Redox Regulation of cAMP-responsive Element-binding Protein and Induction of Manganese Superoxide Dismutase in Nerve Growth Factor-dependent Cell Survival*

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Reactive oxygen species (ROS) act as both signaling molecules and mediators of cell damage in the nervous system and are implicated in the pathogenesis of neurodegenerative diseases. Neurotrophic factors such as the nerve-derived growth factor (NGF) support neuronal survival during development and promote regeneration after neuronal injury through the activation of intracellular signals whose molecular effectors and downstream targets are still largely unknown. Here we present evidence that early oxidative signals initiated by NGF in PC12 cells, an NGF-responsive cell line, play a critical role in preventing apoptosis induced by serum deprivation. This redox-signaling cascade involves phosphatidylinositol 3-kinase, the small GTPase Rac-1, and the transcription factor cAMP-responsive element-binding protein (CREB), a molecule essential to promote NGF-dependent survival. We found that ROS are necessary for NGF-dependent phosphorylation of CREB, an event directly correlated with CREB activity, whereas hydrogen peroxide induces a robust CREB phosphorylation. Cells exposed to NGF show a late decrease in the intracellular content of ROS when compared with untreated cells and increased expression of the mitochondrial antioxidant enzyme manganese superoxide dismutase, a general inhibitor of cell death. Accordingly, serum deprivation-induced apoptosis was selectively inhibited by low concentrations of the mitochondrial targeted antioxidant Mito Q (mitoquinol/mitoquinone). Taken together, these data demonstrate that the oxidant-dependent activation of CREB is a component of NGF survival signaling in PC12 cells and outline an intriguing circuitry by which a cytosolic redox cascade promotes cell survival at least in part by increasing mitochondrial resistance to oxidative stress.

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This paper is dedicated to the memory of Eraldo Antonini, eminent biochemist, prematurely deceased 20 years ago on March 19, 1983.

** Both authors contributed equally to this work.

The abbreviations used are: ROS, reactive oxygen species; Mn-SOD, manganese-dependent superoxide dismutase; CuZn-SOD, copper zinc-dependent superoxide dismutase; DCF-DA, dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; FCS, fetal calf serum; 2-ME, 2-mercaptoethanol; MEF, mouse embryonic fibroblast; Mito Q, mitoquinol/mitoquine; NA.C, N-acetyl-cysteine; NGF, nerve growth factor; FKHR, Forkhead-related transcription factor; CREB, cAMP-responsive element-binding protein; PI, phosphatidylinositol; GFP, green fluorescent protein.

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oxidases and metabolism of arachidonic acid (16), mediates cellular responses to oxidative stimuli. In neurons oxidant species such as NO (17) and ROS regulate signaling events, including PI 3-kinase/Akt pathway, c-Jun NH2-terminal kinase/stress-activated protein kinase pathway, and activation of the nuclear factor xB (18, 19), suggesting a dual role as endogenous toxins and signaling molecules.

Target-derived neurotrophins, including nerve growth factor (NGF) and brain-derived neurotrophic factor act as major regulators of neuronal cell differentiation and survival in the developing nervous system and exert a protective effect after acute neuronal injury (20, 21). Neurotrophins exert their effects through a complex network of intracellular signals involving the activation of the PI 3-kinase/Akt pathway, Ras/mitogen-activated protein kinase pathway, and the small GTPases of the Rho family (Rho, Rac, and CDC42) (20, 22). Many NGF-dependent survival signals converge on the transcription factor cAMP-responsive element-binding protein (CREB), a key molecule responsible for the expression of antiapoptotic genes in sympathetic neurons and cerebellar granule cells (23, 24).

Only few target genes for neurotrophin signaling have been identified so far; because neurotrophins increase cell resistance to oxidative stress (25), and application of NGF to sympathetic neurons reduce the generation of oxygen radicals in mitochondria upon serum withdrawal (26), it is conceivable that these trophic factors modulate cellular antioxidant defense. Transcriptional regulation of the mitochondrial superoxide scavenger Mn-SOD represents a general protective event in response to cellular stress and to pro-apoptotic stimuli (27–30).

Interestingly, a cAMP-responsive element is present within the Mn-SOD promoter, and 12-O-tetradecanoylphorbol-13-acetate-mediated gene expression in lung carcinoma cell line (28) is dependent upon CREB activation. Here we show that induction of Mn-SOD in serum-deprived PC12 cells is NGF- and CREB-dependent and may therefore represent a general modality of neurotrophin signaling. Importantly, the molecular pathway linking NGF to Mn-SOD expression reveals an important role for ROS in triggering CREB activation and gene expression, thereby outlining a redox circuitry in which cytotoxic oxidants are used as messenger intermediates to increase mitochondrial protection from oxidative stress.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, and Chemicals—Myristylated human Akt expression construct was kindly provided by Dr. A. Bellacosa. Expression constructs for CREB-Vp16 and CREB-Vp16/3LZ were obtained from Dr. D. D. Ginty. Rac-1 N17 cDNA was provided by Dr. A. Hall and subcloned in the EcoRI site of the pLPC retroviral expression vector (a gift of Dr. S. Lowe). The retroviral construct encoding the oncogenes V12 H-Ras and E1A on the pLPC backbone was provided by Dr. Lowe. The rat Mn-SOD cDNA was obtained by reverse transcription-PCR from PC12 total RNA and cloned in antisense orientation in the transcription factor cAMP-responsive element-binding protein (CREB), a key molecule responsible for the expression of antiapoptotic genes in sympathetic neurons and cerebellar granule cells (23, 24).

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The following antibodies were used in the present study: anti-phospho-Akt Ser-473, anti-phospho-Akt Thr-308, anti-phospho-FKHR Ser-256, and anti-phospho-extracellular signal-regulated kinase 1/2 (New England Biolabs), anti-extracellular signal-regulated kinase, anti-phospho-CREB Ser-133, anti-Mn-SOD (Upstate Biotechnology), anti-actin (Santa Cruz Biotechnology). Horseradish peroxidase (HRP)–conjugated reagents were from Amersham Biosciences (anti-mouse IgG/HRP), Bio-Rad (anti-rabbit IgG/HRP), and Chemicon (anti-goat IgG/HRP).

Rat nerve growth factor was a kind gift of Drs. D. D. Ginty and D. Mercanti. Dichlorofluorescein diacetate (DCF-DA) and dihydro-DCFH diacetate were purchased from Sigma. The synthetic mitochondrial antioxidant Mito-1 (provided by Dr. D. Ginty), selected in puromycin, and assessed for Trk expression by flow cytometry. Wild-type and mutant cells expressing comparable levels of the receptor were chosen for further experimental analysis.

Cell Transfection and Infection—PC12 cells and E1A/Ras MEFs were transduced with LipofectAMINE (Promega) according to the manufacturer’s instructions. Average transfection efficiency was 15–20%, based on cell positivity for GFP. Cells were left to recover for 24–48 h after transfection before functional or biochemical assays. 293T and Phoenix cells were transfected by calcium-DNA co-precipitation according to the standard procedure (50% efficiency).

For retroviral infection, PC12 cells were grown for 48 h in the supernatant of Phoenix cells previously transfected with retroviral pLPC-Mn-SOD/AS constructs. Transduced cells were enriched by 48-h selection in puromycin (2.5 μg/ml).

For in vitro transformation, SOD2 +/+ and SOD2 −/− MEFs were infected with the pLPC-E1A/H-Ras construct, which harbors no selectable marker, and left in culture for 2 weeks. Transformed foci were then isolated and further expanded.

Measurement of ROS—For detection of reactive oxygen species, cells were maintained for 16 h in low (0.5%) serum. After medium replacement with Hanks’ balanced salt solution or serum-free RPMI, NGF was added, and cells were incubated for an additional 60 or 120 min. During the last 30 min of incubation, DCF-DA (1 μM) or DHE (10 μM) were added to cells. After quick detachment from the plate, cells were immediately subjected to flow cytometry (FL-1 for DCF-DA and FL-2 for DHE) using a COULTER-EPIICS flow cytometer equipped with an argon laser lamp (emission 488 nm). The mean cell fluorescence values were expressed as raw data or as fluorescence variances (stimulated – unstimulated).

In some experiments, cells were plated in serum-free medium with or without NGF, and the fluorescent probes were added directly to the medium 24 h later for 30 min. Cells were then processed as described above.

Assays for Cell Viability—Cells were seeded in serum-free medium with or without NGF and/or antioxidants. In experiments with Mito Q, cells were pretreated with the drug for 8 h in complete medium before serum withdrawal. 48 h later, cell viability was determined by flow cytometry.

Live and dead cells were distinguished by flow cytometry according to two criteria, (a) forward/side scatter profile and (b) exclusion of propidium iodide. The majority of cells positive for propidium iodide (dead cells) fell in a distinct region on the forward/side scatter histogram (R2), which could also be easily identified in the forward/side scatter plot in the absence of the fluorescent marker. This latter population was found to be slightly more abundant than the population of propidium iodide-positive cells, likely due to the presence of early apoptotic cells non permeable to propidium iodide. Cell debris was gated out in all the measurements. The percentage of surviving cells was determined according to the forward/side scatter profile and the following formula: [% cells in the “live” region R1/% cells in “dead” region R2 + % cells in the “live” region R1] × 100.

In some experiments GFP was expressed in cells together with a gene of interest to allow the identification of transfected cells by flow cytometry. Because GFP can leak out of apoptotic cells, the absolute number of GFP-positive cells was determined using fluorescent microparticles (Flow-Count fluorospheres, Coulter) as an internal standard, according to the manufacturer’s recommendations.

Cell Stimulation and Lysis—PC12 cells were stimulated in 0 or 0.5% fetal bovine serum with NGF and in 0% FCS with hydrogen peroxide for the indicated times after 16 h of incubation in low (0.5%) serum. Antioxidants, when necessary, were added either 16 h (N-acetylcysteine (NAC)) or 5 h (Mito Q) before stimulation.

For Western blot analysis cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, leupeptin, and pepstatin, 1 mM sodium.
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NGF Rescues PC12 Cells from Apoptosis Induced by Serum Withdrawal—PC12 cells are dependent on calf serum for survival in vitro and rapidly undergo apoptosis when placed in serum-free medium (25). The addition of NGF to the culture medium largely prevents death induced by serum starvation, with 70% of the cells remaining viable after 48 h of fetal bovine serum withdrawal (Fig. 1, A and B).

NGF activates a multiplicity of signaling cascades in PC12 cells, mainly through the interaction with the tyrosine kinase receptor TrkA and the low affinity receptor p75 (20). As previously shown, NGF addition to serum-deprived PC12 cells dramatically induces the phosphorylation of Akt/protein kinase B and extracellular signal-regulated kinases 1/2 (Fig. 1, A and B), an event mirroring the increase in enzymatic catalytic activity. Although phosphorylation of mitogen-activated protein kinases likely reflects the activation of the Ras/Raf pathway by NGF, Akt is a direct target for the lipid products of the PI 3-kinase, an important mediator of survival signaling by NGF (31). Overexpression of a membrane-targeted, constitutively active mutant of human Akt nearly completely abolishes cell death induced by serum deprivation (Fig. 1D). Interestingly, a negligible or at best marginal effect was upon transfection of an active form of Raf (CAAX-Raf, data not shown), further suggesting that NGF-mediated cell survival in serum-deprived condition is mainly mediated by the PI 3-kinase/Akt pathway (24).

NGF Modulates the Intracellular Content of ROS in PC12 Cells—Reactive oxygen species generated in mitochondria trigger apoptotic cell death in response to many stimuli (32). Moreover, ROS are involved in regulation of gene expression, proliferation, and cell differentiation induced by cytokine and growth factor receptors (7).

NGF treatment of PC12 cells maintained in the absence of serum is initially (15 min to 2 h) accompanied by increased intracellular content of ROS (Fig. 2, A and B), as evaluated by the oxidation of two different redox-sensitive fluorescent probes, DCF-DA (Fig. 2, A and B, a) and DHE (Fig. 2B, b). Because DCF-DA is mainly sensitive to hydrogen peroxide,
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Fig. 2. Modulation of intracellular ROS by NGF in serum-deprived PC12 cells. A, dose-dependent increase of intracellular peroxides in PC12 cells exposed to NGF for 60 min. DCF-DA was added 30 min before reading at 20 μg/ml in Hanks’ balanced salt solution. Probe oxidation was quantified by flow cytometry, as described under “Experimental Procedures.” Values are mean fluorescence (mean ± S.D. of duplicate samples) in arbitrary units (a.u.). The figure is representative of several independent experiments. b, N-acetylcysteine inhibits the early raise of intracellular peroxides induced by NGF. PC12 cells were incubated for 16 h with 10 mM NAC in serum-free medium, stimulated with 100 ng/ml NGF for 30 min, loaded with DCF-DA for an additional 30 min, and subjected to flow cytometry. Values are fluorescence variations (NGF-Control (Ctrl)) (mean ± S.D. of duplicate samples). c, reduced generation of peroxide in response to NGF in PC12 cells expressing an inhibitory mutant of Rac-1 (N17Rac). Cells were retrovirally transduced with RacN17 or the corresponding empty vector (pLPC), left to recover for 24 h, and selected in puromycin for an additional 48 h. After overnight incubation in 0.5% FCS, cells were switched to serum-free medium, stimulated with NGF for 30 min plus an additional 30 min in the presence of 20 μg/ml DCF-DA, and subjected to flow cytometry as described above. Values are variations (stimulated – unstimulated) of mean cell fluorescence and are the mean ± S.D. of duplicate samples. The figure is representative of two independent experiments. It should be noted that the percentage of transduced cells was never higher than 60%, based on GFP expression, even after the selection step in puromycin.

A

B

Whereas DHE detects superoxide (33), it is likely that both reactive species are rapidly produced in response to NGF. During the first 2 h of NGF treatment, the increase of intracellular ROS is concentration-dependent, significant at 10 ng/ml and more pronounced at 100 ng/ml (Fig. 2A). Pretreatment of PC12 cells with the glutathione precursor and radical scavenger NAC drastically reduced early NGF-dependent oxidative events (Fig. 2A, b), and a similar decrease was also observed in cells transfectioned with a dominant negative mutant of GTPase Rac-1 (N17Rac) (Fig. 2A, c).

Interestingly, the early increase of ROS after NGF treatment is followed by a decrease of intracellular oxidants (both peroxides and superoxide) with respect to untreated cells at 24 h after NGF application (Fig. 2B, a and b). The late antioxidant effect of NGF coincides with the onset of morphological signs of death in untreated serum-starved cells. This observation suggests that elevated levels of oxidants detected 24 h after serum deprivation in PC12 cells are causally linked to cell death and are counteracted by application of NGF.

NGF Signaling to Akt and CREB Requires ROS—Upon treatment with NGF, PC12 cells respond with a robust activation of the PI 3-kinase pathway, which couples receptor engagement to cell survival through the activation of Akt/protein kinase B (34, 36). A critical downstream event in the survival signaling initiated by NGF is represented by the phosphorylation of CREB, a transcription factor necessary and sufficient for neurotrophin-dependent survival (23, 35, 36). Accordingly, CREB may be a direct target of Akt (24, 37).

To determine whether the early burst of ROS plays a role in NGF-dependent signaling to Akt and CREB, PC12 cells were treated with glutathione precursor and the ROS scavenger NAC to prevent intracellular oxidations, and cell signaling ability was assessed. Removal of ROS resulted in a dramatic decrease of Akt phosphorylation and activity, as evaluated by Ser-256 phosphorylation of the Akt substrate FKHR (38) (Fig. 3A). Concomitantly, pretreatment of PC12 cells with antioxidant NAC resulted in a decrease of CREB phosphorylation on its critical residue, Ser-133, as revealed by immunoblotting using a phosphorylation site-specific antibody (Fig. 3A). Moreover, inhibition of Rac activity, an event necessary for NGF-dependent redox signaling (Fig. 2A, c), resulted in inhibition of CREB phosphorylation (Fig. 3B). Because CREB phosphorylation...
tion is critical for transcriptional activation (39), our data suggest that CREB activity in response to NGF could also be impaired.

To further test the possibility that redox signals regulates CREB in PC12 cells, we assessed whether exogenous hydrogen peroxide is sufficient to induce CREB activation. Cells exposed to 1 mM H$_2$O$_2$ show increased activation of CREB and Akt, to an extent comparable with NGF (Fig. 3C). Hydrogen peroxide-dependent Akt and CREB phosphorylation is inhibited by the PI 3-kinase inhibitor wortmannin (Fig. 3D), suggesting that oxidants activate multiple survival pathways upstream of PI 3-kinase.

**NGF Induces Mn-SOD Expression in PC12 Cells**—CREB-dependent transcriptional activation of antiapoptotic genes contributes to the survival effect induced by NGF. Late reduction of intracellular ROS in NGF-treated PC12 cells (Fig. 2B, a and b) suggests that neurotrophins might regulate antioxidant enzyme expression. Interestingly, the mitochondrial protein Bcl2, which may have some antioxidant functions, is among the few NGF-dependent antiapoptotic proteins hitherto identified and is modulated in a CREB-dependent fashion.

The mitochondrial antioxidant enzyme and putative survival factor Mn-SOD is induced in many cell types by cytokines (tumor necrosis factor-$\alpha$, interleukin-1) (27, 29) and growth factors (platelet-derived growth factor, vascular endothelial growth factor) (40, 41) through signaling cascades that often involve ROS and redox-sensitive transcriptional regulators (36, 52). Moreover, analysis of the human Mn-SOD gene promoter region has revealed the presence of a perfect CRE, a binding site for CREB, which accounts for gene response to 12-O-tetradecanoylphorbol-13-acetate in a human lung carcinoma cell line (28).

Exposure of serum-deprived PC12 cells to NGF results in a significant increase of expression of Mn-SOD mRNA transcripts of 4.1 and 1.1 kilobases (Fig. 4A). SOD2 mRNA induction is present 1 h after NGF treatment and peaks at 12 and 24 h after induction. An increase in RNA transcripts is mirrored by the increase of both Mn-SOD protein (Fig. 4B) and enzyme activity, as assessed by an in-gel nitro blue tetrazolium-based SOD assay (Fig. 4C) (42). Accordingly, the intracellular concentration of superoxide is significantly decreased 24 h after NGF stimulation (Fig. 4D). Taken together, these data suggest that Mn-SOD is a downstream target of NGF signaling in PC12 cells.

**Modulation of Mn-SOD Expression by PI 3-Kinase and CREB**—PI 3-kinase and the transcription factor CREB play a pivotal role in mediating antiapoptotic signaling generated by NGF. PC12 cell pretreatment with the PI 3-kinase inhibitor...
wortmannin significantly inhibited NGF-induced Mn-SOD expression (Fig. 5A). Similarly, pretreatment of PC12 cells with NAC, which diminished both ROS production and CREB phosphorylation in response to NGF (see Figs. 2 and 3), resulted in a significant decrease of Mn-SOD transcription (Fig. 5A). These data suggest that NGF-dependent induction of Mn-SOD requires both PI 3-kinase activity and oxidants in a fashion that mirrors CREB phosphorylation.

To determine whether CREB is sufficient to induce Mn-SOD mRNAs, we overexpressed a constitutively active form of CREB (VP-16 CREB) (23) in both PC12 (Fig. 5B, a) and 293T cells (Fig. 5B, b). Activation of CREB resulted in a significant increase of Mn-SOD mRNAs when compared with cells overexpressing a mutant form of CREB lacking the DNA binding domain (ΔLZ-VP16, Fig. 5B, b). Taken together these data suggest that CREB is required to regulate Mn-SOD expression. Importantly, induction of Mn-SOD by active CREB is insensitive to NAC (5B, b), suggesting that NAC does not indiscriminately repress Mn-SOD gene and reinforcing the idea that signaling roles for ROS in the NGF cascade are upstream of CREB activation.

**Redox Regulation of NGF-dependent Cell Survival**—Our data suggest that NGF regulates ROS in a complex fashion such that oxygen species generated early after neurotrophin stimulation signal to CREB and act as pro-survival mediators. Later on, when toxic oxidants are generated, NGF contributes to their elimination through up-regulation of compartment-specific, mitochondrial antioxidant, such as Mn-SOD. To assess the physiological relevance of these redox changes in PC12 cells, we used two different antioxidants, the generic, membrane-permeant scavenger NAC and a mitochondria-targeted derivative of ubiquinol (Mito Q) (43). PC12 cells treated with 10 mM NAC, a condition that severely impairs NGF signaling to Akt and CREB (Fig. 3A), showed decreased ability to survive in both the presence and the absence of NGF (Fig. 6, A and B). It is therefore conceivable that NAC promotes cell death by blocking protective redox signals initiated by NGF. Conversely, cell exposure to Mito Q mimicked NGF treatment and resulted in an increased resistance to cell death (Fig. 6A, gray columns). Because Mito Q is present at a low concentration (1 μM) in the culture medium and preferentially accumulates in mitochondria (43), it is unlikely to interfere with oxidative reactions that take place in the cytosol, where early ROS are thought to be produced. Still, Mito Q selectively removes harmful oxidants leaking from the respiratory chain during apoptosis, thereby promoting cell survival. In line with this view, Mito Q, unlike NAC, had no effect on NGF-dependent CREB phosphorylation (Fig. 6C) nor did it block the early rise of ROS in cells treated with NGF (data not shown). Along similar lines of evidence, we found that cell exposure to the recently described SOD inhibitor 2-ME (33) drastically reduced NGF-dependent survival without significantly affecting cells grown in standard medium (10% fetal bovine serum) (Fig. 6D). Taken together, these data suggest that oxidative events generated during cell survival and cell death are distinct and physically compartmentalized between cytosol and mitochondria and that NGF survival signaling is critically dependent on the elimination of intracellular superoxide.

**Mn-SOD Is Required for Trk-mediated Antiapoptotic Signaling**—To confirm that antioxidant defense against mitochon-
drial oxidants is important for antiapoptotic signaling by NGF, we first inhibited expression of Mn-SOD by retrovirus-mediated expression of an antisense cDNA (pBabe/Mn-SODAs), as assessed by Western blot analysis (Fig. 7A, b). PC12 cells expressing low levels of Mn-SOD (Mn-SODAs) were more sensitive to cell death in the presence of NGF when compared with mock-transfected cells (Fig. 7A, a). The important role of Mn-SOD in NGF trophic signaling was further demonstrated by making SOD2-deficient transformed fibroblasts or their wild-type counterparts responsive to the neurotrophin through transfection of the NGF receptor Trk. Transformed fibroblasts are highly sensitive to apoptosis by serum deprivation (44). As indicated in Fig. 7B, NGF signaling through Trk protects SOD2-proficient cells from serum withdrawal, whereas no significant protection was observed SOD2 null cells. A block of trophic signaling in SOD2−/− cells occurred downstream of NGF-induced CREB phosphorylation, which occurred at comparable levels in both cell lines (Fig. 7B, b). Although in part artificial, the MEF/Trk model strongly confirms the involvement of Mn-SOD in NGF signaling, extending the above observations to a non-neuronal cell background. These data are therefore consistent with the idea that elimination of harmful oxygen species from mitochondria through the transcriptional up-regulation of Mn-SOD plays a critical role in NGF-dependent cytoprotection (Fig. 8).

DISCUSSION

Experimental observations described in the present paper lead to two major conclusions as follows. 1) Reactive oxygen species have a role in transducing survival signals released by NGF in PC12 cells. Because PC12 cells respond to NGF at least in part through the Trk-A receptor, the above finding is in line with a growing body of evidence concerning the involvement of ROS in intracellular signaling downstream of PTK-receptor molecules. 2) The up-regulation of mitochondrial superoxide dismutase (Mn-SOD) is part of the antiapoptotic genetic program triggered in PC12 cells by nerve growth factor. This is a novel insight that expands our limited knowledge regarding the transcriptional targets of NGF. Moreover, the above observation indicates that mitochondrial protection from oxidative stress is a potentially general mechanism of action for neurotrophins in physiological and pathological settings.

We have postulated that ROS produced shortly after cell exposure to NGF serve signaling functions downstream of activated NGF receptor(s). This idea is in agreement with recent reports on redox signaling by NGF receptors in neuronal cells (45, 46). Although not biochemically characterized in detail, these oxidant species include probably both hydrogen peroxide and superoxide, based on the data obtained with fluorescent redox probes with different radical specificity (DCF-DA and DHE). It should be noted that probe oxidation in response to NGF was not prevented by NO synthase inhibitors L-NAME and NOS in NGF-dependent generation of oxygen species and in the promotion of NGF-induced CREB phosphorylation, which occurred at comparable levels in both cell lines (Fig. 7B, b). Although in part artificial, the MEF/Trk model strongly confirms the involvement of Mn-SOD in NGF signaling, extending the above observations to a non-neuronal cell background. These data are therefore consistent with the idea that elimination of harmful oxygen species from mitochondria through the transcriptional up-regulation of Mn-SOD plays a critical role in NGF-dependent cytoprotection (Fig. 8).

Fig. 5. Effect of PI 3-kinase and CREB on Mn-SOD gene induction. A, induction of Mn-SOD by NGF is blocked or attenuated by the PI 3-kinase inhibitor wortmannin (Wo) and by NAC. Cells were preincubated with wortmannin (10 μM) for 10 min and with NAC for 16 h before exposure to NGF for the indicated times. The two Mn-SOD transcripts are indicated by arrows. The filter was stripped and re-hybridized for actin, to ensure equal RNA loading. Ctrl, control. B, active CREB is sufficient to induce Mn-SOD in PC12 and 293 T cells. Cells were transfected by LipofectAMINE and calcium/DNA co-precipitation, respectively, and incubated for 48 h in complete medium. 293T cells were also exposed to NAC 10 mM for 24 h before RNA extraction (NAC+). 20 μg total RNA were subjected to Northern blot analysis as in A. In B, b, ribosomal RNA was used as the loading control. Transfection efficiencies for the two cell lines are indicated.

An important point emerging from these studies is that NGF...
signaling to Akt and CREB requires ROS and is severely impaired upon antioxidant treatment. Although experiments with oxidized NAC (data not shown) confirm that the inhibitory effect of N-acetylcysteine on the NGF cascade is directly linked to the capacity of the compound to lower the intracellular concentration of ROS, the link between oxygen species and antiapoptotic signaling is further strengthened by the fact that exogenous hydrogen peroxide, as already reported (50), is sufficient per se to induce site-specific phosphorylation of Akt and CREB.

The redox modulation of CREB phosphorylation/activation represents an intriguing finding whose molecular mechanism deserves further investigation. As a first hypothesis, it is conceivable that Akt, once activated by oxidants, directly phosphorylates CREB, a possibility supported by the observation that both Akt and CREB phosphorylation in response to H_2O_2 are PI 3-kinase-dependent (Fig. 3C and Ref. 47). Another important question is whether redox modulation of CREB operates as well in the process of NGF-dependent neuronal differentiation, because it has been reported that ROS induced by NGF are required for differentiation of PC12 cells (45), a process clearly dependent on CREB activity (51).

The finding that a redox-sensitive antiapoptotic cascade is triggered in PC12 cells by NGF may also have broader pathophysiological implications. In fact, because the nervous system is highly prone to oxidative damage (4), activation of survival pathways by oxidants may represent a sensitive and efficient safeguard mechanism designed to increase cell resistance to environmental stress in response to early changes in the intracellular redox balance.

This hypothesis is supported by the finding here presented that NGF induces expression of Mn-SOD, an enzyme able to remove harmful oxidants, that such induction is dependent on PI 3-kinase activity, and that interference with Mn-SOD expression results in impaired NGF survival signaling in both PC12 cells and Trk-expressing transformed fibroblasts (Fig. 7). Moreover, Mn-SOD, which is a well recognized antiapoptotic enzyme promoting mitochondrial integrity, has already been shown to protect other cell types from apoptosis by serum deprivation (30, 33), and a decrease in mitochondrial superoxide has been proposed as a mechanism for neuroprotection by NGF (26). Furthermore, signs of neurodegeneration have been reported in the brain of SOD2-deficient mice (6). Interestingly, another well established target for NGF survival signaling, Bcl-2 (23), is also located in mitochondria, which underlines the importance of these organelles as targets for neurotrophin signaling and suggests a possible physical and/or functional interaction between the two survival proteins.

Although the mechanism of Mn-SOD induction by NGF deserves further investigation, our data imply CREB in the NGF-dependent regulation of Mn-SOD. In fact, an ideal CREB-responsive element is present at position –1258 of the human Mn-SOD promoter (28), and overexpression of CREB leads to Mn-SOD overexpression in PC12 and 293T cells (Fig. 7). Moreover, cell treatment with NAC drastically decreases Mn-SOD expression in response to NGF concomitantly with a significant reduction in the phosphorylation/activation of CREB. Interestingly, Mn-SOD is induced by a constitutively active mutant of CREB in a ROS-independent fashion, suggesting that this factor is a downstream target for redox signaling in the cascade linking NGF to the up-regulation of Mn-SOD. Further studies utilizing CREB inhibitory molecules will be required to assess

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**Fig. 6.** Redox regulation of NGF-dependent cell survival. A, modulation of serum-starved PC12 cell survival by NAC and Mito Q. Shown are variations in the percentage of cell survival in antioxidant- versus vehicle-treated cells with (black columns) or without (gray columns) NGF 100 ng/ml. NAC reduces cell survival mainly in NGF-treated cells, whereas Mito Q increases cell resistance to serum deprivation, with marginal effects on NGF. B, raw percentages of cell survival in one of two independent experiments. A small inhibitory effect of Me_S0 (vehicle for Mito Q) was consistently observed, maybe due to its properties of radical scavenger. NAC (10 mM) was added to cells upon serum deprivation together with NGF, whereas Mito Q was added 8 h earlier in complete medium to allow accumulation in active mitochondria and removed for the rest of the experiment. C, NAC, but not Mito Q, interferes with NGF signaling to CREB. Cells were treated as in A, except that Mito Q was added for 8 h in serum-free medium. Phosphorylation of CREB (P-CREB) was assessed as described above. Equal protein loading was verified by anti-actin immunoblotting of the same nitrocellulose filter. D, inhibition of NGF rescue by the SOD inhibitor 2-ME. Cells were seeded in either standard medium (10% FCS) or serum-free medium plus 100 ng/ml NGF (SF + NGF), in presence of titrated concentrations of 2-ME. Cell survival was determined 48 h later by flow cytometry. Fig. representative of two independent experiments. Values are mean ± S.D. of duplicate samples.
the relative role of this factor in the induction of Mn-SOD by NGF with respect to other potential candidates such as nuclear factor \( \kappa \)B. However, even though the mechanisms involved are unclear, the evidence of Mn-SOD induction through a ROS-dependent cascade is biologically sound and in line with other findings that oxidants are inducers of antioxidant enzymes in both prokaryotic (52) and eukaryotic cells (53).

The proposed role for ROS as both mediators and targets for survival signaling by NGF may appear contradictory. We suggest that oxygen species involved in early NGF signaling are different from those responsible for cell death in both their timing of production and their subcellular sites of production. Although NGF-induced ROS are produced rapidly after receptor stimulation through Rac-1-dependent mechanisms, late generation of ROS likely takes place in mitochondria and reflects the onset of mitochondrial dysfunction. These latter oxi-
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dants may be eliminated by Mn-SOD, which is in turn induced by NGF (Fig. 8). Importantly, although Mn-SOD activity is expected to lead to peroxide accumulation, decreased DCF-DA fluorescence in concomitance with elevated intracellular SOD is not surprising and is consistent with important recent reports (54, 55).

Although in part speculative, the idea that NGF-triggered redox signaling is intracellularly compartmentalized is in line with similar findings concerning survival signaling by tumor necrosis factor-α (56) and is supported by data displayed in Fig. 6, which shows the opposite effect of a generic (NAC) versus a mitochondrially targeted (Mito Q) antioxidant in cell response to serum deprivation and to NGF. Although Mito Q well mimics the compartment-specific effect of Mito Q-SOD, NAC freely diffuses throughout the cell, inhibiting survival signaling. On the other hand, because NAC can also remove harmful oxidants from mitochondria, this effect could partially mitigate the upstream inactivation of ROS-dependent antiapoptotic signals, thereby explaining the discrepancy between the profound inhibition of Akt, CREB, and Mn-SOD and the limited decrease in cell survival observed in cells treated with NAC (compare Figs. 3 and 6). One final consideration deals with the significance of the PC12 model with respect to the general mechanisms of neuroprotection by NGF. Although NGF plays a key role in target-determined cell survival within the developing nervous system (4), the effect of the neurotrophin on undifferentiated, serum-starved PC12 cells depicts a more general situation of cell rescue from an acute insult. How-
Redox Regulation of cAMP-responsive Element-binding Protein and Induction of Manganese Superoxide Dismutase in Nerve Growth Factor-dependent Cell Survival
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