Differential Effects of Superoxide Dismutase Isoform Expression on Hydroperoxide-induced Apoptosis in PC-12 Cells*

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The current study examines the contribution of mitochondria-derived reactive oxygen species (ROS) in tert-butyl-hydroperoxide (TBH)-induced apoptotic signaling using clones of undifferentiated pheochromocytoma (PC-12) cells that stably overexpress the human mitochondrial Mn-SOD (1), and the cytoplasmic copper zinc superoxide dismutase (CuZn-SOD). The involvement of the mitochondria in apoptotic signaling is a generally accepted paradigm for the activation of cellular apoptosis mediated by oxidants (1). However, the contribution of mitochondrial generated ROS to the initiation of apoptotic signaling and the roles that the mitochondrial antioxidant enzyme, manganese superoxide dismutase (Mn-SOD), and the cytoplasmic copper zinc superoxide dismutase (CuZn-SOD) play in modulating oxidant-induced cell apoptosis are not completely resolved. Previous studies in animal and cell models of SOD overexpression have demonstrated that overexpression of CuZn-SOD and Mn-SOD can both be beneficial and detrimental. For instance, different studies have shown that CuZn-SOD overexpression rendered mice more susceptible to infection (2), induced muscle aberrations (3), and destruction of axons in neuromuscular junctions (4) and death of trisomy 16 neuronal cells (5), yet other studies showed that enzyme overexpression decreased myocardial ischemia/reperfusion injury (6). In comparison, overexpression of Mn-SOD has generally exerted protective effects, such as protection of murine fibroblastic cells from 5-azacytidine-dependent apoptosis (7), attenuation of hyperglycemic-induced bovine endothelial cells oxidative stress (8), and protection against adriamycin-induced acute cardiac toxicity (9). Moreover, increased oxidative damage has been found to be associated with altered mitochondrial function in heterozygous Mn-SOD knockout mice (10). However, increased invasiveness of tumor metastasis has been associated with Mn-SOD overexpression (11).

The quantitative significance of SOD overexpression in cell apoptosis is poorly defined. Moreover, a direct link between SOD overexpression and the intracellular antioxidant status, such as GSH, has not been vigorously explored. Previous studies have shown that as compared with control animals, transgenic mice overexpressing the heterozygous form of Mn-SOD (Mn-SOD+/-) exhibited a decrease of 30–50% reduced GSH in several tissues, such as the lung, brain, and muscle (12). Notably, as these animals age, Mn-SOD transgenic mice exhibited increased muscle apoptosis when compared with age-matched controls (12). In other studies, an overall increase in tissue GSH content was found to be associated with transgenic mice overexpressing CuZn-SOD (13). Thus, the relationship between overexpression of the two different isoforms of SOD and the intracellular GSH status and how these changes contribute to oxidant-induced initiation of cell apoptosis has not been thoroughly investigated.

The objective of the current study was designed to address these questions using tert-butyl-hydroperoxide (TBH) as a model hydroperoxide and undifferentiated pheochromocytoma (PC-12) cells, a cell model that has been previously characterized by Greene and Tischler (14), to study the cellular and molecular aspects of neuronal apoptosis following induction of cell differentiation in culture with nerve growth factor. To address our hypothesis that mitochondrial derived ROS are an important functional mediator of TBH-induced apoptosis in PC-12 cells, we have generated PC-12 clones that overexpress the human mitochondrial Mn-SOD or the human cytoplasmic CuZn-SOD. Given the central role that the mitochondria play in the initiation of the apoptotic cascade mediated by oxidants...
and redox imbalance (1, 15), we determined the contribution of mitochondrial ROS and cellular redox shifts to cell apoptosis in PC-12 cells that stably or transiently overexpress Mn-SOD or CuZn-SOD.

EXPERIMENTAL PROCEDURES

Materials—The following chemicals were obtained from Sigma: agarose, TBG, 4',6-diamidino-2-phenylindole (DAPI), and DNA markers (4X174 HoeIλ and λ EcoRI HindIII). Fetal calf serum and horse serum were obtained from Atlanta Biologicals (Norcross, GA). Monoclonal antibodies against Bax, Bcl-2, and CPP32 were acquired from Transduction Laboratories (Lexington, KY), and the monoclonal antibody against β-actin was bought from Oncogene (Cambridge, MA). Dulbecco’s modified Eagle’s medium was obtained from Invitrogen. Nitrocellulose membranes were acquired from Bio-Rad. The enhanced chemiluminescence system for Western immunoblot (ECL) and the hyperfilm were purchased from Amersham Biosciences. Fluorescent mounting medium was obtained from DAKO Corp. (Carpinteria, CA). 12-mm circle number 1 glass cover slips used for DAPI staining were procured from Fisher. The cDNAs for human Mn-SOD and CuZn-SOD were generously provided by Dr. Sonia Flores (Webb-Waring Institute, Denver, CO). The pRetro-Off expression vectors were obtained from Clontech (Palo Alto, CA). Restriction enzymes were from New England Biolabs (Beverly, MA).

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Preparation of Cell Lysates and Western Analyses of CPP32—PC12 clones were plated at a density of 2 × 10⁶ cells and were harvested at the indicated times (0–6 h) after TBH treatment according to the method of Stefanis et al. (18). Cells were scraped and collected into buffer (25 mM HEPES, pH 7.5, 5 mM EDTA, 2 mM EDTA, 1% Triton X-100, 10 μg/ml each pepstatin and aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 25 mM β-mercaptoethanol). The nuclear pellets were separated from supernatants by centrifugation (1,500 rpm, 5 min). The pellets were stored at −20 °C for Western analyses of PARP and its degradation product. Twenty μg of the protein was resolved on an 8% acrylamide gel and blotted onto nitrocellulose membranes. The membranes were probed with 1:1000 dilution of PARP (rabbit anti-mouse polyclonal antibody) and secondary IgG antibody 1:1000 and then treated with ECL and exposed to film.

Determination of Total Cellular and Mitochondrial Oxidant Production—Total cellular oxidant formation was measured using the oxidant-sensitive nonfluorescent probe, dihydrodihydrodiamine 123 (DHR). Previous studies have utilized oxidation of DHR for the detection of general cellular oxidant production and DHR oxidation is stimulated by biological oxidants like peroxynitrite and a variety of secondary hydroxyl peroxide-dependent intracellular reactions such as H₂O₂-cytochrome c and H₂O₂-Fe²⁺ (19, 20). Intracellular two-electrode oxidation of DHR results in the formation of the fluorescent product, rhodamine 123, which preferentially accumulates in mitochondria according to the trans-mitochondrial potential although DHR can be oxidized in different compartments throughout the cell. Measurement of rhodamine fluorescence at the excitation/emission wavelengths of 505 nm/536 nm provides reasonable quantification of the overall cellular oxidant production. DHR was prepared as a 25 mM stock solution in nitrogen-purged dimethyl formamide and kept stored in the dark at −20 °C. For each experiment, stock DHR was diluted fresh in PBS, and sonicated (Braun-Sonic sonicator, Braun Biotech International, Allentown, PA). Rhodamine 123 accumulation was quantified using a luminescence spectrophotometer (AMINCO Bowman Series 2, Thermo Spectronic, Rochester, NY), at excitation and emission wavelengths of 505 and 538 nm, respectively. Results are expressed as relative fluorescence units/mg of protein.

Mitochondrial oxidant production was assessed using the mitochondria-specific ROS-sensitive fluorescent probe, Mito Tracker Red. PC-12 cells stably transfected with pRetro-Off vector control or Mn-SOD or CuZn-SOD were grown on coverslips and treated with TBH for 0–45 min. Cells were washed twice with PBS and then incubated for 15 min with 50 nM Mito Tracker Red (CM-H₂XROS; Molecular Probes, Inc., Eugene, OR). To verify that mitochondria were the site of ROS formation, co-incubations were performed with 50 nM Mito Tracker Green FM (Molecular Probes), a mitochondria-specific fluorescence probe. Cells loaded with the fluorescence probes were imaged using a Nikon E800 fluorescence microscope, with ×40/1.0 numerical aperture oil or ×60/1.4 numerical aperture oil objectives and excitation/emission wavelength pairs of 490 nm/516 nm and 581 nm/644 nm, for Mito Tracker Green and Mito Tracker Red, respectively. Illumination was provided by a 50-watt Xenon metal-halide source (EFOs, Inc., Mississauga, Canada). An excitation filter was used, and illumination was determined using a photomultiplier tube in the microscope field. All images were captured using a SynSys digital camera (Photometrics, Tucson, AZ) at equal exposure time (500 ms) as determined from the fluorescent emission from untreated pRetro-Off control cells. For the overlaid images, the exposures were made at the same plane of focus for both excitation wavelengths at equal times. Three different fields were computer gradient and analyzed using MetaMod Software (Universal Imaging, Downingtown, PA).

Production of Mammalian Expression Vectors Containing Mn-SOD or CuZn-SOD—The human Mn-SOD cDNA was isolated from pBluescript following NotI and SacI digestion and ligated into the NotI and SacI sites of pBlUESII. This resulting vector was digested with NotI and XmaI and treated with T4 DNA ligase. This fragment of DSOD containing the SacI fragment of 0.45 kb from the SV40 polyadenylation signal. pRetro-Off was digested with BamHI and treated with Klenow to produce blunt end termini. After further digestion of pRetro-Off with NotI, the Mn-SOD NotI-XmaI fragment was ligated into pRetro-Off. The human CuZn-SOD was isolated from...
pBluescript following BamHI digestion and ligated into the BamHI site of pRetro-Off. The orientation of the cDNAs of Mn-SOD and CuZn-SOD in pRetro-Off was confirmed by restriction enzyme analysis and sequencing.

**Transfection of PC-12 Cells**—Transfection of expression vectors was performed by electroporation. The transfection efficiency of PC-12 cells was initially tested by electroporating pGreenlantern (Invitrogen), a plasmid that expresses the green fluorescent protein, into cells using voltages of 150–350 V. After 24–48 h, transfection efficiency was assessed by the percentage of cells expressing green fluorescent protein by flow cytometry. At 200 V, the transfection efficiency was typically 35–40%. Test (Mn-SOD-pRetro-Off or CuZn-SOD-pRetro-Off) or control (pRetro-Off) vectors (10 μg) were mixed in 200 μl of sterile PBS and used to suspend 5 × 10⁵ cells. Cell and DNA mixtures were subjected to 200 V at a capacitance of 960 microfarads. The cells were resuspended in growth media and allowed to grow for 2–3 days. After replating at a population density of 5 × 10⁵/cm², cells were grown in puromycin (1.5 mM/ml) to select for the cells that have integrated the plasmid DNA into their genome. For generation of stable Mn-SOD- or CuZn-SOD-overexpressing cell lines, cells were medium changed every 2 days until single colonies formed. The colonies were isolated and individually expanded to form the respective cell lines. Genomic DNA was prepared from cells using the Qiamp DNA blood minikit (Qiagen) and tested for presence of vector DNA incorporation in the genome using PCR.

**Determination of SOD Activities**—As previously described, total SOD activity (cytoplasmic CuZn-SOD and mitochondrial Mn-SOD) was determined by the ability of cell-free extracts to inhibit the oxidation of cytochrome c by xanthine oxidase in the absence of cyanide (21). The oxidation of cytochrome c was measured by a change in the absorbance at 550 nm. Mn-SOD activity was determined in the presence of cyanide (21). CuZn-SOD activity was measured by a change in the absorbance at 550 nm. Mn-SOD activity was determined in the presence of cyanide. CuZn-SOD activity as compared with the respective enzyme activities of the SOD isoforms. The data were expressed as units/mg protein.

**Protein Assay**—Protein was measured using the Bio-Rad Protein Assay kit according to the manufacturer’s protocol.

**Statistical Analysis**—Results are expressed as mean ± S.E. Data were analyzed using a one-way analysis of variance with Bonferroni corrections for multiple comparisons or using Student’s t test. p values of <0.05 were considered as statistically significant.

**RESULTS**

**TBH-induced PC-12 Apoptosis Is Associated with ROS Generation and the Abrogation by NAC**—For our studies, we have generated three clones of pRetro-Off as well as three clones of Mn-SOD-overexpressing and two clones of CuZn-SOD-overexpressing cells. The results of a complete set of studies using one clone from each of pRetro-Off, Mn-SOD, and CuZn-SOD are presented. Initial experiments show that the other two clones behaved similar to one another and gave reproducible results in response to TBH.

Fig. 1 summarizes the results on the effect of TBH on cellular production of ROS and cell apoptosis in undifferentiated wild type PC-12 cells. Fig. 1A shows that treatment of cells with 100 μM TBH caused a 3-fold increase in rhodamine fluorescence at 30 min, consistent with an early generation of ROS. This TBH-induced DHR oxidation was eliminated by pretreatment of cells with the thiol antioxidant, NAC. The production of ROS caused by TBH elicited ~37% cell apoptosis at 24 h, which was completely prevented by NAC pretreatment (Fig. 1B). Notably, the addition of NAC at 1 h after TBH exposure and at a time after ROS increase did not confer cytoprotection against TBH challenge (Fig. 1B). Collectively, these results are consistent with the suggestions that ROS are a mediator of TBH-induced PC-12 apoptosis and that the initiating signaling occurred within the first 30 min after cells were treated with TBH.

**Mn-SOD and CuZn-SOD Differentially Affect Apoptosis in PC12 Cells**—To determine the intracellular source of ROS production, we generated PC-12 clones that overexpress the human mitochondrial Mn-SOD or the human cytoplasmic CuZn-SOD cDNAs. The insertion of Mn-SOD and CuZn-SOD pRetro-Off mammalian expression vectors into the genome of PC-12 cells was confirmed by PCR (data not shown). The extent of Mn-SOD and CuZn-SOD overexpression was quantified by the respective enzyme activities of the SOD isoforms. The data in Fig. 2A show that transfection with Mn-SOD resulted in a 3.5-fold increase in cellular Mn-SOD enzyme activity, whereas transfection with CuZn-SOD resulted in a 2-fold elevation in CuZn-SOD activity as compared with the respective SOD activities in wild type PC-12 cells and pRetro-Off vector controls. Differential fractionation of cell extracts confirmed that the transfected Mn-SOD and CuZn-SOD proteins were localized to the respective mitochondrial and cytoplasmic compartments (Fig. 2B). In each instance, the endogenous enzyme activity of the other SOD isoform was unaffected by the transfections, indicating that the overexpression of either Mn-SOD or CuZn-SOD did not cause a compensatory up- or down-regulation of the respective isoform.

The results on the effect of TBH on apoptosis of wild-type PC-12 cells and PC-12 clones that stably overexpress Mn-SOD or CuZn-SOD are summarized in Fig. 3. TBH induced ~32% apoptosis in cells transfected with the pRetro-Off vector, a value that was similar to wild type (35%), indicating that the introduction of the vector per se did not influence the effect of TBH. Notably, Mn-SOD-overexpressing cells (hereafter termed Mn-SOD+S cells) were significantly protected from TBH-induced apoptosis, whereas apoptosis was exacerbated in CuZn-SOD-overexpressing cells (hereafter termed CuZn-SOD+S cells). These results suggest that the differential effects of TBH on apoptosis of PC-12 cells overexpressing Mn-SOD or CuZn-SOD were associated with the increases in the respective enzymes in these cells.

**TBH Induces Differential Kinetics of Caspase-3 Activation in Mn-SOD- and CuZn-SOD-overexpressing Cells**—The base-line expression of CPP32 in pRetro-Off vector control and Mn-SOD+S cells (Fig. 4, A and B, respectively) was low, in agreement with wild type PC-12 cells (22, 23). In contrast, base-line procaspase-3 expression was elevated in CuZn-SOD+S cells (Fig. 4C). TBH treatment caused an increase in CPP32 levels in Mn-SOD+S cells at 30 min followed by a decrease at 6 h (Fig. 4B), similar to pRetro-Off control (Fig. 4A) and wild type PC-12 cells (22). In CuZn-SOD+S cells, TBH challenge resulted in a marked decrease in procaspase-3 levels at 30 min, consistent with a rapid cleavage of CPP32 to active caspase-3. The time course of caspase-3 activation directly correlated with the cleavage of its target substrate, PARP, from a native 116-kDa protein to an 85-kDa product. Significant cleavage of PARP...
SOD Expression and PC-12 Apoptosis

Glutathione Levels Are Differentially Affected by TBH in Mn-SOD and CuZn-SOD Cells—To determine whether the TBH-induced cell apoptosis was associated with altered cellular redox, we determined the cellular concentrations of GSH and GSSG. The overexpression of Mn-SOD or CuZn-SOD alone in stably transfected PC-12 cells significantly elevated cellular GSH (3-fold above baseline values) (Fig. 8A), consistent with an increase in GSH synthesis as an adaptive response to elevated SOD levels. TBH challenge in pRetro-Off controls transiently decreased cellular GSH in 5 min but recovered to baseline levels by 30 min (Fig. 8A). Similar kinetics of GSH responses were observed in CuZn-SOD-S cells exposed to TBH (Fig. 8A). In contrast, TBH caused a small decrease in cell GSH in Mn-SOD-S cells at 30 min (Fig. 8A); this delay in GSH loss is consistent with reduced ROS formation in these cells (see Fig. 7). The time course of GSSG increases in pRetro-Off controls (at 5 min) and Mn-SOD-S cells (at 30 min) (Fig. 8B) corresponded to the decreases in GSH in these cells (see Fig. 8A). By 1 h, GSSG levels returned to baseline values. In contrast, TBH caused a marked and sustained elevation in cellular GSSG levels from 5 min to 2 h in CuZn-SOD-S cells (Fig. 8B), indicating an induction of an exaggerated oxidized state in cells that stably overexpress the cytoplasmic form of SOD. The ratio of GSH to GSSG is high in Mn-SOD-stable transfectants, indicative of a highly reduced intracellular milieu that was minimally altered with TBH challenge (Fig. 8A). A substantially lower GSH/GSSG ratio was found in CuZn-SOD-stable transfectants (Fig. 8C), which largely reflected the exaggerated GSSG levels despite high GSH contents, consistent with a high level of base line oxidative stress in these cells.

TBH. Interestingly, CuZn-SOD overexpression was without effect on TBH-induced cellular ROS production, consistent with a minor contribution of cytosolic ROS to cell apoptosis.

Two additional strategies were used to confirm that mitochondrial derived oxidants were responsible for PC-12 cell apoptosis induced by TBH. First, we pretreated pRetro-Off control cells with known inhibitors of mitochondrial sites of ROS production, namely, rotenone, an inhibitor of Complex I (NADH dehydrogenase), and antimycin A, an inhibitor of Complex III (cytochrome bc1 complex). The results (Fig. 6) show that rotenone as well as antimycin A treatment significantly attenuated TBH-induced apoptosis, indicating that blockade of electron flux at Complex I and Complex III can effectively prevent the apoptotic outcome, in agreement with previous observations (22). Second, the mitochondrial source of oxidant production was directly assessed using Mito Tracker Red, a mitochondria-specific ROS sensitive fluorescence probe, and verified with Mito Tracker Green co-incubation. In untreated cells, mitochondria predominantly exhibited green fluorescence, consistent with low ROS generation (Fig. 7, A and E). TBH challenge in pRetro-Off control cells caused rapid and time-dependent increases in red fluorescence relative to green (15–45 min; Fig. 7, B–D), consistent with enhanced ROS production. In contrast, red fluorescence was not detectable at 15 min and minimally increased at 30 min in TBH-treated Mn-SOD-S cells (Fig. 7, F and G), indicating attenuated and kinetically delayed ROS formation. Consistent with this suggestion, the extent of Mito Tracker Red fluorescence at 45 min in Mn-SOD-S cells (Fig. 7H) was similar to control cells at 15 min after oxidant exposure (Fig. 7B). Parallel cell incubations of pRetro-Off controls and Mn-SOD-S cells with 2,7'-dichlorofluorescein acetate, a cytoplasmic specific ROS probe, gave no detectable fluorescence at all time points (data not shown), indicating a lack of ROS generation in the cytoplasm. Taken together, these results demonstrate that mitochondrial ROS production is a critical early step in TBH-induced apoptotic signaling in PC-12 cells.

Glutathione Levels Are Differentially Affected by TBH in Mn-SOD and CuZn-SOD Cells—To determine whether the TBH-induced cell apoptosis was associated with altered cellular redox, we determined the cellular concentrations of GSH and GSSG. The overexpression of Mn-SOD or CuZn-SOD alone in stably transfected PC-12 cells significantly elevated cellular GSH (3-fold above baseline values) (Fig. 8A), consistent with an increase in GSH synthesis as an adaptive response to elevated SOD levels. TBH challenge in pRetro-Off controls transiently decreased cellular GSH in 5 min but recovered to baseline levels by 30 min (Fig. 8A). Similar kinetics of GSH responses were observed in CuZn-SOD-S cells exposed to TBH (Fig. 8A). In contrast, TBH caused a small decrease in cell GSH in Mn-SOD-S cells at 30 min (Fig. 8A); this delay in GSH loss is consistent with reduced ROS formation in these cells (see Fig. 7). The time course of GSSG increases in pRetro-Off controls (at 5 min) and Mn-SOD-S cells (at 30 min) (Fig. 8B) corresponded to the decreases in GSH in these cells (see Fig. 8A). By 1 h, GSSG levels returned to baseline values. In contrast, TBH caused a marked and sustained elevation in cellular GSSG levels from 5 min to 2 h in CuZn-SOD-S cells (Fig. 8B), indicating an induction of an exaggerated oxidized state in cells that stably overexpress the cytoplasmic form of SOD. The ratio of GSH to GSSG is high in Mn-SOD-stable transfectants, indicative of a highly reduced intracellular milieu that was minimally altered with TBH challenge (Fig. 8A). A substantially lower GSH/GSSG ratio was found in CuZn-SOD-stable transfectants (Fig. 8C), which largely reflects the exaggerated GSSG levels despite high GSH contents, consistent with a high level of base line oxidative stress in these cells.
cells. Treatment with TBH further exacerbated the cellular GSH/GSSG imbalance (Fig. 8C). Cells were subjected to digitonin fractionation (24) to determine the mitochondria or cytoplasmic sites of GSH oxidation. Initial results showed that Mn-SOD-S cells exhibited higher mitochondrial GSH content than pRetro-Off vector controls and were minimally altered by TBH treatment. Unfortunately, the values were near the limit of detection (at the optimal cell density of 10^6/ml for this method), which precluded accurate quantification and meaningful conclusions regarding specific intramitochondrial GSH/GSSG changes. Current efforts are aimed at optimizing the digitonin method for handling larger quantities of cells without compromising the effective separation of mitochondrial and cytoplasmic compartments.

Fig. 4. TBH-induced caspase-3 activation and PARP cleavage. pRetro-Off vector and Mn-SOD-S and CuZn-SOD-S PC-12 cells were treated with 100 μM TBH, and at various times, samples were collected and total protein cell lysates were prepared and processed for Western immunoblot of CPP32 expression. In parallel experiments, extracts were collected as described by Stefanis et al. (18) (see “Experimental Procedures”) and analyzed for PARP (116 kDa) and its cleavage product (85 kDa). The immunoblots of CPP32 and PARP are one representative of three separate experiments for pRetro-Off vector control (A), Mn-SOD-S (B), and CuZn-SOD-S (C). Each immunoblot for CPP32 and PARP was reprobed for β-actin, and the results verified equal loading in each lane. Mn-SOD-S and CuZn-SOD-S, cells with stable overexpression of Mn-SOD and CuZn-SOD, respectively.

Fig. 5. Effect of TBH on ROS generation in wild type PC-12 cells and PC-12 clones overexpressing CuZn-SOD or Mn-SOD. PC12 wild type and clones were treated with 100 μM TBH for 30 min in the presence of 5 μM DHR. The oxidation of DHR (a measure of ROS production) was determined as the increase in the fluorescence of rhodamine 123, the oxidation product, at excitation/emission wavelengths of 500 and 536 nm, respectively. Results are expressed as relative fluorescence units (RFU/μg of protein) and presented as mean ± S.E. for six separate preparations. Mn-SOD-S and CuZn-SOD-S, cells with stable overexpression of Mn-SOD and CuZn-SOD, respectively. *, p < 0.05 versus corresponding zero time control; #, p < 0.05 versus TBH-treated wild type, pRetro-Off vector control, and CuZn-SOD-S cells.

Fig. 6. Effect of mitochondrial inhibitors on TBH-induced apoptosis in pRetro-Off vector controls. Cells were grown on cover slips and treated with 100 μM TBH in the absence or presence of 50 μM rotenone (Rot) or 1 μM antimycin A (Anti A) for 24 h. Cells were stained with DAPI, and apoptotic cells were counted. Results are mean ± S.E. for four separate experiments. *, p < 0.05 versus control; #, p < 0.05 versus TBH treatment.
Transient Expression of Mn-SOD and CuZn-SOD Does Not Induce GSH Increases—To test whether the elevation in GSH content per se secondary to stable Mn-SOD expression mediated the protection against TBH-induced apoptosis in Mn-SOD-S cells, we performed transient transfections with Mn-SOD to elevate enzyme activity without a concomitant adaptive increase in cellular GSH content. Cells transiently transfected with Mn-SOD (hereafter termed Mn-SOD-T) exhibited 1.8-fold increases in mitochondrial enzyme activity (2.4 units/mg protein versus 1.2 units/mg protein in pRetro-Off), whereas cells transiently transfected with CuZn-SOD (hereafter termed CuZn-SOD-T) exhibited 1.5-fold increases in cytoplasmic enzyme activity (3.1 units/mg protein versus 2 units/mg protein in pRetro-Off). The cellular GSH levels in Mn-SOD-T were not different from vector controls or wild type cells (22) but were significantly lower than those in stable Mn-SOD transfectants (Fig. 9A). Furthermore, the ratio of GSH to GSSG in Mn-SOD-T resembled that in pRetro-Off vector controls and wild type cells (Fig. 9A). CuZn-SOD-T exhibited base-line levels of GSH and GSH/GSSG ratio similar to controls (Fig. 9, A and B) and wild type cells (22). Whereas the cellular GSH content in stable CuZn-SOD transfectants was elevated, the GSH/GSSG ratio was decreased (Fig. 9B), a consequence of the exaggerated GSSG level in the CuZn-SOD-overexpressing cells.

TBH-induced Apoptosis Is Attenuated in Mn-SOD-T and Exacerbated in CuZn-SOD-T Cells—Fig. 10 summarizes the results on the effect of TBH on apoptosis of Mn-SOD-T and CuZn-SOD-T cells. The transient expression of Mn-SOD resulted in significant protection against TBH-induced apoptosis, but apoptotic death was potentiated in cells transiently transfected with CuZn-SOD, similar to the responses in cells stably transfected with the respective SOD isoforms (see Fig. 3).

DISCUSSION

In a recent study, we have provided evidence that the induction of apoptosis in undifferentiated PC-12 cells by tert-TBH follows a sequence of events that were consistent with mitochondrial signaling in apoptosis (namely TBH-induced ROS generation, loss of redox imbalance, mitochondrial cytochrome c release, and activation of caspase-3) (22). Our current results support a role for mitochondrial ROS in mediating PC-12 apoptosis. This contention is supported by several lines of evidence. The finding that transfection with Mn-SOD afforded protection against TBH-induced apoptosis that directly correlated with a decrease in ROS generation is consistent with the quenching of mitochondrially generated ROS by Mn-SOD. In comparison, the overexpression of CuZn-SOD was without effect. Using mitochondria specific ROS probes, we have verified that mitochondria are the important sites of ROS formation. Moreover, blockade of electron flow at NADH dehydrogenase (Complex I) and cytochrome bc1 complex (Complex III) effectively prevented TBH-induced cell apoptosis, consistent with mitochondrial ROS production being a critical step in apoptotic signaling, in agreement with previous studies (22). We found similar attenuation of lipid hydroperoxide-induced apoptosis in CaCo-2 intestinal cells by pretreatment of cells with rotenone or antimycin A but not with thienyl trifluoroacetate, an inhibitor of succinate dehydrogenase (Complex II). These findings support current paradigms that Complexes I and III, but not Complex II, are major intramitochondrial sources of superoxide formation (25, 26). Taken together, the data support our contention that enhanced mitochondrial production of ROS induced by TBH challenge is an important trigger of the mitochondrial redox signaling events that subsequently lead to PC-12 cell apoptosis. At present, the trigger for mitochondrial ROS generation is unknown, but evidence in the literature suggests a linkage to mitochondrial cytochrome c loss (27).

Regardless of the mechanism, the current results demonstrate that the time window for ROS signaling was within 30–60 min after TBH treatment, since the addition of NAC at 1 h following TBH exposure failed to rescue the cells from the apoptotic outcome at 24 h. In mitochondrial apoptotic signaling, the release of cytochrome c functions as the initiator and mediator of the apoptotic cascade via the activation of caspase-3 (28, 29). Previous studies have shown that the channel-mediated mitochondrial exit of cytochrome c via function of the Bcl-2 families of pro- and antiapoptotic proteins (29) subsequently leads to the activation of caspase-3. Notably, the kinetics of caspase-3 activation and PARP cleavage at 30 min after oxidant challenge in CuZn-SOD-S cells is consistent with the rapid initiation of apoptotic signaling and the exacerbation of apoptosis in these cells. The activation kinetics of caspase-3 and PARP cleavage in Mn-SOD-S cells were substantially slower at 6 h downstream of TBH-induced cellular GSH/GSSG shifts and in conjunction with delayed mitochondrial ROS production. These results support the paradigm that activation of mitochondrial signaling determines the point of no return for the activation of cellular apoptosis for a variety of death signals like oxidants and DNA damage (30).

It is notable that stable transfection with Mn-SOD or CuZn-SOD resulted in significant increases in intracellular GSH concentrations (3–4-fold), consistent with an adaptive synthesis of GSH secondary to the stable overexpression of the SOD isoforms, since transient overexpression of the enzymes did not elicit GSH increases. Increases in GSH with SOD overexpression have been demonstrated (5, 31). Interestingly, despite a
and Mn-SOD-S) were treated with 100 μM TBH, and at 0–6 h, samples were collected and derivatized for analyses of GSH and GSSG by HPLC as described under “Experimental Procedures.”

A

Kinetics of changes in intracellular GSH and GSSG induced by TBH. PC12 clones (pRetro-off vector control, CuZn-SOD-S and Mn-SOD-S) were treated with 100 μM TBH, and at 0–6 h, samples were collected and derivatized for analyses of GSH and GSSG by HPLC as described under “Experimental Procedures.” A, GSH; B, GSSG; C, GSH-to-GSSG ratio. Cellular concentrations of GSH and GSSG are expressed as nmol/mg of protein and presented as mean ± S.E. for six separate experiments. Mn-SOD and CuZn-SOD cells with stable overexpression of Mn-SOD or CuZn-SOD, respectively. A and B, *, p < 0.05 versus pRetro-Off vector control; #, p < 0.05 versus zero time. C, *, p < 0.05 versus pRetro-Off vector control; #, p < 0.05 versus Mn-SOD-S.

similar magnitude of GSH increases, only Mn-SOD-overexpressing and not CuZn-SOD-overexpressing cells were found to be protected against TBH-induced apoptotic death. This indicates that PC-12 cell survival against TBH stress is related to the expression of the mitochondrial SOD isoform rather than to the direct antioxidant effect of elevated GSH content associated with SOD overexpression. However, while independent of changes in cell GSH per se, our current data show that cell survival is linked to the cellular GSH/GSSG ratio status. For instance, the susceptibility of CuZn-SOD-overexpressing cells to TBH-induced apoptosis corresponded to the finding that CuZn-SOD-stable transfectants exhibited high GSSG contents and low cellular GSH/GSSG ratios despite elevated GSH levels, consistent with an exaggerated oxidized state that is exacerbated by TBH challenge. This conclusion is in agreement with previous studies in that the apoptotic end point in wild type PC-12 cells mediated by TBH is dictated by a shift in the redox status toward oxidation (i.e. increase in GSSG relative to GSH) rather than simply by an alteration in cell GSH content per se (22). One explanation for the susceptibility of CuZn-SOD-S cells to oxidative stress may be that overexpression of this SOD isoform results in enhanced ROS production as suggested by earlier studies (3, 32, 33). However, our results (Fig. 5) did not support this contention.

A consistent observation in our study is the low protein expression of procaspase-3 in unchallenged pRetro-Off controls and Mn-SOD-S cells, which was elevated within 30 min of TBH treatment (Fig. 4), an observation similar to wild type PC-12 cells exposed to TBH (22). The reason for the unusual kinetics is unclear and may be related to increased gene induction or, more likely, increased mRNA translation, given the rapidity of
protein expression. It is noteworthy that the time course of CPP32 elevation appeared to be directly correlated with the extent of oxidative stress within cells and how readily these cells ultimately succumbed to apoptosis. For instance, the overexpression of CuZn-SOD exacerbated cell apoptosis and caused a more oxidized intracellular environment than Mn-SOD overexpression or pRetro-Off vector controls, and this phenotype was associated with an elevated basal CPP32 expression even prior to TBH stress (at 0 min) (Fig. 4). In a related study, we have shown that overexpression of Mn-SOD protected this response to their elevated GSSG content and low cellular GSH/GSSG ratio, consistent with a role for redox in cell apoptosis. However, the susceptibility of CuZn-SOD-S cells does correspond to their elevated GSSG content and low cellular GSH/GSSG ratio, consistent with a role for redox in cell apoptosis. Given the different effects that the acute and chronic gene manipulations have on the cellular GSH antioxidant pool, future distinctions are warranted between studies using transient or stable overexpression of SOD.

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