Ascorbic acid suppresses the 2,3,7,8-tetrachloridibenxo-p-dioxin (TCDD)-induced CYP1A1 expression in human HepG2 cells

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ABSTRACT

The mechanisms involving the inhibitory effects of ascorbic acid (AA) on carcinogenesis have not fully defined, except for its free-radical scavenging activity against oxidative DNA damage. In this study, we examined the effects of AA on the expression of the aryl hydrocarbon receptor (AhR)-regulated gene cytochrome P4501A1 (CYP1A1), which catalyzes the activation of genotoxic metabolites that can lead to mutagenesis. Cultured human HepG2 cells were incubated with AA with or without the potent AhR agonist/CYP1A1 inducer 2,3,7,8-tetrachloridibenxo-p-dioxin (TCDD). AA was highly effective at suppressing CYP1A1 induction following coincubation of the cells with 1 nM TCDD. The preventive effects of AA were seen at the level of mRNA and protein expression as well as CYP1A1-specific 7-ethoxyresorufin O-deethylase (EROD) activity. A transient transfection assay using a dioxin response element (DRE)-linked luciferase reporter and an electrophoretic mobility shift assay revealed that AA reduced the amount of AhR that could form a complex with the DRE sequence in the promoter region of the CYP1A1 gene. In addition, AA inhibited the TCDD-induced Ecto-ATPase activity, which is known to be requiring for AhR translocation to the nucleus. These results suggest that AA may exert at least part of its anticarcinogenesis effect by controlling the expression of CYP1A1 at the transcription level.

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1. Introduction

The accumulated evidence shows that people with high dietary intakes of fruits and vegetables are less likely to develop cancer than people who have low dietary intake of these foods (Khan et al., 2008; Tsugane and Sasazuki, 2007). Some dietary phenolic substances have stronger antiproliferative and antioxidant effects than vitamins, but a great deal of effort has focused on vitamin C (ascorbic acid, AA) for its anticarcinogenesis effects. AA, an important water-soluble vitamin, is essential for a range of physiological functions, including as a co-factor for many enzymes, an efficient antioxidant, scavenging of reactive oxygen and nitrogen species, and in protecting cells against free radical-mediated damage (Sánchez-Moreno et al., 2003). Besides exerting antioxidant influence directly, AA is known to modulate various enzymes that are involved in cancer development and progression (Cooke et al., 1998).

The cytochrome P450 (CYP)-dependent monooxygenase system catalyzes oxidative metabolism of a wide variety of drugs, carcinogens, pesticides, and steroid hormones. As a preliminary detoxifi-

cation step, many compounds are first converted to polar metabolites by CYP, which facilitates their elimination. However, some compounds may also be inadvertently bioactivated by CYP to reactive intermediates that produce adverse biological effects (Burke et al., 1985). For example, carcinogenic polyaromatic hydrocarbons (PAH) such as benzo(a)pyrene undergo metabolic activation by CYP and epoxide hydrolase to a chemically reactive carcinogen, diol epoxides (Wang et al., 2005). The CYP1 family, which consists of at least three enzymes (CYP1A1, CYP1A2, and CYP1B1), has been shown to be important in the metabolism of several xenobiotics such as PAH and heterocyclic amines, and the expression of these enzymes is inducible by PAHs such as 2,3,7,8-tetrachloridibenxo-p-dioxin (TCDD) (Kim and Sheen, 2000).

TCDD, a prototypical halogenated aromatic hydrocarbon, is classified as a group I carcinogen by the International Agency for Research on Cancer (IARC, 1997). TCDD is released by the combustion of organic compounds in the presence of chlorine. TCDD, as well as PAH, is known to induce a variety of toxic effects, including immunotoxicity, reproductive disorders, hepatotoxicity, and cancers (Huff et al., 1994). Although the mechanism underlying TCDD-induced carcinogenesis is not completely understood, the aromatic hydrocarbon receptor (AhR) appears to be a key

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transcriptional regulatory protein in TCDD-elicited gene expression and toxicity. Specific binding of TCDD to AhR and the subsequent induction of gene expression seem to have important roles in mediating a variety of toxic effects associated with TCDD (Anwar-Mohamed and El-Kadi, 2008; Davies et al., 2008).

In this work, we have explored the possible inhibitory effect of AA on TCDD-induced expression and activities of CYP1A1 in human HepG2 cells and the molecular mechanisms involved.

2. Materials and methods

2.1. Materials

Unless otherwise specified, all reagents were purchased from Sigma (St. Louis, Mo., USA). The polyclonal CYP1A1 antibody and secondary rabbit antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Amersham Corp. (Arlington Heights, IL, USA), respectively.

2.2. Cell culture and treatment

HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C in 5% CO_2. Cell media and supplements were purchased from GIBCO (Carlsbad, CA, USA). To determine the effect of ascorbic acid (AA) on TCDD-induced CYP1A1, cells were treated with AA before or after exposing with TCDD.

2.3. RNA Preparation and RT-PCR

HepG2 cells in 6-well plates were treated with 1 nM TCDD and 0–1 mM AA in a 1% FBS DMEM medium. After incubation, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad CA, USA). The total RNA (1 μg) was used for first-strand cDNA synthesis using random primers and Superscript reverse transcriptase (Invitrogen). The resulting cDNA was subjected to PCR amplification with primer sets for GAPDH and CYP1A1. The primer sequences used are as follows: GAPDH sense: TCA ACG GAT TTG GTC GTA TT; GAPDH antisense: CGT TGG TCA TGA GTC CTT CC (expected PCR product size: 490 bp); CYP1A1 sense: TCT TTC TCT TCC TGG CTA TC, and CYP1A1 antisense: CTG TCT CTT CCC TTC ACT CT (596 bp). The PCR condition used was as follows: denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s.

2.4. CYP1A1 reporter assay

The transcriptional regulation of CYP1A1 was examined using transient transfection with a reporter construct (pCYP1A1-Luc, Han et al., 2007). The HepG2 cells were plated in a 12-well plate in DMEM supplemented with 10% FBS. At 70–80% confluency, the cells were transiently co-transfected with 0.2 μg of pCMV-β-gal and 1 μg of CYP1A1-Luc using Lipofectamine Plus (Invitrogen) for 24 h. Under the same condition, transfection with control plasmid encoding enhanced green fluorescent protein (EGFP, Clonetec, Mountain View, CA) resulted in fluorescence in 40–50% of the cells. The cells were then pre-treated with various concentration of AA for 1 h. Subsequently, the cells were exposed to TCDD (1 nM) for 8 h, washed with PBS, lysed using 250 μl of 1× lysis buffer (Promega) and spun. The resulting supernatant was assayed for luciferase and β-galactosidase activity. The luciferase activity was determined using a luciferase assay system (Promega) in accord with the manufacturer's instructions using a luminometer (Luminoscan Ascent, Thermo electron Co.). The β-galactosidase assay was car-

ried out in 250 μl of assay buffer containing 120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 20 mM KCl, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 50 μg of O-nitrophenyl-β-galactoside, and 100 μg of the cell extract. The luciferase activity was normalized to the β-galactosidase activity and is expressed as a proportion of the activity detected in the vehicle controls.

2.5. Western blot analyses

The protein extraction and Western blot hybridization were performed as previously described (Chen et al., 2004). The primary antibody preparations used in this study were 1:3000 dilutions of rabbit polyclonal anti-CYP1A1 (SantaCruz). The secondary antibody was horseradish peroxidase-labeled anti-rabbit immunoglobulins from donkeys (Amersham Corp., Piscataway, NJ, USA) used at 1:3000 dilution. The protein bands were visualized using a chemiluminescence kit (Amersham Corp.). The total protein levels were assayed by washing the blotted membrane with a stripping solution (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 62.5 mM Tris–HCl [pH 6.7]) for 30 min at 50 °C, and reprobing the membrane with anti-β-actin mouse monoclonal antibody (Sigma).

2.6. EROD (7-ethoxyresorufin-O-demethylase) assay

EROD activity in HepG2 cells was determined as a measure of CYP1A1 activities, as described by Kennedy and Jones (1994). HepG2 cells were plated onto 24-well plates and exposed to TCDD 1 nM with or without AA for 24 h. An aliquot of 500 μl of EROD buffer (50 mM $MgCl_2$ in 0.1 M KPO_4), 5 μM 7-ethoxyresorufin, and 10 μM dicumarol was added to 10^5 HepG2 cells in 100 μl PBS/EGTA buffer and incubated for 30 min at 37 °C. The reactions were stopped by the addition of a volume of methanol equivalent to the total reaction volume (620 μl), centrifuged at room temperature for 3 min and then transferred to a 96-well plate in triplicate. Each plate also included a standard curve of 20–200 pmol resorufin/well. Resorufin fluorescence generated by ethoxyresorufin conversion by CYP1A was measured at an excitation wavelength of 530 nm and an emission wavelength of 590 nm for 30 min using a Labsystem Fluoroskan Ascent FL (Finland). EROD activity was expressed as picomoles of resorufin formed per minute and per 10^5 cells.

2.7. Electrophoretic Mobility Shift Assay (EMSA) for AhR-DNA binding

The EMSA was carried out using a gel-shift assay system (Promega, Madison, WI, USA). In order to determine the effect of AA, the cells were treated with AA for 1 hr before being exposed to TCDD. The oligonucleotides 5′-gatctgagctcggagttgcgtgagaagagccg-3′ and 5′-cgcctcttctcacgcaactccgagctcagatc-3′ containing the consensus sequence for AhR were end-labeled with [α-^{32}P]adenosine triphosphate (3000 mCi/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK) using T4 polynucleotide kinase, purified in Microspin G-25 columns (Sigma), and used as the probes for EMSA. The nuclear extract proteins were preincubated in binding buffer (10 mM Tris–HCl [pH 7.5], 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 4% [v/v] glycerol, and 0.05 mg/ml poly [deoxyinosine–deoxycytosine]) for 5 min and then incubated with the labeled oligonucleotides in a 5% nondenaturing polyacrylamide gel in 0.5× Tris borate–EDTA buffer at 150 V for 4 h. The gel was dried and subjected to autoradiography.

2.8. Determination of Ecto-ATPase enzymatic activity

To examine the effect of AA on Ecto-ATPase activity, a colorimetric assay utilizing the conversion of p-nitrophenyl phosphate

to p-nitrophenol was used as described previously (Anagnostou et al., 1996). In brief, HepG2 cells were seeded in 12-well plates for 48 h. Thereafter, the cells were treated with TCDD with 0–1 mM AA for 48 h. Cells were then washed with phosphate-free buffer (15 mM Tris, 134 mM NaCl, 3 mM Cacl$_2$, and 3 mM MgCl$_2$) at pH 8.0, and the medium was replaced with 0.5 ml of 0.5 mM p-nitrophenyl phosphate and incubated for 30 min at 37 °C. To stop the reaction, 0.5 ml of 0.2 M NaOH was added to each well. p-nitrophenol in the supernatant was quantified by measuring the absorbance at 405 nm. Enzymatic activity was normalized for cellular protein content, which was determined using BCA reagents.

3. Results

3.1. Effect of TCDD on CYP1A1 expression

Human HepG2 cells were treated with 0.1–1 nM TCDD, and the levels of CYP1A1 mRNA and protein in the cells were determined by RT-PCR and Western blot analyses, respectively. The results showed that the level of CYP1A1 mRNA and protein increased in a dose-dependent manner after incubation of cells with TCDD (Fig. 1A and B). This result is in agreement with previous data where application of TCDD resulted in induction of CYP1A1 in various cell types (Anwar-Mohamed and El-Kadi, 2008; de Waard et al., 2008).

3.2. Effect of AA on TCDD-induced CYP1A1 expression

We examined the effect of AA on the CYP1A1 expression induced by TCDD in HepG2 cells. The data showed that AA prevented the TCDD-induced expression of CYP1A1 mRNA in a dose-dependent manner (Fig. 2A). As shown by Western blot analysis, the TCDD-induced CYP1A1 protein level was also decreased by the AA pre- or post-treatment (Fig. 2B and C). AA did not affect cell viability at the concentration used in this study (data not shown).

3.3. Effect of AA on TCDD-induced CYP1A1 promoter activity

Human HepG2 cells were transiently transfected with a pCYP1A1-Luc reporter plasmid containing four copies of the DRE enhancer sequence located in the 5′-untranslated region. Cells were treated with TCDD in the presence or absence of AA, and luciferase activities were determined. TCDD treatment resulted in a 5-fold increase in the promoter activity compared with the negative control

Fig. 2. Effect of ascorbic acid (AA) on TCDD-induced CYP1A1 in HepG2 cells. (A) The HepG2 cells were incubated with 1 nM TCDD at the indicated concentrations of AA for 8 h, and RT-PCR was performed to determine the expression of the CYP1A1 mRNA. (B) The HepG2 cells pre-treated with the indicated concentrations of AA for 1 h were incubated with 1 nM TCDD for 24 h and Western blot analysis was performed to determine the protein level of CYP1A1. (C) The HepG2 cells incubating with 1 nM TCDD for 24 h treated with 1 mM AA at indicated time point after TCDD addition, and Western bolt analysis was performed.

(DMSO). However, when cells were treated with AA and TCDD, the luciferase activity was decreased in an AA dose-dependent manner (Fig. 3).

3.4. Effect of AA on TCDD-induced AhR activation

The effect of AA on the nuclear accumulation of activated AhR induced by TCDD was examined using an electrophoretic mobility shift assay (EMSA) using nuclear extracts of HepG2 cells. TCDD treatment caused a remarkable increase in the amount of AhR that
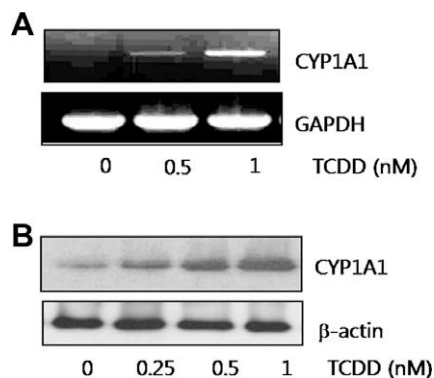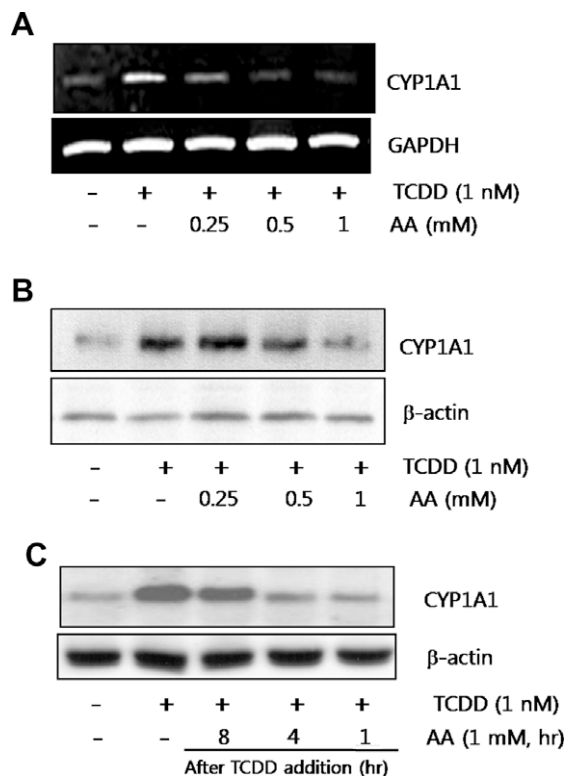

Fig. 1. Induction of CYP1A1 by TCDD in HepG2 cells. (A) The HepG2 cells were incubated with 0–1 nM TCDD for 8 h, and RT-PCR was performed to determine the expression of the CYP1A1 mRNA. (B) The HepG2 cells were incubated with 0–1 nM TCDD for 24 h, and Western bolt analysis was performed to determine the protein level of CYP1A1.
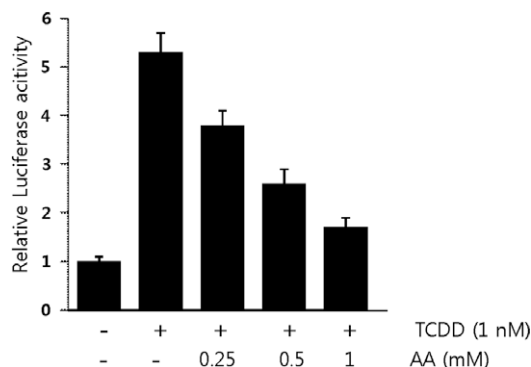

Fig. 3. Effect of AA on TCDD-induced CYP1A1 promoter activity in HepG2 cells. The HepG2 cells transiently transfected with the CYP1A1 reporter construct were incubated with 1 nM TCDD and the indicated concentrations of AA for 8 h. After incubation, the cells were lysed and the luciferase activity was determined using a luminometer. Data represent the mean ± SD from triplicate measurements.
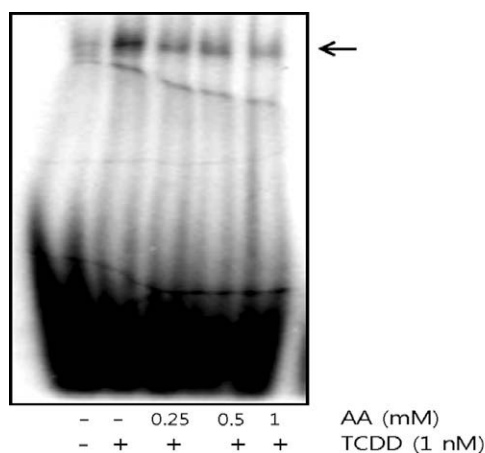
Fig. 4. Effect of AA on TCDD-induced AhR-DRE DNA binding activity in HepG2 cells. The HepG2 cells were incubated with 1 nM TCDD and the indicated concentrations of AA for 24 h. After incubation, the nuclear extracts from the cells were analysed by EMSA to determine the level of AhR using a radiolabeled DRE oligonucleotide probe.

could form a complex with the radiolabeled oligonucleotide probe in EMSA. However, the binding of AhR in the cells pre-treated with AA decreased in a dose-dependent manner (Fig. 4). The specificity of this interaction was verified by the ability of a 200-fold excess of unlabeled oligonucleotide probe to compete with and block TCDD-induced binding of the AhR to the labeled DNA (data not shown).

3.5. Effect of AA on TCDD-induced CYP1A1 activity

The CYP1A1 enzyme activity in HepG2 cells treated with AA in the presence or absence of TCDD was measured using an EROD activity assay. When the HepG2 cells were treated with 1 nM TCDD for 24 h, there was a significant increase in the CYP1A1 enzyme activity. However, TCDD-inducible EROD activities were reduced with AA treatment, in a dose-dependent manner (Fig. 5).

3.6. Effect of AA on TCDD-induced Ecto-ATPase enzyme activity

To further investigate how AA inhibited AhR activation, we examined the effect of AA on Ecto-ATPase activity, an enzyme responsible for the conversion of ATP and ADP to AMP, releasing free energy that is required for AhR translocation. Previous studies have shown that the translocation of the AhR to the nucleus is ATP-dependent (Wang and Safe, 1994). As shown in Fig. 6, TCDD significantly increased the Ecto-ATPase activity, as compared to
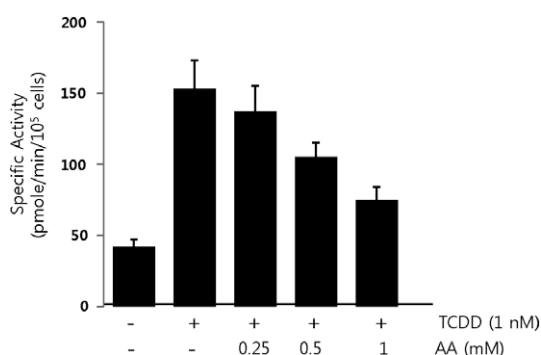

Fig. 5. Effect of AA on TCDD-induced EROD activity in HepG2 cells. The HepG2 cells were treated with 1 nM TCDD and the indicated concentrations of AA for 24 h. After incubation, the EROD activity was determined, as described in Section 2. Data represent the mean ± SD from triplicate measurements.
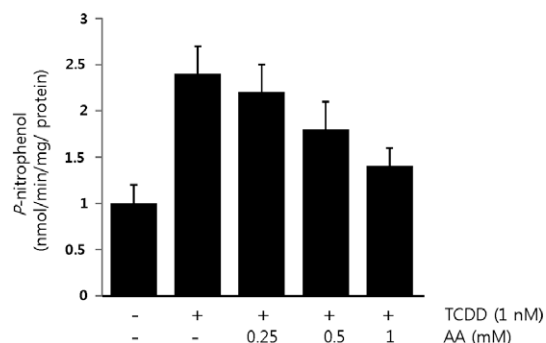

Fig. 6. Effect of AA on TCDD-induced Ecto-ATPase activity. The HepG2 cells were incubated with 1 nM TCDD and the indicated concentrations of AA for 48 h. After incubation, Ecto-ATPase activity was measured in intact living cells using p-nitrophenyl phosphate as a substrate. Data represent the mean ± SD from triplicate measurements.

the control. However, AA prevents the TCDD-induced Ecto-ATPase activities in a dose-dependent manner.

4. Discussion

The notion that ascorbic acid (AA) is involved in resistance to neoplasm, as advocated by Cameron and Pauling (1978), is based on demonstration of low ascorbate reserves in cancer patients and on the results of clinical trials where survival time of cancer patients was prolonged by ascorbate supplementation.

We show here that AA reduces TCDD-induced CYP1A1 mRNA and protein expression in HepG2 cells and that the reduction is elicited by inhibitory effect of AA on CYP1A1 promoter activity. We also demonstrate that AA decreases TCDD-induced AhR activation, CYP1A1 activity and Ecto-ATPase activity. In sum, our findings suggest that AA suppresses TCDD-induced CYP1A1 transcription through reduction in functional AhR.

What is the molecular mechanism by which AA downregulates TCDD-induced CYP1A1 transcription? One explanation is that AA hampers Ecto-ATPase, activity, decreasing the ATP that is required for nuclear translocation of AhR. Wang and Safe (1994) reported that Ecto-ATPase is the main enzyme responsible for releasing energy from ATP that is utilized by the liganded AhR for nuclear translocation. Ziegler et al. (1994) also reported that AA inhibited the ATPase activity. These findings prompted us to investigate the possible role of Ecto-ATPase in the downregulation of CYP1A1 mRNA by AA. Another potential mechanism is that AA might modulate phosphorylation status of the AhR complex, which is supported by the following reports. Chen and Tukey (1996) reported that protein kinase C (PKC) is required for TCDD-induced CYP1A1 transcription in HepG2 cells. In addition, Enan and Matsumura (1993, 1994) showed that TCDD raises protein tyrosine kinase activity in guinea pig adipose tissue. Moreover, Ogiso et al. (2004) argued that phosphorylation of Hsp90, a component of the AhR complex, impedes the ligand-induced release of AhR from Hsp90, leading to decrease in functional AhR Collectively, these reports raise the tempting possibility that AA might positively regulate phosphorylation of the AhR complex, thereby hampering nuclear translocation of AhR. Further studies are required to validate this possibility.

Given that AA decreases TCDD-induced activation of AhR, a key player in TCDD-elicited tumorigenesis and toxicity; it is conceivable that AA could be used for prevention and treatment of acute and chronic TCDD intoxication.

Conflict of interest statement

None declared.

Acknowledgements

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