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# Free Radical-Independent Protection by Nerve Growth Factor and Bcl-2 of PC12 Cells from Hydrogen Peroxide-Triggered Apoptosis[1]

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**To investigate the mechanism of oxidative stress-induced death of PC12 cells, we performed confocal and flow cytometric analysis with a reactive oxygen species (ROS)-specific fluorogen, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (C-DCDHF-DA). Hydrogen peroxide significantly decreased the number of viable PC12 cells after 24 h. Hydrogen peroxide caused membrane blebbing, nuclear condensation and DNA fragmentation, indicating that the PC12 cells died due to apoptosis. The hydrogen peroxide-triggered apoptosis of PC12 cells was associated with enhanced ROS production in a dose-dependent manner by measuring with C-DCDHF-DA. Nerve growth factor (NGF) and Bcl-2 inhibited the hydrogen peroxide-induced apoptosis of PC12 cells. Neither of them, however, reduced the ROS production in PC12 cells. These data suggest that NGF or Bcl-2 protects PC12 cells from hydrogen peroxide-triggered apoptosis independently from ROS production.**

**Key words: apoptosis, Bcl-2, hydrogen peroxide, NGF, reactive oxygen species.**

Recent studies indicated that apoptosis is required for normal development (*1, 2*). Common cellular changes accompany apoptosis. Morphologically, apoptosis, unlike necrosis, is characterized by membrane blebbing, cytoplasmic fragmentation, and nuclear condensation. Biochemically, endonuclease(s) are activated to cleave genomic DNA at internucleosomal regions (*3, 4*).

A rat pheochromocytoma cell line (PC12 cells) constitutes a useful model for studying the mechanism of apoptosis and the prevention of apoptosis. PC12 cells acquire a sympathetic neuron-like phenotype in response to nerve growth factor (NGF) (*5*), and undergo apoptosis in response to several agents. NGF and Bcl-2 effectively prevent some types of cell death (*6-9*). Oxidative stress, such as a high oxygen atmosphere (*10*), antisense nucleotides for Cu/Zn superoxide dismutase (SOD) (*11*), and L-DOPA (*12*), induces apoptosis rather than necrosis in PC12 cells. Although many efforts have been made to clarify the mechanism of oxidative stress-induced neural death *in vivo* and *in vitro*, the cellular and molecular mechanism remains unclear (for a review, *13*). To clarify this issue, we performed confocal and flow cytometric analysis with a ROS-specific fluorescent indicator, C-DCDHF-DA. Our results demonstrated that hydrogen peroxide triggered apoptosis associated with ROS production and that NGF as well as Bcl-2 inhibited the apoptosis without reducing ROS production. These data suggest that NGF or Bcl-2 protects PC12 cells from oxidative stress-induced cell death independently of ROS production.

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## MATERIALS AND METHODS

*Materials*—NGF (2.5S) was prepared from male mouse submandibular glands as described by Bocchini and Angeletti (*14*), with some of the modifications of Suda *et al.* (*15*). Fluorescein diacetate (FDA) was purchased from Polyscience, Hoechst 33,258 and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) (C-DCDHF-DA) from Molecular Probes, insulin from Collaborative, and *N*-acetylcysteine (NAC), transferrin, and progesterone from Sigma, respectively.

*Cell Culture*—Control vector and *bcl-2* expressible DNA-transfected PC12 subclones, PC-pAGE123 and PC-bcl10 (*16*), were maintained in 75 cm² flasks (Costar) using Dulbecco's modified Eagle medium (DME medium; Gibco) containing 5% (v/v) precolostrum newborn calf serum (PNCS; Mitsubishi Kasei) and 5% (v/v) heat-inactivated (56°C, 30 min) horse serum (Gibco), as described by Hatanaka (*17*). NGF was added to a final concentration of 50 ng/ml. TIP (transferrin, insulin, and progesterone) was added at final concentrations of 5 $\mu$g/ml, 5 $\mu$g/ml, and 20 nM, respectively. Twenty-four hours before adding various agents, the cells were spread on 24 well plates (Costar) or Flexiperm discs (Costar) at a density of $2\times10^5$ per/cm² in serum-free DME medium containing TIP.

*FDA, Hoechst 33,258, and C-DCDHF-DA Staining*—FDA or C-DCDHF-DA was added to cell cultures to a final concentration of 10 $\mu$g/ml or 10 $\mu$M, respectively, for 30 min at 37°C. The cells were collected by pipetting without washing and then analyzed with a flow cytometer, CytoAce

350 (Nihon Bunko). The laser was adjusted to emit at 480 nm and a 530 nm long-pass filter was used. The total number of events was 5,000 per sample. Gating was performed to obtain data from viable cells stained with C-DCDHF-DA (*24*).

For confocal analysis, cell cultures were fixed for 20 min in fresh 4% paraformaldehyde in $Ca^{2+}$,$Mg^{2+}$-free phosphate-buffered saline [PBS(−)], rinsed with PBS(−), and then stained for 15 min with 1 μg/ml Hoechst 33,258 in PBS(−). The stained cells were examined under UV illumination using a confocal microscope (RCM 8000; Nikon). The cells stained with C-DCDHF-DA were examined, under blue illumination using a confocal microscope. The cells were viewed within seconds.

*Terminal Deoxynucleotidyl Transferase-Mediated dUTP-Fluorescein Nick End Labeling (TUNEL) Method*—We used an "*In situ* death detection kit" (Boehringer Mannheim) to detect DNA fragmentation *in situ* (*18*). The cells were fixed for 30 min in fresh 4% paraformaldehyde in PBS(−) at room temperature (RT), and then rinsed once with PBS(−). Endogenous peroxidase was inactivated by incubation with 0.3% hydrogen peroxide in methanol for 30 min at RT. The cells were washed with PBS(−) and then incubated in a permeabilizing solution (0.1% sodium citrate and 0.1% Triton X-100) for 2 min at 4°C. The cells were washed twice with PBS(−) and then labeled by incubation with the TUNEL reaction mixture for 60 min at 37°C. The cells were washed twice with PBS(−) and then labeled with peroxidase-conjugated goat antibody (Fab fragment) for 30 min at 37°C. The cells were washed twice with PBS(−) and then stained with a Vectastain ABS kit (Vector Laboratories).

## RESULTS

*Hydrogen Peroxide-Triggered Cell Death via Apoptosis*—We used a PC12 cell subclone transfected with a control vector (PC-pAGE123) throughout this study (*16*). The exposure of PC12 cells to hydrogen peroxide significantly decreased their viability after 24 h, which was confirmed by the FDA fluorescence assay (Fig. 1). The $ED_{50}$ was about 200 μM. Whittemore *et al.* (*19*) reported that 5-min exposure of rat cortical neurons to hydrogen peroxide is sufficient to induce neuronal death. We confirmed that a 30-min exposure was sufficient to produce the same dose-response curve as prolonged exposure (data not shown). We exposed PC12 cells prolongly to hydrogen peroxide to

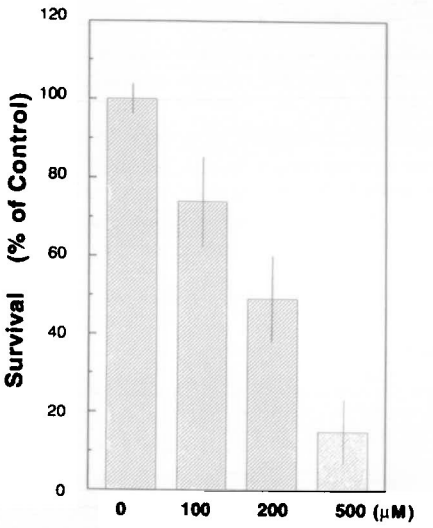


Fig. 1. **Dose-dependent inhibition of cell viability by hydrogen peroxide, in PC12 cells, analyzed using a FDA fluorescence assay.** 0, 100, 200, and 500 μM hydrogen peroxide was added to the culture medium 24 h before FDA (10 μg/ml), and then the cells were analyzed by means of flow cytometry. Values are means ± SD ($n = 4$).

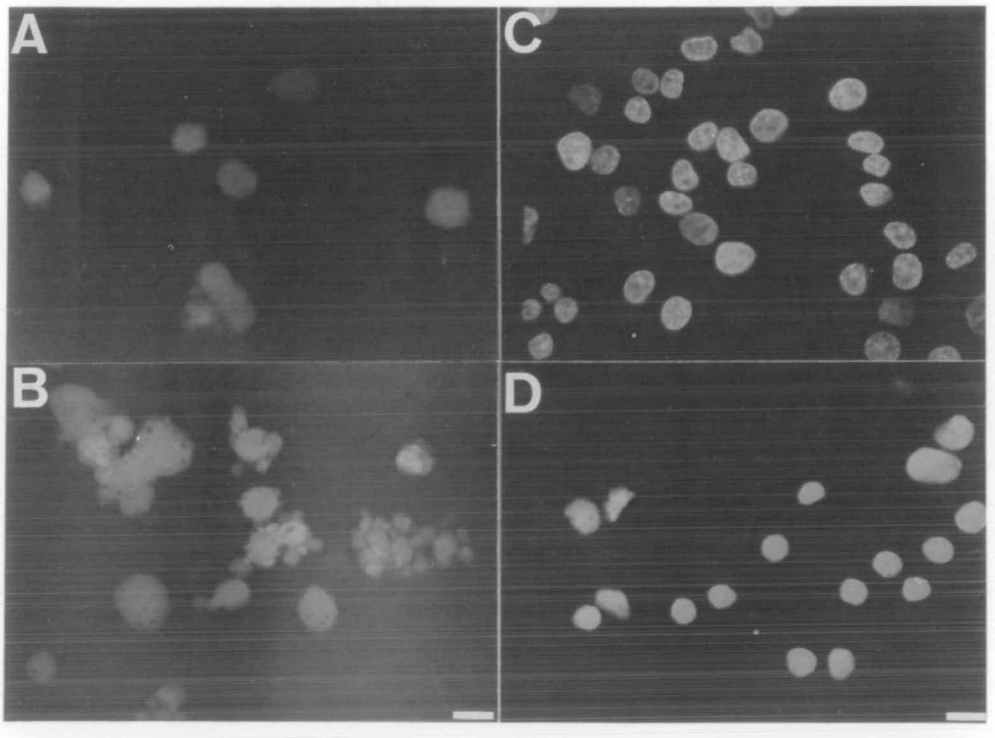


Fig. 2. **Membrane blebbing and cytosolic fragmentation induced by hydrogen peroxide.** Control (A) and hydrogen peroxide (B). FDA (10 μg/ml) was loaded for 30 min and then hydrogen peroxide was added. After 2-h incubation with hydrogen peroxide (200 μM), the cells were examined, under blue illumination by confocal microscopy. (A) and (B) show control and hydrogen peroxide-treated cells. Nuclear condensation induced by hydrogen peroxide detected with Hoechst 33,258. Control (C) and hydrogen peroxide (D). Hydrogen peroxide (200 μM) was added to the culture medium for 24 h before the cells were fixed and stained with Hoechst 33,258. The cells were examined under UV illumination by means of confocal microscopy. All pictures were taken at the same magnification (bar = 12.5 μm).

examine cell viability (Figs. 1 and 6).

One morphological criterion of apoptosis is membrane blebbing. To assess whether or not hydrogen peroxide can induce membrane blebbing, PC12 cells were loaded with FDA (10 $\mu$g/ml) for 30 min, and then hydrogen peroxide (200 $\mu$M) was added and 2 h later the cells were observed under blue illumination using confocal microscope (Fig. 2, A and B). Membrane blebbing and cytosolic fragmentation were obvious in these cells (Fig. 2B). In contrast, control cells exhibited an intact morphology (Fig. 2A).

Another morphological criterion for apoptosis is nuclear condensation. PC12 cells were exposed to hydrogen peroxide (200 $\mu$M) for 24 h, and then fixed and stained with Hoechst 33,258 (Fig. 2, C and D). Chromatin condensation was obvious in the cells exposed to hydrogen peroxide (Fig. 2D). In contrast, control cells exhibited an intact morphology (Fig. 2C).

A biochemical hallmark of apoptosis is the cleavage of DNA at internucleosomal sites to produce DNA fragmentation. As shown in Fig. 3, we detected DNA fragmentation induced by hydrogen peroxide with the TUNEL method. The treatment with hydrogen peroxide (500 $\mu$M, for 5 h) significantly increased the number of TUNEL-positive cells, indicating that DNA fragmentation occurred in response to hydrogen peroxide.

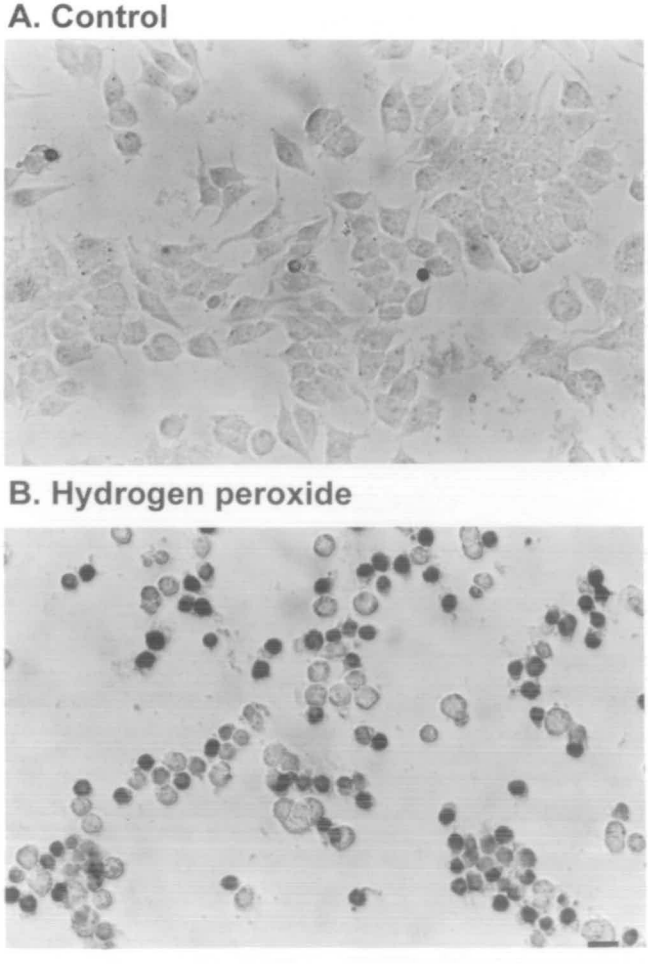


Fig. 3. **DNA fragmentation induced by hydrogen peroxide.** Control (A) and hydrogen peroxide (B). Hydrogen peroxide (500 $\mu$M) was added to the culture medium for 5 h before the cells were fixed and visualized by the TUNEL method. All pictures were taken at the same magnification (bar = 12.5 $\mu$m).

*Hydrogen Peroxide-Enhanced ROS Production*—We performed confocal analysis with C-DCDHF-DA to examine the involvement of ROS in the hydrogen peroxide-induced apoptosis of PC12 cells. C-DCDHF-DA is a lipophilic fluorogen that is converted intracellularly to a fluorescent molecule which, because of its highly polar property, is retained by viable cells. When oxidized by ROS, its fluorescence intensity significantly increases. As shown in Fig. 4, the fluorescence intensity of hydrogen peroxide (200 $\mu$M, 30 min)-treated cells (B) was significantly higher than that of control cells (A). The cells that had high fluorescence intensity also exhibited membrane blebbing, indicating a good correlation with ROS production. We measured this increase in fluorescence by means of confocal image analysis. Figure 4C shows the distribution of the fluorescence intensity of the cells in the fields in Fig. 4, A and B. The number of cells that emitted high fluorescence (>90) greatly was increased among hydrogen peroxide-treated cells. However, further quantification is impossible, especially time-dependent studies, because the fluorescence of C-DCDHF-DA increases upon simple illumination (*20, 21*).

We used flow cytometry with C-DCDHF-DA to overcome the problem of increasing fluorescence on prolonged excitation. C-DCDHF-DA was added to the medium 30 min before hydrogen peroxide. We measured the time-dependence of hydrogen peroxide (200 $\mu$M)-induced ROS production. Samples were obtained at various times and immediately analyzed by flow cytometry. The increase in fluorescence reached a maximum at 20 min. Prolonged exposure of over 180 min did not increase the fluorescence intensity (data not shown).

Figure 5 shows the dose-dependence of hydrogen peroxide-induced ROS production 30 min after exposure. Hydrogen peroxide increased ROS production in a dose-dependent manner. Even at 20 $\mu$M, hydrogen peroxide significantly increased the level of ROS, while the minimal concentration required for cytotoxicity toward PC12 cells was between 50 $\mu$M and 100 $\mu$M. These data indicate that there is a threshold level of ROS in PC12 cells before cytotoxicity occurs.

*NGF and Bcl-2 Protected PC12 Cells without Reducing ROS Production*—In PC12 cells, several types of apoptosis are prevented by NGF (*9, 10*) or Bcl-2 (*22-24, 40*). To determine whether or not NGF and Bcl-2 protect PC12 cells from hydrogen peroxide-triggered apoptosis, we performed an FDA fluorescence assay (Fig. 6, A and B). When PC12 cells were incubated with 200 $\mu$M hydrogen peroxide for 24 h, about half of the FDA-positive cells were lost. In the presence of NGF (50 ng/ml), the number of FDA-positive cells recovered to about 70% of that of the control cells. Bcl-2 (in PC-bcl10) completely blocked hydrogen peroxide-induced apoptosis.

We assessed whether or not NGF and Bcl-2 reduce the ROS production induced by hydrogen peroxide (Fig. 7). Since hydrogen peroxide is a membrane-permeable molecule, its intracellular level rapidly decrease when extracellular hydrogen peroxide is washed out. The fluorescence increase after hydrogen peroxide had been removed predominantly reflects the summation of hydroxyl radical and

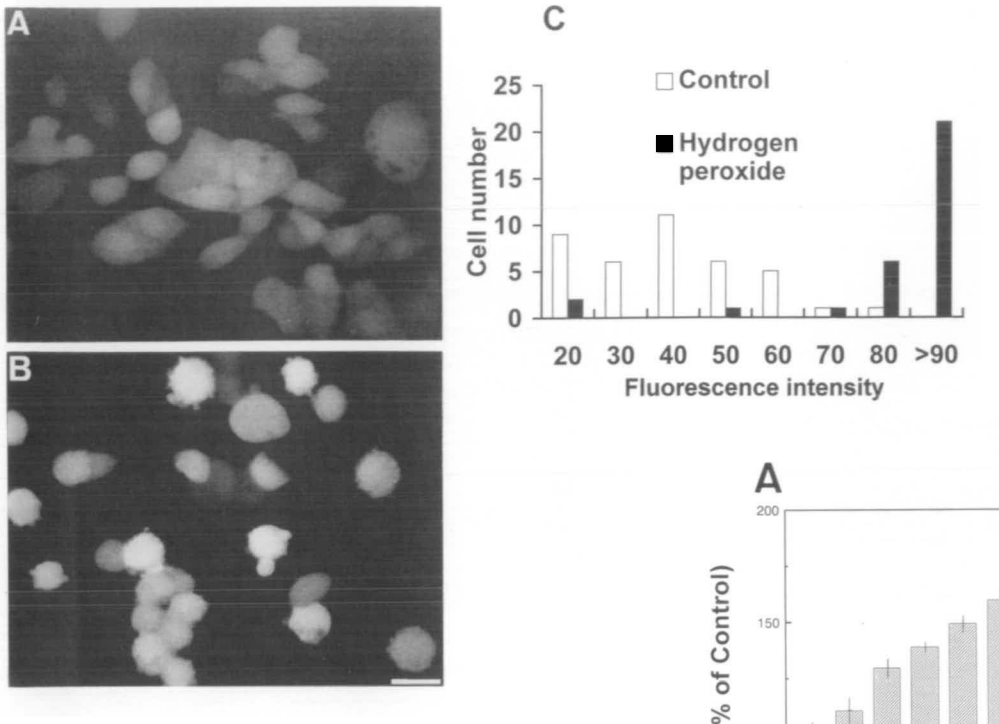


**Fig. 4. Confocal analysis of ROS production detected with C-DCDHF-DA.** Control (A) and hydrogen peroxide (B). C-DCDHF-DA was added to the culture medium 30 min before hydrogen peroxide (200 $\mu$M) was applied. After 30-min incubation, the cells were examined under blue illumination by confocal microscopy. All pictures were taken at the same magnification (bar = 12.5 $\mu$m). (C) Distribution of the fluorescence intensity in the fields in (A) and (B).

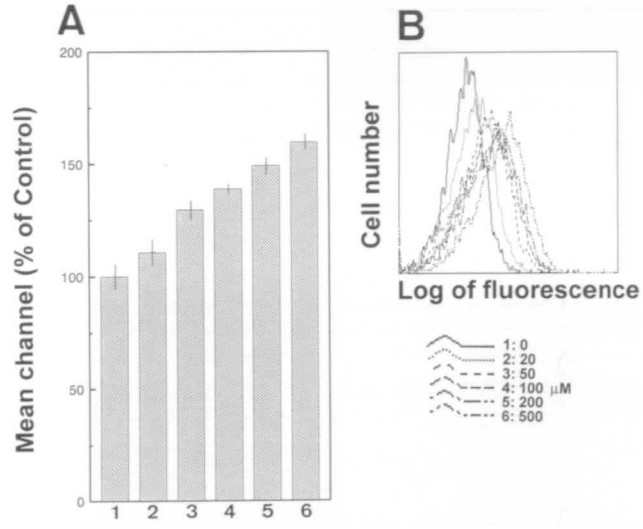


**Fig. 5. The mean channels (A), and typical histograms (B) of dose-dependent enhancement of ROS production in PC12 cells induced by hydrogen peroxide (1: 0, 2: 20, 3: 50, 4: 100, 5: 200, 6: 500 $\mu$M).** C-DCDHF-DA was added to the culture medium 30 min before various concentrations of hydrogen peroxide. After 30-min incubation, the cells were collected and analyzed using flow cytometry. Values are means ± SD ($n = 4$).

endogenous ROS production. In order to discriminate endogenous ROS production from a direct effect of hydrogen peroxide, we studied ROS production for a long time after hydrogen peroxide had been removed. C-DCDHF-DA-loaded PC12 cells were incubated with various concentrations of hydrogen peroxide for 30 min and then the hydrogen peroxide was removed (Fig. 7A). The fluorescence level increased in response to hydrogen peroxide and the enhanced level was maintained for over 120 min at higher concentrations (200 and 500 $\mu$M) after hydrogen peroxide had been removed, while the fluorescence level caused by 100 $\mu$M hydrogen peroxide decreased, indicating that endogenous ROS production occurs independently of external hydrogen peroxide at higher concentrations. The effects of NGF, Bcl-2, and NAC, a radical scavenger, (10 mM) on hydrogen peroxide (200 $\mu$M)-induced ROS production were investigated (Fig. 7B). NAC effectively blocked hydrogen peroxide-induced ROS production (we did not examine the effect of NAC on cell viability because NAC non-specifically induced cell damage at a concentration of over 1 mM). However, NGF and Bcl-2 did not affect the hydrogen peroxide-induced ROS production over 120 min after hydrogen peroxide had been removed.

## DISCUSSION

In PC12 cells and primary neurons, oxidative stress induces apoptosis rather than necrosis (*10, 11, 13, 25, 26, 40*), which must have a significant physiological impact, since the data suggest that ROS production plays an important role in physiologically-occurring cell death (*20*). While $O_2^-$ is the primary product of an $e^-$ attack on $O_2$, it is a rather poorly reactive radical and it is not lipid soluble. Superoxide itself does not result in lipid peroxidation, a common component of cell damage. Most $O_2^-$ is converted to hydrogen peroxide and most damage is supposed to be mediated by the most reactive OH· (hydroxyl) radical.

In this study, we demonstrated that exposure of PC12 cells to the hydroxyl radical producer, hydrogen peroxide, can trigger cell death *via* apoptosis. Hydrogen peroxide-induced cell death is accompanied by the morphological and biochemical features of apoptosis. Morphologically, cells exposed to hydrogen peroxide underwent membrane-blebbing and cytosolic fragmentation (Fig. 2, A and B). The nuclei treated with hydrogen peroxide were condensed (Fig. 2, C and D). Biochemically, the TUNEL method clarified that apoptotic DNA fragmentation occurred in response to hydrogen peroxide (Fig. 3).

The concentration of hydrogen peroxide required for the induction of cell death reported by Jackson *et al.* (*27*) is about five times larger than that reported here. The difference seems to depend on the presence of serum. We have found that serum potently decreases the level of ROS in PC12 cells and cortical neurons, and that serum potently protects PC12 cells from hydrogen peroxide-induced cytotoxicity through decreasing ROS production (*28*). Whitte-

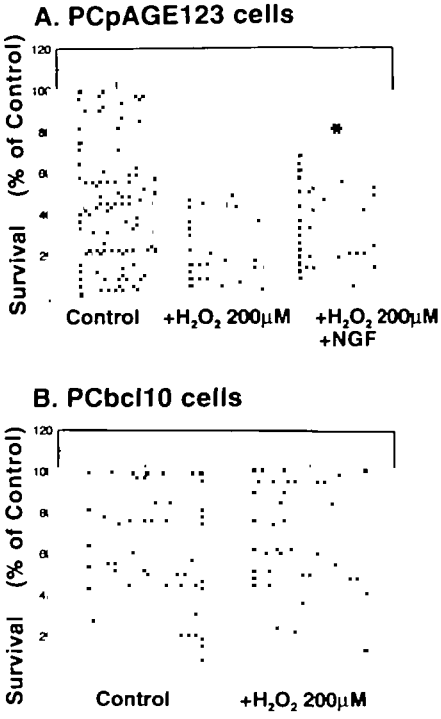


Fig. 6. **NGF (A) and Bcl-2 (B)-induced protection of PC12 cells from hydrogen peroxide (200 $\mu$M)-induced apoptosis.** NGF (50 ng/ml) was added to the culture 30 min before hydrogen peroxide. After 24 h incubation, the cells were loaded with FDA and then analyzed by flow cytometry. No addition, hydrogen peroxide and hydrogen peroxide plus NGF in PC12 (PC-pAGE123) (A) and PC-bcl10 cells (B), respectively. Values were means±SD ($n=4$). Significance values ($^*p<0.01$) are determined *vs.* NGF(−) samples.

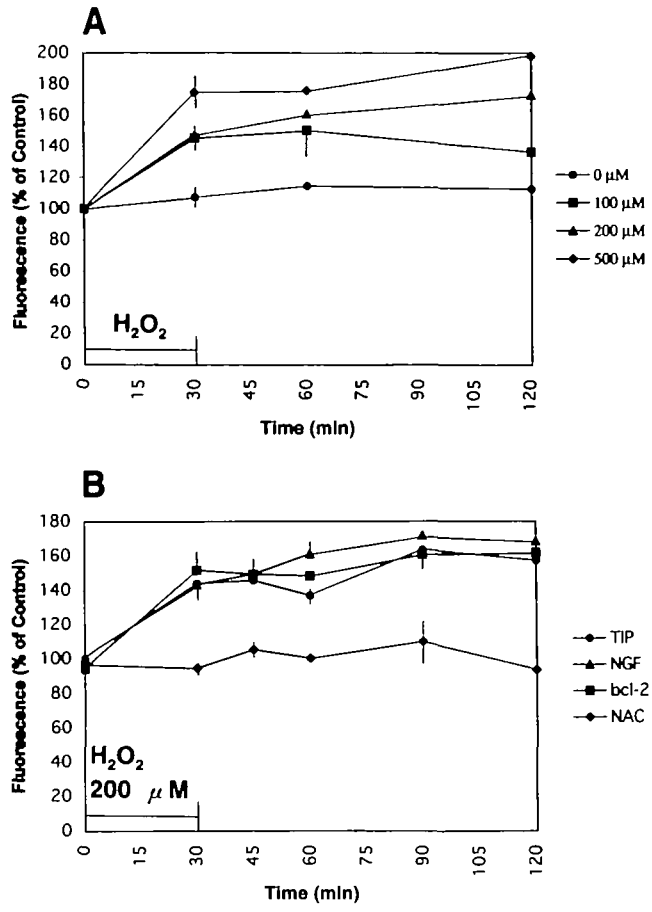


Fig. 7. **The kinetics of ROS production before and after various concentrations of hydrogen peroxide (0, 100, 200, 500 $\mu$M) had been removed (A).** C-DCDHF-DA was added to the culture medium 30 min before various concentrations of hydrogen peroxide were added. After 30 min incubation, the hydrogen peroxide was washed out. At the indicated times, the cells were collected and analyzed using flow cytometry. The effects of NGF, Bcl-2 and NAC, a radical scavenger (10 mM), on the kinetics of the fluorescence increase induced by hydrogen peroxide (200 $\mu$M) (B). C-DCDHF-DA was added to the culture medium 30 min before hydrogen peroxide was applied. After 30 min incubation, the hydrogen peroxide was washed out. At the indicated times, the cells were collected and analyzed using flow cytometry. Values are means±SD ($n=3$).

more *et al.* (*19*) reported that 5-min pulses with hydrogen peroxide kill rat cortical neurons. In PC12 cells, 30-min pulses had the same killing effect as prolonged exposure. The death of PC12 cells needs a ten times higher concentration of hydrogen peroxide that of cortical neurons.

Hydroxyl radicals are mainly generated from hydrogen peroxide through metal-catalyzed reactions: Fenton reactions. Hydroxyl radicals oxidize C-DCDHF-DA to produce fluorescence. Indeed, hydrogen peroxide increased C-DCDHF-DA fluorescence in a dose-dependent manner (Fig. 5). The following evidences indicate that most of the increase in fluorescence induced by hydrogen peroxide is not a direct effect on C-DCDHF-DA but mediated by hydroxyl radicals or endogenous ROS production. First, radical scavengers [NAC and *N,N*-diphenyl-*p*-phenylenediamine (DPPD)] effectively blocked the fluorescence increase induced by hydrogen peroxide. Second, higher, but not lower, concentrations of hydrogen peroxide triggered a sustained increase in the fluorescence even after hydrogen peroxide had been removed. The sustained increase in the fluorescence may reflect the endogenous ROS production independently of external hydrogen peroxide. DPPD, an antioxidant, partially protected PC12 cells from hydrogen peroxide-induced apoptosis by reducing ROS production (unpublished observation), while NAC induced non-specific cell damage at over 1 mM. Lipid hydroperoxide, another oxidant, induces apoptosis of PC12 cells associated with an increasing level of ROS (manuscript in preparation). These data indicate that ROS production is involved in the apoptosis induced by hydrogen peroxide.

We found that there was dissociation between the concentration required for the induction of apoptosis and that for ROS production. Hydrogen peroxide (20 $\mu$M) significantly increased ROS production, whereas up to 100 $\mu$M, it did not induce apoptosis, indicating that the amount of hydroxyl radicals produced by 100 $\mu$M hydrogen peroxide is the threshold level for the induction of apoptosis of PC12 cells.

Confocal analysis revealed that the cells with membrane blebbing were completely identical to those that exhibited high fluorescence intensity, indicating that membrane blebbing is caused by ROS. Since ROS trigger apoptosis, we surmised which target is the most important. One role may be as a signaling molecule that regulates the redox state and affects the activity of several transcription factors (*29-31*), and another may be lipid membrane peroxidation (*32*).

NGF (*10*) or Bcl-2 (*20*) reportedly protects PC12 cells from oxidative stress-induced apoptosis. In this study, NGF and Bcl-2, partially and completely, respectively, blocked hydrogen peroxide-induced apoptosis (Fig. 6). We have reported that the combination of NGF and TIP did not enhance cell survival from serum deprivation-induced apoptosis (*24*). Because TIP medium was used as the basal culture medium in these experiments, the survival effect of NGF was underestimated.

Several investigators suggested that NGF and Bcl-2 reduce the intracellular level of ROS. Hockenbery *et al.* (*32*) reported that NGF withdrawal induces ROS production associated with apoptosis of synpathetic neurons, and Kane *et al.* (*33*) reported that Bcl-2 protected neuronal cells from oxidative stress by reducing the generation of ROS. Furthermore, Mattson *et al.* (*34*) reported that bFGF, a neurotrophic factor, reduces the ROS production induced by NMDA receptor activation. These data suggest that the survival effect of NGF and Bcl-2 as to the hydrogen peroxide-induced apoptosis is due to the reduced generation of ROS. However, in this study, neither NGF nor Bcl-2 reduced hydrogen peroxide-induced ROS production (Fig. 7). In a murine interleukin 3-dependent cell line (FL5.12), Bcl-2 reportedly protects hydrogen peroxide-induced apoptosis without reducing ROS production (*32*). The authors considered that Bcl-2 functions as an anti-oxidant between ROS production and lipid peroxidation. However, two groups have independently reported that Bcl-2 prevents some types of apoptosis without changing the ROS level, indicating that Bcl-2 functions at a point different from an anti-oxidant (*23, 35*). We assume that the lipid peroxidation caused by ROS is the result and not the cause of apoptosis. In this study, Bcl-2 and NGF did not affect ROS production. In a separate experiment we obtained several lines of evidence indicating that ROS are not common mediators of apoptosis in PC12 cells. (i) The calcium ionophore, A23187, induced apoptosis of PC12 cells. (ii) NGF and Bcl-2 prevented A23187-induced apoptosis. (iii) A23187 did not increase ROS production (unpublished observation). These experiments indicated that neither ROS production nor intracellular calcium is a common mediator of apoptosis in PC12 cells. The protease function might be critical for the induction of apoptosis. This notion is consistent with the fact that the *ced-3* gene product, which is similar to the mammalian cysteine protease, interleukin 1$\beta$-converting enzyme (ICE) (*36*), is essential for programmed cell death in *Caenorhabditis elegance* (*37*). The *ced-9* gene, a homologue of *bcl-2* (*38*), appears to regulate the function of *ced-3* in providing protection from cell death (*39*).

In conclusion, our data demonstrate that hydrogen peroxide triggers apoptosis of PC12 cells, and that NGF and Bcl-2 prevent apoptosis without reducing ROS production, indicating that both NGF and Bcl-2 protect PC12 cells independently of ROS production.

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