The neurotransmitter dopamine (DA) induces apoptosis via its oxidative metabolites. This study shows that glutaredoxin 2 (Grx2) from Escherichia coli and human glutaredoxin could protect cerebellar granule neurons from DA-induced apoptosis. *E. coli* Grx2, which catalyzes glutathione-disulfide oxidoreduction via its -Cys-Pro-Tyr-Cys- active site, penetrates into cerebellar granule neurons and exerts its activity via NF-κB activation. Analysis of single and double cysteine to serine substitutions in the active site of Grx2 showed that both cysteine residues were essential for activity. Although DA significantly reduced NF-κB binding activity, Grx2 could stimulate the binding of NF-κB to DNA by: (i) translocating NF-κB from the cytoplasm to the nucleus after promoting the phosphorylation and degradation of IκBα, and (ii) activating the binding of pre-existing nuclear NF-κB. The DNA binding activity of NF-κB itself was essential for neuronal survival. Overexpression of IκB dominant negative gene (IκB-ΔN) in granule neurons significantly reduced their viability, irrespective of the presence of Grx2. Ref-1 expression was down-regulated by DA but up-regulated by Grx2, while treatment of neurons with Ref-1 antisense oligonucleotide reduced the ability of Grx2 to activate NF-κB binding activity. These results show that Grx2 exerts its anti-apoptotic activity through the activation of Ref-1, which then activates NF-κB.

The thiol-disulfide metabolism and balance contributes to the maintenance of the cellular redox (reduction/oxidation) state. Thiol redox control (1) can affect a protein’s synthesis and folding, the assembly into multimeric complexes, the enzymatic activity or the binding activity of transcription factors (Refs. 2 and 3; for review, see Ref. 4). Intracellular redox-active molecules such as thioredoxin, glutaredoxin, and Ref-1, and low molecular weight thiols such as glutathione (GSH), distribute and maintain a reduced cytosolic environment in the normal cell.

Dopamine (DA), the endogenous neurotransmitter of the nigro-striatal pathway, is a powerful oxidant that exerts its toxic potential through its oxidative metabolites. Administration of DA into the rat striatum (5) caused pre- and postsynaptic damage. Intraventricular injection of DA into rats resulted in dose-dependent death of the animals (6). *In vitro* studies have shown that DA can cause cell death in mesencephalic, striatal, and cortical primary neuron cultures (7–11). Moreover, we have shown that DA-induced cell death in sympathetic, cerebellar granule neurons, PC-12 cells, and thymocytes has all the characteristic features of apoptotic cell death (12–14).

Glutaredoxins (Grx) are generally 10-kDa proteins, which catalyze GSH-disulfide oxidoreductions via two redox-active cysteine residues (15). The active site sequence (Cys-Pro-Tyr-Cys) is conserved in a variety of species (16–23). Glutaredoxins can reduce either intramolecular disulphides as in the case of ribonucleotide reductase (24–26), or mixed disulfides between a thiol-containing compound and GSH (25, 27). *Escherichia coli* contains three glutaredoxins, with Grx2 being the predominant in terms of concentration and catalytic activity (80% of total GSH-oxidoreductase activity in *E. coli*) (28). Currently, only one human glutaredoxin has been reported (29). The protein is generally considered intracellular but may also be found in human plasma (29). Apart from its activity with ribonucleotide reductase, human glutaredoxin can reduce the GSH-mixed disulfide of inactive oxidized nuclear factor-1 and thus restore its DNA binding activity (30). Glutaredoxin has a role in reduction of mixed disulfides in cells exposed to oxidative stress (31).

The nuclear factor-κB NF-κB/Rel is one of the transcription factors whose DNA binding activity is regulated by a redox mechanism. Agents that modulate free sulphhydryls such as N-ethylmaleimide and diame inactive the DNA binding of NF-κB, whereas reductants such as dithiothreitol (DTT), β-mercaptoethanol, and thioredoxin enhance its DNA binding activity (for review, see Ref. 4; Ref. 32). NF-κB regulates the expression of various genes related to the immune response, stress, inflammation, and the inhibition of apoptosis (33, 34). Ordinarily, NF-κB proteins are expressed in an inactive form bound to an inhibitory protein referred to as IκB. Following

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appropriate stimuli (UV radiation, inflammatory cytokines, phorbol esters, reactive oxygen species (ROS)), NF-kB is released from the cytoplasmic complex by phosphorylation and ubiquinon-dependent degradation of IκB. The IκB-released NF-kB dimer translocates into the nucleus, where it binds to cognate DNA sequences and activates transcription of specific target genes (35–37). NF-kB is activated in a number of in vivo model systems of brain injury: brain trauma, focal ischemia, and kainate-induced seizure (38–41). Immunohistochemical analysis of brain sections from Alzheimer’s disease patients revealed that NF-kB was activated in the most damaged areas of the brain (42). In Parkinson’s disease patients, the number of neurons with NF-kB-stained nuclei was 70-fold higher than that of control subjects, suggesting that translocation of NF-kB to the nucleus was related to the pathophysiology of the disease (43).

Ref-1 regulates the activity of several transcription factors, including AP-1 elements (Jun-Fos dimers) and NF-kB. In addition, Ref-1 possesses apurinic/apyrimidinic endonuclease DNA repair activity against DNA damage caused by ROS, UV, and IR radiation (44–46). It is expressed in subpopulations of cells in the brain, including granule neurons of the cerebellum (47, 48). Ref-1 itself is subject to redox control and was shown to interact with thioredoxin (49).

The data presented here demonstrate that E. coli and human glutaredoxins could protect cerebellar granule neurons from DA-induced death by activating the DNA binding activity of NF-kB through Ref-1.

MATERIALS AND METHODS
Postnatal day 8 BALB/c mice were obtained from Tel Aviv University Animal Care Facility (Glasberg Animal Research Tower). Culture media, sera, and trypsin (0.25% in 0.05% EDTA) were from Biological Industries Co. (Beit Hemeek, Israel). IκB was obtained from PerkinElmer Life Sciences, Sephadex G-25 from Amersham Pharmacia Biotech (Uppsala, Sweden), and NF-kB inhibitor (SN50) from Promega (Madison, WI). Rabbit antibodies against NF-kB p65 and Ref-1 were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). IκB-ΔN plasmid was a gift from Prof. Yinon Ben-Neriah. E. coli Grx2 was a recombinant preparation purified to homogeneity as described (50). The single and double cysteine to serine mutants in the active site of Grx2 were prepared as described previously (50), but without His tags, and purified to homogeneity with the method for the wild type protein. Antibodies to Grx2 were raised in a rabbit by standard techniques. Recombinant human Grx was prepared as described by (51).

Primary Culture of Cerebellar Granule Neurons—Cultures of highly enriched granule neurons were obtained from postnatal day 8 BALB/c mice (52). Cells were dissociated by trypsinization and plated in standard medium (basal Eagle’s medium, 10% fetal calf serum, 25 mM KCl, 2 mM glutamine, 50 μg/ml gentamycin, and 250 ng/ml amphotericin B, supplemented with 1 ng/ml glucose (53) on dishes coated with poly-1-lysine (cell density, 7 × 10⁵ cells/35-mm diameter dish, 1.5 × 10⁶ cells/well for a 24-well plate, 2.5 × 10⁶ cells/well for a 96-well plate). Cytosine-β-arabinofuranoside (Ara-C) (10 μM) was added to the medium 18–22 h after plating to prevent replication of non neuronal cells (54).

Treatments—DA (3-hydroxytyramine hydrochloride, CH8502, Sigma) was dissolved directly in the proper culture medium. Cerebellar granule neurons were maintained in standard medium for 6–7 days. The medium was then replaced with serum-free standard medium with DA, rhGrx, Grx2, or Grx2 mutants, for various periods of time. Control cultures were maintained in serum-free standard medium.

Prior to addition to the neurons, glutaredoxins were reduced with 2 mM DTT for 20 min at 37 °C. DTT was removed by two spin columns of Sephadex G-25 equilibrated with phosphate-buffered saline (PBS).

Analysis of Neuronal Viability—The viability of the cultures (36-well plates) after treatment with DA and/or glutaredoxins was assessed either by Alamar Blue assay (AccuMed) or by 4,6-diamidino-2-phenylindole (DAPI) staining. Alamar reagent (1:10) was added to the treated cells for 2–2.5 h at 37 °C. The viability was evaluated by subtracting the fluorescence of the medium alone (without cells) from the fluorescence of the cells at 530 nm excitation wavelength and 590 nm emission wavelength. Cells were fixated with 4% paraformaldehyde for 30 min, rinsed with PBS, and incubated with 5 μg/ml DAPI for 5 min. Nuclei were visualized under UV light. Each assay was carried out in triplicate.

Determination of the Apoptotic Commitment Point—Neurons were treated with 600 μM DA. At different time points following DA exposure (1–24 h), the drug was washed out and replaced with fresh medium without DA. Similar sets of untreated cells served as control. Cell survival was monitored 24 h after DA administration, as mentioned above.

Protein Content—Protein cell content was determined according to the method of Bradford (55) using bovine serum albumin as standard. Immunocytochemistry and Nuclear Staining with DAPI—Cerebellar granule neurons were grown on glass coverslips coated with poly-l-lysine hydrobromide (P2636, Sigma). Treated neurons were fixed for 30 min in ice-cold 4% paraformaldehyde/PBS, washed with PBS (pH 7.4), permeabilized, and blocked for 20 min with 0.2% Triton X-100 in 10% normal goat serum. Cells were then incubated with anti-p65 or anti-Ref-1 antibodies (1:50) in PBS containing 2% normal goat serum for 1 h at 37 °C, washed, and then incubated for 1 h with Cy3- or Cy2-conjugated secondary antibody (Jackson Laboratories, Bar Harbor, ME). To identify the cellular location of Ref-1 or p65, the DNA of the cells was stained with DAPI.

Protein Biotinylation—Sulfosuccinimidyl-6-biotinamidohexaenate (catalog no. 21335) (biotin) was purchased from Pierce. Grx2 and Cys-C12S mutant were incubated with biotin (75 μg of biotin/mg of protein) in PBS buffer for 16 h at 4 °C. Residual biotin was removed using Sephadex G-25 column before being given to cultures. Biotinylation was confirmed by Western blot analysis using streptavidin-horseradish peroxidase.

Biotinylated Grx2 and Cys-C12S mutant were incubated for 30 min with the neurons. Thereafter, the slides were washed, fixed for 5 min in methanol (−20 °C) followed by a 2-min incubation in acetone (−20 °C), and blocked with 1% bovine serum albumin and goat γ-globulin (Jack-
A 403 laser (488 and 568 maximum lines) and 10-milliwatt helium-neon laser (Oberkochen, Germany) is equipped with a 25-milliwatt krypton-argon laser-immersion lens (Axiovert 135M, Zeiss) was used for all imaging.

Western Blot Analysis—Western blot analysis was performed as described by Harlow and Lane (57), using 12.5% polyacrylamide gels. Each lane was loaded with an equal amount of protein extracts (30 or 60 μg) (Promega, Madison, WI) was incubated for 30 min with 5 μg/ml of poly(dI-dC), 15,000 cpm of 32P-labeled xB oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Promega, Madison, WI) was incubated for 30 min with 5 μg of nuclear extract. For specificity control, a 50-fold excess of unlabeled probe was applied. In the antibody supershift assay, the reaction mixture minus probe was incubated with 2 μg of p50 and/or p65 antibodies (Santa Cruz Biotechnology). Dried gels were exposed to x-ray film or to phosphor screen (Molecular Dynamics).

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Fig. 2. Biotinylated Grx2 and C9S-C12S mutant penetrate into the granule neurons. Cerebellar granule neurons were incubated for 30 min with 13 μM biotinylated Grx2 or C9S-C12S mutant, washed, fixed, reacted with Cy3-conjugated streptavidin, and analyzed by confocal microscopy. A, cells reacted with Cy3-conjugated streptavidin only (without biotinylated proteins). C and E, detection of intracellular Grx2 and C9S-C12S mutant, respectively. B, D, and F, Nomarski imaging of the cells presented in A, C, and E, respectively. Bar indicates 10 μm.

Nuclear and Cytoplasmic Extracts—Neurons (7 × 10^6 cells) were washed with phosphate-buffered saline, scraped off, pelleted, and resuspended in 30 μl of hypotonic buffer A (10 mM Tris-HCl (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT, and protease inhibitors). After 15 min on ice, Nonidet P-40 was added (0.8%) and the lysates were spun down at 14,000 rpm at 4 °C. The supernatant was removed (cytoplasmic extract), and the nuclear pellet was resuspended in 20 μl of buffer B (20 mM Tris-HCl (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, and protease inhibitors) with frequent vortexing for 30 min at 4 °C. Finally, the nuclear extract was spun at 14,000 rpm for 10 min and the supernatant was used for electrophoretic mobility shift assays (EMSA).

EMSA—The binding reaction mixture containing 10 mM Tris-HCl (pH 7.9), 60 mM KCl, 0.4 mM dithiothreitol, 10% glycerol, 2 μg of bovine serum albumin, 1 μg of poly(dI-dC), 15,000 cpm of 32P-labeled xB oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′) (Promega, Madison, WI) was incubated for 30 min with 5 μg of nuclear extract. For specificity control, a 50-fold excess of unlabeled probe was applied. In the antibody supershift assay, the reaction mixture minus probe was incubated with 2 μg of p50 and/or p65 antibodies (Santa Cruz Biotechnology) for 15 min at room temperature. Products were analyzed on a 5% acrylamide gel made up in 1× TGE (50 mM Tris, 400 mM glycine, 2 mM EDTA). Dried gels were exposed to x-ray film or to phosphor screen (Molecular Dynamics). Quantitative data were obtained using PhosphorImager analysis (Molecular Dynamics). Antisense Oligonucleotide Treatment—Ref-1 antisense oligonucleotide and the complementary sense oligonucleotide were synthesized and high performance liquid chromatography-purified by Sigma Genosys Ltd. The oligonucleotides were labeled with fluorescein at the 5′ end and phosphorotioated at the 3′ end (at the positions marked by *) to confer nuclease resistance. The sequence of the Ref-1 antisense probe
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RESULTS

Glutaredoxin Activity Protects Neuronal Cells from DA-induced Deaths (Fig. 1)—To determine whether glutaredoxin could protect neuronal cells from DA-induced death, cerebellar granule neurons were exposed to 600 μM DA for 5 h, in the presence of various concentrations (2–13.8 μM) of reduced E. coli Grx2, Grx2 mutants C12S (exhibits 70% of the wild type activity) and C9S-C12S (inactive) and recombinant human glutaredoxin (rhGrx) (Fig. 1). In the absence of Grx2, only 20% of the exposed neurons survived. Increasing concentrations of wild type Grx2 rescued exposed neurons from apoptotic death (84%) (Fig. 1A). The protective effect was lower for the Grx2 mutant C12S (52%), whereas no protection was conferred by the double thiol mutant Grx2 C9S-C12S (Fig. 1A). rhGrx showed an almost identical protection profile as E. coli Grx2 (Fig. 1B). Counting condensed and apoptotic nuclei using DAPI staining revealed that Grx2 (4–13 μM) treatment conferred full protection against DA toxicity (Fig. 1C). These results show that glutaredoxins (E. coli and human) protected neurons from DA-induced death. The reduct activity of Grx2 was essential for its protective effect.

Grx2 Penetrates into the Granule Neurons (Fig. 2)—To examine whether Grx2 could penetrate neurons, neurons were incubated with 13 μM Grx2 for 30 min, fixed, and exposed to anti Grx2 antibodies. No cross-reacting material was detected (data not shown). After incubating neurons with biotinylated Grx2, cross-reacting material was observed throughout 100% of the cells (cytoplasm and nucleus, Fig. 2C). No signal was observed from samples in which biotinylated Grx2 was omitted (Fig. 2A). To exclude the possibility that the streptavidin binds to residual levels of free biotin and not to biotinylated Grx2, the neurons were exposed to biotin eluted from Sephadex column (similar to biotinylated proteins), fixed, and then reacted with labeled streptavidin. No signal was obtained under these conditions (data not shown). These results demonstrate that small amounts of wild type Grx2 could penetrate into neurons. Since the inactive double thiol mutant C9S-C12S could also be detected intracellularly (Fig. 2E), it is most likely that the penetration of Grx2 is.


downloads from [http://www.jbc.org/] on July 24, 2018
independent of the redox activity of the protein.

To examine whether coated pit vesicles were involved in Grx2 endocytosis, neurons were exposed to biotinylated Grx2 in the presence of 0.45 M sucrose, which disperses coated pit structures. An indistinguishable pattern of Grx2 permeabilization was observed in the presence or absence of sucrose, suggesting that Grx2 penetrates neurons via mechanisms other than those of coated pit vesicles (data not shown).

DA Decreases whereas Grx2 Increases NF-\(\kappa\)B Binding Activity in Cerebellar Granule Neurons (Figs. 3 and 4)—To determine the duration of exposure to DA required to trigger the apoptotic process, neurons were exposed to DA for various incubation times, followed by removal of DA and re-addition of serum-free medium. Neuronal viability was assessed after 24 h. Cells recovered well after exposure to DA for 1 h. 50% of the neurons did not recover after 1.5–2 h of exposure (data not shown), which was therefore set as the commitment time for apoptosis.

NF-\(\kappa\)B is a transcription factor known to respond to redox signals and could thus be a potential signal molecule to mediate Grx2 activity. We initially examined the DNA binding activity of NF-\(\kappa\)B in neurons exposed to DA and/or Grx2 for 1 and 2 h (2 h being the commitment time). EMSA revealed three distinct bands (Fig. 3). To examine whether these bands were specific to NF-\(\kappa\)B, nuclear extracts were exposed to radiolabeled probes in the presence of a 50-fold excess of unlabeled sequences. All three bands were significantly reduced in the presence of the unlabeled oligonucleotides, indicating that the three distinct bands were NF-\(\kappa\)B-specific. Supershift analysis revealed that the upper shifted band corresponds to the p50/p65 heterodimer, the middle band to the p50/p50 homodimer and the lower band did not contain either p50 or p65 subunits.

Exposure of cerebellar granule neurons to DA for 1 h did not affect the binding activity of NF-\(\kappa\)B. Further exposure led to a significant decrease in the intracellular levels of DNA-bound NF-\(\kappa\)B. On the other hand, incubation of the neurons with Grx2 significantly augmented the DNA binding activity of NF-\(\kappa\)B in a time-dependent manner. Rescuing concentrations of Grx2 could abrogate the DA effect on NF-\(\kappa\)B binding.

The Grx2 C12S monothiol mutant activated slightly the
DNA binding activity of NF-kB (3-fold activation) compared with wild type Grx2 (10-fold activation). The levels of DNA-bound NF-kB remained unchanged when neurons were incubated with the redox inactive C9S-C12S Grx2 mutant. These results demonstrate that only redox-active Grx2 could increase the DNA binding activity of NF-kB.

Grx2 Affects Both the Translocation of NF-kB and Its DNA Binding Activity (Figs. 5 and 6)—At least two separate mechanisms can account for alterations in the binding activity of NF-kB: the redox state of NF-kB and its translocation from the cytoplasm to the nucleus. To investigate whether Grx2 was affecting the redox state of NF-kB, isolated nuclei were exposed to 8.3 μM Grx2 for 30 min at 37°C. Significant stimulation of NF-kB binding activity to DNA was observed (Fig. 5). Treatment of nuclear extracts with 1 mM GSH had almost no effect on NF-kB binding activity. Exposure of isolated nuclei to DA resulted in reduced levels of NF-kB binding, as observed also in whole cell experiments (Fig. 3). NF-kB binding activity decreased also after exposure of cerebellar granule neurons or their nuclear extracts to hydrogen peroxide (30 μM), suggesting that ROS lead to decreased NF-kB DNA binding activity in those cells (data not shown). DA-induced loss of NF-kB binding activity was restored by GSH or Grx2. However, Grx2 (8.3 or 16.6 μM) was more efficient than 1 mM GSH. These results suggest that NF-kB proteins inactivated by oxidative compounds could be reactivated by reducing agents (glutaredoxin, GSH). In addition, a portion of the nuclear NF-kB proteins is not bound to DNA. Upon the appropriate conditions, this portion can be readily activated by Grx2 and bind to DNA.

Next we examined whether Grx2 was affecting the translocation of NF-kB (Fig. 6). Administration of Grx2 (8.3 μM) to neuronal cells caused a 10-fold stimulation (10.6 ± 2.6) in NF-kB binding activity (Fig. 6A). In comparison, treatment of isolated nuclei with Grx2 resulted in a maximum 2-fold stimulation (Fig. 6A, Nuc + Grx2). The differences in the extent of activation of NF-kB between whole cells and isolated nuclei suggested that Grx2 was having an additional cytoplasmic effect. This effect repeated itself upon DA treatment. Exposure of neuronal cells to DA reduced the DNA-bound NF-kB by 25 ± 2.2% (Fig. 6A). When Grx2 was administered to isolated nuclear extracts from DA-treated cells, a maximum 2-fold increase in NF-kB binding was measured (Fig. 6A, DA + Nuc + Grx2). On the other hand, administration of DA and Grx2 to whole neurons resulted in 4-fold increase in NF-kB binding activity (3.75 ± 0.78) (Fig. 6A). Confocal microscopy clearly revealed that Grx2 caused nuclear accumulation of p65 whereas DA reduced nuclear levels of p65 (Fig. 6B). To further analyze the mechanism by which Grx2 affected NF-kB binding activity, we examined whether Grx2 was affecting the levels of IκBα. As shown in Fig. 6C, administration of Grx2 to neurons led to a time-dependent increase in IκBα phosphorylation, which was followed by IκBα degradation. In conclusion, these results show that the major mechanism for NF-kB activation following Grx2 treatment is NF-kB translocation from the cytosol to the nucleus. In addition, nuclear Grx2 could affect the binding of nuclear NF-kB directly or indirectly, presumably by reducing the NF-kB proteins.

NF-kB Activation Is Essential for Neuronal Viability (Figs. 7 and 8)—To explore whether NF-kB activation was important for neuronal survival, neurons were exposed to SN50, a peptide that inhibits NF-kB translocation (59). Even at low concentrations (9 μM), SN50 was very toxic (data not shown), suggesting that inhibition of NF-kB translocation rendered neurons more vulnerable. However, the peptide could have had other nonspecific effects and was thus toxic to neurons through other mechanisms. To specifically inhibit NF-kB translocation, neurons were cotransfected with different amounts of IκB dominant negative gene (60) (IκB-ΔN) and green fluorescent protein (GFP). IκB-ΔN cannot be phosphorylated and thus will not release NF-kB proteins. Around 0.1% of neurons were cotransfected. To assess whether overexpression of IκB-ΔN inhibited NF-kB translocation in GFP-positive neurons, cells were cultured on coverslips, fixed, and reacted with p65 antibodies. Confocal microscopy revealed that the immunoreactivity of nuclear and cytoplasmic p65 levels was reduced in the cotransfected neurons (green cell in Fig. 7), confirming that IκB-ΔN inhibited the translocation of NF-kB to the nucleus.

We next examined whether inhibition of NF-kB translocation affected neuronal viability. Twenty-four hours after transfection, the number of cotransfected green fluorescent neurons was identical to control GFP-transfected cells. Thereafter, the medium was replaced and neuronal viability was determined (Fig. 8). In the presence of 1 μg/0.2 ml IκB-ΔN, neuronal viability was decreased as a function of time and reached a steady level of 65 ± 3.3% S.D. after 5 h. Increasing IκB-ΔN to 5 μg/0.2 ml resulted in a similar rate of neuronal death (58 ± 8.5% S.D.) (Fig. 8A). Cotransfection of the neurons with pRC/CMV plasmid and GFP had no effect on neuronal viability, suggesting that cotransfection per se did not alter the viability. Administration of 600 μM for 5 h resulted in 54% neuronal death. Cotransfected neurons were slightly but not significantly more sensitive to DA (52.6% and 45% neuronal death).
with 1 and 5 μg of I-κB-ΔN, respectively) (Fig. 8A). These results are in agreement with our previous experiments (EMSA of NF-κB and immunolocalization of p65) showing reduced NF-κB activity and accumulation of p65 in the cytoplasm after DA treatment. Moreover, Grx2 was incapable of rescuing cotransfected neurons (Fig. 8B).

**FIG. 9. Ref-1 expression after treatment with Grx2 and/or DA.** Cerebellar granule neurons were treated for 2 h with 8.3 μM Grx2, 600 μM DA and cotreated with Grx2 and DA. The neurons were fixed, reacted with anti-Ref-1 antibodies and Cy2-conjugated goat anti-rabbit antibodies, and analyzed by confocal microscopy. The left panels show immunoreactivity of anti-Ref-1 antibody, the middle panels show nuclear staining with DAPI, and the right panels show superposition of DAPI and anti-Ref-1 immunoreactivity. Bar indicates 10 μm.

Grx2 Regulates NF-κB Binding Activity through Ref-1 (Figs. 9–11)—In untreated cells, Ref-1 was preferentially expressed in the nucleus (Fig. 9, top panel). Two hours of incubation with DA caused slight nuclear condensation and a dramatic reduction in Ref-1 immunoreactivity (Fig. 9, second panel) (in accordance with Western blot analysis, not shown). Grx2 significantly stimulated the expression of both nuclear and cytoplasmic Ref-1 (Fig. 9, third panel). Treating the neurons with Grx2 mutant C98-C128 did not alter Ref-1 expression (data not shown), suggesting that the latter is dependent on a redox-active glutaredoxin. Grx2 could offset the suppression of Ref-1 expression by DA. In the presence of both DA and Grx2, Ref-1 levels were even higher than those of untreated control neurons (Fig. 9, bottom panel).

Since the levels of Ref-1 and NF-κB DNA binding activity were elevated at the same time, it could be that the induction of Ref-1 was important for the activation of NF-κB. Exposure of neurons to Ref-1 antisense oligonucleotide reduced the expression levels of Ref-1, whereas the sense sequence had no effect (Fig. 10A). Similar results were obtained with Western blot analysis (Fig. 10, B and C). Grx2 could not activate NF-κB in the presence of Ref-1 antisense oligonucleotide in a concentration-dependent manner (Fig. 11). However, Grx2 enhanced the DNA binding activity of NF-κB in the absence of Ref-1 antisense, or in the presence of the sense oligonucleotide (Fig. 11). These results demonstrate that Grx2 stimulates the DNA binding activity of NF-κB through Ref-1.

**DISCUSSION**

DA, the endogenous neurotransmitter of the nigro-striatal pathway, is a powerful oxidant that exerts its toxic potential through its oxidative metabolites. Reducing thiols such as DTT, NAC, and GSH are capable of neutralizing DA oxidative metabolites and thereby conferring protection against DA-induced apoptosis (14). In the search of more physiological thiol antioxidant, we found that E. coli Grx2 and human glutaredoxin could protect neuronal cells against DA-induced death by activating the NF-κB-dependent signal transduction pathway through Ref-1. Grx2 penetrated the neurons whether or not it was redox-active (Fig. 2). Our results are consistent with several studies that demonstrated that human Trx and its double thiol mutant were taken up similarly by 3T3 and MCF7 cells (2, 3). The mechanism by which Grx2 penetrates into the neurons is not clear. It does not involve a coated pit-dependent mechanism, perhaps by fluid phase, for example (61). Grx2 internalization per se is not sufficient to initiate signal transduction; it must catalyze GSH-disulfide oxidoreduction in its active form. Similarly, Gasaska et al. (3) have shown that the redox activity of Trx is essential for its effects on cell proliferation. Our study does not rule out the possibility that Grx2 exerts its effects through specific interaction with cell surface receptors.

Next, we looked for the intracellular targets of Grx2. Transcription factors whose DNA binding activity is redox-dependent were good candidates (reviewed in Ref. 4). Such transcription factors are the NF-κB family of proteins, which have conserved cysteine residues in their DNA binding domains (62, 63). Modification of the cysteines with diamide reduced their activity, whereas treatment with reducing agents enhanced DNA binding. In this work, redox-active Grx2 increased the DNA binding activity of NF-κB. The effect was observed in isolated nuclei, suggesting a direct effect on NF-κB (e.g. the reduction of a mixed disulfide between a conserved cysteine and GSH). However, Grx2 also stimulates NF-κB translocation, through a process that involves I-κBα phosphorylation and degradation. Several reports indicated that accumulation of ROS can induce I-κBα phosphorylation and degradation (64–66). Our results show that a reductive agent such as Grx2 can induce I-κBα phosphorylation and/or ubiquitin-dependent degradation. This notion is consistent with the findings that glutathione peroxidase is involved in I-κBα phosphorylation (64). Furthermore, ubiquitin-conjugating enzymes have been shown to be redox-regulated by the ratio between GSH and GSGG (67, 68). Our results are partially consistent with the recent findings obtained by Hirota et al. (69), who showed that hGrx reduced the ability of tumor necrosis factor α to phosphorylate I-κB but on the other hand enhanced NF-κB activation in HEK293 cells.

The fact that Grx2 stimulates NF-κB DNA activity through Ref-1 activation (Fig. 11) demonstrates a multiple signaling role for Grx2. It has been shown that AP-1 transcriptional activity is regulated by a direct association between Trx and Ref-1 (49). Our data show that Ref-1 expression levels are redox regulated. In the presence of DA which causes oxidative stress, Ref-1 levels were dramatically decreased (Fig. 10). Furthermore, no induction in Ref-1 levels was observed in the presence of the double thiol mutant. Ref-1 down-regulation was observed in certain cases of apoptosis, such as the myeloid leukemia cells line HL-60 (70) or following ischemia (71, 72). It is known that, after ischemia and reperfusion, mitochondrial production of superoxide radicals is increased. Thus, the reduced expression of Ref-1 after ischemia or DA treatment may be due to oxidative damage.

Human and E. coli Grx2 differ significantly in their catalytic activity using the mixed disulfide between the β-hydroxyethyl...
disulfide and GSH as a substrate (51). However, their protective profiles against DA-induced apoptosis were almost identical (Fig. 1). This may simply reflect that the two proteins have similar catalytic properties to their nonidentified in vivo substrate.

Monothiol Grx2 could protect neurons from DA-induced apoptosis, but to a lesser extent than the dithiol wild type glutaredoxin. Known glutaredoxins can reduce a disulfide substrate through a monothiol or a dithiol mechanism (73). For the reduction of protein disulfides, both cysteine residues (dithiol mechanism) are required. A single amino-terminal cysteine can specifically reduce protein-GSH mixed disulfides (monothiol mechanism; Refs. 26 and 74). In the latter case, GSH forms a mixed disulfide with glutaredoxin (GrxS-SG), while the protein component is released in its reduced form. The GrxS-SG can be reduced by GSH to give reduced glutaredoxin and oxidized glutathione (GSSG). The ability of monothiol Grx2 to confer increased DNA binding of NF-κB shows that the DNA binding of NF-κB is regulated by a GSH mixed disulfide mechanism.

The contribution of NF-κB to cell death and survival pathways is an intriguing and controversial issue. Our results show that NF-κB activation is important for the viability of cerebellar granule neurons and that Grx2 exerts its protective effects through the activation of NF-κB signaling pathways. First, inhibition of NF-κB translocation and binding by IκB-ΔN overexpression causes neuronal death (Fig. 8). Second, Grx2 could not rescue the neurons overexpressing IκB-ΔN (Fig. 8). Third, DA-induced neuronal death is correlated with a significant reduction in NF-κB DNA binding activity (Fig. 3) and a decrease in p65 levels in condensed and apoptotic nuclei (Fig. 6B). Our findings are in agreement with several recent reports suggesting a role for NF-κB in protection from apoptosis. Abrogation of NF-κB, either by deletion of RelA (p65) or by overexpression of IκB-ΔN, sensitizes immune cells to apoptosis in response to tumor necrosis factor and DNA damaging agents

FIG. 10. Reduction in Ref-1 expression after treatment with Ref-1 antisense. A, cerebellar granule neurons exposed for 2 h to 8.3 μM Grx2 in the presence of Ref-1 antisense (5 or 10 μM) or Ref-1 sense (5 or 10 μM) and were reacted with anti-Ref-1 antibodies. Green staining indicates the presence of antisense/sense oligonucleotides. Left panel shows Ref-1 immunoreactivity and antisense/sense staining, whereas the right panel shows only the Ref-1 immunoreactivity. Ref-1 immunoreactivity in untreated cells and in cells treated with Grx2 is shown in lowest right and left panel, respectively. Bar represents 10 μM. B, Western blot analysis of Ref-1 levels following treatment with Grx2 and antisense (AS, 5 and 10 μM) or sense (S, 5 and 10 μM). C, quantitative analysis of Ref-1 alterations is represented as percentage of untreated cells: **, p < 0.025; *, p < 0.05; –p < 0.05 between Grx2-treated neurons and Grx2-treated neurons that were exposed to Ref-1 antisense. Error bars represent ± S.D. Statistical analyses were performed with two-tailed Student’s t test (n = 5).

FIG. 11. NF-κB binding activity in cerebellar granule neurons following treatment with Ref-1 antisense. A, EMSA showing NF-κB DNA binding activity in nuclear extracts isolated from cerebellar granule neurons treated with 8.3 μM Grx2 or pre-exposed to 5 and 10 μM antisense and 10 μM sense and then treated with Grx2. The first lane shows NF-κB binding activity from untreated granule neurons. Bar represents 10 μM. B, quantitative analysis of NF-κB induction is presented as percentage of untreated cells: **, p < 0.025; *, p < 0.05; –p < 0.05 between Grx2-treated neurons and Ref-1 antisense-treated neurons. Error bars represent ± S.D. Statistical analyses were performed with two-tailed Student’s t test (n = 5).
activation of NF-κB (78). Increased NF-κB activity was found in surviving endothelial cells, whereas apoptotic cells show caspase-mediated cleavage of p65 (79). It was further shown that uncleavable caspase-resistant p65 protected the cells from apoptosis (79). In contrast, NF-κB has been implicated in the death of neurons induced by glutamate and β-amyloid protein (80, 81), by ceramide-induced apoptosis in mesencephalic dopaminergic neurons (43), and in leukemia cells (82). It is possible that the opposing roles of NF-κB in cell survival and death stem from the diversity in subunit expression and subcellular compartmentation. In different cell types, the relative expression of the different members of the NF-κB family may influence the composition of the dimers and, as a consequence, the affinity of the activated complex to specific DNA sequence motifs located in the regulatory region of different genes. Moreover, activation of distinct patterns of genes by the simultaneous activation of other regulatory transcription factors may contribute to opposite cell fates in different cell types as well as within the same cell type. Tamamti et al. (83) showed that tumor necrosis factor protects hippocampal neurons by the induction of bel-2 and bel-x through the activation of NF-κB. These observations strengthen the notion that NF-κB can inhibit apoptosis by inducing the expression of antiapoptotic genes.

Recently, Nakamura et al. (29) showed that Grx and Trx are found in human plasma, and suggested that they may be part of plasma defense mechanisms against oxidative stress. We propose that, besides being part of the plasma defense mechanism, redox-active glutaredoxins function as a signal molecule that penetrates the cells and activates signal pathways by affecting redox regulation.

In summary, our results demonstrate that Grx2 protects cerebellar granule neurons from DA-induced apoptosis by activating NF-κB signaling pathways through Ref-1. Inhibition of NF-κB activity by overexpression of IκB-ΔN causes neuronal death, indicating that the activation of a NF-κB signaling cascade is necessary to granule neuron survival. Studies will be needed to identify Grx- and NF-κB-induced genes that promote neuronal survival, and to clarify the precise role of redox regulation by glutaredoxin in signal transduction and neuronal protection.

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