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Subject Category: Neuroscience

Oligomeric amyloid β induces IL-1β processing via production of ROS: implication in Alzheimer’s disease

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Figure 1  oAβ induces IL-1β release in LPS-primed microglia. Microglia primed with LPS for 3 h were washed with ice-cold PBS and treated with oAβ for varying times; the concentration of IL-1β in the culture supernatant was then measured (a). ***P<0.001, versus 0 h. Western blot analysis of oAβ used in the present study (b). The blot was incubated with mouse anti oAβ monoclonal antibodies (6E10) (1:1000, Chemicon). LPS-primed microglia were treated with oAβ for 48 h, and the concentration of IL-1β in the culture supernatant as well as mRNA expression were measured by ELISA (c) and qPCR (d). Data indicate means ± S.D. for five independent experiments. ***P<0.001, versus LPS-primed microglia

Figure 2  oAβ induces IL-1β secretion/release via caspase-1 activation. LPS-primed microglia were treated with Z-VAD-FMK (a) or Z-YVAD-FMK (b) for 30 min before oAβ stimulation, and IL-1β in the culture supernatant was measured at 48 h. Data indicate means ± S.D. for four independent experiments. **P<0.01, versus LPS-primed microglia as control (ctl). ††† denotes P<0.001, versus LPS-primed microglia without oAβ. (c) After LPS-priming microglia were treated with oAβ for 48 h, Casp-1 p10 in the culture supernatant as well as caspase-1 and β-actin in the cell lysates were assessed by western blotting. Data are representative of two independent experiments. (d) LPS-primed microglia were treated with oAβ for 48 h, and caspase-1 activity was measured. Data indicate means ± S.D. for three independent experiments. **P<0.01, versus LPS-primed microglia without oAβ.
To assess whether oAβ affects the processing of IL-1β, we transiently activated microglia with LPS (1 μg/ml) for 3 h (LPS priming). The cells were then washed twice with ice-cold PBS and further stimulated with oAβ (5 μM) for varying times (0–72 h), and IL-1β concentration in the culture supernatant was measured. We found that oAβ time-dependently increased IL-1β concentration in the culture supernatant when compared with transiently activated microglia with LPS for 3 h, which served as control (Figure 1a). Western blot analysis of oAβ used in the present study was shown in Figure 1b. In addition, oAβ dose-dependently increased IL-1β secretion (Figure 1c). As oAβ alone did not upregulate mRNA levels of IL-1β (Figure 1d), these results indicate that oAβ upregulates processing of IL-1β in LPS-primed microglia. As pro-IL-1β is reported to be processed by a caspase-dependent pathway,15 To determine whether oAβ-induced IL-1β secretion is dependent on caspase, microglia primed with LPS for 3 h were treated with the pan-caspase inhibitor Z-VAD-FMK or caspase-1 inhibitor Z-YVAD-FMK for 30 min before oAβ stimulation. We then measured IL-1β in culture supernatant at 48 h. Both Z-VAD-FMK and Z-YVAD-FMK dose-dependently decreased IL-1β secretion in the culture supernatant (Figures 2a and b). We next assessed the cleaved fraction of caspase-1 (Casp-1 p10) by western blotting and found that oAβ dose-dependently increased the secretion of Casp-1 p10 in the culture supernatant (Figure 2c). Similarly, treatment of oAβ after LPS priming dose-dependently increased caspase-1 activity in microglia (Figure 2d).

To determine whether oAβ-induced IL-1β processing is dependent on NLRP3, we increased the K⁺ concentration in the culture medium, which was previously described to inhibit NLRP3.19 We found that the increased K⁺ concentration, by the addition of KCl, significantly decreased IL-1β release from microglia (Figure 3a). The addition of NaCl did not affect IL-1β release. Furthermore, oAβ stimulation induced the co-localization of caspase-1 with NLRP3 (Figure 3b). NLRP3 is reported to be activated by lysosomal destabilization and release of cathepsin B in response to phagocytosis.22,23,26 To evaluate the requirement of phagocytosis and cathepsin B release in oAβ-induced IL-1β secretion, phagocytosis and cathepsin B were pharmacologically inhibited with cytochalasin D and cathepsin B inhibitor, respectively. We found that cytochalasin D inhibited oAβ-induced IL-1β release and caspase-1 activity as previously described22 (Supplementary Figures 2a and b); however, it had no effect on oAβ-induced caspase-1 activation. LPS-primed microglia were treated with NaCl or KCl before oAβ stimulation, and IL-1β in the culture supernatant was measured at 48 h. Data indicate means ± S.D. for four independent experiments. ***P<0.001, versus LPS-primed microglia (ctl). †††P<0.001, versus LPS priming + oAβ + KCl. (b) LPS-primed microglia were treated with oAβ for 48 h, and co-localization of NLRP3 and caspase-1 were assessed by immunocytochemistry. Data are representative of three independent experiments. Scale bar represents 10 μm.
on Aβ-induced IL-1β secretion and caspase-1 activity (Figures 4a and b). Similarly, cathepsin B inhibitor decreased Aβ-induced IL-1β secretion and caspase-1 activity as previously described (Supplementary Figures 2c and d). ROS are reported to act as danger signal for NLRP3 inflammasome activation. High concentrations of ROS inhibitors are reported to block NF-κB-mediated by priming of NLRP3 inflammasome.

We treated microglia with N-acetylcysteine (NAC), a potent ROS scavenger, for 30 min after LPS priming and before the addition of Aβ. NAC dose-dependently decreased Aβ-induced IL-1β secretion (Figure 5a). Similarly, NAC also inhibited Aβ-induced caspase-1 activity (Figure 5b). Similarly, gp91ds-tat, an NADPH oxidase (NOX)-specific inhibitor, also dose-dependently decreased Aβ-induced IL-1β secretion as well as caspase-1 activity, but not as potently as NAC (Supplementary Figures 3a and b). These results indicate that Aβ-induced IL-1β secretion is partially dependent on NOX.

Mitochondrial ROS are reported to activate NLRP3, so we next determined cellular and mitochondrial ROS production by flow cytometry. Microglia treated with Aβ after LPS priming produced cellular and mitochondrial ROS, which were inhibited by NAC (Figure 5c). We also assessed whether LPS-primed microglia affect neuronal viability. LPS-primed microglia were cocultured with primary cortical neurons and treated with Aβ (5 μM) with or without Z-YVAD-FMK or IL-1ra. Neuronal cultures were also treated with Aβ with or without Z-YVAD-FMK or IL-1ra. We found that treatment of neuronal cultures with Aβ decreased the viability of neurons. The neuronal damage with Aβ was further enhanced in the neurons/LPS-primed microglia cocultures. Although Z-YVAD-FMK or IL-1ra had no effect in the neuronal cultures, it attenuated microglia-induced neurotoxicity in the neuron/LPS-primed microglia cocultures (Figures 6a and b).

Discussion

Microglial-mediated neuroinflammation contributes to the pathogenesis of AD. Indeed, microglial activation and subsequent production of neurotoxic pro-inflammatory molecules have a pivotal role in the progression of AD. However, whether Aβ, a main component of misfolded protein in the AD brain, could induce the production of pro-inflammatory cytokines is controversial. It has been reported that Aβ does not induce IL-1β mRNA in microglia. However, other reports indicate that Aβ induces various inflammatory mediators such as IL-1β, TNF-α, and NO. In this study, we have shown that Aβ alone is not sufficient to induce IL-1β mRNA or

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**Figure 4** Aβ-induced IL-1β release is independent on phagocytosis or cathepsin B. LPS-primed microglia were treated with cytochalasin D for 30 min before Aβ stimulation, and IL-1β (a) in the culture supernatant as well as caspase-1 activity (b) were measured at 48 h. Data indicate means ± S.D. for three independent experiments. ***P<0.001, versus LPS-primed microglia (ctl). ns indicates not significant. LPS-primed microglia were treated with cathepsin B inhibitor for 30 min before Aβ stimulation, and IL-1β (c) in the culture supernatant as well as caspase-1 activity (d) were measured at 48 h. Data indicate means ± S.D. for three independent experiments. ***P<0.001, versus LPS-primed microglia (ctl). ns indicates not significant.
Caspase-1 activity are one representative of three independent experiments microglia. We further showed inflammasome activation in neurons. Thus, oAβ is reported to activate caspase-1 via NLRP3. ROS induce inhibition of K⁺ efflux activating NLRP3. We have also shown that the ROS scavenger NAC (10 μM) for 30 min before oAβ stimulation and cellular (CM-H2DCFDA) and mitochondrial (MitoSOX) ROS were assessed by flow cytometry. Data are one representative of three independent experiments.

Figure 5  oAβ-induced IL-1β release is dependent on ROS. LPS-primed microglia were treated with varying doses of NAC (0.1–10 μM) for 30 min before oAβ stimulation, and IL-1β (a) in the culture supernatant as well as caspase-1 activity (b) were measured at 48 h. Data indicate means ± S.D. for three independent experiments. **P < 0.001, versus LPS-primed microglia (ctl). †, ††, or ††† denotes P < 0.05, 0.01, or 0.001, respectively, versus LPS priming + oAβ + NAC (c). LPS-primed microglia were treated with the ROS scavenger NAC (10 μM) for 30 min before oAβ stimulation and cellular (CM-H2DCFDA) and mitochondrial (MitoSOX) ROS were assessed by flow cytometry. Data are one representative of three independent experiments.

increase IL-1β secretion in unstimulated microglia. oAβ induces IL-1β secretion via activation of caspase-1 when microglia are primed with toll-like receptor (TLR) 4 ligand LPS. We also showed that both mitochondrial as well as NOX2-induced ROS contribute to oAβ-induced caspase-1 activation. Furthermore, oAβ has been shown to induce ROS in microglia by activation of NOX and mitochondria damage. ROS are reported to induce ROS in microglia via activation of caspase-1 when microglia are primed with toll-like receptor (TLR) 4 ligand LPS.

Our results indicate that the mechanism of oAβ-induced IL-1β secretion is different from that induced by fAβ. oAβ-induced IL-1β secretion by microglia was not dependent on phagocytosis and lysosomal disruption with subsequent release of cathepsin B, because we found that the inhibition of phagocytosis by cytochalasin D and cathepsin B inhibitors had no effect on IL-1β secretion. However, fAβ-induced IL-1β secretion is dependent on phagocytosis and subsequent lysosomal disruption. oAβ and fAβ differentially activate microglia and neurons. For instance, oAβ is reported to inhibit phagocytosis, whereas fAβ is reported to stimulate phagocytosis. Moreover, oAβ is reported to be more neurotoxic than fAβ. We have also shown that oAβ induces far greater secretion of IL-1β than fAβ in LPS-primed microglia. We further showed inflammasome activation in microglia increases oAβ-induced neuronal cell death, which is ameliorated by the inhibition of caspase-1 and IL-1β.

Consistent with this observation, genetic deletion of NLRP3 in mice expressing mutant human APP/PS1, an animal model of AD, decreases their disease burden. The role of IL-1β in AD pathology is complex. IL-1β transgenic mice expressing mutant human APP/PS1 are reported to have decreased plaque formation, although the total amount of oAβ is unaltered. However, IL-1β transgenic mice are reported to have learning and memory impairment. IL-1β can also affect synaptic plasticity and inhibit long-term potentiation. It has been shown that secreted mature IL-1β induces the phosphorylation of tau protein and mediates the formation of neurofibrillary tangles. IL-1β can be elevated before the formation of amyloid plaque in patients with Down syndrome, who invariably develop AD-like pathology. Thus, oAβ-induced IL-1β secretion by microglia may augment neuroinflammation, increase neuronal cell death, and contribute to the pathogenesis of AD. Indeed, the infusion of oligomeric human amyloid β in mice lacking IL-1 receptor antagonist (IL-1ra) induces microglial activation and causes neuronal cell death.

In conclusion, our results indicate that oAβ induces the secretion of active IL-1β via increased activation of caspase-1 in LPS-primed microglia, which is dependent on mitochondrial and NOX2-induced ROS production. Secreted IL-1β is involved in neuronal cell death that is ameliorated by inhibiting caspase-1 activation or by neutralization of IL-1β. Thus, the cascade of oAβ-induced IL-1β secretion in microglia may be a target for treating AD.
Materials and Methods

Reagents. LPS, N-acetyl-L-cysteine (NAC) and cytochalasin D were obtained from Sigma-Aldrich (St Louis, MO, USA). Z-VAD-FMK (pan-caspase inhibitor), Z-YVAD-FMK (caspase-1 inhibitor), and Ac-Leu-Val-lysinal (cathepsin B inhibitor) were obtained from Calbiochem (Gibbstown, NJ, USA). Anti-cryopyrin (sc-34410) and anti-caspase-1 antibodies (sc-514) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Gp-91 ds tat was from Anaspec (Fremont, CA, USA). Anti-cryopyrin (sc-34410) and anti-caspase-1 antibodies (sc-514) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IL-1ra was obtained from R&D (Minneapolis, MN, USA).

Cell culture. All animal experiments were conducted under protocols that were approved by the Animal Experiment Committee of Nagoya University. All primary cultures were prepared from C57BL/6 mice (Japan SLC, Hamamatsu, Japan).

Microglia were isolated from primary mixed glial cell cultures prepared from newborn mice on day 14 using the ‘shaking off’ method as previously described.51 Briefly, cortical fragments were dissociated into single cells in dissociation solution (Sumitomo Bakelite, Akita, Japan) and resuspended in nerve culture medium (Sumitomo Bakelite). Neurons were seeded onto 12-mm polyethyleneimine (PEI)-coated glass cover slips (Asahi Techno Glass Corp, Chiba, Japan) at a density of 5 × 10⁴ cells/well in 24-well plates. The purity of the culture was more than 95% as determined by NeuN-specific immunostaining (Merck Millipore, Billerica, MA, USA).

Preparation of oAβ and fAβ. oAβ and fAβ were prepared as previously described.52 To form fAβ synthetic human Aβ1-42 (Peptide Institute, Osaka, Japan) was dissolved in 0.02% ammonia solution at a concentration of 250 µmol/l, diluted to 25 µmol/l in PBS, and incubated at 37 °C for 72 h. Briefly, oAβ1-42 was prepared by dissolving Aβ1-42 to 1 mmol/l in 100% 1,1,1,3,3,3-hexafluoro-2-propanol. 1,1,1,3,3,3-Hexafluoro-2-propanol was dried by a vacuum desiccator and resuspended to 5 mmol/l in DMSO. To form oligomers, amyloid peptide was diluted to a final concentration of 100 µmol/l with Ham’s F-12, incubated at 4 °C for 24 h, and then immediately added to cultures at a final concentration 5 mmol/l. Formation of oAβ was confirmed by western blotting as previously described.30

Measurement of IL-1β and caspase-1 activity. Microglia, seeded at a density of 1 × 10⁵ cells/well in 24-well plates, were treated with LPS for 3 h. The cells were then washed twice and treated with oAβ. Supernatants were collected and the levels of IL-1β in culture supernatant were determined by ELISA according to the manufacturer’s instruction (BD Biosciences). Microglia, seeded at a density of 1 × 10⁵ cells and treated as described above, were measured for caspase-1 activity according to the manufacturer’s instruction (Merck Millipore).

RT-PCR. For quantitative PCR, the total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from total cellular RNA that was denatured for 5 min at 65 °C, followed by a reverse transcription reaction using the SuperScript II (Life Technologies, Carlsbad, CA, USA).

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The cdNA served as a template to amplify genes in quantitative PCRs with TaqMan Gene Expression assays (Applied Biosystems, Foster City, CA, USA), Universal PCR Master Mix (Applied Biosystems), andRotor-GenQ (Qiagen). Expression levels of target genes were calculated using a comparative method and normalization to GAPDH expression levels as previously described.53 The following primers and probes were obtained from Applied Biosystems: IL-1β, Mm00434228_m1; GAPDH, Mm99999915_g1.

Immunocytochemistry. Immunocytochemistry was conducted as previously described. Microglia plated on a glass cover slip were fixed with 4% paraformaldehyde for 10 min. The cells were then permeabilized with 0.05% Triton X-100 for 5 min and blocked with 5% bovine serum albumin for 1 h, followed by incubation with anti-caspase-1 (1:500), and anti-NLRP3 (1:500) antibodies overnight at 4 °C. The cells were then incubated with Alexa 488- or Alexa 568-conjugated secondary antibodies for 1 h. Cells were examined with a deconvolution fluorescence microscope system (Bio Zero, Keyence, Osaka, Japan). Neuronal viability was assessed as previously described.19,52 To determine the viability of neurons in microglia-neuronal co-cultures, microglia were labeled with Cy5 conjugated anti-CD11b (1:250) for 30 min before permeabilization with 0.05% Triton X-100 for 5 min, and blocked with 5% goat serum for 1 h, followed by incubation with anti-4 G8 antibodies (Chemicon, Temecula, CA, USA, 1:1000), and anti-MAP2 antibodies (Merck Millipore, 1:1000) for 2 h at room temperature. Then, the cells were incubated with Alexa 488- or Alexa 568-conjugated secondary antibodies (1:1000) for 1 h. Cells were examined with a deconvolution fluorescence microscope system.

Western blotting. Western blotting was done as previously described.22 Cell culture supernatants were precipitated by centrifugation for 10 min at 20 000 g. The samples were then separated by SDS-PAGE and transferred onto nitrocellulose membranes. Blots were incubated with rabbit polyclonal anti-mouse caspase-1 antibodies. To determine the caspase-1 level in the cell lysate, microglia were lysed with TNES buffer (1 M Tris-HCl, 20% SDS, and 2.5% glycerol) containing phosphatase (Sigma-Aldrich) and dephosphorylated. The samples were separated by a transgenic model of Alzheimer’s disease. Cell Death Disc 2010; 1: e1.

Flow cytometry. Flow cytometry was conducted as previously described.53 Briefly, LPS-primed microglia treated with αβ/ or left untreated were stained with 5-(and-6)-chloromethyl-2,7’-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) or MitoSOX red superoxide indicator (both from Invitrogen, Carlsbad, CA, USA) for 15 min according to the manufacturer’s instruction. After washing twice, cells were analyzed using a Cytomics FC500 (Beckman Coulter, Brea, CA, USA).

Statistical analysis. Statistically significant differences between experimental groups were determined by a one-way ANOVA followed by the Tukey’s test for multiple comparisons. Statistical analysis was performed using the software program Prism 4.0 (GraphPad Software, San Diego, CA, USA). P-values < 0.05 were considered to be statistically significant.

Conflict of Interest
The authors declare no conflict of interest.

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