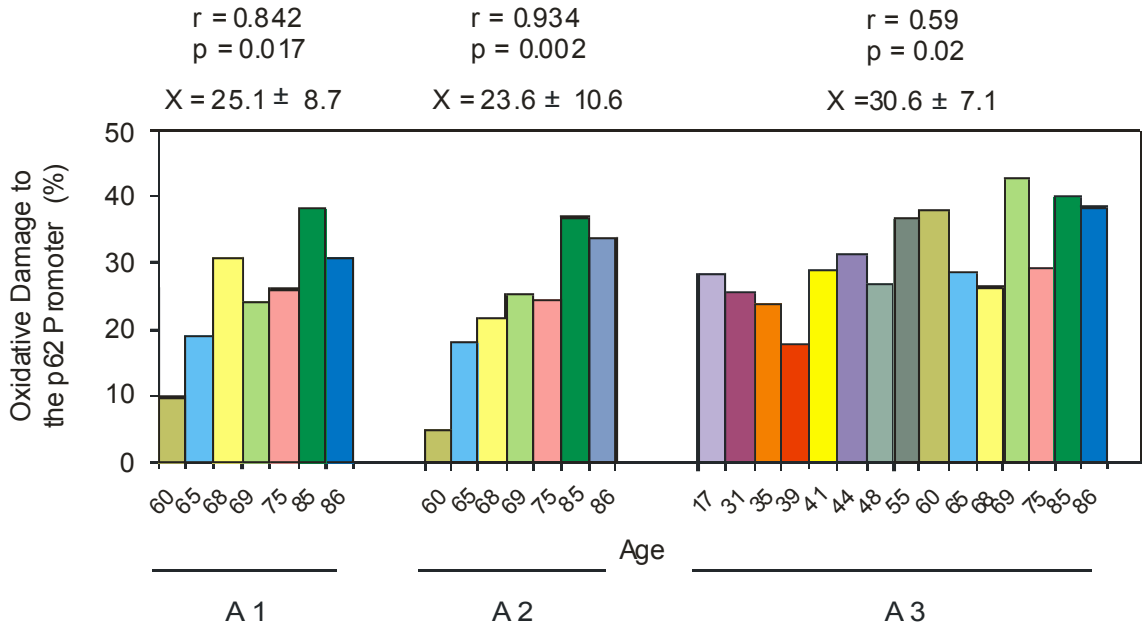


Three amplicons within human p62 promoter

B

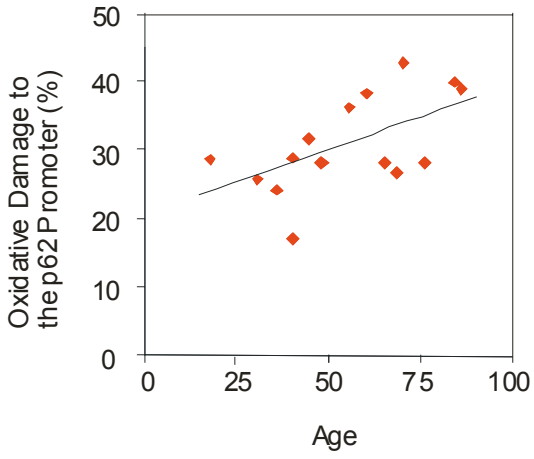


C



D

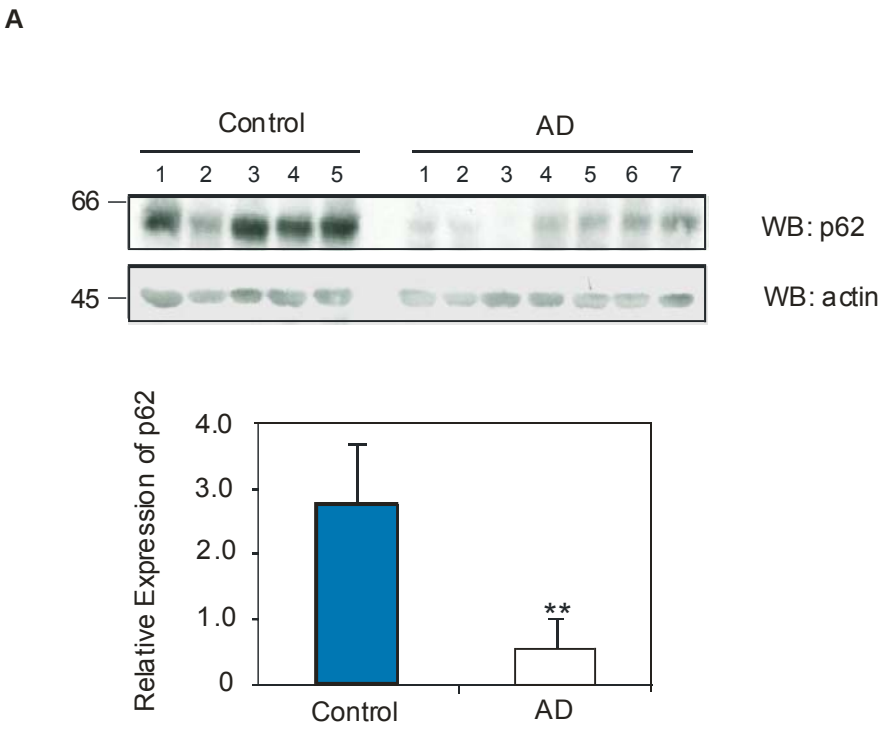
Age	17	31	35	39	41	44	48	55	60	65	68	69	75	85	86	
Damage	28.2	25.1	23.7	17.3	28.5	31.9	27.5	36.4	38	27.4	25.8	42.8	27.5	39.6	39	X =30.6 ± 7.1



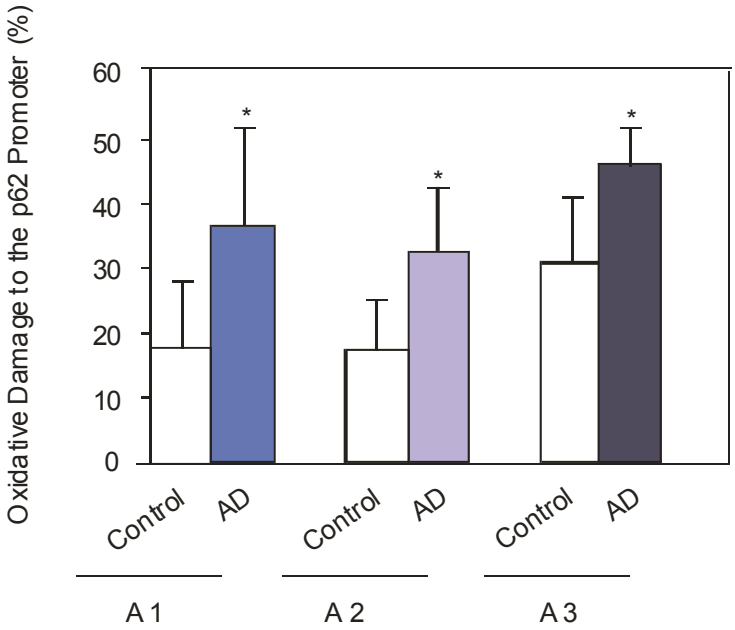
r = 0.589
p < 0.05

Figure 2-3. Oxidative damage to the p62 promoter is age-dependent. **(A)** Diagram of amplicons designed within the mouse and human p62 promoter. The amplicon was designed at -1801~ -1777 of the 5'- flanking region of the mouse p62 gene. Three amplicons, amplicon 1 (-1085 ~ -954), amplicon 2 (-407 ~ -321) and amplicon 3 (-1608 ~ -1484), were designed within the human p62 promoter. **(B)** Oxidative damage to the mouse p62 promoter in WT mice with increasing age. DNA was isolated from 2, 6 and 12 month wild type brain tissue, and then subjected to fpg digestion and Q-PCR with specific primer for the amplicon within the mouse p62 promoter. The oxidative damage to the promoter is expressed as Mean +/- S.D (ANOVA $F = 10.74$, $p < 0.05$) from Tukey's studentized range tests. **(C)** Fpg-treated and non-treated genomic DNA was used as a template and specific primers were used for absolute quantitative real time PCR assay. Oxidative damage to amplicon 3 was examined in 15 human brain tissues from age 17 to 86. **(D)** Oxidative damage was tested in 3 amplicons within the p62 promoter of samples from normal human brains of individuals with various ages and a correlation analysis was conducted between DNA damage in amplicon 3 and age. Correlation coefficients and significance levels are indicated.

Figure 2-4


A
Control
AD
1 2 3 4 5
1 2 3 4 5 6 7
66
45
WB: p62
WB: actin
Relative Expression of p62
4.0
3.0
2.0
1.0
0
**
Control
AD

B


Oxidative Damage to the p62 Promoter (%)
60
50
40
30
20
10
0
*
*
*
Control
AD
Control
AD
Control
AD
A 1
A 2
A 3

C

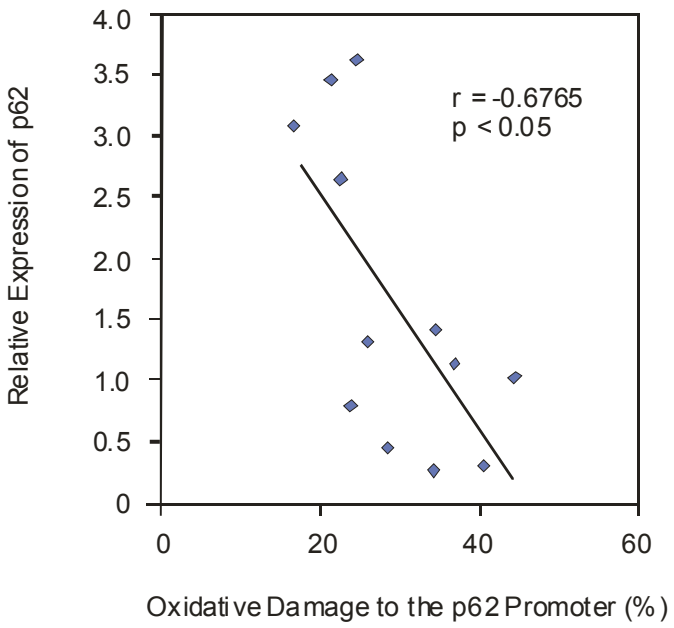

Relative Expression of p62
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
r = -0.6765
p < 0.05
0
20
40
60
Oxidative Damage to the p62 Promoter (%)

Figure 2-4. p62 expression level is reduced in AD brain and correlated with oxidative damage within the p62 promoter region. **(A)** p62 expression in control and AD brains. An equal concentration of tissue homogenate (50 μg) from control and AD brain lysates were subjected to 10% PAGE followed by western blot with anti-p62 and anti-actin antibodies. Expression levels of p62 was determined by scan of the blot. The data are expressed as relative intensity (Mean +/- S.D., ** $p < 0.01$). **(B)** Oxidative DNA damage percentage in 3 amplicons within the human p62 promoter. Total genomic DNA extracted from normal and AD brain tissues were digested by Fpg, then subject to real-time PCR with three sets of specific primers for 3 individual amplicons (Table 2). The Mean +/- S.D. damage is reported (* $p < 0.05$). **(C)** Correlation analysis between oxidative damage within the p62 promoter region and relative p62 expression. Correlation coefficient and significance levels are indicated.

Figure 2-5

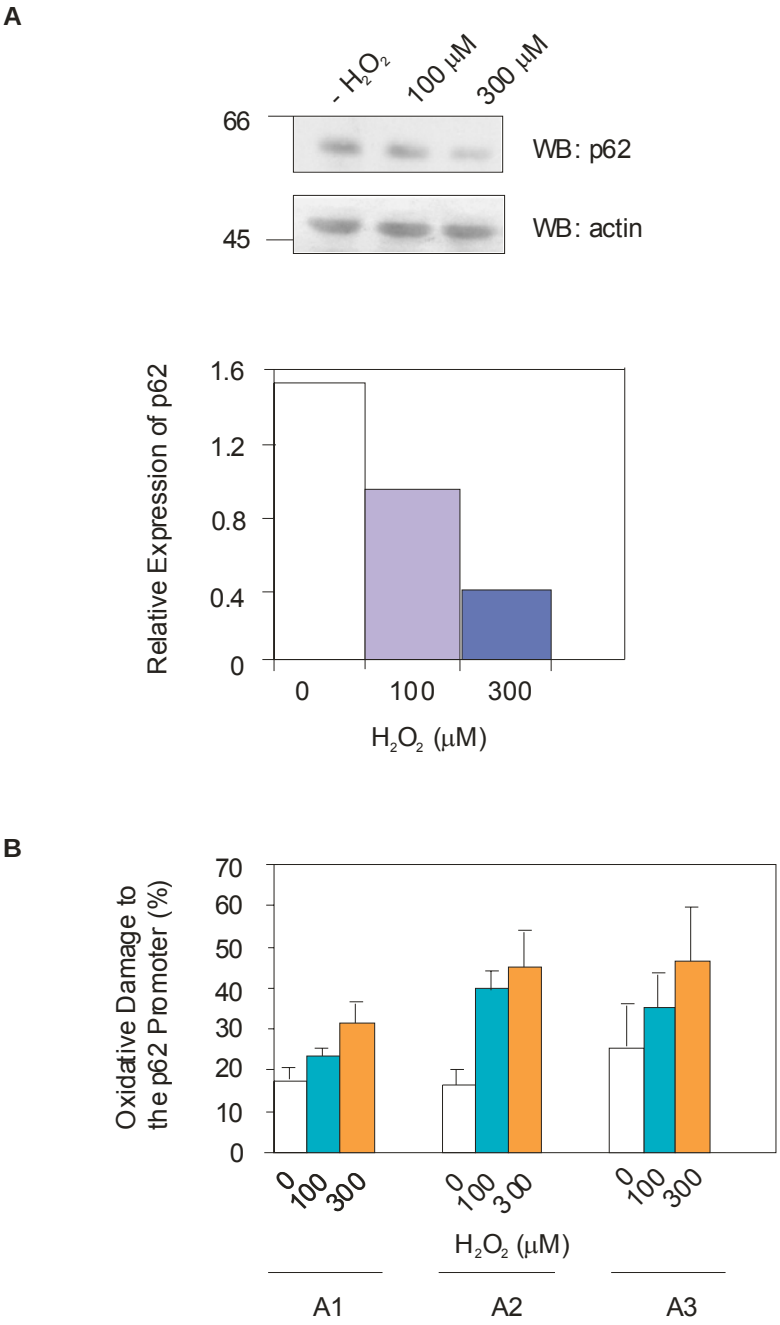
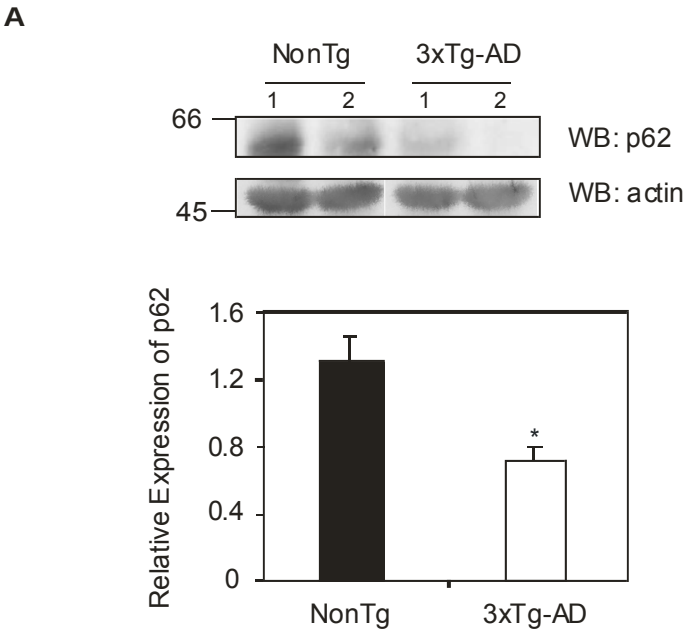

A
- H2O2
100 μM
300 μM
66
45
WB: p62
WB: actin
Relative Expression of p62
1.6
1.2
0.8
0.4
0
0
100
300
H2O2 (μM)
B
Oxidative Damage to the p62 Promoter (%)
70
60
50
40
30
20
10
0
0
100
300
0
100
300
0
100
300
H2O2 (μM)
A1
A2
A3

Figure 2-5. P62 is oxidatively damaged in H_2O_2-treated HEK cells. **(A)** HEK cells were treated with 100 μM, 300 μM and 500 μM H_2O_2 along with 20 μM, 60 μM and 100 μM $FeCl_2$ for 12 h. Equal amount of lysate (40 μg) from non-treated and H_2O_2-treated cells was Western blotted with anti-p62 and anti-actin antibodies. P62 expression level was expressed by a ratio in the relative intensity of the p62 signal to the actin signal.

(B) Profile the oxidative damage to the p62 promoter from H_2O_2-treated and non-treated HEK cells. The percentage of oxidative damage to each of the 3 amplicons within the p62 promoter was measured by QRT-PCR and averaged across all three amplicons. The data is presented as Mean damage +/- S.D (* $p < 0.05$).

Figure 2-6


A
NonTg
3xTg-AD
1
2
1
2
66
45
WB: p62
WB: actin
Relative Expression of p62
1.6
1.2
0.8
0.4
0
*
NonTg
3xTg-AD

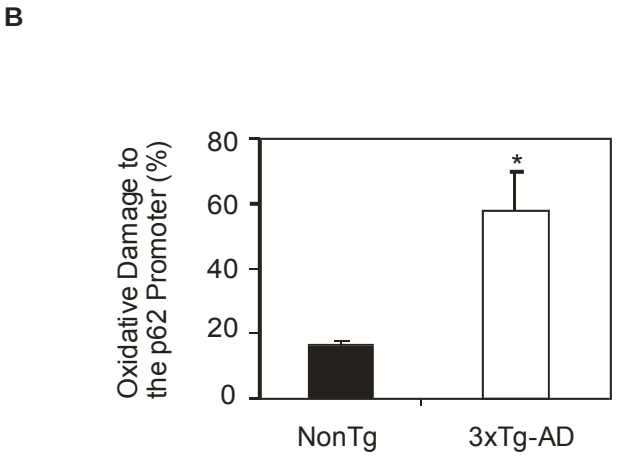

B
Oxidative Damage to the p62 Promoter (%)
80
60
40
20
0
*
NonTg
3xTg-AD

Figure 2-6. Reduced p62 expression and increased damage to the p62 promoter. **(A)** Equal amounts of tissue homogenate protein (50 μg) from WT and transgenic AD mice were western blotted with anti-p62 and anti-actin antibodies. p62 expression levels in normal and AD brains were expressed as the ratio of the relative intensity of the p62 blot to actin blot (Mean +/- S.D., * $p < 0.05$). **(B)** Oxidative damage in the amplicon within the mouse p62 promoter region of NonTg and 3xTg-AD mouse brains. NonTg and 3xTg-AD mice brain tissues were used to prepare genomic DNA. Real-time PCR was performed with specific primers for the amplicon designed within the mouse p62 gene 5'-flanking region. * $p < 0.05$, significant difference.

Figure 2-7

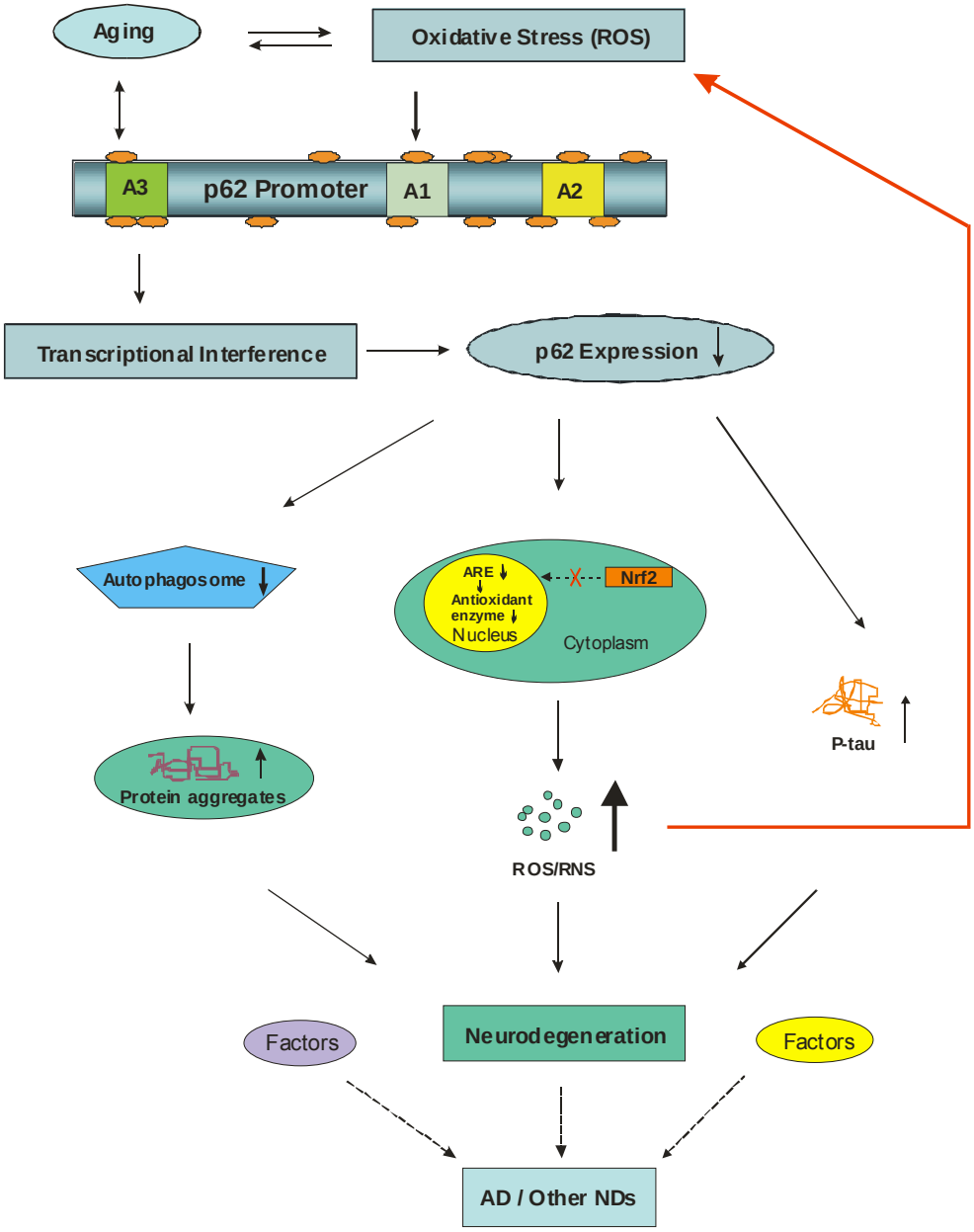

Aging
Oxidative Stress (ROS)
A3
p62 Promoter
A1
A2
Transcriptional Interference
p62 Expression
Autophagosome
ARE
Antioxidant enzyme
Nucleus
Nrf2
Cytoplasm
P-tau
Protein aggregates
ROS/RNS
Factors
Neurodegeneration
Factors
AD / Other NDs

Figure 2-7. Model illustrating the relationship between oxidative damage to the p62 promoter and p62 expression. Accumulating oxidative stress with age leads to the oxidative damage to the p62 promoter, resulting in reduced p62 expression. Decline in p62 levels would result in reduced delivery of substrates to the autophagosome, decreased Nrf2 nuclear translocation, leading to an increased level of protein aggregates, ROS, and misfolded substrates such as tau protein. Decline in p62 promotes a feedforward mechanism to accelerate oxidative damage and reduce phase II detoxification enzymes and antioxidant gene expression. Altogether, decline in p62 expression along with other factors could contribute to age-dependent neurodegenerative diseases such as AD and other neurodegenerative disease (ND).

Table 2-1. Control and AD brain samples

Brain Number	Distributive Dx	Age	Race/ Sex	PMI	ApoE	Other disease	Source
OS00-06	Normal	60	b/f	8	E3/4	-	ADRC, Emory
OS00-23	Normal	68	w/f	11	E3/3	-	ADRC, Emory
OS01-112	Normal	65	w/f	6	E3/3	-	ADRC, Emory
OS02-35	Normal	75	w/f	6	E3/3	-	ADRC, Emory
OS03-299	Normal	69	w/m	2.5	E3/3	-	ADRC, Emory
OS00-05	Mild AD	74	w/m	<6	E2/3	-	ADRC, Emory
OS01-73	Mild AD	76	w/f	15	E3/3	-	ADRC, Emory
OS01-126	Mild AD	87	w/f	32	E3/4	-	ADRC, Emory
OS01-129	Mild AD	92	w/f	NA	E3/3	-	ADRC, Emory
OS00-38	Severe AD	92	w/f	6	E3/3	-	ADRC, Emory
OS01-02	Severe AD	69	w/f	5.5	E4/4	-	ADRC, Emory
OS01-11	Severe AD	80	b/f	5	E3/4	-	ADRC, Emory
BRC 747	Normal	86	-	-	-	-	ADRC, UAB
BRC 576	Normal	85	-	-	-	Aneurysm	ADRC, UAB
BRC 663	Normal	59	-	-	-	Breast Cancer	ADRC, UAB
BRC 511	Normal	59	-	-	-	Adenosarcoma	ADRC, UAB
BRC 399	Normal	39	-	-	-	- Pulmonary Emboli	ADRC, UAB
5727	Normal	48	m	24.32	-	-	HBTRC
5813	Normal	41	m	27.17	-	-	HBTRC
5852	Normal	55	m	35.32	-	-	HBTRC
6951	Normal	17	m	28.92	-	-	HBTRC
5304	Normal	44	m	24.61	-	-	HBTRC
5352	Normal	31	m	32.92	-	-	HBTRC
5358	Normal	35	m	25.67	-	-	HBTRC

Table 2-2. Amplicons and primer pairs for real-time PCR

P62 promoter	Amplicon	Primer sequence	Tm	
Human	Amplicon1	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
Mouse	Amplicon	5'-TTT TCT CGC CAT TTG GCC CGT T-3'	60.2	Sense
		5'-ATG AGC TCT TAG CAG GAA CCC AGT-3'	59.8	Antisense

*CHAPTER III. OXIDATIVE DAMAGE TO THE PROMOTER REGION OF SQSTM/P62 IS COMMON TO NEURODEGENERATIVE DISEASE

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 diseases. 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 H_2O_2 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

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pharmacological means to increase p62 expression may be beneficial in delaying the onset of neurodegeneration.

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 (Shibutain 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 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 Picks disease, but mitochondrial DNA damage has been reported in these two neurodegenerative diseases (Su et al., 2000; Mawrin et al., 2004).

Sequestsome 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, 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 3-1.

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 $MgSO_4$, 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 nitrone (PBN) (Sigma, St. Louis, MO). The high temperature incubation was replaced by 4 hours incubation at 37°C. Following elution with ddH_2O, 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 Chapter II, Table 2-2. 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% H_2O_2 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.

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 16 h 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 10mM EDTA, pH 8.0) twice. DNA was extracted from combined elute by phenol-chloroform and ethanol precipitation, and dissolved in 10 µl ddH_2O. 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 binding site deletion mutation

The Sp-1 element deleted form of the p62 promoter was made by QuikChange 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.

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 (Figure 3-1). No immunoreactivity was present in samples treated with DNase I or in sections incubated without primary antibody (Figure 3- 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 (Figure 3-1, 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 (Figure 3-2A). 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 (Figure 3-2B). 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 the degree of damage to the promoter (Du et al., 2009). Because FTD samples showed enhanced DNA oxidative damage to all three amplicons (Figure 3-2B), we examined if the same correlation existed between the level of p62 expression and the degree of oxidative damage in FTD diseased individuals (Figure 3- 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 (Figure 3-3B). 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 → 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., 2008) 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 3-2A and B). 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 3-2B; Figure 3-4). 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 μM H_2O_2 *in vitro*, transfected into HEK cells, followed by assay to examine p62 basal transcriptional activity (Figure 3-5A). Promoter activity was decreased 40% compared to cells transfected with the non-treated p62 promoter construct (Figure 3-5A). In parallel, oxidative damage to either amplicon 1, 2 or 3 of the H_2O_2-treated p62 promoter was also evaluated. The degree of damage was significantly higher for amplicons 2 and 3 (Figure 3-5B). 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 H_2O_2-treatment of HEK cells led to reduced p62 protein expression (Du et al., 2009).

Deletion of an Sp-1 binding site 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 (Figure 3-2A), with a guanine accessible 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 H_2O_2 treatment of cells (Figure 3-6). The induction of the WT p62 promoter by H_2O_2 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 (Figure 3-6), 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 O_2 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 for mutation analysis. The robust immunostaining for 8-OHdG 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 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 bee 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). Lu et al. examined a large array of genes whose promoters were oxidatively modified, and identified the common element being the high GC content (Lu et al., 2004). 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 Aβ 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 ARE and protect cells from oxidative stress (Liu et al., 2007). p62 can stimulate 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., 2008). 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* H_2O_2 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 mutation with Sp-1 element deletion 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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Figure 3-1

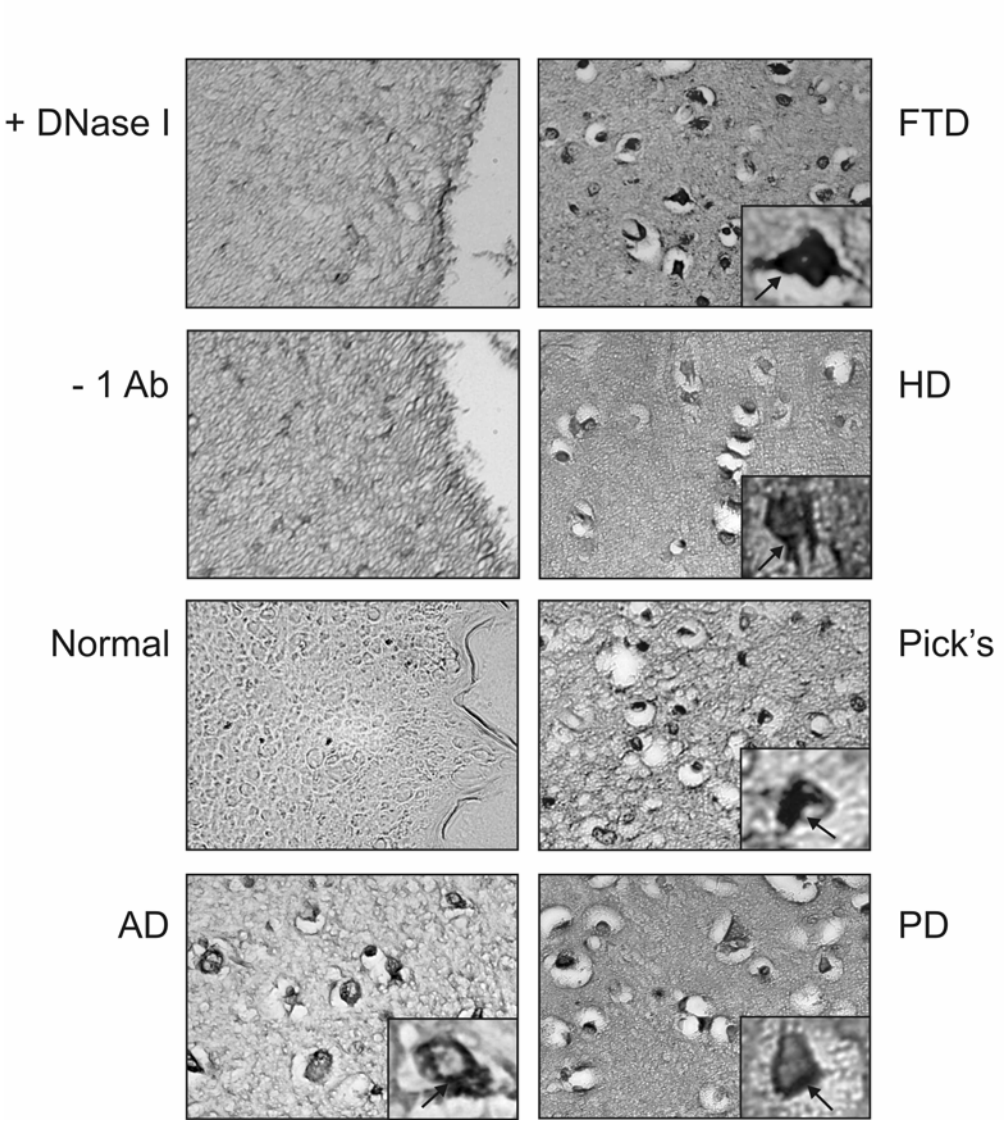
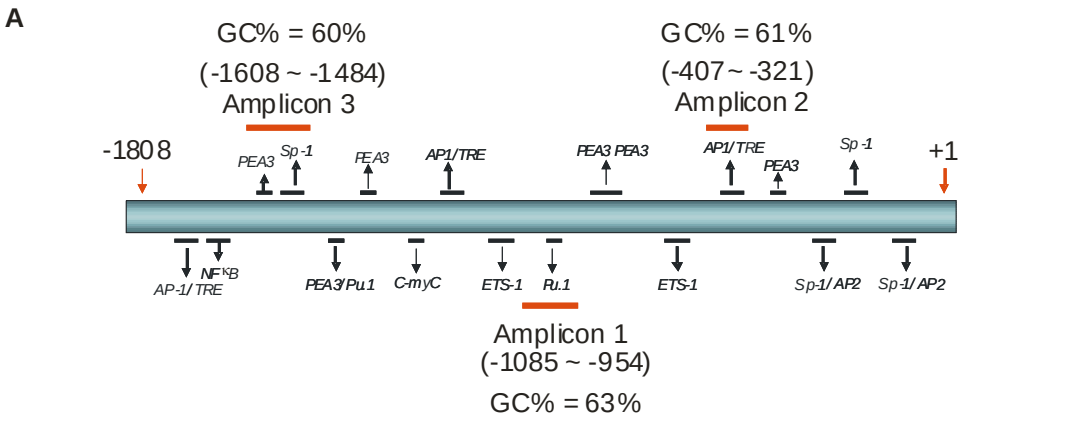
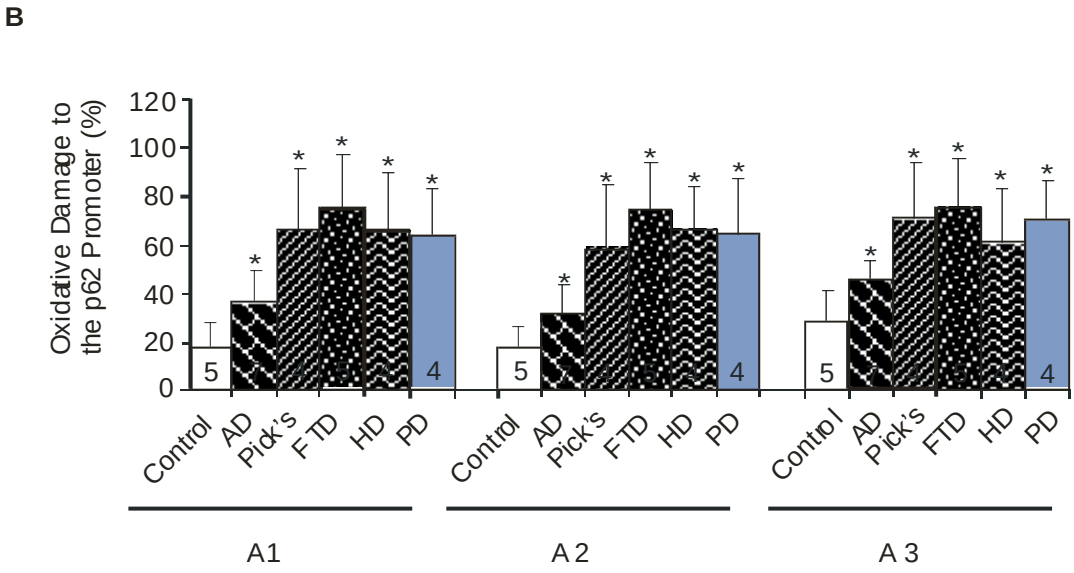


Figure 3-1. Representative immunohisitochemical staining for 8-OHdG in human brain. Sections that were treated with DNase I or incubated without primary antibody did not display any immunoreactivity for 8-OHdG. Little or no immunoreactivity was observed in sections taken from normal brain. More DNA damage 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).

Figure 3-2



Three amplicons within the human p62 promoter



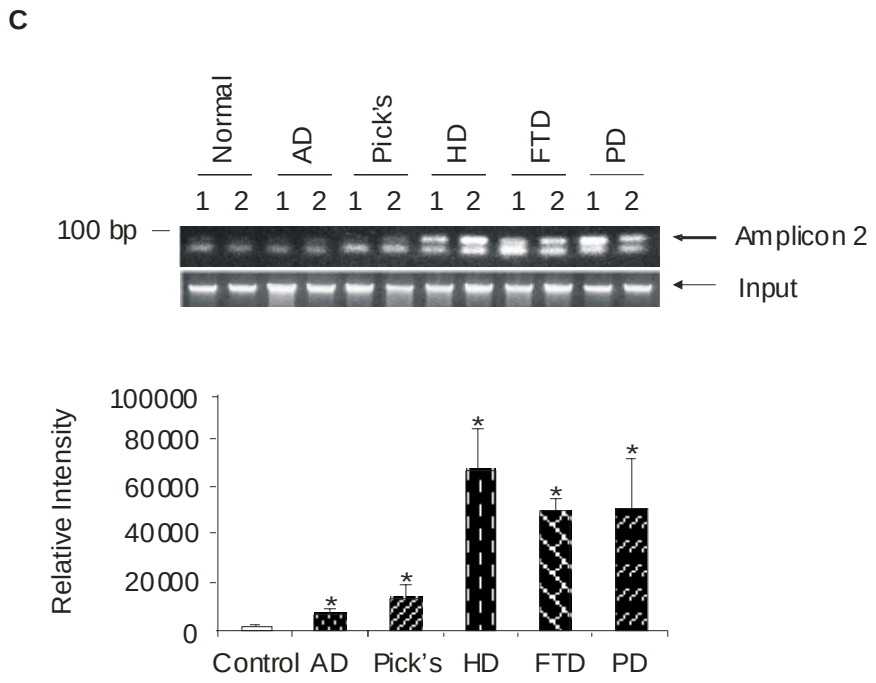


Figure 3-2. Profile of oxidative damage in 3 amplicons within p62 promoter of normal and various samples from neurodegenerative diseased brains. **A.** Three amplicons, amplicon 1 (-1085 ~ -954), amplicon 2 (-407 ~ -321) and amplicon 3 (-1608 ~ -1484), were designed within the human p62 promoter. Amplicon 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 $p < 0.001$).

Figure 3-3

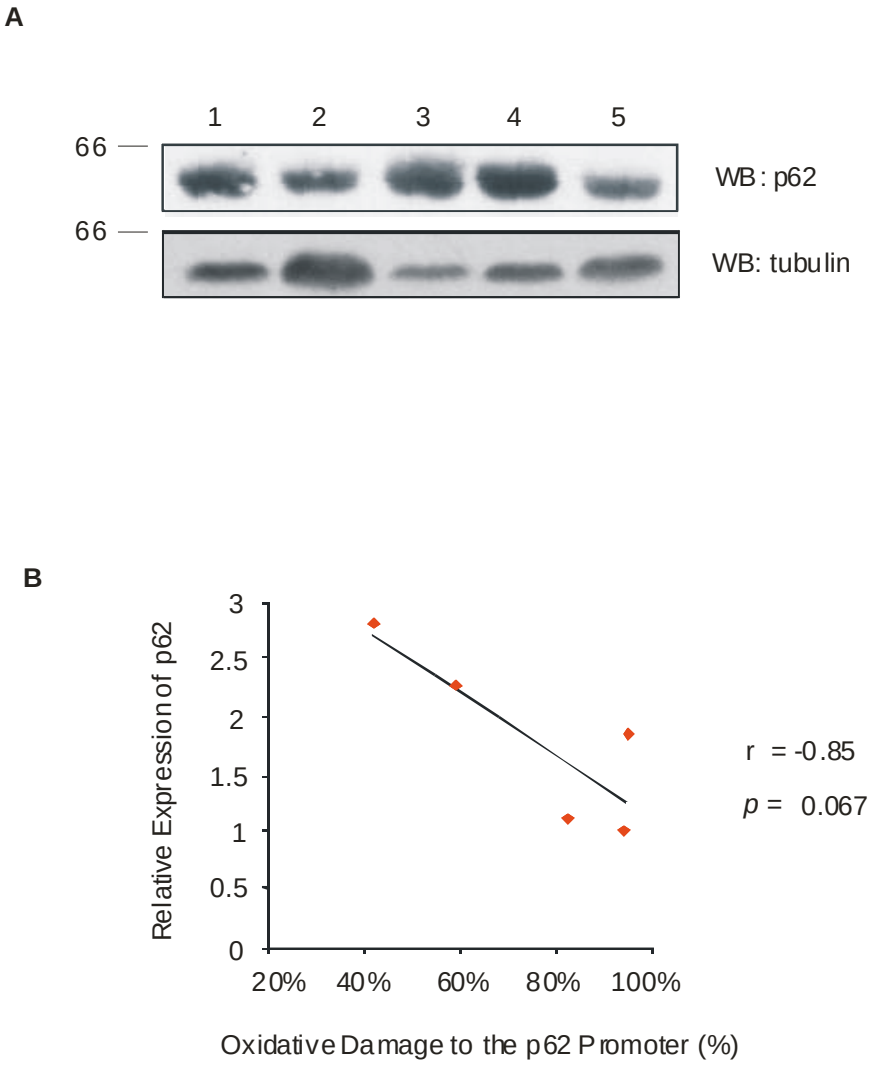

A
1
2
3
4
5
66
66
WB: p62
WB: tubulin
B
Relative Expression of p62
3
2.5
2
1.5
1
0.5
0
20%
40%
60%
80%
100%
Oxidative Damage to the p62 Promoter (%)
r = -0.85
p = 0.067

Figure 3-3. 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 μg) 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 ($r = -0.85$; $p = 0.067$).

Figure 3-4

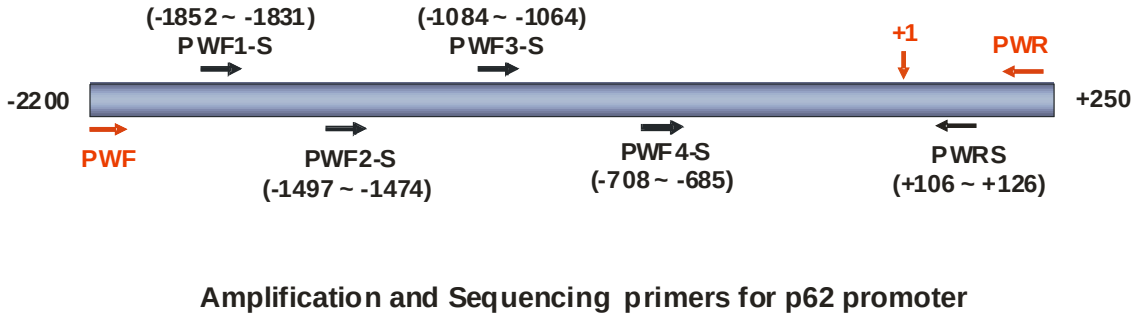


Figure 3-4. Diagram of primers used for p62 promoter amplification and sequencing. Two specific primers, PWF and PWR were used to amplify p62 promoter from genomic DNA. The location of five sequencing primers, PWF1-S, PWF2-S, PWF3-S, PWF4S, and PWR1S were shown.

Figure 3-5

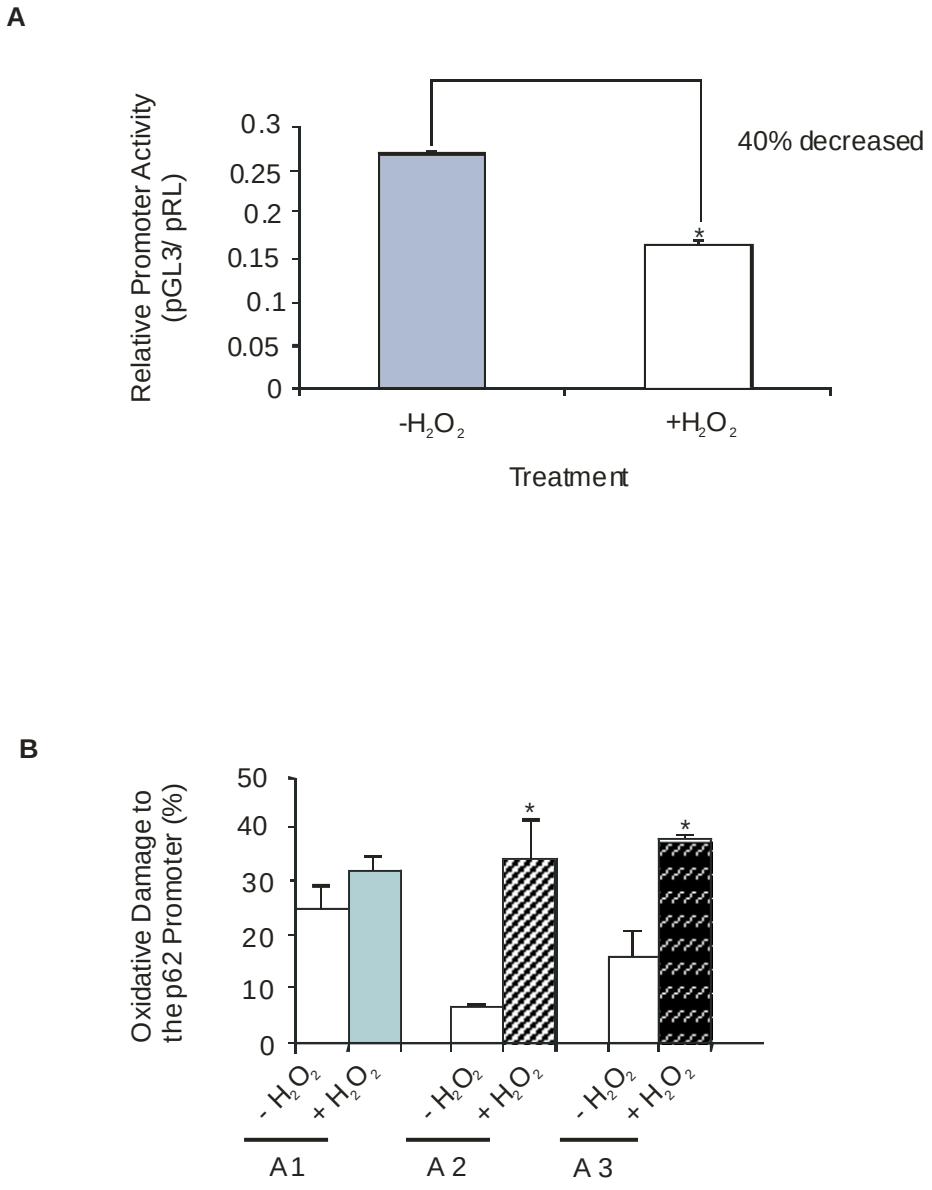

A
Relative Promoter Activity (pGL3/ pRL)
0.3
0.25
0.2
0.15
0.1
0.05
0
40% decreased
*
-H2O2
+H2O2
Treatment
B
Oxidative Damage to the p62 Promoter (%)
50
40
30
20
10
0
*
*
- H2O2
+ H2O2
- H2O2
+ H2O2
- H2O2
+ H2O2
A1
A 2
A 3

Figure 3-5. Oxidative stress reduces p62 promoter activity. **A.** p62 promoter activity decreased upon *in vitro* H_2O_2 treatment. p62 promoter-pGL3 was incubated in the absence or presence of 100 μM H_2O_2 for one hour at 4°C *in vitro*. Non-treated and 100 μM H_2O_2-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 ± S.E.M. (N = 3). **B.** Oxidative DNA damage to 3 amplicons within the p62 promoter upon *in vitro* H_2O_2 treatment. Non-treated p62 promoter-pGL3 construct and *in vitro* 100 μM H_2O_2-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.

Figure 3-6

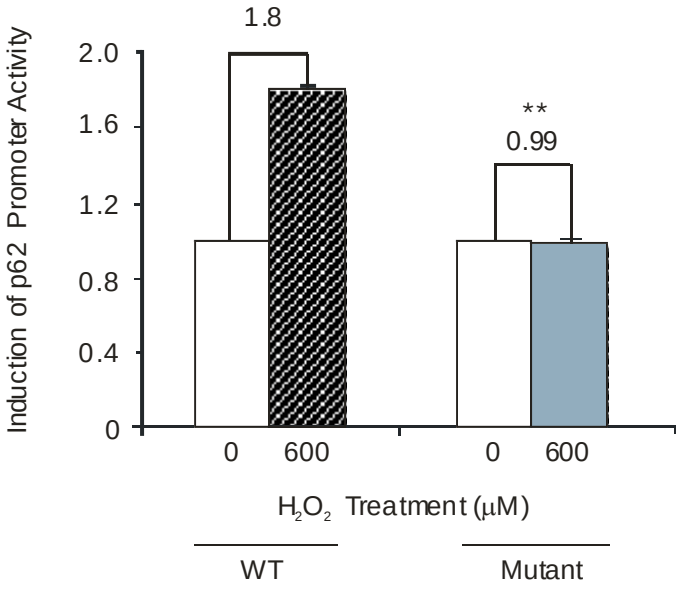


Figure 3-6. Induction of the wild type and Sp-1 binding site deleted p62 promoter upon H_2O_2 treatment. WT p62 promoter-pGL3 and Sp-1 deletion mutant were transfected into HEK cells along with pRL-TK and PDEF. The cells were treated with 600 μM H_2O_2 for 16 h, post-transfection, lysed and subjected 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 3-1. 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
b6812	PD	75	m	19.42		HBTRC

Table 3-2. Samples and primers for amplification and sequencing of the human p62 promoter

A

Samples for sequencing of the human p62 promoter

Sample	1	2	3	4	1	2	3	4	5	6	1	2
Disease	Normal	Normal	Normal	Normal	AD	AD	AD	AD	AD	AD	FTD	FTD
Annealing Temp. (^{O}C)	62	60.5	62	59	59.3	59	60.2	60.2	59.3	59.3	60	60

B

Primers for amplification and sequencing of the human p62 promoter

Primer	Sequence	Tm(^{O}C)	
PWF	5'- TTA CAA CAT GCT GGG CAG CAA CAG -3'	60.3	Sense
PWR	5'- AGC AGA AGC TGA AGC GGC GAA TCT -3'	63.0	Antisense
PWF1-S	5'- AGG TAA GAG GTC ACT GAG ATG GGT -3'	58.9	Sequenceing
PWF2-S	5'- GCC AAA TGG CGA GAA AGC AAA GGA -3'	60.6	Sequenceing
PWF3-S	5'- CAT TCA CAC CTG TGG ACC AGC -3'	58.5	Sequenceing
PWF4-S	5'- TAC CTG CTG AAT TGA GGA GCC CAT -3'	60.2	Sequenceing
PWR1-S	5'- AGA AGC TGA AGC GGC GAA TCT -3'	59.2	Sequenceing

CHAPTER IV. SUMMARY AND FUTURE DIRECTIONS

SUMMARY

Over the past few decades, oxidative stress and aging has been studied intensively, from the proposal of free-radical theory of aging in 1960's to evidences of increased oxidative stress to protein, lipid and DNA with aging in 1990's, and eventually to investigation of the increased lifespan in the mouse model overexpressing antioxidants in the 21st century (Muller et al., 2007). In recent years, it has become clear that oxidative damage is a major contributor to neuronal loss. Oxidative damage to DNA was found to be accumulated during aging and considered a promoter of neurological diseases (Fraga et al., 1990; Poulsen, 2005). However, the exact mechanism by which oxidative DNA damage might lead to neurodegeneration or neuronal cell death still remains unknown.

Oxidative damage to DNA can cause mutations, genome instability and transcription impairment. Findings from transcription profiling of genes from young and aged brains reveals that there are a set of genes whose promoters are selectively damaged in an age-dependent fashion resulting in reduced expression (Lu et al., 2004). Most of these genes play roles in synaptic plasticity, vesicular transport and mitochondrial function, similar to the reported functional role for p62 (Moscat et al., 2007), which is a cyotsolic protein with multiple domains, serving as a scaffold in various cell signaling

and receptor trafficking (Wooten et al., 2006). P62 is oxidatively-induced, and has been localized to aggresomes of various neurodegenerative diseases (Ishii et al., 1997; Zatloukal et al., 2002). Declined p62 expression level was observed in AD brains compared to normal brains, and p62 is transcriptionally regulated during the aggregate formation in neurodegenerative disease (Nakaso et al., 2004), therefore the mechanism and consequences of reduced p62 level in neurodegenerative diseases was the major objective of this project. The presence of mutations in the p62 promoter was determined and no genetic variances were found in the p62 promoter between normal and diseased brains. However, higher 8-OHdG levels, a biomarker of DNA oxidative modification, was observed in sections from various neurodegenerative diseases than that from normal brains suggesting depletion of p62 enhanced oxidative stress (Du et al., 2009b). The oxidative damage to the p62 promoter region was profiled in normal brains with different ages, and various neurodegenerative diseased brains. The damage to three amplicons designed within the p62 promoter in normal brains was age-dependent. Oxidative damage to the p62 promoter increased with aging (Du et al., 2009a). Moreover, this damage is significantly higher in various neurodegenerative diseases including AD, PD, HD, FTD and Pick's Disease than normal brains (Du et al., 2009b). In addition, the defect in p62 increased oxidative stress in the brains and promoted cell death. Furthermore, oxidative damage to the p62 promoter was negatively correlated with the p62 expression level in samples from human brain, mouse brain and HEK cells (Du et al., 2009a). Since oxidative damage to the transcriptional element might abolish the binding of some transcription factors like Sp-1, or NF-κB (Ghosh and Mitchell, 1999), leading to decreased protein expression, p62 promoter activity was examined under oxidative stress.

Oxidative damage to the p62 promoter decreased the promoter activity. Also, the p62 promoter with a Sp-1 element deletion failed to be induced by oxidative stress, suggesting oxidative damage to the p62 promoter decreased the p62 promoter activity due to transcriptional element dysfunction (Du et al., 2009b). The level of 8-OHdG in sections from p62 $^{-/-}$ mice was also elevated compared to sections from wild type mice. Moreover, the 8-OHdG level was increased in age-dependent manner both in wild type and p62 $^{-/-}$ mice. Cell viability of WT MEF cells were significantly higher than p62 $^{-/-}$ cells upon H_2O_2 treatment suggesting p62 protects cells from oxidative damage (Du et al., 2009a). It has been suggested that p62 protects cells against H_2O_2–induced injury through activation of pyruvate dehydrogenase kinase, isozyme 1 (PDK1) and prolonged Akt phosphorylation during the later stages of H_2O_2–induced cell death (Heo and Joung, 2009). Because p62 was reported to activate the antioxidant response element (ARE) and result in nuclear translocation of Nrf2 to regulate antioxidant enzyme expression, Nrf2 level in cytosol was also determined in WT and p62 $^{-/-}$ MEF cells. Nrf2 failed to move from cytosol to nucleus upon H_2O_2 treatment in p62 knock out cells (Du et al., 2009a). Based on these findings, the conclusion could be drawn that oxidative damage to the p62 promoter, leading to decreased p62 promoter activity, results in declined p62 expression, that in turn, causes oxidative stress and cell death involved in development of neurodegenerative disease.

Besides oxidative damage to the promoter, p62 level could be regulated by many other factors like proteasome inhibition, autophagy inactivation, and tumorigenesis. P62 has been identified as a major component of ubiquitinated protein aggregates in various neurodegenerative diseases (Zatloukal et al., 2002; Kuusisto et al., 2001). Actually, p62

did not always co-localize with ubiquitin in the early stage of aggregate formation, but it did in later stages to protect cells from death (Nakaso et al., 2004). This is consistent with the findings that mutant huntingtin aggregation was affected by p62 with UPS flux inhibition in later stage rather than through seeding of aggregates in early stage (Korolchuk et al., 2009). The soluble oligomers of abnormal proteins are harmful and generate ROS to mediate oxidative stress (Tabner et al., 2005). Also, they are formed before formation of harmless aggregates, which induce detoxifying enzyme like GST, NAD(P)H dehydrogenase quinine 1 (Nqo1), and cytochrome P450 families in liver (Tabner et al., 2005; Komatsu et al., 2007b). p62 is required for formation of most ubiquitinated aggregates (Wooten et al., 2006; Nezis et al., 2008), and these aggregates are significantly reduced when p62 is absent (Korolchuk et al., 2009) because p62 binds ubiquitinated protein with UBA domain and oligomerizes with the PB1 domain (Bjørkøy et al., 2005).

There are two main pathways for p62 and ubiquitin positive aggregates clearance: the ubiquitin-proteasome system (UPS) and the autophagy–lysosome pathway. The UPS predominantly degrades short-lived proteins and misfolded proteins, which is usually modified with polyubiquitin. Macroautophagy (referred to as autophagy) is a pathway to engulf cytoplasm, organelles and long-lived proteins in a double-layered membrane vesicle called autophagosome, which in turn fuse with lysosome to degrade the sequestered contents by hydrolytic enzymes. P62 acts as a shuttle protein for targeting protein with K63-linked polyubiquitin chains for proteasome degradation (Seibenhener et al., 2004). However, p62-containing aggregates can also be degraded by autophagy (Bjørkøy et al., 2005). To date, the mechanism involved in sorting of proteins to these

two pathways still remains unclear. Some proteins can be degraded by both proteasome and autophagy like α-synuclein, which form aggregates in PD (Webb et al., 2003). Recent studies suggest that there is a cross-talk between proteasome and lysosome pathway. The proteasome is a target and a regulator of oxidative stress, and the activity of proteasome decreased during aging and in age-related neurodegenerative diseases (Ding et al., 2006; Cecarini et al., 2007). Inhibition of proteasome upregulates p62 expression at the transcriptional level during the formation of lewy body in PD (Nakaso et al., 2004). Long term proteasome inhibition causes the induction of autophagy and some genes important to regulating lysosomal activity (Keller et al., 2004). P62 is required for degradation of polyubiquitin-containing aggregates by autophagy. P62 can also bind directly to LC3, which is the autophagic effector protein, by LC3 interacting region (LIR). LIR located N-terminal to the UBA domain in p62 is acidic and contains 22 amino acids including three glutamate and four aspartate residues (Ichimura et al., 2008). There is an evolutionarily conserved motif in this peptide, which is a negatively charged cluster (Asp 335, 336 and 337) followed by a conserved aromatic residue (Try 340), critical for binding with LC3 (Ichimura et al., 2008; Pankiv et al., 2007). Recently, it was found that autophagy inhibition upregulated the level of p62 and accumulated ubiquitinated proteins because p62 turnover is mediated by autophagy, and p62 is a component of ubiquitin positive inclusions in autophagy-deficient hepatocytes and neurons. Also, loss of p62 abolishes inclusion formation, suggesting that inclusion formation is dependent on the presence of p62 in autophagy-deficient hepatocytes and neurons (Komatsu et al., 2007b). Atg7-deficient mice develop neurodegeneration by causing progressive dystrophy, cell-autonomous, and degeneration of the axon terminals followed by Purkinje cell death and

abnormal mouse behavior (Komatsu et al., 2007a). The mechanism for elevated p62 inclusions due to autophagy inhibition has recently been proposed (Korolchuk et al., 2009). They suggested that autophagy inhibition upregulated p62 levels leading to perturbation of the UPS not by inhibition of proteasome activity but by impaired delivery of UPS substrate to proteasome since accumulated proteins after autophagy inhibition are well-defined UPS substrates. Also, defects of p62 almost completely normalized this result, suggesting p62 is the major mediator of the effect (Korolchuk et al., 2009). Another autophagy receptor, next to breast cancer 1 (NBR1), was also identified (Kirkin et al., 2009). NBR1 was found in p62 and ubiquitin-containing inclusions in patients with liver dysfunction and it can be degraded by autophagy independent of p62. Although p62 and NBR1 are poorly conserved at the primary sequence, NBR1 also contains LIR and UBA domains similar to p62. Moreover, the LIR in NBR1 also contains acidic residues followed by an aromatic residue. Both NBR1 and p62 have been proposed to act as receptors for selective autophagosomal degradation of ubiquitinated targets (Kirkin et al., 2009). In addition, p62 expression is also upregulated in breast caner cells by prostate-derived Ets factor (PDEF) (Thompson et al., 2003).

FUTURE DIRECTIONS

These findings revealed that p62 knockout mice showed higher DNA damage and decreased ability for protecting cells from oxidative stress (Du et al., 2009a and 2009b). It is reasonable to propose that overexpression of p62 will compromise the oxidative damage and provide increased protection to cells from oxidative stress. Therefore, the

next objective will examine the oxidative damage in mice with additional copies of the p62 gene and elucidate the mechanism of oxidative stress regulated by p62 expression. p62-overexpressed transgenic mice have already been generated in our lab. Firstly, the DNA oxidative damage will be studied in sections from p62-overexpressed and WT mouse brain by immunostaining for 8-OHdG. P62 is an activator of the antioxidant response element (ARE), and Nrf2 translocates from cytosol to nuclear fraction upon oxidative stress to activate antioxidant enzymes (Liu et al., 2007). The absence or excess of p62 will affect Nrf2 nuclear translocation and expression of antioxidant gene. In order to investigate which genes are involved in oxidative stress regulated by p62 level, PCR Array will be employed to measure the expression of all genes involved in mouse oxidative stress and antioxidant defense. RNA will be isolated from WT, p62 knockout and p62-overexpressed mouse brain to make cDNA. PCR-array plates with oxidative-related genes are commercially available. Real-time PCR will be performed using cDNA from different mice as templates. Data will be analyzed to compare the gene expression in WT, p62 knockout and p62-overexpressed mouse. This will allow for the discovering of novel genes might be found involved in mechanisms of oxidative stress regulated by p62.

Compared to nuclear DNA, the oxidative damage to mitochondrial DNA (mtDNA) is tenfold severe because mitochondria are the major source of ROS. Oxidative damage to mtDNA will cause mtDNA mutation, deletion and impaired mitochondria integrity (Muller et al., 2007). Many studies have demonstrated that mitochondrial DNA damage increased with aging and is associated with various neurodegenerative diseases (Chan, 2006; Yang et al., 2008), and mitochondrial integrity declines with aging (Balaban et al.,

2005). So far, the mechanism of oxidative damage causing neuronal cell death is still not clear. It was suggested that oxidative DNA damage in neurodegenerative disease might be caused by mitochondrial dysfunction (Trushina and McMurray, 2007). Therefore, other future work is to examine the relationship between defects in mitochondrial functions and p62. The mitochondrial DNA copy number, oxidative damage to mitochondrial DNA, ATP generation, and expression of mitochondrial proteins in WT, p62 knockout, and p62-overexpressed mice will be examined. The copy number and oxidative damage to mitochondrial DNA will be examined by amilfication of a specific short fragment (91 bp) and a long fragment (10 Kb) on mouse mitochondrial DNA. Because the possibility of damage in the long fragment is very high, but the possibility of damage in the short fragment is lower, the PCR product of long fragment represents the oxidative damage in mitochondrial DNA, but the PCR product of short fragment represents the mitochondria copy number. The relative damage in the mitochondrial DNA will be calculated by normalizing the PCR product of long fragment by mitochondria copy number (Acevedo-Torres et al., 2009). ATP generation will be measured by ATP determination kit (Invitrogen, Eugene, OR) and the expression of mitochondrial proteins will be evaluated by western blot. These studies will contribute to further understanding of the mitochondria dysfunction due to p62 deficiency.

The expression of p62 could be regulated by oxidative damage to the promoter, as well as, the inhibition of proteasome or autophagy in aging and neurodegenerative disease. The mechanism, by which p62 impacts oxidative stress and mitochondria dysfunction in neurodegenerative disease, will be further investigated.

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