Oxidative stress is a key apoptotic stimulus in neuronal cell death and has been implicated in the pathogenesis of many neurodegenerative disorders, including Parkinson disease (PD). Recently, we demonstrated that protein kinase C-δ (PKCδ) is an oxidative stress-sensitive kinase that can be activated by caspase-3-dependent proteolytic cleavage to induce apoptotic cell death in cell culture models of Parkinson disease (Kaul, S., Kanthasamy, A., Kitazawa, M., Anantharam, V., and Kanthasamy, A. G. (2003) Eur. J. Neurosci. 18, 1387–1401 and Kanthasamy, A. G., Kitazawa, M., Kanthasamy, A., and Anantharam, V. (2003) Antioxid. Redox. Signal. 5, 609–620). Here we showed that the phosphorylation of a tyrosine residue in PKCδ can regulate the proteolytic activation of the kinase during oxidative stress, which consequently influences the apoptotic cell death in dopaminergic neuronal cells. Exposure of a mesencephalic dopaminergic neuronal cell line (N27 cells) to H2O2 (0–300 μM) induced a dose-dependent increase in cytotoxicity, caspase-3 activation and PKCδ cleavage. H2O2-induced proteolytic activation of PKCδ was mediated by the activation of caspase-3. Most interestingly, both the general Src tyrosine kinase inhibitor genistein (25 μM) and the p60Src tyrosine-specific kinase inhibitor (TSKI; 5 μM) dramatically inhibited H2O2-induced PKCδ and the Parkinsonian toxin 1-methyl-4-phenylpyridinium-induced PKCδ cleavage, kinase activation, and apoptotic cell death. H2O2 treatment also increased phosphorylation of PKCδ at tyrosine site 311, which was effectively blocked by co-treatment with TSKI. Furthermore, N27 cells overexpressing a PKCδNS311F mutant protein exhibited resistance to H2O2-induced PKCδ cleavage, caspase activation, and apoptosis. To our knowledge, these data demonstrate for the first time that phosphorylation of Tyr-311 on PKCδ can regulate the proteolytic activation and proapoptotic function of the kinase in dopaminergic neuronal cells.

Oxidative stress and apoptosis are key mediators of numerous neurodegenerative processes in the nervous system, including Alzheimer (4, 5) and Huntington disease (6, 7), Friedrich ataxia (8, 9), and Parkinson disease (10–12). Oxidative stress has been shown to trigger the apoptotic cell death process through activation of one or more signaling molecules (13–16). In dopaminergic neurons, oxidative stress-induced phosphorylation events involve mitogen-activated protein kinases including p38 mitogen-activated protein kinase (17) and stress-activated protein kinase c-Jun N-terminal kinase kinases (18). Recently, we showed that Parkinsonian toxin MPP+1-induced ROS generation promotes apoptotic cell death in dopaminergic neurons via caspase-3-mediated proteolytic cleavage of protein kinase C-δ (PKCδ) (1). PKCδ is a member of the PKC serine-threonine protein kinase family classified into three groups, namely the classical (α, β, and γ activated by DAG and Ca2+), the atypical (ζ and η/DAG and Ca2+ independent), and the novel (δ, ε, η, and ϕ activated by DAG but Ca2+ independent). PKCδ activation requires either the phosphorylation of its activation loop residues, leading to enzyme translocation, or the proteolytic cleavage of the kinase to yield catalytically active fragments. In the cellular models of Parkinson disease, we observed a caspase-3-mediated activation of PKCδ without any evidence of membrane translocation (1). PKCδ is known to be phosphorylated at tyrosine residues Tyr-52, Tyr-155, Tyr-187, Tyr-311, Tyr-332, and Tyr-565 when activated in response to certain stimuli, particularly to the known oxidative stress-inducing agent hydrogen peroxide (H2O2) (19–21). Src kinase, a member of the nonreceptor protein-tyrosine kinase family, variably modulates PKCδ activity by increasing tyrosine phosphorylation, depending on the cell type and the insulin (22–25). Other members of the Src family of kinases that influence PKCδ activity via phosphorylatory changes are Fyn and c-Abl kinase (26, 27). Furthermore, recent studies have demonstrated that PKCδ, when phosphorylated on the tyrosine residue Tyr-311, exhibits an increased catalytic activity in H2O2-treated cells (28, 29). However, the relationship between PKCδ tyrosine phosphorylation and its proteolytic cleavage has never been explored, particularly whether PKCδ tyrosine phosphorylation can regulate its proteolytic activation and proapoptotic function. Here we demonstrate that phosphorylation of the tyrosine residue Tyr-311 in PKCδ is essential for proteolytic activation, and that inhibition of tyrosine phosphorylation can attenuate oxidative stress-induced apoptotic cell death in dopaminergic neuronal cells.
EXPERIMENTAL PROCEDURES

Chemicals—Hydrogen peroxide (H₂O₂), β-actin antibody (mouse monoclonal), histone H1, β-glycerophosphate, ATP, and protein A-Sepharose were purchased from Sigma. PKCδ rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies to caspase-3 and caspase-7 (monoclonal), histone H1, monoclonal), histone H1, histone H3, and histone H9254 were obtained from Cell Signaling Technology (Beverly, MA). RPMI 1640, fetal bovine serum, 2 mM L-glutamine, 50 units of penicillin, and 50 µg/mL streptomycin were obtained from Invitrogen. Plasmids encoding PKCδ/N27 cells to H₂O₂ (100 µM) were purchased from Upstate Biotechnology, Inc. (Charlottesville, VA). Applied Science. Anti-phosphotyrosine (4G10)-agarose conjugate was obtained from Bachem Biosciences (King of Prussia, PA); and FITC-VAD-FMK was obtained from Cell Signaling Technology (Beverly, MA). The LIVE/DEAD® kit consists of a cocktail of a cell-permeable fluorescent probe that binds to active caspase-3, was used to stain the cells for the duration of the experiment. The cells were removed to the flask by using a cell scraper and centrifuged at 200 g for 5 min, washed with PBS twice, and homogenized as described previously (1). Cell lysates, collected by spinning down the cell fragments at 20,000 g for 10 min to collect the supernatant, were used for immunoprecipitation studies to determine caspase-3 enzyme activity, DNA fragmentation, and PKCδ cleavage. Un-treated cells were grown in the complete medium and used as control samples. For real time fluorescence imaging, the cells were grown in 24-well plates and viewed in the culture wells.

Cytofluorometry—Cell death was determined after exposing the N27 cells to H₂O₂ (100 µM) using the Sytox green cytotoxicity kit and the LIVE/DEAD® viability/cytotoxicity kit (Molecular Probes, Eugene, OR). The Sytox green cytotoxicity assay is based on the principle that Sytox green cannot enter cells with intact membranes (live cells) but permeates cells with compromised plasma membranes and enters the cell to become green-fluorescent. Briefly, N27 cells were grown in 24-well cell culture plates at equal densities and treated with H₂O₂ (0–300 µM) and 1 µM Sytox green fluorescent dye for a period of 4 h. The Sytox green assay allows dead cells to be viewed directly under the fluorescence microscope as well as quantitatively measured with a fluorescence microscope plate reader (excitation 485 nm; emission 530 nm) (SpectraMax Gemini XS model, Molecular Devices, Sunnyvale, CA). The LIVE/DEAD® kit consists of a combination of two dyes: SYTO 10 (green fluorescence), a highly cell permeable cell dye which stains all cells, and an ethidium homodimer (DEAD Red; red fluorescence), a membrane impermeant dye that only permeates cells with compromised plasma membranes. Fluorescent images were taken after exposure to H₂O₂ with a NIKON TE2000 microscope, and pictures were captured with a SPOT digital camera.

In Situ Fluorometric Analysis of Caspase Activity—FITC-VAAD-FMK, a cell-permeable fluorescent probe that binds to active caspase-3, was used as an in situ marker for caspase activity. The entire procedure was performed according to Promega’s CaspACE® kit, as described previously (1). Fluorescent images were captured using a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI).

Enzymatic activity was measured as described previously (32, 38). Acetyl-DEVD-amino-4-methylcoumarin (50 µM) was the fluorometric caspase-3 substrate used for the reaction. Enzymatic activity, measured using a Spectramax microplate reader at 405 nm, was represented as fluorescence units/mg of protein.

Western Blot Analysis—Cells were collected after exposure to 100 µM H₂O₂, resuspended in 300 µL of homogenization buffer (20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 µM/ml aprotinin, and 10 µM/ml leupeptin), sonicated, and then centrifuged at 10,000 × g for 1 h at 4 °C (1). Proteins were separated by 10–12% SDS-PAGE. PKCδ polyclonal (1:2000), PKCδ-Pyr-311 (1:5000), and β-actin (1:5000) antibodies were used to detect the bands. Secondary horseradish peroxidase-conjugated anti-rabbit (1:2000) and anti-mouse (1:2000) were used for antibody detection with an ECL detection kit (Amersham Biosciences).

Immunoprecipitation and Kinase Assay—Immunoprecipitation studies were conducted to determine the phosphorylative changes in the PKCδ protein obtained from H₂O₂-treated N27 cells. Briefly, cells were washed once with 1× PBS and resuspended in 500 µL of lysis buffer (25 mM HEPES (pH 7.5), 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10 mM NaF, and 4 µg/mL of aprotinin and leupeptin) (1). The cell suspension was kept on ice for 30 min and then centrifuged at 13,000 × g for 5 min. The resultant supernatant was collected at the time point and total cellular protein (~200 µg total) was immunoprecipitated overnight at 4 °C using 20–40 µL of anti-phosphotyrosine (4G10)-agarose conjugated antibody. The Sepharose-bound antigen-antibody complexes were washed three times with buffer. Samples were then mixed with 2× SDS-PAGE loading buffer, boiled for 5 min, and then separated on 10–12% SDS-PAGE. For the kinase assay, the immunoprecipitation was done by using a polyclonal rabbit antibody and protein-Sepharose beads. The beads were washed three times with kinase buffer (40 mM Tris (pH 7.4), 20 mM MgCl₂, 20 µM ATP, 2.5 mM CaCl₂). The reaction was started by adding 20 µL of buffer containing 0.4 mg of histone and 5 µC of [γ-32P]ATP (4,500 Ci/mmol). After incubation for 10 min at 30 °C, SDS loading buffer (2×) was added to the samples to terminate the reaction. The reaction products were separated on SDS-PAGE (12.5%), and the 1-H-phospho-histone was detected using a Phospho-ELISA kit (PK model, Bio-Rad Labs) and quantified with Quantity One 4.2.0 software.

DNA Fragmentation Assay—DNA fragmentation was measured using a recently developed Cell Death Detection ELISA Plus assay kit, a fast, highly sensitive, and reliable assay for the detection of early apoptotic death (32, 39). Briefly, after treatment with 100 µM H₂O₂, the cells were spun down at 200 × g for 5 min and washed once with PBS. Cells were detached using a FACS lysis buffer (50 mM Tris-HCl, 25 µL of lysis buffer containing 500 µL of lysis buffer), and spun down again at 5,000 rpm for 10 min to collect the supernatant, which was used to measure DNA fragmentation as per the manufacturer’s protocol. Readings were taken in a Spectramax multiwell plate reader at 405 nm, with 490 nm as a reference reading.

Measurement of ROS Generation—The ROS generation in N27 cells was measured using the fluorescence probe dihydroethidine, as described previously (40). Briefly, N27 cells were plated on 24-well plates at a density of 2 × 10⁶ cells per well for a period of 24 h prior to treatment. After 24 h, the RPMI culture medium was removed from the wells and replaced with clear HBSS medium supplemented with 2 mM CaCl₂, 1 µM dihydroethidine (final concentration) was added to the HBSS for a period of 15 min. The cells were then treated with either 0.01% (v/v) DMSO (alone or along with the tyrosine kinase inhibitor TSKI (5 µM) for a period of 1 h. After the 1-h incubation period, fluorescence was measured by using a microplate reader (Beckman and Coulter). The data were quantified using SpectraMax spectrophotometer analysis software.

Transient Transfections—cDNA encoding PKCδ catalytic fragment (PKCδ-CF) from the eugFFIN vector was subcloned into the lentiviral expression vector p lent/VS-5-TOPO (herein referred to as plenti/PKCδ-CF) by PCR using standard cloning procedures. PKCδ311F encoded in pcDNA3 vector encodes a protein in which the tyrosine residue at position 311 is mutated to phenylalanine. The expression of PKCδ-CF in mammalian cells can be monitored by using an antibody directed against V5 epitope. Transfections of N27 cells with PKCδ311F and TSKI (5 µM) was performed using an AMAXA® NucleofectorTM kit for cell lines (AMAXA GmbH, Germany). Plasmid pCDNA3.1 was used as vector control. Briefly, N27 cells were grown in T-175 flasks at a density of 3 × 10⁶ per mL and harvested for the transfection procedure. The NucleofectorTM solution V was primed by adding a supplement solution (provided by manufacturer) and plasmid DNA. The cells were then resuspended in this DNA-containing solution at an optimal density of 3 million/mL. Electroporation was carried out with AMAXA® NucleofectorTM transfection instrument as per the manufacturer’s protocol. The procedure was repeated for each subsequent sample. 100 µL of cell suspension containing 5 µg of pmax GFP DNA (provided with the kit) was used to determine the transfection efficiency. The transfected cells were then transferred to T-75 flasks or 6-well plates as desired and allowed to grow for a 24-h period before being used for the
Low Dose H$_2$O$_2$ Exposure Induces Dose-dependent Cell Death in Dopaminergic Neuronal Cells—H$_2$O$_2$ is an oxidative insult commonly used to investigate oxidative stress-induced apoptotic signaling in many cell types including dopaminergic neuronal cells (41, 42). To determine the effects of low dose oxidative stress on the dopaminergic system, we treated mesencephalic dopaminergic clonal cells (N27) with low doses of H$_2$O$_2$ (0–300 μM) for a period of 4 h, and cytotoxicity was assayed at 1-h intervals over the entire treatment period. Fig. 1A demonstrates a fluorescent image from a group of N27 cells treated with H$_2$O$_2$ (100 μM) for 4 h and assayed for cell death by a LIVE/DEAD® viability/cytotoxicity kit (Molecular Probes). H$_2$O$_2$ treatment induced a clear increase in the number of dead cells, as evident from the increase in red fluorescence-labeled cells (dead cells) compared with the number of cells exhibiting green fluorescence (live cells). In untreated cells, only a very few red fluorescence-positive cells were observed.

We also measured the cytotoxicity both qualitatively and quantitatively by using another fluorescence dye Sytox® green, which stains only dead/dying cells. Fig. 1B is representative of untreated N27 cells (top row) and cells treated with H$_2$O$_2$ (100 μM) at the end of a 4-h treatment period (bottom row) in both phase contrast and FITC fluorescence imaging. An increase in green fluorescence indicates an increase in cell death in H$_2$O$_2$-treated cells, because the Sytox® green dye only permeates compromised cell membranes to stain the nuclear chromatin. Fig. 1C depicts the quantitative measurement by a microplate reader of Sytox® green fluorescence and demonstrates a dose-dependent increase of cell death in N27 cells treated with varying doses of H$_2$O$_2$ (0–300 μM). H$_2$O$_2$ increased cytotoxicity by 216, 380, 468, and 638% over untreated controls at 10, 30, 100, and 300 μM concentrations 4 h after exposure, demonstrating dose-dependent oxidative stress-induced cell death in N27 cells. Because 100 μM H$_2$O$_2$ consistently induced significant oxidative damage in these cells, we used this concentration for all subsequent experimental analyses in this study.

H$_2$O$_2$ Induces a Time-dependent Increase in Caspase-3-mediated Cellular Apoptosis in N27 Dopaminergic Cells—To determine whether apoptotic cell death occurs during H$_2$O$_2$ treatment, we measured the activity of the key apoptotic cellular enzyme caspase-3 as well as the extent of DNA fragmentation in the treated cells. H$_2$O$_2$ (100 μM) induced a time-dependent increase in caspase-3 activity over a 4-h treatment period (Fig. 2A). Treatment with 100 μM H$_2$O$_2$ induced 260, 438, 1055, and 1369% increases in caspase-3 activity in N27 cells at 90, 120, 150, and 240 min post-exposure. We further confirmed the activation of caspases during oxidative insult by labeling the activated caspase enzyme with the fluorescent substrate Z-VAD-FITC followed by observation under a fluorescence microscope (Fig. 2A, inset). H$_2$O$_2$ treatment induced a significant increase in the fluorescent labeling of the N27 cells after the 4-h post-exposure as compared with the untreated cells.

Cellular apoptosis is often marked by the final precipitating events of chromatin breakdown and DNA fragmentation, which are considered hallmarks of programmed cell death. Therefore, we determined the extent of DNA fragmentation following H$_2$O$_2$ treatment in N27 cells using a DNA ELISA technique. H$_2$O$_2$ (100 μM) induced time-dependent increases in DNA fragmentation of 109, 154, and 392% at 1, 2, and 4 h, respectively, as compared with the untreated cells (Fig. 2B). Together, these data clearly indicate that N27 dopaminergic cells are highly sensitive to low dose oxidative stress and can undergo activation of caspase-3 and subsequent DNA fragmentation.
50 μM of the caspase-3 specific inhibitor Z-DEVD-FMK (Fig. 3B). Together, these results indicate that oxidative stress induces caspase-3-dependent proteolytic cleavage of PKCδ in dopaminergic cells.

**Oxidative Stress-induced Tyrosine Phosphorylation Regulates Proteolytic Cleavage of PKCδ**—To determine whether PKCδ tyrosine phosphorylation modulates its proteolytic cleavage and kinase activity, we tested the effects of tyrosine kinase inhibitors on PKCδ activity in N27 dopaminergic cells following H₂O₂ treatment. Both the broad spectrum Src kinase inhibitor genistein (25 μM) and the specific p60Src peptide inhibitor TSKI (5 μM) inhibited the H₂O₂-induced PKCδ proteolytic cleavage (Fig. 4A). The inhibitory effect of TSKI was complete, as compared with the partial block induced by genistein treatment. To examine whether the observed effects of genistein and TSKI were due to inhibition of PKCδ tyrosine phosphorylation, we measured the level of PKCδ tyrosine phosphorylation in cells treated with H₂O₂ in the presence or absence of genistein and TSKI. Tyrosine-phosphorylated proteins were first immunoprecipitated using phosphotyrosine antibody conjugated with agarose beads. The immunoprecipitates were then separated on SDS-PAGE and immunoblotted with PKCδ antibody. The data indicate that H₂O₂ induced a significant increase in PKCδ-mediated PKCδ proteolytic cleavage during oxidative insult in dopaminergic neuronal cells.
PKCδ tyrosine phosphorylation and proteolytic activation of the kinase.

**TSKI Does Not Promote PKCδ Degradation**—To examine whether TSKI blocks H₂O₂-induced proteolytic cleavage of PKCδ by preventing tyrosine phosphorylation of PKCδ or by promoting the degradation of the cleaved PKCδ fragment, we transiently expressed the 41-kDa PKCδ catalytic fragment with a V5 tag in N27 cells and then exposed them to 5 µM TSKI for 4 h. Western blot analysis with an antibody directed against V5 revealed that the 41-kDa PKCδ catalytic fragment was not degraded in TSKI-treated cells (Fig. 6). Densitometric analysis revealed no significant differences in the 41-kDa PKCδ catalytic fragment expression between control and TSKI-treated N27 cells. This result suggests that the PKCδ catalytic fragment does not undergo degradation by TSKI treatment, but rather TSKI blocks PKCδ tyrosine phosphorylation and subsequent proteolytic cleavage.

**Tyrosine Phosphorylation of PKCδ at Position 311 Is Essential for Oxidative Stress-mediated Cellular Apoptosis in N27 Dopaminergic Cells**—We further investigated whether Tyr-311, which is in close proximity to the PKCδ cleavage site, regulates the pro-apoptotic function of PKCδ. As shown in Fig. 7A, N27 cells treated with H₂O₂ (100 µM) showed a significant increase in PKCδTyr-311 phosphorylation. Src tyrosine kinase inhibitors genistein and TSKI significantly attenuated H₂O₂-induced PKCδTyr-311 phosphorylation (Fig. 7A). We also examined whether inhibition of PKCδTyr-311 phosphorylation by genistein and TSKI significantly attenuated H₂O₂-induced caspase-3 activity (Fig. 7B) and DNA fragmentation (Fig. 7C) in N27 cells. Together, these data clearly indicate that Tyr-311 phosphorylation of PKCδ regulates oxidative stress-induced apoptosis in dopaminergic neuronal cells.

**Tyrosine Phosphorylation of PKCδ Plays a Role in MPP⁺-induced PKCδ Cleavage and DNA Fragmentation**—We had
shown previously (1) that proteolytic activation of PKCδ contributes to the Parkinsonian toxin MPP⁺-induced apoptotic cell death in dopaminergic cells. To confirm the results obtained with the generic oxidant H₂O₂, we tested the effects of the p60Src peptide inhibitor TSKI on PKCδ proteolytic cleavage and apoptotic cell death in N27 dopaminergic cells following a 24-h treatment with 300 μM MPP⁺. TSKI (5 μM) co-treatment inhibited the MPP⁺-induced PKCδ proteolytic cleavage (Fig. 8A) and DNA fragmentation (Fig. 8B). TSKI almost completely inhibited both PKCδ cleavage and DNA fragmentation. These results indicate that PKCδ tyrosine phosphorylation also regulates dopaminergic cell death induced by the Parkinsonian toxin MPP⁺.

Neuroprotective Effect of Src Kinase Inhibitors Genistein and TSKI against H₂O₂-induced Dopaminergic Cell Death—We further determined whether inhibition of tyrosine phosphorylation by genistein and TSKI had any effect on the oxidative stress-induced cellular toxicity in dopaminergic neuronal cells. Genistein (25 μM) and TSKI (5 μM) were co-treated with H₂O₂ (100 μM) for 4 h, and then cytotoxicity was measured by the Sytox® green cytotoxicity assay. Both inhibitors significantly inhibited the cell death induced by H₂O₂ in N27 cells. Fig. 9A is representative of the fluorescent imaging of H₂O₂-treated N27 cells (top panel) compared with those treated with the Src tyrosine kinase inhibitors genistein (middle panel) or TSKI (bottom panel). Quantification of Sytox® green fluorescence revealed that H₂O₂ (100 μM) induced an increase in cell death of 438% over the untreated controls, whereas the cells co-treated with tyrosine kinase inhibitors were almost completely protected against cell death (Fig. 9B). We also tested the effect of c-Abi protein-tyrosine kinase inhibition on H₂O₂-induced dopaminergic cell death. For this purpose we used a specific c-Abi protein kinase inhibitor STI571 (Gleevec-Novartis). Co-treatment with STI571 at various concentrations (10–300 nM) did not afford any neuroprotection in N27 cells exposed to H₂O₂ (100 μM) over a period of 4 h, suggesting that c-Abi does not mediate PKCδ phosphorylation in dopaminergic neuronal cells (supplemental Fig. 1). Taken together, these data indicate that inhibition of PKCδ tyrosine phosphorylation by a select group of pharmacological inhibitors can confer neuroprotection in dopaminergic neuronal cells exposed to oxidative stress.

Tyrosine Kinase Inhibitors Genistein and TSKI Do Not Attenuate H₂O₂-induced Oxidative Stress in Dopaminergic Neurons—Recent studies attributed the protective effect of the soy isoflavone-derived tyrosine kinase inhibitor genistein to the attenuation of oxidative stress, especially in human cortical neuronal cells (43). Therefore, we next determined whether the tyrosine kinase inhibitors used in our studies attenuated cell death via tyrosine kinase inhibition or from their antioxidant properties. Treatment with genistein (25 μM) and TSKI (5 μM) did not alter the H₂O₂ (100 μM)-induced oxidative stress or the basal level of ROS generation (supplemental Fig. 2). However, a higher concentration of genistein (50 μM) significantly attenuated H₂O₂-induced ROS generation (data not shown). This clearly indicates that the neuroprotective effects of the tyrosine kinase inhibitors at the doses selected result from inhibition of tyrosine kinase activity rather than antioxidant effects.

Overexpression of PKCδY311F Mutant Attenuates Proteolytic Cleavage of PKCδ—We used the PKCδY311F mutant to further confirm the role of PKCδ-Tyr-311 phosphorylation in the proteolytic activation and proapoptotic function of the kinase. Tyrosine has been mutated to phenylalanine at position 311 in PKCδY311F, which confers insensitivity to phosphorylation at that amino acid residue. As shown in Fig. 10A, exposure to 100 μM H₂O₂ induced proteolytic cleavage in both normal N27 cells and pcDNA3.1 vector expressing N27 cells, whereas overexpression of PKCδY311F significantly attenuated 100 μM H₂O₂-induced PKCδ proteolytic cleavage, indicating that Tyr-311 is required for the proteolytic cleavage. Also, no cleavage was observed in untreated pcDNA3.1 and PKCδY311F-expressing N27 cells (Fig. 10A). Densitometric analysis of PKCδ expression in vector- and PKCδY311F-transfected cells showed a slight (<7%) but not statistically significant decrease in the PKCδ expression level as compared with the untransfected N27 cells (data not shown). Additionally, we examined whether PKCδY311F-transfected cells are resistant to oxidative stress-induced apoptosis. As shown in Fig. 10, B and C, PKCδY311F-expressing cells showed significantly reduced H₂O₂ (100 μM)-induced caspase-3 activity and DNA fragmentation compared with vector cells. Together, the data clearly indicate that PKCδ tyrosine phosphorylation at Tyr-311 can effectively regulate proteolytic activation and proapoptotic function of the oxidative stress-sensitive kinase in dopaminergic cells.

**DISCUSSION**

The present study reveals that oxidative stress has a profound effect on PKCδ, an important member of a novel PKC isoform family, in dopaminergic neuronal cells. The key findings of this study are as follows: (i) H₂O₂ induces caspase-3-dependent proteolytic activation of PKCδ in dopaminergic neuronal cells; (ii) phosphorylation of the Tyr-311 residue in PKCδ is essential for proteolytic cleavage and activation of the kinase; and (iii) Src tyrosine kinase inhibitors prevent oxidative stress-induced PKCδ proteolytic activation and apoptotic cell death in dopaminergic neuronal cells. These findings are highly significant because biochemical mechanisms in the neurodegenerative process of PD are not clearly understood despite the numerous implications of oxidative damage in the disease pathogenesis (13, 14). The results also suggest that PKCδ may be an attractive target for development of neuroprotective strategies against oxidative damage in PD. H₂O₂ can induce its toxic response by activating the apoptotic cascade in both neuronal and non-neuronal cells (41, 44, 45). H₂O₂-induced cell death in N27 cells, along with the activation of caspase-3 observed in the present study, were consistent with other studies employing various cell types, including PC12 cells (dopaminergic cells), HL-60 cells (human leukemia cells), fibroblasts, SK-N-BE neuroblastoma cells, and cultured hepatocytes (46–50). The upstream events of caspase-3 activation in H₂O₂-induced apoptotic cell death predominantly involve a mitochondrial signaling cascade via cytochrome c re-
lease and caspase-9 activation (51). Activated caspase-3 recognizes a specific sequence, DXXD, on protein substrates to induce proteolytic cleavage of these substrates (52). Some of the known substrates of activated caspase-3 are poly(ADP-ribose) polymerase, DNA-PK, topoisomerases, and lamin B1 (53). These proteins can be either activated (54) or inactivated (55) upon proteolysis by caspase-3, leading to apoptosis by either the induction of DNA damage or the impairment of DNA repair.

PKCδ is a member of the novel PKC family that can be activated in response to numerous cellular stimuli by various mechanisms. Some of the activation pathways include membrane translocation (25, 56), tyrosine phosphorylation (19, 57), and proteolytic cleavage by an activated enzyme (3, 39, 58). Recent studies have shown that H2O2 can activate PKCδ during apoptosis by either membrane translocation (59) or tyrosine phosphorylation (29, 60, 61) but not through proteolytic cleavage-mediated activation. H2O2 treatment has been shown to induce PKCδ translocation to the plasma membrane in CaCo2 cells (59) and to the mitochondrial membrane in NIH3T3 cells (62). However, H2O2 can also induce a remarkable increase in PKCδ kinase activity and tyrosine phosphorylation in cardiomyocytes without any evidence of membrane translocation (28, 60). Although oxidative stress-induced PKCδ activation has been well documented, none of these studies have shown proteolytic cleavage of the kinase as a possible mode of activity, especially in H2O2-induced apoptosis. In dopaminergic neuronal cells, we report here for the first time that H2O2 treatment induces the proteolytic cleavage of PKCδ protein into its catalytically active fragments, which leads to a persistent increase in its kinase activity and apoptosis (Fig. 3 and Fig. 5, A and B). Furthermore, the H2O2-induced PKCδ cleavage was completely abolished by the caspase-3 inhibitor Z-DEVD-FMK (Fig. 3B), indicating that the activation of this kinase was dependent on caspase-3 enzyme activity. PKCδ cleavage induced by caspase-3 activation has been well documented in UV radiation-treated keratinocytes (63), etoposide-treated salivary acinar cells (39, 58, 63), and in methyleclopentadienyl manganese tricarbonyl and dieldrin-treated PC12 cells (64). Recently, caspase-3 independent cleavage of PKCδ in a colon cancer cell line resulted in a kinase-inactive catalytic fragment of PKCδ (65). Furthermore, Konishi et al. (29) also showed that tyrosine phosphorylation of PKCδ increases its kinase activity and promotes H2O2-induced apoptosis in COS cells without any proteolytic cleavage. These findings may be explained by the reported identification of a caspase-3-insensitive PKCδ isoform, PKCδ-II, in certain cell types such as mouse thymocytes (66), which does not undergo proteolytic activation. We have observed a high expression of PKCδ-I isoform in N27 dopaminergic neuronal cells (2). Hence, it appears that different modes of PKCδ activation are highly dependent on type of stimulus, cell line, and the expression characteristics of PKCδ.

Tyrosine phosphorylation of PKCδ has been reported to either increase or decrease the kinase activity during different types of cellular stimulation. The majority of the literature suggests that oxidative stress induces an increase in PKCδ kinase activity. Some of the nonreceptor tyrosine kinases that have been known to phosphorylate PKCδ during H2O2 treatment are Src (67), Lck (68), and Syk (69). The tyrosine residues on PKCδ that can be phosphorylated during oxidative stress...
include Tyr-52 (20) and Tyr-187 (70) on the N terminus of the protein, Tyr-512 and Tyr-523 (26, 71) on the C terminus, and Tyr-311 at the intermediate hinge region (22). Konishi et al. (29) have demonstrated that H2O2 treatment induces the phosphorylation of PKCδ at various tyrosine residues including Ty-311, Ty-332, and Tyr-512 in COS cells, but phosphorylation at Tyr-311 is critical for initiation of PKCδ catalytic activity. PKCδ tyrosine phosphorylation has also been shown to regulate prooxidant etoposide-induced apoptotic cell death in C-6 glial cells (72). Our results demonstrate that phosphorylation of PKCδ by H2O2 treatment induces the phosphorylation of PKCδ at various tyrosine residues including Ty-311, Ty-332, and Tyr-512 in COS cells, but phosphorylation at Tyr-311 is critical for initiation of PKCδ catalytic activity. PKCδ tyrosine phosphorylation has also been shown to regulate prooxidant etoposide-induced apoptotic cell death in C-6 glial cells (72). Our results demonstrate that phosphorylation of PKCδ at Tyr-311 is essential for its caspase-3-dependent proteolytic activation. Because Tyr-311 is in close proximity to the caspase-3 cleavage site, DIPD, in PKCδ, it is likely that phosphorylation of this residue may cause conformational change in the kinase structure to expose its cleavage site to caspase-3. The complete inhibition of H2O2-induced PKCδ cleavage, kinase activity, and tyrosine phosphorylation by the peptide inhibitor TSKI in the present study suggests that p60Src may be an upstream kinase responsible for the Tyr-311 phosphorylation in dopaminergic cells. In this study, we also show that TSKI treatment does not induce degradation of the cleaved PKCδ catalytic fragment as determined by the exogenous expression of the PKCδ catalytic fragment. Recently, Src kinase has been shown to phosphorylate PKCδ on the residue Tyr-311 during H2O2 treatment in primary keratinocytic cells (19, 25), but these studies did not characterize the relationship between tyrosine phosphorylation and the proteolytic cleavage of PKCδ. The important functional consequence of inhibition of PKCδ Tyr-311 phosphorylation by TSKI and the PKCδF311F mutant is the attenuation of H2O2 and MPP+-induced DNA fragmentation in dopaminergic neuronal cells. Together this study clearly demonstrates that PKCδ tyrosine phosphorylation regulates dopaminergic cell death induced by the generic oxidant H2O2 as well as by the Parkinson toxin MPP+. Therefore, this study demonstrates that the PKCδ Tyr-311 phosphorylation site might be a potential target for development of neuroprotective agents against oxidative damage in Parkinson disease. Tyrosine kinase inhibitors derived from soy...
isoflavones like genistein have been shown to be protective in primary cortical neuronal cultures against oxidative stress by scavenging reactive oxygen species (43). It should be noted that the aforementioned study used a higher dose of genistein at 50–100 μM to demonstrate its antioxidant effect. Furthermore, another study demonstrated that genistein did not attenuate oxidative stress at lower doses (20–40 μM) but exhibited antioxidant properties only at higher concentrations (50–100 μM).

In our study we did not observe any attenuation of H$_2$O$_2$-induced oxidative stress by either genistein or TSFI (Fig. 8) at the doses used in the tyrosine kinase inhibitors experiments. Thus, the observed neuroprotective effect of genistein and TSFI in our studies was, in all probability, due to the inhibition of tyrosine kinases and not due to antioxidant action.

Oxidative stress has been described as integral to the pathogenesis of dopaminergic degeneration in PD, but the cellular mechanisms of the selective neurodegenerative processes still remain to be established (14, 33, 74). Our results demonstrate, for the first time, that tyrosine phosphorylation of PKC can regulate the proteolytic activation of the oxidative stress-sensitive kinase PKC in a cell culture model of PD and that modulation of tyrosine phosphorylation can offer neuroprotection against oxidative damage in dopaminergic neuronal cells.

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