Regulation of Nerve Growth Factor mRNA by Interleukin-1 in Rat Hippocampal Astrocytes Is Mediated by NFkB*

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Cytokines such as interleukin-1β (IL-1) are produced in the brain during development and during inflammatory processes that result from lesions or disease. One function of IL-1 in the brain appears to be the stimulation of astrocytes to proliferate and produce a variety of cytokines and trophic factors, including nerve growth factor. The mechanisms by which IL-1 exerts its actions on astrocytes remain poorly defined. We present evidence that this cytokine elicits activation of the NFκB transcription factor and that this transcription factor mediates effects of IL-1 on nerve growth factor mRNA expression. Elucidation of the processes by which cytokines activate astrocytes and influence trophic factor expression may provide insight into mechanisms governing inflammatory processes within the central nervous system.

Astrocytes in the adult brain are normally quiescent; however, proliferation and activation of these cells occur in response to a number of stimuli, such as cytokines, associated with inflammation and disease. One of the many functions subserved by astrocytes may involve a role in mediating immunological events in the brain. Reactive astrocytes can express MHC antigens (1), cytokines such as IL-1, TNFα, IL-6 (2), and colony-stimulating factors (3, 4). Thus, the stimulation of astrocytes by inflammatory molecules may initiate a chain of events leading to increased production of cytokines and growth factors and the ability of astrocytes to mediate immune responses in the brain (5).

Cytokine activation of astrocytes also induces elevated levels of nerve growth factor (NGF) expression (6, 7, 8). NGF is a member of a family of neurotrophic factors, called neurotrophins, which includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Each of these factors is expressed in distinct cellular populations in the brain, both neuronal and glial, and influences specific responsive target cells. NGF is most highly expressed in the hippocampus (9–12). Under quiescent conditions, expression of NGF in the rat hippocampus is primarily detected in neurons (13, 12). However, when activated by dissociation in tissue culture (6, 7), or by a lesion in vivo (14), astrocytes express high levels of NGF mRNA and protein. The induction of glial NGF expression by lesions in the peripheral nervous system was shown to be dependent on interleukin-1 (15). This cytokine was subsequently demonstrated to influence NGF expression in CNS glial cells as well (6–8).

The transduction mechanisms by which IL-1 influences NGF expression have remained largely undefined. The signaling pathway does not appear to involve cAMP, protein kinase C, phosphatidylinositol turnover, or mobilization of intracellular calcium (16). Activation of c-fos (16), and the presence of an AP-1 site in the NGF promoter (17, 18) suggested that this transcription factor in mediating IL-1 stimulation of NGF. Alternatively, the recent identification of the sphingomyelin pathway, leading ultimately to activation of NFκB transcriptional activity by IL-1 and TNF (19, 20), suggested that this pathway might mediate actions of IL-1 in astrocytes.

Cytokine stimulation of gene expression in a variety of cell types is mediated, at least in part, through activation of NFκB transcription factors (21). This has been particularly well studied in cells of the immune system, where the NFκB/rel transcription factor family plays a major role in lymphoid differentiation and activation (reviewed in Refs. 22 and 23). Transcriptionally active protein dimers in the nucleus activate gene transcription by binding to a κB sequence motif in the promoter of responsive genes. Inactive proteins are retained in the cytoplasm as unprocessed precursor proteins (p100 or p105) or as part of an inactive complex containing inhibitor molecules such as the IκB’s. Upon stimulation, the inhibitory molecules are degraded by the ubiquitin-proteosome pathway, freeing the NFκB dimers to translocate to the nucleus and activate transcription. The transcriptionally active NFκB dimer may consist of hetero- and homodimers of the five NFκB/rel proteins or may include novel tissue-specific proteins that recognize κB binding sequences (24). Multiple combinations may exist even within a single cell type. Since numerous cellular genes are transcriptionally regulated by NFκB, the specificity of target gene activation by a given stimulus in a particular cell may be determined in part by the dimer composition. Genes activated by the NFκB/rel transcription factors include those encoding cytokines (IL-1, II-2, II-6, II-8, TNFα, and β-interferon), histocompatibility antigens, and other cellular proteins (e.g. macrophage NO synthase (25)).

NFκB transcription factors have been shown to play an important role in mediating lymphocyte and macrophage cellular responses to a variety of cytokines (22, 23). Recently, IL-1 has also been shown to induce NFκB in astrocytes (2) and astrocytoma cell lines (26). In these studies we have examined whether the NFκB transcription factors play a role in mediating astrocyte activation and nerve growth factor induction by cytokines.
Hippocampal Astrocytes—Hippocampi from embryonic day 21 (E21) rats were dissected under sterile conditions, dissociated by trituration and plated on polylysine-coated 75-cm² flasks. The cells were grown for 1 week in Eagle’s minimal essential medium supplemented with glucose (6 mg/ml), penicillin-streptomycin (0.5 unit/ml-0.5 μg/ml), and 15% fetal calf serum to enhance astrocyte proliferation. Confluent type 1 astrocytes were then separated from other cell types by their differential adhesive properties according to previously published protocols (27, 28). Cells were subjected to several shaking steps on an orbit shaker to eliminate neurons, microglia, oligodendrocytes, and type 2 astrocytes. Cells were then maintained for 3 days in the presence of cytosine arabinoside (0.1 mM) to prevent proliferation of any remaining contaminating populations. Confluent astrocytes were then trypsinized and replated on polylysine-coated 150-mm dishes. When the replated astrocytes were 60–90% confluent, cultures were stimulated by treating with IL-1β (Boehringer Mannheim, 10 units/ml) for 4 h. Cells were then lysed with guanidine isothiocyanate for RNA preparation or harvested for nuclear extract preparation or Western blot analysis.

For NFκB competition experiments, double-stranded, phosphorothioate oligonucleotides were synthesized on an Applied Biosystems 392 DNA synthesizer with the following sequences: wild type (5′-CTG TTT CCC CTC-3′) and mutant (5′-CTC TGC TCA CTT TCC-3′). Each oligonucleotide was annealed to the complementary DNA sequence and was added to astrocyte cultures at a concentration of 7.5 μM 0.5 h prior to IL-1 treatment. For p50-p65 antisense experiments the sequences used were as follows: p50 antisense, 5′-GGG ATC ATC GTC TGC CAT GGT-3′; p65 antisense, 5′-GAG AAA CAA CAC CAT CAT GGT-3′; p50 sense, 5′-ACC ATG GCA GAC GAT CAC GAT CCTG TTT CCC CTC-3′. Oligonucleotides were purchased from Oncor and linked to the Penetratin peptide to facilitate entry into cells (29). Combinations of p50-p65 antisense or p50-p65 sense oligonucleotides were provided to astrocyte cultures overnight at a concentration of 250 nM.

Northern Blot Analysis—RNA from cytokine-treated or untreated astrocytes was isolated by centrifugation through a cesium chloride cushion as described (30) and quantitated spectrophotometrically. Equal amounts of RNA were subjected to electrophoresis through a 1% agarose, 0.7% formaldehyde gel and transferred to nitrocellulose filters (Amersham Hybond-C super). The filters were then hybridized with [32P]P-labeled cDNA probes for NFκB (31), II-6 (obtained from ATCC), NFκB1 (32), or NFκB2 (32). Hybridization was carried out overnight at 42 °C in 4× SSC, 0.1% SDS, 1× Denhardt’s, and 10% dextran sulfate. The filters were washed at 54 °C in 0.1× SSC and 0.1% SDS and exposed to Kodak XAR-5 film at −70 °C.

Electrophoretic Mobility Shift Assays—Nuclear protein was prepared using the extraction method of Dignam et al. (39). 5 μg of nuclear protein was incubated with [32P]P-labeled oligonucleotides (10,000–20,000 cpm) containing the sequence of the NFκB binding sites present in the HIV long terminal repeat (LTR) (33). Competition experiments were performed by inclusion of a 50-fold molar excess of unlabeled specific or nonspecific competitor DNA in the binding reaction. Supershift assays included specific antibodies (Santa Cruz Biotechnology, Inc.) directed against each member of the NFκB/rel family. DNA-protein complexes were resolved by electrophoresis through a 4.5% polyacrylamide gel under nonreducing conditions.

Immunocytochemistry—Astrocytes were grown as described above except that after trypsinization cells were plated onto polylysine-coated 12-well plates. Cells were fixed with 4% paraformaldehyde and washed with PBS. Antiserum to p50 and p65 (Santa Cruz Biotechnology, Inc.) was used in a 1:1,000 dilution in PBS with 0.3% Triton X-100. Cells were preincubated for 10 min in PBS, 0.3% Triton with 5% goat serum and then exposed to the antiserum overnight. Labeled cells were visualized using the avidin-biotin method for peroxidase staining (ABC kit, Vector Laboratories).

Western Blot Analysis—Cultured astrocytes were harvested in PBS, heated in sample buffer consisting of Tris, SDS, and β-mercaptoethanol and subjected to electrophoresis through a 10% polyacrylamide gel. Proteins were transferred electrophoretically to polyvinylidene difluoride membrane, exposed to anti-p65 antiserum (Santa Cruz), and detected by chemiluminescence (Boehringer Mannheim). Blots were stripped of antibody in 2% SDS, 100 mM mercaptoethanol, 62.5 mM Tris at 70 °C, and re-probed with anti-p50 antiserum (Santa Cruz).

RESULTS

II-1 Induction of NFκB mRNAs in Astrocytes—The mechanisms by which II-1 signaling is transduced within a cell to influence gene expression remain poorly defined. This cytokine has been shown to act via an AP-1-dependent mechanism to influence a variety of genes in different cell types (34, 35). However, in contrast to the AP-1 pathway, recent studies have provided evidence for an II-1 signaling pathway similar to that described for TNFα, involving sphingomyelin metabolism and stimulation of a ceramide-dependent kinase leading to activation of the NFκB transcription factor (19). To determine the possible role of this pathway in mediating II-1 actions in astrocytes, we initially examined whether providing the cells with ceramide would induce an increase in NGF mRNA as effectively as II-1. A membrane-permeable form of ceramide, C2- ceramide, was provided to the astrocyte cultures and compared with II-1 for the ability to induce NGF mRNA. Treatment with ceramide resulted in a strong elevation of NGF mRNA (Fig. 1), consistent with the possibility that a ceramide-dependent pathway may mediate regulation of NGF expression by II-1.

II-1 Induction of NGF mRNA Does Not Require Novel Protein Synthesis—To determine whether the II-1-stimulated increase in NGF expression was a direct effect of cytokine treatment, or whether synthesis of additional proteins was required, protein synthesis was inhibited during II-1 treatment of astrocyte cultures. The presence of cycloheximide (CHX) in the medium did not prevent the II-1-induced elevation in NGF expression (Fig. 2), suggesting that the proteins necessary to mediate II-1 actions were already present in the cell. CHX alone elicited a slight induction of NGF mRNA (Fig. 2), which is characteristic of NFκB-dependent genes, since CHX is known to activate nuclear transport of NFκB, presumably by inhibiting synthesis of the relatively labile IκB molecules (36).

To further assess the involvement of an NFκB-mediated pathway, we examined the potential involvement of reactive oxygen intermediates. NFκB is activated by oxygen radicals (37), which are commonly produced during the inflammatory process. The production of oxygen radicals may be a common pathway by which many different stimuli activate NFκB (37). Treatment of cells with an antioxidant, pyrroline dithiocarbamate (PDTC), blocks NFκB activation (38). Astrocyte cultures were treated with PDTC prior to stimulation with II-1, and the antioxidant prevented induction of NGF mRNA (Fig. 3). Induction of NFκB-1 and NFκB-2 mRNAs, both autoregulated NFκB-inducible genes (39, 40), was also prevented by the antioxidant (Fig. 3), similar to the effect on NGF. Thus, a correlation was observed between regulation of NGF expression and that of two NFκB subunits.

II-1 Induces Nuclear NFκB Translocation and Binding Activity in Hippocampal Astrocytes—To directly address whether II-1 treatment of astrocytes leads to NFκB activation and nuclear translocation, nuclear extracts were prepared from II-1-treated or untreated astrocytes. These extracts were incubated with labeled oligonucleotides containing the κB binding sequence from the HIV LTR and were examined by electrophoretic mobility shift assay for NFκB binding activity. A spe-
specific band was detected only in the extracts prepared from cytokine-treated cells, indicating that II-1 treatment induced a nuclear κB binding complex. In competition experiments, this band was eliminated by the presence of excess unlabeled NFκB oligonucleotide, but not by the presence of a nonspecific oligonucleotide competitor, demonstrating the specificity of binding to the NFκB recognition sequence (Fig. 4A).

Supershift experiments using specific antibodies to each of the NFκB/rel proteins were performed to determine the identity of the proteins in the II-1-induced NFκB binding complex. Incubation with antibodies to either NFκB-1 (p50) or relA (p65) either elicited a supershifted complex (NFκB1) or inhibited binding to the NFκB oligonucleotide (relA, Fig. 4B). Antibodies to the other proteins of the NFκB/rel family did not alter binding. These data suggest that acute exposure of astrocytes to II-1 yielded a binding complex containing the classic p50-p65 NFκB heterodimer.

To further demonstrate activation of the p50 and p65 NFκB proteins, nuclear translocation was examined immunocytochemically (Fig. 5). Untreated or II-1-treated astrocytes were fixed and stained with antibodies to p50 or p65. Under basal conditions no nuclear staining was observed for either protein. However, after II-1 exposure all astrocyte nuclei were labeled with the p65 antibody, and a subset of nuclei showed p50 staining, indicating that a subpopulation of hippocampal astrocytes possessed p50 and p65 in the nucleus.

NFκB Mediates NGF Regulation by II-1 in Astrocytes—To demonstrate directly that regulation of NGF expression was dependent on NFκB activation, competing κB binding oligonucleotides were added to the astrocytes (41, 42). These double-stranded, phosphorothioate oligonucleotides contained a tandem repeat of the NFκB binding sequence from the HIV LTR.
(see “Experimental Procedures” for sequence). In the presence of these competing oligonucleotides, induction of NGF mRNA by IL-1 was prevented (Fig. 6A). In contrast, the presence of oligonucleotides containing a mutated κB binding sequence did not prevent the increase in NGF mRNA elicited by IL-1. To confirm that these oligonucleotides effectively blocked NFκB-dependent transcriptional activation, regulation of a known NFκB-dependent gene was examined. Interleukin-6 (IL-6) expression by hippocampal astrocytes increased nerve growth factor expression was provided by the use of antisense oligonucleotides (43). Astrocyte cultures were pretreated overnight with either antisense or sense oligonucleotides to both NFκB-1 (p50) and relA (p65). The cells were subsequently treated with IL-1 for 4 h and expression of NGF mRNA was examined. The antisense oligonucleotides prevented induction of NGF mRNA by IL-1, while the sense oligonucleotide did not influence NGF expression (Fig. 6A). Cultures grown and treated in parallel with the sense and antisense oligonucleotides were analyzed by Western blot for the presence of the p50 and p65 proteins. The antisense oligonucleotides completely eliminated p50 from the cultures and decreased the level of p65 (Fig. 7B). These data further indicate that the NFκB transcription factor plays an important role in IL-1 regulation of NGF expression.

**DISCUSSION**

In these studies we have shown that cytokine stimulation of hippocampal astrocytes increased nerve growth factor expression via an NFκB-dependent pathway. IL-1 treatment induced nuclear NFκB translocation and DNA binding activity. The binding complex consisted of p50/p65 heterodimers as shown by supershift experiments. Several lines of evidence suggested that regulation of NGF expression was correlated with NFκB activation. The inability of cycloheximide, as well as the ability of the antioxidant PDTC, to prevent the IL-1-induced elevation of NGF mRNA, were both consistent with an NFκB-dependent signaling pathway. Moreover, the slight induction of NGF mRNA by CHX alone may be due to the inhibition of IκB synthesis by CHX and is characteristic of NFκB-dependent genes. We investigated the possible role of this transcription factor directly by blocking NFκB activity, either by competing for κB binding or with antisense oligonucleotides to p50 and p65. Inhibition of NFκB prevented the IL-1-induced increase in NGF mRNA. Thus, we have shown that a critical transcription factor, known to mediate cytokine actions in a variety of cell types, is activated in brain astrocytes and mediates induction of trophic factors as well as cytokines. The NGF gene has a complex structure and the promoter has not been well characterized. However, the presence of an AP-1 site in the first intron (17, 18) has been demonstrated and has implicated this transcription factor in mediating regulation of NGF expression (17, 18). Specifically, c-fos seems to be critically involved in the lesion-induced elevation in NGF mRNA in fibroblasts of the
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sciatic nerve (17). However, the ability of the antioxidant PDTC to prevent the II-1 induction of NGF in astrocytes does not support an AP-1-dependent pathway. AP-1 is not activated by reactive oxygen intermediates (37), therefore an AP-1-dependent pathway would not be blocked by the antioxidant treatment. Moreover, the activation of transcription factors and regulation of gene expression may differ in distinct cell types and in response to different stimuli. Recently, the presence of an NFkB site has been demonstrated in the promoter of the human NGF gene. An analogous NFkB site is present in the mouse NGF promoter (GCG data base, locus MUSNGFX, accession number M33683, base pairs 1163–1172). The κB sequence from the human NGF promoter has exhibited binding activity in electrophoretic mobility shift assays and shows supershifted bands in the presence of p50 and p65 antibodies. Thus, the regulation of NGF mRNA which we have observed to be mediated by NFkB in astrocytes is probably due to an analogous site in the rat NGF promoter.

The NFkB/rel family of transcription factors plays a critical role in mediating cellular activation by cytokines. The functional importance of these transcription factors has been well demonstrated in lymphocytes (22, 23, 44). In the brain, response to injury and disease is not mediated by the immune system as in the periphery. Lymphocytes and macrophages cannot enter the CNS unless the blood-brain barrier is compromised. Specialized glial cell types within the brain appear to have functions analogous to peripheral macrophages and lymphocytes. Microglia are phagocytic cells within the CNS. These cells proliferate and aggregate at the site of a lesion, release a variety of cytokines such as II-1, and are considered to be the macrophages of the brain. Astrocytes also become activated by injury and can express MHC antigens (4) and produce a variety of cytokines and trophic factors. This ability may provide an important beneficial environment for neurons consequent to a lesion or disease process. The mechanisms governing astrocyte activation by cytokines such as II-1 remain poorly understood, although II-6 induction in astrocytes is mediated by NFkB (2). Our studies suggest that the NFkB transcription factors may critically mediate additional astrocyte responses to cytokines, including, importantly, induction of trophic factors. Thus, NFkB may function as a common pathway in the pleiotropic astrocyte response to cytokines in the CNS.

The NFkB/rel transcription factors may serve a number of different roles in the CNS. In addition to mediating the effects of cytokines on astrocytes, constitutive nuclear NFkB has been identified in neurons (45) and shown to be responsible for the high levels of HIV LTR promoter activity that can be seen in these cells (46, 47). Neuronal NFkB has been proposed to play a role in normal neuronal gene expression (45), although induction of this transcription factor may also be associated with CNS pathology. The neuronal toxicity of the amyloid β protein was found to correlate with its ability to induce hydrogen peroxide and NFkB (48). NFkB has also been proposed to play a role in other CNS disorders whose pathogenesis involves damage due to oxygen free radicals. Based on our results and those of others (2), it is tempting to speculate that some of the pathologic changes associated with CNS inflammatory processes, such as astrocyte activation, may also be the result of NFkB induction. Although some of these changes, such as NGF induction, may be beneficial in the short term, a prolonged inflammatory process may lead to severe CNS pathology.

The production of trophic factors consequent to a lesion may have a critical impact on surrounding neuronal populations. Lesions of the fimbria-fornix, the pathway connecting the basal forebrain and hippocampus, normally result in extensive cell death in the basal forebrain. Several groups have shown that infusion of NGF after a fimbria-fornix lesion can rescue cholinergic basal forebrain neurons (49–51) Although fimbria transection results in a local increase in hippocampal NGF mRNA and protein (52, 53), the levels of trophic factor that are induced may not be sufficient to prevent the extensive cell death that occurs after a lesion. One possibility is that under conditions of mild trauma, NGF induction in astrocytes may be sufficient to maintain neuronal survival and function; however, upon excessive damage, such as transection of the pathway, additional trophic support is required. Other conditions that compromise the NGF-responsive basal forebrain cholinergic population include progressive diseases such as Alzheimers, in which this population is among the earliest to degenerate, leading to severe memory deficits (54). Animal models using aged rats with memory deficits have shown that NGF is depleted in these animals (55, 56) and that NGF treatment improves performance on memory tasks (57). Furthermore, NGF is currently in clinical use for treatment of Alzheimer patients (58). Therefore, availability of trophic factors to responsive neurons when they are compromised by lesion or disease may critically influence continued survival and function. Thus, it is necessary to understand the mechanisms that mediate NGF induction and to be able to exploit these mechanisms to maximize trophic factor production under appropriate circumstances.

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