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Heptachlor induced mitochondria-mediated cell death via impairing electron transport chain complex III

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ABSTRACT

Environmental toxins like pesticides have been implicated in the pathogenesis of Parkinson's disease (PD). Epidemiological studies suggested that exposures to organochlorine pesticides have an association with an increased PD risk. In the present study, we examined the mechanism of toxicity induced by an organochlorine pesticide heptachlor. In a human dopaminergic neuroblastoma SH-SY5Y cells, heptachlor induced both morphological and functional damages in mitochondria. Interestingly, the compound inhibited mitochondrial electron transport chain complex III activity. Rapid generation of reactive oxygen species and the activation of Bax were then detected. Subsequently, mitochondria-mediated, caspase-dependent apoptosis followed. Our results raise a possibility that an organochlorine pesticide heptachlor can act as a neurotoxicant associated with PD.

1. Introduction

Parkinson's disease (PD) is a second most prevalent neurodegenerative disease characterized by prominent loss of dopaminergic neurons in the subtantia nigra pars compacta (SNpc) and the resulting movement deficits. It has been widely accepted that both genetic and environmental factors are important in the pathogenesis of PD [1]. The environmental factors implicated in the pathogenesis of PD include exposures to neurotoxins like 1-methyl-4-phenylpyridinium (MPTP) and 6-hydroxydopamine or pesticides [2]. Use of pesticides has been reported to be associated with idiopathic PD [3]. Although no specific agent has been implicated consistently, exposures to pesticides including paraquat, rotenone, and maneb have been suggested as a risk factor for PD [3].

Another class of pesticides implicated in sporadic PD pathogenesis is organochlorine compound [4]. Since organochlorine pesticides are very stable and lipophilic, they persist in the environment for decades and tend to bioaccumulate in the lipid rich organs [5]. It was reported that an elevated level of an organochlorine compound dieldrin was found in the post mortem brains of PD patients [6]. More recently, a case-control study reported that there is a correlation between the dieldrin level in the serum collected three decades before the diagnosis and a later development of PD [7]. These observations suggest a possible association between exposures to organochlorine pesticides and PD. An organochlorine pesticide heptachlor was shown to induce neurotoxicity on the striatal dopaminergic pathways in mice [8]. Heptachlor injection at a concentration range of 25–100 mg/kg resulted in the decrease of striatal synaptosome respiration rates but lower dose of injection increased the maximal rate of the striatal dopamine uptake [8]. The increased dopamine uptake can be attributed to the increase of dopamine transporter expression by heptachlor [9]. However, direct neurotoxicity on the dopaminergic neurons or its toxicity mechanism has not been addressed.

In the present study, we investigated whether heptachlor can induce dopaminergic neuronal death and the mechanism of its neurotoxicity in SH-SY5Y cells.

2. Materials and methods

2.1. Antibodies and reagents

Anti-active Bax antibody was purchased from Cell Signalling. Anti-cytochrome c antibody was from Millipore. Heptachlor was purchased from Supelco. Dichlorofluoresceindiacetate (DCF-DA), and MitoTracker Red 580 were from Invitrogen. Carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone (zVAD-fmk) was from Bachem. DPQ (3,4-Dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2*H*)-isoquinoline) was from Calbiochem. Cell culture media and supplements were from JBI. All other reagents were purchased from Sigma unless stated otherwise.

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2.2. Cell culture and viability assay

Human neuroblastoma SH-SY5Y cells were maintained in culture medium consisting of MEM and Ham's F12-K supplemented with 10% fetal bovine serum (JBI) and 1% antibiotics plus antimycotics. To monitor cell death, lactate dehydrogenase (LDH) release assay was performed using CytoTox®96 NON-Radioactive Assay kit (Promega) according to the manufacturer's protocol.

2.3. Immunocytochemistry

Immunostaining was performed by a conventional method. The samples were examined under a fluorescence microscope (Axioplan 2, Zeiss) or a confocal microscope (Leica TCSSP5, Leica).

2.4. Caspase activity assay

To examine enzyme activities of caspases, the cleavage of YVAD-amc, DEVD-amc, IETD-amc or LEHD-amc was monitored in the lysates of SH-SY5Y cells at indicated time points. The assay was carried out as described previously [10].

2.5. Measurement of ATP level

Cellular ATP levels were measured using Luminescence ATP Detection Assay System (PerkinElmer). Cells were incubated with drugs for various time points. Then 100 μl of the ATPlite 1 step reagent was added to the wells and the cell plates were orbitally shaken for 3 min. The plates were read for the emitted luminescence using a microplate luminometer (GloMax 9100-100, Promega).

2.6. Measurement of ROS

SH-Y5Y cells cultured in 24-well plates were treated with appropriate chemicals. After the treatment, cells were washed with Hank's balanced salt solution for two times and incubated with DCF-DA (5 μM) for 30 min. After medium was removed, the cells were washed with PBS and solubilized in 200 μl lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF). After incubation for 10 min on ice, the cells were harvested and transferred to a black 96-well plate. DCF fluorescence was read at λ_{ex} = 485 nm and λ_{exm} = 530 nm using a spectrofluorometer (SpectraMax Gemini EM, Molecular Devices).

2.7. Mitochondrial ETC complex activity assay

Mitochondria were prepared by Percoll density gradient centrifugation method as described by Sims [11]. Mitochondrial ETC complex activity was monitored spectrophotometrically as described by Tatarkova et al. [12]. Absorbance was monitored for the indicated time period before and after addition of specific inhibitors using a spectrophotometer (VersaMax, Molecular Devices). Relative activity of ETC complex was determined from the slope of the absorbance increase or decrease.

2.8. Statistics

For the statistical analysis, all the experiments were repeated at least three times. The results were expressed as means ± SD of at least three independent experiments, unless stated otherwise. The data were evaluated by student's *t*-test.

3. Results

3.1. Heptachlor induced caspase-dependent apoptosis in SH-SY5Y cells

To evaluate the neurotoxicity of heptachlor, SH-SY5Y cells were treated with varying concentrations of heptachlor and the viability of the cells was monitored. As shown in Fig. 1A, heptachlor induced about 50% cell death at a concentration of 40 μM at 18 h after the treatment. To examine the mode of cell death, the cells were preincubated with either a pan caspase inhibitor zVAD-fmk (100 μM) or a potent poly (ADP-ribose) polymerase-1 inhibitor DPQ (50 μM), and then incubated with heptachlor. As shown in Fig. 1A, the cell death induced by heptachlor was effectively suppressed by zVAD-fmk but not by DPQ, suggesting the heptachlor induced caspase-dependent apoptosis in SH-SY5Y.

To investigate which caspases are activated, the heptachlor-treated cells were monitored for different caspase substrates-cleaving activities at indicated times. The cell lysates prepared from the heptachlor-treated cells were incubated with fluorogenic tetrapeptide substrates of caspase-1, -3, -8, and -9 to monitor the changes in the activities of these caspases. As shown in Fig. 1B, the cleavage of LEHD, a substrate for caspase-9 [10], initially peaked at 1 h. Then DEVD-cleaving caspase-3 like activity started to rise at 4 h and remained high until 24 h. The cleavage of YVAD or IETD, a sign of the activation of caspase-1 or -8 respectively [10], did not increase throughout the heptachlor treatment. These results suggest that heptachlor induced apoptosis via an intrinsic

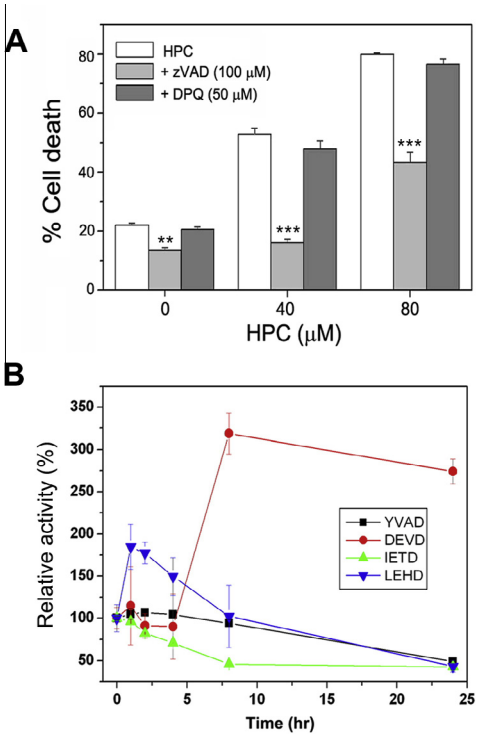

Fig. 1. Heptachlor induced caspase-dependent apoptosis. (A) SH-SY5Y cells were treated with increasing concentrations of heptachlor (HPC) and the viability of the cells was measured by LDH release assay at 18 h after the incubation. The cells were 1 h preincubated with either a pancaspase inhibitor zVAD-fmk (100 μM) or a potent poly (ADP-ribose) polymerase-1 inhibitor DPQ (50 μM) and then further incubated with heptachlor. Values represent mean ± SD for three independent experiments in duplicate. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. respective heptachlor only. (B) The heptachlor-incubated cells were monitored for different caspase substrate-cleaving activities. The cells were treated with heptachlor (80 μM) and the lysates were prepared at indicated time points for the incubation with YVAD-amc, DEVD-amc, IETD-amc, and LEHD-amc. Relative activities of caspases were calculated from the slope of the fluorescence increases (n = 3).

caspase cascade with sequential activation of caspase-9 and then caspase-3.

3.2. Heptachlor induced mitochondrial dysfunction and Bax activation

The sequential activation of caspase-9 and then caspase-3 suggests that heptachlor may induce mitochondria-mediated apoptosis. To examine whether heptachlor induced mitochondrial damage, mitochondrial morphology was first monitored using the mitochondrial dye MitoTracker following heptachlor treatment. As shown in Fig. 2A, heptachlor induced mitochondrial fragmentation as early as 2 h after the treatment. Many of the fragmented mitochondria in the heptachlor-incubated cells were round or donut-shaped whereas mitochondria in the vehicle-treated cells were filamentous or reticular, suggesting heptachlor induced mitochondrial damage. We then examined the changes in the cellular ATP level following heptachlor treatment. Fig. 2B shows that heptachlor induced a significant decrease in the cellular ATP level. At 8 h of heptachlor incubation, the level of cellular ATP reached about 50% of the control level.

It is known that Bax responds to death signals and is activated upon translocation and insertion onto the mitochondrial outer membrane, which promotes the intrinsic caspase-9-mediated apoptosis [13]. To examine whether Bax activation is involved in the heptachlor-induced cell death, the SH-SY5Y cells were incubated with heptachlor and then processed for the immunostaining using an antibody specific for the activated Bax. As shown and quantified in Fig. 2C, active Bax was detected as early as 1 h after heptachlor treatment, suggesting Bax activation may be an early event leading to the mitochondria-mediated apoptosis. To examine whether Bax activation is critical for the heptachlor-induced apoptosis, an effect of furosemide on the heptachlor-induced apoptosis was monitored. Furosemide is known to inhibit the mitochondrial translocation of Bax and subsequent Bax-mediated apoptosis induced by various stimuli [14]. Cells were 1 h pretreated with furosemide, incubated with heptachlor (80 μM) for another 12 h and then assayed for the LDH release. As shown in Fig. 2D, furosemide effectively suppressed the heptachlor-induced cell death, suggesting Bax activation is critical for the heptachlor-induced cell death.

3.3. Heptachlor inhibited the activity of mitochondrial ETC complex III

Previous studies showed that MPTP and rotenone can inhibit mitochondrial ETC complex I and thus exhibit their neurotoxicity [2]. To see if heptachlor can influence mitochondrial ETC complex activities, SH-SY5Y cells were incubated with heptachlor (80 μM) for 2 h and then mitochondria were isolated for the spectrophotometric measurement of mitochondrial ETC complex activities. As shown in Fig. 3A, mitochondria isolated from the heptachlor-incubated cells showed marked reduction in the complex III activity while other complex activities were spared. To further investigate whether heptachlor can act on the mitochondrial complex directly, mitochondria were isolated from mouse brain homogenates and the ETC complex activities were measured in the presence of heptachlor. As shown in Fig. 3B, heptachlor (40 μM) added into the assay buffer induced significant reduction in the complex III activity in accordance with the result obtained from the assay using the mitochondria isolated from the cells preincubated with heptachlor. The reduction of complex III activity induced by heptachlor was comparable to that induced by a known complex III inhibitor, antimycin A (100 nM). However, other complex activities were

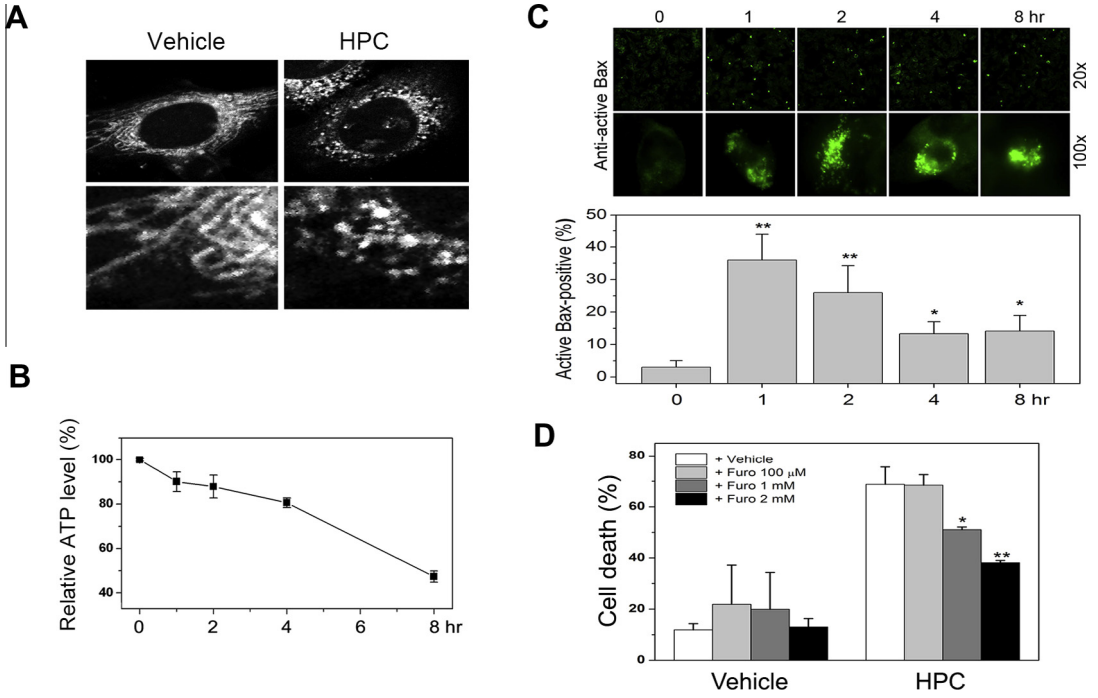

Fig. 2. Heptachlor induced mitochondrial dysfunction and Bax activation. (A) To examine if there is morphological changes in the mitochondria, heptachlor-treated SH-SY5Y cells (HPC, 80 μM, 2 h) were loaded with mitotracker (5 nM) for 30 min, washed and then fixed for the visualization of mitochondria. Note the fragmented mitochondria in the heptachlor-treated cells. Top panels, 40×; bottom panels, magnified from 100× photomicrographs. (B) Cellular ATP levels were measured by ATP-lite assay following heptachlor treatment (80 μM) at indicated time points. (C) To monitor Bax activation, the heptachlor-treated cells were immunostained with anti-active Bax antibody at indicated time points (top panels). Quantification of anti-active Bax-positive cells at indicated time is shown in the bottom panels ($n = 5$, $^{**}p < 0.01$, $^{*}p < 0.05$ vs. zero hour). (D) To examine the effect of inhibition of Bax activation, SH-SY5Y cells were 30 min pretreated with furosemide at indicated concentrations and then treated with heptachlor (80 μM) for 12 h. The cell viability was measured by LDH release assay. Data are expressed as mean ± SD ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. heptachlor only).

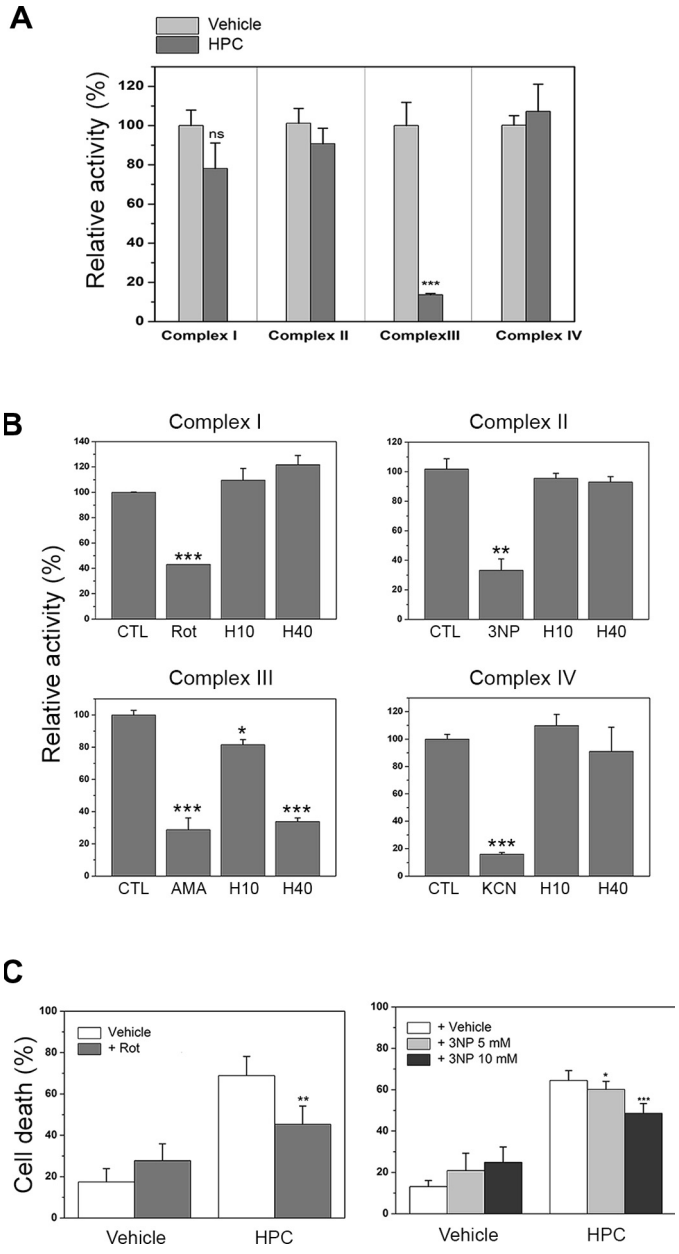

Fig. 3. Heptachlor inhibited the activity of mitochondrial ETC complex III. (A) To examine the effect of heptachlor on the mitochondrial ETC complex activities, SH-SY5Y cells were treated with heptachlor (HPC, 80 μM) for 2 h and then the mitochondria were isolated for the measurement of each complex activity by spectrophotometric reading (Mean ± SD, $n = 3$, $p < 0.001$ vs. vehicle). (B) Fresh mitochondria were isolated from mouse brain and the ETC complex activities were measured spectrophtometrically. Each complex activity of the isolated mitochondria incubated with heptachlor (H, 10 and 40 μM) or inhibitors was expressed as percentage of the vehicle-treated control (CTL) (mean ± SD, $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. CTL). Rot, rotenone at 2 μM; 3NP, 3-nitropropionic acid at 5 mM; AMA, antimycin A at 300 nM; KCN, potassium cyanide at 2 mM.C. To examine the effect of complex I or II inhibition on the cell death induced by heptachlor, the cells were 30 min pretreated with rotenone (Rot, 50 nM) or 3-NP (3NP, 5, 10 mM) and then treated with heptachlor (80 μM) for 12 h. Then the viability was measured by LDH release assay (mean ± SD, $n = 3$ each, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. heptachlor only).

unaffected by heptachlor addition. This result suggests that heptachlor can inhibit mitochondrial respiratory activity by exerting its effect on the complex III.

Previous studies showed that complex III is the major site of ROS production [15] and thus a blockade of electron flow to

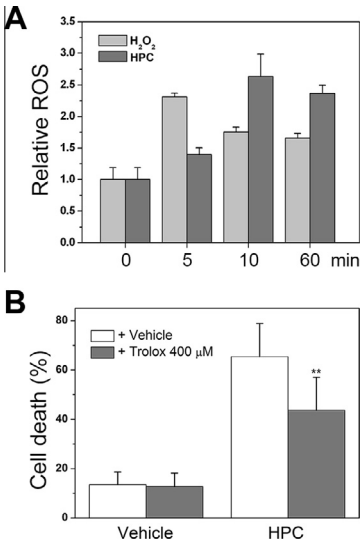

Fig. 4. Heptachlor promoted the generation of ROS. (A) To examine if heptachlor can promote ROS generation, SH-SY5Y cells were treated with heptachlor (HPC, 80 μM) or H_2O_2 (400 μM) and then the cellular level of ROS was measured spectrofluorometrically using DCF-DA (5 μM) at indicated times after the chemical treatment (mean ± SD, $n = 3$). (B) To examine if an antioxidant can inhibit the cell death induced by heptachlor, the cells were 30 min pretreated with Trolox (400 μM) and then incubated with heptachlor (80 μM) for 12 h. The viability was then measured by LDH release assay (mean ± SD, $n = 5$, $^{**}p < 0.01$ vs. heptachlor only).

complex III by complex I or II inhibition attenuated the ROS generated by complex III inhibitor [15–16]. To further test whether the complex III inhibition by heptachlor can be a causative event leading to mitochondria-mediated apoptosis, we examined the effect of complex I or II inhibition on the heptachlor-induced cell death. As shown in Fig. 3C, mitochondrial ETC complex I inhibitor rotenone (50 nM) or complex II inhibitor 3-nitropropionic acid (3-NP, 5 and 10 mM) significantly suppressed the heptachlor-induced cell death. Rotenone or 3-NP alone at these concentrations did not induce cell death significantly. These results suggest that heptachlor may induce neuronal death via inhibiting mitochondrial complex III inhibition and the resulting ROS production.

3.4. Heptachlor promoted the generation of ROS

Attenuation of heptachlor-induced cell death by inhibitors of complex I or II suggests that ROS generated by heptachlor-induced complex III inhibition may be critical for the initiation of cell death process. Thus we sought to examine whether heptachlor promotes the generation of ROS in SH-SY5Y cells. The cells were incubated with heptachlor for the indicated times and the level of cellular ROS was monitored using DCF-DA. As shown in Fig. 4A, heptachlor induced an elevation of cellular ROS level at 10 min after the treatment and the level of ROS remained elevated at 1 h. Addition of hydrogen peroxide induced a comparable level of elevation in cellular ROS with an early peak at 5 min.

To examine if the production of ROS by heptachlor is a critical event leading to apoptosis induced by heptachlor, we tested whether an antioxidant can inhibit the cell death. SH-SY5Y cells were preincubated with an antioxidant Trolox (400 mM) for 30 min and then further incubated with heptachlor for 8 h in the presence of the antioxidant. When the viability was measured, Trolox showed partial but significant protection against the heptachlor-induced toxicities. These results suggest that heptachlor can elevate cellular ROS, which may induce cell death.

4. Discussion

Exposure to pesticides has been consistently suggested to have a significant association with PD [3]. Although it remains to be determined whether pesticide exposure is a causative factor for PD, toxicological evidence from animal models suggests a possibility of causal relationship between pesticide exposure and PD [17]. Until recently, evidence was rather insufficient in epidemiological studies to conclude that specific pesticide compounds have an association with PD. However, recent studies reported that exposures to specific pesticides including organochlorines, paraquat, or manebmay have an association with development of PD [3]. Studies on the organochlorine pesticides as a potential PD-related neurotoxicant are less extensive compared to those on other pesticides. Our study clearly shows that heptachlor can induce Bax-mediated and caspase-dependent apoptosis in SH-SY5Y cells by inhibiting mitochondrial respiration at complex III and promoting ROS generation.

Mitochondrial dysfunction has been implicated in the pathogenesis of PD *in vivo* as well as *in vitro* [18]. Various neurotoxins associated with PD pathogenesis such as MPP^+, paraquat and rotenone have been reported to damage mitochondria by interfering with ETC complex [17]. Although the respiratory chain complex I is known as a major target of the mitochondrial toxins, the rest of the ETC complexes are also inhibited by the neurotoxins [2]. MPP^+ inhibits complex I, III, and V [19], paraquat inhibits complex III and IV [20], and maneb inhibits complex III [21]. Our data indicate that heptachlor inhibits mitochondrial ETC complex III (Fig. 3). Complex I and III of the ETC are the main sites of ROS production [22]. Studies have shown that inhibition of electron transfer upstream of complex III minimizes the ROS production at complex III [15]. Since the heptachlor-induced cell death was suppressed by inhibitors of complex I or II and an antioxidant, it is highly possible that heptachlor induced ROS production by inhibiting the complex III, which initiated the apoptotic cascade. The decrease in ATP level is not likely to be the cause of cell death since the level of ATP was 90% of the control when the activation of Bax and caspase-9 already peaked after the heptachlor treatment. Similarly, a previous study reported that various ETC inhibitors induced apoptosis that can be suppressed by antioxidant whereas ATP depletion by oxidative phosphorylation inhibitors failed to induce apoptosis [23]. Another study showed that anti-apoptotic Bcl-2 suppressed apoptosis induced by a complex III inhibitor antimycin A without restoring cellular ATP level [24], further supporting that ATP depletion is not a major cause for the cell death following complex III inhibition by heptachlor.

Furthermore, time course of the observed events also suggests that the ROS production is the proximal event following heptachlor treatment. The increase in the ROS reached the maximal level as early as 10 min (Fig. 4A), then the activation of Bax and caspase-9 followed at around 1 h (Fig. 1B and 2C).

Activation of Bax following mitochondrial ETC inhibition has been reported in many studies using rotenone, antimycin A [25] and malonate [14]. A possible mechanism of Bax activation following ETC complex inhibition has been suggested as ROS-mediated JNK or p38MAPK activation [14]. However, inhibitors of neither JNK nor p38 suppressed the heptachlor-induced cell death (data not shown), suggesting that Bax activation in heptachlor-induced cell death signaling involves a different mechanism that remains to be studied.

Taken together, our results suggest that heptachlor can be a PD-promoting neurotoxicant since it induced mitochondria-mediated apoptosis via imparing mitochondrial ETC activity and inducing oxidative stress.

Acknowledgments

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