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CRITICAL ROLE OF METHIONINE-722 IN THE STIMULATION OF HUMAN BRAIN G-PROTEINS AND NEUROTOXICITY INDUCED BY LONDON FAMILIAL ALZHEIMER'S DISEASE (FAD) MUTATED V717G-APP$_{714–723}$

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Abstract—We have demonstrated earlier that V717G-APP$_{714–723}$, the membrane fragment of the V717G ("London") familial Alzheimer's disease (FAD) mutant of amyloid precursor protein (APP), is a potent stimulator of G-proteins in human brain membranes. In this study, we tested the hypothesis that Met-722 in the V717G-APP$_{714–723}$ peptide (P2) plays a critical role in the P2-induced oxidative stimulation of G-proteins in the human temporal cortex membranes and in the neurotoxicity of the peptide in differentiated PC12 and cerebellar granular cells. We found that 10 μM P3, the Met-722 sulfoxide analog of P2, produced a twofold lower stimulation of G-proteins ([^{35}S]-GTPγS binding) in control temporal cortex membranes compared with 10 μM P2. The stimulatory effect of 10 μM P4, the Met-722 sulfone analog of P2, was 2.5-fold lower than the effect of P2. In Alzheimer's disease (AD) temporal cortex, the P3 and P4 stimulation of G-proteins was slightly weaker than the P2 stimulation. Substitution of the Met-722 S-atom in P2 by -CH$_2$- group (P5) led to the disappearance of P2 stimulatory effect on G-proteins. Glutathione (GSH), melatonin (Mel), desferrioxamine (DFO) and 17-β-estradiol (17βE) significantly reduced P2 stimulatory effect on G-proteins in human brain. Only DFO and Mel were able to reduce the moderate stimulation of G-proteins by P3, whereas none of the tested antioxidants influenced the weak stimulation by P4. P2 at 100 μM induced a 40% decrease in PC12 cell viability as revealed by MTT assay, the effect being significantly higher than that of P3 or P4, whereas P1 (wild-type APP$_{714–723}$) did not affect cell viability. Trypan Blue exclusion assay demonstrated that 10 μM P2 and P3 induced 3.8- and 3.5-fold death in the cerebellar granular cells as compared with the respective control values. P1 and P4 at 10 μM induced 1.7- and 2.3-fold increase in cell death, respectively. Treatment of the cerebellar granular cells with pertussis toxin decreased the high neurotoxicity of P2 and P3, whereas the low toxicity of P1 and P4 was not influenced. These results support the hypothesis that the G-protein stimulatory effect and neurotoxicity of "London"-mutated V717G-APP$_{714–723}$ (P2) and its Met-722 oxidized analogs involve oxidative-dependent and oxidative-independent mechanisms and the oxidation state of Met-722 plays a critical role in determining the mechanism.

Key words: G-proteins, V717G-APP$_{714–723}$ fragment, methionine-722 oxidation, antioxidants, neurotoxicity.

The currently dominant hypothesis of the familial Alzheimer's disease (FAD) states that mutations in the amyloid precursor protein (APP) and presenilin genes lead to the altered metabolism of APP and over-production of amyloid β-peptides (Aβ), particularly of insoluble A$\beta_{1-42/43}$ (Hardy, 1997; Selkoe, 1997). The latter event serves as a primary cause for the deposition of β-amyloid and subsequent formation of neurofibrillary tangles and neuritic plaques, the hallmarks of Alzheimer's disease (AD) and FAD (Selkoe, 1991; Iwatsubo et al., 1994; Hardy and Selkoe, 2002). Although this "amyloid hypothesis" has been valid for more than 10 years (and has not been challenged by serious alternatives) the other deleterious mechanisms than those initiated by A$\beta_{1-42/43}$ (or its truncated sequences) are thought to be involved in FAD neurodegeneration (Nishimoto, 1998; Neve et al., 2001; Neve, 2003).

Several studies have shown that APP is capable of binding to the brain-specific signal transducing G$_o$-protein (Nishimoto et al., 1993; Brouillet et al., 1999). In addition, V642 ("London") FAD mutants of APP$_{695}$ induce G-protein-mediated DNA fragmentation and cytotoxicity in the nerve cell lines. These processes were shown to be mediated by the G$\beta_2\gamma_2$ complex of G$_o$-protein (Giambarella et al., 1997; Neve et al., 2001) and to occur independently of production the neurotoxic Aβ_{1-42} (Yamatsuji et al., 1996; Niikura et al., 2004).

We have recently demonstrated that the "London"-mutated APP membrane fragments V717F-APP$_{714–723}$ and V717G-APP$_{714–723}$ stimulate G-proteins ([^{35}S]-GTPγS binding) in the aged control and AD brain membranes, the effect of V717G-APP$_{714–723}$ being much stronger than that of V717F-APP$_{714–723}$ or of the wild-type APP$_{714–723}$ (Karelson et al., 2005). Since glutathione (GSH), desferrioxamine (DFO) and other neuroprotectants markedly attenuated the strong stimulatory effect of the mutated V717G-APP$_{714–723}$ on the G-proteins in aging

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Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; DFO, desferrioxamine; FAD, familial Alzheimer's disease; GSH, reduced glutathione; Mel, melatonin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl-tetrazolium bromide; NLE, norleucine; PBS, phosphate-buffered saline; PTX, pertussis toxin; ROS, reactive oxygen species; SFAD, Swedish familial Alzheimer's disease; TE-buffer, 10 mM Tris–HCl and of 0.1 mM EDTA, pH 7.4; 17βE, 17β-estradiol.

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Table 1. Sequences and modifications of the APP membrane domain peptides used in this study

Peptide name	Sequence and methionine-722 side chain structure
Peptide 1 (P1): $APP_{714–723}$	TVIVITLVML-amide $-CH_2-CH_2-S-CH_3$
Peptide 2 (P2): V717G-$APP_{714–723}$	TVIGITLVML-amide $-CH_2-CH_2-S-CH_3$
Peptide 3 (P3): V717G/M722S(O)-$APP_{714–723}$	TVIGITLVM(O)L-amide $-CH_2-CH_2-S(O)-CH_3$
Peptide 4 (P4): V717G/M722S$(O)_2$-$APP_{714–723}$	TVIGITLVM$(O)_2$L-amide $-CH_2-CH_2-S(O)_2-CH_3$
Peptide 5 (P5): V717G/M722NLE-$APP_{714–723}$	TVIGITLV(NLE)L-amide $-CH_2-CH_2-CH_2-CH_3$

brain, we suggested that the stimulation of G-proteins is mediated through the membrane reactive oxygen species (ROS). In the same study, we hypothesized that ROS-mediated stimulation of neuronal G-proteins induces pathogenetic signaling cascades which lead to the neuronal degeneration and death.

Several studies have demonstrated that neurotoxic $A\beta_{1-42/43}$ and its fragments $A\beta_{25–35}$, $A\beta_{31–35}$ can insert themselves into the membrane lipid bilayer via redox active Met-35 residue, possibly initiating the oxidative neurotoxic cascades (Varadarajan et al., 1999; Kanski et al., 2002a; Butterfield and Boyd-Kimball, 2005). In light of these findings, we suggest that Met-722 might be important for the V717G-$APP_{714–723}$-induced potent oxidative stimulation of neuronal G-proteins and perhaps in the subsequent neurotoxicity.

The aim of this study was to compare the effects of the peptides represented in Table 1, P1 (wild-type $APP_{714–723}$), P2 (V717G-$APP_{714–723}$), P3 (Met-722 sulfoxide analog of P2), P4 (Met-722 sulfone analog of P2) and P5 (NLE-analog of P2), on the activity of G-proteins ([^{35}S]-GTPγS binding) in the temporal cortex membranes of the aging subjects as well as of the patients with sporadic AD and Swedish familial Alzheimer's disease (SFAD) carrying the APP670/671 mutation; the temporal cortex was chosen as AD and SFAD-targeted region showing severe neuronal damage and abundance of neuropathological hallmarks (Kalaria et al., 1996; Braak et al., 1998). Our second objective was to test whether neuroprotective compounds, GSH, DFO, 17β-estradiol (17βE) and melatonin (Mel) can affect the putative oxidative mechanism in the stimulation of G-proteins by P2, P3 and P4. Finally, we aimed to compare possible neurotoxic effects of the peptides P1, P2, P3 and P4 in differentiated PC12 cells by using cell viability (MTT reduction) assay and in the cerebellar granular cells by using the Trypan Blue exclusion method.

EXPERIMENTAL PROCEDURES

Materials

Peptides P1, P2, P3, P4 and P5 were purchased from Thermo Hybaid (Ulm, Germany). [^{35}S]-GTPγS (1250 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA, USA). All other reagents were from Sigma (St. Louis, MO, USA). All reagents used were of highest analytical grade.

Brain tissue sampling

Temporal cortex tissues from the medial temporal gyrus (Brodmann area 21, the level of the mamillary bodies) of postmortal human brains were obtained from Huddinge Brain Bank (Huddinger, Sweden). The present study included the tissues from nine age-matched control subjects (seven females and two males) who had no history of neurological or psychiatric disorders. In addition, the region from 11 sporadic AD (nine females and two males) and four SFAD (one female and three males) patients (mean age$\pm$S.D.: 76.0$\pm$9.1, 80.1$\pm$8.1 and 63$\pm$5.3 years for the control, AD and SFAD group, respectively) were used. Our studies showed no gender difference in the effect of the peptides on the temporal cortex G-proteins. The AD and SFAD patients met clinical and histological criteria for AD (Bogdanovic and Morris, 1995). The postmortem time for the brains of control persons and patients was under 24 h. The temporal cortex tissues were rapidly dissected from the brains and kept at -70 °C prior to experiment.

Preparation of temporal cortex membranes

The temporal cortex membranes for the [^{35}S]-GTPγS binding measurement were mainly prepared according to the protocol by Karelson et al. (1995). Briefly, the weighed brain tissues (100–120 mg) were homogenized in six volumes of ice-cold 5 mM Tris–HCl buffer, pH 7.4. The homogenates were 1:10 diluted with the same buffer, stirred on ice for 30 min and centrifuged for 6 min at 1600$\times$g. The membrane pellet was finally resuspended in the standard TE-buffer (10 mM Tris–HCl and of 0.1 mM EDTA, pH 7.4) to give a protein concentration of about 0.8–1.2 mg/ml.

[^{35}S]-GTPγS-binding assay

The temporal cortex membranes with the final protein concentration of 0.04 mg/ml were incubated in a reaction cocktail containing TE-buffer, GDP (1 μM), dithiothreitol (1 mM), $MgCl_2$ (5 mM), NaCl (150 mM) and [^{35}S]-GTPγS (50–70,000 c.p.m. in an aliquot of the reaction cocktail). Incubation was carried out for 2 min at 26 °C in a total volume of 0.1 ml either in the absence (basal value) or in the presence of various concentrations of the peptides. Bound and free [^{35}S]-GTPγS were separated by vacuum filtration through GF/B filters (Whatman International Ltd., Mainstone, UK), which were washed three times with 5 ml of ice-cold TE-buffer. Radioactivity was quantified by "Packard 3255" liquid scintillation counter. In data analysis, the basal [^{35}S]-GTPγS binding was defined as 100%.

Incubation of temporal cortex membranes with antioxidants

To elucidate the effects of antioxidants on P2-P4 stimulation of [^{35}S]-GTPγS-binding to control or AD temporal cortex membranes, 1.5 mM GSH, 0.1 mM Mel, 0.01 mM DFO or 0.01 mM 17βE were added to the medium before the peptide. The antioxidative effect was estimated as a difference in the stimulation of binding in the absence or presence of the respective antioxidant. The effect of antioxidant on the basal [^{35}S]-GTPγS-binding was estimated simultaneously.

PC12 cell cultures and cell viability assay

Rat pheochromocytoma PC12 cells were grown on collagen S-coated dishes in RPMI-1640 medium with 1% L-glutamine supplemented with 10% horse serum, 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were maintained at 37 °C under an atmosphere of 5% CO_2 and saturated humidity.

Cell viability was assessed by using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl-tetrazolium bromide (MTT) assay