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

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Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; DCF-DA, dihydrodichlorofluorescein diacetate; fAβ, fibrillar Aβ; O_2^-, superoxide anion; oATP, oxidized ATP; $P2X_7R$, $P2X_7$ receptor; ROS, reactive oxygen species

Abstract

Present study demonstrated that fibrillar β-amyloid peptide ($fA\beta_{1-42}$) induced ATP release, which in turn activated NADPH oxidase via the $P2X_7$ receptor ($P2X_7R$). Reactive oxygen species (ROS) production in $fA\beta_{1-42}$-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 $P2X_7R$ in microglia, we hypothesized that ROS production in fAβ-stimulated microglia might be mediated by ATP released from the microglia. We therefore examined whether $fA\beta_{1-42}$-induced Ca^{2+} influx was mediated through $P2X_7R$ activation. In serial experiments, we found that microglial pretreatment with the $P2X_7R$ antagonists Pyridoxal-phosphate-6-azophenyl-2',4'- disulfonate (100 μM) or oxidized ATP (100 μM) inhibited fAβ-induced Ca^{2+} influx and reduced ROS generation to basal levels. Furthermore, ATP efflux from $fA\beta_{1-42}$-stimulated microglia was observed, and apyrase treatment decreased the generation of ROS. These findings provide conclusive evidence that fAβ-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 (Aβ) surrounded by reactive microglial cells (Sastre *et al.,* 2006). It has been suggested that fibrillar Aβ (fAβ) stimulates reactive oxygen species (ROS) production from cultured microglial cells via activation of NADPH oxidase (Bianca *et al.,* 1999). In addition, fAβ 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 fAβ-stimulated microglial cells (Bianca *et al.,* 1999; Shimohama *et al.,* 2000). However, the mechanisms responsible for this fAβ-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; Parvathenani *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 $P2X_7$ receptor ($P2X_7R$) induces NADPH oxidase-mediated superoxide generation (Parvathenani *et al.,* 2003). More importantly, upregulated expression of $P2X_7R$ in AD brain and fAβ-treated microglial cells has been recently reported (McLarnon *et al.,* 2006).

Taking these data together, we hypothesized that ROS production in fAβ-stimulated microglia might be mediated by ATP released from the microglia. To explore this hypothesis further, we determined whether fAβ-stimulated microglia release ATP, which could then modulate $[Ca^{2+}]_i$ elevation via $P2X_7R$, resulting in ROS production.

Materials and Methods

Reagents

An Aβ peptide corresponding to human Aβ amino acids 1-42 ($A\beta_{1-42}$) 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'-dihydrodichlorofluorescein 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 Ca^{2+} 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 μg/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 μg/ml streptomycin, and plated on poly-D-lysine-coated T-75 culture flasks, and incubated at 37°C in incubator with 5% CO_2/95% 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 $fA\beta_{1-42}$. For preparing fAβ, the lyophilized $A\beta_{1-42}$ peptides were dissolved in sterile 10% DMSO in PBS at a concentration of 350 μM and aged 5-7 d at 37°C, and then diluted in a culture medium for use in the experiments. After $fA\beta_{1-42}$ treatment, 100 μl of supernatant were transferred to microplate and mixed with 100 μl DMEM without phenol red but containing of 0.5 μM Amplex Red, and 1 U/ml HRP (Amplex Red hydrogen peroxide/peroxidase assay kit). The Amplex Red reagent reacts with H_2O_2 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 μM), oATP (10, 100 μM), 500 μM EGTA, or 5 U of apyrase, and then treated with 0.5 μM $fA\beta_{1-42}$. $fA\beta_{1-42}$-stimulated cells were incubated with 10 μM DCF-DA in HBSS (145 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 1 mM $CaCl_2$, 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).

Ca^{2+} imaging

Intracellular Ca^{2+} concentration was monitored by loading cells with the fluorescent Ca^{2+} indicator Fluo-3/AM, convertible to Fluo-3 in the presence of Ca^{2+}. Cultured microglia plated onto poly-D-lysine-coated 25 mm glass coverslips were incubated with 2 μM 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 μM $fA\beta_{1-42}$. 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 fAβ_{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°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°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 fAβ_{1-42} treatment, cells (3×10^4 cells/well) were plated in 96-well plates, and treated with 0.5 μM fAβ_{1-42} for various times. After incubation, supernatant fluids of individual wells were transferred into sterile tubes and heated at 95°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 ± SEM. A value of $P < 0.05$ was considered statistically significant.

Results

fAβ_{1-42} induces sustained ROS production in microglia

Figure 1A shows that minimum concentration of

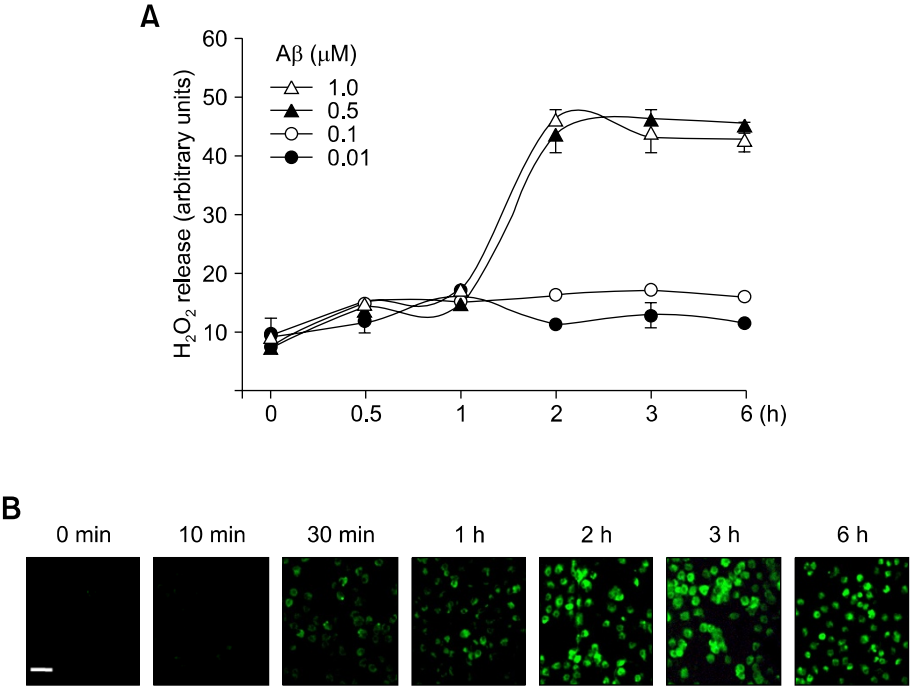

Figure 1. Dose-response and time-response of fAβ_{1-42}-induced ROS production in microglia. Primary rat microglia were plated in 96-well plates (1×10^5 cells/well) or onto coverslips (3×10^4 cells/coverslip). (A) H_2O_2 production in microglia stimulated with various concentrations of fAβ_{1-42} was 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.

$fA\beta_{1-42}$ required for ROS production in microglia is 0.5 μM. Release of H_2O_2 commenced at 1 h, peaked at 2 h after $fA\beta_{1-42}$ treatment, and then continued for 6 h. Next, we evaluated intracellular ROS levels in microglia stimulated with 0.5 μM $fA\beta_{1-42}$ by measuring fluorescence signals from DCF-DA. The DCF fluorescence signals also significantly increased in the interval 0.5-6 h after $fA\beta_{1-42}$ treatment (Figure 1B). These results indicate that ROS production in $fA\beta_{1-42}$-stimulated microglia is initiated slowly, and continues for a long period.

Extracellular Ca^{2+} is required for $fA\beta_{1-42}$-induced ROS production in microglia

Previous studies showed that ROS production in phagocytic cells is mediated by $[Ca^{2+}]_i$ elevation (Baggiolini *et al.,* 1993; Geiszt *et al.,* 1999). We therefore investigated the effect of extracellular Ca^{2+} on ROS production in $fA\beta_{1-42}$-stimulated microglia. In EGTA-containing Ca^{2+}-free buffer, the change in $[Ca^{2+}]_i$ was abolished, whereas $[Ca^{2+}]_i$ increased when buffer was changed to Ca^{2+}-containing buffer, indicating that $fA\beta_{1-42}$ appeared to stimulate Ca^{2+} influx only from extracellular sources (Figure 2A). The DCF fluorescence signals showed that $fA\beta_{1-42}$-stimulated ROS production was blocked to control levels when cells were preincubated in EGTA-containing Ca^{2+}-free medium (Figure 2B). These results clearly suggest that Ca^{2+} influx from the extracellular milieu is necessary for the $fA\beta_{1-42}$-induced ROS production.

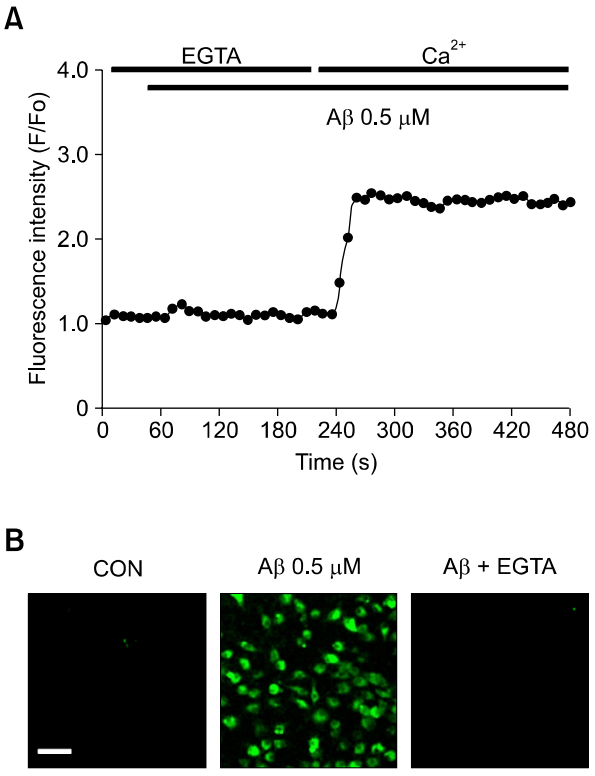

Figure 2. Effects of EGTA on $[Ca^{2+}]_i$ and ROS production in $fA\beta_{1-42}$-stimulated microglia. Microglial cells were plated onto glass coverslips (3×10^4 cells/coverslip) in either HBSS or HBSS without Ca^{2+} containing 0.5 mM EGTA, and then stimulated with 0.5 μM $fA\beta_{1-42}$. (A) Intracellular Ca^{2+} 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 (F_0). (B) Intracellular ROS levels were assayed using 10 μM DCF. Fluorescence (DCF) images were taken using an IX71 confocal microscope (Olympus). Scale bar, 100 μm.

$fA\beta_{1-42}$-induced Ca^{2+} influx and ROS production are mediated by ATP released from microglia through $P2X_7R$ activation

Superoxide generation by Ca^{2+} influx through $P2X_7R$ was observed in microglia (Parvathenani *et al.,* 2003). Therefore, we examined whether $fA\beta_{1-42}$-induced Ca^{2+} influx was mediated through $P2X_7R$ activation. Apyrase treatment prevented $fA\beta_{1-42}$-induced Ca^{2+} influx in a dose dependent manner (Figure 3A), indicating that $fA\beta_{1-42}$-induced Ca^{2+} influx is mediated by ATP released from microglia. Pretreatment of microglia with the $P2X_7R$ antagonists PPADS (100 μM) or oATP (100 μM) inhibited $fA\beta_{1-42}$-induced Ca^{2+} 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 μM. These results suggest that the $fA\beta_{1-42}$-induced Ca^{2+} influx is mediated primarily through the activation of the $P2X_7R$, resulting in ROS generation.

$fA\beta_{1-42}$-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 $fA\beta_{1-42}$ 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 $fA\beta_{1-42}$-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 $fA\beta_{1-42}$-stimulated microglia. The ROS production was abolished by apyrase treatment, but

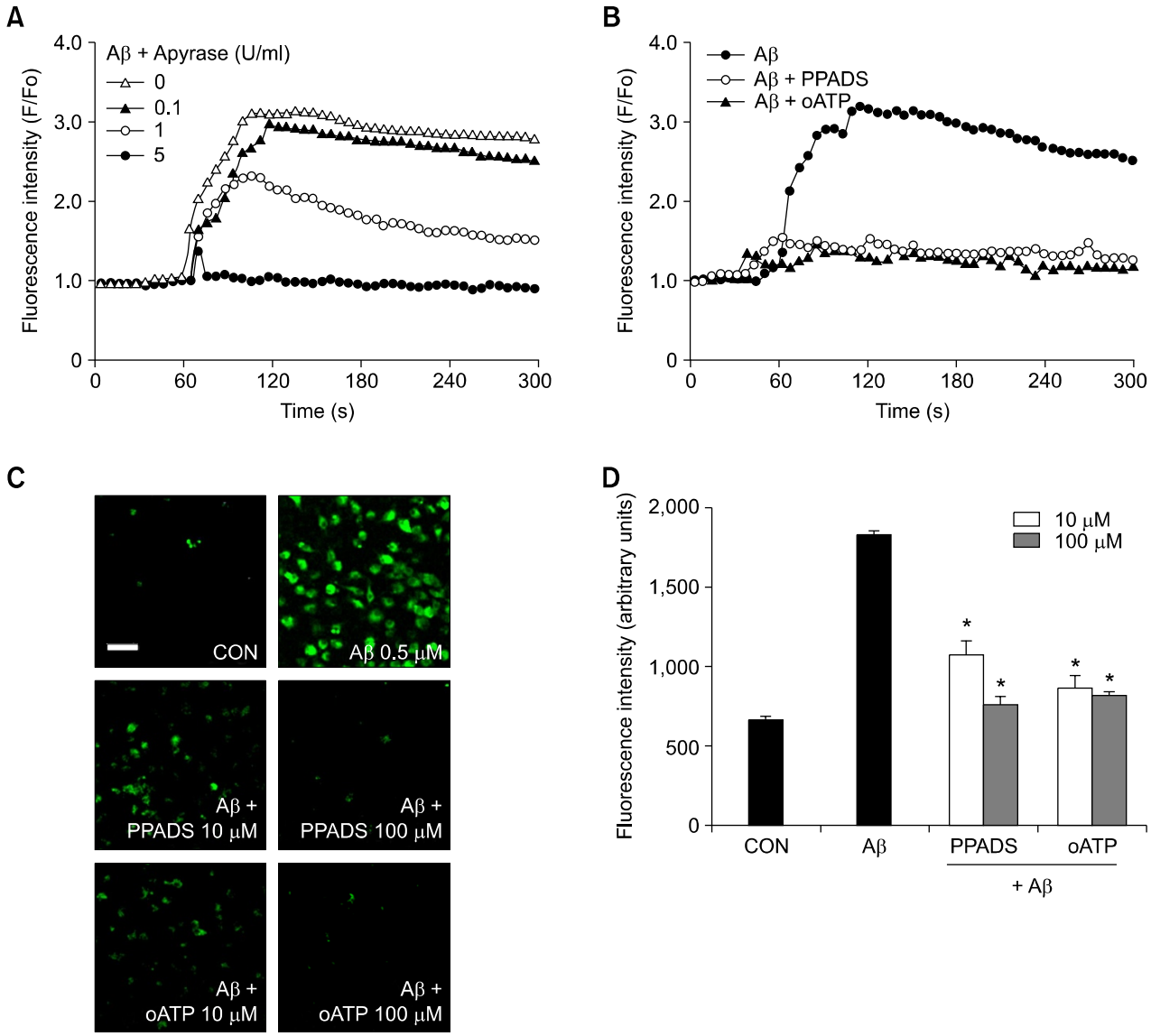

Figure 3. Effects of apyrase or $P2X_7R$ antagonists on $fA\beta_{1-42}$-induced $[Ca^{2+}]_i$ changes in microglia. Microglial cells were plated onto coverslips (3×10^4 cells/coverslip), treated with apyrase (5 U/ml) plus $fA\beta_{1-42}$ (0.5 μM) (A), or pretreated with PPADS (100 μM) or oATP (100 μM) for 1 h, and then treated with 0.5 μM $fA\beta_{1-42}$ (B and C). (A and B) Intracellular Ca^{2+} 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 (F_0). (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 $fA\beta_{1-42}$.

was not affected by boiled apyrase (Figure 4C and D), suggesting that ATP released from $A\beta_{1-42}$-stimulated microglia might trigger ROS production. We also found that the amount of extracellular ATP reached a maximum level 1 h after stimulation with $fA\beta_{1-42}$, and this level was sustained for 6 h (Figure 4E). These results suggest that $fA\beta_{1-42}$-induced $P2X_7R$ activation may be regulated by ATP released from microglia in an autocrine manner.

Discussion

The major new finding in this study is that $fA\beta_{1-42}$ induces ATP release from microglia and that ATP regulates ROS generation through the activation of $P2X_7R$ and Ca^{2+} 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 (O_2^-) can, however, cause serious damage to the host. The phagocyte

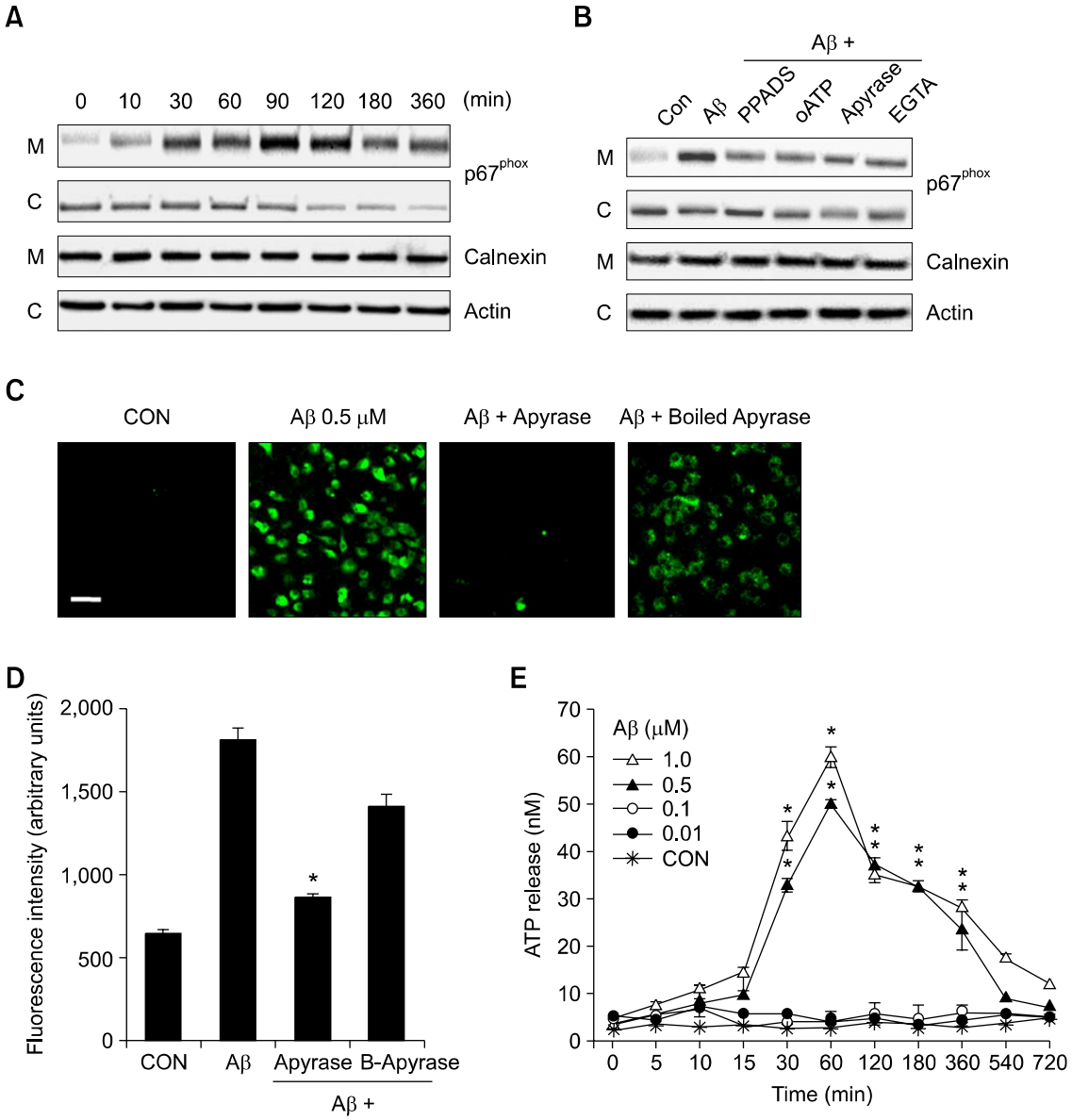

Figure 4. NADPH oxidase activation and ROS production by ATP released from fAβ_{1-42}-stimulated microglia. Microglial cells were plated into 60 mm dishes (2×10^6 cells), and treated with 0.5 μM fAβ_{1-42} for the time periods indicated (A), or pretreated with 100 μM PPADS, 100 μM oATP, 5 U of apyrase, or 0.5 mM EGTA, and then treated with 0.5 μM fAβ_{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/coverslip) were plated onto coverslips, and treated with 5 U/ml of apyrase and 0.5 μM fAβ_{1-42} for 2 h. The effect of apyrase on the production of intracellular ROS was measured by DCF fluorescence. Scale bar, 100 μm. (D) DCF intensities of cells in (C) were measured using Image Gauge 4.0 (Fujifilm). Values are mean ± SEM of 40-50 cells. *$P < 0.001$ compared with fAβ_{1-42}. (E) Time courses of ATP release into the culture supernatants of fAβ_{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 fAβ_{1-42} stimulation. Values are mean ± SEM of triplicate samples. *$P < 0.001$ compared with the control.

NADPH oxidase is a major enzyme catalyzing the non-mitochondrial production of O_2^- (Lambeth, 2004). The activation of NADPH oxidase must therefore be tightly controlled. For example, the bacterial chemoattractant fMet-Leu-Phe (fMLF)-mediated O_2^- production in human polymorphonuclear 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 fAβ_{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 $P2X_7R$-specific agonist, caused Ca^{2+} influx from extracellular sources only (Parvathenani *et al.,* 2003). We therefore examined the possible involvement of $P2X_7R$ 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 $P2X_7R$. A recent study also reported that $P2X_7R$ 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 $P2X_7R$-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 $P2X_7R$ 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 fAβ-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 $P2X_7R$.

Acknowledgments

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