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Neuroprotective Effects of Parkin and Bcl-2 against Dieldrin-induced Endoplasmic Reticulum Stress

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Dopaminergic (DA) cell death in Parkinson's disease (PD) has been attributed to multiple, distinct genetic and environmental factors. In rare familial PD loss of parkin function mutations play a key role in nigral DA neuron-specific pathogenesis primarily via endoplasmic reticulum (ER) stress. In more prevalent sporadic PD, environmental exposure to pesticides has a significant epidemiological role. However, it is largely unknown how environmental exposure to xenobiotics is etiologically linked with the known etiology in familial PD. In the present study biochemical evidence for a common pathogenic mechanism between sporadic and familial PD has been identified employing the recently characterized mesencephalic DA cell line, N27-A. Dieldrin, an organochlorine pesticide epidemiologically implicated in sporadic PD, induced the markers of ER stress response such as a chaperone BiP/Grp78, heme oxygenase-1 and especially, parkin. Accordingly, dieldrin activated the ER resident Caspase-12, a mediator of ER stress-specific apoptosis, during cell death of N27-A cells. Of great interest the dieldrin-induced DA neuronal cell death was synergistically rescued by the overexpression of ER resident neuroprotective proteins, parkin and Bcl-2. The present findings implicate that accumulation of ER stress could be one of common pathogenic mechanisms in idiopathic and familial PD, and some ER proteins, such as parkin and Bcl-2 may effectively attenuate ER stress-mediated N27-A DA cell death.

Key words : Bcl-2, cell death, ER stress, N27-A cells, parkin

Introduction

Parkinson's disease (PD) is caused by multiple environmental risk factors and rare genetic mutations. It is unknown how these distinct environmental factors and specific genetic mutations cause the same neuronal vulnerability in both sporadic and familial PD, respectively. Mounting evidence from epidemiological investigations and twin studies, suggest a significant role of environmental factors, especially pesticide exposure, in some sporadic cases of PD [13, 27]. However, the molecular clues to the etio-pathological link between pesticide and PD remains mostly unknown.

Dieldrin, organochlrorine pesticide, was detected in postmortem brain of PD patients at higher level than that in normal brains [8]. In experimental animals dieldrin caused PD-like symptoms, such as hypokinesia and tremor [14]. Our previous cDNA microarray analysis suggests that dieldrin may cause the neuronal apoptosis via mechanism(s) other than oxidative stress [4]. For instance, dieldrin induced the most prominent level of heme oxygenase-1 (HO-1) despite its production of low reactive oxygen species (ROS), in contrast to the significant ROS activities generated by mitochondrial complex I inhibitors, MPP^+ and rotenone. HO-1 is a known cellular marker for both endoplasmic reticulum (ER) stress and oxidative stress [19]. HO-1 is a constituent of Lewy bodies, a pathological hallmark of PD [24]. Moreover, an antioxidant treatment did not exert any significant neuroprotection against dieldin-induced neuronal cell death [4].

The ER is the site of protein quality control for proper synthesis, folding and maturation in the intracellular secretary pathway. The ER stress response causes an increase in ER chaperones, such as Grp94, BiP/Grp78, pDI, Erp72 and Erp57 by a complex signaling pathway from the ER to the nucleus [10]. Caspase-12 mediates the ER stress-specific apoptosis

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[20]. ER stress inducers, such as β-mercaptoethanol (reducing agent), tunicamycin (glycosylation inhibitor) and thapsigargin (inhibitor of ER Ca^{2+}-ATPase) commonly activate ER resident Caspase-12. Caspase 12-deficient mice are resistant to the ER stress-mediated apoptosis [20].

Deletion mutations in the parkin gene were initially identified as a cause of autosomal recessive juvenile PD in Japan [15]. Parkin protein is distributed in most brain regions and is located in large cytoplasmic vesicles, the ER and the Golgi complex [25]. However, it is unknown why parkin deficit affects primarily, nigral dopaminergic (DA) neurons in familial PD. From the functional study, parkin-deficiency resulted in the pathogenic accumulation of its unfolded substrate, parkin-associated endothelin receptor-like (Pael) receptor, and subsequent ER stress [6]. The present study demonstrated that dieldrin caused the DA cell-specific ER stress and the activation of the ER resident Caspase-12, followed by DA cell death, which was significantly rescued by the increased expression of the ER resident proteins, both pakin and Bcl-2.

Materials and Methods

Cell Culture and Chemical Treatment

The immortalized rat DA neuronal cell line, N27-A, was cultured as described previously [9]. Cells were maintained in RPMI 1640 medium that supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), penicillin-streptomycin and L-glutamine. Cells were usually grown to less than 80% confluence at 37℃ and in 5% CO_2. Usually, one day before any treatment, the culture medium was changed to medium with 0.5% FBS. All chemicals were obtained from Sigma (St. Louis, MO, USA). The doses for dieldrin and thapsigargin (Ca^{2+} ATPase inhibitor) were chosen primarily based on IC_{50} concentrations after 24 hr of incubation. Initial IC_{50} concentrations for rotenone and MPP^+ were established after 48 hr incubation according to our experimental N27-A culture conditions and previous primary mesencephalic culture conditions [4]. The concentrations used were 40 mM for dieldrin, 1 mM for thapsigargin, 1.5 mM for rotenone and 50 mM for MPP^+. Cytotoxic cell death was assessed by both the XTT reduction assay and Trypan blue exclusion.

Immunoblotting

Cells grown under various experimental conditions were washed twice with 1X PBS, lysed by adding 600 ml (per 100 mm plate) of RIPA buffer containing 1% NP40, 0.5% Na deoxycholate, 0.1% SDS, PMSF (100 mg/ml), aprotinin (30 mg/ml) and Na orthovanadate (1 mM). Further cell lysate preparation, electrotransfer to nitrocellulose membrane and detection of proteins by ECL chemiluminescence were done. The primary antibodies from the indicated sources are: HO-1 (1:4,000), BiP (1:2,000), Caspase-12 (1:1,000), Bcl-2 (1:1,000) and actin (1:2,000) (Santa Cruz Biotech., Santa Cruz, CA,USA), parkin (1:1,000) (Cell Signaling Technology, Beverly, MA, USA) and Flag (1:2,000; Sigma).

Caspase Assays

For the colorimetric assay, the specific substrate Ac-LEHD-pNA for Caspase-9, Caspase-3-specific substrate Ac-DEVD-pNA and necessary reagents were purchased as a assay kit from Biomol (Germany). Cleavage of a tetrapeptide substrate was monitored by increased absorption at 405 nm in a 96-well format plate reader. The cell lysates were prepared by using cell lysis buffer (50 mM HEPES, pH7.4, 0.1% CHAPS, imM DTT, 0.1 mM EDTA) as instructed by the company.

Transient Transfection Assay

N27-A cells were plated in culture medium one day before transfection so that cells reached 50 to 70% confluence on the day of transfection. Cells were transfected by using PolyFect reagent (Qiagen, Germantown, MD, USA). Each transfection mixture for a 96-well plate contained 0.5 mg of a DNA expression constructs (pFlag-CMV-4, pFlag-Parkin, and pFlag-Bcl-2) and 0.1 mg of pCMV-β-Gal, 30 ml of medium (without FBS and antibiotics) and 2.5 ml of PolyFect reagent and 150 ml of medium. After mixing, the total volume was transferred to cells in the 96-well plate and incubated for 2-3 hr at 37℃. Then, cells were washed once with PBS and added fresh culture medium without phenol red. Drugs, such as dieldrin or thapsigargin, were added after 24 hr of incubation and then, measured the cell viability with XTT reduction assay. A rat parkin cDNA and a human Bcl-2 cDNA were obtained from Dr. A. Brice (INSERM, France) and Dr. D. Vaux (Walter and Eliza Hall Institute, Australia), respectively [5, 21].

Statistical analysis

The data were analyzed using the GraphPad Prism data analysis program (GraphPad Software, Inc., San Diego, CA, USA). For the comparison of statistical significance between two groups, the Student's *t* test for paired and unpaired data was used. p values <0.05 were considered significant.

Results

Dieldrin Induces DA cell death and ER Stress Response

N27-A cell line is derived from N27 cells to improve the expression of DA neuronal markers [9]. Initial cell viability experiments were performed to evaluate the dieldrin-induced cytotoxicity. As shown in Fig. 1A, 40 mM dieldrin induced gradual cell death over 72 hr period. During dieldrin-mediated N27-A cell death a significant increase in Caspase-3 activation was accompanied with peaked at 48 hr after treatment (Fig. 1B). To investigate the possible role of ER stress during N27-A cell death, the induction of the ER stress makers, BiP and HO-1 [23], and the activation of the ER-specific apoptosis mediator, Caspase-12 [20] were examined. Dieldrin was compared with the mitochondrial complex I inhibitors, MPP^+ and rotenone. Thapsigargin was used as a prominent ER stress pharmacological agent, which also activated procaspase-12 [20]. As shown in Fig. 2, dieldrin caused the robust induction in HO-1 (Fig. 2B) and BiP/Grp78 (Fig. 2C), followed by the activation of Caspase-12 (Fig. 2A), but not Caspase-9 (Fig. 2F) during the apoptotic death of N27-A cells. Bcl-2 expression had no significant changes in our experimental conditions (data not shown). However, the mitochondrial complex I inhibitors, MPP^+ and rotenone, caused neither induction of BiP nor activation of Caspase-12. MPP^+ and rotenone activated Caspase-9, which mediates mitochondrial cytochrome c-mediated apoptotic pathway [17]. As expected, thapsigargin induced both HO-1 and BiP expression, which subsequently activated Caspase-12 in the N27-A cells (Fig. 2). However, thapsigargin did not activate Caspase-9 (data not shown). These results suggest a role of ER stress-mediated activation of Caspase-12 during dieldrin-induced DA cell death, but not during MPP^+ or rotenone-induced death.

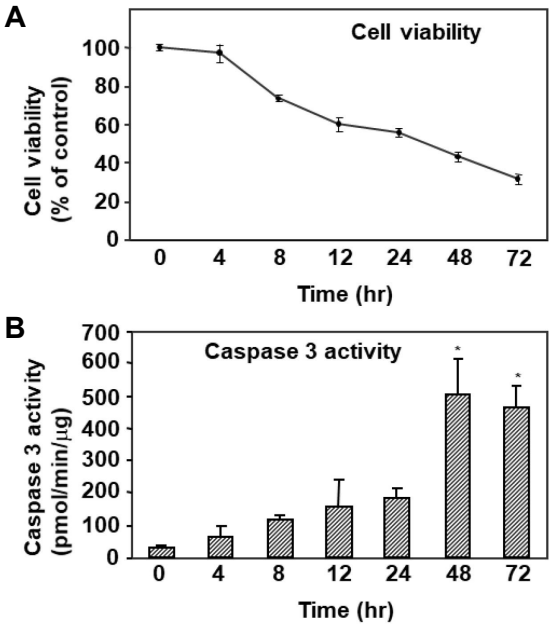

Fig. 1. Induction of apoptotic N27-A cell death by dieldrin, an organochlorine pesticide implicated in sporadic PD. N27-A cells were cultured in the presence of dieldrin (40 mM) treatment. Dieldrin induced significant cell death (~50%) within 24 hr. *In vitro* cytotoxic cell death was assessed by XTT reduction assay and Trypan blue exclusion for 72 hr (A). Activation of Caspase-3 activity induced by dieldrin was measured by colorimetric assay. Caspase-3 was a major downstream executioner caspase. During the cell death Caspase-3 activation was correlated with time-dependently (B). The experiments were performed in triplicate. All values are the mean ± SEM; $*p<0.05$.

Dieldrin Up-regulates Parkin in N27-A Cells

Parkin, an E3 ubiquitin protein ligase, is known to ubiquitinate its own, Pael receptor and other protein substrates [6]. The expression of parkin was reported to be up-regulated by ER stress inducers, such as tunicamycin and β-mercaptoethanol, in SH-SY5Y cells and to exert a partial protection against their toxicity [12]. Therefore, we determined whether dieldrin and thapsigargin can cause an up-regulation of parkin in N27A cells. Indeed, as shown in Fig. 3, parkin was significantly (approximately two-fold) up-regulated by both dieldrin and thapsigargin in N27-A cells. This finding implicates that parkin may have an important functional role during dieldrin-induced DA cell death as a part of ER stress response.

Both Parkin and Bcl-2 Synergistically Protect against Dieldrin-Induced N-27A Cell Death

Parkin was upregulated throughout the dieldrin-induced, ER stress-mediated cell death. The loss of parkin fuction mutations cause ER stress [26]. Thus, we hypothesized that the upregulated expression of parkin is a neuroprotective response against dieldrin-induced neurotoxicity. To test the hypothesis, the N27-A cells were transiently transfected with a mock vector, FLAG-Parkin [21], FLAG-Bcl-2 [5] or both in the presence of dieldrin. Bcl-2 was chosen because of its known anti-apoptotic effect as well as its subcellular abundance in ER besides mitochondria [16]. As shown in Fig. 4A, dieldrin-induced N27-A cell death was partially protected by the overexpression of either parkin (25.1%) or Bcl-2 (24.3 %). Significantly, the synergistic protective effect (43.4%) was exerted when both parkin and Bcl-2 were simultaneously

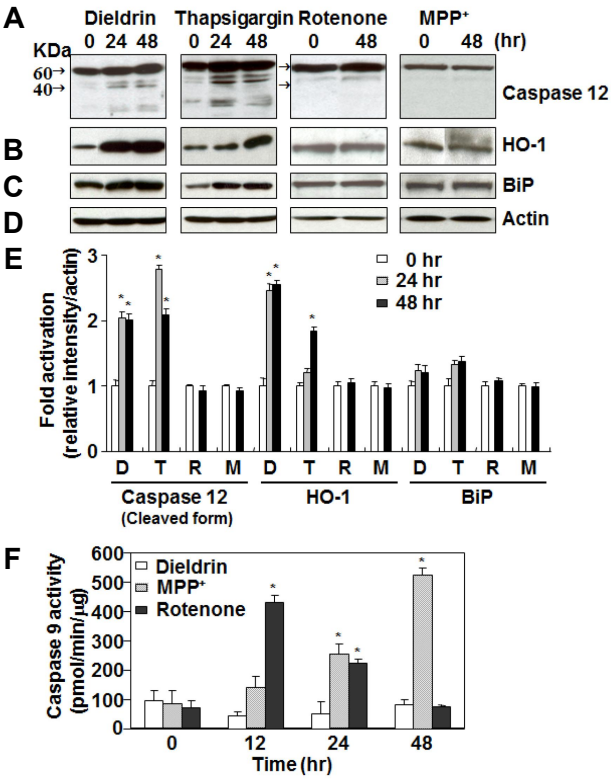

Fig. 2. Activation of Caspase-12 and induction of HO-1 and BiP during dieldrin-induced N27-A cell death. Lysates from N27-A cells were prepared under various treatment conditions with dieldrin (40 mM), thapsigargin (1 mM), rotenone (1.5 mM) or MPP^+ (50 mM) at 0, 24, 48 hr. The 20 mg of lysates were analyzed by immunoblot with antibodies against Caspase-12 (A), HO-1 (B), BiP (C) and actin (D). Both dieldrin and thapsigargin activated Caspase-12 (40 kDa) and induced HO-1 and BiP. However, the mitochondrial complex I inhibitors, MPP^+ and rotenone, neither activated Caspase-12 nor induced BiP and HO-1 under the experimental conditions. (E) The protein expression levels were quantified by densitometric analysis and expressed as fold activation. Actin expression was used for normalization. D: dieldrin; T: thapsigargin; R: rotenone; M: MPP^+. (F) Caspase-9, a mediator of mitochondrial cytochrome c apoptotic pathway, was significantly activated by MPP^+ and rotenone, but not by dieldrin. Thapsigargin did not activated Caspase-9 (data not shown). All values are the mean ± SEM of three independent experiments; *p<0.05, compared with untreated control (0 hr) in each group.

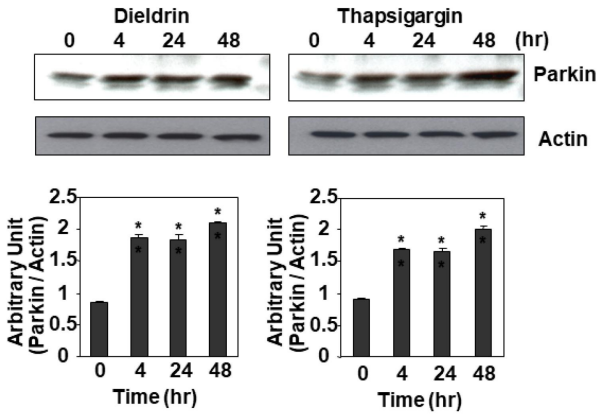

Fig. 3. Up-regulation of parkin by dieldrin and thapsigargin treatment in N27-A cells. To investigate the induction of parkin by dieldrin and thapsigargin in N27-A cells, the cells were harvested after 4, 24 and 48 hr treatments with dieldrin (40 mM) and thapsigargin (1 mM) and 20 mg of lysates were analyzed by immunoblot using parkin antibody. Densitometric scanning of the parkin signals demonstrated approximately 2-fold increase of parkin protein as compared with that of control (0 hr) by normalization to the actin signals. All values are the mean ± SEM for three independent experiments; *p<0.05.

overexpressed. Thapsigargin-induced toxicity was also significantly (21.7%) protected by both parkin and Bcl-2. This result suggests that both parkin and Bcl-2 might be useful for the protection of N27-A neurons against the ER stress-mediated neurotoxicity caused by either dieldrin or loss of parkin function mutations.

Discussion

The present findings provide biochemical evidences that ER stress response in DA neurons might be a common etiopathological mechanism between a specific environmental factor in sporadic PD and recessive parkin mutations in familial PD. Accumulation of ER stress (or unfolded protein stress) by either environmental exposure to peticide dieldrin or loss of parkin function mutations may lead to the same DA neuronal cell death in sporadic and familial PD, respectively. Significantly, the increased expression both parkin and Bcl-2 exerted a significant neuroprotection against the dieldrin-induced N27-A cell death.

A major criticism of the pesticide hypothesis related to sporadic PD is the lack of a molecular mechanism that account for the specific cell death of nigral DA neurons. As an initial attempt to elucidate the underlying molecular mech-

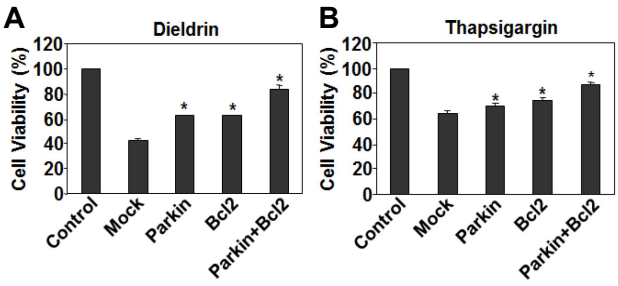

Fig. 4. Neuroprotection against dieldrin-induced N27-A cell death by parkin and Bcl-2. The N27-A cells were transiently transfected with mock vector (pFLAG-CMV4), Flag-Parkin, Flag-Bcl-2 or both in the presence of dieldrin as described in Materials and Methods. The appropriate expressions of the Flag-Parkin and Flag-Bcl-2 were confirmed by immunoblots (data not shown). (A) Dieldrin-induced cell death was partially protected by the parkin and Bcl-2. Significant synergistic protection was demonstrated when parkin and Bcl-2 were overexpressed simultaneously. (B) Thapsigargin-induced cell loss was significantly attenuated by parkin and Bcl-2 co-expression. The experiments were performed in triplicate. All values are the mean ± SEM; *$p<0.05$.

anism(s), we have addressed how the etiologically identified organochlorine pesticide, dieldrin, induces DA cell death, employing the characterized DA cell line, N27-A. The pesticide dieldrin is a very compelling antidote for the investigation of the "pesticide hypothesis" because epidemiological studies and animal and primary mesencephalic culture experiments suggest the relative selectivity in causing DA neuronal cell death of dieldrin [8, 14, 22].

The present study addressed key molecular issues using the in vitro DA cell line model, whether the parkin-related ER functional disturbance is a common pathogenic pathway between sporadic PD and rare familial PD. The selective neurotoxic action of MPP^+ is due to the cell type specific distribution of dopamine transporter in nigral DA neurons [29]. The pesticide rotenone is omnipotent complex I inhibitor, but it exerts a highly specific nigral DA neuronal cell death via unknown DA neuron-specific vulnerability [3]. However, MPP^+ and rotenone are not related to ER stress (Fig. 2). Only nigral DA neurons are significantly affected by ER stress in the recessive parkin gene mutation familial PD [6], despite the widespread expression of both parkin and Pael receptor in brain [15]. Parkin protein is distributed in various brain regions and several peripheral organs. Pael-receptor (or a putative G protein-coupled receptor) is highly expressed in most brain regions, spinal cord, testis, liver and placenta [7]. Therefore, it remains to be determined how the DA neuron-specific vulnerability occurs by ER stress in both these specific cases of sporadic and familial PD.

To further address the functional disturbance of parkin-related signaling during dieldrin-induced DA cell death, it was investigated i) whether parkin is upregulated by dieldrin as a part of ER stress response in DA neuronal cells and ii) whether the increased expression of parkin exerts any neuroprotection against the dieldrin-mediated ER-specific apoptosis. Previous study demonstrated that both parkin mRNA and protein was upregulated as a protective response by ER stress inducers, such as tunicamycin and β-mercaptoethanol, in SH-SY5Y cells [15]. Tunicamycin and β-mercaptoethanol triggered much weaker ER responses than dieldrin and thapsigargin (less than 20% levels of those by thapsigargin) in N27-A cells (data not shown). These results suggest that dieldrin-induced ER stress mechanism(s) share some common signaling with that of thapsigragin, but not those of tunicamycin and β-mercaptoethanol. This mechanistic issue needs to be further elucidated.

When the protective functional role of parkin was assessed during ER stress, parkin overexpression alone exerted significant protection against dieldrin-induced neurotoxicity in N27-A cells. Of interest, Bcl-2 exerted a synergistic neuroprotection with parkin against dieldrin-induced neurotoxicity. Bcl-2, abundant in ER, apart from mitochondria [16], has a known anti-apoptotic function [1], which is consistent with the present observation, demonstrating the anti-apoptotic effect of Bcl-2 against the dieldrin-induced ER stress. Previous study identified that ER Ca^{2+} homeostasis is maintained by Bcl-2 but the precise anti-apoptotic mechanism of Bcl-2 is yet to be elucidated [11]. Thapsigargin causes excessive Ca^{2+} release from the ER and rapid depletion of ER Ca^{2+} stores [28]. Both parkin and Bcl-2 exerted more prominent neuroproetction against dieldrin-induced toxicity than that of thapsigargin. Therefore, dieldrin might disturb not only the Ca^{2+} homeostasis, but also other essential signaling yet to be identified in DA neuronal cells. Recent studies suggested that ER-mitochondria crosstalk is exist and parkin (with PINK1) modulate the crosstalk between ER and mitochondria [2, 18]. Thus, additional study will be necessary to reveal more exact dieldrin/parkin-mediated molecular mechanisms and cellular processes.

In conclusion we have for the first time shown that ER stress could be a principal pathogenic mechanism other than oxidative stress in specific cases of sporadic PD. Moreover accumulation of ER stress in DA neurons might be a common etio-pathological mechanism between the pesticide dielrin-re-

lated sporadic PD and the excessive parkin mutation familial PD. Importantly, the increased level of both parkin and Bcl-2 demonstrated the significant neuroprotection against the dieldrin-induced DA neuronal cell death. Thus, further molecular characterization of both *in vitro* and *in vivo* action mechanisms of dieldrin and parkin in nigral DA neurons will help elucidate DA neuron-specific vulnerability to ER stress and develop a pathogenic mechanism-specific pharmacological intervention for both sporadic and familial PD.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 디엘드린 유도성 소포체 스트레스에서의 parkin과 Bcl-2의 신경보호 효과

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파킨슨병에서의 도파민 신경세포의 사멸 원인은 다양하며 별개의 유전적 요소와 환경적 요소들이 관여한다. 드물게 발생하는 유전성 파킨슨병에서 parkin의 돌연변이와 기능 상실은 주로 소포체 스트레스를 통해 중뇌 흑질의 도파민 신경세포를 특이적으로 손상시킨다. 상대적으로 일반적인 특발성 파킨슨병에서는 살충제 노출이 역학적으로 중요하다. 그러나 환경독성물질에의 노출과 유전성 파킨슨병의 연관성에 대해서는 잘 알려진 바가 없다. 본 연구에서는 잘 확립된 중뇌 유래의 도파민 신경세포주인 N27-A를 사용하여 특발성 파킨슨병과 유전성 파킨슨병 사이의 공통된 발병 기작의 증거를 확인하였다. 특발성 파킨슨병을 유발하는 유기염소계 살충제인 디엘드린은 BiP/Grp78, 헴산화효소-1과 같은 소포체 스트레스 반응 표지자를 발현 유도하였고, 특히 parkin 단백질의 발현을 증가시켰다. 디엘드린이 N27-A 세포를 사멸시키는 과정에서 소포체 스트레스 특이적 세포사를 매개하는 Caspase-12의 활성화가 유의미하게 증가하였다. 흥미롭게도 디엘드린에 의한 N27-A 세포의 사멸이 소포체 단백질인 parkin과 Bcl-2의 과발현시 유의미하게 억제되었다. 본 연구 결과, 소포체 스트레스의 누적이 특발성, 유전성 파킨슨병의 공통의 발병 기작일 가능성이 있으며, 몇 가지 소포체 관련 단백질들이 디엘드린에 의한 도파민 신경세포 손상으로부터 보호 효과를 가지는 것으로 보인다.