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Protection against amyloid beta-peptide (1–42)-induced loss of phospholipid asymmetry in synaptosomal membranes by tricyclodecan-9-xanthogenate (D609) and ferulic acid ethyl ester: Implications for Alzheimer's disease

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Abstract

Amyloid-beta (1–42) [Aβ (1–42)] deposition in the brain is a hallmark of Alzheimer's disease (AD) and has been shown to induce apoptosis and disrupt cellular ion homeostasis. Aβ (1–42) induces membrane lipid peroxidation, and 4-hydroxynonenal (HNE) and 2-propenal (acrolein) are the two reactive products of lipid peroxidation, which structurally modify proteins by covalent interaction and inhibit enzyme function. Phosphatidylserine (PS), an aminophospholipid, is sequestered in the inner leaflet of the plasma membrane in nonstimulated cells. An early signal of synaptosomal apoptosis is the loss of phospholipid asymmetry and the appearance of phosphatidylserine in the outer leaflet of the membrane. The ATP-requiring enzyme, flippase, maintains phospholipid asymmetry of PS. Here, we have investigated the inactivation of the transmembrane enzyme aminophospholipid-translocase (or flippase) by Aβ (1–42). Flippase activity depends on a critical cysteine residue, a putative site of covalent modification by the Aβ (1–42)-induced lipid peroxidation products, HNE or acrolein. The present study is aimed to investigate the protective effects of tricyclodecan-9-xanthogenate (D609) and ferulic acid ethyl ester (FAEE) on Aβ (1–42) induced modulation in phospholipid asymmetry in the synaptosomal membranes. Pretreatment of synaptosomes with D609 and FAEE significantly protected Aβ (1–42)-induced loss of phospholipid asymmetry in synaptosomal membranes. Our results suggest that D609 and FAEE exert protective effects against Aβ (1–42) induced apoptosis. The increase in intracellular Ca^{2+} might not be the sole cause for the loss of flippase activity. Rather, other mechanisms that could modulate the function of flippase might be important in the modulation of phospholipid asymmetry. The results of this study are discussed with relevance to neuronal loss in the AD brain.

Keywords: Alzheimer's disease; Oxidative stress; Lipid asymmetry; Lipid peroxidation; Phosphatidylserine; Synaptosome; Amyloid beta-peptide (1–42); Flippase

1. Introduction

The maintenance of membrane lipid asymmetry is a dynamic process that influences many events over the lifespan of the cell. With few exceptions, most cells restrict the bulk of the aminophospholipids to the inner membrane leaflet by means of specific transporters. The asymmetric distribution of phophatidylserine (PS) over the cellular membrane requires much of the cell's energy and requires the involvement of an ATP-dependent enzymatic activity (aminophospholipid translocase or Flippase) for its maintenance [1,2]. Indeed, if the cells fail to engage mechanisms to maintain asymmetry, aminophospholipids appear at the cell surface. One of the important consequences of altered membrane asymmetry is the recognition and engulfment of phosphatidylserine bearing vesicles and cells by mononu-

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clear phagocytes [3–5]. Loss of phospholipid asymmetry, measured by the exposure of PS on the outer leaflet of the membrane bilayer, is a typical early event that follows apoptotic insult [6].

Previous results have shown that, though still an unsettled question, apoptosis may contribute among the mechanisms of neuronal loss in Alzheimer's disease (AD) brain [7]. The AD brain is under oxidative stress indexed by, among other markers, lipid peroxidation [8–10]. A large body of evidence indicates that lipid peroxidation is directly responsible for the generation of the apoptotic phenotype [11]. Many agents that induce apoptosis are also stimulators of cellular oxidative metabolism [12–14], and many inhibitors of apoptosis have antioxidant activities or enhance cellular antioxidant defenses [15,16].

Recently, D609, a tricyclodecanol derivative of xanthic acid [17], has been reported to have anti-apoptotic and anti-inflammatory properties that are attributed to specific inhibition of phosphatidyl choline-specific phospholipase C [18–21], and ferulic acid (FA) is also known to have anti-oxidation properties [22,23]. Ferulic acid esters have significantly more anti-oxidant properties in comparison to ferulic acid [23]. Hence, the present study was designed to study the protective effect of D609 and ferulic acid ethyl ester (FAEE) against Aβ (1–42)-induced apoptosis in synaptosomes. The results are consistent with the notion that D609 may be acting as GSH mimetic and FAEE as an oxygen radical scavenger, thereby protecting the modulation of phospholipid asymmetry in the synaptosomal membranes.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise. Aβ (1–42) was procured as a dry powder from AnaSpec International (San Jose, CA). NBD-PS was obtained from Avanthi Polar Lipids (Alabaster, AL); Ca^{2+} indicator BAPTA Oregon Green and BAPTA AM were purchased from Molecular Probes (Eugene, OR); mouse anti-glyceraldehyde-3-phosphate dehydrogenase monoclonal antibody (GAPDH) was obtained from Chemicon International (Temecula, CA); and anti-cytochrome *c* was purchased from Stressgen Biotechnologies.

2.1. Synaptosome preparation

Cortical synaptosomes were prepared as described previously [24,25]. Briefly, cortices were isolated from gerbils and homogenized in an isolation buffer (0.32 M sucrose, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 5 μg/ml aprotinin, 20 μg/ml trypsin inhibitor, 0.2 mM PMSF, 2 mM EDTA, 2 mM EGTA, and 20 mM HEPES) by 12 passes with a motorized Teflon pestle. The homogenate was centrifuged at 4000 rpm (1500×*g*) for 10 min at 4 °C. The supernatant was collected and centrifuged at 14 800 rpm (20 000×*g*) for 10 min at 4 °C. The resulting pellet was mixed in a small volume of cold isolation buffer and layered onto discontinuous sucrose gradients containing 10 ml each of 1.18 M (pH 8.5), 1.0 M (pH 8.0) and 0.85 M (pH 8.0) sucrose solutions each with 2 mM EDTA, 2 mM EGTA, and 10 mM HEPES. The gradients were centrifuged in a Beckman L7-55 ultracentrifuge at 22 000 rpm (82 500×*g*) for 2 h at 4 °C. The resulting purified synaptosomal vesicles were removed from the 1.18/1.0 M interface and subsequently washed twice in PBS. The purity and yield of the synaptosomes prepared in this manner have been described previously [25]. Protein concentrations were determined by the Pierce BCA method and the samples were adjusted to a concentration of 2 mg/ml.

2.2. Measurement of loss of PS asymmetry

2.2.1. Annexin V assay

Synaptosomes (0.5 mg) with or without pretreatment with 10 μM BAPTA AM for 30 min were prepared. This agent is a high affinity intracellular Ca^{2+} chelator; thus, free intracellular Ca^{2+} levels are exceedingly low in the presence of this chelator. In this present study, BAPTA AM was used to test the hypothesis that the extent of PS externalization is not affected by the increasing influx of Ca^{2+} induced by Aβ (1–42). The synaptosomes were then washed twice in Locke's buffer, resuspended in the same buffer and incubated for 24 h with 10 μM Aβ (1–42) at 37 °C. After incubation, the synaptosomes were washed twice and resuspended in an annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and treated for 5 min with FITC annexin V at room temperature. Then, the samples were washed twice and the residual fluorescence was recorded on a fluorescence plate reader (Ex/Em: 494/518 nm).

2.2.2. NBD-PS assay

NBD-PS is an alternative specific method (other than annexin V) to study the externalization of phosphatidylserine. This assay was performed according to the procedure described by Comfurius et al. [26]. Synaptosomes were incubated for 1 h with 2 μg/mg protein of the fluorescent phospholipid NBD-PS [1-palmitoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphoserine. After 30 min of incubation, 10 μM BAPTA AM in DMSO was added to half of the samples. After washing with Locke's buffer, aliquots of the synaptosomes were loaded in a fluorescence plate with 10 μM Aβ, covered and incubated at 37 °C for 24 h. Then, the samples in each well were treated with 7.5 mM $Na_2S_2O_4$, which by reduction of the exposed residues leads to a loss of fluorescence of labeled PS. The fluorescence of the residual membrane-resident, non-exposed NBD-PS was recorded with a fluorescence plate reader (Ex/Em: 460/514 nm).

2.3. Measurement of Mg^{2+} ATPase activity

Flippase, an ATP-requiring enzyme, maintains the phospholipid asymmetry of PS. The assay of Mg^{2+} ATPase was performed to investigate the inactivation of the transmembrane enzyme aminophospholipid-translocase (or flippase) by Aβ (1–42). Synaptosomes (500 μg) in Locke's buffer were incubated for 24 h with 10 μM Aβ (1–42). After two washes with Locke's buffer, aliquots of 10 μg were suspended in the ATPase assay buffer (18 mM histidine, 18 mM imidazole, 80 mM NaCl, 15 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, pH 7.1) in a microtiter plate, and were treated with 0.1 mM of the Na^+K^+-ATPase inhibitor ouabain to a final volume of 100 μl. After 10 min of incubation at 37 °C, 3 mM ATP was added and the reaction was allowed to proceed for 60 min at 37 °C. Then, 5 μl of 5% SDS and 125 ml of the color reagent (0.36 g ascorbic acid mixed with 15 ml of molybdate acid solution) were added to each well. The activity of the Mg^{2+} ATPase was measured with a UV plate reader as the amount of phosphate produced by the enzyme [27].

2.4. Measurement of synaptosomal influx of Ca^{2+}

1 mg of synaptosomes, previously treated with the Ca^{2+} indicator BAPTA Oregon Green (Molecular Probes) for 20 min, was rinsed twice with Locke's buffer and resuspended in the presence of 10 μM Aβ (1–42). The influx of Ca^{2+} was detected as an increase in the fluorescence signal of the fluorescent indicator (Ex/Em: 494/523 nm).

2.5. Measurement of Ca^{2+} levels in the presence of Aβ and BAPTA AM

The intrasynaptosomal calcium levels were measured using FURA-2 fluorescence as previously described [28]. FURA-2 stock solution (1 mM) was prepared in dimethyl sulfoxide and 0.5% pluronic acid. In brief, synaptosomes (1 mg) were independently incubated, with and without BAPTA AM (10 μM) for 30 min at 37 °C. The synaptosomes were washed twice with Locke's buffer and resuspended in the same buffer. 10 μM Aβ (1–42) then was added to each of the synaptosomal preparations and further incubated for 24 h at 37 °C. The synaptosomes were washed twice with the control buffer and resuspended in the same buffer (1 ml) containing 5 μM FURA-2. The suspension was incubated with gentle shaking for 40 min at 37 °C. The suspension was then washed in ice-cold Krebs-like buffer and centrifuged to remove the extrasynaptosomal FURA-2. Synaptosomes were then stored on ice and diluted to 300 μg protein in 1 ml Ca^{2+}-containing, Krebs like solution at 37 °C, adjusted to give a final concentration of 1.2 mM Ca^{2+} for 1 ml. Measurements were made by alternating the excitation wavelengths of 340 and 380 nm (the 340:380 nm fluorescence ratio); fluorescent emission was monitored at 510 nm. Calibration of the fluorescence signals was performed at the end of each experiment by adding digitonin (200 μM) to obtain F_{max} followed by EGTA (2 mM) to obtain F_{min} [29]. Extrasynaptosomal FURA-2 was determined for each synaptosomal preparation by adding $MnCl_2$ (10 mM) at the end of each experiment to quench the extracellular fluorescence. Intracellular calcium was calculated by the equation of Grynkiewicz et al. [29], using a K_D of 276 nM for the Ca^{2+}-FURA-2 complex derived under our laboratory conditions by scanning the fluorescent response of FURA-2 to different concentrations of Ca^{2+} (0–39.8 μM).

2.6. Detection of cytochrome c release

In order to address the issue of apoptosis and synaptosome viability, a cytochrome *c* release assay was performed. In brief, synaptosomes (1 mg) were independently incubated, with and without BAPTA AM (10 μM), for 30 min at 37 °C. The synaptosomes were then washed twice with Locke's buffer. Treatment samples were incubated with 10 μM Aβ (1–42) for 24 h at 37 °C. Synaptosomes were then spun at 15000×*g* for 10 min at 4 °C, and the resulting supernatants were used for the detection of the release of cytochrome *c* by Western blotting. The proteins in the supernatants were separated by 4–20% SDS-PAGE, blotted onto a nitrocellulose membrane and probed with sheep anti-cytochrome *c* polyclonal antibody. Blots were developed using SigmaFast tablets (BCIP/NBT) and were quantified using Scion Image (PC version of Macintosh compatible NIH Image) software.

2.7. Statistics

Statistical analysis were obtained using analysis of variance (ANOVA), with significance set as $P<0.05$.

3. Results

3.1. Protection of Aβ (1–42) induced loss of phospholipid asymmetry by D609 and FAEE in synaptosomal membranes, as assessed by annexin V binding assay

Apoptotic cell death is accompanied by a change in plasma membrane structure by surface exposure of phosphatidylserine (PS), while the membrane integrity remains unchallenged. Surface exposed PS can be detected by its affinity for annexin V, a phospholipid binding protein coupled with a fluorescent tag. Synaptosomes treated with 10 μM Aβ (1–42) leads to a loss of lipid bilayer asymmetry ($P<0.0001$) (Fig. 1). D609 and FAEE are antioxidants [30,31] that protect the neurons from oxidative stress. D609, a glutathione mimetic [56], at a concentration of 50 μM significantly protected the Aβ (1–42)-induced oxidative stress in the primary neuronal cells [30]. Similarly, FAEE at a concentration of 25 μM significantly protected the Aβ (1–42)-induced oxidative stress in the primary neuronal

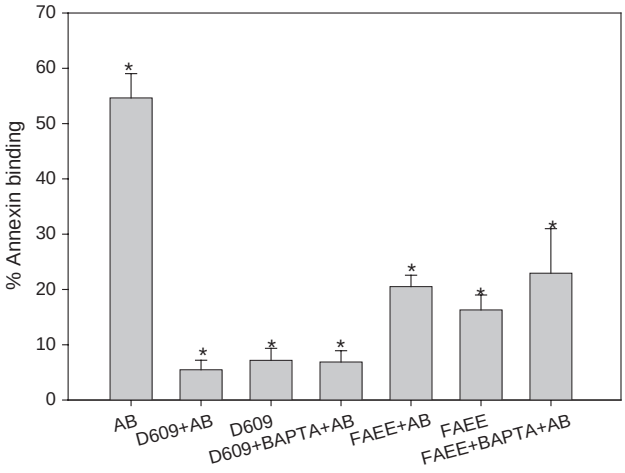

Fig. 1. Annexin V assay. Following incubation with Aβ (1–42) for 24 h, synaptosomes were treated with annexin V in the presence or absence of BAPTA AM Ca^{2+} chelator. The increase in fluorescence indicates increased amount of PS on the outer leaflet of the synaptic membrane even in the presence of the Ca^{2+} chelator. 'AB' refers to Aβ (1–42). The control value was set to 0% and the experimental values were compared to this control. These data are presented as mean±S.E. N=6; *P<0.05.

cells [31]. Synaptosomes (0.5 mg) with or without pretreatment with 10 μM BAPTA AM for 30 min and then D609 (50 μM) and FAEE (25 μM) treatments independently followed by 24 h treatment with 10 μM Aβ (1–42), at 37 °C, yielded protection of Aβ (1–42)-induced loss of phospholipid asymmetry in synaptosomal membranes (Fig. 1). The increase in PS exposure was detected as an increase in fluorescence after the incubation of synaptosomes with Aβ (1–42). Independent treatment of D609 and FAEE prior to the addition of Aβ (1–42), with and without BAPTA AM significantly reduced (P<0.04) the level of PS exposure

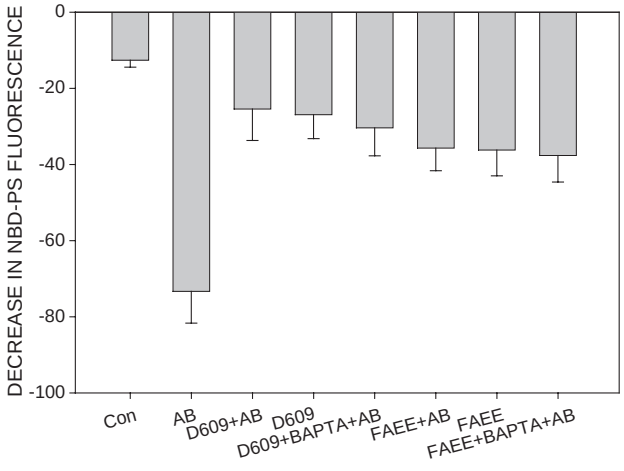

Fig. 2. NBD-PS assay. Synaptosomes, pre-incubated with the fluorescent phospholipid NBD-PS, were treated with Aβ (1–42) for 24 h in the presence or absence of BAPTA AM Ca^{2+} chelator. The decrease in fluorescence is measured after quenching the signal due to the externalized PS. 'AB' refers to Aβ (1–42). These data, in arbitrary units on the ordinate axis, are presented as mean±S.E. N=6, **P<0.0001, *P<0.001.

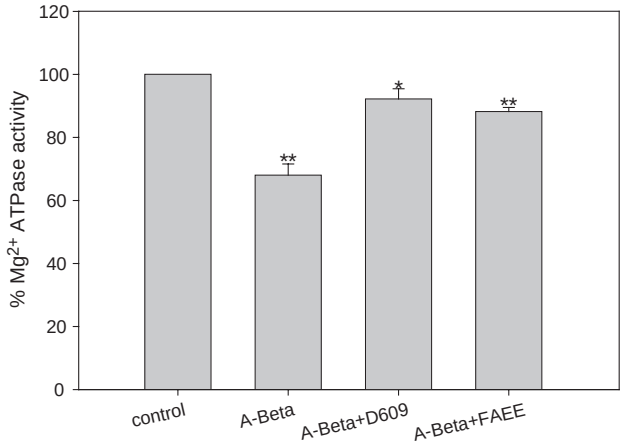

Fig. 3. Measure of the Mg^{2+} ATPase activity. Decreased Mg^{2+} ATPase activity was measured after 24 h treatment with Aβ (1–42). These data are presented as percent of the control (taken as 100%) and are expressed as mean±S.E. N=6; **P<0.0001, *P<0.01.

almost to the level of control. The treatment with D609 and FAEE alone (Fig. 1) suggest that they do not exhibit any synaptosomal cytotoxicity, as the level of PS exposure in this case is the same as the control.

3.2. Protection of Aβ (1–42) induced modulating of phospholipid asymmetry by D609 and FAEE in synaptosomal membranes, as assessed by NBD-PS assay

Another way of measuring the externalization of PS was by fluorescent phospholipid NBD-PS. Pretreatment of synaptosomes with fluorescent NBD-PS allowed the measurement of the extent of phospholipid exposure after quenching the external fluorescence with the reducing agent $Na_2S_2O_4$. The data are represented as the decrease in fluorescence (Fig. 2) as measured after quenching the signal

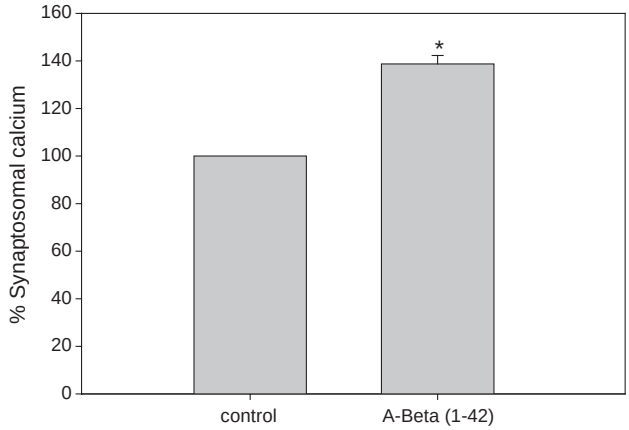

Fig. 4. Measure of Ca^{2+} influx following exposure to 10 μM Aβ (1–42). Pretreatment with the calcium fluorescent indicator BAPTA Oregon Green allowed the measurement of Ca^{2+} influx indexed as increased fluorescence intensity. These data are presented as percent of the control (taken as 100%) and are expressed as mean±S.E. N=6; *P<0.0001.

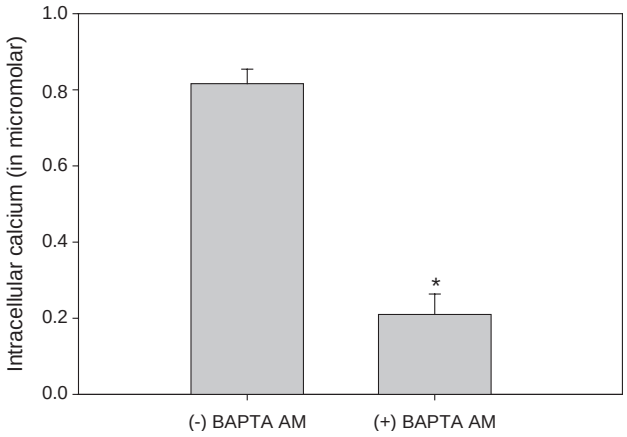

Fig. 5. FURA 2-based determination of intrasynaptosomal Ca^{2+} concentrations following exposure to Aβ (1–42) for 24 h. In the presence of BAPTA AM, significantly decreased Ca^{2+} levels are found compared to that in the absence of BAPTA AM. Thus, the Aβ (1–42) induced loss of flippase activity in synaptosomes may not solely be due to increased intrasynaptosomal Ca^{2+}. These data are presented as mean±S.E. N=6, *P<0.01.

due to the externalized PS. The results, confirming those with annexin V, suggest that there is a significant increase (P<0.0001) in PS exposure with Aβ (1–42) alone. The treatment of synaptosomes with D609 or FAEE prior to the addition of Aβ (1–42), with and without BAPTA AM, significantly reduced (P<0.001) the level of PS exposure to the external leaflet almost to the level of control (Fig. 2). The treatment with D609 and FAEE alone led to slightly elevated PS exposure relative to control, but significantly less than that produced by Aβ (1–42) treatment (P<0.0001).

3.3. Aβ (1–42) induced decreased Mg^{2+} ATPase activity

Mg^{2+} ATPase (flippase) activity in synaptosomes was measured after 24 h treatment with Aβ (1–42), and the results suggest that there is a significant (P<0.0001) decrease in Mg^{2+} ATPase activity in the presence of 10 μM Aβ (1–42), which confirms the inactivation of this ATP-requiring enzyme (Fig. 3). However, we observed that the

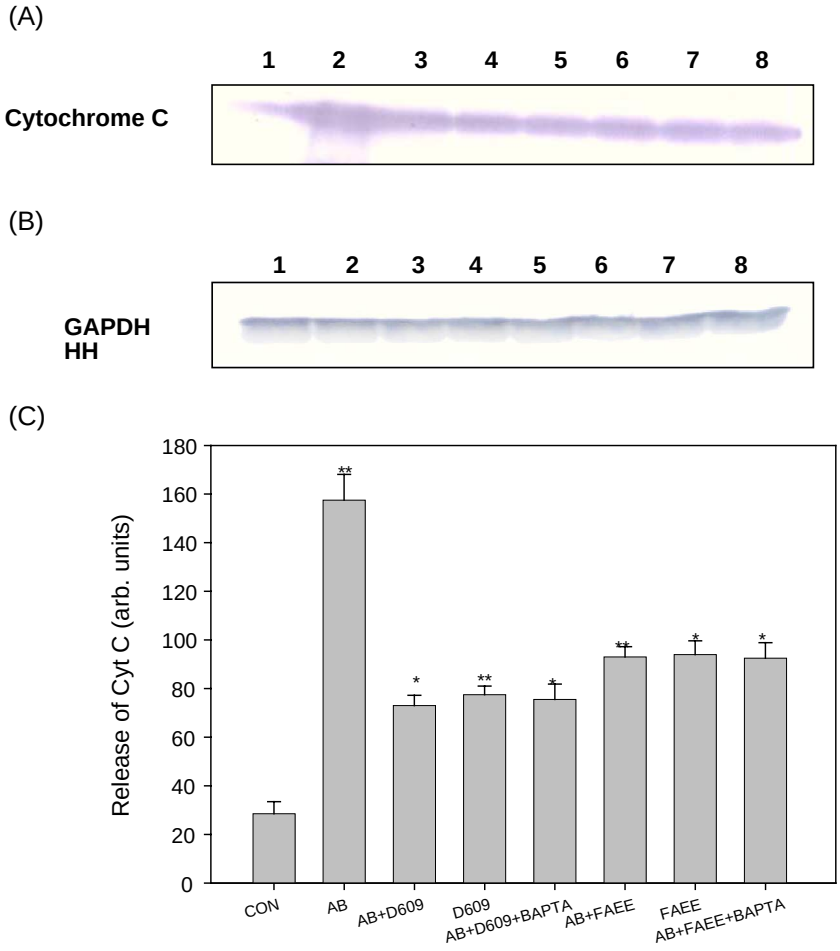

Fig. 6. Cytochrome c release from synaptosomes: (A) Representative Western blots. Lane 1: Synaptosomes without any treatment (control); lane 2: 10 μM Aβ (1–42); lane 3: 10 μM Aβ+D609; lane 4: D609; lane 5: 10 μM Aβ+BAPTA AM+D609; lane 6: 10 μM Aβ (1–42)+FAEE; lane 7: FAEE; lane 8: 10 μM Aβ (1–42)+BAPTA AM+FAEE. (B) Representative Western blot to verify equal loading of protein by analyzing the levels of GAPDH: After respective treatments as in the panel A, 100 μg of the proteins in the synaptosomal supernatants were separated by 12.5% SDS-PAGE, blotted onto a nitrocellulose membrane and probed with anti-GAPDH monoclonal antibody. (C) Bar graph representation of cytochrome c release from the data obtained from Western blotting densitometry analysis. Results shown are means±S.E. obtained for three separate preparations (* P<0.05 and ** P<0.005 vs. control).

Mg^{2+} ATPase activity was not affected in synaptosomes pretreated with D609 and FAEE prior to Aβ (1–42) treatment.

3.4. Aβ (1–42) induces Ca^{2+} influx in synaptosomes

The increase in Ca^{2+} was measured by treating synaptosomes with the Ca^{2+} indicator BAPTA Oregon Green, which fluorescences when chelated to the cation. The results suggest that there is a significant increase ($P<0.0001$) in Ca^{2+} influx into the synaptosomes after treatment with Aβ (1–42) (Fig. 4).

3.5. Decreased intrasynaptosomal Ca^{2+} concentration in the presence of BAPTA AM

The intrasynaptosomal calcium levels in the synaptosomes pre-treated BAPTA AM was measured using FURA-2 fluorescence. The results suggest that, in the presence of Aβ (1–42), there is a significant four-fold decrease ($P<0.01$) in the free intrasynaptosomal Ca^{2+} levels (Fig. 5) in the synaptosomes that had been pre-treated with 10 μM BAPTA AM.

3.6. Cytochrome c release

The release of cytochrome *c* was increased enormously in the supernatant of synaptosomes treated with Aβ (1–42) with respect to that in control experiments (Fig. 6A, C). In contrast, the amount of cytochrome *c* released in the supernatants of synaptosomes pretreated with D609 and FAEE was significantly lower with respect to the supernatant of the synaptosomes treated with Aβ (1–42). The treatment with D609 and FAEE alone led to slightly elevated cytochrome *c* release relative to control, but significantly less (Fig. 6A, C) than that produced by Aβ (1–42) treatment ($P<0.05$). Fig. 6B shows that equal loading of blots was maintained.

4. Discussion

The maintenance of PS asymmetric distribution is crucial for cellular life. PS exposure over the outer leaflet of the red blood cell membrane activates an apoptotic mechanism of death and marks the cell for macrophage-modulated clearance [1,32,33]. Many studies have established the unifying concept that plasma membrane phospholipids are dynamically distributed in an asymmetric fashion such that the majority of the aminophospholipids reside in the cell's inner membrane leaflet with the choline-containing phospholipids being restricted to the outer leaflet [34]. Lipid bilayer asymmetry is maintained by active ATP-dependent process [2], suggesting that it is critical to normal cell function. One of the hallmarks of cells undergoing death, determined by their ability to bind annexin V [35], is the reorientation of PS from the cell's inner to the outer membrane leaflet [6,36], an event that precedes DNA condensation.

Apoptotic cells express aldehyde adducts [37] that also bind annexin V [38]. In principle, aldehydes can react with primary amines on lipids and proteins to generate negatively charged moieties that mimic PS [38]. These lipids have been shown to trigger lipid peroxidation via free radical mechanisms [39] that lead to the generation of 4-hydroxynonenal (HNE) and malondialdehyde (MDA) adducts [40]. Such a mechanism could also be involved in apoptosis [41] and might play a role in phagocyte recognition ligand for phagocytosis [11].

In the present study, the externalization of phosphatidylserine was measured in two different ways. First, the PS binding protein annexin V coupled with a fluorescent tag was used. The increase in PS exposure was detected as increase in fluorescence after the incubation of synaptosomes with Aβ (1–42) and was decreased when pretreated with 50 μM D609 and 25 μM FAEE independently (Fig. 1). In addition, pretreatment of synaptosomes with fluorescent NBD-PS allowed the measurement of the extent of phospholipid exposure after quenching the external fluorescence with the reducing agent $Na_2S_2O_4$ (Fig. 2), which clearly suggests that there is a significant decrease in PS exposure in the synaptosomes pretreated with 50 μM D609 and 25 μM FAEE. Aβ (1–42) is elevated in the AD brain and can cause HNE formation [42,43], which induces neurotoxicity [44–47]. Several studies have reported the high reactivity of HNE and acrolein towards proteins [48], which might be the reason behind the modulation in phospholipid asymmetry in the synaptosomal membrane. The harmful alkenals can diffuse relatively far away from their site of formation [48] and can modify protein structure by covalent binding, especially to cysteine residues [48,49], a likely result of which was the decrease in Mg^{2+} ATPase activity in synaptosomes observed in our study. At least one cysteine residue in the protein primary structure is crucial for the activity of the flippase [32,33], suggesting that the mechanism of inactivation of the enzyme might be related to HNE binding to this residue [50].

The concentration of Aβ (1–42) used was 10 μM, a concentration used in numerous studies involving synaptosomes and cell cultures [8,31,42,43] and a higher concentration than found in the whole CNS in AD. However, at the level of a lipid bilayer, Aβ (1–42) that intercalates to cause lipid peroxidation [8,31,42,43] would have a relatively high concentration. Moreover, it is clear that Aβ (1–42) is critical to the pathogenesis of AD and that lipid peroxidation is found in AD brain [10,42].

One potential concern using annexin V to measure phospholipid asymmetry is the possibility of an unstable system allowing this protein to access the inner leaflet, thereby giving an erroneous interpretation of the loss of phospholipid asymmetry. To address this potential concern, we used a second, independent method to access PS

exposure on the outer membrane leaflet. This second method places fluorescently labeled PS on the outer leaflet, and if the flippase is functioning properly, the fluorescence will remain constant upon the addition of a quencher, since the PS would have been translocated to the inner leaflet. This was not the case following the treatment of synaptosomes with Aβ (1–42). Moreover, the analysis of phospholipid asymmetry by the two methods gave essentially identical results, providing confidence in our interpretation.

D609, a tricyclodecanol derivative of xanthic acid [17], has been reported to have anti-apoptotic and anti-inflammatory properties that are attributed to the specific inhibition of phosphatidyl choline phospholipase C [18–21]. The finding that xanthates efficiently scavenge hydroxyl radicals [51], the mechanism by which D609 may protect against oxidative damage, was further examined is this study. Glutathione is well known for its protection against cellular oxidative damage [52]. The xanthates studied here, especially D609, mimic glutathione (GSH). Previously, studies from our lab demonstrated that D609 inhibits the formation of a PBN spin adduct generated by Fenton chemistry [51], suggesting that D609 is a hydroxyl radical scavenger. One of the substrates for GSH is HNE [53,54]. It was also demonstrated that D609 protects the synaptosome, a functional model of the synapse [55], from free radical induced injury [56]. In addition, D609 binds and thus detoxifies reactive alkenals, thereby preventing the latter from damaging synaptosomes [56]. GSH, acting as a nucleophile, can react with electrophilic aldehydes that are produced by the free radical-mediated oxidation of lipids [10]. D609 scavenges hydroxyl radicals and react with electrophilic products of lipid oxidation (acrolein) in a manner similar to GSH [56]. The antioxidant property of D609 is associated with the free thiol group of xanthate [30,56]. D609 is capable of detoxifying aldehydic products of lipid peroxidation by a mechanism similar to GSH, however, not with the same efficacy as the latter [56]. Moreover, D609 effectively protects neuronal cell cultures from oxidative stress induced by Aβ (1–42) [31]. Hence, in the present study, D609 significantly ($P<0.001$) reduces free radical-induced modulation in phospholipid asymmetry in synaptosomal membrane by supporting its role as an antioxidant. Thus, by acting as a GSH mimic and free radical scavenger, it is plausible that D609 could prevent apoptosis, in addition to inhibiting PC-PLC.

Aβ (1–42) is increased in the AD brain [8,9,10], and also we confirm here that Aβ (1–42) promotes an influx of Ca^{2+} in synaptosomes (Fig. 4). The increase in Ca^{2+} was measured by treating synaptosomes with the Ca^{2+} indicator BAPTA Oregon Green, which fluoresces when chelated to the cation (Fig. 4). The results obtained from both asymmetry assays (Figs. 1 and 2) suggest that the extent of PS externalization, in the presence of Ca^{2+} chelator (BAPTA AM), is not affected by the increasing influx of Ca^2. That is, there are exceedingly low free intracellular Ca^{2+} levels in presence of this chelator, as BAPTA AM has a high affinity for Ca^{2+}. Yet, the degree of loss of phospholipid asymmetry induced by Aβ (1–42) is essentially identical whether or not BAPTA AM is present. This suggests that the Ca^{2+}-related inactivation of flippase may not be the sole reason for the loss of asymmetry. Further studies in this regard was strengthened by using Fura-2 to measure intracellular Ca^{2+} in the presence of Aβ (1–42) and BAPTA AM (Fig. 5), and the results suggest that BAPTA AM is able to block Aβ (1–42)-induced Ca^{2+} elevation nearly four-fold compared to that in the absence of BAPTA AM.

Thus, we conclude that oxidative modification of the flippase by reactive alkenals, induced by Aβ (1–42), which are elevated in the AD brain, likely contributes to the loss of phospholipid asymmetry in the brain. Aβ (1–42), which is elevated in AD brain and can cause HNE formation [42,43], also leads to a loss of phospholipid asymmetry as assessed by the annexin V assay (Fig. 1). These studies conceivably couple lipid peroxidation induced by Aβ (1–42) [8,10] to the loss of phospholipid asymmetry in AD brain.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid), a phenolic compound present in a variety of plants, has potent antioxidant [22,57,58] activity. Ferulic acid (FA) is a potent scavenger of oxygen radicals, as reported previously [59]. FA attenuates iron-induced oxidative damage and apoptosis in cultured neurons [22,60] and protects against lipid peroxidation damage [61]. Previous studies in our laboratory also suggest that ferulic acid protects against free radical mediated changes in the conformation of synaptosomal membrane proteins as monitored by EPR spin labeling techniques [22]. Once inside the brain, the anti-inflammatory and antioxidant properties of FA may be potentially due to its ability to inhibit leukotriene production and reduce oxidative stress [62–64]. In the present study, we have used FAEE as ferulic acid esters have significantly high anti-oxidant properties in comparison to ferulic acid [23], which means that they exhibit high radical scavenging activity. The ester functionality permits the effective passage of the ferulic acid component across the lipid bilayer. Thus, by acting as a lipophilic free radical scavenger, FAEE could prevent the loss in phospholipid asymmetry induced by Aβ (1–42). FAEE also exerts neuroprotective effects by upregulating protective enzymes, such as heme oxygenase-1 and heat shock protein 70 [31,65].

Targeting phospholipids to specific membrane sites is essential for maintaining critical signal transduction cascades, cell shape and homeostasis. In AD brain, an abnormal distribution of phospholipids exists [66]. The modification of flippase by binding to HNE (an Aβ-induced lipid peroxidation product [42]) conceivably might represent an oxidation-related mechanism that causes loss of PS asymmetry. Elevated Ca^{2+} levels also may contribute to the loss of phospholipid asymmetry, but is unlikely the sole determinant of modulation in phospholipid asymmetry. However, in AD brain [8–10], oxidative stress-mediated HNE formation as a result of lipid peroxidation leading to

loss of flippase function may also be important. Aβ (1–42) in the form of small oligomers likely inserts into the lipid bilayer to cause free radical mediated lipid peroxidation [42]. HNE, one of the products of lipid peroxidation, has immediate transmembrane protein targets for covalent modification of these proteins via Michael addition, and we suggest that such processes occur in this study, leading to the oxidative modification and subsequent dysfunction of flippase. Consistent with this notion, the results of the current investigation suggest that D609 (novel GSH mimetic) and FAEE may prevent cellular apoptotic cascades by acting as antioxidants. Thus the antioxidant properties of D609 and FAEE conceivably could be beneficial in the treatment of disease related to oxidative stress.

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