Glutaredoxin Protects Cerebellar Granule Neurons from Dopamine-induced Apoptosis by Dual Activation of the Ras-Phosphoinositide 3-Kinase and Jun N-terminal Kinase Pathways*

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Glutaredoxin 2 (Grx2) from Escherichia coli protects cerebellar neurons from dopamine-induced apoptosis via nuclear factor kappa B (NF-κB) activation, which is mediated by the expression of redox factor-1 (Ref-1). An analysis of the mechanisms underlying Grx2 protective activity revealed dual activation of signal transduction pathways. Grx2 significantly activated the Ras/phosphoinositide 3-kinase/Akt/NF-κB cascade in parallel to the Jun N-terminal kinase (JNK)/AP1 cascade. Dopamine, in comparison, down-regulated both pathways. Treatment of neurons with Ref-1 antisense oligonucleotide reduced the ability of Grx2 to activate Akt and AP-1 but had no effect on the phosphorylation of JNK1/2, suggesting that Akt/NF-κB and AP-1 are regulated by Ref-1. Exposure of the neurons to JNK1/2 antisense oligonucleotide in the presence of Grx2 significantly reduced AP-1 and NF-κB DNA binding activities and abolished Grx2 protection. These results demonstrate that dual activation of Ras/phosphoinositide 3-kinase and AP-1 cascades, which are mediated by Ref-1, is an essential component of the Grx2 mechanism of action.

Excessive production of reactive oxygen species in living cells may damage their biological components. This condition, referred to as oxidative stress, is a common denominator of pathological conditions. Cells have evolved a wide array of antioxidant mechanisms including small reducing molecules (e.g. glutathione, ascorbic acid), antioxidative enzymes (e.g. catalase, superoxide dismutase, glutathione peroxidase; for review, see Refs. 1–4), and oxidoreductase enzymes such as thioredoxin and glutaredoxin (Grx)† (5).

Grx are antioxidant enzymes by virtue of the reducing power of their active site (CXXC), which catalyzes the transfer of electrons from reduced glutathione to disulfides (6). This thiol disulfide interchange reaction is crucial for the maintenance of intracellular redox homeostasis, especially under oxidative stress (6). Mammalian Grx is widely expressed in different cell types, including neurons (7–11). The enzyme can restore the activity of glutathionylated proteins containing mixed disulfides between a protein thiol and GSH (inactive form) by reducing the disulfide bridge to give reduced GSH and the active protein form containing a free thiol. Examples of such GSH-thiol regulation of activity can be found in tyrosine phosphatase 1B (13), phosphofructokinase (14, 15), nuclear factor-I (16), and polyomavirus enhancer-binding protein 2 (17). Thanks to the antioxidant properties of Grx activity, human Grx and Escherichia coli Grx2 can rescue cerebellar granule neurons from dopamine (DA)-induced oxidative stress (18).

DA, the endogenous neurotransmitter of the nigrostriatal pathway, is a powerful oxidant that exerts its toxic effects through its oxidative metabolites. DA-induced oxidations are generally implicated in neurodegenerative processes (19, 20) especially in Parkinson’s disease (21, 22). Administration of DA to rat striatum caused pre- and postsynaptic damage (23). Intraventricular injection of DA in rats resulted in dose-dependent death of the animals (24). In vitro studies have shown that DA can cause cell death in mesencephalic, striatal, and cortical primary neuron cultures (25–29). DA-induced cell death in sympathetic, cerebellar granule neurons, PC-12 cells, and thymocytes has all the features of apoptotic cell death (30–32). Apart from the administration of Grx, the toxic effects of DA can be prevented by the application of small molecular weight antioxidants such as N-acetylcysteine, catalase, ascorbic acid, and dithiothreitol (30, 31, 33, 34).

Little is known about the molecules and signaling pathways involved in DA-induced apoptosis. Enhancement of the DNA binding activity of NF-κB protects neurons from DA-induced apoptosis (18). A survival signal pathway that might activate NF-κB is the Ras/Raf1/Akt/NF-κB cascade (35, 36).

Ras is a family of proteins involved in the regulation of cell proliferation, cytoskeletal rearrangements, and differentiation and survival of different cell types (37, 38). Ras activation is localized on the inner surface of the cell membrane where it

say; GST-RBD, glutathione S-transferase fused to the Ras binding domain of Raf-1.
cycles between two states: a GDP-bound inactive state and a GTP-bound active state. This cycle is regulated by cell surface receptors including tyrosine kinase receptors. Ras-GTP was shown to interact and activate PI3K, a heterodimer composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit. PI3K activity is generally regulated by tyrosine kinase receptors (39). Upon stimulation, PI3K phosphorylates phosphatidylinositol molecules and generates the phosphorylated products phosphatidylinositol (3,4,5)-trisphosphate and phosphatidylinositol (3,4)-bisphosphate, which bind to the pleckstrin homology domain of the serine/threonine kinase Akt (also known as protein kinase B). Akt is then translocated to the plasma membrane where it undergoes phosphorylation by PDK1 and 2 (40–42). Growth factors and cytokines can also activate Akt via the PI3K pathway (43). Activated Akt can phosphorylate and inactivate proapoptotic proteins such as Bad, procaspase 9, glycogen synthase kinase-3, and members of the Forkhead transcription factor family. Alternatively, Akt can activate antiapoptotic proteins such as NF-κB (44–47). Akt is required for nerve growth factor-induced survival in sympathetic neurons (48) and for PI3K-induced survival of cerebellar granule neurons (49–53).

NF-κB and AP-1 are transcription factors that are critically important for cell survival and apoptosis. NF-κB consists of homo- or heterodimers of p50, p52, p65 (RelA), RelB or c-Rel. NF-κB proteins are usually expressed in an inactive form, bound to proteins inhibiting their activity named I-κB. Following the appropriate cellular stimulation, I-κB becomes phosphorylated by the multisubunit I-κB kinase complex, which subsequently targets I-κB for ubiquitination and degradation by the proteosome (54). The free NF-κB dimer translocates into the nucleus and up-regulates transcription of specific genes (55–57). The DNA binding activity of NF-κB is activated in brain injury models (brain trauma, focal ischemia, kainate-induced seizure) (58–61) and in the brains of Alzheimer’s and Parkinson’s disease patients (62, 63).

The AP-1 superfamily consists of several subfamilies including Jun, Fos, and ATF-2, all of which possess a leucine zipper domain that assists their dimerization (64). The AP-1 proteins form homo- or heterodimers before binding to their DNA target sites. The activation of AP-1 is regulated by JNK, extracellular signal-regulated kinase, and p38 mitogen-activated protein kinases (65, 66). Experiments with sympathetic and cerebellar granule neurons, as well as with PC-12 cells, revealed that JNK/c-Jun signaling promotes apoptosis after withdrawal of survival factor (67–69). Studies using JNK (−/−) mice suggested that JNK promotes apoptosis in the hippocampus and in the developing embryonic neural tube. However, in the embryonic forebrain, JNK proteins have the opposite function and are necessary for the survival of developing cortical neurons (70). The JNK pathway is also activated in some experimental models of Parkinson’s disease (71, 72).

Redox factor-1 (Ref-1) is a multifunctional protein that stimulates the DNA binding of numerous transcription factors, among them Fos, Jun, and NF-κB through redox regulation (73). Ref-1 possesses apurinic/apyrimidinic endonuclease DNA repair activity against DNA damage caused by reactive oxygen species, UV and infrared radiation (73–75) and acts as a repressor of genes encoding calcium-responsive elements (76). It is expressed in subpopulations of cells in the brain, including cerebellar granule neurons (77, 78).

Our results show that Grx2 protects neurons against DA toxicity through the activation of both Ras/PI3K/Akt and JNK/AP-1 pathways, which culminate in NF-κB activation. In contrast, DA down-regulated these two pathways.
washed with PBS, 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 (0.6%), 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 200 μl of buffer B (20 mM Tris-HCl, pH 7.9, 25% glycerol, 0.42 mM 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 assay (EMSA).

**EMSA**—The binding reaction mixture contained 10 μM Tris-HCl, pH 7.6, 60 mM KCl, 0.4 mM DTT, 10% glycerol, 2 μg of bovine serum albumin, 1 μg of poly(dI-dC), 15,000 cpm of “-labeled α or AP-1 oligonucleotides (5’-AGTGGAGGGGACTTTCCCAACAGG-3’ and 5’-CGGTTAGATGTCACCCCGA-3’), respectively (Promega, Madison WI) was incubated for 30 min with 5 μg of nuclear extract. For AP-1 binding activity, the reaction was done on ice. For specificity control, a 50-fold excess of unlabeled probe was applied. Products were analyzed by 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 PhosphorImaging (Molecular Dynamics).

**Antisense Oligonucleotide Treatment**—Ref-1 and JNK1/2 antisense oligonucleotides and the complementary sense oligonucleotides were synthesized and high-performance liquid chromatography (HPLC) purified by Sigma Genosys Ltd., Israel. The oligonucleotides were photoroutated at the 3’-end (3 last bases) to confer nuclease resistance. The sequence of the Ref-1 antisense probe was 5’-TTCCCGCCGTGG- GCATGC-3’ and the sense 5’-GGATCCCAAGCGGGGA-3’ (18). The sequence of the JNK1/2 antisense probe was 5’-CGTCTAG- GTCTCGTACTGAC-3’ and the sense 5’-TGAAGGTAAAGCTG- TCAAA-3’ as described by Shan et al. (85). Cerebellar granule neurons were treated with oligonucleotides 6 days after plating. The JNK1/2 antisense was given 2 h before treatment with Grx2 and/or DA.

**PI3K Activity**—Cerebellar granule neurons were washed with PBS and lysed in lysis buffer containing 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.2 mM NaVO₄, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Cleared lysates were normalized for protein content, and 150 μg of protein was rotated with 2 μl of anti-p85 for 2 h at 4 °C. Thereafter protein G-Sepharose beads were added for overnight incubation. The immunoprecipitates were washed twice with 0.5% Nonidet P-40 in PBS and 0.2 mM NaVO₄, once with 0.5 M LiCl, 100 mM Tris-HCl, pH 7.6, 0.2 mM NaVO₄, and once with TNE buffer (10 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.2 mM NaVO₄). Kinase reaction was carried out for 15 min at room temperature in kinase buffer consisting of 5 μM HEPES pH 7.5, 25 mM MgCl₂, phosphatidylinositol (100 μM/ml) sonicated in 10 μM HEPES, 1 mM EGTA, 250 μM ATP, and 5 μCi of [³²P]ATP. The reaction was stopped with 80 μl of 1 N HCl and phosphatases extracted with 160 μl CHCl₃:MeOH (1:1), once with 100 μl of 1 N HCl:MeOH (1:1), and the organic phase was dried under N₂ and resuspended in 10 μl of CHCl₃:MeOH (1:1). Phosphorylated products were resolved on oxalate-imregnated Silica 60 plates (Merck) using CHCl₃:MeOH, and 4 mM NH₄OH (9:7:2) as solvent. Radioactive products were visualized and quantitated by PhosphorImaging.

**Ras Activity Assay**—Activation of Ras was determined by immunoprecipitation of Ras with GST-RBD (86), precluded to glutathione-agarose beads prior to incubation with cell lysates. Cells were treated with FTS or DA (with or without Grx2) for different times, washed with PBS, and lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 20 mM MgCl₂, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 0.1 μg/ml leupeptin, 1 mM benzamidin, and 1 mM DTT. The lysates were spun for 15 min at 14,000 rpm at 4 °C. Precoupled GST-RBD was added to 100–150 μg of supernatant proteins and incubated for 30 min at 0 °C. The beads were collected and rinsed three times in lysis buffer, and bound Ras-GTP proteins were eluted with sample buffer. Samples were analyzed by Western immunoblotting with Pan-Ras as described above.

**RESULTS**

**Grx2 Protection Is Abolished by Ras and PI3K Inhibitors**—To determine whether the protective effect of Grx2 against DA-induced apoptosis was through the activation of the Ras/PI3K signaling pathway, cerebellar granule neurons were exposed to 600 μM DA with or without Grx2 for 5 h in the presence or absence of the Ras inhibitor FTS or PI3K inhibitor wortmannin. Both Ras and PI3K inhibitors blocked the protective effect of Grx2 on DA-treated cells (Fig. 1, 27 ± 8% and 33 ± 6%, respectively). Wortmannin had no apparent effect on the viability of untreated neurons, whereas inhibition of the Ras-dependent pathways by FTS caused a significant reduction in neuronal viability (58 ± 4%). These results suggest that Grx2 protects neurons through the Ras/PI3-kinase pathway.

**Inhibition of the Ras/PI3K Pathway Prevents the Activation of NF-κB by Grx2**—We have shown previously that activation of the DNA binding activity of NF-κB is essential to the viability of cerebellar granule neurons exposed to DA (18). To examine whether the inhibition of Ras and PI3K affected Grx2-dependent NF-κB activation, neurons were exposed to Grx2 for 2 h in the presence or absence of FTS or wortmannin and the NF-κB DNA binding activity analyzed (Fig. 2, A and B). Administration of Grx2 to neuronal cells caused a 10-fold stimulation in NF-κB binding activity (Fig. 2, A and B) and the nuclear accumulation of p65 (Fig. 2C). However, NF-κB activation was completely abolished by FTS and wortmannin (215 ± 180% and 173 ± 53%, respectively) (Fig. 2, A and B). Moreover, wortmannin reduced both cytoplasmic and nuclear levels of p65 and caused nuclear condensation, which is a typical feature of apoptotic nuclei (Fig. 2C). Similarly, treating the neurons with human Grx resulted in NF-κB stimulation, which was abolished by FTS and wortmannin (data not shown). These results suggest that Grx2 and human Grx activated NF-κB through the Ras/PI3K pathway.

**Grx2 Stimulates Ras and PI3K, but DA Inhibits PI3K Activity**—We next examined the effect of Grx2 and DA on active Ras and active PI3K forms (Figs. 3 and 4, respectively). These activities were assayed before the commitment point for DA-induced apoptosis, which was shown previously to be 2 h (18). Both active Ras and active PI3K forms were elevated significantly after exposure of the cells to Grx2 for 1 and 2 h (162 ± 36% and 170 ± 26%, respectively (Ras activation), 124 ± 4.9% and 132 ± 6.8%, respectively (PI3K activation). The effect of Grx2 was not observed before 1 h had elapsed (data not shown). DA had no significant effect on the active Ras form but inhibited PI3K activity significantly (77 ± 7.8% and 35 ± 2% after 1- and 2-h DA exposure, respectively). FTS alone down-regulated active Ras as expected, also causing a small but significant decrease in PI3K activity (88 ± 1.6%, 2-h FTS). In the presence of FTS, Grx2 could not restore active Ras or active PI3K (30 ± 11% and 83 ± 9.2; 56 ± 26% and 79 ± 4% for 1 and 2 h, respectively). FTS and wortmannin attenuated the ability of Grx2 to confer neuronal protection against DA toxicity (Fig. 1). In addition, both compounds inhibited the activation of the Ras/PI3K pathway by Grx2 (Fig. 2). Therefore, the Ras/PI3K
Statistical analyses were performed with a two-tailed Student's t-test.

The Cerebellar granule neurons were exposed to Grx2 alone or with wortmannin. Cerebellar granule neurons were exposed to Grx2 alone or to Grx2 and wortmannin for 2 h, fixed, reacted with anti-p65 antibodies, and analyzed by confocal microscopy. The left frames show nuclear staining with DAPI, the middle frames show superposition of DAPI and anti-p65 immunoreactivity, and the right frames show superposition of DAPI and anti-p65 immunoreactivity. Note the appearance of condensed nuclei after wortmannin treatment and their low level of p65 protein. Bar = 10 μm.

FIG. 2. Inhibition of the Ras/PI3K pathway prevents activation of NF-κB by Grx2. Panel A, EMSA showing the DNA binding activity of NF-κB in nuclear extracts from cerebellar granule neurons incubated with 20 μM Grx2 in the presence/absence of 100 nM wortmannin (W) or 25 μM FTS for 2 h. Identical cultures were incubated in serum-free medium and served as controls. Panel B, quantitative analysis of NF-κB induction is represented as a percent of untreated cells (n = 4). ***p < 0.001; **p < 0.001 versus Grx2 activation. Error bars represent ± S.D. Statistical analyses were performed with a two-tailed Student's t-test.

FIG. 3. Alterations in Ras activity after treatment with Grx2/DA. Panel A, cerebellar granule neurons were treated with 20 μM Grx2, 600 μM DA, 25 μM FTS, or 20 μM Grx2 and 25 μM FTS for 1 and 2 h and assayed for Ras activity as described under “Materials and Methods.” Panel B, quantitative analysis of Ras activity is represented as a percent of untreated cells (n = 4). *, p < 0.005. Error bars represent ± S.D. Statistical analyses were performed with a two-tailed Student's t-test.

C, control.

pathway could be a mechanism by which Grx2 exerts its protective effect on the neurons.

Grx2 Up-regulates the Activity of Akt, but DA Down-regulates It—PI3K can activate Akt, thus promoting the survival of cerebellar granule neurons (49). Furthermore, Akt was shown to promote cell survival via NF-κB activation (87). To investigate whether Akt is involved in the Grx2 protective effect, we monitored the levels of phospho-Akt (the active form of Akt) after Grx2 and DA treatments (Fig. 5). Administration of Grx2 significantly increased the levels of phosphorylated Akt, whereas administration of DA decreased them (240 ± 35% and 44 ± 8% respectively). Grx2-induced phosphorylation was blocked by wortmannin (73 ± 13%), an inhibitor of PI3K. Therefore, the activation of Akt via Grx2 may be dependent on PI3K.

Grx2 Regulates Akt Activity through Ref-1—It was shown that Grx2-induced activation of NF-κB is mediated by upregulating the expression of Ref-1. DA, in comparison, downregulated Ref-1 levels (18). To determine whether the PI3K pathway was involved in the activation of Ref-1, we monitored the expression levels of Ref-1 in neurons treated with Grx2 in the presence or absence of wortmannin (Fig. 6). Grx2 significantly stimulated the expression levels of both nuclear and cytoplasmic Ref-1, whereas wortmannin caused nuclear condensation and a dramatic reduction in Ref-1 immunoreactivity. Western blot analysis (Fig. 6B) revealed a significant increase in Ref-1 levels after exposure to Grx2 only (175 ± 40% after 1 h and 404 ± 46% after 2 h). Wortmannin abolished the ability of Grx2 to elevate Ref-1 levels (105 ± 11 and 31 ± 14% after 1 and 2 h, respectively). Because Ref-1 and Akt activate NF-κB in a PI3K-dependent manner, it could be that one may activate the other. To determine the order of activation, neurons were exposed to Grx2 in the presence or absence of Ref-1 antisense peptides.

FIG. 4. Alterations in PI3K activity after treatment with Grx2/DA. Cerebellar granule neurons were treated with 20 μM Grx2, 600 μM DA, 25 μM FTS, or 20 μM Grx2 and 25 μM FTS for 1 and 2 h and assayed for PI3K activity as described under “Materials and Methods.” Quantitative analysis of PI3K activity is represented as a percent of untreated cells (n = 4). ***, p < 0.001; **, p < 0.002; *, p < 0.05. Error bars represent ± S.D. Statistical analyses were performed with a two-tailed Student’s t test. C, control.

FIG. 5. Grx2 up-regulates but DA down-regulates Akt activity. Panel A, cerebellar granule neurons were treated with 20 μM Grx2 or 600 μM DA in the presence or absence of 100 nM wortmannin (W). Total cellular proteins were extracted and immunoreacted with anti-phospho-Akt antibody, as described under “Materials and Methods.” Panel B, quantitative analysis of Akt phosphorylation is represented as a percent of untreated cells (n = 4). ***, p < 0.001; **, p < 0.001 versus Grx2 activation. Error bars represent ± S.D. Statistical analyses were performed with a two-tailed Student’s t test. No changes in total Akt levels were detected in these experiments (data not shown). C, control.
oligonucleotide (Fig. 7). This Ref-1 antisense oligonucleotide was shown to inhibit Ref-1 expression (18). The Ref-1 antisense oligonucleotide reduced the stimulation of Akt by Grx2 significantly (93 ± 27%). The sense sequence had no effect (228 ± 60%) (Fig. 7). These results demonstrate that Akt is a downstream target of Ref-1.

Fig. 6. Activation of Ref-1 by Grx2 is dependent on PI3K. Panel A, immunolocalization of Ref-1 after treatment with Grx2 and wortmannin (W). Cerebellar granule neurons were exposed to 20 μM Grx2 in the presence or absence of 100 nM wortmannin for 2 h, fixed, and reacted with anti-Ref-1 and Cy2-conjugated goat anti-rabbit antibodies, and analyzed by confocal microscopy. The left frames show the immunoreactivity of anti-Ref-1 antibody, the middle frames show nuclear staining with DAPI, and the right frames show superposition of DAPI and anti-Ref-1 immunoreactivity. Note that the condensed nuclei after wortmannin treatment express low levels of Ref-1. The bar indicates 10 μm. Panel B, Western blot analysis of Ref-1 levels after exposure of cerebellar granule neurons to 20 μM Grx2 in the presence or absence of 100 nM wortmannin for 1 and 2 h. Panel C, quantitative analysis of Ref-1 levels is represented as a percent of untreated cells (n = 4). ***, p < 0.001; **, p < 0.025; *, p < 0.05; †, p < 0.05 versus Grx2 activation. Error bars represent ± S.D. Statistical analyses were performed with a two-tailed Student’s t test.

Fig. 7. Ref-1 antisense blocks Grx2-induced Akt activation. Upper panel, cerebellar granule neurons were treated with 20 μM Grx2 in the presence of Ref-1 antisense (AS) or sense (S) oligonucleotides (5 μM). Total cellular proteins were extracted and immunoreacted with anti-phospho-Akt antibody, as described under “Materials and Methods.” Lower panel, quantitative analysis of Akt phosphorylation is represented as a percent of untreated cells (n = 3). ***, p < 0.001; †, < 0.025 versus Grx2 activation. Error bars represent ± S.D. Statistical analyses were performed with a two-tailed Student’s t test.

Fig. 8. AP-1 binding activity in cerebellar granule neurons after treatment with Grx2, DA, wortmannin, and Ref-1 sense and antisense oligonucleotides. Panel A, EMSA showing AP-1 DNA binding activity in nuclear extracts from cerebellar granule neurons incubated with 20 μM Grx2 in the presence or absence of 100 nM wortmannin (W) or Ref-1 antisense (AS) and sense (S) oligonucleotides (5 μM), and with 600 μM DA for 1 and 2 h. Identical cultures were incubated in serum-free medium and served as control (C). Panel B, quantitative analysis of AP-1 induction is represented as a percent of untreated cells (n = 4). ***, p < 0.001; ††, < 0.001; and ††, p < 0.025 versus Grx2 activation. Error bars represent ± S.D. Statistical analyses were performed with a two-tailed Student’s t test.
binding activity of AP-1, which is known to be involved in cell survival and apoptosis. Exposure of cerebellar granule neurons to Grx2 augmented the DNA binding activity of AP-1 significantly (Fig. 8) as early as 1 h after exposure (240 ± 20%) and returned to basal levels thereafter. In contrast, DA significantly decreased the binding activity of AP-1 in a time-dependent manner (73 ± 5%) (Fig. 8). The DNA binding activity of AP-1 was inhibited by wortmannin or Ref-1 antisense oligonucleotide. Similarly, wortmannin could also inhibit human Grx ability to activate AP-1 binding (data not shown). Similar results were obtained using human Grx (data not shown). These results suggest that Grx2 affected the activation of AP-1 via PI3K and/or Ref-1.

Grx2 Activates JNK, but DA Down-regulates It—Levels of phosphorylated JNK were elevated significantly as early as 10 min (134 ± 2%) after exposure to Grx2 (Fig. 9); they peaked at 1 h (228 ± 45%) and decreased 1 h later. Phosphorylation of JNK was down-regulated by treatment with DA (67 ± 7.5% and 53 ± 4.3% for 1 and 2 h, respectively) (Fig. 9). These results are in accordance with the activation profile of AP-1 (Fig. 8). Wortmannin had no significant effect on the phosphorylation levels of JNK (data not shown).

Grx2 Activates NF-κB through the JNK/AP-1 Signaling Pathway—To evaluate the importance of the JNK/AP-1 pathway in the Grx2 protective mechanism, neurons were exposed to a JNK1/2 antisense oligonucleotide (85) prior to treatment with Grx2. This treatment reduced the expression levels of phospho-JNK (77 ± 32%) and the DNA binding activity of its downstream effector AP-1 (65 ± 45%). The sense sequence had no significant effect. The antisense oligonucleotide also blocked Grx2-induced NF-κB activation (152 ± 93% compared with 1,100 ± 160% Grx2 alone) (Fig. 10, E and F). Accordingly, the JNK1/2 antisense oligonucleotide significantly reduced the ability of Grx2 to confer neuronal protection against DA toxicity (54 ± 9% compared with 98 ± 1%; Fig. 11). Therefore, Grx2 can increase the DNA binding activity of NF-κB through the JNK/AP-1 signaling pathway and protect neurons from DA-induced apoptosis.

**DISCUSSION**

Intracellular redox status has been linked to cellular differentiation, immune response, growth control, tumor progression, and apoptosis (88). Oxidants and antioxidants can act as signaling molecules that modify the function of enzymes such as phosphatases and kinases and directly or indirectly affect the activity of many transcription factors (e.g. c-Jun, c-Fos,

![Figure 9](http://www.jbc.org/)

**Fig. 9.** Grx2 elevates, whereas DA down-regulates, levels of phosphorylated JNK. Panel A, cerebellar granule neurons were treated with 20 μM Grx2 or 600 μM DA for different time points, after which total cellular proteins were extracted and immunoreacted with anti-phospho-JNK antibody, as described under “Materials and Methods.” Panel B, quantitative analysis of JNK phosphorylation is represented as a percent of untreated cells (n = 3). **, p < 0.001; *, p < 0.05. Error bars represent ± S.D. Statistical analyses were performed with a two-tailed Student’s t test. No changes in total JNK levels were detected (data not shown). C, control.

![Figure 10](http://www.jbc.org/)

**Fig. 10.** A JNK antisense oligonucleotide down-regulates the binding activity of AP-1 and NF-κB. Panel A, Western blot analysis of phosphorylated JNK after treatment with JNK1/2 antisense (AS). Cerebellar granule neuron cultures were treated with 20 μM Grx2 in the presence or absence of JNK1/2 antisense and sense (S) oligonucleotides (1 μM) for 2 h. Total cellular proteins were extracted and immunoreacted with anti-phospho-JNK antibody, as described under “Materials and Methods.” Panels C and E, EMSA showing AP-1 and NF-κB binding activity in nuclear extracts from cerebellar granule neurons incubated with 20 μM Grx2 for 1 h (AP-1) and 2 h (NF-κB), in the presence or absence of JNK1/2 antisense and sense oligonucleotides (1 μM) for 2 h. Identical cultures were incubated in serum-free medium and served as control. Panels B, D, and F, quantitative analysis of JNK levels, AP-1 and NF-κB binding (respectively) are represented as a percent of untreated cells (n = 3). ***, p < 0.001; **, p < 0.025; *, p < 0.05; “,” p < 0.01; “,” p < 0.025 versus Grx2 activation. Error bars represent ± S.D. Statistical analyses were performed with a two-tailed Student’s t test. C, control.
Myb, p53) or nuclear receptors such as the glucocorticoid and estrogen receptors ((89) for review, see Refs. 90 and 91). Additional research is needed to clarify the role of redox regulation in the survival of neurons and in neurodegenerative diseases.

Human Grx and E. coli Grx2 can protect neurons from DA-induced apoptosis (18). The effect is specifically related to Grx activity and does not exist in a general reductant molecule. Whereas GSH confers neuronal protection by scavenging DA oxidative metabolites, Grx acts by a different mechanism because it is not a free radical scavenger (data not shown). In addition, GSH at the mM range had a much less protective effect and showed a lower capability of activating NF-κB compared with Grxs at μM concentrations (18). Because human Grx has the same antiapoptotic effect as E. coli Grx2 and stimulates the DNA binding activities of NF-κB and AP-1 to the same extent, we consider that the use of Grx2 in experiments with neurons is biologically relevant.

Our work suggests that Grx2 exerts its protective effects through activation of two separate signaling pathways: the Ras/PI3K/Akt and JNK/AP-1 pathways, which culminate in the stimulation of NF-κB. Ref-1 was essential for the function of both pathways, whereas DA was an inhibitor leading to neuronal attrition.

The mechanism by which Grx2 stimulates Ras and JNK activities is not known. It has been suggested that changes in the redox state may alter Ras and JNK activities directly. Because both enzymes have a critical cysteine residue in their active site, they could be affected by the redox environment (35, 92). For example, in oxidizing conditions, the thiol of the active site, they could be affected by the redox environment (35, 92). Because both enzymes have a critical cysteine residue in their active site, they could be affected by the redox environment (35, 92). For example, in oxidizing conditions, the thiol of the active site, they could be affected by the redox environment (35, 92).

JNK has been implicated in cell death and survival (70 and references therein). We suggest an antiapoptotic role for the JNK/AP-1 pathway which is in agreement with recent works (70, 94–97) but in disagreement with other studies performed on cerebellar granule neurons and experiments in which DA-induced apoptosis was mediated by AP-1 activation (52, 68, 69, 98–102). It may be that activation of NF-κB after inhibition of PI3K (52, 104) is an attempt to activate rescue processes for the prevention of cell death. The apparent dual role of the JNK pathway may reflect the activation of specific Fos/Jun components of AP-1 leading to the transcription of different genes and/or interaction with opposing regulatory signaling pathways.

Our results indicate cross-talk between the Ras/PI3K/Akt and JNK/AP-1 pathways: inhibition of PI3K abolished the ability of Grx2 to stimulate AP-1 binding activity, whereas inhibition of JNK prevented the activation of NF-κB by Grx. Different stimuli that activate NF-κB may also activate the JNK signaling cascade (e.g. tumor necrosis factor-α, UV irradiation) (103, 104). At the same time, several upstream activator proteins of the JNK pathway, such as MEKK1 (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1), Akt-1 (NF-κB-activating protein), CIKS (connection to IκB kinase) and JNK, can activate NF-κB through IκB kinase (103, 105–107). Furthermore, c-Fos and c-Jun were found to interact physically with NF-κB p65 through the Rel homology domain. This complex exhibited enhanced DNA binding and biological function via both the κB and AP-1 response elements (108, 109). IκBα is essential for maintaining basal JNK activation and for regulating the JNK-induced TNF resistance in fibroblasts (110). The existence of proposed cross-talk between the Ras/PI3K/Akt and JNK/AP-1 pathways is supported further by a recent report in which thioredoxin-induced NF-κB

**Fig. 11.** Grx2 neuroprotective activity is attenuated by a JNKI/2 antisense oligonucleotide. Cerebellar granule neurons were incubated with 600 μM DA or 20 μM Grx2 plus 600 μM DA for 5 h in the presence or absence of JNKI/2 antisense (AS) or sense (S) oligonucleotides (1 μM). Cell viability was determined by Alamar Blue assay (n = 4). ***p < 0.001; **, p < 0.025. Error bars represent ± S.D. Statistical analyses were performed with a two-tailed Student’s t test. C, control.

**Fig. 12.** Proposed model for the protective action of Grx2. Grx2 penetrates into the cerebellar granule neurons to activate the Ras/PI3K and JNK/AP-1 signaling pathways. Grx2 elevates the activities of Ras and PI3K, leading to increased expression of Ref-1. Ref-1 activates Akt, which phosphorylates IκB kinase (IKK), in turn phosphorylates IkB, thereby accelerating its ubiquitination and degradation leading to NF-κB activation. In parallel, Grx2 activates JNK and AP-1, the final effect being the stimulation of NF-κB binding activity. Ref-1 stimulates the DNA binding activity of both NF-κB and AP-1. DA blocks PI3K/Akt and JNK/AP-1 pathways and attenuates NF-κB binding activity. Blue arrows represent the activities induced by Grx2; red arrows show the effects of DA. AS, antisense; P-, phospho-.
activation was mediated by the MEKK1/JNK pathway (111). Ref-1 affected both the AP-1 and NF-κB signaling pathways, as AP-1 and NF-κB binding activities were attenuated by the introduction of Ref-1 antisense. This accords with previous studies showing the involvement of Ref-1 in the redox regulation of AP-1 and NF-κB (73–75). The positioning of Ref-1 in the signaling cascade is very likely to be downstream of PI3K but upstream of Akt (Figs. 6 and 7). Interestingly, Akt activation is dependent on newly synthesized Ref-1. In the presence of Ref-1 antisense, which inhibits Ref-1 transcription and translation, Grx2 could not stimulate Akt activity. This finding demonstrates that activation of the Ras/PI3K-Akt pathway is dependent on alterations in gene expression. The type of interaction between Grx2 and Ref-1 is not known. Ref-1 could interact physically with thioredoxin in the nucleus following ionizing radiation and thereby activate AP-1 (112). In addition to increased Ref-1 synthesis as a means of activation, a recent study demonstrated that Ref-1 phosphorylation by cascin kinase II stimulated the redox regulation of AP-1 (112).

In summary (Fig. 12), we demonstrate that Grx2 exerts its protection against DA-induced apoptosis through activation of the Ras/PI3K/Akt and JNK/AP-1 pathways, culminating in NF-κB activation. These pathways are regulated by newly synthesized Ref-1, which stimulates Akt, NF-κB, and AP-1 activities. In contrast, DA down-regulates these pathways. These results point to the critical importance of the redox state for activation of central cellular pathways that determine the fate of the cell.

REFERENCES

1. Holmgren, A. (1985) Annu. Rev. Biochem. 54, 257–271
2. Powis, G., Oblong, J. L., Gasda, P. Y., Breggen, M., Hill, S. R., and Kirkpatrick, D. L. (1994) Oncology Res. 6, 539–544
3. Gasda, J. S., Bergmann, M., and Powis, G. (1995) Cell Growth Differ. 6, 1543–1560
4. Kamata, H., and Hira, H. (1999) Cell Signalling 11, 1–14
5. Holmgren, A. (1989) J. Biol. Chem. 264, 13963–13966
6. Jung, C. H., and Thomas, K. A. (1996) Arch. Biochem. Biophys. 335, 61–72
7. Garcia-Pardo, L., Granados, M. D., Gaytan, F., Padilla, C. A., Martinez-Galisteo, E., Morales, C., Sanchez-Criado, J. E., and Barcena, J. A. (1999) Mol. Hum. Reprod. 5, 914–919
8. Padilla, C. A., Martinez-Galisteo, E., Lopez-Barea, J., Holmgren, A., and Barcena, J. A. (1992) Mol. Cell. Endocrinol. 85, 1–12
9. Rosell, B., Baraona, J. A., Martinez-Galisteo, E., Padilla, C. A., and Holmgren, A. (1993) Endocrinology 131, 524–529
10. Nakamura, H., Vaage, J., Valen, G., Padilla, C. A., Bjornstedt, M., and Holmgren, A. (1998) Free Radic. Biol. Med. 24, 1176–1186
11. Takagi, N., Nakamura, T., Nishiyama, N., Naoki, K., Tanaka, T., Hashimoto, N., and Yodoi, J. (1999) Biochem. Biophys. Res. Commun. 258, 390–394
12. Fritz, G., and Kaina, B. (1999) Oncogene 18, 1033–1040
13. Barrett, W. C., DeGnore, J. F., Konig, S., Fales, H. M., Kony, Y. F., Zhang, Y., and Felsenfeld, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1015–1021
14. Watson, A., Eilers, A., Lallemand, D., Kyriakis, J., Rubin, L. L., and Ham, J. (1998) J. Neurosci. 18, 1713–1724
15. Shinkai, T., Zhang, L., Mathias, S., and Roth, G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 939–944
16. Faucheux, B. A., Agid, Y., and Hirsch, E. C. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5731–5736
17. Yeh, Y. T., and Davis, R. J. (1997) J. Biol. Chem. 272, 751–762
18. Leibovitz, D. I., and Segal, R. A. (1997) J. Neurosci. Res. 55, 557–564
19. Segal, R. A., Kaplan, D. R., and Greenberg, M. E. (1997) Science 275, 1072–1078
20. Shibata, S., Maekawa, T., and Itakura, K. (1996) Biochem. Biophys. Res. Commun. 226, 1309–1315
21. Shino, K., Shi, K., and Nakamura, M. (1999) J. Neurosci. Res. 57, 204–213
22. Lipton, S. A., and Nicoll, R. A. (1999) Trends Pharmacol. Sci. 20, 216–225
23. Xanthoudakis, S., and Curran, T. (1992) J. Biol. Chem. 267, 145–148
24. Koncz, C., and Muzyczka, N. (1996) Trends Biochem. Sci. 21, 1200–1208
25. Xanthoudakis, S., Igarashi, T., and Ogata, E. (1994) J. Biol. Chem. 269, 23071–23076
26. Xanthoudakis, S., and Moriguchi, T. (1995) J. Neurosci. Res. 41, 482–488
27. Xanthoudakis, S., Igarashi, T., and Ogata, E. (1994) J. Biol. Chem. 269, 27855–27862
28. Duguid, J. R., Eble, J. N., Wilson, T. M., and Kelly, M. R. (1995) J. Biol. Chem. 270, 2691–2696
29. Balaban, N. S., and Berggren, P. O. (1996) J. Biol. Chem. 271, 10359–10365
30. Holmgren, A. (1985) Annu. Rev. Biochem. 54, 257–271
85. Shan, B. J., Price, J. O., Gaarde, W. A., Menia, B. P., Krantz, S. B., and Zhao, Z. J. (1999) Blood 94, 4067–4076.
86. Herrmann, C., Martin, G. A., and Wittinghofer, A. (1995) J. Biol. Chem. 270, 2901–2905.
87. Kane, L. P., Shapiro, V. S., Stokoe, D., and Weiss, A. (1999) Curr. Biol. 9, 601–604.
88. Sen, C. K., and Packer, L. (1996) FASEB. J. 10, 709–720.
89. Kamata, H., and Hirata, H. (1999) Cell. Signal. 11, 1–14.
90. Lander, H. M., Hajjar, D. P., Hempstead, B. L., Mirza, U. A., Chait, B. T., Campbell, S., and Quilliam, L. A. (1997) J. Biol. Chem. 272, 4323–4326.
91. Shi, J., Vlamis-Gardikas, A., Åslund, F., Holmgren, A., and Rosen, B. P. (1999) J. Biol. Chem. 274, 36039–36042.
92. Ham, J., Badij, C., Whitfield, J., Pfarr, C. M., Lallemand, D., Yaniv, M., and Rubin, L. L. (1995) Neuron 14, 927–939.
93. Luo, Y., Umegaki, H., Wang, X., Abe, R., and Roth, G. S. (1998) J. Biol. Chem. 273, 3756–3764.
94. Luo, Y., Hattori, A., Munoz, J., Qin, Z. H., and Roth, G. S. (1999) Mol. Pharmacol. 56, 254–264.
95. Luo, Y., Hattori, A., Munoz, J., Qin, Z. H., and Roth, G. S. (1999) Mol. Pharmacol. 56, 254–264.
