

2, 3, 7, 8-Tetrachlorodibenzo-P-Dioxin (TCDD) Induces Premature Senescence in Human and Rodent Neuronal Cells via ROS-Dependent Mechanisms

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Abstract

The widespread environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a potent toxicant that causes significant neurotoxicity. However, the biological events that participate in this process remain largely elusive. In the present study, we demonstrated that TCDD exposure triggered apparent premature senescence in rat pheochromocytoma (PC12) and human neuroblastoma SH-SY5Y cells. Senescence-associated β -galactosidase (SA- β -Gal) assay revealed that TCDD induced senescence in PC12 neuronal cells at doses as low as 10 nM. TCDD led to F-actin reorganization and the appearance of an alternative senescence marker, γ -H2AX foci, both of which are important features of cellular senescence. In addition, TCDD exposure altered the expression of senescence marker proteins, such as p16, p21 and p-Rb, in both dose- and time-dependent manners. Furthermore, we demonstrated that TCDD promotes mitochondrial dysfunction and the accumulation of cellular reactive oxygen species (ROS) in PC12 cells, leading to the activation of signaling pathways that are involved in ROS metabolism and senescence. TCDD-induced ROS generation promoted significant oxidative DNA damage and lipid peroxidation. Notably, treatment with the ROS scavenger N-acetylcysteine (NAC) markedly attenuated TCDD-induced ROS production, cellular oxidative damage and neuronal senescence. Moreover, we found that TCDD induced a similar ROS-mediated senescence response in human neuroblastoma SH-SY5Y cells. In sum, these results demonstrate for the first time that TCDD induces premature senescence in neuronal cells by promoting intracellular ROS production, supporting the idea that accelerating the onset of neuronal senescence may be an important mechanism underlying TCDD-induced neurotoxic effects.

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Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) represents one of the most notorious environmental toxicants and is known to accumulate in both the environment and the human body. One of the important public health concerns related to TCDD is its adverse effect on the neural system. Recent studies have demonstrated that TCDD causes significant neurodevelopmental and neurobehavioral deficits in rodents [1,2]. Consistent with these observations, epidemiological investigations have indicated that accidental exposure to high doses of PCB/TCDD mixtures results in delayed motor development and a higher incidence of hypotonicity in children [3]. In addition, the incidence of many neurological disorders, including sleep disturbances, neuralgia and headache, was markedly elevated in workers that had been accidentally exposed to TCDD [4]. These findings suggest that TCDD may lead to significant neurotoxicity in both humans and rodents.

The majority of the toxic effects of TCDD are related to the role it plays in activating the aryl hydrocarbon receptor (AhR) [5]. The

AhR is a ligand-activated transcription factor that normally exists in a quiescent state in the cytoplasm. Once it has bound to TCDD, the AhR will rapidly translocate into the nucleus and promote the transcription of dozens of target genes. The expression of these genes further activates downstream events that promote the toxic effects of TCDD. In the process, the production of reactive oxygen species (ROS) is regarded as one of the major features underlying TCDD-mediated AhR activation and is believed to be a key determinant of TCDD-induced neurotoxicity [6]. Thus, a better understanding of the role of ROS in mediating neurotoxicity may help clarify the mechanisms underlying TCDD-mediated adverse effects on the neural system.

Decades ago, replicative senescence/permanent cell cycle arrest was identified to be an important mechanism controlling normal cell proliferation and organismal aging. Replicative senescence is a physiological state during which dividing cells gradually lose the ability to proliferate and is accompanied by distinctive morphological changes and the altered expression of senescence-specific markers [7]. Later studies suggested that numerous stimuli, such as ROS, DNA damage, cytokines and oncogenic activation, could

dramatically accelerate the process of cellular senescence, termed stress-induced premature senescence [8]. Premature senescence induced by stress-related conditions has been suggested to play a crucial role in the pathology of various human diseases, such as cancer and neurodegenerative diseases [9,10]. Thus, premature senescence triggered by unfavorable stress-related agents may play a crucial role in the development of several types of human diseases.

Despite the evident toxic effects of TCDD on the neural system, its impact on neuronal cells remains largely elusive. While our studies and some recent reports indicated that cultured neuronal cells underwent rapid apoptosis when exposed to relatively high doses (200–1000 nM) of TCDD, this effect is believed to represent an acute toxic response rather than a common consequence of TCDD-mediated neurotoxicity [11,12,13,14]. In addition, exposure to a much lower dose of TCDD reportedly caused marked ROS accumulation in cultured primary neurons (0.1–10 nM) and brain tissues (46 ng/kg/day), although the precise physiological effects remain largely unknown [15,16]. Because ROS are potent inducers of premature senescence, we speculated that TCDD-induced ROS production may trigger senescence in neuronal cells. We demonstrated that TCDD strongly triggered senescence in neuronal-like cells by promoting ROS accumulation. Our findings may provide greater insight into the mechanisms underlying TCDD-induced neurotoxicity.

Results

TCDD induces premature senescence in differentiated PC12 neuronal cells in both dose- and time-dependent manners

To explore whether TCDD induces neuronal senescence, NGF-differentiated rat pheochromocytoma PC12 neuronal cells were employed as a model. After NGF-induced differentiation, PC12 cells were treated with different doses of TCDD (0, 1, 10, 50 and 100 nM) for 72 h and were then subjected to a senescence-associated β -gal (SA- β -Gal) assay. As shown in Fig. 1A and 1B, the rate of SA- β -Gal staining after TCDD treatment was significantly elevated in a dose-dependent manner. Furthermore, we treated PC12 cells with 50 nM TCDD and analyzed the time-dependency of TCDD-induced SA- β -Gal staining. PC12 cell senescence began approximately 48 h after TCDD exposure and reached a maximum at approximately 72 h (Fig. 1A and 1B).

Despite the fact that SA- β -Gal activity has been widely used as a classical marker of senescence, studies have implied that SA- β -Gal activity may become elevated under conditions that are independent from senescence [7,17]. Thus, we further analyzed whether other senescence markers could be observed in TCDD-exposed PC12 cells. Because senescent cells exhibit dramatic alterations in cell morphology and F-actin assembly, we determined whether changes in F-actin organization could be observed after TCDD exposure. Indeed, we found that TCDD treatment resulted in altered stress fiber distribution (Fig. 1C). Furthermore, the formation of γ -H2AX foci, an alternative senescence marker that has been visualized in aging neurons, was initiated in PC12 cells after TCDD exposure (Fig. 1D) [18,19]. Because TCDD has been reported to induce neuronal apoptosis, we analyzed the levels of active caspase-3 in PC12 cells that had been exposed to different doses of TCDD [11,12]. Consistent with previous reports, TCDD induced apoptosis in PC12 cells at a relatively high concentration (approximately 300 nM), which was distinct from the dose range that induced a senescence response (Figure S1). Taken together, these results demonstrated that TCDD could induce significant premature senescence in PC12 cells.

Altered expression of senescence marker proteins in PC12 cells following TCDD exposure

Next, we analyzed the expression of senescence markers after treatment with different doses of TCDD for 72 h. As shown in Fig. 2A and 2B, both the mRNA and protein levels of p16 were elevated in TCDD-exposed PC12 cells in a dose-dependent manner. In addition, the levels of p21, another important marker protein for senescence, were detected and found to be altered in a similar manner to p16 (Fig. 2C). The expression of the phosphorylated form of Rb protein (p-Rb) was found to be decreased following TCDD exposure. Moreover, the time-dependency of the expression of senescence marker proteins was also analyzed. As compared to DMSO-treated control cells, the expression of p16, p21 and p-Rb in TCDD-treated PC12 cells was significantly altered from the 72 h time point on (Fig. 3). These findings suggested that TCDD exposure significantly influenced the expression of senescence marker proteins in PC12 neuronal cells.

Altered expression profiles of cell phenotype-specific genes following TCDD exposure

We then attempted to clarify the molecular alterations underlying TCDD-triggered neuronal senescence. A number of ROS, senescence, apoptosis and autophagy-related genes were probed for expression alterations after a 50 nM TCDD exposure for 72 h using real-time PCR analysis (Fig. 4). The mRNA expression of many genes involved in ROS metabolism and cell senescence, such as SOD-1, SOD-2, GPX, FOXO1, FOXO3a and p27, was found to be significantly altered after TCDD exposure. However, the expression of autophagy-related genes, including Beclin-1 and ATG5, remained unchanged. Furthermore, although some of the altered genes, such as FOXO transcription factors, p27 and p53, also played critical roles in the regulation of apoptosis, the expression of Bax and Bcl-2 was not substantially altered. More importantly, the increased expression of the genes was mostly attenuated by treatment with the ROS scavenger N-acetylcysteine (NAC). Thus, these data indicated that TCDD exposure could dramatically alter the expression of ROS- and senescence-associated genes in an ROS-dependent manner, implying that ROS may be involved in TCDD-induced PC12 senescence.

TCDD induces mitochondrial ROS production in PC12 cells

Because ROS are considered to be key players in the process of premature senescence, we examined whether ROS production was involved in the occurrence of senescence in PC12 cells. We measured total ROS levels using a DCFH fluorescence assay in TCDD-exposed PC12 cells. As shown in Fig. 5A and 5B, the levels of total ROS fluorescence increased significantly after 50 nM TCDD treatment compared to the DMSO-treated control group.

TCDD-mediated ROS generation has been documented to be primarily related to mitochondrial dysfunction [20,21,22]. We therefore examined whether TCDD could lead to impaired mitochondrial function in PC12 cells. The mitochondrial inner membrane potential was evaluated using the cationic lipophilic dye JC-1, through which we confirmed that TCDD markedly decreased the mitochondrial membrane potential in PC12 cells (Fig. 5B). Furthermore, the mitochondria of control and TCDD-exposed PC12 cells were isolated and subjected to a H₂O₂ production assay using succinate as a substrate. As shown in Fig. 5C, the levels of H₂O₂ production were dramatically elevated in the mitochondria after TCDD exposure. These findings

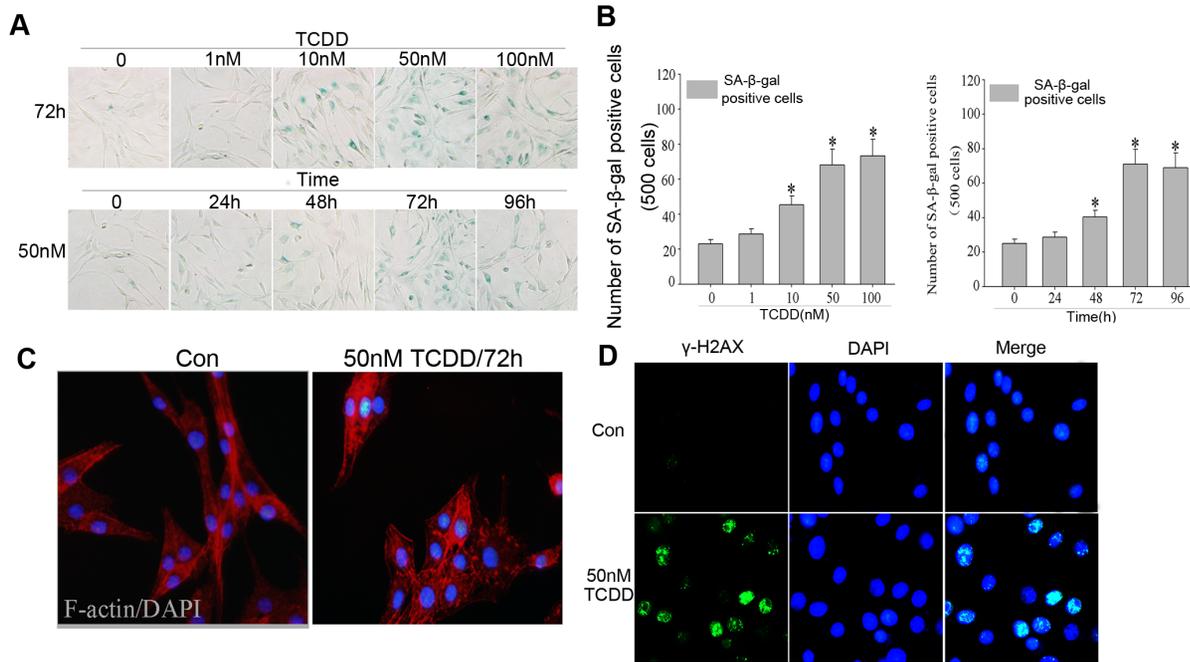


Figure 1. TCDD induces premature senescence in PC12 neuronal cells. (A) Upper panel: PC12 cells were treated with DMSO or 1, 10, 50 and 100 nM TCDD for 72 h and then subjected to an SA- β -Gal staining assay. Lower panel: PC12 cells were treated with 50 nM TCDD for 0, 24, 48, 72 and 96 h and then subjected to an SA- β -Gal staining assay. (B) The number of SA- β -Gal positive cells in each group from Fig. 1A was counted and listed (* $p < 0.05$, significantly different from the DMSO-treated group). (C) PC12 cells were treated with DMSO or 50 nM TCDD for 72 h and then immunostained with FITC-phalloidin to visualize F-actin. (D) PC12 cells were treated with DMSO or 50 nM TCDD for 72 h and were immunostained with a γ -H2AX antibody.

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suggested that mitochondrial dysfunction was involved in TCDD-induced ROS production.

The ROS scavenger NAC abolished TCDD-induced oxidative damage

We analyzed whether ROS accumulation was responsible for TCDD-induced oxidative damage and neuronal senescence. The ROS scavenger NAC was used to eliminate cellular ROS. As shown in Fig. 6A and 6B, pre-incubation with NAC blocked TCDD-induced ROS accumulation in PC12 cells. Reactive free oxygen species often damage many cellular organelles and biomolecules, such as the endoplasmic reticulum, lipids, proteins and genomic DNA. Indeed, immunofluorescence analyses revealed that the formation of 8-oxo-dG, a stable product of oxidative DNA damage, was dramatically elevated in PC12 cells after 50 nM TCDD exposure for 72 h and was abolished by treatment with the ROS scavenger NAC (Fig. 6C). Similarly, TCDD increased the levels of lipid oxidation in an ROS-dependent manner (Fig. 6D). These findings validated ROS generation as a key step in TCDD-induced adverse effects in neuronal cells.

The ROS scavenger NAC attenuated TCDD-triggered neuronal senescence

Because our results suggested that mitochondrial ROS generation was a critical toxic response in TCDD-exposed PC12 cells, we next examined whether treatment with NAC abolished TCDD-induced premature senescence in NGF-differentiated PC12 cells. As shown in Fig. 7A and 7B, treatment with NAC

significantly reduced the number of SA- β -Gal positive cells following TCDD treatment. NAC application also attenuated TCDD-induced elevations in p21 and p16 expression (Fig. 7C and D). Taken together, these data indicated that ROS exerted a crucial role in TCDD-triggered oxidative damage and premature senescence in PC12 cells.

TCDD induces premature senescence in human neuroblastoma SH-SY5Y cells in an ROS-dependent manner

Because the aforementioned studies were performed entirely using rat pheochromocytoma PC12 cells, we were interested in determining whether similar observations could be made in human neuronal cells. Thus, a human neuroblastoma cell line, SH-SY5Y, was employed for these analyses. After challenge with different doses of TCDD for 72 h, SH-SY5Y cells exhibited a senescence response at a starting dose of approximately 10 nM TCDD (Fig. 8). NAC application significantly ameliorated the TCDD-induced senescence phenotype. Consistent with these results, the levels of p21 and p16 were markedly elevated after a 50 nM TCDD treatment and were impaired by the addition of NAC (Fig. 8). Conversely, the expression of p-Rb was decreased in TCDD-exposed PC12 cells and was rescued by NAC treatment. These findings indicated that TCDD induced a similar senescence response in human neuronal cells to that observed in rat neuronal cells.

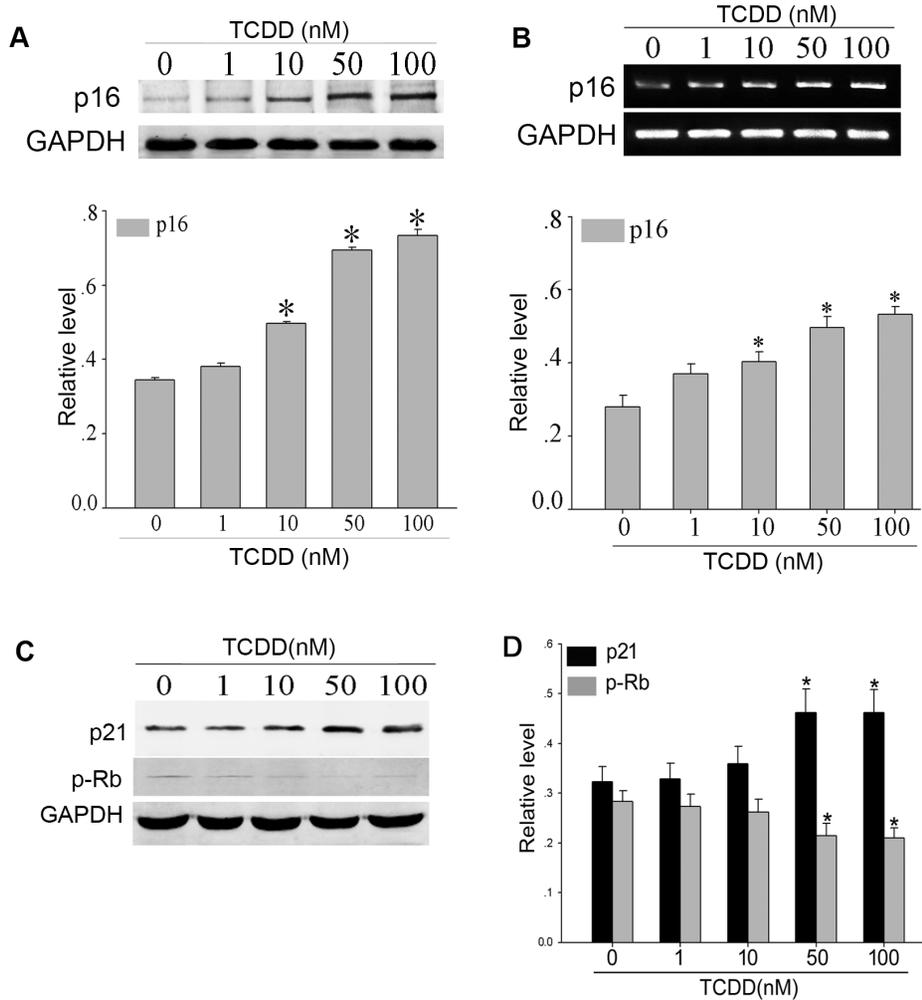


Figure 2. TCDD induced the expression of senescence marker proteins in a dose-dependent manner. PC12 cells were exposed to different doses of TCDD for 72 h. The cells were then harvested and subjected to western blot (A) and semi-quantitative PCR (B) analyses to determine the protein and mRNA levels of p16 (* $p < 0.05$, significantly different from the DMSO-treated group). (C) The expression of p21 and p-Rb was also evaluated using a western blot analysis (* $p < 0.05$, significantly different from the DMSO-treated group). doi:10.1371/journal.pone.0089811.g002

Discussion

The free radical theory of aging regards ROS-mediated DNA damage as an important cause of cellular senescence and human aging [23,24]. Consistent with this hypothesis, recent studies have demonstrated that ROS accumulation and oxidative DNA damage contribute to premature senescence in a number of cell types [25,26]. In the present study, we reported for the first time that TCDD exposure-induced ROS production and oxidative DNA damage strongly stimulate premature senescence of neuronal-type cells, suggesting that ROS-mediated senescence may be an important mechanism underlying TCDD-induced neurotoxicity.

The neurotoxic effects of TCDD remain largely elusive. Although TCDD does not efficiently cross the blood-brain barrier (BBB) and is metabolized slowly in brain tissues shortly after exposure, subchronic exposure to TCDD may cause toxic responses in cerebral vascular endothelial cells and astrocytes, which may eventually result in blood-brain barrier deficits [27,28]. In addition, compared to hepatocytes, brain cells are more sensitive to TCDD exposure [29]. TCDD exposure has been

linked to various alterations in the mammalian CNS and neuronal cells, such as ROS production, elevated intracellular calcium levels, tau phosphorylation and activation of PKC signaling, although the detailed biological processes underlying these molecular alterations remain unclear [30,31]. While some reports have indicated that TCDD decreases neuronal viability and induces neuronal apoptosis, the concentrations that were assayed in those studies were relatively high compared with those examined in other cell types. TCDD has been reported to induce significant apoptosis in human breast carcinoma cells at concentrations as low as 1 nM [32]. The dose range of TCDD that induces apoptotic cell death in some other cell types, such as JAR cells and human granulosa cells, also varies between 1–10 nM [33,34]. In contrast, 100 nM TCDD exposure did not cause significant LDH release in cerebellar granule cells [31]. The neuronal apoptotic responses that were observed in some other studies also emerged at concentrations greater than 100 nM TCDD [12,35]. The results of the present study indicated that human and rodent neuronal cells effectively underwent premature senescence after exposure to TCDD concentrations less than 10 nM, levels that were comparable to the TCDD doses that were

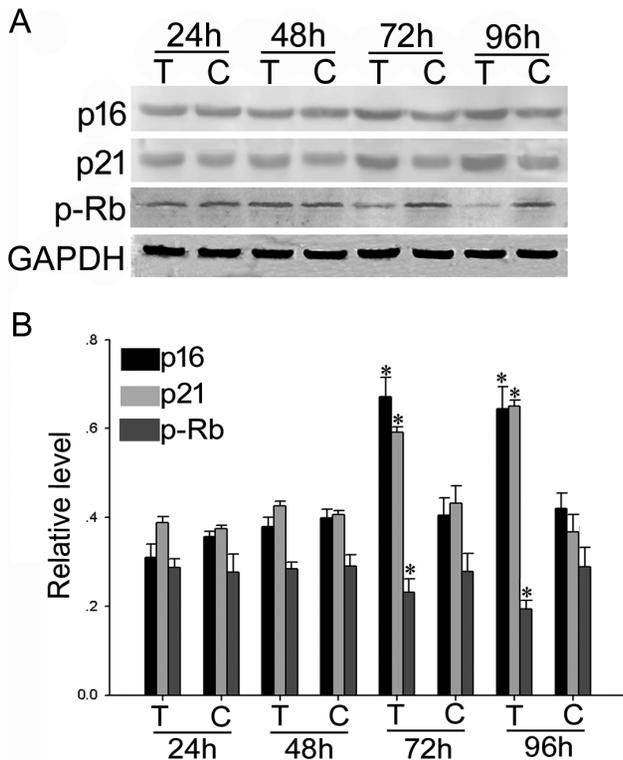


Figure 3. Time-dependency of senescence marker protein expression after TCDD exposure. (A) PC12 cells were exposed to 50 nM TCDD for 24, 48, 72 or 96 h and then harvested for western blot analyses using anti-p16, anti-p21 and anti-p-Rb antibodies. T, TCDD-treated cells; C, control group. (B) Quantitative analysis of the intensity of protein expression relative to GAPDH in the indicated groups (*, # and ^ $p < 0.05$, statistically significant difference from the control group). doi:10.1371/journal.pone.0089811.g003

applied in non-neuronal cells. Taken together, these findings indicated that premature senescence, rather than apoptosis, may be a major toxic response that occurs in neuronal cells following TCDD exposure.

Although numerous studies of cellular apoptosis and proliferation have been conducted, the roles of TCDD and the AhR in modulating senescence have been less extensively investigated. AhR-null MEFs exhibited premature onset of growth cessation and accelerated entry into senescence [36]. Furthermore, TCDD and the AhR have been found to function as negative regulators of senescence by repressing p53 and p16 in keratinocytes [37,38]. However, this type of regulation of p53 has not been observed in PC12 cells (data not shown). TCDD exposure has been found to result in growth arrest in neuronal cells and neural progenitor cells [39,40,41]. Thus, the effects of TCDD on cell proliferation may vary in different cell lineages and are prone to an inhibitory role in neuronal-type cells. Due to the long half-life of TCDD in human organs, TCDD may cause profound alterations in cell proliferation and the expression of cell cycle regulators, providing the opportunity for premature cellular entry into senescence. In addition, given the fact that TCDD has more obvious toxic effects on the developing neuronal system and the fact that TCDD exposure promotes growth arrest in neural progenitor cells, TCDD may also elicit a senescence-promoting function in neural progenitor cells. Further investigation may clarify the mechanisms underlying TCDD-induced neurotoxicity.

ROS and ROS-related signaling pathways have been strongly implicated to play a role in cellular senescence. ROS accumulation has been suggested to be responsible for inducing cellular senescence following a variety of stimulatory processes, including oncogenic activation and TGF- β [42,43]. The generation of ROS is a key step underlying p21-induced senescence, while eliminating ROS strongly abolishes p21-mediated senescence [44]. Recently, p21, ROS and DNA damage responses were reported to form a positive feedback loop to elicit cellular senescence, highlighting the

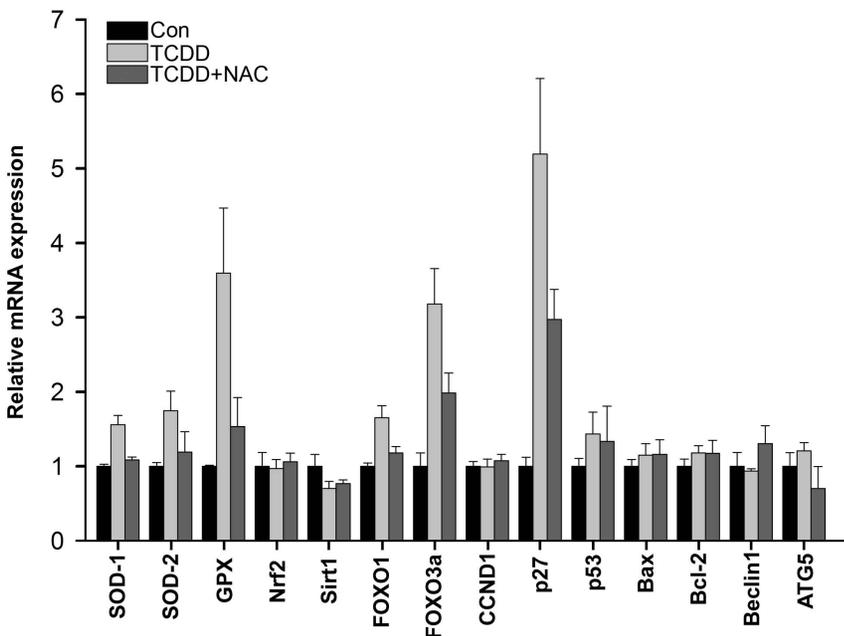


Figure 4. Determination of the mRNA expression levels of phenotype-related genes after TCDD exposure. PC12 cells were treated with DMSO, 50 nM TCDD, or 50 nM TCDD combined with NAC for 72 h. The total mRNA of the cells was then extracted and reverse-transcribed into cDNA. The cDNAs were subjected to real time PCR analyses to detect the relative expression levels of the indicated genes. doi:10.1371/journal.pone.0089811.g004

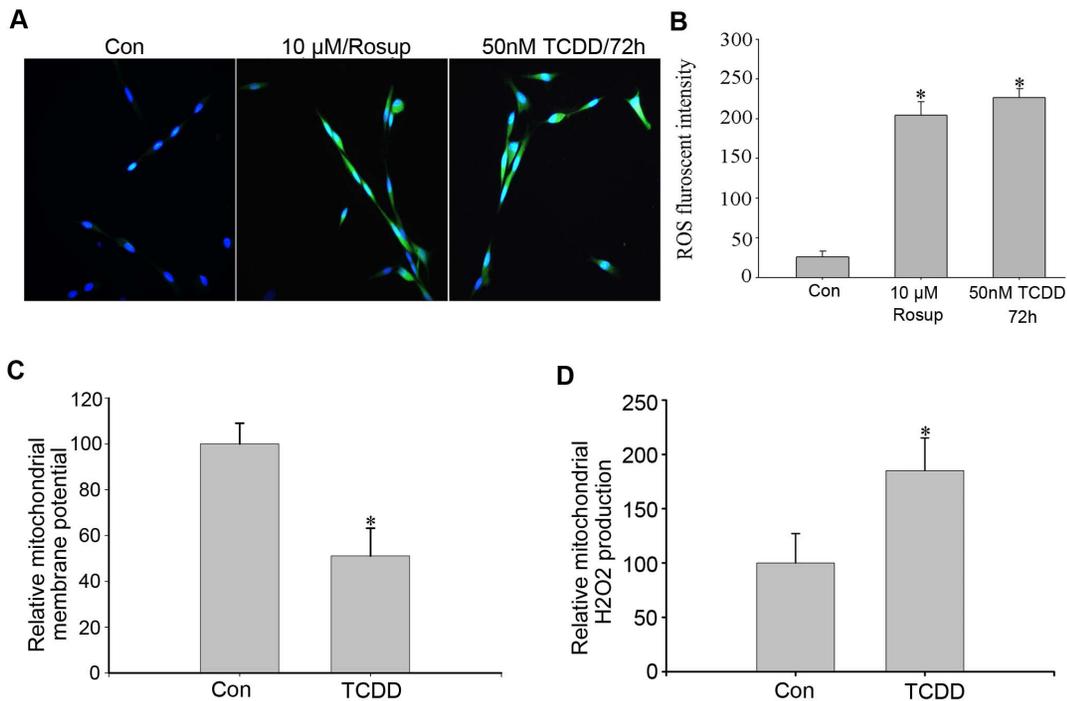


Figure 5. TCDD induces ROS accumulation and DNA damage in PC12 cells. (A) PC12 cells were treated with DMSO or 50 nM TCDD for 72 h. The cells were then stained to examine ROS fluorescence and visualized under a fluorescence microscope. Rosup (10 μmol/L) was used as a positive control. (B) The level of ROS fluorescence in each group was determined using a flow cytometric analysis (* $p < 0.05$, statistically significant difference from the control group). (C) PC12 cells treated with DMSO or 50 nM TCDD for 48 h were analyzed for relative mitochondrial membrane potential using JC-1 fluorescence (* $p < 0.05$, statistically significant difference from the control group). (D) Determination of mitochondrial H₂O₂ production in DMSO or 50 nM TCDD-treated PC12 cells and assayed for H₂O₂ production using succinate as a substrate (* $p < 0.05$, statistically significant difference from the control group). doi:10.1371/journal.pone.0089811.g005

fact that ROS act as fundamental mediators of senescence [45]. Remarkably, mature neurons, which were considered to have permanently exited the cell cycle, also underwent senescence, which was accompanied by increased levels of p21, high ROS production and activation of DNA damage responses, suggesting that neurons exhibit a similar senescence response to proliferative-competent cells [19]. In addition, because ROS production has been directly linked to mitochondrial dysfunction, changes in mitochondrial function may also contribute to TCDD-induced premature senescence. The results of our study demonstrated that TCDD could induce neuronal senescence that was accompanied by rapid ROS production and the occurrence of various ROS-related events, pointing to the pivotal involvement of ROS production in the promotion of cellular senescence. Consistent with these findings, the application of the ROS scavenger NAC attenuated ROS-related alterations and the TCDD-induced senescence response in neuronal cells. Therefore, coinciding with the notion that ROS are key players in premature senescence, our findings indicated that ROS may play vitally important roles in TCDD-induced neuronal senescence. However, much remains unclear regarding the molecular mechanism underlying TCDD-triggered neuronal senescence and the precise role of ROS in this process. Recent studies have suggested that ROS promote the activation of multiple related signaling pathways, such as mTOR signaling, FOXO transcription factors and DNA damage responses, which may also play a causative role in TCDD-induced premature senescence [18,46,47]. In addition to ROS, mTOR activation has been proposed to be an alternative route underlying cellular senescence and human aging [48,49]. Thus, it may be

important to examine whether alterations in these signaling pathways contribute to TCDD-induced neuronal senescence.

In summary, we reported for the first time that the neurotoxic mechanism of action of TCDD may involve the acceleration of neuronal senescence via ROS generation. Because TCDD exposure can lead to significant ROS accumulation in neuronal tissues, the long-term impact of TCDD intake on the onset of neurological aging and neurodegenerative diseases should be carefully assessed.

Materials and Methods

Cell culture, differentiation and TCDD treatment

Human neuroblastoma SH-SY5Y cells and rat pheochromocytoma PC12 cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science. SH-SY5Y cells were cultured using a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) and F12 (Gibco, Grand Island, NY, USA) that was supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, Logan, UT, USA). PC12 cells were grown in DMEM (Gibco, Grand Island, NY, USA) that was supplemented with 10% (v/v) FBS (Hyclone, Logan, UT, USA), 2 mM glutamine (Sigma, St. Louis, MO, USA), 100 units/ml of penicillin and 100 μg/ml of streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. PC12 cells were pre-incubated with DMEM containing 1% FBS and then differentiated using 100 ng/ml of NGF human recombinant nerve growth factor (hrNGF, R&D Systems, Minneapolis, MN, USA) for 5 days before they were subjected to TCDD exposure. The medium was changed every other day.

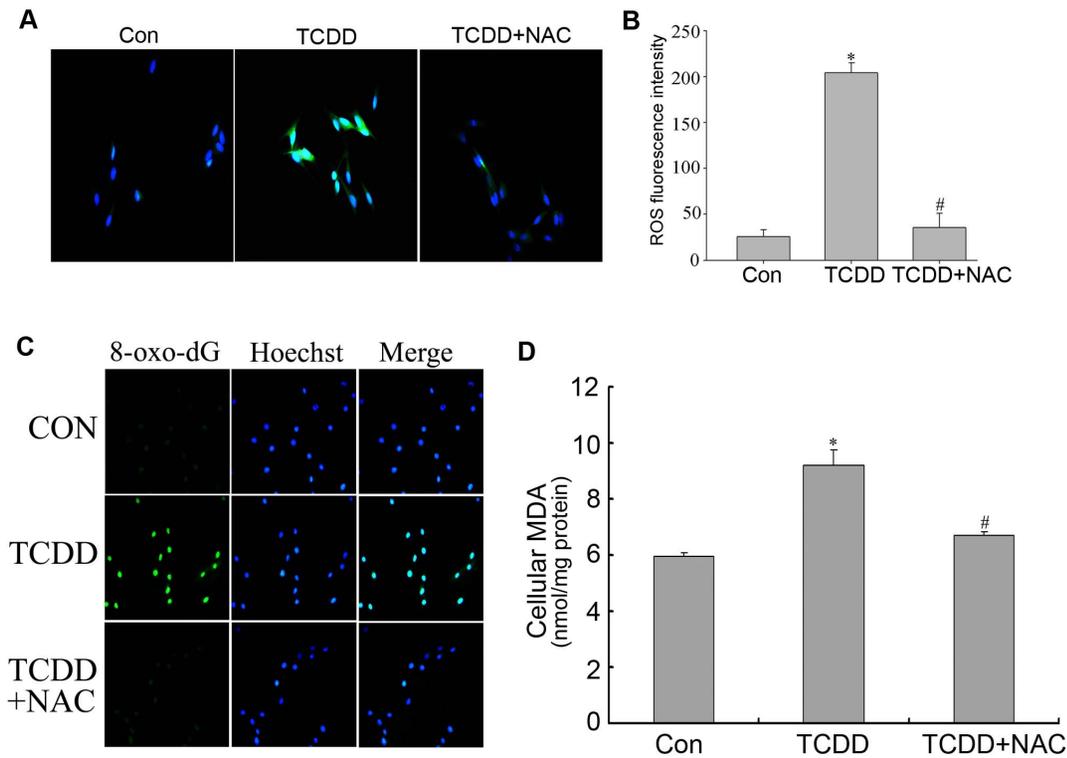


Figure 6. TCDD-induced oxidative DNA and lipid damage were attenuated by the ROS scavenger NAC. (A) ROS fluorescence was examined in PC12 cells that had been treated with DMSO, 50 nM TCDD or 50 nM TCDD plus NAC. (B) Flow cytometric analysis of ROS fluorescence in each group. (C) PC12 cells treated with DMSO, 50 nM TCDD or 50 nM TCDD plus NAC for 72 h were immunostained with an anti-8-oxo-dG antibody. (D) Determination of lipid oxidation by analyzing the MDA content per milligram of total protein of PC12 cells after DMSO, 50 nM TCDD or 50 nM TCDD plus NAC treatment for 72 h (* $p < 0.05$, statistically significant difference from the control group; # $p < 0.05$, statistically significant difference from the TCDD-treated group). doi:10.1371/journal.pone.0089811.g006

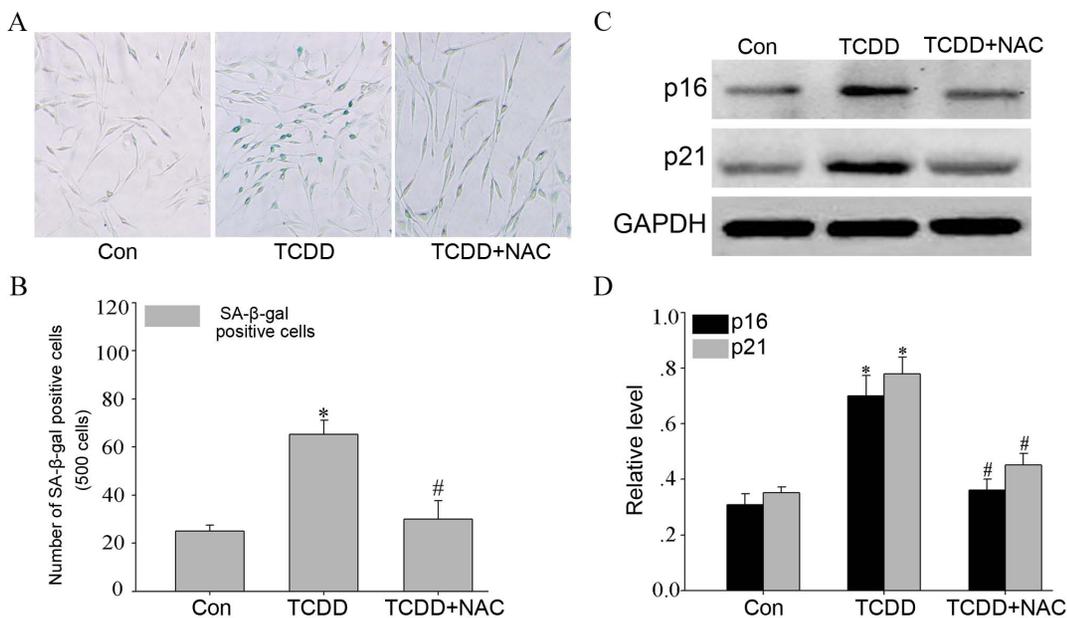


Figure 7. NAC inhibited TCDD-induced senescence in PC12 neuronal cells. (A) PC12 cells were pre-treated with DMSO, 50 nM TCDD, or 50 nM TCDD plus 5 mM NAC for 72 h before SA-β-Gal staining was performed. (B) The number of positive cells out of every 500 cells was counted and presented (* and # $p < 0.05$, statistically significant difference from the control group; # $p < 0.05$, statistically significant difference from the TCDD-treated group). (C) Western blot analysis of the senescence marker proteins, p16 and p21, after the cells had been treated with DMSO, 50 nM TCDD, or 50 nM TCDD and NAC for 72 h. (D) Statistical analysis of the densitometric evaluation of the three independent experiments (* $p < 0.05$, statistically significant difference from the control group; # $p < 0.05$, statistically significant difference from the TCDD-treated group). doi:10.1371/journal.pone.0089811.g007

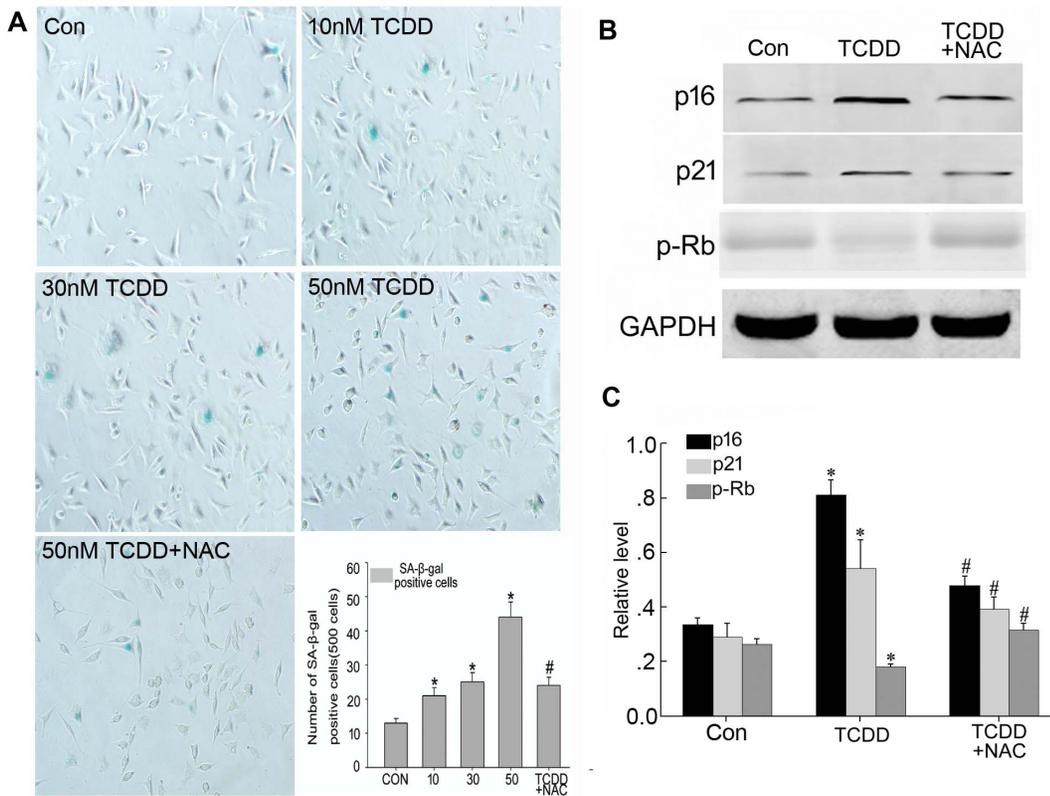


Figure 8. TCDD induces premature senescence in the human neuroblastoma SH-SY5Y cell line. (A) SH-SY5Y cells were incubated with DMSO, 10 nM TCDD (B), 30 nM TCDD, 50 nM TCDD or 50 nM TCDD combined with 5 mM NAC. Seventy-two hours later, the cells were subjected to an SA-β-Gal assay. The percentage of β-Gal-positive cells in each group was counted and presented (* $p < 0.05$, statistically significant difference from the control group; # $p < 0.05$, statistically significant difference from the 50 nM TCDD-treated group). (B) TCDD exposure altered the expression of senescence marker proteins in an ROS-dependent manner in SH-SY5Y cells. SH-SY5Y cells were exposed to DMSO, 50 nM TCDD or 50 nM TCDD+5 mM NAC and then subjected to western blot analyses to determine p16, p21 and p-Rb expression. (C) Quantitative analysis of the intensity of protein expression relative to GAPDH in the indicated groups (* $p < 0.05$, statistically significant difference from the DMSO-treated group; # $p < 0.05$, statistically significant difference from the 50 nM TCDD-treated group). doi:10.1371/journal.pone.0089811.g008

After induction, the NGF-differentiated PC12 cells were seeded on new collagen-coated plates or coverslips with DMEM containing 1% FBS and 100 ng/ml of NGF. Twenty-four hours later, differentiated PC12 cells were starved using serum-free DMEM plus 100 ng/ml of NGF for 24 h and were then either treated with DMSO or the indicated dose of TCDD (1, 10, 50 or 100 nM) for 72 h or were exposed to 50 nM TCDD for 0, 24, 48, 72 and 96 h in DMEM, 1% FBS and 100 ng/ml of NGF. NAC treatment was performed by incubating PC12 cells with 5 mM N-acetylcysteine (NAC; Sigma, St. Louis, MO, USA) for 1 h prior to TCDD exposure.

Senescence-associated β-galactosidase (SA-β-Gal) staining assay

Senescence-associated β-galactosidase (SA-β-Gal) staining was performed using an SA-β-Gal Staining Kit (Genmed Scientifics Inc., Arlington, MA, USA) by following the manufacturer's recommended protocol. Briefly, after TCDD treatment, PC12 cells were fixed in 4% (v/v) formaldehyde for 5 min and stained with SA-β-Gal staining solution at pH 6.0 for 16 h. Cells with a bright blue color were considered to be positive. The number of positive cells was counted under a phase-contrast microscope. The experiment was repeated three times in each group.

ROS measurements and lipid oxidation assay

ROS measurements were performed using a ROS Staining Kit (Genmed, Arlington, MA, USA) in accordance with the manufacturer's recommended protocol. Cells that were treated with 10 μmol/L of Rosup for 30 min were used as a positive control. After the indicated treatments, the PC12 cells were washed three times with serum-free DMEM and incubated with ROS staining solution (DCFH-DA) at 37°C for 20 min. After washing, the nuclei were counterstained with Hoechst-33258. The fluorescent signal was observed using a fluorescence microscope. To quantify the ROS levels, the DCFH fluorescence intensity was analyzed using a flow cytometer (Calibur, BD Biosciences, San Diego, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The experiments were repeated a minimum of three times.

The analysis of lipid oxidation was conducted by measuring the formation of a major lipid peroxidation product, malondialdehyde (MDA). Briefly, PC12 cells were exposed to 0.1% DMSO or 50 nM TCDD for 72 h and then harvested for MDA measurements using a Micro MDA Detection Kit (Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's recommended protocol. The assay was performed using four wells for each group.

Immunofluorescence analyses

PC12 cells were fixed with 4% (v/v) paraformaldehyde in PBS for 30 min and then permeabilized with 1% Triton-X100 for 10 min. Next, the cells were blocked with 1% BSA in PBS for 1 h and incubated with a rabbit polyclonal anti- γ -H2AX (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or a mouse monoclonal anti-8-oxo-dG (Trevigen, Gaithersburg, MD) antibody. Thereafter, the cells were incubated with a FITC-conjugated goat anti-rabbit or anti-mouse antibody (1:300, ICN Cappel, Aurora, Ohio, USA). F-actin staining was performed following incubation with FITC-phalloidin (1:100, Abcam, Cambridge, MA, USA). DAPI was used to visualize the nuclei. The slides were mounted and examined using a confocal microscope (Nikon, Melville, NY, USA).

Measurement of mitochondrial membrane potential and H₂O₂ production

Differentiated PC12 cells were incubated with 0.1% DMSO or 50 nM TCDD for 48 h and then subjected to mitochondrial membrane potential measurements using a JC-1 Fluorescence Kit (Jiancheng Bioengineering Institute, Nanjing, China) by following the manufacturer's recommended protocol. The experiments were repeated a minimum of three times.

Mitochondrial H₂O₂ production was measured in accordance with several previous reports [21,22]. Briefly, differentiated PC12 cells were stimulated with the vehicle (0.1% DMSO) or 50 nM TCDD for 48 h and then subjected to mitochondrial isolation using a Mitochondria Preparation Kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's recommended protocol. Next, 50 μ g of mitochondria was incubated with 5 μ M luminol (Sigma) and 2.5 U of horseradish peroxidase (Sigma) in 1 ml of respiratory buffer (140 mM KCl, 0.1 mM EDTA, 2.5 mM KH₂PO₄, 2.5 mM MgCl₂, 0.05% bovine serum albumin and 5 mM HEPES, pH 7.4). The reaction was initiated by the addition of 6 mM sodium succinate and monitored for luminescence intensity at 37°C using an Infinite M200 PRO Microplate Reader (Tecan, Männedorf, Switzerland).

Western blot analyses

The cells were lysed in lysis buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5% NP-40, 100 mM NaF, 200 μ M Na₃VO₄ and Complete Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland)) for 30 min at 4°C. The lysates were centrifuged at 13,000 \times g for 10 min at 4°C. After determining the protein concentrations using the Bradford assay (Bio-Rad, Hercules, CA, USA), the resulting supernatants (50 μ g of protein) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were then blocked with 5% non-fat milk in TBST for 2 h at room

temperature and incubated with rabbit polyclonal anti-p21, anti-p-Rb (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p16 (1:1,000; Cell Signaling, Beverly, MA, USA) and rabbit anti-GAPDH (1:1,000; Sigma, St. Louis, MO, USA) antibodies. After incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, the protein bands were visualized using an enhanced chemiluminescence system (ECL, Thermo Scientific Pierce, Rockford, IL, USA). ImageJ (NIH) was used to analyze the densities of the bands.

RNA isolation, semi-quantitative PCR and real-time PCR assays

Total RNA was extracted using Trizol reagent (Invitrogen, Grand Island, NY, USA), according to the manufacturer's recommended protocol. Total RNA was reverse-transcribed using the ThermoScript RT-PCR system (Invitrogen, Grand Island, NY, USA). The primers used for semi-quantitative PCR were as follows: p16INK4A (GenBank NM_031550.1), 5'- ATG GAG TCC TCT GCA GAT AGA CTA G -3' and 5'- TTA GCG CTG CTT TGG GGG TT -3' and GAPDH, 5'- ACC ACA GTC CAT GCC ATC AC -3' and 5'- TCC ACC CTG TTG CTG TA -3'.

Real-time PCR was performed using a LightCycler 480 Real-Time PCR System (Roche). The primers used for real-time PCR are shown in Table S1.

Statistical Analyses

All data are presented as the means \pm standard deviation of the mean (SD). Significance testing was performed using a one-way analysis of variance (ANOVA) to compare data from different experimental groups. P<0.05 was considered to be statistically significant.

Supporting Information

Figure S1 The effects of different doses of TCDD on caspase-3 activation in PC12 neuronal cells. PC12 cells that had been exposed to the indicated doses of TCDD for 72 h were probed with an anti-active caspase-3 antibody (* p<0.05, significantly different from the DMSO-treated group). (TIF)

Table S1 Primer sequences for real time PCR analyses. (DOC)

Author Contributions

Conceived and designed the experiments: GX CW. Performed the experiments: CW JL XN SZ. Analyzed the data: JZ ZD. Contributed reagents/materials/analysis tools: CT LL. Wrote the paper: CW GX.

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