Amyloid-β Induces Chemotaxis and Oxidant Stress by Acting at Formylpeptide Receptor 2, a G Protein-coupled Receptor Expressed in Phagocytes and Brain

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Amyloid-β, the pathologic protein in Alzheimer’s disease, induces chemotaxis and production of reactive oxygen species in phagocytic cells, but mechanisms have not been fully defined. Here we provide three lines of evidence that the phagocyte G protein-coupled receptor (N-formylpeptide receptor 2 (FPR2)) mediates these amyloid-β-dependent functions in phagocytic cells. First, transfection of FPR2, but not related receptors, including the other known N-formylpeptide receptor FPR, reconstituted amyloid-β-dependent chemotaxis and calcium flux in HEK 293 cells. Second, amyloid-β induced both calcium flux and chemotaxis in mouse neutrophils (which express endogenous FPR2) with similar potency as in FPR2-transfected HEK 293 cells. This activity could be specifically desensitized in both cell types by preincubation with a specific FPR2 agonist, which desensitizes the receptor, or with pertussis toxin, which uncouples it from Gα-dependent signaling. Third, specific and reciprocal desensitization of superoxide production was observed when N-formylpeptides and amyloid-β were used to sequentially stimulate neutrophils from FPR2−/− mice, which express FPR2 normally. Potential biological relevance of these results to the neuroinflammation associated with Alzheimer’s disease was suggested by two additional findings: first, FPR2 mRNA could be detected by PCR in mouse brain; second, induction of FPR2 expression correlated with induction of calcium flux and chemotaxis by amyloid-β in the mouse microglial cell line N9. Further, in sequential stimulation experiments with N9 cells, N-formylpeptides and amyloid-β were able to reciprocally cross-desensitize each other. Amyloid-β was also a specific agonist at the human counterpart of FPR2, the FPR-like 1 receptor. These results suggest a unified signaling mechanism for linking amyloid-β to phagocyte chemotaxis and oxidant stress in the brain.

In Alzheimer’s disease, progressive dementia and neurodegeneration are associated with a complex pathologic lesion made up of neurofibrillary tangles and aggregated extracellular protein deposits, known as senile plaques, which together are surrounded and infiltrated by activated microglial cells (1). Amyloid-β (Aβ),1 a heterogeneous 39–43-amino acid, self-aggregating peptide produced by sequential cleavage of amyloid precursor protein by the enzymes β-secretase and γ-secretase, is central to the pathogenesis of this disease (2, 3). The main component of senile plaque (4), Aβ is also biologically active and has been proposed to promote neurodegeneration by both direct and indirect mechanisms. It is directly toxic to cultured neurons in vitro (5) and is able to regulate production of the protein tau (6), which accumulates in neurofibrillary tangles. It may also induce neurodegeneration indirectly through its proinflammatory activity (7–10), which includes the ability to directly induce chemotaxis of mononuclear phagocytes (11, 12) as well as production of cytokines and reactive oxygen species (13–19) by microglial cells, monocytes, and neutrophils. Aβ may also induce phagocyte accumulation and activation indirectly, by inducing C5a production through activation of complement (20) or by inducing macrophage colony-stimulating factor release from neurons (21). Consistent with a proinflammatory role, intravenous injection of Aβ causes endothelial cell leakage and leukocyte adhesion and migration in vivo (17). The notion that inflammation is important in the pathogenesis of Alzheimer’s disease is consistent with clinical reports linking nonsteroidal anti-inflammatory drug administration to reduced incidence of disease and milder clinical course in affected patients (22).

The mechanism of Aβ action on cells has not been fully defined yet. Aβ has been reported to bind to several otherwise unrelated receptors, including the receptor for advanced glycation end products (RAGE; Ref. 23), the class A scavenger receptor (19), the p75 neurotrophin receptor (24), glypican (25), neuronal integrins (26), and the N-methyl-d-aspartate receptor (27).

The role of glipicyn, N-methyl-d-aspartate receptors, integrins, and p75 neurotrophin receptor in mediating Aβ action is not defined. RAGE has been implicated in mediating Aβ-induced oxidant stress in endothelial cells and cortical neurons, NF-κB activation in endothelial cells, and induction of tumor necrosis factor-α production, chemotaxis, and haptotaxis of the mouse microglial cell line BV-2 (19); conflicting results have been reported with regard to the role of RAGE in Aβ-induced neurotoxicity (28). Scavenger receptors have been reported to mediate adhesion of rodent microglial cells and human mono-receptor; FPRL1R, FPR-like 1 receptor; LXA4R, lipoxin A4 receptor; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; RAGE, receptor for advanced glycation end products; PTX, pertussis toxin; LPS, lipopolysaccharide; RANTES, regulated upon activation normal T-cell expressed and secreted; SDP-1, stromal cell-derived factor-1; MIP, macrophage inflammatory protein; HBSS, Hank’s balanced salt solution; FMLF, fMet-Leu-Phe; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR.

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‡ The abbreviations used are: Aβ, amyloid β; FPR, N-formylpeptide...
cytes to β2 fibril-coated surfaces, leading to secretion of reactive oxygen species and cell immobilization (18), and to mediate internalization of aggregated β2 protein (29); however, these receptors do not appear to mediate β2 stimulation of peripheral blood monocyte-dependent neurotoxicity (30). Aβ has also been reported to have direct toxic effects on membranes independent of receptors (31). Despite these advances, the precise mechanisms by which Aβ induces chemotaxis and oxidant production in primary phagocytic cells remain undefined.

Most known phagocyte chemotactic receptors are members of the G family of G protein-coupled receptors (GPCRs), which signal through pertussis toxin-sensitive pathways (32). Recently, pertussis toxin was reported to block Aβ induction of interleukin-1β release from the human monocyte cell line THP-1 (14) as well as Aβ induction of calcium flux in HL-60 cells (33). This, together with the fact that calcium flux is strongly associated with GPCR activation by chemotractions, suggested to us that Aβ may act via a GPCR. Since ligand promiscuity is a common property of chemotactic receptors, we tested this hypothesis by examining the ability of cloned phagocyte chemotactic receptors to reconstitute Aβ signaling in a transfected cell line. We also investigated receptors mediating Aβ signaling on mouse phagocytes (reported here) and human phagocytes (reported separately).

MATERIALS AND METHODS

Cell Lines—Construction of human embryonic kidney (HEK) 293 cells lines expressing human formylpeptide receptor (FPR), human formylpeptide receptor-like 1 receptor (FPRL1), mouse FPR, mouse FPRL2, mouse lipoxin A4 receptor (encoded by Fpr-rs1), a mouse orphan receptor encoded by Fpr-rs3, and human CCR5 and CXCR1 has been previously described (34–37). Fpr-rs1 and Fpr-rs3 were tested because of their high structural similarity to the known formylpeptide receptors and because they are also expressed in phagocytes (37). Cells were grown in Dulbecco’s modified Eagle’s medium high glucose medium (Life Technologies, Inc.) containing 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin (Hyclone), and 2 mg/ml G418 (Life Technologies) at 37 °C, 5% CO2, and 100% humidity. A human CCR1-expressing HEK 293 cell line has also been previously described (38); culture conditions were the same except for usage of 200 units/ml hygromycin B (Calbiochem) as the selective antibiotic. A mouse pre-B cell lymphoma cell line (4DE4) expressing human CCR8 has been reported previously (39). These cells were cultured in RPMI 1640 (Life Technologies) containing 10% heat-inactivated fetal bovine serum, 50 μg/ml b-mercaptoethanol (Sigma), and 2 mg/ml G418. The N9 murine microglial cell line was a kind gift from Dr. P. Ricciardi-Castagnoli (Università Degli Studi di Milano-Bicocca, Milan, Italy). These cells express typical markers of resting mouse microglia and have been extensively used as representatives of primary mouse microglial cells (40). The cells were grown in Iscove’s modified Dulbecco’s medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 mg/l b-mercaptoethanol.

Preparation of Mouse Neutrophils—Neutrophils were obtained from the peritoneal cavity of wild type and gene knockout litter mates of F1 to the peritoneal cavity of wild type and gene knockout litter mates of F1—2 mice. One ml of cells was added to the top of a 5-μm pore size membrane. Chemotaxis assays for N9 cells incubated with or without LPS (100 ng/ml) were performed with 48-well chemotaxis chambers (Neuro Probe). Polycarbonate filters with 5-μm pore size and 90-min incubation at 37 °C were used for measurement of microglial cell migration.

Superoxide Production—Mouse neutrophils were suspended in HBSS containing Ca2+ and Mg2+ at 106 cells/ml. 50 μl (5 × 105 cells) were distributed into wells of a 96-well microtiter chemiluminescence plate and incubated at 37 °C for 5 min. Then a mixture of the superoxide-specific chemiluminescence indicator reagent Diogenes (National Diagnostics, Inc., Gaithersburg, MD) for 5 min at 37 °C. Cells were suspended to 1–2 × 106/ml of phosphate-buffered saline containing 2.5 μM Fura-2/AM for 45 min at 37 °C. Neutrophils were washed twice in HBSS and suspended to 1–2 × 106/ml for analysis. Calcium flux was performed with N9 cells preincubated in the presence or absence of 300 ng/ml lipopolysaccharide (LPS) (37 °C, 24 h) using similar procedures.

Chemotaxis—HEK 293 cells were harvested from tissue culture flasks by incubation in trypsin (0.05%/EDTA 0.1%) (Quality Biologicals, Inc., Gaithersburg, MD) for 5 min at 37 °C. Cells were suspended evenly by vigorous pipetting, and excess Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum was then added to block trypsin. Cells were washed twice in Dulbecco’s modified Eagle’s medium and suspended to a concentration of 4 × 104 cells/ml in chemotaxis medium (HEPMI 104 mM Hepes, 100 mM NaCl, 100 mM KCl, 16 mM NaHCO3, and 1% bovine serum albumin (ICN Biomedicals Inc., Aurora, OH). Chemotractions, diluted in chemotaxis medium, were added to the bottom wells of a 96-well chemotaxis plate (Neuro Probe, Inc., Gaithersburg, MD). A 12-μM pore size membrane was placed on top, and 25 μl of cell suspension containing ~100,000 cells was placed in the upper chamber. Cells were incubated for 5 h at 37 °C, 100% humidity, 5% CO2. The membrane was carefully removed, and cells in the bottom well were counted using a hemacytometer. Methods for murine neutrophils were the same except that ~200,000 cells were added to the top of a 5-μM pore size membrane. Chemotaxis assays for N9 cells incubated with or without LPS (300 ng/ml) at 37 °C for 24 h) were performed with 48-well chemotaxis chambers (Neuro Probe). Polycarbonate filters with 8-μM pore size and 90-min incubation at 37 °C were used for measurement of microglial cell migration.

Calcium Flux Analysis—To monitor intracellular Ca2+ concentration, adherent cells were harvested and incubated in phosphate-buffered saline at 37 °C for 15 min and then incubated in phosphate-buffered saline containing 2.5 μM Fura-2/AM at 37 °C for 45 min. Cells were washed twice with HBSS (Life Technologies) and suspended in HBSS at 1× 106/ml. One ml of cells was added to 1 ml of HBSS and stimulated with continuous stirring at 37 °C in a fluorimeter (model MS-III; Photon Technology Inc., South Brunswick, NJ). Data were recorded every 200 ms as the relative ratio of fluorescence emitted at 510 nm following sequential excitation at 340 and 380 nm. The following ligands were evaluated: Aβ (nonfibrillar, human residues 1–42; California Peptide Research; Napa, CA), fMet-Leu-Phe (SMLF, Sigma), ATP (Life Technologies), and the chemokines RANTES, SDF-1, I-309, fractalkine, MIP-1α, and KC (Peprotech, Rocky Hill, NJ). The particular chemokines were tested because of their specificity for phagocyte targets. All chemokines were human with the exception of KC, which is mouse. The receptor targets for these chemokines are as follows: RANTES, FPR, human CCR1, and CCR5; CXCR4, CCR9; fractalkine, CXCR3; MIP-1α, CCR1, and CCR5; CXCXR2. Aβ, chemokines and ATP were dissolved in water and stored at –20 °C. fMLF was dissolved in MeSO and stored at –20 °C. In some experiments, the cells were incubated in 250 ng/ml pertussis toxin (PTX; Calbiochem) for 4 h at 37 °C in medium, harvested, and loaded with Fura-2/AM as described above. Immediately after harvesting, murine neutrophils were incubated in 1–2 × 106/ml of phosphate-buffered saline containing 2.5 μM Fura-2/AM for 45 min at 37 °C. Neutrophils were washed twice in HBSS and suspended to 1–2 × 106/ml for analysis. Calcium flux was performed with N9 cells preincubated in the presence or absence of 300 ng/ml lipopolysaccharide (LPS) (37 °C, 24 h) using similar procedures.
FIG. 1. Amyloid-β is a specific agonist for the G protein-coupled receptor FPR2 (mouse) and its human counterpart FPRL1R. Cells transfected with DNA encoding the receptor indicated at the upper left of each tracing were loaded with Fura-2 and monitored for changes in cell fluorescence, as a reporter of [Ca\(^{2+}\)], in response to 10 \(\mu\)M Aβ. Control agonists were 10 \(\mu\)M ATP (activating an endogenous nucleotide receptor), 10 \(\mu\)M fMLF (for FPRL1R and FPR2), 5 \(\mu\)M fMLF (for human FPR), 10 nM RANTES, 100 nM fractalkine, and 50 nM I-309. Test substances were added at the times indicated by the arrowheads. FPR2 + PTX, FPR2-transfected HEK 293 cells treated with 250 ng/ml pertussis toxin for 4 h before loading with Fura-2. HEK 293, denotes untransfected HEK 293 cells. All results are for HEK 293 cell lines except for CCR8, which is expressed in the mouse pre-B cell lymphoma cell line 4DE4. With the exception of FPR2 and FPRL1R, Aβ given first did not affect the cell response to the control agonist given second (not shown). Cell lines were tested at least three times with the exception of Fpr-rs3, which was tested twice, and Fpr-rs1, CCR8, and CX3CR1, which were all tested once. Complete inhibition of Aβ signaling by pertussis toxin was replicated in three independent experiments implying coupling to \(G_i\).

RESULTS

Mouse FPR2 and Its Human Counterpart FPRL1R Are Receptors for Amyloid-β—Using induction of calcium flux as a highly sensitive and specific real time assay of receptor activation, we screened a panel of stable cell lines transfected with plasmids encoding the known phagocyte formylpeptide receptors (human and mouse FPR, human FPRL1R, and mouse FPR2), four chemokine receptors (human CCR1, CCR5, CCR8, and CX3CR1), the mouse lipoxin A4 receptor (encoded by mouse Fpr-rs1), and an orphan receptor highly related in sequence to formylpeptide receptors (Fpr-rs3), as well as untransfected control cells, for responsiveness to 10 \(\mu\)M Aβ (Fig. 1). This concentration was chosen based on Aβ dose-response studies published previously for human neutrophils and monocytes and rat microglial cells (13). The lipoxin A4 receptor and Fpr-rs3 were included because of their high sequence similarity to the formylpeptide receptors (37).

Aβ induced a response in HEK 293 cells expressing FPRL1R and FPR2, which are human and mouse low affinity formylpeptide receptors, respectively. Activation of each receptor produced a robust transient that was similar in magnitude and duration to the response induced by the prototypical \(N\)-formylpeptide fMLF in the same cells (Fig. 2, A and B) and was similar kinetically to the transients induced by other classical chemoattractants and chemokines (Fig. 1). Aβ was specific for these receptors, since none of the other cell lines tested responded. The CCR1, CCR5, CCR8, and CX3CR1 and the human and mouse FPR (high affinity formylpeptide receptor) cell lines did respond to appropriate known agonists as previously described (34–36). The Fpr-rs1 and Fpr-rs3 cell lines were unresponsive to fMLF but did respond to ATP through an endogenous signaling pathway. Although mRNA for Fpr-rs1 and Fpr-rs3 is present in these two cell lines, we have not yet obtained direct evidence of receptor protein expression.

Aβ signaling could be completely blocked by pretreatment of the cells with pertussis toxin (Fig. 1, column 1, tracing labeled FPR2 + PTX), which inactivates \(G_i\), type G proteins. Pertussis toxin also blocks signaling by other FPR2 agonists (34, 42, 43). When FPR2- and FPRL1R-expressing cells were sequentially stimulated with 10 \(\mu\)M Aβ, they responded to the first but not
concentrations of Aβ responded to fMLF in a concentration-dependent manner, with Aβ cells expressing either mouse or human FPR did not respond to B neutrophils (44), transfected with the indicated receptors, or loaded HEK 293 cells (A) measured as the peak of the change in relative fluorescence of Fura-2. Agonist activity was optimum in FPR2-transfected HEK 293 cells, Aβ induction of calcium flux in mouse neutrophils was completely blocked by pretreatment of the cells with pertussis toxin, indicating a Gβ-dependent signaling pathway (Fig. 4C). Aβ potency was indistinguishable in neutrophils from FPR −/− and +/- mice (Fig. 3B). Although there was a trend toward lower efficacy (maximal response) in cells from FPR −/− mice, this difference was not statistically significant (Fig. 3B).

Aβ Is a Chemotactic Agonist at FPR2—To assess the potential biological significance of Aβ-FPR2 signaling, we used in vitro chemotaxis assays as a model of cell migration. Consistent with the calcium flux results, Aβ induced chemotaxis of FPR2-transfected HEK 293 cells but not mouse FPR-transfected HEK 293 cells; likewise, Aβ induced migration of mouse neutrophils (Fig. 5). In each case, the peak responses occurred at ~10 μM, and the EC50 values were consistent with the values for induction of calcium flux in these cells, 5 μM (Fig. 5, B and C). We have previously shown that the fMLF dose-response curve for chemotaxis in neutrophils from wild type mice has two peaks, one with an optimum at ~500 nM and the other with an optimum at 10 μM. The 500 nM optimum is due to FPR activity, since it is absent in cells from FPR −/− mice (34). The second peak is consistent with FPR2 pharmacology in transfected HEK 293 cells. Since the dose-response curve for Aβ chemotaxis is the same in neutrophils from FPR −/− and +/- mice, Aβ chemotraction of mouse neutrophils is not mediated by FPR. Since in FPR −/− neutrophils the Aβ and FPR2 chemotactic and calcium flux optima are similar and match the Aβ optimum in FPR2-transfected HEK 293 cells, Aβ chemotraction of these cells is most likely mediated by FPR2. Since application of Aβ on both sides of the chemotaxis filter gave net results equivalent to the background control, we conclude that Aβ-induced cell migration was due to chemotaxis, not chemokinesis (Fig. 5A).

Evidence That FPR2 Mediates Induction of Superoxide Generation by Amyloid β—To test whether FPR2 can also mediate production of reactive oxygen species by Aβ, we examined whether Aβ could induce superoxide production in mouse neutrophils and, if so, whether this activity could be desensitized by prestimulation with fMLF. Again, FPR −/− neutrophils were used to eliminate the possibility of cross-desensitization of Aβ activity by fMLF signaling through FPR. As shown in Fig. 6A, Aβ at 10 μM, a concentration that saturated the chemotactic and calcium flux response in mouse neutrophils and FPR2/HEK 293 cell transfecants, induced superoxide production with similar efficacy in FPR −/− and FPR +/- neutrophils. This is consistent with the calcium flux and chemotaxis results. Additional experiments (n = 2) showed a similar graded Aβ dose-response relationship and equivalent potency for FPR −/− versus FPR +/- neutrophils (data not shown). This is

![Fig. 3. Amyloid-β is an equipotent calcium-mobilizing agonist at FPR2, FPRL1R, and mouse neutrophils.](image-url)

The figure shows the peak amplitude (relative fluorescence) plotted against the log [Amyloid-β] (M) for FPR2, FPRL1R, and mouse neutrophils. The graph includes data points for fMLF and Aβ, with a comparison between wild type and FPR−/− mice. The results indicate that FPR2, FPRL1R, and mouse neutrophils respond to Aβ in a concentration-dependent manner, with Aβ inducing calcium flux in both FPR2- and FPRL1R-transfected HEK 293 cells.

The text explains that the second stimulus (Fig. 1, column 1, tracing labeled FPR2, and data not shown) indicating homologous desensitization of the signal transduction pathway, which is characteristic of G protein-coupled receptors (44). Moreover, Aβ and fMLF reciprocally interfered with each other’s signaling at FPR2 (Fig. 2, A and B) in a concentration-dependent manner, providing further evidence that both agonists act at the same receptor. This was specific, since Aβ did not affect signaling by agonists acting at any of the other receptors considered (Fig. 1 and data not shown).

Aβ induced calcium flux in both FPR2- and FPRL1R-transfected HEK 293 cells in a graded concentration-dependent manner, with an EC50 of 5 μM (Fig. 3A). In contrast, HEK 293 cells expressing either mouse or human FPR did not respond to Aβ from 0.5 to 20 μM (Fig. 3A). However, all four cell lines responded to fMLF in a concentration-dependent manner, with EC50 consistent with those previously reported (data not shown; Ref. 34).

To test whether native FPR2 also functions as an Aβ receptor, we first focused on primary mouse neutrophils, which, as we have previously shown, express FPR2 endogenously (34) and which can be analyzed in an FPR-deficient background due to the availability of FPR knockout mice (41). Aβ induced calcium flux in FPR −/− neutrophils with an EC50 of 1 μM, similar to the value for FPR2-transfected HEK 293 cells (Fig. 3, A and B). FPR −/− neutrophils also mimicked FPR2-transfected HEK 293 cells in sequential stimulation experiments; fMLF and Aβ were able to reciprocally cross-desensitize each other (Fig. 4, A and B). Specificity was again confirmed by the lack of cross-desensitization in this assay between Aβ and either SDF-1, MIP-1α, or KC in mouse neutrophils (Fig. 4C). It is important to note that FPR and FPR2 both mediate fMLF signaling in mouse neutrophils (34, 41). However, the desensitization experiments were carried out using neutrophils from FPR knockout mice, which rules out cross-desensitization of Aβ action by fMLF signaling through FPR and strongly implicates Aβ usage of endogenous neutrophil FPR2, the only other known neutrophil fMLF receptor. As with FPR2-transfected HEK 293 cells, Aβ induction of calcium flux in mouse neutrophils was completely blocked by pretreatment of the cells with pertussis toxin, indicating a Gβ-dependent signaling pathway (Fig. 4C). Aβ potency was indistinguishable in neutrophils from FPR −/− and +/- mice (Fig. 3B). Although there was a trend toward lower efficacy (maximal response) in cells from FPR −/− mice, this difference was not statistically significant (Fig. 3B).

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consistent with the chemotaxis and calcium flux results and indicates that Aβ induction of superoxide generation is not mediated by FPR. fMLF also induced superoxide generation in both FPR1/1 and FPR2/2 neutrophils; however, the EC50 was 10-fold lower at FPR2/2 neutrophils, which is consistent with our previous report of weaker potency of fMLF at FPR2 versus FPR for induction of both calcium flux and chemotaxis in both neutrophils and receptor-transfected cells (34).2

The superoxide response of FPR1/1 neutrophils to 10 μM Aβ was markedly attenuated when the cells were pretreated with 5 μM fMLF compared with pretreatment with vehicle alone (Fig. 6B). Likewise, the response to 5 μM fMLF was markedly attenuated when the cells were pretreated with 10 μM Aβ (Fig. 6C). The reduced response is not due to depletion or inactivation of NADPH oxidase by the first stimulation, because PMA could induce large amounts of superoxide production in cells when added after completion of the response to the second stimulus (data not shown). Moreover, costimulation experiments in which PMA was added simultaneously with fMLF or Aβ ruled out scavenging as the mechanism by which each agent reduced superoxide production by the other (data not shown).

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Fig. 4. Shared receptor usage by amyloid-β and fMLF in primary mouse neutrophils: cross-desensitization of calcium flux signaling and pertussis toxin sensitivity. Neutrophils from FPR1−/− and FPR2+/+ mice were loaded with Fura-2 and then monitored for fluorescence changes in sequential stimulation experiments. The name, concentration, and time of the addition of each stimulus are indicated at the arrowheads. Data in A and B are representative of two separate experiments. In C (bottom tracing), cells were incubated in PTX (250 ng/ml) for 4 h and then washed prior to stimulation with Aβ. Data in C are from a single experiment.

Fig. 5. Amyloid-β is a potent chemotactic agonist at FPR2. A, chemotaxis versus chemokinesis. FPR2-expressing HEK 293 cells were applied to the upper well of a chemotaxis chamber with or without 10 μM Aβ present in medium. Lower wells contained medium with or without 10 μM Aβ to distinguish chemotaxis from chemokinesis. The contents of the upper and lower wells of the chemotaxis chamber correspond to the numerator and denominator, respectively, of the fraction below each bar in the graph. B, chemotactic potency of Aβ at mouse FPR-transfected (open bars) and FPR2-transfected (solid bars) HEK 293 cells. C, chemotactic potency of Aβ for neutrophils from FPR1+/+ (open bars) and FPR2−/− (solid bars) mice. All conditions were tested in triplicate, and the results are presented as mean ± S.E. Data in A and B are representative of three separate experiments; data in C are representative of two separate experiments.

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FPR2-selective concentrations of fMLF. Neutrophils from FPR−/− (A–C) and +/+ (A) mice were stimulated as indicated, and the superoxide produced in the 10 min after the addition of the final substance was measured. Each condition was tested in triplicate, and the results are presented as mean ± S.E. A, FPR independence. A representative experiment of two independent experiments is shown. The difference in activity between FPR +/+ and −/− cells was not statistically significant. B and C, desensitization. Cells were stimulated sequentially with 5 μM fMLF and 10 μM Aβ in the order shown. Me2SO and water are the vehicles for fMLF and Aβ, respectively. The differences in B and C were statistically significant (p < 0.05). Representative results of three (B) and two (C) independent experiments are shown.

**FIG. 7. FPR2 gene expression in mouse brain.** Detection of FPR2 mRNA in whole mouse brain by RT-PCR using gene-specific primers is shown. Results are representative of three separate experiments.

Cells (40). Low levels of FPR2 mRNA could be detected in this cell line under resting conditions using RT-PCR; however, the cells did not respond to Aβ either in calcium flux or chemotaxis assays (Fig. 8). Cell activation with LPS induced FPR2 mRNA expression in a time-dependent fashion (Fig. 8A) and rendered the cells responsive to Aβ in a concentration-dependent manner in both calcium flux and chemotaxis assays (Fig. 8, B and C). The potency of Aβ was consistent for both functions and was consistent with the values obtained in studies of mouse neutrophils and FPR2-HEK 293 cells. As we observed with mouse neutrophils and FPR2-transfected HEK 293 cells, fMLF and Aβ were able to reciprocally cross-desensitize each other in sequential stimulation experiments using calcium flux as the functional readout (Fig. 8D). Finally, chemotaxis of LPS-activated N9 cells to Aβ was completely blocked by pretreatment of the cells with pertussis toxin (Fig. 8E), demonstrating a Qγ-dependent signaling pathway. This is consistent with the results obtained using the calcium flux assay in neutrophils and FPR2-transfected HEK 293 cells (Figs. 1 and 4).

**DISCUSSION**

These data directly demonstrate that Aβ, the major component of senile plaque in Alzheimer’s disease, can function as a specific chemotactic agonist at the mouse N-formylpeptide receptor subtype FPR2 expressed in HEK 293 cells. This receptor is constitutively expressed in neutrophils, lung, liver, spleen, and brain and at low levels in macrophages and the mouse microglial cell line N9; FPR2 expression in N9 cells can be induced by LPS. Furthermore, we provide a series of observations that together strongly suggest that Aβ activates mouse phagocytic cells via endogenous FPR2. First, the prototypical FPR2 agonist fMLF can desensitize Aβ action (calcium flux, chemotaxis, and superoxide production) in mouse neutrophils, including neutrophils from mice lacking the other known fMLF receptor subtype FPR. Second, LPS induces responsiveness of the mouse microglial cell line N9 to Aβ in both calcium flux and chemotaxis assays, and fMLF and Aβ can reciprocally cross-desensitize each other in activation of calcium flux in these cells. LPS induction of this activity correlates with the induction of FPR2 mRNA expression. Third, Aβ activates mouse phagocytes and FPR2-transfected HEK 293 cells with similar potency. We have also demonstrated that Aβ can activate the human counterpart of FPR2, FPRL1R, in transfected HEK 293 cells. This receptor is expressed constitutively on human neutrophils and monocytes. Recently, we have reported that human monocyte and FPRL1R-HEK 293 cell chemotactic responses induced by fMLF and Aβ can be reciprocally cross-desensitized, consistent with our observations in mouse cells (45).

Our results suggest a unified molecular mechanism involving FPR2 for Aβ activation of chemotaxis and oxidative stress in phagocytic cells. This signaling system could support beneficial functions such as host defense and tissue repair in settings where Aβ is produced homeostatically (46). In Alzheimer’s disease, where Aβ accumulates pathologically, our results suggest a novel mechanism to explain why activated microglial cells accumulate at senile plaques. Our gene expression data suggest that involvement of the receptor in this process in Alzheimer’s disease would require cell activation, since FPR2 mRNA is expressed at very low levels in brain and N9 microglial cells. Since LPS can induce FPR2 gene expression and responsiveness to Aβ by this cell line, then local production of LPS-sensitive cytokines such as tumor necrosis factor and interleukin-1β in the brains of patients with Alzheimer’s disease could provide such a stimulus in vivo for receptor expression. In this regard, it is important to note that Aβ has been shown to induce interleukin-1β production in THP-1 cells (14).

FPR2 was originally identified as the product of a mouse gene that cross-hybridized with FPR1, a human gene for the high affinity N-formylpeptide receptor FPR (37, 47). FPR1 cross-hybridizes with two other human genes: FPRL1, which encodes the low affinity N-formylpeptide receptor FPRL1R, and FPRL2, which encodes an orphan receptor (48–50). These genes, which are located in a cluster on human chromosome 19q13.3 (51), are expressed in phagocytes (52), and their protein products have 56–69% amino acid identity. FPRL1R has multiple and structurally diverse agonists, which, in addition to Aβ and fMLF (50, 53), include human immunodeficiency virus-1 envelope-derived peptides (54) and two endogenous mediators, serum amyloid A (55) and the eicosanoid lipoxin A4 (56). This receptor has also been called LXA4R, for lipoxin A4 receptor (56). In mice, six FPR-like genes have been cloned...
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(37), and agonists have been identified for three of them: Fpr1, which encodes the mouse FPR orthologue (53), and Fpr-rs1 and Fpr-rs2, both of which encode proteins with ~65% amino acid identity to FPRL1R (34, 37, 57). To date, Fpr-rs1 has been reported to encode a receptor named LXA4R which is specific for lipoxin A4 (57), whereas Fpr-rs2 encodes FPR2, which is specific for the other FPRL1R agonists including Aβ (34, 42, 43). The specificity of FPR2 for lipoxin A4 has not been clearly resolved. Thus, FPRL1R functions may be split between two mouse receptors, FPR2 and LXA4R.

Although several other Aβ receptors have been reported previously, FPR2 and FPRL1R are the only ones that are GPCRs. Consistent with this, most known leukocyte chemokine receptors act via GPCRs (32) and, like FPR2, activate calcium flux. Moreover, FPR2 was already known to function as a chemotactic receptor for serum amyloid A, human immunodeficiency virus-derived peptide T21, and fMLF (34, 42, 43). Aβ had previously been shown to induce calcium flux and interleukin-1β production in a pertussis toxin-sensitive manner (14, 33), suggesting involvement of a Gt-coupled receptor, and FPR2 is such a receptor. RAGE, the receptor for advanced glycation end products and a member of the immunoglobulin superfamily, is also an Aβ-coupled receptor and has been reported to mediate Aβ-induced oxidant stress in endothelial cells and cortical neurons, NF-κB activation in endothelial cells, and induction of tumor necrosis factor-α production, chemotaxis, and haptotaxis of the mouse microglial cell line BV-2 (21, 23). Although RAGE is expressed by primary microglial cells and is found at higher levels in brains from patients with Alzheimer’s disease versus unaffected controls (21), its role in mediating Aβ functional responses in primary microglial cells and other mononuclear phagocytes has not been defined. Aβ has been reported to induce chemotaxis of rodent microglial cells with an EC50 in the low nanomolar range, which is consistent with the reported binding affinity of Aβ for RAGE (21). Yet Aβ functions reported by most other studies have occurred with half-maxi-

![Fig. 8. FPR2 mRNA expression and a shared amyloid β/fMLF signaling pathway are induced by LPS in the mouse microglial cell line N9. A, FPR2 RNA expression. N9 cells were treated with LPS for the number of hours indicated at the top. mRNA was then isolated and amplified by RT-PCR using FPR2- and β-actin-specific primers as detailed under “Materials and Methods.” The reaction product for each time point was diluted as shown and visualized by gel electrophoresis. The size of FPR2 and β-actin PCR products is indicated by the arrowheads at the left. B, induction of calcium flux by amyloid-β (Aβ42) in LPS-activated N9 cells. Resting (LPS(−)) and LPS-activated (LPS(+) N9 cells were loaded with Fura-2 and stimulated with the indicated concentration of Aβ42 at the times indicated by the arrowheads. The results are shown as relative fluorescence in real time. C, induction of chemotaxis by amyloid-β (Aβ42) in LPS-activated N9 cells. The results are expressed as the mean ± S.D. chemotactic index (CI), which represents the fold increase in the number of migrated cells in response to chemotactants over the spontaneous cell migration (to control medium). Data were obtained by counting the number of migrated cells in three high power fields in triplicate samples. The asterisks indicate p < 0.01 for LPS(+) versus LPS(−) at each concentration. D, reciprocal cross-desensitization of calcium flux induction by amyloid-β (Aβ42) and fMLF in LPS-activated N9 cells. Data are real time fluorescence tracings of LPS-activated N9 cells loaded with Fura-2 and stimulated at the times indicated by the arrowheads with the indicated concentrations of agonists. E, Aβ coupling to Gt in N9 microglial cells. LPS-stimulated N9 cells were preincubated in PTX (100 ng/ml) or medium alone at 37°C for 30 min, washed, and then stimulated with the indicated concentrations of Aβ. The asterisk indicates p < 0.01 for the difference between PTX (+) versus PTX (−). Results are representative of three independent experiments.]
mal potency in the low micromolar range. These include Aβ action on mouse neutrophils and N9 microglial cells (present study), Aβ binding to human macrophages (58), Aβ-induced production of tumor necrosis factor by THP-1 (59) and N9 (10) cells, and Aβ induction of reactive oxygen species by human neutrophils (13), human macrophages (13), rat macrophages (60), and rat microglial cells (13). The explanation for this discrepancy is not clear, but it does not appear to simply involve differences in potency of Aβ for activating different cell types or for inducing different cell functions. One possibility is that in primary cells Aβ acts via two or more receptors that differ in affinity for ligand, a paradigm that has many examples, including the fMLF receptors FPR and FPR2 (34).

In summary, we have identified a novel Aβ receptor, FPR2 in mice and FPRL1R in humans, and have presented evidence that FPR2 mediates Aβ action on mouse neutrophils and the LPS-activated mouse microglial cell line N9. In resting N9 cells and in mouse brain, FPR2 mRNA is expressed at low levels. Our combined genetic and pharmacologic analysis of mouse neutrophils strongly suggests that Aβ can induce both chemotaxis and oxidative production via the same receptor, FPR2, which suggests a unified molecular basis for microglial cell recruitment to senile plaques and induction of oxidant stress in microglial cells in Alzheimer's disease. Consistent with this, we have found that FPRL1R RNA is expressed by inflammatory cells infiltrating senile plaques in brain tissues from patients with Alzheimer's disease (45). The role of this receptor in Alzheimer's disease may now be tested further by developing an FPR2 knockout mouse backcrossed onto the amyloid precursor protein transgenic mouse model of Alzheimer's disease, which exhibits the inflammatory aspect of the human disease (61, 62).

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