Lipid raft ER signalosome malfunctions in menopause and Alzheimer's disease

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1. ABSTRACT

The increase in the incidence of Alzheimer's disease (AD) in old women may be attributable to estrogen deficiency, and estrogen replacement therapy may be useful in preventing or delaying the onset of this disease. In neuronal membranes, 17β-estradiol interacts with estrogen receptors (mERs) located in lipid raft signalosomes which trigger neuroprotective responses by anchoring to scaffolding caveolin-1 complexed with other proteins. We suggest that mER-signalosome malfunctions in AD and by menopause due to development of aberrations in these microstructures. Here, we report that mER dissociates from a voltage-dependent anion channel (VDAC), and that progressive dephosphorylation of VDAC1 enhances neurotoxicity. mER dissociates from caveolin-1 and other neuroprotective proteins, including insulin-like growth factor 1 receptor beta. Similar signalosome disarrangements are observed in AD patients. Moreover, in AD, lipid rafts exhibit alterations in lipid composition, and these changes cause an increase in liquid-ordered as compared to controls. Together, the data show that AD and menopause lead to disruption in the lipid raft structure, and disfunctioning of ERalpha and other neuroprotectors integrated into these signalosomes.

2. INTRODUCTION

17β-estradiol plays a crucial role in different functions related to brain preservation and functionality, including neurogenesis, neuronal differentiation, synaptic plasticity and neuroprotection (1-3). Some of these actions are initiated at the plasma membrane, through interaction with estrogen receptors associated with the neuronal membrane (mER) and, in particular, within lipid rafts (4,5). Lipid rafts are plasma membrane microstructures with a distinct lipid composition, and are

known to be the preferential place of signalling proteins involved in cell survival (6,7). Estrogen membrane-related mechanisms, named rapid or non-genomic pathways, take place through the activation of mERs, triggering intracellular responses (8,9). Different non-genomic estrogen mechanisms that exert neuroprotection against a variety of injuries have been reported (10,11). These actions may take place through classic ERs (ERα and ERβ) localized at the plasma membrane (5,8,12,13), although most of the evidences obtained so far suggest that ERα is the main receptor related to these neuronal activities (4,14,15). Furthermore, additional estrogen-binding receptor found at the neuronal membrane is G protein-coupled estrogen receptor (GPR30), a novel ER expressed in the brain associated with cognitive functions (16-18).

The mechanism whereby mERs lacking transmembrane domains are embedded into the neuronal membrane is unclear, and appears to involve receptor S-palmitoylation (19-21). Furthermore, some studies have demonstrated the presence of these receptors in lipid rafts, distinct microstructures of the plasma membrane showing a particular lipid composition that allows the integration of signalling proteins relevant for neuronal functions (22-24). In these microdomains, palmitoylated mERs have been shown integrated in signalosomes, macromolecular complexes formed by scaffolding proteins and other signalling molecules to develop neuroprotective actions (25-27). In particular in cortical and hippocampal neurons, it has been evidenced that ERα is associated with anchoring caveolin 1 (Cav-1) in a macromolecular signalling platform together with, among others, insulin growth factor-1 receptor beta (IGF-IRβ) and a voltage-dependent anion channel (VDAC) (28-30). Membrane ER and IGF-IRβ interactions are crucial for a variety of estrogen roles in the brain, including neuronal survival (31,32). Moreover, increasing evidences indicate that interactions of ERα in this multicomplex orchestrate neuroprotective responses against amyloid beta (Aβ)-induced toxicity (4,32). Thus, it has been suggested that estrogen modulation of plasmalemmal VDAC phosphorylation through mERα interaction may be a hormone mechanism to reduce AD-related pathology (33). In line with this, it has been shown in neuronal cultures that rapid mechanisms of estrogen bound to mERα promotes channel phosphorylation through rapid activation of PKA and Src-kinase signal transduction pathways (34,35). VDAC phosphorylation induced by estradiol maintains the channel inactivated and closed (36-38), preventing apoptosis and neuronal death (35,39). On the contrary, Aβ exposure induces plasmalemmal VDAC dephosphorylation in lipid rafts, as part of a membrane mechanism of neurotoxicity in AD even at early stages (ADI/II) (40). Overall, these data suggest that mER integrated in multimolecular dynamic signalosomes elicits intracellular signalling cascades related to brain preservation against AD.

Lipid rafts represent a pivotal target for protein-lipid interactions at the plasma membrane. In this line of argument, some work has revealed the importance of the particular lipid composition of raft microstructures in order to allow the correct functionality of integrated signalling platforms for neuronal preservation. Indeed, it has been demonstrated that aberrant lipid raft lipid alterations (such as reduction in polyunsaturated fatty acid levels) in cortical and hippocampal brain areas occur during, both, AD and Parkinson's disease (PD) progression (41,42), even at early stages of these diseases (43,44). It has been demonstrated that lipid changes in these microdomains modify their physicochemical properties and alter their local microenvironment, contributing to rearrangements of raft integrated proteins (45-48). In this order of ideas, mER association with plasmalemmal VDAC1 has been shown to be disrupted in cortical and hippocampal areas with the progression of AD (28,44), in parallel with an increase in lipid raft viscosity and liquid order (49). These findings support that functionality of signalosomes lays on optimal composition of particular lipid species in these microdomains. Interestingly, estrogens may act as regulators of lipid membrane stability, suggesting that neuroprotective actions at the plasma membrane not only involve activation of signal transduction but also the modulation of lipid homeostasis (50-52).

Taking into account these evidences, it is feasible to hypothesize that estrogen loss occurring during menopausal stages may indirectly induce changes in membrane integrity that affect raft functionality and neuroprotective responses against AD. Here, we have studied whether estrogen detriment as a consequence of menopause and ageing may alter ER signalosomes in human brain cortical areas. This analysis has been correlated with the results obtained in cortical areas of AD brains of similar age.

3. EXPERIMENTAL

3.1. Materials

The rabbit polyclonal antibodies against APP, Flotillin 1, Caveolin 1, ERα, IGF-1Rβ were, respectively, from Abcam (Cambridge, UK, for APP and Flotillin 1) and Santa Cruz Biotechnologies (Texas, EEUU, for Caveolin 1, ERα and IGF-1Rβ). Mouse monoclonal antibody directed to VDAC1 antibody was from Abcam. The monoclonal anti-amyloid beta peptide antibody and anti-PrPc antibody were purchased from Santa Cruz Biotechnologies (Texas, EEUU). For detection of phosphorylated residues, we used phosphoBLOCKER blocking reagent and phosphoantibody stripping solution that were purchased from Cell Biolabs, Inc (Madrid, Spain). Dynabeads® Antibody Coupling Kit was from Life Technologies. PreCast Mini protean SDS-PAGE and immobilized pH 3-10 and pH 7-10 nonlinear gradient strips for two-dimensional gel electrophoresis were from Bio-Rad Laboratories (Madrid, Spain).

Table 1. Summary of cases

Case	Age (years)	Gender	Post-mortem delay	Neuropathological diagnosis	Braak stage
Control samples with age <50 (group C <50)					
1	24	F	6 h	NL	0
2	46	F	9h 35 min	NL	0
3	46	F	7 h	NL	0
4	47	F	7 h	NL	0
Post-menopausal samples with age >65 (group C >65)					
5	65	F	4 h	NL	0
6	73	F	7 h	NL	0
7	66	F	8 h	NL	0
8	69	F	02 h 30 min	NL	0
9	71	F	08 h 30 min	NL	0
10	81	F	4 h	NL	0
11	64	F	5 h	NL	0
12	75	F	3 h	NL	0
13	78	F	3 h 40 min	NL	0
14	82	F	11 h	NL	0
AD samples (group AD)					
15	82	F	10 h	AD	VC
16	69	F	20 h	AD	VC
17	86	F	10 h	AD+AmA	VC
18	82	F	2 h 30 min	AD	VC
19	72	F	4 h 15 min	AD+AmA	VC
20	96	F	10 h	AD	VC
27	85	F	12 h	AD	VIC
F: Female; NL: No lesions; AD: Alzheimer disease. V/VI: Refers to Braak and Braak stages of AD-related changes; C: Large numbers of senile plaques in the neocortex					

3.2. Human brain samples

Post-mortem human brain tissues were obtained from the Institute of Neuropathology Brain Bank (Bellvitge University Hospital). Samples were manipulated following legal and ethical guidelines for Biomedical Research involving human subjects and approval of the local Ethics Committee. Seven cases were from women suffered from severe dementia of the Alzheimer type (stage V-VI), according to Braak and Braak (53,54). Ten cases were from post-menopausal women of similar age (> 65 years, indicated as >65 y throughout the text), and four cases were from pre-menopausal women (<50 years, indicated as <50 y throughout the text), both groups without any apparent neurological or neuropathological disorders. The postmortem delay was between 4 and 20 h. Frontal cortex (area 8) was dissected free of white matter and used for lipid raft isolation. A summary of these cases is shown in Table 1.

3.3. Lipid raft isolation

Lipid raft fractions were obtained following previous protocols (55) with minor modifications (41). Briefly, frontal cortex tissue was homogenized in buffer A (50 mM Tris-HCl pH 8.0., 10 mM $MgCl_2$, 20 mM NaF, 1mM Na_3VO_4, 5 mM β-mercaptoethanol, 1 mM PMSF), and processed for sucrose gradients differential centrifugation. Six fractions of 2 ml were obtained. Fractions 2 and 3 (F2, F3) were collected and used as lipid raft fractions and fraction 6 (F6) and the resulting pellet were used as non-raft fractions. Characterization of lipid raft and non-raft fractions was previously performed by immunoblotting with a

battery of lipid raft and non-raft protein markers (data not shown).

3.4. SDS-PAGE and two-dimensional gel electrophoresis

For SDS-PAGE, lipid raft fractions were resuspended in SDS loading buffer (625 mM Tris-HCl, 1% sodium dodecyl sulphate, 10% glycerol, 5% β-mercaptoethanol and 0.0.01% bromophenol blue, pH 6.8.). Equal amount of protein (> 4 μg) was loaded on 12.5.% SDS-PAGE using Mini-Protean TGX Gels (456-1034 Bio-Rad). For two-dimensional gel electrophoresis, lipid raft and non-raft samples were resuspended in DeStreak Rehydration Solution (GE Healthcare) (30 μg of protein/125 μL). Protein samples were embedded into immobilized pH gradient 7 cm-length strips (pH 7-10 or pH 3-10), and isoelectrofocused up to 20,000 V/h, following manufacturer's instructions (Bio-Rad). Then, isoelectrofocused strips were equilibrated first in equilibration buffer containing 2% (w/v) ditiothreitol (DTT) for 10 min at room temperature, followed by alkylation in equilibration buffer containing 2.5.% (w/v) iodoacetamide. Strips were processed in 12.5. % SDS-PAGE for second dimension resolution.

3.5. Immunoblotting

Protein extracts resolved by electrophoresis were transferred to PVDF membranes, using Trans-blot Turbo Transfer System (Bio-Rad) following manufacturer's guidelines. Then, PVDF membranes were immersed in Blotting grade blocker diluted in Tris-buffered saline at 5% (BLOTTO), followed by incubation with the different primary antibodies used in the study (overnight at 4ºC with gentle agitation). Antibodies from Abcam were diluted 1:1,000 in BLOTTO, and antibodies from Santa Cruz biotech were diluted 1:200, as recommended. The anti-phosphotyrosine antibody was diluted in 5% phosphate-free bovine serum albumin (BSA). After washing three times for 5 min in Tris-buffered saline (TBS) with 0.1.% Tween-20, membranes were then incubated with horseradish peroxidase conjugated secondary antibodies (diluted 1:5,000 in BLOTTO) for 2 h at room temperature with gentle agitation. Immunospecific signals were visualized by Clarity Western ECL substrate kit (Bio-Rad). Chemiluminescent bands were processed using Chemie-Doc MP Imaging System (Bio-Rad), and analyzed using Image Lab programme.

3.6. Immunoprecipitation assays

Direct immunoprecipitation assays were performed in order to study potential interactions between Caveolin-1, VDAC, Estrogen Receptor-α and IGF-IRβ. Specific anti- Caveolin-1 monoclonal antibody (Abcam) was linked to magnetic beads using the Dynabeads® Antibody Coupling Kit (Life Technologies), following manufacturer's instructions. 1mg beads per 6 μg antibody were used. Lipid Raft and non-Raft fractions from the different subjects were resuspended in cold Immunoprecipitation Buffer (Tris-HCl 50 mm, NaCl 100 mm, EDTA 1 mm, NP40 0.5.% and Protease inhibitor cocktail Complete (Roche)). Antibody-coated beads were added to the samples (2 μg antibody to each sample), and incubated overnight at 4ºC with gentle agitation. After incubation, supernatant was removed out, and beads were washed 3 times in Tris-NaCl Buffer (Tris 20mM, NaCl 100mM, pH 7.4.). Beads were finally resuspended in SDS loading buffer. Protein material unbound to antibody coated beads (supernatant) was precipitated in cold (-20ºC) acetone (four times the volume of protein samples) overnight at -20ºC. After centrifugation (15,000 x g for 15 min), precipitated material was resuspended in SDS in loading buffer. Samples were then analyzed by SDS-PAGE and immunoblotting.

3.7. Lipid analyses

Lipid analyses was performed as explained elsewhere (42,44,56). Lipid classes were analyzed by scanning densitomery on one-dimensional high performance thin layer chromatography plates. Fatty acids from total lipids were analyzed by gas chromatography of fatty acids methyl esters obtained by acid-catalized transmethylation.

3.8. Statistical analyses

Data were assessed by one-way analysis of variance (ANOVA-I) followed by Student-Newman-Keuls t-test or Tukey's *post hoc* tests, where appropriate. Numerical data were represented as mean ± SEM. Statistical significance in Figures is indicated from $p < 0.0.5$. Lipid data was further analyzed using a multivariate approach by means of principal component analyses (PCA).

4. RESULTS

4.1. Distinct patterns of mERalpha-related signalosome in frontal cortex during menopausal stages and AD

Our previous work suggested that the dynamic of raft protein markers embedded in mERα-related signalosomes may be at the basis of neuronal protection against different age-associated neuropathologies (4,26,52). In this order of ideas, profound alterations in raft multiprotein complexes have been shown related to AD (44). Moreover, rearrangements of these signalling platforms may depend on the levels of extracellular estrogen to modulate intracellular homeostatic responses (32). To study whether alterations of circulating estrogen levels occurring during menopause may affect neuronal mERα signalosomes, we analysed by immunoblotting in lipid raft fractions from frontal cortex of post-menopausal subjects (> 65 y) the presence of different protein markers known to modulate some of the molecular aspects of AD. For comparison, we also analysed these same protein patterns in either pre-menopausal samples or AD samples at late stages (V/VI).
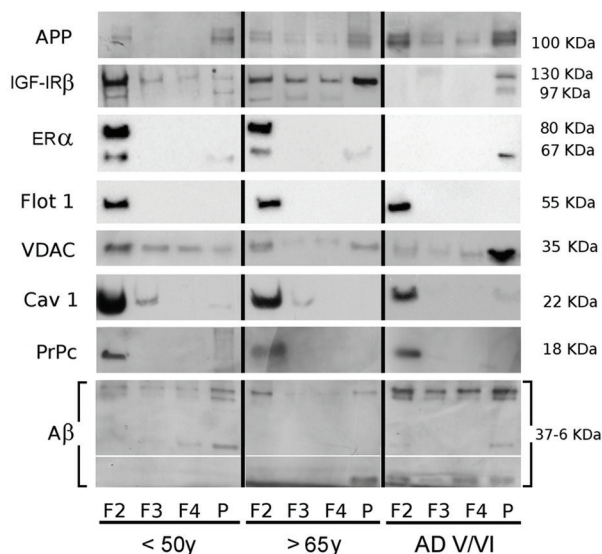
Figure 1. Distinct protein marker distribution in lipid raft and non-raft fractions during menopause and AD. Proteins from lipid raft fractions (F2-F4) and non-raft fraction (NR) from frontal cortex of women below 50 years old without apparent lesions (<50 y WLS), women above 65 years old without apparent lesions (>65 y WLS) and women with AD at late stages of the disease (ADV/VI) were loaded on 12.5.% SDS-PAGE for immunoblotting analysis with specific antibodies directed against the different raft protein markers of the study (see Material & Methods): anti-amyloid precursor protein (APP), anti-insulin growth factor-1 receptor beta (IGF-1Rβ), anti- estrogen receptor alpha (ERα), anti-Voltage dependent anion channel (VDAC), and anti-amyloid beta peptide (Aβ). As a control of lipid rafts, antibodies against anchoring proteins flotillin 1 (Flot 1), caveolin 1 (Cav-1), and Prion protein (PrPc) were also used. Equal amounts of proteins were loaded for the different samples. Apparent Molecular weights expressed in kilodaltons are indicated on the right. Five assays per group.

Thus, lipid raft fractions and non-raft fractions were used for immunoblotting with specific antibodies against the following makers: ERα and IGF-1Rβ, as estrogen modulators of neuroprotection; APP and VDAC1, known to be involved in Aβ formation and toxicity; Flotillin 1 (Flot 1), Caveolin 1 (Cav 1) and PrPc as lipid raft structural proteins (Figure 1). An antibody against Aβ was also used to visualize potential amyloid aggregates. Results show that, as compared with controls without lesions (< 50 y), some differences in the dynamic of VDAC, IGF-1Rβ and ERα were observed in > 65 y group, observing a progressive trafficking from F2-F4 to P, in particular in the case of VDAC1. Interestingly, in AD samples, both, ERα and IGF-1Rβ were absent from lipid raft fractions, whereas the amount of these proteins were only represented in non-raft pellets. The reduced representation of these receptors may be a consequence of low levels of extracellular ligand (i.e. estrogen and insulin growth factor-1) availability. A similar trafficking to non-raft fractions was observed for VDAC. On the contrary, APP appeared to increase in AD lipid raft fractions (F2). This is in agreement with previous results, where APP trafficking into lipid rafts was shown to be a requirement to enhance Aβ processing (57). Indeed,

here using an antibody against different structures of Aβ, a high amount of different morphological populations of Aβ was observed in AD samples, as an indicative of an enhancement of Aβ production and aggregation.

Overall, these results indicate that some rearrangements in ERα-related signalosome may occur as a consequence of the menopause, these changes being exacerbated during AD.

4.2. Disruption of mERalpha-related signalosome during menopausal stages and AD

Previous data has shown that mERα interaction with other raft signalling proteins, such as IGF-1Rβ and VDAC, is regulated by caveolin 1 which is known to play a pivotal anchoring role for the interaction of these protein markers involved in neuroprotection (30). Moreover, alterations in signalosome interactions, such as mERα/VDAC associations, appear to promote neurotoxicity in AD (28). These perturbations are enhanced during ageing, as a consequence of lipid raft structural impairment (43). In order to investigate whether mERα-related signalosome disarrangements may be reflected during menopausal periods, we performed immunoprecipitation experiments with anti-caveolin 1 (CAV 1) polyclonal antibody, using lipid raft and non-raft fractions from the three experimental groups (<50 y, >65 y, and ADV-VI) (Figure 2). Immunoprecipitation assays in lipid raft (LR) fractions induced the coprecipitation of ERα, IGF-IRβ and VDAC in <50 y WLS. However, in neither >65 y or AD experimental groups, VDAC1 was absent of these immunocomplexes, thus corroborating the low levels of VDAC detected in LR fractions. On the contrary, in these two groups there was a progressive coprecipitation of APP in LR fractions, suggesting that the trafficking of this protein into LRs enhances its association with Cav-1. Conversely, as expected in non-raft fractions, no apparent coprecipitation with Cav-1 was observed for any of these markers.

This data indicates that VDAC shifted out of the lipid raft and then lost its association with Cav-1 during both menopause and AD development. This phenomenon may have consequences regarding the regulation of the channel.

4.3. Progressive VDAC dephosphorylation in lipid raft samples from menopausal and AD subjects

Previous findings have demonstrated that estrogen, through binding to mERα, enhances plasma membrane VDAC1 phosphorylation, and inactivation, as part of the alternative mechanisms of neuroprotection against Aβ (35,58). Indeed, disruption of mERα/VDAC complex has been observed in human cortex of AD brains (28). In agreement with this, it has been reported that VDAC1 dephosphorylation is induced by Aβ at the neuronal
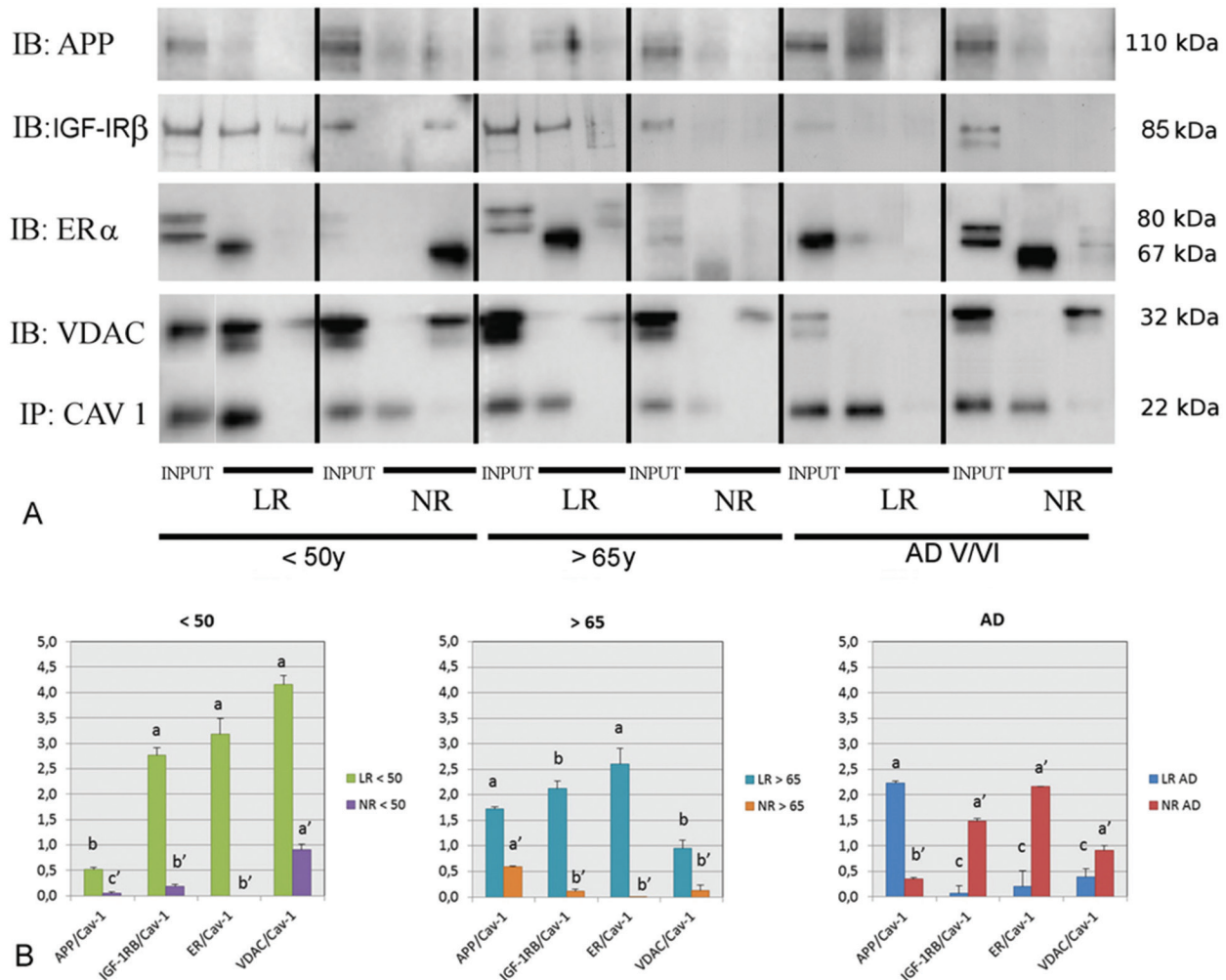
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Figure 2. Both, menopause and late stages of AD promote the disruption of ERα-related signalosome multicomplex in lipid raft microdomains. (A) Lipid raft fractions (LR) and non-raft (NR) fractions from the three experimental groups (women below 50 years old without apparent lesions, <50 y, women above 65 years old without apparent lesions, >65 y, and women at stages ADV/VI) were subjected to immunoprecipitation with anti-caveolin 1 antibody (IP:Cav-1). The resultant immunoprecipitated material was run on 12.5.% SDS-PAGE, and immunoblotted (IB) with corresponding anti-APP, anti-IGF-1Rβ, anti-ERα and anti-VDAC antibodies. Equal amount of protein extracts were used. As immunoblotting controls, inputs of the different raft and non-raft extracts were also loaded. Apparent Molecular weights expressed in kilodaltons are indicated on the right. (B) Normalized densitometry values of the different immunoblotting bands of co-precipitated amyloid precursor protein (APP), insulin growth factor 1 receptor beta (IGF-1), estrogen receptor alpha (ER) and voltage-dependent anion channel (VDAC) relative to the amount of immunoprecipitated caveolin 1 (Cav-1) values in the different lipid raft (LR) and non-raft (NR) fractions. The three experimental groups are represented by < 50 (premenopausal subjects below 50 years old), > 65 (menopausal subjects above 65 years old), and Alzheimer's disease (AD) group. Four assays per group.

membrane as part of the neurotoxicity mechanism (40). In view of these results, it is conceivable that estrogen detriment occurring during menopause may indirectly affect VDAC1 phosphorylation state, making the channel more prone to gating, thereby promoting extrinsic apoptosis (59,60). In an attempt to explore whether the absence of VDAC in lipid rafts of cortical areas in menopausal women may promote VDAC dephosphorylation, we have performed a set of experiments with, both, lipid raft (LR) and non-raft (NR) fractions by protein isoelectrofocusing assays and Western blotting. Thus, LR and NR proteins from the three experimental groups (<50 y, >65 y, ADV/VI) were processed by two-dimensional gel electrophoresis,

and immunoblotted with specific anti-VDAC monoclonal antibody. VDAC1 has been previously reported in lipid rafts from human cortex and hippocampus to be resolved by isoelectrofocusing in four distinct spots (isoelectric points, p/ 7.9., 8.4., 9.5. and 10.0.). Isoforms with p/ 8.4. and 9.5. corresponded to Tyr-phosphorylated forms, and p/ 10.0. to unmodified VDAC1 (40). This same pattern was observed here in control samples <50 y WLS, using pH 7-10 nonlinear gradient strips (Figure 3A). However, in >65 y WLS group, there was a progressive loss of phosphorylated VDAC in lipid raft F2 fraction, in parallel with a progressive displacing of VDAC isoforms to non-raft fraction. In AD, VDAC1 was not significantly
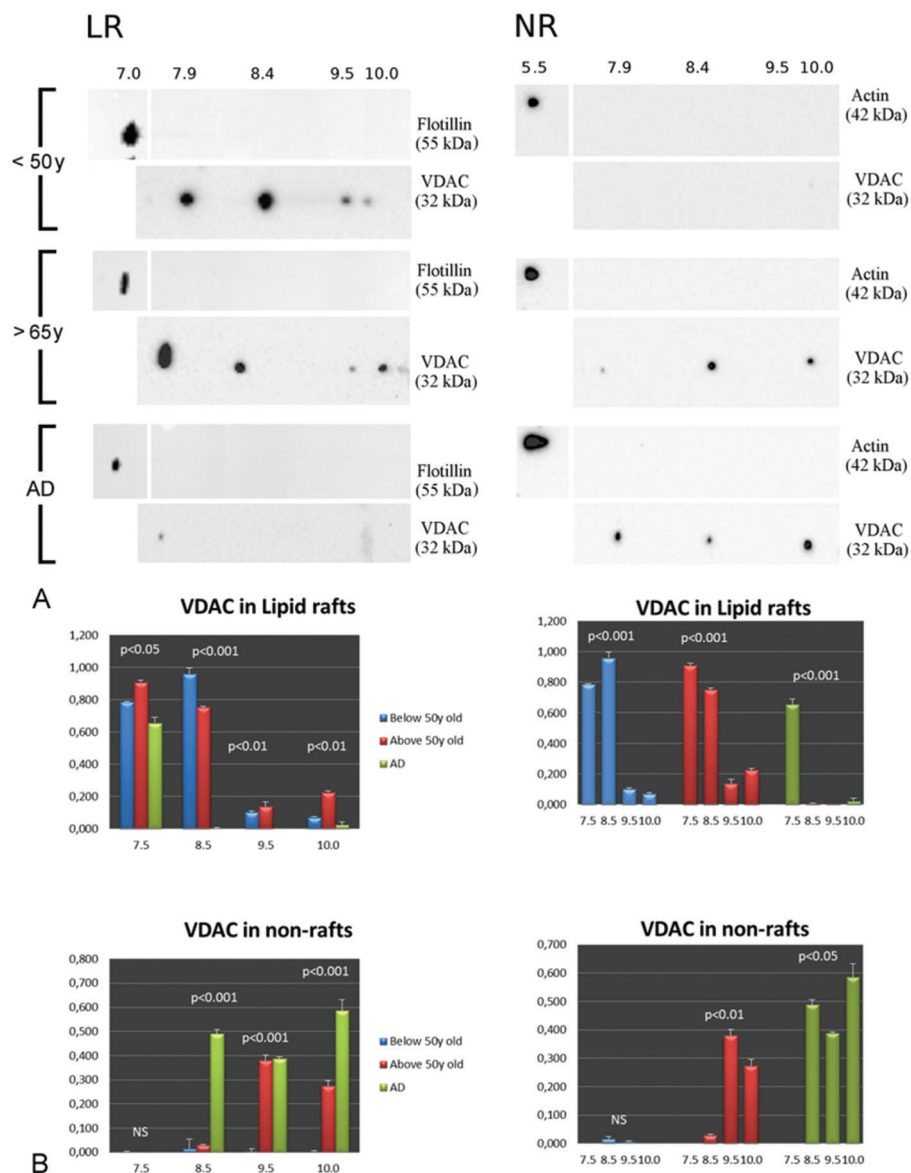
Figure 3. Post-transcriptional pattern of VDAC is altered in cortical lipid rafts in menopausal periods and ADV/VI. (A) Lipid raft (LR) and non-raft (NR) fractions from the different experimental groups (women below 50 years old without apparent lesions, <50 y, women above 65 years old without apparent lesions, >65 y, and women at stages ADV/VI) were electrophoresed in two-dimensional gels using pH 7-10 nonlinear gradient strips. Then, strips were processed on second-dimensional 12.5.% SDS-PAGE, and immunoblotted with monoclonal anti-VDAC specific antibody. Equal amounts of protein extracts were loaded for the different experimental groups: women below 50 years old without apparent lesions, <50 y, women above 65 years old without apparent lesions, >65 y, and women at stages ADV/VI. As a control, another set of two-dimensional electrophoresis with the same protein amount were processed for immunoblotting with anti-flotillin 1 antibody in LR samples, and with anti-actin 1 antibody in non-raft samples. Apparent Molecular weights are indicated on the right side. (B) Normalized densitometric values of the different VDAC spots relative to flotillin and actin in, respectively, lipid raft and non-raft samples of the three experimental groups, as resolved in two-dimensional electrophoresis and immunoblotting. Four assays per group.

represented in LR, and VDAC1 spots were only visible in non-rafts. As a control, the same amounts of the sample were double run on similar two-dimensional gels using pH 3-10 nonlinear gradient strips, and immunoblotted with antibodies directed to flotillin 1 and to actin, as a control of LR and NR. As expected, flotillin 1 was detected as a main large spot at approximately p*I* 7.0. in LR, whereas actin was found at p*I* 5.5. in NR. Figure 3B illustrates

the semiquantitative distribution of the distinct VDAC1 isoforms in each experimental group, and the spot's densitometric values relative to either flotillin 1 (LR) or actin (NR).

These results suggest that estrogen detriment during menopause correlates with progressive dephosphorylation of VDAC in lipid raft fractions,
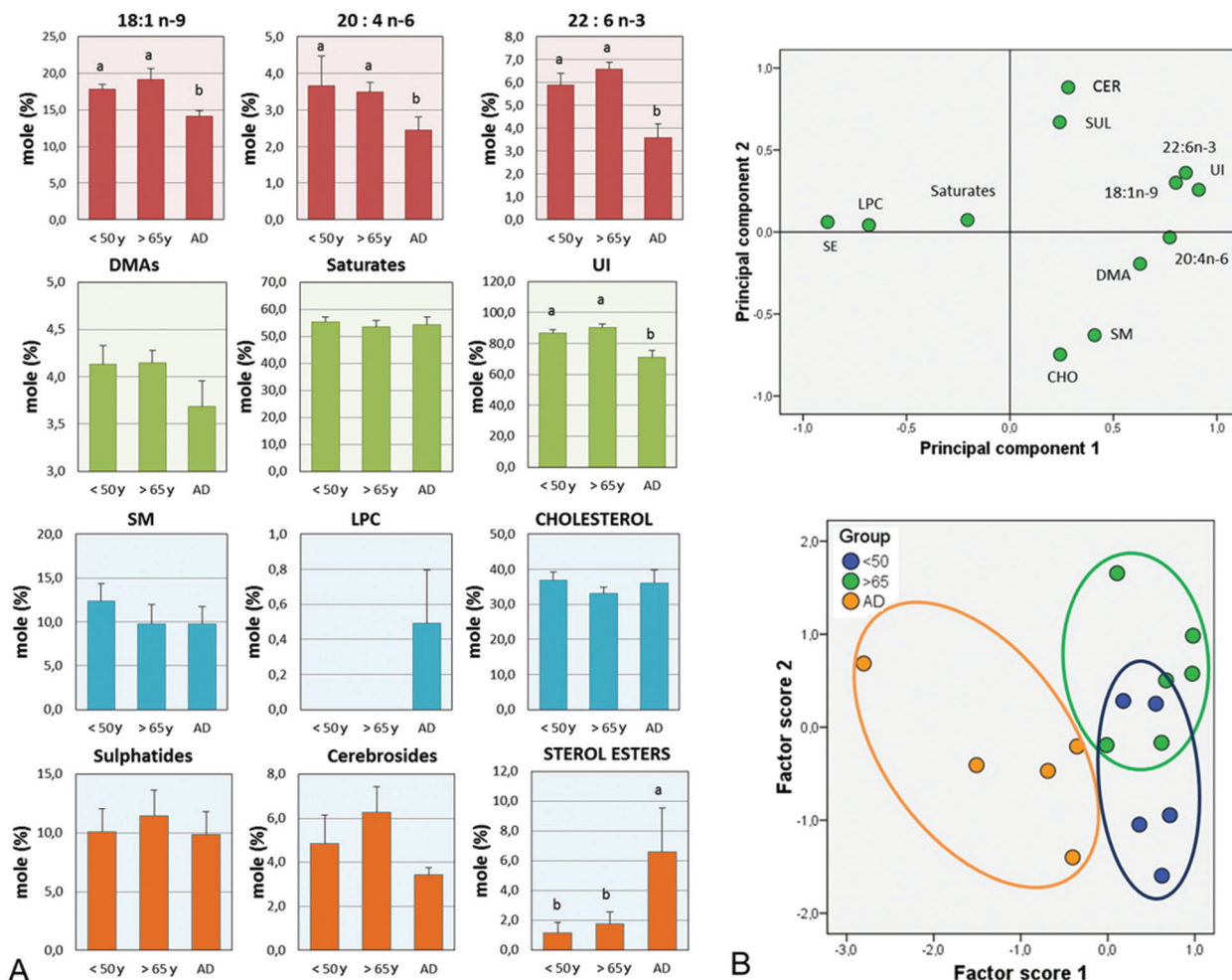
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Figure 4.

concomitantly with a trafficking of the porin out of these microdomains. This phenomenon is exacerbated in late stages of AD.

4.4. Lipid profiles in lipid rafts from pre-menopausal, post-menopausal and AD subjects

Analyses of main lipid classes and fatty acids are shown in Figure 6. Apparently, there were no significant differences between control pre- and post-menopausal women although some trends were observed for increased 18:1n-9, 22:6n-3 and cerebrosides, and decreased cholesterol and sphingomyelin. Conversely, significant differences were detected when comparing post-menopausal and AD women. All three fatty acids illustrated (18:1n-9, 20:4n-6 and 22:6n-3) were notably reduced in AD individuals. The main consequence of these changes was the significant reduction of unsaturation index, which suggest that lipid rafts from AD women are substantially more viscous and liquid-ordered than in age-matched post-menopausal women.

Regarding lipid classes, no changes were observed for prototypical lipid raft constituents, namely sphingomyelin (SM), cholesterol (CHO), saturates, cerebrosides and sulphatides. However, a significant increase was detected for *lyso*-phosphatidylcholine (LPC) and sterol esters in AD lipid rafts.

Principal component analyses using the same lipid combination described above, revealed that PC1, which explains 40.3.% of total variance, was positively correlated to monounsaturated and polyunsaturated fatty acids (more abundant in non-AD women) and negatively to SE and LPC (more abundant in AD women) (Figure 4A). PC2 explained 21% of total variance and positively correlated to cerebrosides and suphatides (which were slightly increased in post-menopausal women) and negatively to cholesterol and sphingomyelin (which, in turn, were slightly decreased in post-menopausal women). Plotting factor scores for the three groups revealed that AD lipid rafts are segregated within a single cluster, while non-AD lipid rafts showed

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some overlapping though exhibiting a certain degree of segregation (Figure 4B). These results indicate that the biochemical structure of lipid rafts is sufficient to discriminate between AD and non-AD women and, to a lesser extent, to differentiate between pre- and post-menopausal women.

5. DISCUSSION

Increasing evidence reveals the impact of lipid rafts in neuroprotective actions of estrogen against different injuries such as AD (4,61,62). These membrane microstructures are key elements that facilitate, among others, lipid-protein and signaling protein interactions to promote brain preservation against AD neuropathology (63,64). Part of estrogen alternative mechanisms of neuroprotection occurs through membrane ERα integrated in these signaling platforms, which interacts in multimolecular complexes with IGF-1Rβ, VDAC1 and raft anchoring caveolin-1 to trigger transduction signals (4,32). Other still poorly characterized members of signalosomes are membrane glutamatergic receptors (mGluR), which have also been shown to interact with mERα in a complex with caveolin 1 (64).

Lipid rafts act as scaffolds for signaling receptors and ion channels that communicate extracellular ligands to intracellular signaling, inducing dynamic rearrangement and modulation of these multimolecular entities. In this sense, integration of mERα in these microstructures supports the fact that extracellular estrogen availability is involved in signalosome homeostatic regulation, a phenomenon which may be relevant in intracellular signaling mechanisms for neuronal survival. Taking into account these premises, in this work, we have demonstrated that lipid rafts from the frontal cortex of post-menopausal women show alterations in the compartmentalization and dynamic of proteins residing in estrogen-responding signalosomes, such as mERα, IGF-1Rβ and VDAC1. Although not the subject of this study, it is worth mentioning that this protein complex may also depend on IGF-1 ligand, as both molecules may cross-talk in these signalosomes to adapt the final intracellular neuroprotective response against different types of injuries (32). In line with this, the efficiency of ERα/IGF-1R interactions also appears to decrease with aging (65,66).

An observed consequence of menopause is the disruption of multimolecular mERα/VDAC/IGF-1Rβ/Cav-1 associations. This phenomenon was exacerbated in similar fractions of age-matched AD samples at late stages of the disease (ADV/VI). This data suggests that one of the effects of the decline in estrogen levels after menopause may be accompanied by disarrangements of mERα-related signalosome in cortical brain areas, a phenomenon that may make neurons more prone to neuropathological events related to aberrant protein clustering and toxic cell signalling.

One of the main alterations in cortical post-menopausal lipid rafts observed in this work is the progressive displacement of VDAC1, which in parallel increases its presence in non-raft pellets, in correlation with the dissociation of mERα/VDAC complex. Previous data has revealed the functional relevance of this molecular complex in lipid rafts, which participates in the protection against Aβ-induced toxicity. Indeed, mERα/VDAC association anchored to caveolin-1 has been shown in lipid rafts from frontal cortex, hippocampus and septum of, both, mouse and human brains (28,58). In agreement, it has been reported an increase in Aβ-induced toxicity concomitantly with dysfunctioning of this macromolecular complex (28). Moreover, part of mERα signalling to promote neuroprotection is related to the inactivation of VDAC1 by modulation of channel phosphorylation (34). This estrogen signalling mechanism involves the rapid activation of, both, PKA and Src-kinase cascades, the latter phosphorylating the channel in tyrosine residues, and blocking channel gating (36,37,67). Hormonal effects on VDAC1 appears to be specific of physiological estrogens since selective estrogen receptor modulators (SERMs) known to bind to ER, such as tamoxifen, induce the opposite effect on VDAC1, and cause the dephosphorylation in tyrosine residues of this channel (34).

Endogenous phosphorylation of VDAC1 in lipid raft fractions appears to be a general phenomenon as it has been observed in immortalized septal and hippocampal cell lines, cortical murine areas, and human cortical and hippocampal areas (34,35,40,60). Furthermore, toxic Aβ mechanism triggered at the plasma membrane induces VDAC1 dephosphorylation, observing an alteration of VDAC1 phosphoisoforms in lipid rafts of AD brains since the early stages (40). Similar results were obtained in this work at stages ADV/VI, where the porin phosphoisoforms were absent of lipid rafts. Indeed, anomalous alterations in VDAC pattern of phosphorylation have also been shown to be related to synaptic impairment in, both, AD patients and Down syndrome (68). Therefore, closing of the channel at the neuronal membrane appears to be crucial for neuronal preservation against Aβ toxicity. In addition, VDAC1 may also participate in Aβ processing, as the porin has been found associated with APP, gamma-secretase and Aβ in human cortical areas (39,44,69). Furthermore, the porin has been shown to be highly abundant at the vicinity of senile plaques and neurofibrillary tangles (28,61).

The functional integrity of the brain protein signalosome depends on the structural stability that a balanced raft lipid homeostasis provides. Lipid composition is a crucial parameter to determine the dynamic and functional properties of these microdomains (70). Particularly abundant in lipid rafts are cholesterol, sphingolipids and saturated fatty acids as key elements of these microdomains, providing peculiar physicochemical properties distinct from the rest of the

plasma membrane (71,72). Although less abundant, it has been claimed that lipid impairment in these membrane microdomains may also be a consequence of abnormally low levels of polyunsaturated fatty acids, in particular docosahexaenoic acid (22:6n-3, DHA). and arachidonic acid (20:4n-6, AA), and monounsaturated fatty acids (mainly oleic acid, 18:1n-9), thereby reducing the unsaturation index, which leads to increased viscosity and liquid order, in parallel with a reduction of lateral mobility and phase transition, that will undoubtedly affect lipid-protein and protein-protein interactions (49,73,74). Our univariate analyses revealed that lipid rafts in AD women exhibit lower levels of polyunsaturated (DHA and AA) and monounsaturated fatty acids (oleic acid), as well as increased sterol esters. AD lipid rafts also contained significant contents of *lyso*-phosphatidylcholine (LPC), which were totally absent in non-AD lipid rafts. The presence of LPC which is generated secondarily (although not exclusively) by free radical-catalyzed oxidation of polyunsaturated phosphatidylcholines, and are indicative of oxidative damage of membrane phospholipids (75). This finding supports the general notion that oxidative stress is an essential part of the pathological process in the progression of AD (76,77). Multivariate analyses revealed that all these variables are extracted in PC1 and account for a complete segregation of AD group from non-AD groups. It is known that abnormal lipid composition in these microstructures may modulate the formation and aggregation of $A\beta$, since it has been observed that APP and β-secretase, key elements of amyloid production, are trafficking into lipid rafts from the early stages of AD (ADI-II) (44). This phenomenon enhances interaction between these key elements of $A\beta$ production, thus providing a mechanism to increase toxicity. On the other hand, alterations of raft lipid matrix appear to occur during normal ageing, although this trend is accelerated since the first stages of AD, inducing protein molecular disarrangements that contribute to neuropathological progression of the disease (43,44,49) which suggest a mechanistic correlation between lipid alterations and toxic amyloidogenesis. Furthermore, we have observed that the main lipid classes represented in PC2 (including sphingomyelin, cholesterol, sulphatides and cerebrosides) allow a certain degree of discrimination between pre- and post-menopausal women. These observations indicate that although no significant differences were observed in these lipid species individually, the whole set of these lipids are subjected to subtle changes that allow pre- and post-menopausic lipid rafts to be distinguished, thereby pointing to an effect of ageing and/or estrogen deprivation.

Interestingly, emerging findings suggest that, apart from triggering rapid signalosome responses, estrogens may play a crucial role in the homeostatic modulation of lipid rafts (52,78). These findings represent a new aspect of estrogen activity in the brain, and may be highly significant regarding beneficial estrogen actions in neurons. So far, very few studies have investigated the potential modulation of estrogens on brain lipids in AD brains (50,79-81). In particular, the hormone appears to regulate brain DHA levels, which is the most abundant n-3 long chain polyunsaturated fatty acid in neural tissues (82). Although this fatty acid is not particularly abundant in lipid rafts, it is essential to maintain a high degree of freedom and molecular disorder in these microstructures to allow macromolecular interactions (83). In support of this, DHA detriment in lipid rafts from cortical and hippocampal brain areas has been reported to increase raft order and viscosity, which is a main factor in AD development (44,49). Furthermore, physiological doses of the hormone appear to be crucial in regulation and bioavailability of brain DHA levels during pregnancy in rats and, conversely, DHA may also act synergistically with estrogen to stabilize brain lipid structure (50,56). This data suggests that an additional role of estrogens in brain preservation against AD neurodegeneration is through the preservation of DHA levels and dynamic in the brain, in particular in brain areas associated with cognitive activities, such as the frontal cortex and hippocampus (50,52). Taken together, these findings point to a dual role of estrogen in lipid rafts. On the one hand, the hormone may be a promoter of healthy intracellular responses modulated by $mER\alpha$-related signalosomes against $A\beta$ neuronal. On the other hand, estrogen may act as a lipostatic modulator to maintain lipid balance and integrity of these membrane microdomains (50).

In view of these reports, it is conceivable that falling estrogen levels that occur after menopause may be a condition that alters lipid raft functionality and integrity. To our knowledge, our observation on $ER\alpha$-related signalosome disruption and subsequent VDAC dephosphorylation reported here is the first demonstration in support of this hypothesis. Indeed, ERs are widely distributed in different brain regions, and estrogen signaling in numerous brain functions has been solidly, including rapid responses (84,85). Numerous studies have documented that women are protected against AD and other dementia relative to men, whereas this tendency is inverted following menopause and ageing (86-88). Among other factors, circulating estrogen decline is coincident with brain metabolic dysfunction and a detriment in bioenergetics may contribute to age-related cognitive decline, and may compromise brain health (66,89). The exact impact of low levels of circulating estrogen that cross the blood-brain barrier is uncertain since there is also a local production of endogenous estrogen in the brain (90,91). However, there is a direct correlation between the fluctuation of estrogen levels during the menstrual cycle and brain activity changes (92), thus indicating a direct and immediate effect of blood estrogen levels on the brain. Therefore, taking into account the minimal age of post-menopausal subjects (>65 y old), that was much higher than the final menstrual period (93,94), it is plausible that

undetectable levels of estrogen may also be expected in the brain of postmenopausal subjects used in this work.

Altogether, these data indicate that the decrease of estrogen occurring during menopause may have significant consequences on ERα-related signalosomes in cognitive areas, such as the frontal cortex. The effects of estrogen detriment on the impairment of these signaling platforms may be dual. On the one hand, it may induce protein-lipid and protein-protein rearrangements and toxic signaling, such as VDAC1 dephosphorylation, which may promote Aβ-induced toxicity. On the other hand, it may provoke alterations in the proportions of distinct lipid classes in lipid raft composition that may contribute to the onset and progression of AD. This information opens up new perspectives in potential indicators of the disruption of membrane lipid rafts that may be detected as peripheral biomarkers to identify women at risk for AD development as a consequence of estrogen detriment during menopause.

6. ACKNOWLEDGEMENTS

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Abbreviations: ERα, Estrogen Receptor alpha; IGF-IRβ, insulin growth factor-1 receptor beta; CSD, caveolar scaffolding domain

Key Words: 17beta-estradiol, Lipid Rafts, Alzheimer's Disease, Menopause, Estrogen Receptor Alpha, Voltage-dependent Anion Channel, Caveolin 1, Insulin Growth Factor-1 Receptor Beta, Membrane Lipids

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