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The H₃ receptor antagonist clobenpropit protects against A β 42-induced neurotoxicity in differentiated rat PC12 cells

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The present study was designed to investigate the effect of the H₃ antagonist clobenpropit on neurotoxicity induced by A β 42 in differentiated rat PC12 cells. PC12 cells were exposed to A β 42 (5 μ M) for 24 h after clobenpropit applied for 18 h. Cell viability, glutamate release or cell surface expression of NMDA receptors were examined. Pretreatment with clobenpropit ameliorated cell impairment induced by A β 42. In the presence of A β 42, clobenpropit increased glutamate release, although there were no differences between the A β 42-treated sample and control. Meanwhile, in the absence of A β 42, clobenpropit increased the surface expression of NMDA receptors when the total expression of NMDA receptors was not influenced. These results indicate that one of the mechanisms by which clobenpropit attenuates A β 42-induced neurotoxicity may act through regulation of glutamate release and NMDA receptor trafficking.

1. Introduction

As a global cognitive disorder, Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by global cognitive decline involving memory, orientation, judgment, and reasoning (Tanzi et al. 2005), and occurs with pathological hallmarks including widespread neuronal degeneration and neuritic plaques containing amyloid- β peptide (A β) (Hardy et al. 1998). Evidence indicates that A β 42 is essential for amyloid deposition in the parenchyma and in vessels (McGowan et al. 2005; Tanzi et al. 2005). In addition to A β , enhancement of excitotoxicity by glutamate receptors, especially NMDA receptors, has also been implicated in the pathogenesis of AD (Doble et al. 1999).

Histamine, which is synthesized by histaminergic neurons located in the tuberomammillary (TM) nucleus, holds a key position in the regulation of many body functions (Schwartz et al. 1991; Haas et al. 2003), including the sleep-waking cycle, energy and endocrine homeostasis, synaptic plasticity, cognition, learning and memory. Histamine participates in pathological processes of many brain diseases. Literature has already shown that in AD, neurofibrillary tangles occur extensively in the TM nucleus, and histamine levels and/or histidine decarboxylase activity decrease in affected areas of the brain. This implies that histamine and/or histamine related drugs may have a potential use for AD, which has been supported by the studies that repeated administration of histamine improves memory retrieval (Zarrindast et al. 2008), and blockade of histamine H₁ receptors has been shown to facilitate spatial memory performance (Zlomuzica et al. 2009). In a previous study we have also found that carnosine, a reservoir of histidine, which is a precursor of histamine, protects against A β 42-induced impairment in differentiated PC12 cells (Fu et al. 2008).

As one of four receptors (H₁, H₂, H₃, H₄) that mediate the diverse biological effects of neurotransmitter histamine (Leurs et al. 2005), the H₃ receptor, which was identified in 1983 by Arrang et al. (1983), was regarded as a presynaptic autoreceptor or heteroreceptor to provide negative feedback to restrict histamine synthesis and release and the release of other transmitters, such as acetylcholine, noradrenaline, glutamate, GABA (Arrang et al. 1983; Brown et al. 2001; Haas et al. 2003). Evidence indicates that H₃ receptor participate in modulating attention and memory processes (Giovannini et al. 1999), and some antagonists of the H₃ receptor were considered to be useful in cognitive disorders (Chen et al. 1999; Huang et al. 2004; Witkin et al. 2004). However, the mechanism is still unclear after decades of study. Therefore, it is interesting to know whether it is still work in the protection against AD. To address this, we investigated the H₃ antagonist clobenpropit in A β 42-induced neurotoxicity in PC12 cells.

2. Investigations and results

2.1. Effect of the H₃ receptor antagonist clobenpropit on A β 42-induced neurotoxicity

A β 42-induced neurotoxicity in PC12 cells was observed after incubating for 24 h in our previous study, and cell viability decreased to $74.4 \pm 4.1\%$ at a concentration of 5 μ M (Fu et al. 2008). Here, we used this model to investigate the effect of clobenpropit in A β 42-induced (5 μ M) neurotoxicity. 18 h after application, the H₃ antagonist clobenpropit at the concentrations of 10^{-6} , 10^{-5} M reversed the A β 42-induced (5 μ M) neurotoxicity by 87.0 ± 5.0 , $94.3 \pm 4.6\%$, respectively (Fig. 1). However, this receptor antagonist alone had no effect at these concentrations (data not shown).

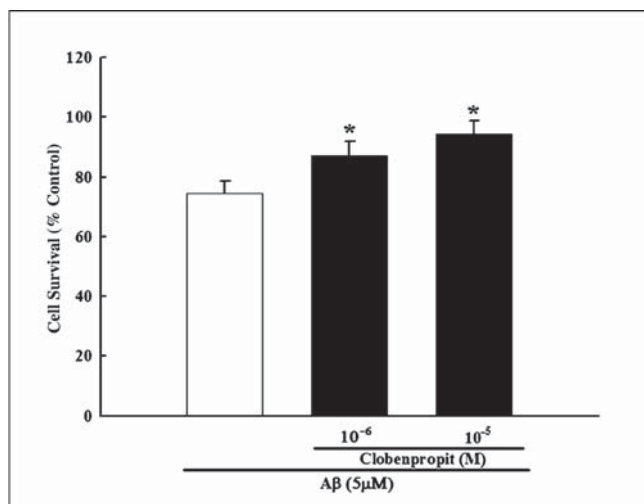


Fig. 1: Effect of clobenpropit on Aβ₄₂-induced neurotoxicity in differentiated PC12 cells. Pretreated with various concentrations of clobenpropit for 18 h, cells were then exposed to 5 μM Aβ₄₂. Cell viability was determined by MTT assay 24 h after application of Aβ₄₂. Values are expressed as percentage of control values. Data are mean ± S.D. of four to six independent experiments with three replicates for each condition. **p* < 0.05, compared with Aβ₄₂ (B) (ANOVA followed by Student-Newman-Keuls test)

2.2. Effect of the H₃ antagonist clobenpropit on glutamate release

We have found that regulation of glutamatergic transmission may participate in the mechanism of neurotoxicity induced by Aβ₄₂ in a previous paper (Fu et al. 2008), so glutamate release was following examined. In the presence of Aβ₄₂, the H₃ receptor antagonist clobenpropit significantly increased glutamate release (Fig. 2). The maximal effect of clobenpropit was at 10⁻⁵ M, when glutamate release was 139.7 ± 2.0% compared to control (Fig. 2). There was no difference between the Aβ₄₂-treated samples and vehicle-treated samples (Fu et al. 2008).

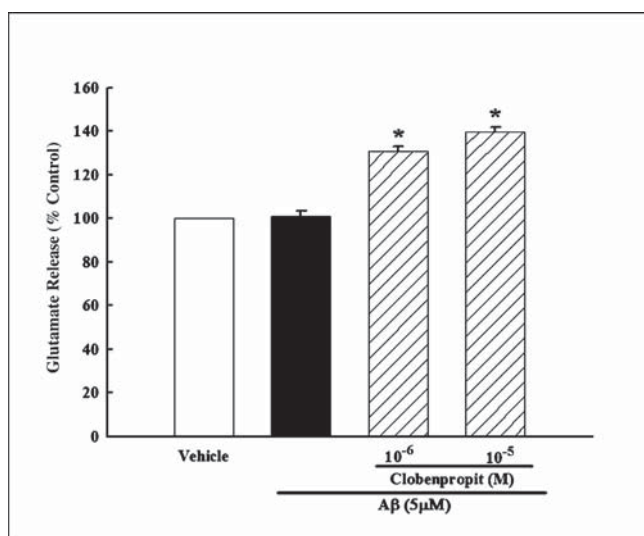


Fig. 2: Effect of clobenpropit on glutamate release. Differentiated PC12 cells were exposed to vehicle or the indicated drugs. Various concentrations of clobenpropit were added 18 h before Aβ₄₂ (5 μM). The amount of glutamate was quantified by HPLC 24 h after application of Aβ₄₂. Values are expressed as percentage of control values. Data are mean ± S.D. of four to six independent experiments with three replicates for each condition. **p* < 0.05, compared with control (ANOVA followed by Student-Newman-Keuls test)

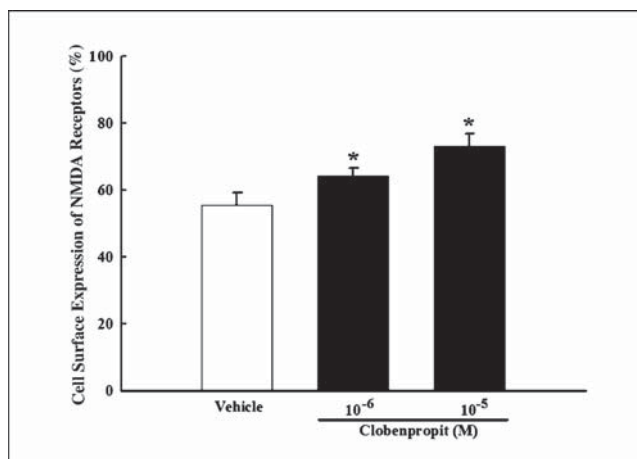


Fig. 3: Effect of clobenpropit on cell surface expression of NMDA receptors. Differentiated PC12 cells were exposed to vehicle or the indicated drugs. Cell surface expression of NMDA receptors was quantified by cell ELISA assay 18 h after application of clobenpropit. Values are expressed as percentage of total values. Data are mean ± S.D. of four to six independent experiments with three replicates for each condition. **p* < 0.05, compared with control (ANOVA followed by Student-Newman-Keuls test)

2.3. Regulation of NMDA receptor trafficking by the H₃ antagonist clobenpropit

Our previous results have already demonstrated that modulation of trafficking of NMDA receptors is one of the most important mechanisms to protect against Aβ₄₂-induced neurotoxicity (Fu et al. 2008). To investigate whether it still works, in this study, cell surface expression of NMDA receptors in PC12 cells was further examined. Expectedly, clobenpropit increased the cell surface expression of NMDA receptors compared to the vehicle control (Fig. 3). And the maximal effect to increase the cell surface expression of NMDA receptors afforded by clobenpropit is ~32% at the concentration of 10⁻⁵ M (Fig. 3). However, clobenpropit had no effect on total expression of NMDA receptors at these concentrations (data not shown).

3. Discussion

There is a preponderance of data supporting a key therapeutic role for the H₃ antagonists in disorders with associated cognitive dysfunction including AD (Medhurst et al. 2007; Passani et al. 2000), which may function to improve cognitive performance in disease states. In this study, we demonstrated that the H₃ antagonist clobenpropit reversed the neurotoxicity induced by Aβ₄₂ in PC12 cells. This is consistent with the finding that *in vivo*, clobenpropit reverses scopolamine-induced deficits in passive avoidance and object recognition tasks, but exerts no precognitive effects in control animals (Haas et al. 2003), and that GSK 19254, a novel H₃ antagonist in AD brain, improves cognitive performance in preclinical models (Medhurst et al. 2007). Study in Apoe^{-/-} mice also support H₃ receptors as potential drug targets in AD (Bongers et al. 2004).

As a heteroreceptor, H₃ receptor is known to modulate various neurotransmitter systems in the brain, which means that an activation of the H₃ heteroreceptor leads to the inhibition of histamine synthesis and release, and release of other neurotransmitters such as acetylcholine, noradrenaline, glutamate, GABA (Arrang et al. 1983; Brown et al. 2001; Haas et al. 2003). H₁ antagonists cause a decline in cognitive functions (Turner et al. 2002), and an indirect modulation of histaminergic brain function through H₁ receptor by H₃ antagonists might be one means to modulate attention and memory process. Secondly, lots of reports indicated that the neuromodulatory role

of the histaminergic system on acetylcholine release might be important in this respect (Haas et al. 2003). Therefore, it is not surprising that the H₃ antagonist clobenpropit protects against A β 42-induced neurotoxicity, which is a key molecule in AD process. Here, it is of huge interest to find out what kind of protective pathway is involved in clobenpropit attenuating A β 42-induced neurotoxicity.

In the current study, we found that clobenpropit significantly increased glutamate release in the presence of A β 42, which suggested that higher glutamatergic activity reverses the impairment induced by A β 42. Since among a number of mechanisms that can give rise to the regionally variable neuronal loss and synaptic dysfunction exhibited in AD, excitotoxicity, especially when induced by glutamate and glutamate receptors, figures prominently (Francis et al. 2008). It is reasonable to hypothesize that histaminergic- glutamatergic interaction contributes to the current protection exerted by clobenpropit. This is supported by our previous study that demonstrates a histaminergic- glutamatergic interaction in the protection of carnosine on A β 42-induced neurotoxicity (Fu et al. 2008), although glutamate has different action in these two models. It is less clear what kind of mechanisms worked in the two models employed, although perhaps these could be attributed to differential neurobiology underlying the models. So far, our results at least suggest that restoring glutamatergic transmission by increasing glutamate release may participate in the protective mechanism of clobenpropit on A β 42-induced neurotoxicity.

Over the past twenty years, the amyloid hypothesis has been strongly supported by a wealth of evidences (Tanzi et al. 2005), but it remains unclear how this peptide causes synaptic dysfunction, synapse loss and neuronal death. Recently, Snyder and colleagues found that A β reduces glutamatergic transmission and NMDA receptor-dependent LTP through internalization of NMDA receptors (Snyder et al. 2005), which suggests a potential therapeutic direction in AD. Our results on increasing cell surface expression of NMDA receptors induced by clobenpropit strongly support the above proposal, just as we found in the carnosine-induced protection on A β 42-induced neurotoxicity (Fu et al. 2008). This also led to our hypothesis which we made above that impairment of glutamatergic transmission induced by A β 42 may be restored by increasing cell surface expression of NMDA receptors afforded by the H₃ receptor antagonists, despite the action of higher glutamate.

In summary, we demonstrated that the H₃ receptor antagonist clobenpropit conferred significant protection against A β 42-induced neurotoxicity in PC12 cells. The mechanism of this protection may attribute to restore the impairment of glutamatergic transmission induced by A β 42 through regulation of glutamate release and NMDA receptor trafficking.

4. Experimental

4.1. Materials

Cell culture plates were from Corning Inc. (NY, USA). Clobenpropit was from Sigma (St. Louis, MO, USA); A β 42 was from Bioscience International (Camarillo, CA); TMB was from Calbiochem (Darmstadt, Germany); anti-NMDAR1 monoclonal antibody was from Chemicon; secondary mouse antibody conjugated to horseradish peroxidase (HRP) was from Rockland (Gilbertsville, RA, USA); and L-glutamate, penicillin, trypsin, streptomycin, poly-L-lysine and Dulbecco's modified Eagle's medium (DMEM) were from Gibco-BRL (Grand Island, NY, USA).

4.2. Handling of β -amyloid peptide

A β 42 was dissolved in sterile, double-distilled water at 1 mg/ml. This solution was stored as 50 μ l aliquots at -20°C until needed. Prior to use aliquots which were from the same lot were placed at 37°C for 1 week to allow aggregation, then directly solubilized at the experimental concentration (5 μM) (Pannaccione et al. 2005). The same lot number was used

throughout the studies unless specified. All peptide batches were tested at graded concentrations (range: 0.01–10 μM) for evaluation of cell toxicity.

4.3. Cell culture

Differentiated PC12 cells were from the Institute of Cell Biology, Chinese Academy of Sciences (Shanghai). Cells were grown in pH 7.4 growth medium consisting of DMEM supplemented with 10% horse serum, 5% heat-inactivated fetal calf serum, 100 U/ml penicillin G, and 100 mg/ml streptomycin, and maintained at 37°C and 5% CO₂ in a humidified incubator. Subculture was performed when the cells reached confluence. Cells were trypsinized at a ratio of 1:3 after confluence using 0.25% trypsin in Hank's containing 0.02% EDTA. Subcultured cells were seeded onto poly-L-lysine coated 96-well plates at densities of 1×10^4 cells/well. Experiments were carried out 24 h after cells were seeded.

4.4. Drug exposure

As a general rule, drugs were dissolved in sterile purified water prior to dilution into DMEM. 18 h after clobenpropit application, PC12 cells were exposed to A β 42 for 24 h.

4.5. Viability studies

PC12 cells were cultured in 96-well plates, with 6 wells in each group. The cells were incubated with MTT (final concentration 0.5 mg/ml) for 3 h at 37°C . Then, the supernatant layer was removed, and 100 μ l of dimethyl sulfoxide was added to each well. MTT metabolism was quantified spectrophotometrically at 490 nm in a Biorad microplate reader. Results were expressed as the percentage MTT reduction, assuming the absorbance of control cells was 100%.

4.6. Neurochemical analysis of glutamate

After stimulation, the supernatant layer was removed and stored at -80°C until assayed, when samples were homogenized in 3% perchloric acid containing 5 mM disodium EDTA and 5-hydro-N^o-methyltryptamine in a Polytron homogenizer (Kinematica, Lucern, Switzerland) at the maximum setting for 20 s in an ice bath. The homogenate was centrifuged at $15,000 \times g$ for 20 min at 4°C , then the supernatant was removed and filtered through a 0.22 μm polyvinylidene difluoride membrane. Glutamate were determined by an HPLC-ECD system consisting of a solvent delivery module (Model 582, ESA, Chelmsford, MA, USA), a 3 mm reversed-phase column (3.0 mm $\times$ 50 mm, CAPCELL PAK C18 MG, Shiseido, Japan), and an HPLC autosampler (Model 542, ESA, Chelmsford, MA, USA); a 4-channel Coul Array electrochemical detector (E1 = +250 mV, E2 = +550 mV) was used for the analysis of glutamate. The mobile phase (0.1 M Na₂HPO₄ in 22% methanol and 13% acetonitrile, pH 6.8 with H₃PO₄) was filtered through a 0.22 μm filter (Millipore, Bedford MA, USA) and degassed before pumping at a flow rate of 0.75 ml/min. The samples were derivatized according to previously described methods (Dibzabtu et al. 1988; Jin et al. 2005; Dai et al. 2006) with minor modifications. The derivatization stock reagent consisted of 27 mg of *o*-phthalaldehyde (OPA, Pickering, Mountain View, CA, USA) dissolved in 1 ml of MeOH with 10 mg thiofluor (Pickering, Mountain View, CA, USA) and 9 ml 0.1 M sodium tetraborate (pH 9.3). The working solution was prepared by diluting 1 ml OPA-thiofluor stock solution with 4 ml 0.1 M sodium tetraborate, pH 9.3. Pre-column amino acid derivatization was performed by mixing 15 μ l volumes of the standard glutamate, histamine or sample and 20 μ l OPA-thiofluor working solution in the autosampler before injection onto the analytical column. Data were collected and analyzed by Coul Array for Windows software (ESA, Chelmsford, MA, USA).

4.7. Cell ELISA assay

Assays were carried out essentially as described previously (Nong et al. 2003). Briefly, PC12 cells were cultured in 96-well plates (approximately 5×10^4 cells per well). After treatment, the cells were washed with PBS for 10 min before fixation for 10 min with 4% paraformaldehyde in PBS, and then half of the samples were permeabilized with 0.1% Triton X-100 in PBS for 5 min. The cells were then rinsed with PBS and incubated with anti-NMDAR1 monoclonal antibody (Chemicon; 1:1000) at room temperature for 1 h after incubation with BSA for 1 h at room temperature. After rinsing with PBS, the cells were incubated for 1 h at room temperature with secondary mouse antibody conjugated to HRP (1:5000; Rockland). After washing with PBS, the HRP substrate TMB was added to produce a colour reaction that was stopped with 0.2 volumes of 3N HCl. The optical density of 1 ml of supernatant was read on a spectrophotometer at 450 nm. The rates of cell surface expression of NMDA receptors were presented as the ratio of colorimetric readings under non-permeabilized conditions *versus* those

under permeabilized conditions. Analysis was based on at least six separate dishes in each group.

4.8. Statistics

The data are given as mean $\pm$ S.D. of four to six experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparison tests (SPSS software 13.0). P values less than 0.05 were considered statistically significant.

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