Mechanisms of Resorcinol Antagonism of Benzo[a]pyrene-Induced Damage to Human Keratinocytes

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Abstract

Benzo[a]pyrene (B[a]P) is a polycyclic aromatic hydrocarbon and ubiquitous environmental toxin with known harmful effects to human health. Abnormal phenotypes of keratinocytes are closely associated with their exposure to B[a]P. Resorcinol is a component of argan oil with reported anticancer activities, but its mechanism of action and potential effect on B[a]P damage to the skin is unknown. In this study, we investigated the effects of resorcinol on B[a]P-induced abnormal keratinocyte biology and its mechanisms of action in human epidermal keratinocyte cell line HaCaT. Resorcinol suppressed aryl hydrocarbon receptor (AhR) activity as evidenced by the inhibition of B[a]P-induced xenobiotic response element (XRE)-reporter activation and cytochrome P450 1A1 (CYP1A1) expression. In addition, resorcinol attenuated B[a]P-induced nuclear translocation of AhR, and production of ROS and pro-inflammatory cytokines. We also found that resorcinol increased nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activity. Antioxidant response element (ARE)-reporter activity and expression of ARE-dependent genes NAD(P)H dehydrogenase [quinone] 1 (NQO1), heme oxygenase-1 (HO-1) were increased by resorcinol. Consistently, resorcinol treatment induced nuclear localization of Nrf2 as seen by Western analysis. Knockdown of Nrf2 attenuated the resorcinol effects on ARE signaling, but knockdown of AhR did not affect resorcinol activation of Nrf2. This suggests that activation of antioxidant activity by resorcinol is not mediated by AhR. These results indicate that resorcinol is protective against effects of B[a]P exposure. The mechanism of action of resorcinol is inhibition of AhR and activation of Nrf2-mediated antioxidant signaling. Our findings suggest that resorcinol may have potential as a protective agent against B[a]P-containing pollutants.

Key Words: Resorcinol, AhR, ARE, HO-1, Nrf2, XRE

INTRODUCTION

The human body is negatively affected by environmental pollutants such as particulate matter, diesel gas, and nicotine smoke (Beamish et al., 2011). Since the main channel through which pollutants enter the body is the respiratory system, this is where they exert the majority of their effects and cardiovascular health issues have been frequently reported (Lee et al., 2018). However, the skin is another highly susceptible channel for exposure to environmental pollutants. Several inflammatory skin diseases have been attributed to pollutant exposure (Mancebo and Wang, 2015). To reduce the damage caused by environmental pollutants, the skin mounts a defense consisting of antioxidant enzymes and reactive oxygen species.
(ROS)-scavenging systems (Manke et al., 2013).

Benzo[a]pyrene (B[a]P) is one of the most common environmental pollutants and is classified as a polycyclic aromatic hydrocarbon. It has been found to exert harmful cytotoxic and carcinogenic effects (Hassan et al., 2011). B[a]P activates the aryl hydrocarbon receptor (AhR), leading to production of ROS (Tsujii et al., 2011). AhR, a xenobiotic chemical sensor, is abundantly expressed in epidermal keratinocytes (Furue et al., 2017). Ligand-bound AhR translocates from the cytoplasm into the nucleus, and binds to its specific DNA recognition sequence, the xenobiotic response element (XRE), in the promoters of its target genes, leading to their upregulation (Beischlag et al., 2008). Cytochrome P450 1A1 (CYP1A1) is a gene with expression dependent on AhR activation (Fujii-Kuriyama and Mimura, 2005). CYP1A1 contributes to both DNA damage by producing ROS (Dietrich, 2016).

To maintain homeostasis, endogenous antioxidant enzymes in the skin act to return elevated ROS to a normal level. These enzymes include heme oxygenase-1 (HO-1) and NAD(P)H dehydrogenase [quinone] 1 (NQO1). Expression of these antioxidant genes is regulated by nuclear factor erythroid 2-related factor-2 (Nrf2), which is a central transcriptional regulator of antioxidant signaling (Kim et al., 2010). Specifically, under physiological conditions, Nrf2 interacts with Kelch-like ECH-associated protein 1 (Keap1) and cullin-3 (CUL3), forming the Nrf2-Keap1-CUL3 complex, which sequesters Nrf2 in the cytoplasm. However, under oxidative conditions, this complex dissociates and Nrf2 freely enters the nucleus, where it binds to antioxidant response elements (AREs) in the promoters of antioxidant genes, inducing their transcription (Nguyen et al., 2009).

Resorcinol (1,3-Benzene diol or m-Dihydroxybenzene) is one of the main components of argan oil (Charrouf and Guillaume, 2009). It has been reported to possess anti-melanogenic, anti-inflammatory, and its potential mechanisms of action have not been examined thus far.

In the present study, we investigated the protective effect of resorcinol on B[a]P-induced damage and its mechanism of action in HaCaT cells, a human keratinocyte cell line.

MATERIALS AND METHODS

Cell culture and materials

HaCaT, a normal human keratinocyte cell line, was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% antibiotics (penicillin/streptomycin) and 10% fetal bovine serum (FBS) in a humidified 37°C and 5% CO2 incubator. The HEK293-TRPV1-luciferase stable cell line was maintained in DMEM supplemented with 10% FBS, 1% antibiotics, and 10% puromycin in a humidified incubator at 37°C and 5% CO2. All TaqMan reverse transcription polymerase chain reaction (RT-PCR) reagents (primers and probes) were obtained from Applied Biosystems (Waltham, MA, USA). Resorcinol (99% purity) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in distilled water. Antibodies including anti-AhR, anti-CYP1A1, anti-NQO1, anti-Nrf2, and anti-β-actin were obtained from Sigma-Aldrich.

DCFDA-cellular ROS detection assay

ROS production was quantitatively measured with the DCFDA-cellular reactive oxygen species detection assay kit (ab113851) using a fluorescence microscope and microplate reader (Nikon Instruments, Inc., Melville, NY, USA). Cells were seeded on 60-mm dishes or 96-well plates. Cultured cells were irradiated with resorcinol or tert-buty hydrogen peroxide (TBHP) solution as a positive control. After 24 h, cells were washed twice in PBS and stained with 25 μM DCFDA in PBS for 3 min at 37°C in the dark. After washing again, the oxidized DCF signal was measured at 485/535 nm (excitation/emission).

Results were calculated as percentage change from control after background subtraction.

Cell viability assay

To determine the cytotoxic effect of B[a]P or resorcinol on HaCaT cell growth in vitro, cells were plated at a density of 1×104 cells/well in six-well plates. Cells were cultured for 24 h, then treated with the indicated compounds for 48 h. After treatment, cell viability was assessed using cell counting kit-8 (CCK-8, Dojindo EU GmbH, Munich, Germany) according to the manufacturer’s instructions; then, the absorbance was read at 450 nm using a microplate reader (BioTek, VT, USA).

The experiment was performed in triplicate.

Small-interfering RNA (siRNA) knockdown of Nrf2 and AhR

ON-TARGETplus SMARTpool human siRNAs were purchased from Thermo Fisher Scientific (Waltham, MA, USA), including siRNA targeting Nrf2 (L-004018-00-0020), targetting AhR (L-004990-00-0020), and non-targeting siRNA (D-001810-10-05). Cells were transfected with 50 nM siRNAs for 24 h using the DharmaFECT transfection agent (Dharmacon Research, Lafayette, CO, USA), according to the manufacturer’s instructions.

Analysis of mRNA expression

Total cellular RNA was prepared from HaCaT cells using the TRizol reagent (Invitrogen, Carlsbad, CA, USA). For cDNA synthesis, Moloney murine leukemia virus reverse transcriptase and random primers (Invitrogen, Carlsbad, CA, USA) were used according to the manufacturer’s protocols. Real-time RT-PCR analysis was conducted using an ABI-7900HT Real-Time PCR Instrument (Applied Biosystems), and pre-designed and optimized Assays-on-Demand (Applied Biosystems) for Nrf2 (ID: Hs00975961_g1), NQO1 (ID: Hs01045993_g1), AhR (ID: Hs00169233_m1), CYP1A1 (ID: Hs01054796_g1), hypoxantheme-guanine phosphoribosyltransferase (HPRT) (Hs02800695_m1), 18S (Hs03003631_g1), and glycolaldehyde-3-phosphate dehydrogenase (GAPDH) (ID: Hs00266705_g1). The PCR cycling parameters were 50°C for 2 min, 60°C for 30 min, 95°C for 5 min, followed by 45 cycles of 94°C for 20 s and 60°C for 1 min (Hwang et al., 2017). ABI Sequence Detector Software version 2.0 (Applied Biosystems) was used to analyze relative mRNA quantity normalized to the expression level of housekeeping genes (GAPDH, 18S, and HPRT). For verification of results, the experiment was performed four times in triplicate.

Luciferase reporter assay

To assay the activity of XRE and ARE-containing promoters, cells were transfected with XRE-luciferase (XRE-Luc)
(Stratagene, La Jolla, CA, USA) or ARE-luciferase (ARE-Luc) reporters (Addgene, MA, USA), and Renilla-luciferase plasmid (1 μg) for normalization (Promega, Madison, WI, USA) using the DharmaFECT™ Duo transfection reagent (Thermo Fisher Scientific) according to the manufacturer’s protocols (Kang et al., 2019). At 24 h post-transfection, resorcinol was added to the cells for a 24 h treatment. The cells were harvested, and luciferase activity was measured using the Dual Luciferase Assay system (Promega) on an LB953 luminometer (Berthold, Germany). Results were verified with three independent transfections.

Analysis of protein levels by ELISA and western blotting

An ELISA kit (Invitrogen) was used to measure interleukin-8 (IL-8) levels according to the manufacturer’s protocol. Absorbance measurements were conducted using a Labsystems Multiskan MS microplate reader (Thermo Bio-Analysis Japan, Tokyo, Japan). The results were confirmed by three independent experiments. In order to measure target protein levels, cell lysates were prepared, electrophoresed, and transferred onto polyvinylidene difluoride membranes. The membranes were probed with antibodies (anti-β-actin, anti-Nrf2, anti-CYP1A1, anti-AhR, or anti-NQO1) and imaged using an enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ, USA). The results were verified by three independent experiments.

Extraction of nuclear and cytoplasmic fractions

Nuclear fractions were isolated to confirm the translocation of transcription factors by western blotting, the B[a]P-induced nuclear translocation of transcription factors was analyzed using antibodies against β-actin, CYP1A1, AhR, Nrf2, NQO1, and α-actin. Western blot analysis was performed for AhR. Three biological experimental replicates were performed. (E) HaCaT cells were incubated with resorcinol (3 mM) in the presence of B[a]P (15 μM) for 24 h, and Western blot analysis was performed for AhR. Three biological experimental replicates were performed. (F) HaCaT cells were incubated with resorcinol (3 mM) in the presence of B[a]P (15 μM) for 48 h, and cell survival assay was performed. Three biological experimental replicates were performed. Data are expressed as mean ± SD. *p<0.05 vs. untreated control, †p<0.05 vs. B[a]P-treated control. RES: resorcinol.

RESULTS

Resorcinol inhibits B[a]P effects on both xenobiotic response element (XRE)-mediated signaling and cell survival in HaCaT cells

To examine the effect of resorcinol (Fig. 1A) on B[a]P-induced damage to human keratinocytes, we performed XRE-Luc reporter assays, Western blotting, and real-time PCR analyses for CYP1A1 in HaCaT cells. As shown in Fig. 1B, resorcinol suppressed B[a]P-induced activation of the XRE reporter in a concentration-dependent manner. In addition, B[a]P-induced expression of CYP1A1 was affected by resorcinol treatment. As shown in Fig. 1C and 1D, protein and mRNA levels of the CYP1A1 gene increased in response to B[a]P treatment, but resorcinol attenuated these effects. As shown by Western blotting, the B[a]P-induced nuclear translocation of AhR was also reduced by resorcinol treatment (Fig. 1E). Furthermore, we examined effects of resorcinol treatment on B[a]P-induced cytotoxicity. As shown in Fig. 1F, while B[a]P

![Fig. 1.](image-url)
Fig. 2. Resorcinol reduces B[a]P-induced production of ROS and IL-8 in HaCaT cells. HaCaT cells were incubated with resorcinol in the presence of B[a]P (15 μM) for 24 h, then subjected to fluorescence image analysis (A) and fluorescence intensity analysis (B). Three biological experimental replicates were performed. Data are expressed as mean ± SD. *p<0.05 vs. B[a]P-treated control. (C) HaCaT cells were transfected with siRNA for the AhR gene using the DharmaFECT® Duo transfection reagent (Thermo Fischer Scientific, Waltham, MA, USA). After incubation for 24 h, the cells were incubated with resorcinol (3 mM) in the presence of B[a]P (15 μM) for 24 h and then subjected to ELISA for IL-8 (C) and Western blot analysis for AhR (D). Three biological experimental replicates were performed. Data are expressed as mean ± SD. *p<0.05 vs. untreated control. RES: resorcinol, NAC: N-acetyl cysteine.

Fig. 3. Resorcinol activates Nrf2-mediated signaling. (A) HaCaT cells were transfected with the ARE-Luc reporter together with a Renilla-luciferase vector using the DharmaFECT® Duo transfection reagent (Thermo Fischer Scientific, Waltham, MA, USA). After incubation for 24 h, the cells were treated with resorcinol under serum-free conditions for 14 h followed by luciferase reporter assay. *p<0.05 vs. untreated control. Three biological experimental replicates were performed. Data are expressed as mean ± SD. (B-D) HaCaT cells were incubated with resorcinol for 24 h and subjected to western blot analysis (B) and real-time PCR analysis (C) for Nrf2, NQO1, and HO-1, and nuclear fractionation to assess Nrf2 translocation (D). Three biological experimental replicates were performed. *p<0.05 vs. untreated control. RES: resorcinol.

Resorcinol reduces B[a]P-induced production of ROS

To investigate whether resorcinol modifies B[a]P-induced ROS production, we performed a DCFDA cellular ROS detection assay. As shown in Fig. 2A, B[a]P increased cellular levels of ROS, but this effect was reduced by resorcinol co-treatment, as evidenced by ROS image analysis (Fig. 2A) and fluorescence intensity assay (Fig. 2B). These data indicate that resorcinol is protective against B[a]P-induced ROS production. In addition, we examined the effects of resorcinol on B[a]P-induced production of proinflammatory cytokines. As shown in Fig. 2C, B[a]P induced IL-8 production, but this effect was reduced by resorcinol co-treatment. In addition, knock-down of AhR by siRNA, and treatment with N-acetyl cysteine, an antioxidant and reducing agent, also reduced B[a]P-induced IL-8 production (Fig. 2C). AhR siRNA was confirmed to successfully knock-down AhR protein in HaCaT cells when compared with control siRNA (Fig. 2D). These data indicate that resorcinol has antioxidant and anti-inflammatory effects, and that the effect of resorcinol on B[a]P-induced IL-8 production may be mediated through reduction of cellular ROS levels.

Resorcinol activates Nrf2-mediated signaling

In the previous experiments, we found that resorcinol suppresses B[a]P-mediated oxidative and inflammatory effects in human keratinocytes. We next examined whether resorcinol also affects the expression of antioxidant genes. First, we performed an ARE-Luc reporter assay. As shown in Fig. 3A, resorcinol increased ARE reporter activity in a concentration-dependent manner. In addition, resorcinol treatment upregulated Nrf2 protein and mRNA levels (Fig. 3B, 3C). As expected, the expression of NQO1 and HO-1, which are Nrf2 target genes,
2.0

Fig. 4. Knock-down of Nrf2 attenuates the resorcinol-induced activation of antioxidant pathways. (A) HaCaT cells were transfected with the ARE-Luc reporter and siRNA for Nrf2 together with a Renilla-luciferase vector using the DharmaFECT® transfection reagent (Thermo Fischer Scientific, Waltham, MA, USA). After incubation for 24 h, the cells were treated with resorcinol (3 mM) under serum-free conditions for 14 h. The cells were then subjected to luciferase reporter assay. *p<0.05 vs. resorcinol-treated control. Three biological experimental replicates were performed. Data are expressed as mean ± SD. (B-D) HaCaT cells were transfected with siRNA for Nrf2 using the DharmaFECT® transfection reagent (Thermo Fischer Scientific). After incubation for 24 h, the cells were incubated with resorcinol (3 mM) under serum-free conditions for 14 h. These cells were then subjected to western blot (B, D) and real-time PCR (C) analyses for Nrf2, NQO1, and HO-1. *p<0.05 vs. resorcinol-treated control. Three biological experimental replicates were performed. Data are expressed as mean ± SD. RES: resorcinol.

also increased with resorcinol treatment (Fig. 3B, 3C). Western blotting showed that resorcinol treatment also increased the nuclear translocation of Nrf2. (Fig. 3D). Furthermore, we found that resorcinol-induced ARE activation is mediated by Nrf2. Knock-down of Nrf2 using siRNA attenuated the effect of resorcinol on ARE activation (Fig. 4A) and upregulation of NQO1 and HO-1 genes (Fig. 4B, 4C). Nrf2 siRNA was confirmed to successfully knock-down Nrf2 protein in HaCaT cells in comparison with control siRNA, which did not show such an effect (Fig. 4D). These data indicate that resorcinol induces ARE-dependent antioxidant gene expression by activating Nrf2.

Resorcinol-induced activation of Nrf2/HO-1 pathway is not mediated by AhR

Some molecules have been reported to induce Nrf2-mediated upregulation of HO-1 via AhR activation (Shin et al., 2007). However, we found that resorcinol did not activate AhR, but rather suppressed its function. Therefore, we investigated the role of AhR in resorcinol induction of HO-1 expression by treating HaCaT with AhR siRNA. As shown in Fig. 5A, resorcinol-induced HO1 upregulation was not significantly different between AhR-knockdown keratinocytes and those transfected with control siRNA. In addition, we examined the involvement of the Nrf2 pathway in resorcinol inhibition of AhR by treating HaCat with Nrf2 siRNA. As shown in Fig. 5B, Nrf2 siRNA did not alter the resorcinol effect on B[a]P-induced CYP1A1 upregulation. These results indicate that resorcinol-induced AhR inhibition and Nrf2 activation occur through different mechanisms.

DISCUSSION

This study demonstrates the protective effects of resorcinol on B[a]P-induced damage to human epidermal keratinocytes. Resorcinol suppressed AhR activity, as shown through suppression of B[a]P-induced XRE reporter activation, CYP1A1 expression, and ROS production in HaCaT cells. In addition, resorcinol treatment activated Nrf2, as demonstrated by ARE reporter activation and increased expression of ARE-dependent genes. We found that the antagonist effects of resorcinol on B[a]P occur through its inhibition of AhR and activation of Nrf2.

AhR is a xenobiotic chemical sensor highly expressed in epidermal keratinocytes (Furue et al., 2017). Various endogenous and exogenous ligands bind to and activate AhR. These include B[a]P, polycyclic aromatic pollutants, dioxins, food metabolites, phytochemicals, and tryptophan photoproduct 6-formylindolo[3,2-b]carbazole (FICZ) (Stejskalova et al., 2011) FICZ, which is generated by UV irradiation, is a well-known high-affinity endogenous ligand for AhR (Murai et al., 2018). Activated AhR translates to the nucleus and binds to XREs upregulating the expression of its target genes, such as cytochrome P450 1A1 (CYP1A1) (Beischlag et al., 2008). CYP1A1 is a member of a multigene family of xenobiotic-metabolizing enzymes that are involved in detoxification (Manfredi et al., 2007). However, CYP1A1 can also exert deleterious effects by generating mutagenic metabolites and ROS (Dietrich, 2016). In our study, we found that B[a]P-induced nuclear translocation of AhR was attenuated by resorcinol treatment.
Resorcinol reduced the expression of CYP1A1, and ROS and IL-8 production induced by [B[a]P] treatment. Therefore, our results suggest that resorcinol contributes to the suppression of the oxidative stress and pro-inflammatory effects induced by [B[a]P].

Nrf2 is also abundantly expressed in epidermal keratinocytes and is a master transcription factor regulating expression of antioxidant genes (Kim et al., 2010). NQO1 and HO-1 are important Nrf2-dependent antioxidant enzymes (Li et al., 2014). Under physiological conditions, cytoplasmic Nrf2 level is controlled by Nrf2-KEAP1-CUL3 complex formation (Bellezza et al., 2018). KEAP1 directly inhibits Nrf2 by binding to it and promoting simultaneous CUL3-catalyzed ubiquitination of Nrf2. Increased levels of oxidative stress lead to dissociation of the complex through oxidation of cysteine residues in KEAP1 that change its conformation. Dissociated Nrf2 then enters the nucleus and induces the expression of antioxidant genes (Velickova and Hasson, 2005). In our study, resorcinol increased the nuclear translocation of Nrf2. The expression of NQO1 and HO-1, target genes of Nrf2, was also upregulated by resorcinol treatment. These data indicate that resorcinol protects cells from oxidative stress through promoting the cellular antioxidant defense system.

Paradoxically, in addition to inducing oxidative stress, AhR has been reported in several recent studies to contribute to antioxidative and protective signaling in response to many ligands, including herbal medicines, azoles, and flavonoids (Furue et al., 2014). For example, while ketoconazole and cinnaropicrin induce the nuclear translocation of AhR by binding to it, they do not lead to production of ROS (Dietrich, 2016). In addition, they activate the Nrf2 pathway, protecting cells from ROS-mediated oxidative damage (Dietrich, 2016). However, cinnamaldehyde inhibits AhR, but activates the Nrf2 pathway and exerts antioxidative activity in an AhR-independent manner (Furue et al., 2018). In our study, similar to cinnamaldehyde, we found that while resorcinol inhibited AhR signaling, it also activated the Nrf2 pathway in an AhR-independent manner.

Nrf2-null mice are reported to have reduced HO-1 expression and a significantly stronger and longer-lasting sunburn reaction to UVB compared with wild-type mice (Kawachi et al., 2008). In humans, Nrf2 expression is downregulated in human malignant skin tumors (Choi et al., 2014) and mutation of the NRF2 gene was associated with some squamous cell carcinoma cases (Kerins and Ooi, 2018). Therefore, resorcinol-induced upregulation of Nrf2 suggests that resorcinol may be beneficial in abnormal skin physiologies such as sunburn and skin tumors.

Resorcinol has been reported to possess anti-melanogenic, anticancer and antibacterial activities. In this study, we demonstrated that resorcinol is protective against [B[a]P]-induced damage to human epidermal keratinocytes and has anti-inflammatory effects. These effects of resorcinol were mediated by inhibiting AhR and activating Nrf2. Our results suggest that resorcinol could be used as an agent for ameliorating the symptoms induced by [B[a]P] in the skin.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.
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