Oxidative Stress Induced Mitochondrial Failure and Cellular Hypoperfusion: Implication in the Pathogenesis of Alzheimer Disease

Uszkodzenia mitochondrialne i hypoperfuzja komórkowa indukowane stresem oksydacyjnym: wpływ na patogenezę choroby Alzheimera

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Summary

Alzheimer disease (AD) and cerebrovascular accidents (CVAs) are two leading causes of age-related dementia. Increasing evidence supports the notion that chronic hypoperfusion is primarily responsible for the pathogenesis that underlies both disease processes. In this regard, hypoperfusion appears to induce oxidative stress, which is largely due to the formation of reactive oxygen species (ROS). Oxidative stress in brain microvessels and/or parenchymal cells results in an accumulation of ROS, thus promoting leukocyte adhesion and increasing endothelial permeability. The chronic injury results in progressive cellular hypometabolism, which is responsible for AD and CVAs, and appear to be a central initiating factor for vascular abnormality, mitochondrial damage and an imbalance in the activity of vasoactive substances such as different isoforms of nitric oxide synthase (NOS), endothelin-1 (ET-1), oxidative stress markers, mtDNA and mitochondrial enzymes in the vascular wall and in brain parenchymal cells. Here we outline recent evidence, as well as our own experimental data, indicating that chronic injury-stimulus induces hypoperfusion in the microcirculation of vulnerable brain regions which leads to energy failure. This energy failure is manifested by damaged mitochondrial ultrastructure, formation of a large number of non-mature or “young” electron dense “hypoxic” mitochondria and overexpression of mitochondrial DNA (mtDNA) deletions. Additionally, these mitochondrial abnormalities coexist with
increased redox metal activity, overexpression of lipid peroxidation markers and RNA oxidation, known to elucidate the oxidative stress that occurs within various cellular compartments and most notably in the vascular endothelium that is associated with atherosclerotic damage. The ultrastructural pathology in the neurovascular regions co-exists with neuronal and glial damage, known to be important in the development of AD. Vulnerable neurons and glial cells show mtDNA deletions and overexpression of oxidative stress markers in regions that only closely associate with damaged vessels. This evidence strongly suggests that chronic hypoperfusion induces the accumulation of the oxidative stress products. Moreover, the degree of vascular wall cell lesions in AD brains is proportional to the degree of neuronal and glial cell damage. We hypothesize that continuous accumulation of oxidative stress products, such as peroxynitrite and large amounts of nitric oxide (NO) generated by the overexpression of the inducible and/or neuronal NO synthase (iNOS and nNOS, respectively), appear to be secondary but accelerating factors for damage and for compromising the blood brain barrier (BBB) in hypoxia/hypoperfusion or AD. Our hypothesis is that pharmacological intervention targeted towards correcting chronic hypoperfusion will change the natural history of the dementing neurodegeneration. Therefore, eliminating this imbalance can be used as an alternate strategy for the treatment of AD.

The association between health risk factors caused by aging and Alzheimer disease

**Vascular abnormalities and ApoE genotype effects on AD**

The association of amyloid beta (Aβ) with cerebral vessels is an intriguing feature of AD. While some degree of cerebral Aβ angiopathy involving the leptomeninges and intraparenchymal vessels occurs in almost all cases of AD, Aβ deposits within the neocortical region is unknown [1]. In addition, the mechanisms behind the effects of several vascular factors and peripheral vascular pathophysiology might promote a late-onset of AD [1-4]. Apolipoprotein E (ApoE), a major risk factor for atherosclerosis [5-7], as well as AD [8, 14, 93, 190], may be linked to AD via its effects on vascular NO and consequently on the vasculature [9-12, 191].

In clinically and pathologically confirmed AD cases studied by Thomas and coworkers, cerebral microvessels in the temporal cortex and parahippocampal gyrus associate with the predominant Aβ1-42 form of the Aβ peptide and may affect microvascular function [1]. Surprisingly, double immunostaining methods reveal that at least 40% of the microvessels in the two brain regions contain Aβ1-42 deposits but no correlation of such localization with the ApoE genotype [1]. However, ε4 homozygotes display a greater Aβ1-40 burden. Moreover, the levels of total serum, low density lipoproteins (LDL), and ApoB correlate with increased deposition of Aβ in demented individuals with neuropathologically confirmed AD [13]. These findings also indicate a key role for vascular abnormalities in the pathogenesis of AD. Since chronic hypoxia/hypoperfusion, Aβ depositions, and AD are maladies with similarities to atherosclerosis, we would expect them to share risk factors [9, 10, 14,93,190-192] and that the same preventive interventions would alleviate their symptoms [13, 14,190,192-194].

**The role of Hyperlipoproteinemia in the development and progression of AD**

Hyperlipoproteinemia, characterized by elevated levels of cholesterol in the blood, is linked with the impairment of NO-mediated, endothelium-dependent dilation [15,191]. Galle and coworkers demonstrate that oxidized lipoprotein impairs endothelium-dependent dilation and is more potent than oxidized LDL in this effect [15]. Comparisons between ventricular fluid (VF) lipoproteins isolated from AD patients and age-matched non-demented patients show that cerebral spinal fluid CSF lipoprotein metabolism is altered in AD patients, thus supporting the hypothesis describing the direct relationship between vascular and lipoprotein abnormalities in people with AD [16,93], (Figure 1).

Additionally, AD and fat intake are relevant [7, 17]. According to Refolo and collaborators cholesterol metabolism mediates the development of AD in a transgenic (Tg) mouse model [18]. Diet-induced hypercholesterolemia results in significantly increased levels of formic acid-extractable Aβ peptides in the central nervous system (CNS) in AD mice. The total level of Aβ strongly correlates with the level of cholesterol in both plasma and the CNS and with the number and size of amyloid deposits [18].
Vascular changes and their influence in the pathology seen in AD

Recent findings demonstrate that there is a similarity between the ultrastructural features of both vascular lesions and mitochondria in brain vascular wall cells from human AD brain biopsies, human short postmortem brain tissues, yeast artificial chromosome (YAC R140) and C57B6/SJL Tg+ mice overexpressing Aβ precursor protein (PP) [14,93]. Performing in situ hybridization using mtDNA probes for human wild type, 5kb deleted and mouse mtDNA, and immunocytochemistry using antibodies against APP, 8-hydroxy-2'-guanosine (8OHG) and cytochrome c oxidase subunit 1 (COX) provide congruent ultrastructural localization [14,93]. A higher degree of amyloid deposition in the vascular walls of the human AD, YAC and C57B6/SJL Tg (+) mice exists compared to aged-matched controls [14,93]. Severely damaged vessels exhibit immunopositive staining for APP. More mitochondrial abnormalities are present in human AD, YAC and C57B6/SJL Tg (+) mouse microvessels where lesions occur [14,93]. Undamaged regions of human AD tissues, YAC and C57B6/SJL Tg (+) mouse tissues and in aged-matched control subjects lack these features, while damaged vessels manifest cells possessing clusters of wild and deleted mtDNA-containing positive probes [14]. Our observations demonstrate that vascular wall cells, especially their mitochondria, appear to be central targets for oxidative stress-induced damage before the development of AD pathology [14,93]. On the other hand, the positive correlation between AD and cholesterol levels suggests that antioxidant therapy and cholesterol-lowering drugs could delay the occurrence of AD [14, 19, 20,93]. However, despite their frequencies, the pathophysiological and morphological changes in brain microcirculation that accompany AD remain poorly understood, and the specific factors controlling vascular tone in AD remain unknown (see Figure 1-2).

![Diagram of Presumptive Pathway Involved in Ischemic Stroke](image)

**Fig. 1.** Hypothesizes the development of atherosclerosis can lead to stroke following chronic brain hypoperfusion (CBH). Low stress shear from CBH induces endothelial cell conformational (rounder EC shape) changes inducing transitory eNOS upregulation and increased vascular NO release. Following temporary blood flow homeostasis, depleted vascular NO production results in brain blood flow dyshomeostasis and eventual ATP decline (from reduced glucose/oxygen delivery). Continues chronic blood flow dyshomeostasis generates a neuronal energy crisis coupled to oxidative stress. The outcome of this abnormal cascade is manifested as stroke or as transient or temporary ischemic attacks (TIAs) that appeared to be main initiator for the development of mild cognitive impairment (MCI) and then severe mental retardation and dementia.
The consequences of tobacco smoking on the nicotinic receptors
Smoking tobacco results in cardiovascular, cerebrovascular, pulmonary diseases and cancer. The effects are linked to the damaging actions of free ROS. Supporting evidence suggests AD and cardiovascular disease have a positive correlation [3, 9, 12, 14, 21-24]. Abnormal AβPP metabolism and cholinergic dysfunction interact during AD progression. These major features of the disease both occur at restricted loci during normal aging, a potential model for early AD pathology.

Nicotinic acetylcholine receptors (nAChR), a class of ligand-gated channels composed of α and β subunits with specific structural, functional and pharmacological properties, are focal during the development of disease conditions and participate in physiological and behavioral effects of acetylcholine by mediating responses to nicotine [25, 26, 28]. The number of high-affinity nicotine binding sites in specific regions of the human brain changes during aging, and age-associated neurodegenerative diseases, including AD [27]. During development, aging, and diseases including autism, schizophrenia, AD, Parkinson disease (PD) and Lewy body dementia (LBD) neurotransmitter expression varies [25, 28]. Deficits in α4β2 and α7 correspond respectively to disorders of abnormal brain maturation such as autism, and schizophrenia. In aging and age-related neurodegenerative disorders nAChR deficits are predominantly linked to α4-containing receptors, although some studies also indicate the involvement of the α3 and α7 subunits. While aging appears to be associated with reductions in subunit mRNA as well as protein expression, only protein loss is evident during AD [25, 28]. The entorhinal cortex is particularly vulnerable to beta-amyloidosis, and compared with other cortical areas has high density of nicotinic (3H-nicotine) receptor binding as compared to cholinergic or glutamate binding receptors [25, 26, 29].
During aging (between 40 and 100 years) high affinity nicotine binding in the frontal cortex decreases in parallel with glutamate NMDA receptor binding ([3H] MK801) [26]. Nicotine binding also declines with age in the hippocampal formation and the entorhinal cortex, but NMDA receptor binding remains unchanged [26]. This reduction may predispose the neo- and archicortex to the loss of nAChRs observed in age-associated neurodegenerative conditions. In contrast, no age-related loss of nAChR binding is observed in the thalamus. Only after the 7th decade is some loss observed in the striatum [26]. However, deficits in nAChRs are observed in AD, PD and LBD and may be matched with specific disease-related processes [26]. Nevertheless, α-3 mRNA density correlated negatively with age in the entorhinal cortex of both Alzheimer’s and normal subjects. This observation thus suggests a potentially extensive decay in α-3 -expressing neurons or loss of α-3 -containing receptors in intact neurons of the entorhinal cortex [27]. Nicotinic receptor binding in the entorhinal cortex declines with increasing age. However, muscarinic M1 and non-M1, glutamate NMDA and non-NMDA receptors are spared [29]. Normal elderly individuals, distinguished by the absence of beta A4 immunoreactive plaques in this area, differ from those with plaques by higher nicotine binding [29].

Individuals with an established history of smoking tobacco possess elevated nicotinic receptor binding and hippocampal choline acetyltransferase compared with non-smokers, and these individuals have a reduced density of cortical plaques [29]. These findings are consistent with the hypothesis that down regulation of the nicotinic cholinergic receptor-ligands gated calcium channel controls the expression of neurotrophin, a chemical that plays a role in the evolution of Alzheimer-type pathology [29]. In addition, the role of nicotinic receptors in AD as a potential therapeutic target has been considered recently [30]. We speculate that cigarette smoking probably plays a role as a pathogenic cofactor in the initiation of AD. However, the role of tobacco smoking as a hypoperfusion and oxidative stress factor that may act as a pathogenic factor in the development of cerebrovascular and neuronal lesions in AD has not yet been considered or given proper attention. Detailed ultrastructural studies elucidating the mechanisms behind the development of Aβ depositions, along with investigations into the possible accelerating effects of chronic hypoxia in an animal model will likely open new avenues of treatment for Alzheimer patients. However, more research is needed to determine the exact nature of this role (see Figures 1-2).

Features that influence the development and prognosis of AD

Interactions between cerebrovascular diseases and AD

ROS are generated at sites of injury and/or inflammation (Figure 1-2). The vascular endothelium, which regulates the passage of macromolecules and circulating cells from blood to tissue, is a major target of oxidant stress and plays a critical role in the pathophysiology of several vascular diseases. In addition, the vascular endothelium, neurons, and glia can synthesize, store, and release ROS and vasoactive substances in response to certain stimuli, especially to chronic hypoxia/hypoperfusion. Their contribution to the pathophysiology of stroke, cerebrovascular disease and AD is extremely important. Moreover, the role of hypoperfusion as a key factor for vascular lesions that causes oxidative stress, appears to be widely accepted as an initiator of AD [9, 10]. This idea is based on a positive correlation between AD and cardiovascular diseases.

Specifically, accumulated oxidative stress increases vascular endothelial permeability and promotes leukocyte adhesions, which is coupled with alterations in endothelial signal transduction and redox-regulated transcription factors. Based on these findings we hypothesize that the cellular and molecular mechanisms by which hypoperfusion-induced ROS accumulation impairs endothelial barrier function and promotes leukocyte adhesion, induces alterations in normal vascular function and results in the development of AD. The sustained hypoperfusion and oxidative stress of brain tissues could also stimulate the secondary overexpression of iNOS and nNOS and endothelin-1 (ET-1) in brain cells (Figure 2). Also, the increased accumulation of oxidative stress products probably contributes to the decompensation of the BBB and the damage to brain parenchymal cells. Therefore, determining the mechanisms behind these disturbances in experimental animals may provide crucial information in the development of new, more effective therapies for the treatment of cerebrovascular and neurodegenerative diseases, including AD [14, 65, 93, 190-194].
Many common underlying risk factors play key roles in the development of cardiovascular, cerebrovascular and neurodegenerative diseases [6, 9, 10]. Cigarette smoking causes chronic hypoxic conditions and the formation of a large amount of free oxygen radicals that appear to be key factors in the development of these diseases. Latest evidence indicates that continuous free oxygen radicals induce cellular damage and decreases antioxidant defenses. Several recent studies indicate that cigarette smoking is a cofactor in the initiation of AD via its effect on the vasculature, as previously discussed. Nicotine via nicotinic receptor activation may counter these effects in part. The role of vascular insufficiency/hypoperfusion has been considered as a pathogenetic factor in the development of AD, and the positive relationship between cerebrovascular diseases such as stroke and especially cerebrovascular atherosclerosis indicates the latter may also be linked to the pathogenesis of AD. However, the role of tobacco smoking in the pathogenesis of AD is still unclear and controversial.

The influence of oxidative stress on the function of brain microvessels in AD

ROS can function as signaling intermediates at low levels and regulate fundamental cell activities including growth and adaptive responses (Figure 2). However, at higher concentrations, ROS can cause cell injury and death. Vascular endothelium modulates the passage of macromolecules and circulating cells from blood to tissue and is a major target of oxidant stress [31]. Specifically, oxidative stress increases vascular endothelial permeability and promotes leukocyte adhesions, which are coupled with alterations in endothelial signal transduction and redox-regulated transcription factors [31]. Based on these recent findings we hypothesize that impairing endothelial barrier function and promoting leukocyte adhesion also induce alterations in normal vascular endothelial cell (EC) function, resulting in AD progression (for the detail see Figure 2).

Compared to other organs or tissues, the brain is more vulnerable to ROS-induced damage due to its high rate of oxygen consumption [65,190-194], high polyunsaturated lipid content, and relative paucity of classic antioxidant enzymes [32]. The AD brain contains increased regional levels of oxidative stress indicators [7, 33-38]. Studies demonstrate a decline in polyunsaturated fatty acids (PUFA) [39-41], increased levels of lipid peroxidation markers [37, 39], as well as protein oxidation [42, 43], DNA oxidation [44-46] and RNA oxidation [14, 47-49] during AD. Additionally, the presence of advanced glycation end products (AGE), glycoxidative end products (N-ε-carboxy-methyl-lysine and lipid peroxidation adducts) are present in both neurofibrillary tangles (NFT) and senile plaques (SP) in AD [14, 34, 36, 37, 42, 43, 47-52] and in post-ischemic tissues [53-57].

Vascular aging correlates with both structural and functional changes that can take place at the level of the endothelium, vascular smooth muscle cells (vSMC) and the extracellular matrix of blood vessels [192-193]. In the endothelium, reduced vasodilatation in response to agonists occurs in large conduit arteries, as well as in resistance arteries as a result of aging [58]. Furthermore, enhanced oxidative stress by hypoperfusion contributes significantly to the deleterious effects of aging on the endothelium by means of NO breakdown due to ROS. The relative contribution of the above phenomenon to age-related endothelial dysfunction is highly dependent on the species and the type of vascular bed involved [5, 6, 58, 59, 93, 192].

Cortical and subcortical gray matter and in meningeal and gray matter blood vessels (congophilic angiopathy) contain Aβ deposits, one of the prominent features of AD [3, 5, 6, 23, 58, 59]. In vitro experimental evidence shows that these Aβ deposits induce cerebrovascular dysfunction in the rat brain [60], and that the Aβ peptide produces endothelial dysfunction in cerebral microvessels via ROS. This occurs when the ROS superoxide-scavenging enzyme, superoxide dismutase, prevents acetylcholine-induced endothelium-dependent vasodilation [60]. In addition, accumulating evidence supports the idea that the Aβ peptide is responsible for the cerebrovascular effects of AβPP overexpression [61, 62].

A study by Iadecola and coworkers shows how Tg mice overexpressing AβPP have a profound and selective impairment in endothelium-dependent regulation of neocortical microcirculation. Moreover, peptides derived from APP processing may contribute to the alterations in cerebral blood flow (CBF) and neuronal dysfunction during AD [61]. The study confirmed that Aβ1-40 did not influence the increasing CBF produced by the endothelium-independent vasodilators and hypercapnia. In contrast, Aβ1-42 did
not reduce resting CBF or the increasing CBF produced by endothelium-dependent vasodilators. The superoxide scavengers, SOD and MnTBAP, reversed the cerebrovascular effects of Aβ1-40. This data strongly suggests that Aβ1-40, but not Aβ1-42, produces the cerebrovascular alterations seen in AβPP, and thus, Aβ1-40 could play a role in the cerebrovascular alterations observed in AD [23, 62]. Moreover, this study supports recent evidence that microvessels isolated from the AD brain kill neurons in vitro [63]. However, despite all the research on the effects of Aβ, the source of the ROS in vivo and link to hypoperfusion is not completely understood. Pathological features of brain cerebrovascular lesions and AD.

Several morphometric features of BBB dysfunction are present in pathologically confirmed AD patients [64]. Accumulation of Aβ deposits around vessels in AD brain biopsy samples may be an indication of progressive damage of BBB during AD [14, 64, 65]. Our findings [14] demonstrate that structural or physiologic abnormalities of the BBB itself may represent a seminal pathogenic event during AD development, leading to vascular amyloid deposition in the brain [41, 64, 66]. The heterogeneous pathology of AD arises from the variability in nature and severity of vascular lesions. Additionally, Aβ co-exists with cerebrovascular diseases such as cerebrovascular arteriosclerosis (CVA) [12]. For example, significantly higher densities of Aβ immunoreactive plaques are present in AD+CVA as compared to AD alone [12].

The Aβ deposits in SP and cerebrovascular angiopathy are derived from AβPP expressed in neurons and in a variety of non-neuronal cells (some outside of the central nervous system) [67-71]. Perivascular Aβ deposition may be a risk factor for reduced regional CBF [72]. The age-related loss of mechanisms/cells that are capable of removing Aβ deposits involve subtle molecular alterations in the components that bind Aβ protecting the basement membrane from cellular degradation [73]. The activation of non-neuronal cells including microglia further contribute to neuronal damage [74]. Several factors that may ameliorate AD either improve CBF or prevent CBF decline [72]. Ultrastructural studies revealing widespread penetration of Aβ deposits by degenerating microvessels reflect direct relationship between vascular changes in brain and the AD pathology [3, 23]. According to numerous morphometric studies EC contact with the vast majority of SP occurs randomly. It is also clear that a certain subpopulation of SP shows a real and intimate relationship with the vasculature [75, 76]. It is likely that SP have more than one origin [33], and that vessels are one of their direct targets. In over 90% of AD cases, Aβ can be detected in at least some vessels [77], and the source of this Aβ is likely vascular EC and SMC rather than neurons, since EC and SMC show abundant APP immunoreactivity [14, 75, 78, 79]. Ultrastructural studies on blood vessels with Aβ deposits indicate their intermittent associations with membrane abnormalities of SMC [33]. Indeed, in AD cases with a clinical history of cerebral bleeding, Aβ deposits completely replace the muscle layers [75, 78, 79]. This finding suggests that vascular wall cell alterations such as EC damage and muscle cell atrophy may occur in AD, even in the absence of visible Aβ depositions, and indicates that the vascular system appears to be a primary target for the development of this disease [14, 93].

The role of mitochondrial abnormalities during the development of AD

In aerobic cells 90-95% of the total amount of ATP production requires oxygen. The synthesis of ATP via the mitochondrial respiratory chain is the result of electron transport across the electron transport chain coupled to oxidative phosphorylation [80,194]. The main radical produced by mitochondria is superoxide anion. Intramitochondrial antioxidant systems scavenge this radical to avoid oxidative damage which can lead to impaired ATP production [81-83]. During aging and some neurodegenerative diseases, including AD, damaged mitochondria are unable to maintain the energy demands of the cell [65, 84, 93, 190-194]. This can lead to an increased production of free radicals, inducing the interruption of oxidative phosphorylation, and resulting in decreased levels of ATP [83]. Both processes, defective ATP production and increased oxygen radicals, may induce mitochondria-dependent cell death [83, 93, 193-194].

Animal studies using mitochondrial toxins provide the association between neurodegeneration with mitochondrial dysfunction and oxidative damage [83]. These consequences are implicated in the pathogenesis of human as well as animal models of neurodegenerative diseases [85-88], particularly
AD [14, 65, 81, 82, 84, 89, 90]. After long-term ischemia/reperfusion the mitochondria ultrastructure disintegrates in vivo and in vitro [6, 56, 57]. Apoptosis of degenerating neurons occurs in association with the accumulation of perikaryal mitochondria and oxidative damage to the nucleus [114]. This same pattern of mitochondrial lesions is observed in human AD brain biopsy samples [65, 84]. The reduced expression of both mtDNA and nuclear DNA encoded genes is consistent with a physiological down-regulation of the mitochondria respiratory chain in response to declining neuronal activity [81, 82, 88, 90, 92]. However, the role of somatic cells and mtDNA mutations in the pathogenesis of mitochondria failure during AD is still controversial [82, 88, 90].

The deleted mtDNA increases at least 3 fold in AD cases as compared to controls in humans [84]. Moreover, mtDNA isolated from the brains of AD patients includes oxidative modifications containing 8-hydroxy-2'-deoxyguanosine (8OHdG) [44-46]. Studies using in situ markers for 8OHdG showed that RNA oxidation is a prominent feature of damaged neurons in AD [14,47-49]. Quantitative analysis reveal a strong positive correlation between mtDNA deletions and 8OHG (r = 0.934) [84]. However, given that mtDNA (even DNA containing the 5kb deletion) is spared relative to 8OHG, we suspect that mitochondrial abnormalities correlate, but do not directly produce ROS. Therefore, it is important to recognize that 8OHG is formed by the direct attack of •OH. These •OH radicals have only a 2 nm sphere of diffusion and are unable to diffuse through the mitochondrial membrane [14, 65, 84, 93].

Polarographic studies by Cormier and coworkers describe the effects of nicotine on respiratory chain in rat brain mitochondria [96]. The measurements of oxygen consumption show significant concentration-dependent inhibition by nicotine. Nicotine binds to complex I of the respiratory chain, inhibits the NADH-Ubiquinone reductase activity, and competes with NADH for complex I [96]. Furthermore, nornicotine, but not cotinine, the main nicotine metabolite, inhibit mitochondrial respiration. Complex I generates the superoxide anion, nicotine, and was able to inhibit this ROS generation [96]. This may explain a part of the beneficial and protective effects of nicotine in a few neurodegenerative diseases, as suggested by many epidemiological studies [96]. However, future studies should focus on elucidating the effect of nicotine on the mitochondria functions as well as DNA overexpression and/or deletion during the development of neurodegenerative disorders including AD. The exact cellular mechanisms behind vascular lesions and their relation to oxidative stress markers identified by RNA oxidation, lipid peroxidation, or mtDNA deletion remain unknown. Future studies comparing the spectrum of oxidative stress-induced damage during reperfusion injury or, more importantly, during hypoxia/hypoperfusion, with AD damage are warranted.

**Subcellular mechanisms involved in the development and maturation of human AD**

In a comparison between AD brain biopsies and age-matched controls, ultrastructural features of the microvessels in EC and perivascular cells did not show visible changes. Mitochondria in the EC were intact. Conversely, microvessels from AD brain biopsies were characterized by the differential degree of damage. However lesions were heterogeneous [14, 65, 84, 93, 95]. In some areas, microvessels show multiple lesions. Examples of such lesions include a cluster of mitochondria-derived lysosomes and necrotic changes in the ultrastructure of both the vascular EC and perivascular cells. Very often, the capillary endothelium showed the presence of “giant” sized lipid vacuoles in the cellular matrix. All brain cellular compartments exhibit evidence of mitochondria transforming to the mitochondria-derived lysosomes [14, 65, 84, 93, 95]. In addition, EC occupied only a small part of the vessel wall. Perivascular cells showed the presence of large numbers of the mitochondria-derived vacuoles in their matrix. Sometimes microvessels within the endothelium, at the early stages of AD, did not show any damage in their ultrastructure. However, the luminal plasmalemma of this EC sharply protruded into the vessel lumen, indicating the effect of hypoperfusion before any visible ultrastructural damage. Cellular organelles including mitochondria remained intact. Perivascular spaces contained some vacuolar degenerative structure [14, 65, 84, 93, 95]. AD affected regions very often characterized the presence of vascular endothelium, and perivascular cells contained giant-sized vacuolar degenerative structures in their matrix. These abnormalities co-existed with the formation of mitochondria-derived lysosomal structures [14, 65, 84, 93, 95]. Perivascular cells from lesioned vessels showed the presence of a giant-sized, lipid-laden vacuolar degenerative structure with amyloid deposition in the cytoplasmic
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matrix. The cytoplasmic matrix contained only residues of the cytoplasmic organelles (e.g. membranous structure) [14, 65, 84, 93, 95]. We demonstrate in recent work that cortical neurons from AD brain biopsies have selective localization of mitochondria abnormalities in the cell body [14, 65, 84, 93, 95]. The majority of the neurons, which closely associate with the lesioned vessels, possess a different degree of ultrastructural abnormality [14, 65, 84, 93, 95]. In the neuronal cell body, the presence of partially and completely damaged mitochondria, correlate with lipofuscin formation, and mitochondria appear to be a major substrate for this process [14, 65, 84, 93, 95]. A large number of electron dense hypoxic mitochondria with abnormal cristae are evident throughout the cell body. In many cases, the neuronal cell body shows an absence of cellular organelles. Different stages of mitochondrial abnormality, such as formation of mitochondria-derived lysosomes and lipofuscin, are present in damaged but not in normal neurons. The mitochondria-derived lysosomes vary in size and density, as well as lipofuscin deposits. Mitochondrial lesions and lipofuscinogenesis are also present in other cellular compartments of the brain parenchyma. Glial cells at the damaged area, also characterized by the accumulation of lipofuscin and mitochondria-derived lysosomes appear to be a major component and source for these substrates. In addition, glial cells also show the intracellular accumulation of different sized amyloid deposits, and they are accompanied by the presence of giant-sized lipid-laden vacuoles and mitochondria-derived lysosomes [14, 65, 84, 93, 95].

Quantitative morphometric measurements of the percentage of the different types of mitochondria (normal, partially damaged and completely damaged) indicate that aged-matched control groups have a significantly higher percentage of normal mitochondria, compared to completely damaged mitochondria from AD cases [14, 65, 84, 93, 95]. No significant differences between partially damaged mitochondria are seen in both groups, indicating that aging induces damage to mitochondria. However, the main differences between the aged-matched controls and AD cases appear to be significant differences in the percentage of the normal and completely damaged mitochondria.

Cytological in situ hybridization studies using human wild-type and 5kb deleted mtDNA probes reveal that mtDNA signals associate with severely damaged or mitochondria-derived lysosomal structures [65, 93]. However, the areas containing lipofuscin show no mtDNA containing positive signals [65, 93]. Clusters of 5kb deleted mtDNA containing gold particles (17 nm) are commonly found in mitochondria-derived lysosomal structures [14, 65, 93], indicating that mtDNA deletions and turnover occur at late stages of mitochondrial lesion formation. Similarly, 5kb deleted mtDNA positive signals are not present in lipofuscin-containing areas of the neuronal cell body [65, 93]. In contrast to this observation, aged-matched control, hippocampal neuronal mitochondria did not show mtDNA positive immunoreactivity nor contain gold particles in their matrices. Infrequently, a few wild type mtDNA positive signals containing gold particles are seen [65]. Our application of pre-embedding immunocytochemistry methods using antibodies against free lipoic acid demonstrate that the mitochondrial abnormalities in neurons couple with increased markers of lipid peroxidation. Free lipoic acid-containing colloidal gold particles are located in the membrane of the partially and/or completely damaged mitochondria [14]. The external membrane of the damaged, but not normal mitochondria, exhibits clusters of free lipoic acid-containing immunopositive gold particles [14]. However, the matrix of lipofuscin deposits or amyloid like structure is free from lipoic acid-containing gold particles (17 nm). Clusters, of free lipoic acid-containing gold particles bind with the membrane of the granular vacuolar degenerative structure [14]. Increases in the immunoreactivity of lipid peroxidation markers are associated with the RNA oxidation (staining by 8-OHG). The clusters of 8-OHG containing immunopositive gold particles (17 nm) localize in the matrix of completely and/or partially damaged mitochondria [14, 93].

Cofactors for oxidative stress-induced cerebrovascular lesions

Hypoperfusion-induced oxidative stress as a key factor for the development of AD

Hypoperfusion-induced oxidative stress in vascular abnormalities coincides with the pathogenesis of AD (Figure 1-2). Several studies conclude that chronic cerebral hypoperfusion in AD is secondary to oxygen reduction [10, 97-100]. However, recent evidence reveals that a greater fraction of oxygen is removed from the vasculature in AD patients as compared to non-AD controls [15]. This suggests that low vascular blood flow is a prominent feature of the brain during chronic hypoxia/hypoperfusion
and may be a main initiating factor during the development of AD [21, 101]. An impairment of energy metabolism characterizes the AD brain [89]. Positron emission tomography (PET) reveals a decline in the cerebral metabolic rate of the parietal and temporal lobes during AD [43, 102]. These metabolic defects are present before AD symptoms develop in ApoE ε4 homozygote patients [43, 190]. De la Torre [21] proposes that advanced aging with a comorbid condition, such as a vascular risk factor that further decreases cerebral perfusion, promotes a critically attained threshold of cerebral hypoperfusion (CATCH). With time, CATCH induces brain capillary degeneration and suboptimal delivery of energy substrates to neuronal tissue [21]. Because glucose is the main fuel of brain cells, its impaired delivery, together with a deficient delivery of oxygen, compromise neuronal stability because the substrates for aerobic glycolysis fail to meet brain tissue demand. The outcome of CATCH is a metabolic cascade that involves, among other things, mitochondrial dysfunction, oxidative stress, decreased adenosine triphosphate (ATP) production and increased calcium entry, abnormal protein synthesis, cell ionic pump deficiency, signal transduction defects, and neurotransmission failure. These events contribute to the characteristic progressive cognitive decline of patients with AD, as well as regional anatomic pathology, consisting of synaptic loss, SP, NFT, tissue atrophy, and neurodegeneration. CATCH characterizes the clinical heterogeneous pattern of AD and provides compelling evidence that a multitude of etiopathophysiologic vascular risk factors, in the presence of advanced aging, can lead to AD [21, 103, 192-193]. Therefore, we hypothesize that taken together with vascular EC and SMC atrophy, hypoperfusion is a key factor in the development of AD.

Cerebrovascular lesions observed during ischemia/reperfusion induced oxidative stress

The risk for Alzheimer dementia and stroke are known to increase at comparable rates with age. Recent advances suggest that vascular risk factors linked to cerebrovascular disease and stroke in the elderly significantly increase this risk [23]. Although some vascular lesions such as cerebral amyloid angiopathy, endothelial degeneration, and periventricular white matter lesions are evident in most AD cases, one third will exhibit cerebral infarction. Longitudinal clinical studies suggest that stroke and AD occur in tandem more often than randomly [2]. Strokes often occur in patients with AD and have been linked to the pathogenesis of dementia [23]. Nevertheless, the nature of this relationship remains unexplored. Cerebral ischemia is a possible causal factor for AD. Irrespective of the ultimate pathogenic mechanism, these funding suggest that managing vascular disease is important in the treatment and prevention of AD [9, 10] or mixed dementia [23].

Chronic hypoxia can alter cerebral microvessels ultrastructure, but this effect is heterogeneous and in some cases capillaries can respond to hypoxia independently of the arteriole [104]. Exposure to three weeks of hypobaric hypoxia results in increased capillary density in rat models [105]. Capillary segment elongation plays a role in this increase in the deeper layers of the cerebral cortex [105, 107]. Therefore, prolonged hypoxia results in structural and functional adaptive responses that improve tissue oxygen delivery [106]. Mitochondria of brain capillary EC maintain normal density in hypoxia, but the number of mitochondria in the surrounding neuropil decreases significantly about 30% [107]. Moreover, exposure to hypobaric hypoxia yields an increase in basic fibroblastic growth factor (bFGF) mRNA in brain tissue [108]. During moderate hypobaric hypoxia, increased brain vasculature is associated with increased density of the brain capillary glucose transporter (Glut-1). However, this change is reversible and dependent on hypoxia exposure time [109]. This same pattern has been observed in the microvascular system of the human AD brain [14, 93-95]. Based on these findings, the relationship between oxidative stress markers and extracellular matrix binding ligands in the hypoxic brain during stroke and AD deserves further investigation. In addition, the injury induced by reperfusion after chronic hypoxia is important to note because the oxidative products that accumulate during hypoxia induce more tissue and cellular damage than the hypoxia itself.

Ischemia/reperfusion is a systemic process affecting the whole organ or tissue. Different types of blood cells may contribute to the pathogenesis of ischemia/reperfusion, including platelets, monocytes, neutrophils and others [53]. According to Bednar and coworkers [110] neutrophils might be important contributors to ischemia-induced brain injury whereas the role of platelets is more nebulous. In fact, systemic depletion of neutrophils reduced the volume of cerebral infarct after transient middle cerebral artery occlusion in the rat [111]. EC affected by ischemia during the early stages is completely
reversible and dependent upon reperfusion. Eventually, however, injured tissue passes a “point of no-return” and the damage becomes irreversible [112]. Initially, cells strive to increase their surface area for gas and nutrient exchange by expressing cytoplasmic microvilli [6, 53, 54, 56, 57] or by extending membrane protrusions into the vessel lumen [6, 53, 54, 56, 57, 113]. The appearance of these microvascular changes corresponds to the duration of the ischemia and may be an adaptive EC response to altered hemodynamic conditions [6, 113]. The functional significance of microvilli, microblebs and other morphological changes is not clear, but they may have a role in the production of delayed, post-ischemic hypoperfusion by increasing vascular resistance [6, 113]. The extent of EC injury depends on the duration of ischemia and on the metabolic needs of the affected vascular system. The duration of experimental ischemia or acute anoxia required to cause damage varies for different organs. It takes approximately 10-15 minutes for irreversible damage to occur in brain [113-115]. After long-term ischemia and the following reperfusion, the decreased number of active capillary vessels is proportional to the ultrastructural lesions in ischemic vessels and underlying tissues and cells [6, 53, 54, 56, 57]. Cada and coworkers demonstrate that decreased CBF in aging rats produces deficits in visuospatial behavior after permanent surgical occlusion of both carotid arteries [116]. This deficit is coupled with metabolic abnormalities of the brain as visualized by quantitative COX histochemical mapping [116]. These results suggest that deficits in visuospatial learning are not exclusively the result of hippocampal dysfunction, but may be directly involved with altered oxidative energy metabolism in other integrative visuomotor regions identified in this study. They also suggest that chronic cerebrovascular ischemia in this aged rat model produces neurometabolic and behavioral alterations that may be relevant risk factors for the development of AD [116].

**The potential role of vasoactive substances in the endothelial content during ischemia/reperfusion**

The synthesis and release of vasoactive substances, such as the endothelium-derived vasodilator NO and vasoconstrictor ET-1, regulate EC’s role in controlling vascular tone [96, 117-119, 120] (Figure 1). Aside from vascular tone NO regulates platelet aggregation, leukocyte adhesion, SMC proliferation, synaptic neurotransmission and cytotoxic/cytostatic actions of macrophages [107, 119, 121-127].

This labile molecule may carry out important biological roles both within the cell in which it is synthesized, and by interacting with nearby cells and molecules [128, 129]. Three distinct isoforms of NOS derived from different genes generate NO: nNOS, iNOS, and endothelial NOS (eNOS) [117-119, 130-132]. These isoforms are similar in structure and function [117, 129, 131, 132]. eNOS was first purified and cloned from vascular endothelium, but is found in cardiac myocytes, blood platelets, brain cells [118, 119, 125, 127, 131, 133] and in cellular compartments such as mitochondria [134, 135]. The activity of eNOS is a major determinant of vascular tone and blood pressure. It is altered in diseases such as hypertension, diabetes, atherosclerosis, ischemia/reperfusion [5, 93, 125, 126, 136] and AD [22, 95] (Figure 1).

Excess NO is produced during excitotoxicity, inflammation and ischemia/reperfusion injury [161], and the oxidation products of NO, namely peroxynitrite and peroxynitrate. Also, ONOO− can generate the highly reactive OH• radical, a more powerful oxidant than NO itself [35, 123, 161]. The increased nitrotyrosine immunoreactivity in AD is present in the neuronal cytoplasm of the cerebral cortex within regions of neurodegeneration, yet it is undetectable in corresponding control regions [35]. This distribution is essentially identical to that of free carbonyls [34]. The widespread occurrence of nitrotyrosine immunoreactivity in neurons [35] suggests that chronic oxidative damage is not restricted to long-lived polymers such as NFTs, but instead, reflects a generalized oxidative stress contributing to the pathogenesis of AD.

NOS positive neurons are present in subgroups throughout many regions of the brain [123, 191]. Immunostaining for reduced NADPH-diaphorase, as well as nNOS and eNOS, reveals their presence in dendritic and axonal terminals that closely interact with the middle cerebral artery and cerebral microvessels [122-124, 136]. The presence of L-arginine in astrocytes in vivo suggests that glia may store this chemical for NO production in brain [122, 136, 138]. Moreover, glial cells exhibit an inflammatory response during infection or ischemic disease. They also release pro-inflammatory cytokines and synthesize and release NO [138]. The large amount of NO that is released from glial cells via the expression of iNOS
after their stimulation is neurotoxic, because it induces oxidative stress, mitochondrial dysfunction and excitotoxicity [122, 127, 162,191,194]. Hypoxic brain injury (acute or chronic) is associated with the formation of both NO [123, 138, 159, 163-165] and the superoxide anion, which may react to form free radicals [35, 127] and cause neurotoxicity [122-124, 127, 159, 163, 166, 167, 170,191]. Further investigations into determining the exact ultrastructural localization of the different NOS isoforms in the brain vascular tree, neurons and glia in post-hypoxic and AD brain are warranted [191].

**eNOS involvement in the cerebrovascular tone**

A dynamic balance of relaxing and constricting factors regulates cerebrovascular tone (Figure 1). Constitutively produced NO normally influences basal cerebral vascular tone, and mediates vascular responses to diverse stimuli [137] and cerebral vasodilation [138]. Vasorelaxation of brain microvessels is a feature of some diseases including chronic hypertension, diabetes, hypercholesterolemia, sub-arachnoid hemorrhage (SAH), and ischemia [5, 137, 138]. NO is also involved in regulating the cerebral circulation during hypercapnia [139, 140] and focal [136, 141-143] or global brain ischemia [140, 144-148]. Furthermore, arginine-derived NO mediates the powerful effects of CO₂ on cerebral circulation. NO synthesized by the action of nNOS participates in regulating basal CBF and is the major contributor to the hypercapnic CBF response [149]. Chronic inhibition of constitutive NO production increases EC permeability during various vascular diseases [9, 93, 115, 118, 119, 131,191]. Due to its vascular effect, NO might improve tissue perfusion and exert a protective action. Moreover, overproduction, either by activation of nNOS by excitatory amino acids [150], or by induction of iNOS in glial, vascular, or blood cells [145-147] during the ischemic episodes, might be deleterious. Mice with eNOS gene knock-out exhibit a decrease in vascular relaxation. Thus, NO synthesized by eNOS protects against ischemic damage by increasing CBF, whereas NO produced by nNOS contributes to lesions [151, 152]. The inhibition of NO synthesis by EC leads to increased intracellular oxidative stress, which induces neutrophil-EC interactions [6, 55] and may promote the development and progression of vascular diseases such as atherosclerosis [5, 126] and ischemia/reperfusion injury [6, 53, 54, 137, 138, 153].

**nNOS expression and regulation**

Modification of nNOS expression in the entorhinal cortex and hippocampus occurs during AD [154]. Tissues containing the constitutive forms of NOS, like brain, kidney, and endothelium express dimethylargininase [155-157,191]. It regulates NO production by hydrolyzing free methylated arginine derivatives (effective endogenous inhibitors of NOS) [158]. The expression of dimethylargininase dramatically increases during AD [38]. Dimethylargininase abnormalities in the AD are the result of elevated levels of nitration from effective oxidants peroxynitrite or peroxynitrate [35, 159, 160]. However, the ultrastructural localization of dimethylargininase immunoreactivity in different cellular compartments of the AD brain or in Tg animal models of AD has yet to be described [191].

**iNOS as a mediator of oxidation during AD**

A variety of cells express iNOS in response to lipopolysaccharides, certain cytokines and ROS generators [9, 117, 119, 122-125, 133, 136, 138,191]. iNOS may be an important mediator of cytotoxicity in the brain because it produces much greater amounts of NO than either eNOS or nNOS [124, 128,191]. Thorns and collaborators suggest that iNOS plays a role in the formation of NFT [154].

Iadecola and coworkers propose that iNOS contributes to ischemic brain damage [146]. The catalytic activities of iNOS enzymes or mRNA expression are evident in brain tissue after 2 hours of transient focal ischemia or 1-2 days after permanent focal ischemia [145, 147].

**ET-1 role as a vasoconstrictor in AD brains**

ET-1 appears to be a vasodilator at physiologically relevant concentrations, and a potent vasoconstrictor in several pathologies associated with a rise in ET-1 plasma and tissue levels [120, 168-171]. Several immunoreactivity studies identify augmented ET-1 levels in human atherosclerotic vessel wall cells [5, 169-172], post-ischemic vascular lesions [168], aged rats [173], and during other diseases such as metastatic adenocarcinoma of the prostate [174] and human colorectal liver metastases [175, 176]. Thoracic EC from human and animal models of atherosclerotic vessels and express increased ET-1 production [5, 94, 169, 171, 172, 177]. This increase correlates inversely with the depression of eNOS.
immunoreactivity [5, 94, 173, 175, 178]. An imbalance between endothelium-derived vasorelaxant and vasoconstrictor substances may play a key role in the development of chronic brain hypoxia and in the adaptive response of the brain to oxidative stress in ischemia and AD.

Transgenic animal models as powerful tools to understand Alzheimer Pathobiology

Transgenic animals as models of study for cerebrovascular and neuronal lesions in AD

Developing an animal model is crucial for investigating the molecular and cellular etiology of AD [67-69, 93, 179-182, 190-192]. There are a number of Tg animals that overexpress normal AβPP or AβPP with familial AD (FAD) mutations or fragments of AβPP [67-69, 179-182]. Heterogeneous genetic and environmental factors are the causes of clinical and neuropathological phenotypes of AD [93]. Indeed, several identified genes appear to responsible for most familial forms of the disease. Conversely, the ε4 allele of ApoE is a significant risk factor for late onset forms of AD [10, 14, 67-69, 103, 179-182, 190]. Impairment of spatial memory in mice overexpressing wild type AβPP751 or wild type AβPP 695 and the neuropathology in mice expressing Aβ1-42 are documented [53]. Expression of AβPP in FAD mutant mice results in deposition of Aβ, while mice expressing the carboxyl terminus 100 or 104 (C100 or C104) amino acids of AβPP demonstrate both neurodegeneration and specific impairment of spatial memory [183].

Calhoun and coworkers [184] link the formation of amyloid plaques leading to region-specific loss of neurons in a Tg mouse. In addition, mice overexpressing the human mutant amyloid precursor protein (hAβPP) show learning deficits, but the apparent lack of a relationship between these deficits and the progressive Aβ plaque formation that the hAβPP mice display is puzzling [18, 185]. Using a new water-maze training protocol, that PDAPP mice also exhibit a separate age-related deficit in learning a series of spatial locations [185].

This learning impairment correlates with Aβ plaque burden and is evident in both cross-sectional and longitudinal experimental designs. These findings indicate that Aβ overexpression and/or Aβ plaques parallel with the disturbed cognitive function. They also suggest that some but not all forms of learning and memory are suitable behavioral assays of the progressive cognitive deficits associated with AD-type pathologies [185]. Later studies demonstrate that AβPP expression also occurs in different pathological conditions such as after the global ischemia, even without the presence of a genetic abnormality [186]. This finding indicates a central and crucial role of the chronic injury stimuli (e.g. ischemia, hypoxia, virus, toxins, etc.) in the pathogenesis of AD [93].

Cerebral amyloid (CA), thought to be produced in the lysosomes of EC [182], was first proposed as the cause of BBB breakdown, allowing neurotoxic serum proteins access to neuronal cells and beginning the cascade of neurodegeneration. We demonstrate that the C57B6/SJL Tg mouse model, which overexpresses AβPP [187] with FAD mutations contains an Aβ deposition patterns similar to those seen in cases of AD. In addition, the C57B6/SJL Tg mouse possesses a beta fibroblast growth factor (bFGF) binding pattern similar to that seen in AD. When tissues from these mice are placed through immunohistological assays, the cores of amyloid plaques show intense staining for the antibody against Aβ (4G8). Additionally, bFGF binding was greatly diminished by heparinase pretreatment [95, 188].

We further report that mitochondrial abnormalities such as electron dense (ED) mitochondria, mitochondrial-derived lysosomes, and lipofuscin appear to be features of damaged neurons in aged C57B6/SJL Tg (+) mice [93]. This indicates that the vascular abnormalities correspond with the selective damage of cortical neurons, raising questions about the relationships between vascular abnormalities, BBB breakdown, neuronal loss and amyloid deposition during the maturation of AD-like pathology in these Tg mice [14, 93]. However, no serum amyloid protein (SAP) immunoreactivity is found in the Tg mouse brain [188]. Since only peripheral organs synthesize SAP, its presence in the AD brain suggests impairment of the BBB [60]. These results suggest that the pathogenesis of BBB impairment in this mouse model differ from that in AD [188].
Recently, a yeast artificial chromosome (YAC) Tg mouse model that overexpress Aβ was developed [60, 67-69, 179-181]. The YACs contain the entire ~400kbp human gene encoding AβPP. The gene harbors either 1) the asparagine for lysine and leucine for methionine FAD substitution at codons 670 and 671 (APP\textsubscript{K670N/M671L}), 2) the isoleucine for valine FAD substitution at codon 717 (APP\textsubscript{V717I}), or 3) a combination of both substitutions [60, 67-69, 179-181]. Lowered levels of α-secretase-generated soluble AβPP derivatives are observed in these mice [67-69, 179, 180]. Moreover, there are elevated levels of extended Aβ peptides (species terminating at amino acids 42/43) in YAC Tg mice that express human AβPP\textsubscript{V717I}, suggesting that these mice should be appropriate for detailed analysis on the \textit{in vivo} effects of AβPP metabolism and Aβ production. Therefore, since YAC Tg approaches avoid the problems regarding regional and temporal specificity of molecular pathogenesis, they may provide unique insights into the mechanisms behind the progression of AD in humans [14, 93].

The changes of the vascular wall in YAC AβPP mice, but not age-matched controls, reveal a different degree of amyloid deposition present in vascular wall cells [14, 93]. These vessels also exhibit immunopositive staining for amyloid APP and are characterized by the presence of a large number of lipid-laden vacuoles in the matrix of endothelial and perivascular cells [14, 93, 95]. Neuronal cell bodies of parietal cortical neurons from aged YAC AβPP mice include clusters of APP-containing immunopositive gold particles [93, 95]. Aβ deposits around the microvessels are commonly present within the ultrastructural abnormalities in vascular wall cells and neurons [14, 93, 95]. This data indicates that disruption of BBB function in vascular EC may be a major factor in lipid accumulation and amyloid deposition during the development of AD-like pathology in YAC AβPP mice [14, 93].

Furthermore, damage to the vascular endothelium (induced by chronic hypoperfusion) acts as a primary key factor for oxidative stress and contributes to potential neuronal lesions. Non-reversible changes in neurons that induce AβPP overexpression and Aβ depositions are also consequences and permanent features of AD [14, 93, 95, 172]. These findings raise questions regarding the direct relationship between vascular abnormalities, BBB breakdown, neuronal loss, mitochondrial lesions and Aβ deposition during the maturation of AD-like pathology [14, 95, 172]. Therefore, cellular and subcellular investigations into both the mechanisms behind Aβ deposition development and the possible accelerating effects of environmental factors such as chronic hypoxia/reperfusion may open the door to new pharmacological treatments of AD.

\section*{Transgenic mouse model as a tool for comparing the mechanisms involved in AD pathology}

Recently we applied the C57B6/SJL Tg mouse model overexpressing Aβ to assess the binding of bFGF and SAP to Aβ as a measure of BBB integrity. Adjacent sections of brain were stained with 4G8, a monoclonal antibody to amyloid, with bFGF binding followed by 48.1, a monoclonal antibody against bFGF. The binding of bFGF in this model is similar to that of AD cases in which bFGF binds specifically to Aβ neuritic plaques and the basement membrane (BM) of cerebral microvessels [188]. In addition, the cores of amyloid plaques are intensely stained with the 4G8, and bFGF binding is co-localized with amyloid immunoreactivity as visualized by polyclonal antiserum to amyloid.

Our ultrastructural study indicates that bFGF immunostaining in aged C57B6/SJL mice appear to be that of immunopositive peroxidase-anti-peroxidase (PAP) products or gold particles that bind with damaged, but not normal neurons [14, 93]. Moreover, this corresponds with the different degree of Aβ immunostaining in the neuronal cell body and vascular wall cells [14]. In addition, we have found the degree of mitochondrial abnormality, such as electron dense (ED) mitochondria-derived lysosomes and lipofuscin. Together, they appear to be features of damaged neurons in aged (24 m old) C57B6/SJL TG(+) but not age-matched control mice. Consequently the selective damage of cortical neurons manifests the degree of vascular abnormality. The direct relationships between vascular abnormality, BBB breakdown, neuronal loss and amyloid depositions during the maturation of AD like pathology remain fully elucidated [14, 93].

The ultrastructural features of vascular lesions and mitochondrial changes in neuronal cell bodies in aged YAC AβPP and non-Tg age-matched control mice are analyzed following perfusion fixation [93]. EM immunocytochemistry using monoclonal antibody reveals different sizes of fibrils and extracellular
types of amyloid deposits of brain tissues in YAC AβPP Tg mice [14, 93]. In addition, the amyloid depositions support the formation of parietal helical filamental (PHF) structures, which is a permanent feature of neuronal lesions in AD brains [93]. These vessels also manifest immunopositive staining for APP and the presence of a large size of lipid-laden vacuoles in the matrix of EC and perivascular cells [14]. Moreover, these changes reflect those similar to cortical microvessels in AD [14]. The ultrastructural abnormalities of vascular wall cells depend on the presence of Aβ deposits around the microvessels [14]. In contrary to these observations, age-matched control mice brain vessels did not show any particular changes in the ultrastructure of vascular EC at different levels of microcirculation. Only infrequently a minute amount of lipid droplets appear in the matrix of perivascular cells [14]. These data clearly indicate that disruption of BBB function in vascular EC may be major factor in lipid accumulation and amyloid deposition during the development of AD-like pathology in YAC AβPP Tg (+) mice without cholesterol feeding [14, 95].

Different degrees of ultrastructural alterations in mitochondrial structures characterize the similar cortical neuronal cell bodies in YAC AβPP mice as AD [65, 93]. Giant and ED mitochondria are permanent features of the neuronal abnormality as a result of APP overexpression [93]. We also observe the similarity in AD brains during different stages of mitochondrial lesions. The cytoskeleton appears to correlate with the absence of microtubule and lipofuscin formation [189]. Age-matched control mice did not show any particular changes in their neuronal ultrastructure [93]. In situ hybridization analysis with mouse and human mtDNA probes detect abundant deleted mtDNA in YAC AβPP compared to age-matched controls [14, 93]. Moreover, the majority of mtDNA deletion localizes in mitochondria-derived lysosomes. These regions correspond to lipofuscin deposits, thereby suggesting that proliferation, deletion and duplication of mtDNA occurs in mitochondria. Many of these mitochondria fuse with lysosomes, in YAC AβPP mice [93].

In addition, neurons in samples from AD are dominated by abnormal mitochondria as compared to the control group [84]. By in situ hybridization analyses, with a chimeric cDNA probe to the 5kb common deletion [84], deleted mtDNA increases at least 3 fold for the AD cases as compared to the controls. In quantitative analysis of the mtDNA deletion and 8OHG in the same cases, a strong positive correlation (r = 0.934; Fig. 10) is determined. Ultrastructural localization of mtDNA using in situ hybridization with colloidal gold manifests the location of deleted mtDNA within abnormal mitochondria,

(those lacking cristae, swollen and in many cases fused with lipofuscin) [84]. These findings suggest that the mtDNA in situ hybridization detected mtDNA proliferation, deletion and duplication in abnormal mitochondria, many of which fused with lysosomes, thus indicating turn over of such mitochondria.

The detailed analysis of 8-OHG immunostaining demonstrates that only vulnerable neurons show immunopositive staining for 8-OHG in AD, but not in age-matched controls. By using ultrastructural analysis we discover that 8-OHG immunostaining is selectively present in vulnerable neurons and microvessels of AD brain [14, 84]. The 8-OHG immunogold labeling (17 nm) is seen throughout the cytoplasm, including the damaged mitochondria or electron dense abnormal mitochondria [14, 84].

However, we did not find 8-OHG in normal mitochondria or in lipofuscin (see Fig. 9). The capillary EC and perivascular pericytes show the high intensity of 8OHG immunostaining [14]. We speculate that the oxidative stress markers seen in the AD brain selectively affect the population of vulnerable neurons, vascular EC and perivascular cells. These observations suggest that hypoperfusion-induced oxidative stress plays a key role in the pathogenesis of vascular and non-vascular cell lesions during the maturation of AD.

Detailed immunocytochemical analyses using colloidal gold probes indicate that the vascular wall in YAC AβPP Tg mice possesses atherosclerotic lesions, while control and non-damaged vessels from YAC AβPP mice do not show APP immunopositivity [14]. Very often the clusters of APP positive immunoreactivity were observed in the neuronal cell bodies of parietal cortical neurons from aged YAC AβPP Tg mice [14, 93]. In situ hybridization using wild and deleted mtDNA probes (human and mouse specific) reveal mtDNA containing gold particles in YAC AβPP, but not in control mouse hippocampus [93]. The
main source of the mtDNA probes is located in damaged mitochondria and mitochondria-derived lysosomes, but not in lipofuscin. We have found that wild and chimeric mtDNA were also detectable in YAC AβPP Tg (+) mouse microvessels but not in control, age-matched brain tissue [14]. In addition, vessels with atherosclerotic lesions show that endothelium and perivascular cells contain clusters of wild and deleted mtDNA containing positive signals [14]. These observations suggest that the key role of hypoperfusion, mitochondrial abnormality and oxidative stress in the pathogenesis of vascular and non-vascular cells lesions during the development of AD-like pathology in YAC AβPP mice [14] and at the many point overlap with the neuropathology of human AD.

Conclusion

We believe that the evidence garnered from this review will focus our future direction in investigation and will provide us with a clearer understanding of the relationship between a number of age-related disorders including atherosclerosis, ischemia/reperfusion, stroke, and neurodegenerative diseases such as AD. Furthermore, we indicate that chronic vascular hypoperfusion is a part of their common underlying mechanisms, as it appears to be a central initiating factor for vascular abnormality, mitochondrial damage and an elucidator of the imbalance in the activity of NOS isoforms, ET-1, oxidative stress markers, mtDNA and mitochondrial enzymes in the vascular wall and in brain parenchymal cells, where it is predominant in CVA and AD. We hypothesize that an imbalance between NOSs and ET-1, along with antioxidant system deficiencies, are a predominant feature in the brains of stroke and AD patients (Figure 2). Elevated chronic hypoperfusion and physical distortion of the tissue are likely to contribute to the collapse of post-ischemic/hypoxic or AD vessels. The sustained hypoperfusion and oxidative stress of brain tissues could also stimulate the expression of NOSs and ET-1 in brain cells and probably increase the accumulation of oxidative stress products, therefore contributing to BBB breakdown and brain parenchymal cell damage. Therefore, determining the mechanisms behind these imbalances may provide crucial information in the development of new, more effective therapies for stroke and AD patients in the near future. Future studies must seek to answer the following questions:

1) What are the major factors altering and/or controlling CBF during accumulation of chronic hypoperfusion and/or the development of atherosclerotic changes in brain microvessels?

2) What are the roles of vasoactive substances (namely NO and ET-1) during the development of these changes?

3) Does chronic hypoperfusion with concomitant oxidative stress accelerate the vascular and neuronal lesions (including the mtDNA deletions) during normal aging and/or when the brain is exposed to chronic hypoxia? Resolving these issues will allow for novel therapeutic approaches that will modify the natural history of these chronic age onset disorders.

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