Mitochondrial alterations are detected in most neurodegenerative disorders and may contribute to the dysfunction and demise of neuronal cells. Because glucose synthase kinase-3 (GSK-3) is considered to be a critical factor in regulating neuronal cell survival and death, we studied the effects of modulating GSK-3 activity in cultured neurons treated with the mitochondrial inhibitor, rotenone. Interestingly, chronic inhibition of GSK-3 protects against rotenone-induced apoptosis in cultured neuronal cells. In an attempt to elucidate the molecular mechanisms underlying this neuroprotection, we demonstrated that chronic inhibition of GSK-3 reprograms the metabolism of neuronal cells, leading to an enhancement of glycolysis. This effect was accompanied by the induction and accumulation of hexokinase II (HKII) in the mitochondria. Interfering with either the mitochondrial binding of HKII or HKII expression significantly diminished the neuroprotection evoked by GSK-3 inhibition, and importantly, HKII overexpression is sufficient to protect against rotenone-induced cell death. Thus, mitochondrial HKII is a promoter of neuronal survival under the regulation of GSK-3. Furthermore, the neuroprotective effect of HKII may be relevant to neurodegenerative diseases in which glucose hypometabolism and mitochondrial dysfunction are prominent features.

Most neurodegenerative diseases share certain common features, including mitochondrial dysfunction (reviewed in Refs. 1, 2) and glucose hypometabolism (3, 4), which may contribute to the selective loss of specific neuronal cell populations (5). Rotenone is an inhibitor of the mitochondrial complex I that has been used extensively as a pesticide (6) and is suspected to be an environmental trigger of Parkinson disease (7, 8). Rotenone has also been employed to develop animal models for Parkinson disease, because animals exposed to rotenone display a parkinsonian-like phenotype with nigrostriatal degeneration and the formation of Lewy body inclusions (9–11). Furthermore, rotenone has also been used to establish cell models of mitochondrial dysfunction and induced neurodegeneration, because there is compelling evidence showing that neurons exposed to rotenone undergo apoptosis (12–14).

Glucose synthase kinase-3 (GSK-3) is a multifunctional protein kinase that is involved in several cellular processes, such as the regulation of cell metabolism, apoptosis, transcription, and cytoskeleton dynamics (15, 16). It is well known that inhibition of GSK-3 protects neuronal cells from distinct pro-apoptotic stimuli, such as trophic factor withdrawal, β-amyloid exposure, excitotoxicity, or prion peptide-induced cell death (17–20). Thus, GSK-3 has been proposed as a possible therapeutic target for neurodegenerative diseases (21).

In this study, we show that chronic inhibition of GSK-3 induces significant protection against rotenone-triggered neuronal cell death. We also demonstrate that this neuroprotection is accompanied by an increase in the translation of hexokinase II (HKII) to mitochondria, as well as enhanced glycolysis. Furthermore, stripping the mitochondria of HKII partially abrogated the neuroprotection derived from GSK-3 inhibition, while silencing HKII expression with short interfering RNAs drastically impaired the neuroprotection provoked by GSK-3 inhibition. Finally, HKII overexpression was capable of mimicking the neuroprotective effect of GSK-3 inhibition, whereas knock down of the voltage-dependent anion channel-1 (VDAC1), the presumed docking protein for HKII at mitochondria, completely blocked the effect derived from HKII overexpression. Altogether, our results emphasize the pivotal role of mitochondrial HKII in neuroprotection.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Matrigel™ Basement Membrane Matrix and the monoclonal antibodies against Bax clone 6A7 (1:400) and GSK-3β (1:1000) were obtained from BD Biosciences. Neurobasal Medium, GlutaMax I, B-27, and B-27...
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Minus Anti-Oxidant supplements and fetal bovine serum were from Invitrogen. Dibutylryl-cAMP, retinoic acid, rotenone, 2-deoxyglucose (2-DGlc), clotrimazole, and the monoclonal antibody against β-actin (1:2000) were from Sigma (Madrid, Spain). Recombinant human brain-derived neurotrophic factor was from Alomone Laboratories (Jerusalem, Israel), the phosphatidylinositol 3-kinase inhibitor LY-294002 and the monoclonal antibody against VDAC1 (porin 31-HL) were from Calbiochem (La Jolla, CA), and SB-415286 was from Tocris Bioscience (Bristol, UK). Polyclonal antisera against hexokinase I and hexokinase II (all at a dilution of 1:100 for immunofluorescence; anti-HKII was used at 1:500 for western blot) were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Cell Lysis and Western Blot Analysis—Cells were washed once with phosphate-buffered saline (PBS), placed on ice and then homogenized in a buffer containing: 20 mM HEPES, pH 7.4; 100 mM sodium chloride (NaCl); 100 mM sodium fluoride (NaF); 1% Triton X-100; 1 mM sodium orthovanadate (Na3VO4); 5 mM EDTA; and the Complete™ protease inhibition. Vehicle-treated cells did not show any significant differences in cell viability.

HSV-1-amplicon Vectors—Packaging and titration of HSV-1-amplicon vectors was performed by transfection of the vector plasmid into the 2–2 packaging cell line and the subsequent superinfection with 5dl1.2 disabled HSV-1 strain as described previously (25, 26). The pHSVlac plasmid, encoding the Escherichia coli β-galactosidase, was used as a reporter of gene transfer (27). Plasmids pHSVSK3WT and pHSVSK3K85R, encoding the wild-type and K85R dead-kinase mutant of GSK-3β, respectively, have been described elsewhere (22, 28, 29). The placHK2 amplicon vector was generated by inserting the HK2 cDNA under the control of the cytomegalovirus immediate early promoter as an Ahdl/XbaI fragment derived from pcDNA3-HK2 (a kind gift from Dr. John E. Wilson) into pHLC plasmid (30). Multiplicity of infection (m.o.i.) was determined for each cell type known to transduce over 90% of the cells: 2.0 for SH-SY5Y (23) and 5.0 for brainstem primary neurons (data not shown).

Lentiviral Vectors—Lentiviral vectors encoding scrambled short hairpin RNA (scrambled shRNA MISSION shRNA™ vector library) or short hairpin RNA against HKII (HK2 shRNA, MISSION shRNA™ vector library) and VDAC1 (VDAC1 shRNA MISSION shRNA™ vector library) were purchased from Sigma. Lentiviral stock production was accomplished by calcium phosphate-mediated transient transfection of three plasmids as described previously (the vector plasmid, the packaging plasmid pCMVΔR8.74, and the VSVG envelope protein-coding plasmid pMD2G (31, 32)). The viral supernatant was subsequently collected, and the minimal volume of viral supernatant to achieve efficient knockdown of HKII and VDAC1 was determined experimentally.

Cell Lysis and Western Blot Analysis—Cells were washed once with phosphate-buffered saline (PBS), placed on ice and then homogenized in a buffer containing: 20 mM HEPES, pH 7.4; 100 mM sodium chloride (NaCl); 100 mM sodium fluoride (NaF); 1% Triton X-100; 1 mM sodium orthovanadate (Na3VO4); 5 mM EDTA; and the Complete™ protease inhibitor mixture (Roche Applied Science). After determining the protein content using the Bradford Assay, samples containing the same amount of protein were mixed with electrophoresis buffer containing SDS, boiled for 5 min, and separated by gel electrophoresis in the presence of SDS on 8–15% acrylamide gels. The proteins were then transferred to nitrocellulose membranes following standard procedures, and the membranes were blocked with 10% nonfat dried milk in PBS, 0.2% Tween 20 (PBST). The blocked membranes were incubated overnight with primary antibodies diluted in blocking solution at 4 °C, the filters were rinsed three times in PBST and then incubated with the corresponding peroxidase-conjugated secondary antibody for 1 h at room temperature. The immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences), and subsequent densito-
metric analysis was performed with an imaging densitometer (GS-710 model, Bio-Rad, Hercules, CA).

Subcellular Fractionation and Mitochondria Isolation—Mitochondria isolation was performed following a previously described procedure (33). Cells were washed twice with PBS, and they were then collected by centrifugation at 600 × g for 10 min. The pellet was resuspended in 5 volumes of isolation buffer (0.25 M sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol). Cells were homogenized in this solution with a glass-glass homogenizer with 20 strokes of the pestle. The homogenate was then centrifuged at 750 × g for 10 min, the supernatant was collected, and the pellet was resuspended in isolation buffer and recentrifuged. Supernatants from both centrifugations were pooled and then centrifuged at 10,000 × g for 15 min. The mitochondria-enriched pellet (crude mitochondrial fraction) was then resuspended in lysis buffer. Both the lysed crude mitochondrial fraction and the cytosolic supernatant were mixed with electrophoresis buffer, boiled for 5 min, and analyzed by Western blotting. All procedures were carried out at 4 °C.

Lactate Determination—Lactate production and release into the extracellular culture medium was determined as a measure of glycolytic influx (34). This assay is based upon the reversibility of lactate dehydrogenase activity in Reaction 1.

$$\text{Pyruvate} + \text{NADH} + H^+ \leftrightarrow \text{lactate} + \text{NAD}^+$$

**REACTION 1**

NADH generation may be determined as the increase in optical density of the sample at 340 nm. To force the reaction to completion in the direction of lactate consumption—NADH generation, newly formed pyruvate was trapped with hydrazine. Accordingly, a 5-mg NAD vial was resuspended in 2 ml of Gly-0.6 M hydrazine buffer, 4 ml of milli-Q water, and 100 μl of bovine lactate dehydrogenase. 580 μl of reaction mix was combined with 20 μl of culture medium and left at 37 °C for 30 min. NADH generation was determined as an increase in optical density at 340 nm. Known concentrations of L-lactate were used to determine the final amount of lactate produced by the cells, and lactate production was normalized against the total amount of protein.

Immunofluorescence—Cell cultures were fixed with PBS containing paraformaldehyde (4%) for 30 min. After several washes with PBS, the cells were permeabilized in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin for 1 h. After a brief wash with PBS, the cells were incubated for 2 h at room temperature with the primary antibodies diluted in the same blocking solution. Following extensive washes with PBS, the cultures were then incubated for 45 min with the appropriate secondary antibody conjugated to Alexa-488 or Alexa-594. After washing the cells, they were mounted with Fluoromount-G (Southern Biotech Assoc. Inc., Birmingham, AL) and examined under a either a Zeiss Axiovert 200 fluorescence microscope or with a laser scanning confocal microscope (LSM510 Meta) coupled to a Zeiss Axiovert 200 microscope. Image acquisition as well as the quantification of fluorescence intensity and co-localization was performed using MetaMorph v6.2r6 software as previously described (23). For these measurements, each complete set of samples was processed in parallel, and confocal images were taken in one single session. All images were captured fixing all detection settings (pinhole opening, laser intensity, gain, binning, and gamma). At least 10 cells per field, 15 fields per sample, were selected at random for analysis. In the case of HKI or HKII/Complex V double-staining, co-localization degree was determined by selecting a whole cell and processing both overlaying images through the co-localization analysis tool from MetaMorph software. All fluorescence intensity data were subjected to background normalization by selecting a blank area in each field and subtracting its intensity average from each single cell intensity.

**Determination of Cell Viability**—Cell viability was assessed by calcein-propidium iodide uptake (35). Calcein/acetoxyethyl ester is taken up and cleaved by esterases present in living cells, yielding yellowish-green fluorescence. In contrast, propidium iodide is only taken up by dead cells, which then exhibit orange-red fluorescence. Briefly, cells were incubated for 30 min at 37 °C with 2 μM propidium iodide (Sigma) and 1 μM calcein/acetoxyethyl ester (Molecular Probes, Eugene, OR). The cultures were then rinsed once with Hanks’ balanced salt solution containing 2 mM CaCl$_2$, and then they were visualized by fluorescence microscopy using a Zeiss Axiovert 200 inverted microscope. Three randomly selected fields were analyzed per well (300 – 400 cells/field) in at least three independent experiments. Cell viability was expressed as the percentage of calcein-positive cells with respect to the total number of cells.

**Detection of DNA Fragmentation: TUNEL Staining**—Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed with the FragEL™ kit (Onco gene Research Products, San Diego, CA), according to the manufacturer’s instructions. Briefly, cells were cultured on coverslips, and upon terminating the experiment, they were fixed with 4% paraformaldehyde. Samples were then permeabilized with proteinase K at room temperature for 5 min before incubating the cells for 10–30 min with equilibration buffer. The cells were nick-labeled for 1–1.5 h at 37 °C in the dark and finally, the coverslips were washed with Tris-buffered saline and mounted on glass slides with mounting media with 4’,6-diamidino-2-phenylindole (supplied with the kit).

Using a Zeiss Axiovert 200 inverted microscope, the total cell population was visualized with a UV filter, and fluorescein-labeled cells were visualized with a fluorescein filter. To analyze cell survival, three fields per coverslip (300 – 400 cells/field) were selected at random from at least three independent experiments. Cell death was expressed as the percentage of fluorescein-labeled cells compared with the total number of cells.

**Statistical Analysis and Data Processing**—The mean ± S.E. values from at least three independent experiments are represented in the graphs. Statistical comparison of the data sets was performed using two-tailed Student’s t test. The differences are presented with their corresponding statistical significance or p value, the probability that the observation occurred merely by chance under the null hypothesis. Protection was estimated as follows: 100% protection was established as the proportion of cells surviving in the absence of treatment, whereas 0% protection was the survival rate when exposed to rotenone alone. The rest of the values were normalized within
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RESULTS

Chronic Inhibition of GSK-3 Protects Neuronal Cells from Rotenone-triggered Cell Death—Because rotenone-treated neurons undergo apoptosis (12–14), we determined whether inhibition of GSK-3 protects neurons against rotenone-evoked cell death, both in human dopaminergic SH-SY5Y neuroblastoma cells differentiated into neuron-like cells (23) and in primary brainstem neurons. Thus, GSK-3 activity was inhibited in these neuronal cells with either chemical inhibitors (2 mM LiCl (36); 5 μM SB-415286 (37) supplemental Fig. S3) or by expressing a GSK-3 “dead-kinase” mutant K85R (supplemental Figs. S2 and S3), which has previously been described to act as dominant negative mutant (19, 20, 22, 29, 38, 39).

Differentially SH-SY5Y cells (Fig. 1A) and primary brainstem neurons (Fig. 1D) were exposed to two distinct chemical inhibitors of GSK-3 (LiCl, 2 mM; SB-415286, 5 μM) for 5 days, and then challenged with rotenone (2 μM) for 24 h, and cell viability was determined. A clear protective effect of GSK-3 inhibitors against rotenone neurotoxicity is observed. We next set out to inhibit GSK-3 using a genetic approach. To that end, we employed a dead-kinase mutant of GSK-3, K85R, compared with the expression of the wild-type form of GSK-3 and the expression of E. coli β-galactosidase, with no biological effect (the characterization of the expression of these genes encoded in herpesviral ampli con constructs is detailed in supplemental Fig. S2). We expressed the above mentioned constructs in either differentiated SH-SY5Y cells or primary brainstem neurons for 5 days. After that, cells were exposed to rotenone (2 μM) for 24 h before assessing cell survival. C, protection against rotenone-induced cell death was estimated after chronic inhibition of GSK-3 by different approaches in human neuron-like cells reaching levels up to 50%. D and E, brainstem primary neurons were cultured for 2 days and then subjected to chronic inhibition of GSK-3 for additional 5 days either with chemical inhibitors LiCl (2 mM) and SB-415286 (5 μM) (D) or by expressing dead-kinase mutant of GSK-3, K85R (m.o.i. 5.0) (E). Subsequently, they were challenged with rotenone (2 μM) for 24 h, and cell viability was assessed. F, protection against rotenone-elicited cell death in brainstem primary neurons previously subjected to chronic inhibition of GSK-3. The mean values ± S.E. from at least three independent experiments are shown. *, p < 0.05; **, p < 0.005 (Student’s t test).

that scale. Consequently, negative protection values represent an increase in cell death over that achieved when exposed to rotenone.

FIGURE 1. Protection against rotenone-induced cell death in neuronal cells after chronic inhibition of GSK-3. Human neuron-like cells (A–C) and brainstem primary neurons (D–F) were subjected to chronic inhibition of GSK-3 for 5 days and then exposed to 2 μM rotenone for 24 h. A, human neuron-like cells were treated either with lithium chloride (2 mM) or SB-415286 (5 μM), and they were then exposed to rotenone (2 μM) for 24 h. After that, cell viability was assessed. B, human neuron-like cells were transduced with pH5Vlac, pH5VGSK3WT, or pH5VGSK3K85R (encoding a dead-kinase mutant of GSK-3, K85R) at m.o.i. 2.0, they were allowed to express the constructs for 5 days, and they were then exposed to rotenone (2 μM) for 24 h before assessing cell survival. C, protection against rotenone-induced cell death was estimated after chronic inhibition of GSK-3 by different approaches in human neuron-like cells reaching levels up to 50%. D and E, brainstem primary neurons were cultured for 2 days and then subjected to chronic inhibition of GSK-3 for an additional 5 days either with chemical inhibitors LiCl (2 mM) and SB-415286 (5 μM) (D) or by expressing dead-kinase mutant of GSK-3, K85R (m.o.i. 5.0) (E). Subsequently, they were challenged with rotenone (2 μM) for 24 h, and cell viability was assessed. F, protection against rotenone-elicited cell death in brainstem primary neurons previously subjected to chronic inhibition of GSK-3. The mean values ± S.E. from at least three independent experiments are shown. *, p < 0.05; **, p < 0.005 (Student’s t test).
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Chronic Inhibition of GSK-3 Prevents the Transduction of the Cell Death Cascade Triggered by Rotenone—We have studied some of the events that take place during rotenone-induced apoptosis, and we have analyzed how modulating GSK-3 activity affects these events (Fig. 2). In particular, we focused on the translocation of Bax to the mitochondria, the activation of caspase-3, and the fragmentation of nuclear DNA as canonical steps of mitochondrial dysfunction-driven cell death (40, 41). Differentiated SH-SY5Y cells treated with rotenone (2 μM) for 4 h were fractionated, and each cellular fraction was analyzed by Western blotting (Fig. 2A). In this way, we found that the translocation of Bax to the mitochondria-enriched fractions following exposure to rotenone was partially attenuated by prior chronic inhibition of GSK-3. In these cells, the levels of Bax remained unaltered in the cytosolic fraction, suggesting that thus protecting neuronal cells from cell death induced by mitochondrial dysfunction.

Chronic Inhibition of GSK-3 Enhances Glycolysis, which Is Crucial for Neuroprotection—It is well known that lithium increases glucose uptake and metabolism through glycolysis in rat brain (44, 45), as well as inhibiting GSK-3 (36). Thus, we explored the possibility that chronic GSK-3 inhibition may lead to an increase in glucose consumption through glycolysis. Therefore, we determined lactate production as a measure of the glycolytic rate of cells (34), both in the basal state and when inhibition of GSK-3 activity had been down-regulated by chemical inhibitors (lithium or SB-415286) or through the expression of a dead-kinase mutant effectively blocks the apoptotic cascade caused by rotenone, up-regulation of Bax transcription, and translation during apoptosis (42) may also be prevented by chronic inhibition of GSK-3.

Cleavage and the subsequent activation of caspase-3 occurs during rotenone-elicted cell death in neuronal cells (12). Thus, we exposed human neuron-like cells to rotenone (2 μM) for 24 h before examining the activation state of caspase-3 by Western blot with a specific antibody against the active 17- and 19-kDa fragments of caspase-3. Significant attenuation of caspase-3 activation was observed in cells subjected to chronic inhibition of GSK-3 prior to the rotenone challenge (Fig. 2B).

Fragmentation of nuclear DNA is a late event in the cell death process, and because many 3′-OH ends become available, it can be readily assessed by TUNEL labeling (43). TUNEL staining of differentiated SH-SY5Y cells after a 24-h challenge with rotenone (2 μM) demonstrated that nuclear DNA undergoes fragmentation upon rotenone treatment. In agreement with all our earlier data, this fragmentation was partially blocked in cells subjected to chronic inhibition of GSK-3 prior to rotenone exposure (Fig. 2, C and D). In summary, we conclude that chronic inhibition of GSK-3 by either specific chemical inhibitors or gene transfer of a dead-kinase mutant effectively blocks the apoptotic cascade caused by rotenone.

Cell death was also significantly augmented in wild-type cells that overexpress GSK-3, consistent with previous evidence suggesting a pro-apoptotic role for GSK-3 (Fig. 1, B and E).

Thus, we have shown that chronic inhibition of GSK-3 by chemical inhibitors or gene transfer of a dead-kinase mutant effectively blocks the apoptotic cascade caused by rotenone.

In conclusion, we have demonstrated that chronic inhibition of GSK-3 by chemical inhibitors or gene transfer of a dead-kinase mutant effectively blocks the apoptotic cascade caused by rotenone.

To determine whether this enhanced glycolysis contributes to neuroprotection, we carried out a cell viability assay in which...
human neuron-like cells were treated with rotenone in the presence of 2-DGlc, which blocks glycolysis (46). We exposed human neuron-like cells to both rotenone (2 μM) and 2-DGlc (2 mM) for 24 h, and found that the neuroprotective effect against rotenone induced by chronic inhibition of GSK-3 was completely abolished in the presence of 2-DGlc (Fig. 3, B–E). Together these results suggest that the increase in glycolysis resulting from chronic GSK-3 inhibition may be required for neuroprotection, possibly by compensating the bioenergetic deficit associated with rotenone-triggered mitochondrial dysfunction.

**Chronic Inhibition of GSK-3 Drives the Re-organization of the Glucose Metabolism-related Protein HKII**—To gain some insight into the molecular mechanisms responsible for the enhanced glycolysis following GSK-3 inhibition, we analyzed the expression and subcellular localization of hexokinase isoenzymes, which catalyze the first reaction in glucose metabolism. Not only was a significant increase in the immunofluorescence intensity for HKII observed (Fig. 4), but we also found a marked increase in the localization of HKII in mitochondria, from 50% at the basal state to 80% levels after chronic GSK-3 inhibition (Fig. 5, B and C). In contrast, no significant differences were observed in the expression and subcellular distribution of HKI (supplemental Fig. S4).

*Mitochondrial HKII Is Involved in Neuroprotection*—Because mitochondrial HKII may be more active than cytosolic HKII (47) and it might also play an anti-apoptotic role in some tumor cell lines (48–52), we explored how the mitochondrial association of HKII might influence the protection against rotenone neurotoxicity in neuronal cells (Fig. 5). Silencing HKII expression using a short hairpin RNA interference encoded in a lentiviral vector (supplemental Fig. S2) virtually abolished the neuroprotective effect of chronic GSK-3 inhibition (Fig. 5A), highlighting the crucial role of HKII in neuroprotection. Additionally, pre-treatment of neuron-like cells with clotrimazole, an antifungal azole that strips HKII off mitochondria without directly affecting its kinase activity (50, 52, 53) (supplemental Fig. S5), also impaired the neuroprotective effect of GSK-3 inhibition (Fig. 5B), emphasizing the importance of the mitochondrial localization of HKII for neuroprotection.

These results suggested that mitochondrial HKII is essential for chronic GSK-3 inhibition to exert a neuroprotective effect. To test whether mitochondrial HKII is indeed sufficient for
neuroprotection, we overexpressed HKII in neuronal cells. Overexpression of HKII protected both human neuron-like cells and primary brainstem neurons against a rotenone insult (Fig. 5, C and D, respectively), indicating that HKII per se is capable of mimicking the neuroprotective effect derived from GSK-3 inhibition. An enhanced glycolysis was also observed after HKII overexpression (Fig. 5E), thus suggesting that HKII is a rate-limiting step in glycolysis. Interestingly, the neuroprotective effect of HKII overexpression was partially inhibited by detaching HKII from mitochondria using clotrimazole, although the enhancement of glycolysis was not significantly affected by clotrimazole (Fig. 5, E and F). Together these results suggest that an increased glycolysis might be necessary but is not sufficient to protect against rotenone neurotoxicity, whereas mitochondrial localization of HKII seems to be crucial for neuroprotection.

Additionally, we explored the role of VDAC1 in HKII-elicited neuroprotection against rotenone. Compelling evidence supports the view that VDAC1 is the major docking protein for hexokinases at the mitochondria (48, 54), so the dynamic inter-

**FIGURE 4.** Subcellular distribution of HKII after chronic inhibition of GSK-3. Human neuron-like cells were subjected to chronic inhibition of GSK-3 for 5 days, with either a chemical inhibitor (LiCl, 2 mM; SB-415286, 5 μM) or by expressing the dead-kinase mutant K85R. The cells were subsequently fixed and immunostained with an anti-HKII (green) antibody. The cells were counterstained with an antibody against mitochondrial complex V (red) to identify the mitochondria. A, confocal microscopy was performed to visualize the subcellular distribution of HKII with respect to mitochondria. Scale bar: 20 μm. B and C, computer processing of the images obtained with MetaMorph v6.2r6 software, showing the fluorescence intensity of HKII normalized to complex V (B) or the degree of HKII co-localization with complex V (C). At least 15 cells/field from 5 random fields per experiment were analyzed. The mean ± S.E. from at least three independent experiments are shown. *, p < 0.05; **, p < 0.005 (Student’s t test).

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1. **A**
2. **B**
3. **C**
4. **FIGURE 4.** Subcellular distribution of HKII after chronic inhibition of GSK-3. Human neuron-like cells were subjected to chronic inhibition of GSK-3 for 5 days, with either a chemical inhibitor (LiCl, 2 mM; SB-415286, 5 μM) or by expressing the dead-kinase mutant K85R. The cells were subsequently fixed and immunostained with an anti-HKII (green) antibody. The cells were counterstained with an antibody against mitochondrial complex V (red) to identify the mitochondria. A, confocal microscopy was performed to visualize the subcellular distribution of HKII with respect to mitochondria. Scale bar: 20 μm. B and C, computer processing of the images obtained with MetaMorph v6.2r6 software, showing the fluorescence intensity of HKII normalized to complex V (B) or the degree of HKII co-localization with complex V (C). At least 15 cells/field from 5 random fields per experiment were analyzed. The mean ± S.E. from at least three independent experiments are shown. *, p < 0.05; **, p < 0.005 (Student’s t test).
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FIGURE 5. Role of HKII in neuroprotection. The degree of protection against rotenone neurotoxicity is shown under the different conditions examined. A, human neuron-like cells were subjected to chronic inhibition of GSK-3, and a set of cells were concomitantly exposed to a lentiviral vector encoding a short hairpin interfering RNA against HKII (HK2 shRNA) to knock down HKII expression. A scrambled short hairpin RNA (scrambled shRNA) was used as negative control. The cells were then exposed to 2 μM rotenone for 24 h, and cell viability was assessed. B, human neuron-like cells were subjected to chronic inhibition of GSK-3 for 5 days and then exposed to rotenone (2 μM) for 24 h. Prior to rotenone exposure, a set of cells was preincubated for 2.5 h with clotrimazole (20 μM), which was maintained with the rotenone for another 24 h. C and D, human neuron-like cells (C) and brainstem primary neurons (D) were transduced at m.o.i. s 2.0 and 5.0, respectively, with an HKII-expressing vector placHK2 (using pHSVlac as a control), and 12 h later they were exposed to 2 μM rotenone for 24 h. E, human neuron-like cells were transduced with placHK2. The culture medium was replaced 24 h later with fresh medium, and small aliquots were collected at different time points up to 24 h. Shown are values corresponding to lactate production and release at 24 h. As shown, overexpression of HKII significantly enhanced lactate production, thus indicating an increase in glycolysis. F, human neuron-like cells were transduced with placHK2 and subsequently exposed to rotenone (2 μM) for 24 h. A subset of cells was pre-treated with clotrimazole (20 μM) for 2.5 h prior to rotenone exposure. Clotrimazole was maintained with rotenone for another 24 h. Cell viability was then determined. Clotrimazole partially but significantly diminished HKII overexpression-induced neuroprotective effect. G, expression of VDAC1 was knocked down in human neuron-like cells by transducing them with a VDAC1 shRNA encoded in a lentiviral vector. Then the cells were transduced with placHK2 to overexpress HKII. Cell viability was determined after challenging the cells with rotenone (2 μM) for 24 h. A scrambled shRNA-encoding vector and pHSVlac were used as controls of transduction. In all cases, the mean ± S.E. values from at least three independent experiments are shown. *, p < 0.05; **, p < 0.003 (Student’s t test).

DISCUSSION

A thorough understanding of the molecular mechanisms underlying the actions of different neuroprotective agents will be important to discover novel therapeutic targets and eventually develop more effective therapies for neurodegenerative diseases. Recent studies have emphasized the importance of GSK-3 as a possible therapeutic target, because inhibitors of this kinase may help to protect neuronal cells from several different pro-apoptotic stimuli (reviewed in Ref. 21). Here we demonstrate that GSK-3 inhibition protects both human neuron-like dopaminergic cells and rodent brainstem primary neurons against rotenone toxicity, an established trigger of mitochondrial dysfunction and neurodegeneration. In fact, our data show that chronic, but not acute, inhibition of GSK-3 prior to rotenone exposure is required to achieve the greatest neuroprotective effect. These results suggest that chronic inhibition of GSK-3 may help to generate a cellular state that is more resistant to apoptosis, which is consistent with previous studies demonstrating a similar effect of GSK-3 inhibition in non-differentiated neuroblastoma cells (56, 57).

Some recent studies have pointed to interesting molecular routes that can be modulated by GSK-3 and action of HKII with VDAC1 could be an important modulator of cell death (reviewed in Ref. 55). As another approach to address the relevance of HKII binding to mitochondria, we studied the impact of knocking down VDAC1 expression on HKII-derived neuroprotection (Fig. 5G). Silencing the expression of VDAC1 with RNA interference techniques completely blunted the neuroprotective effect of overexpressing HKII. These results are consistent with the view that the binding of HKII to VDAC1 on the mitochondrial outer membrane is crucial for the neuroprotective effect of HKII against rotenone-triggered neuronal cell death.
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that may play crucial roles in the regulation of cell death and survival. GSK-3 has been shown to accumulate in the mitochondria and nucleus in response to apoptotic stimuli (58), and it can phosphorylate the pro-apoptotic protein Bax, targeting it to the mitochondria to promote permeabilization (59). Likewise, GSK-3 may also favor the interaction between Bax and mitochondria by phosphorylating the voltage-dependent anion channel (VDAC), thus disrupting its interaction with HKII and rendering VDAC more accessible to Bax in HeLa cells (60). The anti-apoptotic Bcl-2 protein Mcl-1 may also be phosphorylated by GSK-3 (61). Finally, it has recently been shown that GSK-3 activity may impair the xenobiotic and antioxidant cell response by phosphorylating the transcription factor Nrf-2 promoting its subsequent nuclear exclusion (39).

We now demonstrate that chronic inhibition of GSK-3 also significantly enhances glucose metabolism through glycolysis in neuronal cells. This increased glycolysis is consistent with previous studies demonstrating an increase in glucose uptake and metabolism in the brain of lithium-treated rodents (44, 45).

More importantly, our results also demonstrate an increase in the association of HKII with mitochondria after chronic inhibition of GSK-3. This association may play a dual role as the increase of HKII at the mitochondria may initially account for the enhancement of glycolysis (47, 62). This increased glycolysis may partially counteract the bioenergetic deficit derived from mitochondrial dysfunction after rotenone treatment. Secondly, binding of HKII to mitochondria may play an anti-apoptotic role by preventing the translocation of Bax to mitochondria and, hence, the subsequent release of pro-apoptotic factors (48, 52). Our results suggest that this anti-apoptotic role of mitochondrial-bound HKII is crucial for neuroprotection. Activation of GSK-3 has recently been reported to disrupt the interaction of HKII with VDAC in HeLa cells, also suggesting that acute inhibition of GSK-3 may reinforce the attachment of HKII to mitochondria (60). Additionally, it has been recently shown that Akt may also target HKII for binding to mitochondria in cardiomyocytes (63). However, lithium treatment may also diminish the detachment of HKII from mitochondria in melanoma cells (64). In our post-mitotic neuron-like cells, the translocation of HKII to mitochondria did not occur immediately after inhibition of GSK-3, and it was only observed after chronic inhibition of GSK-3. Therefore, it seems likely that GSK-3 activity may modulate the interaction between HKII and mitochondria through distinct mechanisms, which may significantly vary depending on the cell type.
Mitochondrial Hexokinase II in Neuroprotection

The model we propose for neuroprotection, depicted in Fig. 6, relies upon a shift in metabolism orchestrated after chronic inhibition of GSK-3. Interestingly, the increased association of HKII with mitochondria through its interaction with VDAC1, as well as the increase in lactate production, strongly suggests a raise in glycolysis upon GSK-3 inhibition. More importantly, the most significant feature of our study shows that the mitochondrial localization of HKII is essential for neuroprotection, maybe by preventing Bax translocation to mitochondria. In this regard, the crucial role of VDAC1 as a docking site for HKII at the mitochondria is highlighted by the fact that the knock down of VDAC1 blocks the neuroprotective effect achieved by HKII overexpression.

A close connection has been revealed in a variety of cell types and organisms between glucose metabolism and cell death pathways (65, 66). Interestingly, HKII and an abnormally high glycolytic rate are widely considered to favor cell survival and rapid cell growth in tumor cells, thereby stimulating tumor progression (62, 67). Our results now suggest that HKII is also a potent promoter of neuronal cell survival, and it can therefore be considered as a promising therapeutic target in the context of neurodegenerative diseases (3, 4, 68). Indeed, it has been suggested that detachment of hexokinase from the mitochondria may occur in neurodegenerative diseases (69, 70). Therefore, experimental strategies directed toward maintaining mitochondrial HKII may be effective both in enhancing glucose metabolism, and in blocking mitochondrial-dependent apoptosis in neuronal cells, which might eventually slow down neurodegeneration in neurological diseases.

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