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Rosiglitazone inhibits chlorpyrifos-induced apoptosis via modulation of the oxidative stress and inflammatory response in SH-SY5Y cells

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ABSTRACT

Oxidative stress can lead to expression of inflammatory transcription factors, which are important regulatory elements in the induction of inflammatory responses. One of the transcription factors, nuclear transcription factor kappa-B (NF-κB) plays a significant role in the inflammation regulatory process. Inflammatory cell death has been implicated in neuronal cell death in some neurodegenerative disorders such as Parkinson's disease (PD). In this study, we investigated the molecular mechanisms underlying apoptosis initiated by chlorpyrifos (CPF)-mediated oxidative stress. Based on the cytotoxic mechanism of CPF, we examined the neuroprotective effects of rosiglitazone (RGZ), a peroxisome proliferator-activated receptor gamma (PPAR-γ) agonist, against CPF-induced neuronal cell death. The treatment of SH-SY5Y cells with CPF induced oxidative stress. In addition, CPF activated the p38, JNK and ERK mitogen-activated protein kinases (MAPKs), and induced increases in the inflammatory genes such as COX-2 and TNF-α. CPF also induced nuclear translocation of NF-κB and inhibitors of NF-κB abolished the CPF-induced COX-2 expression. Pretreatment with RGZ significantly reduced ROS generation and enhanced HO-1 expression in CPF-exposed cells. RGZ blocked the activation of both p38 and JNK signaling, while ERK activation was strengthened. RGZ also attenuated CPF-induced cell death through the reduction of NF-κB-mediated proinflammatory factors. Results from this study suggest that RGZ may exert an anti-apoptotic effect against CPF-induced cytotoxicity by attenuation of oxidative stress as well as inhibition of the inflammatory cascade via inactivation of signaling by p38 and JNK, and NF-κB.

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Introduction

Chlorpyrifos (CPF), an acetylcholinesterase (AChE) inhibitor, is a widely used organophosphate pesticide, and has become one of the most commonly used insecticides in agricultural, industrial, and domestic applications. Recent studies have shown that CPF generates oxidative stress and lipid peroxidation in different cell types and in a rat model and also causes neuronal damage via elevated production of reactive oxygen species (ROS), DNA damage, and lipid peroxidation in the central nervous system (Mansour and Mossa, 2009; Saulsbury et al., 2004; Verma et al., 2007; Lee et al., 2012b). In particular, recent experimental evidence has shown that CPF-mediated neuronal cell death is highly dependent on ROS and the associated signaling pathways, such as those of the mitogen activated protein kinases (MAPKs) which include p38, c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) cascades (Lee et al., 2012b). In addition, ROS function as modulators of pro-inflammatory processes in microglia-associated neurodegenerative diseases (Pawate et al., 2004; Peng et al., 2005). Thus, generation of ROS is often associated with inflammation-associated pathogenic effects including neurodamage (Kim et al., 2002). However, relatively little is known about the involvement of CPF in ROS-dependent inflammatory signaling pathways.

Inflammation, including COX activation, is a key mechanism associated with the initiation and progression of neuronal cell damage. It has been reported that inflammation is up-regulated in PD patients, and large amounts of reactive microglia have also been found in these patients (McGeer et al., 1988). Increasing evidence has showed that microglia become activated and release various neurotoxic factors, such as TNF-α, interleukin-1ß (IL-1ß), prostaglandin E2 (PGE2), NO, and ROS, which work in concert to trigger neurodegeneration (Block and Hong, 2005; Liu and Hong, 2003). These inflammatory factors from activated microglia can induce dopaminergic neuronal cell death and inhibition

Abbreviations: COX-2, cyclooxygenase-2; CPF, chlorpyrifos; ERK, extracellular signal-regulated kinase 1/2; HO-1, heme oxigenase-1; JNK, c-Jun N-terminal kinase; NAC, *N*-acetyl cysteine; NF-κB, nuclear transcription factor kappa-B; p38, p38 MAP kinase; ROS, reactive oxidative species.

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of microglia activation could protect dopaminergic neurons (Hunter et al., 2007; Liu et al., 2003). In particular, it has been reported that rotenone induces microglial activation *in vitro* and *in vivo* (Gao et al., 2002; Zhou et al., 2007). Indeed, a single exposure to paraquat in mice induces activation of microglia even in the absence of observable dopaminergic neuron damage (Purisai et al., 2007).

Cyclooxygenase-2 (COX-2) plays a key role in the inflammatory process via the formation of reactive oxygen species and is increased in the PD ventral midbrain compared to control (Teismann et al., 2003a). In addition, PGE, the major enzymatic product of COX-2, was higher in the SN of PD patients (Mattammal et al., 1995). In fact, COX-2 has been shown to be one of the earliest markers of oxidative stress within its environment (Madrigal et al., 2003). Studies from other groups have demonstrated protective actions of COX-2 inhibitors in various neurological disorders including epilepsy, stress and PD related pathologies (Akula et al., 2008; Dhir et al., 2007; Hunter et al., 2008). The findings suggest that the expression of COX-2 exacerbates microglia activation and induces neuroinflammation. However, little is known about the molecular mechanisms mediating CPF-induced COX-2 expression.

Nuclear factor kappa-B (NF-κB) is a key regulator of inflammation associated with COX-2 activity and is also involved in cellular differentiation, proliferation, apoptosis, oxidative response and immune response (Janssen-Heininger et al., 2000; Won et al., 2005). In addition, increased NF-κB levels have been found in the postmortem PD brain (Hunot et al., 1997) and NF-κB dimers were translocated from cytoplasm to nucleus in LPS-activated microglia *in vitro* (Xing et al., 2007). Furthermore, the activation of NF-κB is known to be mediated by wide upstream protein kinases. MAPKs have been involved in cellular responses to proinflammatory and other stress signals. Numerous reports have demonstrated that MAPKs played a critical role in microglia-mediated neuronal death in neurodegenerative disease and inhibition of MAPKs could protect dopaminergic neurons (Lee et al., 2005; Xie et al., 2004; Xing et al., 2007).

Several studies have found that anti-inflammatory drugs have beneficial effects in the prevention of neurodegenerative disease. Rosiglitazone (RGZ), an insulin-sensitizing drug with selective agonistic activity at peroxisome proliferator-activated receptor-γ (PPARγ), has been shown to improve neuronal survival by exerting a broad spectrum of anti-inflammatory effects *in vitro* and in animal models of PD by inhibiting expression of COX and several interleukins as well as activation of microglia (Drew et al., 2006; Lee et al., 2012a; Kiaei et al., 2005; Luo et al., 2006; Yan et al., 2003).

Based on the previous evidence, in this study we aimed to determine whether activation of NF-κB has a role in the COX-2-induced neuronal cell death by CPF. Therefore, we were designed to investigate the effects of RGZ on CPF-induced neuronal cell death and to elucidate the related signaling pathways. We found that, RGZ had neuroprotective effects through the inhibition of COX-2 expression via blockade of NF-κB translocation cytosol to nucleus. In addition, we revealed the antioxidant effect of RGZ on CPF-induced oxidative stress.

Materials and methods

Reagents. Antibodies used were as follows: anti-COX-2 from Abcam (Cambridge, UK), anti-p-NFκB, TNF-α, caspase-3, PPAR-γ, HO-1, p-p38, p-JNK and p-ERK from Cell Signaling (MA, USA). Selective inhibitors were as follows: rosiglitazone from Cayman (MI, USA). Selective inhibitor was SN50 from Calbiochem (EMD Biosciences, Inc. San Diego, CA, USA). All other chemicals were obtained from Sigma-Aldrich.

Cell culture and treatment. SH-SY5Y cells were obtained from the American Type Culture Collection (ATCC, VA, USA) and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), and 25 μg/μl penicillin–streptomycin. Cells were seeded onto plates and grown at 37 °C in a humidified 5% CO_2 atmosphere. Cells used for western blot analysis were grown in six-well cluster dishes, whereas those used for cell viability assays were grown in 96-well plates. Cells were plated at a density of 5×10^4 cells (96-well plate) and allowed to attach overnight. Culture media were replaced every 3 days. The cells were used for experiments prior to passage 20.

Drug treatment. CPF was obtained from Sigma-Aldrich (MO, USA). The incubation time for CPF (0–200 μM) treatment ranged from 0 to 24 h as indicated in the figures and figure legends. Because CPF is a lipophilic molecule, we were concerned that binding of CPF to serum proteins might compromise its activity and thus, cells were transferred to low serum medium containing 0.1% FBS before CPF treatment to prevent excessive serum retention of CPF. CPF, SN50 and RGZ were dissolved in dimethyl sulfoxide (DMSO), which served as the vehicle control for these agents. SN50 and RGZ were administered 30 min and 2 h before CPF treatment, respectively.

Cell viability. Cell viability was measured by MTS assay (CellTiter96 AQueous One Solution Cell Proliferation Assay, Promega, WI, USA). Briefly, MTS was added to SH-SY5Y cells in 96-well plates and the plates were incubated at 37 °C for 4 h in a humidified 5% CO_2 atmosphere. Metabolically active cells convert the yellow MTS tetrazolium compound to a purple formazan product. The latter is soluble in tissue culture medium and the quantity of formazan product as measured by absorbance at 490 nm is directly proportional to the number of living cells in culture. Results were expressed as a percentage of the controls.

Lactate dehydrogenase (LDH) release measurement. An LDH cytotoxicity detection kit (Takara, MK401, Japan) was used to measure the leakage of soluble cytoplasmic LDH into the extracellular medium due to cell death. LDH converts pyruvate to lactic acid in the presence of reduced β-nicotinamide adenine dinucleotide (NADH), and any pyruvate not converted to lactic acid produces a brightly colored phenylhydrazone when treated with 2,4-dinitrophenylhydrazine. After incubation in the presence of either CPF or vehicle, culture medium was collected and centrifuged at 4000 ×g for 10 min at 4 °C. The resulting supernatant was used to measure LDH activity, following the manufacturer's instructions. The reaction was run in the dark for 30 min prior to measurement, and the absorbance was measured with a multi-plate reader at 490 nm.

Measurement of intracellular ROS. The conversion of non-fluorescent chloromethyl-DCF-DA (2,7-dichlorofluorescindiacetate) to fluorescent DCF was used to monitor intracellular ROS production. Cells plated in coated six-well plates were grown in EMDM medium and treated with 100 μM CPF or DMSO as a control for 6 h, with or without pretreatment with the antioxidant, *N*-acetyl cysteine (NAC). Because 24 h incubation with CPF leads to a significant reduction in cell density at higher concentrations, ROS production varies in a CPF concentration-dependent manner, and ROS itself has a short half-life, cells were incubated with various concentrations of CPF for 6 h. The medium was removed and cells were washed with PBS. Then, 200 μlof DCF-DA (10 μM) was added followed by incubation for 30 min at 37 °C in the dark and a subsequent washing with PBS to remove excess dye. Cells were detached with trypsin and washed in PBS. After centrifugation, the cell pellet was suspended in 500 μl PBS. Intracellular ROS production was detected by the signal obtained using flow cytometry (BD FACSCalibur; BD Bioscience; CA; CellQuest Software) and fluorescent images were acquired with an Olympus microscope.

Nuclear morphology assessment by fluorescence microscopy. Nuclear morphological changes were measured using Hoechst 33342 (Invitrogen, Carlsbad, CA, USA). The cells were grown on cover slips in 24-well plates coated with poly-L-lysine. After being treated with CPF for 24 h, the cellular monolayer in 24-well plates was fixed with 4% paraformaldehyde for 20 min and stained with 5 μg/ml Hoechst 33342 solutions in the dark for

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