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

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Exogenous application of β-amyloid (Aβ25-35, a fragment of Aβ1-42) significantly elevated levels of reactive oxygen species (ROS) in C6 astroglialoma cells, as measured by confocal microscopic analysis of H2O2-sensitive 2′,7′-dichlorofluorescin fluorescence. Subsequent characterization of the signalling pathway revealed that expression of RacN17, a dominant-negative Rac1 mutant, completely blocked Aβ25-35-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 A2 (cPLA2) and 5-lipoxygenase (5-LO) contributed to the response and found that inhibition of either enzyme completely blocked Aβ25-35-induced ROS generation, indicating its dependence on arachidonic acid synthesis and metabolism to leukotrienes (e.g. leukotriene B4). Consistent with those findings, Aβ25-35 Rac-dependently stimulated translocation of 5-LO to the nuclear envelope and increased intracellular levels of leukotriene B4, while exogenous application of leukotriene B4 increased intracellular H2O2 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 Aβ25-35-induced H2O2 generation. Our findings thus demonstrate that PI 3-kinase, Rac, cPLA2, and 5-LO are all essential components of the β-amyloid signaling cascade leading to generation of ROS.

Key words: Aβ, cytosolic phospholipase A2, leukotriene B4, signal transduction.

INTRODUCTION

Alzheimer’s disease (AD) is characterized neuropathologically by the accumulation of plaques containing β-amyloid (Aβ) fibrils, reactive astrocytes and activated microglia [1–2]. Moreover, a number of studies have shown that Aβ may provide a linkage between oxidative stress and AD-associated neuronal cell death. For example, Aβ 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 Aβ-evoked ROS generation appears to be critical to the pathogenesis of AD. H2O2, in particular, appears to be an important intermediate in Aβ neurotoxicity [8]. Beyond that, however, little is known about the intracellular signalling mediators involved in Aβ-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 catalyse the synthesis of leukotrienes (LTs) from arachidonic acid (AA), a principal product of cytosolic phospholipase A2 (cPLA2) [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 Aβ-evoked signalling to ROS generation has never been characterized. Rac activation leads to stimulation of PLA2, in particular the cytosolic isoform (cPLA2), which was therefore postulated to be a major downstream mediator of Rac signalling within cells [10–12]. For example, activation of cPLA2 and the resultant release of AA have been implicated in Rac-mediated stimulation of Ca2+ influx and Rho-dependent cytoskeletal reorganization in fibroblasts [11,13]. In addition, we previously showed that this Rac-cPLA2-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-cPLA2-5-LO-linked cascade may mediate Aβ-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 Aβ and leading to ROS generation in C6 astroglialoma cells.

EXPERIMENTAL

Chemicals

Aβ25-35 (a fragment of Aβ1-42 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, Aβ25-35 was dissolved in

Abbreviations used: AA, arachidonic acid; AA681, 2-(12-hydroxydocoeca-5,10-dienyl)-3,5,6-trimethyl-1,4-benzoquinone; AACOCF3, arachidonyl trifluoromethyl ketone; Aβ, β-amyloid; AD, Alzheimer’s disease; BLT, leukotriene B4 receptor; cPLA2, cytosolic phospholipase A2; DCF(DA), 2′,7′-dichlorofluorescin (diacetate); DMEM, Dulbecco’s modified Eagle’s medium; DPL, diphenyleine iodonium; FBS, fetal bovine serum; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; LTB4APA, leukotriene B4-3-aminopropylamide; 5-LO, 5-lipoxygenase; Lts, leukotrienes; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzoypyrain-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. Di-phenylethylene iodonium (DPI), arachidonyl trifluoromethyl ketone (AACOCF3) and LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzoopyran-4-one, an inhibitor of PI 3- and 4-kinases] were purchased from Calbiochem (La Jolla, CA, U.S.A.). 2(12-Hydroxydocosatetraenoyl)-5,10-dienoyl)-3,5,6-trimethyl-1,4-benzoquinone (AA861, a 5-LO inhibitor), N-acetylcysteine and LTB₄ were from Sigma (St. Louis, MO, U.S.A.). LTB₄-aminopropylamide (LTB₄-APA) 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, cPLA₂, 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′-GTGCTGGCCA-GGCATCTGGGT and 3′-GGCACGCCCAAGGCGC-

Cell culture and transfections
C6 astroglial 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/CO₂ (19:1) mixture. C6 clones stably expressing RacN17 were prepared by co-transfection of pEXV-RacN17 and pcDNA-Neo⁰ 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 × 10⁵ 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 Na₂HPO₄/2.7 mM KCl/1.5 mM K₃PO₄/2.5 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 (ECL⁰) kit (Amersham).

Measurement of intracellular H₂O₂
Intracellular H₂O₂ was measured as a function of dichlorofluorescin (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 H₂O₂, cells were then incubated for 7 min with the H₂O₂-sensitive fluorophore DCFDA (5 μg/ml), which, when taken up, fluorescently labels intracellular H₂O₂ 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 H₂O₂ 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/ (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.

LT₄ assays
Cells (3 × 10⁶) 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 LT₄, the plates were rinsed twice with cold PBS, mixed with 4 ml of 100% ethanol and left at 4°C for 30 min. The resulting precipitate was removed by centrifugation at 100 000 × g for 30 min at 4°C. LT₄s were isolated from the ethanol 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 LT₄ using an ELISA (RPN 224; Amersham) as instructed by the manufacturer. The enzyme immunoassay was calibrated with standard LT₄ over the range 0.31–40 pg/well. The sensitivity, defined as the minimal amount of LT₄ needed to reduce zero dose binding, was 0.3 pg/well or 6 pg/ml. Statistical significance of LT₄ 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 × 10⁶ 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 MgCl2 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 astroglia

Aβ-induced generation of ROS was confirmed by directly measuring ROS levels as a function H2O2-sensitive DCF fluorescence. After serum starvation for 48 h, DCFDA-loaded C6 astrocytes were exposed to the indicated concentrations of Aβ25–35 for 15 min (Figure 1A) or to 100 nM Aβ25–35 for the indicated durations (Figure 1B). Application of Aβ25–35 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 Aβ25–35 concentration of 100 nM. Application of higher concentrations (results not shown) or exposure for longer periods (Figure 1B) diminished responses to Aβ25–35. Similar patterns of ROS generation was observed by applying Aβ1–42 (results not shown), indicating that the biological activities of both Aβ25–35 and Aβ1–42 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 Aβ25–35-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 Aβ25–35-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 cPLA2, NADPH oxidase and PI 3-kinase in Aβ signalling to ROS generation

We previously showed that PI 3-kinase and cPLA2 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 Aβ25–35, we analysed the effects of LY294002 and AACOCF3, specific inhibitors of the activities of PI 3-kinase and cPLA2 respectively. Pretreatment of C6 cells with either 20 μM LY294002 or 10 μM AACOCF3 completely blocked Aβ25–35-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 cPLA2 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 astroglia

(A) C6 cells were serum-starved for 48 h and then stimulated with the indicated concentrations of Aβ25–35 for 5 min. (B) Serum-starved cells were stimulated with 100 nM Aβ25–35 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 ± 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 cPLA2 by Aβ

Upon their activation, Rac1 and cPLA2 are translocated from the cytosolic fraction of cells to the particulate membrane fraction [18–20]. Therefore, to further confirm that activation Rac and cPLA2 are involved in the intracellular signalling by Aβ, the effects of Aβ on the distribution of these proteins was
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 Aβ25–35. Likewise, Aβ25–35 also induced redistribution of cPLA2 to the membrane fraction.

Role of 5-LO in Aβ-induced ROS generation

The dependence of Aβ-signalling to ROS generation on activation of cPLA2 implies a role for AA or its metabolites in that process. We previously showed that synthesis LTB4 by 5-LO metabolism of AA is situated downstream of the Rac–cPLA2 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 Aβ25–35-induced generation of ROS.

The role of 5-LO in Aβ-signalling was further confirmed by examining the effect of Aβ25–35 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 Aβ25–35 (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 cotransfecting cells with a RacN17 expression vector (Figure 5B), but not the empty vector itself (results not shown).

The above results encouraged us to test whether the level of LTs, such as LTB4, 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 LTB4 (approx. 7.5-fold increase over 0 h) was observed at 24 h.
in response to Aβ25–35 at C6 cells, whereas no noticeable increased LTB4 was detected in RacN17-expressing cells, supporting again a mediatory role of Rac1 in Aβ-induced 5-LO stimulation (Figure 5C).

Exogenous LTB4 induces ROS generation

The capacity of LTs, such as LTB4, to induced ROS generation was then directly assessed by examining the effects of their exogenous application to C6 astrocytes. Application of 100 nM LTB4 significantly increased intracellular ROS levels (Figure 6A), whereas application of a mixture of cysteinyll-LTs (LTC4/LTD4/LTE4) 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 LTB4 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 6B, expression of a BLT1 with the expected size was indeed detected in C6 cells. Moreover, pretreating the cells with the BLT antagonist LTB4APA completely blocked the increase in DCF fluorescence otherwise elicited by Aβ (Figure 6C). Apparently,
LTB₄ 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 astroglia cells via a PI 3-kinase–Rac-cPLA₂–5-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 cPLA₂ 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 LTB₄ levels induced by Aβ (Figures 5B and 5C) and by the capacity of exogenous LTB₄ to act via BLTs to increase ROS levels (Figure 6C).

Consistent with our findings, increasing evidence supports a signalling link between cPLA₂-mediated AA metabolism and ROS generation. For example, Li et al. [24] and Lindsay et al. [25] respectively suggest that LTB₄ 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 LTB₄ and leading to ROS generation is not known. Nonetheless, the present findings show that blockade of BLT₁, a G protein-coupled, cell surface LTB₄ receptor, using a specific antagonist (LTB₄APA) 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 LTB₄ 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 LTB₄ is not clear, but it may serve to amplify its own action and, in turn, Aβ signalling. Moreover, the capacity of LTB₄ 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 LTB₄ mediates inflammatory reactions in response to Aβ.

There is substantial evidence indicating the presence of a cPLA₂–5-LO-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 O₂⁻ 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, cPLA₂ 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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**β-Amyloid**$_{25–35}$ signalling to reactive-oxygen-species generation via a Rac1-linked cascade


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