N-Acetylcysteine-promoted Survival of PC12 Cells Is Glutathione-independent but Transcription-dependent*

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Our prior work established that comparable concentrations of N-acetylcysteine (NAC) both block the proliferation of PC12 cells and prevent death of trophic factor-deprived sympathetic neurons and PC12 cells. The present work addresses several aspects of the mechanisms of these actions. NAC increases intracellular levels of glutathione (GSH) by approximately 10-fold in PC12 cells. However, blockade of this increase by treatment with buthionine sulfoximine did not affect either promotion of survival or inhibition of DNA synthesis. Thus, these actions of NAC are independent of its effects on intracellular GSH. NAC’s actions in our system do not appear to be dependent on its anti-oxidant/radical scavenger properties, but may be due to its activity as a reductant. Consistent with this, several other reducing agents, the most effective of which was 2,3-dimercaptopropanol, mimicked NAC in blocking DNA synthesis and suppressing death of PC12 cells and sympathetic neurons. Finally, we observed that in striking contrast to nerve growth factor and a number of other trophic agents, the survival-promoting effects of NAC on PC12 cells are blocked by actinomycin D. This suggests that NAC may act by inducing specific gene expression.

Apoptotic cell death is a normal aspect of development as well as a consequence of cellular injury or degeneration (1, 2). In the nervous system, developmental neuronal apoptotic death appears in part due to competition for limited amounts of target-derived trophic factors (1). As judged by their palliative effects when administered in experimental models, trophic factors may also be involved in regulation of nerve cell death associated with brain injury or neurodegenerative disease (3–5). In an effort to understand the basic mechanisms by which trophic agents prevent death of neuronal and non-neuronal cells and to devise drugs that may be useful for amelioration of maladies characterized by cell death, extensive efforts have been made to identify small molecules that mimic the survival-promoting actions of trophic factors.

Two model systems that have been used to study regulation of neuronal cell death by trophic factors and small molecules are cultured neonatal sympathetic neurons and the PC12 pheochromocytoma cell line. Cultured neonatal rat sympathetic neurons can be maintained in the presence of the trophic factor NGF† and undergo apoptotic death when the factor is withdrawn (6–8). PC12 cells do not require NGF when grown in serum-containing medium, but rapidly die by an apoptotic mechanism when deprived of serum (9–12). Under such serum-free conditions, NGF prevents PC12 cell death (9, 10); conversely, withdrawal of NGF under serum-free conditions triggers PC12 cell apoptotic death (12).

Exploitation of the sympathetic neuron and PC12 cell systems has uncovered a variety of small molecules that mimic NGF in preventing death (11, 13, 14). Recently, we reported that N-acetylcysteine (NAC) effectively maintains the long term survival of sympathetic neurons and PC12 cells in the absence of NGF (14). NAC also has been found to have survival promoting actions in several other cell systems (15–17). Exposure of cells to NAC leads to a large increase in intracellular levels of glutathione (GSH) (18), and it has been generally assumed that the survival-promoting actions of NAC are due to its direct or indirect (via intracellular GSH) action as an anti-oxidant or free radical scavenging agent.

In our prior work (14), we made several observations that are relevant to the mechanism by which NAC might prevent cell death caused by withdrawal of trophic support. First, NAC was unexpectedly found to inhibit PC12 cell proliferation with a dose-response curve similar to that for which it prevents cell death. This raised the possibility that capacity of NAC to prevent cell death is due to its anti-proliferative activity and is consistent with the hypothesis that death of PC12 cells and sympathetic neurons (as well as of other cell types) caused by trophic factor withdrawal is due to an aberrant attempt to complete or re-enter the cell cycle (19–22). Second, several other anti-oxidants and free radical scavenging agents did not share with NAC the capacity to prevent PC12 cell and sympathetic neuron death or to inhibit DNA synthesis. This suggested a mechanism for NAC other than as an anti-oxidant or free radical scavenger. Third, the β-stereoisomer of NAC, which should not be metabolized, was as effective as L-NAC in preventing apoptosis and blocking proliferation, thereby indicating that a metabolic product of NAC was not responsible for these actions. Last, both L- and β-NAC increased PC12 cell levels of GSH by at least 10-fold. This showed that the effect of NAC on GSH levels was not via direct metabolic conversion as suggested previously, but rather by reduction of extracellular cystine to increase available levels of cysteine, a precursor amino acid of GSH. This observation also raised the possibility that NAC actions, as often suggested, are mediated by GSH.

In the present work, we have continued our investigations of the mechanism of NAC actions. We directly tested whether the induced increase in intracellular GSH is indeed required for the survival and anti-mitotic properties of NAC. We also ascer-

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§ The abbreviations used are: NGF, nerve growth factor; NAC, N-acetylcysteine; BSO, buthionine-(S,R)-sulfoximine; DMP, 2,3-dimercaptopropanol; BME, β-mercaptoethanol; LNAC, N-acetyl-L-cysteine.
obtained whether NAC’s actions might be related to its effectiveness as a reducing agent. Last, we determined whether RNA synthesis is required for NAC’s survival-promoting activities.

**EXPERIMENTAL PROCEDURES**

**Materials—**Human recombinant NGF was a generous gift from Genentech and was used at a concentration of 100 ng/ml. N-Acetyl-L-cysteine, thioglycolate, 2,3-dimercaptopropional, β-mercaptoethanol, mouse NGF, and anti-mouse NGF antiserum were obtained from Sigma and directly dissolved in RPMI 1640.

**Cell Culture—**PC12 cells were cultured as described previously (23) in rat tail collagen-coated dishes with RPMI 1640 medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum.

For survival experiments, PC12 cells were washed extensively with serum-free RPMI 1640 medium as described previously (10) and plated in 0.5 ml of medium in collagen-coated 24-well plates at a density of 10–15 × 10^3/well. To feed the cells, and to avoid loss of floating live cells, 0.2 ml of fresh medium was added every other day.

Primary cultures of dissociated sympathetic neuronal cultures were obtained from the superior cervical ganglia of P1-P2 rat pups, dissociated in 0.25% trypsin, and plated in collagen-coated 24-well plates at the density of ~0.5 ganglion/well in RPMI 1640 medium supplemented with 10% horse serum and mouse NGF (100 ng/ml). A mixture of uridine and fluorodeoxyuridine (10 μM) was added to the culture on the second day to eliminate non-neuronal cells. After 3–5 days, the cultures were washed three times with RPMI medium plus 10% horse serum to remove the mouse NGF. The wells were then cultured in the same medium (0.5 ml/well), with the indicated additives, in the presence of NGF or anti-NGF rabbit antibodies at a dilution of 1:200.

**Cell Counts—**To determine the number of living PC12 cells in 24-well dishes, we removed the medium and replaced it with 0.1 ml of lysis buffer (25). This buffer lysed the cell membrane and leaves the nuclei intact. We then counted the nuclei in a hemacytometer. Broken or damaged nuclei were not counted. All counts were carried out in triplicate and are expressed as relative to cell counts obtained at day 0 ± 5 E.

For sympathetic neurons, cell counts were carried out by strip counting (26). Briefly, a strip was placed over the dish area, examined under high-power phase-contrast microscopy. Cells with neuron-like morphology that were located on this strip were scored. The same strip was counted on each day. Each well was scored individually and the survival expressed relative to the initial cell number in the same well (designated as 100).

**Thymidine Incorporation—**Triplicate cultures were plated in collagen-coated 24-well plates in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal bovine serum or with 3 μg/ml insulin and treated as described for 24 h. The wells were then incubated with 0.5 μCi/well of [3H]thymidine (DuPont NEN) for 1 h, and washed three times with ice-cold phosphate-buffered saline. The acid-soluble material was extracted with 0.3 ml of ice-cold 5% trichloroacetic acid (w/v) and discarded. The acid-insoluble material was solubilized with 0.150 ml of 1 N NaOH, neutralized with the same volume of 1 N HCl and subjected to scintillation counting.

**GSH Measurement—**Measurements of intracellular glutathione were carried out according to a modification of the method of Tietze (27). Briefly, cells were cultured at a concentration of 0.8–1.0 × 10^5/well in six-well dishes, washed three times with phosphate-buffered saline, lysed with 0.1 ml of 3% perchloric acid for 15 min at 4 °C, centrifuged, and the supernatant neutralized with 0.9 ml of 0.1 M NaH₂PO₄ buffer (pH 7.5). Glutathione contents were measured by the rate of colorimetric change of 600 μM 5,5′-dithiobis(nitrobenzoic acid) at 412 nm in the presence of 1 unit of glutathione reductase (Boehringer Mannheim) and 0.21 mM NADPH (Boehringer Mannheim). The perchloric acid pellet was resuspended in 1 N NaOH, and the solubilized protein measured by the Bradford method. Glutathione contents were normalized to total cell protein.

**RESULTS**

**The Survival-promoting and Anti-mitotic Actions of NAC Are Independent of GSH Levels—**A past study has shown that exposure of PC12 cells to 40–60 mM NAC protects them from apoptosis caused by trophic-factor withdrawal and increases their intracellular GSH levels by approximately 10-fold (14). To test whether the intracellular GSH increase caused by NAC is necessary for its survival-promoting effects, we treated PC12 cells with NAC and buthionine(S,R)-sulfoximine (BSO) for up to 3 days. BSO inhibits γ-glutamylcysteine synthase, the rate-limiting enzyme involved in GSH synthesis, and by this means causes the depletion of intracellular GSH (28). Fig. 1A shows that treatment with NAC alone resulted in a substantial increase in GSH at both time points. Fig. 1A further illustrates that the NAC-mediated increase in intracellular levels of GSH...
was effectively inhibited by 0.2 mM BSO, even after 3 days of continuous exposure. Dose-response experiments (over the range of 0.1–10 mM) established that 0.2 mM BSO is not toxic to the cells in the presence of serum. The data in Fig. 1B show that despite its inhibition of GSH accumulation, BSO did not significantly affect the capacity of NAC to rescue PC12 cells from death triggered by withdrawal of trophic support. This was equally true at 1 and 3 days of treatment. As shown in Fig. 2, the serum-deprived cells treated with NAC and BSO maintained a healthy appearance, in contrast to the untreated control cells which were dead. BSO administered alone did not maintain cell survival or inhibit cell proliferation.

Our past work (14) showed that the same concentrations of NAC that prevent PC12 cell apoptosis also inhibit DNA synthesis. To test whether the latter effect requires elevation of GSH levels, we assessed incorporation of [3H]thymidine in PC12 cell cultures treated with NAC alone or with NAC plus BSO. Fig. 1C shows that BSO did not affect NAC-induced inhibition of DNA synthesis. Taken together, these findings indicate that elevation of intracellular GSH does not account for the mechanism by which NAC rescues cells from death or inhibits their proliferation.

Reducing Agents Delay the Death of PC12 Cells and Sympathetic Neurons and Inhibit DNA Synthesis—In addition to its effect on intracellular GSH, NAC is an anti-oxidant. However, we noted in the past that other anti-oxidants such as vitamin E do not mimic NAC’s actions on cell survival and DNA synthesis (14). Another property of NAC is to function as a reducing agent able to mediate thiosulfate exchange reactions (29). We therefore tested whether any of the GSH-independent actions of NAC might be due to its reducing activity. To carry this out, we assessed several additional reducing agents known to act in biological systems, including thioglycolate, β-mercaptoethanol and 2,3-dimercaptopropanol (DMP, also known as British anti-Lewisite). Dose-response experiments were performed to evaluate the protective and toxic actions of these compounds on serum-deprived PC12 cells maintained with or without NGF; these revealed that the optimal concentrations that conferred protection at 24 h, but that were not toxic, were 15 mM thioglycolate, 1 mM BME, and 150 μM DMP. At these concentrations, the reducing agents provided partial protection from cell death for 24 h in the absence of trophic support (Fig. 3A). DMP, the most effective compound tested, significantly delayed cell death up to approximately 5 days (Fig. 4). None of the other compounds provided long-term protection from death (data not shown). DMP increased intracellular GSH levels by 5-fold at 24 h of treatment, but as in the case of NAC, its ability to promote survival was not affected by the presence of BSO (control: 14% ± 1 survival; DMP: 70% ± 4; DMP + BSO: 74% ± 5). Thus, reducing agents in addition to NAC can at least delay cell death and appear to do so by mechanisms independent of GSH elevation.

Our past experiments suggested that withdrawal of trophic support leads to apoptosis by resulting in uncoordinated cell cycle progression and showed correlation between the capacity of NAC to block cell death and to inhibit DNA synthesis. We therefore tested whether other reducing agents also affect cell division as assessed by inhibition of [3H]thymidine incorporation. PC12 cells were cultured in serum-free medium with insulin, which supports proliferation and full survival, and treated with the reducing agents at their respective optimal concentrations. As shown in Fig. 3B, thioglycolate and DMP, which were the most effective in maintaining cell survival at 24 h of serum deprivation, also inhibited a substantial fraction of thymidine incorporation at 24 h of treatment. When the potencies of DMP for promoting survival and for suppressing DNA synthesis at 24 h were examined in further detail, this revealed a reciprocal relationship with maximal survival occurring at concentrations yielding maximal inhibition of thymidine incorporation at 24 h of treatment. When the potencies of DMP for promoting survival and for suppressing DNA synthesis at 24 h were examined in further detail, this revealed a reciprocal relationship with maximal survival occurring at concentrations yielding maximal inhibition of thymidine incorporation (Fig. 5). In contrast, BME, which was relatively ineffective in promoting survival, had relatively little effect on DNA synthesis (Fig. 3B). These findings indicate that as for NAC, there is a good correlation between the capacities of several reducing agents to promote survival and to inhibit the synthesis of DNA.

We next tested whether DMP, like NAC, can suppress the death of sympathetic neurons brought about by withdrawal of NGF. In this system, the highest concentration of DMP that did
not cause morphological changes (i.e. cell detachment from the substrate) in the presence of NGF was 85 μM. As shown in Figs. 6 and 7, at this concentration, DMP significantly delayed neuronal death by up to 5–12 days; we observed the same effect at the concentrations of 50 and 75 μM. However, as in the case of NAC, DMP did not elicit or maintain neurite outgrowth, nor did it interfere with NGF’s ability to promote neurite outgrowth.

The Survival-promoting Actions of NAC Require Transcription—Previous work has established that NGF and other trophic substances, including cAMP analogs, do not require transcription to promote the survival of “naive” (NGF-untreated) PC12 cells (10). To determine whether this is the case for NAC, PC12 cells were washed free of serum; preincubated for 1 h with or without 10 μM of the transcriptional inhibitor actinomycin D; exposed to NAC, NGF, or chlorophenylthio-cAMP in the continued presence of actinomycin D; and evaluated 1 day later for survival. As shown in Fig. 8, NAC-promoted survival was inhibited by actinomycin D. This effect could not be attributed to toxicity because, as reported previously, actinomycin D did not affect promotion of survival by NGF or chlorophenylthio-cAMP. Similar results were achieved with DMP (data not shown). These observations thus suggest that the actions of NAC and DMP on survival are dependent on de novo synthesis of RNA.
DISCUSSION

NAC Actions and GSH Levels—In a previous study, we showed that NAC protects PC12 cells and sympathetic neurons from apoptotic death triggered by withdrawal of trophic support and inhibits PC12 cell proliferation (14). Here, we have sought to further elucidate the mechanisms by which NAC exerts these effects.

One well described property of NAC is to increase intracellular levels of GSH (18), and its protective actions have often been attributed to this effect (30, 43). We show here that BSO treatment completely blocks the NAC-induced increase in intracellular GSH, but has no effect on the capacity of NAC to protect PC12 cells from apoptotic death or to inhibit their synthesis of DNA. Thus, in this system, enhanced GSH levels cannot account for NAC's actions. This conclusion is consistent with several other observations in the literature. Jones et al. (31) recently reported that BSO treatment of T cells blocked approximately half of the increase in GSH levels generated by exposure to NAC, but had no effect on the capacity of NAC to protect these cells from apoptosis induced by exposure to anti-CD3 antibodies. NAC has been shown to inhibit viral replication and in this regard, Mihm et al. (32) showed that although exposure of Molt-4 cells to NAC or to GSH brought about comparable increases in intracellular GSH levels, only NAC suppressed cellular production of HIV. NAC also protects cells from apoptosis induced by Sindbis virus in N18 neuroblastoma cells, and this effect appears to be independent of increased intracellular GSH.2

NAC and Other Reducing Agents—Because NAC does not act in our system through GSH, we have considered alternate mechanisms. One possibility is that trophic factor withdrawal causes formation of reactive oxygen species which are suppressed by NAC acting as an antioxidant or free radical scavenger. However, several other anti-oxidants/free radical scavengers were unable to mimic NAC's survival-promoting and anti-mitotic actions in our system (14). Moreover, unlike vitamin E (an anti-oxidant and free radical scavenger), NAC does not protect PC12 cells from apoptotic death caused by diminution of their levels of SOD1 (33).3 In the latter case, death appears to be dependent on formation of reactive oxygen species.3 Taken together, these observations suggest that NAC's actions in our system are unlikely to be due to reaction with reactive oxygen species.

Another possibility is that a metabolic product of NAC mediates its actions in our experiments. However, this appears to be ruled out by the findings that the d-stereoisomer of NAC, which is presumably not metabolized, fully maintains cell survival and inhibits proliferation (14).

An additional mechanism we considered is that NAC effects are due to its actions as a reducing agent. The efficacy of NAC as a reducing agent in our system is reflected by our observation that d-NAC enhances intracellular GSH levels as well as

2 R. Ratan, personal communication.
3 Troy, C. M., Derossi, D., Prochiantz, A., Greene, L. A., and Shelandski, M. L. J. Neurosci., in press.
Reducing Agents Promote PC12 and Neuronal Survival

L-NAC (14), indicating that it reduces extracellular cysteine to cysteine. To assess this notion, we tested several other reducing agents in our paradigm. Both thioglycolate and dimercaptopropanol suppressed apoptotic death and inhibited DNA synthesis. Like NAC, DMP was also found to delay death of NGF-deprived sympathetic neurons and showed close correlation of the dose-response curves for promotion of survival and inhibition of thymidine incorporation. BME, in contrast, was less effective at both preventing death and suppressing synthesis of DNA. These findings are consistent with prior observations regarding the actions of reducing agents and indicate that the efficacy of a given reducing agent may vary with cell type (34–37). For instance, BME has been reported to enhance the survival of neurons cultured in serum-free medium (36, 37) and to decrease concanavalin A-induced proliferation of human lymphocytes (34).

Our observations with reducing agents raise several points. One is that none of the agents we have tested is as effective as L- or D-NAC in maintaining long term survival. The reason for this is unclear, but could include factors such as kinetics of entry and accumulation in cells, reducing potential or additional activities. A second point is that our data provide further correlation between the capacities of agents to inhibit DNA synthesis and to promote neuronal cell survival. Thioglycolate and DMP suppressed both death and thymidine incorporation, while BME was only partially effective in each case. This correlation supports the hypothesis that apoptosis caused by withdrawal of trophic support is due to an inappropriate attempt to re-enter or progress through the cell cycle. Finally, it must be acknowledged that the reducing agents we employed are also anti-oxidants. Thus, we cannot definitively rule out that this property contributes to their activities in our experiments (but see above).

NAC and Transcription—We show here that NAC-mediated rescue of PC12 cells from apoptotic death occurs through a transcription-dependent mechanism. This is in striking contrast to survival in this system mediated by NGF and cAMP derivatives as well as by FGF and IGFS, all of which occur by synthesis-independent mechanisms (10). We could not assess whether NAC-promoted survival of sympathetic neurons also requires transcription, because inhibition of protein or RNA synthesis in such cultures alone is sufficient to promote survival (38).

The mechanism by which NAC might alter transcription to promote survival is presently unclear. One possibility is via reduction of NGF or NGF receptors to modulate survival of PC12 cells (13). In contrast, the data presented here suggest that NAC may promote neuronal survival by a transcription-dependent mechanism. We tested this for the Trk NGF receptor and found that NAC promoted the survival of PC12 cells by increasing levels of Trk mRNA (1879–1887). The respective correlation between the capacities of agents to inhibit DNA synthesis and for valuable advice and Drs. S. E. Farinelli and A. Rukenstein for helpful discussion.

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