Bcl-2 Attenuation of Oxidative Cell Death Is Associated with Up-regulation of γ-Glutamylcysteine Ligase via Constitutive NF-κB Activation

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Oxidative stress induced by reactive oxygen intermediates often causes cell death via apoptosis, which is regulated by many functional genes and their protein products. The evolutionarily conserved protein Bcl-2 blocks apoptosis induced by a wide array of death signals. Despite extensive research, the molecular milieu that characterizes the anti-apoptotic function of Bcl-2 has not been fully clarified. In this work, we have investigated the role of bcl-2 in protecting against oxidative death induced by H2O2 in cultured rat pheochromocytoma PC12 cells. Transfection with the bcl-2 gene rescued PC12 cells from apoptotic death caused by H2O2. Addition of NF-κB inhibitors such as pyrrolidine dithiocarbamate and N-tosyl-L-phenylalanine chloromethyl ketone to the medium aggravated oxidative cell death. PC12 cells overexpressing bcl-2 exhibited relatively high constitutive DNA binding and transcriptional activities of NF-κB compared with vector-transfected control cells. Western blot analysis and immunocytochemistry revealed that bcl-2-transfected PC12 cells retained a higher level of p65 (the functionally active subunit of NF-κB) in the nucleus compared with vector-transfected controls. In addition, sustained activation of ERK1/2 (upstream of NF-κB) was observed in bcl-2-overexpressing cells. In contrast, the cytoplasmic inhibitor IκBα was present in lower amounts in cells overexpressing bcl-2. The ectopic expression of bcl-2 increased the cellular glutathione level and γ-glutamylcysteine ligase expression, which were attenuated by NF-κB inhibitors. These results suggest that NF-κB plays a role in bcl-2-mediated protection against H2O2-induced apoptosis in PC12 cells through augmentation of antioxidant capacity.

Oxidative stress refers to the mismatched redox equilibrium between the production of reactive oxygen intermediates (ROIs) and the ability of the cell to defend against them.

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The abbreviations used are: ROIs, reactive oxygen intermediates; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone; PDTC, pyrrolidine dithiocarbamate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCF-DA, dichlorofluorescein diacetate; TMRE, tetramethylrhodamine ethyl ester; GCL, γ-glutamylcysteine ligase; GCLC, GCL catalytic subunit; GCLM, GCL modulatory subunit; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/ERK kinase; MEKK, MEK kinase.
mechanisms underlying the anti-apoptotic effect of NF-κB have not been clarified. In this work, we investigated whether Bel-2 can protect against H₂O₂-induced apoptosis through activation of NF-κB in cultured rat pheochromocytoma PC12 cells. For this purpose, we compared the extent of NF-κB activation and levels of antioxidative defense capacity, especially glutathione metabolism, in Bel-2 transfected and vector-treated control cells to link Bel-2 and the NF-κB signaling pathways in the context of their commitment to cellular protection against oxidative insults.

**EXPERIMENTAL PROCEDURES**

**Chemical and Biochemical Reagents**—Poly-l-lysine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), PTDTC, and TPCK were purchased from Sigma. H₂O₂ was a product of Junsei Chemical Co., Ltd. (Tokyo, Japan). Dulbecco’s modified Eagle’s medium, Hanks’ balanced salt solution, fetal bovine serum, horse serum, Geneticin (G418), nutrient mixture P-12, and N-2 supplement were provided by Invitrogen. Dichlorofluorescein diacetate (DCF-DA), 5,6,6′,6″-tetrachloro-1,3,3′,3″-tetraethylbenzimidazolocarbocyanine iodide (JC-1), and tetramethylrhodamine ethyl ester (TMRE) were obtained from Molecular Probes, Inc. (Eugene, OR). The in situ cell death detection kit was supplied by Roche Diagnostics (Mannheim, Germany). Oligonucleotides and the luciferase assay kit with reporter lysis buffer were purchased from Promega (Madison, WI). γ-32P-ATP was the product of PerkinElmer Life Sciences. The NF-κB and γ-glutamylcysteine ligase (GCL) promoter-luciferase constructs were kindly provided by Dr. Young Mi Kim (University of Ulsan Biomedical Science, Seoul, Korea) and Dr. Shelly C. Lu (University of Southern California School of Medicine), respectively.

**Cell Culture**—PC12 cells transfected with a eukaryotic expression vector containing the human cytosolengavirus major immediate-early enhancer/promoter followed by a full-length human Bel-2 cDNA sequence were kindly provided by Dr. Young J. Oh (Yonsei University, Seoul) and maintained in our laboratory. Briefly, DNA transfection was performed with 1 × 10⁵ PC12 cells cultivated on poly-l-lysine-coated 100-mm Petri dishes by adding a transfection mixture of 2 μg of plasmid/10 μl of LipofectAMINE (Invitrogen) in Dulbecco’s modified Eagle’s medium. Subsequently, single neomycin-resistant colonies were selected and expanded in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 5% horse serum, and 500 μg/ml G418. Stable PC12 cell lines overexpressing bel-2 were characterized by immunoblot analysis. PC12 cells transfected with a eukaryotic expression vector without a human bel-2 DNA sequence were utilized as a control cell line. The subsequent cultures were conducted as reported previously (6).

**Assessment of Cell Viability**—PC12 cells were plated at a density of 4 × 10⁴ cells/300 μl in 48-well plates, and cell viability was determined using the conventional MTT reduction assay. After incubation, cells were treated with MTT solution (1 mg/ml final concentration) for 2 h. The dark blue formazan crystals formed in intact cells were solubilized with lysis buffer (20% SDS in 50% aqueous N,N-dimethylformamide), and absorbance at 540–585 nm was measured with a microplate reader (Molecular Devices, Inc., Sunnyvale, CA). Results are expressed as percent MTT reduction.

**Terminal Deoxynucleotidyltransferase-mediated dUTP Nick End Labeling (TUNEL)**—The commercially available in situ death detection kit was utilized to assess DNA fragmentation. PC12 cells (5 × 10⁶ cells/3 ml on a chamber slide) were fixed for 30 min in 10% neutral buffered formalin solution at room temperature. Endogenous peroxidase activity was inhibited by incubation with 0.3% hydrogen peroxide in methanol for 30 min at room temperature and further incubated in a permeabilizing solution (0.1% sodium citrate and 0.1% Triton X-100) for 2 min at 4°C. The cells were incubated with the TUNEL reaction mixture for 60 min at 37°C, followed by labeling with peroxidase-conjugated antibody. Following the addition of the reaction mixture and visualized with x-ray film.

**Measurement of the Mitochondrial Transmembrane Potential**—To measure the mitochondrial membrane potential (ΔΨm), the lipophilic cationic dyes JC-1 and TMRE were used. The green fluorescent JC-1 probe undergoes a conformational change at higher potential, JC-1 forms red fluorescent J-aggregates that exhibit a broad excitation spectrum. Following treatment with H₂O₂ for 6 h, cells (5 × 10⁵ cells/3 ml on a chamber slide) were rinsed with PBS, and JC-1 (10 μg/ml) was loaded. After a 20-min incubation at 37°C, cells were examined under a confocal microscope with excitation at 488 nm and emission at 530/590 nm. Determination of ΔΨm was also carried out using TMRE, which rapidly equilibrates between cellular compartments due to potential differences. Thus, a decrease in fluorescence is indicative of reduced ΔΨm. Following the incubation, the cells were treated with TMRE (150 nM) for 30 min, rinsed, and examined by confocal microscopy in the same manner as done for JC-1, except that the fluorescent signal was measured at 570 nm.

**Measurement of Intracellular ROI Accumulation**—To monitor net intracellular accumulation of ROS, the fluorescent probe DCF-DA was used. After treatment with H₂O₂ for 30 min, cells (1 × 10⁶ cells/3 ml in 6-well plates) were rinsed with Krebs-Ringer phosphate solution, and 10 μM DCF-DA was loaded. Following an additional incubation for 15 min at 37°C, cells were examined under a confocal microscope equipped with an argon laser (488 nm, 200 milliwatts).

**Preparation of Nuclear Proteins**—After treatment with H₂O₂, cells (1 × 10⁶ cells/7 ml in a 100-mm dish) were washed with PBS, centrifuged, and resuspended in ice-cold isotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride, followed by incubation in 0°C for 20 min. After vortex mixing, the resulting suspension was centrifuged, and the supernatant was stored at −70°C for the NF-κB DNA binding assay. The protein concentration was determined using the BCA protein assay kit.

**Electrophoretic Mobility Shift Assay**—A synthetic double-strand oligonucleotide harboring the NF-κB-binding domain was labeled with [γ-32P]ATP using T4 polynucleotide kinase and separated from unincorporated [γ-32P]ATP by gel filtration using a nick spin column (Amersham Biosciences). Prior to incubation in the solution of the γ-32P-labeled oligonucleotide (100,000 cpm), 10 μg of the nuclear extract was kept on ice for 15 min in gel shift binding buffer (4% glycerol, 1 mg/ml EDTA, 1 mM dithiothreitol, 100 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.1 mg/ml sonicated salmon sperm DNA). DNA-protein complexes were resolved by 6% nondenaturing PAGE at 200 V for 2 h, followed by autoradiography.

**Western Blot Analysis**—Treated cells (1 × 10⁵ cells/7 ml in a 100-mm dish) were collected and washed with PBS. After centrifugation, cell lysate was carried out at 4°C by vigorous shaking for 15 min in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM dithiothreitol, 1 mM Na₃VO₄, and protease inhibitors). After centrifugation at 23,000 × g for 15 min, the supernatant was separated and stored at −70°C until used. The protein concentration was determined using the BCA protein assay kit (Pierce). After addition of sample loading buffer, protein samples were electrophoresed on a 12.5% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride blot at 300 mA for 3 h. The blots were blocked for 1 h at room temperature in fresh blocking buffer (0.1% Tween 20 in Tris-buffered saline (pH 7.4)) containing 5% nonfat dry milk. The blots were incubated overnight at 4°C. Following three washes with 0.1% Tween 20 in PBS (PBST), the blots were incubated with horseradish peroxidase-conjugated secondary antibodies in PBS with 3% nonfat dry milk for 1 h at room temperature. The blots were washed again three times with 0.1% PBST, and transferrin-proteins were incubated with ECL substrate solution (Amer sham Biosciences) for 1 min according to the manufacturer’s instructions and visualized with x-ray film.

**Preparation of Nuclear Proteins**—After treatment with H₂O₂, cells (1 × 10⁵ cells/7 ml in a 100-mm dish) were washed with PBS, centrifuged, and resuspended in ice-cold isotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM phenylmethylsulfonyl fluoride, followed by incubation in 0°C for 20 min. After vortex mixing, the resulting suspension was centrifuged, and the supernatant was stored at −70°C for the NF-κB DNA binding assay. The protein concentration was determined using the BCA protein assay kit.
ary antibody in 0.1% PBST with 3% bovine serum albumin for 1 h at room temperature. Cells were rinsed with PBS, and stained cells were analyzed under a confocal microscope and photographed.

**Transient Transfection and Luciferase Assay**—One day before transfection, PC12 cells were subcultured at a density of 1 × 10^5 cells/60-mm dish to maintain ~60–80% confluency. They were transiently transfected with the NF-κB or GCL promoter-luciferase construct using the transient transfection reagent N-[1-(2,3-bis(dialkylamino)propyl)-N,N,N-trimethylammonium] (Roche Diagnostics) according to the instructions supplied by the manufacturer. After overnight transfection, the treated cells were harvested and lysed with reporter lysis buffer (Promega luciferase assay system). The cell extract (20 μl) was mixed with 100 μl of the luciferase assay reagent and analyzed with a luminometer (AutoLumat LB953, EG&G Berthold, Bad Wildbad, Germany). The β-galactosidase assay (Promega β-galactosidase enzyme assay system) was done according to the supplier’s instructions to normalize the luciferase activity.

**Assessment of Intracellular GSH Levels**—The intracellular GSH levels were assessed using the commercially available colorimetric assay kit BIXOYTECH GSH-400 (OXIS Research, Portland, OR). Cells were harvested and homogenized in meta-phosphoric acid working solution. After centrifugation, 50 μl of R1 solution (solution of the chromogenic reagent in HCl) was added to the 700-μl supernatant, followed by gentle vortex mixing. Following addition of 50 μl of R2 solution (30% NaOH), the mixtures were incubated at 25 ± 3 °C for 10 min. After centrifugation, the absorbance of the clear supernatant was read at 400 nm. The protein concentration was determined using the BCA protein assay kit.

**Reverse Transcription-PCR**—Total RNA was lysed, extracted with TRIzol (Invitrogen), and converted to cDNA by Moloney murine leukemia virus reverse transcriptase (Promega) according to the manufacturer’s instructions. Reverse transcription-PCR was performed following standard procedures. Specific DNA sequences were amplified with a PCR mixture (HyMed, Seoul). Each PCR primer used in this study was as follows: GCL catalytic subunit (GCLC), 5′-GCC AAG GTC ATC- CAT GAC AAC-3′ (sense) and 5′-AGT GAT GCC GAC CAT GAC CTT-3′ (antisense); GCL modulatory subunit (GCLM), 5′-AGA CCG GGA A- CCC TGC TCA AC-3′ (sense) and 5′-CAT CAC CCT GAT GCC TAA GC-3′ (antisense); glyceraldehyde-3-phosphate dehydrogenase, 5′-AGT G- TA GCC CAG GAT GCC CTT-3′ (sense) and 5′-GCC AAG GTC ATC A- AT GAC AAC-3′ (antisense). The reaction conditions were 25 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s. After amplification, the products were resolved by electrophoresis on 1.0% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light.

**RESULTS**

**bcl-2-Overexpressing PC12 Cells Are Less Susceptible to the Cytotoxic Effects of H2O2**—To address whether ectopic expression of Bcl-2 can protect against H2O2-induced cell death, PC12 cells were stably transfected with a plasmid harboring the bcl-2 gene. The effects of bcl-2 overexpression on cell survival following exposure to H2O2 were then examined by the MTT reduction assay. As illustrated in Fig. 1A, bcl-2 overexpression rescued PC12 cells from H2O2-induced cytotoxicity.

**Bcl-2 Overexpression Attenuates Apoptotic Cell Death Induced by H2O2**—One of the distinctive biochemical hallmarks of apoptotic cell death is the unique occurrence of internucleosomal DNA fragmentation. Apoptotic cells were confirmed by TUNEL staining, which is widely used to detect DNA fragmentation in situ. Bcl-2 overexpression reduced H2O2-induced DNA fragmentation as revealed by the decreased proportion of TUNEL-positive cells (Fig. 1B). H2O2 treatment also led to the fragmentation as revealed by the decreased proportion of internucleosomal DNA of apoptotic PC12 cells.

**bcl-2 Mitigates the Dissipation of the Mitochondrial Transmembrane Potential and Intracellular Accumulation of Hydroperoxide in H2O2-treated PC12 Cells**—When PC12 cells were exposed to H2O2 (250 μM), the mitochondrial membrane became rapidly depolarized, as shown by an increase in green fluorescence and the concomitant disappearance of red fluorescence derived from the JC-1 dye (Fig. 2A, panels a and c). Bcl-2 overexpression reduced the changes in mitochondrial membrane transition (ΔΨ_m) as indicated by repression of green fluorescence and restoration of red fluorescence (Fig. 2A, panels b and d). These findings were further supported by use of another voltage-sensitive dye, TMRE. Again, H2O2-induced dissipation of ΔΨ_m (Fig. 2A, panel e) was found to be blocked by bcl-2 overexpression (panel f). Accumulation of intracellular hydroperoxide was detected by use of DCF-DA, which is freely permeable to cell membranes. Once inside cells, the compound is hydrolyzed by an esterase activity to DCF and trapped intracellularly. DCF is then able to interact with peroxides to form fluorescent 2′,7′-dichlorofluorescin, which is readily detectable by confocal microscopy. The activation of DCF is relatively specific for the detection of H2O2 and secondary or tertiary peroxides such as lipid peroxides. H2O2-derived intracellular peroxide accumulation decreased in PC12 cells overexpressing bcl-2 (Fig. 2B).
NF-κB Inhibitors Render PC12 Cells More Vulnerable to H$_2$O$_2$-induced Cell Death—NF-κB activation in PC12 cells was assessed by electrophoretic mobility shift assay with an oligonucleotide harboring a consensus NF-κB-binding element. Treatment of PC12 cells with H$_2$O$_2$ (100, 250, and 500 μM) caused a concentration-dependent increase in NF-κB DNA binding activity in these cells (Fig. 3A). PDTC, an antioxidant reported to effectively block the IκB degradation pathway (24, 25), reduced the DNA binding activity of NF-κB in a concentration-dependent manner (Fig. 3B). To examine the possible role of NF-κB in protecting against H$_2$O$_2$-induced cytotoxicity, cells were exposed to H$_2$O$_2$ for 9 h in the absence and presence of PDTC (10 μM) or another NF-κB inhibitor, TPCX (5 μM), and cell viability was assessed by the MTT reduction assay. Both PDTC (Fig. 3C) and TPCX (Fig. 3D) exacerbated the H$_2$O$_2$-induced cytotoxicity. Neither of these NF-κB inhibitors exhibited apparent toxicity to PC12 cells at the concentrations used in this experiment.

Ectopic Expression of Bcl-2 Leads to Constitutive Activation of NF-κB through Stimulating the Degradation of Cytoplasmic IκBa—in PC12 cells, the DNA binding activity of NF-κB was transiently enhanced by H$_2$O$_2$ treatment (Fig. 4A). PC12 cells overexpressing bcl-2 exhibited relatively high levels of constitutively activated NF-κB compared with vector-transfected control cells (Fig. 4A). Since the NF-κB DNA binding activity is largely regulated by IκBa, which sequesters NF-κB in the cytoplasm, we determined whether the observed increase in nuclear NF-κB binding activity in bcl-2-overexpressing PC12 cells is due to increased IκBa degradation. Protein extracts of both the nucleus and cytoplasm were subjected to Western blot analysis to measure p65, IκBα, or phospho-IκBα. Cytoplasmic IκBα levels were profoundly reduced, whereas phospho-IκBα and nuclear p65 levels were constitutively increased in PC12 cells overexpressing bcl-2 compared with control cells (Fig. 4B). We also verified the nuclear accumulation of p65 by immunocytochemistry using anti-p65 antibody (Fig. 4C). The transcriptional activity of NF-κB was also constitutively increased in bcl-2-transfected cells as assessed using an NF-κB-reporter plasmid containing the consensus NF-κB-binding DNA site linked to a luciferase reporter gene (pELAM-Luc). As illustrated in Fig. 4D, the base-line transcriptional activity of NF-κB was found to be approximately six times higher in bcl-2-transfected cells than in vector-transfected control cells. To elucidate a molecular target for NF-κB-mediated potentiation of cellular defense against oxidative insult in bcl-2-overexpressing cells, we examined the effect of GSH on H$_2$O$_2$-induced cell death. GSH, a ubiquitous tripeptide thiol, is a vital intra- and extracellular antioxidant against oxidative stress. N-Acetyl-l-cysteine undergoes a rapid deacetylation in cells.
Transcriptional activity of NF-κB is increased in bcl-2-overexpressing PC12 cells, which was aggravated by depletion of GSH by L-buthionine-(SR)-sulfoximine (Fig. 5B). The increased NF-κB DNA binding activity, suggesting that NF-κB activation in bcl-2-overexpressing PC12 cells is mediated partly via the ERK signaling pathway. Consistent with the above gel shift data, the increased nuclear p65 and decreased cytoplasmic IκBα in bcl-2-overexpressing cells was assessed at 3 h. Both pharmacological and genetic inhibition of ERK abrogated the NF-κB DNA binding activity, suggesting that NF-κB activation in bcl-2-overexpressing cells is mediated partly via the ERK signaling pathway. Consistent with the above gel shift data, the increased nuclear p65 and decreased cytoplasmic IκBα levels in bcl-2-transfected PC12 cells were reduced by U0126 treatment (Fig. 7B).
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**DISCUSSION**

Several protein families highly conserved during evolution are considered to be specifically involved in regulating programmed or apoptotic cell death. Examples are Bax and Bcl-2, which form homo- and heterodimers in vivo. The relative expression of these two proteins determines cell death or survival. When Bcl-2 is in excess, the Bcl-2 homodimer predominates and promotes cell survival. It has been reported that overexpression of bcl-2 or the related gene bcl-x<sub>L</sub> protects a variety of mammalian cells from apoptosis induced by diverse death stimuli, including chemotherapeutic agents, radiation, tumor necrosis factor-α, glucocorticoids, glutamate, and withdrawal of serum or growth factors (9, 26–28). Bcl-2 has been proposed to prevent apoptosis by regulating an antioxidant response to oxidative stress and those involved in providing signals (32, 33). In this work, Bcl-2 mitigated not only distinct morphological as well as biochemical changes related to apoptotic death, but also intracellular accumulation of ROS in H<sub>2</sub>O<sub>2</sub>-treated PC12 cells.

The molecular events and genetic programs activated in response to oxidative stress and those involved in providing cells with resistance against oxidative insults remain to be unraveled. Accumulating evidence supports a role of the ubiquitous eukaryotic transcription factor NF-κB in regulating oxidative stress-induced cell damage (34). Consistent with this notion, our present study has revealed that the DNA binding of NF-κB and its transcriptional activity are constitutively increased in Bcl-2-overexpressing PC12 cells compared with vector-transfected control cells. Overexpression of NF-κB/Rel promotes cell survival by hampering the induction of apoptosis (35–37). High constitutive NF-κB activation also confers resistance to oxidative stress in neuronal cells (20). Conversely, NF-κB inhibitors have been found to decrease cell viability by stimulating apoptosis. Inhibition of p65 nuclear translocation by PDTC, which is capable of blocking IκB phosphorylation, and the peptide proteasome inhibitor or addition of SN50, a
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and putative binding sites for NF-κB have been identified in the promoter region of the heavy subunit (42, 43). In our study, Bcl-2-overexpressing PC12 cells exhibited an increased GSH pool and increased GCLC expression. Moreover, blocking the activation of NF-κB led to down-regulation of GCLC and subsequent depletion of GSH. Based on these findings, we propose that NF-κB fortifies the cellular protection against oxidative stress through augmentation of antioxidative capacity, particularly GSH biosynthesis. Additional studies will be necessary to search for antioxidant molecules or enzymes other than GCL that are involved in cellular antioxidant defense mechanisms regulated by NF-κB in PC12 cells overexpressing bcl-2.

Because Bcl-2 possesses no inherent kinase activity, we hypothesized that bcl-2 possibly impacts on cellular factors that directly or indirectly lead to NF-κB activation. The critical regulatory step in the activation of NF-κB is the phosphorylation of IκBα and other IκB proteins, which is mediated by a high molecular mass multiprotein complex called IκB kinase. In bcl-2-overexpressing cells, the relatively high constitutive NF-κB activity we observed was associated with enhanced degradation of IκBα, consistent with findings in mature murine B-cells (44). The N-terminal region of IκBα has been proposed to be an important regulatory site for Bcl-2 (45, 46). However, the molecular mechanism by which Bcl-2 mediates NF-κB activation through interaction with IκB is not completely clarified. One possibility is that Bcl-2 directly or indirectly modulates IκB activity by interacting with one of the cellular factors that are involved in the activation of IκB kinase. IκB kinase is phosphorylated and activated by one or more upstream activating kinases, which are likely to be the members of the MAPK kinase family of enzymes (also known as MAP3Ks and MEKKs). MEKK1, which phosphorylates the upstream kinase of MAPKs, was shown to bind and phosphorylate IκB kinase (47). Alternatively, MEKK2 and MEKK3 also have the potential to activate IκB kinase and thereby stimulate NF-κB activation (48). Other evidence supports that regulation of the IκB activity by Bcl-2 may be mediated by a mechanism that involves the Raf-1/MEKK1 signaling pathway (49). This study suggests that Bcl-2, through the Bcl-2 homology 4 domain, interacts with Raf-1, leading to the downstream activation of MEKK1 and subsequent IκB kinase-dependent NF-κB activation. However, under certain experimental conditions, bcl-2 overexpression can negatively regulate the activation of NF-κB (50, 51). It depends on the conditions of the manipulated cells, including the transfection system and differentiation status. It has been reported that a sustained low level of bcl-2 expression resembles stable transfected cell lines; however, transiently increased high level expression of bcl-2 may result in immediate cellular alterations, which have not yet been characterized in stable clonal cell lines (52). The basal activity of NF-κB in nerve growth factor-treated cells is high, which is required for the survival of neurons (53), so bcl-2 overexpression can differentially regulate stress stimuli-induced NF-κB activation.

Recently, ROIs have gained special attention because of their potential role as second messengers in the intracellular signaling network (54, 55). Although oxidative stress may directly activate redox-sensitive transcription factors including NF-κB, ROIs may function as secondary messengers in various cellular signaling cascades. Exogenous H₂O₂ has been demonstrated to activate MAPKs such as ERK, JNK, and p38 MAPK (56, 57). However, the roles of MAPKs in cell death or survival are controversial. In general, activation of ERK occurs in response to growth factor stimulation (58), whereas JNK and p38 MAPK are activated after exposure to environmental stress such as ROIs, UV irradiation, hyperosmolarity, and endotoxins (59).
ERK is regarded as an anti-apoptotic kinase, although it may control proliferation of certain cells, either positively or negatively, depending on the duration of its activation (60). In our study, treatment of PC12 cells with H2O2 led to transient activation of ERK, which was constitutively up-regulated in bcl-2-overexpressing PC12 cells. However, bcl-2 overexpression did not cause any substantial alterations in the phosphorylation of p90RSK, whereas JNK activation was slightly diminished. The ERK signaling cascade has been implicated in NF-κB activation through phosphorylation of inhibitory IκB (61). The association between the ERK signaling cascade and NF-κB activation is also supported by the finding that the ERK-regulated kinase p90RSK phosphorylates and thereby inactivates IκB in response to mitogenic stimuli (62). Overexpression of bcl-2 in the PC12 cell line leads to phosphorylation of c-Jun at Ser73 via the ERK pathway, which contributes to the anti-apoptotic function of bcl-2 (63). bcl-2 overexpression increases expression of neural differentiation-associated genes through the TrkA/MEK/ERK pathway (64). Neuroprotection by transforming growth factor-β1 involves activation of NF-κB through the Akt/protein kinase B and ERK1/2 signaling pathways, further supporting that NF-κB is a target of ERK signaling (65).

In summary, ectopic expression of bcl-2 in PC12 cells protected these cells from apoptotic death induced by H2O2. Of particular interest is that PC12 cells overexpressing bcl-2 exhibited relatively high levels of constitutively activated NF-κB and the upstream kinase ERK1/2 compared with vector-transfected control cells. Pharmacological inhibition of NF-κB or ERK1/2 aggravated H2O2-induced PC12 cell death, suggesting that both NF-κB and ERK are responsible for protection of PC12 cells from oxidative stress.

Taken together, these findings suggest that constitutive activation of the redox-sensitive transcription factor NF-κB is part of a self-defense program that enables neuronal cells to protect themselves against oxidative stress. Considering the notion that some neuronal cells can survive accumulating oxidative damages and degenerative processes, an understanding of the molecular mechanisms that can alleviate the vulnerability of neurons and consequently increase their resistance to oxidative stress is of great interest in the context of establishing therapeutic strategies for the management of neurodegenerative disorders.

REFERENCES

1. Markey, W. R. (1997) Free Radic. Biol. Med. 23, 134–147
2. Drugo, W. (2002) Physiol. Rev. 82, 47–85
3. Wood, K. A., and Youle, R. J. (1998) Annu. N. Y. Acad. Sci. 860, 400–407
4. Jacobson, M. D. (1996) Trends Biochem. Sci. 21, 83–86
5. Slater, A. F., Nobel, C. S., and Orrenius, S. (1995) Biochem. Biophys. Acta 1271, 59–62
6. Kim, H.-J., So, Y. J., Jang, J.-H., Lee, J.-S., Oh, Y. T., and Suh, Y.-J. (2001) Mol. Pharmacol. 60, 440–449
7. Burlacu, A. (2003) J. Cell. Mol. Med. 7, 249–257
8. Moncayola, D. J. (1998) Toxicol. Lett. 95, 157–168
9. Zhong, L. T., Sarafian, T. A., Anton, R., Hahn, G., Grulla, E. B., Valentine, J. S., Ord, T., Brewster, D. E. (1993) Science 262, 274–277
10. Lawrence, M. S., Ho, D. Y., Sun, G. H., Steinberg, G. K., and Sapolsky, R. M. (1996) J. Neurosci. 16, 486–488
11. Paixão, K. S., Schlapbach, R., and Fontana, A. (1998) Eur. J. Immunol. 28, 4398–4408
12. Liu, Q., and Garrit, Y. (2003) Blood 101, 4105–4114
13. Schonbroodse, S., and Piette, J. (2000) Biochem. Pharmacol. 60, 1075–1083
14. Foo, S. Y., and Nolan, G. P. (1999) Trends Genet. 15, 229–235
15. Oya, M., Ohtsubo, M., Takayanagi, A., Tachibana, M., Shimizu, N., and Murai, M. (2001) Oncogene 20, 3888–3896
16. Bhakar, A. L., Tannis, L. L., Zendeider, C., Russo, M. P., John, C., Park, D. S., MacPherson, S., and Barker, P. A. (2002) J. Neurosci. 22, 8466–8475
17. Mitsiades, N., Mitsiades, C. S., Polisik, V., Chauhan, D., Richardson, J., Piddington, T., Munshi, N., Treon, S. P., and Anderson, R. K. (2002) Blood 90, 4079–4086
18. Dringen, R., and Hirrlinger, J. (2003) Biochem. 384, 505–516
19. Fulton, I., and MacNee, W. (2000) Free Radic. Biol. Med. 28, 1405–1420
20. Kondo, T., Higashiyama, Y., Goto, S., Iida, T., Cho, S., Iwanaga, M., Mori, K., Tani, M., and Urata, Y. (1999) Free Radic. Res. 31, 325–334
21. Yang, H., Wang, J., Huang, Z. Z., Ou, X., and Lu, S. C. (2001) Biochem. J. 357, 417–455
22. Mulcahy, R. T., and Gipp, J. J. (1995) Biochem. Biophys. Res. Commun. 209, 227–233
23. Davis, R. E., Brown, K. D., Siebenlist, U., and Staudt, L. M. (2001) J. Exp. Med. 194, 1811–1874
24. de Moosac, D., Mustapa, S., Greenberg, A. H., and Kirshenbaum, L. A. (1998) J. Biol. Chem. 273, 2394–23951
25. de Moosac, D., Zheng, H., and Kirshenbaum, L. A. (1999) J. Biol. Chem. 274, 29505–29509
26. Nemoto, S., DiDonato, J. A., and Lin, A. (1998) Mol. Cell. Biol. 18, 7336–7343
27. Zhao, Q., and Lee, F. S. (1999) J. Biol. Chem. 274, 8355–8358
28. Regula, K. M., Ems, K., and Kirshenbaum, L. A. (2000) J. Biol. Chem. 275, 38676–38682
29. Massaad, C. A., and Tagliatalata, G. (2003) Neuroreport 14, 1167–1170
30. Song, Y. S., Park, H. J., Kim, S. Y., Lee, S. H., Yoo, H. S., Lee, H. S., Lee, M. K., Oh, K. W., Kang, S. K., Lee, S. E., and Hong, J. T. (2004) Neurosci. Res. 49, 46–50
31. Seyfried, J., Evert, B. O., Schwarz, C. S., Schuapp, M., Schulz, J. B., Klockgether, T., and Willner, U. (2003) Free Radic. Biol. Med. 34, 1517–1530
32. Magyar, S. B., Ramires, S., Tong, N., Gelbard, H. A., and Dwershur, S. (2000) J. Neurochem. 74, 527–539
33. Finkel, T. (1998) Curr. Opin. Cell Biol. 10, 248–253
34. Rao, S. G. (1999) Exp. Mol. Med. 31, 53–59
35. Wang, X. M., Martin, J. L., Liu, Y., and Hoolbrook, N. J. (1998) Biochem. J. 333, 291–300
36. Bhat, R. N., and Zhang, P. (1999) J. Neurochem. 72, 112–119
37. Xia, Z., Dickens, M., Rassendu, J., Davis, R. J., and Rosenberg, M. E. (1995) Science 270, 1236–1313
38. Hagemann, C., and Blank, J. L. (2001) Cell. Signal. 13, 863–875
39. Ishikawa, Y., and Kittamura, M. (1999) Biochem. Biophys. Res. Commun. 264, 696–701
40. Chen, B. C., and Lin, W. W. (2001) Br. J. Pharmacol. 134, 1055–1065
41. Schouten, G. J., Vertegeal, A. C., Whiteside, T. S., Israel, A., Toches, M., Dorman, J. C., van der Eb, A. J., and Zantema, A. (1997) EMBO J. 16, 3134–3144
42. Schwarz, C. S., Seyfried, J., Evert, B. O., Klockgether, T., and Willner, U. (2002) Neuroreport 13, 2349–2354
43. Liang, Y., Mirnic, Z. K., Yan, C., Nylander, K. D., and Schor, N. F. (2003) Oncogene 22, 5515–5518
44. Zhu, Y., Cullmore, C., Klump, S., and Krieglstein (2004) J. Neurosci. 24, 897–906
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