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Phosphorylation of p38 MAPK Induced by Oxidative Stress Is Linked to Activation of Both Caspase-8- and -9-mediated Apoptotic Pathways in Dopaminergic Neurons*

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We evaluated the contribution of p38 mitogen-activated protein kinase and the events upstream/downstream of p38 leading to dopaminergic neuronal death. We utilized MN9D cells and primary cultures of mesencephalic neurons treated with 6-hydroxydopamine. Phosphorylation of p38 preceded apoptosis and was sustained in 6-hydroxydopamine-treated MN9D cells. Co-treatment with PD169316 (an inhibitor of p38) or expression of a dominant negative p38 was neuroprotective in death induced by 6-hydroxydopamine. The superoxide dismutase mimetic and the nitric oxide chelator blocked 6-hydroxydopamine-induced phosphorylation of p38, suggesting a role for superoxide anion and nitric oxide in eliciting a neurotoxic signal by activating p38. Following 6-hydroxydopamine treatment, inhibition of p38 prevented both caspase-8- and -9-mediated apoptotic pathways as well as generation of truncated Bid. Consequently, 6-hydroxydopamine-induced cell death was rescued by blockading activation of caspase-8 and -9. In primary cultures of mesencephalic neurons, the phosphorylation of p38 similarly appeared in tyrosine hydroxylase-positive, dopaminergic neurons after 6-hydroxydopamine treatment. This neurotoxin-induced phosphorylation of p38 was inhibited in the presence of superoxide dismutase mimetic or nitric oxide chelator. Co-treatment with PD169316 deterred 6-hydroxydopamine-induced loss of dopaminergic neurons and activation of caspase-3 in these neurons. Furthermore, inhibition of caspase-8 and -9 significantly rescued 6-hydroxydopamine-induced loss of dopaminergic neurons. Taken together, our data suggest that superoxide anion and nitric oxide induced by 6-hydroxydopamine initiate the p38 signal pathway leading to activation of both mitochondrial and extramitochondrial apoptotic pathways in our culture models of Parkinson's disease.

Parkinson's disease (PD)[1] is a common neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra. Although its precise etiology is unknown, such factors as oxidative stress, impairment of mitochondrial respiration, and abnormal protein aggregation may play roles in its pathogenesis (1, 2). Although the role of apoptosis in the process of dopaminergic neuronal death has been highlighted in studies using postmortem brains and experimental models of PD, other evidence implicates both apoptosis and non-apoptotic death in PD (3). Regardless, it is not clearly understood which signals may be involved in determining the mode of dopaminergic neuronal death.

Among the upstream signals leading to neuronal degeneration, one may include the stress-activated protein kinases. These are c-Jun N-terminal kinases (JNK) and p38 kinases, which are activated in response to various stimuli and are potent effectors of neuronal apoptosis (4). Most interesting, the phosphorylated forms of JNK and p38 are expressed in postmortem brains of PD (5). Moreover, evidence from experimental models of PD has implicated JNK in the process of dopaminergic neuronal death. For example, activation of JNK has been demonstrated in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. Blockade of JNK by JNK-interacting protein-1 or by a synthetic inhibitor of the mixed lineage kinase family is neuroprotective (6–8). Early JNK activation is found in various culture models following 1-methyl-4-phenylpyridinium (MPP^+) treatment (9–11). Blockade of JNK in cultures has also been shown to be neuroprotective (12, 13). Although its role is less defined, p38 activation has been investigated following treatment with dopamine, MPTP, or MPP^+ (14–16). As typified in the MPTP mouse model and in MPP^+-treated culture models, protection of neuronal degeneration by minocycline is mediated by inhibiting drug-induced glial inducible NOS expression and nitric oxide

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[1] The abbreviations used are: PD, Parkinson's disease; JNK, c-Jun N-terminal kinases; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP^+, 1-methyl-4-phenylpyridinium; NO, nitric oxide; 6-OHDA, 6-hydroxydopamine; BAF, *t*-butoxycarbonyl-(Asp)-fluoromethyl ketone; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone; AMC, 7-amino-4-methylcoumarin; AFC, 7-amino-4-trifluoromethylcoumarin; *p*NA, *p*-nitroanaline; DAF-FM diacetate, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; Mn-TBAP, Mn(III)tetrakis(4-benzoic acid)porphyrin chloride; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase; ROS, reactive oxygen species; TH, tyrosine hydroxylase; phospho-p38, phosphorylation of p38; ANOVA, analysis of variance; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid.

(NO)-induced phosphorylation of p38 (15). However, a role for the p38 signal in dopaminergic neuronal degeneration is controversial (17–19). Furthermore, cellular events upstream and downstream of p38 activation leading to dopaminergic neuronal death are not yet understood.

Here, we attempted to evaluate the following: (i) to what extent p38 contributes to 6-hydroxydopamine (6-OHDA)-induced death; (ii) whether p38 is linked to activation of the mitochondrial and extramitochondrial apoptotic pathways; and (iii) the temporal sequence of cell death with regard to oxidative stress, and the activation of p38 and caspases using MN9D dopaminergic neuronal cells and primary cultures of mesencephalic neurons. Our data show that superoxide anion and NO induced by 6-OHDA activate p38 that, in turn, activates both the caspase-8 and -9-dependent apoptotic pathways in culture models of PD.

EXPERIMENTAL PROCEDURES

Materials—*t*-Butoxycarbonyl-(Asp)-fluoromethyl ketone (BAF), Z-LEHD-fmk, and Z-IETD-fmk were obtained from Enzyme Systems Products (Livermore, CA). Fluorogenic caspase substrates Ac-DEVD-AMC and Ac-LEHD-AFC were obtained from Calbiochem-Novabiochem. The colorimetric assay kit for caspase-8-like activity using IETD-*p*NA was from R&D Systems (Minneapolis, MN). The reactive oxygen species-selective indicators used included dihydroethidium (a superoxide anion indicator; Molecular Probes, Eugene, OR) and 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM diacetate; a nitric oxide indicator; Molecular Probes). Fetal bovine serum, minimum Eagle's medium, and laminin were from Invitrogen. Inhibitor of p38 (PD169316) was from Calbiochem-Novabiochem. Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (Mn-TBAP; a superoxide dismutase mimetic) was from Calbiochem-Novabiochem, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO; a nitric oxide scavenger) was from Molecular Probes. Culture dishes and plates were from Corning Glass. All other reagents including 6-OHDA and *N*-acetyl-L-cysteine were purchased from Sigma.

Cell Culture, Drug Treatment, and Survival/Death Assay—MN9D dopaminergic neuronal cells were established by a somatic fusion between embryonic mesencephalic neurons and N18TG cells (20). MN9D cells were transfected with a eukaryotic expression vector (pcDNA3) containing FLAG epitope-tagged dominant negative p38 (MN9D/p38DN, T180A, and Y182F; see Ref. 21) and selected in the presence of 500 μg/ml G-418 as described previously (22). Both MN9D and MN9D/p38DN cells plated on 25 μg/ml poly-D-lysine-coated culture dishes or plates were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in an atmosphere of 10% CO_2 for 2–3 days, and switched to N-2 serum-free defined medium containing 100 μM 6-OHDA in the presence or the absence of various experimental reagents. Following drug treatment, the rate of cell survival was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay as described previously (22). The rate of cell death was quantitatively assessed by the measurement of lactate dehydrogenase (LDH) released by damaged cells into the bathing medium (23).

Immunoblot Analysis—MN9D cells, MN9D/p38DN cells, or primary cultures of dopaminergic neurons were treated with 6-OHDA in the presence or the absence of various inhibitors. Following drug treatment, cells were washed with ice-cold phosphate-buffered saline, lysed in a buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM dithiothreitol, 0.1% w/v bromphenol, 2 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 5 mM sodium vanadate for 15 min, and triturated with a syringe bearing a 26-gauge needle on ice. An equal amount of proteins from each sample was separated on a 12.5% SDS-PAGE gel, blotted onto pre-wetted polyvinylidene difluoride nitrocellulose filters, and processed for immunoblot analysis. Primary antibodies used included rabbit polyclonal antibodies that recognize p38, phosphorylated form of p38, cleaved forms of caspase-3, -7, and -9 (all antibodies above were used at 1:1000; Cell Signaling, Beverly, MA), cleaved form of caspase-8 (1:1000, Pharmingen, San Diego, CA), and caspase-8-cleaved form of Bid (1:3000, a generous gift from Dr. S. J. Krajewski at the Burnham Institute). For detecting the mitochondrial release of cytochrome *c* into the cytosol, cells were treated with 100 μM 6-OHDA in the presence or the absence of the indicated inhibitors, lysed, and subsequently fractionated as described previously with minor modifications (24). Primary antibody used was a mouse monoclonal anti-cytochrome *c* (1:3000; Pharmingen). A mouse monoclonal anti-FLAG conjugated with peroxidase (1:1000, Sigma) was used to check the expression level of FLAG epitope-tagged dominant negative form of p38. For loading controls, a mouse monoclonal anti-cytochrome *c* oxidase subunit IV (1:1000; Molecular Probes) and a rabbit polyclonal anti-IκB (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) were utilized. Specific bands were visualized by enhanced chemiluminescence (Amersham Biosciences).

Measurement of Reactive Oxygen Species (ROS)—MN9D cells and MN9D/p38DN cells were incubated with 100 μM 6-hydroxydopamine alone or in combination with 20 μM PD169316. Cells were then incubated with 1 μM dihydroethidium for 30 min at 37 °C and washed twice with a buffer containing 144 mM NaCl, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM KCl, 10 mM D-glucose as described previously (25). Cells were also incubated with 5 μM DAF-FM diacetate for 40 min at 37 °C, washed twice with phosphate-buffered saline, and further incubated in 37 °C for 15 min. Cells were quantified at Ex530/Em590 nm (excitation/emission wavelength for dihydroethidium) or at Ex485/Em520 nm (for DAF-FM diacetate) using a FL 600 plate reader (Bio-Tek, Winooski, VT).

Caspase Substrate Assays—Caspase activity was measured by either a fluorogenic or colorimetric substrate assay. Briefly, following treatment with 100 μM 6-OHDA in the presence or the absence of 20 μM PD169316, MN9D cells were lysed in a buffer containing 50 mM Tris, pH 7.0, 2 mM EDTA, 1.0% Triton X-100. Cellular lysates (10 μg) were incubated with 25 μM Ac-DEVD-AMC for caspase-3-like activity, 25 μM Ac-LEHD-AFC for caspase-9-like activity, or 100 μM IETD-*p*NA for caspase-8-like activity for 1 h at 37 °C. The reactions were carried out in a buffer containing 100 mM HEPES, pH 7.4, 10% sucrose, 5 mM dithiothreitol, 0.1% CHAPS for caspase-3- or -9-like activity assay, or the reaction buffer provided by the manufacturer for caspase-8-like activity assay. Activity was measured at Ex360/Em460 nm for Ac-DEVD-AMC and Ex360/Em520 nm for Ac-LEHD-AFC or with a wavelength at 405 nm for IETD-*p*NA using a FL 600 plate reader.

Primary Cultures of Mesencephalic Neurons and Counting of Tyrosine Hydroxylase (TH)-positive Neurons—For primary cultures of mesencephalic neurons, ventral mesencephalic areas were removed from the brains of embryonic day 14 fetal Sprague-Dawley rats (Daehan Biolink, Daejeon, Korea). Dissociated cells were then plated and maintained as described previously in our laboratory (26). At 5 or 6 days *in vitro*, cultures were washed with minimum Eagle's medium, switched to N-2 serum-free defined medium, and treated with 20 μM 6-OHDA alone or in combination with various experimental reagents. To determine the number of surviving dopaminergic neurons following drug treatment, primary cultures were subjected to immunocytochemical localization of TH as described previously (26). Manual counts by an individual blind to the treatment conditions were made of all the TH-positive neurons having neurites longer than twice the length of the soma.

Immunocytochemistry—For immunocytochemical localization of the phosphorylated form of p38, MN9D cells were fixed with 4% paraformaldehyde for 20 min, permeabilized in phosphate-buffered saline containing 1% bovine serum albumin, 0.1% Triton X-100 for 30 min at room temperature, and incubated with a rabbit polyclonal anti-phosphorylated form of p38 antibody (1:1000) overnight at 4 °C. After three washes with 20 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 0.1% Tween 20 (TBST), cells were incubated with Alexa Fluor® 568 goat anti-rabbit IgG (1:200; Molecular Probes) for 1 h at room temperature. For a double immunocytochemical localization of TH and phosphorylated form of p38 or cleaved caspase-3, primary cultures of mesencephalic neurons treated with 20 μM 6-OHDA alone or in combination with 5.0 μM PD169316 were processed as described (26). The primary antibodies used were a mouse monoclonal anti-TH (1:7500; Pel-Freez, Rogers, AR), a rabbit polyclonal anti-phosphorylated form of p38 antibody (1:500), and a rabbit polyclonal antibody that recognizes cleaved caspase-3 (1:200). Following three washes with TBST, cultures were further incubated with Alexa fluor® 568 goat anti-rabbit IgG and Alexa Fluor® 488 goat anti-mouse IgG for 1 h at room temperature. Stained cells were visualized under an Axiovert 100 Microscope equipped with epifluorescence and a digital image analyzer (Carl Zeiss, Zena, Germany). Manual counts by an individual blind to the treatment conditions were made of all the phosphorylated forms of p38- or cleaved caspase-3-positive neurons among TH-positive neurons.

Statistics—Data were means ± S.E. from the indicated number of experiments. Significance of difference between 6-OHDA treated groups and groups co-treated with various reagents was determined by one-way ANOVA and post hoc Student's *t* test. Values of $p < 0.05$ were taken as being statistically significant.

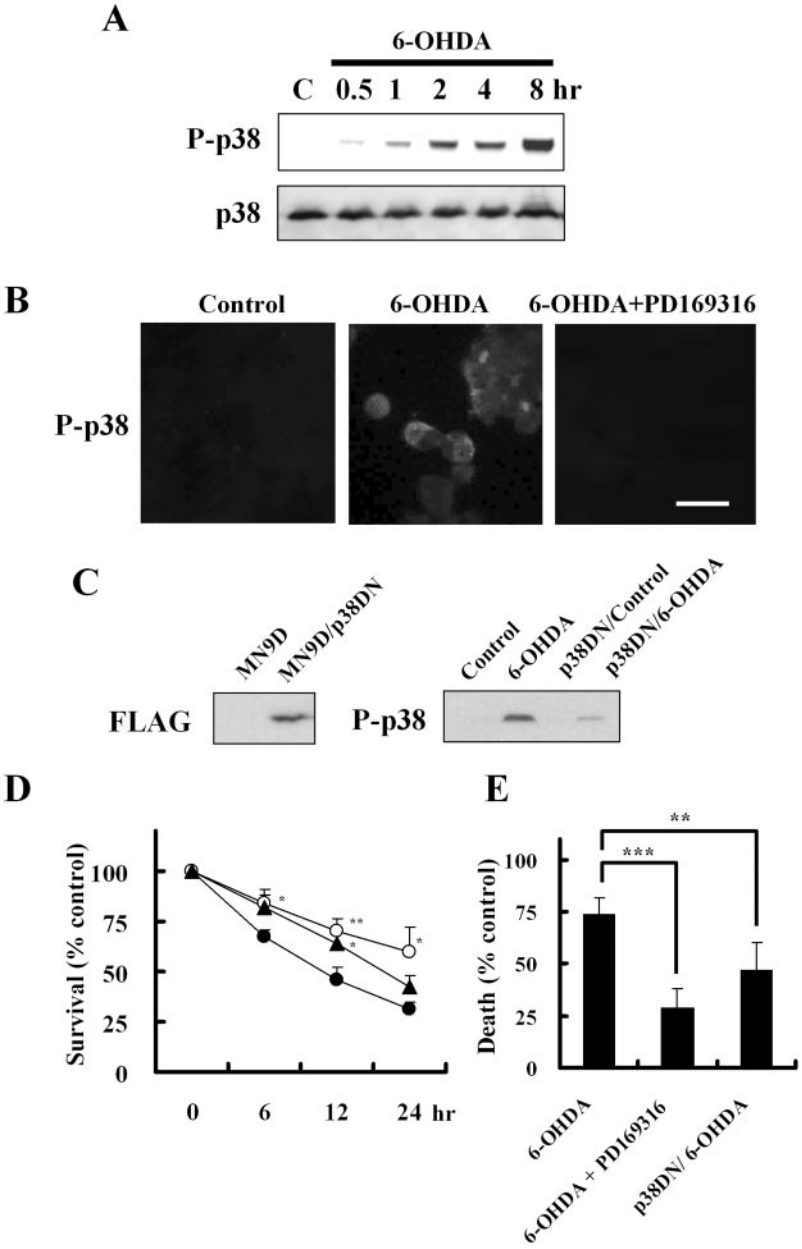

FIG. 1. **Phosphorylation of p38 is induced and inhibition of p38 is neuroprotective in MN9D dopaminergic neuronal cells following 6-OHDA treatment.** *A,* MN9D cells were treated with 100 μM 6-OHDA for the times indicated. Equal amounts of proteins from the whole cellular lysates (50 μg) were separated on 12.5% SDS-PAGE and transferred to pre-wetted polyvinylidene difluoride nitrocellulose filters. Blots were immunolabeled with a rabbit polyclonal anti-p38 or the anti-phosphorylated form of p38 (*P-p38*). Specific bands were detected by enhanced chemiluminescence. *B,* MN9D cells treated with 100 μM 6-OHDA for 3 h in the presence or the absence of 20 μM PD169316 were then subjected to immunocytochemical localization of phospho-p38. *Scale bar,* 50 μm. *C,* MN9D cells were transfected with a eukaryotic expression vector containing FLAG epitope-tagged dominant negative p38 (*MN9D/p38DN*) and characterized by immunoblot analysis using a peroxidase-conjugated mouse monoclonal anti-FLAG. Both MN9D cells and MN9D/p38DN cells were treated with 100 μM 6-OHDA for 4 h. Cellular lysates were processed for immunoblot analysis using anti-phospho-p38. *D* and *E,* MN9D cells and MN9D/p38DN cells were treated with 100 μM 6-OHDA in the presence or the absence of 20 μM PD169316 (a p38 inhibitor). The rate of cell survival/death was measured by (*D*) the MTT reduction assay in MN9D cells treated with 6-OHDA alone (*closed circles*) or in combination with PD169316 (*open circles*), and in MN9D/p38DN cells treated with 6-OHDA (*closed triangles*) for the times indicated or (*E*) LDH assay at 24 h after 6-OHDA treatment. Values from each treatment were expressed as a percentage of the untreated control for MTT or full kill for LDH assay. Each *bar* represents the mean ± S.E. from 3 to 4 independent experiments done in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ANOVA with *post hoc* Student's *t* test.

RESULTS

Inhibition of p38 Is Neuroprotective Following 6-OHDA Treatment—We demonstrated previously (25–28) that 6-OHDA induces changes typical of apoptosis. Typically, ~70% of MN9D cells were found to be dying following treatment with 100 μM 6-OHDA for 24 h. As such, this dose of neurotoxin was selected to determine whether and to what extent p38 contributed to neurotoxin-induced cell death. As shown in Fig. 1*A*, phosphorylation of p38 (phospho-p38) in MN9D cells was detectable as early as 30 min following 6-OHDA treatment and continued to increase in a time-dependent manner. Ten to twelve hours after 6-OHDA treatment, levels of phospho-p38 started to decrease. The levels of p38 remained largely the same (Fig. 1*A*). As shown in Fig. 1*B*, immunocytochemistry also indicated that MN9D cells were positive for phospho-p38 following 6-OHDA treatment. Among the concentration ranges of PD169316 (an inhibitor of p38; 1.0–80 μM), we found the maximum dose of p38 inhibitor that blocked 6-OHDA-induced generation of phospho-p38 without causing cytotoxicity, 20 μM PD169316. The appearance of 6-OHDA-induced phospho-p38 was significantly blocked in the presence of 20 μM PD169316 or in MN9D cells expressing a dominant negative p38 (MN9D/p38DN; Fig. 1, *B* and *C*). Co-treatment with 20 μM PD169316 or expression of a dominant negative p38 significantly blocked 6-OHDA-induced cell death as determined by MTT reduction assay and LDH assay (Fig. 1, *D* and *E*).

Activation of p38 Is Downstream of the Oxidative Burst and Upstream of Caspase Activation Following 6-OHDA Treatment—We and others (25–29) have previously raised the possibility that generation of ROS at an early phase and subsequent ROS-mediated signal pathways play an essential role in 6-OHDA-induced cell death in MN9D cells and primary cultures of cortical and mesencephalic neurons. We hypothesized therefore that ROS induced by 6-OHDA contributes to the appearance of phospho-p38 in MN9D cells. First, we measured levels of ROS by using cell-permeable dihydroethidium (a superoxide anion indicator; see Ref. 30) and DAF-FM diacetate (an NO indicator; see Ref. 31) following 6-OHDA treatment in the presence or the absence of 20 μM PD169316 or in MN9D/p38DN cells. As shown in Fig. 2*A*, generations of superoxide anion and NO were induced regardless of treatments. Quantitative studies demonstrated that co-treatment with 20 μM

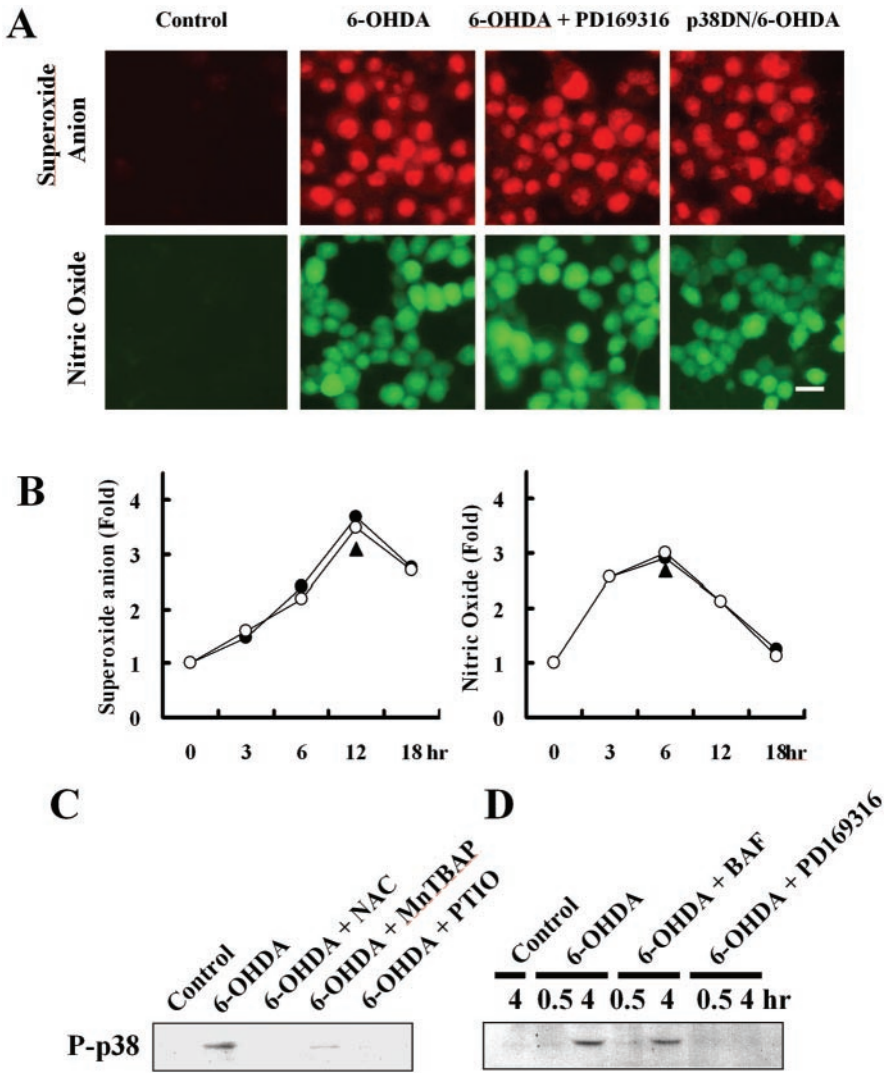

FIG. 2. **Phosphorylation of p38 is downstream of ROS generation and upstream of caspase activation during 6-OHDA-induced MN9D cell death.** *A*, both MN9D cells and MN9D/p38DN cells were treated with 100 μM 6-OHDA for 10 h in the presence or the absence of 20 μM PD169316. Cells were then incubated with 1 μM dihydroethidium (a superoxide anion indicator) or 5 μM DAF-FM diacetate (an NO indicator), washed, and photographed. *Scale bar*, 50 μm. *B*, the amounts of dihydroethidium-sensitive superoxide anion or DAF-FM-sensitive NO generated in MN9D cells treated with 100 μM 6-OHDA alone (*closed circles*) or in combination with 20 μM PD169316 (*open circles*) for the times indicated were quantified using a fluorescence plate reader. At 12 h, the levels of superoxide anion or NO were measured in MN9D/p38DN following 100 μM 6-OHDA treatment (*closed triangles*). Each *point* represents the mean from two independent experiments in triplicate. MN9D cells were treated with 100 μM 6-OHDA for 4 h in the presence or the absence of either 1 mM *N*-acetyl-L-cysteine (*NAC*), 25 μM Mn-TBAP (a superoxide dismutase mimetic), or 100 μM carboxy-PTIO (*PTIO*, an NO scavenger) (*C*), or for the time indicated in the presence or absence of either 100 μM BAF or 20 μM PD169316 (*D*). Cellular lysates (50 μg) were subjected to immunoblot analysis using a rabbit polyclonal anti-phospho-p38 (*P-p38*).

PD169316 or expression of a dominant negative p38 did not affect the kinetics of superoxide anion and NO generation following 6-OHDA treatment for up to 18 h (Fig. 2*B*). We then tested whether co-treatment with antioxidants inhibits the 6-OHDA-induced appearance of phospho-p38. As determined by immunoblot analysis (Fig. 2*C*), appearance of phospho-p38 following 6-OHDA treatment was largely inhibited in the presence of *N*-acetyl-L-cysteine (1.0 mM), a superoxide dismutase mimetic (Mn-TBAP; 25 μM) or by an NO scavenger (carboxy-PTIO; 100 μM), indicating that ROS comprises one of the essential triggers of the p38 signal. Since caspase activation is involved in 6-OHDA-induced MN9D cell death, we next attempted to establish the temporal cell death sequences in relation to p38 and caspases. As shown in Fig. 2*D*, co-treatment with a pan-caspase inhibitor, BAF (100–300 μM) did not inhibit the 6-OHDA-induced appearance of phospho-p38. Similarly, co-treatment with another pantothenate-caspase inhibitor (Z-VAD-fmk; 100–300 μM) was without effect (not shown).

Phospho-p38 Is Linked to Both Caspase-8 and -9-mediated Apoptotic Pathways Following 6-OHDA Treatment—It has been demonstrated that both caspase-8 and -9 are among the keys initiating caspase cascades that eventually lead to activation of an execution phase of caspases including -3, -6, and -7 (32). Once we established the appearance of phospho-p38 as upstream of caspase activation following 6-OHDA treatment, we then attempted to examine whether 6-OHDA-induced phospho-p38 may be linked to the activation of the caspase-8 and -9-mediated apoptotic pathways. First, caspase substrate assays were carried out in MN9D cells treated with 6-OHDA for various times using Ac-DEVD-AMC (for caspase-3-like activity; caspase-3, -6, -7, -8, and -10), Ac-LEHD-AFC (for caspase-9-like-activity), or IETD-*p*NA (caspase-8-like activity) in the presence or the absence of 20 μM PD169316. As shown in Fig. 3*A*, following 6-OHDA treatment, caspase-3-, -8-, and -9-like activities were elevated over the untreated control in a time-dependent manner up to 12 h. Co-treatment with 20 μM PD169316 significantly blocked 6-OHDA-induced activation of these caspases. To more specifically determine how p38 signal affects the activation profiles of caspases following 6-OHDA treatment, cellular lysates were subjected to immunoblot analysis using antibodies recognizing the cleaved forms of caspases. As shown in Fig. 3*B*, slight but detectable levels of the cleaved form of caspase-8 appeared as early as 6 h following 6-OHDA treatment and continued to increase thereafter. Although very slight levels of the cleaved forms of caspase-9 first appeared as early as 8 h following 6-OHDA treatment, a marked increase in the cleaved forms of caspase-9 occurred by 10–12 h. In addition, immunoblot analysis indicated that the appearance of cleaved forms of caspase-3 and -7 were first detected at 6 h following 6-OHDA treatment. Inhibition of p38 signal by 20 μM PD169316 or by expression of a dominant negative p38 largely diminished 6-OHDA-induced generation of cleaved forms of caspases with a slightly different efficacy (Fig. 3, *B* and *C*).

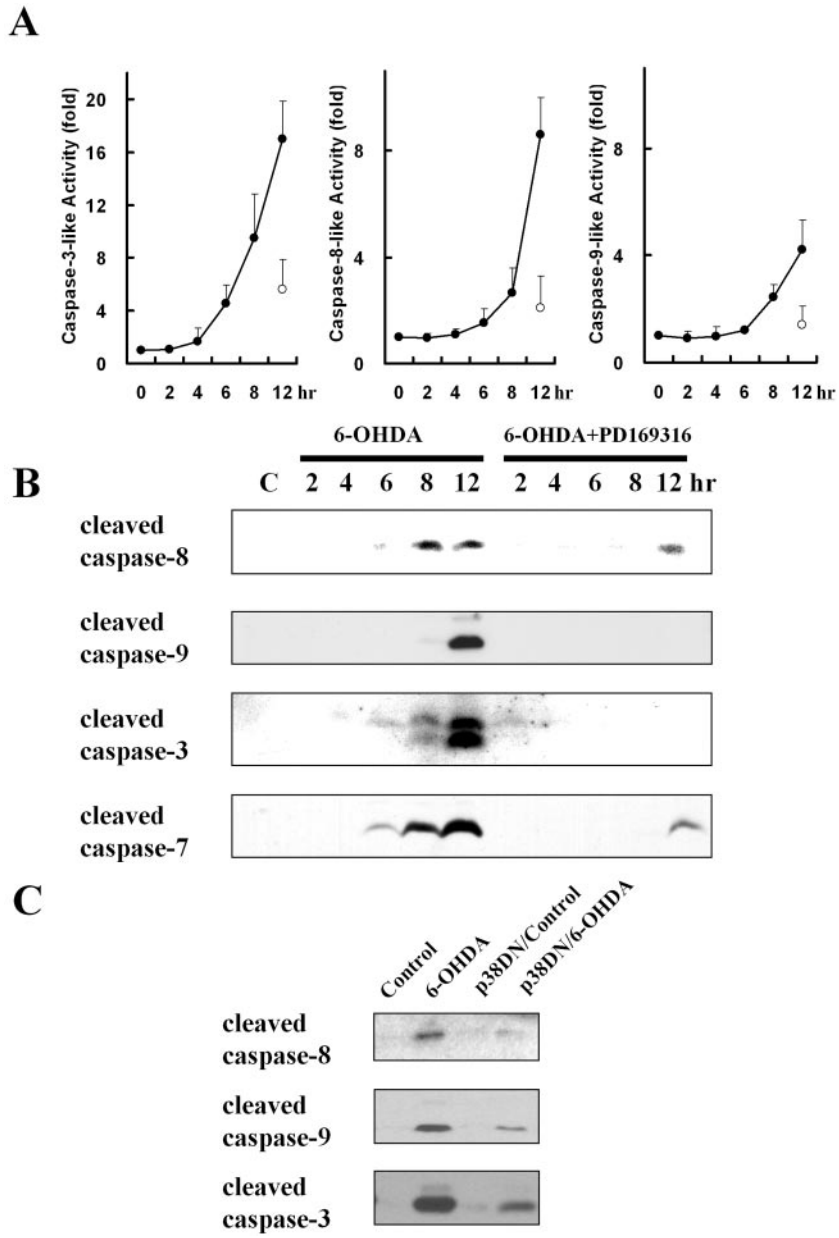

FIG. 3. **Activation of p38 signal is linked to both capsase-8- and -9-mediated apoptotic pathways in MN9D cells treated with 6-OHDA.** *A*, MN9D cells were treated with 100 μM 6-OHDA in the presence (*open circles*) or the absence 20 μM PD169316 (*closed circles*) for the times indicated. Cellular lysates (10 μg) were incubated with 25 μM Ac-DEVD-AMC (caspase-3-like activity), 100 μM IETD-*p*NA (caspase-8-like activity), or 25 μM Ac-LEHD-AFC (caspase-9-like activity) and processed for fluorogenic or colorimetric caspase substrate assays. Data represent the mean ± S.E. from three independent experiments done in triplicate. *B*, cellular lysates (50 μg for caspase-3 and -7; 100 μg for caspase-8 and -9) obtained from MN9D cells treated with 100 μM 6-OHDA alone or in combination with 20 μM PD169316 for the times indicated were subjected to immunoblot analysis with a rabbit polyclonal antibody that recognizes cleaved forms of caspase-8, -9, -3 or -7. *C*, 12 h after 100 μM 6-OHDA treatment, cellular lysates obtained from both MN9D cells and MN9D/p38DN cells were subjected to immunoblot analysis.

Mitochondrial Apoptotic Signal Can Be Activated by Caspase-8-cleaved tBid-dependent and -independent Routes Following 6-OHDA Treatment—In response to extracellular cues and internal insults, mitochondria release various apoptogenic molecules including cytochrome *c* into the cytosol and trigger the activation of the caspase-9-mediated, mitochondrial apoptotic pathways. In addition, caspase-8-mediated cleavage of Bid (tBid) promotes mitochondrial release of cytochrome *c* into the cytosol and eventually leads to the activation of caspase-9 (32). MN9D cells were treated with 100 μM 6-OHDA in the presence or the absence of 20 μM PD169316. The appearance of tBid was first detected by 6 h following 6-OHDA treatment and continued to increase thereafter (Fig. 4*A*). Co-treatment with PD169316 inhibited 6-OHDA-induced generation of tBid. Appearance of tBid was also inhibited in the presence of 100 μM Z-IETD-fmk (a caspase-8 inhibitor) but not by 100 μM Z-LEHD-fmk (a caspase-9 inhibitor; Fig. 4*B*); this indicates that p38-mediated activation of caspase-8 leads to generation of tBid following 6-OHDA treatment. As shown in Fig. 4, *C* and *D*, 6-OHDA-induced release of cytochrome *c* into the cytosol was largely inhibited by 20 μM PD169316 but not by 100 μM Z-IETD-fmk. Although it remains to be directly confirmed, this suggests that following 6-OHDA treatment, the mitochondrial release of cytochrome *c* into the cytosol may be induced by caspase-8-cleaved tBid-dependent and -independent routes. Co-treatment of MN9D cells with 100 μM Z-IETD-fmk or with 100 μM Z-LEHD-fmk protected cells equally from death as determined by the MTT reduction assay (46.1 and 45.8%, respectively, *versus* 27.8% survival in 6-OHDA-treated group; Fig. 5). Although it was not statistically significant, the extent of the protection was greater when 100 μM Z-IETD-fmk and Z-LEHD-fmk were combined (55.0%) and quite comparable with that in the PD169316-treated group (59.9%). This indicates that the majority of the p38 signal induced by 6-OHDA is linked to caspase-8 and -9-mediated apoptotic pathways in MN9D cells.

p38 Is Also Activated in Primary Cultures of Mesencephalic Neurons Following 6-OHDA Treatment—Once we established the temporal sequence of cell death in MN9D cells with regard to ROS, and activation of p38 and caspases, we tried to confirm these findings using primary cultures of mesencephalic neurons. As we demonstrated previously in our laboratory (26),

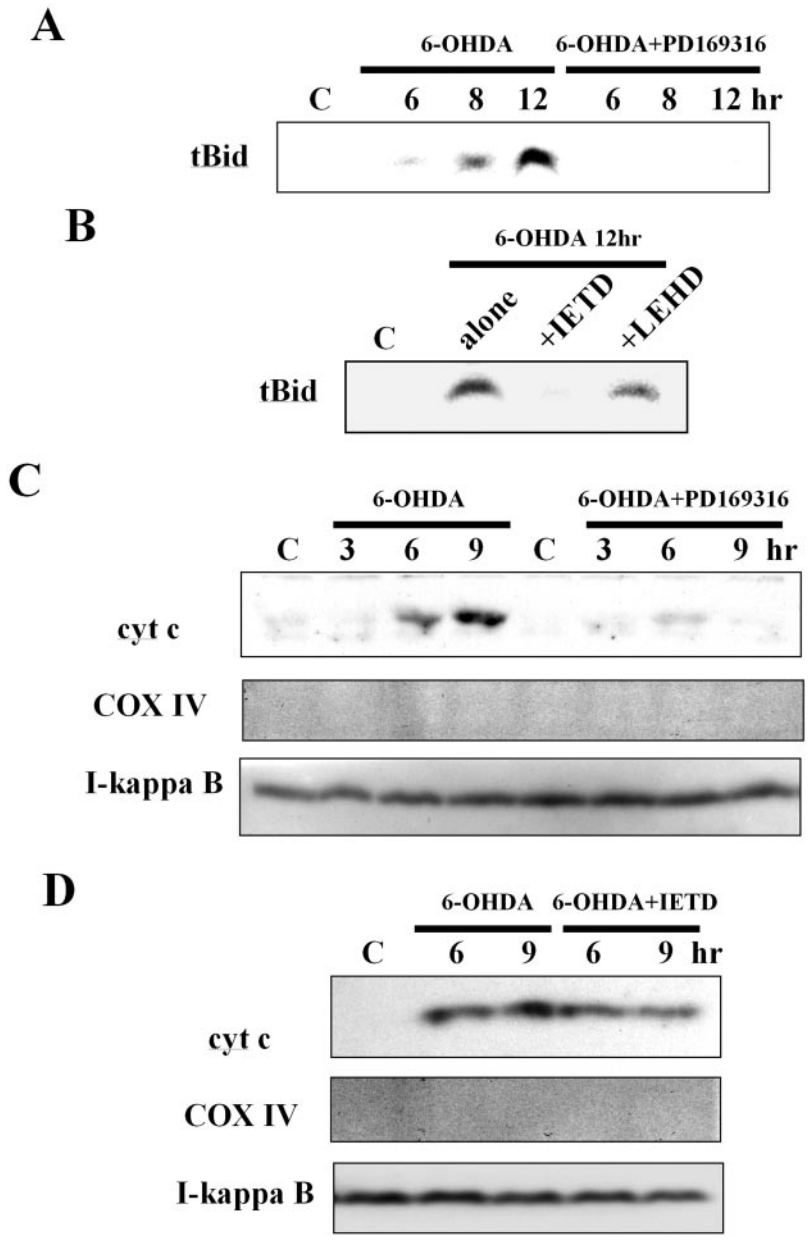

FIG. 4. **Mitochondrial release of cytochrome *c* is mediated both by caspase-8-cleaved tBid-dependent and -independent routes.** Inhibition of p38 blocks these routes in 6-OHDA-treated MN9D cells. MN9D cells were treated with 100 μM 6-OHDA for the times indicated in the presence or the absence of 20 μM PD169316 (*A* and *C*) or either 100 μM Z-IETD-fmk (*IETD*; a caspase-8 inhibitor) or 100 μM Z-LEHD-fmk (*LEHD*; a caspase-9 inhibitor) (*B* and *D*). *A* and *B*, cellular lysates (50 μg) were subjected to immunoblot analysis with an antibody that recognizes the truncated form of Bid (*tBid*). *C* and *D*, cytosolic proteins (75 μg) obtained as described previously (23) were subjected to immunoblot analysis with a mouse monoclonal anti-cytochrome *c* (*cyt c*). Duplicate blots were immunolabeled with a mouse monoclonal anti-cytochrome *c* oxidase subunit IV (*COX IV*) to confirm the absence of mitochondrial contamination. Similarly, blots were also immunolabeled with a rabbit polyclonal anti-IκB to confirm that the loading of the cytosolic proteins in each lane was relatively equal.

treatment with 20 μM 6-OHDA for 24 h resulted in a loss of ~70% of TH-positive neurons. Therefore, this concentration of neurotoxin was chosen for further study. As shown in Fig. 6*A*, appearance of phospho-p38 was increased in a time-dependent manner. In addition, a double-immunofluorescent localization of TH and phospho-p38 was performed in primary cultures of mesencephalic neurons treated with 20 μM 6-OHDA. Fig. 6*B* shows representative fluorescent photomicrographs of untreated control or 6-OHDA-treated cultures. Following 6-OHDA treatment, dying TH-positive neurons having retracted neurites were positive for phospho-p38. The phospho-p38-positive neurons among TH-positive neurons were apparent by 6 h following 6-OHDA treatment (Fig. 6*C*). At 18 h, ~15.0% of TH-positive neurons within the cultures were positive for phospho-p38. Less than 2–3% of TH-positive cells were positive for phospho-p38 in untreated control. Virtually none of glial cells were positive for phospho-p38.

Inhibition of ROS-mediated p38 Signal Is Neuroprotective in Primary Cultures of Mesencephalic Neurons Following 6-OHDA Treatment—In our previous study (26), we demonstrated that co-treatment with 0.5 mM *N*-acetyl-L-cysteine alleviates the loss of TH-positive neurons induced by 6-OHDA. Therefore, we then examined whether an antioxidant prevents the 6-OHDA-induced appearance of phospho-p38 and inhibition of p38 blocks 6-OHDA-induced loss of TH-positive neurons. As shown in Fig. 7*A*, the appearance of phospho-p38-positive neurons induced by 20 μM 6-OHDA treatment was largely abolished in the presence of such antioxidants as 0.5 mM *N*-acetyl-L-cysteine, 25 μM Mn-TBAP, and 100 μM carboxy-PTIO (3.8, 5.6, and 4.8%, respectively, *versus* 12.9% for 6-OHDA alone). Furthermore, 6-OHDA-induced loss of TH-positive neurons was significantly blocked in the presence of PD169316 (Fig. 7*B*). Among the concentration ranges of PD169316 (1.0–20 μM), we found 5 μM PD169316 conferred the maximum protection without causing cytotoxicity. At 5 μM, PD169316 significantly inhibited the 6-OHDA-induced loss of TH-positive neurons (23.9 *versus* 60.6%). Although phospho-p38 was present at around 13–15% in 6-OHDA-treated, TH-positive neurons, PD169316 significantly rescued TH-positive neurons. This may indicate that only a small subset of TH-positive neurons is transiently undergoing p38 activation at any given time.

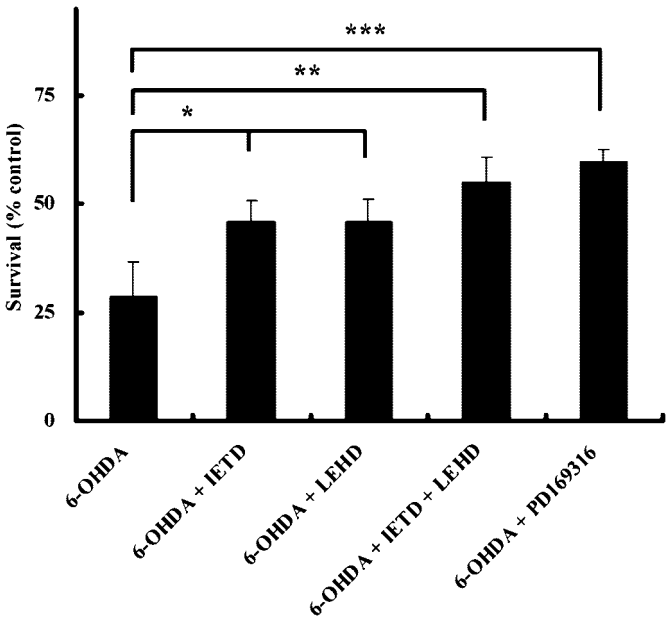

FIG. 5. **Co-treatment with caspase-8 inhibitor and/or caspase-9 inhibitor is neuroprotective in 6-OHDA-induced MN9D cell death.** MN9D cells were treated with 100 μM 6-OHDA for 24 h in the presence or the absence of either 100 μM Z-IETD-fmk (*IETD*), 100 μM Z-LEHD-fmk (*LEHD*), the combination of 100 μM IETD and LEHD, or 20 μM PD169316. Cell viability was measured by the MTT reduction assay. Values from each treatment were expressed as a percentage of the untreated control (100%). Each *bar* represents the mean ± S.E. from three independent experiments done in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ANOVA with post hoc Student's *t* test.

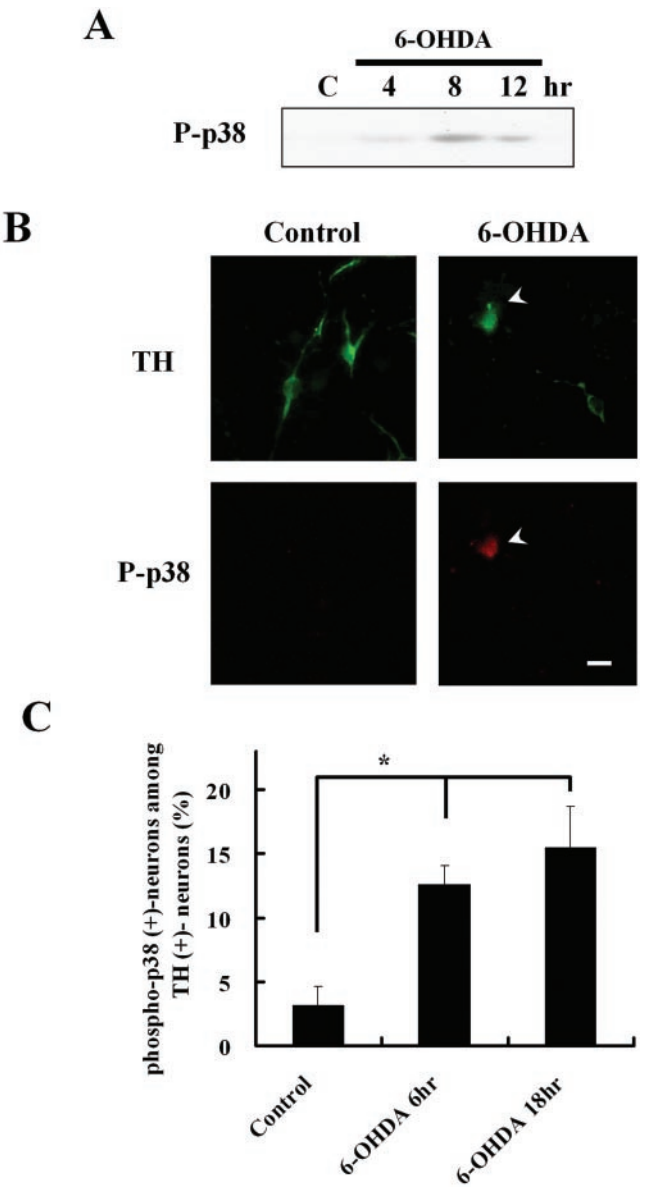

FIG. 6. **Dying TH-positive neurons exhibit phospho-p38 following 6-OHDA treatment.** Primary cultures derived from the mesencephalon of E14 rat embryos were treated with 20 μM 6-OHDA for the times indicated. *A,* cellular lysates were subjected to immunoblot analysis with a rabbit polyclonal anti-phosphorylated form of p38 (*P-p38*). *B,* cultures were then double immunolabeled with a mouse monoclonal anti-TH and a rabbit polyclonal anti-phosphorylated form of p38 following 6-OHDA treatment for 12 h. This was then followed by incubation with Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 568 goat anti-rabbit IgG. Cultures were examined with an Axiovert inverted microscope equipped with epifluorescence and digital image analyzer. Between two TH-positive neurons, *arrowhead* indicates dying TH-positive neuron that is also positive for phospho-p38. *Scale bar*, 20 μm. *C,* neurons that were positive for phospho-p38 among TH-positive neurons were counted. As reported previously (25), ~300–400 TH-positive neurons in 8-mm aclar film were counted in untreated controls. Data represent the mean ± S.E. from 3 to 4 independent experiments. *, $p < 0.005$; ANOVA with post hoc Student's *t* test.

Inhibition of p38 Blocks Caspase Activation in Primary Cultures of Mesencephalic Neurons Following 6-OHDA Treatment—To determine whether inhibition of the p38 signal prevents 6-OHDA-induced caspase activation, primary cultures of mesencephalic neurons were treated with 20 μM 6-OHDA in the presence or the absence of 5 μM PD169316. Fig. 8*A* shows representative fluorescent photomicrographs of the untreated control and 6-OHDA-treated cultures for 24 h followed by a double immunofluorescent localization of TH and cleaved caspase-3. Both 6-OHDA-induced retraction of neurites and the appearance of cleaved caspase-3 were prevented in the presence of PD169316. As demonstrated previously in our laboratory (26), ~12% of the cleaved caspase-3-positive neurons among TH-positive neurons were detected following 6-OHDA treatment for 24 h (Fig. 8*B*). At 5 μM PD169316, only 4.1% of TH-positive neurons were immunoreactive for cleaved caspase-3. We then investigated whether the caspase-8 and -9 pathways are involved in 6-OHDA-induced, p38-mediated apoptosis in primary cultures of mesencephalic neurons. Among the concentration ranges of caspase -8 and -9 inhibitor tested (12.5–100 μM), we found 25 μM of each inhibitor conferred the maximum protection. This concentration is lower than that used by others in primary cultures of mesencephalic neurons (33). At 25 μM, Z-IETD-fmk or Z-LEHD-fmk significantly blocked 6-OHDA-induced loss of TH-positive neurons (49.2 and 50.4%, respectively, *versus* 30.3% TH-positive neurons in 6-OHDA alone; Fig. 9). Although not statistically significant, combination of 25 μM caspase-8 and -9 inhibitors conferred better protection than either alone (60.7%). This protection rate was quite compatible to that by 5 μM PD169316 (see Fig. 7*B*) or by 50 μM Z-VAD (26).

DISCUSSION

We demonstrated previously that 6-OHDA induces ROS-dependent apoptosis in the MN9D dopaminergic neuronal cell line (25, 27), in primary cultures of dopaminergic neurons (26), and in cortical neurons (28). Based on our findings, we propose that the p38-mediated death signal initiated at least by superoxide anion and NO plays a critical role in 6-OHDA-induced apoptosis in *bona fide* mesencephalon-derived dopaminergic neurons. Furthermore, we have demonstrated that the p38 signal in response to 6-OHDA is linked to activation of both caspase-8- (extramitochondrial) and caspase-9-mediated (mito-

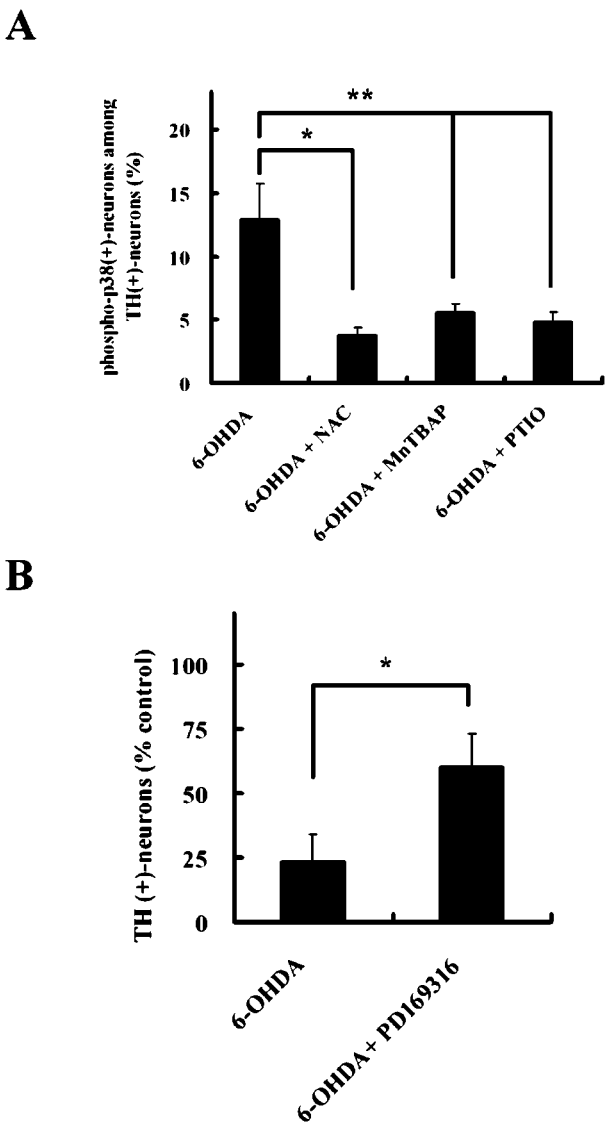

FIG. 7. **Antioxidants attenuate 6-OHDA-induced phosphorylation of p38.** The inhibition of the p38 signal is neuroprotective in primary cultures of mesencephalic neurons following 6-OHDA. *A,* primary cultures of mesencephalic neurons were treated with 20 μM 6-OHDA in the presence or absence of either 0.5 mM *N*-acetyl-L-cysteine (*NAC*), 25 μM Mn-TBAP, or 100 μM carboxy-PTIO for 12 h. Cultures were double-immunolabeled with a mouse monoclonal anti-TH and a rabbit polyclonal anti-phosphorylated form of p38. This was followed by incubation with Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 568 goat anti-rabbit IgG. Neurons that were positive for phospho-p38 among TH-positive neurons were counted. Data represent the mean ± S.E. from three independent experiments. *, $p < 0.005$; **, $p < 0.01$; ANOVA with *post hoc* Student's *t* test. *B,* primary cultures of mesencephalic neurons were treated with 20 μM 6-OHDA for 24 h in the presence or the absence of 5 μM PD169316. Cultures were then immunostained for TH, and the number of surviving TH-positive neurons was counted manually as described under "Experimental Procedures." Values from each treatment were expressed as a percentage of the untreated control (100%). Data represent the mean ± S.E. from at least three independent experiments. *, $p < 0.01$; ANOVA with *post hoc* Student's *t* test.

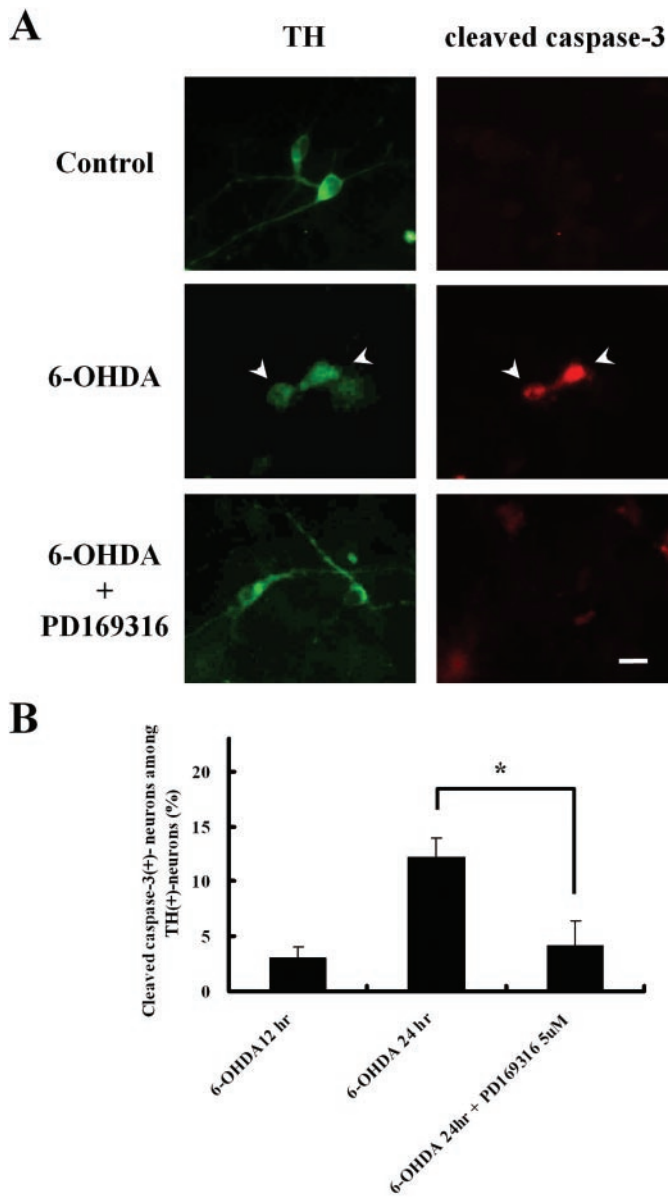

FIG. 8. **Inhibition of p38 signal prevents 6-OHDA-induced caspase activation.** Primary cultures of mesencephalic neurons were treated with 20 μM 6-OHDA for 24 h in the presence or the absence of 5 μM PD169316. Cultures were double-immunolabeled using a mouse monoclonal anti-TH and a rabbit polyclonal anti-cleaved caspase-3. This was followed by incubation with Alexa Fluor® 488 goat anti-mouse IgG and Alexa Fluor® 568 goat anti-rabbit IgG. *A,* typical photomicrographs of the untreated control or 6-OHDA-treated cultures in the presence or the absence of PD169316 are shown. *Arrowheads* indicate neurons that are positive for both cleaved caspase-3 and TH. *Scale bar*, 20 μm. *B,* neurons that were positive for cleaved caspase-3 among TH-positive neurons were counted as described under "Experimental Procedures." Data represent the mean ± S.E. from three independent experiments. *, $p < 0.01$; ANOVA with *post hoc* Student's *t* test.

chondrial) apoptotic pathways. Most interesting, in 6-OHDA-induced apoptosis, the appearance of cleaved forms of caspase-8, -3, and -7 preceded the appearance of cleaved forms of caspase-9 by at least 2 h (Fig. 3). This raises interesting possibilities as both caspase-8- and -9-initiated apoptotic pathways converge independently to activate the execution phase of caspase-3, -6, and -7 (32). In 6-OHDA-treated cells, generation of tBid was blocked in the presence of caspase-8 inhibitor. We are tempted to propose that the caspase-8-cleaved tBid-related route may play an important role in 6-OHDA-induced neuronal cell death. Therefore, inhibition of the p38 signal significantly abolishes 6-OHDA-induced cascades of caspase activation and subsequent cell death. We have largely confirmed the presence of this death sequence with regard to ROS-mediated activation of p38 and subsequent activation of caspase-8- and -9-mediated pathways in primary cultures of mesencephalic neurons treated with 6-OHDA. The difference in the rate of cell death protection (Fig. 1, *D* and *E*) and inhibition of caspase activation (Fig. 3, *B* and *C*) seen between MN9D cells co-treated with

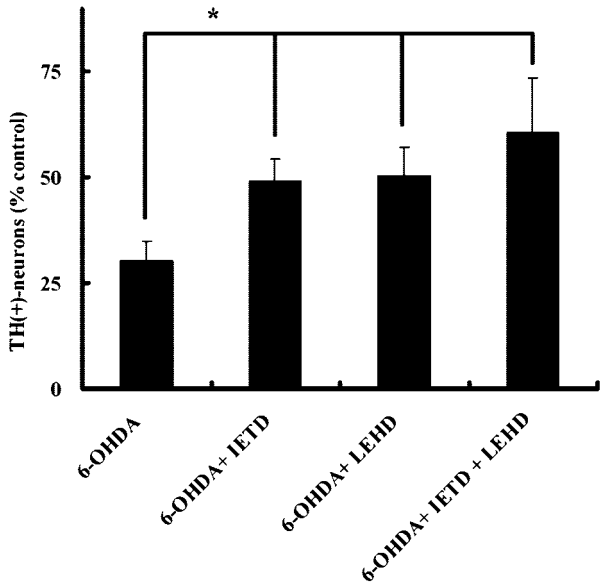

FIG. 9. **Inhibition of caspase-8 and/or -9 is neuroprotective following 6-OHDA treatment.** Primary cultures of mesencephalic neurons were treated with 20 μM 6-OHDA for 24 h in the presence or absence of 25 μM Z-IETD-fmk (*IETD*) or 25 μM Z-LEHD-fmk (*LEHD*) either alone or in combination. Cultures were then immunostained for TH, and the number of surviving TH-positive neurons was counted manually. Values from each treatment were expressed as a percentage of the untreated control (100%). Data represent the mean ± S.E. from at least three independent experiments. *, $p < 0.01$; ANOVA with *post hoc* Student's *t* test.

PD169316 and MN9D/p38DN cells may reflect the presence of additional targets of the p38 inhibitor. An alternative explanation may be that a dominant negative form of p38 does not completely suppress endogenous p38 activation in MN9D cells (Fig. 1*C*).

It is widely accepted that oxidative stress contributes to the cascade leading to degeneration of dopaminergic neurons in PD. There is considerable evidence that oxidative damage in PD may result from the action of NO (2). Among the proposed mechanisms of action of NO, Du *et al.* (15) indicated that NO induces phosphorylation of p38 (phospho-p38) and that the p38 inhibitor therefore blocks NO toxicity in cerebellar granular neurons. Similarly, inhibition of p38 abolishes NO-induced apoptotic death in SH-SY5Y, in primary cultures of cortical neurons, and in neural progenitor cells (34, 35). Although a contrasting report suggests a protective role of NO in 6-OHDA-induced cell death in PC12 cells (36), our data demonstrate that co-treatment with an NO scavenger (carboxy-PTIO) prevents 6-OHDA-induced phospho-p38 and subsequent cell death supporting a cytotoxic role of NO via p38 signaling pathway in dopaminergic neurons. Furthermore, we raise the possibility that not only NO but also superoxide anion is responsible for the 6-OHDA-induced activation of the p38 signal. Although there is scanty evidence for increased superoxide anion and for its neuropathological role in PD, overexpression of Cu/Zn-SOD in transgenic mice and cultured dopaminergic cells attenuates 6-OHDA- as well as MPP^+-induced cell death (37–39). A significant decrease in Cu/Zn-SOD and Mn-SOD mRNA is detected following 6-OHDA-induced destruction of the nigrostriatal pathway (40). In MN9D cells, we have observed previously (25) a dramatic reduction of Cu/Zn-SOD following 6-OHDA. In the present study, co-treatment with the superoxide dismutase mimetic (Mn-TBAP) significantly prevents 6-OHDA-induced phospho-p38 and subsequent cell death in MN9D cells and primary cultures of dopaminergic neurons. Most interesting, inhibition of superoxide anion or NO generation protects cells equally from 6-OHDA-induced death (see Fig. 7*A*). Although inconclusive, one might speculate that following 6-OHDA treatment, superoxide anion and/or perhaps peroxynitrite are also responsible for the activation of the p38 signaling pathway.

Recent studies indicate that such MAPK as ERK and stress-activated protein kinase (JNK and p38) signaling is implicated in the mechanisms underlying neurodegeneration in such disorders as stroke and Alzheimer's disease (41, 42). Therefore, inhibition of p38 signaling might serve as a therapeutic target in acute brain insults and in various degenerative disorders (43, 44). However, there is no direct evidence to indicate that the p38 signal is involved in the pathogenesis of PD nor is there unifying evidence for its neuropathological role in MPTP/MPP^+-treated models of dopaminergic neuronal degeneration. For example, Ferrer *et al.* (5) demonstrated that phospho-p38 immunoreactivity is found in cytoplasmic granules in the vicinity of Lewy bodies or in association with irregularly shaped or diffused α-synuclein deposits in a small percentage of neurons of the brain stem in PD. Several studies using culture models (*e.g.* SH-SY5Y neuroblastoma cells, N2a neuroblastoma cells, cerebellar granular neurons, and mesencephalic dopaminergic neurons) raised the possibility of a role for p38 in neuronal apoptosis (14–16, 45–48). In these cases, inhibition of the p38 signal activated by MPP^+, dopamine, glutamate, lipopolysaccharide, peroxynitrite, or serum withdrawal is neuroprotective. By contrast, there are conflicting results on whether MPTP/MPP^+ induces activation of p38 and inhibition of the p38 signal thereby serving a neuroprotective function. Gomez-Santos *et al.* (17) demonstrated that, in parallel with the increase in α-synuclein and apoptotic death in SH-SY5Y cells treated with MPP^+, activation of ERK but not JNK and p38 is observed; inhibition of ERK reduces the damage caused by MPP^+. Furthermore, inhibition of p38 signal in PC12h and SH-SY5Y cells *increases* cell death in response to MPTP/MPP^+ treatment (18, 19). Although this controversy is not understood, our data from 6-OHDA-treated MN9D cells and primary cultures of mesencephalic neurons appear supportive of p38 activation and its role during 6-OHDA-induced dopaminergic neuronal cell death.

Recent human postmortem studies demonstrated that active forms of caspase-8 are immunohistochemically localized in dying dopaminergic neurons (33, 49). Although it is not clearly understood whether activation of caspase-8 in PD is mediated by the cell death receptor superfamily, there is growing evidence suggesting that caspase-8 can be activated by intrinsic triggers independent of the death receptor pathway in both *in vivo* and *in vitro* paradigms (50–52). Holtz and O'Malley (53) demonstrated that death ligand and receptors (*e.g.* FAS, TNF-R1, TRAIL-R1, and TRAIL-R2) are absent in MN9D cells as determined by microarray. In our preliminary study, exogenous treatment with TNF or Fas combined with cycloheximide did not kill MN9D cells (not shown). As demonstrated previously (51), activation of caspase-8 by p38 in our culture models of PD seems to be death receptor-independent.

Taken together, our findings support an important role of p38 signaling pathway leading to a cascade of caspase activation during dopaminergic neuronal death. As a site of pharmacological intervention, targeting the p38 pathway has been proposed as one of several effective tools that slow the progression of PD (15, 54). Further studies including examining the potential mediator(s) linking ROS to p38 activation to caspase activation may expand our opportunities to develop novel therapeutics for PD. As inhibition of p38 signal confers partial protection in our culture models of PD, further identification of p38-independent death signal(s) is also worth investigating.

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