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Tetrachlorobenzoquinone induces Nrf2 activation *via* rapid Bach1 nuclear export/ubiquitination and JNK-P62 signaling

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ABSTRACT

Our previous studies demonstrated that tetrachlorobenzoquinone (TCBQ), an active metabolite of pentachlorophenol, has effects on the generation of reactive oxygen species (ROS) and oxidative stress *in vitro* and *in vivo*. Nuclear factor erythroid-derived 2-like 2 (Nrf2) is a cellular sensor of electrophilic or oxidative stress that regulates the expression of antioxidant enzymes and defensive proteins. We have illustrated that TCBQ activates Nrf2 signaling by promoting the formation of the Kelch-like ECH-associated protein 1 (Keap1) cross-linking dimer and the formation of an ubiquitination switch from Nrf2 to Keap1. The activation of Nrf2 by TCBQ may serve as an adaptive response to a TCBQ-induced oxidative insult. BTB and CNC homolog 1 (Bach1) compete with Nrf2, leading to the negative regulation of the antioxidant response element (ARE). In this report, we propose that TCBQ induces the dynamic inactivation of Bach1. We observed a rapid nuclear efflux of Bach1 and an accumulation of Nrf2 in nuclei upon TCBQ treatment that precedes the binding of Nrf2 with ARE. We found that the nuclear export of Bach1 is dependent on its chromosomal region maintenance 1 (Crm1) interaction and tyrosine phosphorylation. Although TCBQ induces the ubiquitination of Bach1, TCBQ also increases the mRNA and protein levels of Bach1, returning Bach1 to normal levels. Moreover, we found that TCBQ-induced activation of Nrf2 involves c-Jun N-terminal kinase (JNK)-P62 signaling.

1. Introduction

Tetrachlorobenzoquinone (TCBQ) is an active metabolite of pentachlorophenol (PCP). PCP hydroxylase, which is isolated from several bacteria growing in soil and groundwater contaminated with PCP, catalyzes the hydroxylation of PCP, yielding TCBQ (Yadid et al., 2013). TCBQ is an effective inducer of ROS and oxidative stress with a strong affinity towards soft nucleophiles, such as cysteine residues in glutathione and proteins (Song et al., 2009; Su et al., 2015; Waidyanatha et al., 1996).

The generation of reactive oxygen species (ROS) and the induction of oxidative stress represent the typical toxic mode-of-action of environmental pollutants (Poljsak and Fink, 2014). ROS cause molecular damage when they react with macromolecules, thereby disrupting the intracellular redox status, which is responsible for the pathogenesis of diverse diseases and aging (Lam, 2015; Santilli et al., 2015). To maintain the cellular redox homeostasis, cells induce an antioxidant battery of protective enzymes, such as heme oxygenase-1 (HO-1) (Satoh et al., 2013).

HO-1 is one of the major defense enzymes against oxidative stress, and it is predominately regulated by nuclear factor erythroid 2-related factor 2 (Nrf2). Under unstressed conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1), which facilitates the proteasomal degradation of Nrf2 (Baird and Dinkova-Kostova, 2011). When stresses occur, Nrf2 is released from Keap1 "kidnapping" and then translocates into the nucleus, ultimately triggering the expression of antioxidant enzymes.

Previously, we demonstrated that TCBQ significantly stimulates HO-1 expression (Su et al., 2015). One of the possible mechanisms

Abbreviations: ARE, antioxidant response element; Bach1, BTB and CNC homolog 1; bZip, basic region leucine zipper; ChIP, chromatin immunoprecipitation; CHX, cycloheximide; Crm1, chromosomal region maintenance 1; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; FITC, fluorescein isothiocyanate; Crm1, chromosome region maintenance 1; HepG2, human hepatoma; HO-1, heme oxygenase-1; JNK, c-Jun N-terminal kinase; Keap1, Kelch-like ECH-associated protein 1; LMB, leptomycin B; NQO1, NAD(P)H/quinone oxidoreductase 1; Nrf2, nuclear factor erythroid 2-related factor 2; PCP, pentachlorophenol; ROS, reactive oxygen species; siRNA, small interfering RNA; TCBQ, tetrachlorobenzoquinone.

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for the release of Nrf2 by TCBQ involves the modification of Keap1. Specifically, the nucleophilic characteristics of TCBQ enable its covalent binding to Keap1, yielding Keap1-TCBQ conjugate. Alternatively, the pro-oxidative properties of TCBQ enhance cellular oxidative stress, resulting in the formation of disulfide bonds between Keap1 cysteine residues. Both mechanisms boost Keap1 dimer formation, leading to a conformational change in Keap1 and a weakened binding affinity between Keap1 and Nrf2. Meanwhile, modified Keap1 is classified as an "unwanted" component in cells and becomes the target for ubiquitination, suggesting a different machinery for Keap1-dependent Nrf2 control (Hong et al., 2005; Zhang et al., 2005).

However, a BTB and CNC homolog 1 (Bach1)-mediated mechanism for the regulation of Nrf2 has also been reported. Bach1 contains a BTB domain and a well-conserved basic region leucine zipper (bZip) domain that facilitate its binding to other bZip-containing proteins (Oyake et al., 1996). Normally, Bach1 forms heterodimers with the small Maf proteins to bind to antioxidant response element (ARE) sequences that are also separately bound by Nrf2 (Oyake et al., 1996; Sun et al., 2002). Essentially, Bach1 is a transcriptional repressor of Nrf2. Previous studies indicated that Bach1-deficiency is sufficient for the transcriptional induction of HO-1 that leads to a significant protective effect in different disease models (Reichard et al., 2008; Tanimoto et al., 2009). When stimulated, *e.g.,* by oxidative stress, Bach1 is released from ARE sites (Sun et al., 2004) and facilitates Nrf2 translocation and HO-1 gene activation (Itoh et al., 1999). Bach1 contains different nuclear export signals that respond to specific stimuli such as cadmium (Suzuki et al., 2003), arsenite (Reichard et al., 2008), heme (Suzuki et al., 2004) or antioxidants (Kaspar and Jaiswal, 2010). However, the effect of the organic pro-oxidant TCBQ on the nuclear export of Bach1 is unknown.

P62 (sequestosome-1) is known as a substrate of autophagy (Jain et al., 2010). Additionally, P62 has been found to interact with protein kinase C and function as an adaptor protein in nuclear factor κB (NF-κB) signaling pathways (Sanchez et al., 1998). Interestingly, recent studies demonstrated that P62 activates the stress responsive transcription factor Nrf2 (Komatsu et al., 2010; Lau et al., 2010) and stimulates the expression of genes containing an ARE in their promoter regions (Liu et al., 2007) *via* the inactivation of Keap1. On the other hand, Jain et al. mapped the ARE in the P62 promoter region that is responsible for P62 activation *via* Nrf2 (Suzuki et al., 2003). Collectively, p62 and Nrf2 create a positive feedback loop by inducing ARE-driven gene transcription. Therefore, the expression of anti-oxidative proteins may be determined by the regulation of transcription factors Nrf2 and Bach1 (Goven et al., 2008). Previous studies have also found that P62 was regulated by c-Jun N-terminal kinase (JNK) activation by treatment with a phenolic antioxidant (Zou et al., 2012), herbal medicine (Kim et al., 2014), or an endogenous hormone (Vegliante et al., 2016). In view of this, we studied the role of JNK in the regulation of P62 protein after treatment with TCBQ.

In the current study, we found that TCBQ induces the rapid export of Bach1 from the nucleus to the cytoplasm, where it is subsequently degraded in the cytoplasm, allowing Nrf2 to translocate into the nucleus. Then, Nrf2 binds to ARE and induces the expression of antioxidant proteins. Moreover, we also found that TCBQ induces the activation of Nrf2 through JNK-P62 signaling.

2. Materials and methods

2.1. Materials

Tetrachlorobenzoquinone (TCBQ) CAS#118-75-2, with a purity >98% (chemical structure is shown in Fig. 1), was obtained from Aladdin Reagent Database Inc. (Shanghai, China). Antibodies against Bach1, P62, chromosomal region maintenance 1 (Crm1), HO-1, NAD(P)H/quinone oxidoreductase 1 (NQO1), Lamin B and β-actin were purchased from Proteintech group, Inc. (Wuhan, China). Rabbit IgG antibody, the nuclear/cytosol fractionation kit and the EasyBlot ECL kit were supplied by Sangon Biotech Co., Ltd. (Shanghai, China). Antibodies against JNK, *p*-JNK, phosphotyrosine and the Bach1/Cy3 conjugated antibody were purchased from Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Antibodies against Nrf2 and ubiquitin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goldview nucleic acid stain and PCR amplification primers of Bach1 and ARE were synthesized by Dingguo Biotechnology Co., Ltd. (Beijing, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and genistein were supplied by Musheng Biotechnology (Chongqing, China). The proteasome inhibitor MG132 was obtained from Selleck Chemicals (Shanghai, China). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), fluorescein isothiocyanate (FITC)-conjugated AffiniPure goat anti-rabbit IgG (H + L), leptomycin B (LMB), chromatin immunoprecipitation (ChIP) assay kit, the JNK inhibitor SP600125 and Protein A agarose beads were obtained from Beyotime Institute of Biotechnology (Nanjing, China). Nrf2, Bach1, P62 and control small interfering RNA (siRNA) were synthesized by GenePharma Co., Ltd. (Shanghai, China). Cycloheximide (CHX) was purchased from Yuanye Biotechnology Co., Ltd. (Shanghai, China).

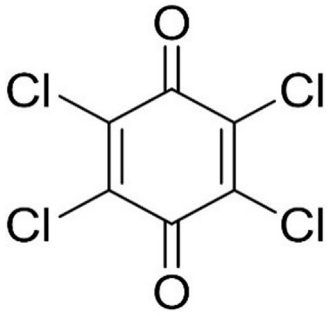

Fig. 1. Chemical structure of TCBQ.

2.2. Cell culture

Human hepatoma (HepG2) cells were supplied by the Third Military Medical University (Chongqing, China). Cells were grown in DMEM supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 mg/mL) and were then transferred onto plates in an incubator at 37 °C in 95% air and 5% CO_2 overnight before the experiments.

2.3. Cytosolic and nuclear proteins extraction

Cytosolic and Nuclear proteins were extracted by the nuclear/cytosol fractionation kit according to its manufacturer's instructions, and the cytosolic and nuclear extracts were stored at −20 °C and −80 °C, respectively.

2.4. Western blotting

The whole cell lysates, cytosolic and nuclear protein fractions and the immunoprecipitation products were separated by SDS-PAGE and were then transferred onto a PVDF membrane. Membranes were blocked with 5% bovine serum albumin (BSA) for 3 h and were then incubated with the indicated primary antibodies at 4 °C overnight and then with the secondary antibody for 2 h. The proteins were detected by the ECL system, and