Rotenone Induces Apoptosis via Activation of Bad in Human Dopaminergic SH-SY5Y Cells

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ABSTRACT

Chronic complex I inhibition caused by rotenone induces features of Parkinson’s disease in rats, including selective nigrostrial dopaminergic degeneration and Lewy bodies with α-synuclein-positive inclusions. To determine the mechanisms underlying rotenone-induced neuronal death, we used an in vitro model of human dopaminergic SH-SY5Y cells. In rotenone-induced cell death, rotenone induced Bad dephosphorylation without changing the amount of Bad proteins. Rotenone also increased the amount of α-synuclein in cells showing morphological changes in response to rotenone. Because Bad and α-synuclein are known to bind to 14-3-3 proteins, we examined the effects of rotenone on these complexes.

Whereas a decreased Bad amount bound to 14-3-3 proteins, rotenone increased α-synuclein binding to these proteins. Because dephosphorylation by calcineurin activates Bad, we examined the possible involvement of Bad activation in rotenone-induced apoptosis by using the calcineurin inhibitor tacrolimus (FK506). Tacrolimus suppressed two rotenone-induced actions: Bad dephosphorylation and apoptosis. Furthermore, the inhibition of caspase-9, which functions downstream from Bad, completely suppressed rotenone-induced apoptosis. Our findings demonstrate that Bad activation plays a role in rotenone-induced apoptosis of SH-SY5Y cells.

Parkinson’s disease is characterized by selective nigrostrial dopaminergic degeneration and the formation of ubiquitin- and α-synuclein-positive cytoplasmic inclusions known as Lewy bodies (Spillantini et al., 1997). The etiology of the nigral dopamine neuron degeneration is unknown, although both genetic mutations and environmental factors have been identified as contributing to certain forms of this disorder (Shastryl, 2001). An established hallmark of Parkinson’s disease is a reduction in the activity of brain mitochondrial enzyme complex I (Schipirila et al., 1990). Conversely, complex I inhibitors, such as 1-methyl-4-phenylpyridinium ion and rotenone, have been shown to injure nigral dopaminergic neurons and cause parkinsonian motor dysfunction (Heikkila et al., 1985; Forno et al., 1993; Greenamyre et al., 2001). These observations suggest that a defect in mitochondrial complex I activity may contribute to the neurodegenerative process in Parkinson’s disease. The neuronal death in Parkinson’s disease could be apoptotic; complex I inhibition in dopaminergic cells induces apoptosis (Hartley et al., 1994). Moreover, there is evidence to suggest that nigral neurodegeneration occurs in Parkinson’s disease, at least in part, via a caspase-3-mediated apoptotic mechanism (Mochizuki et al., 1996; Tompkins et al., 1997; Hartmann et al., 2000). To identify potential targets for therapeutic intervention in this disorder, it is important to determine signaling pathways linking complex I impairment and the induction of programmed cell death in dopaminergic neurons.

α-Synuclein belongs to a protein family consisting of α-, β-, and γ-synuclein (Jakes et al., 1994; Clayton and George, 1998). α-Synuclein is expressed in neurons as well as in a variety of somatic cells (Ueda et al., 1993). In neurons, α-synuclein is enriched in presynaptic terminals, where it is distributed between a soluble pool and a vesicle-bound pool of proteins. Recent research suggests that α-synuclein contributes to the pathophysiology of many neurodegenerative disorders. In Parkinson’s disease, α-synuclein accumulates in Lewy bodies. A number of studies have suggested that α-synuclein can be toxic to some cells. Incubation of the neuronal cells with α-synuclein, SK-SY5Y cells, leads to apoptosis (El-Agnaf et al., 1998). Our current knowledge of α-synuclein biology is not, however, sufficient to understand how α-synuclein might affect cell survival.

The 14-3-3 proteins constitute a family of protein chaperones that are particularly abundant in the brain, like α-synuclein. The 14-3-3 protein family consists of seven
known mammalian isoforms (Fu et al., 2000). It exhibits a remarkable degree of sequence conservation, among both species and isoforms. 14-3-3 is known for its ability to bind many different proteins, most of which contain phosphorylated serine residues (Muslin et al., 1996). An emerging role for 14-3-3 is that of an effector of prosurvival signaling (Fu et al., 2000), suggested in part by the large number of 14-3-3 binding proteins involved in apoptosis (Vincenz and Dixit, 1996; Brunet et al., 1999; Zhang et al., 1999). 14-3-3 also binds to phosphorylated Bad, which seems to stabilize Bad maintenance in the cytoplasm (Zha et al., 1996). Bad is a member of the BH3-only subfamily of Bcl-2 apoptosis-regulating proteins, regulated extensively by phosphorylation of serine residues. At least three sites on Bad can be phosphorylated, including S112, S136, and S155 of the murine protein (Zha et al., 1996). Dephosphorylated Bad is localized to the mitochondria along with Bcl-2 and Bcl-XL, where it can induce apoptosis (Zha et al., 1996). When S112 and S136 of Bad are phosphorylated, Bad remains in the cytosol, as it is bound to 14-3-3 proteins rather than Bcl-2 or Bcl-XL (Zha et al., 1996). This phosphorylated form of Bad does not promote apoptosis. Mutation of any of the phosphorylation sites enhances the cell-killing ability of Bad, suggesting that Bad phosphorylation is a critical mechanism for inhibiting its activity.

In this study, we found that rotenone, which is known to inhibit mitochondrial complex I, induces α-synuclein, Bad dephosphorylation, caspase-9 activation, and ultimately apoptosis in human dopaminergic SH-SY5Y cells. Tacrolimus (FK506) was used as a reagent for inhibiting Bad dephosphorylation, caspase-9 activation, and ultimately apoptosis in human dopaminergic SH-SY5Y cells. Tacrolimus (342500) was from Calbiochem (San Diego, CA), and rotenone (R8875), N-benzoyloxy carbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK) (C2105), Z-Leu-Glu(O-Me)-His-Asp(O-Me) fluoromethyl ketone (Z-LEHD-FMK) (C1355), and anti-actin antibody (A5060) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-α-synuclein antibody (AB5038) and anti-tyrosine hydroxylase antibody (MAB318) were obtained from Chemicon International (Temecula, CA). Anti-14-3-3 (FL-246), Bad (C-7), and p-Bad (Ser136) antibody were purchased from Santa Cruz Biotecnology, Inc. (Santa Cruz, CA), and mouse anti-tyrosine hydroxylase antibody (clone 7H3.19) was from Calbiochem. (San Diego, CA).

**Cell Culture.** SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C under 5% CO₂ in air.

**Analysis of DNA Fragmentation by Agarose Gel Electrophoresis.** The detection of DNA fragmentation using agarose gel electrophoresis was performed as described previously (Watabe et al., 1996). Cells were collected by centrifugation and washed with phosphate-buffered saline (PBS). The washed cells were lysed in a solution of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate (SDS), and 0.1% (w/v) RNase A, with incubation for 60 min at 50°C. The lysate was incubated for an additional 60 min at 50°C with 1 μg/ml proteinase K and then subjected to electrophoresis for 60 min at 50 V in a 1% (w/v) agarose gel in 40 mM Tris acetate, pH 7.5, which contained 1 mM EDTA. After electrophoresis, DNA was visualized by staining with ethidium bromide.

**Materials and Methods.**

**Materials.** Rotenone (R8875), N-benzoyloxy carbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK) (C2105), Z-Leu-Glu(O-Me)-His-Asp(O-Me) fluoromethyl ketone (Z-LEHD-FMK) (C1355), and anti-actin antibody (A5060) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-α-synuclein antibody (AB5038) and anti-tyrosine hydroxylase antibody (MAB318) were obtained from Chemicon International (Temecula, CA). Anti-14-3-3 (FL-246), Bad (C-7), and p-Bad (Ser136) antibody were purchased from Santa Cruz Biotecnology, Inc. (Santa Cruz, CA), and mouse anti-tyrosine hydroxylase antibody (clone 7H3.19) was from Calbiochem. (San Diego, CA).

**Immunoblot Analysis.** Immunoblotting was performed as described previously (Watabe et al., 1996). Cells were lysed in a buffer containing SDS and mercaptoethanol, and the cell lysate was then boiled. Denatured proteins were separated on a polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (American Biosciences UK Ltd., Buckinghamshire, Little Chalfont, UK). The membrane was incubated with a blocking solution [2% skim milk (Invitrogen, Carlsbad, CA)] dissolved in PBS containing 0.2% Tween 20 for 1 h at room temperature, washed with PBS containing 0.2% Tween 20, and incubated for 1 h with a primary antibody dissolved in the blocking solution overnight at 4°C. After washing, the membrane was incubated for 1 h with horseradish-linked secondary antibody. Immunoreactive proteins were detected with an enhanced chemiluminescence system (Amersham Biosciences UK, Ltd.).

**Immunofluorescence Microscopy.** Immunocytochemical study was performed as described previously (Watabe et al., 1997, 2000). Cells were washed with PBS and fixed with 3.7% formaldehyde for 20 min. Cells were permeabilized with PBS containing 0.2% Triton X-100 for 5 min and then washed three times with PBS. Incubation with primary antibody was carried out for 1 h at room temperature. Excess antibody was washed three times with PBS. This was followed by incubation with an appropriate fluorophore-labeled second-
ary antibody for 1 h at room temperature in an area protected against light. After washing out the excess antibody three times with PBS, the specimens were mounted using PBS containing 1 mg/ml Hoechst33258 (Sigma-Aldrich). Images were collected by fluorescence microscopy.

Immunoprecipitation. Immunoprecipitation was performed as described previously (Watabe et al., 2004). After treatment with rotenone, the cells were pelleted, washed twice in PBS, and lysed in lysis buffer [20 mM Tris-Cl (pH 7.4), 140 mM NaCl, 10% glycerol, 1% Triton X-100, and 2 mM EDTA] containing a protease inhibitor mixture (Roche Molecular Biochemicals). The cell lysates were then used for immunoprecipitation in the presence of an anti-14-3-3 antibody. Immune complexes were precipitated using Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc.) and washed four times in lysis buffer. The precipitate was resuspended in buffer containing SDS and mercaptoethanol, boiled, and immunoblot analysis was then performed.

Results

We first examined the effect of rotenone on apoptosis of human dopaminergic SH-SY5Y cells. A dose-dependent increase in histone-associated DNA fragmentation caused by rotenone was detected (Fig. 1A). Rotenone treatment induced nuclear fragmentation (Fig. 1B). These results confirm that rotenone induces apoptosis in SH-SY5Y cells. To investigate the mechanisms underlying rotenone-induced apoptosis, we examined the effects of rotenone on the expressions of various proteins, including α-synuclein, Bcl-2, Bax, 14-3-3, and tyrosine hydroxylase. Rotenone treatment markedly increased α-synuclein expression after 24 h (Fig. 2). Immunohistochemical analysis revealed rotenone treatment to increase the amount of cellular α-synuclein in apoptotic cells (Fig. 3A, arrow). Bad is a member of the BH3-only subfamily of Bcl-2 apoptosis-regulating proteins, and its activity is regulated extensively by phosphorylation of serine residues. In the case of Bad, two of its three known phosphorylation sites, S112 and S136, lie within potential 14-3-3 binding sites. When S112 and S136 of Bad are in a phosphorylated state, Bad is found in the cytosol and bound to 14-3-3 proteins rather than to Bcl-2 or Bcl-X<sub>L</sub> (Zha et al., 1996). On the other hand, α-synuclein shares physical and functional homology with 14-3-3 and α-synuclein also binds to 14-3-3 proteins. Therefore, we examined the effects of rotenone on the complex formation of these proteins. As shown in Fig. 3B, rotenone increased the amount of 14-3-3-bound α-synuclein and decreased the amount of 14-3-3-bound Bad. This is consistent with the data in Fig. 2, which shows that rotenone induced Bad dephosphorylation with no change in the amount of Bad.
protein. Because the protein phosphatase calcineurin is known to activate Bad via its dephosphorylation (Wang et al., 1999), we examined the effect of the calcineurin inhibitor tacrolimus on rotenone-induced Bad dephosphorylation. Tacrolimus treatment inhibited rotenone-induced Bad dephosphorylation (Fig. 4A) and decreased the amount of 14-3-3-bound Bad (Fig. 3C). However, tacrolimus treatment had no effect on the rotenone-increased amount of 14-3-3-bound α-synuclein (Fig. 3C). Under these conditions, we examined the effect of tacrolimus on rotenone-induced apoptosis. As shown in Fig. 4B, tacrolimus treatment inhibited rotenone-induced apoptosis. To further investigate the possibly essential role of Bad activity in the initiation of rotenone-induced apoptosis, we examined the effects of the inhibitors of caspase-9, which functions downstream from Bad. Z-VAD-FMK is an irreversible caspase inhibitor with a broad spec-
ificity against various caspases and Z-LEHD-FMK is an irreversible specific inhibitor of caspase-9. When the cells were pretreated with either of these caspase inhibitors, DNA fragmentation caused by rotenone was suppressed (Fig. 4C). Together, these results suggest rotenone treatment of SH-SY5Y cells increases 14-3-3-bound α-synuclein, induces Bad activation via dephosphorylation, activates caspases, and ultimately induces apoptosis.

Discussion

In this study, we demonstrated for the first time that rotenone, known to inhibit mitochondrial complex I, leads to the activation of Bad by dephosphorylation and induces apoptosis. There is evidence to suggest that nigral neurodegeneration involves apoptosis in Parkinson’s disease (Mochizuki et al., 1996; Tompkins et al., 1997). We present herein new information on rotenone-induced apoptosis, which was used as a model of nigral degeneration in Parkinson’s disease.

Rotenone induced Bad dephosphorylation with no change in the amount of Bad protein. To investigate whether Bad dephosphorylation is a key event in rotenone-induced apoptosis, we attempted to inhibit this dephosphorylation of Bad. Cyclosporin A and tacrolimus are immunosuppressive compounds, which exert their actions through binding to immunophilins such as the cyclophilins or FK binding proteins (Galat and Metcalfe, 1995). When complexed with cyclosporin A and tacrolimus, the properties of the immunophilins change, leading to the functional inhibition of proteins including the phosphatase calcineurin (Liu et al., 1991). Calcineurin dephosphorylates Bad protein in a calcium-dependent manner. However, cyclosporin A, but not tacrolimus, prevents mitochondrial permeability transition by blocking translocation of the mitochondrial matrix-specific cyclophilin-D to the inner mitochondrial membrane (Connor and Halestrap, 1994). Therefore, we used tacrolimus, rather than cyclosporin A, as the calcineurin inhibitor in our study. Indeed, tacrolimus inhibited both rotenone-induced Bad dephosphorylation and apoptosis.

The observation that overexpression of human α-synuclein causes dopaminergic neuronal death suggests α-synuclein to play a role in the pathogenesis of Parkinson’s disease (Zhou et al., 2002). In the present study, α-synuclein increased with rotenone treatment during the process of apoptosis induction. Indeed, this increase was observed in the cells damaged by rotenone. α-Synuclein is known to be a component of 14-3-3 proteins as well as Lewy bodies in Parkinson’s disease (Ubl et al., 2002). 14-3-3 is known for its ability to bind many different proteins, most of which contain phosphoserine (Muslin et al., 1996). An emerging role for 14-3-3 is that of an effector of prosurvival signaling (Fu et al., 2000), suggested in part by the large number of 14-3-3 binding proteins involved in apoptosis, such as A20 (Vincenz and Dixit, 1996), ASK1 (Zhang et al., 1999), Bad (Zha et al., 1996), and FKHRL1 (Brunet et al., 1999). Experiments using dominant negative forms of 14-3-3 in cultured cells (Zhang et al., 1999) and transgenic animals (Xing et al., 2000) support this notion. Therefore, we speculate that the increase in α-synuclein by rotenone may interfere with the prosurvival signaling of 14-3-3. Indeed, rotenone induced an increase in 14-3-3-bound α-synuclein with no change in the total amount of 14-3-3, suggesting its prosurvival signaling capacity to be decreased.

There are at least two possible sequences of events involving the association of α-synuclein with 14-3-3 Bad dephosphorylation and its dissociation from 14-3-3. One possibility is that α-synuclein displaced phosphorylated Bad from 14-3-3 and calcineurin then dephosphorylated Bad after dissociation from 14-3-3. If this was the case, the amount of phosphorylated Bad bound to 14-3-3 would be decreased even in the presence of tacrolimus. However, because the 14-3-3 family of proteins consists of seven known mammalian isoforms (Fu et al., 2000) and no clear evidence has been presented as to the specificity of each 14-3-3 isoform to α-synuclein and Bad, this speculation is not supported at present. The other possibility is that the α-synuclein increase and Bad dissociation are independent events. In other words, calcineurin is capable of dephosphorylating Bad still bound to 14-3-3. If this is the case, the amount of phosphorylated Bad bound to 14-3-3 would not decrease in the presence of tacrolimus. In addition, tacrolimus treatment indicated no effect on the amount of 14-3-3-bound α-synuclein increased by rotenone. Our data favor the latter hypothesis. Therefore, the precise role of α-synuclein in rotenone-induced apoptosis requires further investigation. To precisely elucidate the mechanisms underlying α-synuclein biology, chemicals such as phalloidin (Lee et al., 2004), which binds to α-synuclein and thereby induces a structural change in the protein, might be useful.

To clarify the role of the Bad-dependent pathway in rotenone-induced apoptosis, we used inhibitors of each key event. First, tacrolimus significantly inhibited rotenone-induced Bad dephosphorylation and apoptosis. Second, caspase-9 inhibitors also inhibited rotenone-induced apoptosis. Because caspase-9 lies downstream from Bad activation in the mitochondrial-mediated apoptotic pathway, these results suggest that rotenone promotes Bad dephosphorylation and activation, and subsequently activates caspase-9. Although we cannot exclude the involvement of pathways other than the Bad system, our results show Bad to play a role in rotenone-induced cell death in SH-SY5Y cells. As for SH-SY5Y cells, we speculate that rotenone-induced α-synuclein affects many apoptosis-related proteins such as Bad through interaction with 14-3-3. Then, dephosphorylated Bad dissociates from 14-3-3 and translocates to mitochondria, where Bad functions as an apoptotic inducer. Subsequently, cytochrome c is released, caspase-9 is activated, and apoptosis ensues.

Because there are no reports on familial Parkinson’s disease with Bad or 14-3-3 mutations, the roles of these proteins in familial Parkinson’s disease pathogenesis remain uncertain. However, a few reports have suggested another calcineurin inhibitor, cyclosporin A, to have a neuroprotective effect against 6-hydroxydopamine- and 1-methyl-4-phenylpyridinium ion-induced neuronal death (Matsuura et al., 1997; Seaton et al., 1998). Therefore, it is possible that the Bad system plays a role in the pathogenesis of sporadic Parkinson’s disease.

In summary, we have demonstrated the activation of Bad to be proapoptotic in rotenone-induced apoptosis in human dopaminergic SH-SY5Y cells.

References


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