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The acute exposure of tetrachloro-*p*-benzoquinone (a.k.a. chloranil) triggers inflammation and neurological dysfunction *via* Toll-like receptor 4 signaling: The protective role of melatonin preconditioning

Juanli Fu, Xiaomin Xia, Zixuan Liu, Yawen Wang, Yuxin Wang, Qiong Shi, Xiufang Song, Erqun Song, Yang Song*

Key Laboratory of Luminescence and Real-Time Analytical Chemistry (Southwest University), Ministry of Education, College of Pharmaceutical Sciences, Southwest University, Chongqing, 400715, People's Republic of China

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ABSTRACT

This study is aimed to investigate the inflammation and neurological dysfunction induced by tetrachloro-*p*-benzoquinone (TCBQ) through Toll-like receptor 4 (TLR4) signaling. We also investigated the protective role of melatonin as an antioxidant and anti-inflammatory agent. In vitro model was established by rat pheochromocytoma PC12 cells, meanwhile, TLR4 wild-type (C57BL/6) and knockout mice (C57BL/10ScNJ TLR4$^{-/-}$) were used as in vivo model. In vitro study showed TCBQ exposure enhanced the expression of TLR4, myeloid differentiation factor 88 (MyD88) at both transcriptional and post-transcriptional levels. By contrast, melatonin decreased TLR4 and MyD88 expressions. Moreover, our result indicated that melatonin disrupted the formation of TLR4/MyD88/MD2/CD14 complex. In addition, melatonin terminated TCBQ-mediated phosphorylation of c-Jun N-terminal kinase (JNK), p38, and extracellular regulated protein kinase (ERK) signaling and hampered its downstream pro-inflammatory cytokine releases. In vivo result also indicated TLR4 deficiency partially protected against TCBQ-induced morphological and neuropathological changes in mice brain, suggested the role of TLR4. In conclusion, melatonin modulates TCBQ-mediated inflammatory genes through TLR4/MyD88-dependent signaling pathway. Our current study, to the best of our knowledge, is the first time show melatonin not only disrupt the binding of TLR4 and MyD88, but also restricted the formation of TLR4/MD2/CD14 complex, suggesting that melatonin supplementary may represent a valuable therapeutic strategy for inflammatory neurological dysfunction.

1. Introduction

Environmental pollutants-caused health problems have drawn much attention nowadays. Pentachlorophenol (PCP) has been extensively used as herbicide and insecticide in the agriculture and industry due to its broad applicability, however, it is recognized as a priority environmental contaminant. PCP has been listed as a group 2B (possibly carcinogenic to humans) carcinogen by the International Agency for Research on Cancer (IARC) and a group B2 carcinogen (probable human carcinogen) by U.S. Environmental Protection Agency (EPA). Pentachlorophenol (and its salts and esters) were classified as persistent organic pollutants (POPs) by Stockholm Convention in 2015 (Stockholm Convention, 2015). Tetrachloro-*p*-benzoquinone (TCBQ) is an oxidative metabolite of PCP (Yadid et al., 2013). TCBQ, also known as chloranil, is a widely-used fungicide as well. In China, the annual production of TCBQ is

Abbreviations: CCK-8, cell counting kit-8; Co-IP, co-immunoprecipitation; ERK, extracellular regulated protein kinase; IL-1, interleukin-1; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; MAPK, melatonin receptors protein kinase; MT1R, melatonin receptors MT1; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-kappa B; NGF, Nerve growth factor; PCP, pentachlorophenol; RAGE, receptor for advanced glycation end-products; ROS, reactive oxygen species; RT-qPCR, real-time quantitative PCR; TBST, tris-buffered saline containing 0.1% Tween 20; TCBQ, tetrachloro-*p*-benzoquinone; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor alpha.

* Corresponding author at: College of Pharmaceutical Sciences, Southwest University, Beibei, Chongqing, 400715, People's Republic of China.
E-mail addresses: songyangwenrong@hotmail.com, ysong@swu.edu.cn (Y. Song).

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approximately 2000 tons in recent years (Liu et al., 2012b). Thus, TCBQ may pose a considerable humans and environmental health risk. Although PCP has been extensively investigated, the toxic mode-of-action of TCBQ has not been unveiled.

Quinones are a group of toxicological substances, which are able to generate hazardous effects in vitro and in vivo, such as cytotoxicity, immunotoxicity, and carcinogenesis. It is believed that the production of reactive oxygen species (ROS) play an important role for TCBQ to exert its toxic effects (Bolton et al., 2000; Monks and Jones, 2002). The characteristics of TCBQ-derived semiquinone and hydroxyl radicals has been previously reported (Song et al., 2008, 2009). Recently, TCBQ has been shown to be a pro-oxidative and pro-inflammatory agent in our in vivo and in vitro studies (Dong et al., 2014; Xu et al., 2014a,b). Meanwhile, the possible mechanism of quinone toxicity includes covalent binding to macromolecules to form adducts (Nguyen et al., 2005; Song et al., 2009; Waidyanatha et al., 1994).

Melatonin is predominantly synthesized and released from the pineal gland at night (Stehle et al., 2011). It is well known as a transmitter which involved in the regulation of circadian, as well as seasonal rhythms (Hardeland et al., 2012). Besides, accumulating evidence indicated that melatonin have numerous physiological and pharmacological activities, including anti-inflammatory, anti-oxidative, anti-apoptotic properties (Zhang and Zhang, 2014). Melatonin has been proved to be protective in several ROS-associated neurological dysfunctions, for example neurodegenerative diseases like Alzheimer's disease and Parkinson's disease (Mayo et al., 2005; Rosales-Corral et al., 2012). The protective effects of melatonin on these conditions are ascribed to its modulation of neuro-inflammation, through the downregulation of both early and late phase nuclear factor-kappa B (NF-κB) activation. Consistently, the expressions of inflammatory related genes, including interleukin-1 (IL-1), IL-6, IL-12, IL-10 and tumor necrosis factor alpha (TNF-α), were also manipulated by melatonin. Although a broad range of pharmacological activities of melatonin has been suggested, insufficient information concerned about its anti-inflammatory capability against environmental pollutants-caused neurological dysfunction.

Herein, we attempted to evaluate the inhibitory role of melatonin in TCBQ-induced oxidative stress and inflammatory effects. Although the parent compound of TCBQ, PCP showed unambiguous neurological toxicity, the effect of TCBQ needs further investigation. Thus, a well-established in vitro model, PC12 cell line, has been selected in the current study. Our group has recently demonstrated that TCBQ promotes ROS-dependent apoptotic (Hu et al., 2015) and inflammatory responses via the activation of IKK/IκB/NF-κB pathway in PC12 cells (Fu et al., 2016b), which suggest its neurotoxicity. In mammals, Toll-like receptor (TLR) play a critical role in innate of inflammatory process (Takeda and Akira, 2004; Wang et al., 2014). There are 10 members in TLR family, each TLR recognizes specific components of pathogens. One of the most studied members of the TLR family, TLR4, recognizes its receptor and recruits the adapter molecule myeloid differentiation factor 88 (MyD88) (Takeda and Akira, 2004). In turn, it leads to the activation of NF-κB and the mitogen-activated protein kinase (MAPK) transduction cascades. Previous studies have shown that TLRs are also involved in the cellular response to organic, inorganic compounds, or nano-size materials (Roy et al., 2014; Testa et al., 2014; Tsou et al., 2013; Ze et al., 2014). Thus, we analyzed the anti-inflammatory role of melatonin through regulating TLR4-mediated signaling. In this study, the mechanisms underlying the actions of melatonin in TCBQ-stimulated TLR4-signaling were explored. This study provides important information for melatonin as a potential candidate against TCBQ-induced inflammatory actions.

2. Materials and methods

2.1. Chemicals and reagents

TCBQ (>98% purity) was purchased from Aladdin Reagent Database Inc. (Shanghai, China). Nerve growth factor (NGF) and melatonin were purchased from Sigma-Aldrich Co. LLC. (Shanghai, China). Cell counting kit-8 (CCK-8) was obtained from Vazyme biotech Co. Ltd. (Nanjing, China). Rabbit IL-6, c-jun N-terminal kinase (JNK), p-JNK, extracellular signal-regulated kinase (ERK1/2), c-jun, β-actin, Lamin B polyclonal antibodies, mouse TNF-α polyclonal antibody, and goat anti-mouse IgG-HRP-conjugated secondary antibody were obtained from Proteintech (Wuhan, China). Rabbit IL-1β, p-ERK1/2, p38, p-p38, melatonin receptors MT1 (MT1R) and MT2R polyclonal antibodies, goat anti-rabbit IgG-HRP-conjugated secondary antibody, total protein extraction kit and nuclear/cytoplasmic extraction reagents were obtained from Sangon Biotech Co., Ltd (Shanghai, China). Rabbit NeuN, Iba-1, MyD88, c-fos and c-jun polyclonal antibodies were purchased from Bioss Biotech Co., Ltd. (Beijing, China). Rabbit AP-1 antibody was obtained from Ruiying Biological (Suzhou, China). TLR4 and MyD88 siRNA were purchased from GenePharma Co., Ltd. (Shanghai, China). All other chemical substances used were of highest commercial grade.

2.2. Cell culture

Rat pheochromocytoma PC12 cell line was purchased from Nanjing KeyGEN Biotech. Co., Ltd. (Nanjing, China). Cells were maintained in DMEM supplemented with 10% newborn calf serum (Zhejiang Tianhang Biological Technology Co., Ltd.), 100 U/ml penicillin and 100 U/ml streptomycin in an incubator aerated with 5% CO_2 at 37 °C. For differentiation, after plating cells for 24 h, a concentrated stock of NGF was added to the culture medium at a final concentration of 50 ng/ml. Fresh media containing 50 ng/ml NGF was replaced every other day for 8 days.

2.3. Cell viability

PC12 cell viability was determined by CCK-8 according to the manufacture's instruction. In this assay, water-soluble tetrazolium salt (WST-8) is reduced by dehydrogenase activities in cells to give an orange-color formazan dye, which reflects the number of living cells. Cells were incubated with different concentrations of melatonin for 1 h, then exposed to 25 μM TCBQ for 6 h. After that, existing medium in every well was removed and replaced with 10 μl of CCK-8 solution, followed by incubation at 37 °C for 3 h. Supernatants were transferred to 96-well plates after incubation, the absorbance of the colored solution was measured using a microplate reader (BioTek ELX800) at a wavelength of 450 nm (maximum absorption wavelength of formazan).

2.4. Western blotting

Cells were lysed with RIPA lysis buffer and debris was removed by centrifugation. Protein concentrations were determined using the Bradford protein assay kit. Protein was separated by 10% or 12.5% SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in 50 mM Tris-buffered saline containing 0.1% Tween 20 (TBST) at 37 °C for 1.5 h and then incubated appropriate primary antibodies overnight at 4 °C. After washing in TBST three times, the membranes were incubated with secondary antibody at 37 °C for 2 h. The membranes were washed three times with TBST and visualized with ECL substrate system (Beyotime, Shanghai, China). Representative blots were chosen from three independent experiments.