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The Roles of Dopamine Oxidative Stress and Dopamine Receptor Signaling in Aging and Age-Related Neurodegeneration

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ABSTRACT

Aging is accompanied by a decline of functions controlled by the central dopaminergic system, such as reduced locomotor activity, motivation, impairment of memory formation, and learning deficits. The molecular mechanisms underlying age-related impairment of dopaminergic functions are unknown. Current literature and our own recent work, which are reviewed and summarized in the present paper, suggest that dopamine oxidative stress and its subsequent signaling may contribute to the aging of dopaminergic system. Antiox. Redox Signal. 2, 449–460.

INTRODUCTION

DOPAMINE (DA) is a neurotransmitter under physiological conditions and plays a fundamental role in locomotor movement, motivation, and learning and memory (Maldonado *et al.*, 1997; Picetti *et al.*, 1997). However, DA also produces reactive oxygen species (ROS) and toxic quinone species through autoxidation and enzyme-catalyzed reaction. These DA oxidative metabolites are accumulated in aging and subsequently result in impairment of the functions controlled by central DAergic system. In this article, we summarize our recent work about apoptotic signaling caused by DA oxidative stress and effect of DA on glial cells.

DOPAMINE NEUROPHYSIOLOGY AND ITS AGE-RELATED CHANGES

DA is a neurotransmitter that controls a variety of physiological functions. In the central nervous system (CNS), dopaminergic neurons are originally from the ventral tegmental area and the substantia nigra and consist of four dopaminergic pathways: the nigrostriatal, the mesolimbic, the mesocortical, and tuberoinfundibular pathways. These pathways are involved in locomotor movement, motivation, reward, memory, and hormone synthesis and release (Maldonado *et al.*, 1997; Picetti *et al.*, 1997). Dysfunction of these pathways will result in neurological, psychological, and endocrinological diseases (Picetti *et al.*, 1997). For example, the nigrostriatal pathway is involved in Parkinson's disease (PD). PD is a progressive age-related DAergic neurodegeneration in the nigrostriatal area that results in significant depletion of over 80% of striatal DA. Like PD, normal human aging is frequently linked with impairment of locomotor control (Newman *et al.*, 1985; Morgan *et al.*, 1994) that is accompanied with DAergic neuron loss within the substantia nigra (Fearnley and Lees, 1991). In rhesus monkeys, a good correlation of motor

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deficits with the loss of nigral DA neurons in aging is demonstrated by using unbiased stereologic technique (Emborg *et al.*, 1998). Aging is also associated with a decline in cognitive abilities. Studies in animals and in patients with PD suggest that the mesolimbic and mesocortical pathways may be involved in cognitive function (Simon *et al.*, 1980; Scatton *et al.*, 1983). An age-related decrease of DA neurotransmition was reported in both human frontal cortex (de Keyser *et al.*, 1990) and rat hippocampus (Bach *et al.*, 1999).

DA mediates its physiological functions through interaction with its receptors. DA receptors can be divided into at least five subtypes. The D1 and D5 receptors share similar molecular structure and pharmacological profile and belong to D1 receptor family. The D1 receptor family stimulates cAMP formation through coupling to the Gs subtype of G proteins. The signaling of D1/D5 receptors in hippocampus, including activation of protein kinase A and phosphorylation of cAMP response element binding protein (CREB), is suggested to play a positive role in memory consolidation (Hersi *et al.*, 1995; Bernabeu *et al.*, 1997). Although the density of D1/D5 receptors in the hippocampus is not changed with aging (Hersi *et al.*, 1995; Levin *et al.*, 1997), there are reports of decrease in DA levels (Godefroy *et al.*, 1989; Luine *et al.*, 1990) and increasing activity in phosphodiesterase type IV, which promotes degradation of cAMP (Tohda *et al.*, 1996). These observations suggest that hippocampal memory-associated D1/D5 neurotransmition is impaired during aging. D2, D3, and D4 receptors share a high degree of structural homology, show similar pharmacological profiles, and are defined as the D2 receptor family. In addition, there are two isoforms of D2 receptors, D2L and D2S, which are produced by alternative splicing of same gene. D2 receptors belong to members of the pertussis toxin (PTX)-sensitive G protein (including Gi and Go)-coupled receptor superfamily and are expressed in both neurons and glial cells. On the basis of studies of D2 receptor knockout mice, D2 receptors have been suggested to play a crucial role in the dopaminergic system. For example, mice lacking D2 receptors show a parkinsonian-like locomotor impairment characterized by akinesis and bradykinesis in behavior tests and dramatically decreased spontaneous movement (Baik *et al.*, 1995), which are probably produced by abnormal synaptic plasticity in the corticostriatal pathway (Calabresi *et al.*, 1997).

Electrophysiological studies show that D2 receptors are major autoreceptors in dopaminergic neurons (Mercuri *et al.*, 1997). Recently, the D2 receptor agonist, pramipexole, has been shown to be efficacious to treatment of early and advanced PD patients. Administration of the D2 DA receptor agonist, but not the D1 receptor agonist, can protect against ischemia-induced hippocampal neurodegeneration (O'Neill *et al.*, 1998). During aging, loss of D2 receptors has been observed in rodents, as well as primates including humans, which may contribute to age-related decline of motor function (Han *et al.*, 1989; Roth and Joseph, 1994).

DA AS A NEUROTOXIN

Accumulating evidence suggests that a high availability of DA, in addition to serving as a neurotransmitter, may act as a neurotoxin and thereby participate in neurodegenerative processes. These include ischemia, hypoxia (Akiyama *et al.*, 1991; Buisson *et al.*, 1992), local exposure to neurotoxins such as high concentrations of excitatory amino acids (Filloux and Wamsley, 1991), and methamphetamine (Schmidt *et al.*, 1985), all of which result in a significant increase in DA concentration in striata. For example, in the case of the gerbil ischemic model, the local striatal extracellular DA concentration can reach as high as 0.2 m*M* (Slivka *et al.*, 1988). Depletion of endogenous DA by surgical destruction of the nigrostriatal pathway attenuates ischemic damage to the striatum (Globus *et al.*, 1987; Buisson *et al.*, 1992). Treatments with DA D2 receptor agonists also prevent hippocampal neuron death in the gerbil ischemic model (O'Neill *et al.*, 1998). Moreover, direct intrastriatal DA injection results in a selective DAergic neurodegeneration (Hastings *et al.*, 1996). In *in vitro* cell cultures, application of DA, 6-OH DA, and other monoamines also show neurotoxic effects (Michel and Hefti, 1990; Ziv *et al.*, 1994; Zilkha-Falb *et al.*, 1997).

The DA neurotoxicity is highly linked to oxidative metabolism. Chemically, DA contains an unstable catechol moiety. DA can oxidize spontaneously *in vitro* or through an enzyme-catalyzed reaction *in vivo* to form ROS and quinones (Cohen and Heikkila, 1974; Graham, 1978; Hastings, 1995). These oxidation products can damage cellular components such as lipids, proteins, and DNA. DA quinones can bind to cysteine or cysteinyl residues on proteins, thereby resulting in imbalance of glutathione homeostasis. Both free and protein-bound cysteinyl DA can be detected in the striata of the brain (Fornstedt *et al.*, 1989). During aging, these DA oxidative metabolites accumulate significantly (Fornstedt *et al.*, 1989, 1990). Direct intrastriatal DA injections in rats increase DA oxidation products (Hastings *et al.*, 1996). Moreover, quinones can further polymerize to form another neurotoxin, neuromelanin, which occurs in the DA-containing neurons of the substantia nigra (Jellinger *et al.*, 1992).

These DA oxidation products, which result from DA oxidative stress, can induce programmed cell death, or apoptosis. Apoptosis is a controlled form of cell death and has been suggested to participate in the cascade of some neurodegenerative diseases, *e.g.*, stroke, Alzheimer's disease, and PD (Mochizuki *et al.*, 1997). The ability of DA to induce apoptosis has been demonstrated in both *in vitro* cell cultures (Luo *et al.*, 1998b; Ziv *et al.*, 1994) and following *in vivo* intrastriatal DA injections in rats (Hattori *et al.*, 1998; Luo *et al.*, 1999a). In *in vitro* studies, a variety of cell types have been shown to be sensitive to DA toxicity. These include both neuronal and noneuronal cells, such as primary neonatal rat striatal cell cultures (Cheng *et al.*, 1996; Shinkai *et al.*, 1997), primary chick sympathetic neurons (Ziv *et al.*, 1994), a cloned catecholaminergic cell line (CATH.a) derived from the CNS (Masserano *et al.*, 1996), human neuroblastoma NMB cells (Simantov *et al.*, 1996), neuronal PC12 cells (Walkinshaw and Waters, 1994), and even mouse thymocytes (Offen *et al.*, 1995) and human embroynic kidney 293 cell line (Luo *et al.*, 1998b). The apoptotic cells induced by DA are characterized by condensed chromatin, DNA fragmentation, and shrinkage in cell shape. DA-induced apoptosis is associated with ROS because it can be effectively inhibited by application of anti-oxidants, such as *N*-acetylcysteine, catalase, glutathione (GSH), and dithiothreitol (DTT) (Ziv *et al.*, 1994; Gabbay *et al.*, 1996; Luo *et al.*, 1998b; Shinkai *et al.*, 1997). In human neuroblastoma NMB cells, application of DA transport blockers also prevents DA neurotoxicity (Simantov *et al.*, 1996).

DA oxidative stress-induced apoptosis may be implicated in normal brain aging and age-related DAergic neurodegeneration. In aging, DA oxidative metabolites are increased in DA-enriched brain area, such as substantia nigra and striata (Fornstedt *et al.*, 1989, 1990). Apoptotic cells are also detected in aged monkey substantia nigra (Emborg *et al.*, 1998) and aged rat striata (Zhang *et al.*, 1995). Although the cause of age-related DAergic neuron death in PD is unknown, the DA oxidative stress-involved neurotoxicity is believed to be a major pathological factor. In the "free radical hypothesis," the neuronal loss in PD is considered to result from high exposure of these dopaminergic neurons to ROS, especially generated by oxidation of DA (Adams and N, 1991; Olanow and Arendash, 1994). This hypothesis is supported by postmortem studies showing that in the substantia nigra of PD brain, there are increased indices of oxidative stress, increased levels of iron, increased lipid peroxidation, decreased mitochondrial complex I activity, and decreased levels of GSH (Hirsch *et al.*, 1991; Jenner, 1993). Apoptotic nigral neurons are also observed in PD brain (Anglade *et al.*, 1997; Hunot *et al.*, 1997).

DA OXIDATION APOPTOTIC SIGNALING

A next important question is how DA induces apoptosis. Recent evidence suggests that the c-Jun NH_2-terminal kinase (JNK, also called SAPK) pathway may play an important role in events of apoptosis responding to a variety of cellular stresses, such as heat shock, ultraviolet irradiation, changes in osmolarity, inflammatory cytokines (tumor necrosis factor-α, interleukin-1), and protein synthesis inhibitors (cyclohexamide and anisomycin) (Dérijard *et al.*, 1994, 1995; Kyriakis *et al.*, 1994; Verheij *et al.*,

1996). JNK/SAPK is strongly activated in response to above oxidative stresses. Activation of the JNK/SAPK pathway involves an orderly activation of the proteins MEKK1, SEK1, JNK, and c-Jun (Dérijard *et al.*, 1994, 1995; Ichijo *et al.*, 1997). SEK1 is an upstream kinase of JNK, whereas c-JUN, which is located in nuclei, is a downstream substrate of activate JNK. Overexpression of negative dominant mutants of components in JNK pathway, such as SEK1 (K → R), and c-Jun Δ169, can effectively prevent apoptosis (Ham *et al.*, 1995; Xia *et al.*, 1995; Verheij *et al.*, 1996). Furthermore, transfection of the constituted activated forms of SEK1, or c-Jun results in apoptotic cell death (Ham *et al.*, 1995; Xia *et al.*, 1995; Verheij *et al.*, 1996; Ichijo *et al.*, 1997). We first tested the hypothesis that DA-induced apoptosis may be involved in activation of c-Jun-containing nuclear transcription factor in cell cultures and then extended our observations to the rat *in vivo* model.

We have used human embryo kidney 293 cells and neonatal striatal cell cultures as noneuronal and neuronal cells, respectively, to observe the apoptotic DNA laddering induced by dopamine. We first used 293 cells to find optimal conditions for DA-induced apoptotic DNA fragmentation due to the limited amount of material available from neonatal rat striatal cell cultures. As expected, DA induced a typical apoptotic DNA ladder with a 200-bp range increase in a time- and concentration-dependent manner. In the presence of 500 μM DA, DNA laddering was observed beginning at 16 hr and reaching a maximum at 30 hr. Within 30 hr of exposure, DA proportionally induced DNA fragmentation at concentrations from 100 μM to at least 500 μM. Under a fluorescence microscope using 4,6-diamidino-2-phenylindole (DAPI) staining, DA-induced nuclear changes exhibited typical apoptotic characteristics, such as nuclear condensation and fragmentation. The nuclei in control cells showed intact uniform staining. These apoptotic cells represented $70 \pm 5.3\%$ ($n = 4$) of total cells when exposured to 500 μM DA. By using this maximal stimulation with 500 μM DA for 30 hr, apoptotic cells in rat neonatal striatal cell cultures represented about $31 \pm 4.6\%$ ($n = 5$) of the total population.

DA strongly stimulated the JNK pathway in a time- and concentration-dependent manner. JNK activity was determined with an anti-JNK1 immunocomplex kinase assay. In 293 cells, application of 500 μM DA induced JNK activity up to 9.3-fold compared to controls, when the cells were stimulated for 3 hr. This DA-stimulated JNK activation was sustained for at least 27 hr in our time-course studies. Following the activation of JNK1, phosphorylation of c-Jun, a target of JNK1, and amount of c-Jun protein were also increased during DA stimulation. Both phosphorylated c-Jun and c-Jun were detected using immunoblottings of antispecific phospho-c-Jun and anti-c-Jun, respectively. The activation of the JNK pathway by DA, including JNK activity, phosphorylation of c-Jun, and synthesis of c-Jun were detectable at 100 μM, and increased at a 500 μM concentration. DA at concentrations from 1 to 500 μM had no or a little effect on amount of JNK1 protein determined by anti-JNK1 immunoreactivity. DA also stimulated the JNK pathway in neonatal rat striatal cell cultures in a similar pattern to 293 cells. Please note that stimulation of the JNK pathway occurs before apoptotic cell death. In both 293 cells and striatal cell cultures, activation of the JNK pathway appeared after 3–4 hr of exposure to 500 μM DA. Apoptotic cell death occurred after 16 hr. The DA dose-response of the JNK activation is parallel to its ability to induce cell death.

We assessed roles of the SEK1-JNK-c-Jun signaling in DA-induced apoptosis using a dominant negative SEK1 (K → R) mutant (Verheij *et al.*, 1996) and a c-Jun negative mutant FlagΔ169 (Brown *et al.*, 1994; Ham *et al.*, 1995). Using 293 cells, we have transiently transfected this SEk1 negative mutant at an efficiency about 30–50%. Over-expression of this SEK1 (K → R) mutant inhibited DA (500 μM for 3 hr)-induced endogenous JNK activity by $49 \pm 6.7\%$ ($n = 4$), phosphorylation of JNK, and following phosphorylation of c-Jun compared with the control group. Consequently, the transfection of the SEK1 mutant also significantly reduced DA-induced DNA laddering and apoptotic cells from 68.6% (DA + empty vector) to 30.5% (DA + mutant). The transfection of SEK1 mutant did not affect JNK1 expression. FLAGΔ169 is a mutant that was generated from the mouse c-Jun cDNA by deleting sequences that encode

the amino-terminal c-Jun transactivation domain (amino acids 1–168) (Brown *et al.*, 1994; Ham *et al.*, 1995). This c-Jun Δ169 lacks the ability to be phosphorylated and to activate transcription, but it is still capable of dimerization and binds to DNA (Hirai *et al.*, 1989). In 293 cells, c-Jun Δ169 was transfected with an efficiency of about 30–50% as determined by cotransfection with pCMVβ vector expressing β-galactosidase. Compared with empty vector-transfected 293 cells, c-Jun Δ169 effectively reduced DA-induced DAN laddering. In rat neonatal striatal cell cultures, the expression of Δ169 was confirmed by anti-M2 immunocytochemistry that recognizes a FLAG epitope tagged in the amino-terminal Δ169. We did not observe apoptotic morphology in the anti-M2 positive staining cells that were treated with 500 μM DA for 24 hr, although there were some adjacent cells showing apoptotic chromatin condensation with DAPI staining. In the empty vector control group, treatment with DA (500 μM) significantly increased apoptotic cells ($34 \pm 3\%$; $n = 5$), indicated by chromatin condensation and DNA fragmentation. The transfected Δ169 group showed significant reduction of apoptotic cells from $34 \pm 3\%$ (DA + empty vector) to $12 \pm 2\%$ (DA + Δ169). Both the empty vector and the Δ169 groups show only a low background cell death. Thus, DA-induced apoptosis requires activation of SEK1-JNK-c-Jun pathway.

Next we extended the above findings to a rat *in vivo* intrastriatal DA injection model. Intrastriatal injections of DA in amounts from 1 to 2 μmol result in apoptotic cell death, as indicated by terminal deoxynucleotidyl transferase (TdT) labeling of DNA strand breaks and Klenow polymerase-catalyzed [^{32}P]dCTP-labeled DNA laddering (Hattori *et al.*, 1998; Luo *et al.*, 1999a). It is noted that the DNA fragmentation induced by DA could not be detected by conventional ethidium bromide staining after agarose electrophoresis, but it can be easily found by using high-sensitivity Klenow polymerase-catalyzed [α-^{32}P]dCTP labeling. Injections of same amount of NaCl as DA in contralateral striatum did not show an obvious ^{32}P-labeled DNA ladder. Instrastriatal DA injections resulted in rapid and sustained activation of transcription factor activated protein 1 (AP-1) determined by an electrophoretic mobility shift assay (EMSA). At 8 and 48 hr after 2 μmol DA administration, AP-1 activity was increased 2.2-($\pm$0.2, $n = 4$) and 3.0-($\pm$0.2, $n = 4$) fold compared to 2 μmol NaCl control injection at the 8-hr time point. Application of NaCl had little effect on AP-1 binding. AP-1 binding complexes in DA-stimulated samples contain c-Jun, phosphorylated c-Jun, and c-Fos, as the AP-1 binding can be inhibited in the presence of antibodies against the DNA-binding region of either c-Fos or c-Jun. Preincubation of the extracts with anti-phospho (ser 63)-specific c-Jun supershifted the AP-1 binding band. Immunoblotting assays in whole striatal tissue lysates further confirmed the existence of phosphorylation of c-Jun, as well as c-Jun and c-fos. The phosphorylated c-Jun had a slow migration with a molecular size about 45 kD. The molecular weight of c-Jun per se was about 39 kD. The phosphorylation of c-Jun in whole lysates was increased from 8 to 48 hr after DA injection. At the 24- and 48-hr time points after DA injection, the 45-kD protein migrated more slowly than those at 8 hr, suggesting that multiple phosphorylation of c-Jun protein occurred. This time course of formation of multiple phosphorylation of c-Jun was parallel to the time-course of DA-induced AP-1 binding activity and apoptosis. Taken together, these *in vivo* results agree with those from *in vitro* cell cultures.

Nuclear factor-κB (NF-κB) is an inducible transcription factor in response to oxidative stress. Using this *in vivo* rat model, we also examined the role of NF-κB in DA-induced apoptosis. DA activated NF-κB in a time- and concentration-dependent manner. The time-course studies reveal that the onset of NF-κB activation was at about 24 hr, which differed from the kinetics of DA-induced AP-1 activation that was observed 8 hr after administration of 2 μmol DA. This kinetic difference may reflect that a different signaling pathway for activation of NF-κB is required from that for stimulation of AP-1. Activation of NF-κB was proportionally increased when rat striata received 1–2 μmol of DA.

To determine whether activation of AP-1 and NF-κB might contribute to the process for DA-induced apoptosis *in vivo*, we first examined

the effect of curcumin on DA-induced AP-1 activity and subsequent apoptosis. In non-neuronal cells, curcumin can inhibit both c-Jun/AP-1 activation (Huang *et al.*, 1991) and NF-κB activity (Singh and Aggarwal, 1995), depending on the dosage. At intrastriatal injection of 1 μmol, curcumin dramatically inhibited AP-1 binding without affecting NF-κB activity induced by DA. The DA-induced DNA laddering was greatly reduced with preinjection of 1 μmol of curcumin, although curcumin itself at this dosage showed some cytotoxicity as indicated by the appearance of a slight DNA ladder. The curcumin toxicity may be caused by interruption of normal c-Jun physiological functions. We then examined the role of NF-κB activation in DA toxicity by using SN50. SN50 is a cell-permeable inhibitory peptide and has been shown to block specifically translocation of the NF-κB active complex into the nucleus (Lin *et al.*, 1995). In *in vivo* rat striatal injection studies, SN50 was shown to inhibit NF-κB activity specifically without affecting activation of AP-1 and OCT-1 induced by quinolinic acid (Qin *et al.*, 1998). DA-induced NF-κB activity was greatly inhibited by preinjection of SN50 (20 μg), but was not affected by application of same amounts of SN50M, an inactive peptide that has the same peptide sequence as SN50 except for Lys-363 to Asn and Arg-364 to Gly in the region of nuclear localization signal of NF-κB. Blocking of NF-κB translocation by SN50 also greatly reduced DNA laddering induced by DA. Similar to injection of curcumin alone, SN50 itself had a cytotoxic effect as indicated by a weak DNA ladder, suggesting that SN50 interrupts the physiological NF-κB function. Thus, both AP-1 and NF-κB contribute to the processes of DA neurotoxicity.

Anti-oxidants can block both DA-induced AP-1 and NF-κB activation and subsequent apoptosis. DA neurotoxicity is thought to be mediated by its oxidative stress (Ziv *et al.*, 1994; Luo *et al.*, 1998b, 1999a). In the rat *in vivo* model, the DA oxidative metabolites in striata are easily detected by formation of free and protein bound cysteinyl DA, by which the endogenous GSH is greatly exhausted (Hastings *et al.*, 1996). On the basis of this finding, we examined the roles of GSH in DA-induced apoptosis *in vivo*. As expected, administration of exogenous GSH (0.2 μmol) prevented both 1 μmol DA-induced AP-1 and NF-κB activation and subsequent apoptosis. Preinjection of this dosage of GSH also greatly reduced DNA laddering by 2 μmol DA. In *in vitro* cell cultures, application of anti-oxidants, such as *N*-acetylcysteine and catalase, effectively inhibited DA-induced JNK activation and subsequent apoptosis (Luo *et al.*, 1998b). Thus, DA oxidation-associated activation of AP-1 and NF-κB is required for DA-induced apoptosis.

EFFECT OF DA ON ASTROCYTIC CELLS

It is known that astrocytes are the intimate partners of neurons and play important physiological roles in the maintenance of the microenvironment of neurons (Wilkin *et al.*, 1990). In the rat *in vivo* model, intrastriatal DA injections, in addition to resulting in neurodegeneration, also activate astrocytes as indicated by an increase of glial fibrillary acidic protein (GFAP) immunocytochemical staining and astrocyte proliferation determined by [^{3}H]R05-4864 binding to peripheral benzodiazepine receptors on astrocytes (Filloux and Townsend, 1993). In the rat ischemic model, activated striatal astrocytes are suggested to have a protective role for DA-innervated neurons (Zoli *et al.*, 1997). In *in vitro* cell cultures, striatal astrocytes appear to play a protective effect against hydrogen peroxide toxicity to DAergic neurons (Langeveld *et al.*, 1995). Nevertheless, the molecular events underlying DA activation of astrocytes are unknown.

We chose C6-D2L cells to examine the effect of DA on glial cells for the following reasons: (i) C6 cells were originally cloned from a rat astrocytoma (Benda *et al.*, 1968) and have been widely used as a model of glial phenotype. (ii) C6-D2L cells stably express recombinant D2L DA receptors (Neve *et al.*, 1989). Recent evidence suggests that D2 receptors are also expressed in striatal astrocytes. Physiologically, DA can induce membrane hyperpolarization in the majority of astrocytes from striatum, which can be inhibited by application of domperidone, a D2 receptor antagonist (Hosli *et al.*, 1987). D2 antagonists [^{3}H]domperidone and [^{3}H]spiperone can specifically label striatal as-

trocytes (Hosli and Hosli, 1986). D2 receptor mRNA can be detected by either *in situ* hybridization or polymerase chain reaction from astrocytes in striatum, an area enriched with DAergic termini, but not astrocytes in the cerebellum, which receives little DAergic innevation (Bal *et al.*, 1994). (iii) The expression level of D2 receptors in C6-D2L cells is about 188 fmol/mg protein, which is close to that in striatal membranes (~400 fmol/mg protein) (Leve *et al.*, 1991; Luo *et al.*, 1998a). (iv) The D2 receptors in the C6-D2L cells are functionally coupled to pertussis toxin-sensitive G proteins, resulting in inhibition of adenylate cyclase (Watts and Neve, 1996) and activation of both extracellular signal-regulated kinases (ERKs) and JNK in this cell line (Luo *et al.*, 1998a). Thus, this C6-D2L cell line serves as a good *in vitro* cell culture model for studies of DA regulation of glial response.

Unlike DA neurotoxicity, micromolar levels of DA stimulated mitogenesis in C6-D2L cells (Luo *et al.*, 1999b). The mitogenesis, which was determined by DNA [^{3}H]thymidine incorporation and occurred about 29 hr after DA exposure, was linearly increased from 0.1 μM to 500 μM DA. This DA-stimulated [^{3}H]thymidine incorporation could be completely inhibited by aphidicoline, a potent DNA polymerase A inhibitor. Flow cytometry analysis indicated that DA increased the percentage of the cells in S phase and decreased the percentage of cells in G_1 phase. The DA-stimulated mitogenesis requires activation of D2 receptors because it can be completely inhibited with 10 μM (+)-butaclamol, a potent D2 receptor antagonist. Application of 10 μM (−)-butaclamol, an inactive form of (+)-butaclamol, had no effect on the DA-stimulated mitogenesis. Quinpirole, a specific D2 receptor agonist, also promoted cell progression through the cell cycle. Furthermore, exposure of wild-type C6 cells without expression of D2L receptors to DA in a concentration range of 0–500 μM did not show a mitogenic effect. Instead, DNA [^{3}H]thymidine incorporation in wild-type C6 cells was decreased about 20–26% at DA concentrations from 10 to 500 μM.

The DA-stimulated mitogenesis requires D2 receptor-activated protein tyrosine phosphorylation. Micromolar levels of DA strongly stimulated protein tyrosine phosphorylation in a time- and concentration-dependent manner. In the presence of 200 μM DA, protein tyrosine phosphorylation, which was determined by immunoblotting with monoclonal anti-phosphotyrosine IgG (PY99), rapidly increased within 5 min, reached a maximum between 15 and 30 min, then decreased by 1 hr. Most tyrosine-phosphorylated proteins were located within the molecular size range of 50–200 kDa. Consistent with the DA concentrations for stimulation of mitogenesis, DA-stimulated tyrosine phosphorylation was evident at DA concentrations ranging from 10 to 500 μM. Preincubation of C6-D2L cells with (+)-butaclamol prevented DA-induced protein tyrosine phosphorylation. Application of same amount of (−)-butaclamol failed to block the tyrosine phosphorylation. Quinpirole from 10 to 200 μM also increased tyrosine-phosphorylated proteins. In wild-type C6 cells, no significant changes in tyrosine-phosphorylated proteins were observed when exposed to DA concentrations from 10 to 500 μM. This D2 receptor-mediated tyrosine kinase activation is required for DA-stimulated mitogenesis because genistein, a potent tyrosine kinase inhibitor, could inhibit both DA-induced tyrosine phosphorylation and subsequent mitogenesis.

DA stimulated mitogenesis through regulation of an intracellular redox-tyrosine kinase cascade. Diphenylene iodonium (DPI) is an inhibitor of flavonoid-containing oxidases that can catalyze the formation of superoxide from oxygen with NADH/NADPH as an electron donor (Ruppersberg *et al.*, 1991; Lo *et al.*, 1996). NAC is an antioxidant that can scavenge free radicals. Application of either 1 μM DPI or 20 mM NAC to C6-D2L cells greatly reduced tyrosine phosphorylation and consequent mitogenesis induced by DA. Moreover, exogenous application of a low concentration of hydrogen peroxide (H_2O_2) can enhance the ability of DA to stimulate protein kinases and mitogenesis. Thus, it appears that DA stimulates D2 receptors and increases intracellular ROS, which serve as second messengers, resulting in activation of protein tyrosine kinase and mitogenesis.

In addition to promoting cell mitogenesis, micromolar levels of DA also significantly

stimulated GFAP expression, a sensitive parameter serving as another criterion of reactive astrocytes (Major *et al.*, 1997). Unlike the dose-response data of DA-induced mitogenesis, GFAP production was maximal at the 200 μM DA for 24 hr. By using a paradigm similar to that for DA-stimulated mitogenesis in C6-D2L cells, We, surprisingly, found that the GFAP stimulation did not appear to be regulated by D2 receptor-redox-kinase pathway. The DA-stimulated GFAP expression was not influenced by D2 receptor antagonist (+)-butaclamol, 10 μM genistein, or 20 mM NAC.

GFAP expression stimulated by DA is involved in activation of p38 MAPK. Pretreatment of cells with SB 203580, a selective p38 MAPK inhibitor (Lee *et al.*, 1994, Cuenda *et al.*, 1995) completely blocked the increases in GFAP generation stimulated by DA at concentrations ranging from 10 to 200 μM. Moreover, DA (100 μM) rapidly stimulated P38 MAPK activity, which was determined by using anti-phospho-specific p38 MAPK immunoblotting. Phosphorylation of p38 MAPK is required for p38 MAPK activity (Raingeaud *et al.*, 1995). Corresponding to the range of DA concentrations for GFAP stimulation, p38 MAPK activity was greatly increased. (+)-Butaclamol had no effect on DA-stimulated phosphorylation of p38 MAPK. Thus, these data suggest that DA-activated GFAP synthesis may be mediated by D2 receptor independent p38 MAPK pathway.

SUMMARY

In addition to serving as a neurotransmitter, DA also produces ROS and toxic quinone species that are accumulated in aging and selectively result in neurodegeneration of the central DAergic system. Thus, this DA oxidative stress-induced neurotoxicity is suggested to participate in both processes of normal age-associated decline of locomotor control and the pathologic etiology of Parkinson's disease. By using a rat *in vivo* model of intrastriatal DA injection and *in vitro* cell cultures, we have studied the molecular events involved in DA toxicity. DA can induce apoptosis both *in vivo* and in cell cultures. In cell cultures, DA strongly and persistently activates the JNK pathway, including increases in JNK activity, phosphorylation of c-Jun, and increase in c-Jun protein. Transfection of dominant negative mutants SEK1(K $\rightarrow$ R) or FLAGΔ169, an upstream kinase and a downstream target of the JNK, respectively, significantly reduces DA-induced activation of JNK pathway and subsequent apoptosis. In the *in vivo* rat model, injections of DA produce a strong and prolonged AP-1 activity that contains c-Fos, c-Jun, and phosphorylated c-Jun protein. DA also produces a delayed activation of NF-κB, an oxidative stress responsive transcription factor. Application of curcumin at a dose that selectively inhibits the AP-1 activation without affecting NF-κB activity attenuates DA-induced DNA laddering. Administration of SN50, a specific permeable recombinant NF-κB translocation inhibitor peptide, also decreases NF-κB activation and apoptosis induced by DA. Moreover, preinjection of the antioxidant GSH results in a significant inhibition of activation of transcription factors AP-1 and NF-κB and subsequent apoptosis stimulated by DA. Thus, these data suggest that DA-oxidative stress, which is increased in aging and may disturb GSH homeostasis, triggers neuronal apoptosis via activation of transcription factors AP-1 and NF-κB pathways. Using a rat C6 glioma cell line stably expressing recombinant D2L receptors, we have also studied the effect of DA on glial cells. Micromolar levels of DA stimulate mitogenesis and GFAP expression, both serving as parameters of reactive gliosis. The DA-stimulated mitogenesis requires DA D2 receptor-mediated intracellular redox-tyrosine kinase activation. The GFAP expression induced by DA requires a D2 receptor-independent p38 MAPK activation. Although these *in vitro* results from C6-D2L cells have not been demonstrated in the *in vivo* model, our results suggest that glial cells may play neuroprotective roles. The roles of glial cells during DA oxidative stress induced neurodegeneration need further studies.

ABBREVIATIONS

AP-1, activated protein 1; CNS, central nervous system; CREB, cAMP response element binding protein; DA, dopamine; DAPI, 4,6-di-

amidino-2-phenylindole; DTT, dithiothreitol; DPI, diphenylene iodonium; EMSA, electrophoretic mobility shift assay; ERKs, extracellular signal regulated kinases; G protein, guanine nucleotide-binding protein; GFAP, glial fibrillary acidic protein; GSH, glutathione; H_2O_2, hydrogen peroxide; JNK, c-Jun NH_2-terminal kinase (also called SAPK); MAPK, mitogen-activated protein kinase; SEK1, SAPK/Erk kinase 1; NAC, *N*-acetylcysteine; NF-κB, nuclear factor-κB; PD, Parkinson's disease; PTX, pertussis toxin; ROS, reactive oxygen species; SB 203580, a selective p38 MAPK inhibitor; SN, substantia nigra; TdT, terminal deoxynucleotidyl transferase.

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