Apolipoprotein E isoform-specific disruption of phosphoinositide hydrolysis: protection by estrogen and glutathione

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Abstract The mechanism(s) by which the E4 isoform of apolipoprotein E (apoE4) influences Alzheimer's disease (AD) are not fully known. We report that apoE4, but not apoE3, disrupts carbachol-stimulated phosphoinositide (PI) hydrolysis in SH-SY5Y neuroblastoma cells. Carbachol responses were also disrupted by β-amyloid (Aβ) (1–42) and apoE4/Aβ(1–42) complexes, but not by apoE3/Aβ(1–42). Glutathione and estrogen protected against apoE4 and Aβ(1–42) effects, as well as those of H_2O_2. Estrogen protection was partially blocked by wortmannin, suggesting the involvement of phosphatidylinositol 3-kinase. An apoE4-induced disruption of acetylcholine muscarinic receptor-mediated signalling may explain the lower effectiveness of cholinergic replacement treatments in apoE4 AD patients. Also, the beneficial effect of estrogen in AD may be partially due to its ability to protect against apoE4- and Aβ(1–42)-mediated disruption of PI hydrolysis.

Key words: Alzheimer's disease; Apolipoprotein E; β-Amyloid; Phosphoinositide; Estrogen; Glutathione

1. Introduction

A wealth of genetic, histological and biochemical evidence has implicated apolipoprotein E (apoE) in the pathogenesis of Alzheimer's disease (AD). Of the three different apoE isoforms (E2, E3 and E4), the dose of apoE4 both increases the risk and reduces the age of onset for familial and sporadic AD. A number of hypotheses have been proposed to explain the isoform-specific association of apoE and AD. ApoE isoforms may contribute differentially to the disease through isoform-specific interactions with β-amyloid (Aβ), the major component of plaques. ApoE isoforms may also differentially bind and sequester the tau protein component of neurofibrillary tangles to prevent its phosphorylation (for review, see [1]). Accumulating data support the possibility that apoE may also directly influence intracellular signalling cascades. ApoE has been shown to increase intracellular free Ca^{2+} in rat hippocampal astrocytes and neurons [2], and apoE4 has been found to modulate activation of cAMP response element-binding protein through the extracellular signal-regulated kinase cascade [3]. We recently reported that apoE and apoE/Aβ complexes can also induce protein kinase C-α translocation in human neuroblastoma cells and fibroblasts [4].

The neurochemical pathology of AD includes the impairment of a number of signal transduction pathways, including those mediated by acetylcholine. Acetylcholine muscarinic agonist stimulation of phosphoinositide (PI) hydrolysis is reported to be approximately 50% lower in the prefrontal cortex of AD brains as compared to controls [5]. Also, Ladner and colleagues reported a disruption of muscarinic M1 receptor–G-protein coupling in AD brain regions showing extensive senile plaque accumulation [6].

It has also been shown that there is an association between inheritance of apoE4 and the extent of cholinergic dysfunction in AD. ApoE4 carriers with AD show a greater deficit in cholinesterase activity in the hippocampus and cortex [7,8]. Also, the total number of cholinergic neurons is more severely reduced in apoE4 AD subjects [7]. Although muscarinic receptor levels do not differ significantly between AD patients with different apoE genotypes, apoE4 carriers are reported to have poorer responses to acetylcholinesterase inhibitor therapies, as compared to non-apoE4 subjects [8].

Increasing evidence indicates that oxidative stress also plays an important role in AD pathogenesis (for review, see [9]), and indeed may contribute to the cholinergic disruption seen in the disorder, as well as to the mechanism of action of apoE. Aβ has been shown to disrupt agonist-induced muscarinic cholinergic signal transduction by a free radical-mediated mechanism [10]. Miyata and Smith [11] reported a differential antioxidant ability of apoE isoforms to protect against Aβ- and H_2O_2-induced oxidative stress. Recently, Pedersen et al. [12] reported that apoE also has an isoform-specific capability of protecting against lipid peroxidation by isoform-specific binding to 4-hydroxynonenal (HNE), one of its toxic products. In both reports, the different apoE isoforms were seen to be protective with the rank order of E2 > E3 > E4. These in vitro findings are in agreement with AD post-mortem brain studies in which protein modification by HNE [13] and higher oxidative insults [14] are associated with inheritance of the apoE4 allele.

The present study tests the hypothesis that apoE isoforms

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Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; AO, antioxidants; apoE, apolipoprotein E; GSH, glutathione (reduced form); HNE, 4-hydroxynonenal; KHB, Krebs–Henseleit bicarbonate buffer; MEM, minimum essential medium; PG, *n*-propyl gallate (3,4,5-trihydroxybenzoic acid *n*-propyl ester); PI, phosphoinositide; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PLC, phospholipase C

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exert differential effects on acetylcholine muscarinic receptor-mediated signal transduction as a possible consequence of increased oxidative stress. To this end we investigated the effects of variant apoE isoforms on basal and carbachol-stimulated PI hydrolysis in human SH-SY5Y neuroblastoma cells. We also determined the effects of apoE isoforms in complex with Aβ(1–42) as well as Aβ(1–42) alone. Finally, the ability of several antioxidants (AO) to protect against the potential effects of apoE and Aβ(1–42) was studied.

2. Materials and methods

2.1. Materials

Chemicals and isotopes were purchased from the following companies: *myo*-[2-^{3}H]inositol (10 Ci/mmol) from NEN Du Pont Europe; carbamylcholine chloride (carbachol), glutathione (reduced form) (GSH), 17β-estradiol (estrogen), vitamin E (α-tocopherol), *n*-propyl gallate (3,4,5-trihydroxybenzoic acid *n*-propyl ester) (PG) and Dowex 1X8-200 (chloride form) were from Sigma-Aldrich, Sweden; Aβ(1–42) was purchased from U.S. Peptide (CA, USA). Apolipoprotein E3 and E4 isoforms were from Panvera (Madison, WI, USA).

All other chemicals were standard laboratory reagents.

2.2. Cell culture

Human SH-SY5Y neuroblastoma cells were cultured at 37°C, 5% CO_2, in minimum essential medium (MEM) with Earle's salts containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco-BRL Life Technologies, European Division).

2.3. Treatments

Treatments with apoE, Aβ(1–42) and apoE/Aβ(1–42) complexes were done as described in previous studies [4,15]. In brief, Aβ(1–42) was dissolved in serum-free MEM (pH 7.4) at a concentration of 10 μM and 'aged' for 72 h by incubation at 37°C. Human recombinant apoE isoforms were dissolved in serum-free MEM to a concentration of 10 nM. The apoE/Aβ complexes were made by co-incubation at 37°C in neutral pH for 24 h. A concentration of 10 nM apoE (E3 or E4 isoform) was added to 48 h 'aged' 10 μM Aβ(1–42), mixed and incubated together for 24 h at 37°C.

GSH (325 μM), vitamin E (50 μg/ml), and PG (50 μM) were dissolved in serum-free media. Estrogen and wortmannin were first dissolved in dimethyl sulfoxide (DMSO) and then in serum-free medium to 10 μM and 100 nM concentrations, respectively. The final DMSO dilution in the medium was 1:10^4 (v/v). An alternative control group of cells treated with this DMSO concentration was included in the experiments with estrogen and wortmannin.

2.4. PI hydrolysis assay

Cells were cultured until confluence. One day prior to the experiment, cells were changed to serum-free medium containing 5 μCi/ml *myo*-[2-^{3}H]inositol and incubated for 19 h. The medium was then replaced with that for the different conditions plus 5 μCi/ml *myo*-[2-^{3}H]inositol. Cells were incubated in the presence of all treatments for 5 h.

PI hydrolysis was measured essentially as described by Fowler et al. [16]. After treatment, cells were harvested by scraping with a rubber policeman in 4 ml phosphate-buffered saline (PBS). Contents were centrifuged at 1500 rpm for 15 min. Pellets were washed twice with 37°C PBS and re-suspended in 3 ml 37°C Krebs–Henseleit bicarbonate buffer containing 10 mM LiCl (KHB/Li), gassed with 5% CO_2/95% O_2 and centrifuged again (15 000 rpm, 15 min). Cell pellets were re-suspended in 210 μl KHB/Li, regassed and 50 μl added to glass centrifuge tubes containing 250 μl KHB/Li buffer with or without 100 μM carbachol. The tubes were incubated at 37°C under an atmosphere of 5% CO_2/95% O_2 with gentle agitation for 25 min. Incubations were stopped by adding 940 μl of chloroform:methanol (1:2). Tubes were incubated on ice for 30 min and phases separated by adding 310 μl chloroform and 310 μl water followed by vortexing and centrifugation. 750 μl of the aqueous phase was removed and labelled inositol phosphates (IPs) separated from *myo*-[^{3}H]inositol by Dowex chromatography. The chloroform phase was extracted with 75 μl HCl and tubes were vortexed again for 20 s, followed by 5 min centrifugation. The chloroform phase was removed, placed into scintillation vials and allowed to evaporate before determination of 'lipid dpm' by scintillation spectroscopy. Results are expressed as dpm IPs/(dpm IPs+dpm lipid). This unit is independent of the number of cells aliquoted in each tube and of the degree of labelling of inositol phospholipids [16].

2.5. Statistical analyses

Analyses of differences were carried out by ANOVA followed by Fisher's PLSD post-hoc test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of apoE3, apoE4, Aβ(1–42) and apoE/Aβ complexes on PI hydrolysis

Carbachol (100 μM) induced an approximate 2.5-fold elevation in the cytosolic accumulation of IPs in SH-SY5Y neuroblastoma cells (Fig. 1A). Five hour treatment with 10 nM apoE3 had no effect on either basal or carbachol-stimulated PI hydrolysis. In contrast, 5 h treatment with 10 nM apoE4 significantly reduced the carbachol-stimulated accumulation of IPs, with no effect on basal responses (Fig. 1A). Carbachol-stimulated PI hydrolysis responses were also reduced by 10 μM Aβ(1–42). Complexes of apoE4/Aβ significantly reduced both basal and carbachol-stimulated PI hydrolysis, whereas apoE3/Aβ complexes were without effect (Fig. 1A,B).

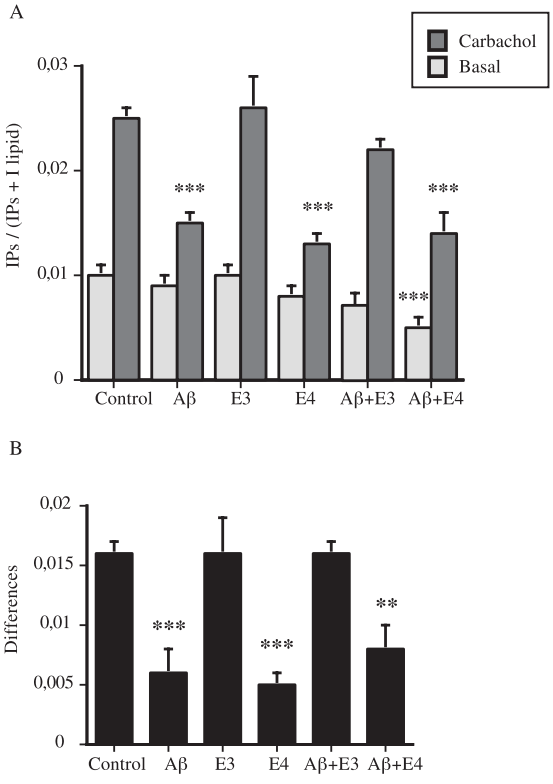

Fig. 1. Effects of 10 μM Aβ(1–42), 10 nM apoE3, 10 nM apoE4, apoE3/Aβ(1–42), and apoE4/Aβ(1–42) complexes on basal and carbachol (100 μM)-stimulated PI hydrolysis in SH-SY5Y neuroblastoma cells. Cells were treated for 5 h. Histograms show mean ± S.E.M. of at least five independent experiments. A: Basal and carbachol-stimulated PI hydrolysis. B: The differences (carbachol-stimulated minus basal). Statistical analysis of the results was carried out using ANOVA followed by Fisher's post-hoc test. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to controls (untreated cells).

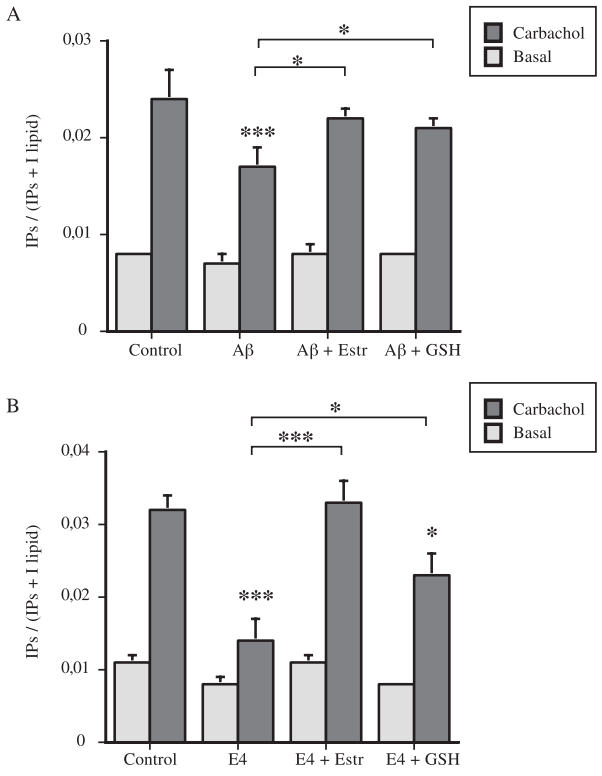

Fig. 2. Estrogen (10 μM) and glutathione (GSH) (325 μM) protect against the disruption of carbachol-stimulated PI hydrolysis due to Aβ(1–42) (10 μM) (A) and apoE4 (10 nM) (B). Histograms represent means ± S.E.M. of three independent experiments with data of basal and carbachol-stimulated PI hydrolysis. Significances were determined using ANOVA followed by Fisher's post-hoc test. $*P < 0.05$, $***P < 0.001$.

3.2. Estrogen and glutathione protect against Aβ(1–42)- and apoE4-mediated disruption of PI hydrolysis

To determine whether the effects of Aβ(1–42) and apoE4 on PI hydrolysis were likely due to induction of oxidative stress, we used a number of AO compounds in co-treatment with either 10 μM Aβ or 10 nM apoE4 for 5 h. Neither vitamin E (50 μg/ml) nor PG (50 μM) was able to reverse the deleterious effects of either Aβ(1–42) or apoE4 on carbachol-stimulated PI hydrolysis (data not shown). In contrast, both 10 μM estrogen and 325 μM GSH gave protection against the disruptive effects of Aβ(1–42) (Fig. 2A) and apoE4 (Fig. 2B). No effects were found in cells treated with either estrogen or GSH alone (data not shown). DMSO ($1{:}10^4$, v/v) had no effect on basal or carbachol-stimulated PI hydrolysis (data not shown).

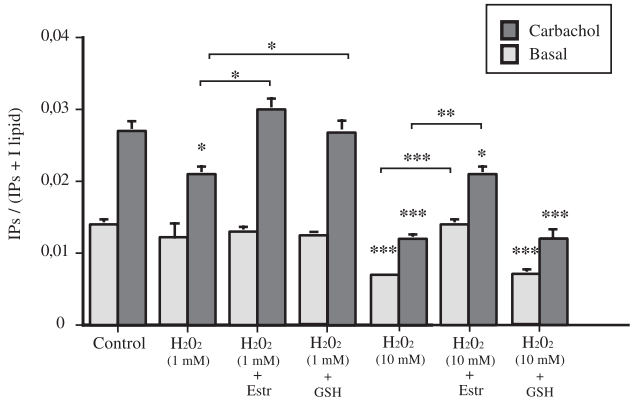

Fig. 3. Estrogen (10 μM) and glutathione (GSH) (325 μM) effects on H_2O_2-mediated disruption of PI hydrolysis. Histograms represent means ± S.E.M. of three independent experiments with data of basal and carbachol-stimulated PI hydrolysis. Significances were determined using ANOVA followed by Fisher's post-hoc test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

We then tested whether the protective effects of estrogen were likely due to its antioxidant properties. For this we used the oxidative agent H_2O_2, which has been shown to inhibit carbachol-stimulated PI hydrolysis in SH-SY5Y neuroblastoma cells [17]. Five hour treatment with 1 mM H_2O_2 significantly reduced the response to carbachol with no effect on basal IP levels. A higher concentration of H_2O_2 (10 mM) reduced both basal and carbachol-stimulated PI hydrolysis (Fig. 3).

GSH (325 μM) gave protection against the H_2O_2 (1 mM)-mediated disruption of PI hydrolysis, but not against that induced by 10 mM H_2O_2 (Fig. 3). Estrogen (10 μM) protected against the disruption of carbachol-stimulated PI hydrolysis induced by 1 mM H_2O_2 and also partially that caused by 10 mM H_2O_2 (Fig. 3).

Since it has been reported that activation of phosphatidylinositol 3-kinase (PI3K) mediates the neuroprotective effect of estrogen against glutamate-induced neurotoxicity [18], we also

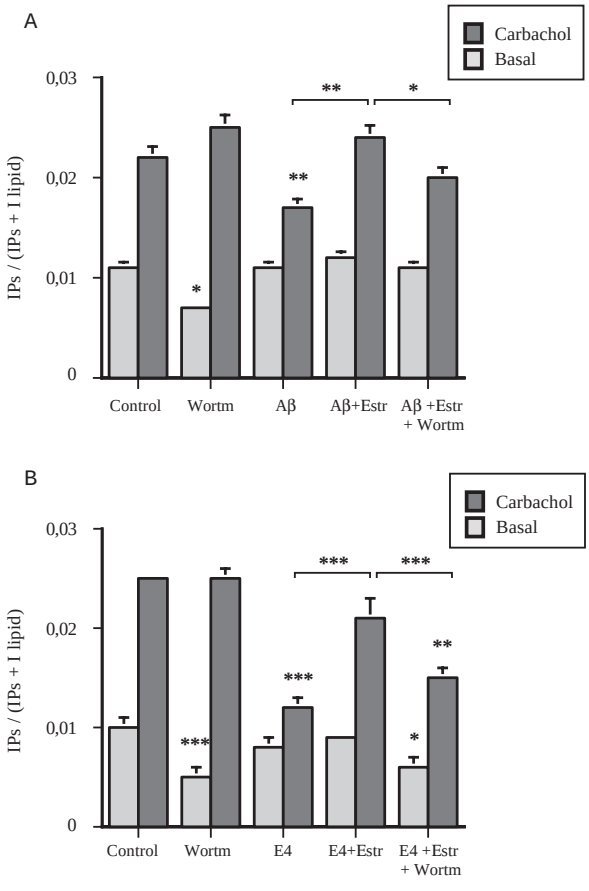

Fig. 4. Wortmannin (100 nM) blocks the protective effects of estrogen (10 μM) against the disruption of carbachol-stimulated PI hydrolysis caused by Aβ(1–42) (10 μM) (A) and apoE4 (10 nM) (B). Histograms represent means ± S.E.M. of three independent experiments with data of basal and carbachol-stimulated PI hydrolysis expressed. Significances were determined using ANOVA followed by Fisher's post-hoc test. $*P < 0.05$, $***P < 0.001$.

performed experiments with the PI3K inhibitor wortmannin (100 nM) to determine whether a similar mechanism mediated the protective effect of estrogen in our paradigm. As shown in Fig. 4, treatment of SH-SY5Y cells with wortmannin alone decreased basal, but had no effect on carbachol-stimulated PI hydrolysis. Data from cells treated with the combination of 10 μM estrogen, 100 nM wortmannin and either 10 nM apoE4 or 10 μM Aβ(1–42) showed that basal PI hydrolysis was significantly decreased, whereas the protective effect of estrogen on apoE4- or Aβ(1–42)-induced disruption of carbachol-stimulated PI hydrolysis was partially reversed by wortmannin (Fig. 4A,B).

4. Discussion

The association of apoE4 with sporadic and familial AD is well demonstrated, although the mechanism(s) by which apoE4 influences the pathology and onset of disease are not fully understood. In the complexity of the AD scenario, both cholinergic impairment and increased oxidative stress are believed to play important roles. Muscarinic agonist stimulation of PI hydrolysis is impaired in AD brain, and can also be disrupted by Aβ in vitro. The present study addresses the issue of whether variant apoE isoforms differently influence acetylcholine muscarinic receptor-mediated signalling.

We first demonstrated that a physiological concentration (10 nM) of apoE4 could disrupt carbachol-induced PI hydrolysis in human SH-SY5Y neuroblastoma cells. In contrast, apoE3 was without effect, indicating selectivity for the pathogenic E4 variant of the protein.

Independent studies have shown that presynaptic cholinergic markers, such as choline acetyltransferase activity and immunoreactivity, as well as levels of nicotinic and nerve growth factor receptors are reduced to a greater extent in apoE4 AD patients, as compared to non-apoE4 individuals [7,8]. The association between cholinergic dysfunction and apoE4 was also found to have an impact on responses to therapies based on the enhancement of cholinergic neurotransmission [8]. An impaired lipid delivery to the cholinergic system in apoE4 carriers has been proposed as one mechanism for this association [7]. Our data indicate that the apoE4 isoform may also have a direct negative effect on cholinergic signalling.

To further investigate these effects in the context of AD, we studied the effects of both apoE isoforms on the Aβ(1–42)-induced disruption of PI hydrolysis. Aβ has been previously found to decrease carbachol-stimulated signalling in cultured rat cortical neurons [10]. In agreement with this, we found that 10 μM Aβ(1–42) significantly decreased the responses to carbachol in human SH-SY5Y neuroblastoma cells. No effect was seen when Aβ(1–42) was in complex with apoE3 (10 nM), suggesting that apoE3 may exert some protective mechanism against the Aβ(1–42)-mediated disruption of PI hydrolysis. In contrast, apoE4/Aβ(1–42) complexes decreased both basal and carbachol-stimulated PI hydrolysis in SH-SY5Y cells, suggesting some synergy of their respective effects.

We also hypothesized that the different ability of variant apoE isoforms to disrupt carbachol-stimulated PI hydrolysis could be due to differential abilities to induce oxidative stress. Oxidative agents have been shown to inhibit acetylcholine receptor-stimulated phosphoinositide signalling and even to reduce the G-protein sensitivity of muscarinic receptors in a number of in vitro models (for review see [19]), including in SH-SY5Y neuroblastoma cells [17]. Another line of investigation proposes that the mechanism of apoE isoform association with AD involves a differential involvement of variant apoE isoforms on neurotoxicity [20]. Post-mortem studies have shown that protein modification by HNE [13] and higher oxidative insults [14] in AD are associated with inheritance of the apoE4 allele. ApoE4 has also been shown to be more sensitive to oxidation than E3 [21]. On the other hand, apoE3 has been shown to have a better antioxidant ability than E4 [11,12]. In view of this, it is reasonable to suggest that a different involvement of oxidative stress could explain the differential effects of apoE isoforms on PI hydrolysis. In this context, apoE3 would be expected to protect against the effects of Aβ(1–42) due to its reported antioxidant properties.

To determine if oxidative stress could mediate the apoE4- and Aβ(1–42)-induced disruption of carbachol-stimulated PI hydrolysis, we tested the ability of a number of compounds with known AO properties to protect against these insults. We found that GSH and estrogen were effective, whereas neither vitamin E, an inhibitor of lipid peroxidation, nor PG, an inhibitor of lipid peroxidation and of superoxide radical-induced oxidation, showed any protective effect. Others have shown that pretreatment (16 h) of cortical neurons with vitamin E or PG can prevent Aβ(25–35)-induced impairment of carbachol-stimulated calcium responses in rat cortical neurons [10]. We chose not to pretreat cells with AO so as to avoid potential effects on the incorporation of *myo*-[2-^{3}H]inositol that could complicate comparison of the PI hydrolysis data. The fact that GSH, an important intracellular AO against a variety of oxidative species, protected completely against Aβ(1–42) and partially against apoE4 disruptions of carbachol-stimulated PI hydrolysis demonstrates that oxidative stress is involved in the effects of those two molecules. Both GSH and estrogen can protect human fibroblasts against the disruptive effects of Aβ(1–42) and apoE4 on bradykinin-stimulated PI hydrolysis (data not shown), suggesting that effects may be consistent between different cell types.

The potent protective effect of estrogen against the detrimental effects of apoE4 and Aβ(1–42) on PI hydrolysis is in contrast to the fact that only GSH (used at a super-physiological concentration of 325 μM) and not the other classical AOs vitamin E and PG showed protection. This finding could have particular importance since estrogen replacement therapy in postmenopausal women is associated with a reduced risk for AD (for review, see [22]), and also enhances the responses to acetylcholinesterase inhibitor treatments in women with AD [23]. Estrogen is a multifaceted hormone that has many cellular effects, including interaction with second messenger cascades and neuroprotection. We investigated if the potent protective effect of estrogen could be mediated by mechanisms other than its AO properties. The AO effects of estrogen are generally seen with μM concentrations, while other neuroprotective effects are often found with significantly lower concentrations (for review, see [22]).

The possibility that PI3K could mediate the protective effects of estrogen was tested using the specific inhibitor wortmannin. In PI metabolism, phosphatidylinositol 4,5-bisphosphate (PIP2) is a substrate for both phospholipase C (PLC) and PI3K. PI3K phosphorylates PIP2 to phosphatidylinositol 3,4,5-trisphosphate (PIP3), whereas PLC hydrolyzes this lipid to produce diacylglycerol and IP_3. Molecular cloning has revealed three major families of PLC, β, γ and δ (for review, see

[24]). Estrogen has also been shown to use PLC-β to increase intracellular calcium concentrations via rapid IP_3 formation [25]. Recent works have demonstrated cross-talk between both signalling pathways. The PI3K product, PIP3, binds to PLC-γ and enhances its activity [26]. In addition, PLC-γ can be regulated by tyrosine protein kinases that also activate PI3K (for review, see [24]). It has also been reported that tyrosine kinase inhibitors can block the rapid generation of IP_3 induced by estrogen in a human hepatoma cell line [27]. Furthermore, activation of PI3K mediates the neuroprotective effect of estrogen against glutamate-induced neurotoxicity in cultured cortical neurons [18]. We found that wortmannin blocked the protective effect of estrogen against both apoE4- and Aβ(1–42)-induced disruption of carbachol-stimulated PI hydrolysis. This demonstrates that estrogen protection is mediated at least in part by activation of PI3K. The decrease in basal PI hydrolysis after wortmannin treatment is also consistent with the inhibition of PLC-γ-mediated production of IPs.

In summary, the present report shows that apoE has isoform-specific effects on acetylcholine muscarinic receptor-stimulated PI hydrolysis. We show that apoE4 impairs this signalling pathway, while apoE3 alone is without effect and may indeed have a protective effect against Aβ(1–42)-mediated disruption. We also show that estrogen prevents the apoE4- and Aβ(1–42)-induced impairment by a mechanism which likely involves its AO effect as well as PI3K activation.

This is to our knowledge the first study showing that apoE has isoform-specific effects on acetylcholine muscarinic signal transduction. Although we are aware of the limitations of an in vitro model, our findings may help to explain the lower effectiveness of cholinergic replacement therapies in apoE4 AD patients. This study also provides new insight into the mechanism of action of estrogen.

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