p,p′-DDE Induces Apoptosis through the Modulation of Tumor Necrosis Factor α in PC12 Cells

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · FEBRUARY 2014

Impact Factor: 3.53 · DOI: 10.1021/tx4003963 · Source: PubMed

4 AUTHORS, INCLUDING:

Cui Wang
Zhejiang Chinese Medical University
40 PUBLICATIONS **324** CITATIONS
SEE PROFILE

Quan Zhang
Zhejiang University
26 PUBLICATIONS **217** CITATIONS
SEE PROFILE

Meirong Zhao
Zhejiang University of Technology
71 PUBLICATIONS **1,061** CITATIONS
SEE PROFILE

Chemical Research in Toxicology

Article

pubs.acs.org/crt

p,p′-DDE Induces Apoptosis through the Modulation of Tumor Necrosis Factor α in PC12 Cells

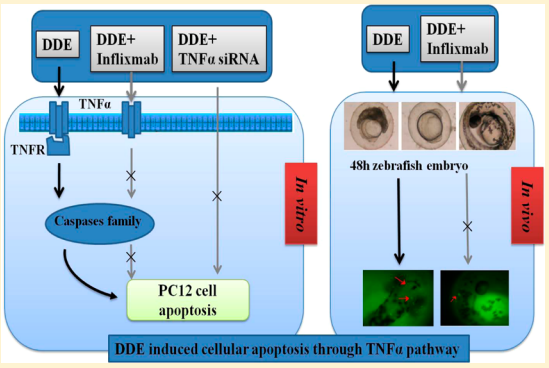
Cui Wang,[†,‡,§] Quan Zhang,[†,‡,§] Yi Qian,[‡] and Meirong Zhao*[,‡]

[†]College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, China

[‡]College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou 310032, China

Supporting Information

ABSTRACT: p,p′-DDE, the main metabolite of DDT, is notorious for its persistent and bioaccumulation. It has detrimental effects on the nervous system, while the mechanism is unclear. We sought to investigate the mechanism of p,p′-DDE-induced neurocytic apoptosis in PC12 cells by cytoflow and screen the potential target gene by microarray and ELISA. Co-incubation with antagonist and SiRNA were applied to confirm the effect of the selected molecular. Results were also confirmed in zebrafish embryo. Results showed that p,p′-DDE induced apoptosis in PC12 cells at a concentration of $\geq 2 \times 10^{-5}$ mol/L. Microarray results indicate that the TNF family plays a key role in p,p′-DDE-induced apoptosis among 84-apoptotic genes. In particular, the protein level of TNFα increased 4-fold. When incubated with TNFα antibody (infliximab), the number of apoptotic cells attenuated by 50%, and both activities of caspases 8 and 9 decreased. SiRNA silencing of TNFα showed similar trends. Furthermore, p,p′-DDE induced neuronal apoptosis in zebrafish embryos in a dose-dependent manner.This effect was partially reversed by infliximab, too. Overall, the present study herein indicated that the TNFα signaling pathway is involved in p,p′-DDE-induced neurocyte apoptosis. These data could be expanded to other cases of OCP-induced apoptosis and would support the need for scientific intervention to address the neurotoxicity of these chemicals.



INTRODUCTION

The environmental burden of DDT (1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane) can largely be attributed to its previous widespread use and its reintroduction into some areas to control the prevalence of malaria. p,p′-DDE (1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene) is the main metabolite found in the environment as a result of DDT decomposition.[1] This compound is biologically and environmentally persistent, capable of long-range transport, and can undergo significant biomagnification (up to 70,000-fold). As a result, p,p′-DDE is frequently detected in the soil, air, water, organisms, and humans that have been exposed to DDT.[2] After DDT was banned a few decades ago, p,p′-DDE remains the major residual congener in developed countries. As an indispensable component of insecticides used for indoor residual spraying (IRS) against malaria, people in developing countries are being increasingly exposed to this toxin. In most of these areas, the residue level of p,p′-DDE is up to parts per million (ppm) levels. The presence of p,p′-DDE in human milk samples in Noushahr was up to 2.936 μg/g lipids.[3] This level reached up to 190 μg/g lipid in chickens in malaria-controlled areas.[4] High levels of DDT exposure has been documented to cause immune system impairment in houses sprayed with the chemical.[5] Though the prevalent method for malaria control involves the indoor spraying of DDT,[6] a global assessment on other toxic effects when exposure at high levels is still lacking.

Although DDT, p,p′-DDE, and other organochlorine pesticides (OCPs) have neurotoxic properties, health concerns have focused on endocrine disruption for the past several decades. As an antiandrogen compound, animal studies using mice and birds confirmed that p,p′-DDE induce neuro-developmental toxicity and disruption of nocturnal activity, respectively.[7] The association between p,p′-DDE exposure and neurodevelopment has been controversial. Some reports showed that p,p′-DDE could damage psychomotor and mental development or cause hyporeflexia.[8] Another study on the critical window of p,p′-DDE exposure *in utero* also indicated that psychomotor development may be its target.[9] Moreover, laboratory studies, ecological studies, case-control investigations, and cohort research have found an association between OCPs and neurodegenerative disease.[10] Although the relationship between p,p′-DDE and Parkinson's disease (PD) has not been verified, two cohort studies found high levels of p,p′-DDE in the substantia nigra of patients with neurodegeneration.[11,12] In spite of the currently limited evidence, the neurotoxic nature of p,p′-DDE should not be neglected.

Apoptosis is controlled by a diverse range of signaling and plays an important role in homeostasis under normal physiological conditions. Defective apoptotic processes may

Received: October 3, 2013
Published: February 27, 2014

lead to a variety of diseases, including neurodegeneration and neural development toxicity.[13,14] The positive association between *p,p′*-DDE exposure and cellular apoptosis has been widely observed in children, rats, and *in vitro* models.[15−18] These reports suggest that *p,p′*-DDE elevates the mRNA levels of both the death receptor family (Fas, FasL) and caspases and that it disrupts mitochondrial function in sertoli cells, which leads to testicular apoptosis in *in vivo* and *in vitro*. The phosphorylation of p44/p42 mitogen-actived protein kinase is the only available signaling information on DDE-induced neuronal apoptosis.[19] However, this protein is not found in the typical apoptotic pathways.

Our present study aimed to investigate the signals that mediate *p,p′*-DDE-induced neurocyte apoptosis. First, *p,p′*-DDE-induced apoptosis in neurocytes was determined using a cell viability assay and flow cytometry in PC12 cells. To screen for potential target molecules involved in apoptosis, we used a microarray containing 84-apoptotic related molecules. After verification of protein expression, antibody blocking and siRNA techniques were applied to confirm the contribution of specific molecules to *p,p′*-DDE-induced apoptosis. We also used zebrafish embryos as an *in vivo* model to verify *p,p′*-DDE-related apoptotic signaling. We report here for the first time a novel signaling pathway involved in *p,p′*-DDE-related neurocyte apoptosis. The data presented herein also support the neurotoxic properties of OCPs.

MATERIALS AND METHODS

Materials. *p,p′*-DDE [98.5%, 1,1-bis-(4-chlorophenyl)-2,2-dichloroethylene] was purchased from Bestown (Beijing, China). Dulbecco's modified Eagle's medium (DMEM)/F12 and fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) were obtained from Katimesbio (Hangzhou, China). The reagent kits for caspase 3, caspase 8, caspase 9, and tumor necrosis factor α (TNFα) were purchased from Beyotime, China. Infliximab (Johnson & Johnson, USA) was purchased from BaoZhiLi (Guangzhou, China). Lipofectamine 2000 and Opti-MEM were purchased from Invitrogen (California, Carlsbad, CA, USA).

Cell Culture and Treatment. Highly differentiated rat pheochromocytoma (PC12) cells were obtained from the cell bank of the Chinese Academy of Sciences. The procedure for cell culture was according to our previous research.[20]

PC12 were seeded in culture plates (Costar) at a density of 1×10^5 cells/mL. After adherence, the experimental medium containing 2% FBS with or without different doses of *p,p′*-DDE was added for up to 24 h. To evaluate the effect of *p,p′*-DDE on cell viability, apoptosis, and caspase activity, infliximab (antagonist of TNFα) was added 1 h prior to *p,p′*-DDE treatment.

Assessment of Cell Viability. Cell viability was measured with the CellTiter 96 Aqueous one Solution (Promega). After pesticide incubation, 20 μL of the reagent was added to each well. The plate was incubated at 37 °C for 1 h, and then the absorbance at 490 nm was recorded by a Bio-Rad Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Apoptotic Microarray Assay. Total RNA was extracted using high-purity RNeasy Mini kits (Qiagen, Catalog #74104). The quality of RNA samples used in the microarray met the standard of the kits. The real-time PCR for the rat apoptosis microarray assay was performed using the RT2 Profiler PCR microarray (SuperArray Bioscience, Frederick, MD). For details, see ref 20.

Assessment of Apoptosis. Apoptosis levels were assayed with an Annexin V/PI apoptosis kit (Multisciences, China) according to the protocol. The performance details are according to Wang et al.[20]

Detection of TNFα Production and the Activity of Caspases 3, 8, and 9. TNFα protein was detected using a TNFα enzyme linked immunosorbent assay (ELISA) kit (Biovalue, China). Caspase activity was determined with a caspase active assay kit (Beyotime, China). The whole cell protein extract and procedure were according to the ref 20.

RNA Interference of TNFα. The sequence of TNFα siRNA sense is CGGAGCUAAACUACCAGCU, and the antisense sequence is AGCUGGUAGUUUAGCUCCG. The sequence for the negative control sense is CCUACGCCAAUUUCGU, and the antisense sequence is ACGAAAUUGGUGGCGUAGG. PC12 cells were seeded into 30-mm dishes at a density of 4×10^5/mL. TNFα siRNA and 3 to 5 μL of lipfectamine 2000 were diluted into 125 μL of Opti-MEM I reduced serum medium. After 5 min, the samples were gently mixed to a final volume of 250 μL and incubated at room temperature (RT) for 20 min. The mixture was added to the incubation medium to obtain transfection solution concentrations of 20, 40, 60, or 80 nM of siRNA. After a 6 h of incubation at 37 °C in a humidified incubator, the complete medium with 10% FBS was used to replace the transfection medium. To detect the efficiency of transfection, cells were harvested and lysed by Trizol regent after 24 h based on the experimental data; we chose 60 nM TNFα siRNA as the optimum concentration for subsequent assays.

Zebrafish Embryo Treatment and TUNEL Staining. Healthily fertilized zebrafish (*Danio rerio*) embryos were collected from mating adult fish as previously described.[21] Eggs were placed in 96-well culture plates by a pipet (one embryo in each well) and housed at 28 °C. Embryos were exposed to 0, 1×10^{-5}, 2×10^{-5}, or 3×10^{-5} mol/L of *p,p′*-DDE beginning at the sphere stage 4 h postfertilization (hpf). Each treatment consisted of 12 embryos, and the experiment was performed in triplicate.

Apoptosis was detected using the DeadEnd Colorimetric TUNEL System (Promega, Madison, USA). After 48 h, embryos were collected in a 1.5 mL tube (each dose/tube), fixed in 4% PFA overnight, and then stored in methanol at −20 °C. The following day, embryos were kept at RT for approximately 5 min and then rehydrated in a series of methanol−PBS solutions. The embryos were then digested with 10 μg/mL proteinase K. After a 25 min digestion, the embryos were incubated in equilibration buffer for 1 h at RT. Next, the embryos were incubated in the TDT enzyme at 4 °C overnight and at 37 °C for 1 h the next day. The reaction was stopped by adding 2 × SCC. Samples were analyzed using a fluorescent microscope.

Statistical Analysis. All data are represented as the mean ± standard deviation (SD). Comparisons between the treatment groups and control groups were completed using one-way analysis of variance (ANOVA). The significance level was set at $p < 0.05$. All experiments were performed three independent times.

RESULTS

PC12 Cell Viability and Apoptosis Induced by *p,p′*-DDE. Cell viability after *p,p′*-DDE exposure was measured using the MTS assay. The reduction in cell viability was dose-dependent. A sharp reduction in viability occurred at 2×10^{-5} mol/L and was approximately 50% of the control after exposure to 3×10^{-5} mol/L *p,p′*-DDE (Figure S1, Supporting Information). On the basis of these data, apoptosis was assayed using flow cytometry after cells were exposed to 2×10^{-5} or 3×10^{-5} mol/L *p,p′*-DDE. The four-quadrant figure shows 6.1% and 7.8% apoptotic cells at 2×10^{-5} mol/L and 3×10^{-5} mol/L, respectively. These levels were significantly higher than those of the control (Figure S2, Supporting Information).

Microarray Screening of Apoptotic Molecules. To examine the potential target molecules of *p,p′*-DDE-induced apoptosis, an 84-apoptotic molecule RT-PCR microarray was used. Samples were processed after exposure to 3×10^{-5} mol/L *p,p′*-DDE. A minimum of a 1.4-fold change in transcription level was considered significant (Table S1, Supporting Information). *p,p′*-DDE-induced neuron cellular apoptosis triggered mainly five families including the Bcl2 family, caspase family, TNF family, CARD family, and the other family (Figure 1). Three members of the Bcl-2 family and CARD family,

dx.doi.org/10.1021/tx4003963 | Chem. Res. Toxicol. 2014, 27, 507−513

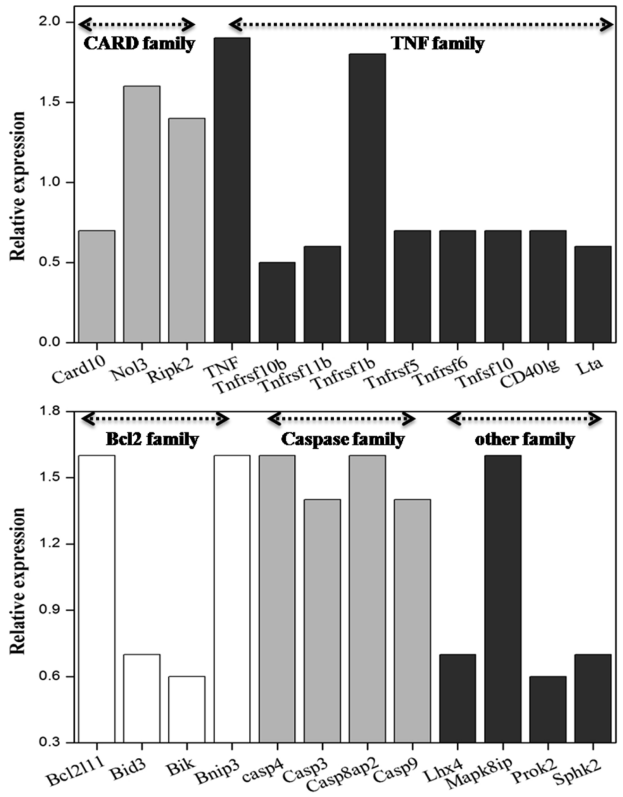


Figure 1. Relative expression of the 5 families of apoptotic genes after treatment with *p,p′*-DDE ($p<0.05$). Cells were exposed to 3×10^{-5} mol/L of *p,p′*-DDE for 24 h. After exposure, the RNA was extracted, and real-time PCR for apoptosis microarray assays was performed using the RT2 Profiler PCR microarray.

including Bcl2l11, Bik, Bid3, Card 10, Nol3, and Ripk2, were significantly changed. Four apoptosis executors (caspase family) including caspase 3, 4, 8ap2, and 9 were upregulated for more than 1.4-fold. The late phase of apoptosis is also active in other genes such as Lhx4, Prok2, Sphk2, and MapK8iP. The extracellular TNF family seems to play a critical role in *p,p′*-DDE-induced cell apoptosis. Significant changes were found in TNF and its receptor superfamily, including the death ligand, CD40lg, TNFrsf 6, 1b, 10b, and 11b, etc. The relative genetic expression of TNFα was 1.8-fold high. The TNF family is the trigger for the extracellular apoptosis signaling pathway. Considering the critical role of TNFα in this family, we assessed whether TNFα was one of the molecular targets of *p,p′*-DDE.

Confirmation of the Target Molecule with ELISA. ELISA kits were used to measure the protein level of TNFα in cells. The ELISA measurements displayed in Figure 2 indicated that the TNFα was significantly activated after exposure to 2×10^{-5} mol/L (4-fold) and 3×10^{-5} mol/L (2.5-fold) *p,p′*-DDE for 24 h (Figure 2). Although apoptosis was induced in a dose-dependent manner, TNFα levels show a nonlinear relationship with *p,p′*-DDE concentration. Overall, TNFα was upregulated during *p,p′*-DDE-induced neurocyte apoptosis.

Preventing *p,p′*-DDE-Induced Neurocyte Apoptosis with Antibody Blocking of TNFα. To confirm the role of TNFα in *p,p′*-DDE-induced apoptosis, we blocked the activity of TNFα using infliximab. After incubation with multiple doses of the inhibitor, we determined that a concentration of 325 μM was nontoxic to PC12 cells and that it significantly attenuated *p,p′*-DDE-induced toxicity. Initially, the effects of infliximab

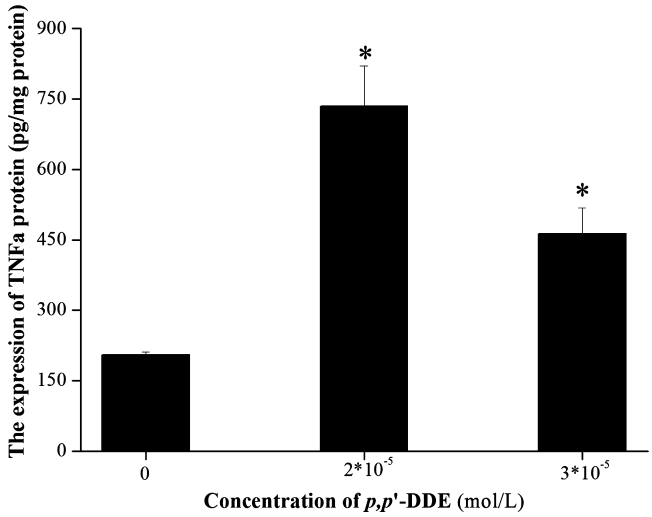


Figure 2. ELISA kit analysis to manifest the expression of TNFα protein in PC12 cells after 24 h of exposure to *p,p′*-DDE at different doses. PC12 cells were exposed to 3×10^{-5} mol/L of *p,p′*-DDE for 24 h. Then, whole-cell protein was extracted, and the levels of testing protein synthesized were quantified using the ELISA kit (R & D system, USA). The asterisk above each bar indicates statistical significance compared to a negative control ($p < 0.05$).

were assayed by the MTS viability assay. The dose-dependent effects of *p,p′*-DDE-induced cytotoxicity in PC12 cells is depicted in Figure 3. The effects of 2×10^{-5}, 2.5×10^{-5}, and 3×10^{-5} mol/L *p,p′*-DDE exposure were remarkably attenuated with prior incubation of cells with infliximab (Figure 3).

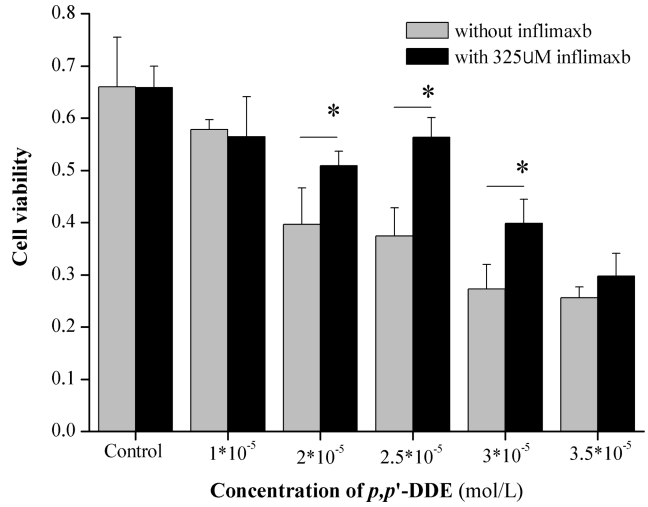


Figure 3. Inhibition of TNFα expression attenuates *p,p′*-DDE-induced cell death. PC12 cells were pretreated for 1 h with infliximab (325 μM) or vehicle control, followed by a different dose of *p,p′*-DDE for 24 h of treatment. After exposure, cell viability was measured by the MTS assay. The asterisk above each bar indicates statistical significance between treatment groups with or without the TNFα antagonist ($p < 0.05$).

Similarly, increased cell viability coincided with a decrease in apoptosis after TNFα inhibition. As shown in Figure S3 (Supporting Information), the percentage of apoptotic cells was decreased from 4.1% to 2.8% and from 8.3% to 5.2% after infliximab pretreatment when compared with 2×10^{-5} and 3×10^{-5} mol/L *p,p′*-DDE exposure.

dx.doi.org/10.1021/tx4003963 | *Chem. Res. Toxicol.* 2014, 27, 507−513

Three apoptotic executioners were measured according to the data from the microarray. Activity of the cleaved caspases (caspases 3, 8, and 9) was detected with or without pretreatment with the TNFα inhibitor (Figure 4). Only cells

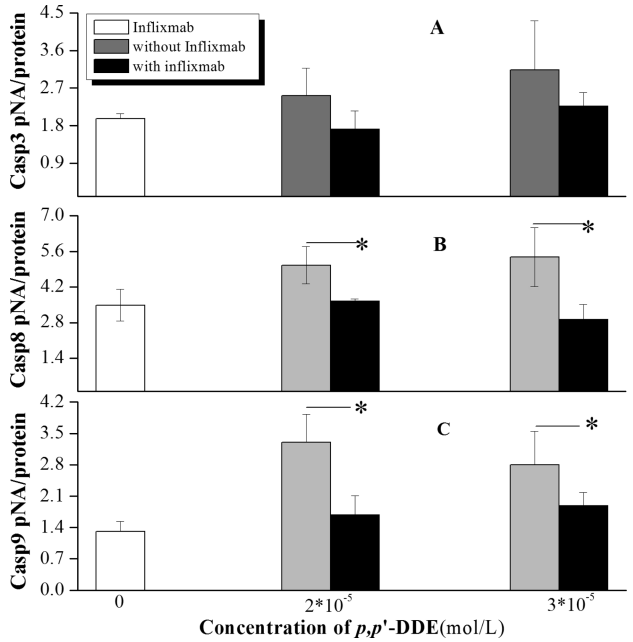


Figure 4. Caspase activation reductions by infliximab protection: (A) caspase3, (B) caspase8, and (C) caspase9. ELISA kit analysis of PC12 cells following exposure to different doses of *p,p′*-DDE with or without infliximab for 24 h. The asterisk above each bar indicates statistical significance between treatment groups with or without the TNFα antagonist ($p < 0.05$).

exposed to *p,p′*-DDE showed changes in the activity of caspases 3 ($p > 0.05$) (Figure 4A), 8 ($p < 0.05$) (Figure 4B), and 9 ($p < 0.05$) (Figure 4C) when compared with the control. Remarkably, the activity of caspase 8 and caspase 9 was attenuated when cells were pretreated with the TNFα inhibitor (Figure 4B,C).

Verification of the Role of TNFα in *p,p′*-DDE-Induced Cell Apoptosis Using siRNA. To determine the role of TNFα signaling in *p,p′*-DDE-induced apoptosis, we used siRNA targeted against TNFα in PC12 cells. Real-time PCR analysis was used to detect the transcription efficiency of TNFα siRNA. After incubation with 60 nM TNFα siRNA for 24 h, the TNFα mRNA level was decreased by 45%, and the protein level declined 38% when compared with the negative control oligonucleotide transfection (Figure S4a,b, Supporting Information). The silence efficiency of TNFα was not significantly changed after incubating with *p,p′*-DDE (Figure S4a,b, Supporting Information). Cells treated with TNFα siRNA show a partial protection from *p,p′*-DDE induced apoptosis (Figure 5).

Assessment of Neurocyte Apoptosis Using TUNEL Staining in Zebrafish Embryos. Visualization of vital dyes is a key tool for the assessment of toxicity on zebrafish embryos because of its transparent characteristics. A recent report suggests that zebrafish can be used as a predictive model for neurotoxicity because compounds that induce neurotoxicity in mammals have induced similar effects in zebrafish.[22] By 48 hpf, zebrafish have completed neural differentiation and brain ventricle formation.[23,24] To assess the effects of *p,p′*-DDE on neurocyte apoptosis, we performed TUNEL staining on zebrafish embryos after 48 h of exposure. Figure 6 shows that severe neurocyte apoptosis (bright green dots) was observed after *p,p′*-DDE exposure. The staining in the control sample was negative, while the number of positive spots in the treatment group was increased with the exposure dose of DDE. To investigate the role of TNFα in *p,p′*-DDE-induced neurotoxicity *in vivo*, infliximab was coincubated with DDE (Figure 6). Our results suggest that *p,p′*-DDE-induced neurocyte apoptosis in zebrafish embryos can be partially attenuated by infliximab, supporting the role of the TNFα signaling pathway.

DISCUSSION

The health effects of large exposures to DDT and its main metabolite *p,p′*-DDE is a critical problem that one needs to face in controlling malaria. Evidences on the health risks of DDT were mainly collected in North America and Europe, where the exposure level is now much lower than those active IRS areas.[25] Here, we show for the first time, a potential mechanism of neurocyte apoptosis induced by *p,p′*-DDE using doses typically found in malaria-controlled areas.

Although most of the DDT-like compounds are endocrine disruptors, high doses of DDTs induce other toxicological effects, including reproductive toxicity,[16−18] immunotoxicity,[26,27] and neurotoxicity.[28] These toxic effects go through novel signaling pathways rather than the classical estrogen receptor-mediated pathway. Specific extracellular and intracellular signaling pathways are involved in cell apoptosis. The mitochondrial pathway and the interaction of extracellular membrane receptors with their ligands are the two major players involved in DDT- and *p,p′*-DDE-induced toxicity. Among the TNF superfamily, the Fas/FasL pathway was stimulated in rat sertoli cells and induced testicular cell apoptosis after exposure to *p,p′*-DDE in *in vivo* and *in vitro* models.[15,18] Reproductive cell apoptosis and the mitochondrial pathway were triggered by the release of cytochrome *C* and the upregulation of Bax and Bak after exposure to *p,p′*-DDE.[15,18] Previous data suggest that the p38 mitogen activated protein kinase (MAPK) cascade played a critical role in DDT-related endometrial Ishikawa and human embryonic kidney 293 cell apoptosis.[29] Our previous study indicated that acetofen, another member of the OCPs, induces immune cell apoptosis through the mitochondrial pathway.[30] Nevertheless, only one study has reported an association between the phosphorylation of p44/42 MAPK and apoptosis in undifferentiated PC12 cells.[19] The MAPK signaling cascade is often involved in stress-mediated signaling pathways, which modulate several cell processes, including proliferation, differentiation, and apoptosis. Therefore, the MAPK signaling pathway is not specific to apoptosis. We now report that TNFα, a potent extracellular signaling inducer of apoptosis, participates in OCPs-induced neurocyte apoptosis.

Activation of TNFα stimulates both cell apoptosis and survival, whereas apoptosis occurs after the upregulation of the caspase cascade.[31] TNFα is a well-known stimulator of caspases 3, 8, and 9, and the activation of these proteins is required for TNF-mediated apoptosis.[32] Our present study shows that the activation of caspases 8 and 9 can be markedly attenuated by blocking TNFα, confirming the relationship between caspases and TNFα. The crosstalk between the TNF family and caspase family has been previously reported. Shi et al. observed that FasL, caspase 3, and caspase 9 were activated in *p,p′*-DDE-

dx.doi.org/10.1021/tx4003963 | *Chem. Res. Toxicol.* 2014, 27, 507−513

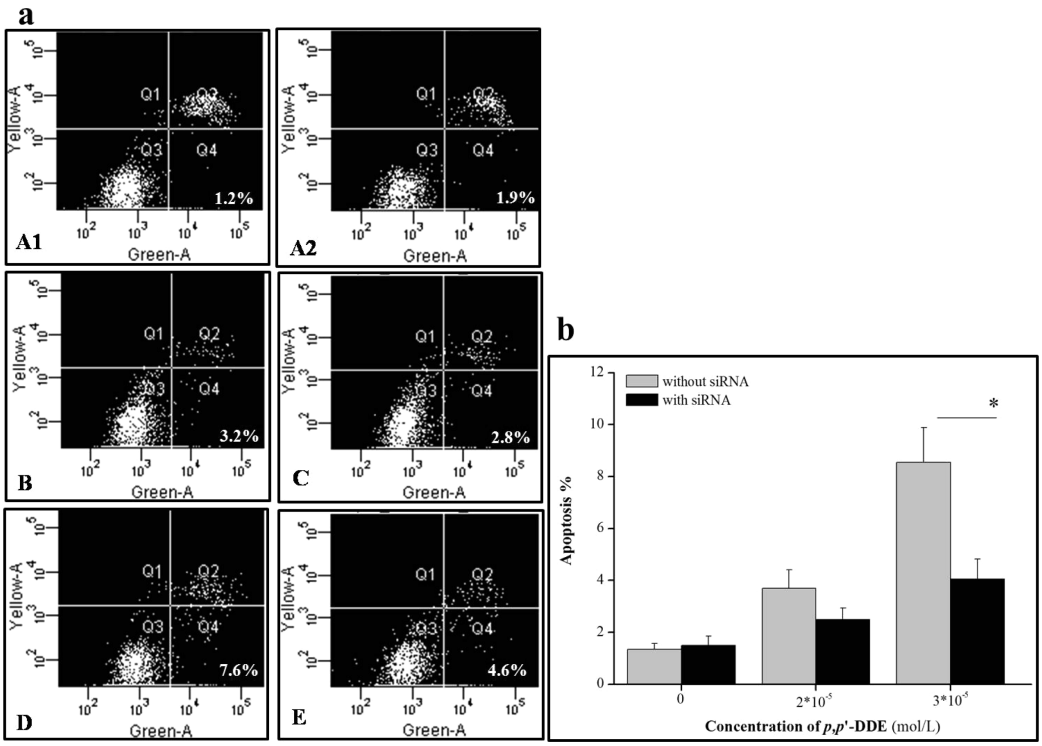


Figure 5. Small interfering TNFα (SiTNFα)-attenuated *p,p′*-DDE-induced cell apoptosis. (a) (A1) control, (A2) SiTNFα, (B) 2×10^{-5} mol/L *p,p′*-DDE, (C) 2×10^{-5} mol/L *p,p′*-DDE + SiTNFα, (D) 3×10^{-5} mol/L DDE, and (E) 3×10^{-5} mol/L *p,p′*-DDE + SiTNFα. PC12 cells were transformed with/without 60 nM SiTNFα. After 24 h of incubation, except for the negative control or control group, all cells were treated with 2×10^{-5} or 3×10^{-5} mol/L of *p,p′*-DDE. For the next 24 h, cellular apoptosis was assayed by cytoflow. (b) The percentage of apoptotic cells followed by the different treatments.

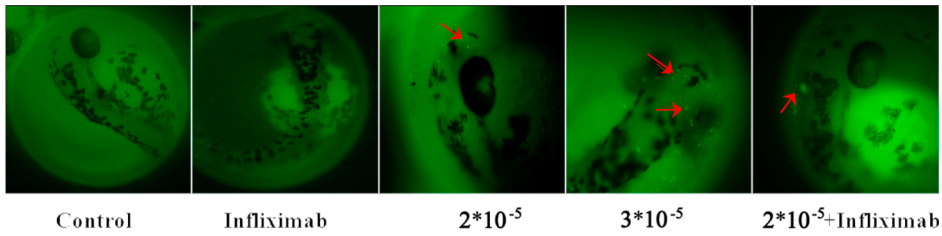


Figure 6. Inhibition of TNFα expression attenuates *p,p′*-DDE-induced apoptosis of neuron cells in zebrafish embryo. 4-hpf zebrafish embryos were incubated with different doses of *p,p′*-DDE with or without infliximab. After 48 h of treatment, cell apoptosis in the heads of embryos was determined by the TUNEL assay. The arrowhead indicates the apoptotic cells.

induced testicular cell and setori cell apoptosis.[16,17] Our current data suggest that the TNFα and caspase family play a key role in neurocyte apoptosis induced by OCPs.

The neuronal injury and disease associated with OCP exposure needs to be further studied. Excessive cellular apoptosis can account for neurodegenerative diseases.[33] Since neuronal injury in retired malaria control workers and neurodevelopment impairment in children have been addressed in previous reports,[34,8,9] we speculated that neurocyte apoptosis or necrosis may contribute to chemical-mediated developmental toxicity and neurodegenerative disease. Detailed testing of currently used chemicals to determine the mechanism of toxicity should be pursued when screening tests reveal neurotoxic effects.[35] Some researchers have suggested that molecules, such as caspases and excitatory amino acids, can contribute to neuronal damage in response to ischemia; therefore, therapeutic intervention may be possible using a multiple-hit strategy.[36] According to our study, chemicals that alter TNFα expression may be a viable option for the treatment of OCPs-related neuronal injury.

For POPs and some OCPs, their complete effects on human health as well as environmental risk assessments are still missing. Research on mechanisms can provide strong evidence for the qualitative assessment of the toxic effects of these chemicals. Since occupational hazards and sequential exposure may result in particularly high levels in certain individuals because of the high lipophilicity and degradation resistance of POPs, it is necessary to investigate the effects of these compounds at high dosages too.

■ ASSOCIATED CONTENT

Supporting Information

Microarray results for 84-apoptotic genes, cell viability, and cellular apoptosis measured by cytoflow. This material is available free of charge via the Internet at http://pubs.acs.org.

dx.doi.org/10.1021/tx4003963 | *Chem. Res. Toxicol.* 2014, 27, 507−513

AUTHOR INFORMATION

Corresponding Author

*Tel: +86 571 8832 0265. Fax: +86 571 8832 0265. E-mail: zhaomr@zjut.edu.cn.

Author Contributions

§C.W. and Q.Z. made equal contributions to this work.

Funding

This study was supported by the National Natural Science Foundations of China (21077094, 21377119, and 21337005).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS

IRS, indoor residual spraying; OCPs, organochlorine pesticides; TNFα, tumor necrosis factor α; siRNA, small interfering RNA; ELISA, enzyme linked immunosorbent assay; PFA, paraformaldehyde; RT, room temperature; SD, standard deviation

REFERENCES

(1) The Agency for Toxic Substances and Disease Registry (ATSDR) (2002) *Toxicological Profile for DDT, DDE, and DDD*, Agency for Toxic Substances and Disease Registry, Atalanta, GA.

(2) Turusov, V., Rakitsky, V., and Tomatis, L. (2002) Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence, and risks. *Environ. Health Perspect. 110*, 125−128.

(3) Behrooza, R. D., Saria, A. E., Bahramifarc, N., and Ghasempouri, S. M. (2009) Organochlorine pesticide and polychlorinated biphenyl residues in human milk from the Southern Coast of Caspian Sea, Iran. *Chemosphere 74*, 931−937.

(4) Van Dyk, J. C., Bouwman, H., Barnhoorn, I. E. J., and Bornman, M. S. (2010) DDT contamination from indoor residual spraying for malaria control. *Sci. Total Environ. 408*, 2745−2752.

(5) Aneck-Hahn, N. H., Schulenburg, G. W., Bornman, M. S., Farias, P., and De Jager, C. (2007) Impaired semen quality associated with environmental DDT exposure in young men living in a malaria area in the Limpopo province, South Afica. *J. Androl. 28*, 423−434.

(6) World Health Organization (WHO) (2007) *The Use of DDT in Malaria Vector Control*, WHO Position Statement, World Health Organization, Geneva, Switzerland.

(7) Mahoney, J. J., Jr. (1975) DDT and DDE effects on migratory condition of white-throated sparrows fed DDT for 5 and 11 weeks. *J. Wildl. Manag. 39*, 520−527.

(8) Ribas-Fitó, N., Torrent, M., Carrizo, D., Muñoz-Ortiz, L., Julvez, J., Grimalt, J. O., and Sunyer, J. (2006) In utero exposure to background concentrations of DDT and cognitive functioning among preschoolers. *Am. J. Epidemiol. 164*, 955−962.

(9) Torres-Sanchez, L., Rothenberg, S. J., Schnaas, L., Cebrian, M. E., Osorio, E., Hernadez, M. C., Carcia-Hemandez, R. M., del Rio-Garcia, C., Wolff, M. S., and Lopez-Carrillo, L. (2007) In utero *p,ṕ*-DDE exposure and infant neurodevelopment: a perinatal cohort in Mexico. *Environ. Health Perspect. 115*, 435−439.

(10) Hatcher, J. M., Pennell, K. D., and Miller, G. W. (2008) Parkinson's disease and pesticides: a toxicological perspective. *Trends Pharmacol. Sci. 29*, 322−329.

(11) Corrigan, F. M., Wienburg, C. L., and Shore, R. F. (2000) Organochlorine insecticides in substantia nigra in Parkinson's Disease. *J. Toxicol. Env. Health A 59*, 229−234.

(12) Fleming, L., Mann, J. B., Bean, J., Briggle, T., and Sanchez-Ramos, J. R. (1994) Parkinson's disease and brain levels of organochlorine pesticides. *Ann. Neurol. 36*, 100−103.

(13) Liang, Q. L., and Zhang, J. H. (2009) *Essentials of Apoptosis: Apoptosis in Neurodegerative Disease*, pp 479−503, Humana Press, Totowa, NJ.

(14) Bornman, M. S., Pretorius, E., Marx, J., Smit, E., and van der Merwe, C. F. (2007) Ultrastructural effects of DDT, DDD and DDE on neural cells of the chicken embryo model. *Environ. Toxicol. 22*, 328−336.

(15) Song, Y., Liang, X. M., Hu, Y. F., Wang, Y. N., and Yang, K. D. (2008) *p,p′*-DDE induces mitochondria-mediated apoptosis of cultured rat sertoli cells. *Toxicology 253*, 53−61.

(16) Shi, Y. Q., Wang, Y. P., Song, Y., Li, H. W., Liu, C. J., Wu, Z. G., and Yang, K. D. (2010) *p,p′*-DDE induces testicular apoptosis in prepubertal rats via the Fas/FasL pathway. *Toxicol. Lett. 193*, 79−85.

(17) Shi, Y. Q., Song, Y., Wang, Y. N., Liang, X. M., Hu, Y. F., Guan, X., Cheng, J., and Yang, K. D. (2009) *p,p′*-DDE induces apoptosis of rat sertoli cells via a FasL-dependent pathway. *J. Biomed. Biotechnol.*, DOI: 10.1155/2009/181282.

(18) Shi, Y. Q., Li, H. W., Wang, Y. P., Liu, C. J., and Yang, K. D. (2013) *p,p′*-DDE Induces Apoptosis and mRNA expression of apoptosis-associated genes in testes of pubertal rats. *Environ. Toxicol. 28*, 31−41.

(19) Shinomiya, N., and Shinomiya, M. (2003) Dichlordiphenyltrichloroethane suppresses neurite outgrowth and induces apoptosis in PC12 pheochromocytoma cells. *Toxicol. Lett. 137*, 175−183.

(20) Wang, C., Li, Z. Y., Zhang, Q., Zhao, M. R., and Liu, W. P. (2013) Enantioselective induction of cytotoxicity by *o,p′*-DDD in PC12 cells: implications of chirality in risk assessment of POPs metabolites. *Environ. Sci. Technol. 47*, 3909−3917.

(21) Jin, M. Q., Zhang, Y., Ye, J., Huang, C. J., Zhao, M. R., and Liu, W. P. (2010) Dual enantioselective effect of insecticide bifenthrin on locomotor behavior and development in embryo-larval zebrafish. *Environ. Toxicol. Chem. 7*, 1561−1567.

(22) Parng, C. L., Roy, N. M., Ton, C., Lin, Y. X., and McGrath, P. (2007) Neurotoxicity assessment using zebrafish. *J. Pharmacol. Toxicol. Methods 55*, 103−112.

(23) Jiang, Y. J., Brand, M., Heisenberg, C. P., Beuchle, D., Furutani-Seiki, M., Kelsh, R. N., Warga, R. M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Mullins, M. C., Odenthal, J., van Eeden, F. J., and Nusslein-Volhard, C. (1996) Mutations affecting neurogenesis and brain morphology in the zebrafish, Danio rerio. *Development 123*, 205−216.

(24) Schier, A. F., Neuhauss, S. C., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D. Y., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangimi, Z., Yang, H., and Driever, W. (1996) Mutations affecting the development of the embryonic zebrafish brain. *Development 123*, 165−178.

(25) Van den Berg, H. (2009) Global status of DDT and its alternatives for use in vector control to prevent disease. *Environ. Health Perpect. 117*, 1656−1663.

(26) Alegría-Torres, J. A., Díaz-Barriga, F., Gandolfi, A. J., and Pérez-Maldonado, I. N. (2009) Mechanisms of *p,p′*-DDE-induced apoptosis in human peripheral blood mononuclear cells. *Toxicol. in Vitro 23*, 1000−1006.

(27) Pérez-Maldonado, I. N., Athanasiadou, M., Yanez, L., Gonzalez-Amaro, R., Bergman, A., and Diaz-Barriga, F. (2006) DDE-induced apoptosis in children exposed to the DDT metabolite. *Sci. Total Environ. 370*, 343−351.

(28) Bornman, M. S., Pretorius, E., Marx, J., Smit, E., and van der Merwe, C. F. (2007) Ultrastructural effects of DDT, DDD, and DDE on neural cells of the chicken embryo model. *Environ. Toxicol. 22*, 328−336.

(29) Frigo, D. E., Vigh, K. A., Struckhoff, A. P., Elliott, S., Beckman, B. S., Burow, M. E., and McLachlan, J. A. (2005) Xenobiotic-induced TNF-α expression and apoptosis through the p38 MAPK signaling pathway. *Toxicol. Lett. 155*, 227−238.

(30) Zhao, M. R., Zhang, Y., Wang, C., Fu, Z. W., Liu, W. P., and Gan, J. (2009) Induction of macrophage apoptosis by an organochlorine insecticide acetofenate. *Chem. Res. Toxicol. 22*, 504−510.

(31) Rath, P. C., and Aggarwal, B. B. (1999) TNF-induced signaling in apoptosis. *J. Clin. Immun. 19*, 350−364.

(32) Fan, T. J., Han, L. H., Cong, R. S., and Liang, J. (2005) Caspase family proteases and apoptosis. *Acta Biochim. Biophys. Sin. 37*, 719−727.

(33) Dash, P. Apoptosis, Basic Medical Sciences, St. George's University of London, www.sgul.ac.uk/dept/immunology/~dash.
(34) Van Wendel de Joode, B., Wessling, C., Kromhout, H., Monge, P., Garcia, M., and Mergier, D. (2001) Chronic nervous nervous-system effects of long-term occupational exposure to DDT. *Lancet* 357, 1014−1016.
(35) Landrigan, P. J. (1992) *Environmental Neurotoxicology: Testing for Neurotoxicity*, pp 53, National Academy Press, Washington, D.C.
(36) Roberts, R. (2000) *Apoptosis in Toxicology: Role of Apoptosis in Neuronal Toxicology*, pp 152, New York Taylor & Francis Routledge Press, London, U.K.