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# Regulation of Reactive Oxygen Species by Nerve Growth Factor but not Bcl-2 as a Novel Mechanism of Protection of PC12 Cells from Superoxide Anion-Induced Death[1]

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**Although neurotrophins protect PC12 cells and neurons from oxidative stress-induced death, the molecular mechanism of this effect is largely unknown. Xanthine (XA)+ xanthine oxidase (XO) increased the production of the superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$), and the death of PC12 cells. Catalase but not superoxide dismutase (SOD) nor a NO scavenger protected PC12 cells from death, indicating that $H_2O_2$ is the main effector responsible for this cell death. Both nerve growth factor (NGF) and Bcl-2 protected PC12 cells from $O_2^-$-induced toxicity. NGF enhanced the production of $O_2^-$ and suppressed that of $H_2O_2$, suggesting that it inhibits the conversion of $O_2^-$ to $H_2O_2$, while Bcl-2 had no such effect. These results suggested that NGF protected the cells from oxidative stress by altering the composition of the reactive oxygen species (ROS) without affecting their total level.**

**Key words: apoptosis, neurotrophic factor, oxygen radical, superoxide dismutase, xanthine.**

Nerve growth factor (NGF), a member of the neurotrophin family, enhances the survival of CNS and peripheral neurons (*1*). NGF protects PC12 cells from serum/NGF deprivation, high oxygen, and hydrogen peroxide (*2-7*). NGF binds to and activates TrkA, resulting in TrkA tyrosine phosphorylation (*1*). The tyrosine-phosphorylated TrkA serves as a scaffolding for the recruitment of several enzymes and effectors including phospholipase C$\gamma$ (*8*), phosphatidylinositol 3-kinase (PI3-K) (*9*), and Shc (*10*). The RAS-MAP kinase pathway initiated by Shc may play a role in neuronal differentiation, while PI3-K may play a role in the promotion of survival (*11*). Bcl-2 has also been reported to be a regulator of the cell survival of CNS and peripheral neurons (*12, 13*). Bcl-2 protected PC12 cells from serum/NGF deprivation, high oxygen and hydrogen peroxide (*5-7, 14-16*). Bcl-2 is present in the outer membrane of mitochondria, and is supposed to inhibit the loss of mitochondrial membrane potential (*17*) and to form a dimer with the *bax* gene product that enhances cell death (*18*). Although Bcl-2 was previously reported to function as an anti-oxidant (*19, 20*), this protective effect appeared not to involve alterations in reactive oxygen species (ROS) levels (*21, 22*). Both NGF and Bcl-2 potently protected PC12 cells from death by increasing the generation of ROS (*5-7, 15*). The expression of Bcl-2 has been reported to mediate some portions of the protective effect of NGF, suggesting that Bcl-2 and NGF may share the same mechanism (*23-25*). However, the molecular mechanisms of their protective effects are not clear. The most fundamental question is whether they can modulate ROS levels or not.

$O_2^-$ is constitutively produced by mitochondria through aerobic metabolism. Superoxide dismutase (SOD) converts $O_2^-$ to $H_2O_2$ and then catalase or glutathione peroxidase removes $H_2O_2$. Protection from $O_2^-$ is critical for cell survival, since increased production of $O_2^-$ resulted in cell death with features characteristic of apoptosis (*7, 26-31*). There are two distinctive pathways of $O_2^-$-induced cell death, one is mediated by $H_2O_2$, and the other is mediated by NO and $ONOO^-$. XA+XO induced the death of cerebellar granule neurons through increased production of $H_2O_2$ (*28*), while SOD1 antisense nucleotides triggered death through increased production of $ONOO^-$ (*30*). Another important finding is that lipoxygenase 12 activation is involved in cysteine depletion-induced neuronal death (*32*). In the present study, we examined the effects of XA+XO, and the protective effects of NGF and Bcl-2 in PC12 cells.

Here, we found that NGF enhanced $O_2^-$ activated by

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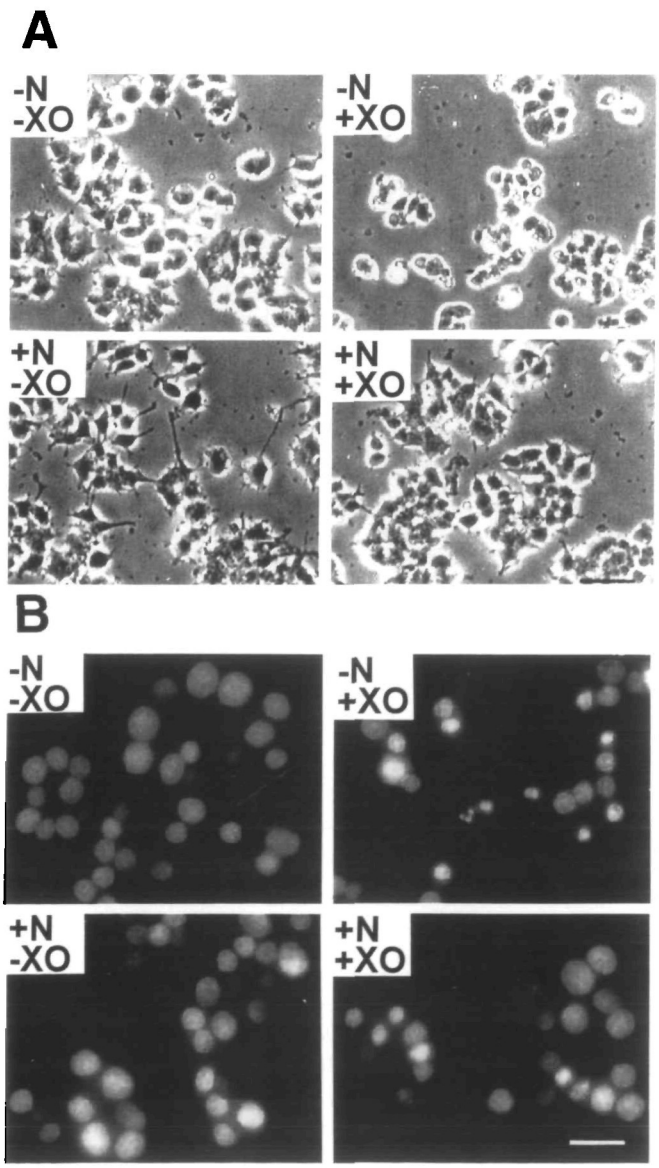
Abbreviations: Carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline 1-oxyl-3-oxide, potassium salt; C-CDHF-DA, 6-carboxy-2′,7′-dichlorofluorescein, di(acetoxymethyl ester); DHR123, dihydrorhodamine 123; FDA, fluorescein diacetate; HEt, hydroethidine; NGF, nerve growth factor; ROS, reactive oxygen species; SOD, superoxide dismutase; XA, xanthine; XO, xanthine oxidase.

XA + XO and suppressed $H_2O_2$, while Bcl-2 had no such effect. Both NGF and Bcl-2 potently protected PC12 cells from $O_2^-$-induced toxicity. These results suggested that decreased conversion of $O_2^-$ to $H_2O_2$ is a novel mechanism by which NGF but not Bcl-2 protects cells from oxidative stress.

## MATERIALS AND METHODS

*Materials*—NGF (2.5S) was prepared from male mouse submandibular glands as described by Bocchini and Angeletti (*33*), with some modifications according to Suda *et al.* (*34*). Fluorescein diacetate (FDA) was purchased from Polyscience, Hoechst 33,258, 6-carboxy-2′,7′-dichlorofluorescein, di(acetoxymetyl ester) (C-DCDHF-DA), hydroethidine (HEt), and dihydrorhodamine 123 (DHR123) from Molecular Probe, SOD and catalase from Worthington Biochemical, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline 1-oxyl-3-oxide, potassium salt (carboxy-PTIO: a NO scavenger) from Funakoshi, XA from Dohjin, and XO from Biozyme.

*Cell Culture and Measurement of Cell Survival*—A PC12 subclone, PC12h (*35*), was used in the present study except for the experiments involving Bcl-2 (Fig. 5) in which PC-pAGE123 (control vector-transfected PC12 cells) and PC-bcl10 (Bcl-2 expression vector-transfected PC12 cells) (*36*) were used. The cells were maintained in 75 $cm^2$ flasks (Costar) using a 1:1 mixture of Dulbecco's Modified Eagle medium (DMEM) and F12 medium supplemented with 5% (v/v) of heat-inactivated (56°C, 30 min) horse serum (GIBCO) and 5% (v/v) of precolostrum newborn calf serum (Mitsubishi Kasei) (5/5 DF). The cells were transferred to collagen-coated 24 well plates (Costar) at a density of $5 \times 10^4$ cells/$cm^2$ and then incubated in 5/5 DF for 24 h. The medium was changed to serum-free DMEM (*37*) supplemented with 5 μg/ml of human transferrin (Sigma), 5 μg/ml of bovine insulin (Collab. Res.), 20 nM progesterone (Sigma), 100 μM XA (TIP/XA-DMEM), and then NGF or BDNF (50 ng/ml) were added, followed by incubation for 24 h. SOD, catalase or carboxy-PTIO was added 30 min



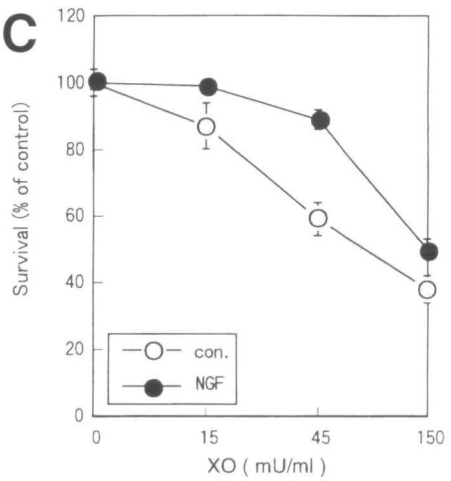




Fig. 1. **Protection of PC12 cells by NGF.** PC12h cells were transferred to collagen-coated 24-well plates at a density of $5 \times 10^4$ cells/$cm^2$ and then incubated in 5/5 DF for 24 h. The medium was changed to TIP/XA-DME with or without NGF, followed by incubation for 24 h. The cells were treated with XA + XO for 12 h. (A) Cell morphology was examined by phase contrast microscopy. Bar = 50 μm. (B) Nuclear morphology was examined by Hoechst 33258 staining. The cells were fixed in 4% paraformaldehyde, stained with 1 μg/ml Hoechst 33,258 and then examined under UV illumination using a fluorescence microscope (Nikon). Bar = 25 μm. (C) FDA (10 μg/ml) was added, followed by incubation for 30 min. Then the cells were collected by pipetting, and FDA-positive cells were counted by flow cytometry. The values are means ± SD ($n = 4$).

before XO was added to the medium. The cells were treated with XA+XO for 12 h, and then FDA (10 $\mu$g/ml) was added, followed by incubated for 30 min. Then the cells were collected by pipetting, and FDA-positive cells was counted by flow cytometry (CytoAce 350: Nihon Bunko) on FL-1 (excitation: 480 nm, emission: 530 nm) (*4*).

*Hoechst 33,258 Staining*—The cells were fixed in 4% paraformaldehyde at room temperature for 20 min, stained with 1 mg/ml Hoechst 33,258 in PBS(−) for 15 min, and then examined under UV illumination using a fluorescence microscope (Nikon).

*Measurement of HEt, C-DCDHF-DA, and DHR-123 Fluorescence*—We followed the previously reported procedure for the detection of intracellular ROS using HEt, C-DCDHF-DA, and DHR123 (*28*). HEt, C-DCDHF-DA, or DHR123 was added to cultures at a final concentration of 1 $\mu$g/ml, 10 $\mu$M, or 10 $\mu$M, followed by the addition of XO and incubation for 15 min. The cells were collected by

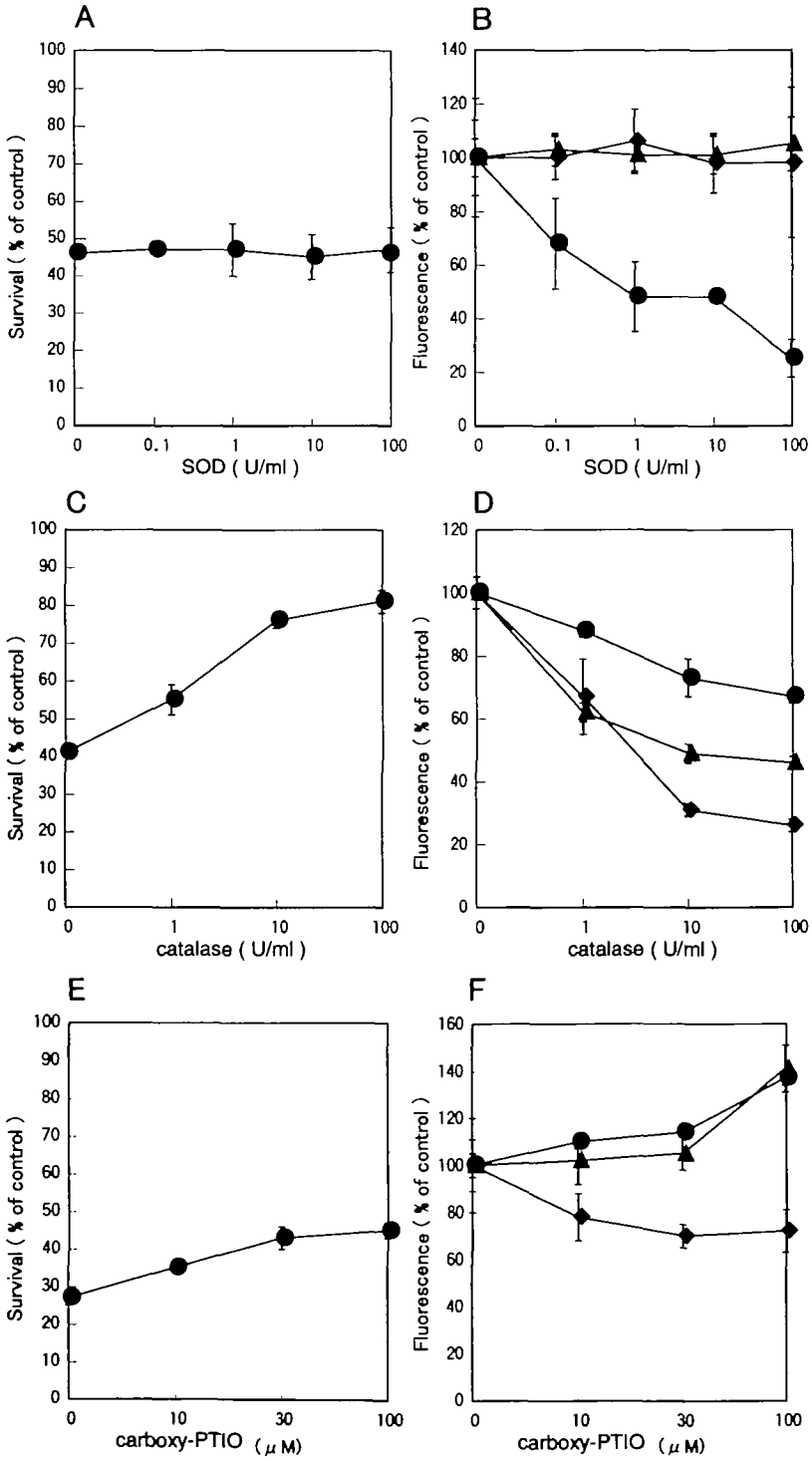



Fig. 2. **Effects of SOD, catalase, and carboxy-PTIO on cell survival and ROS levels.** (A, C, and E) PC12h cells were incubated in 5/5 DF for 24 h, and then the medium was changed to TIP/XA-DME, followed by incubation for 24 h. Various concentrations of SOD (A), catalase (C), or carboxy-PTIO (E) were added 30 min before XO (45 mU/ml). After incubation for 12 h, FDA (10 $\mu$g/ml) was added, followed by incubation for 30 min. Then the cells were collected by pipetting, and FDA-positive cells were counted by flow cytometry. (B, D, and F) PC12h cells were incubated in 5/5 DF for 24 h, and then the medium was changed to TIP/XA-DME, followed by incubation for 24 h. Various concentrations of SOD (B), catalase (D), or carboxy-PTIO (F) were added to the cultures for 30 min, then C-DCDHF-DA (10 $\mu$M) (diamonds), HEt (1 $\mu$g/ml) (circles), or DHR123 (10 $\mu$M) (triangles) was added to the cultures for 30, 15, and 20 min, respectively, and finally XO (45 mU/ml) was added. The cells were incubated for 15 min and collected by pipetting, and then fluorescence was measured by flow cytometry. The values are means ± SD ($n$=4).

pipetting without washing and then analyzed by flow cytometry (CytoACE 350; Nihon Bunko). The laser was adjusted to FL2, FL1 and FL1 for HEt, C-DCDHF-DA and DHR123, respectively. The total number of events was 1,000 per sample. Mean channels were used as indicators of fluorescence intensity.

## RESULTS

*NGF Protected PC12 Cells from XA+XO-Induced Death*—Exposure of PC12 cells to $O_2^-$ generated by XA+XO significantly decreased their viability to less than 40% after 12 h, which was confirmed by FDA fluorescence assay (Fig. 1A). Based on the results of morphological examination, the cell death seemed to be apoptotic rather than necrotic since the cell bodies underwent shrinkage and the nuclei became condensed (Fig. 1, A and B). Furthermore, the presence of cycloheximide (3 $\mu$M) or actinomycin D (0.3 $\mu$M) significantly suppressed the death (data not shown). However, high concentrations (150 mU/ml) of XO produced more necrotic cells with swollen cell bodies (data not shown). The presence of NGF (50 ng/ml) significantly protected the cells from the $O_2^-$-induced toxicity (Fig. 1). The protective effect was most potent at the XO concentration of 45 mU/ml, and was decreased at the high concentration of 150 mU/ml (Fig. 1C). Most NGF-treated PC12 cells were morphologically intact and had sparse nuclei even in the presence of XA+XO (Fig. 1, A and B).

*$H_2O_2$ as the Main Effector of XA+XO-Induced Toxicity*—We used three ROS-sensitive fluorogens, HEt, DHR123 and C-DCDHF-DA, for the detection and discrimination of $O_2^-$ and $H_2O_2$. C-DCDHF-DA fluorescence increased mainly by $H_2O_2$ (*19, 28*), as well as $O_2^-$ (*38*), NO and $ONOO^-$ (*39*), produces fluorescein. HEt is a fluorogen specific to $O_2^-$ and produces ethidium which intercalates with DNA (*28, 40*). DHR123 reacts with $H_2O_2$ and is converted to rhodamine 123 in the presence of peroxidase, $Fe^{2+}$ and cytochrome *c* which is localized to mitochondria (*41, 42*). Fluorescence microscopy demonstrated that XA+XO potentiated broad green fluorescence in C-DCDHF-DA-treated cells, red fluorescence in nuclei and small particles in the cytoplasm of HEt-loaded cells, and green fluorescence in particles in the cytoplasm of DHR123 cells (data not shown), indicating that the $O_2^-$ and $H_2O_2$ levels increased in response to XA+XO in PC12 cells.

We examined the effects of specific scavengers, SOD ($O_2^-$ scavenger), catalase ($H_2O_2$ scavenger) and carboxyl-PTIO (NO and $ONOO^-$ scavenger), to identify the main effector of the XA+XO-induced cell death (Fig. 2). SOD showed no protective effect at any concentration used (1-100 U/ml) and specifically decreased the HEt fluorescence without affecting the C-DCDHF-DA or DHR123 fluorescence. Catalase completely prevented the death, and effectively decreased the DHR123 and C-DCDHF-DA fluorescence. Carboxyl-PTIO had a very slight protective effect and decreased the C-DCDHF-DA fluorescence. These results suggested that the main effector of the XA+XO-induced cell death was $H_2O_2$. NO and $ONOO^-$ may be involved to a small extent in the death, while $O_2^-$ itself seemed to make no contribution.

*NGF Enhanced $O_2^-$ Production and Suppressed $H_2O_2$*—Next, we examined the intracellular mechanism of the protective effect of NGF against XA+XO-induced cell death. We examined whether or not NGF modulates ROS levels during $O_2^-$-induced toxicity (Fig. 3). The presence of NGF did not effect the C-DCDHF-DA fluorescence activated intensity by XA+XO (Fig. 3A) (*6, 7, 28*). However, NGF potently enhanced the HEt fluorescence (Fig. 3B). In contrast, it depressed the DHR123 fluorescence (Fig. 3C). These results indicated that NGF enhanced the $O_2^-$ level and depressed the $H_2O_2$ level.

*Bcl-2 Had a Protective Effect but Did Not Modulate the*

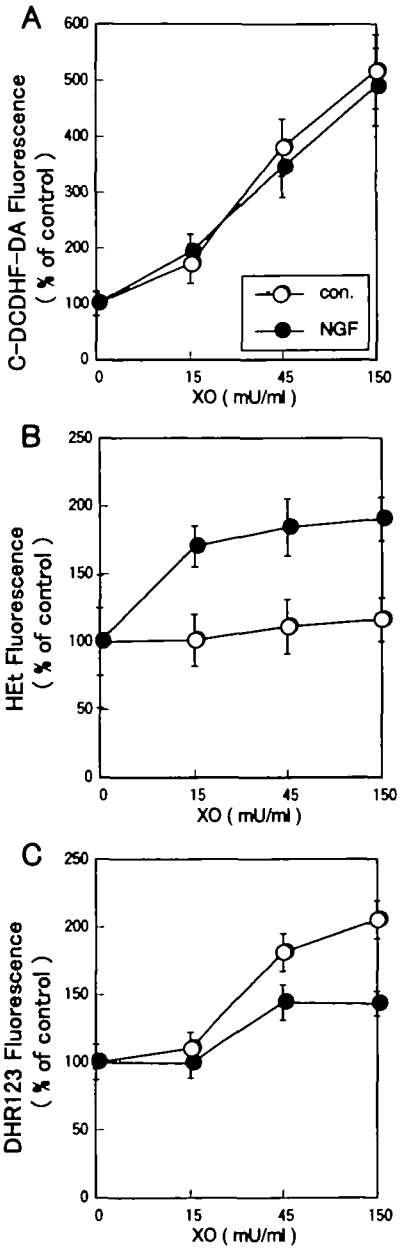



Fig. 3. **Modulation of ROS by NGF.** PC12h cells were spread on 24-well plates at a density of $5\times10^4$ cells/cm$^2$ and then incubated in 5/5 DF for 24 h. The medium was changed to TIP/XA-DME with or without NGF (50 ng/ml), followed by incubation for 24 h. C-DCDHF-DA (10 $\mu$M) (A), HEt (1 $\mu$g/ml) (B), or DHR123 (10 $\mu$M) (C) was added to the cultures for 30, 15, and 20 min, respectively, and then XO (45 mU/ml) was added for 15 min. Then the cells were collected by pipetting, and fluorescence was measured by flow cytometry. The values are means±SD ($n=4$).

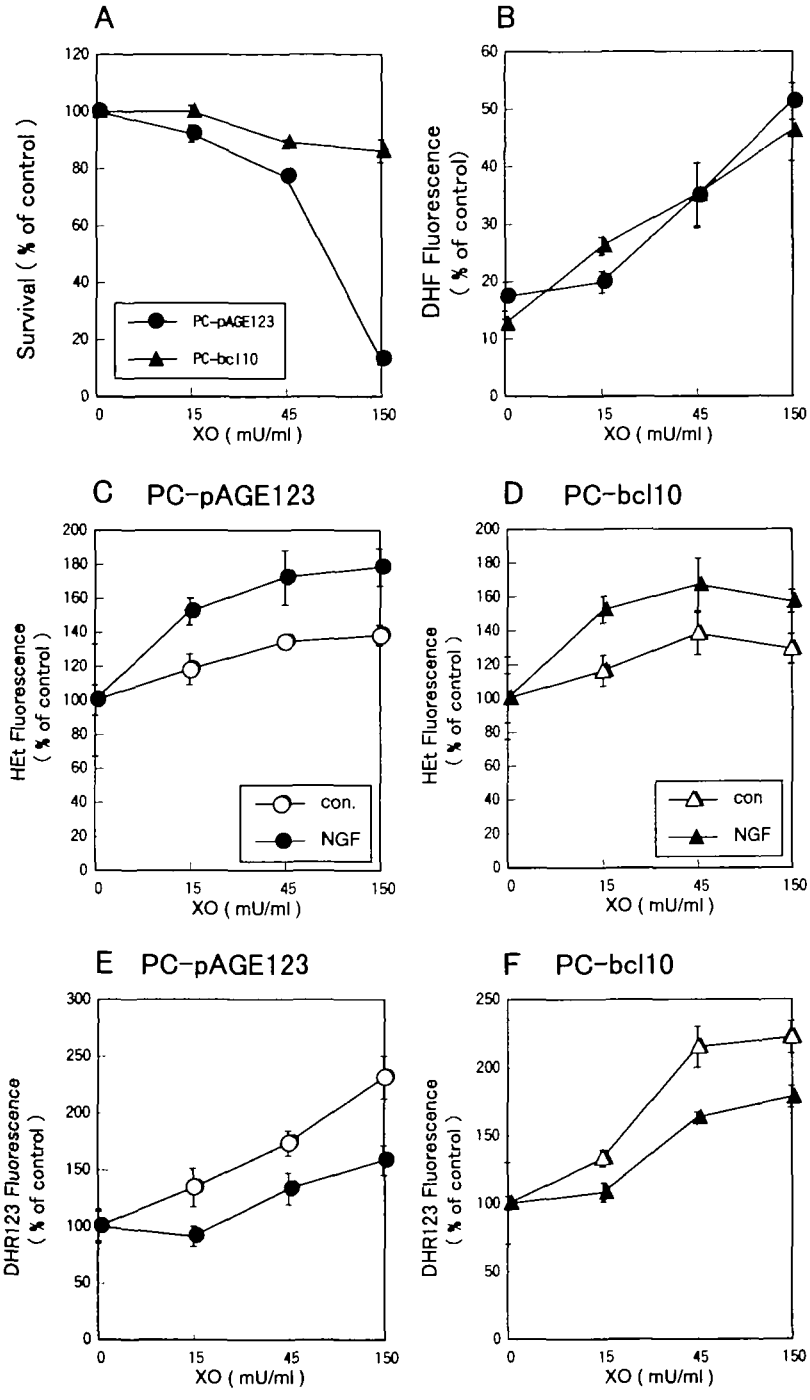



Fig. 4. **Effects of Bcl-2.** (A) PC12 cells (PC-pAGE123 [Bcl-2(−)] and PC-bcl10 [Bcl-2(+)] were incubated in 5/5 DF for 24 h, then the medium was changed to TIP/XA-DME, followed by incubation for 24 h, and finally XO (45 mU/ml) was added. After incubation for 12 h, FDA (10 $\mu$g/ml) was added, followed by incubation for 30 min, and then the cells were collected by pipetting, and FDA-positive cells were counted by flow cytometry. (B, C, D, E, and F) PC12 cells were incubated in 5/5 DF for 24 h. The medium was changed to TIP/XA-DME with or without NGF (50 ng/ml), followed by incubation for 24 h. C-DCDHF- DA (10 $\mu$M) (B), HEt (1 $\mu$g/ml) (C and D), or DHR123 (10 $\mu$M) (E and F) was added to the cultures for 30, 15, and 20 min, respectively, and then XO (45 mU/ml) was added for 15 min. Then the cells were collected by pipetting, and fluorescence was measured by flow cytometry. The values are means±SD ($n$=4).

*$O_2^-$ or $H_2O_2$ Level*—Bcl-2 inhibits the cell death induced by oxidative stressors such as XA+XO (*38*), hydrogen peroxide (*4*), lipid hydroperoxide (*7, 43*), and other types of death (*15, 16*). We used PC12 subclones stably expressing Bcl-2 (PC-bcl10) as well as ones transfected with a control vector (PC-pAGE123) (*36*). The presence of Bcl-2 completely protected the cells from XA+XO-induced cell death (Fig. 4A). To determine whether or not Bcl-2 modulates ROS such as NGF, we examined the effects of Bcl-2 on the C-DCDHF-DA, HEt and DHR123 fluorescence activated by XA+XO (Fig. 4, B, C, and D). The presence of Bcl-2 did not modulate the C-DCDHF-DA fluorescence activated by XA+XO (Fig. 4B) (*4-7*). In contrast to NGF, Bcl-2 did not affect the levels of HEt and DHR123 fluorescence activated by XA+XO (Fig. 4, C, and D). Furthermore, NGF showed additive effects on the HEt and DHR123 fluorescence. NGF potently enhanced the HEt fluorescence and depressed the DHR123 fluorescence even in PC-bcl10 cells (Fig. 4, C, D, E, and F). These results indicate that Bcl-2 has no effect on the levels of $O_2^-$ and $H_2O_2$.

## DISCUSSION

*$O_2^-$-Induced Cell Death*—Energy production in the brain is highly dependent on aerobic metabolism, which constitutively produces $O_2^-$. This $O_2^-$ is released from the cells through anion transporters, or removed by protective enzymes such as SOD, catalase or glutathione peroxidase (*44*). However, increased production of $O_2^-$ in degenerative diseases, ischemia or trauma induces neuronal damage. In cultured neurons, increased production of $O_2^-$ under conditions such as high oxygen, cysteine depletion or XA+XO induced neuronal cell death with morphological and biochemical features characteristic of apoptosis (*14, 26, 27, 29*). $O_2^-$ also induces apoptotic cell death associated with the loss of mitochondrial membrane potential (*7*) and the release of L-glutamate from cerebellar granule neurons (*28*). In the present study, XA+XO induced nuclear condensation and macromolecule synthesis inhibitors inhibited this death (Fig. 1), indicating that XA+XO induced apoptosis rather than necrosis (*28*).

Catalase but not SOD effectively protected the cells from XA+XO-induced death, indicating that $H_2O_2$ is the main effector for this cell death (Fig. 2) (*28*). Carboxyl-PTIO had a slight protective effect against the death (Fig. 2), indicating slight but significant involvement of NO and $ONOO^-$ in the death. Troy *et al.* (*45*) reported that antisense nucleotides for SOD1 induced cell death through increased production of $ONOO^-$. We observed that SOD and carboxyl-PTIO completely inhibited the high oxygen-induced cell death (data not shown), indicating that the death induced by SOD1 deletion or high oxygen is mediated by NO and $ONOO^-$. These results suggested that there are two types of $O_2^-$-induced cell death, one mediated by $H_2O_2$ (Fig. 2) (*28*), and the other by NO- and $ONOO^-$ (*45*).

Catalase reduced the C-DCDHF-DA and DHR123 fluorescence more effectively than the HEt fluorescence (Fig. 2B), whereas SOD reduced the HEt fluorescence but not the C-DCDHF-DA or DHR123 fluorescence (Fig. 2C). These results indicated that the HEt fluorescence originates mainly from $O_2^-$, and that the C-DCDHF-DA and DHR123 fluorescence originates mainly from $H_2O_2$ (*19, 28*). However, carboxyl-PTIO, a scavenger of NO, partially reduced the C-DCDHF-DA fluorescence, indicating that NO or $ONOO^-$ also increased the C-CDHF-DA fluorescence (Fig. 2D) (*39*). Greenlund *et al.* (*38*) reported that $O_2^-$ as well as other ROS increased C-DCDHF-DA fluorescence. These results indicated that DHR123 is rather specific to $H_2O_2$ and that C-DCDHF-DA exhibits broad reactivity to ROS.

*NGF Protection*—NGF allows PC12 cells to differentiate into a sympathetic neuron-like phenotype (*35, 46*) and protects the cells from various stimuli (*3-6, 15, 47*). NGF potently protected the cells from the XA+XO-induced cell death (Fig. 1). NGF enhanced the HEt fluorescence and suppressed the DHR123 fluorescence, while it did not change the C-DCDHF-DA fluorescence (Fig. 3). It has been demonstrated that C-DCDHF-DA exhibits a broad reactivity to ROS (Fig. 2), suggesting that its fluorescence can be regarded as an indicator of the total amount of ROS. We reported previously that NGF protected cells from the $H_2O_2$-induced toxicity without affecting ROS levels measured with C-DCDHF-DA (*5*). It is likely that NGF did not change the total amount of ROS activated by XA+XO (Fig. 4A). However, NGF seems to modulate the composition of ROS by enhancing the $O_2^-$ level and depressing the $H_2O_2$ level. Modulation of ROS by NGF may play a role in the survival-promoting effect of NGF, because ROS production is involved in the death induced by not only oxidative stress (*26, 28, 29*), but also other stimuli, such as NGF or serum removal (*6, 38, 48*). However, the precise mechanism of ROS modulation is unclear. One possible interpretation is inhibition of SOD, resulting in the accumulation of $O_2^-$ and a decrease in $H_2O_2$ level. An increased level of $O_2^-$ itself does not seem to be toxic in PC12 cells (Fig. 2) or cerebellar granule neurons (*28*), or may promote cell survival (*49, 50*). The decrease in $H_2O_2$ must contribute to enhancement of cell survival since we clarified $H_2O_2$ is the main effector of the death (Fig. 2) (*28*). Another possible interpretation is activation of catalase. Jackson *et al.* (*47*) reported that NGF protected PC12 cells from the $H_2O_2$-induced toxicity through increased catalase activity. However, only a single mechanism of catalase activation does not seem to be sufficient, since the addition of catalase induced a significant decrease in the level of $O_2^-$ as well as that of $H_2O_2$ (Fig. 2).

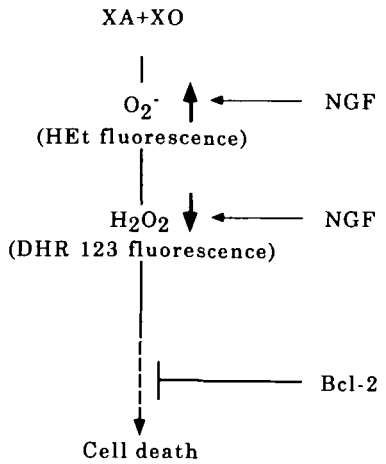



Fig. 5. **The distinct protection between by NGF and by Bcl-2 against the XA+XO-induced death of PC12h cells.**

*Bcl-2 Protection*—Bcl-2 was reported to prevent the death of PC12 cells induced by various stimuli (*5-7, 15, 16, 22, 28*). Kane *et al.* (*20*) and Hockenbery *et al.* (*51*) proposed that Bcl-2 functions as an anti-oxidant that prevents apoptosis. However, Bcl-2 was reported to prevent apoptosis without affecting ROS, indicating that it is not simply an anti-oxidant (*6, 7, 17, 22, 47*). Petit *et al.* (*17*) proposed the permeability transition hypothesis in which distinct apoptosis inducing agents are suggested to converge at the mitochondrial level to induce the permeability transition which is subject to regulation by Bcl-2. The permeability transition releases an apoptosis-inducing factor (AIF), which causes nuclear condensation or cytoplasmic fragmentation. We reported that Bcl-2 prevented the oxidative stress-induced death of PC12 cells without affecting ROS levels (C-DCDHF-DA fluorescence), but was associated with a loss of mitochondria membrane potential (*6, 7*). In the present study, Bcl-2 did not modulate the $O_2^-$ or $H_2O_2$ level activated by XA+XO (Fig. 4). From these results, Bcl-2, in contrast to NGF, did not seem to modify the composition of ROS.

In the present study, we demonstrated that XA+XO induced the death of PC12 cells associated with increased production of $O_2^-$ and $H_2O_2$. We summarized the result of distinct protection between by NGF and by Bcl-2 against the XA+XO-induced death of PC12h cells, in Fig. 5. The main effector of the death seems to be $H_2O_2$, not $O_2^-$ or NO-$ONOO^-$. NGF and Bcl-2 protected the cells from the $O_2^-$- induced cell death. NGF, but not Bcl-2, enhanced $O_2^-$ production and suppressed $H_2O_2$ production. These results suggested that NGF protected PC12 cells through a mechanism distinct from that in the case of Bcl-2.

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