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Paraquat induces cyclooxygenase-2 (COX-2) implicated toxicity in human neuroblastoma SH-SY5Y cells

Wonsuk Yang[a], Evelyn Tiffany-Castiglioni[c], Mi-Young Lee[d], Il-Hong Son[a,b,*]

[a] Inam Neuroscience Research Center, Department of Neurology, Sanbon Medical Center, College of Medicine, Wonkwang University, Sanbon, 435-040, Republic of Korea
[b] Department of Neurology, College of Medicine, Wonkwang University, Sanbon, Republic of Korea
[c] Department of Integrative Biosciences and Faculty of Toxicology, Texas A&M University, College Station, Texas, USA
[d] Department of Medical Biotechnology, College of Medical Sciences, Soonchunhyang University, Asan, Republic of Korea

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ABSTRACT

Paraquat produces dopaminergic pathologies of Parkinson's disease, in which cyclooxygenase-2 (COX-2) is implicated. However, it is unclear whether paraquat induces toxicity within dopaminergic neurons through COX-2. To address this, human neuroblastoma SH-SY5Y cells were treated with paraquat and then the involving mechanism of COX-2 was investigated. We initially examined the involvement of COX-2 in paraquat-induced toxicity. Data suggest that COX-2 is implicated in paraquat-induced reduction of viability in SY5Y cells. Then, to confirm the presence of COX-2 in SY5Y cells, we examined COX-2 mRNA and protein levels, which are regulated by NF-κB. Data indicate that paraquat activates NF-κB and up-regulates COX-2. We then checked quinone-bound proteins as quinones produced by COX-2 bind to intracellular proteins. Paraquat obviously forms quinone-bound proteins, in particular, quinone-bound DJ-1 and this formation is attenuated by meloxicam. Finally, we investigated antioxidant system including nuclear factor erythroid-related factor 2 (Nrf2), gamma glutamylcysteine synthetase (γGCS), and glutathione (GSH) as DJ-1 is linked to Nrf2 and Nrf2 regulates γGCS expression and γGCS is a GSH synthesis enzyme. Paraquat decreases protein levels of Nrf2 and γGCS and intracellular GSH level and these decreases are alleviated by meloxicam. Therefore, collectively, our data indicate that paraquat induces COX-2 implicated toxicity in SY5Y cells. In conclusion, current findings support the idea that paraquat might produce toxicity in dopaminergic neurons through COX-2.

1. Introduction

Paraquat (1,1′-dimethyl-4,4′ bipyridium dichloride) belongs to a class of bipyridyl herbicides commonly used as a weed controller, defoliant, and pre- or post-harvest desiccant, (EPA R.E.D. Facts, 1997). Though its use is restricted in many countries, the occupational, accidental, or intentional exposure of humans to paraquat still occurs (Madeley, 2002). Brain damages are found in patients, who drank paraquat to commit suicide (Hughes, 1988). Of particular concern, numerous studies suggest a link between exposure to paraquat and an increased risk for Parkinson's disease (PD) development (Dinis-Oliveira et al., 2006). Paraquat is structurally similar to MPP+, the active metabolite of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), which is known to produce parkinsonian symptoms in drug users (Langston et al., 1999) and PD pathologies in animals (Burns et al., 1983). In addition, a recent epidemiologic study shows the possible correlation in between exposure to paraquat and PD development in humans (Costello et al., 2009). This hypothesis is supported by laboratory studies demonstrating that paraquat reproduces the cardinal PD pathologies such as loss of dopaminergic neurons (Kuter et al., 2007), dopamine deficiency in the striatum (Liou et al., 1996), and protein aggregation in dopaminergic neurons (Manning-Bog et al., 2002) as well as other pathologies that include oxidative stress (McCormack et al., 2005), proteasome dysfunction (Yang et al., 2007), and mitochondrial dysfunction (Castello et al., 2007). Therefore, paraquat is classified as an environmental etiologic factor of PD in humans (Brown et al., 2006).

One mechanism by which dopaminergic neurons may be killed or damaged in Parkinson's disease or its models is via cyclooxygenase-2 (COX-2). COX-2 catalyzes the conversion of arachidonic acid into prostaglandins (O'Banion, 1999) and also oxidizes dopamine into dopamine quinones, which produce dopaminergic neurotoxicity (Miyazaki and Asanuma, 2009). COX-2 is nearly undetectable in most tissues under normal physiological condition, but is rapidly induced by various stimuli such

* Corresponding author at: Wonkwang University, Neurology 1142, Sanbon-dong, Gunpo-city, Gyeonggi-do, Sanbon 435-040, Republic of Korea.
Tel.: +82 2 31 390 2410; fax: +82 2 31 390 2414.
E-mail address: sonih@wonkwang.ac.kr (I.-H. Son).

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as pro-inflammatory cytokines (Narita et al., 2008), growth factors (Tamura et al., 2002), and bacterial endotoxins (Lacroix and Rivest, 1998). In brain, its expression leads to the production of prostaglandins, which in turn, induce toxicity in neurons (Manabe et al., 2004) and thus, COX-2 is suspected to be pathologically linked to neurological diseases including stroke, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) (Liang et al., 2007), and PD.

Several lines of evidence link COX-2 to dopaminergic cell death in PD. COX-2 protein levels increase in dopaminergic neurons in PD brain tissue (Teismann et al., 2003) and the levels of prostaglandin E2, a major product of COX-2, are higher in the substantia nigra of PD brain, compared to normal brain (Mattammal et al., 1995). In vivo studies show that the pharmacological inhibition and genetic deletion of COX-2 attenuate dopaminergic neuronal death in mice treated with MPTP (Teismann and Ferger, 2001; Vijitruth et al., 2006). In addition, a recent report demonstrates that COX-2 produces dopaminergic neuronal loss through prostaglandin E2 and its receptor EP1 in mice treated with 6-hydroxydopamine (6-OHDA) (Carrasco et al., 2007). Furthermore, Litteljohn et al. (2008) recently reported that the formation of 3,4-dihdroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA) is attenuated in striatum of COX-2 deficient mice treated with paraquat, implicating COX-2 in paraquat-induced pathology. Thus, ample evidence supports the hypothesis that dopaminergic neuronal death induced by paraquat is mediated by COX-2.

In order to test this hypothesis, we examined the toxic mechanisms by which COX-2 contributed to cell death in the SH-SY5Y human neuroblastoma cells treated with paraquat. This cell culture model has been previously used by our group to elucidate dopaminergic pathologies of PD (Yang and Tiffany-Castiglioni, 2005, 2007) as these cells express the phenotypes of human dopaminergic neurons (Xie et al., 2010). Endpoints measured in the current study, were protein levels of I kappa B alpha (IκBα), COX-2, nuclear factor erythroid-related factor 2 (Nrf2), and gamma glutamylcysteine synthetase (γGCS), as well as mRNA levels of COX-2. We also examined the DNA binding activity of p65, the formation of quinone-bound DJ-1, the levels of quinone-bound protein and the levels of intracellular glutathione (GSH) in paraquat-treated SY5Y cells.

2. Materials and methods

2.1. Materials

T-75 flasks and culture dishes (100 mm × 20 mm) were purchased from SPL Life Sciences (Seoul, Korea). 96-well plates were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Korea (Seoul, Korea). Meloxicam was purchased from Calbiochem (San Diego, CA, USA). Monochlorobimane (MCB) was purchased from Invitrogen (Carlsbad, CA, USA). A lactate dehydrogenase (LDH) cytotoxicity assay kit was purchased from BioVision (Mount view, CA, USA). A cell proliferation reagent WST-1 was purchased from Roche Applied Science (Indianapolis, IN, USA). A Nuclear Extraction Kit and NF-kB transcription factor assay kit were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The ready gradient gels (4–15%), protein or DNA size marker and iScript one step reverse transcription-polymerase chain reaction (RT-PCR) kit with SYBR Green were purchased from Bio-Rad Korea (Seoul, Korea). An RNA extraction kit NucleoSpin RNA II (Macherey-Nagel) was purchased from Power Lab (Suwon, Korea). Rabbit polyclonal antibodies to IκBα and tyrosine hydroxylase, respectively, were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibodies to COX-2 or Nrf2 and an anti-rabbit IgG antibody were purchased from Santa Cruz (Santa Cruz, CA, USA). A rabbit polyclonal antibody to DJ-1 was purchased from Abcam (Cambridge, UK). A rabbit polyclonal antibody to γGCS was purchased from Thermo Fisher Scientific Inc (Fremont, CA, USA). A mouse monoclonal antibody to β-actin was purchased from Sigma (St Louis, MO, USA). ECL Western blotting detection reagent was purchased from Animal Genetics, Incorporated (Tallahassee, FL, USA). Unless specified, all other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture and chemical treatment

Human neuroblastoma SH-SY5Y cells were purchased from the Korean Cell Line Bank (KCLB). The expression of tyrosine hydroxylase was confirmed in these cells by Western blotting (data not shown), indicating that SY5Y cells used in this study possess the dopaminergic phenotype. Stock cultures of SH-SY5Y cells (passage 25–35) were maintained in T-75 flasks. Unless specifically mentioned, cultures used for treatments were seeded at 3×10^6 cells in 100 mm × 20 mm culture dishes, grown for 2 days until they reach 70% confluence, and then treated with test chemicals. SY5Y cells were treated with paraquat (0.5 mM, PQ) for 48 h to examine COX-2 mediated toxicity in this study. We have previously shown that this PQ treatment regimen significantly induces cytotoxicity in SY5Y cells (Yang and Tiffany-Castiglioni, 2005, 2007). Various other toxicants and inhibitors were used as positive and negative controls. For example, meloxicam was used to inhibit COX-2 activity (Chae et al., 2007, 2008) and tetrahydrobiopterin (BH4) was used to confirm the generation of quinone-bound protein (Han et al., 2007). The dopaminergic neurotoxicant 6-OHDA was as a positive control used to confirm NF-κB activation (Levites et al., 2002; Zhang et al., 2007), COX-2 induction (Lin et al., 2007), and DJ-1 oxidation (Nunome et al., 2008) by PQ. N-acetyl cysteine (NAC) was used to scavenge quinones in order to inhibit the formation of quinone-bound protein (Xu et al., 1996; Bagh et al., 2008). L-Buthionine-sulfoximine (BSO) was used to inhibit γGCS activity to deplete glutathione (Yang and Tiffany-Castiglioni, 2005). In preliminary experiments, meloxicam and vehicles (saline and DMSO) themselves had no effect on NF-κB activation, mRNA and protein levels of COX-2, levels of quinone-bound protein, levels of DJ-1, Nrf2 and γGCS protein, and GSH level for up to 50 h (data not shown) and thus, this exposure regimen was applied to pre-treat or treat SY5Y cells. These chemicals also had no toxic effect on SY5Y cells for up to 50 h, according to formazan formation and lactic dehydrogenase activity assays (data not shown).

2.3. Formazan formation

Cytotoxicity was measured with a cell proliferation reagent WST-1 according to manufacturer's protocol. In brief, 3×10^4 cells per well were seeded into a 96-well plate in duplicates and the next day, were pre-incubated with vehicles (saline for paraquat; DMSO for meloxicam) for 2 h and then treated with paraquat (0.5 mM) or meloxoicam (5–40 μM), respectively, for 48 h. Additional cultures were pre-treated with meloxicam (5–40 μM) for 2 h and then further treated with paraquat (0.5 mM) for 48 h. Vehicle controls were incubated with vehicles for 50 h. The formazan levels were determined by measuring the absorbance of reaction mixture at 440 nm with a Synergy 2 Multi-Detection microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

2.4. Lactate dehydrogenase (LDH) activity

Cell membrane integrity was measured by the activity of LDH released into the culture medium according to manufacturer's protocol. 3×10^4 cells per well were seeded into a 96-well plate in duplicates and the next day, were pre-incubated with vehicles (saline for paraquat; DMSO for meloxicam) for 2 h and then treated with paraquat (0.5 mM) or meloxoicam (5–40 μM), respectively for 48 h. Additional cultures were pre-treated with meloxicam (5–40 μM) for 2 h and then further treated with paraquat (0.5 mM) for 48 h. Vehicle controls were incubated with vehicles for 50 h. The LDH activity was determined by measuring the absorbance of reaction mixture at 490 nm with a Synergy 2 Multi-Detection microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

2.5. NF-κB (p65) DNA binding activity

The DNA binding activity of NF-κB (p65) was measured with a NF-κB transcription factor assay kit according to manufacturer's protocol. Cells grown in culture dishes (100 mm × 20 mm) were pre-incubated with a vehicle (saline) for 2 h and then treated with paraquat (0.5 mM) for up to 48 h. Vehicle controls were incubated with saline for 50 h. For a positive control, cells were treated with 6-OHDA (100 μM) for 24 h. The p65 DNA binding activity was determined by measuring the absorbance of reaction mixture at 450 nm with a Synergy 2 Multi-Detection microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

2.6. Quantitative reverse transcription (RT)-polymerase chain reaction (PCR)

Cells grown in culture dishes (100 mm × 20 mm) were pre-incubated with a vehicle (saline) for 2 h and then treated with paraquat (0.5 mM) for up to 48 h. Vehicle controls were incubated with saline for 50 h. Positive control cultures were treated with 6-OHDA (100 μM) for 24 h. After treatment, 1.5 μg of RNA isolated from cells were mixed with the solutions of a One-Step RT-PCR kit and 300 nmol (final concentration) each of forward and reverse primers in a 96-well optical plate. The mixture was adjusted to a final volume of 50 μl with nuclease-free water. The sequences of the primers used in this study are as follows: human COX-2 (forward: 5′-ACCAGAGCAGGCAGATGAAATACC-3′/reverse: 5′-CTACCAGAAGGGCAGGATACAGC-3′) and human β-actin (forward: 5′-GCACCACACCTTCTACAATGAGC-3′/reverse: 5′-TAGCACAGCCTGGATAGCAACG-3′). The cDNA was synthesized at 50 °C for 10 min and then reverse transcriptase was inactivated at 95 °C for 5 min. PCR amplification