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LYSOSOMAL INVOLVEMENT IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE

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Cover picture: confocal microscopy images of APPswe cells double immunostained for Aβ oligomers (A11 antibody, red fluorescence) and LAMP-2 (green fluorescence). Nuclei were stained by DAPI (blue fluorescence).

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Dedicated o my dear family

献给我最亲爱的家人

ABSTRACT

Alzheimer's disease (AD), the major cause of senile dementia, is associated with progressive formation of neurofibrillary tangles and extraneuronal plaques composed of amyloid beta peptide (Aβ). Aβ has been also found within Alzheimer neurons in association with the lysosomal system, an acidic vacuolar compartment possessing numerous hydrolytic enzymes. Lysosomes have been shown to be involved in both the formation of Aβ and its toxicity to neurons. Another line of evidence implicates oxidative stress as an important factor in the development of AD. It is reported that oxidative damage is one of the earliest changes in AD and plays an important role in the development of the disease. Although both the lysosomal system and reactive oxygen species are involved in AD, the mechanisms of this involvement are not well understood.

To gain insight into the relationship between oxidative stress and the lysosomal system in AD pathogenesis, we focused our study on: 1) the effect of oxidative stress on intracellular distribution of Aβ; 2) the role of endogenous Aβ in oxidant-induced apoptosis; 3) the role of autophagy and APP processing in oxidant induced damage; and, 4) the intraneuronal localization of Aβ and its relationship to the lysosomal system.

In our study, hyperoxia (40% versus 8% ambient oxygen) was used as a model of mild oxidative stress in vitro, while transfected cells producing different amounts of Aβ were used to assess toxicity due to endogenous Aβ. It was found that: 1) oxidative stress induces autophagic uptake of Aβ, resulting in its partial accumulation within lysosomes; 2) oxidative stress can induce neuronal death through macroautophagy of Aβ and consequent lysosomal membrane permeabilization; 3) increased cellular Aβ production is associated with enhanced oxidative stress and enhanced macroautophagy, resulting in increased intralysosomal Aβ accumulation and consequent apoptosis; and, 4) in normal conditions, intracellular Aβ shows primarily cytosolic distribution, not related to lysosomes and other acidic vacuoles, endoplasmic reticulum, Golgi complexes, synaptic vesicles or mitochondria. Only a minor portion of Aβ shows partial colocalization with cellular organelles. Inhibition of secretion significantly increased Aβ colocalization with endoplasmic reticulum, Golgi complexes, synaptic vesicles and lysosomes, as well as the amount of mitochondrial and cytosolic Aβ.

Oxidative stress induces intralysosomal autophagy-generated Aβ accumulation, consequently causing lysosomal membrane permeabilization and apoptosis. Our findings provide a possible explanation of the interactive role of oxidative stress and lysosomal system in AD pathogenesis, and may be helpful for a future therapeutic strategy against AD.

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ABBREVIATIONS

Aβ	amyloid β-peptide
AD	Alzheimer's disease
AICD	APP intracellular domain
APH-1	anterior pharynx defective-1
APLP	amyloid precursor-like protein
APOE	apolipoprotein E
APP	amyloid precursor protein
APPwt	wild-type APP695
APPswe	Swedish KM670/671NL double mutation
ATG	autophagy related protein
AVs	autophagic vacuoles
BACE	β-site APP cleaving enzyme
CamKII	calmodulin-dependent kinase II
Cdc2	cell division cyclin 2
Cdk5	cyclin dependent kinase 5
CMA	chaperone-mediated autophagy
CNS	central nervous system
CTF	C-terminal fragment
CTFα	C-terminal α fragment
CTFβ	C-terminal β fragment
CysC	cystatin C
C83	CTFα
C99	CTFβ
DAPI	4' 6-diamidino-2-phenylindole;
DAPT	LY-374973, N-[N-(3, 5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester
DCF	carboxy-H2DCFDA
EE	early endosomes
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK1/2	extracellular regulated kinase 1/2
E64d	(2S, 3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester, EST
FAD	Familial AD
GSK-3β	glycogen synthase kinase 3β
HEK	human embryonic kidney
H_2O_2	hydrogen peroxide
iEM	immunoelectron microscopy
IDE	insulin degrading enzyme
ECE	endothelin-converting enzymes
LAMP	lysosome-associated membrane protein
LC3	microtubule-associated protein light chain 3
LE	late endosomes
MPRs	mannose 6-phosphate receptors
mTOR	mammalian target of rapamycin
MVB	multivesicular body
M6P	mannose-6-phosphate
NCT	nicastrin
NH4Cl	ammonium chloride
NFT	neurofibrillary tangles

·NO	nitric oxide
NOS2	nitric oxide synthase 2
NT	nontransfected
$OH^{\cdot}$	hydroxyl radical
$O_2^{\cdot -}$	superoxide
PAS	pre-autophagosomal structure
PBS	phosphate-buffered saline
PCD	programmed cell death
PEN-2	PS enhancer-2
PHF	paired helical filaments
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PP	phosphatase
PS	presenilin
RA	retinoic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAD	sporadic AD
sAPPα	soluble α-APP fragments
sAPPβ	soluble β-APP fragments
SP	senile plaques
SOD	superoxide dismutase
TeNT	tetanus toxin
TGN	*trans*-Golgi network
UPS	ubiquitin-proteasomal system
α-APP	α-secretase-processed APP
α2M	α2-macroglobulin
3MA	3-methyladenine

LIST OF PUBLICATIONS

I. **Zheng L,** Roberg K, Jerhammar F, Marcusson J and Terman A, Autophagy of amyloid beta-protein in differentiated neuroblastoma cells exposed to oxidative stress. Neuroscience Letters 394 (2006) 184-189

II. **Zheng L*,** Kågedal K*, Dehvari N, Benedikz E, Cowburn R, Marcusson J, Terman A. Oxidative stress induces macroautophagy of amyloid beta-protein and ensuing apoptosis. Free Radical biology and Medicine 46 (2009) 422–429

III. **Zheng L**, Terman A, Hallbeck M, Dehvari N, Cowburn R, Benedikz E, Kågedal K, Cedazo-Minguez A, Marcusson J. Macroautophagy-generated increase of lysosomal amyloid β-protein mediates oxidant-induced apoptosis of cultured neuroblastoma cells. Autophagy 7 (2011) 1528-1545

IV. **Zheng L**, Cedazo-Minguez A, Hallbeck M, Jerhammar F, Hultenby K, Marcusson J and Terman A. Intracellular localization of amyloid beta peptide in SH-SY5Y neuroblastoma cells. Manuscript.

*Equal contribution

INTRODUCTION

ALZHEIMER'S DISEASE

Alzheimer's disease: basic characteristics

With longer life expectancy, the population of the worldwide elderly is increasing, especially in the western industrialized countries. The need to address the growing problem of health care for the elderly underscores the importance of this area for research. Alzheimer's disease, as the most common forms of dementia (50-70% of dementia cases), not only has tragic influence on the patients and the closest family, but it also presents a heavy burden for society. The increased prevalence of AD and ineffectual treatment raise the issue: What are the mechanisms that cause Alzheimer's disease? How can AD be prevented or, perhaps, its progress slowed? And, finally, what treatments can be made available? These are huge questions for researchers worldwide, and pose financial challenge for the world community as well.

Alzheimer's disease (AD) is an age-related neurodegenerative disorder primarily characterized by progressive cognitive dysfunction, followed by personality change, impaired learning, decline in motor skills, and eventual language loss [1]. It has been reported that 34.4 million people are diagnosed AD worldwide and estimated costs ran up to $422 billion in 2009 [2]. Moreover, AD is predicted to affect one of every 85 persons globally by 2050 without a efficient solution [3]. This would seriously burden health-care systems due to its persistent, disabling and costly burden.

Risk factors

Not one single factor has been identified as a cause for Alzheimer's disease. It is likely that a combination of factors, including age, genetic inheritance, environmental factors, diet and overall general health, contributed.

AD is classified as either sporadic or familial. More than 95% of AD cases belong to sporadic, late-onset AD (SAD, > 65 years old), while fewer than 1% of AD cases are familial early-onset AD (FAD, < 65 years old) [4].

Familial AD (FAD) is associated with mutation of three genes: APP, presenilin1 (PS1) and presenilin2 (PS2), which are localized on chromosomes 21, 14 and 1, respectively [5]. APP mutations either increase the ratio of $A\beta_{42}/A\beta_{40}$ or total accumulation of Aβ production; or, alternatively, they generate highly fibrillogenic Aβ while PS mutations cause increase in the ratio of $A\beta_{42}/A\beta_{40}$ [6]. Down syndrome patients with trisomy 21 (three copies of APP) develop AD pathology as early as the age of 20 years [7].

SAD is associated with several genes. The best known genetic risk factor of SAD is having the gene encoded for APOE (on chromosome 19) [8]. Apolipoprotein E (APOE) plays a vital role in the metabolism and clearance of Aβ along with α2-macroglobulin (α2M) and low-density lipoprotein receptor. APOE has three isoforms of alleles, APOE ε2, APOE ε3, and APOE ε4. The risk associated with ε4> ε3 >ε2. The more common APOE ε3 (40-90% of the population) and the rare APOE ε2 have been relatively protective against AD, while APOE ε4 increased Aβ aggregation, decreased Aβ clearance, and is carried more often by AD patients. Besides these, some evidence indicates other genes are associated with SAD, including: insulin degrading enzyme (IDE), which is active in the degradation of Aβ and may predispose individuals to the disease [9]; ubiquilin-1 (UBQLN1), which affects intracellular APP trafficking [10]; SORL1, which encodes for a neural receptor of APOE [11]; and CALHM1, which encodes for a transmembrane protein influencing calcium levels and Aβ production [12].

Aging is the most important risk factor, though other possible risk factors include stroke, high blood pressure in mid-life, obesity, depression, diabetes, excessive alcohol consumption, high cholesterol levels in mid-life, chronic stress, head trauma, low vitamin B_{12} levels, smoking [13, 14].

Neuropathology

The major pathological hallmarks of AD, first described by Dr. Alois Alzheimer, are including: 1) extraneuronal deposits of Aβ in both *senile plaques* (SPs) and blood vessels, 2) intraneuronal *neurofibrillary tangles* (NFTs), and, 3) *loss of neurons and synapses* [15-17]. The neuron loss and astrocyte proliferation result in gross *atrophy* in the affected regions, including cerebral cortical atrophy, particularly of the temporal and frontal lobes, and associated ventricular dilation.

Both NFTs and SPs are found in normally aging brains, though their quantitative excess is pathognomic in the diagnosis of AD. Study has shown that greater abundance of these plaques is associated with symptom severity in almost all AD patients, and that such plaques tend to proliferate with aging.

The senile plaques are made up of amyloid β peptides (Aβ), peptides of 39-43 (4 kDa) amino acids. There are two forms of amyloid plaques in the AD brain*: neuritic plaques* (also called *senile plaques*) and *diffuse plaques*. The *senile plaques* have a diameter ranging between 10 and 160 µM and appear as radiating bundles of amyloid, having a dense central core. They are extracellularly deposited insoluble fibrillar Aβ ($A\beta_{40/42}$) surrounded by dystrophic neurites (axons and dendrites) that accumulate tau protein, activated microglia, and reactive astrocytes. The *diffuse plaques*, commonly referred as "preamyloid deposits", are much less dense and consist of nonfibrillary forms of Aβ ($A\beta_{42}$). The diffuse plaques are believed to be an immature formation of senile plaques. Moreover, Aβ is also deposited in blood vessel walls, referred to as *amyliod angiopathy*.

The *NFT*s are mainly composed of paired helical filaments (PHFs), formed by abnormally hyperphosphorylated and glycosylated form of tau protein [18, 19]. Normally tau binds and stabilizes mircrotubules, which are important for neurotransmitter and cell structure. Hyperphosphorylated tau will, instead, self-aggregate and not bind to microtubules, leading to damaged microtubules and defects in axonal transport, synapse loss and eventual cell death. NFT accumulation is thought to start in the transentorhinal region of the brain and then spreads to the hippocampus, amygdala and, finally, the neocortex. This pattern of spread of pathology positively correlates with cognitive decline in AD. Neurofibrillary tangles (NFTs) are not specific to AD since they occur in aging, and in other neurodegenerative diseases.

Tau pathology in AD is thought to be induced by an imbalance of either protein kinase and/or phosphatase (PP) activity, either of which is responsible for tau phosphorylation. The protein kinases involved in tau phosphorylation include glycogen synthase kinase 3β (GSK-3β), cyclin dependent kinase 5 (Cdk5), extracellular regulated kinase ½ (ERK1/2), calmodulin-dependent kinase II (CamKII), protein kinase A (PKA) and cell division cyclin 2 (Cdc2). Further, PP-2A has been suggested to be the major phosphatase involved in dephosphorylation of tau in AD. Reduced activity of PP2A in AD brain has been shown (Reviewed in [20]).

Plaques and tangles are present, mainly, in brain regions involved in learning and memory and emotional behaviors, such as the entorhinal cortex, hippocampus, basal forebrain and amygdala. Abnormal accumulation of the associated proteins in brain leads to synaptic and neuronal, and consequent volumetic shrinkage in neocortex, entorhinal area, hippocampus, amygdala, and other regions of brain. Associated with this neurodegeneration symptoms eventually appear, including: memory impairment and other cognitive deficits, behavioral changes and emotional disturbances [4].

The α-synuclein, a protein involved in Parkinson's disease, can self-aggregate into oligomers and into larger inclusions in neurons, known as Lewy bodies. By definition, all patients with AD have many plaques and tangles; most patients also have Lewy bodies. There is also abnormal accumulation of lipid-carrier protein APOE in the nervous system in AD pathogenesis [21].

Amyloid β protein

Aβ is 39-43 (4 kDa) amino acid peptide generated from a large transmembrane amyloid precursor protein (APP) by normal proteolytic cleavage by β and γ secretases. The most common isoforms are $A\beta_{40}$ (90%) and $A\beta_{42}$ (10%). The latter is more hydrophobic, more prone to aggregate, more fibrillogenic, more resistant to degradation, more toxic, and most often appears in senile plaques. $A\beta_{42}$, specifically, is more abundant in all forms of FAD [22, 23]. Finally, while the former is more concentrated in cerebrovascular plaques, the latter is concentrated in neuritic plaques [24].

Aβ is produced in the brain and other organs throughout life in healthy individuals, though its physiological role of Aβ is not well known. One possibility being explored is that Aβ, normally, plays a role in a negative feedback system that may prevent neuronal hyperactivity [25]. Usually Aβ is quickly removed from our brains by clearance mechanisms. When its concentration is increased by overproduction or defective clearance, Aβ monomers self-aggregate into assemblies of various types, including: oligomers to protofibrils, fibrils and amyloid plaques [26]. Aβ monomers can be neuroprotective, though the aggregation of Aβ might, on the other hand, contribute to AD pathology [27]. Soluble Aβ oligomers have recently been reported as being more toxic than monomeric or fibrillar Aβ, and may induce synaptic loss, which eventually contribute to the development of AD and cognitive deficits [28-30].

Although extracellular Aβ has shown neurotoxicity, more recent attention has been placed on intraneuronal Aβ. First, it has been recently reported that Aβ-related synapse damage and memory impairment in AD-transgenic mice correlated with intracellular levels of Aβ but not with plaque burden [31]. Second, cultured neurons from Tg mice carrying a human AD causing mutation, showed reduced secretion and enhanced intracellular accumulation of Aβ [32]. Third, accumulation of intracellular Aβ has been pointed out to be involved in the early stage of disease, directly causing neurotoxicity and initiating AD pathology [33]. Fourth, Much evidence support that the lysosomal system, a vacuolar compartment with acidic pH (3.5-6.0) containing various hydrolytic enzymes, is associated with Aβ generation and neurotoxicity [34-37]. In addition, it has been proposed that senile plaques originate from intraneuronal Aβ as a result of its secretion into extracellular space [38], or its release after neuronal death [39].

Sites of Aβ generation and location. Aβ generation from APP is thought to occur in a variety of organelles where APP, β- and γ-secretase reside. In particular, it has been reported that Aβ is produced in endoplasmic reticulum (ER) [40-43]; medial Golgi saccules [44]; trans-Golgi network (TGN) [35, 45, 46]; autophagic vacuoles [35, 47] and endosomes of the endocytic pathway [48-50]. Besides, Aβ is focused in multivesicular bodies/late endosomes and lysosomes [51], as well as in the secretory pathway, mitochondria [52] and the cytosol [29, 53-56]. The intracellular location of Aβ is presented in **Figure 1**.

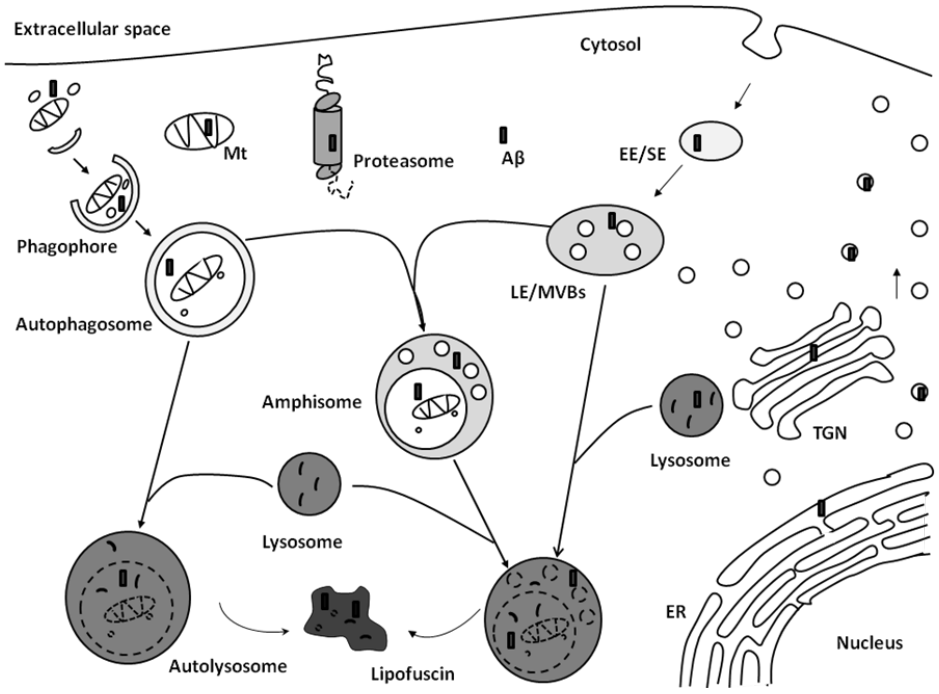

Figure 1. The intracellular location of Aβ. Amyloid-β (Aβ) is localized with endoplasmic reticulum (ER), Golgi complex, secretory pathway, early endosomes/sorting endosomes (EE/SE), late endosomes/multivesicular body (LE/MVB), lysosomes, mitochondria (Mt) and cytosol. TGN, trans Golgi network.

In AD and experimental AD models, Aβ has been identified at four intraneuronal compartments associated with the lysosomal system, including: 1) rab5-positive endosomes [57]; 2) autophagic vacuoles [47]; 3) lysosomes [58-60]; and 4) multivesicular bodies (MVBs) [61], which could be related to either the endocytic or autophagic pathways. Theses cellular compartments may generate Aβ, not only because of their cathepsins, but also because they contain rich sources of APP and its processing enzymes such as β- and γ-secretases.

Aβ degradation. Aβ degradation is mediated by zinc metallopeptidases, including: neprilysin; insulin degrading enzyme (IDE); and the endothelin-converting enzymes ECE1 and ECE2. Besides the cysteine protease, cathepsin B in lysosomes also degrades Aβ peptides, especially the aggregation-prone species, $A\beta_{42}$.

Amyloid precursor protein and its processing

APP is a ubiquitously expressed and evolutionary conserved type I transmembrane protein. It contains a large extracellular N-terminal domain, a transmembrane domain and a short intracellular-cytoplasmic C-terminal domain. APP has three isoforms: APP695, APP751 and APP770. APP695 is expressed in neurons while the other two are non-neuronal isoforms. There are two similar proteins to APP: Amyloid precursor-like protein1 (APLP1) and APLP2. The APLPs are not known to be involved in AD. The physiological role for APP is in synaptic formation and repair, cell signaling, long-term potentiation, neuroprotection and cell adhesion, trafficking of vesicles in the axon [4, 62, 63].

APP processing. APP is metabolized via a non amyloidogenic pathway or an amyloidogenic pathway. Along the amyloidogenic pathway, APP is first cleaved by β-secretase, producing soluble β-APP fragments (sAPPβ) and C-terminal β fragment (CTFβ, C99). C99 is further cleaved by γ-secretase, producing APP intracellular domain (AICD) and Aβ. On the other hand, most APP is hydrolytically cleaved along the non amyloidogenic pathway. It is first cleaved by α-secretase within Aβ domain, generating soluble α-APP fragments (sAPPα) and C-terminal fragment α (CTFα, C83). C83 is further cleaved by γ-secretase, producing non-toxic P3 and AICD. Cleavage of APP at the α-secretase site prevents Aβ production. The sAPPα is a neuroprotective/neurotrophic protein, while sAPPβ is shown to trigger neuronal death. AICD is believed to interact with the transcription of genes in nucleus. The APP processing is presented in **Figure 2**.

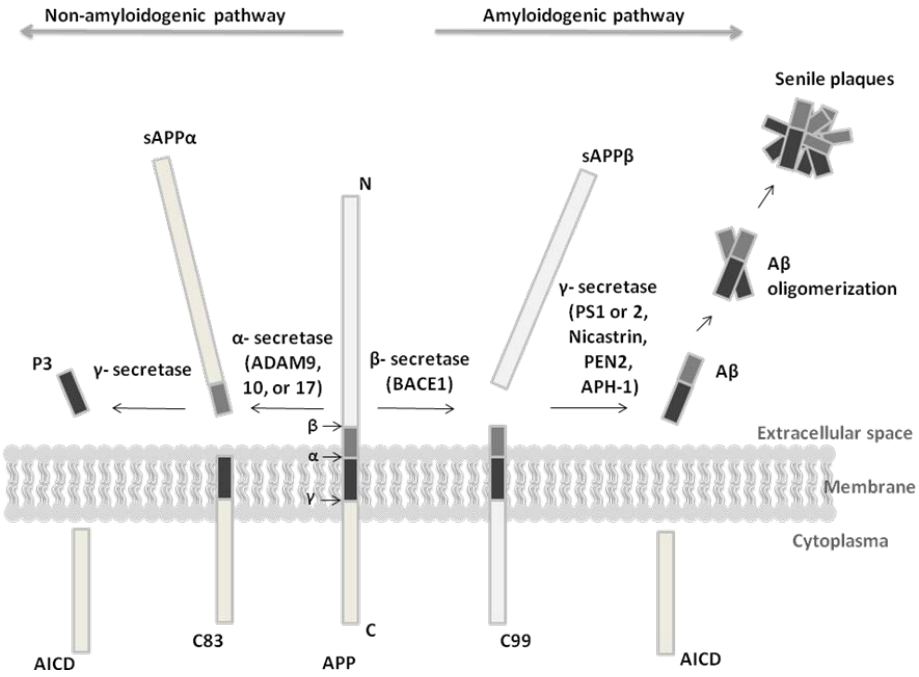

Figure 2. APP processing. APP is metabolized via a non amyloidogenic or amyloidogenic pathway. In the amyloidogenic pathway, APP is first cleaved by β-secretase (BACE1), producing soluble β-APP fragments (sAPPβ) and C-terminal β fragment (CTFβ, C99). C99 is further cleaved by γ-secretase complex (PS1 or 2, Nicastrin, PEN2 and APH-1), producing APP intracellular domain (AICD) and Aβ. On the other hand, most APP is hydrolytically cleaved along the non amyloidogenic pathway. It is first cleaved by α-secretase (ADAM9, ADAM10 and ADAM17) within Aβ domain, generating soluble α-APP fragments (sAPPα) and C-terminal fragment α (CTFα, C83). C83 is further cleaved by γ-secretase, producing non-toxic P3 and AICD. PS, presenilin; PEN2, PS enhancer-2; APH-1, anterior pharynx defective-1.

Both pathways are active in normal metabolism. Three enzymes of the ADAM family (a disintegrin and metalloproteinase family of enzymes), ADAM9, ADAM10 and ADAM17 (tumor necrosis factor converting enzyme), are known to have α-secretase activity [64]. The β-site APP cleaving enzyme 1 (BACE1), the transmembrane aspartyl-protease, has β-secretase activity. γ-secretase complex consists of presenilins (PS1 or PS2), nicastrin (NCT), anterior pharynx defective 1 (APH-1) and PS enhancer-2 (PEN-2) [65]. In addition to its role in APP processing, the γ-secretase complex is important in the cleavage of notch, a widely expressed transmembrane protein involved in cell communication.

BACE1 cleavage occurs mostly in the late Golgi/TGN and in endosomes [49]. Further, γ-secretase components have been found in many subcellular compartments, such as the ER [66], ER-Golgi intermediated compartment [67], Golgi, TGN, endosomes [68] and at the plasma membrane [69]. Moreover, PS was found in synaptic compartments [70, 71]. In addition, all four γ-secretases components were found in phagosomes [72]. PS1, nicastrin, and APP were localized in the outer membranes of lysosomes [73, 74]. The site of active γ-secretases has been reported in the plasma membrane [69, 75], lysosomal membranes [73, 74] and mitochondria [76].

Mutations in three genes - APP, PS1 and PS2 - produce different effects on APP processing according to the mutation type. These include: the Swedish APP 670/671 double mutation, which leads to an increased Aβ production due to increased cleavage of APP by β-secretase [77, 78]; APP 717 mutation, which leads to an increased production of longer Aβ peptides having an increased propensity to form fibrils [79]; Arctic mutation, which causes rapid Aβ protofibril formation resulting in accelerated build up of insoluble Aβ intra and/or extracellularly [80]; mutations in the presenilins, such as the PS1M146V mutation, that increase levels of $A\beta_{42}$ [81, 82]. Increased dosage of the APP gene also results in AD [83, 84]. Down syndrome, in which triplication of chromosome 21 (on which APP resides) occurs, leads to Aβ accumulation early in life [33, 85].

APP trafficking. APP is normally synthesized in the endoplasmic reticulum (ER) and transported to the Golgi and trans-Golgi network (TGN). Only 10% of APP reaches the plasma membrane by the secretory pathway while the rest of APP is in the Golgi and TGN. Some of APP is predominantly cleaved by α-secretase at the cell surface [86], releasing sAPPα into the extracellular space and leaving C83 within the membrane; some is transported from plasma membrane to retromer recycling endosomes before recycling back to Golgi, as regulated by the sortilin related receptor SORL1 [11]; others are reinternalized within the endosomal-lysosomal system via endocytosis and cleaved by BACE1 with optimal pH [40-42], resulting in C99. C99 either shuttles back to the ER to be processed into Aβ by the ER γ-secretase, or shuttles back to the plasma membrane to be cleaved by γ-secretase, or processed into Aβ within endosome/lysosomal system [29]. In addition, APP is also localized to mitochondrial membrane [87].

Hypotheses of AD pathogenesis

AD may be caused by multifactorial disease mechanisms and, accordingly, different hypotheses that explain its pathogenesis of the disease have been proposed.

The cholinergic hypothesis, the earliest proposed, states that decreased cholinergic transmission plays a major role in the expression of cognitive, functional and possibly behavioral symptom in AD. This hypothesis is based on the selective vulnerability and disruption of the cholinergic system in AD. The nucleus basalis of Meynert, a specific population of neurons in the basal forebrain, which provides most of the acetylcholine to the cerebral cortex, was shown to be selectively degenerated in AD [88, 89]. However; this hypothesis is weakened by a lack of finding that a cholinergic deficit is observed in early stages of AD or in patients with mild cognitive impairment.

At present, *the amyloid cascade hypothesis*, first proposed by John Hardy and Gerald Higgis in 1992 [90], is the most dominant theory to explain the etiology and pathogenesis of AD. The core hypothesis of the amyloid cascade model is that accumulation of Aβ is an early event leading to neurodegeneration [91]. The pathogenesis of AD is initiated by an alteration in the expression or processing of APP. Imbalance between Aβ production and clearance leads to increased level of Aβ, results in Aβ oligomerization, fibril formation and accumulation in senile plaques. Further, Aβ accumulation induces microglial and astrocytic activity, pro-inflammatory response, oxidative injury, altered kinase/phosphatase activity, followed by formation of NFTs, finally neuronal death. Support for the amyloid cascade hypothesis hypothesis (**Figure 3)** may be based on the cytotoxicity effect of Aβ to neurons and synapse; the fact that Down syndrome patients who possess an extra copy of chromosome 21 develop early cerebral amyloidosis, and the fact that APP and PS mutations lead to increased Aβ deposition.

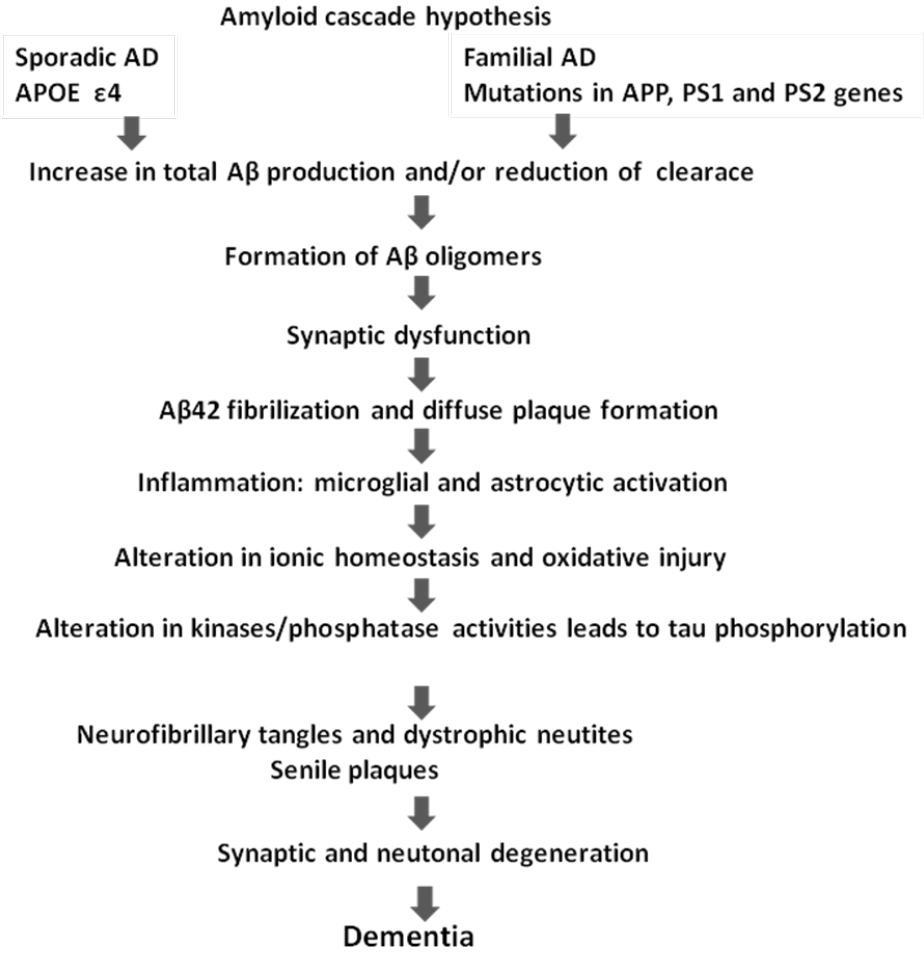

Figure 3. Amyloid cascade hypothesis (references are given in the text).

In opposition to the Amyloid cascade hypothesis, *the tangles hypothesis* is proposed as the central pathogenic feature of AD [92]. Tau hyperphosphorylation decreases the binding of tau to microtubules, resulting in increased free cytoplasmic tau self-aggregation that then forms PHF, leading to loss of cytoskeletal function and cell death. In support of this hypothesis, the presence of tangles correlates better with the status of AD than does the presence of plaques [93]. Further, tangle formation starts in brain areas known to be critical for memory [94]. The discovery of tau mutations in families with fronto-temporal dementia demonstrated that tau pathology can provoke dementia, and that tau can be a pathogenic protein. However, the absence of plaque deposition in tau mutation cases and the presence of both plaque and tangles in APP mutation cases suggest that amyloid buildup precedes tau hyperphosphorylation in AD.

Other hypotheses include: the *calcium hypothesis*, which holds that the alterations in calcium signaling cause both amyloid formation and tau hyperphosphorylation [95-97]; the *cholesterol hypothesis*, which states that AD pathogenesis results from disruption of cholesterol uptake and metabolism that, in turn, results in abnormal trafficking of membrane protein critical to normal neuronal function and synaptic plasticity [98, 99]; the *ApoE hypothesis*, which states that ApoE is associated with all the biochemical disturbances characteristic of AD, such as Aβ deposition, tangle formation, oxidative stress, neurodegeneration, lipid dysfunction, loss of synaptic plasticity and cholinergic dysfunction [100]; and the *oxidative stress hypothesis* that is based on aging as a major risk factor for AD, that Aβ aggregates in the presence of free radicals, and that oxidative damage is widespread in AD brain [101, 102]; the *mitochondrial hypothesis*, supported by the finding that mitochondria are the prime site for production of oxidative species and an early target for ROS. Mitochondrial dysfunction presents a common theme for several neurodegenerative disorders including AD [103].

Our hypothesis with regard to the involvement of oxidative stress and the lysosomal system in the pathogenesis of AD is discussed in detail below.

OXIDATIVE STRESS

Reactive oxygen species and oxidative stress

Oxidative stress represents an imbalance between the production of reactive oxygen species (ROS) /reactive nitrogen species (RNS) and the biological system's ability to readily detoxify the reactive intermediates, or to repair the resulting damage (antioxidant systems).

Oxidants, such as ROS and RNS are a part of normal physiological process and produced at low levels in all aerobic organisms as a consequence of normal respiration. ROS include superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). Mitochondria are the major place to produce ROS through the respiratory chain. The most important targets of ROS damage are nucleic acids (DNA/RNA damage), carbohydrates, lipids (lipid peroxidation) and proteins (protein oxidation) [104].

Reactive nitrogen species (RNS) are a family of antimicrobial molecules derived from nitric oxide (·NO) and superoxide ($O_2^{\cdot-}$) produced via the enzymatic activity of inducible nitric oxide synthase 2 (NOS2) and NADPH oxidase respectively [105]. Reactive nitrogen species act together with reactive oxygen species (ROS) to damage cells, causing nitrosative stress [106]. Therefore, these two species are often collectively referred to as ROS/RNS. RNS are also continuously produced in plants as by-products of aerobic metabolism or in response to stress [107].

To defend against ROS- and RNS-mediated injury, cells develop several antioxidant system responses that prevent the formation, detoxification or scavenging of oxidant species. Antioxidants include both enzymes (superoxide dismutase (SOD), catalase, glutathione peroxidase and several sulfur-containing enzymes like (thioredoxin and glutaredoxin) and low molecular weight compound (glutathione and NADPH) [104]. Glutathione peroxidase and catalase detoxify hydrogen peroxide (H_2O_2), which generates hydroxyl radixals ($OH^{\bullet}$) in the presence of transition metals (Fe^{2+}). In addition to other antioxidants are vitamins (e.g. α-tocopherol, ascorbic acid and β-carotene), synthetic (e.g. butylated hydroxytoluene), natural (e.g. plant-derived polyphenols) and inorganic (e.g. selenium). Some act as chain-breaking molecules because they prevent the propagation of or stop radical chain reactions (i.e. α-tocopherol) [108, 109].

In the case that the production of oxidant species exceeds the endogenous antioxidant defending system, an oxidative imbalance occurs. This results in cellular oxidative stress and subsequently leads to molecular oxidative damage, which can result in altered cellular functions and eventually cell death [110].

Oxidative stress and aging

Aging is defined as "gradual irreversible changes in structure and function of an organism that occur as a result of the passage of time." These changes are commonly harmful, decreasing normal functioning and adaptability, and simultaneously increasing the probability of death. Regarding cellular aging, or senescence, the emphasis is usually placed on the decreased ability to proliferate as a result of either exceeded proliferative limit (replicative senescence) [111] or cellular stress (stress-induced senescence) [112].

Free radicals were first associated with aging when Denham Harman presented the "the free radical theory of aging" in 1956 [113]. According to the theory, biologic aging (senescence) occurs because of the accumulation of oxidatively damaged macromolecules. Today, although other factors may also be involved in the aging process (e.g., evolution, somatic mutations, errors in protein synthesis, accumulation of waste products, neuroendocrine and immunologic disturbances), the role of free radicals to aging is considered to be an important contributor in various biologic species ranging from yeast to humans [114-117].

Oxidative stress and AD

Ample evidence implicates oxidative stress as an early event that is widespread in the AD brain, and which plays an important role in the pathogenesis of AD [101, 117, 118]. Increased ROS/RNS [119, 120] and the dysfunctional antioxidant system [102, 110] might lead to further increase of ROS, thereby causing oxidatively damaging biomolecules, including proteins, lipids, carbohydrates, DNA and RNA [121]. ROS are responsible for progressive age related neuronal damage involving the accumulation of aberrant proteins, defective mitochondria and lipofuscin-loaded lysosomes [122]. These changes may, finally, culminate in neuronal apoptosis and release of Aβ from dying cells [61, 123].

Moreover, oxidative stress upregulating APP processing leads to the increase in intracellular content of Aβ [124, 125]. Aβ is aggregated in the presence of free radicals and acts as a pro-oxidant by generating more free radicals, thus inducing cell death by a ROS-mediated mechanism [101, 126, 127]. In AD, levels of oxidative stress and protein oxidation increase predominantly in cognition-associated Aβ-rich regions, such as the cortex and hippocampus [128]. Aβ has been shown to exert neurotoxicity by increasing neuronal sensitivity to oxidative stress [129-131]. Furthermore, there is extensive evidence that redox-active transition metals are involved in AD pathogenesis [132]. AD brains have increased concentrations of metals that catalyze the production of free radicals, including iron [133] and aluminum [134]. Other metals such as copper and zinc may also be involved. Copper is reduced in the hippocampus of AD brains and it is essential for the activity of many enzymes such as cytochrome-c oxidase [135]. Both copper and zinc bind APP; and it is believed that this can modulate the functional properties of the molecule [136].

In addition, the source of oxidant species in central nervous system (CNS) includes altered mitochondrial function, the Aβ peptides and the presence of unbound transtiton metals [137]. These factors are related to each other. In early stages of the disease, Aβ could enter the mitochondria where it would increase the generation of ROS and induce oxidative stress. Interestingly, Aβ and APP found in mitochondrial membranes can block transport of protein and disrupt the electron transport chain with final, irreversible cell damage.

Evidence indicates that accumulating effects of long and gradual oxidative damage precedes the appearance of clinical and pathological AD symptoms, including Aβ deposition, neurofibrillary tangle formation, metabolic dysfunction, and cognitive decline [138]. The markers of oxidative stress such as protein, DNA, RNA oxidation or lipid peroxidation have been identified in the AD brain, which supports the 'oxidative stress hypothesis' [102, 139] [140]. Consistent with the role of oxidative stress in AD pathogenesis, some studies reported positive effects of antioxidant intake that lowered the risk for AD [141].

Genetic mutations of APP or PS1 increase Aβ formation. Oxidative stress can increase APP levels or modulate the activity, elevating levels of β-secretase (BACE) and γ-secretase, influence Aβ formation. Aβ is pro-oxidant factor and can induce more oxidative stress, creates positive feedback on APP levels and on its proteolytic pathway. The elevated levels of Aβ oligomers

favor the phosphorylation of tau protein. With time, Aβ oligomers are deposited in the extracellular space forming senile plaques (SPs), whereas inside neurons, the hyperphosphorylated tau form neurofibrillary tangles (NFTs). Both lesions trigger further oxidative stress reactions and sustained inflammatory responses, which ultimately will result in irreversible cell damage, slow degeneration and eventual cell death. These cell-biologic events will clinically manifest with progressive cognitive decline, early signs of dementia and, finally, full clinical AD [108].

LYSOSOMAL SYSTEM

Cellular degradation processes

There are two major cellular degradative pathways: 1) Autophagic-lysosomal pathway, also called autophagy or autophagocytosis, by which most long-lived proteins and all organelles are digested in the lysosomal compartment; and, 2) ubiquitin-proteasomal pathway, by which short-lived proteins in the nucleus and cytosol are ubiquitinized, and degraded mainly by calpains and proteasomes. Cellular degradation pathways are presented in **Figure 4**. The proteasomal and lysosomal systems can together compensate for degradation. Moreover, mitochondria possess their own proteolytic system, which includes Lon, Clp-like proteases, and AAA proteases. Furthermore, irreversibly damaged cells are removed by self-killing programs, including apoptotic (caspase dependent) programmed cell death (PCD-I), autophagic cell death (PCD-II), or, occasionally, through necrosis (PCD-III).

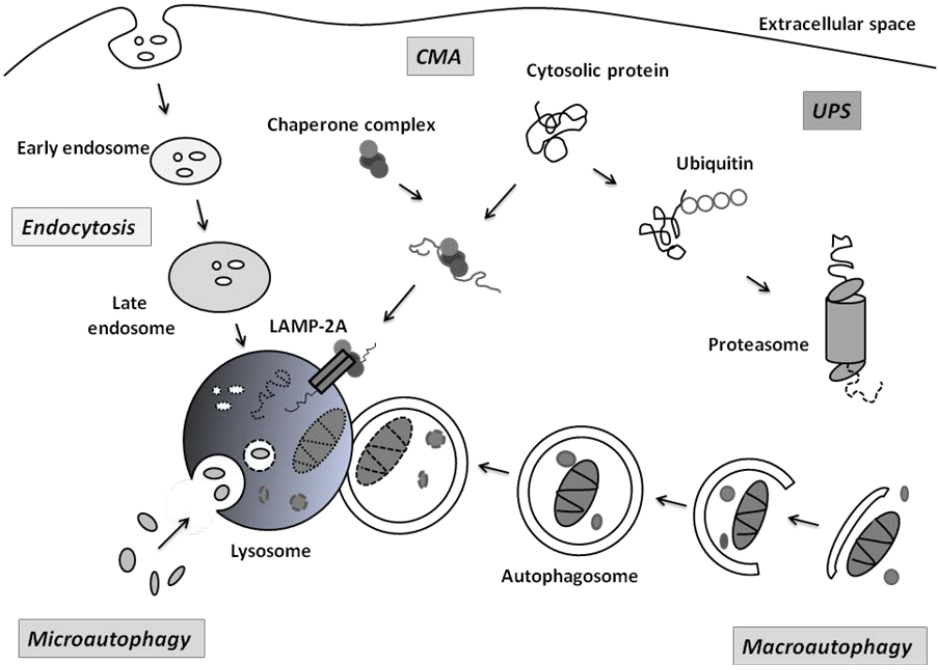

Figure 4. Cellular degradation processes. There are two major cellular degradative pathways: 1) Autophagic-lysosomal pathway, also called autophagy or autophagocytosis, by which most long-lived proteins and all organelles are digested in the lysosomal compartment; and, 2) ubiquitin-proteasomal pathway, by which short-lived proteins in the nucleus and cytosol are ubiquitinized, and degraded mainly by calpains and proteasomes. Three kinds of autophagy are recognized in mammalian cells: 1) CMA (chaperone-mediated autophagy), in which the cytoplasmic proteins are selectively delivered into lysosome by recognizing their specific motifs (KFERQ) through lysosomal receptors LAMP-2A; 2) microautophagy, in which the cytoplasmic proteins are directly engulfed by lysosome for degradation; 3) macroautophagy, which involves the sequestration and transport of complete regions of the cytoplasm within double membrane-bounded vacuoles, lysosomal degradation and recycling by lysosomal hydrolases. UPS, ubiquitin-proteasomal system.

Lysosomes

The lysosomal system is defined as a family of communicating acidic compartments (pH:3.5-6.0), which contain over 80 'lysosomal' acid hydrolases, including proteases, nucleases, phosphatases, lipases, and glycosidase [142]. The lysosomal compartment is rich in active acid hydrolases, has acidic environment (pH~4-5), receives materials from different pathways for degradation, and plays an important role in maintaining cellular homeostasis. Among a wide spectrum of hydrolytic enzymes in lysosomes, cathepsins are the most important group. Lysosomal cathepsins (pH optima ~5) can be divided as cysteine (cathepsins B, C, F, H, K, L, O, S, V, W, and X), aspartic (cathepsins D and E) and serine (cathepsin G) proteases [143-145]. Cysteine protease and aspartic peptidases are also involved in β and γ-secretase activity, respectively [146].

In mammalian cells, the diameter of individual lysosome is about 0.5 μm, with 10 nm thick membrane [147], a total cell volume of 0.5-15%, and they are concentrated near microtubules [148]. Lysosomes are receiving material through three different pathway: 1) biosynthesis pathway, which delivery lysosomal hydrolases from TGN to lysosomes; 2) heterophagy (receptor-mediated endocytosis, pinocytosis and phagocytosis), by which the materials from extracellular space or on the membrane will be delivered to lysosomes; 3) autophagy (microautopahgy, macroautophagy, and chaperone-mediated autophagy), which degrades intracellular material including damaged mitochondria and other organelles, such as ribosomes, endoplasmic reticulum (ER), and the proteasome microorganelles, as well as long-lived proteins.

Along the biosynthesis pathway, lysosomal hydrolases are synthesized in the ER, tagged with mannose-6-phosphate (M6P) at the cis-Golgi area, and then enclosed in transport vesicles (sometimes named primary lysosomes, and having a neutral pH) in the TGN with the help of M6P receptors. The hydrolases-containing vesicles are then transported to slightly acidic (pH~6) late endosomes, which arise from early endosomes containing endocytosed material. The lysosomal hydrolases are then activated when they release M6P receptors that are recirculated to the Golgi apparatus. Finally, the late endosomes mature to lysosomes, which by then lack M6P receptors, though are rich in acid hydrolases, have a pH of 4–5, and contain material to be degraded. Late endosomes differ from mature lysosomes by the presence of M6P receptor in their membrane.

Through degradative pathways, lysosomes fuse with autophagosomes/endosomes (fusion) to form ‘‘hybrid’’ organelles containing material that, in the course of degradation, originates both from the outside and the inside of the cell. After completed degradation of the enclosed material, lysosomes turn into ‘‘resting’’ organelles (fission) that, in turn, are ready for new rounds of fusion. The pronounced fusion and fission activity is a typical characteristic of the lysosomal compartment [149], which allows hydrolytic enzymes and other lysosomal contents to be distributed between different lysosomes.

Endosomal-lysosomal degradation pathway

Extracellular materials and membrane proteins are internalized by receptor-mediated (clathrin) endocytosis or bulk-phase endocytosis (pinocytosis) into early endosomes/sorting-endosomes (Rab5 positive). After sorting in early endosomes, some materials are sent back to the plasma membrane via recycling endosomes, some go to the trans-Golgi network for further packaging and trafficking, others reach late endosomes (LE)/multivesicular bodies (MVB, Rab7 positive), which contains hydrolase vesicles delivered from TGN by shuttle vesicles. The LE/MVB either fuses with the autophagosome to form amphisome and then fuse with the lysosome to form autolysosome degradation; or it fuses directly with a lysosome to form autolysosome for degradation. The mechanism of the endosomal-lysosomal degradation is presented in **Figure 5**.

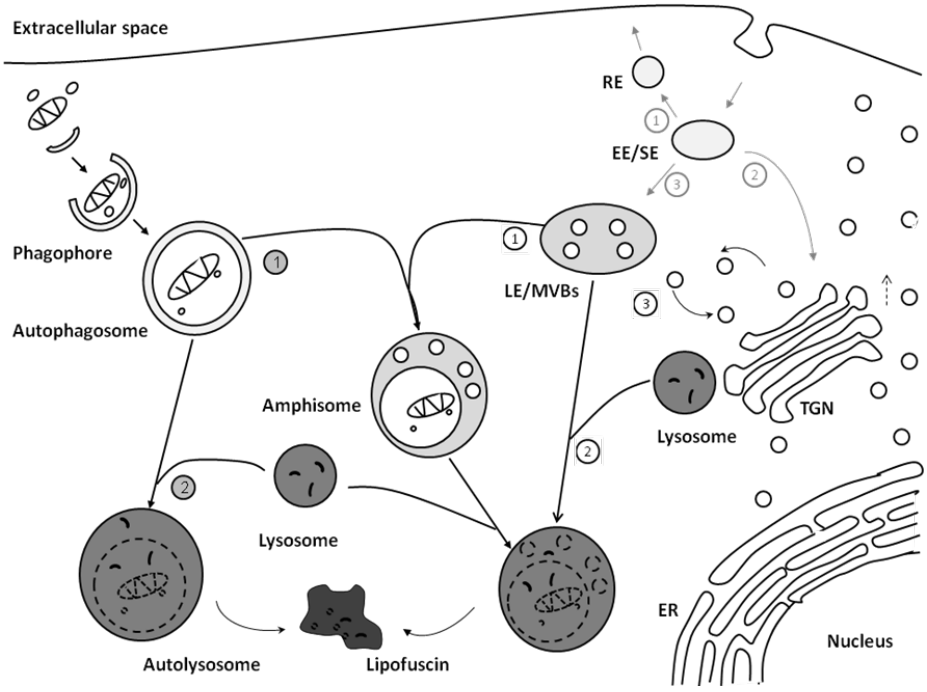

Figure 5. The mechanism of lysosomal degradation. In endosomal-sysosomal degradation pathway, extracellular materials and membrane proteins are internalized by receptor-mediated (clathrin) endocytosis or bulk-phase endocytosis (pinocytosis) into early endosomes/sorting-endosomes (EE/SE). After sorting in early endosomes, some materials are sent back to the plasma membrane via recycling endosomes (RE), some go to the trans-Golgi network for further packaging and trafficking, others reach late endosomes (LE)/multivesicular bodies (MVBs), which contain hydrolase vesicles delivered from TGN by shuttle vesicles. The LE/MVB either fuse with the autophagosomes to form amphisomes and then fuse with lysosomes to form autolysosomes; or they fuse directly with lysosomes forming autolysosomes where material is degraded. In autophagosomal-lysosomal pathway, first, cytoplasm is sequestered to form an isolation membrane, also known as a pre-autophagosomal structure (PAS), or "phagophore". Second, autophagosome formation is achieved as the membrane elongates and the edges of the membrane fuse, resulting in the formation of enclosed double-membrane vacuoles termed autophagosomes, which hold cytoplasmic contents. The autophagosomes themselves are devoid of lysosomal enzymes and have a limited degradation capacity. Thus, the autophagosomes need the lysosomes for effective clearance of their content. Third, autophagosomes either fuse with lysosomes or other mature autophagic vacuoles (AVs) to form autophagolysosomes (also called autolysosomes), in which they acidify and acquire proteolytic enzymes; or they fuse with MVB/late endosomes through alternative endosomal pathway to form amphisomes, which will later fuse with lysosomes to form autolysosomes for degradation. Finally, lipofuscin, which is composed of lipid-containing residues of lysosomal digestion, is formed.

Autophagy

Autophagy (also called autophagocytosis), “self-eating”, is an evolutionary conserved cellular pathway for delivery of the cell’s own constituents (long-lived proteins and organelles) to lysosomes for degradation, turnover and reutilization. During autophagy, cells can degrade unessential components, and, thereby, generate new substrate for energy and cellular remodeling, which may help cells maintain homeostasis [150].

The autophagy occurs in all eukaryotes during starvation, cell and tissue development, and cell death [151]. It is important for development, growth, longevity and cellular homeostasis [152, 153], and that defects in autophagy leads to the intracellular accumulation of proteins in toxic complexes [153, 154]. Autophagy has also been connected with aging processes, characterized by accumulation of cellular components with limited or no activity (loss-of-function) due to oxidative damages or decreasing effectiveness of the cell turnover and house-keeping functions [155]. To maintain intracellular homeostasis, balance in the autophagy and proteasome biosynthesis, degradation reactions are required. Autophagy is normally regulated to certain basal levels, but during conditions of starvation, cell damage, hormonal stimulation and under influence of some chemicals, a non-selective degradation of the cytoplasm is undertaken to generate energy, and, for other purposes, specific degradation of cytoplasm and damaged organelles is induced.

Depending on the pathway for delivery of material for lysosomal degradation, there are three kinds of autophagy: 1) *CMA (chaperone-mediated autophagy)*, in which the cytoplasmic proteins are selectively delivered into lysosome by recognizing their specific motifs (KFERQ) through lysosomal receptors LAMP-2A; 2) *microautophagy*, in which the cytoplasmic proteins are directly engulfed by lysosome for degradation; 3) *macroautophagy*, which involves the sequestration and transport of complete regions of the cytoplasm within double membrane-bounded vacuoles, lysosomal degradation and recycling by lysosomal hydrolases [156-158]. Macroautophagy is a regulated nonselective process for cellular turnover or elimination of cell components including aggresomes, dysfunctional mitochondria and proteasomes, as well as long-lived soluble proteins, in order to supply energy and protect the cell, activated during developmental stages and under certain conditions of cellular stress.

Autophagic pathways are schematically presented in **Figure 4**. Among these, macroautophagy (hereinafter autophagy) is the most prevalent form.

The autophagy pathway involves a series of ordered steps: 1) First, *initiation/nucleation*, the formation of an isolation membrane, also known as a pre-autophagosomal structure (PAS) or "phagophore," is performed. This sequesters a region of cytoplasm; 2) Second, *autophagosome formation* is achieved as the membrane elongates (elongation) and the edges of the membrane fuse, resulting in the formation of enclosed double-membrane vacuoles termed autophagosomes (completion), which hold cytoplasmic contents. The autophagosomes themselves are devoid of lysosomal enzymes and have a limited degradation capacity. Thus, the autophagosomes need the lysosomes for effective clearance of their content; 3) The third step is *trafficking/maturation*. Autophagosomes either fuse with lysosomes or other mature autophagic vacuoles (AVs) to form autophagolysosome (also called autolysosome), in which they acidify and acquire proteolytic enzymes; or they fuse with MVB/late endosomes through alternative endosomal pathway to form amphisomes, which will later fuse with lysosomes to form autolysosome for degradation. Autophagic-lysosomal degradation is also presented in **Figure 5**; 4) Finally, the fourth step is the *recycling/release of macromolecules*. Both engulfed cytoplasma and inner membrane of autophagosome are degraded by the required hydrolases in lysosomes and the macromolecules are released to cytosol to be reused for the metabolism.

Different autophagy-related genes (ATG) are involved in different stages of autophagic pathway [159-161]. The first step, *induction/nucleation* of autophagy, is triggered by signals of starvation or stress. At this step two kinases are important: Ser/Thr protein kinase mammalian target of rapamycin (mTOR) and the class III phosphatidylinositol 3-kinase (PI3K) complex. mTOR is regulated by stimuli (insulin/insulin-like growth factor, cell energy status, nutrients, and stress) [162]. Upon specific stimulus to TOR, autophagy is inhibited; conversely, an inhibitory stimulus to mTOR, such as nutrient deprivation, stimulates autophagy. Inhibition of mTOR indirectly results in dephosphorylation of ATG13, allowing ATG13 to interact with ATG1 to initiate autophagy (induction of the isolation membrane) [163, 164]. On the other hand, PI3K complex, an mTOR-independent pathway, is a stimulatory regulator at the nucleation step of autophagy. The complex contains three highly conserved proteins, the protein kinase vacuolar protein sorting (Vps)-15, Vps34 and Beclin 1/ATG6 [165]. Moreover, a*utophagosome formation* occurs

by functioning of two ubiquitin-like conjugation systems. First, ATG5-ATG12-ATG16 conjugation system, which is engaged during the forming of the autophagosome until its completion.ATG7 and ATG10 act as E1 and E2 ligases, respectively for conjugation [159, 160]. Second, LCII/ATG8, conjugated by ATG7 (E1 ligases) and ATG3 (E2 ligases), regulates the elongation, curvature and closure of autophagosome membraine [166-168]. The transmembrane protein ATG9 is also known to be involved in the formation of autophagosome. Furthermore, *maturation of autopahgosomes* is regulated by small G protein [169], and *fusion* with lysosomes is associated with the soluble N-ethylmaleinide-sensitive factor attachment protein receptors and Rab proteins, specifically Rab7 [152, 170] . Finally, *lysosomal degradation and macromolecules release to cytosol* is mediated by ATG22 [171]. Autophagy pathway and related gene are presented in **Figure 6**.

mTOR forms two multiprotein complexes known as mTOR complex (mTORC) 1 and 2 [162]. mTORC1 controls cellular homeostasis, and its activity is inhibited by rapamycin; in contrast mTORC2 is insensitive to rapamycin and controls cellular shape by modulating actin function [162, 172]. By regulating both protein synthesis and degradation, mTOR plays a key role in controlling protein homeostasis and hence brain function; indeed, mTOR activity has been directly linked to learning and memory [173-175]. Additionally, genetic and pharmacological reduction of mTOR activity has been shown to increase the lifespan in different organisms including yeast, Drosophila, and mice [176-181].

LC3 (microtubule-associated protein light chain 3) is one of three mammalian homologues of yeast Atg8, which is localized in autophagosome membrane after processing. LC3 is not only necessary for the formation of autophagosome, but also participates in the formation of autophagosomal membrane [161, 166]. Following synthesis, the C-terminus of LC3 is cleaved by Atg4 to produce LC3-I (resides in the cytosol; free form), LC3-I is then further converted to LC3-II (membrane bound form) by Atg7 and Atg3. The amount of LC3-II is correlated with the extent of autophagosome formation and is often used as a marker of autophagy induction.

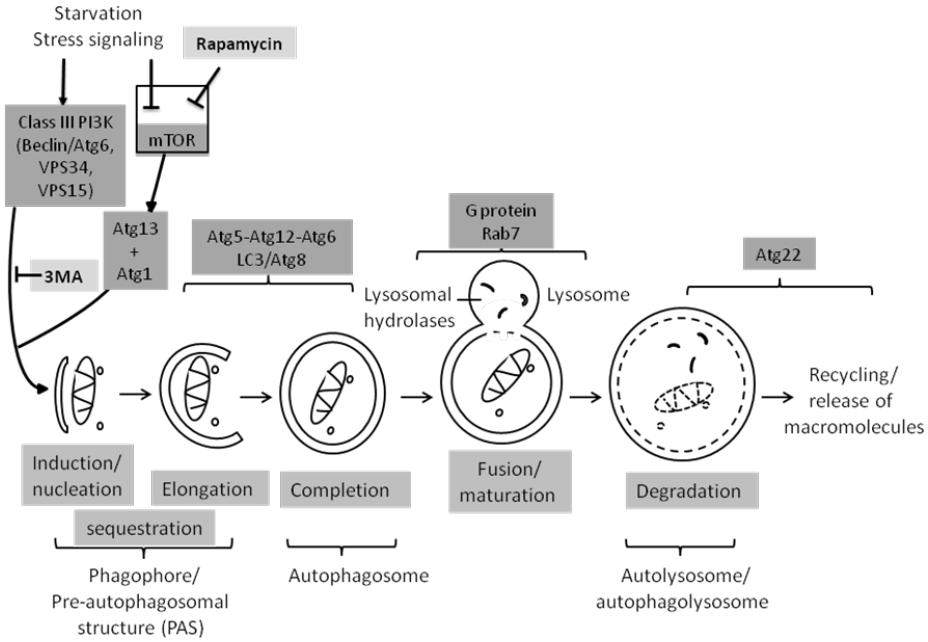

Figure 6. Autophagic pathway and its related genes. The autophagy pathway involves a series of ordered steps. 1) *initiation/nucleation*, the formation of an isolation membrane, also known as a pre-autophagosomal structure (PAS) or "phagophore," is performed. This sequesters a region of cytoplasm. 2) *autophagosome formation* is achieved as the membrane elongates (elongation) and the edges of the membrane fuse, resulting in the formation of enclosed double-membrane vacuoles termed autophagosomes (completion), which hold cytoplasmic contents. The autophagosomes themselves are devoid of lysosomal enzymes and have a limited degradation capacity. Thus, the autophagosomes need the lysosomes for effective clearance of their content. 3) *trafficking/maturation*. Autophagosomes fuse with lysosomes to form autophagolysosome (also called autolysosome), in which they acidify and acquire proteolytic enzymes for degradation. 4) *recycling/release of macromolecules*. Both engulfed cytoplasma and inner membrane of autophagosome are degraded by the required hydrolases in lysosomes and the macromolecules are released to cytosol to be reused for the metabolism. Different autophagy-related genes (ATG) are involved in different stages of autophagic pathway. The first step, *induction/nucleation* of autophagy, is triggered by signals of starvation or stress. At this step two kinases are important: Ser/Thr protein kinase mammalian target of rapamycin (mTOR) and the class III phosphatidylinositol 3-kinase (PI3K) complex. mTOR is regulated by stimuli (insulin/insulin-like growth factor, cell energy status, nutrients, and stress). Upon specific stimulus to TOR, autophagy is inhibited; conversely, an inhibitory stimulus to mTOR, such as nutrient deprivation, stimulates autophagy. Inhibition of mTOR indirectly results in dephosphorylation of ATG13, allowing ATG13 to interact with ATG1 to initiate autophagy (induction of the isolation membrane). On the other hand, PI3K complex, an mTOR-independent pathway, is a stimulatory regulator at the nucleation step of autophagy. The complex contains three highly conserved proteins, the protein kinase vacuolar protein sorting (Vps)-15, Vps34 and Beclin 1/ATG6. Moreover, a*utophagosome formation* occurs by functioning of two ubiquitin-like conjugation systems. First, ATG5-ATG12-ATG16 conjugation system, which is engaged during the forming of the autophagosome until its completion. Second, LCII/ATG8, conjugated by ATG7 (E1 ligases) and ATG3 (E2 ligases), regulates the elongation, curvature and closure of autophagosome membraine. The transmembrane protein ATG9 is also known to be involved in the formation of autophagosome. Furthermore, *maturation of autopahgosomes* is regulated by small G protein, and *fusion* with lysosomes is associated with the soluble N-ethylmaleinide-sensitive factor attachment protein receptors and Rab proteins, specifically Rab7. Finally, *lysosomal degradation and macromolecules release to cytosol* is mediated by ATG22.

Autophagy has roles in both normal cellular homeostasis and disease states. The activation of autophagy induced by nutrient starvation results in enhanced protein degradation accompanied by an increase in the amino acid pool, which provides an energy source and allows for necessary protein synthesis [182]. The constitutively active autophagy (basal level) is believed to be especially critical for cells that are postmitotic, such as neurons, because of their inability to dilute aberrant components through cell division [183-185]. Loss of basal autophagy in central nervous system causes an accumulation of ubiquitinated protein inclusions and neurodegeneration, suggests a neuroprotective role of autophagy.

Lysosomal involvement in AD

Some genetic mutations influence both AD likelihood and the lysosomal system include: 1) A mutation of APP plays a role in upregulation of endocytosis leading to abnormal endosomes and endosomal storage Aβ alters MVB trafficking [57, 186, 187]; 2) Mutation of PS1 slows autopahgic protein turnover of long-lived proteins [188]; 3) Inheritance of the ε4 allele of APOE exacerbates endocytosis upregulation and potentiates $A\beta_{1-42}$-induced lysosome destabilization [189-191]; 4) SORL1 releases APP into the endocytic increasing Aβ generation [11]; 5) carriers of CatD T-224C polymorphism that alters pro-Cat trafficking, increasing AD risk [192]; 6) Cystatin C (cysC) increases autophagy associated with lower AD risk.

The endosomal-lysosomal system has been shown to involve, in early stage of AD, pathogenesis and, specifically, Aβ-amyloidogenesis. Several studies have recognized the endosomal-lysosomal pathway as an important regulator of the processing of APP [49, 73, 193]. Early endosomes produce Aβ from APP in normal cells and mediate the uptake of Aβ and soluble APP. In the AD brain, activation of endocytic pathway is the earliest noted intracellular manifestation of the disease, and neurons in susceptible brain areas exhibit progressive abnormalities in the endocytic pathway, such as increase in size and volume of early endosomes [34, 194]. Aβ has also been detected in these enlarged endosomes that are immunopositive for the early endosomal marker rab5 [57]. MVBs/late endosomes are relatively rich in APP and APP secretases, and those in AD brain and mouse model of AD contain Aβ peptide [39, 61].

Abnormalities in the lysosomal pathway also occur early in AD pathogenesis before the appearance of neurofibrillary tangles or senile plaques. The upregulation in the lysosomal system

occurs in vulnerable cell populations and results in increased numbers of lysosomes with elevated expression of lysosomal hydrolases [195]. These hydrolases include cathepsins that are directly [196] and indirectly [34, 197] involved in Aβ formation. As AD pathogenesis progresses, lysosomal dysfunction appears to occur with the buildup of vacuolar structures and the accumulation of $A\beta_{42}$. The degeneration of the compromised neurons leads to the release of these structures into the extracellular space, where they associate with deposits of Aβ [198].

More recent accumulative evidences show that the autophagic-lysosomal system, the principal self-clearance machinery [199-201], plays an important role in AD process. First, neurons from AD patients contain increased numbers of autophagosomes, autolysosomes, and early or late autophagic vacuoles [202] and lysosomes [35]; and they show increased expression of lysosomal hydrolases [36], indicating activation of the autophagic-lysosomal system in this disorder. Second, these numerous AVs contain the components for Aβ generation, APP, and highly active γ-secretase [35, 47], suggests Aβ generation has been detected within autophagosomes following activation of macroautophagy. Third, Aβ shows partial accumulation within neuronal lysosomes in transgenic mice expressing both human mutant APP and mutant presenilin-1 [58]. Fourth, exogenous $A\beta_{1-42}$ is internalized by cultured cells and accumulates within lysosomes, causing lysosomal membrane permeabilization and ensuing apoptotic cell death [37, 203] , in accordance with the previously demonstrated involvement of lysosomes in apoptosis [204, 205] .

Two competing hypotheses have been proposed to explain the involvement autophagy in AD pathogenesis [206]. One hypothesis links autophagic inhibition to AD pathogenesis. Autophagy could be inhibited in the AD brain by a reduction in beclin 1 or by the HSV1 protein, and consequent neurotoxicity. Another hypothesis focuses on aberrant autophagy induction and/or defective lysosomal fusion and clearance. Oxidative stress has been proposed to cause increases Aβ by upregulating autophagy or by affecting its capacity for degradation and clearance. In the early stage of AD, autophagy is enhanced under stress of mutant APP, oxidative stress, and injured organelles, such as mitochondria. In the late stage of the disease, transport, maturation and degradation of autophagosomes are blocked due to the microtubule disruption caused by tau hyperphosphorylation. Furthermore, lysosome enzyme dysfunction also interrupts autopahgosome-lysosome fusion in AD. All these defects in the autophagic pathway contribute

to the accumulation of AVs and AD associated molecules, which directly increases the intracellular level of Aβ and lipofuscin leading to neuronal degeneration.

SPECIFIC AIMS

The aims of the study are to determine:

- The effect of oxidative stress on intracellular distribution of beta amyloid (Paper I).
- The role of endogenous amyloid beta-protein in oxidant-induced apoptosis (Paper II).
- The role of autophagy and APP processing in oxidant-induced damage (Paper III).
- Intracellular localization of amyloid beta peptide and its relationship to the lysosomal system (Paper IV).

MATERIALS AND METHODS

Cell culture

Nontransfected (NT) human SH-SY5Y neuroblastoma cells (Papers I and IV) were obtained from American Type Culture Collection (ATCC). HEK293 human embryonic kidney cells (Paper II) and human SH-SY5Y neuroblastoma cells (Paper III and IV) were obtained from ATCC and stably transfected with an empty pcDNA 3.1 vector containing a cytomegalovirus promoter, or wild-type APP695 (APPwt), or APP Swedish KM670/671NL double mutation (APPswe) using Lipofectamine 2000 according to the manusfacturer's instruction (Invitrogen). NT cells were cultured in Minimum Essential Medium with Glutamax (MEM) containing 10% fetal bovine serum 50 IU/ml penicillin G and 50 mg/ml streptomycin in the atmosphere of 8% O_2, 87% N_2 and 5% CO_2 at 37^{o}C. For selection of transfected cells, 200 μl/ml geneticin was added to cultured medium instead of penicillin G and streptomycin.

Treatments

Induction of oxidative stress

Normobaric hyperoxia is known as an *in vitro* model of mild chronic oxidative stress and accelerates age-related changes [207, 208]. In our study (Papers I, II, and III), hyperoxia (40% O_2, 55% N_2 and 5% CO_2) was used as a chronic oxidative stress model; normoxia (8% O_2, 87% N_2 and 5% CO_2) was used as a normal condition.

Serum withdrawal suppressed mitotic activity and allowed longer cultivation under hyperoxia, which cells in complete medium survived poorly. Serum withdrawal also induced cell starvation and active autophagy. Serum free OptiMeM was used in our study during exposure to normoxia and hyperoxia.

Inhibition of lysosomal function

Different lysosomal inhibitors were applied to suppress the lysosomal function by different mechanisms. Chloroquine (Paper I) was used to suppress the activity of acid hydrolases due to the rise of lysosomal pH; bafilomycin A (Paper II) was used to inhibit the lysosomal proton

pump; ammonium chloride (Papers II and III) was used to increases lysosomal pH; leupeptin (Papers II and III) was used to inhibit cysteine cathepsins; pepstatin A (Paper II and III) was used to inhibit aspartic cathepsins; E64d (Paper III) was used to inhibit cysteine protease.

Inhibition of exocytosis

Tetanus toxin (TeNT) is an exocytosis inhibitor which blocks the transportation of secretory vesicles to the plasma membrane. In Paper IV, 5 nM TeNT was used to study the involvement of exocytosis in AD pathogenesis.

Inhibition of γ- secretase

To study the possible involvement of local APP processing in Aβ accumulation within lysosomes, a γ- secretase inhibitor DAPT (500 nM) was administrated prior or parallel with autophagy induction (Paper III).

Inhibition of autophagy

3-methyladenine (3MA, Papers I, II and III) and ATG5 siRNA (Paper III) were used to inhibit autophagy.

3MA is an inhibitor of the class III phosphatidylinositol 3-kinase (PI3K), which is required for sequestration of autophagosomes, the initial stage of autophagy [209]. Besides this, RNA interference of autophagic genes, siRNA for ATG5 (involved in formation of Autophagosome), is also used to suppress autophagy.

Detection of autophagy

In our thesis (Papers I, II and III), detection of autophagy was performed by several methods: 1). Western blotting of LC3 (performed on 15% SDS page gels). The density of LC3-II band (16 KDa) was chosen to reflect autophagy. 2). Immunocytochemistry of LC3. The increased punctuate LC3 immunofluorescence indicates the upregulation of autophagy. 3). The ratio of Phospho-P70S6K to P70S6K, represents induction of autophagy. 4). Autophagy flux was evaluated by treating the cells with 10 μM E64d (an inhibitor of cysteine protease) and 10 μg/ml pepstatin A (an inhibitor of acid proteases (aspartyl peptidases). 5). Transmission electron microscopy was used to observe the morphology of autophagosomes.

Western blot analysis

This methods was performed as previously described [210], equivalent amount of protein were separated by SDS-PAGE. Proteins were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech AB, Uppsala, Sweden) at 200 mA overnight. Blocking was performed in TBS buffer (10 mM Tris-HCL pH 7.5, 150 mM NaCl) with 0.1% tween-20 and 5% (w/v) dry milk powder. Membranes were incubated with the primary antibodies at the following concentrations: 1:500 (P70 S6 Kinase and Phospho-p70 S6K), 1:1000 (6E10, LN27, LC3, CD107b/LAMP-2 and GAPDH), 1:5000 (Actin). The immunoblots were subsequently washed 3 times x 10 min in TBS containing 0.05% Tween-20 and incubated for 1 h with HRP-linked anti-rabbit or anti-mouse IgG (Amersham Pharmacia Biotech, 1:2000). Secondary antibody was detected by ECL or ECL plus detection systems (Amersham Pharmacia Biotech). To semi-quantify the specific proteins, the relative density of immunoreactive bands was calculated from the optical density multiplied by the area of the selected band (Papers II and III).

Enzyme-linked immunosorbent assay (ELISA)

Secreted $A\beta_{1-42}$ or $A\beta_{1-40}$ was measured by using $A\beta_{1-42}$ or $A\beta_{1-40}$ colorimetric ELISA kit (BioSource), respectively, by following the manufacturer's instructions (Paper II).

Measurement of intracellular reactive oxygen species production

Intracellular reactive oxygen species (ROS) production was detected by carboxy-H2DCFDA (DCF) oxidation that was assessed by flow cytometry (Paper III).

Detection of cell death

For fixed cells, DAPI (Papers I, II, and III) was used to study apoptotic cells by counting condensed and/or fragmented nuclei. In addition, cells with punctate Bax staining (Paper II) were used to indicate an early stage of apoptosis.

For living cells (Paper II), MTT was used to assess cell viability.

Measurement of lysosomal membrane integrity

Lysosomal membrane integrity was measured by using 50 nM LysoTracker Green DND-26 which was assessed by flow cytometer. Cells with reduced lysosomal membrane proton gradient (pale cells) were gated, and their percentage was calculated (Paper II).

Immunoelectron microscopy (iEM)

Cells were fixed in 3 % paraformaldehyde in 0.1 M phosphate buffer. Cells were washed and centrifuged to a pellet and embedded in 10 % gelatin. Samples were then infiltrated into 2.3 M of sucrose and frozen in liquid nitrogen. Sectioning was performed at -95°C and placed on carbon-reinforced formvar-coated, single hole Nickel grids. Immunolabelling was performed as follows: Grids were placed directly on drops of 0.1 M phosphate buffer followed by incubation in 2% BSA (Sigma fraction V) and 2% Fish gelatin (GE Healthcare, Buckinghampshire, UK) in 0.1 M phosphate buffer to block non-specific binding. Sections were then incubated with the primary antibody diluted 1:20 in 0.1M of phosphate buffer containing 0.1% BSA + 0.1% Gelatin over night in a humidified chamber at room temperature. The sections were thoroughly washed in the same buffer and bound antibodies were detected with protein A coated with 10 nm gold (Biocell, BBInternational, Cardiff, England) at a final dilution of 1:100. Sections were rinsed in buffer and fixed in 3% paraformaldehyde and contrasted with 0,05% uranyl acetate and embedded in 1% methylcellulose and examined in a examined in a Tecnai G2 Bio TWIN (FEI company, Eindhoven, The Netherlands) at 100 kV. Digital images were taken by a Veleta camera (Soft Imaging System GmbH, Műnster, Germany) [211] (Paper IV).

Low temperature dehydration (LTE)

Cells, embedded in gelatine were dehydrated by stepwise increased concentration of methanol and, in each step, the temperature was gradually lowered to -40°C in a Lecia EMAFS (Leica microsystem, Wien, Austria) and embedded and polymerized in Lowicryl K11M (Polysciences, Warrington, United States) at -40°C. Ultrathin sections were cut at room temperature and placed on carbon formvar-coated nickel grids. [212]

Immunolabelling procedure for K11M sections were performed as described above with the exception of contrasting which was done by 2 % uranyl acetate followed by lead citrate.

Immunocytochemistry and fluorescence microscopy

To investigate the intralysosomal colocalization of Aβ or APP, cells were double immunostained with Aβ (Aβ $_{1-40}$, Aβ $_{1-42}$, or oligomeric Aβ) or APP and LAMP2 or Cathepsin D (lysosomal marker), or LC3 (a marker for autophagic vacuoles), respectively. The nucleus was labeled by DAPI.

To quantify the intralysosomal accumulation of Aβ or APP, the cells with one or more $A\beta_{42}$ or APP positive autophagosomes/lysosomes (usually exceeding 1 μm in diameter) were counted under a Nikon Microphot-SA microscope using both phase contrast and fluorescence illuminations. The percentage of these cells was calculated for each specimen and averaged within each experimental group (n=3). At least 300 randomly selected cells in each specimen (900 cells in one group) were counted.

Double immunstaining of Aβ (Aβ $_{1-40}$, Aβ $_{1-42}$, or oligomeric Aβ) and Rab5 (a marker for early endosomes) was used to investigate the involvement of endocytosis in hyperoxia-induced intralysosomal Aβ.

To study the intracellular colocalization of Aβ, double immunostaining of Aβ (Aβ $_{1-40}$, Aβ $_{1-42}$, or oligomeric Aβ) and different vacuolar markers were used. These included: lysosomal-associated membrane protein 2 (LAMP-2, marker for lysosomes and late endosomes), Rab5 (marker for early endosomes), Golgin97 (marker for Golgi complex), M6P (mannose 6-phosphate receptors, marker for transport vesicles between Golgi and late endosomes), Rab9 (for TGN and late endosomes), Rab8 (for TGN and Golgi-derived secretory vesicles), Synaptobrevin/VAMP2 and Rab3 (for synaptic vesicles).

Mitochondria were vitally stained with 200 nM MitoTracker Red CMXRos (Molecular Probes,) for 30 minutes at 37 ^{o}C followed by fixation in 4% buffered formaldehyde.

The images were taken with a Nikon Eclipse E600 W confocal microscope using a 488 nm argon laser (green fluorescence), 543 nm helium-neon laser (red fluorescence) and 405 nm Diode laser (blue fluorescence)

Image analysis

The Aβ colocalization with subcellular structures was measured using Image J program with colocalization plugin (http://rsbweb.nih.gov/ij/) at display value =250, threshold for red and green

channels =50, ratio =50. For each cell, the total area of colocalization points was measured and its ratio to the total area corresponding Aβ granules was calculated. The experiments were repeated tree times. The total of thirty cells from each group was analyzed.

Statistical analysis

The results were analyzed for statistical significance using Mann-Whitney U test for two-group comparisons and Kruskal-Wallis test for multi-group comparisons. P values ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

I. Autophagy of amyloid beta-protein in differentiated neuroblastoma cells exposed to oxidative stress.

To determine whether oxidative stress has any influence on the relationship between lysosomes and $A\beta_{1-42}$ (the most toxic form of Aβ), we studied the effect of hyperoxia (40% versus 8% ambient oxygen) on the intracellular localization of $A\beta_{1-42}$ (assessed by immunocytochemistry) in retinoic acid differentiated SH-SY5Y neuroblastoma cells maintained in serum-free OptiMEM medium. In control cells, $A\beta_{1-42}$ were not found in the early endosomes and lysosomes. However, numerous large $A\beta_{1-42}$-containing lysosomes (over 1 μm) were detected after 5 days exposure of cells to hyperoxia (chronic oxidative stress), while still not detected in early endosome. Furthermore, 3MA, an inhibitor of autophagic sequestration, prevented the accumulation of $A\beta_{1-42}$-positive lysosomes due to hyperoxia. In parallel experiments, the autophagy of $A\beta_{1-40}$ following oxidative stress was found as well.

This finding indicates that oxidative stress induces translocation of endogenously formed Aβ into lysosomes, due to enhanced autophagy instead of endocytosis. Our finding suggests a novel pathogenic mechanism for the involvement of ROS in AD. Conceivably, ROS-induced damage to cellular structures results in the compensatory activation of autophagy promoting intralysosomal accumulation Aβ within neurons. Being a toxic substance, in particular a pro-oxidant, Aβ (especially $A\beta_{1-42}$) can induce lysosomal membrane rupture, and so, release of acid hydrolases into the cytosol and apoptotic death [205, 213]. This agrees with previous in vitro experiments showing toxicity of exogenous $A\beta_{1-42}$ towards cultured cortical neurons and neuroblastoma cells [37, 203]. This hypothesis is also in agreement with enhanced autophagy demonstrated for Alzheimer neurons [202].

II. Oxidative stress induces macroautophagy of amyloid beta-protein and ensuing apoptosis

To investigate how oxidant enhanced autophagy promote cell death, we compared the effects of hyperoxia (40% ambient oxygen) in cultured HEK293 cells that were transfected with either an empty vector (Vector), or wild-type APP (APPwt), or Swedish KM670/671NL double mutation (APPswe). Exposure to hyperoxia for five days increased the number of cells with Aβ-containing

lysosomes, as well as the number of apoptotic cells compared to normoxic conditions. The rate of apoptosis in all three cell lines demonstrated dependence on intralysosomal Aβ content (Vector<APPwt<APPswe). Furthermore, the degree of apoptosis positively correlated with lysosomal membrane permeabilization, while inhibitors of macroautophagy and lysosomal function decreased oxidant-induced apoptosis and diminished differences in apoptotic response between different cell lines.

We have demonstrated that HEK cells transfected with APPwt and APPswe (both generating increased quantities of Aβ) are more sensitive to hyperoxia than vector control cells. This increased sensitivity to prolonged oxidative stress, manifested by activation of macroautophagy (consistent with the increase in the number of large lysosomes) and apoptosis induction (as is evident from nuclear fragmentation and translocation of Bax to mitochondria) is particularly pronounced in APPswe cells. These cells are especially rich in intralysosomal Aβ, including its toxic oligomeric form, suggesting that oxidative stress-induced cell death might be mediated by Aβ accumulation within lysosomes. Consistent with lysosomal involvement in oxidant-induced cell death, exposure of APPswe cells to hyperoxia is associated with the pronounced loss of lysosomal membrane integrity. Although the role of lysosomes in mediating Aβ toxicity has been previously demonstrated by exposing cultured cells to exogenous Aβ [37], here we show that intralysosomal accumulation of endogenous cellular Aβ results in cell death.

Overall, our results demonstrate that intralysosomal accumulation of endogenous Aβ through macroautophagy can promote apoptosis, which is associated with release of the lysosomal protease cathepsin D to the cytosol and is prevented by inhibitors of macroautophagy and lysosomal function. This suggests that intralysosomal Aβ accumulation can cause lysosomal membrane permeabilization with ensuing cell death, a possible mechanism of neuronal loss in AD. Considering that macroautophagy is a continuous repair process that protects cells, in particular, from minor oxidative damage during normal oxygen metabolism, Aβ would accumulate intralysosomally. This, in turn, would promote cell death, especially if APP processing is increased, such as in FAD. Oxidative stress conditions would additionally enhance molecular damage and, consequently, macroautophagy of Aβ and cell death, as is consistent with a recognized role of oxidative stress in AD.

III. Macroautophagy-generated increase of lysosomal amyloid β-protein mediates oxidant-induced apoptosis of cultured neuroblastoma cells

The relationship between oxidative stress and the autophagic-lysosomal system in AD is not well understood. We have previously shown that mild chronic oxidative stress (normobaric hyperoxia) results in increased numbers of autophagic vacuoles and intralysosomal accumulation of Aβ in retinoic acid (RA) differentiated neuroblastoma cells [59]. Furthermore, using human embryonal kidney (HEK) cells, we demonstrated that increased cellular Aβ production is associated with enhanced oxidant-induced intralysosomal Aβ accumulation, causing apoptotic cell death through lysosomal destabilization [60]. In this study, we investigated the effects of normobaric hyperoxia and APP overexpression on lysosomal Aβ accumulation and cell viability, using RA-differentiated SH-SY5Y neuroblastoma cells. We show that SH-SY5Y cells overexpressing APP are characterized by both enhanced oxidative stress and enhanced macroautophagy, resulting in increased intralysosomal accumulation of monomers and oligomers of Aβ and consequent apoptosis. Moreover, specific inhibition of the lysosomal degradation and γ-secretase suggest that intralysosomal accumulation of Aβ resulted to a large extent from its macroautophagic uptake, although APP processing within autophagic vacuoles can occur as well [47]. The deleterious effects of normobaric hyperoxia were more pronounced in cells overexpressing the Swedish FAD mutation (APPswe) compared to those with wild type APP (APPwt), and were prevented by the inhibition of macroautophagy using 3-methyladenine (3MA) or siRNA against the autophagy-related protein ATG5. These data provide further support for the interactive roles of oxidative stress and autophagy in AD.

In summary, our results indicate that macroautophagy-generated intralysosomal increase of Aβ is essential for oxidant-induced apoptosis, providing additional support for the interactive role of oxidative stress and the lysosomal system in AD-related neurodegeneration.

IV. Intracellular localization of amyloid beta peptide in SH-SY5Y neuroblastoma cells.

We have previously shown that in differentiated SH-SY5Y neuroblastoma cells cultured under normal conditions the majority of Aβ is localized extralysosomally, while oxidative stress significantly increases intralysosomal Aβ content through activation of macroautophagy. It is, however, not clear what cellular compartments contain the majority of Aβ in intact SH-SY5Y cells.

In this study, we demonstrated that, in differentiated non-transfected neuroblastoma cells cultured under normal conditions in vitro, Aβ (including $A\beta_{1-40}$, $A\beta_{1-42}$ and Aβ oligomers) is mainly localized to the cytosol, and only a minor part of Aβ species (9, 10 and 13%, respectively) shows colocalization with organelles, including endoplasmic reticulum, Golgi complexes, early and late endosomes, lysosomes, mitochondria, and exocytotic vesicles. In APPswe cells the percentage of Aβ colocalized with organelles is somewhat higher than in NT cells (16, 17 and 21% for $A\beta_{1-40}$, $A\beta_{1-42}$ and Aβ oligomers, respectively), but still its major part shows cytosolic localization. These findings are supported by iEM data showing labelling in clusters in the cytoplasm.

Overall, our findings demonstrate that intracellular Aβ (including $A\beta_{1-42}$, $A\beta_{1-40}$ and Aβ oligomers) in normal cells is mainly located in non-membrane-bounded cytosolic structuresand only a relatively little portion of it is colocalized with organelles. In cells overproducing Aβ, the colocalization of Aβ with subcellular compartments is increased, but still, the majority of it is in the cytosol. Inhibition of secretion induces Aβ colocalization with secretory compartments, and lysosomal compartment as well, which suggests that most Aβ in the subcellular compartment is secreted through the exocytotic pathway.

The presence of Aβ in the cytosol has been previously demonstrated [52, 53]. It was suggested that Aβ formed in the endoplasmic reticulum (and possibly other luminal compartments) is transported to the cytosol where it can be partially degraded by proteasomes [54, 55]. Our results suggest that cytosolic Aβ can constitute a substantial portion of its total cellular content. Further studies are needed to better understand how this normal Aβ distribution and the metabolism of Aβ shift during AD pathogenesis.

CONCLUSIONS

Normally most of intracellular Aβ (including monomeric $A\beta_{1-42}$, $A\beta_{1-40}$ and oligomeric Aβ) is mainly localized to the cytosol, and only a relatively minor part of it (totally 9 to 13% in our model) shows colocalization with organelles, such as endoplasmic reticulum, Golgi complexes, early and late endosomes, lysosomes, mitochondria, and exocytotic vesicles. Oxidative stress initiates the accumulation of Aβ in the lysosomes, which is a characteristic of Alzheimer neurons. Oxidative stress enhances both the macroautophagic import of APP and, probably, the APP processing, as well as the macroautophagic import of Aβ, resulting in the amassing of endogenous Aβ in the lysosomal compartment. As a pro-oxidant, Aβ (especially $A\beta_{1-42}$ and oligomeric Aβ) can induce lysosomal membrane rupture and release of acid hydrolases into the cytosol, leading to apoptotic cell death. This study links oxidative stress and lysosomal system in AD, and demonstrates the role of the autophagic-lysosomal system in SAD pathogenesis. Our hypothesis on the role of oxidative stress and autophagy in the endogenous Aβ-mediated neurotoxicity is presented in **Figure 7.**

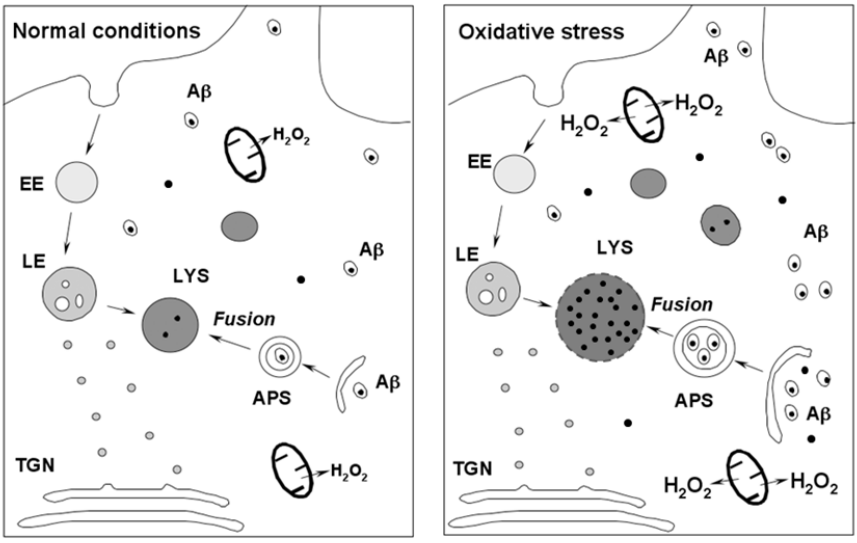

Figure 7. Tentative scheme illustrating the roles of oxidative stress and autophagy in the intralysosomal accumulation of Aβ within neurons. Aβ forms as a result of APP cleavage (the site of Aβ production is still not clearly identified) and is transported throughout the cytoplasm within specific vacuoles. During autophagy, cytoplasmic components (apparently including Aβ granules) are sequestered within double membrane-bounded autophagosomes (APS) that fuse with lysosomes (LYS) where autophagocytosed material is exposed to hydrolytic enzymes. Lysosomal enzymes, initially conjugated with mannose-6-phosphate receptors (MPR), are transported from trans-Golgi network (TGN) to late endosomes (LE) that develop into mature lysosomes upon further acidification and the loss of MPR. Oxidative stress (indicated as an increased diffusion of hydrogen peroxide through mitochondrial membranes) results in the activation of reparative autophagy and ensuing accumulation of Aβ within lysosomes. Oxidative stress might also enhance intralysosomal deposition of Aβ by interfering with lysosomal degradation as well as by increasing overall Aβ production (shown as increased numbers of Aβ-containing vacuoles) through the activation of amyloidogenic APP processing. EE, early endosome.

Aberrant induction of autophagy (upregulation or inhibition), defective transport and maturation of autophagosomes, and defective lysosomal degradation due to lysosomal enzyme dysfunction and/or protein oxidation, leads to increased amyloidogenesis, defective clearance of Aβ and consequent Aβ accumulation within cells that can result in apoptosis. Possible therapies for AD based on modulation of autophagy will require careful targeting of specific steps of the pathway to achieve more efficient digestion. Moreover, regulating the balance of the autophagic-lysosomal system for maintaining cellular homeostasis require further investigation.

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