Scavenger receptors internalize chemically modified low density lipoprotein particles (ac-LDL) and other ligands through the process of receptor-mediated endocytosis. During this investigation using amyloid-β as a natural ligand for the SR, we studied under a ligand-induced oxidative stress condition, changes in protein expression of several adaptor proteins important in the organization of the endocytic machinery in microglia and macrophages. Differential expression experiments of β-adaptin, α-adaptin, SR-AI, and SR-BI in RAW (macrophages) and EOC (microglia) cells were performed according to dosage and exposure time to amyloid-β. Our results show that according to dosage, amyloid-β produces an oxidative stress state that importantly affects the availability of β-adaptin. Under these conditions, RT-PCR assays show that β-adaptin mRNA is normally synthesized, reason why protein translation or protein structure of β-adaptin might be altered. These observations might have impact in the understanding of the mechanisms microglia employ to process amyloid-β in the brain.

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Scaevenger receptors (SR) divided into six classes consist of a broad family of membrane receptors that internalizes chemically modified low density lipoproteins (LDL) and several other ligands [1]. These receptors are localized in macrophages and similar cells such as microglia, as well as in hepatocytes, ovary, testicle, suprarenal gland, and astrocytes [2].

SR-AI is able also to bind oxidized LDL (ox-LDL), methylated bovine serum albumin (M-BSA), polyninosinic acid, polyguanosin acid, polysaccharides such as dextran sulfate, fucoidin, lipoteichoic acid (LTA), lipopolysaccharide (LPS), and advanced glycosylation end-products (AGE) [3]. Another important ligand for this receptor is the amyloid-β peptide (Aβ) [4]. Aβ in high concentrations acquires an insoluble β-folded structure that leads to fibril formation, process directly related to extracellular peptide accumulation, formation of neurite plaques, development of oxidative stress, and cell death [5].

SR-AI endocytosis is carried out through the formation of coated pits on the cell surface [6]. The VXFD amino acid sequence found in the cytoplasmatic tail of SR-AI is fundamental for the internalization of this receptor, in a similar fashion with specific sequences identified in other receptors important for endocytosis [7].

The class B type I scavenger receptor (SR-BI) was identified due to its binding to ac-LDL, ox-LDL, and native LDL. It also binds M-BSA, anionic phospholipids, apoptotic cells, Aβ, very low density lipoproteins (VLDL), and high density lipoproteins (HDL) [8]. SR-BI has been localized in diverse cell types, including glia and microglial cells [4,9].

Although SR-BI plays a fundamental role in reverse cholesterol transport due to its binding capacity for
HDL, it does not internalize lipoprotein particles through the classical process of receptor-mediated endocytosis, but rather selectively transfers lipids from the cell membrane to lipoproteins [10]. Notwithstanding, when ligands are of a proteic character, SR-BI is considered an endocytic receptor since it promotes uptake and degradation of AGE-BSA, M-BSA, ox-LDL, and ac-LDL [11].

The mechanism followed by SR-BI internalization is not known with certainty, but it is believed to occur through caveolae, since approximately 60% of SR-BI within a cell is found in cholesterol-rich lipid domains associated to these structures. Although the remaining 40% of SR-BI is localized in caveolin-poor zones [12], there is also evidence that endocytosis through this receptor might be carried out through clathrin-coated pits [13]. On the other hand, CD36, a class B scavenger receptor, has been reported to be expressed on microglia in Alzheimer disease brains and mediates production of reactive oxygen species (ROS) [14].

Nearly 30 proteins involved in clathrin-mediated endocytosis have been described; many of these are accessory molecules that aid in the formation of the coated pit. Among these, in addition to clathrin, the clathrin assembly lymphoid myeloid leukemia protein AP180/CALM, Eps15, dynamin, and amphiphysin stand out [15].

Clathrin monomers are arranged within a structure denominated triskelion, composed of three heavy and three light chains that bind in polyhedral structures, fundamental in the coated pit formation. The N-terminal end of the clathrin heavy chain interacts with proteins involved in endocytosis [16]. α-adaptin, which makes up together with the β-, µ-, and σ-adaptin part of the AP2 complex is important for the binding of this heterotetramer to the plasma membrane, as well as for its interaction with CALM and amphiphysin [17]. On the other hand, β-adaptin possesses two clathrin-binding sites [18] and intervenes in the selection of the vesicle cargo molecule [19]. AP180/CALM presents binding sites for clathrin, AP2 [20], and for PtdIns(4,5)P2-containing membranes [21]. Interestingly, the epidermal growth factor (EGF) receptor pathway sub- strate clone 15 (Eps 15) is a protein that interacts with AP180/CALM, epsin, α-adaptin, as well as several other accessory proteins involved in endocytosis [22].

From our laboratory, previous work employing tert-butylhydroperoxide (TBH) has established that SR-AI-transfected CHO cells under oxidative stress present the ability to bind ac-LDL, while lipoprotein internalization practically disappears [23]. Similar results have been found with C6 (glia) and PC12 (pheochromocytoma) cells, which possess SR-BI in a natural form (Aguilar-Gaytán et al., unpublished data). In both cases, oxidative stress did not modify either mRNA or SR protein expression levels.

Therefore, the main objective of this study has been to evaluate possible modifications in the expression of several of these endocytic proteins during an episode of natural oxidative stress provoked by Aβ. Together with the possible consequences this situation might have upon SR func-

tion, for the first time we have been able to study the interactions between the cytoplasmic region of the SR and endocytic proteins in response to binding of Aβ to the SR.

The experiments were conducted with mouse macrophages (RAW) and mouse microglia (EOC) cells, two cell lines that possess SR-AI and SR-BI naturally. Macrophages were used as control cells and microglia as resident macrophages of the brain that present a well-established physiological relationship with Aβ deposits.

Materials and methods

Materials. Salts and buffers were purchased from Sigma (St. Louis, MO). High-purity synthetic peptides were obtained from PeptidoGen Research & Co., Inc. (Livermore, CA). Reagents for cell culture (GIBCO-Invitrogen, Carlsbad, CA). Rat anti-mouse SR-AI/II antibody (Serotec, Raleigh, NC). Rabbit anti-mouse SR-BI antibody (Calbiochem-Merck, Darmstadt, Germany). Anti-endocytic proteins, goat polyclonal anti-Aβ and secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The ECL Plus kit as well as protein G Sepharose 4 Fast Flow (Amersham Bioscience, Uppsala, Sweden). 6-Carboxy-H2DCFDA [6-carboxy-2,7’-dichlorodihydrofluorescein diacetate, di (acetoxyethyl ester)] (Molecular Probes, Eugene, OR).

Amyloid-β fibril formation. Aβ1-42 peptide (Gly-Ser-Asn-Lys-Gly-Ala-Ile-Ile-Ile-Ile-Glu-Leu-Met) considered the most toxic fragment from the original 40-42 amino acid peptide (Asp-Ala-Glu-Phg-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Glu-Ser-Azn-Lys-Gly-Ala-Ile-Ile-Ile-Ile-Glu-Leu-Met) was used to induce oxidative stress. Aβ1-42 was solubilized in deionized water at 10 µg/ml and stored at −20 °C prior to use. To induce fibril formation, Aβ1-42 peptide was resuspended in serum-free culture media and incubated for 3 days at 37 °C. Fibril formation was confirmed by electron microscopy (data not shown). In control assays the reverse Aβ42-35 peptide was used (Met-Leu-Gly-Ile-Ile-Ala-Gly-Lys-Asn-Ser-Gly).

Cell culture. RAW cells were grown in Minimum Essential Media (MEM), and EOC cells in Dulbecco’s modified Eagle’s medium (DMEM), containing penicillin (50 U/ml)/streptomycin (50 µg/ml) and supplemented with 10% fetal bovine serum at 37 °C in a 5% CO2/95% air atmosphere. EOC cell medium was supplemented with 20% LADMAC conditioned media (produced from the LADMAC cell line, rich in growth factor colony stimulating factor 1).

Oxidative stress assays. 6-Carboxy-H2DCFDA was resuspended in DMSO at 10 mM concentration and stored at −20 °C. RAW and EOC cells were exposed at Aβ25-35 (0–200 µg/ml) and 1 or 20 µM 6-carboxy-H2DCFDA for RAW and EOC cells, respectively, during 24 h in serum-free culture medium. Cell fluorescence intensity was measured on a FAC-Scalibur instrument from Becton–Dickinson (NJ, USA).

Immunobots. Cells were treated during 24 h or indicated times with different doses of Aβ25-35 in serum-free media. After this procedure, cells were washed twice with PBS and lysed for 45 min at 4 °C in lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1% Triton X-100, 0.5% NP40, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 10 mM benzami- dine, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 250 µM PMSF). Lysed cells were centrifuged at 1500 g for 10 min at 4 °C and the supernatant recovered. Protein concentration was determined using the Micro-BCA protein assay (Pierce, Rockford, IL) and samples (10 µg/lane) from the total protein fraction were analyzed by SDS-PAGE on 10% gels further transferred to nitrocellulose paper (Bio-Rad, Hercules, CA). Membranes were blocked overnight at room temperature with a solution containing 5% fat-free milk and 1% Tween 20 in Tris-buffered saline (TBS), pH 7.6. The following antibodies each diluted 500-fold were used: goat polyclonal anti-heavy chain of clathrin, β2 and α2-adaptins and a rabbit polyclonal anti-Eps15. A rat monoclonal anti-mouse scavenger receptor AI/II antibody and a rabbit polyclonal anti-mouse scavenger receptor BI antibody.
were also used diluted 100-fold. Secondary antibodies consisted of a donkey anti-goat IgG, a goat anti-rabbit IgG both conjugated to horseradish peroxidase, and a goat anti-rat horseradish peroxidase mouse-absorbed. Secondary antibodies were used employing 5000-fold dilutions and were incubated for 1 h at 37 °C in blocking buffer. The membranes were washed with TBS-Tween 1%, and HRP activity was detected using the ECL Plus kit according to the supplier’s instructions. Band images were scanned and analyzed using ImageJ software (National Institute of Health, Bethesda, Maryland, USA).

RT-PCR. RAW and EOC cells were treated with Aβ<sub>25-35</sub> (0–200 μg/ml) for 24 h in serum-free medium. RNA was isolated with Trizol (Invitrogen Co.), according to the manufacturer’s instructions. The level of transcripts from encoding RNAs for scavenger receptor AI and BI, and endocytic proteins were determined by RT-PCR starting with 2 μg of total RNA using the GeneAmp RNA Kit (Applied Biosystems). cDNAs were derived using 2.5 μM random hexanucleotides and oligo(dT)<sub>18</sub>, and 2.5 U MuLV of reverse transcriptase at 42 °C for 45 min followed by denaturation at 99 °C for 5 min. cDNAs were amplified by PCR using specific primers (0.2 μM) and 2.5 U of AmpliTaq DNA polymerase during 30 cycles; denaturation at 99 °C for 2 min, annealing at indicated temperature for each primer pair (60–63 °C) for 30 s, and extension at 72 °C for 1 min. SR-AI sense primer was 5′-CTGGAC AACTG GTCCAC CT-3′ and antisense primer 5′-TCCCCC TTCTCT CCCCTT GT-3′. SR-BI sense primer 5′-CAGGCT GTGGGA ACTCTA GC-3′ and antisense primer 5′-GAAGAA GGCACA GATACA GC-3′. β-adaptin sense primer 5′-TTTGTT GGCATT GGACTG TA-3′ and antisense primer 5′-AGAGC CC GTGCAT AACTGA CC-3′. β-adaptin sense primer 5′-CGCTCC TTCACC TACTCC TG-3′ and antisense primer 5′-GTGAGT AAACGT CCCCCGA AA-3′. Clathrin sense primer 5′-GTCGAGC ACAAGGA ATCTGC AA-3′ and antisense primer 5′-CAATCA ACACCT GCACTG CT-3′. β-actin sense primer 5′-GGAGCA AGGAGA TTTCGA TC-3′ and antisense primer 5′-GGTCTC AACCAT GACCTG GG-3′.

Immunoprecipitations. Cells were treated with Aβ<sub>25-35</sub> (20 μg/ml) for 0, 5, 10, 15, and 30 min, and lysed as previously described. Five micrograms of cellular protein lysate was incubated with the goat polyclonal antibody anti-β2-adaptin (1:50) for 1 h at 4 °C, and immune complexes were precipitated with protein G-Sepharose. Immunoprecipitated proteins were washed twice with lysis buffer and resuspended in 40 μl of 5X SDS loading buffer. Twenty microliter samples were run on a 10% denaturing SDS-polyacrylamide gel for immunodetection of associated proteins in the complex.

Results and discussion

Oxidative stress

In order to determine, whether cells in contact with Aβ<sub>25-35</sub> present evidence of an oxidative stress state, we utilized the 6-carboxy-H<sub>2</sub>DCFDA reagent, a molecule that easily passes through the cell membrane and reacts with reactive oxygen species (ROS). Fig. 1A shows fluorescence intensity of RAW cells exposed to increasing doses of Aβ<sub>25-35</sub>. We observe that RAW control cells without serum present a basal fluorescence similar to the one shown by control cells with serum, indicating a low stress level due to its absence. Once exposed to Aβ<sub>25-35</sub>, RAW cell fluorescence increases gradually. EOC cells treated with increasing doses of Aβ<sub>25-35</sub> (0–200 μg/ml) in the presence of 20 μM 6-carboxy-H<sub>2</sub>DCFDA and cultured in the absence of serum for 24 h showed the same tendency in fluorescence increase (Fig. 1B). Since in the past microglia have shown to be relatively resistant to Aβ-induced cell death, which must have probably takes place through the development of different mechanisms against the production of ROS [24], in our hands, using incubation times up to 24 h and maintaining cell viability above 50%, success was achieved in establishing an oxidative stress state in this cell type. Moreover, the amount of Aβ employed had to be maintained relatively high in order to obtain clearcut responses. This set of results supports the fact that RAW and EOC cells develop an Aβ<sub>25-35</sub> dependent oxidative stress state, consistent with previous reports showing that this peptide produces high levels of ROS not only in rat cortical neurons [25] and hippocampus neurons [26], but also in microglia [27].

Cell viability of RAW and EOC cells was evaluated by the trypan blue method when in contact with different doses of Aβ<sub>25-35</sub> during 24 h. Viability percentages decreased up to 50% in the 200 μg/ml dose in both cell lines. Viability shown by control cells grown with or without serum was carried out in order to avoid possible interference with other receptors, since assays were always serum-free. Control experiments performed with the reverse Aβ<sub>35-25</sub> peptide showed that cell viability was not affected in either of the experiments carried out with or without the presence of serum (data not shown).

Protein expression and endocytosis

We evaluated Aβ<sub>25-35</sub> internalization in RAW and EOC cells through immunofluorescence assays with an anti-Aβ antibody and a rhodamine-coupled secondary antibody. During these assays, we observe according to the dosage employed that cells internalize Aβ<sub>25-35</sub> and, found distributed throughout the cytoplasm. Although, we observed high fluorescence levels at the higher doses of peptide, fluorescence is lost when using 200 μg/ml. It is possible that exposure of cells to high concentration of Aβ<sub>25-35</sub>, as further explored in our study, promotes cells to lose the capacity to internalize Aβ through the SR, a phenomenon apparently directly related to the disruption of the endocytic machinery (data not shown).

To date, there are few data concerning the influence Aβ exerts upon proteins that participate in the endocytic machinery [28]. Therefore, this study, for the first time, explores the effect of an oxidative stress state produced by exposure to Aβ<sub>25-35</sub> upon the expression of proteins involved in the process of endocytosis. This condition might be considered of special interest when studying cells that normally present the SR and under physiological conditions can be found in direct contact with Aβ deposits. To study this phenomenon, we analyzed the expression levels of SR-AI, SR-BI, α-adaptin, β-adaptin, and Eps15 all involved in receptor-mediated endocytosis.

First, we used total protein extracts from RAW and EOC cells treated for 24 h with different dosages of control peptide Aβ<sub>35-25</sub> (0–200 μg/ml). These immunotransfers show that cell treatment with the control peptide does not alter the levels of α-adaptin, β-adaptin, Eps15, SR-AI or SR-BI (Fig. 2A and C). Nevertheless, expression levels of the same proteins studied with RAW and EOC cells
treated for 24 h with Aβ25–35 (0–200 μg/ml) show an important decline of β-adaptin levels in direct correlation to the Aβ25–35 concentration employed (Fig. 2B and D). A clear decrease in the level of β-adaptin at a moderate concentration of Aβ25–35 (50 μg/ml) is evident. Protein band analysis from these experiments was consistent with this important decrease (data not shown).

The importance of β-adaptin in this process lies on the fact that it shows two clathrin-binding sites: a clathrin-box motif in the hinge region and a specific site in the ear region [18] that participates in the selection of vesi-
cle-charged molecules [19]. On the other hand, RAW and EOC cells treated for 24 h with Aβ25–35 show an increased level of SR-BI in relationship with the concentration of Aβ employed (Fig. 2B and D). Moreover, EOC cells also show an increase in α-adaptin according to the Aβ25–35 dosage concentration used. We believe changes in the level of expression of this accessory protein, as well as those observed for SR-BI, might be considered as compensatory mechanisms in response to the important decrease in the level of β-adaptin during an oxidative stress state.

Fig. 1. Cell oxidative stress. Fluorescence intensity analyzed by flow cytometry of (A) RAW and (B) EOC cells exposed for 24 h to Aβ25–35 (0–200 μg/ml). RAW and EOC monolayers were grown in the presence of Aβ25–35 and exposed to (A) 1 μM or (B) 20 μM 6-carboxy-H2DCFDA, washed, scraped, and resuspended in PBS buffer. Fetal bovine serum (FBS). Values in this set of results are expressed as means of four different experiments ± standard error (*p < 0.05).

Fig. 2. Endocytic proteins. Expression levels of endocytic proteins α- and β-adaptin, Eps15, SR-AI, and SR-BI. (A) RAW cells treated with different doses (0–200 μg/ml) of Aβ35–25 (control peptide), (B) RAW cells incubated with Aβ25–35 (0–200 μg/ml), (C) EOC cells treated with Aβ35–25, and (D) EOC cells exposed at Aβ25–35. All experiments were performed in serum-free medium after an incubation period of 24 h. Representative experiment of a series of 3.
Interestingly, when ac-LDL was used as a ligand, the levels of β-adaptin remained stable (data not shown). This result seems to be of relevance since although both ligands are recognized by the SR, only one is capable of affecting β-adaptin levels. This might be related to the fact that Aβ25–35 is capable to initiate and maintain an oxidative stress condition, in contrast to ac-LDL.

RT-PCR

Our study for the first time reports the effect of Aβ25–35 exposure upon the SR, α-adaptin, β-adaptin, and clathrin transcript levels (Fig. 3). Interestingly, an increase in mRNA expression of α-adaptin and SR-BI is observed in accordance with the Aβ25–35 dosage employed in both RAW and EOC cells. We believe this phenomenon again might be considered as a cell response in an attempt to render endocytosis in an effective way as a compensatory mechanism to the decrease in the availability of β-adaptin.

Since immunoblot assays indicate an important decrease in the signal shown by β-adaptin in both cell types (Fig. 2B and D) and mRNA expression assays do not show any changes when cells are exposed to an oxidative stress state, our results indicate the possibility that damage is exerted upon β-adaptin at the translational or post-translational level.

Immunoprecipitation assays

Although several interactions between diverse proteins of the endocytosis machinery are known in direct relationship to a series of receptors, there are no specific data of this phenomenon with respect to the SR. To conduct more detailed experiments of the interactions that occur between proteins during the process of clathrin-mediated endocytosis of Aβ25–35 through the SR, we carried out immunoprecipitation assays.

These assays were performed with RAW and EOC cells during 0, 5, 10, 15, and 30 min using a single concentration of Aβ25–35 (20 μg/ml), and immunoprecipitating with anti-β-adaptin antibody. We found that β-adaptin immunoprecipitates with α-adaptin in both cell lines even in the absence of Aβ (Fig. 4A and B). From this result, we conclude that β-adaptin must probably interact directly with α-adaptin, in support of results that show these two proteins form part of the AP-2 complex [17,29].

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**Fig. 3.** Protein transcript levels. Ethidium bromide-stained gels of RT-PCR assays of α- and β-adaptin, clathrin, SR-AI, and SR-BI from RAW and EOC cells treated for 24 h with Aβ25–35 (0–200 μg/ml). Representative experiment of a series of 3.

**Fig. 4.** Interaction of endocytic proteins. Immunoprecipitation assays using anti-β-adaptin antibody of (A) RAW and (B) EOC cells exposed to Aβ25–35 (20 μg/ml) for 0, 5, 10, 15, and 30 min in serum-free medium. Representative immunoblots of a series of three independent experiments.
β-adaptin interacts with Eps15 also, independently of the presence of the ligand in EOC cells, it seems that in the case of RAW cells, Eps15 does not communoprecipitate with β-adaptin.

On the other hand, SR-AI immunoprecipitates with β-adaptin in the absence of ligand in RAW cells (Fig. 4A). In contrast, the β-adaptin antibody is not able to communoprecipitate SR-AI in EOC cells. Therefore, in the first case, this interaction can be considered a preformed condition that does not change in response to ligand binding. Moreover, SR-BI immunoprecipitates with β-adaptin in both cell lines only when Aβ25-35 is used as a ligand (Fig. 4A and B). In this case, the interaction between β-adaptin and SR-BI seems to be promoted by Aβ (Fig. 4A and B).

In sum, we report β-adaptin as an important molecule of the protein machinery involved in clathrin-mediated endocytosis of the SR. The availability of β-adaptin decreases notably in response to Aβ25-35 binding that in turn mediates a state of oxidative stress. Since binding of ligands such as LDL at concentrations like the ones used in this study does not unchain oxidative stress by itself, in this case we observe β-adaptin levels to remain constant. It is interesting to mention that although the degree of similarity between the scavenger A and scavenger B receptors is non-existent, both receptors decrease their endocytic capacity under the action of high concentrations of Aβ. Since ligand binding is not affected, this result together with previous findings from our laboratory [23], suggests that an impaired internalization of Aβ is a consequence of the lack of organization of the endocytic machinery due to β-adaptin alteration, secondary to the establishment of an oxidative stress state. Since RT-PCR assays show that β-adaptin mRNA is normally synthesized in both cell lines studied under oxidative stress, our results put forward the possibility that this condition might be affecting protein translation or directly altering the protein structure of β-adaptin. These possibilities are currently being explored in our laboratory.

Our finding identifying β-adaptin as a key molecule in the internalization of Aβ advances the understanding of the possible mechanisms involved in the accumulation of Aβ in the extracellular space of neurons from patients suffering of Alzheimer disease. The possibility this phenomenon might apply to other diseases such as atherosclerosis, a process started to be considered as an amyloid related disease, remains also to be studied.

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