The Effects of Different Antioxidants on the Activity of Cerebrocortical MnSOD and Na,K-ATPase from post mortem Alzheimer’s Disease and Age-matched Normal Brains

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Abstract: Among the markers and targets of the early phase of Alzheimer’s disease (AD) pathogenesis MnSOD (mitochondrial dysfunction) and Na-pump (disturbances in function/regulation) are often highlighted. This paper focused on comparison of the effects of three antioxidants on the activity of cerebrocortical MnSOD and Na,K-ATPase from post mortem Alzheimer’s disease and age-matched normal brains. Antioxidant compounds with different origins: natural glutathione, synthetic UPF peptides (glutathione analogues) and phytoestrogen genistein were investigated. Firstly, MnSOD and Na,K-ATPase activities were found to be decreased in the post mortem AD brains compared with age-matched controls. Secondly, GSH had no effect on MnSOD activity, but decreased Na,K-ATPase activity both in the control and AD brains. Thirdly, UPF1 and UPF17 increased MnSOD activity, and UPF17 suppressed Na,K-ATPase activity. Further studies are needed to clarify, if the inhibitory effect of UPF17 on Na,K-ATPase could abolish the beneficial effect gained from MnSOD activation. Both the antioxidative potential of genistein and its potency to up-regulate Na,K-ATPase activity make it an attractive candidate substance to suppress the early phase of the pathogenesis of AD.

Keywords: Alzheimer’s disease, genistein, MnSOD, Na,K-ATPase, oxidative stress, UPF peptides.

INTRODUCTION

Alzheimer’s disease (AD) is the most frequent neurodegenerative disease which causes dementia in the elderly. Intensive aerobic metabolic activity and high content of polyunsaturated fatty acids and iron make brain especially susceptible to oxidative damage. Oxidative stress (OxS), an imbalance between free radical generation and elimination, is involved in AD pathogenesis in multiple ways, including disturbed mitochondrial function, the pro-oxidant role of amyloid-β (Aβ) peptide, and redox active transition metals [1-3]. It has been suggested that OxS occurs in the early phase of development of AD, before the appearance of pathological hallmarks, such as extracellular Aβ plaques and intracellular neurofibrillary tangles (NFT). These pathological deposits are considered to be an adaptive mechanism in the first stage of disease progression [4]. Based on 8-hydroxyguanosine levels, it has been shown that after the formation of Aβ plaques and NFT the level of OxS decreases [5]. Neurons with aforementioned kind of oxidative damage have increased mitochondrial degradation products, such as mitochondrial DNA and proteins in cytoplasm [6]. These findings relate early OxS to concurrent mitochondrial dysfunction. In addition, a large body of evidence reviewed by several authors underlines the importance of mitochondrial dysfunction and impaired energy metabolism in the early phase of AD progression [7, 8]. Mitochondrial dysfunction is even suggested to be the trigger for AD pathology and concomitant overproduction of mitochondrial superoxide to be a key player in AD [9, 10]. Manganese-dependent superoxide dismutase (MnSOD, SOD2) is a primary and crucial defense mechanism against superoxide radical produced by mitochondrial respiratory chain as it reduces superoxide radicals to hydrogen peroxide, which is further converted to water by catalase or glutathione peroxidase. Investigations in transgenic AD mice have shown that partial deficiency of MnSOD increases the formation of Aβ plaques and NFT, and accelerates the appearance of behavioral changes [11-13]. On the other hand, MnSOD over-expression results in decreased levels of oxidized proteins, reduced plaque formation and improved memory [14]. Taken together, all these evidences suggest that increasing MnSOD activity should have a preventive impact on AD pathology. However, it must be considered that the antioxidative defense system works as a network and potent up-regulation of a single compound could have a rather deleterious effect.

Impaired mitochondrial function through attenuated ATP production may cause changes in the functionality of Na,K-ATPase which is present at very high concentrations in the brain membranes and consumes about 50-60% of the ATP generated in the tissue [15]. It maintains sodium and potassium transmembrane gradients needed for the functionality of the nervous tissue. Previous studies have shown impairment of Na,K-ATPase in AD brain [16, 17]. It seems that a reduction in Na,K-ATPase activity may be caused directly by Aβ deposits [18]. Several clinical studies indicate that estrogen therapy in postmenopausal women may have beneficial effects on AD risk and symptoms [19]. Among other
mechanisms, it has been shown that the positive effect of estrogen therapy is caused by reducing amyloid-β toxicity [20]. These results lead to the hypothesis that if estrogen decreases amyloid-β toxicity, which in turn disturbs the function of Na,K-ATPase, then could estrogen-like natural compounds (like exogenous antioxidant genistein) have an effect on Na,K-ATPase activity? Genistein is a major phytoestrogen in soybean and a relatively selective estrogen receptor β-agonist, which is hoped to be an alternative to estrogen with less side effects in AD prevention [21]. Therefore, we used genistein to modulate Na,K-ATPase activity.

As the functionality of MnSOD is an important prerequisite for the maintenance of normal functionality of Na,K-ATPase, we compared the character of modulation of both enzyme activity by different antioxidants. The selected antioxidants were: a major natural cellular antioxidant glutathione (GSH), its synthetic peptidic analogues (UPF1 and UPF17; the sequences differ by one residue – UPF17 contains γ-glutamyl, while UPF1, similarly to GSH, γ-glutamyl moiety) and exogenous antioxidant genistein.

MATRALS AND METHODS

Peptide Synthesis

UPF1 (Tyr(Me)-γ-Glu-Cys-Gly) and UPF17 (Tyr(Me)-Glu-Cys-Gly) were synthesized manually by solid phase peptide synthesis using Fmoc-chemistry and by machine using tert-Boc-chemistry as described previously [22, 23]. The purity of the peptides was >99% as demonstrated by HPLC on an analytical Nucleosil 120-3 C18 reversed-phase column (0.4 cm x 10 cm) and the peptides were identified by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass-spectrometry (Voyager DE Pro, Applied Biosystems).

Preparation of Human Brain Mitochondrial Fraction

All human brain tissues were obtained from the Hudinge Brain Bank, Huddinge University hospital, Sweden. The brain autopsy was made between 12 and 24 h postmortem. A previous study has shown that oxidative stress parameters do not depend on postmortem delay time during this period [24]. Age-matched control subjects (86–92 years) were with no history of neurological or psychiatric disorders. AD patients met the clinical DSM-IV and neuropsychological CERAD criteria [25, 26]. Brain tissue sampling details have been described previously [24]. Mitochondria were isolated from the post mortem human temporal cortex using the method based on centrifugation of diluted mitochondrial fraction in discontinuous Percoll density gradient published by Rajapakse et al. with some modifications [27, 28].

Measurement of MnSOD Activity in the Temporal Cortex Mitochondria

MnSOD activity in untreated mitochondria fromagematched controls and the temporal cortex of AD brain was measured. The temporal cortex was chosen as previous investigations have shown it to be a region with the highest lipid peroxidation and the most attenuated antioxidant defense among cerebrocortical areas [24]. MnSOD activity was measured with a commercially available Ransod kit (Randox Laboratories Ltd, Ardmore, UK). To inhibit possible CuZn-SOD activity, 5 mM KCN was added to the reaction mixture. Mitochondrial preparation from the AD brain temporal cortex was incubated with GSH at concentration 10 nmol/mg protein for 40 min. UPF1 and UPF17 were used at concentrations 1.0 and 5.0 nmol/mg protein with the incubation time of 40 min. The protein content in the enzyme preparations was determined by the method of Lowry et al. [29].

Measurement of Na,K-ATPase Activity in the Frontal Cortex

The membrane preparations of Na,K-ATPase were isolated from the post mortem human frontal cortex and homogenized as described previously [30, 31]. The Na,K-ATPase preparations from subjects without and with AD were incubated with 3 mM GSH and with UPF17 at concentration range from 10 nM to 0.1 mM in vitro for 40 minutes. In addition, the enzyme preparations from subjects with AD were incubated with 80 μM Na,K-ATPase inhibitor ouabain alone or in combination with 80 μM UPF17. In this experiment, the membrane fractions were incubated for 5 minutes with UPF17 followed by 5 minutes incubation with ouabain and vice versa. The ouabain concentration was chosen based on previous experiments where 80 μM solution caused 50% Na,K-ATPase inhibition. Also UPF17 has shown biological effects in the chosen concentration range [32]. The membrane fractions from the AD brains and age-matched control brains were incubated with soybean phytoestrogen genistein at concentrations of 10 and 100 μM for 5 minutes and 25 minutes. For the Na,K-ATPase activity measurements 30–40 μg of the membrane proteins were taken and the experiment was performed in a medium containing 100 mM NaCl, 20 mM KCl, 4 mM MgCl₂, 4 mM Tris-ATP and 25 mM imidazole-HCl (pH 7.4 at 37 °C). After 10 minutes, the reaction was stopped by 3.5% SDS. The Na,K-ATPase activity was measured spectrophotometrically according to a difference between the release of inorganic phosphate (P₀) from ATP with or without NaCl and KCl in the reaction medium. The protein concentrations in the supernatants were determined by Lowry’s method, using bovine serum albumin as a standard [29]. All the chemicals including GSH, ouabain and genistein were obtained from Sigma, Sigma-Aldrich, Germany.

Statistical Analysis

The results on the graphs are presented as the mean ± standard error of the mean (SEM). Significant differences were identified using the unpaired Student’s t-test (p < 0.05). Data were analyzed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

MnSOD Activity

MnSOD Activity in the Untreated Temporal Cortex Mitochondria

MnSOD activity in the mitochondria of AD brain was decreased to 40.3% (1.01 U/mg protein) compared with age-matched controls (Co; corresponds to 100% – 2.54 U/mg protein) (Fig. 1A).
The Effects of Different Antioxidants  

**MnSOD Activity in the Temporal Cortex Mitochondria Incubated with GSH**

GSH had no effect on MnSOD activity neither in the Co brain nor in the AD brain mitochondrial preparations (Fig. 2).

**MnSOD Activity in the Temporal Cortex Mitochondria Incubated with UPF1 and UPF17**

UPF1 and UPF17 showed no statistically significant effect on MnSOD activity in the Co brain. In the mitochondria of AD brain, UPF1 at concentration of 1 nmol/mg protein increased the MnSOD activity by 19% ($p = 0.0766$) and at concentration of 5 nmol/mg protein by 28% ($p < 0.05$), respective values for UPF17 were 27% ($p < 0.05$) and 39% ($p = 0.0618$) for incubation time 40 minutes (Fig. 2).

**Na,K-ATPase Activity**

**Na,K-ATPase Activity in the Untreated Frontal Cortex**

Na,K-ATPase activity in the membrane preparations of AD brains was decreased to 73.8% compared with the Co (corresponds to the 100%) (Fig. 1B). Respective Na,K-ATPase activities were 0.76 μmol P/min/mg for AD brain and 1.02 μmol P/min/mg for Co.

**Na,K-ATPase Activity in Frontal Cortex Incubated with GSH**

GSH decreased Na,K-ATPase activity in the Co brain by 21% and by 17% in the AD brain for 40 minutes (Fig. 3).

**Na,K-ATPase Activity in the Frontal Cortex Incubated with UPF17**

UPF17 as a more effective antioxidant compared with UPF1 was used in the Na,K-ATPase activity studies in comparison with GSH. In addition, UPF1 inhibited Na,K-ATPase at a very low concentration in a concentration-independent way, which is indicative of a non-specific inhibitory mechanism (data not shown). Na,K-ATPase activity in the Co brain was decreased by 44% after 40 minutes incubation with UPF17, whereas the decline of the enzyme activity in the AD brain was 19% (Fig. 2). The inhibition of Na,K-ATPase activity by GSH and UPF17 did not progress and reached a plateau at concentration range from 10 nM to 0.1 mM. The results in (Fig. 3) are shown at peptide concentration of 10 nM. To elucidate the mechanism by which UPF17 inhibits Na,K-ATPase activity it was used together with cardiac glycoside ouabain, a classical Na,K-ATPase inhibitor. After 5 minutes incubation with UPF17 at concentration of 80 μM the Na,K-ATPase activity in the AD brain decreased by 25%. At the same concentration, ouabain inhib-
ited the enzyme activity by 52%. Treatment of enzyme preparations with UPF17 followed by ouabain resulted in a decline of Na,K-ATPase activity by 63%, whereas addition of ouabain before UPF17 resulted in a similar 62% inhibition (Fig. 4).

**DISCUSSION**

In this paper we compared the effects of three different antioxidants (exogenous natural, exogenous synthetic and endogenous antioxidants) on the activity of MnSOD and Na,K-ATPase in AD human brain. Many studies have focused on the changes of MnSOD activity and mRNA expression in mouse models of AD, however there is less information about human brain tissue. Our first finding was that MnSOD activity in the post mortem temporal cortex of AD patients was decreased 2.5-fold compared with the Co (Fig. 1). Different studies have shown that MnSOD expression is reportedly augmented in AD hippocampal astrocytes and in normal aging brain [33, 34]. This contradiction to our data could be explained by the circumstance that OxS is more pronounced in the beginning of AD progression, which probably leads to an increase in MnSOD activity as an adaptive defense response. The other hypothesis is that the decreased MnSOD activity in the later stages of AD can cause an increase in mRNA expression of the enzyme.
activity, whereas UPF17 attenuated the CuZnSOD activity [39]. In the current study on MnSOD activity we did not notice any analogous effects in opposite directions.

Na,K-ATPase activity is suppressed under pathological conditions which are closely related to Oxs, including AD [40, 41]. According to our results, the Na,K-ATPase activity was decreased in the frontal cortex of the AD brain 1.35-fold compared to the Co (Fig. 1). Incubation with GSH reduced Na,K-ATPase activity both in the Co brain and in the AD brain. This result is consistent with the experiment on rat cerebellar granule cells showing that Na,K-ATPase activity is redox sensitive and suppressed by both decrease and increase of the intracellular glutathione level [41]. However, pretreatment with NAC, known as a thiol containing precursor of glutathione, showed a small, but not significant inhibitory effect on brain Na,K-ATPase activity in a lipopolysaccharide induced Oxs model in rats [42].

In the Co brain the inhibitory effects of UPF17 were more pronounced. The decrease in Na,K-ATPase was about twice as large as in the AD brain, being basically on the same level with the enzyme activity on AD brain incubated with UPF17 (Fig. 3). We speculate that GSH and UPF17 may have a common inhibitory mechanism, which could be a thiol interaction with the thiol groups of Na,K-ATPase. There are several free thiol groups in Na,K-ATPase to where the investigated peptides could potentially interact. Substitution of individual cysteine residues by alanine and serine in Na,K-ATPase a1 subunit, except of Cys 242, resulted in functional enzymes [43]. However, addition of a large molecule, such as GSH or UPF17, to a thiol group would probably cause changes in the enzyme activity. It is possible that in the Co brain UPF17 has an additional/different inhibitory mechanism, whereas in the AD brain there is only one aforementioned thiol-interaction mechanism. Differences in the inhibitory mechanisms in the Co and AD brains may be caused by steric changes and hindered access of UPF17 to Na,K-ATPase in the AD brain. To clarify the interaction of UPF17 with the enzyme, a treatment in combination with ouabain, a selective Na,K-ATPase inhibitor, was tested. Treatment with a combination of ouabain and UPF17 showed a stronger inhibitory effect compared to the single compounds, independently of the order of addition (Fig. 4). This result suggests that UPF17 and ouabain have synergistic effects but different binding sites. Ouabain binds to an extracellular domain of Na,K-ATPase and has an allosteric effect on phosphorylation of the cytoplasmatic catalytic site [44]. Another possibility to explain the effect of UPF17 is the fact that neural cell membranes in the AD brain have altered gycerophospholipid composition and increased rigidity due to polyunsaturated fatty acids oxidation [45, 46]. UPF17 has an aromatic ring in the structure and through hydrophobic interaction it could contribute to altered membrane dynamics and Na,K-ATPase activity.

The third substance we chose to test on Na,K-ATPase activity was a natural exogenous phytoestrogen genistein. Genistein has been shown to have an antioxidative effect mediated by direct radical scavenging or, similarly to estrogens, by the up-regulation of MnSOD expression through MAP kinase activation, which in turn activates NF-xB [47, 48]. The results of current paper show that genistein augments Na,K-ATPase activity in the frontal cortex of the AD brain, increasing it near or even up to the respective value for normal aging brain, depending on the used concentration and incubation time. In the control brain genistein induced a decrease in Na,K-ATPase activity after using a lower concentration and a longer incubation time or a higher concentration and a shorter incubation time (Fig. 5). This result in the control brain is in accordance with the study by Wang et al. where genistein as a tyrosine kinase inhibitor attenuated the Na,K-ATPase inward and outward currents in the cortical neurons and moderately inhibited the enzyme [49]. Na,K-ATPase is redox sensitive, whereas shifts both into a more reduced and oxidized states cause the suppression of Na,K-ATPase activity [41]. S-glutathionylation of Na,K-ATPase regulatory subunit, S-nitrosoylation, oxidation of free thiol groups of Na,K-ATPase and its regulatory kinases are the redox-sensitive mechanisms to decrease Na,K-ATPase activity [50, 51]. These events could be evoked by elevated oxidative stress level in the AD brain which causes shift in redox balance. We speculate that genistein as antioxidant may change redox balance back towards the reduced state and help to improve Na,K-ATPase activity in the AD brain. In the control brain the activity is reduced probably because Na,K-ATPase needs an optimal redox ratio. Intriguingly, genistein affects Na,K-ATPase activity in the control and AD brains in opposite directions, whereas GSH and UPF17 have an inhibitory effect in both cases (see Fig. 3 and 5). UPF17 as a GSH analogue may potentially bind to free thiol groups instead of GSH and decrease Na,K-ATPase activity both in the control and AD brains. S-glutathionylation protects Na,K-ATPase from irreversible oxidation, decreases its activity and helps to save ATP in the case of energy deficit [50].

Considering that UPF17 increases MnSOD activity and decreases Na,K-ATPase, in vivo experiments should answer the question, could the inhibitory effect of UPF17 on Na,K-ATPase be critically prevailing and abolish the beneficial effect gained from MnSOD activation. Genistein is an antioxidant which potentially up-regulates Na,K-ATPase activity. These properties would make it an especially attractive substance to suppress the pathogenesis of AD.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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