Oxidative Stress-induced Mitochondrial DNA Overproliferation and Deletion, Cellular Hypoperfusion and Brain Hypometabolism In the Context of Cerebrovascular and Alzheimer Disease: Offer New and Successful Targets for the Drug Delivering and Treatment

Nadmierny rozrost i zanik mitochondrialnego DNA spowodowane stresem tlenowym, zmniejszeniem perfuzji i metabolizmu tkanki mózgowej w chorobie naczyniowej mózgu i Alzheimera: nowa oferta skutecznej terapii.

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Scientific problem

Atherosclerosis and stroke (including cerebrovascular athero- and arteriosclerosis with neurological consequence such as Alzheimer disease (AD)) are two leading causes of age-associated disability, dementia and death. Four million people suffer from AD in the United States and this figure will balloon to 16 million persons affected, mostly the elderly, by the year 2040. Conventional wisdom for the last 20 years has decreed that AD is a ‘neurodegenerative’ disorder caused primarily by abnormal deposition in brain tissue protein called ‘amyloid-beta’). Neurodegenerative disorders are characterized by loss of cognitive function and inappropriate death of nerve cells in areas of the brain that control such functions as memory and language. The trigger for nerve cell death is unknown in the case of AD.

A rapidly growing body of evidence indicates that increased oxidative stress resulting from reactive oxygen radicals is associated with the aging process and age-related degenerative disorders such as atherosclerosis, ischemia/reperfusion, arthritis, stroke, and neurodegenerative diseases. Reactive oxygen species (ROS) are generated at sites of inflammation and injury, and at low levels they can function as signaling intermediates in the regulation of fundamental cell activities such as growth and adaptation responses. At higher concentrations, ROS can cause cell injury and death. The vascular endothelium, which regulates the passage of macromolecules and circulating blood to cells and tissue, is a major target of oxidative stress, playing a critical role in the pathophysiology of vascular diseases. Since the vascular endothelium, neurons and glia are all able to synthesize, store and release ROS and vascular active substances in response to certain stimuli, their contribution to the pathophysiology of atherosclerosis, stroke, other non-atherosclerotic cerebrovascular disease and neurodegenerative syndrome such as mild cognitive impairment (MCI) and AD is extremely important. In addition, abnormalities in cholesterol metabolism, oxidative stress and vascular lesions are important factors in the pathogenesis of late-onset forms of AD, forms of mental retardation, stroke and MCI. This idea is based on the positive correlations found between stroke, MCI, AD and cardiovascular diseases. New evidence indicates that continuous formation of free ROS induces cellular damage and decreases antioxidant defenses. Specifically, oxidative stress increases vascular endothelial permeability and promotes leukocyte adhesion, all of which are coupled with alterations in endothelial signal transduction and redox-regulated transcription factors. We theorize that the cellular and molecular mechanisms, by which cholesterol metabolism abnormalities induce the formation of large amounts of ROS, decrease endothelial barrier function via the overexpression of inducible nitric oxide synthase (iNOS) and promote leukocyte adhesion. Chronic injury stimuli has the action of inducing decompensation and or alterations in normal vascular function, which results in the development of cerebrovascular arterio- and atherosclerosis that further manifest as stroke, MCI and/or AD.

Current theory and our proposal

The finding of amyloid-beta (Aβ) deposition in AD brains after death led to the so-called “amyloid hypothesis”. For over a decade, the amyloid hypothesis has so influenced and guided research in the field of Alzheimer dementia that many workers consider it as the gold standard of scientific investigation. We have extensively reviewed the literature which claims that AD is caused by the deposition of Aβ within structures called senile plaques that invade AD brains and that such plaque formation then leads to further abnormalities within the nerve cells, eventually killing them. We have found little evidence to support this claim and ample to question it. For example, the amyloid hypothesis has been criticized because its research findings up to now have not generated any benefits in the clinical management and treatment of AD patients nor to an understanding of how the elderly are preferentially affected. The three main flaws of the amyloid hypothesis appear to be that: 1) Aβ deposition has not been found to be toxic or cause damage and death of nerve cells in human or animal brain; 2) the brains of many cognitively normal aged individuals show abundant Aβ containing senile plaques but no clinical signs of Alzheimer disease, 3) and since there is general agreement that senile plaques containing Aβ are the products of degenerating neurons, they can not be the cause, since it is axiomatic that a product is the result not the cause of some activity. By contrast, there is now considerable and still growing evidence from the fields of epidemiology, pharmacology, neuroimaging, clinical medicine, microscopic anatomy, and molecular biology which indicate that non-genetic AD is a vascular disorder whose underlying cause
is impaired blood flow to the brain during advanced aging. This evidence can be summarized as follows: (1) numerous epidemiologic studies link AD risk factors such as stroke, heart disease, hypertension and atherosclerosis to reduced cerebral blood flow; (2) evidence that AD and vascular dementia (VaD), an acknowledged vascular disorder, share practically all the same risk factors and may benefit from the same treatments; (3) drug therapy reported to improve AD symptoms (including prescriptive drugs now available for AD) all increase blood flow to the brain; (4) people who are likely to develop AD but do not yet show dementia symptoms can be identified by using brain blood flow measurements and brain PET scans; (5) the clinical symptoms are very similar in most AD and VaD patients; (6) parallel abnormalities occur in brain vessels and brain tissue including Aβ laden plaques in AD and VaD patients; (7) low levels of brain blood flow in aged humans and animals can lead to abnormal cell metabolism, tissue damage and memory problems independent of Aβ; (8) mild cognitive impairment (a term used to describe a preliminary stage leading to AD) can convert equally to AD or VaD; (9) and small vessel damage is present in the majority of AD brains after death.

For these reasons, it is suggested that AD be re-classified as an oxidative stress induced vascular disorder and described as a "vasocognopathy". The term aptly describes the origin of the disease (vaso: vessel blood flow), its primary effect on a system (-cogn: relating to mental ability) and its clinical course (-pathy: disorder). Re-classification of AD from a neurodegenerative to a cerebrovascular disorder would speed the development of truly beneficial treatments or a cure, improve patient management, provide earlier diagnosis, and reduce the number of AD cases in the future by aggressively treating the risk factors that can turn on this dementia. In conclusion, a bare-bones examination of the literature reveals no compelling evidence that Aβ deposition causes AD or that it results in significant damage to brain cells. By contrast, the findings that support AD as a primary oxidative stress induced cerebral metabolic misbalanced vascular disorder appear substantially more convincing. Determining the mechanisms behind these imbalances in experimental animals will provide crucial information in the development of new, more effective therapies for the treatment of atherosclerosis, including cerebrovascular athero- and arteriosclerosis. Cerebrovascular pathology found in mild cognitive impairment (MCI) and AD with the consequence of the mental deterioration and progressive neurodegeneration deserves special attention. Therefore, pharmacological intervention aimed at correcting chronic oxidative stress induced energy crisis that initiate brain hypoperfusion will also be useful for treating and preventing dementing neurodegeneration.

Background

It is widely accepted that during neuronal energy crisis, cerebral hypometabolism and vascular hypoperfusion are major and potentially treatable contributors to the loss of function in patients with stroke as well as Alzheimer disease (AD). Based on our previous and current research, we have found that the mitochondria of the brain cellular compartments (neurons, glia, microglia and vessels wall cells) are a primary target of brain damage due to their high energy demand and susceptibility to oxidation, leading to energy failure, and resulting in cognitive impairment and memory decline. Moreover, mounting evidence indicates that targeting mitochondria with antioxidants and metabolites is a powerful treatment capable of restoring cell integrity and eliminating damage in the brain, resulting in significantly restored cognitive function and spatial memory. In addition, we have recently found an unexpected capacity for gene expression modification supplement (Aminocare A10) to retard and slow down the general aging process via gene expression modification and aid in the production of energy in the process. To achieve the goal of preventing brain dysfunction, we hypothesize that selective mitochondrial antioxidants (Acetyl-L-Carnitine and R-α-Lipoic acid (ALCAR+LA), supplement that is able to retard and slow down the general aging process via gene expression modification (Aminocare- A10) plus brain longevity substances [Aminocare Brain Longevity Forte (BLF)- as a supplement for brain aging that slow down of AD and cognitive decline] can be used as a new and more effective therapeutic approach in the treatment of stroke and AD patients.
Goal

We have determined the cellular and subcellular features of vascular lesions and mitochondria in brain vascular wall cells as well as neurons from human AD brain biopsies, human short postmortem brain tissues, rat model of 2 vessel occlusion (2-VO), yeast artificial chromosome (YAC), and C57B6/SJL transgenic positive (Tg+) mice overexpressing amyloid beta precursor protein (AβPP). We expand our models towards the E4 isoform of apolipoprotein E (ApoE) which is involved in cardiovascular and cerebrovascular disorders and is the most prevalent risk factor for late onset of sporadic AD. Moreover, ApoE4 transgenic (Tg+) mice are appropriate models for studying the pathogenesis and preclinical treatment of ApoE-related cognitive deficits associated with late onset and sporadic AD. We have also determined the potential therapeutic effects of using a combination of a selective mitochondrial antioxidant plus A10 and BLF both in combination with our recently developed brain exercise program in patients with stroke and AD (moderate and severe AD symptoms).

Methods

In situ hybridization, using mitochondrial DNA (mtDNA) probes for human wild type, 5kb deleted and mouse mtDNA, was performed in conjunction with immunocytochemistry using antibodies against AβPP, 8-hydroxyguanosine, all three isoforms of nitric oxide synthase (neuronal, inducible and endothelial NOS), GRK-2 and cytochrome c oxidase. We have also measured age-dependent effects of the human ApoE4 on cerebral blood flow (CBF) using ApoE4 transgenic mice compared to age-matched wild-type (WT) mice by use of [14C] iodoantipyrine autoradiography. Spatial memory and temporal memory tests were also employed to determine the potential protective effects of ALCAR+LA as a selective mitochondrial antioxidants treatment. Our animal study applies the vascular dementia paradigm to ApoE4 Tg+ mice in order to analyze the effects of the selective mitochondrial antioxidants ALCAR+LA on cerebral blood flow (CBF), neuropathology, brain and vessel ultrastructural abnormalities and behavior. Moreover, these studies can apply to the brain hypometabolism and mitochondrial failure paradigm to stroke and AD patients in order to analyze cognitive function in patients who receive ALCAR+LA, Omega-3-6-9 Fish, Flax, Borage oil as well as Coenzyme Q-10 and A10+BLF, along with diet changes in combination with our recently developed brain activation program (a home-based protocol involving mild physical exercise and cognitive training). The average age of the patients was 72. The patients were evaluated at baseline and in one year post treatment.

Results

A significant higher degree of mitochondrial damage was found in neurons and cerebrovascular cell walls in AD and in animal models used when compared to age-matched controls and non-treated subjects. These abnormalities coexist with the over expression of GRK-2, AβPP and inducible NOS immunoreactivity in these cells, and closely related to amyloid deposition in the same regions. They were also characterized by the presence of large, lipid-laden vacuoles in the cell body of severely damaged neurons and cytoplasmic matrix of the vascular endothelium. In situ hybridization revealed deleted mtDNA positive signals in the damaged mitochondria of neurons, vascular endothelium and perivascular cells. Moreover, brain microvessels with atherosclerotic lesions revealed endothelium and perivascular cells, which stained positively and in clusters when probed with wild and deleted mtDNA probes. These mtDNA deletions were associated with increased amounts of immunoreactive GRK-2, AβPP, 8OHG, and COX in the same cellular and subcellular compartments. Moreover, GRK overexpression appeared to be a selective hallmark for mitochondrial damage at the earlier but not late stages of neuronal and other brain cellular compartment lesions. ApoE4 associated factors reduced the CBF gradually and created brain hypoperfusion when compared to the WT and the differences in CBF were greatest as animals aged from 6 weeks to 12 months. Transmission electron microscopy (TEM) with colloidal gold immunocytochemistry and in situ hybridization using human and mouse DNA probes showed structural damage and mitochondrial DNA overproliferation and/or deletion in the young and aged microvessels endothelium of ApoE4 animals, extending to the cytoplasm of perivascular cells, perivascular nerve terminals, hippocampal neurons, and glial cells. These blood flow changes associates with severe structural lesions in young and aged microvessels endothelium of ApoE4 animals extended to the cytoplasmic matrix of...
perivascular cells, perivascular nerve terminals and hippocampal neurons and glial cells in the damaged regions of the brain. Moreover, mitochondrial structural alterations coexist with mitochondrial DNA overproliferation and/or deletion in all brain cellular compartments. Most likely, further development of these alterations can lead to blood brain barrier (BBB) failure and breakage during the development of AD. In contrary to this observation, the animals that received selective mitochondrial antioxidants (ALCAR+LA) treatment showed an absence of any cellular or subcellular abnormality in brain cellular compartments. Spatial and temporal memory tests showed a trend in improving cognitive function in ApoE4 Tg+ mice that were fed with the selective mitochondrial antioxidants (ALCAR+LA).

Our clinical results showed that patients who received ALCAR+LA, Omega-3-6-9 Fish, Flax, Borage oil as well as Coenzyme Q-10 and A10+BLF, along with diet changes in combination with our recently developed brain activation program exhibited the maximum cognitive improvement at the end of 12 months of treatment. The maximum cognitive improvement was seen with the combined treatment in MMSE, attention, memory, naming, construction, clock drawing, verbal fluency, and Ruff Frontal Fluency tests. By the end of 12 months of treatment, significant improvement was observed, especially in attention, construction and clock drawing, when patients received the combined treatment. In addition, this group also showed that in all categories there were no signs of a decrease and/or decline below the base line for the entire period of treatment.

Conclusion

Our conclusion is that for the first time we were able to demonstrate the potential pharmacologic modulation of brain hypometabolism and therefore the cognitive improvements by using combination of selective mitochondrial antioxidants/metabolites, a gene expression modification substance (A10) and supplement for brain aging (BLF) with diet changes and brain exercise training. This represents a completely new and more effective strategy to treat stroke, Alzheimer and/or other types of dementia. Moreover, further increase in the examination of the ultrastructural degeneration caused by aging, especially under cardio- and cerebrovascular disease complications, is likely to contribute to our understanding of neurodegenerative etiology and will indicate a new avenue for the development of novel prophylactic and treatment strategies by offering selective mitochondrial antioxidants like ALCAR+LA and gene expression modification substrate (A10) and brain aging supplement (BLF) to the stroke, AD and/or other demented patients.
Learning objective

**Project 1:**

1. Determine the effect of aging in animals genetically lacking LDL receptor and ApoE gene as a model of CBH on regional cerebral blood flow (rCBF), and functional state of the hippocampal endothelium and neighboring neuronal tissue.

2. Investigate the effect of additional cholesterol supplementation (1, 2, and 3 months after feeding) and chronic vessel occlusion (2 vessel occlusion, 2-VO) in an animal with genetic dyslipidaemia or ApoE gene lacking on rCBF, amyloid production and activities of NOS isoforms and EC markers.

3. Evaluate the effect of selective pharmacological inhibition of iNOS, nNOS, and eNOS after cholesterol supplementation with additional 2-vessel occlusion at 1, 2, and 3 months before and after the administration of the hydroxymethylglutaryl coenzyme A reductase inhibitors atorvastatin and simvastatin, of the NOS donor S-nitroso-N-acetylpenicillamine (SNAP), the angiotensin II type 1-receptor blocker candesartan, A10 and BLF on hippocampal Aβ accumulation and on oxidative stress markers and rCBF.

**Project 2:**

1. To determine how nicotine exposure and hypoxia/reperfusion independently affect CBF and Spatial Memory in ApoE transgenic (Tg+) mice before any amyloid deposition and/or any pathology is accountable.

2. Investigate the cellular, subcellular and biochemical pattern of the changes in NOS, ET-1, and nAChR activity in the brain of the mouse overexpressing ApoE after the nicotine or hypoxia/reperfusion exposure.

3. Determine the biochemical, cellular, and subcellular features of the pattern on the oxidative stress markers, RNA oxidation, mitochondrial abnormalities (mtDNA overproliferation and/or deletion, mitochondrial enzyme activity, and structural changes) and nAChR receptors activity in ApoE Tg+ mice brain after nicotine treatment or hypoxia/reperfusion exposure.

4. Determine the functional, biochemical, and structural features of the changes in NOS, ET-1, nAChR activity, mitochondrial pathology, and oxidative stress markers when ApoE Tg+ mice are exposed to chronic nicotine treatment or hypoxia/reperfusion with additional drug treatment (A10+BLF).

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