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Caspase inhibitors prevent cisplatin-induced apoptosis of auditory sensory cells

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IN VITRO **studies tested the efficacy of three caspase inhibitors, Ac-VAD-cmk (caspase-1 inhibitor), z-DEVD-fmk (caspase -3 inhibitor) and B-D-fmk (BOCDFK, a general inhibitor), for protecting auditory sensory cells from cisplatin-damage induced loss. Treatment of 3-day-old rat organ of Corti explants with these caspase inhibitors protected > 80% of the auditory hair cells from cisplatin-damage initiated apoptosis. Dissociated cell cultures of 3-day-old rat spinal ganglia treated with any of these three caspase inhibitors in addition to exogenous neurotrophin have highly significant increases in neuronal survival following cisplatin exposure. These results indicate that loss of auditory sensory cells as a result of cisplatin-induced damage involves apoptosis and that blocking of this cell death pathway at the caspase level effectively rescues both hair cells and neurons.** ***NeuroReport*** **9: 2609–2614 © 1998 Rapid Science Ltd.**

Key words: Apoptosis; Auditory sensory cells; Caspases; Caspase inhibitors; Cisplatin damage; Protection

Introduction

Apoptosis has been shown to be an important process by which damaged cells are removed from injured tissues. Apoptosis can be initiated in a cell by a variety of insults, including loss of growth factor support, toxins and damage caused by chemotherapeutic agents or radiation. Cellular damage caused by these noxious stimuli triggers the release of cytochrome C from the mitochondria into the cytoplasm of the cell. Cytochrome C can then bind to a protein named apoptotic protease activating factor-1 (Apaf-1) and activate caspase 9, which results in the activation of downstream cascade of proteases and the initiation of apoptosis.[1] Alternative pathways of apoptosis activation include tumor necrosis factor (TNF) binding to its receptor that results in the activation of an upstream cascade of proteases.[1,2] The ultimate effectors of this cascade are a group of proteases first discovered in the nematode, *C. elegans.* As a result of mutations in the *ced-3* gene, organisms fail to undergo their normal developmental pattern of programmed cell death, and contain an excess of normal cells.[3] Ced-3 is a cysteine protease whose mammalian homologs are the interleukin 1β converting enzyme (ICE) family of caspases.[4,5] The homologous caspases that have been identified in mammalian models of apoptosis share a common peptide sequence surrounding the active site of the enzyme.[6] Targets of apoptosis include poly ADP-ribose polymerase (PARP), an enzyme involved in DNA repair, as well as nuclear lamins A, B, C, a subunit of the U1 small ribonucleoprotein and β actin.[7]

Activation of caspases (ICE-like proteases) results in the enzymatic destruction of DNA by endonuclease and histological changes characteristic of apoptosis.[8,9] Injection of caspases or overexpression of the genes that encode these caspases results in the induction of apoptosis.[10] Blocking the release of cytochrome C from mitochondria by either Bcl-2 or Bcl-x proteins[11] or by inhibiting the members of the ICE family of caspases by synthetic inhibitors or virus-produced proteins, e.g. poxvirus serpine CrmA and baculovirus p35,[12] that act as caspase inhibitors, aborts or prevents apoptosis in a number of different systems. Numerous inhibitors of the ICE family of caspases have been developed, allowing for a correlation between a specific casapse and its role in the activation of apoptosis in different cell types.[13,14]

Auditory damage due to ototoxins such as cisplatin is mediated by free radical generation, membrane lipid peroxidation damage, and is thought to be due to the initiation of apoptosis.[15–19] Apoptosis has been demonstrated to be responsible for the loss of both auditory neurons following loss of neurotrophin support[20] and vestibular hair cells following damage by a vestibulotoxic antibiotic.[21]

Cisplatin is an important chemotherapeutic agent that induces the death of malignant cells through a variety of mechanisms.[18] Studies on auditory neurons,[22] as well as auditory hair cells[15] in postnatal rat spiral ganglion cell cultures and organ of Corti explants, suggest that cisplatin induces synthesis of reactive oxygen intermediate (ROI) generated free radicals which have been shown to trigger activation of the apoptosis cascade. To investigate whether the apoptosis induced by cisplatin-damage to auditory hair cells and neurons could be prevented through the interruption of the apoptosis pathway by inhibition of caspases, we pretreated 3-day-old (P3) rat organ of Corti explants and dissociated cell cultures of P3 spiral ganglia with caspase inhibitors. The effectiveness of inhibition of different members of the ICE family of caspases on cisplatin-induced apoptosis was determined by hair cell counts and neuron counts in explants and cell cultures respectively and counts of TUNEL positive nuclei in the hair cell area of the organ of Corti explants.

Materials and Methods

Animals: All Wistar rats used in these experiments were housed in our AAA LAC-accredited animal facility and all procedures were in accordance with NIH and IACUC guidelines.

Organ of Corti explants: P3 Wistar rat pups were anesthetized in a methoxyflurane chamber, decapitated, their calvaria divided with the membranous labyrinths excised from each half of the divided skull. Organ of Corti explants with attached auditory ganglion were obtained by dissecting out the modiolus and striping off the stria vascularis from base to apex. 96-well culture plates (NUNC) were used as the culture system with each well receiving 100 μl Dulbecco's modified Eagle medium (DMEM, Gibco) containing a N1 cocktail (Sigma), supplemented with glucose to a final concentration of 6 g/l. Explants, one per well, with or without caspase inhibitors (i.e. caspase-1 inhibitor II or caspase-3 inhibitor II, Calbiochem or BOCDFK inhibitor, Enzyme Systems Products) were initially placed in a humidified incubator at 37°C with 5% CO_2 for 24 h, then 10 μg/ml CDDP (Bristol-Myers Squibb) was added to the media with or without caspase inhibitors (200 μM) for an additional 48 h of incubation.

Hair cell counting: At the end of the 72 h culture period, explants were fixed with 4% paraformadelhyde and stained with fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma) to identify the stereociliary bundles and the cuticular plates of the auditory hair cells. Phalloidin-FITC stained explants were observed with a Zeiss Axiophot epifluorescent microscope. Base, middle and apical segments of these explants were counted for the presence of inner and outer hair cells. Hair cell number was quantified per 0.1 mm of cochlear duct length using an ocular grid system and a ×20 objective lens. The minimum criteria for a countable hair cell was a phalloidin-FITC stained intact cuticular plate in the CDDP-exposed and caspase inhibitor treated/CDDP-exposed explants while a stained intact cuticular plate with a normal stereocilia bundle was required for control explants ($n = 20$ explants/condition).

TUNEL labeling of nuclei: Organ of Corti explants were fixed with 10% neutral-buffered formalin for 1 h at RT. TUNEL labeling was performed by using an ApopTag apoptosis detection kit (Oncor). The organ of Corti explants were observed and photographed with a Zeiss Axiophot microscope. TUNEL positive cells of basal, middle and apical segments of the explants were counted under ×20 magnification within a 30 grid box area (i.e. 3×10 boxes) positioned over the hair cell area of the organ of Corti explants. The number of TUNEL positive nuclei are expressed per 0.1 mm of cochlear duct ($n = 6$ explants/condition).

Dissociated cell cultures of auditory ganglia: Dissected P3 rat auditory ganglia were placed in a petri dish containing 0.25% trypsin (Gibco) and incubated at 37°C for 20 min. The enzymatic action of the trypsin was stopped by adding medium (DMEM + N1 + glucose) supplemented with 20% fetal bovine serum (Gibco). The ganglion cell suspension was transferred into a sterile centrifuge tube, spun down and resuspended in culture medium. The ganglion cell suspension density was adjusted to a concentration of 60 000 cells/ml, 100 μl of this suspension was seeded into each culture well (i.e. 6 000 cells/well). The culture wells of the 96-well NUNC plate were precoated with 0.1 mg/ml poly-D-lysine (1 h at RT) and 0.01 mg/ml laminin (1 h, 37°C) prior to seeding. Ganglion cell cultures were pretreated with neurotrophins, i.e. 50 ng/ml hrBDNF and 50 ng/ml hrNT-3 (Regeneron), and 200 μM of either caspase-3 inhibitor II or BOCDFK inhibitor for

24 h; 6 μg/ml CDDP was then added to some of the cultures for 48 h with both neurotrophins and caspase inhibitors at the aforementioned concentrations.

Neuron counting: At the end of 72 h cultures were fixed with methanol + acetone (1:1) for 20 min at –20°C. An anti-neurofilament antibody, α NF-66kDa, immunostained these neurons *in situ*[22]. Anti-NF-66kDa-stained neurons were counted for each culture well to determine neuronal survival. The criteria for a viable neuron was neurofilament-positive immunostained cell body and neuritic processes and projection that are more than 3 times the width of the neuronal cell body ($n = 20$ wells/condition).

Statistical analysis: All comparisons for statistical significance between experimental groups were performed by the Student's *t*-test with $p < 0.05$ considered significant.

Results

Caspase inhibitor protection of auditory hair cells: In control explants phalloidin-FITC stained specimens show a normal configuration of a single row of inner hair cells and three rows of outer hair cells in the area of the middle turn, as depicted in Fig. 1A. A similar middle turn area of a control explant after TUNEL labeling also defines the normal condition observed in these organ of Corti after 72 h *in vitro* with no TUNEL-labeled nuclei present (Fig. 1D). The hair cell counts (Fig. 2) and counts of TUNEL positive nuclei (Fig. 3) define the normal hair cell number and level of TUNEL-positive nuclei observed in these control cultures. Exposure to CDDP (10 μg/ml) for 48 h had a profound effect on both hair cell survival (Fig. 1B) and the number of TUNEL-positive nuclei in the hair cell area (Fig. 1E) of these middle turn segments of the explants. The hair cell number of the CDDP-exposed explants decreases by 62% (Fig. 2) along with a dramatic increase (100-fold) in TUNEL-positive nuclei present in the hair cell area of the CDDP-exposed explants (Fig. 3).

Treatment of the CDDP-exposed organ of Corti explants to an inhibitor of caspase (200 μM caspase-1 inhibitor II, 200 μM caspase-3 inhibitor II or 200 μM BOCDFK inhibitor) resulted in a dramatically different response of these explants for both hair cell survival (Fig. 1C) and TUNEL labeling of nuclei (Fig. 1F). Treatment of the organ of Corti explants with any one of the three caspase inhibitors tested prevented the loss of CDDP-damaged hair cells by apoptosis in a highly significant manner (Figs 2, 3) but did not protect the hair cells from damage to their sensory hair bundles initiated by exposure to CDDP (compare Fig. 1E with 1A). There was a slight advantage to using a combination of two caspase inhibitors (i.e. 100 μM caspase-1 inhibitor II + 100 μM BOCDFK inhibitor) to protect from CDDP-induced apoptosis, as is evident in the increased level of suppression of TUNEL-positive nuclei in the hair cell area of the caspase inhibitor protected explants, where TUNEL labeling was at a control culture level (Fig. 3).

Caspase inhibitor protection of auditory neurons: The neuronal survival data from dissociated cell cultures of P3 auditory ganglia were normalized to control for inter-experiment culture well variability. The level of all control culture wells of each experimental series was considered one and therefore results are presented on a log scale. The auditory neurons in these dissociated cell cultures were particularly sensitive to the neurotoxic effect of CDDP. At the 6 μg/ml level of CDDP exposure, over a 48 hr period, it is evident that neither neurotrophins (BDNF or NT-3) nor caspase inhibitor pretreatment could effectively prevent CDDP-damage induced loss of auditory neurons (Fig. 4). Only when these CDDP-exposed cell cultures were protected by treatment with a combination of both neurotrophins and any one of the three caspase inhibitors was CDDP-damage induced apoptosis of these neurons prevented in a highly significant manner.

Discussion

We have demonstrated that CDDP damage to cells can induce apoptosis in organ of Corti explants by monitoring for the nuclei of apoptotic cells with staining for DNA fragmentation using a TUNEL labeling kit (ApopTag). Explants exposed to CDDP showed a greater accumulation of TUNEL-positive nuclei in the area of the hair cells than can be accounted by the original hair cell population, which suggests that other cell types (e.g. Support cells) in hair cell area are also being lost via CDDP-initiated apoptosis. Treatment of CDDP-exposed explants both prior to and during CDDP exposure with either caspase-1 inhibitor II and/or BOCDFK inhibitor resulted in an almost complete absence of TUNEL labeling, indicating that no ApopTag-detectable DNA degradation was taking place. The absence of TUNEL-labeled nuclei in the caspase inhibitor-protected explants correlates strongly with an increase in hair cell survival in the inhibitor-treated explants (compare Figs 2 and 3). The prevention of hair cell loss from caspase inhibitor-protected organ of Corti explants, however, did not prevent the CDDP from damaging the sensory hairs of the

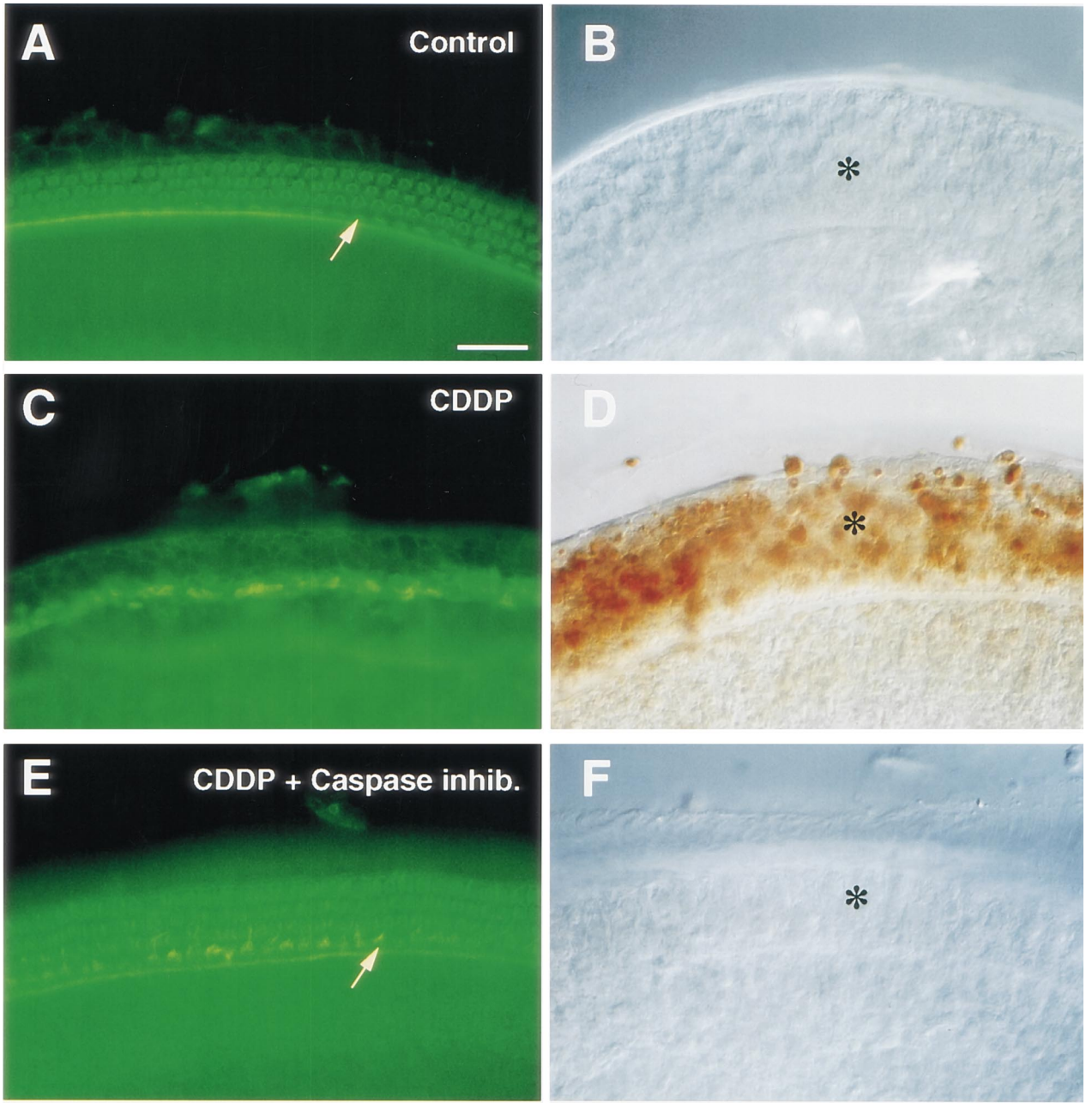

FIG. 1. P3 rat organ of Corti explants, 72 h *in vitro*. Middle turns segments: (**A**,**C**,**E**) phalloidin-FITC stained hair cells; (**B**,**D**,**F**) TUNEL labeling of nuclei. (A,B) Control, 72 h normal medium; no loss of hair cells, no TUNEL-positive nuclei in the hair cell area. (C,D) Cisplatin (CDDP)-exposure: normal medium 24 h; 10 μg/ml CDDP, 48 h. Extensive loss of auditory hair cells and many TUNEL-positive nuclei in the hair cell area. (E,F) Caspase inhibitor protection: 100 μM caspase-1 inhibitor + 100 μM BOCDFK, 72 h; 10 μg/ml CDDP, 48 h. Extensive hair cell stereocilary bundle damage but no hair cell loss, no TUNEL-positive nuclei in the hair cell area. Arrows in (A) and (E) indicate FITC–phalloidin-stained inner hair cells and the asterisks in (B), (D) and (F) indicate the hair cell area of the TUNEL-stained explants. Bar = 30 -μm (in A, also applies to C and E) and 15 μm (B,D,F).

stereocilia bundles (compare Fig. 1A and 1C), suggesting that CDDP-induced cellular damage still occurs with only the final cellular response of apoptosis being prevented by treatment with caspase inhibitors. Some studies on apoptosis have suggested that activation of a second cascade of enzymes that are responsible for degradation of actin.[2] Another recent report that supports the separation of actin proteolysis from the process of cell death is the finding that calpain inhibitors not caspase inhibitors can prevent actin proteolysis during apoptosis.[23] Our observations are consistent with such a scenario with cell loss being prevented but actin damage at the stereocilia level still occurring.

The result of this study demonstrates that apoptosis clearly plays an important role in the induction of auditory sensory cell death after CDDP exposure. CDDP-induced loss of auditory neurons is

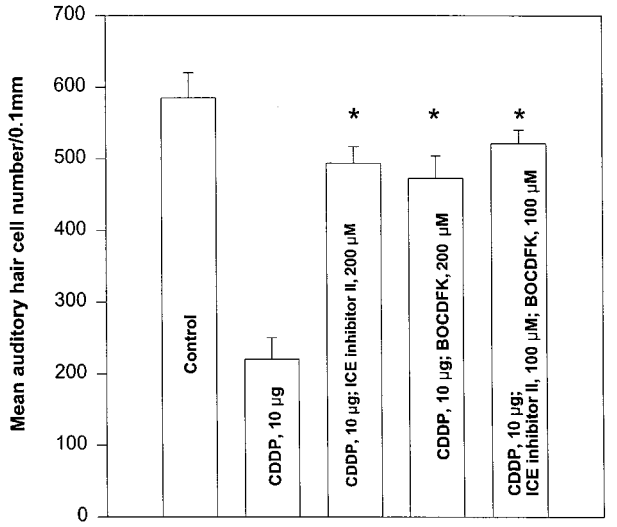

FIG. 2. Effect of caspase inhibitors on the hair cell density of P3 rat organ of Corti explants following CDDP-exposure. Control explants: untreated 72 h; CDDP-exposed explants: untreated 24 h, CDDP, treated 48 h; Caspase inhibitor protected explants, caspase inhibitor treated 72 h, CDDP-treated 48 h. Total culture period 72 h. Mean hair cell number represents total hair cell count values averaged for base, middle and apical segments counted for all explants examined. The asterisks denote a significant difference ($p < 0.05$) between the indicated columns and the CDDP only column. $n = 20$ explants/condition.

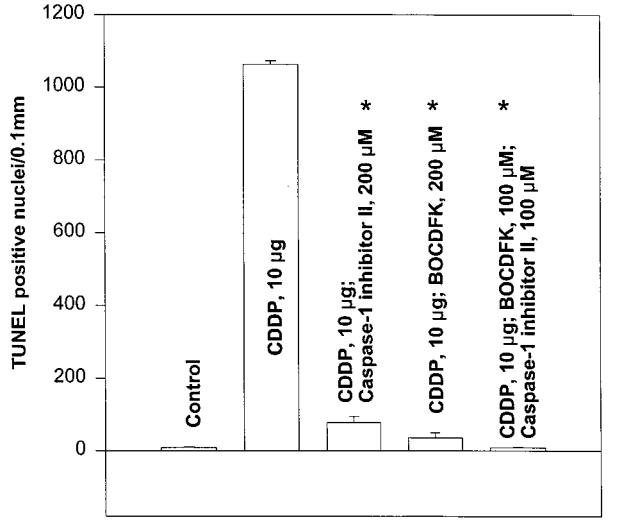

FIG. 3. Effect of caspase inhibitor on TUNEL-positive nuclei in the hair cell area of P3 rat organ of Corti explants following CDDP exposure. Control explants, untreated 72 h; CDDP-treated explants, untreated 24 h, CDDP treated 48 h; Caspase inhibitor-protected explants, caspase inhibitor-treated 72 h, CDDP-treated 48 h. Total culture period 72 h. The asterisks denote a significant difference between the indicated columns and the CDDP only column. $n = 6$ explants/condition.

also mediated by ROI-generated free radicals,[22] resulting in activation of an apoptosis cascade of cellular caspases. Inhibition of these caspases by caspase inhibitors prevented an apoptotic cell death cascade in our neuronal cell cultures if also treated with neurotrophins but not if treated by either neurotrophin alone or a caspase inhibitor alone (Fig. 4). This requirement for both a caspase inhibitor and neurotrophin to prevent apoptosis may be the result of the combination of stresses placed on these neurons since they are both placed in dissociated cell culture disrupting their normal spiral ganglion cell-cell relationships. Additionally these cultures are exposed to the neurotoxic effects of CDDP for longer (i.e. 48 h) than in a previous study[22] in which a neurotrophin alone was shown to protect the neurons from the neurotoxicity by a lower concentration (4 g/ml) of CDDP after 24 h of CDDP treatment. Neurotrophins may play a role in repairing some of the CDDP-induced cellular damage in these neurons, thereby augmenting the ability of the caspase inhibitor in the prevention of neuronal cell loss. CDDP damage induces the apoptotic cell death of both auditory neurons and auditory hair cells. The results of the current study (Figs 1–4) clearly demonstrate that caspase inhibition can act to prevent the CDDP-damage initiated cell death of these auditory sensory cells but does not prevent damage to the auditory hair cells (Fig. 1C) and perhaps the neurons since neurotrophins are required in combination with the caspase inhibitor to prevent neuronal losses (Fig. 4).

This blocking of cell death but not of CDDP-induced cell damage may be a useful model to study the ability of damaged auditory hair cells to self-

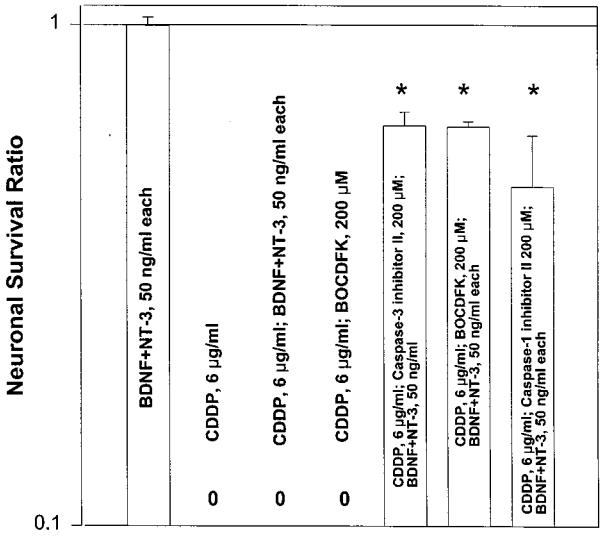

FIG. 4. Effect of caspase inhibitor and neurotrophin treatment on neuronal survival in dissociated cell cultures of P3 rat auditory ganglia following CDDP-exposure. Control cultures, untreated 72 h; CDDP-treated cultures, untreated 24 h, CDDP-treated 48 h; Neurotrophin-protected cultures, neurotrophin-treated 72 h, CDDP-treated 48 h. Caspase inhibitor cultures, caspase inhibitor-treated 72 h, CDDP-treated 48 h; Caspase inhibitor + neurotrophin-protected cultures, caspase inhibitor + neurotrophin-treated 72 h, CDDP-treated 48 h. Total culture period 72 h. The asterisks denote a significant difference ($p < 0.05$) between the indicated columns and the CDDP only, the CDDP +neurotrophins, and the CDDP + caspase inhibitor columns. $n = 20$ cultures/condition.

repair and the role of trophic molecules such as transforming growth factor-α in this process. Studies are underway to explore these possibilities for auditory hair cells and auditory neurons.

Conclusion

Apoptosis plays a major role in the loss of cisplatin-damaged sensory cell from the auditory receptor. Blocking the action of members of the interleukin 1β converting enzyme (ICE) family of caspases with inhibitors prevents loss of auditory sensory cells from cisplatin-induced apoptosis *in vitro*. Caspase inhibitor-rescued auditory hair cells survive but are damaged from CDDP-exposure and are in need of repair, e.g. growth factor treatment.

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