Excitotoxin Through Distinct Pathways Impart Neuroprotection to an Inflammatory Cytokines IL-1α, IL-1β, IL-6, and TNF-α Impart Neuroprotection to an

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Inflammatory Cytokines IL-1α, IL-1β, IL-6, and TNF-α Impart Neuroprotection to an Excitotoxin Through Distinct Pathways

Noel G. Carlson,* Whitney A. Wieggel, † Jian Chen, † Annalisa Bacchi, † Scott W. Rogers,* †§ and Lorise C. Gahring2*‡

The proinflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α are produced within the CNS, and, similar to the periphery, they have pleotropic and overlapping functions. We have shown previously that TNF-α increases neuronal survival to a toxic influx of calcium mediated through neuronal N-methyl-d-aspartic acid (NMDA) glutamate-gated ion channels. This process, termed excitotoxicity, is a major contributor to neuronal death following ischemia or stroke. Neuroprotection by this cytokine requires both activation of the p55/TNF receptor type I and the release of TNF-α.

Many of the inflammatory cytokines commonly associated with the peripheral immune system are also found and produced within the CNS (1–4). Peripheral sources of CNS cytokines include immune cells such as macrophages, T lymphocytes, and B lymphocytes, but also a variety of other cells such as fibroblasts and keratinocytes. Access of cytokines produced in the periphery to cells in the CNS has been suggested to occur through a variety of routes. First, access of cytokines to the CNS can occur through regions of the brain that possess a poor blood brain barrier (BBB) with fenestrated capillaries from which molecules as large as 500,000 m.w. can escape to adjacent brain parenchymal cells (5). These regions include the circumventricular organs (organum vasculosum of the lamina terminalis, median eminence, subfornical organ, and area postrema). Second, a saturable transport system has been proposed to allow passage of cytokines through the BBB (6, 7), and finally BBB permeability can increase during systemic inflammatory responses that induce fever and even following epileptic seizure or trauma (8–10).

Cytokines present in the CNS originate not only from the immune system but also through endogenous production by cells of the brain, including astrocytes and neurons (11) whose production can be stimulated by peripheral cytokines. In the CNS, cytokines exert their function through both traditional engagement of their receptors, which are expressed by both glial and neuronal cells (11–14), and through less traditional means such as modulation of neurotransmitter receptor function (15, 16). For example, IL-1 modulates γ-aminobutyric acid responsive neurotransmitter receptors to enhance inhibitory responses (16). Both IL-1 (17, 18) and IL-6 also modulate synaptic plasticity through inhibiting formation of long-term potentiation (19, 20). Further, we (21) and others (22–24) have reported that TNF-α functions in the CNS to modulate responses of neurons to a class of ionotropic glutamate receptors (Glur) known for their activation by the agonist N-methyl-d-aspartic acid (NMDA).3 Excessive activation of NMDA receptors results in the death of neurons through a process termed excitotoxicity (25, 26). Excitotoxicity is a major pathway (27) of neuronal cell death that is associated with ischemia, trauma, and neurodegenerative diseases and results from an uncontrolled elevation in intracellular calcium that enters the cell through chronically activated NMDA receptors. Agents such as antioxidants, growth factors, and certain cytokines protect against excitotoxicity, either through directly modulating receptor function or indirectly through inhibiting key metabolic steps subsequent to Glur activation. TNF-α has been demonstrated to protect cultured neurons against an excitotoxic death induced by the GluR agonist NMDA (21, 23). Further, in an animal model of stroke, mice deficient for TNF-α receptors have enhanced sensitivity to ischemic brain damage following arterial occlusion (23), again alluding to the neuroprotective role of TNF-α in the brain.

We have previously demonstrated (21) that: 1) the neuroprotective activity of TNF-α in cultured cortical neurons is mediated through the p55/TNF receptor type I (TNFRI), 2) the release of TNF-α from neurons plays a role in neuroprotection, and 3) the
plant alkaloid nicotine inhibits TNF-α-induced neuroprotection. In this report, we demonstrate that IL-1 (both α and β) and IL-6 are also neuroprotective cytokines. However, the mechanisms of neuroprotection induced by IL-1 and IL-6 are distinct from TNF-α-induced neuroprotection. These studies support an important role of cytokines in the CNS and suggest that neuroprotective cytokine networks in the CNS function through distinct but overlapping mechanisms.

Materials and Methods

Reagents

Recombinant human TNF-α (hTNF-α), hamster monoclonal anti-p55/TNFRI agonist Ab, and recombinant murine IL-1α were purchased from Genzyme (Cambridge, MA). Recombinant murine IL-1β and recombinant murine IL-6 were purchased from BioSource International (Camarillo, CA). Mouse monoclonal anti-nerve growth factor (NGF)-neutralizing Ab and the TUNEL apoptosis detection kit were purchased from Boehringer Mannheim (Indianapolis, IN). Rabbit polyclonal (purified IgG) anti-mouse TNF-α-neutralizing Ab was purchased from Endogen (Cambridge, MA).

Murine IL-1 receptor antagonist (IL-1ra) was a generous gift from Dr. Michael Bienkowski (Department of Cell Biology, Upjohn, Kalamazoo, MI). Nicotine, NMDA, and α-bungarotoxin were obtained from Research Biochemical International (Natick, MA). To stain dead cells, ethidium homodimer was used according to the manufacturer’s instructions (Molecular Probes, Eugene, OR).

Cortical cell cultures

Enzymatically dissociated cells from cortices of E14-16 embryos (mouse strain CD1; The Jackson Laboratory, Bar Harbor, ME) were plated onto 35-mm diameter Corning (Corning, NY) culture dishes coated with poly-L-lysine at a density of 1.3 × 10⁶ trypan blue-excluding cells/plate. Cells were plated in 2 ml of MEM with Earle’s salts/5% horse serum (heat-inactivated) and 5% FBS (heat-inactivated) and grown at 37°C in humidified chambers with 5% CO₂. Every 2–3 days, cultures were replenished with growth media (MEM with Earle’s salts/10% horse serum, 30 mM glucose, 2 mM glutamine). Arabinose-cytosine was added for 24 h (final concentration of 10 mM) 7 days after plating to limit growth of mitotic cultures. Cells were allowed to recover for 1 day after feeding before using them in excitotoxicity experiments. Cultures were used between days 16 and 21 after plating.

Excitotoxicity assay

Neuronal cell death was induced by prolonged NMDA (20–25 μM) exposure and quantitated by morphological inspection of three to four fields (~200 neurons per field before NMDA) per culture dish before and after NMDA treatment. If lower concentrations of NMDA were applied to cultures, less neuronal death occurred. Our experiments were designed to use an amount of NMDA sufficient to impart 85–90% neuronal death. This paradigm allows for a consistent ability to count viable cells accurately and in turn minimizes sampling variability. In each experiment, the concentration of NMDA required to elicit 85–90% death of neurons was determined just before the actual experiment using an NMDA concentration response curve on cultures from the same cell preparation. Culture dishes were marked with reference points, or etched cover slips were used (CELLocate, Eppendorf) so that the same cell fields could be relocated and photographed before and 20 h after NMDA treatment. The criteria for scoring live cells was verified by assuring that these cells were not positive for staining by either the TUNEL detection stain for apoptosis (Boehringer Mannheim) or the ethidium homodimer dead cell stain (Molecular Probes). Neuroprotective agents or vehicle controls were added directly into the growth media at the final concentrations listed and incubated at 37°C for 24 h (unless otherwise specified), whereupon NMDA was added directly into the same growth media. During the time frame of the experiments (maximum of 48 h), no neuronal death was detected in the absence of NMDA or in the presence of cytokines alone, nicotine alone, or any of the reagents used in these experiments (e.g., anti-NGF or anti-mTNF-α). Therefore, the basal level of neuronal survival is defined as 100% and the basal level of death as 0. All values are expressed as a percentage of the total number of cells that were counted in the culture before treatment. Each of the experiments presented was repeated as independent experiments a total of three or more times.

Results

The neuroprotective effects of recombinant cytokines against assault by the glutamate receptor agonist NMDA were determined using the assay diagrammed in Fig. 1A. Murine cortical cultures were pretreated for 24 h with the agent to be tested (e.g., TNF-α) before addition of NMDA. Before the addition of NMDA (25 μM), four fields of cells were photographed and counted, and these same fields were photographed and counted 20 h later. Neuronal survival following NMDA treatment is expressed as percent (%) survival that equals: [(the number of live neurons counted after NMDA) / (the number of live neurons present before NMDA)] × 100. B. Phase microscopy of murine cortical neurons in culture before and 20 h after the addition of 25 μM NMDA. This field of neurons represents approximately one-ninth of a field of neurons in the schematic above. White arrows indicate examples of some neurons that survived the NMDA treatment. C. Concentration-dependent response of neuronal cultures to TNF-α. Cultures were treated with between 5 and 200 ng/ml of hTNF-α for 24 h before the addition of NMDA. Control cultures were treated with the vehicle for TNF-α (Veh, 0.9% saline). Neuronal fields were photographed and counted (no difference in viability between Veh and TNF-α-treated cultures was detected) and then treated with NMDA for 20 h. Neuronal survival following NMDA was determined to be optimal using 100 ng/ml of TNF-α and significantly (**, p < 0.01) better than vehicle-treated. *s, p < 0.05.
therefore chose to use IL-1 alpha (10–50 ng/ml), murine IL-1 beta (10–50 ng/ml), or murine IL-6 (2.5–10 ng/ml) to add to neuronal cultures 24 h before the addition of NMDA (25 μM), as described in Fig. 1. Neuronal survival was determined for each concentration of cytokine and compared with that observed in vehicle-treated cultures (Veh). None of the cytokines tested induced cell death in the absence of NMDA over the 48 h of this assay. Neuroprotection was optimal and significant with IL-1 alpha at 25–50 ng/ml, IL-1 beta at 25–50 ng/ml, and IL-6 at 10 ng/ml. In experiments described subsequent to this, we therefore chose to use IL-1 alpha and IL-1 beta at 25 ng/ml and IL-6 at 10 ng/ml.

photographed and scored for surviving neurons (see Materials and Methods). In the absence of NMDA, no evidence of cell death was apparent after addition of any of the cytokines (IL-1 alpha, IL-1 beta, IL-6, or TNF-alpha) over the same time course as determined by: 1) visual inspection as above, 2) ethidium homodimer dye exclusion, and 3) TUNEL analysis to measure apoptosis. This chronic NMDA treatment paradigm results in the death of ~85–90% of the neurons, but not monolayer cells consisting mostly of glial cells that lack NMDA receptors. The concentration of NMDA used was deliberately selected to induce high, but not complete, neuronal death (~85% of the neurons died, leaving 15% survival). This provides for the accurate quantitation of surviving neurons. Under these conditions, TNF-alpha pretreatment routinely increased the percent of surviving neurons to 25–30% (Fig. 1B, and below). In Fig. 1B, a subregion of a neuronal field (approximately one-ninth of one field of neurons) was photographed under phase microscopy both before and after treatment with NMDA. Representative live cells are depicted by white arrows (other live cells are also present). Also visible is the monolayer on which the neurons lie.

TNF-alpha-induced neuroprotection against an excitotoxic challenge with NMDA was concentration-dependent, with maximal neuroprotection observed with pretreatment of 100 ng/ml of recombiant human TNF-alpha (Fig. 1C). hTNF-alpha was chosen for these experiments because it interacts only with the mouse p55/TNFRI (and not mouse p75/TNFRII), which is the major receptor for signal transduction of TNF-alpha-induced events in our system. We have also shown that an anti-mouse p55/TNFRI-specific agonist Ab (see below) also induces neuroprotection similar to mouse TNF-alpha and hTNF-alpha (21).

Other inflammatory cytokines have been tested for their neuroprotective properties. IL-1 alpha, IL-1 beta, or IL-6 when added to neuronal cultures 24 h before the addition of NMDA were also neuroprotective (Fig. 2). This observation lead us to test the relatedness of mechanisms through which these individual cytokines induce neuroprotection. Cultured neurons express TNF-alpha, as measured by immunocytochemistry, and this immunoreactivity diminishes upon stimulation with hTNF-alpha (or anti-p55/TNFRI agonist Ab), consistent with release of mature TNF-alpha (11, 21). Further, TNF-alpha-induced neuroprotection is blocked by addition of an anti-mouse-specific TNF-alpha-neutralizing Ab to cultures immediately before p55/TNFRI activation. Therefore, we determined whether neuroprotection conferred by IL-1 alpha, IL-1 beta, or IL-6 also required the release of murine TNF-alpha. To test this possibility, neutralizing Ab to mouse TNF-alpha was added to cultures 1 h before the addition of either IL-1 alpha, IL-1 beta, or IL-6. Concentrations of anti-mouse TNF-alpha-neutralizing Ab, sufficient to block neuroprotection by hTNF-alpha (21) or p55/TNFRII agonist Ab (Fig. 3), had no effect on the neuroprotection conferred by IL-1 alpha, IL-1 beta, or IL-6 (Fig. 3). This indicates that IL-1 and IL-6 are not dependent on mTNF-alpha for their neuroprotective effects.

To determine whether IL-1, IL-6, or TNF-alpha neuroprotection is mediated through the IL-1 receptor, we tested whether these cytokine-mediated neuroprotective effects could be blocked with IL-1ra. To block IL-1-induced responses effectively, a large excess of IL-1ra over IL-1 is required (29). We found that addition of 100 μg/ml of IL-1ra (1 h before IL-1) was required for optimal inhibition of neuroprotection by IL-1alpha or IL-1beta. Pretreatment with IL-1ra (100 μg/ml) sufficient to block IL-1-mediated neuroprotection, also blocked IL-6-mediated neuroprotection, but did not affect TNF-alpha-mediated neuroprotection (Fig. 4). These results demonstrate that, in contrast to TNF-alpha, the neuroprotective activities of IL-1 (α and β) as well as IL-6 involve the engagement of an IL-1 receptor.
this concentration inhibit IL-6-induced responses to any further extent (data not shown). Possible explanations for this result are discussed in the next section.

One feature of the TNF-α-induced neuroprotective pathway is its relationship with the neuronal nicotinic acetylcholine receptor (nAChR) system. By itself, nicotine (10 μM) is neuroprotective against NMDA-mediated excitotoxicity; however, the coapplication of TNF-α and nicotine to cultured neurons 24 h before the addition of NMDA completely abrogates the neuroprotective properties induced by either agent alone (Ref. 21, and Fig. 6). Both the neuroprotective properties of nicotine and its antagonistic effect toward TNF-α can be reversed by 10 nM α-bungarotoxin, a compound that is a potent and highly specific antagonist of nAChRα7 subunit-containing neuronal nAChRs (31, 32). To determine whether activity of other cytokines is also modulated by nAChR, nicotine was coapplied with IL-1α, IL-1β, or IL-6. A concentration of nicotine sufficient to block TNF-α-mediated neuroprotection had no effect on neuroprotection conferred by IL-1α, IL-1β, or IL-6 (Fig. 6). Therefore, the antagonistic relationship between nicotine and TNF-α does not extend to the other inflammatory cytokines and, again, supports the hypothesis that these cytokines impart neuroprotection through distinct intracellular pathways or mechanisms.

Discussion

We have shown that treatment of cortical neuronal cultures with recombinant IL-1 (α or β) or IL-6 confers a concentration-dependent neuroprotective effect against an excitotoxic challenge with NMDA. Specificity, and in some cases overlap, between these respective pathways in imparting neuroprotection can be demonstrated by antagonizing components unique to the cellular response imparted by each inflammatory cytokine. The TNF-α neuroprotective pathway requires activation of p55/TNFRI receptor, release of neuronal stores of endogenous TNF-α, and is sensitive to inhibition by nicotine activation of nAChRα7-containing receptors (21). By contrast, IL-1-mediated neuroprotection was inhibited by IL-1ra and by anti-NGF neutralizing Abs, agents that had no effect on neuroprotection mediated by TNF-α. Combined, these results suggest that neuroprotection mediated by IL-1 or TNF-α proceeds through independent pathways. Neuroprotection conferred by IL-6, as measured in the present experiments, also appears to be distinct from that of TNF-α, but requires activation of the IL-1 receptor. Nevertheless, neutralizing Abs to NGF only partially inhibited neuroprotection induced by IL-6. Several possibilities can be postulated to explain these results. First, while IL-1 receptor-mediated events are required for IL-6-induced neuroprotection (as determined by IL-1ra inhibition), other mediators stimulated by IL-6 (33) may contribute to neuroprotection by this cytokine. In this case, partial block of neuroprotection by anti-NGF-neutralizing Ab would be expected. Second, it has been suggested that IL-6 may enhance NGF activity (34, 35). Consequently, the amount of α-NGF-neutralizing Ab added to cultures may not be sufficient to completely inhibit NGF. However, doubling the amount of anti-NGF Ab added to the cultures had no greater effect on inhibition of IL-6-mediated neuroprotection (data not shown). A third possibility could reflect a nontraditional interaction of this cytokine with other neurotransmitter receptors. For example, as noted in the introduction, we and others (16, 36) have shown that...
IL-1α or β both have the ability to enhance the function of certain inhibitory γ-aminobutyric acid-activated receptors. The possibility that IL-6 or combinations of inflammatory cytokines may have an equivalent nontraditional direct role in modulating neurotransmitter receptor function (4, 37), or possibly an indirect modulatory role, as may occur through the stimulation of specific modulators of neurotransmission (e.g., NO production (38), or arachidonic acid metabolites (39)), has not yet been investigated in our system, but their contribution cannot be ruled out as a potential mechanism through which neuroprotection can be conferred.

Of note is the finding that nicotine inhibits TNF-α-induced, but not IL-1- or IL-6-induced neuroprotection. Nicotine stimulates neurons via activation of nAChRs and, specifically for neuroprotection, those nAChR composed of α7 subunits (31, 32). Activation of this ligand-gated ion channel induces a flux of calcium into the cell as well as other intracellular events (40–43). How these intracellular events interfere with TNF-α-induced effects is unknown. Numerous signal transduction pathways activated by TNF-α through p55/TNFFR1 have been reported (44–48). For example, TNF-α stimulates sphingomyelinas(e) activity to enhance the hydrolysis of sphingomyelin to ceramide and sphingosine (49, 50). In fact, ceramide is a major mediator of TNF-α-induced cellular events (51), and cell permeable analogues of ceramide can mimic the effect of TNF-α and confer neuroprotection to β-amyloid toxicity (52, 53). IL-1 has also been shown to generate ceramide (54, 55), but it is not yet known if ceramide generated subsequent to IL-1 receptor activation contributes to neuroprotection. However, phospholipase A2-associated release of arachidonic acid and metabolism by lipoxygenase accompanying activation of the IL-1 receptor has been shown to play an integral part in IL-1 stimulation of NGF secretion in astrocytes in culture (56).

Since TNF-α has been shown by many groups (1–4, 45) to induce IL-1 and IL-6 production in peripheral cells, why is IL-1 or IL-6 neuroprotection not conferred subsequent to TNF-α stimulation? First, the TNF-α-stimulated protein production may not be temporally compatible with the likely narrow window in which these cytokines must function to impart protection (21). Second, normal caspase (e.g., IL-1 converting enzyme) activation could be altered in our system, resulting in inappropriate protein processing or release. This latter point is particularly crucial when examining inflammatory cytokine function in cellular systems, since cytokine-mediated neuroprotection does not occur when concentrations are either too low or too high. Resolution of these issues will await further kinetic and biochemical analysis of the pathways defined in this report.

This study outlines the beneficial effects of IL-1 and IL-6 in promoting neuronal survival in an in vitro system. There are, however, numerous studies in vivo indicating that these cytokines may have a deleterious effect on neuronal survival. Notably, most of these reports are in the context of pathological conditions such as cerebral ischemia, where the expression of both IL-1 and IL-6 is induced and the increased expression of IL-1 appears to contribute to neuronal death (57). Further, increased expression of IL-1α can be neuroprotective in some instances (1, 2, 58). In contrast to our studies, where we see IL-1 as a neuroprotective agent, the concentrations of IL-1 are considerably higher when used as an agent of neuronal death. Further, while IL-6 has also been reported by others to diminish excitotoxic neuronal death in vitro (59) and in vivo following cerebral ischemia (60), in transgenic animals, overexpressing IL-6 pathogenic alterations in the CNS are readily apparent (61–63). These findings underscore the importance of understanding the effects of CNS cytokines and cytokine networks in various physiological contexts, including the presence and identity of participating cytokines, their concentration, and their participation in neurotransmitter function.

References
