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ORIGINAL RESEARCH

Effects of Acetylcholine on β-Amyloid-Induced cPLA2 Activation in the TB Neuroectodermal Cell Line: Implications for the Pathogenesis of Alzheimer's Disease

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Abstract The role of β-amyloid (Aβ) in the pathogenesis of Alzheimer's disease (AD) is still considered crucial. The state of Aβ aggregation is critical in promoting neuronal loss and neuronal function impairment. Recently, we demonstrated that Acetylcholine (ACh) is neuroprotective against the toxic effects of Aβ in the cholinergic LAN-2 cells. In biophysical experiments, ACh promotes the soluble Aβ peptide conformation rather than the aggregation-prone β-sheet conformation. In order to better understand the biological role of ACh in AD, we studied the effect of Aβ on the phosphorylation of the cytosolic phospholipase A2 (cPLA2) in the TB neuroectodermal cell line, which differentiates toward a neuronal phenotype when cultured in the presence of retinoic acid (RA). We chose the phosphorylated form of cPLA2 (Ser505, Phospho-cPLA2) as a biomarker to test the influence of ACh on the effects of Aβ in both undifferentiated and RA-differentiated TB cells. Our results show that TB cells are responsive to Aβ. Moreover, in undifferentiated cells 1 h treatment with Aβ induces a 2.5-fold increase of the Phospho-cPLA2 level compared to the control after 24 h in vitro, while no significant difference is observed between Aβ-treated and non-treated cells after 4 and 7 days in vitro. The RA-differentiated cells are not sensitive to Aβ. In TB cell line ACh is able to blunt the effects of Aβ. The ability of ACh to protect non-cholinergic cells against Aβ reinforces the hypothesis that, in addition to its role in cholinergic transmission, ACh could also act as a neuroprotective agent.

Keywords Alzheimer's disease · β-Amyloid · Phospholipase A2 · Acetylcholine · TB cell line · Differentiation

Introduction

Until the early 1980s, Alzheimer's disease (AD), the most common form of age-related dementia (Fiandaca et al. 2014) was attributed to a cholinergic deficit due to degeneration of the cholinergic projections from the basal forebrain (nucleus basalis magnocellularis of Meynert) to the cortex and hippocampus (Davies and Maloney 1976). Subsequently, when it became clear that the pathogenesis of the disease was more complex than a simple neurotransmitter deficit, the so-called amyloid cascade hypothesis (Hardy and Higgins 1992) became the main research paradigm in AD pathogenesis. A more recent notion of amyloid hypothesis claims that AD is the result of the overproduction and/or impaired clearance of β-amyloid protein (Aβ) that represents the principal component of senile plaques (SP) (Selkoe 2001). However, amyloid-independent hypothesis has also been proposed (Pimplikar et al. 2010); (Sorrentino et al. 2008).

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Aβ is the product of the proteolytic cleavage of the amyloid precursor protein (APP), a ubiquitous, glycosylated, sulfated, and phosphorylated integral membrane protein (Weidemann et al. 1989). It was recently demonstrated that in cerebrospinal fluid (CSF) at least 18 different forms of Aβ are present. However, $A\beta_{1-40}$ and $A\beta_{1-42}$ have been the primary focus for AD pathogenesis (Portelius et al. 2006). Aβ peptides are soluble molecules that, in response to environmental factors, may aggregate into soluble, low molecular weight oligomers and higher molecular weight protofibrillar oligomers, which, in turn, give rise to the insoluble fibrils that form SP (Baglioni et al. 2006); (Kayed et al. 2009). Aβ oligomers have been recognized as the primary toxic species responsible for the neuronal loss in AD (Haass and Selkoe 2007). Although the role of Aβ in AD pathogenesis is supported by different lines of evidence, its molecular basis is yet to be cleared. There is considerable evidence that the extracellular Aβ accumulation triggers a variety of membrane events. Aβ activates phospholipases A2, C, and D in the cholinergic human neuroblastoma cell line LAN-2 (Singh et al. 1997); (Kanfer et al. 1998). This observation has been recently confirmed (Desbène et al. 2012). The systematic activation of these three phospholipases by Aβ is expected to liberate several biologically active lipidic second messengers. Sustained phospholipase activation also increases the rate of phospholipid catabolism (Kanfer et al. 1999). The continuously increased second messenger generation and phospholipid breakdown would eventually become deleterious to the cell (Abramov et al. 2004). Hence, there is increasing interest about the identification of new AD biomarkers based on the detection of phospholipids in the plasma (Mapstone et al. 2014).

The pathophysiological properties of Aβ can be studied through the use of synthetic peptides. The specific folding of these peptides is critical, and in experimental studies shorter Aβ can be used. $A\beta_{25-35}$ is a synthetic peptide of 11 amino acids that corresponds to a fragment of $A\beta_{1-40}$ and $A\beta_{1-42}$, and is an intermembrane domain of APP (Kang et al. 1987). This shorter peptide may represent the core and the biologically active region of the full-length toxic peptide $A\beta_{1-42}$ because it shows similar fibrillization through β-sheet formation (Naldi et al. 2012). $A\beta_{25-35}$ is often selected as a model for full-length Aβ because it retains both its physical and biological properties, while its short length readily allows derivatives to be synthesized and studied (Hughes et al. 2000). For a comprehensive review on the topic see also (Kaminsky et al. 2010).

Among the different classes of phospholipases, the phospholipase A2 (PLA2) plays an important role in the manifestation and progression of AD, mediating processes such as inflammation (Zhu et al. 2006); (Sanchez-Mejia et al. 2008), oxidative stress (Lee et al. 2011), mitochondrial dysfunction (Lee et al. 2011), intracellular trafficking, differentiation, proliferation, and apoptosis (Sun et al. 2004). In particular, the cytosolic calcium-dependent phospholipase A2 (cPLA2) and the calcium-independent one (iPLA2) regulate the loss of mitochondrial membrane potential, the mitochondria swelling, and the production of reactive oxygen species (ROS) induced by Aβ (Zhu et al. 2006); (Lee et al. 2011).

The amyloidogenic and cholinergic hypotheses, while being apparently different, share molecular pathways that converge at several points. The cholinergic hypothesis has therefore been revisited and studies have demonstrated that deficits in cholinergic neurotransmission are generally correlated with the impairment of neuronal homeostasis in the hippocampus in AD brain (Bartus 2000); (Craig et al. 2011). Moreover, Aβ negatively regulates the synthesis and release of acetylcholine (ACh) from the basal forebrain cholinergic system (Kar et al. 1996); (Vaucher et al. 2001). It has been shown that the aggregation of Aβ peptides to form Aβ oligomers compromises ACh transmission, inducing cellular dysfunction, an imbalance in neurotransmitter signaling and, ultimately, the appearance of neurological signs (Sorrentino et al. 2014). Several cholinesterase inhibitors are used in anti-Alzheimer therapy eliciting anti-amyloid effects even by a regulation of Aβ oligomerization (Wattmo et al. 2012).

Based on the amyloid cascade hypothesis, many substances able to control the Aβ conformation have been screened (Stains et al. 2007). These possible drug candidates interact with Aβ monomers to prevent aggregation or to accelerate the formation of fibrils in order to reduce the lifetime of toxic oligomers. Using the circular dichroism analysis, we have recently showed that ACh prevents the formation of the toxic oligomeric/fibrillar species. Moreover, we demonstrated that ACh is able to blunt the effects of Aβ on cPLA2 in the cholinergic neuroblastoma LAN-2 cells (Grimaldi et al. 2016).

We have previously established a neuroectodermal cell line (TB) from a cerebrospinal fluid specimen of a patient with clinical diagnosis of primary leptomeningeal melanomatosis (Sorrentino et al. 1999). In TB cells no Choline Acetyltransferase (ChAT) activity was found, whereas significant amounts of serotonin (5HT) and its metabolite, 5-hydroxyindoleacetic acid (5HIAA), were detected. These cells are a good model of differentiation toward a neuronal phenotype, as they express only the low molecular weight neurofilament protein (NF-L) in the basal condition, while the treatment for 4 and 7 days with retinoic acid (RA), but not with NGF, induces the appearance of the middle (NF-M) and the high molecular weight neurofilaments (NF-H), respectively. Therefore, TB cells represent a suitable model system to study the effect of pharmacological molecules occurring during the critical phases of neuronal differentiation in vitro.

The present work deals with the influence of the neuronal differentiation in a non-cholinergic cell line such as TB cells, on both the Aβ-induced cPLA2 activation and the ability of ACh to blunt this activation. More specifically, we compare the effect of Aβ on cPLA2 in RA-differentiated and undifferentiated TB cells to better understand the mechanisms of action of Aβ and ACh. Furthermore, we studied the effects of ACh on cPLA2 activation induced by Aβ in the function of the neuronal phenotype.

Materials and Methods

TB Cell Line

TB cells were isolated from a cerebrospinal fluid specimen obtained by a lumbar puncture performed for diagnostic purposes on a patient with clinical diagnosis of primary leptomeningeal melanomatosis (Sorrentino et al. 1999). They were grown in Leibovitz's L-15 Medium, GlutaMAX Supplement (*Gibco*), to which 15% Fetal Bovine Serum (FBS, *Gibco*) and 1% antibiotic solution containing 10,000 U/mL of penicillin and 10,000 µg/mL of streptomycin (*Gibco*) were added. Cells were grown in 25 cm^2 flasks (*Sarstedt*) and incubated at 37 °C in the presence of 5% CO_2.

When a highly uniform population of growing cells was observed (doubling time: 18 ± 2 h), we collected and determined the number of TB cells using a Burker counting chamber. The cells were seeded 24 h before the treatment with retinoic acid (RA) in number of 1.5×10^6, 5×10^5, and 2×10^5 cells/flask when they were grown in vitro for 1, 4, and 7 days, respectively.

Treatment with Retinoic Acid, $A\beta_{25–35}$, and ACh

After 24 h in vitro, the growing medium was replaced with fresh medium containing 10 µM RA in 0.1% ethanol to differentiate the cells for 7 days. Cells treated with 0.1% ethanol were used as control (undifferentiated). After 1, 4, and 7 days in vitro, TB cells were incubated for 1 h with 20 µM $A\beta_{25–35}$. $A\beta_{25–35}$ is the shortest toxic fragment corresponding to the amino acids 25–35, which encompasses the β-sheet of the full protein and is considered the biologically active region of the full-length peptide $A\beta_{1–42}$ (Abramov et al. 2004); (Kaminsky et al. 2010). The $A\beta_{25–35}$ amyloid peptide, GSNKGAIIGLM, was manually synthesized by conventional solid-phase chemistry using the Fmoc/t-Bu strategy (Chan and White 2000) and its lyophilized pellet (100 µg) was dissolved in 10 mM phosphate buffered saline (PBS) pH 7.4 up to a concentration of 100 µM Aβ. TB cells were treated with 5 or 20 µM Aβ and 25 µM ACh for 1 h. In fact, it is known that in a physiological solution ACh interacts with the peptide Aβ, by altering its conformation and favoring its more soluble and toxic form, the monomers (Grimaldi et al. 2016). Cells treated with PBS were used as control. After 1 h treatment by incubating cells at 37 °C in a 5% CO_2 environment, TB cells were collected mechanically and all collected pellets were stored at −20 °C.

Furthermore, cell viability was assessed using Trypan Blue Solution, 0.4% (*Gibco*) for the dye exclusion test after 24 h in vitro with 10 µM RA and then adding 20 µM Aβ for 1 h treatment.

Protein Extraction

Total proteins were isolated from collected pellets using a lysis buffer composed of 1X Protease Inhibitor Cocktail Tablets (*Roche*), 10 mM Hepes pH 7.5, 0.5 mM Dithiothreitol, 1.5 mM $MgCl_2$, 10 mM KCl. We used 5 volumes of lysis buffer to extract total proteins from the cells, then the samples were kept on ice for 10 min and centrifuged at 16,000×*g* for 5 min. The supernatant was recovered and the protein concentration was measured using the Bradford assay.

Bradford Assay

To determine total protein concentrations, the reagent Bio-Rad Protein Assay Dye Reagent Concentrate 5X (*Bio-Rad*) was used.

Protein Electrophoresis under Denaturing Conditions

After determining the concentration of total protein lysates, total proteins were separated according to their molecular weight by electrophoresis on a pre-formed gel in a polyacrylamide gradient (Bolt 4–12% Bis–Tris Plus Gel, *Life Technologies*) under denaturing conditions (SDS-PAGE).

We prepared samples containing 20 µg of total proteins, to which a loading buffer (Bolt LDS Sample Buffer 4X, *Life Technologies*) was added. Samples were incubated at 70 °C for 10 min to facilitate the denaturation process, then they were kept on ice for 5 min and centrifuged at 16,000×*g* for 1 min before loading them on the gel. A marker of molecular weight composed of ten bands in the range 4–250 kDa (SeeBlue Plus2 Pre-Stained Standards, *Life Technologies*) was used to follow the electrophoretic run and to identify the bands of interest (about 100 kDa for Phospho-cPLA2, about 30 kDa for RPL7). The electrophoretic run was carried out at constant voltage of 165 V for 30–40 min in a support Bolt Mini Gel Tank (*Life Technologies*), using the 20X Bolt MES SDS Running Buffer (*Life Technologies*) at a final concentration of 1X.

Western Blotting

The phosphorylated form of the cytosolic phospholipase A2 (Ser505, Phospho-cPLA2) has been chosen as a biomarker in our investigation. In fact, Ser505 is a consensus site for phosphorylation by mitogen-activated protein kinases (MAPKs), which activates cPLA2 (Sun et al. 2004) allowing its multiple functions. After protein separation, they were transferred from the polyacrylamide gel to a nitrocellulose membrane using the iBlot Gel Transfer Stacks Nitrocellulose Mini kit (*Life Technologies*). We used iBlot by *Invitrogen* for a dry transfer, applying a constant potential difference of 20–25 V for 8 min. After the transfer of the proteins, the nitrocellulose membrane was incubated in a blocking solution composed of 5% milk powder (Blotting Grade Blocker, *Bio-rad*) dissolved in PBS for 1 h at room temperature under gentle agitation. Subsequently, the membrane was incubated overnight at 4 °C with the primary antibodies Anti-PLA2G4/PLA2G4 Antibody (phospho-Ser505) (*LSBio,* diluted 1:500) and Anti-RPL7/L7 Antibody (aa 199-248) IHC-plus (*LSBio*, diluted 1:1000). Anti-rabbit IgG HRP conjugated by *R&D Systems* diluted 1:1000 was used as secondary antibody, incubating the membrane for 1 h at room temperature. The detection of the proteins on the membrane was performed using the Clarity Western ECL Substrate kit (*Bio-rad*). The signals were captured using the Alliance Mini (*UVITEC Cambridge*) system. The quantization of the bands was carried out by the UVITEC software and the intensity of each band of interest (Phospho-cPLA2) was normalized comparing it to the housekeeping ribosomal protein RPL7, used as loading control. Subsequently, the intensity of each tested band was compared to negative controls and any change was expressed as a percentage.

Statistical Analysis

The experiments were performed in triplicate and repeated at least three times. The data were expressed as the mean ± SD. The differences between treatments were examined using Student's *T* test and statistical significance was set at $p < 0.05$ (*) and $p < 0.01$ (**).

Results

The Influence of Differentiation on the Level of Phospho-cPLA2

In order to understand whether the differentiation process towards a neuronal phenotype alone could affect the level of phospho-cPLA2, we measured the phosphorylated form of cPLA2 in RA-differentiated and undifferentiated TB cells. TB cells were grown in the presence of 10 μM RA up to 7 days.

The Western blot analysis using a primary antibody specific for the phosphorylated form of cPLA2 (Ser505) showed no significant differences between undifferentiated and RA-differentiated TB cells up to 7 days (Fig. 1). Nevertheless, the level of phosphorylation of cPLA2 decreased in function of the days in vitro. Our results showed that the differentiation process toward a neuronal phenotype of TB cells did not produce any significant effect on the level of the phosphorylated form of cPLA2.

Sensitivity of TB Cells to Aβ

We recently observed that in cholinergic neuroblastoma LAN-2 cells, 1 h treatment with 5 μM Aβ increases the phosphorylated form of cPLA2 2.5 fold compared to the control (Grimaldi et al. 2016). We now tested the sensitivity of the non-cholinergic TB cell line to the effect of Aβ. Two different concentrations of Aβ (5 and 20 μM) were used on undifferentiated TB for 1 h treatment after 24 h in culture. 5 μM Aβ failed to produce any significant effect on the level of the phosphorylated form of cPLA2, while 20 μM Aβ increased the level of the enzyme roughly 1.5 fold as compared to the control (Fig. 2). These results would suggest that the serotoninergic TB cells are responsive to Aβ although to a lower extent than the cholinergic LAN-2 cells.

The Influence of Differentiation on Aβ-Induced cPLA2 Activation

The possible influence of the differentiation process on the responsivity to Aβ of TB cells was evaluated assaying the levels of the phosphorylated form of cPLA2 in RA-differentiated and undifferentiated TB cells treated with Aβ.

We tested the effect of 20 μM Aβ on TB cells grown in vitro with and without RA up to 7 days. In undifferentiated cells, Aβ increased the phosphorylation of cPLA2 2.5 fold compared to the control after 24 h in vitro, while after 4 and 7 days in vitro no significant difference was observed between Aβ-treated and non-treated cells. In RA-differentiated cells, Aβ did not affect the level of cPLA2 phosphorylation at any of the three tested time points (Fig. 3). Our data showed that the stronger effect of Aβ on cPLA2 was present in the undifferentiated cells grown in vitro for 1 day compared to the cells grown for longer time (4 and 7 days). Moreover, the undifferentiated cells were more responsive to Aβ effect than differentiated ones. Furthermore, we assessed cell viability by the dye exclusion test using Trypan Blue solution 0.4% after 24 h in vitro with 10 μM RA (85% viability, data not shown) and then adding 20 μM Aβ for 1 h treatment (80% viability, data not shown).

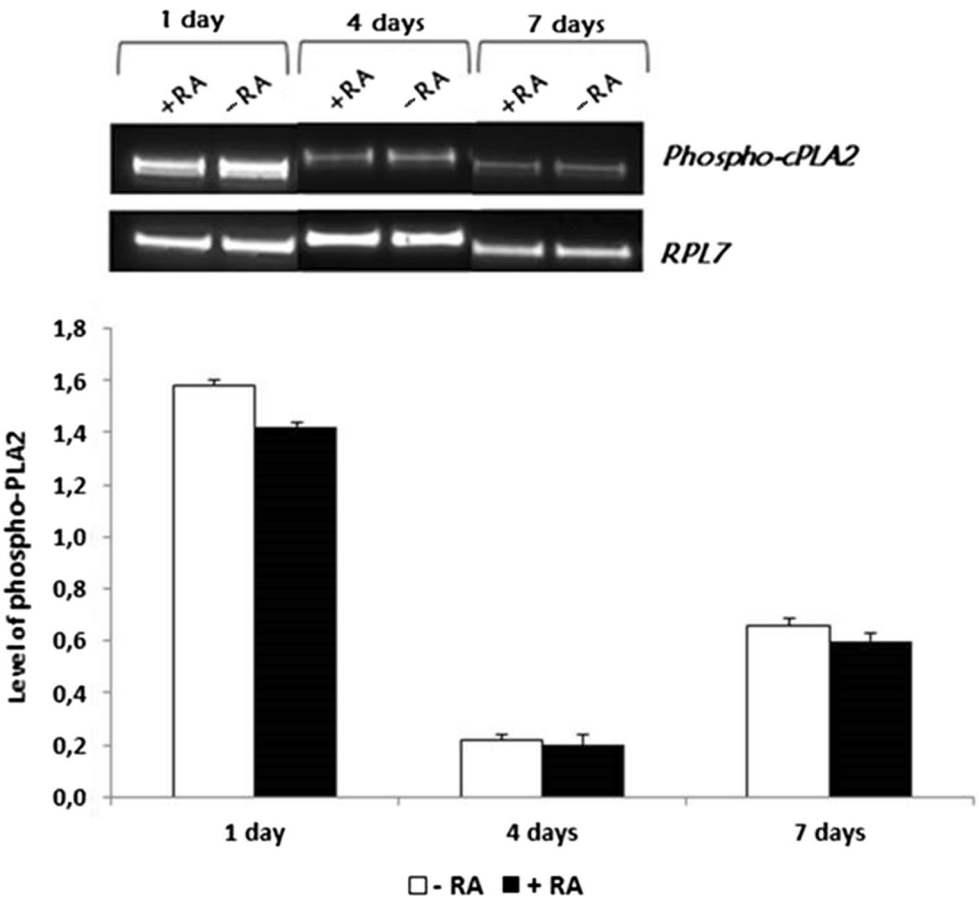

Fig. 1 Influence of differentiation on the level of phospho-cPLA2. Total proteins extracted from each sample were loaded (20 µg) on a pre-formed gel in a polyacrylamide gradient under denaturing conditions (SDS-PAGE). The Western blot analysis using a primary antibody specific for the phosphorylated form of cPLA2 (Ser505, Phospho-cPLA2) showed no significant differences between undifferentiated (−RA) and 10 µM RA-differentiated (+RA) TB cells up to 7 days in culture. The housekeeping ribosomal protein RPL7 was used as loading control. Details in the text

Role of ACh on Aβ-Induced cPLA2 Activation

We have previously shown that in the cholinergic LAN-2 cell line, 25 µM ACh was able to blunt the Aβ-induced increase of the phosphorylated form of cPLA2 (Grimaldi et al. 2016). We verified whether, even in a non-cholinergic cell line such as TB cells, ACh interferes with the ability of Aβ to increase the activation of cPLA2. We evaluated the role of ACh on Aβ effects in TB cells grown without RA for 24 h and then treated for 1 h with Aβ (Fig. 4). In these experimental conditions 20 µM Aβ increased the phosphorylation of cPLA2 by about 60% compared to the control, while 25 µM ACh blunted this cytotoxic effect. ACh alone had no effects on cPLA2 activation (Fig. 4).

This experiment was repeated on 24 h RA-differentiated TB cells. As we have seen before, in RA-differentiated cells Aβ did not affect the level of cPLA2 phosphorylation. ACh alone had no effects on TB cells (Fig. 5).

These experiments showed that in TB cell line ACh, similarly to LAN-2 cells, is able to blunt the ability of Aβ to increase the level of phospho-cPLA2.

Discussion

We have previously provided evidence that ACh favors the soluble conformation of the Aβ peptide, possibly through a reciprocal direct interaction, and exerts a neuroprotective effect against the neuroinflammatory and toxic effects of Aβ. More specifically, ACh is able to blunt the Aβ-induced cPLA2 activation in the LAN-2 cholinergic human neuroblastoma cell line (Grimaldi et al. 2016). In the present work, our interest was to evaluate the biological behavior with respect to both the ability of Aβ to activate cPLA2 and that of ACh to blunt this activation in the TB cell line (that, differently from LAN-2 cells, is non-cholinergic and acquires a neuronal phenotype as a function of time when cultured with RA).

We tested the effect of differentiation per se on the level of cPLA2. In TB cells, the acquisition of a more mature neuronal phenotype did not determine any increase of phospho-cPLA2. Differently, in the cholinergic LAN-2 cells, not only choline acetyltransferase (ChAT), but also cPLA2 activity gradually increased throughout 8 days in vitro (DIV), both

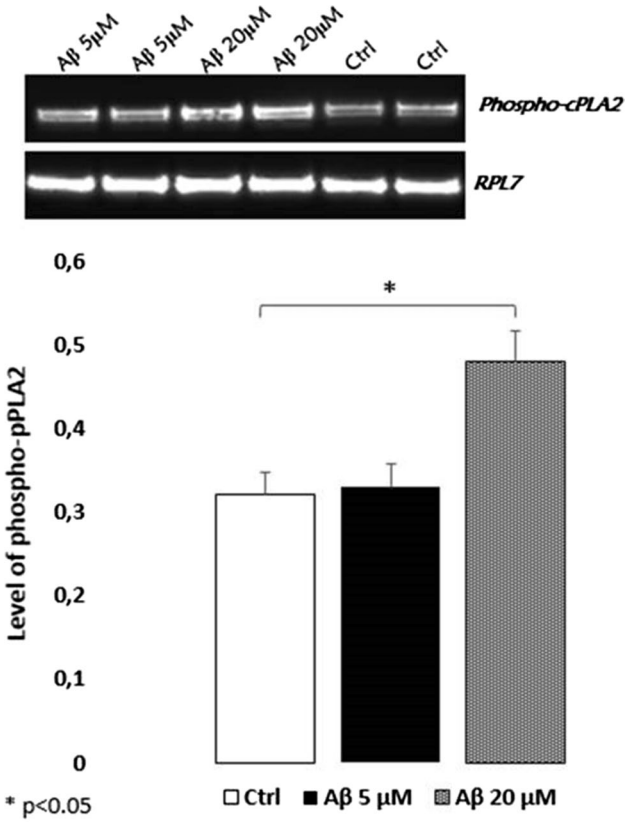

Fig. 2 Sensitivity of TB cells to Aβ. Western blot analysis on the total proteins extracted from undifferentiated TB cells treated for 1 h with 5 μM and 20 μM $A\beta_{25–35}$ after 24 h in culture. 5 μM Aβ failed to produce any significant effect on the level of the phosphorylated form of cPLA2, while 20 μM Aβ increased the level of the enzyme roughly 1.5 fold compared to the control (Ctrl). The housekeeping ribosomal protein RPL7 was used as loading control. Details in the text

in the untreated control and, to a major extent, in the RA-differentiated cultures (Singh et al. 1990). Many evidences support, directly or indirectly, the notion that specific gene expression in a cell line depends not only on the state of differentiation, but also on the cell line itself (Chan et al. 2017); (Rancic et al. 2017); (Suebsoonthron et al. 2017). The observation that in TB cells the level of cPLA2 is unsensitive to the differentiation process makes these cells as well as the selected biomarker (cPLA2) even more suitable to study the effect of Aβ during differentiation.

As compared to the LAN-2 cells, the TB cell line displayed less sensitivity to the effect of Aβ as suggested by the observation that 5 μM Aβ did not produce any effect on the level of phosphorylation in cPLA2, whereas it was necessary to increase the Aβ concentration up to 20 μM to have a significant increase of phospho-cPLA2. The work of Rönicke et al. appears to be in accordance to our data (Rönicke et al. 2008). The authors tested the toxic effect of different Aβ species, measured by the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in several cell lines and tissues. The treatment with Aβ induced a reduction of MTT, which was evident either in neurons, microglia, and astrocytes, but to different extents. In contrast, Aβ does not produce any effect on MTT level in a hippocampal slice culture. These observations, in agreement with our data, suggest that several effects of Aβ may be cell and/or tissue specific.

The main aim of our work is to check if the process of differentiation has an influence on the ability of Aβ to increase the phosphorylation in cPLA2. Surprisingly, while in undifferentiated TB cells 20 μM Aβ produced a 2.5 fold increase of phospho-cPLA2 after 1 DIV (at 4 and 7 DIV no Aβ effect was detectable), in the RA-treated cells Aβ treatment did not show significant effects at any time point. Interestingly, it was recently demonstrated that neuronal cells show higher resistance to amyloid toxicity (Cecchi et al. 2008). For instance, RA-differentiated SH-SY5Y cells were significantly more resistant against $A\beta_{1–40}$ and $A\beta_{1–42}$ toxicity as compared to undifferentiated cells. However, the topic is quite complex because Aβ also directly affects cellular differentiation. For instance, the Amyloid Precursor Protein (APP) increases the differentiation of human neuronal stem cells and shifts them toward glial rather than neuronal differentiation (Kwak et al. 2006). Interestingly, it was noted that $A\beta_{1–40}$ preferentially enhances neurogenesis of neuronal stem cells (NSC), while $A\beta_{1–42}$ favors gliogenesis. $A\beta_{25–35}$ was found not to influence NSC fate (Fonseca et al. 2013). However, it was recently demonstrated that modulators of the γ-secretase, that would reduce endogenous $A\beta_{1–42}$ levels, do not alter neuronal differentiation (D'avanzo et al. 2015). On the other hand, previous evidences showed that phenserine, a cholinesterase inhibitor that reduces APP levels, also increases the neuronal differentiation of NSC (Marutle et al. 2007).

Finally, we confirmed and reinforced our previous results. We proved that ACh is able to blunt the activation of cPLA2 induced by Aβ not only in a cholinergic cell line, such as LAN-2 cells, but also in a non-cholinergic one (TB cell line). ACh stabilizes the soluble secondary structure of $A\beta_{25–35}$, counteracting the formation of β-strand structures over time.

However, Aβ displays a complex and controversial role on cPLA2. For example, it was observed that in cultured cortical neurons, pre-mixing $A\beta_{1–40}$ with $A\beta_{1–42}$ significantly reduces the activation of cPLA2, maybe because $A\beta_{1–40}$ forms oligomers with $A\beta_{1–42}$ and these are less toxic than $A\beta_{1–42}$ alone (Bate and Williams 2010).

It has been recently demonstrated that one of the native physiological functions of Aβ is the allosteric modulation of the intrinsic catalytic efficiency of cholinesterases (Kumar et al. 2015). Additionally, an increase in the protein and transcript levels of the non-cholinergic "readthrough" AChE (AChE-R) variants has been found in AD patients compared to controls. The differential

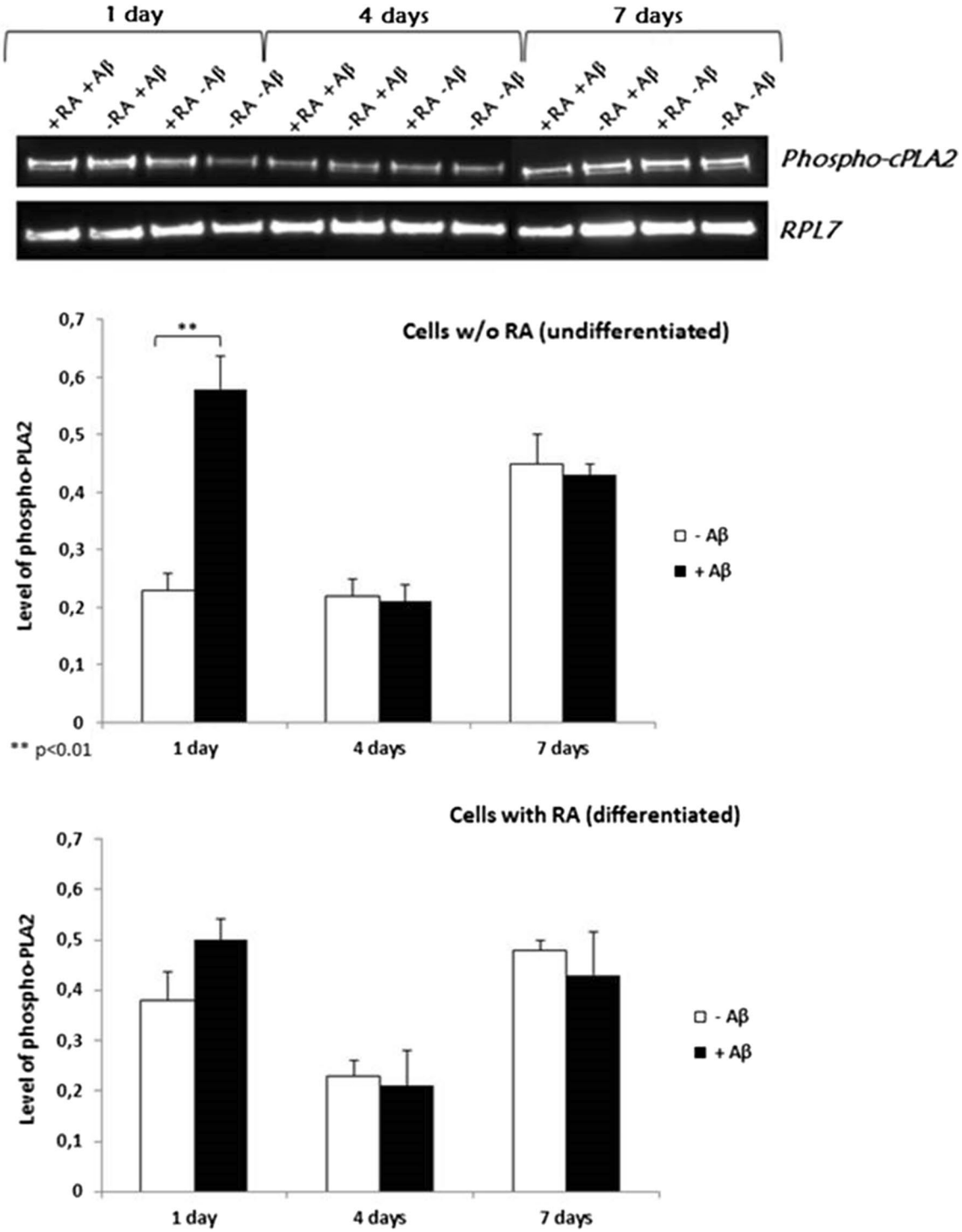

Fig. 3 Influence of differentiation with retinoic acid on Aβ-induced cPLA2 activation. Western blot analysis on total proteins extracted from undifferentiated (−RA) and 10 μM RA-differentiated (+RA) TB cells grown up to 7 days in vitro and treated for 1 h with 20 μM $A\beta_{25–35}$ (+Aβ) after 1, 4, and 7 days in culture. In undifferentiated cells, Aβ increased the phosphorylation of cPLA2 2.5 fold compared to the control (−Aβ) after 24 h in vitro, while after 4 and 7 days in vitro no significant difference was observed between Aβ-treated and non-treated cells. In RA-differentiated cells, Aβ did not affect the level of cPLA2 phosphorylation at any of the three tested time points. The housekeeping ribosomal protein RPL7 was used as loading control. Details in the text

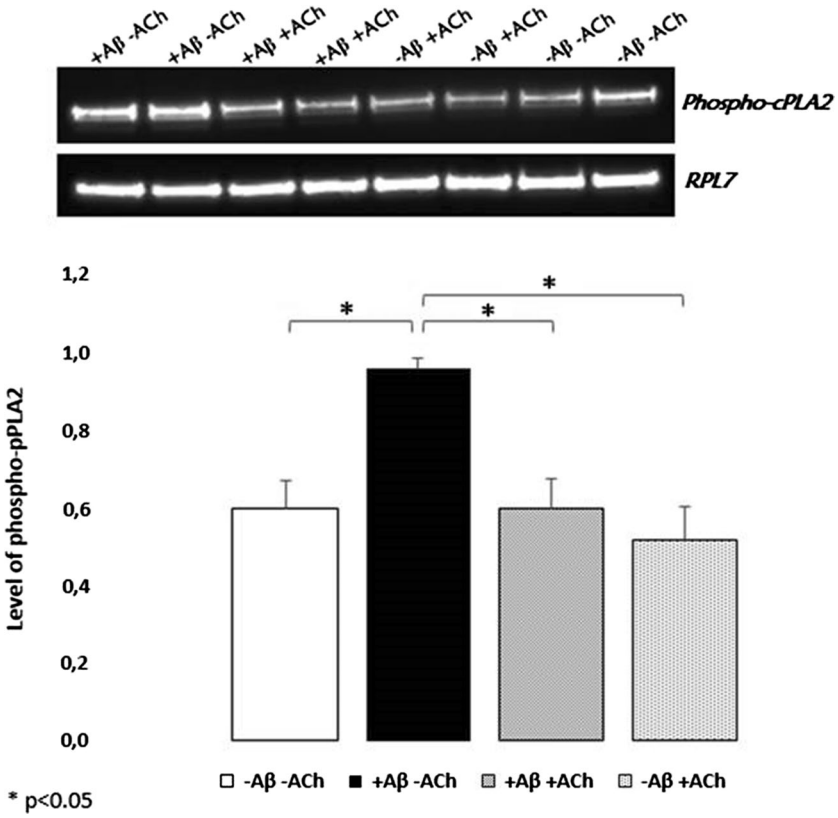

Fig. 4 Effects of ACh on Aβ-induced cPLA2 activation. Western blot analysis on total proteins extracted from TB cells treated for 1 h with 20 μM $Aβ_{25–35}$ and 25 μM ACh after 24 h in vitro without RA. Aβ (+Aβ −ACh) increased the phosphorylation of cPLA2 by about 60% compared to the negative control (−Aβ −ACh), while 25 μM ACh blunted this cytotoxic effect (+Aβ +ACh). ACh alone (−Aβ +ACh) had no effects on cPLA2 activation. The housekeeping ribosomal protein RPL7 was used as loading control. Details in the text

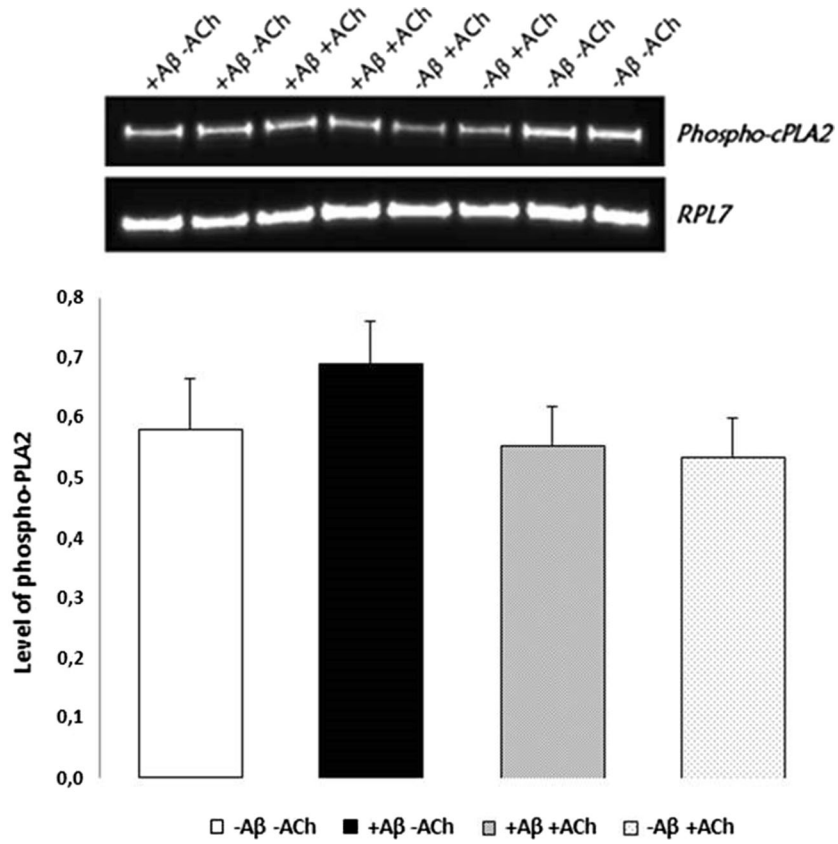

Fig. 5 Effects of ACh on Aβ-induced cPLA2 activation. Western blot analysis on total proteins extracted from TB cells treated for 1 h with 20 μM $Aβ_{25–35}$ and 25 μM ACh after 24 h in vitro in presence of RA. In this conditions Aβ (+Aβ −ACh) did not affect the level of cPLA2 phosphorylation compared to the negative control (−Aβ −ACh). ACh alone (−Aβ +ACh) had no effects on TB cells. The housekeeping ribosomal protein RPL7 was used as loading control. Details in the text

expression of the AChE-R variant in AD may reflect changes in the pathophysiological role of AChE, independent of cholinergic impairment or its role in degrading acetylcholine (Campanari et al. 2016). The data reported in the present work, showing that ACh, in addition to its role in cholinergic transmission, protects non-cholinergic cells against Aβ peptide, are in accordance with the hypothesis that Aβ may interfere with the cholinergic neurotransmission, determining different final effects, independently of its primary biological role.

In conclusion, we demonstrated that the $A\beta_{25–35}$ peptide exerts its cytotoxic effects, activating cPLA2, not only in human cholinergic cells (Grimaldi et al. 2016), but also in the TB human non-cholinergic cell line. Furthermore, we proved that the differentiation with 10 μM RA makes TB cells less sensitive to Aβ and that ACh can blunt the cytotoxic effects of $A\beta_{25–35}$.

A limitation of our work arises from the fact that we used a concentration of Aβ higher than that found in brain tissues. However, it should be considered that the effect of Aβ in an in vitro model, such as the one we used, is tested for a very short time (minutes), as opposed to the very long course (decades) that takes place during the real pathophysiological processes.

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Author Contributions Authors made substantial contributions to conception and design, acquisition of data, analysis, and interpretation of data. Authors participated in drafting the article or revising it critically and they gave final approval of the version to be submitted. *Study conception and design* A. Polverino, A.M. D'Ursi, G. Sorrentino; *Acquisition of data* A. Polverino, M. Grimaldi; *Analysis and interpretation of data* A. Polverino, M. Grimaldi, P. Sorrentino, F. Jacini, A.M. D'Ursi, G. Sorrentino; *Drafting of manuscript* A. Polverino, M. Grimaldi, P. Sorrentino, F. Jacini, A.M. D'Ursi, G. Sorrentino; *English Quality revision* P. Sorrentino; *Critical revision* G. Sorrentino, A. Polverino.

Compliance with Ethical Standards

Conflict of interest The authors declare that there is no personal or institutional conflict of interest related to the presented research and its publication.

Ethical Approval All procedures performed in studies involving human were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments.

Informed Consent Informed consent was obtained from the relatives of the patient involved in the study.

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