Implication of the small GTPase Rac1 in the generation of reactive oxygen species in response to β-amyloid in C6 astroglioma cells

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Exogenous application of β-amyloid ($A\beta_{25-35}$, a fragment of $A\beta_{1-42}$) significantly elevated levels of reactive oxygen species (ROS) in C6 astroglioma cells, as measured by confocal microscopic analysis of H_2O_2-sensitive 2′,7′-dichlorofluorescin fluorescence. Subsequent characterization of the signalling pathway revealed that expression of RacN17, a dominant-negative Rac1 mutant, completely blocked $A\beta_{25-35}$-induced generation of ROS, which is indicative of the crucial role played by Rac GTPase in this process. To better understand the downstream mediators affected by Rac, we assessed the degree to which inhibition of cytosolic phospholipase A_2 ($cPLA_2$) and 5-lipoxygenase (5-LO) contributed to the response and found that inhibition of either enzyme completely blocked $A\beta_{25-35}$-induced ROS generation, indicating its dependence on arachidonic acid synthesis and metabolism to leukotrienes (e.g. leukotriene B_4). Consistent with those findings, $A\beta_{25-35}$ Rac-dependently stimulated translocation of 5-LO to the nuclear envelope and increased intracellular levels of leukotriene B_4, while exogenous application of leukotriene B_4 increased intracellular H_2O_2 via BLT, its cell-surface receptor. In addition to the aforementioned downstream mediators, inhibition of phosphoinositide 3-kinase (PI 3-kinase), an enzyme situated upstream of Rac, also completely blocked $A\beta_{25-35}$-induced H_2O_2 generation. Our findings thus demonstrate that PI 3-kinase, Rac, $cPLA_2$ and 5-LO are all essential components of the β-amyloid signaling cascade leading to generation of ROS.

Key words: $A\beta$, cytosolic phospholipase A2, leukotriene B_4, signal transduction.

INTRODUCTION

Alzheimer's disease (AD) is characterized neuropathologically by the accumulation of plaques containing β-amyloid ($A\beta$) fibrils, reactive astrocytes and activated microglia [1–2]. Moreover, a number of studies have shown that $A\beta$ may provide a linkage between oxidative stress and AD-associated neuronal cell death. For example, $A\beta$ stimulates microglia to secrete pro-inflammatory cytokines and reactive oxygen species (ROS), which damage neurons [3–6], which in turn stimulates astrocytes to up-regulate chemokine release, cytokine expression and ROS production, thereby triggering an inflammatory cascade [6,7]. Such $A\beta$-evoked ROS generation appears to be critical to the pathogenesis of AD. H_2O_2, in particular, appears to be an important intermediate in $A\beta$ neurotoxicity [8]. Beyond that, however, little is known about the intracellular signalling mediators involved in $A\beta$-evoked ROS generation, especially in astrocytes.

There are a number of potential sources of cellular ROS, including NADPH oxidase and 5-lipoxygenase (5-LO), which catalyses the synthesis of leukotrienes (LTs) from arachadonic acid (AA), a principal product of cytosolic phospholipase A_2 ($cPLA_2$) [2]. While Rac, a Rho family GTPase, is known to function as a regulator of intracellular ROS generation in non-phagocytic cells [9,10], its role in $A\beta$-evoked signalling to ROS generation has never been characterized. Rac activation leads to stimulation of PLA_2, in particular the cytosolic isoform ($cPLA_2$), which was therefore postulated to be a major downstream mediator of Rac signalling within cells [10–12]. For example, activation of $cPLA_2$ and the resultant release of AA have been implicated in Rac-mediated stimulation of Ca^{2+} influx and Rho-dependent cytoskeletal reorganization in fibroblasts [11,13]. In addition, we previously showed that this Rac–$cPLA_2$–AA cascade mediates nuclear signalling in response to tumour necrosis factor (TNF)-α or ceramide, a product of sphingomyelin hydrolysis elicited in response to various stresses and proinflammatory cytokines [12,14], and that Rac signalling to ROS generation and c-Jun N-terminal kinase (JNK) stimulation is dependent upon metabolism of AA to LTs by 5-LO [15,16].

These findings led us to hypothesize that a Rac–$cPLA_2$–5-LO-linked cascade may mediate $A\beta$-induced ROS generation. The results of the present study strongly suggest that all three, as well as phosphoinositide 3-kinase (PI 3-kinase), are essential components of the intracellular signalling cascade triggered by $A\beta$ and leading to ROS generation in C6 astroglioma cells.

EXPERIMENTAL

Chemicals

$A\beta_{25-35}$ (a fragment of $A\beta_{1-42}$ that shows a similar activity to the whole molecule) was purchased from Bachem (Bubendorf, Switzerland) and freshly prepared by dissolving it in distilled water (1 mM stock). Alternatively, $A\beta_{25-35}$ was dissolved in

Abbreviations used: AA, arachidonic acid; AA861, 2-(12-hydroxydodeca-5,10-dienyl)-3,5,6-trimethyl-1,4-benzoquinone; AACOCF3, arachidonyl trifluoromethyl ketone; $A\beta$, β-amyloid; AD, Alzheimer's disease; BLT, leukotriene B_4 receptor; $cPLA_2$, cytosolic phospholipase A_2; DCF(DA), 2′,7′-dichlorofluorescin (diacetate); DMEM, Dulbecco's modified Eagle's medium; DPI, diphenylene iodonium; FBS, fetal bovine serum; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; LTB_4APA, leukotriene B_4-3-aminopropylamide; 5-LO, 5-lipoxygenase; LTs, leukotrienes; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PI 3-kinase, phosphoinositide 3-kinase; ROS, reactive oxygen species; RT-PCR, reverse-transcription PCR; TNF-α, tumour necrosis factor-α.

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DMSO (100 mM), then diluted to 1 mM with distilled water and stored at 4 °C for up to 2 weeks before treatment. Diphenylene iodonium (DPI), arachidonyl trifluoromethyl ketone (AACOCF3) and LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one, an inhibitor of PI 3- and 4-kinases] were purchased from Calbiochem (La Jolla, CA, U.S.A.). 2-(12-Hydroxydodeca-5,10-dienyl)-3,5,6-trimethyl-1,4-benzoquinone (AA861, a 5-LO inhibitor), *N*-acetylcysteine and LTB_4 were from Sigma (St. Louis, MO, U.S.A.). LTB_4-3-aminopropylamide (LTB_4APA) was from Biomol (Plymouth Meeting, PA, U.S.A.). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), non-essential amino acids were from Gibco BRL (Gaithersburg, MD, U.S.A.). Antibodies to c-Myc, $cPLA_2$ and Rac1 were purchased from Santa Cruz Biotechnology (Santa Cruz. CA, USA). 2′,7′-Dichlorofluorescin diacetate (DCFDA) was from Molecular probes (Eugene, OR, U.S.A.).

Plasmids

pEXV, a Myc-tagged expression vector, and pEXV-RacN17, an expression vector encoding RacN17, a dominant-negative mutant of Rac1, were gifts from Dr Alan Hall (MRC Laboratory for Molecular Cell Biology, University College London, London, U.K.). pEGFP-C2-5-LO DNA was from Dr Colin Funk (Center for Experimental Therapeutics and Department of Pharmacology, University of Pennsylvania, Philadelphia, PA, U.S.A.). Oligonucleotide primers for BLT1 were purchased from Geno-Tech (Taejeon, Korea); the sequences were 5′-GTGCTGGCA-GGCATCTGGGT and 3′-GGCAGCCACAAGGCGGCGAA.

Cell culture and transfections

C6 astroglioma cells were obtained from the American Type Culture Collection (A.T.C.C., CCL-107) and were grown in DMEM supplemented with 10 % (v/v) FBS and 0.1 mM non-essential amino acids at 37 °C under a humidified air/CO_2 (19:1) mixture. C6 clones stably expressing RacN17 were prepared by co-transfection of pEXV-RacN17 and pcDNA-Neo^R plasmids, after which transfectants were selected for 2–3 weeks in the presence of G418 antibiotics (400 μg/ml). Expression of RacN17 protein in the selected clones was confirmed by Western-blot hybridization using anti-(c-Myc epitope) antibody as a probe. Transient transfection was carried out by plating approx. 3×10^5 cells in 60-mm-diameter dishes for 24 h, after which calcium phosphate/DNA precipitates (6 μg of DNA/dish) were added as described previously [14]. To control for variations in cell number and transfection efficiency, all clones were co-transfected with 0.3 μg of pCMV-β-GAL.

Subcellular fractionation of cell lysates and Western blotting

C6 cells were serum-starved for 12 h in serum-free DMEM and then exposed to Aβ for 10 min. The medium was then removed, and the cells were washed twice with ice-cold PBS, scraped off, harvested by microcentrifugation and resuspended in 0.5 ml of buffer A [137 mM NaCl/8.1 mM Na_2HPO_4/2.7 mM KCl/1.5 mM K_2PO_4/2.5 mM EDTA/1 mM dithiothreitol/ 0.1 mM PMSF/ leupeptin (10 μg/ml), pH 7.5]. Cytosolic and particulate fractions were prepared and analysed by Western hybridization as described previously [16]. Blots were incubated overnight with primary antibody in a blocking solution and then for 1 h with a horseradish peroxidase-conjugated secondary antibody. The bands were visualized using an enhanced chemiluminescence (ECL®) kit (Amersham).

Measurement of intracellular H_2O_2

Intracellular H_2O_2 was measured as a function of dichlorofluoroscin (DCF) fluorescence as described by Ohba et al. [17]. Briefly, cells were grown on coverslips for 1 day and then serum-starved in DMEM/0.5 % FBS for an additional 2 days. The cells were then stabilized in serum-free DMEM without Phenol Red for at least 30 min before exposure to agonists (LTs) for the indicated times. When assessing the effects of inhibitors, cells were pretreated with the respective inhibitor for 20 or 30 min prior to addition of the agonist. To measure intracellular H_2O_2, cells were then incubated for 7 min with the H_2O_2-sensitive fluorophore DCFDA (5 μg/ml), which, when taken up, fluorescently labels intracellular H_2O_2 with DCF. The cells were then immediately observed under a laser-scanning confocal microscope (Carl Zeiss LSM 410). DCF fluorescence was excited at 488 nm using an argon laser, and the evoked emission was filtered with a 515 nm long-pass filter. DCF fluorescence was measured in 30 randomly selected cells. Values represent means ± S.D. of DCF fluorescence intensity in three independent experiments. Statistical significance of H_2O_2 measurements was assessed with unpaired Student's *t* tests. Values of $P < 0.05$ were considered significant.

Translocation of 5-LO

Cells were grown on coverslips for 1 day and then co-transfected by incubation for 3 h with green-fluorescent-protein (GFP)-tagged 5-LO DNA and pEXV or pEXV-RacN17 using LIPOFECTAMINE™(Gibco BRL). The cells were supplemented with DMEM/5 % FBS and incubated for another 3 h, after which the media were changed to DMEM/0.5 % FBS for 36 h. The serum-starved cells were then exposed to Aβ_{25-35} (100 nM) for 10 min, washed with ice-cold PBS and fixed in 4 % paraformaldehyde. The photomicrographs shown were obtained using a Carl Zeiss Axiovert 25 microscope equipped for fluorescence microscopy; the excitation wavelength was 480 nM, the emission wavelength was 520 nM.

LTB_4 assays

Cells (3×10^5) were plated on 60-mm-diameter dishes and incubated first for 24 h in DMEM/10 % FBS and then for an additional 24 h in DMEM/0.5 % FBS, after which they were treated with Aβ for the indicated durations. To measure intracellular levels of LTB_4, the plates were rinsed twice with cold PBS, mixed with 4 vol. of 100 % ethanol and left at 4 °C for 30 min. The resulting precipitate was removed by centrifugation at 100000 ***g*** for 30 min at 4 °C. LTs were isolated from the ethanolic supernatant using a C2 reverse-phase column (RPN 1903; Amersham). The methyl formate in the eluted samples was removed by evaporation under vacuum, and the samples reconstituted in assay buffer were stored under argon at 50 °C until assay for LTB_4 using an ELISA (RPN 224; Amersham) as instructed by the manufacturer. The enzyme immunoassay was calibrated with standard LTB_4 over the range 0.31–40 pg/well. The sensitivity, defined as the minimal amount of LTB_4 needed to reduce zero dose binding, was 0.3 pg/well or 6 pg/ml. Statistical significance of LTB_4 assays was assessed by analysis of variance. Values of $P < 0.05$ were considered significant.

RNA isolation and reverse transcription (RT)-PCR

Cells were plated at a density of 1×10^6 cells on to 100-mm-diameter plates and maintained in DMEM/10 % FBS and for 24 h, after which they were collected, and their total cellular RNA

was extracted using RNAzol B (Tel-test, Friendswood, TX, U.S.A.), dissolved in diethyl pyrocarbonate-treated water and quantified by UV scanning. Samples (1 μg) of total RNA were then reverse-transcribed at 37 °C for 60 min in 20 μl of buffer (containing 10 mM Tris, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$ and 1 mM each of dATP, dCTP, dGTP, and dTTP) in the presence of 5′ and 3′ primers. Hot-start PCR was used to increase the specificity of the amplification. The PCR products were subjected to electrophoresis in 1.8 %-(w/v)-agarose gels, and the resultant bands were visualized by ethidium bromide staining and photographed using Polaroid 667 film.

RESULTS

Aβ induces intracellular ROS generation in C6 astroglioma

Aβ-induced generation of ROS was confirmed by directly measuring ROS levels as a function H_2O_2-sensitive DCF fluorescence. After serum starvation for 48 h, DCFDA-loaded C6 astrocytes were exposed to the indicated concentrations of A$\beta_{25–35}$ for 15 min (Figure 1A) or to 100 nM A$\beta_{25–35}$ for the indicated durations (Figure 1B). Application of A$\beta_{25–35}$ significantly increased DCF fluorescence intensities in a concentration-dependent manner, with the maximum ROS generation (an approx. 2.3-fold increase over control) being observed within 5 min and at an A$\beta_{25–35}$ concentration of 100 nM. Application of higher concentrations (results not shown) or exposure for longer periods (Figure 1B) diminished responses to A$\beta_{25–35}$. Similar patterns of ROS generation was observed by applying A$\beta_{1–42}$ (results not shown), indicating that the biological activities of both A$\beta_{25–35}$ and A$\beta_{1–42}$ are quite similar to each other, at least as regards ROS generation.

Essential role of Rac in Aβ-induced ROS generation

Rac plays a key role in mediating ROS generation in response to TNF-α in many non-phagocytic cells [9,10]. To investigate whether Rac is also involved in A$\beta_{25–35}$-evoked ROS generation, C6 clones stably expressing RacN17, a dominant-negative form of Rac1, were prepared. The expression of RacN17 protein was confirmed in several selected clones (colony numbers 6, 7, 8 and 9) by Western analysis using anti-(c-Myc) antibody as a probe (Figure 2A). Expression of the dominant-negative mutant completely blocked A$\beta_{25–35}$-induced increases in DCF fluorescence in all four of the C6-RacN17 clones (Figure 2B), strongly suggesting that Rac mediates the Aβ-induced signalling to ROS generation.

Roles of cPLA$_2$, NADPH oxidase and PI 3-kinase in Aβ signalling to ROS generation

We previously showed that PI 3-kinase and cPLA$_2$ respectively serve as upstream and downstream mediators of Rac in the signalling to ROS generation [12,16]. To test whether the same elements might participate in the generation of ROS by A$\beta_{25–35}$, we analysed the effects of LY294002 and AACOCF3, specific inhibitors of the activities of PI 3-kinase and cPLA$_2$ respectively. Pretreatment of C6 cells with either 20 μM LY294002 or 10 μM AACOCF3 completely blocked A$\beta_{25–35}$-induced ROS generation, as compared with their vehicle controls (Figure 3), and a similar inhibitory effect was exerted by DPI, a flavoprotein inhibitor. Together, these results indicate that the activities of cPLA$_2$ and PI 3-kinase, as well as a DPI-sensitive flavoprotein (e.g. NADPH oxidase), are required for Aβ signalling to ROS generation.

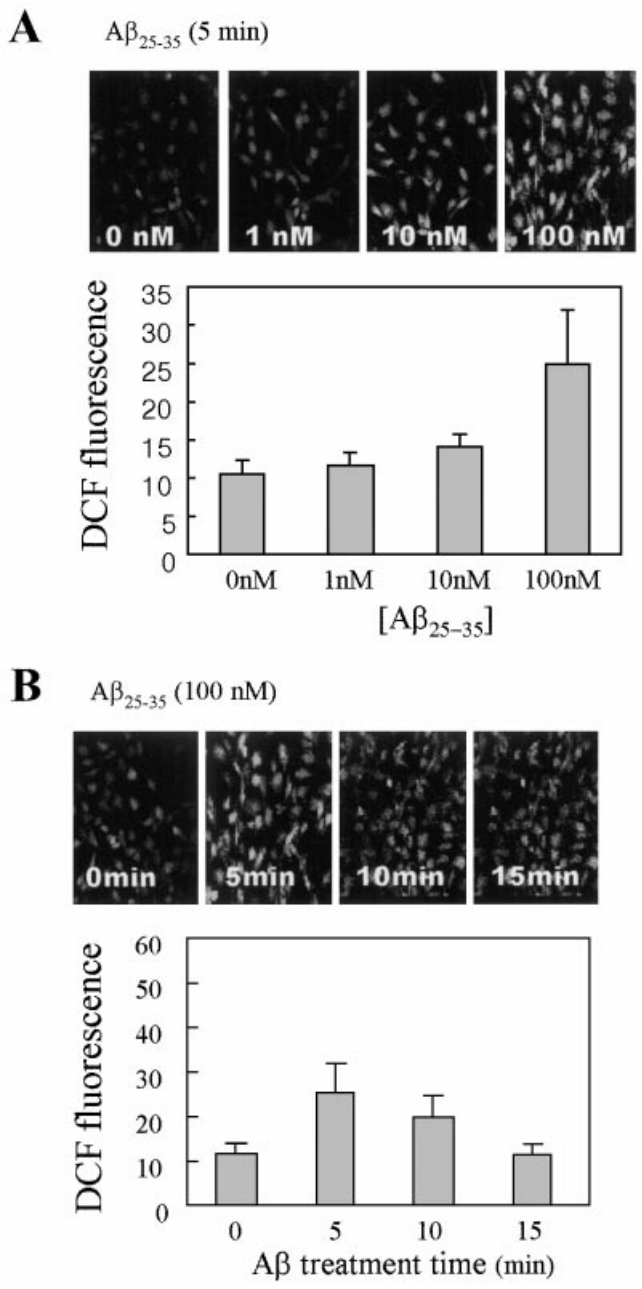

Figure 1 Aβ induces generation of ROS in C6 astroglioma

(**A**) C6 cells were serum-starved for 48 h and then stimulated with the indicated concentrations of A$\beta_{25–35}$ for 5 min. (**B**) Serum-starved cells were stimulated with 100 nM A$\beta_{25–35}$ for the indicated times. DCF fluorescence, reflecting the relative levels of ROS (arbitrary units), was imaged using confocal laser scanning fluorescence microscopy and then quantified as described in the Experimental section. Results are expressed as means $\pm$ S.D. ($n = 30$ cells). Statistical significance of changes in DCF fluorescence was assessed using unpaired Student's t tests ($P < 0.01$).

Translocation of Rac1 and cPLA$_2$ by Aβ

Upon their activation, Rac1 and cPLA$_2$ are translocated from the cytosolic fraction of cells to the particulate membrane fraction [18–20]. Therefore, to further confirm that activation Rac and cPLA$_2$ are involved in the intracellular signalling by Aβ, the effects of Aβ on the distribution of these proteins was

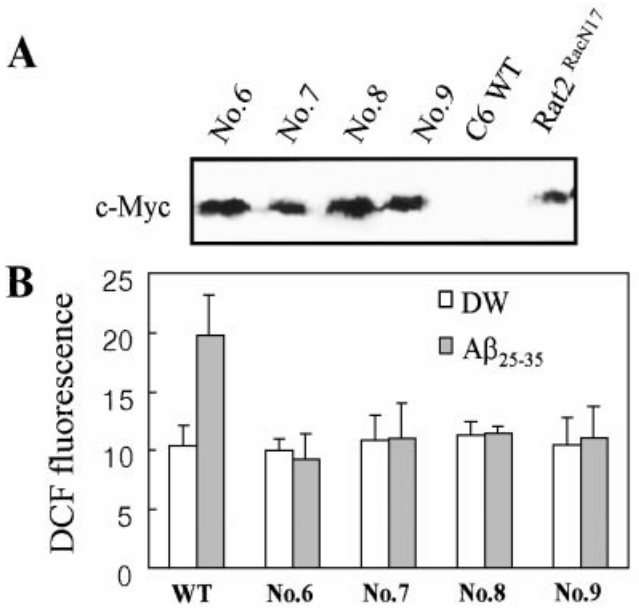

Figure 2 Aβ-induced ROS generation is dependent on Rac1

(**A**) Lysates of C6 cells (WT, wild-type) and selected C6-RacN17 stable clones were analysed for RacN17 expression by Western blotting using an anti-(c-Myc) antibody as a probe. Rat2-RacN17 cells [33], stably expressing RacN17, served as a positive control. (**B**) Serum-starved C6 and C6-RacN17 cells (clone nos. 6, 7, 8 and 9) were stimulated with 100 nM $A\beta_{25-35}$ or distilled water (DW) for 5 min. DCF fluorescence was immediately imaged and quantified as described in Figure 1. Results are expressed as means $\pm$ S.D. ($n = 30$ cells). Statistical significance of changes in DCF fluorescence was assessed using unpaired Student's t tests ($P < 0.01$).

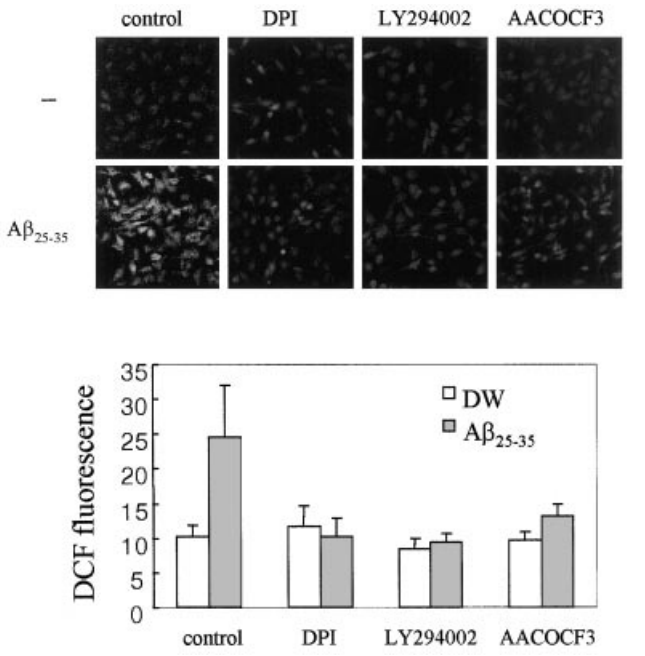

Figure 3 Aβ-induced ROS generation is dependent on cPLA$_2$, NADPH oxidase and PI-3 kinase

Serum-starved C6 cells were exposed to 100 nM $A\beta_{25-35}$ for 5 min in the presence or absence of DPI (10 μM), LY294002 (20 μM) or AACOCF (10 μM), which are inhibitors of flavoproteins (e.g. NADPH oxidase), PI 3-kinase and cPLA$_2$ respectively. The inhibitors were added 20 min prior to the addition of $A\beta_{25-35}$. DCF fluorescence was imaged (upper panels) and quantified (lower panels) as described in Figure 1. Results are expressed as means $\pm$ S.D. ($n = 30$ cells). Statistical significance of changes in DCF fluorescence was assessed using unpaired Student's t tests ($P < 0.01$).

examined. Figure 4 shows that most Rac1 was localized in the cytosolic fraction of unstimulated cells and that a significant component of Rac1 was translocated to the membrane fraction in response to 100 nM $A\beta_{25-35}$. Likewise, $A\beta_{25-35}$ also induced redistribution of cPLA$_2$ to the membrane fraction.

Role of 5-LO in Aβ-induced ROS generation

The dependence of Aβ-signalling to ROS generation on activation of cPLA$_2$ implies a role for AA or its metabolites in that process. We previously showed that synthesis LTB$_4$ by 5-LO metabolism of AA is situated downstream of the Rac–cPLA$_2$ cascade in TNF-α signalling to ROS generation [10]. Therefore, we next determined whether 5-LO is involved in Aβ-induced ROS generation. As shown in Figure 5(A), pretreating cells with 1 μM AA861 or 0.05 μM MK886, two specific inhibitors of 5-LO, almost completely abolished $A\beta_{25-35}$-induced generation of ROS.

The role of 5-LO in Aβ-signalling was further confirmed by examining the effect of $A\beta_{25-35}$ on the distribution of 5-LO, as this enzyme is reportedly translocated to the region of the nuclear envelope upon stimulation [19]. C6 astrocytes were transiently transfected with a GFP-tagged 5-LO expression plasmid, after which the distribution of 5-LO was determined by fluorescence microscopy. As shown in Figure 5(B), exposing cells to $A\beta_{25-35}$ (100 nM, 10 min) caused a rapid translocation of 5-LO from the cytosol to the area of the nuclear envelope, thereby forming a ring-like structure. Pretreatment with DPI or LY294002 abolished Aβ-induced 5-LO translocation, as did co-transfecting cells with a RacN17 expression vector (Figure 5B), but not the empty vector itself (results not shown).

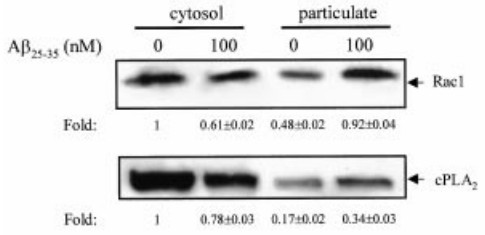

Figure 4 Aβ induces translocation of Rac1 and cPLA$_2$ from the cytosolic to the particulate fraction of C6 cells

The cells were serum-starved for 12 h and then exposed to 100 nM $A\beta_{25-35}$ for 10 min, after which the cytosolic and particulate fractions were prepared as described in the Experimental section. The processed sample extracts were subjected to SDS/PAGE and then Western blotting using anti-Rac and anti-cPLA$_2$ antibodies as probes. The results shown are representative of at least three independent experiments. Quantification of band intensity was carried out by Image J version 1.24 software (developed at the National Institutes of Health, Bethesda, MD, U.S.A.) and expressed as fold increases $\pm$ S.D. of control from three independent experiments. Statistical significance of quantification was assessed using unpaired Student's t tests ($P < 0.05$).

The above results encouraged us to test whether the level of LTs, such as LTB$_4$, is indeed enhanced by Aβ in C6 cells. After incubation in DMEM with 0.5% FBS for 24 h, C6 cells or C6-RacN17 cells stably expressing RacN17 were exposed to 100 nM Aβ for the indicated time periods (12 and 24 h). Consistent with the above results, a significant increased levels of LTB$_4$ (approx. 7.5-fold increase over 0 h) was observed at 24 h

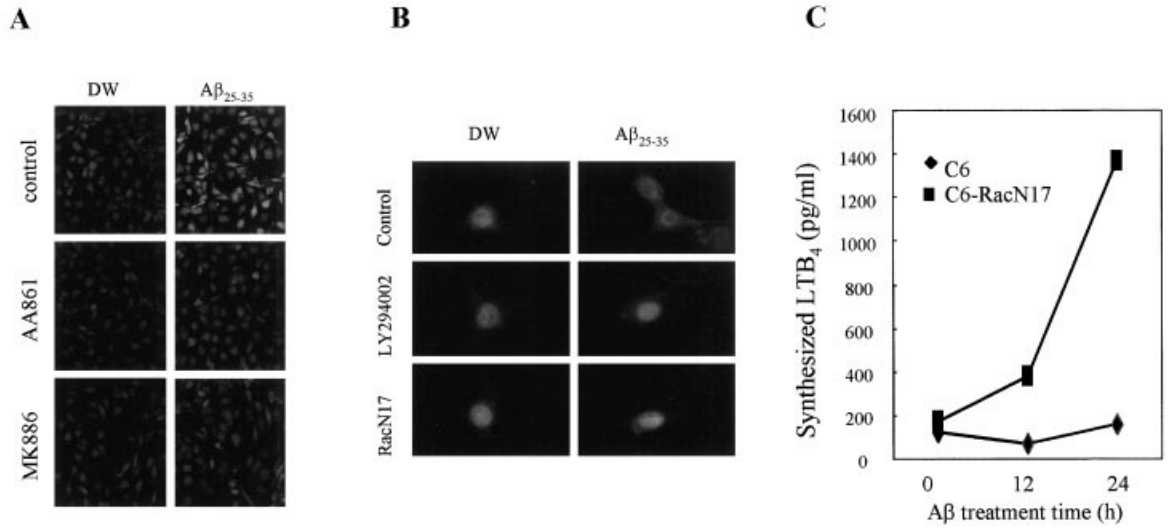

Figure 5 Aβ-evoked ROS generation is dependent on 5-LO

(**A**) Serum-starved C6 cells were exposed to 100 nM A$\beta_{25–35}$ for 5 min in the presence or absence of AA861 (1 μM) or MK886 (0.05 μM), which are two specific inhibitors of 5-LO. The inhibitors were added 30 min prior to the addition of A$\beta_{25–35}$. DCF fluorescence was imaged using confocal laser scanning fluorescence microscopy. (**B**) C6 cells were transiently co-transfected with GFP-tagged 5-LO and pEXV (top and middle panels) or pEXV-RacN17 DNA (bottom panels) using LIPOFECTAMINE^{TM} reagent. The transfectants were treated with 100 nM A$\beta_{25–35}$ or distilled water (DW, a vehicle control) for 5 min, washed with ice-cold PBS and fixed in 4% paraformaldehyde. DPI (10 μM) or LY294002 (20 μM) were then added 30 min prior to the addition of A$\beta_{25–35}$. Results are representative of three independent experiments. (**C**) C6 and C6-RacN17 cells were exposed to 100 nM Aβ for 12 h or 20 h, after which intracellular LTB$_4$ was assayed as described in the Experimental section. Results are the means for three independent experiments.

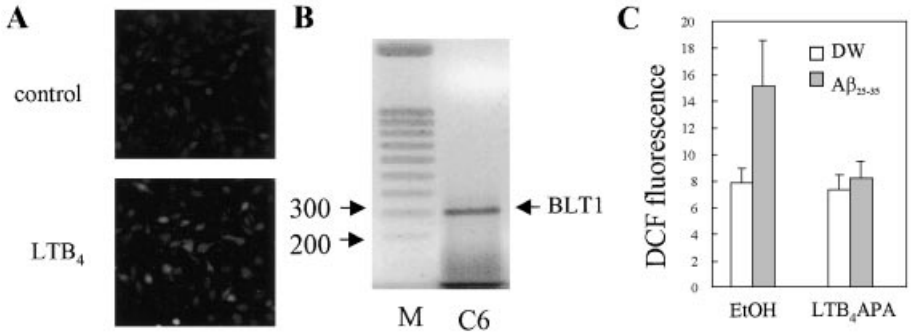

Figure 6 Role of BLT in Aβ-induced ROS generation

(**A**) C6 cells were serum-starved for 48 h and then stimulated with 100 nM LTB$_4$ for 3 min. DCF fluorescence was imaged using confocal laser scanning fluorescence microscopy. (**B**) RT-PCR was performed in C6 cells using BLT1-specific primers (lane C6); lane M, DNA size markers. (**C**) Serum-starved C6 cells were exposed to 100 nM A$\beta_{25–35}$ in the presence or absence of LTB$_4$APA (50 ng/ml), a BLT antagonist or ethanol (EtOh, vehicle control). The inhibitor and ethanol were added 20 min prior to the addition of Aβ. DCF fluorescence was quantified as described in Figure 1. Results are expressed as means $\pm$ S.D. ($n = 30$ cells). Statistical significance of ROS measurements was assessed using unpaired Student's t tests ($P < 0.01$).

in response to A$\beta_{25–35}$ at C6 cells, whereas no noticeable increased LTB$_4$ was detected in RacN17-expressing cells, supporting again a mediatory role of Rac1 in Aβ-induced 5-LO stimulation (Figure 5C).

Exogenous LTB$_4$ induces ROS generation

The capacity of LTs, such as LTB$_4$, to induced ROS generation was then directly assessed by examining the effects of their exogenous application to C6 astrocytes. Application of 100 nM LTB$_4$ significantly increased intracellular ROS levels (Figure 6A), whereas application of a mixture of cysteinyl-LTs (LTC$_4$/LTD$_4$/LTE$_4$) at the same concentration produced no noticeable effect (results not shown). However, a higher concentration of the cysteinyl LT mixture (1 μM) evoked a similar level of ROS generation (results not shown).

It has been suggested that LTB$_4$ acts in an autocrine factor via BLT, its specific cell-surface receptor [21–23]. To determine whether BLT is expressed in C6 astrocytes, we carried out RT-PCR using BLT1-specific primers. As shown in Figure 6(B), expression of a BLT1 with the expected size was indeed detected in C6 cells. Moreover, pretreating the cells with the BLT antagonist LTB$_4$APA completely blocked the increase in DCF fluorescence otherwise elicited by Aβ (Figure 6C). Apparently,

LTB_4 acts via BLT to exert an autocrine effect leading to ROS generation.

DISCUSSION

The results of the present study indicate that Aβ stimulates ROS generation in C6 astroglioma cells via a PI 3-kinase–Rac-$cPLA_2$–5-LO-linked cascade. By comparing the effects Aβ on ROS generation in C6 and C6-RacN17 cells, the role of Rac1 was demonstrated (Figure 2); activation of Rac1 by Aβ was then confirmed when its rapid translocation from the cytosolic to the membrane fraction was observed upon exposing cells to Aβ (Figure 4). The capacity of specific inhibitors of $cPLA_2$ and 5-LO (AACOCF3 and AA861/MK886 respectively) to block the effects of Aβ on ROS generation is indicative of the requirement for synthesis and metabolism of AA (Figure 3 and Figure 5A). The key role played by 5-LO was further confirmed by its translocation to the nuclear envelope in response to Aβ; by the rise in cellular LTB_4 levels induced by Aβ (Figures 5B and 5C) and by the capacity of exogenous LTB_4 to act via BLTs to increase ROS levels (Figure 6C).

Consistent with our findings, increasing evidence supports a signalling link between $cPLA_2$-mediated AA metabolism and ROS generation. For example, Li et al. [24] and Lindsay et al. [25] respectively suggest that LTB_4 activates ROS generation in neutrophils and eosinophils. Moreover, transcription of the 5-LO gene is up-regulated during the neurodegeneration seen in AD, as well as in normal aging [26–27], and the ratio of 5-LO contents of the membrane and cytosol is larger in the brains of older rats than younger ones. Together, these findings point to the involvement of 5-LO in the pathobiology of neurodegenerative diseases like AD [28], and perhaps in normal aging.

The molecule targeted by LTB_4 and leading to ROS generation is not known. Nonetheless, the present findings show that blockade of BLT1, a G protein-coupled, cell surface LTB_4 receptor, using a specific antagonist (LTB_4APA) markedly inhibited the effects of Aβ (Figure 6C). Although expression of BLT1 has been thought to occur mainly in polymorphonuclear leukocytes [28], we have been able to detect it in C6 cells (Figure 6B) and in a number of other cell types (results not shown). It is thus likely that LTB_4 serves an autocrine/paracrine function in the pathway via which Aβ induces ROS generation in astrocytes. In fact, action through an autocrine stimulatory loop was previously shown to be crucial for the ROS generation in response to TNF-α in non-phagocytic fibroblasts [10].

We also found that pretreatment with pertussis toxin significantly diminished Aβ-induced ROS generation by C6 cells (results not shown), which is consistent with a previous report that Aβ activates a pertussis toxin-sensitive G-protein-coupled chemoattractant receptor [19]. The precise function of the autocrine action of LTB_4 is not clear, but it may serve to amplify its own action and, in turn, Aβ signalling. Moreover, the capacity of LTB_4 to act as a potent chemoattractant in such diseases as bronchial asthma, inflammatory bowel disease and psoriasis, as well as in AD [29–32], may explain the mechanism by which LTB_4 mediates inflammatory reactions in response to Aβ.

There is substantial evidence indicating the presence of a $cPLA_2$–5LO-linked cascade downstream of Rac; on the other hand, the upstream components affecting Rac activity are less well understood. The present finding that Aβ stimulates Rac-dependent ROS generation via PI 3-kinase (Figure 3) is in accordance with our previous observation that PI 3-kinase acts upstream of Rac in TNF-α signalling to ROS generation [12]. Likewise, it was also shown that, in microglia cells, Aβ-induced ROS formation requires the activity of both PI 3-kinase and NADPH oxidase [3]. Usually, Aβ-induced production of ROS in non-phagocytic astrocytes is only 1–2 % of that seen in phagocytes, which produce large amounts of O_2^- as part of the body's defence against disease processes [33]. Consequently, generation of ROS in response to Aβ appears not to be involved in Aβ cytotoxicity; indeed no cytotoxicity was detected under our experimental conditions (results not shown).

In summary, the results presented are indicative of the crucial roles played by PI 3-kinase, Rac, $cPLA_2$ and 5-LO in the Aβ-induced generation of ROS. Although the specific function of Rac in the pathological progress of AD is not yet clear, characterization of an Aβ-activated Rac-linked signalling cascade leading to ROS generation in astroglial cells should contribute substantially to our basic understanding of the pathobiological processes underlying AD.

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