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β-Amyloid Stimulation of Inducible Nitric-oxide Synthase in Astrocytes Is Interleukin-1β- and Tumor Necrosis Factor-α (TNFα)-dependent, and Involves a TNFα Receptor-associated Factor- and NFκB-inducing Kinase-dependent Signaling Mechanism*

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In Alzheimer’s disease, β-amyloid (Aβ) plaques are surrounded by activated astrocytes and microglia. A growing body of evidence suggests that these activated glia contribute to neurotoxicity through the induction of inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor-α (TNFα) and the production of neurotoxic free radicals, mediated in part by the expression of inducible nitric-oxide synthase (iNOS). Here, we address the possibility that Aβ-stimulated iNOS expression might result from an initial induction of IL-1β and TNFα. We find that in Aβ-stimulated astrocyte cultures, IL-1β and TNFα production occur before iNOS production, new protein synthesis is required for increased iNOS mRNA levels, and the IL-1 receptor antagonist IL-1ra can inhibit nitrite accumulation. Likewise, dominant-negative mutants of tumor necrosis factor-α receptor-associated factor (TRAF) 6, TRAF2, and NFκB-inducing kinase (NIK), intracellular proteins involved in IL-1 and TNFα receptor signaling cascades, inhibit Aβ-stimulated iNOS promoter activity. Our data suggest that Aβ stimulation of astrocyte iNOS is mediated in part by IL-1β and TNFα, and involves a TRAF6-, TRAF2-, and NIK-dependent signaling mechanism.

Astrocytes and microglia are glial cells that play a major role in the inflammation observed in Alzheimer’s disease (AD) as well as many other neurodegenerative diseases (1–5). Upon stimulation from various agents or insults such as cytokines or trauma, these cells adopt a reactive phenotype, a morphological hallmark in AD pathology, during which they themselves may produce still more inflammatory cytokines and neurotoxic free radicals. One potentially detrimental free radical is the highly reactive nitrogen species peroxynitrite, which is a derivative of nitric-oxide (NO) production (6). In the brain, astrocytes are able to produce NO during inflammatory conditions by the enzyme inducible nitric-oxide synthase (iNOS or NOS2) (7, 8).

Increased levels of iNOS production in astrocytes surrounding amyloid plaques (9), and significant peroxynitrite damage to neurons have been observed in AD brain (10). Peroxynitrite can also induce alterations in synaptosomal membranes of neurons, and is thus a potent source for oxidative stress in AD brain (11). Several lines of similar in vitro evidence also show that β-amyloid, the primary plaque component in AD brain, can stimulate NO production from astrocytes (12–15), and that activated astrocytes can produce NO levels that are detrimental to neurons (12, 16).

While the 42-amino acid β-amyloid peptide (Aβ42) is capable of activating astrocytes in vitro and in vivo, resulting in the activation of NFκB and the induction of iNOS (17), the molecular pathway(s) and participants governing astrocyte iNOS induction by Aβ42 have not yet been described. In addition to activation of astrocytes, Aβ42 can also activate microglia, leading to the production of interleukin (IL)-1β (15, 18) and tumor necrosis factor (TNF) α (12, 19, 20). Because IL-1β and TNFα can themselves stimulate iNOS production in astrocytes (21, 22), Aβ42 stimulation of iNOS in astrocytes could be a direct effect on the astrocyte or an indirect result of Aβ42 stimulation of IL-1β and TNFα induction in microglia.

IL-1β is a critical inflammatory cytokine in AD. Microglia localized to amyloid plaques stain positively for IL-1, and increased numbers of IL-1β-expressing microglia are associated with AD progression (23). IL-1β is also capable of stimulating astrocytes to produce additional pro-inflammatory cytokines such as IL-6, another inflammation marker associated with neurodegeneration (24–26).

A signal transduction pathway activated by IL-1β that ultimately leads to NFκB activation has been described (27–29) and involves the TNFα receptor-associated factor-6 (TRAF6). Similarly, TNFα activates NFκB via TRAF2 (28, 30). Both TRAF2 and TRAF6 participation can then lead to the activation of NFκB-inducing kinase (NIK), the common mediator of IL-1β and TNFα activation of NFκB (31), and NIK can complex with and activate the IkB kinase signalsome complex (α-β-γ) (32). Activated IkB kinase signalsome complex phosphorylates IkB, the inhibitor of the transcription factor NFκB, directing the inhibitor for proteasome-mediated degradation and allowing NFκB to translocate to the nucleus, where it binds to specific promoter response element sequences and stimulates
gene transcription (for reviews, see Refs. 33 and 34). Activated NFκB has been observed in AD brain (35, 36), and we have shown previously that NFκB activation is necessary for Aβ42-stimulated iNOS induction in astrocytes (17).

The molecular events that coordinate the neurotoxic inflammatory response in neurodegenerative diseases such as AD are crucial to understand. To determine if IL-1β induction is required for the Aβ42 stimulation of iNOS in astrocytes, we used a series of approaches. We examined the temporal pattern of Aβ42 stimulation of IL-1β and iNOS production in mixed glial cultures, and whether new protein synthesis was required for iNOS induction. The participation of IL-1β signal transduction pathways in iNOS activity was blocked at the ligand/receptor interface with the IL-1β receptor antagonist (IL-1ra). Finally, we inhibited either IL-1β-mediated activation of NFκB by using the dominant-negative truncated form of TRAF6, or TNFα and IL-1β-mediated activation of NFκB by the kinase-inactive form of NIK. We report here that inhibition of IL-1β signal transduction (and, to a lesser extent, TNFα signal transduction) resulted in decreased Aβ42-stimulated nitrite production and iNOS promoter activity, demonstrating that Aβ42-stimulated iNOS induction is mediated at least in part by inflammatory cytokine production.

MATERIALS AND METHODS

Cell Culture—Rat primary cortical glial cultures were prepared and maintained as described previously (16). Briefly, cells were grown in αMEM supplemented with 10% fetal calf serum (HyClone) and 1% penicillin/streptomycin (Life Technologies, Inc.). At least 24 h prior to transfection, the cDNA for murine TRAF2 and murine TRAF6 cDNAs cloned into an SR vector and driven by the herpes simplex virus immediate early promoter from the herpes simplex virus, allowing for the signal transduction (and, to a lesser extent, signal transduction) resulted in decreased Aβ42-stimulated nitrite production and iNOS promoter activity, demonstrating that Aβ42-stimulated iNOS induction is mediated at least in part by inflammatory cytokine production.

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TRANSDUCTION AND REPORTER ASSAYS—Plasmid DNA was prepared by endotoxin-free Maxi-Preps (Qiagen) before transfection. Transfection of astrocyte cultures has been described previously (17). Briefly, for all transfections, the cationic lipid reagent Test-50 (Promega) at a 1:1 (charge:μg of DNA) ratio was used. For transfections using the iNOS-Luciferase (Promega), 104 cells/well were plated in a 24-well tissue-culture plate and transfected with a total of 1 μg of DNA/well (0.5 μg of expression construct and 0.5 μg of reporter construct, total amount of DNA kept constant as described above).

Luciferase assays were performed with the Luminite reagent (Packard) and read on a LumiCount luminometer (Packard) as described previously (17). Chemiluminescent SEAP assays were performed with the Phospha-Light assay reagents (Tropix) by the manufacturer’s protocol and read on the same luminometer. Relative light units for both assays were calculated as a percentage of appropriate controls. At least eight replicates were conducted per experiment, and statistical significance (p < 0.05) was determined by Student’s t test.

Western Blotting—Cell lysates for Western blotting were prepared in SDS-containing sample buffer and separated on 10% SDS-polyacrylamide gels. After transfer to nitrocellulose membranes (Im mobilon-P, Millipore), blots were probed with antibodies to IL-1β (polyclonal goat anti-IL-1β diluted 1/2000; R&D/Genzyme), the astrocyte-specific marker glial fibrillary acidic protein (GFAP, monoclonal anti-pig GFAP diluted 1/10, Sigma), and iNOS (monoclonal mouse monoclonal anti-mouse maciNOS diluted 1/2000, Transduction Laboratories). ECL detection of protein bands was performed using LumioGLO reagent (Nuclon Diagnostics).

Northern Blotting—Northern blots were performed as described previously (14). Briefly, total RNA was isolated from cells using RNeasy spin columns (Qiagen). Equal amounts of total RNA (800 ng/sample) were slot blot-loaded onto Durulon-UV membrane (Stratagene) and immediately UV cross-linked. Partial cDNA probes were specific for the iNOS promoter (RdG/Genzyme) or were prepared from the murine iNOS cDNA sequence by PCR (Promega). Each of the amplified products was subcloned into the expression vector pBluescript KS+ (Stratagene). Each construct, termed dnNIK, was verified by DNA sequence across the mutated site and by restriction analysis. Plasmids containing cloned partial cDNA fragments of rat IL-1β, rat iNOS, and rat GAPDH have been described previously (14).

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Axiophot2 microscope using Spot camera (Diagnostic Instruments) and used at 1/400 dilutions. Images were digitally captured from a Zeiss goat IgG and donkey anti-mouse IgG (Jackson ImmunoResearch) were dilution. Secondary fluorochrome-conjugated antibodies donkey anti-rat IL-1 was not detected until 6 h after A was secreted into the conditioned medium and detectable by ELISA. Immediately after the last time point was collected, cytokine levels in the conditioned media were measured by ELISA (rat IL-1β or rat TNFα assay kit, R&D Systems) and performed by the manufacturer’s protocol.

Immunofluorescence—Identification of cell types expressing either IL-1β or iNOS was done by immunofluorescence as described previously (41) using the same antibodies as for the Western blots. Polyclonal goat anti-rat IL-1β antibody and monoclonal mouse anti-macNOS were used at 1/200 dilution. Secondary fluorochrome-conjugated antibodies donkey anti-goat IgG and donkey anti-mouse IgG (Jackson ImmunoResearch) were used at 1/400 dilutions. Images were digitally captured from a Zeiss Axio photot2 microscope using Spot camera (Diagnostic Instruments) and Metamorph software.

RESULTS

Aβ42-stimulated Cytokine Production Occurs before iNOS Production in Vitro—To determine the temporal sequence of events during Aβ42 activation of glial cultures, a time-course Western blot profile was used to demonstrate that IL-1β protein production (proIL-1β form associated with cellular content prior to secretion) occurs before iNOS protein production (Fig. 1). Aβ42-stimulated IL-1β production was detected by 3 h, reached a peak by 6 h, and continued to be present through 36 h after stimulation. However, protein production of iNOS was not detected until 6 h after Aβ42 stimulation, and iNOS levels steadily increased through 36 h. In cells treated with diluent alone, there was a delayed, minor IL-1β production detected at 6–12 h, but no iNOS protein detected. GFAP levels did not increase at any time point examined, consistent with previous findings in Aβ42 stimulation of astrocytes (14). To determine if Aβ42 stimulates IL-1β (active form) secretion from these cells, an IL-1β ELISA was performed on conditioned medium (Fig. 2A). Similar to the Western blot time-course profile of Aβ42-stimulated proIL-1β production, active IL-1β was secreted into the conditioned medium and detectable by 3 h, and active IL-1β levels accumulated through 12 h.

Another cytokine rapidly induced in glia by Aβ42 is TNFα. Because TNFα is readily secreted from the cells, an ELISA was performed on cell conditioned media to measure the time course of TNFα production from Aβ42-stimulated cells. As shown in Fig. 2B, there was a rapid increase in cytokine production upon addition of Aβ42, with TNFα protein detected as early as 1 h after stimulation and high levels of TNFα sustained through 12 h. These time courses demonstrate that IL-1β and TNFα responses are early, rapid inflammatory responses to Aβ42 with protein production occurring within the first hr of exposure to Aβ42, and support the possibility that Aβ42-stimulated iNOS production may be a response to an initial IL-1β and/or TNFα production.

Protein Synthesis Inhibitor Blocks Aβ42-stimulated iNOS mRNA Levels—If cytokine production is necessary for Aβ42-stimulated iNOS expression, then we would expect that cells pre-treated with cycloheximide, a protein synthesis inhibitor, should fail to express iNOS when treated with Aβ42. Northern blot analysis (Fig. 3) of cells stimulated with 10 μM Aβ42 for 12 h in the presence of 1 μM cycloheximide demonstrates that IL-1β mRNA can still be detected strongly, but iNOS mRNA levels are reduced. The Northern blots were stripped and then re-probed with GAPDH for normalization (data not shown). Quantitation of several experiments by phosphorimaging densitometry showed that the Aβ42-stimulated iNOS mRNA levels were reduced in the presence of cycloheximide by approximately 50% compared with the mRNA levels seen in the Aβ42-stimulated cells in the absence of cycloheximide (Fig. 3). In cells treated with cycloheximide and stimulated with 1 mM Bt-cAMP, which can directly induce iNOS expression, there was no decrease in iNOS mRNA levels, but in fact a dramatic increase in iNOS mRNA levels (data not shown). These data indicate that the reduction of Aβ42-stimulated iNOS mRNA in the presence of cycloheximide is not due to cell toxicity, and suggest that new protein synthesis is important for iNOS induction.

IL-1β Receptor Antagonist Decreases the Levels of Aβ42-stimulated iNOS and Nitrite Production—As a more specific means of inhibiting the effects of IL-1β, we stimulated cells with Aβ42 for 18 h in the absence or presence of the IL-1 receptor antagonist (IL-1ra). IL-1ra attenuated Aβ42-stimulated levels of iNOS protein as determined by Western blot analysis (Fig. 4A),
but did not affect GFAP protein levels. Similarly, we used increasing concentrations of IL-1ra and measured the levels of nitrite, the stable metabolite of NO, that accumulated in the conditioned medium. Cells pre-treated for approximately 5 min with increasing concentrations of IL-1ra showed a dose-dependent decrease in nitrite levels in both Aβ42-stimulated cells and IL-1β-stimulated cells (Fig. 4B). Complete inhibition of IL-1β-stimulated nitrite production was not observed as the concentration of receptor antagonist necessary for such an effect was not reached (IL-1ra concentrations were initially optimized for inhibition of Aβ42-stimulated nitrite levels, which require less receptor antagonist than direct IL-1β administration). IL-1ra did not inhibit the levels of nitrite produced by 1 mM Bt2cAMP-stimulated astrocytes, demonstrating the specificity of the receptor antagonist as cAMP can stimulate iNOS transcription via molecular mechanisms different from IL-1β (42, 43).

### Dominant-negative TRAF Proteins Can Block NFκB Activation in Astrocytes—In many cell types, the IL-1β signaling pathway stimulates the activation of NFκB via TRAF6, and the TNFα signaling pathway stimulates NFκB activation via TRAF2. Dominant-negative TRAF6 (N-terminal truncation, leaving only the amino acids from 301 to 531; termed dnTRAF6-C) has been reported to be able to inhibit NFκB activation by IL-1β; similarly, dominant-negative TRAF2 (amino acids 87–501; termed dnTRAF2-C) can inhibit NFκB activation by TNFα (27). We tested the effects of these dominant-negative constructs on Aβ42 stimulation of astrocytes. First, we overexpressed dnTRAF6-C and dnTRAF2-C in the cells and verified that each construct can inhibit NFκB activity, as measured by a co-transfected NFκB SEAP reporter construct. As expected, dnTRAF2-C was able to inhibit TNFα but not IL-1β activation of the NFκB reporter in astrocytes (Fig. 5A), and conversely, dnTRAF6-C was able to inhibit IL-1β but not TNFα activation of the NFκB reporter (Fig. 5B). Interestingly, it has recently been reported that dnTRAF6-C but not dnTRAF2-C can inhibit LPS-stimulated NFκB activity in an endothelial cell line and a monocyte cell line (44), suggesting the importance of TRAF6 in LPS stimulation of NFκB.

### Dominant-negative TRAF6 Inhibits iNOS Promoter Activation by Aβ42—While the participation of TRAF2 and TRAF6 in NFκB activation by specific cytokines is now well identified, the involvement of TRAF2 and TRAF6 in stimulation of gene expression is less well studied. Only recently has TRAF6 been implicated in IL-8 promoter activity in macrophages (45). To address the possibility that iNOS promoter activation by Aβ42 is mediated by TNFα or IL-1β, we co-transfected cells with the iNOS promoter-luciferase reporter construct and increasing amounts of dnTRAF6-C or dnTRAF6-C. As shown in Fig. 6, increasing amounts of dnTRAF6-C reduced Aβ42-stimulated iNOS promoter activity by approximately 80%, and increasing amounts of dnTRAF6-C reduced Aβ42-stimulated iNOS promoter activity by approximately 60%, suggesting that IL-1β, and to some extent TNFα, are critical mediators of Aβ42-stimulated iNOS activation. In contrast, dnTRAF2-C and dnTRAF6-C decreased Bt2cAMP-stimulated iNOS promoter activity by only ~25% (Fig. 6), suggesting only a minor role for IL-1β and TNFα in Bt2cAMP-stimulated iNOS activity.

Because NIK is involved in mediating both IL-1β and TNFα signaling pathways, an over-expressed kinase-inactive NIK (dominant-negative NIK, termed dnNIK) should inhibit both IL-1β- and TNFα-stimulated NFκB activation (46). When cells were co-transfected with the iNOS promoter reporter construct and increasing amounts of dnNIK, Aβ42-stimulated iNOS activity was inhibited by approximately 80%, whereas the Bt2cAMP-stimulated iNOS promoter activity was not significantly reduced (Fig. 7). The dnNIK inhibition curve for Aβ42-stimulated iNOS promoter activity is similar to the dnTRAF6-C inhibition curve of the iNOS promoter activity, suggesting that TRAF6 (and thus IL-1β) is the major mediator of Aβ42-stimulated iNOS production in these rat glial cells, and that TRAF2 (and thus TNFα) is a secondary mediator of Aβ42-stimulated iNOS production.

### IL-1β Localizes to Microglia and iNOS Localizes to Astrocytes in Aβ42-stimulated Glial Culture—To identify the cell type(s) responsible for IL-1β and iNOS production in the rat glial cultures, cells were stimulated with Aβ42 for 12 or 36 h and then IL-1β and iNOS localization monitored by FITC immunofluorescence. Double labeling for GFAP with Texas Red immunofluorescence was done to allow for astrocyte identification. As shown in Fig. 8A, IL-1β localizes to non-astrocytic cells (no overlapping FITC/Texas Red fluorescence). The IL-1β-positive cells were primarily microglia, as determined by OX42 staining (data not shown). In contrast, as shown in Fig. 8B, iNOS was detected in astrocytes (overlapping FITC/Texas Red fluorescent cells). These data suggest that Aβ42 stimulates IL-1β production by activated microglia, which in turn can participate in the activation of astrocytes to produce iNOS.

### DISCUSSION

We demonstrate here that in rat glial cultures, the amyloid plaque component Aβ42 stimulates IL-1β and TNFα cytokine production in microglia prior to astrocyte iNOS production, and that the Aβ42-stimulated iNOS promoter activity and NO production occur in an IL-1β-dependent (and to a lesser extent, TNFα-dependent) manner via a signal transduction pathway that involves TRAF6 and NIK. We not only therefore define a clear linkage among Aβ42, inflammatory cytokines, and putative peroxynitrite neurotoxicity, but we also demonstrate for the first time that TRAF6, TRAF2, and NIK can mediate an inflammatory response that is relevant to AD. Because there is an imperative need to understand inflammation in AD, identi-
fying each component such as these in a signal transduction pathway that ultimately leads to deleterious effects in the central nervous system such as peroxynitrite-mediated damage to neurons is important from a therapeutic standpoint.

The pro-inflammatory cytokine IL-1 is a critical neurotoxic component to such an inflammatory pathway, and is found in activated microglia localized to amyloid plaques in AD (47). Evidence that IL-1β can mediate neuronal injury has been shown recently in transgenic mouse models of IL-1β-converting enzyme (ICE). Significantly less ischemic brain injury occurs in ICE-knockout mice (48) as well as in dominant-negative ICE transgenic mice (49).

**Fig. 4.** Inhibition of Aβ42-stimulated iNOS and nitrite production by IL-1 receptor antagonist. A, Western blot analysis of cultures stimulated for 18 h with either 10 μM Aβ42 or 10 μM Aβ42 plus 50 ng/ml recombinant murine IL-1 receptor antagonist (IL-1ra). IL-1ra attenuates Aβ42-stimulated iNOS protein levels, but does not affect GFAP protein levels. The blot shown is a representative of 4 independent experiments. B, cultures were stimulated for 30 h with 5 μM Aβ42, 100 ng/ml recombinant rat IL-1β, or 1 mM Bt2cAMP in the absence (0) or presence of increasing concentrations (3, 10, 30 ng/ml) of IL-1ra. Nitrite levels in the conditioned medium were quantitated by Griess assay, and expressed as percentage of control, where control is the maximum amount of stimulated nitrite production in the absence of receptor antagonist. Values represent mean ± S.E. of eight experiments.

**Fig. 5.** Demonstration of selective functionality for dominant-negative TRAFs in NFκB activation. Cultures were co-transfected with the reporter construct pNFκB-SEAP and increasing amounts (0.05, 0.15, or 0.5 μg) of dnTRAF2-C or dnTRAF6-C (in all transfections, the total amount of DNA transfected per well was kept constant with the addition of pCMV2-FLAG, the control vector). After 48 h, cells were stimulated for 12 h with either 20 ng/ml rat TNFα (A) or 50 ng/ml rat IL-1β (B), and then conditioned medium was collected. NFκB activity was determined by secreted alkaline phosphatase (SEAP) assays on equal amounts of conditioned medium. Activity is expressed as percentage of control, where control is the maximum amount of stimulated SEAP activity in the absence of dominant-negative construct. Data represent mean ± S.E. of eight independent experiments.

**Fig. 6.** Dominant-negative TRAFs and iNOS promoter activation. Cultures were co-transfected with the iNOS-Luc reporter construct and increasing amounts (0.6 or 2.0 μg) of dnTRAF2-C or dnTRAF6-C (total amount of DNA transfected equalized with control vector as in Fig. 5). After 48 h, cells were stimulated with either 10 μM Aβ42 (A) or 1 mM Bt2cAMP (B) for 12 h, and the luciferase activity in cell lysates was determined. Data are expressed as percentage of control, where control is the maximum amount of stimulated luciferase activity in the absence of dominant-negative construct. Data represent mean ± S.E. of 10 independent experiments.

**Fig. 7.** Dominant-negative NIK and iNOS promoter activation. Cultures were co-transfected with the iNOS-Luc reporter construct and increasing amounts (0.6 or 2.0 μg) of dnNIK (in all transfections, the total amount of DNA transfected per well was kept constant with the addition of pcDNA3-HA, the control vector). After 48 h, cells were stimulated with either 10 μM Aβ42 or 1 mM Bt2cAMP for 12 h, and then luciferase activity in cell lysates was determined. Data are expressed as percentage of control, where control is the maximum amount of stimulated luciferase activity in the absence of dnNIK. Data represent mean ± S.E. of eight independent experiments.

Aβ Stimulates Astrocyte iNOS via Cytokines, TRAF Signaling

enzyme (ICE). Significantly less ischemic brain injury occurs in ICE-knockout mice (48) as well as in dominant-negative ICE transgenic mice (49). Similarly, adenoviral-directed cerebral
expression of IL-1ra in mouse brain also resulted in reduced ischemia-induced brain injury (50). A major consequence of IL-1β production in the brain is the stimulation of astrocytic iNOS activity. IL-1β stimulation of iNOS in rat, mouse, and human astrocytes as well as in other cell types that can participate in inflammation, such as endothelial cells, hepatocytes, and macrophages, has been well characterized (for reviews, see Refs. 51 and 52), and such IL-1β-stimulated iNOS activity has been suggested to be detrimental in several neurodegenerative disorders including AIDS dementia complex, the murine multiple sclerosis model of experimental autoimmune encephalitis, and AD (53). This detrimental effect of iNOS activity is supported by evidence from knockout mice studies, which demonstrate that under ischemic conditions, there is a delayed reduction of brain injury and neurological deficits in iNOS-null mice (54). Previously, we showed (17) that Ap42, the primary amyloid plaque component in the brain, can activate glia in vitro in a dose- and time-dependent manner, as determined by morphological response and IL-1β expression, and that Ap42 stimulates iNOS activity in astrocytes via the transcription factor NFκB. Whether this astrocyte iNOS activity is a direct result of Ap42 stimulation of astrocytes or an indirect result, requiring the participation of other glial factors or inflammation mediators such as IL-1β had not been defined previously. While studies consistent with ours (12, 13, 18) have shown that different forms and preparations of Ap42 can stimulate glial cells to produce cytokines or NO, there have been no previous reports to demonstrate and define the linkage between Ap42-stimulated microglial IL-1β (or TNFα) and astrocytic iNOS.

Our results demonstrating that Ap42-stimulated astrocyte iNOS mRNA production requires new protein synthesis is consistent with previous studies using cycloheximide to inhibit LPS-stimulated iNOS mRNA production in fetal hepatocytes (55). Cycloheximide was partially able to reduce Ap42-stimulated iNOS mRNA production. The partial reduction of iNOS mRNA may reflect the fact that the 1 μM cycloheximide concentration is not enough to completely inhibit protein synthesis. Higher concentrations of cycloheximide were not used because they proved to be toxic to the primary astrocyte cultures; the 1 μM cycloheximide concentration did not promote a strong inflammatory response or toxic response in the astrocytes. A slight increase in iNOS mRNA was detected in cells treated with control buffer alone in the presence of cycloheximide, but this weak stimulation has been seen before in other systems with a similar concentration of cycloheximide (55). Similarly, the observation that Ap42-stimulated nitrite could not be inhibited completely with increasing concentrations of IL-1ra is consistent with previous studies using human fetal astrocytes stimulated with IL-1β (56), where the effective concentration of receptor antagonist needed to completely inhibit IL-1β-stimulated iNOS mRNA production was several orders of magnitude higher than the concentration of stimulus.

The ability of dnTRAF2-C to inhibit TNFα- but not IL-1β-stimulated NFκB activation in cultured astrocytes, and the ability of dnTRAF6-C to inhibit IL-1β- but not TNFα-stimulated NFκB activation in cultured astrocytes are consistent with previous studies with 293 cells, which demonstrate TRAF-specificity in cytokine stimulation of NFκB (27). In addition, we have extended our investigations beyond the NFκB response element reporter construct, and using the iNOS promoter reporter construct, we show, as has recently been shown for TRAF2 and the IL-8 promoter (45), that TRAF- and NIK-dependent signaling can also produce direct consequences in models of inflammation, emphasizing the need to characterize further how TRAFs and NIK interact in signal transduction mechanisms.

There are other potential signal transduction pathways activated during inflammation, which may also participate in Ap42-stimulated iNOS production in astrocytes. For example, the p38 MAPK and c-Jun N-terminal kinase/stress-activated protein kinase stress kinase pathways that target the AP-1 transcription factor could contribute to iNOS promoter activity. In mixed glial cultures, it has been reported (57) that p38 MAPK can mediate TNFα- and IL-1β-stimulated iNOS activation in astrocytes, based on the observation that the p38 inhibitor SB203580 is able to inhibit cytokine-stimulated iNOS activity and nitrite production. Whether or not p38 MAPK plays a role in Ap42-stimulated IL-1β production in microglia or regulates iNOS expression in astrocytes in cooperation with NFκB has not been addressed. A recent study showed that p38 MAPK was important in LPS-stimulated IL-1β transcription in murine macrophages (58), so it is possible that in a mixed glial culture containing both microglia and astrocytes, suppressing p38 MAPK leads indirectly to iNOS inhibition via an IL-1β inhibition in microglia. Indeed, it has recently been demonstrated that activated p38 MAPK can be immunolocalized to microglia that are associated with amyloid plaques in AD brain (59), and amyloid fibrils can activate p38 MAPK in microglia in vitro (60).

Altogether, our data substantiate a model of inflammation observed in AD where Ap42 activates microglia to produce pro-inflammatory cytokines such as IL-1β and TNFα. These cytokines in turn activate surrounding astrocytes, which exacerbate the inflammation with the production of neurotoxic mediators such as iNOS. The resultant peroxynitrite ultimately damages local neurons and contributes to the neurodegeneration observed in AD.

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