Inhibition of Choline Acetyltransferase as a Mechanism for Cholinergic Dysfunction Induced by Amyloid-β Peptide Oligomers*

Received for publication, November 9, 2011, and in revised form, March 15, 2012. Published, JBC Papers in Press, April 13, 2012, DOI 10.1074/jbc.M111.321448

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Background: Cholinergic dysfunction is an early feature of Alzheimer disease (AD).

Results: Soluble oligomers of the amyloid-β peptide (Aβ) bind to cholinergic neurons and inhibit choline acetyltransferase (ChAT) activity before any cell death or lesion.

Conclusion: ChAT inhibition might impair acetylcholine production and cholinergic function in AD brains.

Significance: This novel effect of Aβ oligomers may be relevant in early stage AD pathology.

Dysregulated cholinergic signaling is an early hallmark of Alzheimer disease (AD), usually ascribed to degeneration of cholinergic neurons induced by the amyloid-β peptide (Aβ). It is now generally accepted that neuronal dysfunction and memory deficits in the early stages of AD are caused by the neuronal impact of soluble Aβ oligomers (AβOs). AβOs build up in AD brain and specifically attach to excitatory synapses, leading to synapse dysfunction. Here, we have investigated the possibility that AβOs could impact cholinergic signaling. The activity of choline acetyltransferase (ChAT, the enzyme that carries out ACh production) was inhibited by ~50% in cultured cholinergic neurons exposed to low nanomolar concentrations of AβOs. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, lactate dehydrogenase release, and [3H]choline uptake assays showed no evidence of neuronal damage or loss of viability that could account for reduced ChAT activity under these conditions. Glutamate receptor antagonists fully blocked ChAT inhibition and oxidative stress induced by AβOs. Antioxidant polyunsaturated fatty acids had similar effects, indicating that oxidative damage may be involved in ChAT inhibition. Treatment with insulin, previously shown to down-regulate neuronal AβO binding sites, fully prevented AβO-induced inhibition of ChAT. Interestingly, we found that AβOs selectively bind to ~50% of cultured cholinergic neurons, suggesting that ChAT is fully inhibited in AβO-targeted neurons. Reduction in ChAT activity instigated by AβOs may thus be a relevant event in early stage AD pathology, preceding the loss of cholinergic neurons commonly observed in AD brains.

Alzheimer disease (AD)§ is a progressive neurodegenerative disorder and the main cause of dementia in the elderly. AD is clinically characterized by memory deficits and progressive cognitive decline, which appears as a result of synaptic and neuronal damage (1).

The pathogenesis of AD has long been linked to the amyloid-β (Aβ) peptide, which accumulates markedly in diseased brains, forming insoluble structures known as senile plaques. Although plaques may play a role in AD pathology, during the past decade much of the focus has turned to neurologically active soluble oligomers of the Aβ peptide, also known as Aβ-derived diffusible ligands (for review, see Refs. 2, 3). Oligomers build up in affected human brains and in transgenic mouse models of AD and may account for previously puzzling aspects of the disease, such as the imperfect correlation seen between plaque burden and disease progression or its brain region specificity (4, 5).

The cholinergic neurons of the nucleus basalis of Meynert constitute a group of nerve cells that are preferentially damaged during the course of AD (6). A cholinergic hypothesis of AD was in fact proposed nearly 30 years ago, supported then by extensive data, ranging from biochemical and pharmacological studies to electrophysiology, showing that cholinergic dysfunction is a prominent feature of AD (7). Although recent experiments have challenged this hypothesis, there is general consensus that cholinergic impairment is a very important feature of AD pathogenesis and cognitive decline (8).

Using primary neuronal cultures enriched in cholinergic neurons, we have previously shown that choline acetyltransferase (ChAT; EC 2.3.1.6) the enzyme responsible for acetylcholine production, is negatively modulated by excitatory amino acids (EAA)s (9). Cultures chronically exposed to EAA show a marked down-regulation of ChAT activity before any detectable cell death or lesion and in the absence of changes in total ChAT levels. This effect was shown to be dependent on calcium influx, nitric oxide (NO) production, and possibly ox-

DNQX, 6,7-dinitroquinoxaline-2,3-dione; EAA, excitatory amino acid; H, DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NR2B, NMDA receptor type 2B; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species.
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dative damage to the enzyme (9). ChAT inhibition was highly selective, leaving intact the high affinity choline transporter and most, if not all, other neurotransmitter systems expressed by the cultured neurons.

Considering the well established connection between the neurotoxicity of Aβ oligomers (AβOs) and aberrant activation of glutamate receptors (10–15), we have investigated the hypothesis that AβOs might cause ChAT inactivation in cultured cholinergic neurons. We show that nanomolar concentrations of AβOs induce a marked inactivation of neuronal ChAT, which is mediated by activation of EAA receptors. Moreover, agents that protect cells from oxidative stress also protect ChAT from the neuronal impact of AβOs.

EXPERIMENTAL PROCEDURES

Materials—Aβ peptide (Aβ_1-42) was from Bachem (Torrance, CA). MK-801, DNQX, α-linolenic acid, arachidonic acid, stearic acid, oleic acid, memantine, insulin, DAPI, hemicholinium-3, ifenprodil, choline, and anti-α-tubulin antibody were purchased from Sigma-Aldrich. [3H]Choline and [3H]acetyl-CoA were from GE Healthcare. MEM, fetal calf serum (FCS), and H_2DCFDA were from Invitrogen. Anti-ChAT antibody was from Millipore. NU4 antibody was prepared and characterized as described (16). All other reagents were of the highest analytical grade available.

Cell Culture—Avian retinas were used as a source of cholinergic neurons. The retina cholinergic system is entirely contained within the tissue and has been used previously as a model to study pharmacological effects of EAs on cholinergic activity (9, 17). All cholinergic cells in the retina are amacrine neurons, which project their neurites to the inner plexiform layer where they form two well defined sublamina of cholinergic terminals (17–20). All cholinergic markers can be analyzed in this tissue without interference by contaminants from other regions of the nervous system. Primary cultures of embryonic avian retina develop neurochemical properties typical of the intact tissue, making this a suitable model to study the cholinergic system (21, 22). Neuron-enriched cultures were obtained from the retinas of 9-day-old chick embryos, as described previously (23). Briefly, retinas were dissected, digested with 0.05% trypsin, and dissociated by mechanical aspiration through a large bore pipette. Plating density and serum levels were kept relatively low. Approximately 10^6 cells/ml of minimum essential medium (MEM) supplemented with 1% fetal calf serum (FCS) were plated in standard 24-wells plastic culture plates (0.5 ml/well) previously coated with poly-d-lysine. Under such conditions, glial proliferation was low, and cultures became greatly enriched in neurons, of which ~15% stained positively for ChAT. Cultures were maintained for 4–5 days at 37 °C in a humidified atmosphere containing 5% CO_2/95% air.

Enzyme Activity Assays—ChAT was measured by monitoring conversion of [3H]acetyl-CoA into [3H]acetylcholine, using the method described by Fonnum (24). Acetylcholinesterase (AChE) activity was measured using the colorimetric method of Ellman et al. (25). Data were normalized by protein content, measured with the Lowry method (26) using bovine serum albumin (BSA) as a standard. Results were expressed as percentage of control (vehicle-treated) values. Basal ChAT activity in control cultures was 1350 ± 120 pmol of acetylcholine formed per min per mg of protein (n = 25), similar to the activity observed in intact tissue at the equivalent developmental stage. Average AChE activity was 5.70 ± 0.49 nmol of acetylcholine hydrolyzed per min per mg of protein (n = 9).

Immunocytochemistry—After a brief (30-min) incubation with oligomers, cultures grown on poly-d-lysine coated glass coverslips were washed with phosphate-buffered saline (PBS) and fixed for 10 min with 4% paraformaldehyde containing 1% picric acid in PBS. Fixed cultures were blocked with 5% BSA and 5% normal donkey serum for 1 h, followed by overnight incubation with mouse anti-Aβ-derived diffusible ligands primary antibody NU4 (1:800). For permeabilization, cells were washed with PBS containing 0.01% Tween 20 and incubated again overnight, with goat anti-ChAT primary antibody (1:50). Secondary donkey anti-mouse Alexa Fluor 488 (1:500) and anti-goat Alexa Fluor 555 (1:500) antibodies were used. Nuclei were stained briefly with 4’,6-diamidino-2-phenylindole (DAPI) and coverslips mounted on slides using n-propylgallate.

Viability Assays—MTT reduction assay was performed as previously described (27). Lactate dehydrogenase release was measured using a Promega kit (Madison, WI).

Western Blotting—After treatments, cultures were washed with ice-cold PBS and scraped in radioimmunoprecipitation assay buffer with standard protease inhibitors. Harvested cells were sonicated briefly and centrifuged at 10,000 × g for 5 min at 4 °C. Supernatants were collected and protein concentrations determined using the Bradford method (28). For each sample, 30 μg of protein were run on 12% SDS-polyacrylamide gels, transferred to PVDF membranes, and probed with anti-ChAT antibody (1:500). An anti-α-tubulin antibody (1:50,000) was used as loading control.

Preparation of AβOs—AβOs were prepared weekly as originally described (29) with minor modifications (30). Aβ_1-42 (Bachem, Torrance, CA) was prepared in aliquots as a dried hexafluorosilicopropanol film and stored at −20 °C. The film was resuspended to 5 mM Aβ concentration in anhydrous, sterile dimethyl sulfoxide, diluted in ice-cold PBS to a final concentration of 100 μM, and maintained at 4 °C for 24 h. The preparation was then centrifuged at 14,000 × g for 10 min at 4 °C to remove insoluble aggregates, and the supernatant containing oligomers was stored at 4 °C and used within 2 days. Concentration was determined using the BCA assay and BSA as a standard and is expressed in terms of total Aβ monomer concentration.

Neuronal Oxidative Stress—Formation of reactive oxygen species (ROS) was measured in living neurons using H_2DCFDA, a fluorescent probe sensitive to various ROS species, including hydrogen peroxide, hydroxyl radical, peroxyl radicals, and peroxynitrite, as described previously (10, 31). Cultures incubated for 3 h at 37 °C with 500 nm oligomers or vehicle were loaded with 10 μM H_2DCFDA during the last 40 min of incubation. Cells were rinsed three times with MEM and were immediately imaged on a Zeiss microscope. Analysis of integrated H_2DCFDA fluorescence intensity was carried out using Imagej (National Institutes of Health) (32). In each experiment, two low magnification (∗×200) images were analyzed per experimental condition to allow determination of changes in ROS levels.
**RESULTS**

Our initial experiments aimed to determine whether AβOs could modulate ChAT activity in cultured cholinergic neurons. Exposure of retinal neuronal cultures to increasing concentrations of oligomers for 17 h resulted in dose-dependent inhibition of ChAT activity. Maximal inhibition amounted to ~50% of total ChAT activity and was observed following treatments with oligomer concentrations of 100 nM or higher (Fig. 1A). Shorter incubation periods revealed that inhibition was time-dependent, with maximal inhibition observed after 12 h of exposure to 500 nM AβOs (Fig. 1B). Given the potential cytotoxicity of the Aβ peptide, we examined the possibility that loss of cell viability in cultures might account for loss of enzyme activity. Lactate dehydrogenase release and MTT reduction assays indicated no differences between AβO-exposed and control cultures, even after a longer (24-h) incubation period (Fig. 2, A and B). Inspection of the cultures under the microscope also showed no morphological changes induced by oligomers (Fig. 2E). Moreover, AChE activity and the high affinity hemicholinium-3-sensitive uptake of [3H]choline were unaffected by exposure to AβOs (Fig. 2C and F). These results indicate that cholinergic neurons remained viable throughout the incubation period with oligomers.

Decreased ChAT activity as a result of reduced enzyme expression has been reported previously in postmortem AD brain (34). To evaluate the possibility that a decrease in ChAT expression might be responsible for the decrease in activity observed under our experimental conditions, we compared ChAT levels in cultures exposed for 24 h to 500 nM oligomers (which produces maximal inhibition of enzyme activity) (Fig. 1) versus vehicle-treated control cultures. No differences were found between ChAT levels in oligomer-exposed or control cultures (Fig. 2D).

Overactivation of glutamate receptors has been shown to induce a similar inhibition of ChAT activity (9). In addition, several recent reports indicate that glutamate receptors of both NMDA and non-NMDA types play important roles in the neuronal impact of AβOs (10, 12, 13, 15, 35–37). Thus, we next asked whether the inhibition of ChAT activity by AβOs might be prevented by the NMDA receptor blocker MK-801 and the AMPA receptor antagonist, DNQX. ChAT inhibition was fully prevented in the simultaneous presence of both antagonists (Fig. 3A) as well as by clinically relevant doses of memantine, an NMDA receptor blocker used to treat AD (Fig. 3B). Recent reports have shown that memantine preferentially blocks extrasynaptic over synaptic NMDA receptors, possibly due to their different activity patterns (38). To address the relevance of NMDA receptor type to oligomer-induced ChAT inhibition, we tested ifenprodil, a selective NR2B antagonist (39). At concentrations of 1 and 10 μM, ifenprodil fully blocked the effect of AβOs on ChAT activity.

Excessive production of ROS has been reported as an important mediator of synaptic damage induced by AβOs (10, 13, 40). ROS have been reported to inactivate a number of enzymes, often through the formation of reactive nitrogen species (41) and could possibly be involved in the inactivation of ChAT by EAAs (9). Using the ROS-sensitive fluorescent probe H$_2$DCFDA, we found that incubation with AβOs stimulated ROS formation in retinal neuronal cultures and that this effect was dependent on glutamate receptor activation, as it could be completely blocked by MK-801 and DNQX (Fig. 4).
Consistent with the above findings, polyunsaturated fatty acids (PUFAs), generally regarded as effective antioxidants (42), blocked ChAT inhibition by AβOs. Preincubation with α-linolenic or arachidonic acid completely blocked ChAT inhibition, whereas monounsaturated oleic acid and saturated stearic acid had no effect (Fig. 5A). α-Linolenic acid also prevented oligomer-induced ROS formation (Fig. 4).

Activation of insulin receptor signaling was recently shown to block AβO binding to cultured hippocampal neurons by down-regulating their binding sites on the neuronal surface.
Interestingly, we now found that the inhibition of ChAT activity by oligomers was completely blocked by insulin (Fig. 5B).

As noted above, inhibition of ChAT activity by AβOs was saturable and reached a maximum of ~50%. To investigate the possibility that this could be related to selective inhibition of a subpopulation of cholinergic neurons, we analyzed oligomer binding to neurons in our cultures. Approximately 50% of cultured neurons showed oligomer binding (Fig. 6, A and B). A similar proportion of oligomer-binding neurons was found within the cholinergic population, identified by double labeling with a ChAT antibody, indicating no apparent preferential binding of oligomers toward this neuronal phenotype.

DISCUSSION

Here, we show a marked impact of AβOs on the differentiated cholinergic phenotype of cultured neurons. Oligomers induce a major reduction in ChAT activity in the absence of changes in neuronal viability or in total ChAT expression, whereas other elements of the cholinergic synapse remain unaffected.

Although the exact mechanism of inhibition of ChAT remains to be fully elucidated, oxidative and nitrative stress-related reactions, such as oxidation of cysteine and nitration of tyrosine residues, appear as a possibility. In line with previous findings (10, 13) oligomers instigated an NMDA receptor-mediated robust increase in neuronal ROS levels. Moreover, neuronal nitric-oxide synthase (nNOS) can be readily activated by calcium influx through the NMDA receptor, favoring the formation of strong oxidants, such as peroxynitrite (41). Consistent with this view, antioxidant PUFAs prevented ChAT inhibition, whereas saturated and monounsaturated fatty acids were ineffective. The fact that the number of unsaturations is relevant for the protective effect supports the idea that direct reaction of PUFAs with ROS underlies the protective mechanism. However, indirect protective pathways cannot be ruled out because PUFAs are involved in complex intracellular signaling in the nervous system, promoting neuroprotection through modulation of inflammatory responses (43, 44).

ChAT inhibition was clearly dependent on glutamate receptors, particularly of the NMDA subtype, as it was successfully prevented by MK-801 and memantine, a clinically tolerated NMDA receptor blocker, currently used in AD patients. Notably, the concentration of memantine required to completely prevent inhibition of ChAT by oligomers was 10 μM, the same concentration estimated to be physiologically present after therapeutic dosing (38) and that effectively blocks neuronal oxidative stress induced by oligomers in hippocampal cultures (10). Consistent with recent findings in mouse brain slices, ifenprodil, a selective NR2B antagonist, also prevented ChAT inhibition in oligomer-treated cultures, when used in the low micromolar range, suggesting the participation of NR2B-containing extrasynaptic receptors in this effect (45).

Considering that NMDA receptors have recently been proposed to be part of the receptor complex that binds oligomers (10, 13), it is important to note that none of the antagonists used prevent oligomer binding nor seem to compete for its binding site (10, 46).

It has recently been reported that insulin signaling downregulates oligomer binding sites in cultured hippocampal neurons (40). This drove us to test whether insulin would protect cholinergic neurons from oligomer-induced ChAT inhibition. Interestingly, we found that insulin indeed prevented ChAT inhibition in oligomer-treated cultures. Although it is possible
that down-regulation of oligomer binding sites, as seen in hippocampal cultures, may be at play here, we cannot discard the possibility of alternative mechanisms, including an increase in ChAT expression or activity. Further experiments should address these questions. In any case, these results suggest that bolstering neuronal insulin signaling should be further investi-

**FIGURE 4.** $A_b$Os increase ROS levels in cultured neurons. A, representative images from cultures treated for 3 h with 500 nM $A_b$Os displaying a 4–5-fold increase in ROS levels, as detected by dichlorodihydrofluorescein fluorescence. Pretreatment with either a combination of 5 $\mu M$ MK-801 and 100 $\mu M$ DNQX or 20 $\mu M$ $\alpha$-linolenic acid blocked the increase in ROS induced by oligomers (scale bar, 20 $\mu M$). B, average results from experiments with four independent cultures ($n = 4$; *, $p < 0.01$, one-way ANOVA, Bonferroni’s post hoc test). Error bars, S.E.

**FIGURE 5.** Treatment with PUFAs or insulin prevents ChAT inhibition induced by $A_b$Os. A, cultures pretreated for 1 h with PUFAs ($\alpha$-linolenic acid or arachidonic acid, 20 $\mu M$) retained control levels of ChAT activity after a 17-h exposure to 500 nM oligomers. Pretreatment with 20 $\mu M$ saturated stearic acid or 50 $\mu M$ monounsaturated oleic acid failed to prevent ChAT inhibition caused by oligomers ($n = 3$–10; *, $p < 0.05$, one-way ANOVA, Bonferroni’s post hoc test). Error bars, S.E. B, cultures pretreated for 1 h with insulin (0.1 or 1 $\mu M$) showed no significant loss of ChAT activity after a 17-h exposure to 500 nM oligomers ($n = 3$; *, $p < 0.01$ one-way ANOVA, Bonferroni’s post-hoc test).
gated as a useful approach to prevent cholinergic dysfunction in early stage AD.

Although, under physiological conditions, ChAT is not rate-limiting for acetylcholine release (47), severe inhibition of ChAT might have functional consequences with respect to acetylcholine levels in presynaptic terminals. The fact that 50% of cholinergic neurons in our cultures are attacked by oligomers and that overall ChAT inhibition also reaches 50% suggests that ChAT may actually be completely inhibited in affected neurons. Whether this is the case or not, ChAT inhibition/inactivation induced by AβOs reported here may be an important feature of early stage AD pathology.

FIGURE 6. AβOs bind to cholinergic neurons in culture. Cultures grown on glass coverslips were treated with 500 nM oligomers for 3 h and processed for immunocytochemistry. A–C, oligomer binding was clearly detected on ChAT neurons. Scale bar, 15 μm. D, approximately 50% of total neurons exhibited oligomer binding, the same proportion seen in the ChAT neuronal population (n = 3).

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doi: 10.1074/jbc.M111.321448
originally published online April 13, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M111.321448

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