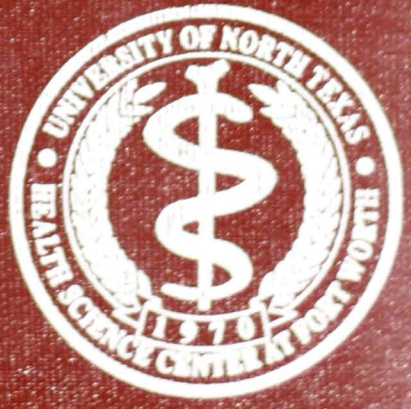
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Identification of oxidized
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Oxidative modification of specific proteins is central to the pathology of Alzheimer's disease (AD). The purpose of this study was to identify the oxidation-sensitive proteins in neuronal cells, fibroblasts from AD subjects, and in the blood of AD patients. In all cases, age-matched non-Alzheimer's samples were used as controls. Proteomic methods were used to isolate and characterize the oxidized proteins. These included two-dimensional gel electrophoresis, immunolocalization of oxidized proteins and identification by MALDI-TOF mass spectroscopic methods.

It was hypothesized that knowledge of these critical oxidation-sensitive proteins would shed light on the underlying mechanism of the disease. In addition, it was postulated that these proteins might prove to be biomarkers for early detection and monitoring the progress of the disease.

The results show that two different oxidative stressors (H_2O_2 generated enzymatically, or the amyloid beta peptide, $A\beta_{25-35}$) induce apoptotic cell death and oxidation of specific proteins (heat shock protein 60 and vimentin) in skin fibroblasts from AD subjects and in neuronal cells. In addition, the results indicate that susceptibility of these two proteins to oxidative stress is increased in fibroblasts from AD patients, compared to non-AD controls. Pretreatment with antioxidants (e.g., vitamin E or flavonoids) protect these proteins from oxidative damage. Both heat shock protein 60 and vimentin, have been

suggested to function as antiapoptotic proteins. Thus, their oxidative damage could lead to the apoptotic neuronal cell death in Alzheimer's disease.

In the blood plasma of AD subjects, isoforms of fibrinogen gamma chain and alpha-1 antitrypsin were found to be oxidized. These proteins exhibited a two- to six-fold greater specific oxidation index in plasma from AD subjects when compared to controls. Both these proteins have been suggested to be implicated in oxidation-mediated damage of inflammation in the AD brain.

IDENTIFICATION OF OXIDIZED PROTEINS IN ALZHEIMER'S DISEASE

Joungil Choi, B.S., M.S.

APPROVED:

Major Professor

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IDENTIFICATION OF OXIDIZED PROTEINS IN ALZHEIMER'S DISEASE

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas
Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Joungil Choi, B.S., M.S.
Fort Worth, Texas
August, 2002

ACKNOWLEDGEMENTS

I would like to express my gratitude and sincere appreciation to those who assisted me to succeed to accomplish my dream:

To my advisors, Dr Robert.R, Gracy for his help, guidance support for my research.

To Dr. Conrad, C.C. for his encouragement, friendship, development of original idea for this project.

To the other faculty of the Department of Molecular Biology and Immunology for serving as my committee members, Drs. B. Harris, A Lacko, E. Isenberg, M.C. Wu, and M. Smith.

To my husband, Kyu-Sang Jung, my son, Ji-Yul Jung, my parents, Kyu-Whan Choi and Suk-Jae Lee, my parents –in-law, Sung-Do Jung and Malye Lee, for their love, support, patience and encouragement.

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ABBREVIATIONS

Alpha-2-Macroglobulin (**A2M**)
Alzheimer's Disease (**AD**)
Amyloid Beta Peptide (**Aβ**)
Amyloid Precursor Protein (**APP**)
Apolipoprotein E (**ApoE**)
Bicinchoninic acid (**BCA**)
Computerized Cooled Digital camera(**CCD**)
Cyclin-Dependent-Kinase 5 (**cdk5**)
Cytochrome c (**Cyto c**)
Dulbecco's Modified Eagle Medium (**DMEM**)
2,4-dinitrophenol (**DNP**)
2,4-dinitrophenylhydrazine (**DNPH**)
Electrospray ionization (**ESI**)
Familial Alzheimer's Disease (**FAD**)
Fetal Bovine Serum (**FBS**)
Glucose oxidase (**GO**)
Heat shock protein 60 (**HSP 60**)
High performance liquid chromatography (**HPLC**)
Horseradish Peroxidase (**HRP**)
Hydrogen Peroxide (**H_2O_2**)
Integrin Associated Protein (**IAP**)
Immobilized pH Gradient (**IPG**)
Matrix-assisted-laser-desorption and Ionization mass spectrometry time of flight (**MALDI-TOF**)
3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (**MTS**)
NF-κB Inducing Kinase (**NIK**)
Neurofibrillary Tangles (**NFT**)
One-dimensional Polyacrylamide Gel Electrophoresis (**1-D PAGE**)
Phenazine methosulfate (**PMS**)
Phosphate Buffered Saline (**PBS**)
Polyvinylidene difluoride (**PVDF**)
Presenilin-1 (**PS1**)
Presenilin-2 (**PS2**)
Reactive Oxygen Species (**ROS**)
Scutellaria baicalensis Georgi Extract (**SbE**)
Sodium Dodecyl Sulfate (**SDS**)
Two-dimensional polyacrylamide gel electrophoresis (**2-D PAGE**)
Tumor Necrosis Factor (**TNF**)
Transferase mediated dUTP nick end labeling (**TUNEL**)

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INTRODUCTION

Alzheimer's Disease (AD) is the most common cause of dementia-type neurodegenerative disease and was first diagnosed by the German psychiatrist, Alois Alzheimer in 1906. The clinical features of AD include progressive memory loss, change in behavior and personality, and impairments in insight, judgment, and other cognitive functions. Eventually, Alzheimer's patients become completely dependent on a caregiver and develop other illnesses and/or infections. Most AD patients die of pneumonia at the final stage of disease [99]. The pathological features of Alzheimer's disease are the presence of extracellular senile plaques and intracellular neurofibrillary tangles, massive loss of neurons, and altered neurotransmitters in selected brain regions [61, 121].

No one yet knows exactly what causes Alzheimer's disease. Although aging is a major risk factor for AD, dementia is not a part of normal aging. In the normal aging brain, the numbers of changes occur modestly: (1) only some neurons die, but not massively; (2) mitochondria become more susceptible to damage; (3) inflammation increases; (4) oxidative stress increases. However, in AD brain, some of these changes are much more severe and result in dramatic loss of neurons and cognitive function [21, 86, 91, 99, 104].

Currently, AD research is divided into three areas: diagnosis, cause/risk factors, and treatment of disease. The only way to definitively diagnose AD is from the presence of amyloid senile plaque and neurofibrillary tangles at autopsy. As a result, clinical diagnosis as 'probable AD' totally depends on a thorough examination of medical

conditions, psychiatric symptoms, and medical history. Although clinical diagnosis can achieve 75-80% accuracy, by this stage of the disease it is already irreversible and no effective treatment for AD is available so far.

Four million Americans suffer from this illness, and one of 20 adults aged 65 and older are afflicted. The incidence of the disease increases dramatically with age. For example, approximately half of the individuals over the age of 85 are reported to have dementia-most of which appears to be AD. AD has become the 4th leading cause of death in the United States and is one of the most expensive diseases. The annual cost of caring for one AD patient ranges from $20, 000 to $36, 000 depending on the stage of disease, and the cost of caring for AD patients in the U.S. exceeds $100 billion annually [43]. As a result of the increase in life expectancy, 14 million Americans are expected to have Alzheimer's disease by the middle of this century. The cost for care of Alzheimer's patients will be unsustainable [99].

Etiology of Alzheimer's Disease

There are two general categories of AD, familial (early-onset, < 65 years old) and sporadic (late-onset, > 65 years old). The familial AD (FAD, comprising less than 5% of total AD cases) is caused by genetic mutation of genes coding for the amyloid precursor protein (APP), presenilin 1 (PS-1), or presenilin 2 (PS-2). These mutations result in an increased production of amyloid beta protein (Aβ) in the senile plaque [120]. Although the APOE e4 allele of the APOE gene has been identified as a major risk factor for sporadic AD (comprising >95% of total AD cases), there are no known genetic mutations related to this type of AD (Table 1) [91].

Amyloid Precursor Protein (APP)

APP is a type-I transmembrane glycoprotein encoded by a gene on chromosome 21. It is composed of a 590-680 amino acid long extracellular amino terminal domain, a 23 residue transmembrane domain, and an approximately 55 amino acid cytoplasmic tail that contains an intracellular trafficking signal. There are three major isoforms of APPs (APP695, APP751, and APP750) in the central nervous system, which are produced by alternative mRNA splicing. APP751 contains a Kunitz-protease inhibitor (KPI) domain; and APP770 contains both the KPI domain and an MRC-OX2 antigen domain, in addition to the APP695 sequence [53, 75]. Although APP has been suggested to play a role in the outgrowth or maintenance of nerve terminals, its exact function remains unclear.

APP can be cleaved by α- and γ-secretases (endoproteases), which produce non-amylodogenic fragments. On the other hand, cleavage of APP by β- and γ-secretases generates $A\beta_{1\text{-}40/42}$, which deposits in the senile plaques in the brain of subjects with AD (Fig.2). Several mutations in the APP gene have been found in familial (early-onset) AD (Fig.1) [26]. Each of the FAD mutations in the APP gene alters the frequency of enzymatic cleavage of APP by the secretases (α, β, and γ). Mutations at codon 716 (Florida) and 723 (Australian) cause an increased proportion of γ-secretase cleavage at position 42 or 43 in the amyloid sequence [40, 85]. A mutation found at codons 670 and 671 (Swedish) results in increased β-secretase cleavage, whereas a point mutation at codon 612 (Flemish) inhibits α-secretase cleavage [20, 56]. The consequence of these APP mutations is to increase the processing of APP via the β-secretase and γ-secretase

	Gene	Chromosome	Effects	Reference
Causal	APP	21	Amyloid deposition	[26]
	PS 1	14	Increased production of $A\beta_{1-42}$ Apoptosis	[107]
	PS 2	1	Increased production of $A\beta_{1-42}$ Apoptosis	[107]
Risk	Tau	17	Main component of neurofibrillary tangles	[144]
	APOE	19	Amyloid deposition Oxidative stress	[118]
	Mitochondrial DNA	--	Oxidative stress	[104]
	Alpha-2 macroglobulin	12	Amyloid deposition	[14]
	Synuclein	4	Neurotoxicity	[83]
	IL-1	2	Inflammation	[100]

Table 1. Genes involved in Alzheimer's disease. Mutation in three genes (APP, PS1, and PS2) are linked to early-onset AD. Other genes are risk factors for late-onset AD. Abbreviations: amyloid precursor protein (APP), presenilin 1 (PS 1), presenilin 2 (PS 2), apolipoprotein E (APOE), interleukin 1 (IL-1).

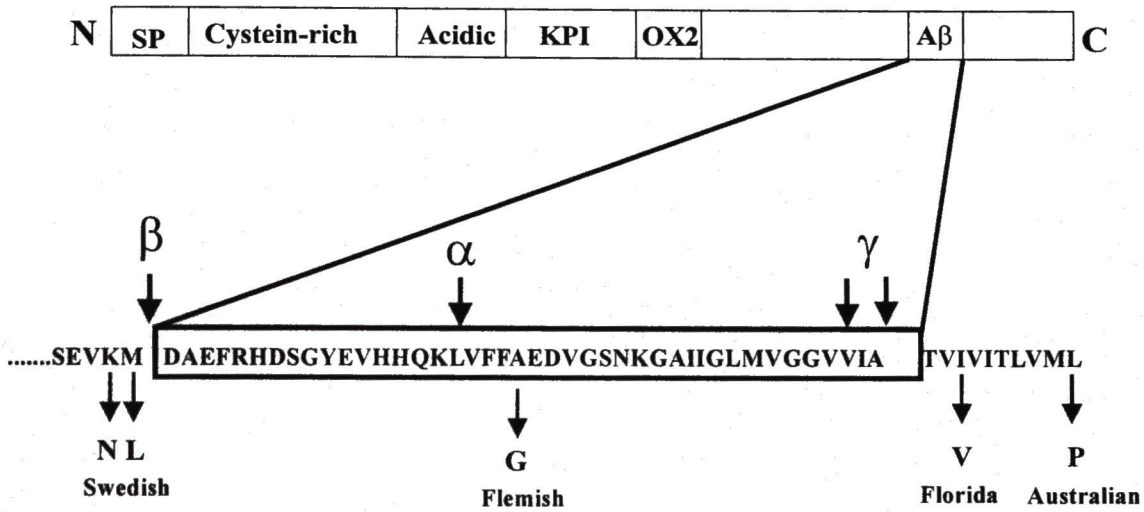


Figure 1. Schematic representation of APP and location of familial-type Alzheimer's disease (FAD)-linked mutations in APP (i.e. Swedish, Flemish, Florida, Australian). All of these mutations cause the increased production of $A\beta_{1\text{-}40/42}$. [107]. The sites of cleavage of the α-, β-, and γ-secretase are also shown. Cleavage by the β and γ secretases generates the amylodgenic $A\beta_{1\text{-}40/42}$ peptide.

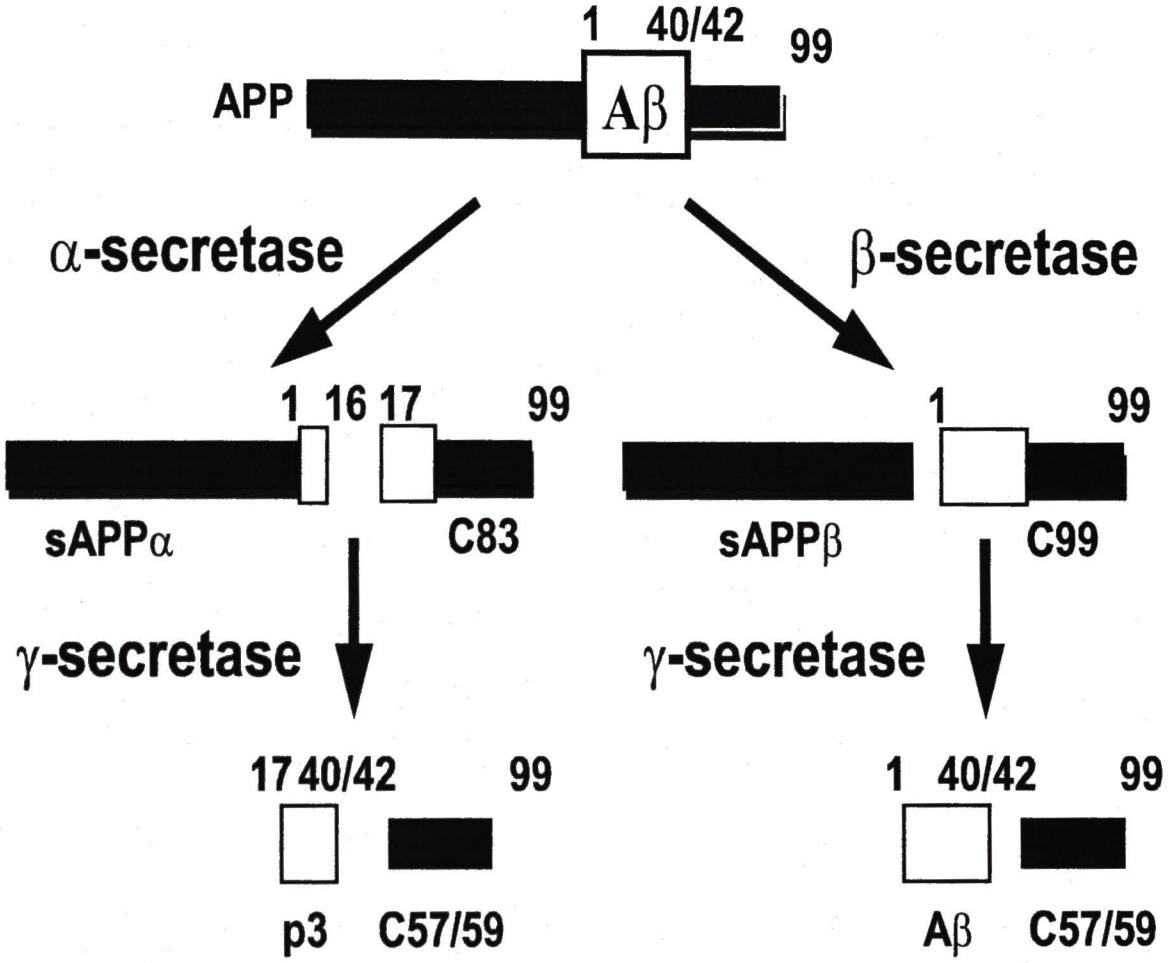


Figure 2. Two alternative processing of APP. In non-amyloidogenic pathways, APP is cleaved by α-secretase, which cleaves the protein in the middle of the amyloid peptide region thereby preventing the production of the neurotoxic peptide, followed by γ-secretase clipping. In amyloidogenic pathways, β-secretase cleaves at the ectodomain of APP followed by intramembranous γ-secretase clipping, which results in the production of the toxic fragment of APP ($A\beta_{1\text{-}40/42}$) that is precipitated in amyloid plaques of AD brain [60].

pathway. This results in increased production of the neurotoxic $A\beta_{1\text{-}40/42}$, which is a major component of senile plaque in the AD brain (Fig.2) [60, 113, 142]. $A\beta_{1\text{-}40/42}$ has been shown to undergo a slow aggregation, which results in the production of fibrils, which appear to be crucial to the neurotoxicity [63, 92, 105-106]. However, the relevance of in vitro models of Aβ toxicity for human AD has not been established.

Presenilins 1 and 2 (PS1 and PS2)

PS1 is a transmembrane protein composed of seven to nine transmembrane domains. PS2, with a similar configuration, contains 60% sequence identity with PS1, with the exception of the cytoplasmic domain, which shows < 10 % identity. The mutations in the genes that code for PS1 and PS2 account for almost 40% of cases of familial AD [107]. One of the definitive effects of PS1 and PS2 mutations is an alteration of APP processing, favoring the production of the toxic fragment of APP, i.e. $A\beta_{1\text{-}40/42}$ [120]. Although the precise function of PS has been unknown, a study of PS1-knockout mice has reported a defect in γ-secretase [45].

Tau

Neurons, and in particular axons, use microtubules to transport substances between the center of the neuron and its outer portions. The assembly and structural integrity of microtubules are dependent upon several proteins called 'tau'. Microtubules in the neurites of neurons are reinforced by tau proteins that hold it together like the crosspieces on a stretch of railroad track. When tau protein is abnormally phosphorylated, it cannot perform its function; thus microtubules start to disintegrate.

The neuritis then shrinks and the whole neuron withers and dies [89].

Hyperphosphorylated tau is the main component of neurofibrillary tangles, of which the number and distribution are correlated with the degree of dementia in AD [43, 144]. Mutations in tau cause front-temporal dementia, along with Parkinsonism associated with chrosome 17 (FTDP-17), suggesting that aberrant forms of tau can give rise to neurodegeneration [69, 144]. However, tau mutations have not been found in cases of Alzheimer's disease. It has been suggested that activation of protein kinase cdk5 by the increased calcium concentration caused by Aβ deposits may contribute to both tau phosphorylation and neuronal apoptosis [89].

APOE

The e4 allele of the APOE gene has been identified as a major risk factor for the sporadic (late-onset) AD [110]. APOE is a unique apolipoprotein in terms of having a special relevance to neuronal tissue. It is involved in the mobilization and redistribution of cholesterol and phospholipid during membrane remodeling associated with synaptic plasticity in the central nervous system.

Three major isoforms of ApoE (e4, e3, and e2) are expressed from multiple alleles at a single ApoE genetic locus (chromosome 19). These isoforms differ from each other by cysteine-arginine interchanges at positions 112 and 158. Lipoproteins associated with the e4 isoform are cleared more efficiently than the ones containing e3 and e2. A recent study of transgenic mice with the human e4 allele showed the increased Aβ deposit, as well as a large increase in the amount of fibrillar Aβ compared to the mice with the human e3 gene.

Although the mechanism by which the e4 alleles impose a major risk for AD is still unknown, it appears to be related to its decreased binding of Aβ [67, 110, 118, 132].

Oxidative Stress

Reactive oxygen species (ROS) can arise from both enzymatic and nonenzymatic sources in biological systems (Table 2) [27]. Under normal conditions, there is a delicate balance between production and elimination of reactive oxygen species inside cells. However, a disturbance of oxidative balance occurs under pathological conditions; so that ROS production exceeds the cellular antioxidant defensive ability and oxidative damage of cellular components, including DNA, protein, lipid, etc, occurs [22, 44, 55]. Many ROS are free radicals, which have unpaired electrons, and are thus extremely reactive. Inside cells, many ROS (e.g. H_2O_2) can modify a variety of cellular components in the presence of other cofactors, such as certain metal ions [27, 38, 130].

Oxidative Stress in AD

One of the theories of the etiology of both familial and sporadic Alzheimer's disease involves oxidative stress [90, 126]. Oxidative damage is not unique to the brain. However, the brain is particularly vulnerable to oxidative processes because of its high glucose-driven metabolic rate; the presence of low levels of antioxidant defense enzymes; its high concentrations of polyunsaturated fatty acids, which are potential substrates for lipid peroxidation; and its high contents of transition metals [43].

There is a great deal of evidence showing the importance of oxidative stress to the pathogenesis of AD: (1) Iron is increased in two major neuropathological regions, senile

Cellular ROS Production	Cellular Antioxidant Defense System
(A) Enzymatic Sources	
1. Xanthine oxidase	1. Cu/Zn-SOD (cytosol)
2. Monoamine oxidase	2. Mn-SOD (mitochondria)
3. Mitochondrial electron transport	3. GSH peroxidase
4. Nitric oxide synthase	4. GSH-S-transferase
5. Activated macrophages	5. GSSG reductase
	7. Catalase
	8. Ascorbate
	9. GSH
(B) Nonenzymatic Sources	10.Urate
	11.Catecholamines

Table 2. Major cellular sources of oxyradicals, other oxidizing species, and cellular antioxidant defense system [27].

plaques and neurofibrillary tangles. Iron is a major cofactor for production of the most reactive ROS, the hydroxyl radical from hydrogen peroxide [56]. (2) Aβ has been shown to generate ROS directly [9]. (3) Abnormalities in the mitochondrial genome or deficiencies in key metabolic enzymes (e.g. cytochrome-c oxidase), which are a major source of hydrogen peroxide inside cells, have been reported in AD brain [32]. (4) Increased protein oxidation, measured by carbonyl content, has been observed in AD brain [124].

It is believed that beta-amyloid-induced oxidative stress plays a central role in AD pathology [13, 64, 95, 156]. Reactive Oxygen Species (ROS) are generated from the environment, and from cells in the brain (e.g. inflammation), and lead to the aggregation of the amyloid beta peptide (Aβ). These aggregates generate further ROS, cause oxidative damage to other proteins, and leads to the pathological cascade that ultimately results in neuronal apoptosis and dementia. Aβ has been shown to cause both neuronal membrane peroxidation and protein oxidation, and its toxicity is prevented by antioxidants and by catalase that degrades hydrogen peroxide [8-9, 18]. Although the neurotoxic activity of Aβ has been shown to be mediated by production of hydrogen peroxide and other ROS [9, 62, 125], the cellular targets of Aβ-mediated oxidations remain unclear.

Antioxidants

As a consequence of the involvement of oxidative stress in the pathogenesis of AD, many different antioxidants for treatment or prevention of this disease have been suggested.

These include both fat and water soluble vitamins such as E and C, many botanical organics (ginkgo biloba, melatonin, flavonoids carotenoids) and hormones (e.g. estrogen) [3, 8, 48, 81, 114, 117]. In studies with Aβ, H_2O_2, and glutamate, antioxidants protected neuronal cells from oxidative stress-induced cell death [8, 57]. In clinical trials of AD patients with moderately severe impairment, vitamin E slowed the progression of the disease [99].

Protein Oxidation in AD

In both sporadic and familial AD, oxidative damage to proteins appears to play a central role in the pathology [127-129]. Oxidative modification of proteins may have several consequences: (1) The damaged protein may remain in the organelle and disrupt the normal function. For example, if a protein in the mitochondrial respiratory chain is damaged, the flow of electrons may be interrupted and cause massive radical production instead of ATP. (2) The removal of damaged molecules involved in signaling processes (e.g. NFκB) disrupts the intracellular signaling cascade. (3) The inability to remove damaged proteins results in the buildup of inactive material that increase the solute pool. This increases the osmotic pressure and molarity of the cytosol. Therefore, the cell has to work harder to maintain its shape and function. (4) It is also possible that there will be either no change in message expression for replacement of this protein or a decrease in message expression, depending on how the system is regulated. (5) Oxidatively modified proteins may form aggregates, which are not removed by the proteasome and thus may accumulate and exhibit toxic properties.

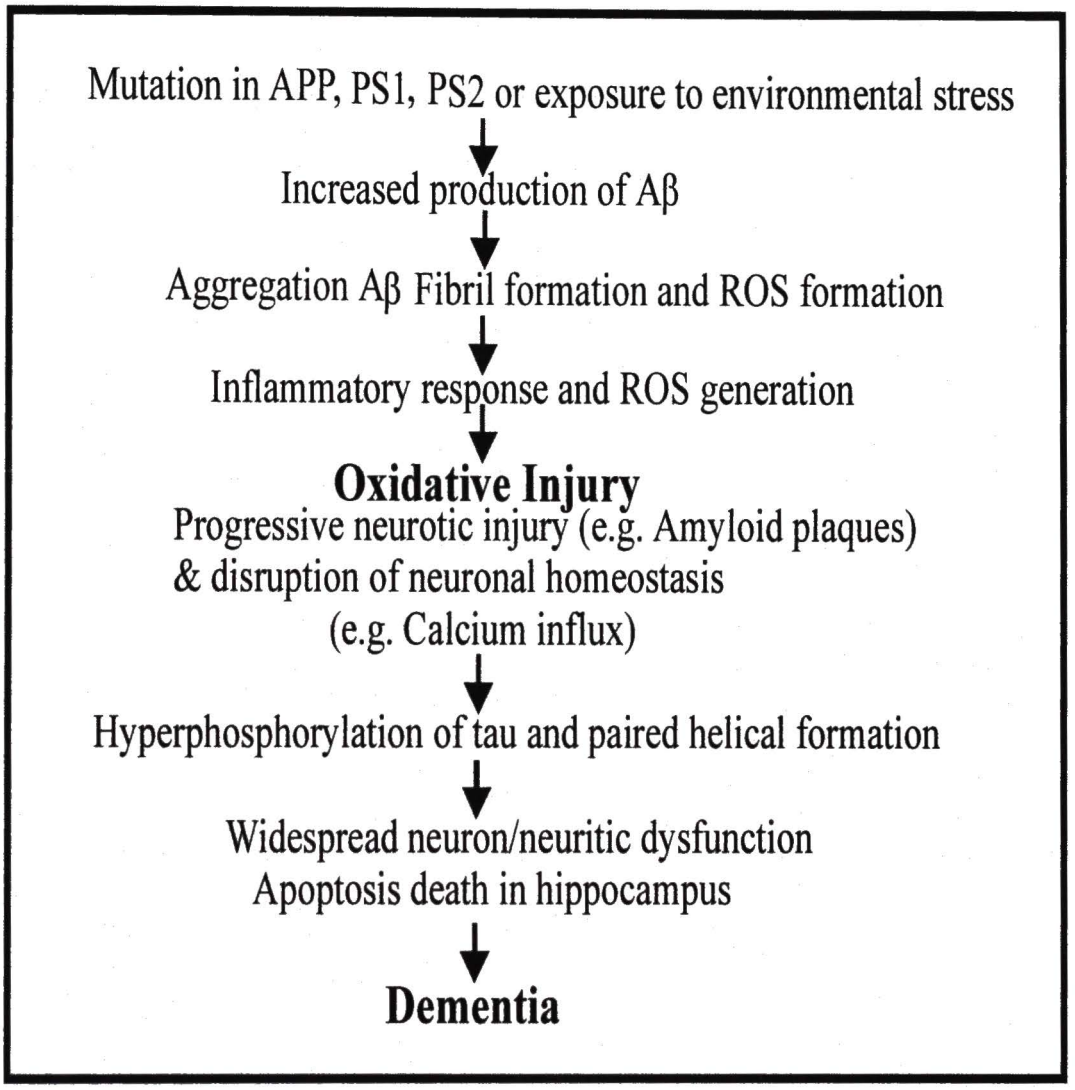


Figure 3. Hypothetical pathological cascade occurred in Alzheimer's disease. Genetic mutation and environmental stress can lead to Aβ deposition, which cause the production of reactive oxygen species, thus apoptotic cell death of neurons and dementia [64, 118].

Oxidative damage to proteins can occur via different mechanisms and result in peptide cleavage, cross-linking, and/or the modification of the side chain of virtually every amino acid [127]. However, not all proteins are equally sensitive to oxidative damage, and oxidation susceptibility depends on the structure of the protein (e.g. sequence motifs and environmentally exposed residues) and bound ligands (e.g., metal atoms). For example, glutamine synthetase and alcohol dehydrogenase are oxidation sensitive, but hexokinase is highly resistant to in vitro oxidation [13, 122].

Evidence in both prokaryotes and eukaryotes suggests that oxidation sensitive proteins (OSP) may act as second messengers activating or inhibiting transcription factors and other regulatory elements [37,66, 134]. In addition, OSPs may provide an antioxidant "buffer" or defense from reactive oxygen species (ROS) by protecting other more "critical" proteins from oxidative damage [122]. Thus, the ability to identify specific OSPs should provide insight to understanding mechanisms of the age related diseases resulting from chronic oxidative stress.

Detection of Protein Oxidation

Protein carbonylation is an irreversible oxidative modification of proteins. The number of carbonyl groups within a protein correlates with protein damage caused by oxidative stress [87]. Carbonyl groups are composed of stable C=O functional groups (e.g., ketones, aldehydes, carboxylic acids, and esters) [112]. Protein carbonyls are the principal products of metal-catalyzed oxidation of proteins. Carbonyls are not the only oxidation products formed from exposure to ROS, but they can be easily detected by derivatizing with 2,4-dinitrophenylhydrazine (DNPH).

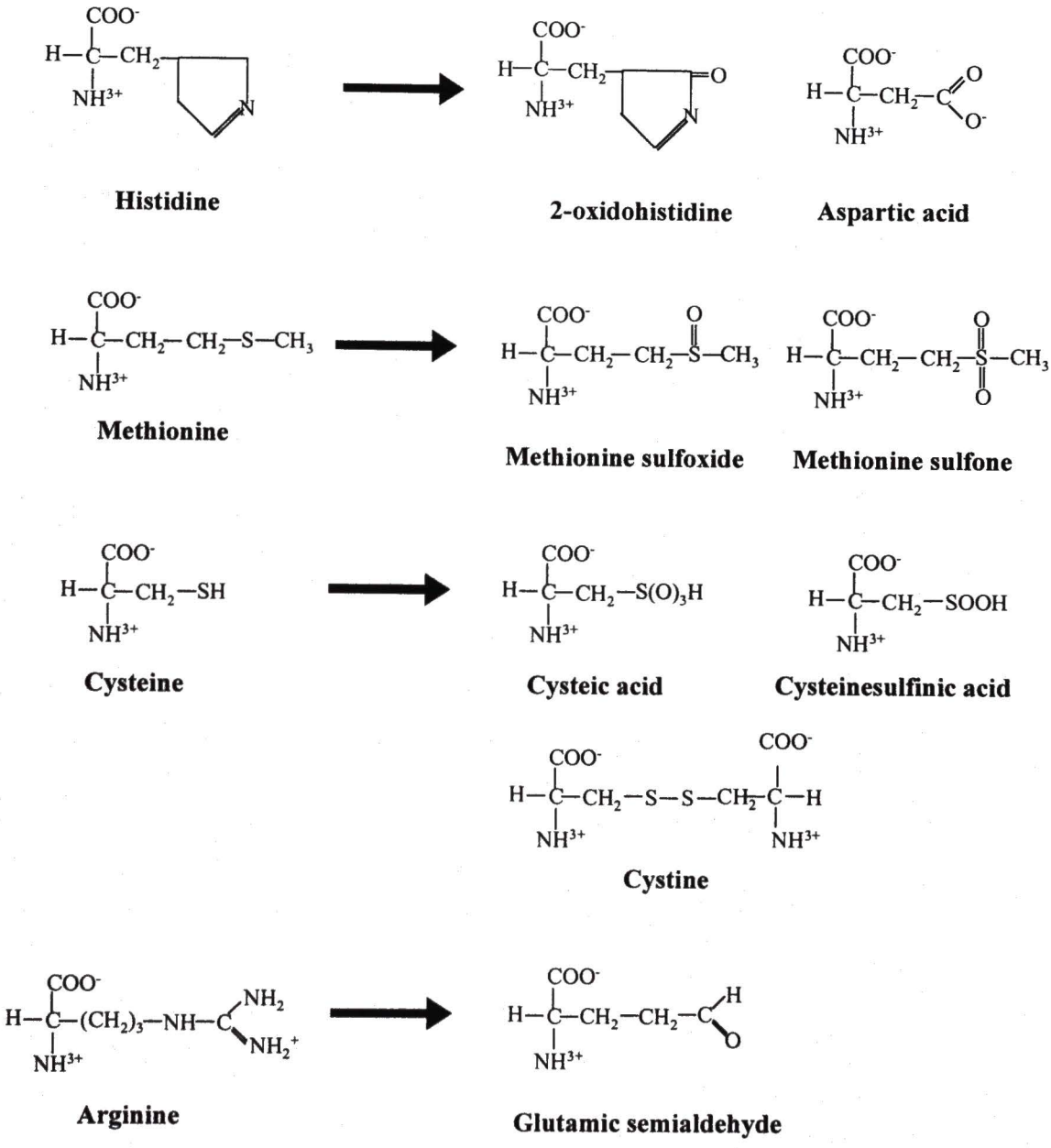
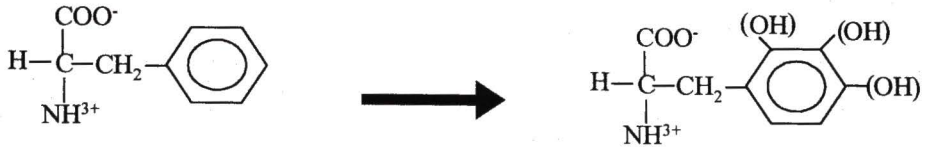
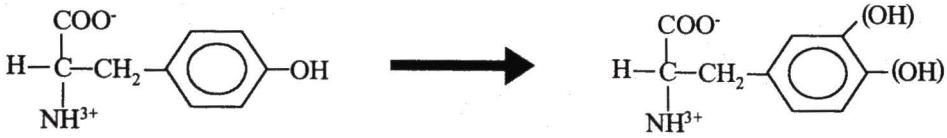


Figure 4. Amino acid modifications that may occur during protein oxidation [129].



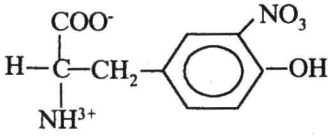
Phenyalanine

2-, 3-, 4-hydroxyphenylalanine

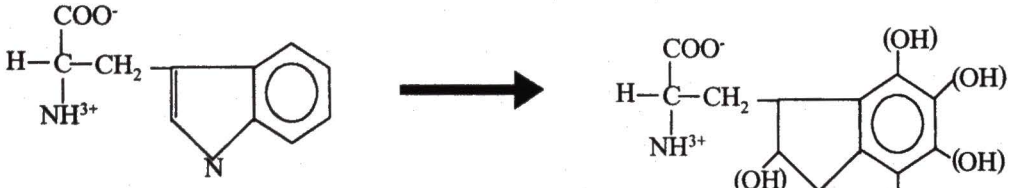


Tyrosine

3-, 4-dihydroxyphenylalanine

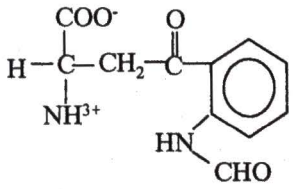


3-nitrotyrosine



Tryptophan

2-, 4-, 5-, 6-, 7-hydroxytryptophan



Formal kynurenine

Figure 4. Amino acid modifications that may occur during protein oxidation [129] (continued)

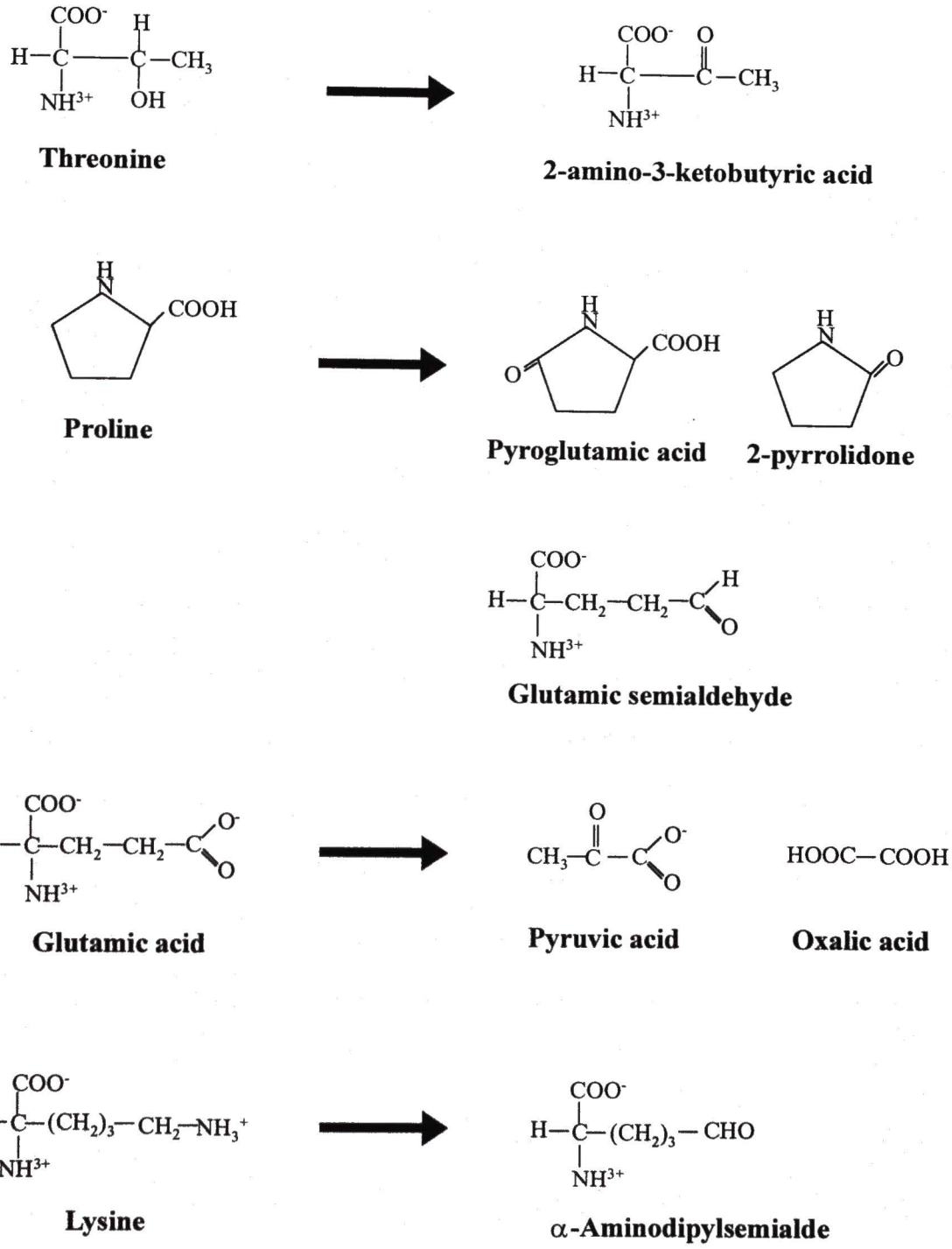


Figure 4. Amino acid modifications that may occur during protein oxidation [129]. (continued)

High-resolution two-dimensional polyacrylamide gel electrophoresis (2-DE) of the DNP-tagged oxidized proteins can be utilized to identify specific OSPs [31].

Apoptosis in Alzheimer's Disease

Massive neuronal cell death in regions of the brain that are essential to learning, memory, attention, and judgment characterize AD [34, 135]. Therefore, an important focus of research into the etiology of AD has focused on the mechanisms of neuronal apoptosis. A leading hypothesis is that neuronal apoptosis is mediated by neurotoxicity of $A\beta_{1\text{-}40/42}$. Although the mechanism by which Aβ induces apoptotic cell death remains unclear, it could involve excitoxicity, oxidative stress and the formation of free radicals, or competition for the low-affinity nerve growth factor receptor [18, 57]. Apoptosis is believed to have several different initiation points and propagation pathways, but key events focus on mitochondrial function. Pro- and anti-apoptotic mitochondrial signals trigger or inhibit generation of ROS and their related downstream effects. These events include changes in electron transport, loss of mitochondrial transmembrane potential, and release of cytochrome c (i.e. caspase activator), which culminate to alter the redox state of the cell [101].

A number of molecules that regulate neuronal apoptosis have been identified. Of particular importance are the Bcl-2 family of proteins and caspases that cleave polypeptides at specific sites following aspartate residues [79, 101, 103]. Both the Bcl-2 and the caspase family of apoptosis modulators are regulated by the redox state of the cell. The Bcl-2 family proteins have a central role in the intracellular apoptotic signaling. This protein family composed of anti-apoptotic (Bcl-2 and Bcl-x_L) and pro-apoptotic

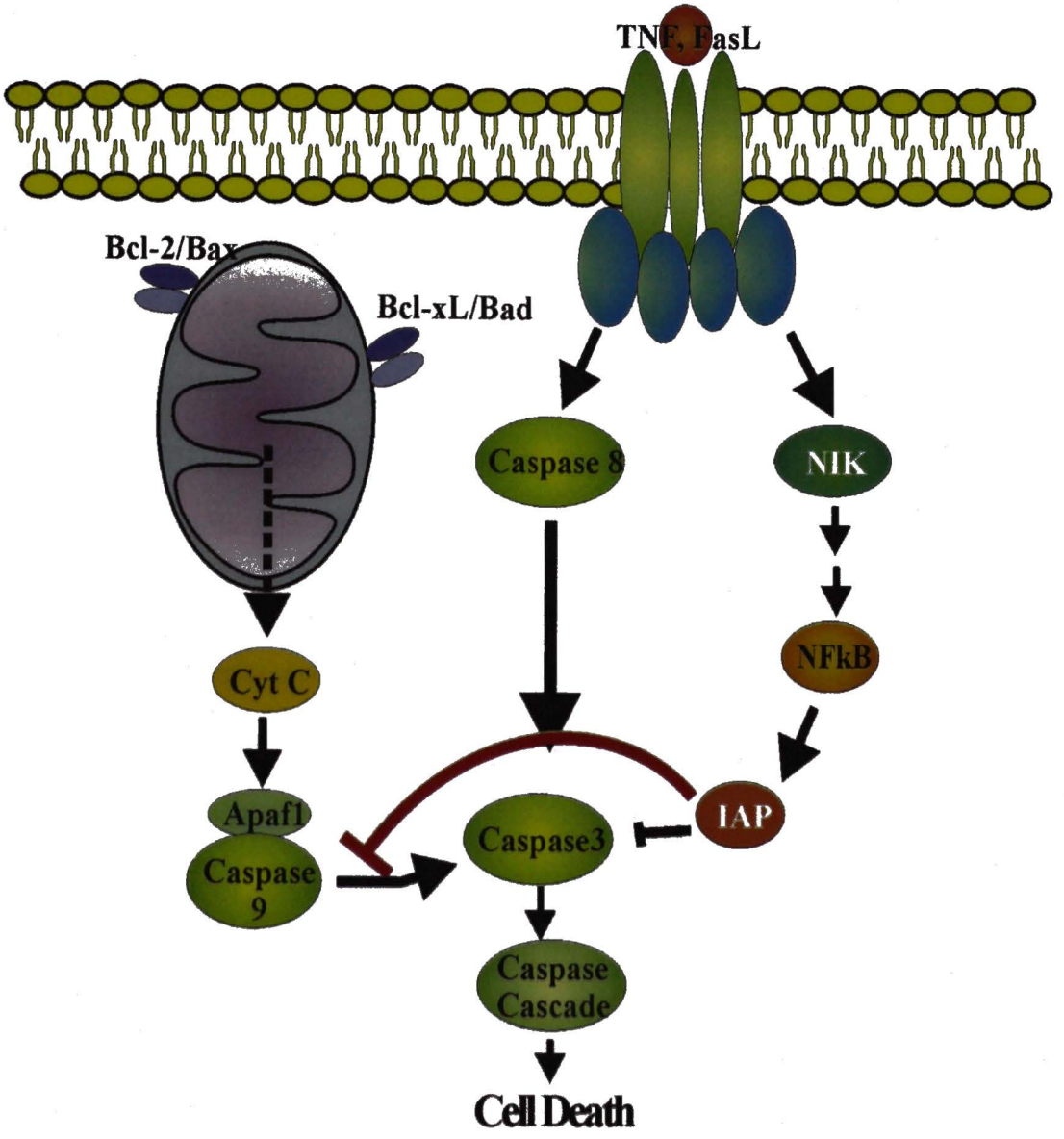


Figure 5. Schematic diagram of apoptotic pathway. In living cells, caspases exist as inactive zymogens that are activated by proteolytic cleavage. There are two pathways that lead to caspase activation, thus cause cell death. One pathway involves death receptors, such as Fas, and a tumor necrosis factor (TNF) receptor at the cell surface, leading to the activation of caspase-8 intracellularly. Activated caspase-8 will cleave and activate other downstream caspases. Another means of caspase activation is through the release of cytochrome *c* from the mitochondria. Released cytochrome *c* forms a multimeric complex with Apaf-1 and caspase-9. Activated caspase-9 will cleave the downstream caspases, thus leads cell death. Abbreviation: NF-κB Inducing Kinase (NIK); Integrin associated proteins (IAP); Cytochrome *c* (Cyt C).

(Bax and Bcl-x_S) proteins that contain one or more Bcl-2 homology (BH) domains [150]. A major regulatory step for caspase activation in the intracellular signaling cascade is the cytochrome ^{c} release from mitochondrial matrix to cytosol. Cytochrome ^{c} release results in not only activation of caspases through the interaction with the Apaf-1, but also damage of the electron transfer chain, thus causing reduced energy generation and increased ROS due to incomplete reduction of oxygen [111]. The release of cytochrome c is regulated by interaction of proteins in the Bcl-2 family (Fig. 5). For example, overexpression of anti-apoptotic Bcl-2, or Bcl-x_L blocks cytochrome ^{c} release induced by a variety of apoptotic stimuli such as oxidative stress [80, 155]. In contrast, Bax have been shown to cause release of cytochrome ^{c} [73]. The mechanism of cytochrome c release by Bcl-2 family proteins remains unknown. One possible mechanism is that changes in mitochondrial membrane permeability induce swelling of mitochondria, thus cause rupture of the mitochondrial outer membrane [82].

Activation of microglial cells, which may be another contributor to neuronal cell death, is a prominent feature of the inflammatory response occurring in the AD brain, [58]. Microglial cells are normally in a resting state, exhibiting a low turnover and no macrophage-like characteristics [5]. However, after activation, microglial cells are clustered within amyloid plaques in the AD brain, exhibit an amoeboid morphology, are phagocytic, and express many macrophage-like pro-inflammatory molecules such as MHC II, cytokines, chemokines, complement, and neurotoxins [133]. It has been known that microglial cells in the AD brain upregulate their expression of the macrophage scavenger receptor and the receptor for advanced glycation end products, both of which appear to have Aβ as ligands [77, 154].

Adhesion of microglia to Aβ fibrils via scavenger receptors leads to immobilization of the cells, thus induces production of reactive oxygen species and activates numerous signaling cascades within microglia that are common to peripheral inflammatory responses [77]. These include tyrosine kinase-based cascades, calcium-dependent activation of the PKC pathway, and ERKs MAP kinase cascades. All of these events lead to the activation of transcription factors responsible for subsequent pro-inflammatory gene expression, the production of ROS through NADPH oxidase, and to the secretion of neurotoxins and excitoxins (e.g. glutamate) [154, 94, 28-29].

Hypothesis and Significance of This Study

The fundamental question "how does Aβ- and ROS- generated oxidative damage lead to neuronal apoptosis in AD?" must be answered before mechanism-based interventions for the disease can be realized. These issues are the focus of the research described in this dissertation. The studies described here test the hypothesis that:

In AD, specific oxidation-sensitive-proteins (OSPs) become oxidized, accumulate, and may play a central role in the apoptotic pathology leading to neuronal cell death. Identification of these OSPs should provide important insight into mechanisms for preventing or treating the disease. In addition, the identity of such AD specific OSPs may yield new diagnostic insight into predicting the onset of the disease or monitoring its progression.

In order to test this hypothesis we have sought to identify the critical OSPs, which occur in three different AD models: (A) hippocampal neuronal cells exposed to oxidative stress, (B) fibroblasts from normal and AD-subjects, and (C) plasma of subjects diagnosed with AD.

MATERIALS & METHODS

Materials

Cell culture media, other cell culture reagents, sodium dodecyl sulfate (SDS); 2,4-dinitrophenylhydrazine (DNPH); urea; acrylamide; HCl; glutaraldehyde; silver nitrate; formaldehyde; Tris-base; and other general chemicals suitable for use with electrophoresis were purchased from either Fisher Scientific (Houston, TX) or VWR International. Amyloid beta protein was obtained from American Peptide Company. Glucose oxidase was supplied by ICN and *Scutellaria baicalensis Georgi* extract was provided by Chun-Su Yuan from the University of Chicago. Anti-DNP antibody and goat-anti-rabbit secondary antibody were supplied by Molecular Probes and Sigma respectively. Anti-Bcl_2 and anti-Bax antibodies were supplied by Santa Cruz Company.

Cell Culture

HT-22 hippocampal cell lines were obtained from Dr. David Schubert at the Salk Institute (San Diego, CA). These cells were cultured as described previously [139] using standard Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) fetal bovine sera and 2.5% (w/v) gentomycin. Cells were maintained in T-25 flasks until they were confluent and ready for use.

Human skin fibroblasts were obtained from Coriell Cell Repository (Camden, NJ). Fibroblasts of AD were obtained from autopsy-confirmed Alzheimer's disease patients. These cells were cultured as described previously [139] using standard

Dulbecco's Modified Eagle Medium (DMEM) with 10% (w/v) fetal bovine sera and 2.5% (w/v) gentomycin. Cells were maintained in T-75 flasks until they were confluent and ready for use.

Subjects & Blood Samples

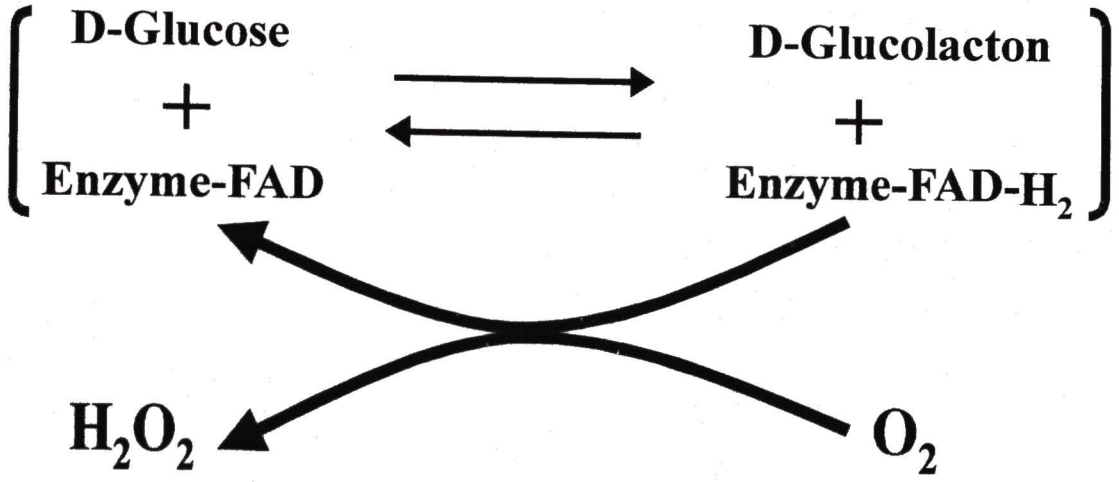
Blood samples were collected from patients diagnosed with AD aged 70-89 (n=9); and healthy, elderly, non-demented controls aged 74-89 (n=9). Of the AD subjects, two were confirmed to have Alzheimer's by neuropathology at autopsy. The others were clinically diagnosed with Alzheimer's. Blood plasma was collected into "lavender cap" (K_3) EDTA Vaccutainer tubes, centrifuged for 15 min, and separated from pelleted erythrocytes through serum separators (Fisher Scientific, Houston, TX). Plasma protein concentrations were determined using the BCA protein assay kit from Pierce.

Generation of Reactive Oxygen Species

Glucose Oxidase

Glucose oxidase (GO) from *Aspergillus niger* (ICN, Aurora, OH) was used to generate a continuous production of hydrogen peroxide (approximately 1 mU H_2O_2 per min) [17, 76]. The amount of hydrogen peroxide generated by the action of glucose oxidase in Buffer A (PBS containing 5.0 mM glucose) was estimated by following the oxidation of *o*-dianisidine in the presence of horseradish peroxidase at 436 nm. Cells were exposed to increasing concentrations of glucose oxidase (0-12 mUnits total) for various time periods (0-24 hours) to determine the optimal conditions for the oxidation

reaction. Cell viability, apoptosis, and protein oxidation were then determined (see below).



Generation of hydrogen peroxide by enzymatic reaction of glucose oxidase

Beta-Amyloid

$A\beta_{25\text{-}35}$ peptide was dissolved in sterile water to a concentration of 1 mg/ml and diluted with media to the desired concentration. For survival assays, cells were plated in 30mm plates at a density of 125,000 cells per plate the day before. For 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE), cells were plated in 100mm culture dishes at a density of 2 million cells per plate.

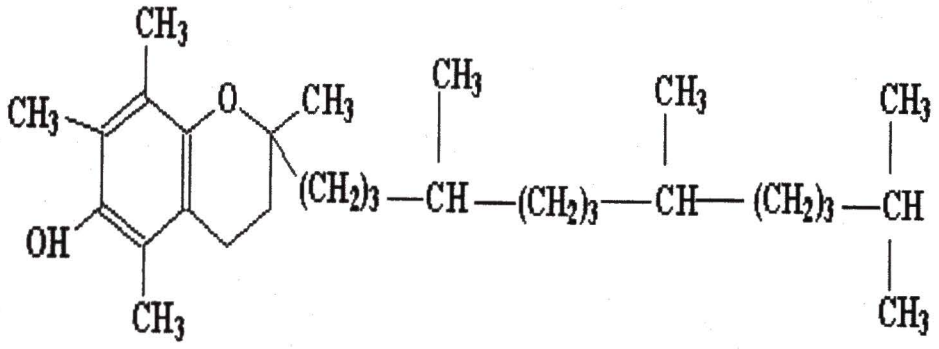
N Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Gly-Leu-Met C

Sequences of $A\beta_{25\text{-}35}$

Antioxidant Treatment

Vitamin E (Vit E)

A 10X stock (10 mM) was prepared by dissolving Vit E in 100% ethanol. Subsequent dilutions were prepared in cell culture media. Control cells treated with a similar concentration of ethanol without Vit E did not affect cell viability. Cells were pretreated with Vit E for a minimum of 12 hours prior to oxidative insult.

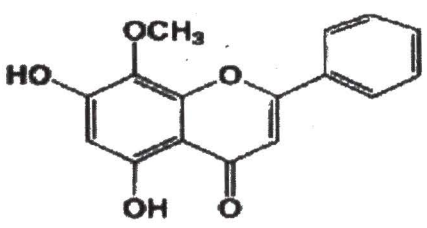


Structure of Vitamin E

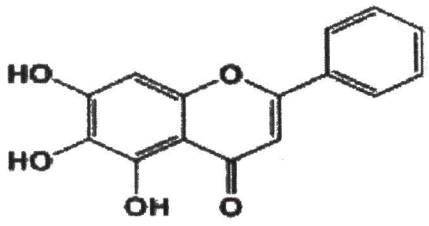
Scutellaria baicalensis Georgi Extract (SbE)

The root of *S. baicalensis* was obtained from the Shanghai Chinese Herbal Medicine Company. The extract that was used for all experiments was prepared as described previously [123]. The plant roots were first cut into small pieces and then soaked in 4 °C water for 2 hours. The mixture was heated to 95 °C and stirred constantly for 1 hour. The hot water soluble fraction was filtered (<11 μm) and then lyophilized. For each experiment, the dried powder was suspended in a balanced salt solution (BSS), and centrifuged for 5 min at 10,000 x g. Fresh preparations of the supernatant were used

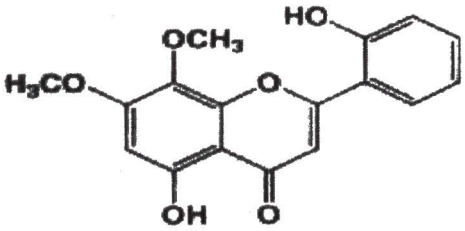
for the experiments and denoted as "*S. baicalensis* extract" (SbE). The constituents of the SbE were identified by liquid chromatography/mass spectrometry (LC/MS) (Hitachi M1000, Hitachi Denshi, Ltd., Tokyo, Japan) utilizing an atmospheric pressure chemical ionization interface. The mobile phase consisted of 14 mM ammonium acetate in acetonitrite (1:99, v/v). Ten milligrams of dried powder was dissolved in 10 ml of deionized water. The sample (150 µl solution) was injected while the flow rate was 0.8 ml/min. Analysis of the extract showed that it contained the following four flavones: wogonin (51.5%, w/v), baicalein (35.6%, w/v), skullcapflovone I (4.8%, w/v), and skullcaplavone II (8.3%, w/v). Baicalein (Sigma, St Louis, MO) was used as a standard to quantify the four flavones in SbE, as described previously [123].



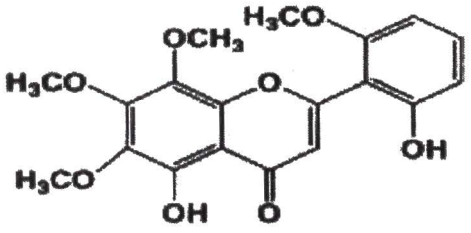
Wogonin



Baicalein



Skullcapflavone I



Skullcapflavone II

Structure of four major flavonoids found in SbE

Cell Viability Assay

Triplicate samples of HT-22 cells (approximately 5,000) and fibroblast (approximately 4, 000) were seeded in 96 well plates and subjected to oxidative stress/antioxidant conditions as described in the text. Cell viability was measured using a formazan assay kit (Promega, Madison, WI) [33]. The conversion of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt] into the aqueous soluble formazan by dehydrogenases in metabolically active cells was quantified from the absorbance at 490 nm.

Measurement of Scavenging Ability of Flavones

Scavenging ability of flavones for reactive oxygen species (ROS) in cells was assessed using the probe 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes Inc., Eugene, OR). DCFH-DA is freely permeable, and enters the cell, where cellular esterases hydrolyze the acetate moieties, making a polar structure that remains in the cell. ROS in the cells oxidize the DCFH, yielding the fluorescent product 2′, 7′-dichlorofluorescin (DCF) [143]. Cells were seeded into 96-well plates at a density of 5,000 cells per well one day prior to an experiment. On the day of the experiment, DCFH-DA loading medium was added at a final concentration of 50 μM. Then cells were incubated in 5% CO_2/95% air at 37 °C for 45 min. After removing DCFH-DA, cells were washed twice with PBS and incubated in phenol red-free DMEM medium containing 10% (v/v) FBS, with various combination of antioxidants and oxidative stresses. The fluorescence of cells from each well was measured at suitable time points

with a Bio-Tek microplate fluorescence reader FL-600. The excitation filter was set at 485±20 nm and emission filter was set at 530±25 nm.

Measurement of Apoptosis

TUNEL Assay (Transferase-mediated dUTP Nick End Labeling)

Cleavage of genomic DNA during apoptosis yields double-stranded low molecular weight DNA fragments, as well as single strand breaks ("nicks") in high molecular weight DNA. Such strand breaks were identified by enzymatic labeling of free 3'-OH termini with modified nucleotide (fluorescein-dUTP). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes the template independent polymerization of deoxyribonucleotides to the 3′ end of single- and double-stranded DNA. The TUNEL assay of apoptosis utilized an apoptosis kit (Boehringer Mannheim) described previously [50]. The cells used as positive controls were grown under serum starvation conditions. The negative control contained the nucleotide mixture in reaction buffer but without the terminal transferase (TdT).

Analysis of DNA Fragmentation in Agarose Gels

HT-22 cells (5 x 10^6) were lysed in 1 ml of DNA extraction buffer containing 5 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% (w/v) Triton X-100. The lysates were centrifuged at 13,000 x g for 30 min, incubated with 5 μg/ml DNase-free RNase A at 37 °C for 1 hr, then incubated with 100 μg/ml proteinase K in 1% (w/v) sodium dodecyl sulfate for 2 hours at 50 °C. After incubation, 1 ml of phenol:chloroform (1:1, v/v) was mixed with the cell lysates, then centrifuged at 20,000 x g for 10 min. DNA in the

aqueous phase was collected by the precipitation with 2 volumes of absolute ethanol/3 M NaOAc (20:1, v/v). After centrifugation, the DNA pellets were washed with 70% (v/v) ethanol and dried. The DNA was dissolved in 10 mM Tris-HCl and 1 mM EDTA, and its concentration was measured at 260 nm. DNA was separated on 2% (w/v) agarose gels containing 1 μg/ml ethidium bromide, and DNA fragments were visualized by exposing the gel to UV irradiation [10].

Protein Extraction

Approximately 2 X 10^6 cells were added into 100 mm plates and allowed to attach for 12 hours. Cells were then exposed to the different experimental conditions (e.g. control, H_2O_2, or H_2O_2 plus flavones). Cells were then trypsinized, collected via centrifugation (1000 x g, 10 min.), washed 3 times in PBS and recollected by centrifugation. The cellular pellet was then dissolved in dSDS buffer [0.3% (w/v) SDS, 1% (w/v) BME, 0.05 M Tris-HCl (pH 8.0)] and passed through a 22-gauge needle 10 times. The extract was placed in boiling water for 3 min, and then transferred to ice for 10 min. Protease inhibitor cocktail (Complete™, Fisher, Houston, TX), DNase (M610A: Promega), and RNase (732-6349: Bio-rad) were added, and then placed at 37 °C for 1 hr. Particulates were removed by centrifugation (15,000 x g, 15 min) and the supernatant solution was collected. Because the dSDS solution interferes with protein quantification, this was determined by TCA precipitation of a 20 μl aliquot, and the protein precipitate reconstituted in BCA protein reagent (Pierce, Rockford, IL) [88].

Western Blot Analysis

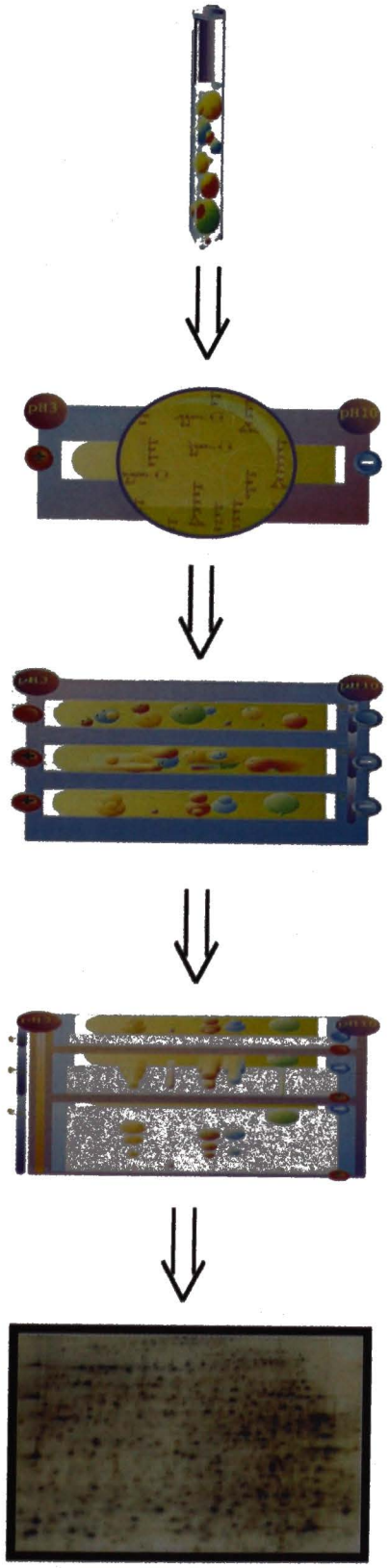
Thirty-five micrograms of proteins were dissolved in sample buffer [50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1% (w/v) SDS, and 100 mM NaCl]. The samples were boiled for 3 min and separated on 4-20% porosity gradient gels. After the separated proteins were transferred onto PVDF membrane (Pall Gelman, Ann Arbor, MI), the membrane was incubated in blocking buffer [1% (w/v) BSA in Tris-buffered saline] for 30 min at room temperature, and then probed with a mouse monoclonal anti-Bcl-2 (SC-7382: Santa Cruz Biotechnology) or anti-Bax antibody (SC-7480: Santa Cruz Biotechnology) in 0.25% (w/v) BSA/ Tris-buffered saline containing 0.3% (v/v) Tween 20 (TBS-Tween) for 2 hours at room temperature. The membrane was washed 3 times in Tris-buffered saline containing 0.3% (w/v) Tween 20 (TBS-Tween), and then probed with anti-mouse IgG1- or IgG2b-horseradish peroxidase conjugated secondary antibody (1:5000; Santa Cruz Biotechnology) in 5% (w/v) milk/TBS-Tween for 2 hours at room temperature. The membrane was washed 3 times in TBS-Tween, and then probed with anti-mouse IgG1- or IgG2b-horseradish peroxidase conjugated secondary antibody (1:5000; Santa Cruz Biotechnology) in 5% (w/v) milk/TBS-Tween for 1 hour at room temperature. The membrane was washed three times in TBS-Tween. A chemiluminescence kit (SuperSignal® West Femto Maximum Sensitivity Substrate, Pierce) was used to visualize the immunostained blots. Following exposure to the chemiluminescent chemicals, the membranes were placed in a light-tight cabinet; and a cooled computerized CCD camera-based imaging system (Alpha Innotech) was used to capture the light produced by the chemical reaction and to record the stained proteins.

Two-Dimensional Gel Electrophoresis

Protein samples (350 μg) were absorbed onto 18 cm immobilized-pH-gradient (IPG) strips (pH 3-10) (Bio Rad) using sample buffer containing 8 M Urea, 2% (w/v) CHAPS, 2% (v/v) IPG buffer pH 3-10L, 0.3% (w/v) DTT, and trace bromophenol blue, in an IPG re-swelling tray. The IPG strips, containing samples, were then electrophoresed on a Multiphor II (Amersham Biosciences) for 24,000 Volt/Hours. Following isoelectric focusing the IPG strips were immediately DNP-derivatized (as described below), then subjected to equilibration for 15 min in 50 mM Tris-HCl (pH 8.8) containing 6M urea, 2% (w/v) SDS, 30% (v/v) glycerol, and 1.0 % (w/v) dithiothreitol. Strips were then re-equilibrated for 15 min in the same buffer containing 2.5 % (w/v) iodacetamide in place of dithiothreitol. In all cases, molecular weight separation was achieved using the ISO-DALT slab gel SDS-PAGE systems (Amersham Biosciences) [139].

Derivatization of Protein Carbonyls

Protein samples were derivatized by the in-strip DNP derivatization method described by Conrad, et al [30]. Following sample re-hydration and isoelectric focusing, the immobilized pH gradient (IPG) strips were placed in 25 ml test tubes then incubated for 15 min in 2N HCl/10 mM DNPH at 25°C. After the reaction, the samples were washed once with 2 M Tris-base/30 % (v/v) glycerol for 15 min. Sample IPG strips were then prepared for molecular weight separation, as described above.



(A) Sample collection and Protein concentration

(B) Re-hydration of IPG

(C) Electropocusing of IPG an Derivatization of IPG with DNPH

(D) MW separation of proteins

(E) Visualization of blots/gels

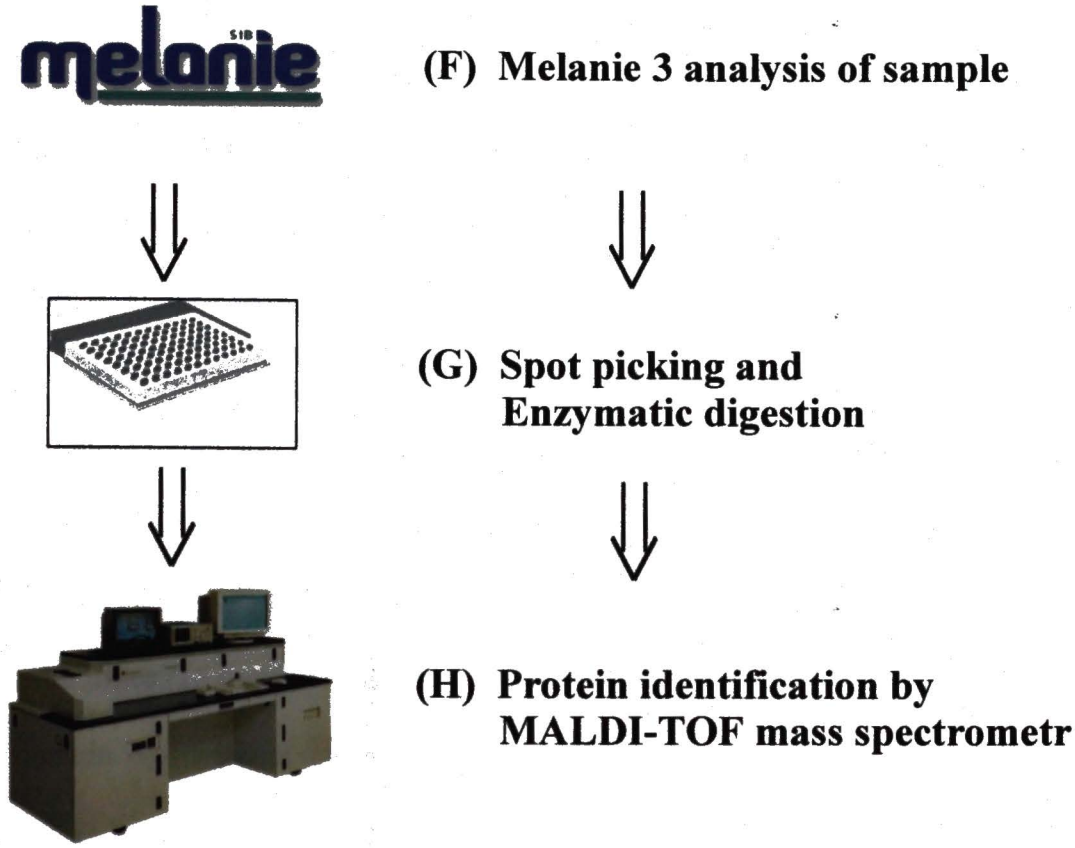
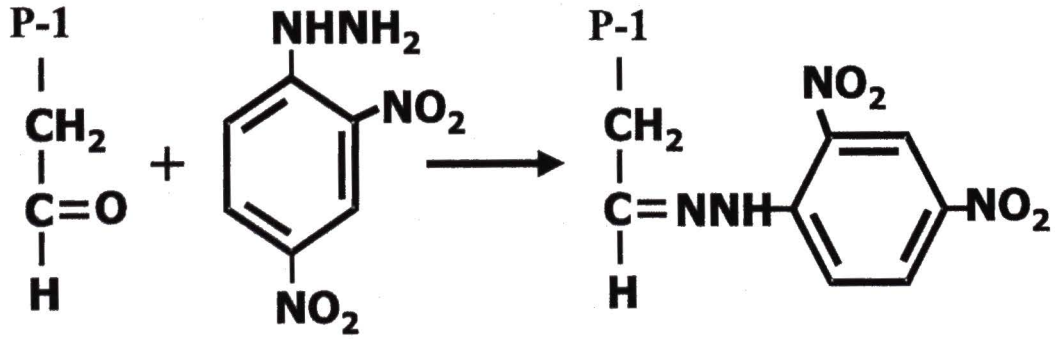


Figure 6. Protein separation by 2-D Gel Electrophoresis and Protein identification by MALDI-TOF mass spectrometry

DNP-Immunostaining

The PVDF membranes were removed from the ISO-DALT electroblotting apparatus and incubated with 5% (w/v) milk in phosphate buffered saline (PBS) and Tween (3%, v/v) for one hr. Membranes were incubated overnight at 4 °C with a 1:16,000 dilution of the primary antibody (Molecular Probes) in PBS-Tween containing 5% (w/v) milk as described previously [30]. The membranes were washed three times (15 min/each) with PBS-Tween solution and incubated 1 hour at 4 °C with a 1:16,000 dilution of the goat –anti-rabbit (horseradish peroxidase-HRP-conjugated) secondary antibody (Sigma) in PBS-Tween containing 5% (w/v) milk. The membranes were washed 3 times (15 min/each) in PBS-Tween solution. Membranes were visualized as described in Western blot analysis.



2,4-DNPH

Derivatization of protein carbonyl with 2,4-DNPH for detection of oxidized protein by anti-DNP antibody

Silver Staining

Duplicate samples of the derivatized proteins were separated on pore gradient polyacrylamide gels (e.g. 9-15% porosity polyacrylamide). Proteins in one gel were silver stained, while proteins from the other gel were electroblotted to polyvinylidene difluoride (PVDF) using the ISO-DALT system. The silver staining procedure was a modification of a method described previously [108]. Proteins were fixed in the gel using ethanol:acetic acid (40:1, v/v) overnight. Two separate sensitizing solutions [glutaraldehyde (1%, w/v)/sodium acetate (0.5M); and 2,7-naphthalene disulfonic acid (0.05%, w/v)] were used sequentially to increase sensitivity. Silver diamine staining solution (500 mM $AgNO_3$, 500 mM NH_4OH, 20 mM NaOH) and the developer solution [citric acid (0.01%, w/v)/formaldehyde (0.1%, v/v)], followed by a stopping solution [tris base (5%, w/v)/ acetic acid (2%, v/v)], were used to visualize the proteins. Silver stained gels were digitized using a ScanMaker 4 optical scanner (Microtek) at a resolution of 300 x 300 dpi.

SyproRuby Staining

Duplicate samples of the derivatized proteins were separated on SDS-gradient polyacrylamide gels (e.g. 9-15% porosity acrylamide and 0.1% [w/v] SDS). Proteins in one gel were SyproRuby-stained, while proteins from the other gel were electroblotted to polyvinylidene difluoride (PVDF) using the ISO-DALT system. Proteins were fixed in the gel using methanol:acetic acid (40:1, v/v) for 30 min. Protein gel was incubated in SyproRuby Staining Solution (Bio-Rad) for overnight, and destained using methanol:acetic acid (1:0.6, v/v) for 45 min. The membranes were placed in a light-tight

cabinet and a cooled computerized CCD camera-based imaging system (Alpha Innotech) was used to capture the fluorescence.

Protein Image Analysis and 2-DE Database

The digitized images from silver stained gels and immunostained blots were analyzed using the 2-DE gel analysis program *Melanie 3* (Geneva Bioinformatics, Geneva, Switzerland). A comparison report of qualitative and quantitative differences between specific proteins for each gel set of data was then generated.

Tryptic Digestion and Mass Spectrometry Analysis

Spots of interest were excised from the gel, digested in situ with trypsin (Promega, modified) as described below. The gel pieces, in a 0.5 ml microcentrifuge tube, were washed with 0.4 ml of 50% acetonitrile in 25 mM ammonium bicarbonate buffer at pH 8.0, and allowed to stand for 10 min with frequent vortexing. After repeating this wash twice more to further destain the gel band, the gel pieces were soaked in 100 µl of 100% acetonitrile until the gel bands dehydrated and turned opaque. They were then dried in a Speed-Vac for 15-20 min. The gel pieces were reswollen by adding 15 µl of trypsin (Promega) solution (10 µg/ml in 25 mM NH_4HCO_3 buffer, pH 8.0), covered by adding 25 mM NH_4HCO_3 / 0.02% Zwittergent and incubated 16-24 hrs at 37 °C. The digested protein fragments were extracted by addition of 20 µl 2.5% trifluoroacetic acid. Two µl of the peptide extracts were analyzed by mass spectrometry (MALDI-TOF/MS using an Applied Biosystems Voyager DE-STR and capillary-HPLC-ESI/MS/MS on a Thermo Finnigan LCQ). The peptide mass maps produced by MALDI-

TOF/MS were searched against the published databases by means of the MS-Fit module in Protein Prospector (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm). The uninterpreted HPLC-ESI/MS/MS data were analyzed by SEQUEST, a component of the LCQ software.

RESULTS

I. NEURONAL CELL MODEL (HT-22)

Vit E Protects Neuronal Cells from Oxidative Stress

Exponentially dividing cells were dissociated with trypsin and plated into 96-well microtiter plates in DMEM with FBS. The cell viability was measured by MTS assay and the percent survival was corrected for the growth in 24 hours. The survival of HT-22 cells exposed to the enzymatic production of H_2O_2 is shown in Figure 7. Oxidative stress for 12 hours caused a significant ($27 \pm 5\%$) decreased survival compared to controls. Nearly all oxidation stressed cells were dead by 24 hours. In contrast, pretreatment with Vit E increased survival almost to the level of the non-stressed controls.

The TUNEL assay was performed to assess apoptosis following exposure to the oxidative stress. The control cells appeared flat and remained attached to the cell culture plate (Fig. 8A). The cells incubated with 4.3 μM of VitE only showed a similar appearance (data not shown). Cells subjected to H_2O_2 stress by 3 mUnits of glucose oxidase were attached to the plate but rounded in appearance and were TUNEL positive (Fig. 8A). This was contrasted to the relatively normal appearance of cells pretreated with Vit E.

Another measure of apoptosis assessed Ca^{2+}-dependent endonuclease cleavage of DNA into histone-associated DNA fragments (mono- and oligo-nucleosomes) prior to the appearance of morphological changes. We observed such DNA fragmentation patterns in the oxidatively stressed cells, but not in controls nor in H_2O_2 + Vit E treated cells (Fig.

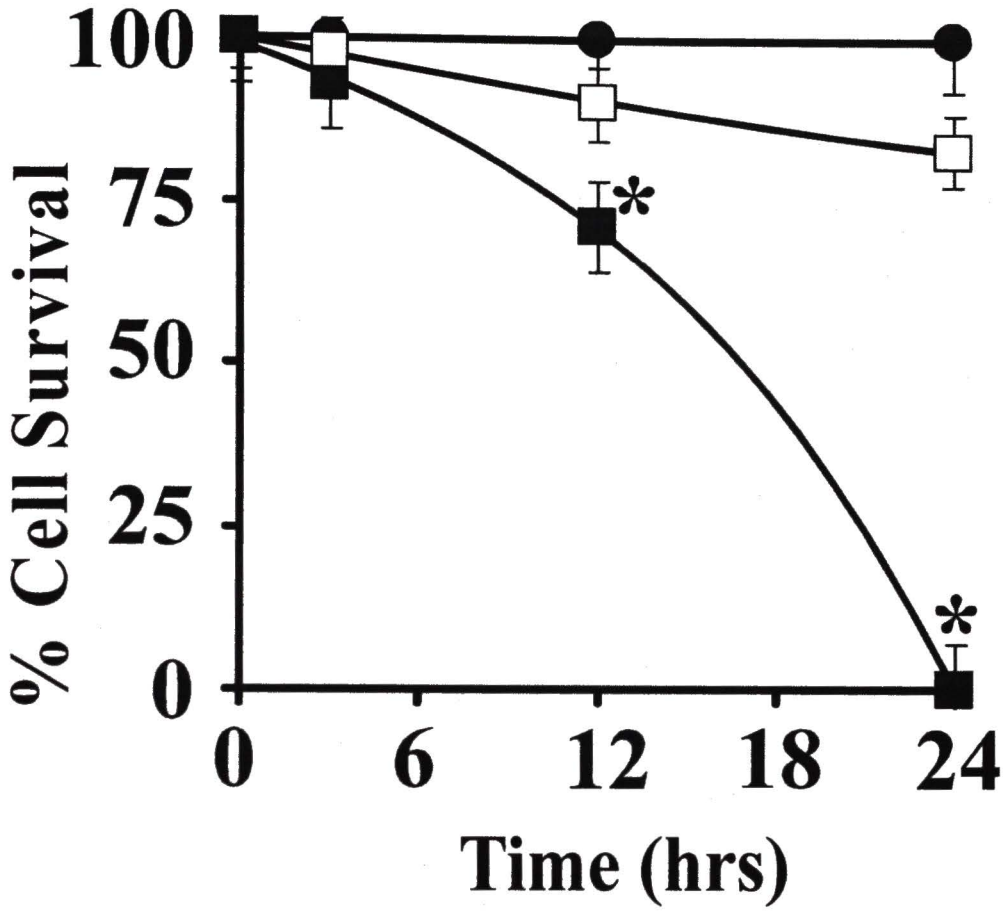


Figure 7. Effect of Vit E on the cytotoxic responses of HT-22 cells exposed to H_2O_2. Exponentially dividing cells were dissociated with trypsin and plated into 96-well microtiter plates in DMEM with FBS. Control cells (●) were not exposed to the oxidative stress. Stressed cells were exposed to 3.0 mUnits of glucose oxidase alone (■) or in the presence of 4.3 μM Vit E (□). The cell viability was assessed and the percent survival was corrected for the growth in 24 hours. Each value represents the mean ± SEM of 3 different samples. Values that represent significant differences ($P < 0.05$) within the same time point are indicated by asterisks (*).

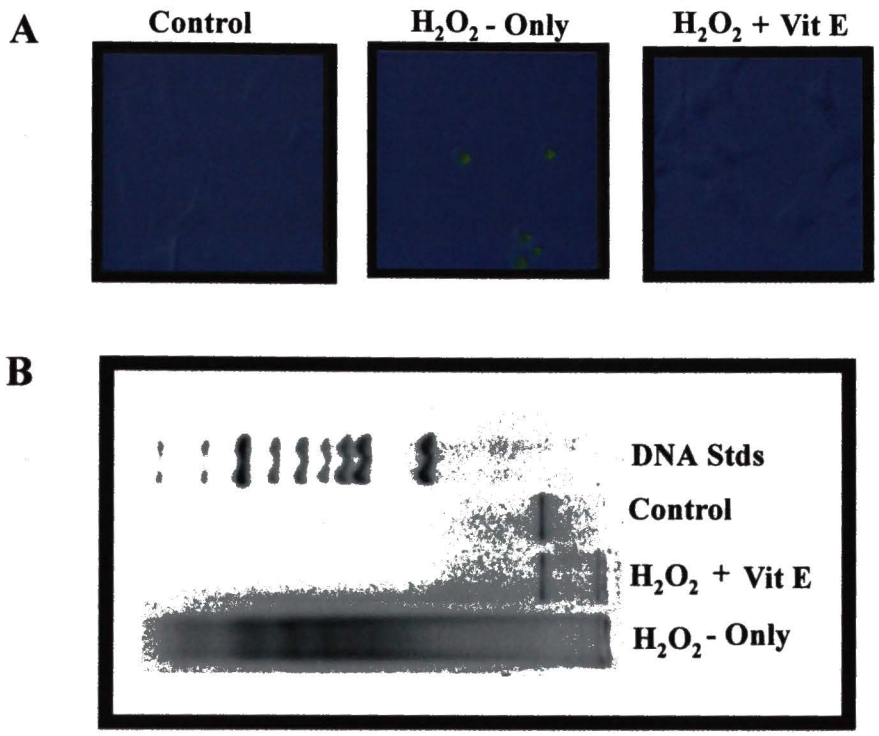


Figure 8. Vitamin E pretreatment prevents oxidative stress induced apoptosis. (A) Following exposure of cells to different oxidative stress and/or oxidative stress + 4.3 μM VitE conditions. *In situ* detection of apoptotic cells was determined using the TUNEL assay and fluorescence microscopy, as described in methods. No TUNEL positive staining (i.e. green/yellow) cells are visible in control cells or in the cells pretreated with Vit E. However, oxidation stress catalyzed by glucose oxidase caused appearance of TUNEL positive cells. (B) Following a 12 hour exposure of cells to H_2O_2 generated from 3.0mUnits of glucose oxidase or H_2O_2 + 4.3 μM Vit E conditions, the DNA was isolated and separated on a 2% agarose gel. DNA banding patterns were visualized using ethidium bromide and UV light. DNA fragmentation was observed in the cells treated with oxidative stress, but not in control cells and cells pretreated with Vit E.

8B). Thus, Vit E protected the cells from apoptosis induced by H_2O_2.

Flavones of SbE Protects Neuronal Cells from Oxidative Stress

Fig. 9 shows a similar experiment to that described in Fig. 7. Similar to the case with Vit E, flavones from extracts of *Scutellaria baicalensis* Georgi protected the neuronal cells from H_2O_2 – induced cell death. This cell death was shown also to be due to apoptosis as shown in the TUNEL assay and DNA ladder formation assays. Serum starvation was used as a positive control. Negative control cells were treated similarly to positive controls; however, terminal deoxynucleotidyl transferase was omitted when cells were processed. Sixty-seven percent of cells exposed to 3 mUnits of glucose oxidase were TUNEL positive. This was significantly higher than the 0% of TUNEL positive cells observed when cells were treated with both 3 mUnits of glucose oxidase and the flavones. When the concentration of glucose oxidase was increased to 6 mUnits, a 40% decrease in the total number of cells was observed in cells not treated with flavones. Cells not treated with the flavones were 70% TUNNEL positive. Cells exposed to 12 mUnits of glucose oxidase alone were all detached. In contrast, cells also treated with flavones were attached to the plate, but 100% TUNEL positive. Higher magnification (40X) was used to produce the data presented in Table 3 and shown in Figure 10 (panel A). The control cells appeared flat and well attached to the cell culture plate (Fig. 10A). Cells treated with 3 mUnits of glucose oxidase were attached to the plate, but rounded in appearance and TUNEL positive, compared to the relatively normal appearance of cells pretreated with flavones (Fig. 10A). DNA ladder formation after oxidative stress was observed on agarose gel electrophoresis; but not for controls, nor for oxidative stress +

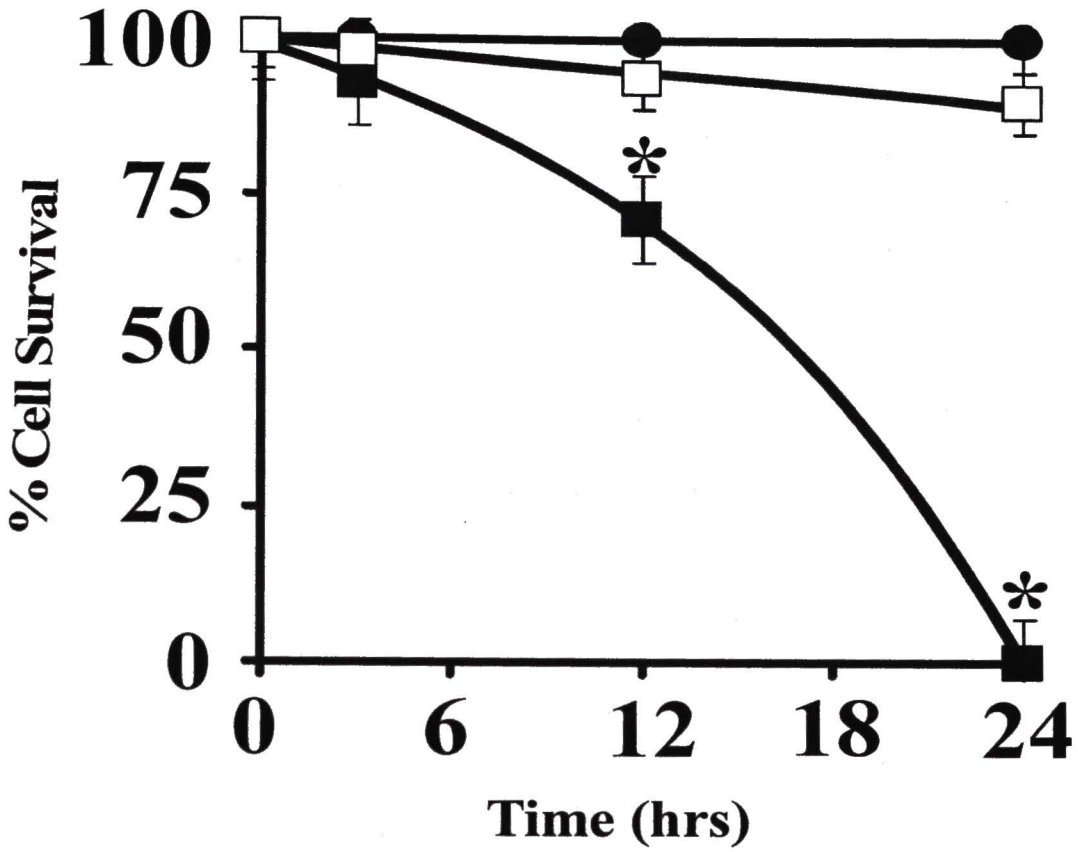
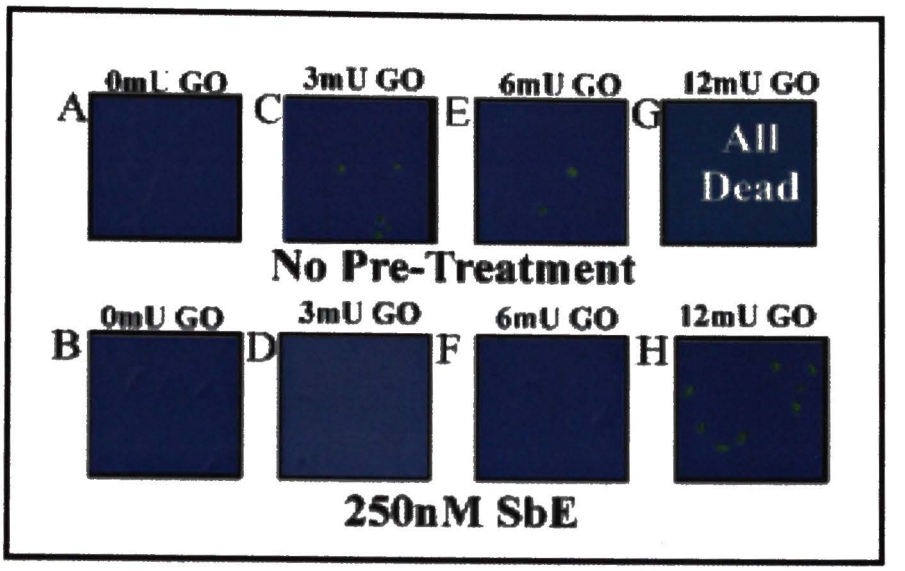


Figure 9. Cytotoxic responses of HT-22 cells exposed to H_2O_2 and flavones of SbE. Exponentially dividing cells were dissociated with trypsin and plated into 96-well microtiter plates in DMEM containing FBS. Control cells were not exposed to glucose oxidase generated H_2O_2 (●). The other cells were exposed to 3.0 mUnits of glucose oxidase alone (■) or after addition of 50 μg/ml SbE (□). The % survival was corrected for the growth in 24 hours by dividing the absorbance value for each data point by the absorbance value of the control for the same time point. Each value represents the mean ± SEM of 4 different samples. Values that represent significant differences ($P < 0.05$) within the same time point are indicated by asterisks (*).



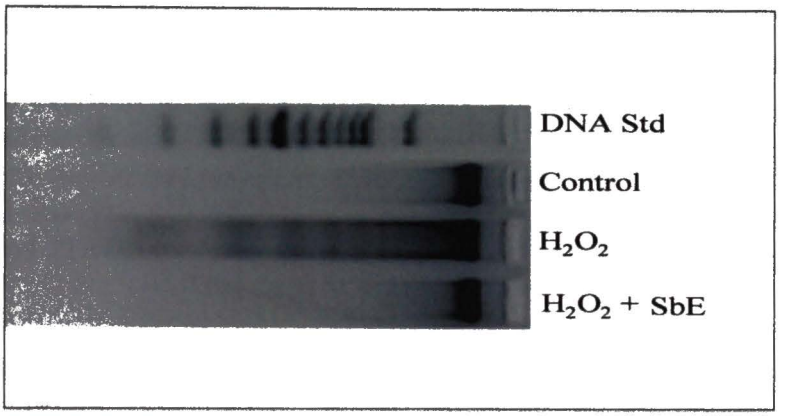


Figure 10. Effect of SbE pretreatment on apoptosis in neuronal cells exposed to oxidative stress. (Top panel) Exponentially dividing HT-22 cells were dissociated with trypsin and approximately 4,000 cells were plated onto circular cover slips in six-well cell culture plates and allowed to attach. Following exposure of cells to different oxidative stress and SbE conditions, *in situ* detection of apoptotic cells was performed using the TUNEL assay and fluorescence microscopy. No TUNEL positive staining (i.e. green/yellow) cells are visible in control cells. However, when cells were treated with glucose oxidase virtually all SbE-untreated cells exposed to glucose oxidase-treated cells were TUNEL-positive. (Bottom panel) Exponentially dividing cells were dissociated with trypsin, and approximately 2 x 10^6 cells were plated into 100 mm plates and allowed to attach. Following a 12 hour exposure of cells to GO, or GO + SbE, the DNA was isolated and separated on a 2% agarose gel. DNA banding patterns were visualized using ethidium bromide and UV light.

Treatment	Total Cells	Apoptotic Cells	Percent Apoptosis
Control	13 ± 1	none	0%
Control (negative)	13 ± 1	none	0%
Control (positive)	14 ± 1	4 ± 1	14 ± 6
3mUnits GO	11 ± 1	8 ± 1	67 ± 8
3mUnits GO + SbE	15 ± 1	none	0%
6mUnits GO	7 ± 1	5 ± 1	70 ± 12
6mUnits GO+ SbE	12 ± 1	none	0%
12mUnits GO	all dead	all dead	0%
12mUnits GO+ SbE	18 ± 3	18 ± 3	100 ± 1

Table 3. The apoptotic responses of HT-22 cells were determined by TUNEL assay following a 12 hr exposure to different concentreations of glucose oxidase and/or *Scutellaria baicalensis* extract (flavones). Control cells were cultured under normal (control) or serum-starvation (positive control). Negative control cells were incubated without terminal-deoxyneucleotidyl transferase in TUNEL mixture. Mean values represent total and TUNEL positive staining cells in four separate fields of view at 10X magnification.

flavone treated cells (Fig. 10B). Thus, these studies clearly indicate that the flavones protect neuronal cells from apoptotic cell death induced by oxidative stress.

The ability of flavones to scavenge reactive oxygen species was assessed using fluorescent probes. Cells were incubated in the presence of 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) with 3 mU glucose oxidase and a series of different concentrations of flavones. Addition of flavones (0-75 μg/ml) caused concentration-dependent attenuation of the DCF fluorescence, indicating that the flavones were effective in scavenging the reactive oxygen species (Fig. 11).

Flavones mediate oxidative changes in Bax and Bcl-2

To gain further information on the mechanisms of oxidative induced cell death and antioxidant protection by flavones or Vit E, we examined levels of the key regulators of apoptosis, Bcl-2 and Bax. Both Bcl-2 and Bax protein levels were decreased less than 10 % (compared to the control) when subjected to H_2O_2 - induced oxidative stress. Pretreatment with flavones plus oxidative stress increased the Bcl-2 protein significantly, whereas Bax protein level was decreased approximately 2-fold (Fig. 12). Similar results were obtained from treatment with flavones alone.

There are several phosphorylation sites in both Bcl-2 and Bax, which are important for their function. Therefore, the effect of the flavones on phosphorylation of Bcl-2 and Bax was measured. Most of the Bcl-2 focused with pI value of approximately 8.0 (Fig. 13A). Upon exposure of cells to flavones (in the presence or absence of oxidative stress), most of the Bcl-2 exhibited a pI value of approximately 5. This suggested the phosphorylation of Bcl-2. This was confirmed by phosphatase treatment

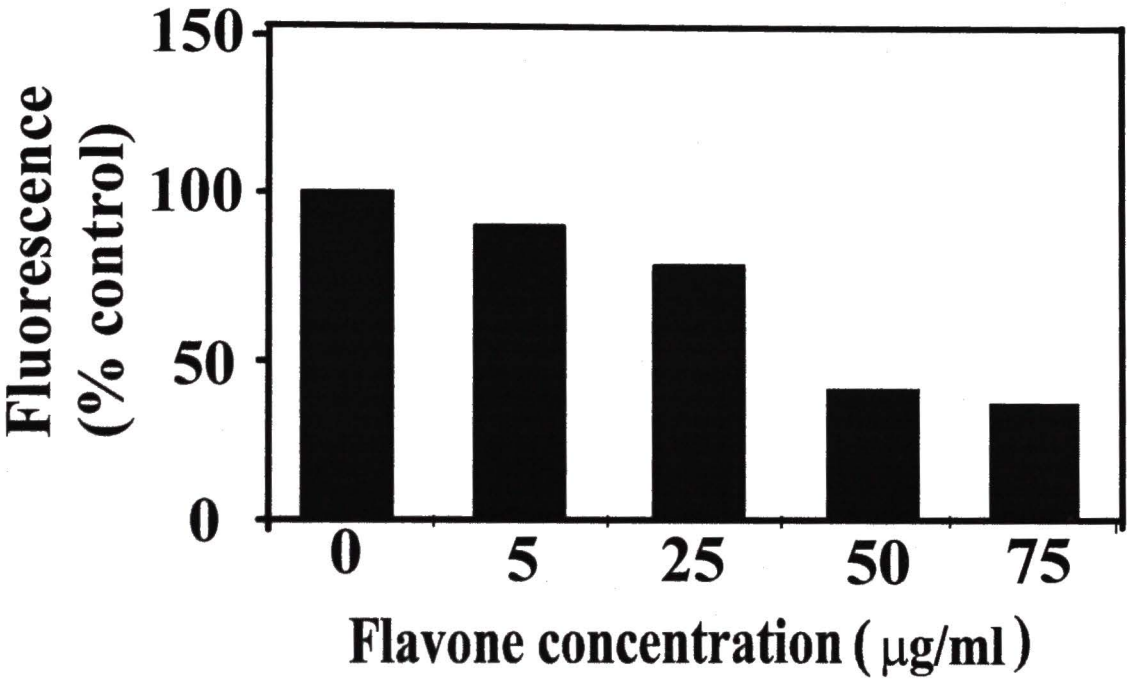


Figure 11. Scavenging effects of flavones on reactive oxygen species generated by glucose oxidase.Experiment was done as described in Materials and Methods. Cells were treated with a series of flavone concentrations and incubated with 3 mU of glucose oxidase. The control value 100% represents the level of fluorescence produced in the absence of the flavones. Values are means of four different determinations.

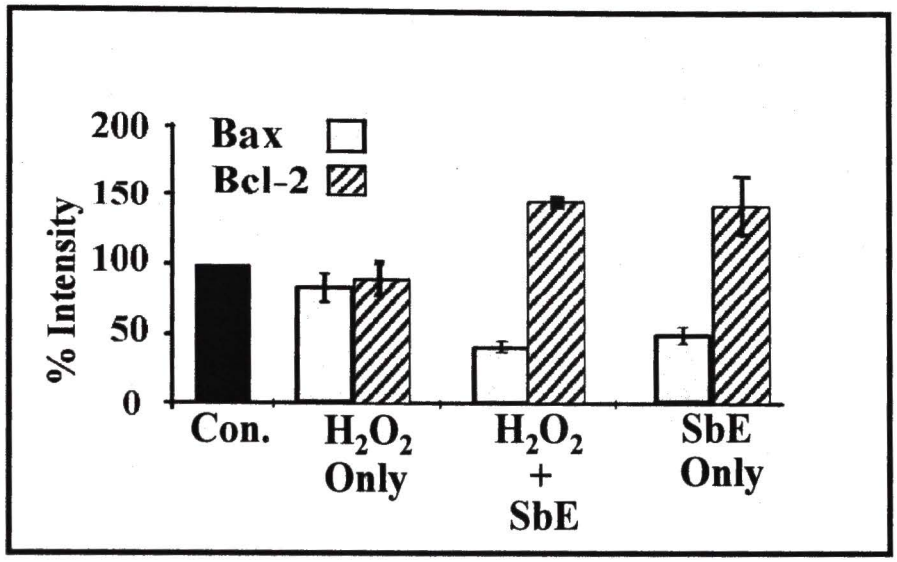


Figure 12. Effect of SbE pretreatment on the Bcl-2- and Bax-protein levels. Exponentially dividing cells were dissociated with trypsin; and approximately 1,000,000 cells were plated into 100 mm plates and allowed to attach. Following a 12-hour exposure of cells to oxidative stress by generation of H_2O_2 via the glucose oxidase system and SbE conditions, cell extracts were collected. The equal amounts of protein were separated on 4-20% gradient gel and transferred to PVDF membrane. The blots were probed with anti-Bcl-2 and the membrane then reprobed with anti-Bax. Visualization of the proteins was performed with the SuperSignal chemiluminescence reagent. The level of Bcl-2 and Bax is expressed as the intensity of the immunostain signal per mg of protein. Values are mean ± S.D. (n=3).

of the sample, which caused conversion to the isoform with a pI of 8.0 (Fig. 13C).

Vit E and Flavones Protect Specific Proteins from Oxidation

To determine the appropriate time point for detection of oxidized proteins, neuronal cells were grown with the H_2O_2 oxidative stress generated by glucose oxidase. Following incubation, cells were collected at the 0-, 3-, 7-, and 12-hour incubation time points; and the proteins were extracted, derivatized with DNPH, visualized, and quantified, as described in Material and Methods. The left panel (Fig. 14) shows Coomassie stain-visualized total proteins, and the right panel (Fig. 14) shows the immunoblot from the one-dimensional gel. Elecrotransferred proteins have been reacted against anti-DNP antibody, which binds to the derivatized carbonyl groups of the oxidized proteins. Significant increases in the level of oxidized proteins started to appear after the 7-hour time point and continuously accumulated through the 12-hour time point of incubation time with oxidative stress.

In order to evaluate which proteins were oxidized, and whether Vit E could prevent the oxidation of specific proteins, we utilized 2-DE coupled with immunostaining for protein carbonyls, as described in methods. Figure 15A shows a typical example of HT-22 cell extracts separated by 2-DE and silver stained for total proteins. Over 300 proteins are clearly visible. These proteins were transferred to PVDF membranes, and then analyzed using oxidation specific immuno-probes (Fig. 15A "oxidized proteins"). Figure 15B shows three 2-DE immunostains of proteins from a specific region in the 2-DE blots (MW = 40-90 kDa, pI = 4.5-7.5). Overall, the total number of proteins visible (i.e. silver stained) on HT-22 cellular extracts was relatively consistent in the control,

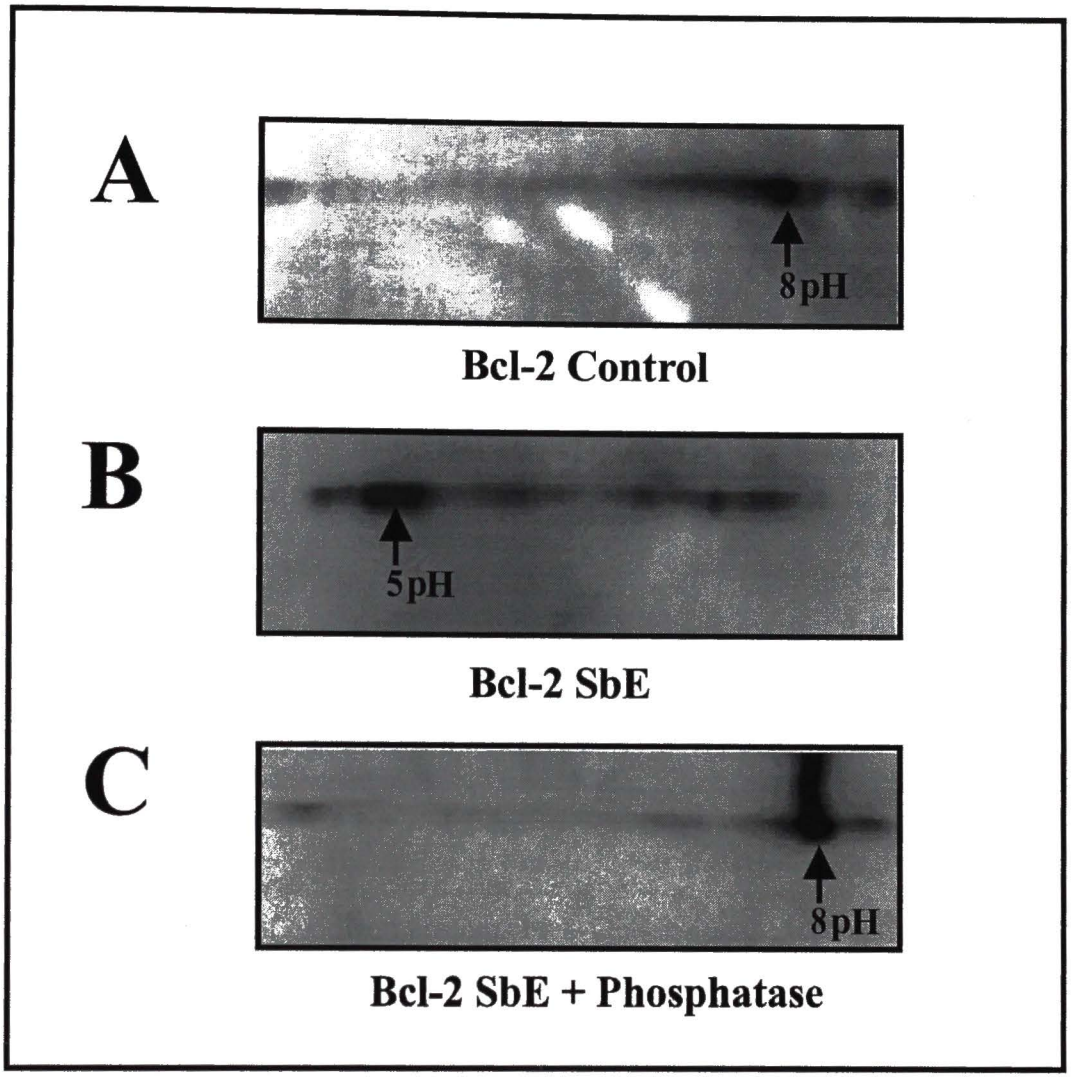


Figure 13. Effect of SbE pretreatment on the phosphorylation of Bcl-2 and Bax proteins. Exponentially dividing cells were dissociated with trypsin and approximately 1,000,000 cells were plated into 100 mm plates and allowed to attach. Following a 12-hour incubation, controls (panel A) or SbE treated cells (panel B) were collected, then subjected to isoelectric focusing (7cm IPG strips, pH 3-10) and molecular weight separation. Samples were analyzed by immunoblotting with anti-Bcl-2 antibody. The SbE-incubated sample (50 μg) was treated with 50 units of phosphatase (λ-PPase, Calbiochem) in buffer (50 mM Tris-HCl, 5 mM DTT, 2 mM $MnCl_2$, 100 μg /ml BSA) for 30 min, separated on 2-DE gel, and blotted with anti-Bcl-2 antibody (panel C).

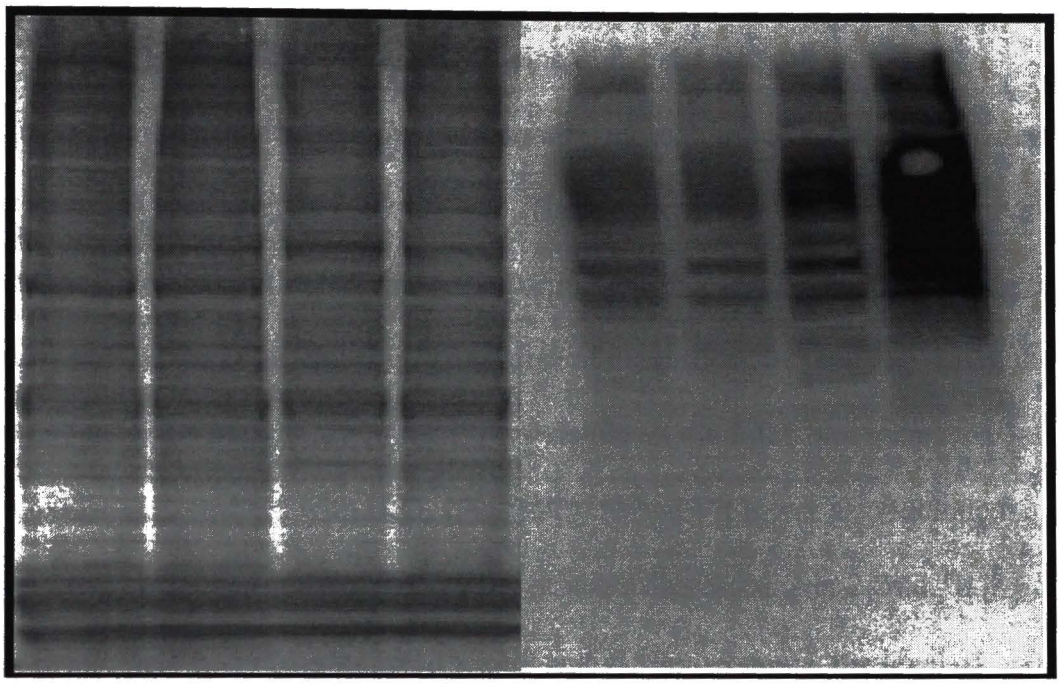
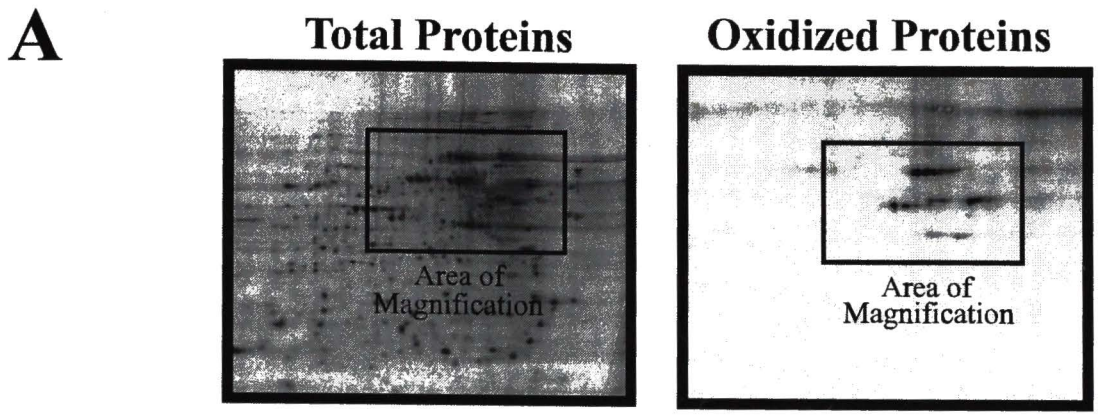


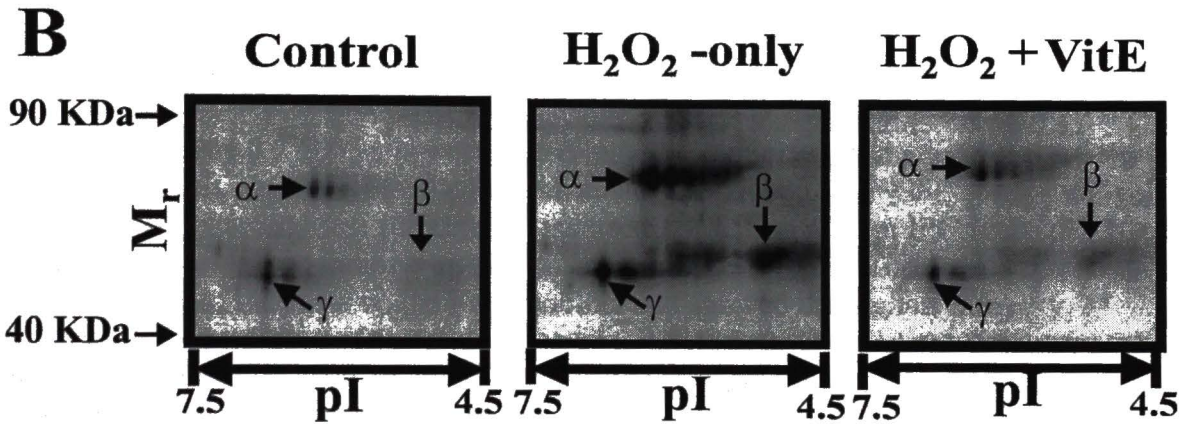
Figure 14. Visualization of oxidized proteins by 1-Dimensional Electrophoresis. Exponentially dividing cells were dissociated with trypsin, and approximately $2x10^6$ cells were plated into 100mm plates and allowed to attach. Following a 12-hour exposure of cells to 6 mUnits of glucose oxidase, cell extracts were collected each time points, and subjects to molecular weight separation. Left panel shows a total protein stain done by Coomassie. Right panel shows an immunostain to detect oxidized proteins.

stressed, and stressed + Vit E or flavones cells (330 $\pm$ 30 proteins –approx 9% variance) (Fig. 15C).

In contrast, the number of oxidized proteins from cells stressed with H_2O_2 increased over fifty-five percent (55%). Furthermore, more than half of all the oxidized proteins appear in a small area of the 2-DE gel. These data indicate that only a relatively low percentage (10-15%) of the proteins are susceptible to this level of oxidation (Fig. 15C). In this area of the 2-DE blot (Fig. 15A-"area of magnification" and Fig. 15B), the intensities and numbers of oxidized protein spots visible on the 2-DE blot increased 3-fold when oxidatively stressed with H_2O_2 (Fig. 15C). When Vit E was also included prior to oxidative stress, the number of oxidized proteins decreased over 2-fold. It is interesting to compare specific proteins (e.g. α, β and γ). For example, the level of oxidation of "α" increased over 6-fold when treated with H_2O_2 (Fig. 15C). However, if Vit E was also present, oxidation levels of this protein remained near the control levels. A similar situation was seen for oxidized protein "β". In contrast, oxidized protein γ showed much lower increase in oxidation suggesting that oxidized proteins "α", and "β" are more sensitive to oxidation than protein "γ". Pretreatment of flavones also protects the neuronal cells from oxidative stress. The intensity of the oxidized proteins increased nearly 8-fold from control levels for α and γ; and almost 14-fold for β (Fig. 16C). However, if flavones were also present during the oxidative stress, oxidation levels remained near control levels. Thus, the protein "β" is the most sensitive to oxidation among those three proteins chosen here; and the proteins.

Figure 15. Effect of Vit E pretreatment on specific protein oxidation visualized by 2-Dimensional Electrophoresis. Exponentially dividing cells were dissociated with trypsin, and approximately 2 x 10^6 cells were plated into 100 mm plates and allowed to attach. Following a 12-h. exposure of cells to H_2O_2 generated from 6 mUnits of glucose oxidase and/or glucose oxidase + Vit E conditions, cell extracts were collected, then subjected to isoelectric focusing (18 cm IPG strips pH 3-10) and molecular weight separation, as described in Materials and Methods. Panel A shows a 2-DE gel that was silver stained to visualize all proteins, and its corresponding blot immunostained to visualize oxidized proteins. Panel B represents enlarged views of the areas on the immunoblots that showed the highest intensity staining; three specific proteins have been highlighted (α, β, and γ). Panel C shows a table indicating the numbers of total and oxidized proteins found on the 2-DE silver stains and immunoblots.



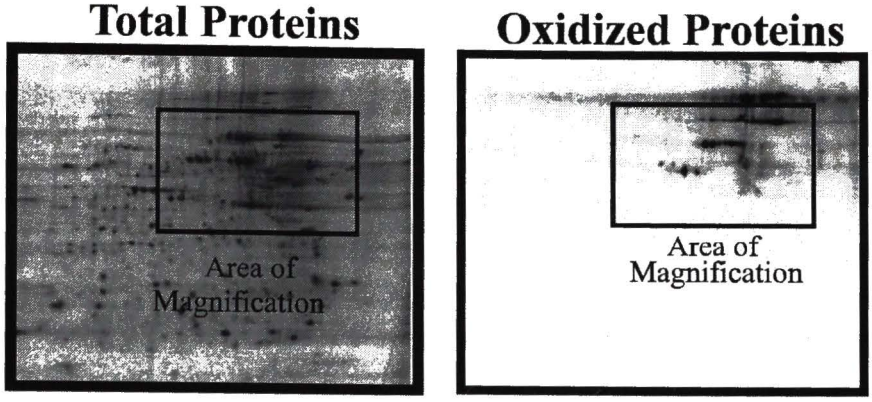


C

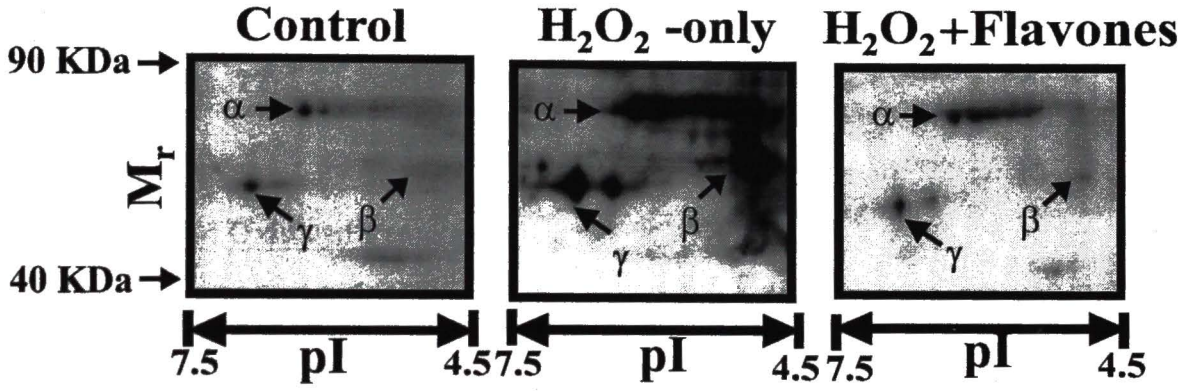
Gel Name	Whole Gel: Total spots	Whole Gel: Oxidized Spots	Magnified Area Only: Total spots	Magnified Area Only: Oxidized Spots	Specific Oxidation Index: α	Specific Oxidation Index: β	Specific Oxidation Index: γ
Control	328	24	77	12	306	227	324
H_2O_2 -only	360	53	87	34	1846	1322	1126
H_2O_2 + VitE	304	27	83	16	354	247	349

Figure 16. Effect of SbE pretreatment on specific protein oxidation visualized by 2-Dimensional Electrophoresis. Exponentially dividing cells were dissociated with trypsin, and approximately 2 x 10^6 cells were plated into 100 mm plates and allowed to attach. Following a 12-h. exposure of cells to 9 mUnits of GO and/or GO + SbE conditions, cell extracts were collected, then subjected to isoelectric focusing (18 cm IPG strips pH 3-10) and molecular weight separation on 9-15% gradient SDS-PAGE. Top panel represents enlarged views of the areas on the immunoblots that showed the highest intensity staining; three specific proteins have been highlighted (α, β, γ, δ, and ε). Bottom panel shows a table indicating the numbers of total and oxidized proteins found on the 2-DE silver stains and immunoblots.

A



B



C

Gel Name	Whole Gel: Total spots	Whole Gel: Oxidized Spots	Magnified Area Only: Total spots	Magnified Area Only: Oxidized Spots	Volume: α	Volume: β	Volume: γ
Control	328	24	77	12	513	301	470
H_2O_2-only	360	53	87	34	4011	4388	4309
H_2O_2+ Flavones	304	27	83	16	790	382	654

Proteins Oxidized by Oxidative Stress Generated by $A\beta_{25-35}$

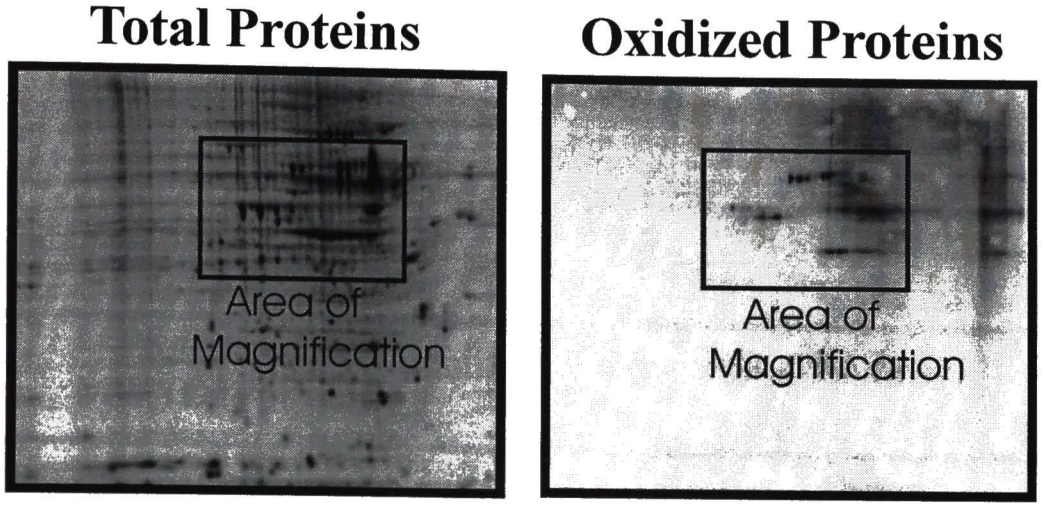
Neuronal cells were incubated with the beta-amyloid peptide $A\beta_{25-35}$ for 3 days, and the proteins were extracted and resolved on 2-Dimensional Electrophoresis as described in Materials and Methods. Similar protein-oxidation profiles were observed with oxidative stress generated by either $A\beta_{25-35}$ or glucose oxidase generated H_2O_2. The oxidation levels of three protein spots on the immunostained membrane blot from cells exposed oxidative stress generated by $A\beta_{25-35}$ were increased 4.5 to 7-fold from that of control cells (Fig. 17).

Identification of Oxidized Proteins

Three protein spots (α, β, and γ) were excised from the gels, digested in situ with trypsin (Promega, modified), and the resulting digests analyzed by mass spectrometry (MALDI-TOF/MS using an Applied Biosystems Voyager DE-STR and capillary-HPLC-ESI/MS/MS on a Thermo Finnigan LCQ). The peptide mass maps produced by MALDI-TOF/MS were searched against the published databases by means of the MS-Fit module in Protein Prospector (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm) (Table 6). These three proteins, which were the most easily oxidized, were identified as a heat shock protein 60, a vimentin, and an unknown neuronal protein that matched with hemoglobin alpha chain in the protein database (Table 4).

Figure 17. Effect of beta-amyloid treatment on specific protein oxidation visualized by 2-Dimensional Electrophoresis. Exponentially dividing cells were dissociated with trypsin, and approximately 2 x 10^6 cells were plated into 100 mm plates and allowed to attach. Following a 3 day exposure of cells to 200 μM beta-amyloid condition, cell extracts were collected, then subjected to isoelectric focusing (18 cm IPG strips pH 3-10) and molecular weight separation, as described in Materials and Methods. Panel A shows a 2-DE gel that was SyproRuby stained to visualize all proteins, and it's corresponding blot immunostained to visualize oxidized proteins. Panel B represents enlarged views of the areas on the immunoblots that showed the highest intensity staining; three specific proteins have been highlighted (α, β, and γ). Panel C shows a table indicating intergrated intensity of protein spots in immunoblot.

A



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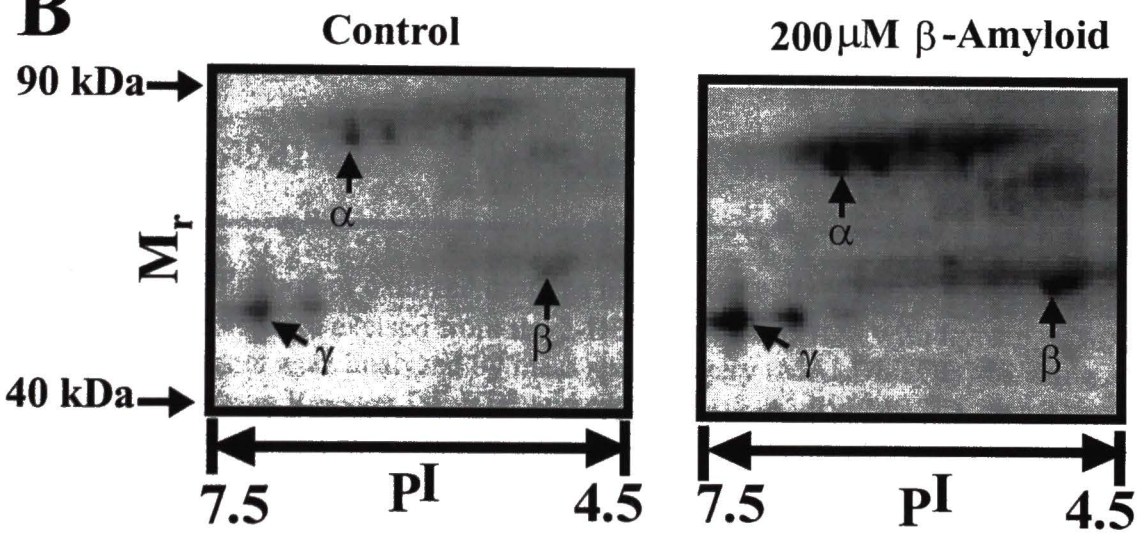


C

Gel Name	Whole Gel: Total Spot	Whole Gel: Oxidized Spots	Magnified Area Only: Total Spot	Magnified Area Only: Oxidized Spots	Specific Oxidation Index: α	Specific Oxidation Index: β	Specific Oxidation Index: γ
Control	318	39	53	14	247	230	330
100 μM β-amyloid	315	85	55	24	1760	1352	1508

Spot name	Protein MW (Da)/pI	Identification (accession number)
α	60979/6.3	Heat shock protein 60 (284763)
β	53630/5.09	Vimentin (281012)
γ	14981/8.6	Hemoglobin alpha chain (A45964)

Table 4. Identification of oxidized proteins in neuronal cells (Fig. 15, Fig. 16, and Fig. 17). Protein spots were excised from the 2-DE gels and digested with trypsin. Two μl of the peptide extracts were analyzed by mass spectrometry (MALDI-TOF/MS using an Applied Biosystems Voyager DE-STR and capillary-HPLC-ESI/MS/MS on a Thermo Finnigan LCQ). The peptide mass maps produced by MALDI-TOF/MS were searched against the published databases by means of the MS-Fit module in Protein Prospector (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm). The uninterpreted HPLC-ESI/MS/MS data were analyzed by SEQUEST, a component of the LCQ software.

III. FIBROBLAST MODEL

Effects of $A\beta_{25-35}$ on Protein Oxidation in the Fibroblasts of AD Subjects

Table 5 shows the cell survival of human fibroblasts from Alzheimer's disease patients and age-matched controls. $A\beta_{25-35}$ was not noticeably toxic to either the human fibroblasts from AD or the age-matched control until more than 2 days had elapsed. Both types of cells began to experience cytotoxicity after a 3-day incubation with $A\beta_{25-35}$. Thereafter, the AD-derived fibroblasts were significantly ($P < 0.001$) more sensitive to oxidative induced cell death than the age matched controls. Throughout the four days, control cells exhibited typical elongated, flat fibroblast morphology. However, by day three some of the AD cells exposed to $A\beta_{25-35}$ began to exhibit changes: became round up, and these cells were dead by the forth day (Fig. 18).

The levels of oxidative stress generated by $A\beta_{25-35}$ (expressed as DCF fluorescence per level of cell viability) were similar in fibroblasts from both AD and age-matched control (Table 6). This indicates that fibroblasts from AD and controls do not differ in their ability to take up the DCFH; thus the different susceptibility of these cells to the oxidative stress is not due to differential production of reactive oxygen species by $A\beta_{25-35}$ treatment.

Initial evaluations of oxidation of proteins were carried out by incubating cells in the presence and absence of $A\beta_{25-35}$, withdrawing samples and analyzing for total proteins and oxidized protein (DNP-immunoassay) after one-dimensional gel electrophoresis. While the AD cells exhibited slightly greater levels of oxidized proteins after 1 and 2 days, by day 3 the levels of oxidation of proteins in the fibroblasts from the AD subjects were approximally three-fold greater than from the controls.

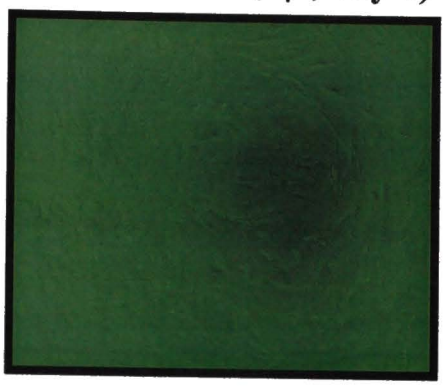
Cell line ID #	Diagonosis	Donor age	% Cell survival in the presence of $A\beta_{25-35}$			
			Incubation time			
			24hr	48hr	72hr	96hr*
AG 14048	Control	71	1.08	1.04	0.89	0.77
AG 13245	Control	68	0.99	1.0	0.86	0.75
AG 13983	Control	71	0.98	1.0	0.92	0.83
AG 07374	AD (Clinically)	73	1.05	1.02	0.81	0.54
AG 08243	AD (Clinically)	72	0.96	1.06	0.85	0.46
AG 08527	AD (Autopsy-confirmed)	61	0.99	0.98	0.82	0.47

Table 5. Cytotoxicity of $A\beta_{25-35}$ to fibroblasts from Alzheimer's subjects. The % cell survival for each time point was corrected for the growth of the cells by dividing the absorbance value of the treated cells ($A\beta_{25-35}$) by the absorbance value of the corresponding non-treated cells. The value of % cell survival is the mean of seven samples. Cell death of the fibroblasts from AD increased significantly ($p < 0.001$) compared to control fibroblasts at 96-hour time point.

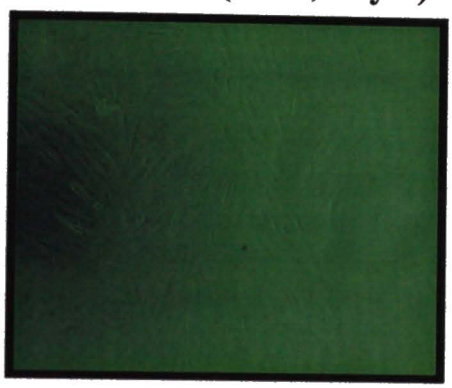
Figure 18. Representative microscopic images of AD fibroblasts and age-matched control fibroblasts. Exponentially dividing cells were dissociated with trypsin and plated into 96-well microtiter plates in DMEM containing FBS. Three AD fibroblasts and three age-matched control fibroblasts were incubated with 100 μM beta-amyloid each over a period of 4 days. Similar fibroblast samples were incubated without beta-amyloid. Pictures of the cells were obtained daily by light microscopy (10X magnification).

AD fibroblast (none, day 0)

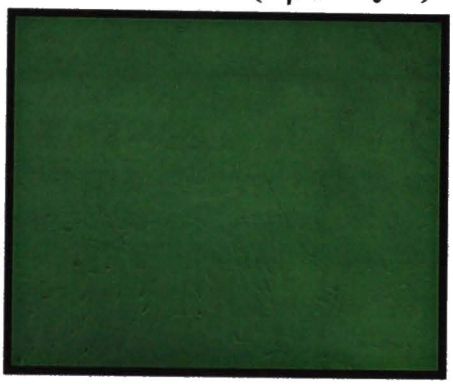
AD fibroblast (Aβ, day 3)



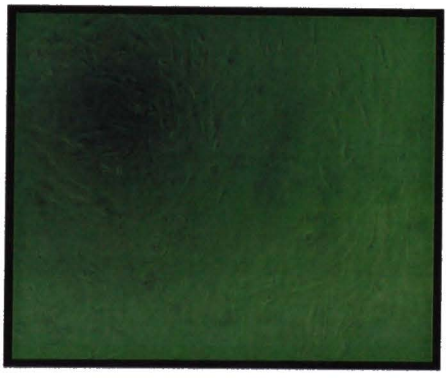
AD fibroblast (none, day 4)



AD fibroblast (Aβ, day 4)



Control fibroblast (Aβ, day 4)



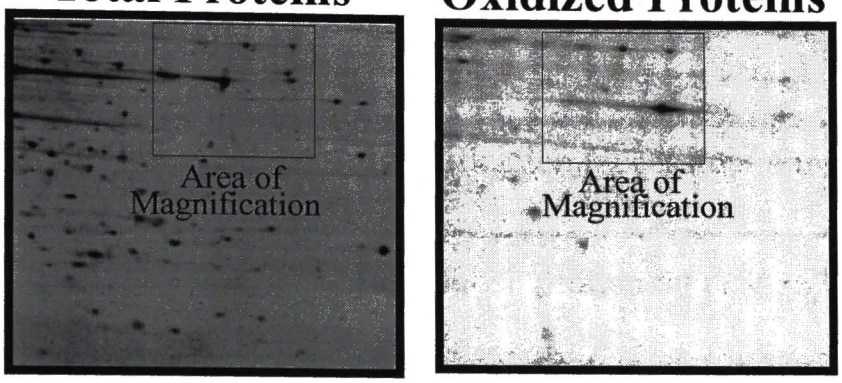
Fibroblasts	2,7-DCF Fluorescence / MTS (Ratio of beta-amyloid treated / control)
Control	1.26 ± 0.05
AD	1.24 ± 0.037

Table 6. Reactive oxygen species generation. Fibroblats were incubated in 100 μM $A\beta_{25-35}$ for 3 days. Levels of cellular oxidative stress generated by beta-amyloid were measured using the fluorescence of DCF as described in Materials and Methods and are expressed as fluorescence intensity per levels of cell viability measured by MTS assay. Values are means ± S.E.M of nine samples.

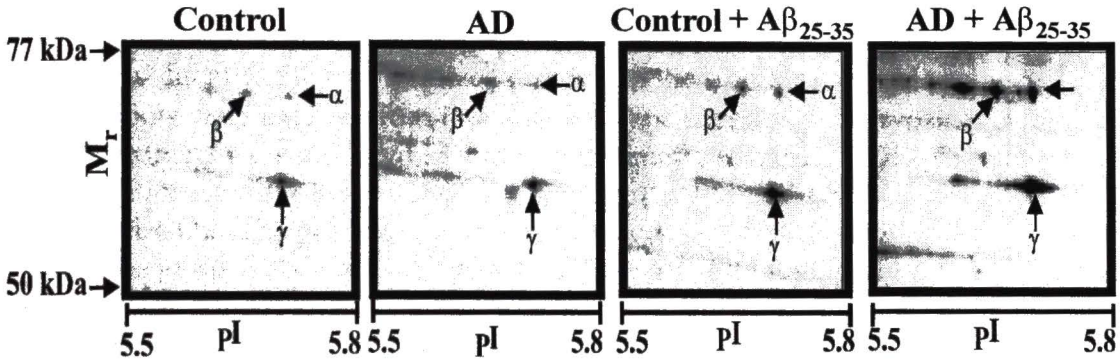
In order to determine which specific proteins were oxidized, we utilized 2-Dimensional Electrophoresis (2-DE). Figure 19A (left panel) shows a typical example of human skin fibroblast extracts separated by 2-DE and SyproRuby stained for total proteins. Figure 19A (right panel) shows the immunostained protein profile on the Western blot after utilizing anti-DNP antibody to detect the oxidized proteins. Figure 19B represents the immunostained protein profile from the defined area of magnification in Figure 19A left panel. Only a few of the proteins were oxidized and they were found in the area: pI = 5-6, MW = 50-80 kDa. Fibroblasts from both AD and age-matched control showed basal levels of oxidation of these particular oxidation sensitive proteins even when there was no exposure to $A\beta_{25-35}$. The level of oxidation of these proteins in the fibroblasts from AD subjects after exposure to $A\beta_{25-35}$ for 3-days was increased almost 9-fold over levels of controls for the proteins designated "α"and "β", and 4.5-fold for "γ" (Fig 19C). In contrast, in the fibroblast from age-matched controls under the same conditions mentioned above, these proteins showed approximately 2-fold increase in oxidation. This indicates that the same proteins are susceptible to oxidation in fibroblasts from both AD and age-matched controls. However, the levels of oxidation of all three proteins ("α", "β", and "γ") were much higher in the fibroblasts from AD compared to control after incubation with $A\beta_{25-35}$. Protein spots ("α", "β", and "γ") were excised from the gels and identified by MALDI-TOF/MS. These three proteins, which were the most easily oxidized, were identified as a heat shock protein 60 ("α" and "β") and vimentin ("γ") (Table 7).

Figure 19. Effect of beta-amyloid treatment on specific protein oxidation visualized by 2-dimensional electrophoresis. Exponentially dividing cells were dissociated with trypsin; and approximately 1,000,000 cells were plated into 100-mm plates and allowed to attach. Following a 0-3 day exposure of cells to 100 μM beta-amyloid, both treated and nontreated cell extracts were collected, then subjected to isoelectric focusing (18-cm IPG strips, pH 5-6) and molecular weight separation, as described in Materials and Methods. Panel A shows a 2-DE gel of a treated AD fibroblast sample that was SyproRuby stained in order to visualize all proteins, and a blot immunostained to visualize oxidized proteins. Panel B represents enlarged views of the area that showed the highest intensity immunostaining: three specific proteins are highlighted (α, β, and γ,). Panel C shows a table indicating the values of integrated intensity of the oxidized proteins found on the immunostained membrane.

A



B



C

Gel Name in panel B	Treatment	Specific Oxidation Index α	β	γ
Control fibroblast	None	60	63	214
AD fibroblast	None	55	62	246
Control fibroblast	100 μM β-amyloid	132	118	472
AD fibroblast	100 μM β-amyloid	559	593	1113

Spot name	Protein MW (Da)/pI	Identification (accession number)
α	61055/5.7	Heat shock protein 60 (284763)
β	61055/5.7	Heat shock protein 60 (284763)
γ	53686/5.1	Vimentin (P08670)

Table 7. . Identification of oxidized proteins in the fibroblasts of AD subjects (Fig. 19). Protein spots were excised from the 2-DE gels and digested with trypsin. Two μl of the peptide extracts were analyzed by mass spectrometry (MALDI-TOF/MS using an Applied Biosystems Voyager DE-STR and capillary-HPLC-ESI/MS/MS on a Thermo Finnigan LCQ). The peptide mass maps produced by MALDI-TOF/MS were searched against the published databases by means of the MS-Fit module in Protein). The peptide mass maps produced by MALDI-TOF/MS were searched against the published databases by means of the MS-Fit module in Protein Prospector (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm). The uninterpreted HPLC-ESI/MS/MS data were analyzed by SEQUEST, a component of the LCQ software.

III. HUMAN PLASMA MODEL OF AD SUBJECTS

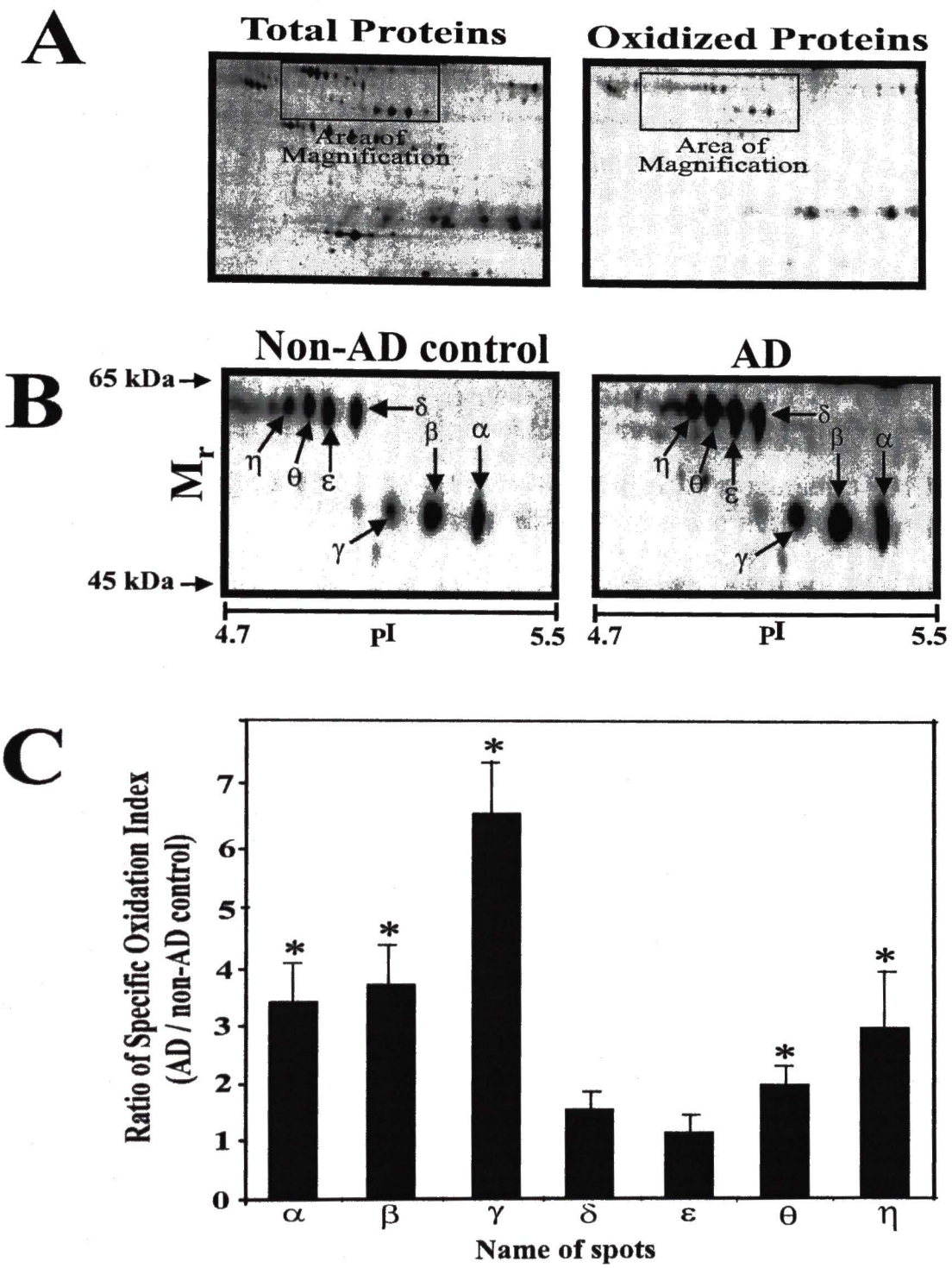
Identification of Oxidized Plasma Proteins in AD

The two previous chapters demonstrated that specific oxidation sensitive proteins can be identified and that such proteins may be directly linked to the apoptotic mechanism and to the pathology of Alzheimer's disease (see discussion). Therefore, it was of great interest to determine if such oxidation sensitive proteins could be identified in the blood plasma of subjects with AD.

To identify uniquely oxidized proteins characteristic of AD, we compared plasma from 9 AD and 9 non-AD controls by 2-DE, followed by immunological staining for protein carbonyls. Figure 20A (left panel) shows a total protein stain of a portion of the 2-DE gel, demonstrating the high-resolution separation of the proteins and the low background staining. Fig 20A (right panel) shows the corresponding immunostain, with the oxidized proteins detected by the DNP-antibody. Fig 20B shows the immunoblots for the "area of magnification" in Fig 20A. Seven oxidized protein spots electrofocused with pI values from 4.7 to 5.5 and molecular weight from 65 to 45 kDa. These oxidized proteins were observed in the blood of both AD subjects and non-AD controls. However, the level of oxidation of these protein spots was markedly increased in the AD samples. For example, level of oxidation of spot 'γ' was increased 7-fold in plasma from AD subjects. The spots 'α', 'β', and 'θ' showed about a 3- to 4-fold increase in oxidation in the blood of AD subjects. This increased oxidation was not a generalized phenomenon of all oxidized proteins since the spots 'δ' and 'ε'showed only slight differences in oxidation between the AD subjects and non-AD controls. Thus, this increase in oxidation of the seven spots appears to be specific. For example, in the total protein stain profile,

more than 300 spots were detected, but only less than 20 spots were positive by immunostaining with anti-DNP antibody. Furthermore, only seven spots were intensively oxidized and six spots among seven showed significant ($P < 0.05$) differences between the plasma of AD subjects and non-AD controls. These seven protein spots were excised from the 2-DE gel, digested in situ with trypsin, and analyzed by MALDI-TOF/MS. Spots 'α' and 'β' were identified as fibrinogen gamma chain precursor protein (Table 8). Protein spots designated as 'δ', 'ε', 'θ', and 'η' were also identified as isoforms of alpha-1 antitrypsin (Table 8).

Figure 20. Oxidation of specific proteins in the plasma of Alzheimer's disease. (A) A 2-DE gel that was silver stained in order to visualize all proteins (left panel) and immunostained with anti-DNP antibody to visualize oxidized proteins of plasma (right panel); (B) Enlarged views of the immunostained 'Area of magnification' showing the oxidized protein spots of interest: Seven spots are highlighted (α, β, γ, δ, ε, θ, and η); (C) Ratio of integrated intensity values of the spots (α, β, γ, δ, ε, θ, and η). These values represents the specific oxidation index of each protein spot (i.e. the immunointensity of the oxidation divided by the intensity of the protein stain) [27]. Values represent means ± SEM for 9 AD and 9 non-AD individuals. The data were analyzed statistically by ANOVA. The means showing significant differences ($P < 0.05$) are identified by an asterisk (*).


A
Total Proteins
Oxidized Proteins
Area of Magnification
Area of Magnification
B
Non-AD control
AD
65 kDa
45 kDa
M_r
δ
β
α
η
θ
ε
γ
4.7
pI
5.5
C
Ratio of Specific Oxidation Index (AD / non-AD control)
0
1
2
3
4
5
6
7
*
α
β
γ
δ
ε
θ
η
Name of spots

Spot name	Protein MW (kDa)/pI	Identification (accession number)
α	51 / 5.4	Fibrinogen gamma chain precursor (P02679)
β	51 / 5.3	Fibrinogen gamma chain precursor (P02679)
γ	51 / 5.24	Fibrinogen gamma chain precursor* (P02679)
δ	60 / 5.15	Alpha-1-antitrypsin precursor (P01009) (Alpha-1 protease inhibitor)
ε	60 / 5.13	Alpha-1-antitrypsin precursor (P01009) (Alpha-1 protease inhibitor)
θ	60 / 5.08	Alpha-1-antitrypsin precursor (P01009) (Alpha-1 protease inhibitor)
η	60 / 5.07	Alpha-1-antitrypsin precursor (P01009) (Alpha-1 protease inhibitor)

Table 8. Identification of oxidized proteins in the plasma of AD subjects (Fig. 20). Protein spots were excised from the 2-DE gels and digested with trypsin. Two μl of the peptide extracts were analyzed by mass spectrometry (MALDI-TOF/MS using an Applied Biosystems Voyager DE-STR and capillary-HPLC-ESI/MS/MS on a Thermo Finnigan LCQ). The peptide mass maps produced by MALDI-TOF/MS were searched against the published databases by means of the MS-Fit module in Protein Prospector (http://prospector.ucsf.edu/ucsfhtml3.4/msfit.htm). The uninterpreted HPLC-ESI/MS/MS data were analyzed by SEQUEST, a component of the LCQ software.
*Spot was not unequivocally identified by MALDI-TOF/MS. However, the MS data and the 2-D mapping coordinate were consistent with 'fibrinogen gamma chain precursor'.

DISCUSSION

Recent evidence in the field of Alzheimer's disease research has emphasized the importance of oxidative stress in the pathogenesis. Aβ, which is found in the senile plaque of AD brain, is toxic to the cells through production of free radicals and oxidative damage of cellular components, especially proteins [44, 124]. Protein oxidation has been suggested as playing a pivotal role in AD-associated neuronal cell death and loss of cognitive functions. Oxidation of proteins can result in many types of loss of normal biological function. This can include loss of catalytic activity, altered specificity, or the accumulation of aggregates, which may be cytotoxic. For example, modified proteins that form aggresomes have been implicated in several neurodegenerative disorders including Alzheimer's, Huntington's, and prion diseases [93]. However, the relationship between oxidatively damaged proteins and neurodegeneration remains unclear. Therefore, the identification of the specifically modified proteins and the nature of these modifications are critical for developing rational mechanisms for the prevention or treatment of the diseases. Since at least some oxidative damage may be prevented by antioxidants in cell culture and animal models [57], the protective effects of antioxidants on protein oxidation was also a topic of this dissertation.

Oxidative Stresses

Beta-amyloid has been shown to produce ROS, thus causing the accumulation of oxidatively damaged proteins, which ultimately lead to apoptotic cell death [9, 18]. Although it is generally recognized that ROS cause apoptosis, the mechanisms remain unclear. Increasing evidence suggests that proteins with specific "oxidation sensitive" amino acid sequences can regulate cellular transcription events, and may play a substantial role in apoptotic neurodegeneration [36, 130]. For example, when the cytoplasmic transcription factor complex (NF-κB / IκB -α) is exposed to ROS, the dimer dissociates and the inhibitor is proteolytically degraded, allowing the translocation of NF-κB to the nucleus [7, 51, 78 102, 115, 136, 157, 147]. Because oxidatively damaged proteins can readily lose or change functionality, oxidation-sensitive proteins (OSPs) may provide a secondary messenger signaling system capable of regulating cellular functions, including apoptosis [1, 65]. Thus, the identification of specific redox-responsive elements would be a significant advance for quantitatively assessing the relationship between increased ROS generation and apoptosis. Furthermore, the lesions in the AD brain reveal oxidative damage by free radicals [44, 124]. Although oxidized proteins and protein aggregates have been detected extensively in AD brain, the identity of these modified proteins has not been investigated in detail. To answer these questions, hippocampal neuronal cells were systematically treated with oxidative stresses generated by enzymatic production of H_2O_2 or by the addition of the beta-amyloid peptide $A\beta_{25-35}$.

Beta-amyloid can exist as either soluble or fibril forms. The aggregated fibril form (which is self-generated from several-days incubation of soluble form) exhibits greater cytotoxicity due to increased production of ROS [63, 106]. In our studies,

fibroblast cells were not noticeably affected until after two-day incubation with beta-amyloid. These results were observed in the cell survival assay, in light microscopy, and from analysis of levels of oxidized proteins. The measurement (by DCF-fluorescence method) of ROS produced inside cells showed a negligible difference between non-treated and beta-amyloid treated samples through the first two days of incubation with beta-amyloid. However, after third-day beta-amyloid incubation, the levels of DCF-fluorescence were increased in the beta-amyloid treated cells compared to the non-treated cells. These findings may be due to the lag time required for the formation of aggregated beta-amyloid (approximately 3 days) [106]. The levels of DCF-fluorescence, however were similar in fibroblasts from both AD and controls, indicating no difference in ability to produce reactive oxygen species inside cells by $A\beta_{25-35}$. The different susceptibility (shown by cell survival, cell morphology, and protein oxidation) of $A\beta_{25-35}$ -treated fibroblasts from AD subjects compared to the treated fibroblasts from age-matched controls suggests inherent differences in the cells from subjects with AD (e. g. diminished antioxidant capacity).

Effects of Antioxidants on Cell Protection and Protein Oxidation

A large number of antioxidants (vitamin E and flavones) have been proposed to produce beneficial effects on human health. Flavonoids are major components contained in fruits, nuts, and certain vegetables, and have been shown to have antioxidant actions. The four major flavone components of the medicinal plant *Scutellaria baicalensis* Georgi are polyphenols, and have been reported to possess surprising antioxidant properties [4, 109, 123]. Extracts of *Scutellaria baicalensis* Georgi (SbE) have been used to treat a

variety of clinical disorders and may be beneficial for supplementation in disorders that are suspected to have increased oxidative stress as a causative factor [4].

Pretreatment of the cells with vitamin E (Vit E) protected the neuronal cells from apoptotic cell death and increased cell survival to almost that of the non-stressed controls. The induction of apoptosis that results from increased oxidative stress has been established in cell culture and animal models [25, 34]. The attenuation of apoptosis by the antioxidant properties of Vit E and the flavones is consistent with this mechanism of apoptosis. Also, protein oxidation caused by reactive oxygen species was also prevented by the pretreatment with antioxidants.

Compounds with cellular antioxidant effects may act by inhibiting the formation of ROS, or by enhancing cellular antioxidant mechanisms. Results from other laboratories suggest that flavones can scavenge ROS directly [48, 123]. We confirmed this observation by using 2′, 7′-dichlorofluorescin diacetate (DCFH-DA).

There are three important structural requirements of flavonoids for protection from oxidative stress: hydroxyl groups on one particular aromatic ring, unsaturation of one of the rings, and hydrophobicity. The unsaturated aromatic ring allows electron delocalization across the molecule for the stabilization of the free radical. In general, the more hydrophobic a flavonoid is, the more protective it is against the oxidative stress. This may be due to high capability for penetration by the hydrophobic antioxidants through the plasma membrane into the cytoplasm, in which they perform the antioxidant functions.

Since the material used in these studies is a mixture of four flavones, the question can be asked which particular flavone(s) is providing this novel chemoprotective ability.

At this point, it is not possible to fully answer this question. However, previous studies [123] have shown that baicalein has the ability to scavenge superoxide radicals, hydroxyl radicals, or to degrade hydrogen peroxide prior to its reduction to hydroxyl radicals. Thus, the direct antioxidant effects can certainly be, at least in part, attributed to this component. Whether this, or one of the other flavones, is responsible for the changes in Bcl-2 and Bax is not known. Also, the antioxidant activity of SbE containing the four flavones was observed to be greater than that of pure baicalein [123]. Thus, it is very possible that these flavones may be acting synergistically.

In this study, we showed that and flavonoids in SbE protect the cells by scavenging free radicals directly and by altering the Bcl-2/Bax ratio. Other groups have reported that flavonoids protect neuronal cells from oxidative stress (e.g. glutamate toxicity) by increasing intracellular glutathione level, scavenging free radicals, and preventing the influx of Ca^{2+} despite high level of ROS. Therefore, flavonoids appear to protect the cells from oxidative stress by a combination of several functions [48, 81, 92]. Alteration of the ratio of anti- and pro-apoptotic protein levels and posttranslational modification of these proteins are other major mechanisms of antioxidant protection. Our results showed that the Bcl-2 protein level was increased and the proteins post-translationally modified (phosphorylated), whereas the Bax protein level was decreased after exposure to the flavones. In 2-DE gels, several high molecular weight proteins immunostained with antibodies to Bax were observed (data not shown). These proteins may be Bax proteins post-translationally modified by ubiquitination, homodimerization, or heterodimerization with unknown Bcl-2 family proteins.

The Bcl-2 family proteins consist of proapoptotic (Bax, Bcl-x_s) and anti-apoptotic (Bcl-2, Bcl-x_L) proteins, which play critical roles in programmed cell death. Bcl-2 is a mitochondrial membrane protein capable of protecting cells from oxidative stress. Bax, on the other hand, antagonizes the Bcl-2 inhibition of apoptosis. The overexpression of Bcl-2 protects against hydrogen peroxide-induced cell death, whereas overexpression of Bax leads to increased apoptosis in neuronal cells [66]. Therefore, the ratio of the Bcl-2 to Bax proteins is important for determining the cellular fate. In addition, it is believed that post-translational modifications of Bcl-2 family proteins are important in determining their active or inactive conformations. For example, phosphorylation of Bcl-2 is required for its antiapoptotic function; and the heterodimerization of Bax with Bcl-2 abolishes its anti-apoptotic function [103, 138].

Detection of Protein Oxidation

We initially measured the total protein carbonyl content of cellular extracts from the cells. Surprisingly a random assortment of oxidized proteins was not found throughout the mass and charge spectra. High-resolution 2-Dimensional Electrophoresis and Western blotting showed that more than half of the oxidized proteins were located in one specific region of the blots (MW 50-70, pI 6-7.5). These data indicate that not all proteins are equally sensitive to oxidative stress. This is reasonable since specific amino acid sequence motifs appear to be susceptible to oxidation [119]. For example, the Aβ peptide contains 40 amino acids, of which only Met 35 is readily oxidized [148]. In peptide sequences that contain the same amino acids, but in reversed or scrambled sequences, the methionine is not oxidized [156].

To identify the specific proteins that are most easily oxidized and that might be important for initiation of apoptotic cell death caused by oxidative stress, high-resolution 2-DE separation of proteins, coupled with immunostaining of protein carbonyl and MALDI-TOF mass spectrometry were used.

The identities of the three major proteins, which were oxidized in the neuronal cells, were identified by mass spectrometry as heat shock protein 60 (HSP 60), vimentin, and hemoglobin alpha chain. Both heat shock protein 60 and vimentin were also oxidized in the fibroblasts from AD subjects. The two isoforms of HSP 60 differ only in charge (isoelectric point) properties and thus are most likely produced by different degrees of oxidation.

Vimentin

Fig. 21 shows a proposed model for the involvement of oxidized vimentin in the mechanism of Alzheimer's disease. Vimentin is a component of intermediate filaments, and has been suggested as a critical cytoskeleton protein that is cleaved by caspases at an early stage of apoptosis [19]. The overexpression of a caspase-resistant vimentin attenuates apoptotic cell death caused by oxidative stress in vitro [11]. Therefore, vimentin appears to play a role as an anti-apoptotic protein.

Vimentin has an interesting feature related to the aggresome that appears in the pathology of Alzheimer's disease [23, 149]. When the accumulation of misfolded proteins (e.g. from oxidative modification) exceeds the ability of cellular degradation systems (e.g. proteasome) to remove them, these damaged proteins form aggregates that resistant to degradation. These result in the accumulation of morphologically and

biochemically distinct intracellular and extracellular protein aggregates, some of which are ubiquitinated, along with the deposition of disordered intermediate filament proteins, especially vimentin [93]. Vimentin is a critical component of aggresome formation, which forms a cage surrounding a pericentriolar core of aggregated, ubiquitinated protein [71]. The presence of aggresome in intracellular and extracellular lesions is associated with cell death in the Alzheimer's disease.

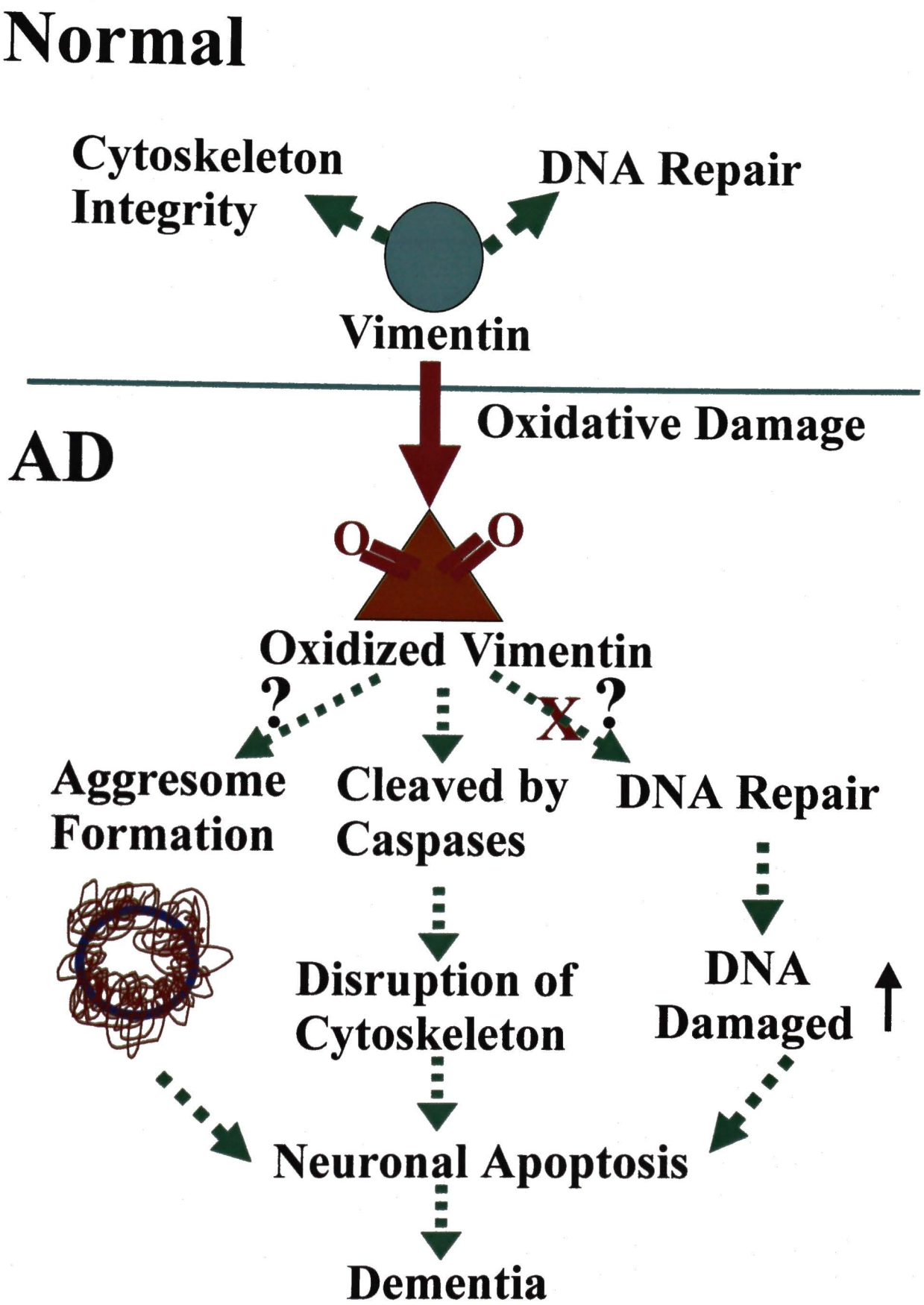
Vimentin has also been implicated in delaying senescence and in the spontaneous immortalization of mouse embryo fibroblasts. Vimentin exhibits affinity for, and forms crosslinkage products with, recombinogenic nuclear, as well as mitochondrial, DNA in intact cells, and has been suggested to play a role in the recombinogenic repair of oxidative damage [67, 140]. Oxidative damage of vimentin may cause the loss of this function. This would result in accumulation of potentially toxic damaged DNA and proteins.

HSP 60

Fig. 22 shows a proposed model for the involvement of oxidized HSP 60 in the mechanism of Alzheimer's disease. Similar to vimentin, the oxidative damage of HSP 60 is believed to be involved in apoptotic cell death. It has been reported that overexpression of the HSP 60 protects myocytes from the apoptosis [84]. Therefore, oxidative damage of HSP 60 may cause the loss of antiapoptotic function and thus leads to neuronal cell death.

The most important function of HSP 60 is as a molecular chaperon that is responsible for correction of misfolded proteins. Protein aggregation is an inevitable

Figure 21. Proposed model for toxicity caused by oxidative damage of vimentin. Vimentin is the critical component of the cytoskeleton and has been known to play a role in recombinogenic DNA repair. Oxidative modification of vimentin causes the loss of its function, thus results in an accumulation of DNA damage that should be harmful to the cells. Cleavage of vimentin by caspases in the early apoptotic cascades causes the disruption of the cytoskeleton and integrity of neuron. Also, vimentin is found in aggresome structures in which aggregated proteins formed by oxidative modification are transported to the microtubule-organizing center, where they are unsheathed by the intermediate filament protein, e.g. vimentin. Thus, oxidative modification of vimentin appears to cause neuronal cell death.

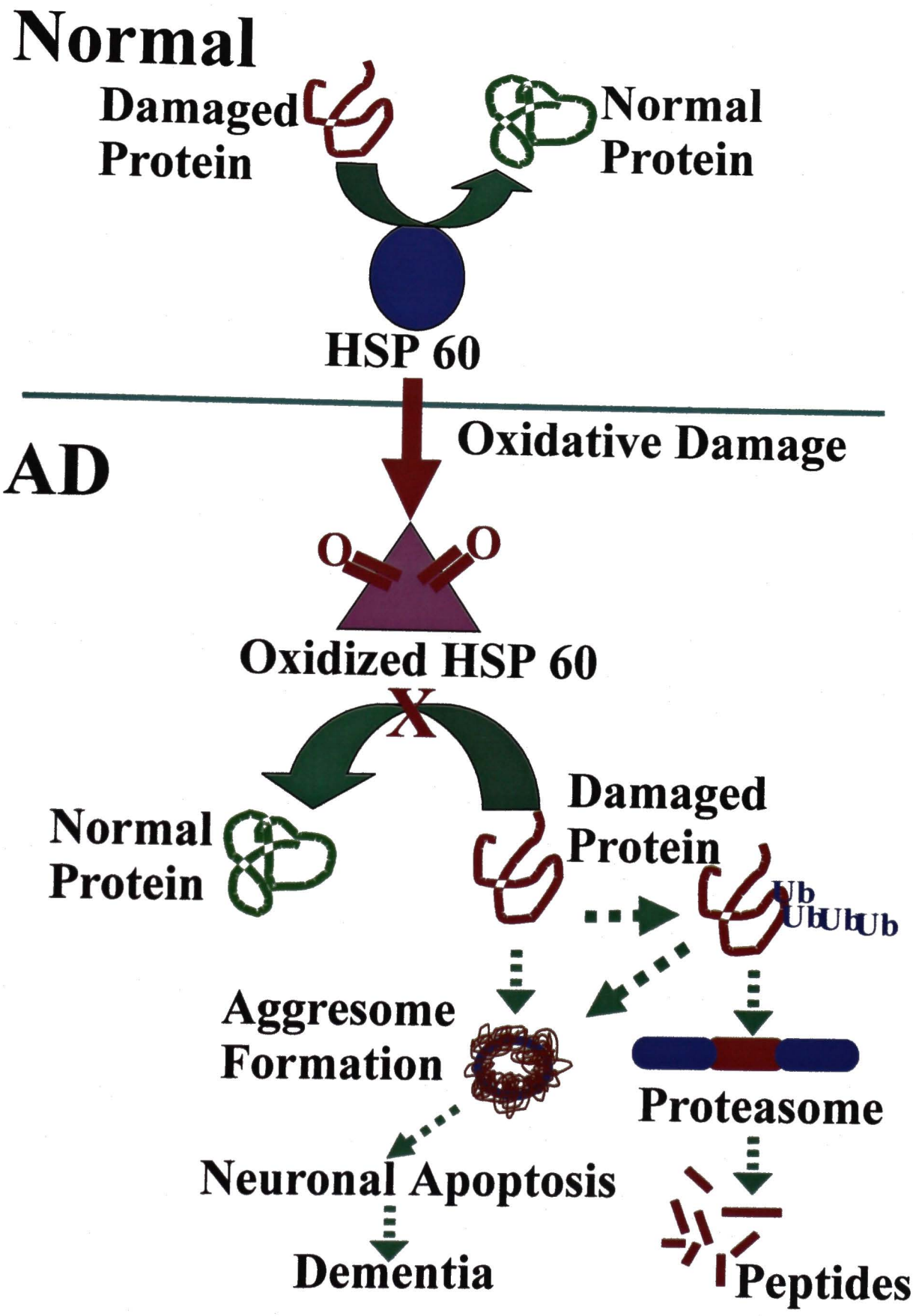

Normal
Cytoskeleton Integrity
DNA Repair
Vimentin
AD
Oxidative Damage
O
O
Oxidized Vimentin
?
X
?
Aggresome Formation
Cleaved by Caspases
DNA Repair
Disruption of Cytoskeleton
DNA Damaged
Neuronal Apoptosis
Dementia

consequence of cellular existence. In unstressed cells, protein aggregates do not accumulate, despite their continued production, due to balanced quality control machinery, such as chaperons (e.g. HSP 60) and proteasome, which correct or selectively degrade improperly folded polypeptides before they can aggregate. However, protein aggregates accumulate in stressed conditions such as oxidative stress, due to failure of this quality control system [137]. Also, aggresomes are enriched with molecular chaperons (HSPs) in addition to a major aggregated protein species shown by immunohistochemical analysis, indicating that HSPs are directly involved in aggresome formation [49]. Our finding of HSP 60 as a prime target for oxidation indicates the failure of one of the most important components of cellular quality control machinery, which in turn causes further accumulation of damaged protein aggregates. Taken together, the two most easily oxidized proteins, HSP 60 and vimentin, appear to be involved in not only apoptotic cell death but also the formation of protein aggregates, both of which are major pathologies of Alzheimer's disease. The loss of function caused by oxidative damage of these two proteins, HSP 60 and vimentin would appear to clearly fit into the mechanism of apoptosis in Alzheimer's disease, which is accelerated by oxidative stress.

Oxidized Plasma Proteins in AD

Oxidized proteins were also found to be elevated in plasma of subjects diagnosed with AD. While vimentin and HSP 60 were not found, two specific oxidized proteins were identified as isoforms of alpha-1 antitrypsin and fibrinogen gamma chain. In both cases, the isoforms differed only in their charge properties (i.e. pI value), which would be

Figure 22. Proposed model for toxicity caused by oxidative damage of HSP 60. HSP 60 is one of the molecular chaperons that are responsible for correction of misfolded proteins in the cells. Oxidative modification of HSP 60 results in accumulation of damaged proteins, which become ubiquitinated and destined for proteosomal degradation, or else become aggregated and form aggresome. Aggresome is one of the major pathological structures found in brains of Alzheimer's patients and has been suggested to toxic to the cells by producing reactive oxygen species.


Normal
Damaged Protein
Normal Protein
HSP 60
AD
Oxidative Damage
O
O
Oxidized HSP 60
X
Normal Protein
Damaged Protein
Ub
UbUbUb
Aggresome Formation
Proteasome
Neuronal Apoptosis
Dementia
Peptides

expected from oxidative modification. It is interesting that both of these proteins have been previously implicated in the etiology of Alzheimer's.

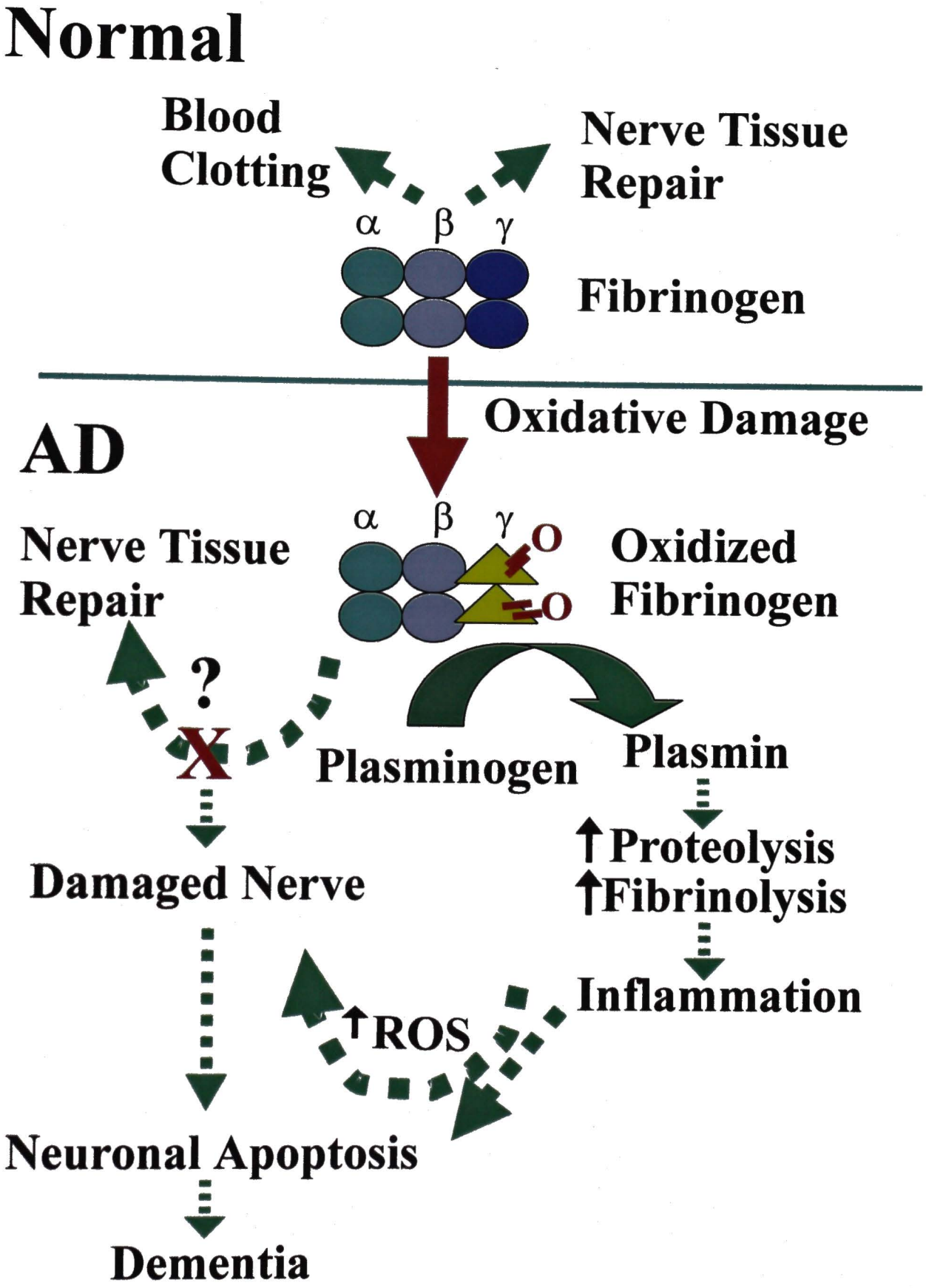
Fibrinogen

Fibrinogen oxidation may be relevant to the mechanism as shown in Fig. 23. All six chains of fibrinogen (α_2, β_2, γ_2) must be intact for normal fibrinogen; thus, oxidative damage to the γ-chain would likely causes altered fibrinogen function. The conformational changes of fibrinogen caused by oxidation increased the binding affinity to the tissue type plasminogen activator (t-PA) and resulted in increased activation of plasminogen by t-PA, thereby contributing to fibrinolysis and proteolysis in areas of inflammation [131]. In fact, metal-ion catalyzed oxidation of fibrinogen produces dityrosine formation and loss of tryptophan residues, resulting in diminished platelet aggregation and adhesion [12]. Blood coagulation and fibrinolysis have been reported to play pivotal roles in inflammation in extravascular tissue related to the Alzheimer's disease [2, 133], and components of the coagulation system, and adhesion molecules have been identified with altered levels in senile plaques [41, 46].

Alpha-1-Antitrypsin

Similarly, alpha-1-antitrypsin appears to be relevant to the disease (Fig. 24). Alpha-1 antitrypsin, one of the major serine proteinase inhibitors (serpins) in human plasma [141], functions to inhibit overexpressed proteinases during inflammation [74]. Proteinase activity is tightly regulated by these inhibitors under normal physiological conditions. However, under some pathological conditions, proteinase activity may

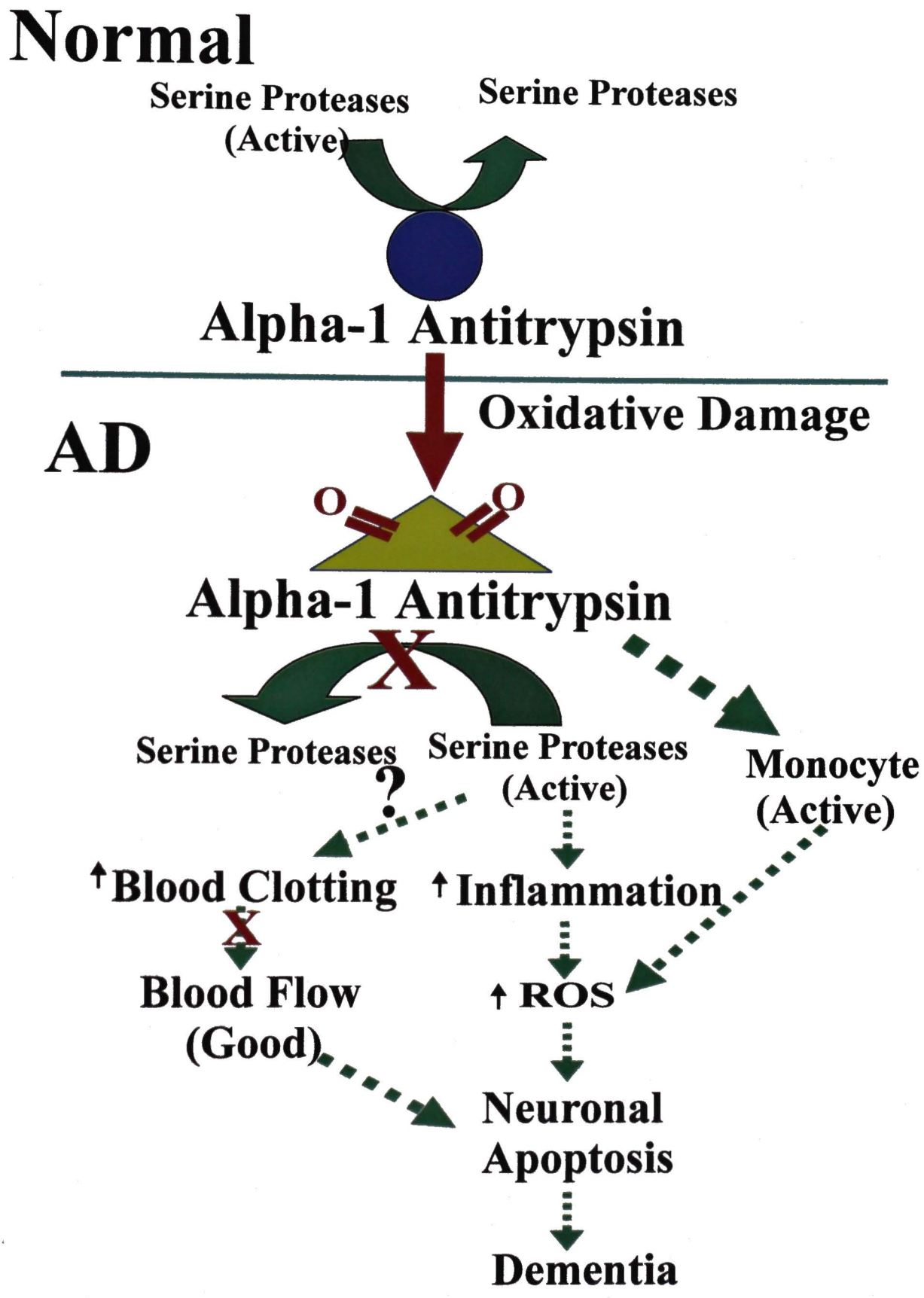
Figure 23. Proposed mechanism for the role of the oxidation of fibrinogen in the plasma of AD subjects in the neuropathology of the brain. Oxidative modification of fibrinogen causes conversion of plasminogen to plasmin, a potent proteolytic enzyme with a broad spectrum of activity including triggering of fibrinolysis and proteolysis, and thus results in increased inflammation and neuronal cell death. Furthermore, oxidized fibrinogen is accumulated in the brain tissue by leakage through the blood brain barrier and causes inflammation and nerve tissue damage due to loss of its function as a player in the repair of nerve tissue damage. After a nerve injury, it seems that a fibrinogen leaked from the blood into nerve cells is converted into fibrin. Fibrin has two effects on the repair processes of nerves. As it does in other types of injury, the protein helps stop bleeding and choreograph the repair process. Therefore, oxidized fibrinogen in the plasma of the AD subjects may contribute to neuronal cell death and dementia.


Normal
Blood Clotting
Nerve Tissue Repair
α β γ
Fibrinogen
Oxidative Damage
AD
Nerve Tissue Repair
α β γ
O
O
Oxidized Fibrinogen
?
X
Plasminogen
Plasmin
Damaged Nerve
↑Proteolysis
↑Fibrinolysis
Inflammation
↑ROS
Neuronal Apoptosis
Dementia

exceed the capacity of such proteinase inhibitors as alpha-1-antitrypsin. This could be caused by oxidation of the inhibitor [16, 39 141]. Isoforms of alpha-1 antitrypsin have been observed with altered properties that result from oxidation, e.g.peptide cleavage, polymerization, and complexation with proteases or other ligands [68]. Human alpha-1 antitrypsin contains nine methionine residues, and two of them (Met 351 and Met 358) are easily oxidized. Methionine oxidation in the alpha-1 antitrypsin results in loss of its anti-neutrophil elastase activity and uncontrolled degradation of connective tissues in rheumatoid arthritis and pulmonary emphysema [72, 97, 137, 151]. Also, the oxidized isoforms contribute to inflammation by activating primary human monocytes [98] and may therefore contribute to the inflammatory processes in neurodegenerative disorders such as Alzheimer's [35, 145]. In AD brain, alpha-1 antitrypsin and alpha-1 antichymotrypsin are localized in neurofibrillary tangles and senile plaques, and their involvement in the pathogenesis of the lesions of AD is thus implied [54]. Alpha-1 antichymotrypsin forms complexes with the amyloid peptide $A\beta_{1-42}$ [96, 152].

Reduced blood flow to the brain is another pathology of AD. Blood flow is essential for nutrient delivery to the brain. The better the blood flow, the more likely a person is to have good cognitive function [146]. Interestingly, these two proteins, fibrinogen and alpha-1 antitrypsin, are involved in the blood clotting process and thus regulate blood flow. Alpha-1 antitrypsin inhibits thrombin conversion of fibrinogen to fibrin, which is the critical step in pathways of blood clotting. Therefore, increased oxidation of these two proteins in the plasma of AD patients may be related to the alteration of blood flow to the brain, thus contributing to the neuropathology of the disease.

Figure 24. Proposed mechanism for neuronal cell death caused by oxidative damage of alpha-1 antitrypsin. Alpha-1 antitrypsin is synthesized in the brain tissue and may play an important role in maintenance of integrity of brain tissue. The activity of serine proteases is regulated by serine protease inhibitors. Serine proteases play important roles in inflammation and blood clotting. For example, one of the serine proteases, thrombin, is responsible for conversion of fibrinogen to fibrin, which are the essential steps in the blood-clotting pathway is inactivated by alpha-1 antitrypsin. Oxidation of alpha-1 antitrypsin causes increased inflammation and reduced blood flow to brains due to loss of anti-protease activity, which is critical for regulating blood coagulation, fibrinolysis, and tissue protection from proteases. Also, alpha-1 antitrypsin activates human monocytes upon oxidative modification, thus cause inflammation by generating reactive oxygen species (ROS). Increased inflammation in the brain causes leakage of the blood brain barrier that is critical for protection of brain from lots of components harmful to the nerve cells in the blood. And decreased blood flow to the brains results in the lack of oxygen and nutrients that are essential for survival of the brain cells.


Normal
Serine Proteases
(Active)
Serine Proteases
Alpha-1 Antitrypsin
AD
Oxidative Damage
O
O
Alpha-1 Antitrypsin
X
Serine Proteases
?
Serine Proteases
(Active)
Monocyte
(Active)
↑Blood Clotting
↑Inflammation
X
Blood Flow
(Good)
↑ ROS
Neuronal
Apoptosis
Dementia

Conclusion

This study shows that oxidative stresses from H_2O_2 or $A\beta_{25-35}$ are damaging to neuronal cells and fibroblasts. Pretreatment with antioxidants protects cells from apoptotic cell death by (a) scavenging reactive oxygen species directly (b) by altering ratio of anti-apoptotic to pro-apoptotic proteins (Bcl-2 and Bax families) or (c) by affecting posttranslational modification of these proteins. When neuronal cells or AD fibroblasts are exposed to oxidative stresses, vimentin and heat shock protein 60 was oxidized (carbonylated). These two proteins appear to be involved in apoptotic cell death, and may play a role in the pathology of Alzheimer's disease. In our studies, these proteins were protected from oxidation by antioxidants. Two proteins that were extensively oxidized in the plasma from AD subjects are alpha-1 antitrypsin and the gamma chain of fibrinogen. These proteins have been shown to be involved in inflammation processes associated with Alzheimer's disease. These oxidized proteins may be of utility as specific AD-biomarkers. Our finding of specific oxidized proteins in the cultured cells and plasma of AD subjects offers insight into the pathological mechanism of AD and may also provide biomarkers for the early diagnosis of the disease and for monitoring antioxidant therapy in clinical trials.

Limitation

Two-dimensional gel electrophoresis is a powerful technique but remains a fairly cumbersome method for characterization and identification of proteins of low abundance, including those that may be also oxidized. Carbonyl moieties can be introduced into proteins not only by direct oxidation of amino acid side chains, but also by reaction with

aldehydes such as 4-hydroxynonenal and malonaldehyde formed from oxidation products of polyunsaturated fatty acids. Furthermore, some amino acids are converted into specific oxidation products without production of carbonyl groups, e.g. the conversion of phenylalanine residues to *o*-tyrosine and of tyrosine to dityrosine. Despite this caveat, assay of carbonyl groups in proteins provides a convenient technique for detecting and quantifying oxidative modification of proteins.

Future Directions

The results reported here suggest several interesting research areas.

1) How are the two most easily oxidized proteins, heat shock protein 60 (HSP 60) and vimentin, related to initiation of the oxidative stress induced apoptotic cell death?

2) How are these two proteins, HSP 60 and vimentin, related to the pathogenesis of Alzheimer's disease?

3) Are these two proteins, HSP 60 and vimentin, also the most easily oxidized in other cell types of age-related diseases, e.g. Parkinson's- or Progeria cells?

4) Are these two proteins, HSP 60 and vimentin, found in the brains of animals and as components of aggresome structure in that brain under oxidative stress?

5) After oxidation of these two proteins, are there any gained functions that may be important to neuropathology of Alzheimer's disease?

6) How are the two oxidized plasma proteins in the blood of an AD patient, alpha-1 antitrypsin and fibrinogen gamma chain, related to the inflammation occurred in the AD brain?

7) How is the degree of oxidation of these two plasma proteins related to the progression of AD?

8) What is the source of oxidized alpha-1 antitrypsin in the plasma of AD subjects? Does increased oxidized form of this protein in the plasma come from the brain through leakage of the blood brain barrier?

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