The contrasting roles of ICE family proteases and interleukin-1β in apoptosis induced by trophic factor withdrawal and by copper/zinc superoxide dismutase down-regulation

(oxidative stress/neuronal cells/nerve growth factor/antisense oligonucleotides/cell death)

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ABSTRACT We compare here the mechanisms of apoptotic death of PC12 cells induced by down-regulation of Cu²⁺,Zn²⁺ superoxide dismutase (SOD1) and withdrawal of trophic support (serum/nerve growth factor). Our previous results indicated that the initiating causes of death are different in each paradigm. However, bcl-2 rescues cells in either paradigm, suggesting common downstream elements to the cell death pathway. To determine whether the ICE [interleukin 1β converting enzyme] family of proteases, which is required for apoptosis on trophic factor withdrawal, is also required for apoptosis induced by oxidative stress, we have developed a novel peptide inhibitor that mimics the common catalytic site of these enzymes and thereby blocks their access to substrates. This differs from the more usual pseudosubstrate approach to enzyme inhibition. Blockade of ICE family proteases by either this inhibitor or by a permeant competitive ICE family antagonist rescues PC12 cells from apoptotic death following apoptosis induced by down-regulation of SOD1, as well as from trophic factor/nerve growth factor deprivation. SOD1 down-regulation results in an increase in interleukin 1β (IL-1β) production by the cells, and cell death under these conditions can be prevented by either blocking antibodies against IL-1β or the IL-1 receptor antagonist (IL-1Ra). In contrast, trophic factor withdrawal does not increase IL-1β secretion, and the blocking antibody failed to protect PC12 cells from trophic factor withdrawal, whereas the receptor antagonist was only partially protective at very high concentrations. There were substantial differences in the concentrations of pseudosubstrate inhibitors which rescued cells from SOD1 down-regulation and trophic factor deprivation. These results suggest the involvement of different members of the ICE family, different substrates, or both in the two different initiating causes of cell death.

Members of the family of cysteine proteases related to the interleukin 1β converting enzyme (ICE) have been shown to be necessary for programmed cell death in a number of biological systems (1). For example, mutations of the ICE homologues, ced3, inhibit cell death which normally occurs during development in Caenorhabditis elegans (2), and overexpression of ICE or the ICE-like proteases NEDD-2/ICH-1 and Yama/apopain/CPP32 induces apoptosis in primary neurons, rat fibroblasts, and insect cells (3–7). Mice lacking ICE are resistant to apoptosis induced by Fas antibody (8). In the mammary gland, ICE mRNA is expressed during involution, when apoptosis occurs in this tissue (9). The pox virus product crmA, a serpin-like pseudosubstrate for ICE and ICE-like proteases, protects sensory neurons and fibroblasts from trophic factor withdrawal-induced death (3, 4), whereas Fas/APO-1 mediated apoptosis is blocked by the ICE-inhibitory pseudosubstrate peptide YVAD, as well as by crmA (10–13). Normal motor neuron loss in development is also blocked by YVAD, a pseudosubstrate which mimics the pro-interleukin 1β (IL-1β) cleavage site and thus inhibits ICE-like proteases (14). Whereas ICE cleaves pro-IL-1β to produce IL-1β, the role of IL-1β itself in apoptosis is unresolved, and it has been suggested that other substrates may be critical in cell death (15–17).

In the clonal rat pheochromocytoma line PC12, a commonly used model for neuronal differentiation and cell death (18–22), both trophic factor withdrawal (23) and oxidative stress induced by down-regulation of Cu²⁺,Zn²⁺ superoxide dismutase (SOD1) result in apoptosis (24, 25). The former is inhibited by cAMP analogues (19), N-acetylcysteine (20), and a variety of growth factors including nerve growth factor (NGF; refs. 19, 21, and 22), but not by vitamin E (20) or inhibitors of nitric oxide synthase (26). The latter, in contrast, is insensitive to cAMP analogs, N-acetylcysteine, and growth factors, but is blocked by vitamin E and, consistent with a role for peroxynitrite generation, by nitric oxide synthase inhibitors (24, 25). In spite of these differences, the finding that bcl-2 overexpression rescues PC12 cells from death induced by either trophic factor deprivation (27) or SOD1 down-regulation (25) suggests that these apoptotic pathways ultimately converge. Inhibition of ICE-like proteases has been shown to block apoptosis induced by trophic factor deprivation, but the role of these enzymes in oxygen-radical induced death has not been previously explored. The studies presented here show an obligate role for ICE-like proteases in both paradigms and place them, together with bcl-2, on the shared branch of the apoptotic pathway. However, these studies also show that IL-1β itself can play a critical role in death initiated by SOD1 down-regulation but only a minor role in apoptosis caused by withdrawal of trophic support, and these studies therefore suggest involvement of different members of the ICE family in the two initiating causes of cell death.

MATERIALS AND METHODS

Cell Culture. PC12 Cells. PC12 cells were grown as described (18) on rat-tail collagen-coated dishes in RPMI 1640 medium containing 5% fetal calf serum and 10% heat-inactivated horse serum (complete medium). NGF-primed PC12 cells were grown in 1% horse serum and exposed to NGF before use.

Abbreviations: NGF, nerve growth factor; IL-1β, interleukin 1β; ICE, IL-1β converting enzyme; SOD1, Cu²⁺,Zn²⁺ superoxide dismutase; V-ASOD1, vector-linked antisense oligonucleotide to SOD1; FMK, fluoromethylketone; CMK, chloromethylketone; V-ICE, vector peptide linked to the hexapeptide IOACRG ICE family inhibitor.

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for at least 7 days in RPMI 1640 medium with 1% horse serum and NGF (100 ng/ml). For cell survival assays involving trophic factor deprivation, cells (either naïve or NGF-pretreated) were extensively washed in serum-free RPMI 1640 medium and replated on fresh collagen-coated 24-well dishes as described (19) in RPMI 1640 medium lacking serum or NGF. For SOD1 down-regulation survival assays, cells were replated in complete medium with vector-linked antisense oligonucleotide to SOD1 (V-ASOD1; 50 nM) as described (25). Various concentrations of ICE inhibitors were included in the medium as indicated. Numbers of viable cells per culture were determined by quantifying intact nuclei as previously described (19). Counts were performed in triplicate and reported as means ± SEM.

Sympathetic Neurons. Sympathetic neuron cultures were prepared from 2-day-old rat pups as described (20). Cultures were grown in 24-well collagen-coated dishes in RPMI 1640 medium supplemented with 10% horse serum with mouse NGF (100 ng/ml). One day following plating, uridine and 5-fluorodeoxyuridine (10 μM each) were added to the cultures and left for 3 days to eliminate nonneuronal cells. On the sixth day following plating, NGF was removed by washing the cultures three times with RPMI 1640 medium with 10% horse serum, followed by the addition of medium containing anti-mouse NGF (1:200; Sigma) with or without ICE inhibitors. Each culture was scored, as described (28), as numbers of living, phase-bright neurons at various times. Three replicate cultures were assessed for each condition, and data were normalized to numbers of neurons present in each culture at the time of NGF withdrawal and reported as means ± SEM.

Assay of ICE Activity. Recombinant human pro-IL-1β was purchased from Citron (Pine Brook, NJ) as a 10 μg/ml solution in 10 mM Tris (pH 8.1), 0.1% Triton X-100, 0.1 mM EDTA, and 10% glycerol. The assay of pro-IL-18 cleavage was carried out in buffer containing 100 mM Hepes (pH 7.5), 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), 10 mM DTT, and 10% sucrose. Ten nanograms of pro-IL-1-B was incubated with or without 1 mM IQACRG (a hexapeptide) for 30 min at 37°C. Three units of recombinant human ICE (kindly provided by Nancy Thornberry, Merck, Rahway, NJ) were then added, and the reaction mixtures were incubated for 30 min at 25°C (29). Reactions were stopped with 2× Laemmli sample buffer (30) containing 10 mM DTT, and the samples were boiled for 3 min and subjected to SDS/15% PAGE followed by immunoblotting. The blot was probed with a monoclonal antibody to human IL-1β (1 μg/ml, kindly provided by the National Cancer Institute, Frederick, MD) and then visualized by enhanced chemiluminescence (Amersham) using anti-mouse IgG peroxidase as secondary antibody.

Coupling of Antennapedia Peptide (Vector Peptide) with the Hexapeptide IQACRG ICE family inhibitor. IQACRG, synthesized by Chiron, was resuspended in tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) buffer, an equimolar ratio of NPyS-pAntp43-58 peptide (Penetratin 1; Oncor), hereafter called the vector peptide, was added, and the mixture was incubated at 20°C for 2 hr (31). The yield of the reaction, estimated by SDS/PAGE followed by Coomassie blue staining, was routinely >50%. Control peptides (GRCAQI and ICGRAQ) were coupled to the vector peptide in the same way.

Assay of IL-1β. IL-1β was quantified by ELISA using the Interstel-1X kit (Genzyme). PC12 cells were grown as described above, on 24-well plates in 500 μl of medium. After a 1-day incubation, medium was removed, the amount of IL-1β was measured following the manufacturer's instructions, and the number of viable cells in each well was quantified.

Additional Materials. ZVAD (Z-Carbobenzyloxy)-fluoromethylketone (FMK) was from Enzyme Systems Products (Dublin, CA), and Ac-TYVAD-chloromethylketone (CMK) was from Bachem. Monoclonal human and murine IL-1β antibody (3ZD) was kindly provided by the National Cancer Institute, as was recombinant human IL-1β. Blocking monoclonal hamster anti-mouse IL-1β was purchased from Genzyme, and blocking antimurine IL-1α was from R & D Systems. Mouse IL-1 receptor antagonist was kindly provided by David Hirsh (Columbia University, New York).

RESULTS

In these studies we used three paradigms to induce apoptosis of cultured PC12 cells. In the first, naïve PC12 cells without NGF exposure were induced to die by the withdrawal of serum. In the second, PC12 cells that have been "primed" by NGF pretreatment in serum-free medium for a week and that have a neuronal morphology underwent apoptosis upon withdrawal of NGF and serum. The third model induced apoptosis by down-regulating SOD1 in either primed or naïve cells by exposure to an SOD1 antisense oligonucleotide. Withdrawal of serum resulted in the death of 50–85% of the cells within 24 hr, and NGF/serum deprivation and SOD1 down-regulation resulted in 50–60% mortality by this time (19, 20, 24, 25).

To investigate the role that ICE-like proteases play in apoptosis induced by these treatments, we used a peptide that mimics the conserved active site, IQACRG, of the ICE-family of proteases (5) and that was anticipated to bind to substrates and thereby block their cleavage. IQACRG should inhibit activity regardless of the specific substrate, avoiding the problem posed by differences in the preferred substrates for individual members of the ICE family. A search of the Swiss Protein Bank revealed that only ICE family members have this sequence. This approach therefore minimizes the chance of blocking cysteine proteases other than those in the ICE-family.

To verify that IQACRG blocks ICE activity, we tested the capacity of recombinant ICE to cleave recombinant pro-IL-1β

![Fig. 1](image-url)
in the presence or absence of the peptide. As shown in Fig. 1A, the peptide effectively inhibits ICE cleavage of pro-IL-1β in vitro. There is some cleavage in the presence of the inhibitor but much less than in the presence of ICE alone. There was no inhibition seen with a scrambled sequence of the peptide (data not shown). Cellular uptake of the IQACRG peptide was facilitated by linking it to the highly penetrant 16-aa Antennapedia peptide (Fig. 1B), which greatly enhances cellular uptake of peptides as well as antisense oligonucleotides (25, 31, 32). The Antennapedia vector peptide (V) was linked to IQACRG (ICEinh) by a reducible peptide bond to form V-ICEinh. Previous studies have shown that after uptake, reduction of the S-S bond releases free peptide within the cell (31).

Inhibitors of ICE-Like Proteases Protect PC12 Cells and Sympathetic Neurons from Death Induced by Withdrawal of Serum and NGF. We anticipated from the reported inhibition of cell death in NGF-deprived sensory neurons by crmA (3) that if V-ICEinh significantly inhibits ICE-family proteases in vivo, it should block cell death caused by withdrawal of serum from naive PC12 cells and serum and NGF from neuronally differentiated PC12 cells. The data in Fig. 2A show that this is the case with complete protection at 24 hr obtained with 200–400 nM peptide. Treatment with V-ICEinh provided partial protection of naive and primed PC12 cells (Fig. 2B) for at least 8 days. To control for possible nonspecific actions of V-ICEinh, we also tested reversed and scrambled V-linked peptides in the PC12 cell system and found neither to be effective or toxic over the same concentration range (data not shown). To further explore the role of ICE proteases in this system, the permeant competitive inhibitor ZVAD-FMK was also assessed. This also blocked cell death, but it required much higher concentrations (50 μM) to be fully protective (Fig. 2C). The peptide Ac-YVAD-CMK (15), an additional competitive inhibitor of ICE family proteases, was only partially effective at 250 μM (data not shown).

In parallel experiments, NGF-deprived sympathetic neurons exposed to V-ICEinh (50 nM) were completely protected from death for 3 days, and death was retarded for at least 6 days (Fig. 3A). Although a second addition of V-ICEinh after 24 hr somewhat prolonged survival time, subsequent treatment at daily intervals did not. As reported with crmA protection (3), cell bodies but not neurites are maintained with V-ICEinh (Fig. 3B–D). ZVAD-FMK (100 μM) also protected from NGF deprivation, but Ac-YVAD-CMK had no effect, even at 250 μM (data not shown).

V-ICEinh Protects PC12 Cells from Death Induced by Down-Regulation of SOD1. Exposure of PC12 cell cultures to the antisense oligonucleotide ASOD1 results in rapid down-regulation of SOD1 activity and the death of 50–60% of the cells within 24 hr (24). As shown in Fig. 4A, V-ICEinh protects both naive and NGF-treated PC12 cells from death in this paradigm. Control, scrambled, and reversed V-linked peptides were, in contrast, without effect (data not shown). V-ICEinh had no effect on the capacity of ASOD1 to lower cellular SOD1 (data not shown). Protection was the same whether the experiment was done in complete serum-containing medium or in serum-free RPMI 1640 medium supplemented with 3 μM insulin (data not shown). The protective effects of inhibiting ICE family proteases were confirmed using the inhibitory ZVAD-FMK peptide (Fig. 4B). The dose of V-ICEinh (25–50 nM) required for maximal protection was again considerably lower than that of ZVAD-FMK (6 μM). However, the concentrations of both V-ICEinh and ZVAD-FMK required to protect cells from SOD1 down-regulation were significantly lower than those needed to block apoptosis caused by trophic factor withdrawal (compare Figs. 2A and 4A). Moreover, Ac-YVAD-CMK afforded full protection from SOD1 down-regulation at 50 μM (data not shown), in contrast to its partial protection from trophic factor withdrawal at 250 μM.

Both Antibodies Against IL-1β and an IL-1 Receptor Antagonist Protect Fully Against SOD1 Down-Regulation but Not Trophic Factor Withdrawal. The inhibition of apoptosis by ICE inhibitors in each of our paradigms raised the question of whether IL-1β itself plays a direct role in cell death. Exposure of V-ASOD1-treated (SOD1-deficient) PC12 cells to blocking an-
tibodies against mouse (m) IL-1β at 30 μg/ml (Fig. 5A) or to the recombinant mL-1 receptor antagonist (rmIL-1Ra) at 100 ng/ml (Fig. 5B) completely suppressed cell death. Similar concentrations of antibodies against mL-1α and a nonblocking antibody to mL-1β failed to confer protection under these conditions. In contrast to the results in cells in which SOD1 had been down-regulated, the mL-1β blocking antibody failed to protect cells from trophic factor withdrawal at concentrations up to 100 μg/ml (Fig. 5A). Furthermore, the IL-1 receptor antagonist improved survival under these conditions only modestly and

![Graph A](image)

**Fig. 3.** V-ICE<sub>inh</sub> protects cultured sympathetic neurons from apoptotic death induced by NGF withdrawal. (A) Time course of protection of sympathetic neurons by V-ICE<sub>inh</sub> (50 nM). (B-D) Photomicrographs of sympathetic neurons: NGF (B), anti-NGF (C), and anti-NGF plus V-ICE<sub>inh</sub> (50 nM) (D). Sympathetic neuron cultures were prepared from 2-day-old rat pups. On the 6th day following plating, NGF was removed by washing the cultures three times with RPMI 1640 medium with 10% horse serum, followed by the addition of medium containing anti-mouse NGF (1:200). V-ICE<sub>inh</sub> (50 nM) was added to certain cultures as indicated. Numbers of surviving neurons was assessed by counting the number of intact, phase-bright neurons in each well by strip counting. This determination was made on the initial day of NGF deprivation and then on subsequent days. Results are expressed as the percentage of neurons present relative to that present immediately following NGF withdrawal.

then only at a concentration of 10,000 ng/ml (Fig. 5B), two orders of magnitude greater than the dose that fully protected cells from SOD1 down-regulation.

The disparity in effects afforded by blocking IL-1β or the IL-1 receptor in the two paradigms raised the issue of whether IL-1β secretion is differentially affected. When SOD1 is down-regulated, there is an almost 3-fold increase in IL-1β secretion per cell at 24 hr, an increase that is blocked by V-ICE<sub>inh</sub> (Fig. 5C). Upon withdrawal of trophic support, there is no increase in IL-1β secretion at 24 hr; in contrast, cells treated with NGF manifest a 4-fold increase in IL-1β secretion (Fig. 5C). Similar trends were observed at 6 hr of treatment in both paradigms.

The effectiveness of V-ICE<sub>inh</sub> in protecting cells against SOD1 down-regulation led us to question whether exogenous IL-1β could reverse this action. As shown in Fig. 5D, exogenous IL-1β potentiates cell death caused by V-ASOD1 and abrogates the protection provided by V-ICE<sub>inh</sub>. In contrast, in the absence of SOD1 down-regulation, exogenous IL-1β had no significant effect on survival. These observations support the notion that IL-1β is a necessary, but not sufficient, component in apoptotic death induced by SOD1 down-regulation.

**DISCUSSION**

It has been previously demonstrated that inhibition of ICE family proteases by CrmA protects neurons from trophic factor
withdrawal (3). By using a novel substrate-directed protease inhibitor designed to inhibit all members of the ICE family, we have now been able to show that ICE-like proteases are necessary for apoptosis induced by SOD1 down-regulation. This finding of commonality is in agreement with earlier data showing that expression of bcl-2 also protects in both paradigms (25, 26), as well as in a variety of systems in which cell death is initiated by different means (33, 34). Our recent findings indicate that PC12 cell death initiated by SOD1 down-regulation is dependent on generation of nitric oxide and therefore, apparently, on formation of peroxynitrite (25). The inhibition of cell loss by ICE family inhibitors suggests that free radicals such as peroxynitrite themselves do not directly cause apoptotic death and that they more likely function as signals that initiate a common death pathway.

Despite the presence of what appears to be a broadly shared final route to apoptosis, our data indicate that even this "final pathway" may show individuality, depending on the initiating causes of death. For example, we noted significant distinctions in the concentrations of ICE family inhibitors at which protection was obtained in our different paradigms. Cells were protected from SOD1 down-regulation at concentrations of V-ICEinh nearly an order of magnitude lower than those required to save them from trophic factor withdrawal. There was a comparable difference in the potency of ZVAD-FMK in the two systems. In addition, Ac-YVAD-CMK fully protected from SOD1 down-regulation while providing only partial protection from trophic deprivation, even at 5-fold higher levels. For sympathetic neurons, cell death evoked by NGF deprivation was abrogated by V-ICEinh and ZVAD-FMK but was unaffected by Ac-YVAD-CMK. It should be noted that Ac-YVAD-CMK would be expected to be more specific as an inhibitor of ICE, while ZVAD-FMK may more generally inhibit ICE family proteases (17). These differences in the efficacy of these compounds in the two paradigms could reflect the involvement of different members of the ICE family of proteases in each pathway, the availability of different substrates, or a combination of the two.

The demonstration that the ICE family of proteases is involved in apoptosis in our models led us to investigate the role of IL-1β itself. Once again differences in the final pathway are apparent. Both a blocking antibody to IL-1β and the naturally occurring IL-1 receptor antagonist, IL-1ra, provide almost complete protection against SOD1 down-regulation. In contrast, the blocking antibody failed to protect against trophic factor withdrawal, and IL-1ra protected partially but only at extremely high levels. Although there is an increase of IL-1β release after SOD1 down-regulation, there was no detectable change after withdrawal of trophic support. This rules out the possibility that loss of trophic support triggers a massive release of IL-1β that can be blocked only by enhanced concentrations of ICE inhibitors or that
cannot be blocked by the levels of antibody or receptor antagonist that we employed. We further noted that the largest increase in IL-1β release occurs after exposure to NGF. Because NGF prevents rather than causes death of PC12 cells deprived of trophic support, it appears that enhanced IL-1β production is not sufficient to evoke death in our system. Consistent with this, addition of 1 μg of recombinant human IL-1β to PC12 cultures in the presence of NGF, insulin, or serum did not produce cell death (human IL-1β has been shown to be effective in rat; ref. 35). Therefore, apoptosis triggered by down-regulation of SOD1 does not appear due to increased secretion of IL-1β alone but rather due to a decreased vulnerability to this cytokine. This could stem from a variety of mechanisms, including enhanced responsiveness to IL-1β or increased nitric oxide production in response to IL-1β. IL-1β has been reported to induce nitric oxide production in pancreatic cells (36). Therefore it is possible that the increase in IL-1β release with either SOD1 inhibition or NGF treatment results in increased nitric oxide production and that, as we have discussed, nitric oxide is toxic in the first paradigm (25) and protective in the second (26). Therefore, protection by blocking antibody would occur only on SOD1.

Although it is tempting to exclude IL-1β as a major factor in death caused by trophic factor deprivation in our system, we cannot formally rule out the possibility that loss of trophic support makes the cells so exquisitely sensitive to even basal levels of this interleukin that blockade can be achieved only by extremely high antibody and receptor antagonist levels.

In spite of the differences in the specifics of the final steps leading to cell death in the two paradigms tested here, our data show the possibility of designing agents, which could block cell loss in neurodegenerations of diverse etiology. For example, the novel inhibitor V-ICE inh, which we used in these experiments, would be expected to inhibit all ICE family proteases by denying them access to their substrates. This differs from pseudosubstrate inhibitors such as those based on the motifs of peptides YVAD or DVED, which distinguish between individual members of the ICE protease family by mimicning the cleavage site of the substrate (17). In additional contrast to pseudosubstrate inhibitors, V-ICE inh should also avoid the problem of inhibiting non-ICE cysteine proteases. Although we have purposely designed a general substrate-directed inhibitor of ICE family proteases (V-ICE inh), the Antennapedia-linked vector should also be useful for facilitating internalization of pseudosubstrate ICE family inhibitors. Thus, this technology might be useful to target either all members or specific members of the ICE protease family. V-ICE inh may therefore be viewed as a prototype of a potential new class of therapeutic agents.

In addition to the general importance of free radicals in neurodegenerative disorders (37), the demonstration of a role for IL-1β in apoptosis induced by free radicals might have particular relevance to Alzheimer disease. The brains of patients dying from this disease have been reported to have elevated levels of IL-1 (38). Several recent reports have raised the possibility that free radical generation by glycated tau (39), amyloid (40), or both can occur in these brains. If this is the mechanism by which cell damage and loss occurs, it is likely that agents such as V-ICE inh, which block ICE activity and anti-inflammatory agents that block IL-1, might be useful in the treatment of the disease. The latter have already been reported to show promise in preliminary clinical studies (41–43).

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