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Covalent binding of quinones activates the Ah receptor in Hepa1c1c7 cells

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ABSTRACT — Highly reactive quinone species produced by photooxidation and/or metabolic activation of mono- or bi-aromatic hydrocarbons modulate cellular homeostasis and electrophilic signal transduction pathways through the covalent modification of proteins. Polycyclic aromatic hydrocarbons, but not mono- or bi-aromatic hydrocarbons, are well recognized as ligands for the aryl hydrocarbon receptor (AhR). However, quinone species produced from mono- and bi-aromatic hydrocarbons could potentially cause AhR activation. To clarify the AhR response to mono- and bi-aromatic hydrocarbon quinones, we studied Cyp1a1 (cytochrome P450 1A1) induction and AhR activation by these quinones. We detected Cyp1a1 induction during treatment with quinones in Hepa1c1c7 cells, but not their parent compounds. Nine of the twelve quinones with covalent binding capability for proteins induced Cyp1a1. Cyp1a1 induction mediated by 1,2-naphthoquinone (1,2-NQ), 1,4-NQ, 1,4-benzoquinone (1,4-BQ) and tert-butyl-1,4-BQ was suppressed by a specific AhR inhibitor and was not observed in c35 cells, which do not have a functional AhR. These quinones stimulated AhR nuclear translocation and interaction with the AhR nuclear translocator. Interestingly, 1,2-NQ covalently modified AhR, which was detected by an immunoprecipitation assay using a specific antibody against 1,2-NQ, resulting in enhancement of xenobiotic responsive element (XRE)-derived luciferase activity and binding of AhR to the Cyp1a1 promoter region. While mono- and bi-aromatic hydrocarbons are generally believed to be poor ligands for AhR and hence unable to induce Cyp1a1, our study suggests that the quinones of these molecules are able to modify AhR and activate the AhR/XRE pathway, thereby inducing Cyp1a1. Since we previously reported that 1,2-NQ and tert-butyl-1,4-BQ also activate NF-E2-related factor 2, it seems likely that some of quinones are bi-functional inducers for phase-I and phase-II reaction of xenobiotics.

Key words: Electrophile, Aryl hydrocarbon receptor, Quinone, CYP1A1

INTRODUCTION

Aromatic hydrocarbons are widely distributed in the environment; for example, mono- and bi-aromatic hydrocarbons, such as benzene and naphthalene, are present in gasoline and fuel oil, and naphthalene is commercially produced as an insect repellent. These chemicals are also found as contaminants in diesel engine exhaust, burning coal, and cigarette smoke (Wallace, 1989; Batterman et al., 2012).

The aryl hydrocarbon receptor (AhR) is a transcription factor, which in the 1970’s was found to be a protein that has high binding affinity for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds (Poland et al., 1976). Several classes of polycyclic aromatic hydrocarbons, such as biphenyls, polyhalogenated dibenzo-p-dioxins, and dibenzofurans, are reported to be efficient ligands for AhR, which is known as a sensor for polycyclic aromatic hydrocarbons (Waller and McKinney, 1995). A number of structure activity relationship studies with polycyclic aromatic hydrocarbons suggest that the size of the hydrocarbon is important to be able to fit the binding pocket of AhR, which has a size of 14 × 12 × 5 Å (Waller and McKinney, 1995). Conversely, kynurenine, and curcumin, which are non-classical ligands for AhR, are electrophilic and can modify protein nucl
ophiles and are known to induce cytochrome P450 1A1 (CYP1A1) through AhR activation (Opitz et al., 2011; Quattrochi and Tukey, 1993; Ciolino et al., 1998). These findings suggest that there may be ligand-independent electrophiles that can activate AhR. However, aromatic hydrocarbons with a ring number of one or two (e.g., benzenes and naphthalenes) cannot activate AhR, except for halogenated naphthalene that has ligand activity for AhR (Waller and McKinney, 1995). Thus, mono- and bi-aromatic hydrocarbons do not affect downstream proteins, such as CYP1A1, because they are too small to act as ligands (Badham and Winn, 2007; Genter et al., 2006).

Of toxicological interest, it is well known that benzene and naphthalene undergo conversion by photooxidation and/or metabolic activation to yield quinone species that are able to modify protein thiols, leading to the formation of protein adducts (Kumagai et al., 2012). A variety of quinones, including 1,4-benzoquinone (1,4-BQ), 1,2-naphthoquinone (1,2-NQ), and 1,4-NQ are found in diesel engine exhaust, burning coal, and cigarette smoke (Eiguren-Fernandez et al., 2010; Batterman et al., 2012; Wallace, 1989) and as metabolites during exposure of experimental animals and cultured cells (Wilson et al., 1996). Butylated hydroxyanisol (BHA) and tert-butyl-1,4-benzoquinone (TBQ), which are used as a food and cosmetic additive to preserve oils, are metabolized to tert-butyl-1,4-benzoquinone (TBQ) (Verhagen et al., 1989; Snyder and Hedli, 1996). We found that 1,2-NQ and TBQ with α,β-unsaturated carbonyl groups, are able to modify cellular proteins with reactive thiol groups and activate electrophilic signal transduction pathways, such as protein phosphatase 1B (PTP1B)/epidermal growth factor receptor (EGFR) signaling and Kelch-like ECH-associated protein 1 (Keap1)/NF-E2-related factor 2 (Nrf2) pathways (Kumagai et al., 2012). These observations led us to the hypothesis that chemical modification of cellular proteins could modulate their function(s), thereby changing cellular homeostasis processes mediated by them. A preliminary microarray analysis to identify a sensor protein for quinone compounds showed that TBQ up-regulated genes downstream of AhR in human pulmonary A549 cells (Abiko et al., unpublished observation), suggesting that aromatic hydrocarbon quinones, such as TBQ, could activate AhR. In the present study, we assessed induction of Cyp1a1 with aromatic hydrocarbons and their 12 quinone species (ring number = 1-2) in Hepa1c1c7 cells. Using quinones to induce Cyp1a1, we examined the translocation of AhR into the nucleus, interaction of AhR with aryl hydrocarbon receptor nuclear translocator (ARNT), and the covalent modification of cellular AhR, using a Cyp1a1 promoter assay and chromatin immunoprecipitation (ChIP) analysis. Our present study indicates that arylation of AhR is, at least in part, involved in its activation, resulting in induction of Cyp1a1.

MATERIALS AND METHODS

Materials
1,2-NQ (97.4% purity determined by HPLC), 1,4-NQ (98% purity determined by GC), TBQ (98% purity determined by GC), TBHQ (98% purity determined by GC), 1,4-BQ (98% purity determined by iodometric titration), 5,8-dihydroxy-1,4-NQ (79.8% purity determined by HPLC), and 2-hydroxy-1,4-NQ (99.9% purity determined by HPLC) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 5-Hydroxy-1,4-NQ (95% purity determined by HPLC), 2-chloro-1,4-BQ (95% purity determined by GC), 2,3,5,6-tetramethyl-1,4-BQ (97% purity determined by GC), 2-methyl-1,4-BQ (98% purity determined by HPLC) and 2-hydroxy-3-(3-methyl-2-butynyl)-1,4-NQ were obtained from Sigma-Aldrich (St. Louis, MO, USA). 2-Methyl-1,4-NQ (98% purity determined by HPLC) and BHA (98% purity determined by GC) were obtained from Nacalai (Kyoto, Japan). AhR antagonist, 1-methyl-N-[2-methyl-4-[2-(2-methylphenyl)diazencyl]phenyl]-1H-pyrazole-5-carboxamide (CH223191), was purchased from ChemBridge (San Diego, CA, USA). 1,2-NQ antibody was prepared as reported previously (Miura and Kumagai, 2010). All other reagents were of the highest grade available.

Determination of electrophilicity index

Lowest unoccupied molecular orbital energy (E_LUMO) and highest occupied molecular orbital energy (E_HOMO) were determined using Gaussian09 (ver. 8.0) software (Conflex, San Diego, CA, USA) to predict electrophilicity of chemical compounds. The ground state equilibrium geometries of each structure were calculated with density-functional RB3LYP 6-31G(d). The global hardness (H) was calculated as H = (E_LUMO - E_HOMO)/2. The total hardness was calculated as H = (E_HOMO + E_LUMO)/2. The electronic chemical potential (µ) was calculated as µ ≈ (E_HOMO + E_LUMO)/2. The electrophilicity index (ω) was given as ω = µ³/2η (Parra et al., 1999).

Covalent binding of quinones to protein detected by biotin-PEAC₅-maleimide (BPM) assay

The BPM precipitation assay was performed according to procedures described previously, with a minor modification (Abiko et al., 2015; Toyama et al., 2013). A homogenate of liver from C57BL/6J male mice was centrifuged at 600 x g for 10 min, the supernatant was centrifuged at
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9000 × g for 20 min, and then the lysate obtained was stored frozen under liquid nitrogen and kept at -80°C before use. The lysates were incubated with the quinones in 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (HCl) (pH 8.0) at 37°C for 1 hr, an aliquot (20 μg of protein) was reacted with 2 nmol of BPM in 25 mM sodium hydrogen carbonate (pH 9.5) at 37°C for 30 min, and then was subjected to western blot analysis with an horse radish peroxidase (HRP)-linked anti-biotin antibody (Cell Signaling Technology, Beverly, MA, USA). The bands were quantified using ImageJ software, and the 50% effective concentration (EC50) values were determined.

Cells and cell culture

Mouse hepatoma Hepa1c1c7 cells and its variant c35 cells, which have no functional AhR, from the American Type Culture Collection were cultivated in α-minimal essential medium (αMEM; Wako, Osaka, Japan) supplemented with 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Human lung adenocarcinoma epithelial A549 cells (RIKEN Cell Bank, Ibaraki, Japan) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Wako) containing 10% fetal bovine serum, 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and 2 mM L-alanyl-L-glutamine (Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO2. The cells were cultured in serum-free medium overnight to avoid that serum decreases the effect of electrophiles, and then exposed to each of the compounds.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted with ISOGEN (Wako) according to the manufacturer’s protocols, and complementary DNAs (cDNAs) were synthesized from 2 μg of total RNA using the High Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Grand Island, NY, USA). The resulting cDNA products were dissolved in a final volume of 200 μL, and a 5 μL aliquot was used as a template for subsequent quantification by RT-PCR. PCR was conducted in duplicate in a total volume of 20 μL using SYBR Green PCR Master Mix (Applied Biosystems) and 0.1 μM of primer, Cyp1al (5’-GTGTCGTTACTTGGACAACTG-3’ and 5’-AACATGGACATGCAAGC-3’), Gapdh (5’-TCAAACGAGCACTTCTTCCA-3’ and 5’-ACCCCTGAGCTTAGCCCTATCA-3’), CYP1A1 (5’-CCGGGACATCAACAGACACC-3’ and 5’-CCAGCTCAGAAGTGTTCAACA-3’), and GAPDH (5’-GGGCTGCTTTTAACTCTGGTAA-3’ and 5’-GGGCTGCTTTTAACTCTGGTAA-3’). Amplification was performed in an ABI 7500 Real Time PCR (Applied Biosystems); thermal cycling parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Relative differences between samples were determined by the ∆∆Ct method using each threshold cycle (Ct) values. The Ct values were normalized to values for Gapdh to determine ΔCt values. The ∆∆Ct, which were calculated by subtracting control ΔCt values from the corresponding ΔCt values of experimental samples, were converted to fold changes over control by 2(-∆∆Ct). Melting curve analysis was conducted to ensure a single PCR product.

Western blot analysis

The cells were seeded in 35 mm dishes and incubated for 24 hr, then incubated for a further 24 hr in serum-free medium. The cells were exposed to the chemicals and washed with ice-cold phosphate-buffered saline (PBS), then collected by scraping into 2% sodium dodecyl sulfate (SDS) and heated at 95°C for 20 min. Protein concentrations were determined using the BCA Protein Assay (Pierce, Rockford, IL, USA). Each sample was mixed with a half-volume of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (62.5 mM Tris-HCl, pH 6.8; 6% SDS; 24% glycerol; 15% 2-mercaptoethanol; and 0.015% bromophenol blue) and incubated at 95°C for 5 min. The cellular proteins were then separated using SDS-PAGE and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween 20 (TTBS, 20 mM Tris-HCl, pH 7.5; 150 mM sodium chloride (NaCl); and 0.1% Tween 20) and then incubated with anti-AhR or anti-ARNT antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as primary antibodies for 2 hr at room temperature in TTBS. To detect immunoreactive proteins, we used HRP-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) and an enhanced chemiluminescence system (Chemi-Lumi One; Nacalai). Representative blots are shown from three independent experiments.

Luciferase assay

Transient transfection of plasmid DNA was performed using Attractene (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. Briefly, Hepa1c1c7 cells were seeded at 2 × 10⁵ cells/cm² in 12-well plates. After the cells were incubated overnight, 0.8 μg of pAHRTKLuc3 cDNA, which was constructed according to procedures reported previously (Chang and Puga, 1998), and 0.08 μg of pRL-TL cDNA or Attractene reagent were mixed with OPTI-MEM
(Invitrogen) and incubated for 15 min at room temperature to allow the formation of complexes. These solutions were then added to the cells and incubated for 24 hr, followed by a further 24 hr incubation in serum-free medium. The cDNA-transfected cells were then exposed to the quinones, washed with PBS, and lysed with Passive Lysis Buffer (Promega, Madison, WI, USA). Luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

**Immunoprecipitation**

Cells were washed twice with PBS and lysed for 30 min on ice with 100 μL of radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.1% SDS; 0.5% deoxycholic acid; 1% NP-40) containing 1% protease inhibitor cocktail. The nuclei were pelleted, resuspended in nucleus lysis buffer (50 mM Tris-HCl, pH 8.0; 10 mM EDTA; 1% SDS), and then boiled in SDS-PAGE loading buffer. Proteins were detected by western blot analysis as described above.

**ChIP assay**

The ChIP assay was performed, with a minor modification, according to procedures described previously (Schnekenburger et al., 2007). Briefly, the cells were seeded in 15 cm dishes, incubated for 24 hr, and then incubated for a further 24 hr in serum-free medium. The cells were exposed to the chemicals, fixed with 1% formaldehyde for 20 min, and 2.5 M glycine was added to the cells. The cells were washed with ice-cold PBS, and then collected by scraping into 2% SDS

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BPM precipitation assay

The BPM precipitation assay was performed according to procedures described previously (Toyama et al., 2013). Briefly, A549 cells were exposed to 25 or 50 μM of TBQ or 1 μM MC after pretreatment of MG132. The cell lysates, which were collected into RIPA buffer, were incubated with BPM at 37°C for 30 min, then reacted with avidin-agarose at 4°C overnight. After centrifugation at 13,000 × g for 5 min, the agarose were washed with RIPA buffer and incubate at 95°C for 20 min. The samples were centrifuged at 13,000 × g for 10 min and the supernatant were boiled in SDS-PAGE loading buffer without 2-mercaptoethanol. AhR and ARNT were detected by western blot analysis as described above.

Statistical analysis

Statistical significance was assessed using the ANOVA; p < 0.05 was considered significant.

RESULTS

Cyp1a1 induction mediated by mono- and bi-aromatic hydrocarbon quinones

Exposure of mouse hepatoma Hepa1c1c7 cells to 1,2-NQ (5 μM, 6 hr) indicated that Cyp1a1 was a mostly induced mRNA (3.36 times increase) in the microarray data, by 1,2-NQ among 34,017 genes, which we examined (data not shown). Nqo1 (2.39 times increase) and Aldh3a1 (2.01 times increase) in the data (data not shown), which were downstream genes of AhR induced by 1,2-NQ (Nebert et al., 1993; Prochaska and Talalay, 1988). This result suggests that aromatic hydrocarbon quinones with electrophilic properties are capable of inducing Cyp1a1. To address whether or not such a chemical reactivity is associated with its capability to modified cellular proteins, five mono- and seven bi-aromatic hydrocarbon quinones with global electrophilicities (ω) ranging from 6.15 to 8.5 eV were used in this study. As shown in Fig. 1, 2-hydroxy-1,4-NQ (ω = 6.15 eV), 2,3,5,6-tetramethyl-1,4-BQ (ω = 6.36 eV), 2-methyl-1,4-NQ (ω = 6.39 eV), and 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-NQ (ω = 6.52 eV) had negligible chemical modification capacities as evaluated by the BPM assay (Toyama et al., 2013; Abiko et al., 2015), whereas other quinones arylated proteins in 9000 × g supernatant of mouse liver (Fig. 1). These results suggest that quinones with ω values of less than approximately 6.5 have negligible ability to enable covalent modification of the proteins. 2-Methyl-1,4-BQ with a ω value of 7.4 was the most potent arylation agent for the protein preparation. However, there

| No | Compound | R¹ | R² | R³ | R⁴ | ω (eV) | EC₅₀ (μM) |
|----|----------|----|----|----|----|--------|----------|
| 1  | 1,4-BQ   | H  | H  | H  | H  | 7.76   | 12.7     |
| 2  | 2-Methyl-1,4-BQ | CH₃ | H  | H  | H  | 7.40   | 6.7      |
| 3  | 2-Chloro-1,4-BQ | Cl  | H  | H  | H  | 8.50   | 13.9     |
| 4  | TBQ      | C(CH₃)₂ | H  | H  | H  | 7.32   | 16.4     |
| 5  | 2,3,5,6-Tetramethyl-1,4-BQ | CH₃ | CH₃ | CH₃ | CH₃ | 6.36   | > 100    |
| 6  | 1,2-NQ   | H  | H  | -  | -  | 7.01   | 38.5     |
| 7  | 1,4-NQ   | H  | H  | H  | H  | 6.58   | 18.1     |
| 8  | 2-Methyl-1,4-NQ | CH₃ | H  | H  | H  | 6.39   | > 100    |
| 9  | 5-Hydroxy-1,4-NQ | H  | H  | OH | H  | 7.61   | 16.4     |
| 10 | 2-Hydroxy-1,4-NQ | OH | H  | H  | H  | 6.15   | > 100    |
| 11 | 5,8-Dihydroxy-1,4-NQ | H  | H  | OH | OH | 8.50   | 12.1     |
| 12 | 2-Hydroxy-3-(3-methyl-2-butenyl)-1,4-NQ | CH₂CHC(CH₃)₂ | OH | H  | H  | 6.52   | > 100    |

Fig. 1. Electrophilicity values calculated from structures, and EC₅₀ values against proteins determined by BPM assay in mouse liver, of a variety of quinones.
was little correlation between covalent protein modification capacity and electrophilicity index under these conditions.

When Hepa1c1c7 cells were treated with the 12 quinones as described above, Cyp1a1 mRNA levels were enhanced by 2-methyl-1,4-BQ, 2-chloro-1,4-BQ, TBQ, 1,2-NQ, 1,4-NQ, 5-hydroxy-1,4-NQ, and 5,8-dihydroxy-1,4-NQ in a concentration-dependent manner (Figs. 2A and 2B). We used lower concentration of bi-aromatic hydrocarbon quinones (1 or 5 μM) than mono-aromatic hydrocarbon quinones (10 or 25 μM) because bi-aromatic hydrocarbon quinones showed higher toxicity in Hepa1c1c7 cells (data not shown). With bi-aromatic hydrocarbon quinones it was observed that increasing the electrophilicity index up to approximately 7.5 eV was associated with increased induction potency of Cyp1a1 gene expression; however, this mRNA level was enhanced by 5,8-dihydroxy-1,4-NQ with a high electrophilicity index (ω = 8.5 eV) by 2.1 times (1 μM) and 5.4 times (5 μM) compare to dimethyl sulfoxide (DMSO) (data not shown).

As shown in Fig. 2C, 1,4-BQ, TBQ, 1,2-NQ, and 1,4-NQ mediated gene expression of Cyp1a1 in Hepa1c1c7 cells, whereas the parent chemicals, including benzene, hydroquinone, naphthalene, BHA, and TBHQ, had no effect on Cyp1a1 expression even in higher concentration without toxicity (Fig. 2D).

Role of AhR in Cyp1a1 induction by mono- and bi-aromatic hydrocarbon quinones

To determine whether or not mono- and bi-aromatic hydrocarbon quinone-mediated Cyp1a1 induction is attributable to AhR activation, we used a specific inhibitor for AhR and c35 cells, which have low AhR activity because of a mutation in the AhR (Sun et al., 1997). TBQ- and 1,2-NQ-dependent Cyp1a1 induction was marked-

![Fig. 2. AhR-related increase in Cyp1a1 level during exposure of Hepa1c1c7 cells to quinones. Hepa1c1c7 cells were treated with (A) mono- or (B) bi-aromatic hydrocarbon quinone compounds for 4 hr. The total RNA was converted to cDNA, and then quantitative-PCR was performed using Cyp1a1 primer. 1, 1,4-BQ; 2, 2-methyl-1,4-BQ; 3, 2-chloro-1,4-BQ; 4, TBQ; 5, 2,3,5,6-tetramethyl-1,4-BQ; 6, 1,2-NQ; 7, 1,4-NQ; 8, 2-methyl-1,4-NQ; 9, 5-hydroxy-1,4-NQ; 10, 2-hydroxy-1,4-NQ; 11, 5,8-dihydroxy-1,4-NQ; 12, 2-hydroxy-3-(3-methyl-2-butenyl)-1,4-NQ and C, control in these figures. (C) The cells were treated with TBQ, 1,4-BQ, 1,2-NQ, or 1,4-NQ for 4 hr or 8 hr. (D) Hepa1c1c7 cells were treated with butylated hydroxyanisol (BHA), tert-butyl-1,4-hydroquinone (TBHQ), naphthalene (NP), benzene, 1,4-hydroquinone (HQ), and 3-methylcholanthrene (MC) for 4 hr. The total RNA was converted to cDNA, and then quantitative-PCR was performed using Cyp1a1 primer. Each value is the mean ± S.E. of three determinations. *Significantly different from DMSO control at p < 0.05 and ***, p < 0.01 (ANOVA).
ly blocked by pretreatment with CH223191, which is an AhR inhibitor (Fig. 3A). It is likely that CH223191 blocks AhR nuclear translocation whether it is classical ligand-mediated or non-classical ligand-mediated. In addition, the concentration-dependent induction of Cyp1a1 during treatment with TBQ, 1,4-BQ, 1,2-NQ, 1,4-NQ, and MC, which is a positive control of AhR activation, observed in Hepa1c1c7 cells, was completely abolished in c35 cells (Fig. 3B).

When AhR is activated by chemicals, AhR translocates into the nucleus. Then, this transcription factor heterodimerizes with ARNT in the nucleus and the resulting complex binds to the XRE on DNA (Fujisawa-Sehara et al., 1987; Soshilov and Denison, 2008). Consistent with this, treatment with TBQ, 1,2-NQ, 1,4-NQ, and 1,2-NQ all caused translocation of AhR into the nucleus in Hepa1c1c7 cells detected by immunostaining and Western blotting using nuclear fraction (data not shown). When 1,2-NQ was used as a model for bi-aromatic hydrocarbon quinones, interaction of AhR with ARNT was enhanced by 1,2-NQ in a concentration-dependent manner (Fig. 4A). Under the same conditions, an immunoprecipitation assay with an anti-1,2-NQ antibody revealed that AhR undergoes covalent modification by 1,2-NQ in a concentration-dependent manner (Fig. 4A). TBQ, 1,2-NQ, and 1,4-NQ induced XRE-dependent luciferase activity (Fig. 4B), and binding of AhR and RNA pol II, which is one of the important factors to initiate transcription (Schnekenburger et al., 2007), to the Cyp1a1 enhancer region (−1.3 kbp) and proximal promoter region (−0.1 kbp) were also detected with TBQ and 1,2-NQ (Figs. 5A and 5B). Mouse IgG and rabbit IgG, as a negative control, did not bind to the DNA region between −2.9 and +0.6 kbp of the mouse Cyp1a1 promoter (Figs. 5C and 5D).

**CYP1A1 induction mediated by TBQ in human pulmonary epithelial cells (A549 cells)**

We confirmed that mono-aromatic hydrocarbon quinone also induced CYP1A1 mRNA in human cell line and cells from a different organ using A549 cells, in which Nrf2 was constitutively activated because of mutation of its negative regulator Keap1 protein, resulting in permanent activation of Nrf2. Exposure of A549 cells to TBQ under non-toxic concentrations induced CYP1A1 mRNA (Fig. 6A) and enhanced interaction of AhR with ARNT (Fig. 6B). Under these conditions, XRE-driven luciferase activity was enhanced by TBQ exposure (Fig. 6C). Next, we performed BPM precipitation assay to detect electrophilic modification of TBQ to AhR protein and found that binding of BPM to AhR was decreased in a concentration of TBQ dependent manner, indicating that TBQ modified thiol groups of AhR protein (Fig. 6D).

**Cyp1a1 induction mediated by 1,2-NQ in vivo**

To confirm whether or not 1,2-NQ could induce Cyp1a1 in mouse liver in vivo, we treated with 1,2-NQ with different doses and MC as a model for Cyp1a1 inducer. Oral injection of MC (1 mg/kg) into mice resulted in a 1.57-fold increase Cyp1a1 mRNA level in the liv-

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**Fig. 3.** Quinone-mediated increase in Cyp1a1 level in the presence of an AhR antagonist and in c35 cells. (A) Hepa1c1c7 cells were treated with 10 μM CH223191 for 1 hr, and then the cells were treated with 10 μM TBQ, 5 μM 1,2-NQ, or 10 nM TCDD for 4 hr. The total RNA was converted to cDNA, and then quantitative-PCR was performed using Cyp1a1 primer. (B) Hepa1c1c7 cells and c35 cells were treated with indicated concentration of TBQ, 1,4-BQ, 1,2-NQ and 1,4-NQ for 4 hr. The total RNA was converted to cDNA, and then quantitative-PCR was performed using Cyp1a1 primer. C, control in these figures. Each value is the mean ± S.E. of three determinations. *Significantly different from control at p < 0.05 (ANOVA).
Fig. 4. Quinone-mediated transactivation of AhR. (A) Hepa1c1c7 cells were treated with 1,2-NQ for 90 min, and then the association of AhR with ARNT and modification of 1,2-NQ to AhR in whole cell lysates was detected with an immunoprecipitation assay using indicated antibodies. (B) Cyp1a1-promoter luciferase cDNA transfected cells were treated with TBQ, 1,2-NQ, 1,4-NQ, and MC for 6 hr, then luciferase activity was measured. C, control in these figures. Each value is the mean ± S.E. of three determinations. *Significantly different from DMSO control at \( p < 0.05 \) and **, \( p < 0.01 \) (ANOVA).

Fig. 5. Quinone-mediated interaction of AhR and RNA pol II with Cyp1a1 promoter region. Hepa1c1c7 cells were treated with 10 \( \mu \text{M} \) TBQ, 5 \( \mu \text{M} \) 1,2-NQ, and 10 \( \mu \text{M} \) MC for 90 min and a ChIP assay was performed with indicated antibody. A, anti-AhR antibody; B, anti-RNA pol II antibody; C, rabbit IgG; D, mouse IgG. Each value is the mean ± S.E. of three determinations. *Significantly different from DMSO control at \( p < 0.05 \) and **, \( p < 0.01 \) (ANOVA).
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DISCUSSION

The present study indicates that mono- and bi-aromatic quinones with electrophilicity indexes of more than approximately 6.7 are able to activate AhR and induce Cyp1a1, a member of the phase-I detoxification enzyme family, in Hepa1c1c7 cells. Such a phenomenon was not observed with the parent compounds, including benzene (ω = 1.6 eV), hydroquinone (ω = 1.7 eV), naphthalene (ω = 2.7 eV), BHA (ω = 1.2 eV), and TBHQ (ω = 1.7 eV) as reported by others (Genter et al., 2006; Badham and Winn, 2007; Buetler et al., 1995; Liu et al., 1994). Thus, a difference in electrophilic properties...
between aromatic hydrocarbons and their quinone species enables the quinones to modify protein nucleophiles. For example, mono- and bi-aromatic hydrocarbon quinones that had minimal chemical modification capacities for 9,000 × g supernatant of mouse liver as determined by a BPM assay did not induce \( \text{Cyp1a1} \) expression (Figs. 1, 2A and 2B). Although the electrophilicity indexes of 2-methyl-1,4-NQ and 2-hydroxy-1,4-NQ are 6.39 and 6.15 eV, respectively, these bi-aromatic hydrocarbon quinones were not able to modify proteins and thus did not induce \( \text{Cyp1a1} \) in the present study. This discrepancy may be explained by the poor electrophilicity of 2-hydroxy-1,4-NQ, because of the high electron density at the C3 position (Ollinger and Brunmark, 1991). Nevertheless, our present study suggests that the electrophilicity of mono- and bi-aromatic hydrocarbons might be an essential factor in \( \text{Cyp1a1} \) induction coupled to AhR activation. 1,2-NQ- and/or TBQ-mediated \( \text{Cyp1a1} \) inductions were also observed in A549 cells (Fig. 6), human hepatocellular carcinoma (HepG2 cells), mouse primary hepatocytes (Abiko et al., unpublished observation), and even mouse liver in vivo (Fig. 7). Collectively, these findings suggest that quinone-mediated induction of \( \text{Cyp1a1} \) is not cell-specific.

It is generally considered that efficient AhR ligands, such as TCDD, MC, and benzo(a)pyrene exhibit a high affinity for this transcription factor and thus cause a conformational change in AhR, leading to facilitation of its translocation into the nucleus in cells (Henry and Gasiwiecz, 2003; Harper et al., 1991). In the present study, 1,4-BQ, TBQ, 1,2-NQ, and 1,4-NQ also translocated AhR into the nucleus and induced \( \text{Cyp1a1} \) in Hepa1c1c7 cells, whereas benzene and naphthalene without electrophilic properties did not translocate AhR in the cells because of their poor ligand characteristics (Badham and Winn, 2007; Genter et al., 2006). These results suggest that there is little metabolic activation of benzene or naphthalene to yield 1,4-BQ, 1,2-NQ and 1,4-NQ in Hepa1c1c7 cells and that chemical modification of AhR by quinones plays a role in the facilitation of AhR nuclear translocation. Consistent with this notion, 1,2-NQ did modify AhR, thereby enhancing the interaction of AhR with ARNT (Fig. 4A) and the binding of AhR to \( \text{Cyp1a1} \) enhancer region (Fig. 4B), leading to recruitment of RNA pol II to the promoter region and increased XRE transactivation (Fig. 5A and 5B). We speculate that such arylation of AhR by 1,2-NQ may promote the conformational change in AhR involved in its activation. In our preliminary study, we found that non-classical ligands for AhR such as curcumin (Ciolino et al., 1998) were able to bind to proteins as determined by a BPM assay, whereas little appreciable chemical modification of carbaaryl (Denison et al., 1998) and oltipraz (Miao et al., 2003) was seen, even at concentrations of 100 μM (Abiko et al., unpublished observation). Further studies are needed to understand the association of the modification capacity of non-classical ligands for AhR with its activation.

We have used 1,2-NQ as a model for an environmental electrophile and observed that 1,2-NQ covalently binds to PTP1B through Cys121 and binds to Keap1.

**Fig. 7.** 1,2-NQ-mediated increase in \( \text{Cyp1a1} \) level in vivo. Mice were orally exposed to 1,2-NQ. After 6 hr, liver was removed and RNA in the liver was extracted. The total RNA was converted to cDNA, and then quantitative-PCR was performed using \( \text{Cyp1a1} \) primer. Each value is the mean ± S.E. of three determinations. *Significantly different from control at \( p < 0.05 \) (ANOVA).

**Fig. 8.** \( \text{Cyp1a1} \) expression during exposure of WT or C327S AhR transfected c35 cells to 1,2-NQ. The transfected cells were treated with 1,2-NQ for 4 hr. *Significantly different from WT of mAhR cDNA transfected cells at \( p < 0.05 \) (ANOVA).
through Cys151, Cys253, Cys273, Cys288, and Cys489, thereby activating EGFR and Nrf2 (Iwamoto et al., 2007; Kobayashi et al., 2009) because PTP1B and Keap1 are known to be negative regulators for EGFR and Nrf2, respectively (Itoh et al., 1999; Barford et al., 1995). These findings strongly indicate that 1,2-NQ is a bi-aromatic hydrocarbon that potentially activates EGFR and Nrf2 following covalent modification of PTP1B and Keap1 protein, respectively, through these thiol groups. Heat shock protein (HSP) 90, which negatively regulates interaction of AhR with ARNT, is covalently modified by 1,4-NQ in A431 cells resulting in activation of HSF1, thereby up-regulating HSPs (Shinkai and Kumagai, unpublished observation). Taken together, it is postulated that 1,4-NQ-mediated chemical modification of HSP90 as well as AhR participate, at least in part, in the induction of Cyp1a1. However, the binding of 1,2-NQ or 1,4-NQ to Hsp90 was not observed using precipitation assay with geldanamycin-biotin probe and avidin-agarose, which can precipitate Hsp90 in Hepa1c1c7 cells (data not shown). Further study is needed to determine involvement of electrophilic modification of Hsp90 during quinones-mediated activation of AhR.

Mouse AhR consisting of a basic region/helix-loop-helix motif, Per-Arnt-Sim domain, and Q-rich domain, possesses 17 cysteine residues, including Cys327 corresponding to human Cys333 in the ligand binding pocket of AhR (Denison et al., 2002; Li et al., 2011). Mutation of Cys327 repressed TCDD-specific binding to AhR (Pandini et al., 2007), suggesting that arylation of Cys327 may be a factor for 1,2-NQ-mediated activation of AhR. Consistent with this, mutation of this cysteine to serine diminished 1,2-NQ-mediated induction of Cyp1a1 in transfected c35 cells (Fig. 8). Overall, it seems likely that 1,2-NQ affects the conformational alteration of AhR presumably through covalent modification of Cys327. On the other hand, it is well recognized that reactivity of a given Cys thiol strongly depends on the effect of its surrounding chemical milieu on its pKa. The pKa of the thiol of Cys is 8.33, with most protein-associated Cys thiols displaying a pKa between 8.2 and 8.5. This translates to relatively low protein thiol reactivity at a typical intracellular pH of 7.4, where only 10-20% of thiols are deprotonated to the reactive thiolate anion (S). This species is the more readily oxidized and alkylated state compared with its protonated form. The pKa of a Cys thiol is decreased when it is proximal to basic amino acids, such as histidine, lysine, and arginine (Jones, 2008). This facilitates deprotonation to the more reactive thiolate anion and promotes more facile Michael addition and oxidation reactions. Of all Cys residues in AhR, Cys213, Cys216, Cys220, Cys310, Cys327, and Cys494 are proximal to Arg212, Arg215 and Arg217, Arg119, Arg312, His326, and Lys493, respectively. Further study is required to elucidate whether some of 1,2-NQ-mediated covalent modification of these cysteine thiols could be associated with substantial activation of AhR, leading to CYP1A1 gene expression.

Aromatic hydrocarbons such as naphthalene invaded into cells undergo oxidation by CYP isozymes to substantially produce these quinones referred to as “phase-I reaction” (Fig. 9 and Scheme 1). The reactive species undergo 2-electron reduction mediated by aldo-keto reductases and subsequent conjugation with hydrophilic low molecular compounds (e.g., glucuronic acid) by UDP-glucuronosyltransferases to yield polar metabolites referred to as “phase-II reaction” (Scheme 1B). Finally, these detoxified metabolites are excreted into extracellular space through ATP-binding cassette transporters such as multi-drug resistance associated proteins referred to as “phase-III reaction” (Scheme 1B). Of interest, this phase-II and phase-III reactions are cooperatively regulated by transcription factor Nrf2 (Itoh et al., 1999; Maher et al., 2007). We previously reported that quinones such as 1,2-NQ and TBQ cause activation of Nrf2, thereby up-regulation of its downstream genes involved in phase-II and phase-III reactions (Miura et al., 2011; Abiko et al., 2011) (Scheme 1A). Other groups also found that 1,4-BQ and 1,4-NQ activate Nrf2/ARE pathways (Motahari et al., 2015; Khan et al., 2011). Some of compounds such as Sudan III, β-naphthoflavone, and indole-3-carbinol are known to be bi-functional inducers, which up-regulate phase-I and phase-II enzymes, and BHA and quinones were reported to activate Nrf2, recognizing that these chemicals are regarded as mono-functional inducers (Prochaska and Talalay, 1988; Nioi and Hayes, 2004). In this study, however, we report here, for the first time, that a variety of quinones such as 1,4-BQ, TBQ, 1,2-NQ and 1,4-NQ with a covalent binding capability are able
to activate transcription factor AhR as well, resulting in up-regulation of a phase-I enzyme Cyp1a1. This suggests that these quinones are bi-functional inducers through covalent modification (Scheme 1A). As shown in Scheme 1B, there is little doubt that activation of both AhR and Nrf2 mediated by NQs derived from naphthalene causes increased levels of phase-I enzymes (e.g., CYP1A1), phase-II enzymes (e.g., aldo-keto reductases and UDP-glucuronosyltransferases) and phase-III transporters (e.g., multi-drug resistance associated proteins). We therefore speculate that NQs would be unique metabolites that facilitate eventually the metabolism of its parent compound (i.e., naphthalene) associated with detoxification and excretion into extracellular space (Abiko et al., 2011; Kumagai et al., 2012; Miura et al., 2011) (Scheme 1B).

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**Scheme 1.** Quinones as a bi-functional inducer for AhR and Nrf2 (A) and naphthoquinones as a facilitator for substantial naphthalene metabolism (B). AKRs, aldo-keto reductases; AhR, aryl hydrocarbon receptor; ARE, antioxidant responsive element BHA, butylated hydroxyanisol; 1,4-BQ, 1,4-benzoquinone; CYPs, cytochrome P450; CYP1A1, cytochrome P450 1A1; DHDH, dihydrodiol dehydrogenase; EH, epoxide hydrolase; MRPs, multi-drug resistance associated proteins; Nrf2, NF-E2-related factor 2; NQs, naphthoquinones, TBQ, tert-butyl-1,4-benzoquinone; UGTs, UDP-glucuronosyltransferases; XRE, xenobiotic responsive element.
Conflict of interest---- The authors declare that there is no conflict of interest.

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