

ATP released from β -amyloid-stimulated microglia induces reactive oxygen species production in an autocrine fashion

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Abbreviations: A β , β -amyloid peptide; AD, Alzheimer's disease; DCF-DA, dihydrodichlorofluorescein diacetate; fA β , fibrillar A β ; O $_2^-$, superoxide anion; oATP, oxidized ATP; P2X $_7$ R, P2X $_7$ receptor; ROS, reactive oxygen species

Abstract

Present study demonstrated that fibrillar β -amyloid peptide (fA β_{1-42}) induced ATP release, which in turn activated NADPH oxidase via the P2X $_7$ receptor (P2X $_7$ R). Reactive oxygen species (ROS) production in fA β_{1-42} -treated microglia appeared to require Ca $^{2+}$ influx from extracellular sources, because ROS generation was abolished to control levels in the absence of extracellular Ca $^{2+}$. Considering previous observation of superoxide generation by Ca $^{2+}$ influx through P2X $_7$ R in microglia, we hypothesized that ROS production in fA β -stimulated microglia might be mediated by ATP released from the microglia. We therefore examined whether fA β_{1-42} -induced Ca $^{2+}$ influx was mediated through P2X $_7$ R activation. In serial experiments, we found that microglial pretreatment with the P2X $_7$ R antagonists Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (100 μ M) or oxidized ATP (100 μ M) inhibited fA β -induced Ca $^{2+}$ influx and reduced ROS generation to basal levels. Furthermore, ATP efflux from fA β_{1-42} -stimulated microglia was observed, and apyrase treatment decreased the generation of ROS. These findings provide conclusive evidence that fA β -stimulated ROS generation in microglial cells is regulated by ATP released from the microglia in an autocrine manner.

Keywords: adenosine triphosphate; Alzheimer's disease; amyloid β -protein; calcium; microglia; NADPH oxidase; purinoceptor P2Z; reactive oxygen species

Introduction

A neuropathological characteristic of Alzheimer's disease (AD) is the appearance of neuritic plaques consisting of extracellular beta-amyloid peptide (A β) surrounded by reactive microglial cells (Sastre *et al.*, 2006). It has been suggested that fibrillar A β (fA β) stimulates reactive oxygen species (ROS) production from cultured microglial cells via activation of NADPH oxidase (Bianca *et al.*, 1999). In addition, fA β mediated neurotoxicity in mixed neuron-microglia cultures by causing the production of ROS (Qin *et al.*, 2002). Furthermore, NADPH oxidase activation has been identified in AD brains (Shimohama *et al.*, 2000). These findings suggest that microglia-mediated oxidative stress, via NADPH oxidase activation, plays a critical role in the pathogenesis of AD.

NADPH oxidase is a multicomponent enzyme that is composed of the cytosolic components, p40^{phox}, p47^{phox}, p67^{phox} and the small G-protein Rac, and the plasma membrane components p22^{phox} and gp91^{phox} (Lambeth, 2004). Activation of NADPH oxidase occurs when inflammatory stimuli bring about the phosphorylation of p47^{phox} and p67^{phox}, and GDP/GTP exchange on Rac, causing these proteins to translocate to the membrane, where they assemble with p22^{phox} and gp91^{phox} (DeLeo and Quinn, 1996). The translocation of both the p47^{phox} and p67^{phox} subunits from the cytosol to the membrane has been observed in AD brain and fA β -stimulated microglial cells (Bianca *et al.*, 1999; Shimohama *et al.*, 2000). However, the mechanisms responsible for this fA β -stimulated NADPH oxidase activation remain largely unknown.

ATP is known to be released by neurons and glia under conditions such as trauma, ischemia/hypoxia, and inflammation, and such release is a potential cause of neurodegeneration (Burnstock, 2007). Extracellular ATP has been reported to stimulate ROS production via P2 purinergic receptors in eosinophils and microglia (Dichmann *et al.*, 2000; Parvathani *et al.*, 2003). In eosinophils, elevation of intracellular calcium concentra-

tion ($[Ca^{2+}]_i$) plays a crucial role in the regulation of ROS production (Dichmann *et al.*, 2000). In microglia $[Ca^{2+}]_i$ increases caused by the stimulation of the P2X₇ receptor (P2X₇R) induces NADPH oxidase-mediated superoxide generation (Parvathenani *et al.*, 2003). More importantly, upregulated expression of P2X₇R in AD brain and fA β -treated microglial cells has been recently reported (McLarnon *et al.*, 2006).

Taking these data together, we hypothesized that ROS production in fA β -stimulated microglia might be mediated by ATP released from the microglia. To explore this hypothesis further, we determined whether fA β -stimulated microglia release ATP, which could then modulate $[Ca^{2+}]_i$ elevation via P2X₇R, resulting in ROS production.

Materials and Methods

Reagents

An A β peptide corresponding to human A β amino acids 1-42 (A β ₁₋₄₂) was purchased from the American Peptide Company (Sunnyvale, CA). Pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS), adenosine 5'-triphosphate 2', 3'-acylic dialcohol (oxidized ATP; oATP), apyrase (an ATP-hydrolyzing enzyme), 2',7'-dihydrodichloro-fluorescein diacetate (DCF-DA), pluronic F-127, DNase I, an ATP bioluminescence assay kit, and a protease inhibitor mixture, were all purchased from Sigma (St. Louis, MO). The Amplex Red hydrogen peroxide/peroxidase assay kit and the Ca²⁺ indicator Fluo-3/AM were from Molecular Probes (Eugene, OR).

Microglial cell culture

Microglial cultures were prepared from the brains of 3-day-old Sprague-Dawley rats as described previously (Kim *et al.*, 2002). Briefly, whole brains were dissected into small cubes, incubated in D-PBS (JBI, Taegu, Korea) containing 0.1% trypsin and 40 μ g/ml DNase I for 15 min at 37°C, and dissociated into single cells by gentle pipetting. Dissociated cells were suspended in DMEM (JBI) containing 5% horse serum, 5 mg/ml glucose, 100 U/ml penicillin and 100 μ g/ml streptomycin, and plated on poly-D-lysine-coated T-75 culture flasks, and incubated at 37°C in incubator with 5% CO₂/95% air atmosphere. After 2-4 weeks of growth in flasks, microglia floating in the medium were collected and grown in separate 96-well plates or on coverslips.

Detection of ROS

(1) Measurement of extracellular ROS: Microglial cells were plated at approximately 1×10^5 cells/well in 96-well plates, and then stimulated with fA β ₁₋₄₂. For preparing fA β , the lyophilized A β ₁₋₄₂ peptides were dissolved in sterile 10% DMSO in PBS at a concentration of 350 μ M and aged 5-7 d at 37°C, and then diluted in a culture medium for use in the experiments. After fA β ₁₋₄₂ treatment, 100 μ l of supernatant were transferred to microplate and mixed with 100 μ l DMEM without phenol red but containing of 0.5 μ M Amplex Red, and 1 U/ml HRP (Amplex Red hydrogen peroxide/peroxidase assay kit). The Amplex Red reagent reacts with H₂O₂ in the presence of HRP to produce the fluorescent compound resorufin (Zhou *et al.*, 1997). Fluorescence intensities were measured at indicated times using a LS-55 luminescence spectrophotometer (Perkin Elmer Instruments, Wellesley, MA).

(2) Measurement of intracellular ROS: Intracellular ROS levels were measured using the fluorescent dye DCF-DA, which is readily converted to a fluorescent product in the presence of ROS in cells. In brief, cells were preincubated with PPADS (10, 100 μ M), oATP (10, 100 μ M), 500 μ M EGTA, or 5 U of apyrase, and then treated with 0.5 μ M fA β ₁₋₄₂. fA β ₁₋₄₂-stimulated cells were incubated with 10 μ M DCF-DA in HBSS (145 mM NaCl, 2.5 mM KCl, 1.8 mM MgCl₂, 1 mM CaCl₂, 10 mM D-glucose and 20 mM HEPES; pH 7.4) for 30 min. The cells were then washed extensively with D-PBS to remove extracellular DCF-DA, and fluorescence images were taken using an IX71 confocal laser scanning microscope (Olympus; Tokyo, Japan).

Ca²⁺ imaging

Intracellular Ca²⁺ concentration was monitored by loading cells with the fluorescent Ca²⁺ indicator Fluo-3/AM, convertible to Fluo-3 in the presence of Ca²⁺. Cultured microglia plated onto poly-D-lysine-coated 25 mm glass coverslips were incubated with 2 μ M of the acetoxymethyl ester of Fluo-3 (Fluo-3/AM) and 0.02% pluronic F-127 in HBSS for 30 min at 37°C, and then washed with HBSS. Fluo-3-loaded cells were placed in a perfusion chamber mounted on the stage of a confocal laser-scanning microscope and stimulated with 0.5 μ M fA β ₁₋₄₂. To measure the intracellular calcium concentration, a confocal laser-scanning microscope (IX71, Olympus) equipped with an Argon/Keron laser (15 mW; Coherent, Santa Clara, CA) was used. Fluo-3 was excited by the 488 nm line of an argon laser and the fluorescence was measured at an emission wavelength above 510 nm.

Western blot analysis

Microglial cells treated with $fA\beta_{1-42}$ were lysed with lysis buffer (10 mM Na_2HPO_4 , 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% NP 40; pH 7.5). Lysates were centrifuged at $13,000 \times g$ for 10 min at $4^\circ C$ and supernatants were collected. An aliquot of each sample containing 20 μg total protein was loaded onto a 10% acrylamide gel, and then transferred to a PVDF membrane. The blots were incubated with blocking buffer [0.5% skim milk in TBST (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20, pH 7.5)] at room temperature for 1 h, and incubated with primary monoclonal antibodies against the $p67^{phox}$ (1:500; BD Biosciences, San Diego, CA), and HRP conjugated anti-mouse secondary antibody (1:1,000; Amersham Pharmacia).

Cell fractionation

Microglial cells were harvested and resuspended in a cold hypotonic solution (0.25 M sucrose, 10 mM Tris-HCl, and 5 mM $MgCl_2$; pH 7.4) including a protease inhibitor mixture, and centrifuged at $600 \times g$ for 10 min. The supernatant was ultracentrifuged at $100,000 \times g$ for 1.5 h at $4^\circ C$. The resulting supernatant was removed and saved as the cytosolic fraction, and the membrane pellet was resuspended in hypotonic solution containing 1% Triton X-100. Samples were analyzed by Western blotting using antibodies against the NADPH

oxidase components, as described above.

ATP efflux measurement

To examine whether extracellular ATP was released from microglial cells by $fA\beta_{1-42}$ treatment, cells (3×10^4 cells/well) were plated in 96-well plates, and treated with 0.5 μM $fA\beta_{1-42}$ for various times. After incubation, supernatant fluids of individual wells were transferred into sterile tubes and heated at $95^\circ C$ for 2 min. Extracellular ATP in supernatants was immediately measured using the luciferase-luciferin assay of the ATP bioluminescence assay kit following the instructions of the manufacturer using a luminometer (TD2020; Turner Designs, Sunnyvale, CA).

Statistical analysis

All statistical comparisons in this study were performed using one-way ANOVA with the Tukey-Kramer multiple comparisons test, and data are expressed as mean \pm SEM. A value of $P < 0.05$ was considered statistically significant.

Results

$fA\beta_{1-42}$ induces sustained ROS production in microglia

Figure 1A shows that minimum concentration of

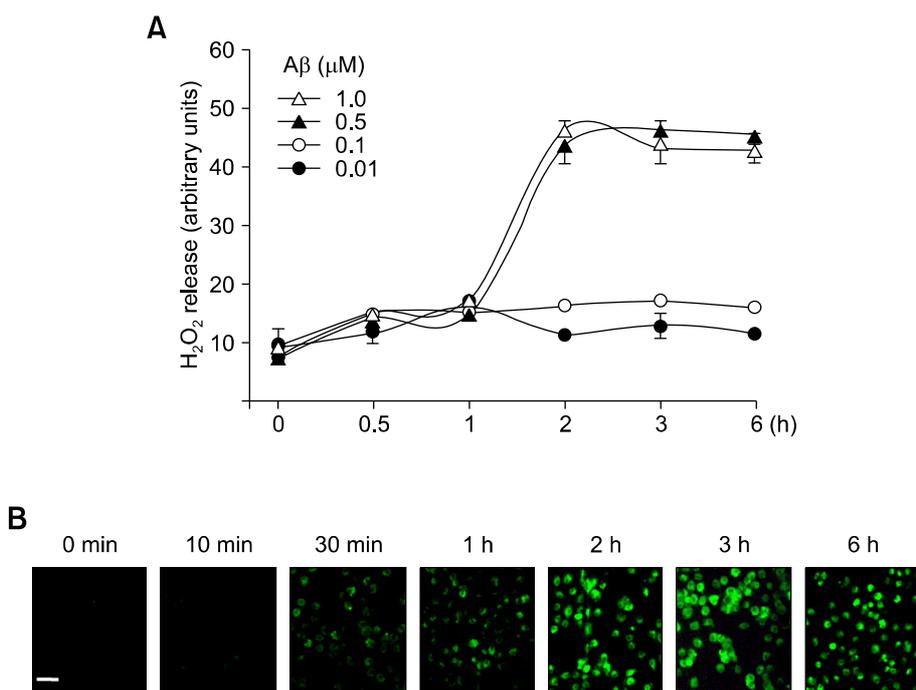


Figure 1. Dose-response and time-response of $fA\beta_{1-42}$ -induced ROS production in microglia. Primary rat microglia were plated in 96-well plates (1×10^5 cells/well) or onto coverslips (3×10^4 cells/coverslip). (A) H_2O_2 production in microglia stimulated with various concentrations of $fA\beta_{1-42}$ was determined over 6 h. The amounts of H_2O_2 released into media were measured using Amplex Red. (B) Intracellular ROS levels were assayed using 10 μM DCF as described in Materials and Methods. Fluorescence (DCF) images were taken using an IX71 confocal microscope (Olympus). Scale bar, 100 μm .

fA β_{1-42} required for ROS production in microglia is 0.5 μ M. Release of H₂O₂ commenced at 1 h, peaked at 2 h after fA β_{1-42} treatment, and then continued for 6 h. Next, we evaluated intracellular ROS levels in microglia stimulated with 0.5 μ M fA β_{1-42} by measuring fluorescence signals from DCF-DA. The DCF fluorescence signals also significantly increased in the interval 0.5-6 h after fA β_{1-42} treatment (Figure 1B). These results indicate that ROS production in fA β_{1-42} -stimulated microglia is initiated slowly, and continues for a long period.

Extracellular Ca²⁺ is required for fA β_{1-42} -induced ROS production in microglia

Previous studies showed that ROS production in phagocytic cells is mediated by [Ca²⁺]_i elevation (Baggiolini *et al.*, 1993; Geiszt *et al.*, 1999). We therefore investigated the effect of extracellular

Ca²⁺ on ROS production in fA β_{1-42} -stimulated microglia. In EGTA-containing Ca²⁺-free buffer, the change in [Ca²⁺]_i was abolished, whereas [Ca²⁺]_i increased when buffer was changed to Ca²⁺-containing buffer, indicating that fA β_{1-42} appeared to stimulate Ca²⁺ influx only from extracellular sources (Figure 2A). The DCF fluorescence signals showed that fA β_{1-42} -stimulated ROS production was blocked to control levels when cells were preincubated in EGTA-containing Ca²⁺-free medium (Figure 2B). These results clearly suggest that Ca²⁺ influx from the extracellular milieu is necessary for the fA β_{1-42} -induced ROS production.

fA β_{1-42} -induced Ca²⁺ influx and ROS production are mediated by ATP released from microglia through P2X₇R activation

Superoxide generation by Ca²⁺ influx through P2X₇R was observed in microglia (Parvathenani *et al.*, 2003). Therefore, we examined whether fA β_{1-42} -induced Ca²⁺ influx was mediated through P2X₇R activation. Apyrase treatment prevented fA β_{1-42} -induced Ca²⁺ influx in a dose dependent manner (Figure 3A), indicating that fA β_{1-42} -induced Ca²⁺ influx is mediated by ATP released from microglia. Pretreatment of microglia with the P2X₇R antagonists PPADS (100 μ M) or oATP (100 μ M) inhibited fA β_{1-42} -induced Ca²⁺ influx to baseline levels (Figure 3B). Next, we measured DCF fluorescence signals in microglial cells pretreated with PPADS or oATP. As can be seen in Figure 3C and D, DCF fluorescence signals were significantly suppressed by PPADS and oATP at 10-100 μ M. These results suggest that the fA β_{1-42} -induced Ca²⁺ influx is mediated primarily through the activation of the P2X₇R, resulting in ROS generation.

fA β_{1-42} -induced NADPH oxidase activation is mediated by ATP released from microglia

We examined the activation of NADPH oxidase by monitoring the membrane translocation of p67^{phox} over time. Following fA β_{1-42} treatment, the amounts of p67^{phox} in the membrane fraction increased up to 90 min post-treatment, and then decreased over the next 6 h (Figure 4A). In addition, PPADS, oATP, EGTA, and apyrase (an ATP-hydrolyzing enzyme) prevented the fA β_{1-42} -induced membrane translocation of p67^{phox} (Figure 4B). Translocation of the cytosolic factor p67^{phox} to the plasma membrane started at about 30 min post-stimulation, and was prevented by apyrase treatment. Based on these findings, we wondered whether ATP is released from fA β_{1-42} -stimulated microglia. The ROS production was abolished by apyrase treatment, but

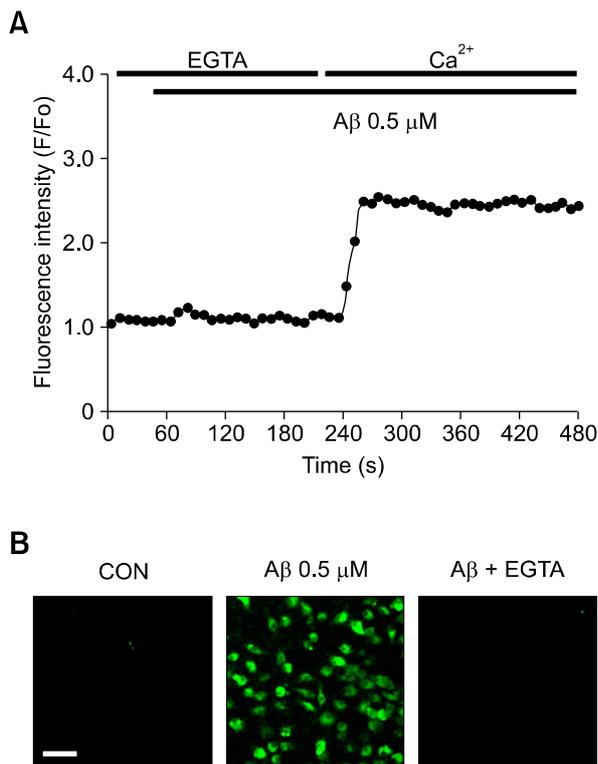


Figure 2. Effects of EGTA on [Ca²⁺]_i and ROS production in fA β_{1-42} -stimulated microglia. Microglial cells were plated onto glass coverslips (3×10^4 cells/coverslip) in either HBSS or HBSS without Ca²⁺ containing 0.5 mM EGTA, and then stimulated with 0.5 μ M fA β_{1-42} . (A) Intracellular Ca²⁺ concentration was measured by Fluo-3 as described in Materials and Methods, and represented by the ratio between the fluorescence intensity after treatment (F) and fluorescence in the resting state (F₀). (B) Intracellular ROS levels were assayed using 10 μ M DCF. Fluorescence (DCF) images were taken using an IX71 confocal microscope (Olympus). Scale bar, 100 μ m.

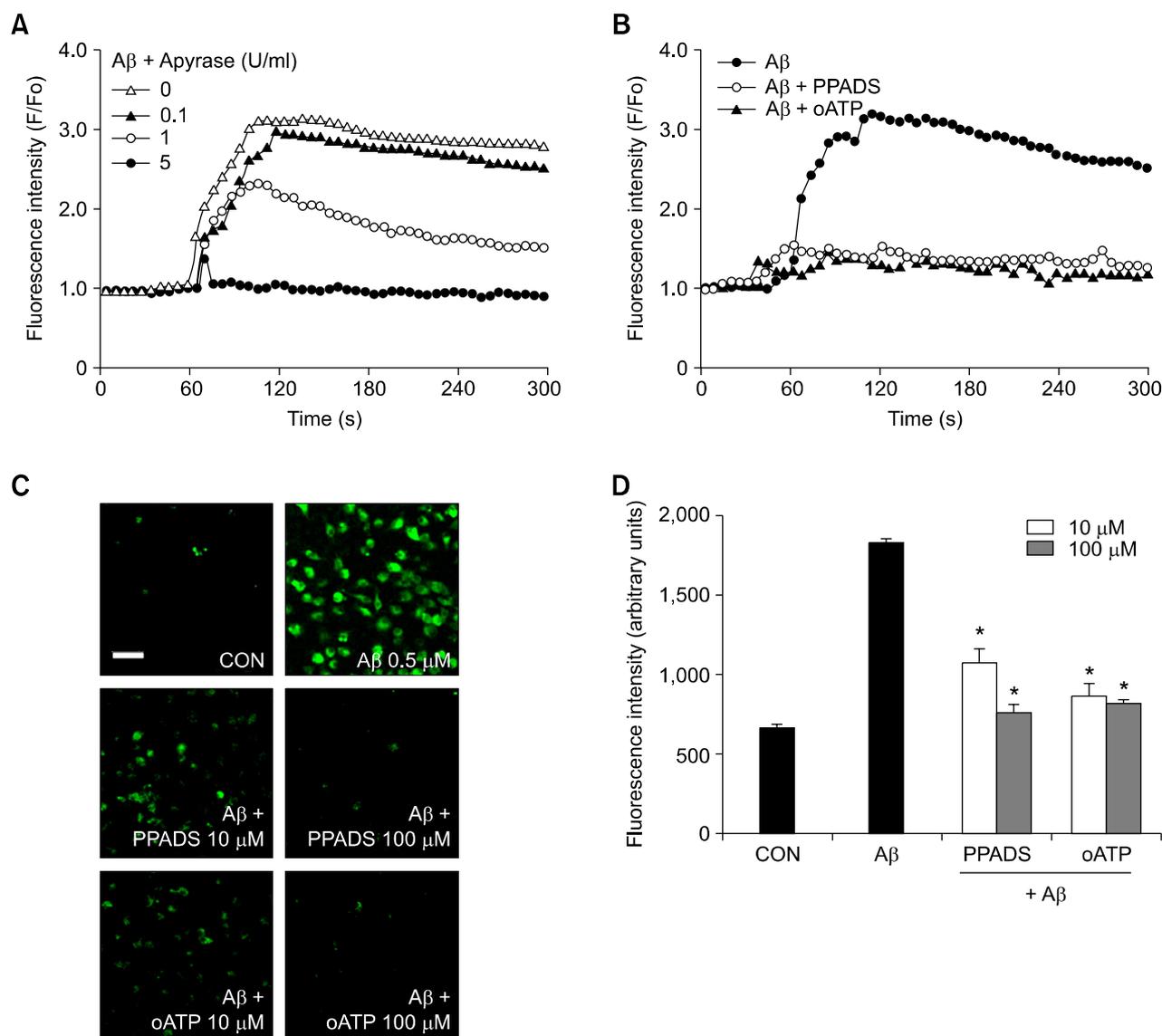


Figure 3. Effects of apyrase or P2X₇R antagonists on fAβ₁₋₄₂-induced [Ca²⁺]_i changes in microglia. Microglial cells were plated onto coverslips (3 × 10⁴ cells/coverslip), treated with apyrase (5 U/ml) plus fAβ₁₋₄₂ (0.5 μM) (A), or pretreated with PPADS (100 μM) or oATP (100 μM) for 1 h, and then treated with 0.5 μM fAβ₁₋₄₂ (B and C). (A and B) Intracellular Ca²⁺ concentration was measured by Fluo-3 as described in Materials and Methods, and represented by the ratio between the fluorescence intensity after treatment (F) and fluorescence in the resting state (F₀). (C) Intracellular ROS levels were assayed using 10 μM DCF. Fluorescence (DCF) images were taken using an IX71 confocal microscope (Olympus). Scale bar, 100 μm. (D) DCF intensities of cells in (C) were counted using Image Gauge 4.0 (Fujifilm). Values are mean ± SEM of 40-50 cells. *P < 0.001 compared with fAβ₁₋₄₂.

was not affected by boiled apyrase (Figure 4C and D), suggesting that ATP released from Aβ₁₋₄₂-stimulated microglia might trigger ROS production. We also found that the amount of extracellular ATP reached a maximum level 1 h after stimulation with fAβ₁₋₄₂, and this level was sustained for 6 h (Figure 4E). These results suggest that fAβ₁₋₄₂-induced P2X₇R activation may be regulated by ATP released from microglia in an autocrine manner.

Discussion

The major new finding in this study is that fAβ₁₋₄₂ induces ATP release from microglia and that ATP regulates ROS generation through the activation of P2X₇R and Ca²⁺ influx. It is believed that phagocytes including neutrophils and macrophages produce ROS to counter invading pathogens, in a phenomenon known as the respiratory burst. An excess of the superoxide anion (O₂⁻) can, however, cause serious damage to the host. The phagocyte

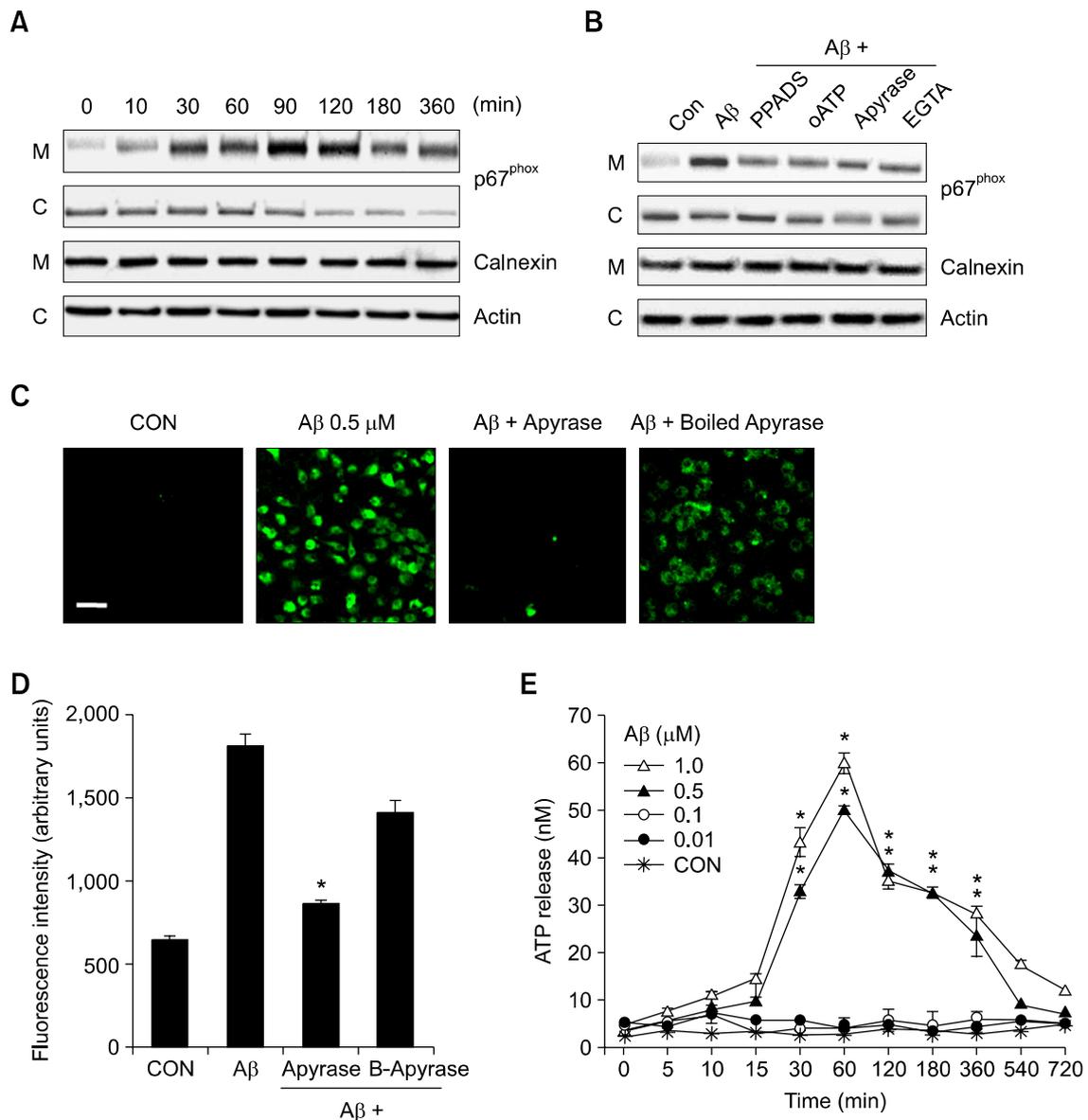


Figure 4. NADPH oxidase activation and ROS production by ATP released from fA β ₁₋₄₂-stimulated microglia. Microglial cells were plated into 60 mm dishes (2×10^6 cells), and treated with 0.5 μ M fA β ₁₋₄₂ for the time periods indicated (A), or pretreated with 100 μ M PPADS, 100 μ M oATP, 5 U of apyrase, or 0.5 mM EGTA, and then treated with 0.5 μ M fA β ₁₋₄₂ for 90 min (B). (A and B) Lysates were fractionated, and p67^{phox} distributions in cytosolic and membrane fractions were analyzed by Western blot analysis. The cytosol protein actin and the membrane protein calnexin were used as internal standards to normalize experimental data. C, cytosolic fraction; M, membrane fraction. (C) Microglial cells (3×10^4 cells/cover slip) were plated onto coverslips, and treated with 5 U/ml of apyrase and 0.5 μ M fA β ₁₋₄₂ for 2 h. The effect of apyrase on the production of intracellular ROS was measured by DCF fluorescence. Scale bar, 100 μ m. (D) DCF intensities of cells in (C) were measured using Image Gauge 4.0 (Fujifilm). Values are mean \pm SEM of 40-50 cells. * P < 0.001 compared with fA β ₁₋₄₂. (E) Time courses of ATP release into the culture supernatants of fA β ₁₋₄₂-stimulated microglia. Microglial cells (3×10^4 cells/well) were plated into 96-well plates. ATP concentrations in the culture supernatants were determined at the indicated time points after fA β ₁₋₄₂ stimulation. Values are mean \pm SEM of triplicate samples. * P < 0.001 compared with the control.

NADPH oxidase is a major enzyme catalyzing the non-mitochondrial production of O₂⁻ (Lambeth, 2004). The activation of NADPH oxidase must therefore be tightly controlled. For example, the bacterial chemoattractant fMet-Leu-Phe (fMLF)-mediated O₂⁻ production in human polymorpho-

nuclear leukocytes peaked at 45 s, and then declined rapidly to 50% of maximal by 2 min (McPhail and Snyderman, 1983). In contrast, ROS production in our experiments was initiated slowly and continued for a long period. This implied that fA β ₁₋₄₂-stimulated ROS production in microglia

employed a process different from the mechanisms used for transient ROS generation in phagocytic cells.

The best-characterized signaling pathway for ROS production in neutrophils is NADPH oxidase activation resulting from elevation in $[Ca^{2+}]_i$ through a process termed store-operated Ca^{2+} influx (Thelen *et al.*, 1993). The fMLF-induced activation of the G-protein-coupled formyl peptide receptor triggers Ca^{2+} release from intracellular stores, followed by an influx of Ca^{2+} through the plasma membrane (Granfeldt *et al.*, 2002). In contrast, our experiments showed that $fA\beta_{1-42}$ stimulation provoked Ca^{2+} influx but not Ca^{2+} release from intracellular stores, and that ROS production was decreased to control levels by blocking Ca^{2+} influx. These differences in methods of $[Ca^{2+}]_i$ increase may be correlated with the sustained production of ROS in our experiment compared with transient ROS generation in phagocytic cells. In fact, a recent study showed that BzATP, a P2X₇R-specific agonist, caused Ca^{2+} influx from extracellular sources only (Parvathenani *et al.*, 2003). We therefore examined the possible involvement of P2X₇R in $fA\beta_{1-42}$ -mediated $[Ca^{2+}]_i$ changes and ROS generation, and found that $fA\beta_{1-42}$ -induced ROS generation in microglia was entirely attributable to the activation of P2X₇R. A recent study also reported that P2X₇R expression was upregulated in primary microglia obtained from an AD brain, and that ATP abnormally triggered the Ca^{2+} response (McLarnon *et al.*, 2005).

A putative mechanism explaining P2X₇R-dependent ROS generation in $fA\beta_{1-42}$ -stimulated microglia is that $fA\beta_{1-42}$ stimulates the secretion of ATP from microglial cells, and that the released ATP subsequently stimulates P2X₇R activity. In support of this hypothesis, our results showed that both the translocation of p67^{phox} and ROS production were prevented by EGTA, PPADS, oATP, or apyrase treatment. Furthermore, ATP release from $fA\beta_{1-42}$ -stimulated microglia was confirmed. Notably, the time course of ATP release agreed with the kinetics of p67^{phox} translocation and ROS production, providing conclusive evidence that $fA\beta_{1-42}$ -stimulated NADPH oxidase activation in microglial cells is regulated by ATP released from microglia. Recently, it has been recognized that ATP can be released from LPS-stimulated microglial cells (Ferrari *et al.*, 1997; Seo *et al.*, 2004), and a recent study reported that ATP release from glutamate-stimulated microglia was mediated by intracellular Ca^{2+} release (Liu *et al.*, 2006). Our data show Ca^{2+} influx from extracellular sources only, implying that $fA\beta_{1-42}$ -stimulated ATP release from microglia occurs by a mechanism distinct

from that involved in glutamate-stimulated ATP release. Future work on identifying the mechanisms involved in $fA\beta$ -stimulated ATP release from microglia will provide a better understanding of the role of microglia in pathological conditions of AD.

In conclusion, our study describes a novel mechanism of ROS production by microglia in AD by demonstrating that $fA\beta_{1-42}$ indirectly stimulates NADPH oxidase activation in microglia through ATP release and subsequent activation of P2X₇R.

Acknowledgments

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