Amyloid Fibrils Activate Tyrosine Kinase-Dependent Signaling and Superoxide Production in Microglia

Douglas R. McDonald,1 Kurt R. Brunden,2 and Gary E. Landreth1
1Alzheimer Research Laboratory, Department of Neurology and Neurosciences, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106, and 2Gilatech, Incorporated, Cleveland, Ohio 44122

Alzheimer’s disease (AD) is a devastating neurological disorder characterized by loss of cognitive skills and progressive dementia. The pathological hallmark of AD is the presence of numerous senile plaques throughout the hippocampus and cerebral cortex associated with degenerating axons, neurofibrillary tangles, and gliosis. The core of the senile plaque primarily is composed of the 39–43 amino acid β-amyloid peptide (Aβ), which forms fibrils of β-pleated sheets. Although considerable genetic evidence implicates Aβ in the pathogenesis of AD, a direct causal link remains to be established.

Senile plaques are foci of local inflammatory processes, as evidenced by the presence of numerous activated microglia and acute phase proteins. Aβ has been shown to elicit inflammatory responses in microglia; however, the intracellular events mediating these effects are largely unknown. We report that exposure of microglia and THP1 monocytes to fibrillar Aβ led to time- and dose-dependent increases in protein tyrosine phosphorylation of a population of proteins similar to that elicited by classical immune stimuli such as immune complexes. The tyrosine kinases Lyn, Syk, and FAK were activated on exposure of microglia and THP1 monocytes to Aβ, resulting in the tyrosine kinase-dependent generation of superoxide radicals. The present data support a role for oxidative damage in the pathogenesis of AD, provide an important mechanistic link between Aβ and the generation of reactive oxygen intermediates, and identify molecular targets for therapeutic intervention in AD.

Key words: Alzheimer’s disease; β-amyloid; microglia; THP1 monocytes; signal transduction; tyrosine kinase; inflammatory; superoxide; piceatannol; RAGE; scavenger receptor

Dementia of the Alzheimer type is the most prevalent form of dementia of the aged. The pathological hallmark of Alzheimer’s disease (AD) is the presence of numerous senile plaques associated with degenerating neurons, neurofibrillary tangles (Selkoe, 1991), and marked gliosis throughout the hippocampus and cerebral cortex (Itagaki et al., 1989). The senile plaque is composed primarily of the β-amyloid protein (Aβ; Glenner and Wong, 1984; Masters et al., 1985). Aβ is a 39–43 amino acid peptide derived from the larger amyloid precursor protein (APP) as a result of proteolytic processing (Cole et al., 1989; Golde et al., 1992). Aβ forms fibrils that aggregate and form deposits comprising the core of senile plaques. Considerable genetic evidence has implicated Aβ in AD pathogenesis (Selkoe, 1996); however, the relationship between Aβ and neuronal death and gliosis is incompletely understood.

It has been postulated that the progressive pathology associated with AD is a consequence of local inflammatory reactions. This view has been supported by clinical studies demonstrating the efficacy of anti-inflammatory drug treatments in reducing the incidence of dementia (McGeer and McGeer, 1996). Microglia, the main immune effector cells within the brain (Leong and Ling, 1992), are the predominant glial cell type present within senile plaques (Itagaki et al., 1989). Microglia that are in direct contact with senile plaques exhibit an activated phenotype, as evidenced by elevated expression of HLA-DR, complement receptors, and immunoglobulin receptors (McGeer et al., 1989, 1993). In addition, acute phase proteins (Abraham et al., 1988; Griffin et al., 1989; McGeer et al., 1989; Cataldo and Nixon, 1990; Bauer et al., 1991) are present in AD-afflicted brain tissue at significantly elevated levels and are known to be secreted by reactive microglia (Araujo and Cotman, 1992). A critical question concerning the pathogenesis of AD is whether Aβ is directly capable of eliciting a local inflammatory response that is damaging to neurons.

The presence of reactive microglia and their secretory products within senile plaques suggest that microglia respond to constituents of the plaques, leading to the acquisition of an activated phenotype. The signal transduction pathways subserving the phenotypic changes mainly are unknown. Importantly, microglia that are in direct contact with senile plaques exhibit high levels of tyrosine-phosphorylated proteins (Wood and Zinsmeister, 1991), suggesting sustained activation of intracellular signaling processes. The activation of tyrosine kinases is the initial step in regulating a variety of cellular processes, including proliferation, differentiation, and inflammatory responses. Numerous inflammatory stimuli are known to activate tyrosine kinases such as Lyn and Syk in monocytes and macrophages, resulting in the release of cytokines and superoxide (Pfefferkorn and Fanger, 1989; Agarwal et al., 1993; Ghazizadeh et al., 1994; Crowley et al., 1996).

We report here that exposure of microglia and THP1 monocytes to fibrillar forms of Aβ resulted in the activation of tyrosine kinase-dependent intracellular signaling systems and the generation of superoxide radicals. These responses, however, are not linked to scavenger receptors or the receptor for advanced glycation end products, which recently have been shown to bind Aβ.
These findings support a role for oxidative damage in the pathophysiology of AD and provide a mechanistic link between Aβ and the acquisition of an activated phenotype in microglia and the generation of local inflammatory responses. Moreover, identifying signal transduction pathways that are activated on exposure of the cells to Aβ provides molecular targets for therapeutic interventions in AD.

MATERIALS AND METHODS

Materials. The anti-phosphotyrosine antibody PY20 and the anti-panxillin mAb were obtained from Transduction Labs (Lexington, KY). The anti-phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-FcyRI (mAb A23.22) and anti-FcyRII (mAb IV.3) were obtained from Medarex (Annendale, NJ). Affinity-purified polyclonal antisera to Lyn, Syk, and FAK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibody was obtained from Boehringer Mannheim (Indianapolis, IN). Goat anti-mouse F(ab)2 was obtained from Cappel (West Chester, PA). Peptides corresponding to amino acids 25–35 of Aβ (Aβ25–35), amino acids 1–28 of Aβ (Aβ1–28), and Substance P were obtained from American Peptide (Sunnyvale, CA). Nonfibrillar Aβ1–40 (Bachem, Philadelphia, PA) was prepared by dissociating fibrils in hexafluoroisopropanol, followed by precipitation of insoluble material in sterile distilled water, and then used immediately. Fibrillar Aβ1–40 was prepared by reconstitution of the lyophilized peptide in sterile distilled water, followed by incubation for 1 week at 37°C. Aβ1–42 and scrambled Aβ25–35 (SC25–35; NAMGKILSGIG) were synthesized at Gliatech (Cleveland, OH). All peptides were solubilized in sterile distilled H2O. Serum amyloid A, human LDL, and acetylated human LDL were kind gifts of Dr. Frederick DeBeer (University of Kentucky). LPS, ferrocytochrome C, type III, nitroblue tetrazolium (NBT), and superoxide dismutase were obtained from Sigma (St. Louis, MO). Protein A-agarose, fatty acid-free BSA, and picatinnanol were obtained from Boehringer Mannheim. BSA was maleylated as previously described (Haberland and Fogelman, 1985).

Cell culture. THP-1 cells were maintained in RPMI-1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS), 5 × 10−5 M 2-mercaptoethanol, 5 mM HEPES, and 1.5 μg/ml gentamicin in an atmosphere of 5% CO2. Jurkat cells were maintained in the the same medium but without 2-mercaptoethanol. Microglia and astrocytes were derived from the brains of neonatal rats as previously described but with some modifications (Giulian and Baker, 1986). Cerebral cortices were isolated from postnatal day 0 (P0) Sprague Dawley rats, and meninges and blood vessels were removed completely. Cortices were minced with a sterile razor blade, and cells were dissociated in PBS containing 0.25% trypsin and 1 mM EDTA for 30 min at 25°C. Digestion was terminated by adding an equal volume of DMEM/F12 medium (Life Technologies, Gaithersburg, MD) containing 20% FCS, and cells were centrifuged to obtain a single-cell suspension. Cells were plated in 75 mm flask coated with poly-L-lysine (0.1 mg/ml; Sigma) at a density of 5 × 106 cells per flask. Media were replaced the next day with DMEM/F12 containing 20% FCS. Cells were grown for 7 d without changing the medium to allow microglial proliferation. Microglia were harvested by shaking for 30 min on a rotary shaker at 120 rpm. Purity of cultures was determined by staining with the microglial marker Griffonia simplicifolia I lectin B4 (Sigma). Astrocytes were recovered after removal of microglia and passed three times, generating highly enriched cultures of astrocytes.

Cell stimulation. Tissue culture dishes were coated with nitrocellulose (Lagenaur and Lemmon, 1987) and derivatized by adding 48 pmol/mm2 Aβ peptides in distilled water and allowed to dry. Microglia (5 × 105 cells) and astrocytes (1 × 105 cells) were seeded at 1 × 104 cells (1 × 105 cells) were resuspended in 1 ml of HBSS and added to undervirginized dishes or dishes derivatized with immobilized peptide for 5 min. In some cases the cells were resuspended in additions of stimulants in solution. High-affinity (FcγRI) and low-affinity (FcγRII) immunoglobulin receptors were cross-linked by incubation of 1 × 105 THP1 monocytes with 2 μg of anti-FcγRI or anti-FcγRII mAb (Becton Dickinson) for 30 min. Cells were pelleted and resuspended in 1 ml Jurkat buffer (1 M) in the absence or presence of 10 μg of goat anti-mouse F(ab)2 for 5 min. Then cells were lysed in 0.5 ml of ice-cold Triton buffer [1% Triton X-100 and (in mm): 10 Tris, pH 7.4, 140 NaCl, 1 Na3VO4, 10 NaF, 1 EDTA, 1 EGTA, and 2 PMSF]. Insoluble material was removed by centrifugation at 10,000 g at 4°C for 10 min. Laemmli sample buffer was added to RIPA lysates, which were resolved by SDS/PAGE under reducing conditions. The proteins were transferred to PolyScreen membranes (DuPont NEN, Boston, MA) and then blocked in TBS-T (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.05% Tween 20) containing 3% BSA overnight at 4°C. The blots were incubated with the appropriate primary antibody for 1 h, washed three times in TBS-T and incubated for 1 h with goat-anti-rabbit or goat-anti-mouse antibodies conjugated to HRP in TBS-T plus 5% nonfat dried milk, and washed three times in TBS-T, followed by detection with an enhanced chemiluminescence (ECL) detection system (DuPont NEN). In some instances blots were stripped by incubation for 30 min at 50°C in stripping buffer (62.5 mM Tris, pH 6.8, 100 mM β-mercaptoethanol, and 2% SDS) and reprobed with other antibodies.

Tyrosine kinase assays. Tyrosine kinase assays were stimulated with Aβ25–35, and tyrosine-phosphorylated proteins were immunoprecipitated with PY20 as described above. Tyrosine kinase activity then was assayed by phosphorylation of polyGluTyr (PGT; 4:1 Glu, Tyr; Sigma) (Guan and Shalloway, 1992). PGT assays were performed by adding 10 μg (0.1 μg) aliquots of phosphotyrosine-containing proteins eluted with 100 μl of TBS-5% FCS containing 3% BSA and 0.25 μg of PGT kinase (50 mM Tris, pH 7.4, 10 mM MnCl2, 10 μM ATP, and 30 cpm/ml [γ-32P]ATP) and incubated at 25°C for 30 min. Reactions were terminated by adding Laemmli sample buffer and were boiled for 5 min. Samples were resolved by SDS/PAGE under reducing conditions, and radioactivity was quantitated by Cerenkov counting.

In vitro kinase assays were performed with THP1 monocytes, which were grown for 2 d in media alone or supplemented with 50 ng/ml lipopolysaccharide (LPS; Sigma), and then stimulated as described above and lysed in Triton buffer (Ghazalizadeh et al., 1994). Phosphotyrosine-containing proteins were immunoprecipitated, and the immunoprecipitates were washed three times in Triton buffer and then once in HEPES buffer containing (in mm) 25 HEPES, pH 7.4, 150 NaCl, and 1 Na3VO4 followed by incubation in kinase buffer (25 mM HEPES, pH 7.4, 10 mM MgCl2, 10 mM ATP, and 30 cpm/ml [γ-32P]ATP) and incubated at 25°C for 60 min. Reactions were terminated by adding Laemmli buffer and were boiled for 5 min. Incorporated radioactivity was quantitated by Cerenkov counting of the excised gel lane.

The enzymatic activity of Lyn was analyzed from unstimulated THP1 monocytes or THP1 monocytes stimulated on 60 pmol/mm2 surface-bound Aβ25–35 (Ghazalizadeh et al., 1994). [γ-32P]Labeled anti-phosphotyrosine immunocomplexes were obtained as described for in vitro kinase assays. Reactions were terminated, and immune complexes were dissociated by adding 70 μl of Triton buffer containing 3% SDS and were boiled for 3 min. Eluted proteins were diluted 10-fold with Triton buffer and immunoprecipitated with 1 μg of anti-Lyn antisera and 10 μl of protein A-agarose for 3 h. Proteins were resolved by SDS/PAGE under reducing conditions, and [32P]labeled proteins were detected by autoradiography.

Measurement of superoxide production. O2− production was measured by the reduction of ferricynochrome C, as previously described (Pick, 1986). Microglia (2 × 105 cells) were added to 48-well tissue culture dishes and allowed to adhere overnight, whereas THP1 monocytes (5 × 105 cells) were added to dishes in 0.5 ml HBSS containing 80 μM ferricynochrome C type III immediately before use. Media were removed from microglia, and stimulants were added in 0.5 ml of HBSS containing 80 μM ferricynochrome C type III in duplicate and incubated for 90 min. The media was heated to 4°C before measurement of the THP1 monocytes in duplicate and incubated for 90 min. The specificity of the reaction was verified by adding 40 μg of superoxide dismutase (Sigma) to one set of samples. In some instances THP1 monocytes and micro-
glia were pretreated for 1 hr with 25 μg/ml picatannol, followed by stimulation of the cells with peptides in the presence of picatannol. The medium was collected, and production of superoxide was determined spectrophotometrically by measurement of the reduction of ferricytochrome C at 550 nm and converted to moles of O$_2\,$ using an extinction coefficient of $21 \times 10^3 \text{ M}^{-1}\text{ cm}^{-1}$. Production of O$_2\,$ is expressed as nanomole O$_2\,$ per 90 min per 2 \times 10^6$ cells. Intracellular production of O$_2\,$ was assayed by the reduction of NBT, as previously described (Pick, 1986). THP1 monocytes $(1 \times 10^6)$ were suspended in 0.5 ml of HBSS containing 1 mg/ml NBT. Stimulants were added to cells, followed by incubation at 37°C for 10 min. Cells were pelleted rapidly, supernatants were removed, and cells were lysed by sonication in RIPA buffer to release reduced NBT precipitates. Reduction of NBT was measured by the change in absorbance at 550 nm. Cells treated with vehicle only served as blanks. Assays were performed in duplicate.

RESULTS

**Aβ stimulates tyrosine phosphorylation in microglia and THP1 monocytes**

We tested whether Aβ could initiate intracellular signaling events via the stimulation of the activity of tyrosine kinases by exposing primary cultures of rat microglia and astrocytes, as well as human THP1 monocytes and Jurkat cells, to Aβ peptides that had been immobilized on the surface of tissue culture dishes. The interaction of microglia and THP1 monocytes with Aβ1–40 or a peptide derived from the C terminus of Aβ, Aβ25–35, elicited a rapid and dramatic increase in tyrosine phosphorylation of a similar population of proteins (Fig. 1A, B). Astrocytes and Jurkat cells were unresponsive, demonstrating that Aβ-stimulated protein tyrosine phosphorylation was specific to cells within the microglial lineage (Fig. 1A).

Aβ contained within senile plaques primarily consists of Aβ1–42 and Aβ1–40 in a fibrillar β-pleated sheet conformation (Masters et al., 1985). THP1 monocytes were exposed to fibrillar Aβ1–42 and Aβ1–40, which had been immobilized on tissue culture dishes. Aβ induced markedly elevated levels of protein tyrosine phosphorylation (Fig. 1B). Then the biologically active domain within Aβ was defined with peptides derived from the N terminus (Aβ1–28) and C terminus (Aβ25–35) of Aβ. The active domain of Aβ was found to be restricted to amino acids 25–35, a region capable of forming β-pleated sheets (Terzi et al., 1994). Control peptides included scrambled Aβ25–35 (SC25–35), substance P (SP), fibronectin, and serum amyloid A (SAA). These peptides elicited no alteration in protein tyrosine phosphorylation.

The dependence of the conformation of Aβ on its ability to induce tyrosine phosphorylation in THP1 monocytes was examined. A fibrillar conformation of Aβ25–35 was far more effective in stimulating tyrosine phosphorylation than nonfibrillar Aβ25–35 (Fig. 1C). Similarly, the activation of tyrosine kinase activity was dependent on exposure of the cells to fibrillar Aβ1–40 (Fig. 1D). Exposure of the cells to nonfibrillar Aβ1–40 had no effect on the enzymatic activity of tyrosine kinases. These observations are consistent with previous studies, which have shown that a fibrillar confirmation of Aβ is critical for biological responses such as neurototoxicity (Pike et al., 1990) and stimulation of IL-1β release from THP1 monocytes (Lorton et al., 1996).

**Time course and dose–response of Aβ-stimulated tyrosine phosphorylation**

Exposure of THP1 monocytes to surface-bound Aβ rapidly stimulated protein tyrosine phosphorylation, reaching maximal levels in 5 min and returning to basal levels by 30 min, as measured by Western blot analysis with anti-phosphotyrosine antibodies. Aβ-induced tyrosine kinase activity was measured in parallel assays by an enzymatic assay of the tyrosine kinases by phosphorylation of the substrate PGT. Aβ stimulated rapid activation of tyrosine kinase activity with similar kinetics, reaching peak levels in 5 min (Fig. 2A). We did not consistently observe significant increases in tyrosine kinase activity from 30 to 60 min.

The dose–response of Aβ-stimulated tyrosine phosphorylation was examined. The response of THP1 monocytes to surface-bound fibrillar Aβ or fibrillar Aβ6 in solution was compared. Fibrillar Aβ25–35 added in solution to THP1 monocytes stimulated maximal tyrosine phosphorylation between 80–100 μM Aβ25–35, and surface-bound Aβ25–35 stimulated maximal levels of tyrosine phosphorylation in THP1 monocytes at a peptide density between 80–100 pmol/mm$^2$ (Fig. 2B). However, fibrillar Aβ added in solution consistently stimulated greater increases in tyrosine phosphorylation at lower quantities of peptide than surface-bound Aβ. This observation may suggest that Aβ in solution is more potent than surface-bound Aβ or that similar quantities of Aβ may be more accessible to cells in solution than bound to a surface. We did not consistently detect any further stimulation of tyrosine phosphorylation at higher levels of peptides (data not shown). The maximal response was elicited by similar quantities of Aβ25–35 presented to THP1 monocytes in suspension (1 ml, 80 μM = 80 nmol) or surface-bound (80 pmol/mm$^2$ = 77 nmol). Repeated examination of Aβ-stimulated tyrosine phosphorylation did not reveal significant qualitative or quantitative differences in the populations of tyrosine-phosphorylated proteins observed in THP1 monocytes when stimulated with maximal quantities of Aβ in solution or bound to a surface.

**Aβ and activation of immunoglobulin G receptors elicits tyrosine phosphorylation of a common population of proteins**

Monocytes respond to a variety of immune stimuli by activation of tyrosine kinases. A direct comparison of the response of THP1 monocytes to Aβ and to activation of the high- and low-affinity immunoglobulin receptors (FcγRI and FcγRII) revealed that these stimuli induced phosphorylation of a similar population of proteins, suggesting that Aβ activates common elements within an inflammatory response pathway (Fig. 3). Therefore, we initiated studies to establish the identities of several of the tyrosine kinases activated on exposure of microglia and THP1 monocytes to Aβ.

**Identification of Aβ-stimulated tyrosine-phosphorylated proteins**

Activation of monocytes by immune stimuli, such as FcγRI and FcγRII cross-linking, results in the activation of Src family tyrosine kinases such as Lyn (Kiener et al., 1993; Ghazizadeh et al., 1994). To determine whether Aβ activated Lyn, we exposed microglia and THP1 monocytes to Aβ25–35. Src family tyrosine kinases have been shown to associate with the Triton-insoluble cytoskeleton (Clark and Brugge, 1993), so Lyn was extracted from the cytoskeleton by incubation in RIPA buffer. RIPA lysates were analyzed by Western blot with anti-phosphotyrosine mAb, followed by stripping the blot and reprobing with anti-Lyn antisera. In parallel assays, the effect of Aβ25–35 on the enzymatic activity of Lyn was examined from Triton lysates of THP1 monocytes that were sonicated briefly to disrupt cytoskeleton. Lysates were immunoprecipitated with anti-phosphotyrosine mAb, and immunoprecipitates were incubated with [$^{32}$P]ATP. $^{32}$P-labeled proteins
were eluted from immune complexes by boiling 3 min in Triton buffer containing 3% SDS and were diluted 10-fold in Triton buffer. The proteins were reimmunoprecipitated with anti-Lyn antisera, and immunoprecipitates were resolved by SDS-PAGE and analyzed by autoradiography. Exposure of microglia and THP1 monocytes to Aβ peptides leads to increased protein tyrosine phosphorylation. Western blot analysis of tyrosine phosphoproteins was performed on phosphotyrosine immunoprecipitates from primary rat microglia and astrocytes, human THP1 monocytes, and Jurkat T-lymphocytes. The cells were exposed for 5 min to Aβ peptides (48 pmol/mm²) immobilized on the surface of tissue culture dishes or to an underivatized surface. The broad band at 55 kDa is IgG heavy chain. B, Ligand specificity of Aβ-stimulated tyrosine phosphorylation in THP1 monocytes. Tissue culture dishes were underivatized (c) or derivatized with 48 pmol/mm² Aβ1–42, Aβ1–40, Aβ1–28, Aβ25–35, scrambled Aβ25–35 (SC25–35), NAMGKILSGIG, substance P (SP), serum amyloid A (SAA), or 20 µg/ml fibronectin (Fn). THP1 monocytes were exposed to the immobilized substrates or an underivatized surface for 5 min, followed by Western blot analysis with anti-phosphotyrosine antibodies. The broad band at 55 kDa is IgG heavy chain. C, Stimulation of proteintyrosinephosphorylation in THP1 monocytes by fibrillar Aβ25–35, but not nonfibrillar Aβ25–35. THP1 monocytes were incubated for 5 min in the absence (−) or presence (+) of 20 µM fibrillar (F) Aβ25–35 or nonfibrillar (NF) Aβ25–35 in suspension. Western blot analysis of tyrosine phosphoproteins was performed on phosphotyrosine immunoprecipitates. The broad band at 55 kDa is IgG heavy chain. D, Fibrillar Aβ1–40 stimulates tyrosine kinase activity in THP1 monocytes. Tyrosine kinase activity in THP1 monocytes was measured after a 5 min exposure of the cells to fibrillar Aβ1–40 or nonfibrillar Aβ1–40 (Aβ1–40NF) immobilized on a dish. Tyrosine kinase activity was measured from phosphotyrosine immunoprecipitates, with polyGluTyr as a substrate. Proteins were resolved by SDS-PAGE, the gel was dried, and the lanes were cut and subjected to Cerenkov counting. The positions of the molecular weight standards (kDa) are indicated.

Figure 1. Fibrillar Aβ peptides stimulate increased tyrosine phosphorylation and tyrosine kinase activity in microglia and THP1 monocytes. A, Exposure of microglia and THP1 monocytes, but not astrocytes and Jurkat cells, to Aβ peptides leads to increased protein tyrosine phosphorylation. Western blot analysis of tyrosine phosphoproteins was performed on phosphotyrosine immunoprecipitates from primary rat microglia and astrocytes, human THP1 monocytes, and Jurkat T-lymphocytes. The cells were exposed for 5 min to Aβ peptides (48 pmol/mm²) immobilized on the surface of tissue culture dishes or to an underivatized surface. The broad band at 55 kDa is IgG heavy chain. B, Ligand specificity of Aβ-stimulated tyrosine phosphorylation in THP1 monocytes. Tissue culture dishes were underivatized (c) or derivatized with 48 pmol/mm² Aβ1–42, Aβ1–40, Aβ1–28, Aβ25–35, scrambled Aβ25–35 (SC25–35), NAMGKILSGIG, substance P (SP), serum amyloid A (SAA), or 20 µg/ml fibronectin (Fn). THP1 monocytes were exposed to the immobilized substrates or an underivatized surface for 5 min, followed by Western blot analysis with anti-phosphotyrosine antibodies. The broad band at 55 kDa is IgG heavy chain. C, Stimulation of proteintyrosinephosphorylation in THP1 monocytes by fibrillar Aβ25–35, but not nonfibrillar Aβ25–35. THP1 monocytes were incubated for 5 min in the absence (−) or presence (+) of 20 µM fibrillar (F) Aβ25–35 or nonfibrillar (NF) Aβ25–35 in suspension. Western blot analysis of tyrosine phosphoproteins was performed on phosphotyrosine immunoprecipitates. The broad band at 55 kDa is IgG heavy chain. D, Fibrillar Aβ1–40 stimulates tyrosine kinase activity in THP1 monocytes. Tyrosine kinase activity in THP1 monocytes was measured after a 5 min exposure of the cells to fibrillar Aβ1–40 or nonfibrillar Aβ1–40 (Aβ1–40NF) immobilized on a dish. Tyrosine kinase activity was measured from phosphotyrosine immunoprecipitates, with polyGluTyr as a substrate. Proteins were resolved by SDS-PAGE, the gel was dried, and the lanes were cut and subjected to Cerenkov counting. The positions of the molecular weight standards (kDa) are indicated.

The cytosolic tyrosine kinase Syk is an important signaling component that is tyrosine-phosphorylated and enzymatically ac-

were eluted from immune complexes by boiling 3 min in Triton buffer containing 3% SDS and were diluted 10-fold in Triton buffer. The proteins were reimmunoprecipitated with anti-Lyn antisera, and immunoprecipitates were resolved by SDS-PAGE and analyzed by autoradiography. Exposure of microglia and THP1 monocytes to Aβ25–35 stimulated both the tyrosine phosphorylation and enzymatic activity of Lyn (Fig. 4A,B).

The cytosolic tyrosine kinase Syk is an important signaling component that is tyrosine-phosphorylated and enzymatically ac-
proximal catalytic elements comprising a signal transduction pathway mediating the activation of these cells.

Microglia are the predominant glial cell type found within the senile plaques (Itagaki et al., 1989), suggesting that microglia are likely to migrate to and interact with these structures. The cytosolic tyrosine kinase, FAK, has been shown to be important in cellular adhesion, migration, and inflammatory responses (Furuta et al., 1995; Hamawy et al., 1995; Ilic et al., 1995). Examination of phosphotyrosine-labeled proteins from Aβ-stimulated microglia and THP1 monocytes revealed a prominent 125 kDa phosphoprotein, the tyrosine phosphorylation of which was stimulated markedly by exposure to Aβ bound to surfaces or added in solution (Fig. 2B). This protein was identified as p125FAK by immunoprecipitation with anti-FAK antisera, followed by anti-phosphotyrosine blot (Fig. 4A,B).

One of the primary targets of FAK and the src family tyrosine kinases such as Lyn is the cytoskeletal-associated protein, paxillin (Minoguchi et al., 1994; Bellis et al., 1995). Paxillin has been shown to be involved in linking membrane proteins and signaling molecules to the actin cytoskeleton and colocalizes at focal adhesions and phagolysosomes in macrophages (Greenburg et al., 1994). Exposure of microglia and THP1 monocytes to Aβ25–35 resulted in a dramatic stimulation of the tyrosine phosphorylation of paxillin in both cell types (Fig. 4A,B).

**Stimulation of respiratory burst in microglia and THP1 monocytes by Aβ**

To establish whether Aβ is capable of directly activating microglia and stimulating the release of potentially harmful inflammatory products, we studied the effects of Aβ on the production of superoxide radicals in microglia and THP1 monocytes. Adding Aβ peptides to microglia and THP1 monocytes resulted in the production of O2−, which was blocked by the presence of superoxide dismutase (Fig. 5A). The stimulation of O2− release was
dependent on addition of fibrillar Aβ1–40 and Aβ25–35. Adding nonfibrillar Aβ25–35 to microglia stimulated 50% less O2 production than fibrillar Aβ25–35, and this modest response is most likely a consequence of aggregation of Aβ25–35 during the 90 min time course of the assay (Fig. 5B; Terzi et al., 1994). Importantly, pretreatment of microglia and THP1 monocytes with piceatannol, the Syk-selective tyrosine kinase inhibitor, blocked the production of superoxide, demonstrating that Aβ-stimulated superoxide production is linked to the activation of Syk or kinases downstream of Syk (Fig. 5C).
Effect of scavenger receptor and advanced glycation end product receptor ligands on tyrosine phosphorylation and respiratory burst

Recently, Aβ was shown to interact at the cell surface with class A scavenger receptors and the receptor for advanced glycation end products (RAGE; El Khoury et al., 1996; Yan et al., 1996), both of which have been linked to the production of reactive oxygen species. RAGE is postulated to immobilize Aβ at the cell surface, where Aβ then generates reactive oxygen species extracellularly (Hensley et al., 1994). The scavenger receptor, which binds both Aβ and advanced glycation end products, has been shown to mediate adhesion of microglia to Aβ, resulting in the generation of reactive oxygen species. The intracellular signal transduction pathways activated by scavenger receptors are not well described, and RAGE has not been shown to be linked to signaling pathways or to elicit cellular effects. We tested whether binding of Aβ to

Figure 5. Fibrillar Aβ peptide stimulates respiratory burst in microglia and THP1 monocytes. A, Rat microglia were incubated in the absence (c) or presence of 60 μM fibrillar Aβ1–40, Aβ25–35, or scrambled Aβ25–35 (SC25–35). The peptides were added alone or in the presence of 40 μg of superoxide dismutase (SOD), and O2− production was measured. THP1 monocytes were incubated in absence (c) or presence of 60 μM Aβ25–35. B, Fibrillar, but not nonfibrillar, Aβ25–35 stimulated release of O2− from rat microglia. Microglia were incubated with 40 μM fibrillar Aβ25–35 or 40 μM nonfibrillar Aβ25–35 (Aβ25–35NF), 1 μg/ml phorbol myristate acetate (PMA), or vehicle only (c) in duplicate wells for 90 min. C, Aβ25–35-stimulated release of O2− from THP1 monocytes and microglia is blocked by piceatannol. Microglia and THP1 monocytes were pretreated for 1 hr with 25 μg/ml piceatannol (P). Cells were unstimulated (con) or stimulated for 90 min with 60 μM Aβ25–35 ± 25 μg/ml piceatannol. Supernatants were collected, and production of superoxide was determined spectrophotometrically by measurement of the reduction of ferricytochrome C at 550 nm and converted to moles of O2−, using an extinction coefficient of 21 × 10^3 M^−1 cm^−1. Production of O2− is expressed as nanomole O2− per 90 min per 2 × 10^5 cells.
these receptors was likely to be responsible for activation of tyrosine kinases and subsequent generation of reactive oxygen species. THP1 monocytes were exposed to the scavenger receptor ligands, maleylated-BSA and acetylated-LDL, and the RAGE ligand, glycated BSA in combination with iron-saturated lactoferrin. These ligands failed to elicit de novo increases in protein tyrosine phosphorylation. Conversely, Aβ stimulated dramatic increases in protein tyrosine phosphorylation of multiple proteins (Fig. 6A). Because Aβ and glycated proteins have been postulated to generate oxygen radicals (Sakurai and Tsuchiya, 1988; Hensley et al., 1994) spontaneously, the intracellular reduction of NBT was used to assay cell-dependent generation of superoxide (Pick, 1986). Aβ was a potent stimulus of generation of intracellular superoxide radicals. We were unable to detect scavenger receptor ligand and RAGE ligand-stimulated intracellular superoxide production (Fig. 6B). We were, however, able to measure a modest level of scavenger receptor-mediated superoxide production from phorbol ester-differentiated THP1 monocytes (data not shown). These data indicate that Aβ is likely to activate different signaling pathways in monocytic cells than classical scavenger receptor and RAGE ligands. Also, we have been unable to implicate FcγRI, FcγRII, and tachykinin receptors directly in this response (data not shown). The identity of the microglial receptor(s) subserving these rapid effects of fibrillar Aβ remains unknown.

**DISCUSSION**

We report that microglia are able selectively to detect fibrillar forms of Aβ, resulting in the activation of intracellular signaling cascades. These observations provide an important mechanistic link in understanding how these cells acquire an activated phenotype in the AD brain. It is of particular interest that the signaling pathways activated in response to Aβ also are used by these cells (and other cells of this lineage) to respond to conventional immune stimuli and result in common biological effects such as cytokine secretion (Debetz et al., 1988) and superoxide production (Pfefferkorn and Fanger, 1989). Macrophages and microglia express a complex set of cell-surface proteins that mediate inflammatory responses to a wide variety of immune stimuli. Scavenger receptors or RAGE does not seem to mediate the rapid effects of Aβ on the activation of tyrosine kinases and downstream signaling events leading to the generation of superoxide. These data provide evidence that Aβ may interact with other membrane proteins linked to intracellular signal transduction pathways. These findings also suggest that scavenger receptor and RAGE occupancy elicit the production of reactive oxygen species through mechanistically distinct pathways that have not been defined.

Activation of tyrosine kinases of the src family, such as Lyn and Lck, is an initial step in signal transduction cascades of several...
immune responses in monocytes, macrophages, and lymphocytes. Stimulation of microglia and THP1 monocytes with Aβ also has led to activation of Lyn. Lyn is associated constitutively with the cytoplasmic domain of the FcγRII (Ghazizadeh et al., 1994) receptor as well as the gamma subunit of FcγRI and FcεRI (Wang et al., 1994) and becomes activated on clustering of the receptors by immune complexes. Lyn then phosphorylates these receptors on two tyrosine residues within the tyrosine activation motif (TAM), allowing the interaction and activation of downstream effector molecules such as members of Syk/ZAP70 tyrosine kinase family (Burkhardt et al., 1994; Shiue et al., 1995).

The tyrosine kinases of the Syk/ZAP70 family are also critical components for several immune responses in monocytes, macrophages, and lymphocytes and have been shown to be essential to B-cell maturation (Cheng et al., 1995; Turner et al., 1995; Crowley et al., 1996). Syk is activated by binding to phosphoryrosine residues within TAMs present on the cytoplasmic domain of the receptor or receptor subunits (Shiue et al., 1995). It is also possible that Syk may be activated by a direct interaction with Lyn, because Syk has been shown to be coimmunoprecipitated with Lyn (Sidorenko et al., 1995).

Syk has been shown to be essential for phagocytosis of immune complexes (Indik et al., 1995). Exposure of microglia and THP1 monocytes to Aβ led to tyrosine phosphorylation of p72Syk. It is not known, however, whether the Aβ receptor as well as the gamma subunit of FcγRI within this signaling pathway represents molecular targets for inhibition of the activity of Syk or other elements such as members of Syk/ZAP70 tyrosine kinase family (Burkhardt et al., 1994; Shiue et al., 1995).

The tyrosine kinase inhibitor, piceatannol, has been shown to inhibit Syk (Oliver et al., 1994) selectively. The specific action of this drug has established the requirement for Syk in the IgE-stimulated release of serotonin from RBL-2H3 rat mast cells. We have observed that one of the biological consequences of microglial interactions with Aβ is the activation of an inflammatory response, evidenced by the generation of superoxide radicals. Superoxide radicals are produced via the action of NADPH oxidase, a multisubunit complex, the assembly of which is stimulated by extracellular stimuli. Significantly, protein tyrosine phosphorylation has been shown to be critical for the activation of NADPH oxidase, presumably via activation of downstream signaling cascades, including the MAP kinases (Dusi et al., 1994). Phosphorylation of p47phox is essential for the assembly of the NADPH oxidase complex (Curnutte et al., 1994). The Aβ-stimulated production of superoxide radicals was arrested by pretreatment of microglia and THP1 monocytes with piceatannol. Therefore, inhibition of the activity of Syk or other elements within this signaling pathway represents molecular targets for blockade of microglial inflammatory responses to Aβ.

FAK has been shown to be involved in regulating integrin-mediated cellular adhesion and migration. Activation of FAK is known to occur at focal adhesions and mediates the assembly of large protein complexes linking the cytoskeleton to membrane signal transduction machinery in numerous cell types (Clark and Brugge, 1995), including monocytes (Lin et al., 1995). Microglia are the predominant cell type found within the core of senile plaques, suggesting that microglia must migrate to and interact with components of the plaques. We have demonstrated that the interaction of microglia and THP1 monocytes with either surface-bound fibrillar Aβ or fibrillar Aβ in solution stimulated the tyrosine phosphorylation of FAK. Thus, it is likely that activation of FAK is involved in microglial adhesion to and migration on Aβ within senile plaques. In addition, Aβ-stimulated tyrosine phosphorylation of FAK may affect its interactions with other components of the Aβ-stimulated signal transduction pathway by serving as a site for the formation of signaling complexes (Clark and Brugge, 1995).

A consequence of microglial Aβ interactions is the activation of a complex signal transduction cascade resulting in the production of superoxide, which was found to be dependent on interaction of the cells with fibrillar conformations of Aβ. This finding is significant, because AD pathology is associated with mature senile plaques possessing Aβ in a fibrillar or β-pleated sheet conformation. In addition, other investigators have found that the biological effects of Aβ, such as neurotoxicity (Pike et al., 1990) and stimulation of IL-1β release from THP1 monocytes (Lorton et al., 1996), are dependent on a fibrillar conformation of the peptide. It is unlikely that the responses to fibrillar Aβ described here are mediated by RAGE, because it was identified on the basis of its ability to bind Aβ monomers.

Importantly, previous activation of microglia and THP1 monocytes was not necessary for Aβ to induce release of superoxide. The ability of Aβ to elicit the production of reactive oxygen species is consistent with a previous report showing that Aβ stimulated the release of NO2- from primary cultures of rodent microglia (Meda et al., 1995). However, the latter response was entirely dependent on priming microglia with INFγ. In addition, the role of NO2- in human macrophage responses to inflammatory stimuli remains controversial (Albina, 1995), and recent studies have failed to detect iNOS mRNA in cultures of activated human microglia (Walker et al., 1995). The present data demonstrate that Aβ alone is sufficient to activate complex intracellular signaling processes in microglia, resulting in the release of toxic inflammatory products, and support a role for oxidative damage in the pathogenesis of AD.

Recently, RAGE and scavenger receptors have been shown to interact with Aβ at the cell surface, where the peptide may elicit potentially harmful responses in microglia and neurons via generation of reactive oxygen species (El Khoury et al., 1996; Yan et al., 1996). However, occupancy of these receptors with saturating quantities of their specific ligands does not stimulate changes in protein tyrosine phosphorylation or intracellular O2- production, in contrast to the effect of Aβ, RAGE is believed to act as a tether that binds Aβ to cell surfaces, where reactive oxygen species are generated extracellularly by an undefined mechanism. In contrast, the data presented here demonstrate that fibrillar Aβ activates signal transduction pathways in microglia, leading to intracellular generation of superoxide as well as its release in the extracellular space (Figs. 5, 6). Although Aβ has been shown to interact with scavenger receptors and RAGE, the present data demonstrate that these receptors do not mediate the activation of tyrosine kinases and generation of superoxide detected here. Moreover, these data provide evidence for the existence of other Aβ-interactive species that are linked to the signal transduction pathways in these cells. However, it remains possible that fibrillar Aβ may bind scavenger receptors or RAGE but activate intracellular processes distinct from natural ligands, including acetylated LDL and glycated BSA, respectively.

These data provide support for the view that the pathogenesis of AD comprises a series of events initially characterized by the production of Aβ, aggregation of Aβ into fibrils, and deposition of Aβ fibrils as extracellular plaques within the brain. Microglia...
initially interact with Aβ fibrils and initiate a rapid, complex cellular response, resulting in the elaboration of potentially toxic products, including reactive oxygen intermediates. The elaboration of cytokines and other mediators of inflammation, such as complement components, proteases, and protease inhibitors, then may generate a feed-forward inflammatory process. The progressive neuropathological changes in the AD brain and the accompanying deterioration in cognitive ability are likely to be, in part, a consequence of ongoing local inflammatory responses in the brain mediated by microglia. Inflammation, generation of reactive oxygen intermediates, and cytokine release are recurring mechanisms in the pathophysiology of many neurodegenerative diseases (Halliwell, 1992; Brown et al., 1996; Lorton et al., 1996). Indeed, the hypothesis that AD pathogenesis involves an ongoing inflammatory response is supported by recent epidemiological analysis demonstrating that long-term anti-inflammatory drug treatment is correlated with a lower incidence of dementia (McGeer and McGeer, 1996).

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