Abstract. There is an endemic cardiomyopathy currently occurring in China, termed, Keshan disease (KD). The authors previously compared mitochondrial-associated gene expression profiles of peripheral blood mononuclear cells (PBMCs) derived from KD patients and normal controls, using mitochondria-focused cDNA microarray technology. The results detected an upregulation of the enzyme-associated CYP1A1 gene, (ratios ≥2.0). The aryl hydrocarbon receptor (AhR) regulates the expression of numerous cytochrome P450 (CYP) genes including members of the CYP1 family; CYP1A1 and CYP1A2. Several previous studies have suggested roles for the aryl hydrocarbon receptor (AhR) and the genes that it regulates. An example involves cytochrome P4501A1 (CYP1A1), in the pathogenesis of heart failure, cardiac hypertrophy and other cardiomyopathies. Mitochondria comprise ~30% of the intracellular volume in mammalian cardiomyocytes, and subtle alterations in mitochondria can markedly influence cardiomyopathies. The present study investigated alterations in the activity and functions of mitochondria following AhR-induced overexpression of CYP1A1. AC16 cells were treated with the CYP1A1 inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and cytotoxicity was then evaluated in MTT assays. Reverse transcription-quantitative polymerase chain reactions, western blot analysis and 7-ethoxyresorufin O-deacylase assays were performed to analyze the mRNA and protein levels, and the enzymatic activity of CYP1A1. Mitochondrial activity and mass were analyzed using an inverted fluorescence microscope and a fluorescence microplate reader. Reactive oxygen species (ROS) activity was analyzed using flow cytometry. The results of the current study demonstrated that TCDD gradually increased mRNA and protein levels of AhR and CYP1A1, in addition to the enzymatic activity. Mitochondrial activity and the quality of mitochondrial membranes were also significantly attenuated, and mitochondrial ROS levels were elevated in the TCDD-induced cardiomyocytes. The results indicate the involvement of the AhR/CYP1A1 signaling pathway in the mechanism of action of TCDD in human cardiomyocytes. The present findings may provide an explanation for myocardial injuries caused by polycyclic aromatic hydrocarbons. The authors conclude that exposure to TCDD results in regulatory alteration to the expression of detoxification genes that ultimately affect the metabolic activation and function of cardiomyocytes.

Introduction

The aryl hydrocarbon receptor (AhR) is a cytosolic receptor and ligand-activated transcriptional factor. In the absence of a ligand, cytoplasmic AhR is present in a primarily dormant state in an inactive complex that contains two 90-kDa heat-shock proteins (HSP90), hepatitis B virus X-associated protein, p23 and the AhR inhibitory protein (1) It has been hypothesized that HSP90 maintains AhR in a conformation that is capable of high-affinity ligand binding to prevents its nuclear translocation (2). AhR ligands consist primarily of polycyclic aromatic hydrocarbons (PAHs) (3). Upon binding a ligand, AhR is activated. AhR is able to dissociate from HSP90, translocate into the nucleus and form a heterodimer with the closely associated nuclear protein aryl hydrocarbon receptor nuclear translocator (ARNT) (4). The AhR-ARNT
heterodimer creates a specific DNA recognition sequence, GCGTG, within a responsive element, known as the xenobiotic responsive element (XRE). XREs are located in the promoter regions of a number of receptor-regulated genes, including CYP1A1, CYP1A2, CYP1B, and CYP2E1 (1). CYP1A1 is of particular interest because it is the most active of the group of cytochrome P450s that metabolize ligands of AhR, PAHs, into reactive species.

Cardiac CYP1A1 transcription was significantly induced by treatment with AhR ligands, including 2,3,7,8-tetrachlorodibenzofuran (TCDD), benzo(a) pyrene (BaP), 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126), β-naphthaflavone (bNF) and 3-methylcholanthrene, in a variety of mammalian species (5,6). Previous studies investigating the expression of constitutive CYP1A1 mRNA have demonstrated that TCDD and BaP are the most potent inducers of CYP1A1 expression (3). It has been suggested that CYP1A1 mRNA can be superinduced by treatment with TCDD, which is the most potent AhR ligand described (7). TCDD is a pervasive environmental contaminant that induces hepatic and myocardial oxidative stress (8,9).

CYP1A1 belongs to the cytochrome P450 family (family 1, subfamily A, polypeptide 1). CYP1A1 was initially thought to be localized only to the endoplasmic reticulum, until a mitochondrial form of CYP1A1 was characterized in the livers of rats that were pre-treated with bNF and, more recently, on inner mitochondrial membranes (10). CYP1A1 has been demonstrated to produce elevated levels of reactive oxygen species (ROS) in fish and rodent mitochondria and microsomes when challenged with the PAH compounds 3,3′,4,4′-tetrachlorobiphenyl (11) and PCB 126 (12). It has been previously indicated that the CYP1A1 metabolism serves a major role in detoxifying foreign chemicals and metabolic activation, which leads to oxidative damage (3). A previous study established a link between CYP1A1 and oxidative stress (8). However, little is currently understood regarding the mechanisms underlying mitochondrial oxidative stress in human cardiomyocytes.

In the present study, it was investigated whether the AhR-induced overexpression of CYP1A1 causes mitochondrial oxidative stress-related effects in the human cardiac-derived AC16 cell line. The present study may aid to determine the involvement of AhR and its downstream gene product, CYP1A1, in the mechanisms underlying dioxin-induced subacute levels of mitochondrial oxidative stress.

Materials and methods

Chemicals. The reagent, TCDD, was purchased from AccuStandard, Inc. (New Haven, CT, USA). A pre-calculated volume of TCDD (10 µl) was first dissolved in dimethyl sulfoxide (DMSO; 300.5 µl). A concentration of 100 nM TCDD stock liquid of TCDD was obtained. The appropriate stock solutions of TCDD were added directly to the culture media to achieve a final concentration of 0.1, 1, 5 and 10 nM of TCDD. The concentration of DMSO was not allowed to exceed 0.05% (v/v). Dulbecco's modified Eagle's medium (DMEM)/F-12 was purchased from Beijing Aipoo Huamei Biotechnology Co., Ltd. (Beijing, China). Fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A High-Capacity cDNA Reverse Transcription kit and SYBR-Green Super Mix were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The reverse transcription- quantitative polymerase chain reaction (RT-qPCR) primers were synthesized by AuGCT DNA-SNY Biotechnology, Inc. (Beijing, China). Rabbit anti-human CYP1A1 (catalog no. sc-39397)/AhR (catalog no. sc-0816R) polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell cultures and treatments. The AC16 human myocardial cell line (Land Biology, Inc., Guangzhou, China) was cultured in standard DMEM that was supplemented with 10% FBS and 1% penicillin-streptomycin (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) in 25 cm² plastic culture flasks. The cells were grown in the culture flasks at 37°C in a 5% CO₂ humidified incubator. The appropriate stock solutions of TCDD were added directly to the culture media to achieve a final concentration of 0.1 nM or 10 nM of TCDD. To measure the effects of TCDD on cell viability and cardiomyocyte mitochondrial membrane oxidative injury, cells were grown at a density of 7.5x10⁴ cells/well in 96-well tissue culture plates. For the analyses used to determine mRNA and protein levels and the intracellular production of oxidants, the cardiomyocytes were seeded into 6-well plates at a density of 1.5x10⁶ cells/well. To measure mitochondrial activity, the cells were cultured in 24-well tissue culture plates at a density of 1.5x10⁶ cells/well.

The effect of TCDD on cell viability. The cytotoxic effects of TCDD on AC16 myocardial cell viability were determined by measuring the capacity of the reducing enzymes present in viable cells to formazan crystals. Briefly, cardiomyocytes were treated with various concentrations of TCDD (0, 0.1, 1, 5 or 10 nM) for 24 h. A volume of 20 µl MTT solution (5 mg/ml in PBS) was then added, and the cells were incubated for 4 h at 37°C. The supernatants were then aspirated, and 150 µl DMSO was added to each well. The plates were shaken for 10 min to thoroughly dissolve the formazan crystals. The absorbance at 570 nm was measured for each well using a microplate reader (Infinite™ M2000; Tecan, Männedorf, Switzerland).

RNA extraction and RT-qPCR. Following incubation of the AC16 cardiomyocytes with various concentrations of TCDD (0, 0.1, 1, 5 or 10 nM) for 24 h, total RNA was extracted using an E.Z.N.A Total RNA kit I (R6834-01; Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's instructions. The concentration and purity of the total RNA was evaluated by measuring the 260/280 nm absorbance ratio using a Thermo Nanodrop 2000 (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). To obtain cDNA, total RNA (0.5 µg) was reverse transcribed using a PrimeScript™ RT reagent kit (DRR037A; Takara Bio, Inc., Otsu, Japan), three times. RT-qPCR was then performed using a Bio-Rad IQ5 PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with SYBR Premix Ex Taq™ II (RR820A; Takara Bio Inc.) according to the manufacturer's instructions. The following specific primer sequences were used: CYP1A1 forward, 5'-CTCTTCTAACAACCTCGCTC-3' and reverse, 5'-AGAAATTTGACGAGGACAT-3'; CYP2C19 forward, 5'-GGTCCTTTGTGCTGTCTTCT-3' and reverse, 5'-CAT
ATCCATGCAGCACCCC-3'; GAPDH forward, 5'-TGC ACCACCAACTGTAGC-3' and reverse, 5'-GGCATG GACTGTGGTCATAG-3'.

**Protein extraction and western blot analysis.** Cardiomyocytes were pre-treated with various concentrations of TCDD (0, 0.1, 1, 5 or 10 nM) for 24 h. The cells were then rinsed with ice-cold PBS, and the cytoplasmic and nuclear proteins were lysed using radioimmunoprecipitation buffer (HEART Biological Technology Co., Ltd., Xi'an, China). A total of 25 µg protein was separated from each treatment group using 12% SDS-PAGE, and the separated proteins were then electrophoretically transferred onto polyvinylidene difluoride membranes. The membranes were blocked in 0.75 g skim milk dissolved in 15 ml tris-buffered saline with Tween-20 (TBST) and probed with primary polyclonal rabbit anti-human antibodies against CYP1A1 (catalog no. sc-39397; 1:500), AhR (catalog no. sc-166586; 1:800) and β-actin (catalog no. sc-130656; 1:400; Santa Cruz Biotechnology, Inc.) at 4°C overnight. The primary antibodies were then discarded and the membranes were washed three times with TBST and incubated with the appropriate horseradish peroxidase-conjugated goat anti-rabbit IgG (catalog no. A0216; 1:400; Beyotime Institute of Biotechnology, Haimen, China) for 2 h with shaking at room temperature. The bands were visualized using enhanced chemiluminescence according to the manufacturer's instructions(EMD Millipore, Billerica, MA, USA).

**Determination of CYP1A1 enzymatic activity.** The activity of 7-ethoxyresorufin O-deacylase (EROD) was used as a diagnostic marker of CYP1A1 enzymatic activity. The 7-ethoxyresorufin generated resorufin when it was catalyzed by EROD. The wavelengths used to analyze excitation and emission (λex, 488 nm and λem, 505 nm) were 530 and 590 nm, respectively. The rate of the change in fluorescence was recorded prior to the experiment and after the addition of a known quantity of resorufin using a microplate reader.

**Effect of TCDD on intracellular ROS production.** To determine whether TCDD-induced CYP1A1 overexpression resulted in an increase in intra-cellular ROS production, intracellular oxidant production was analyzed using the non-fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology). Cardiomyocytes were seeded into 6-well plates at a density of 1.5x10^6 cells/well and treated with various concentrations of TCDD. The cells were incubated with diluted DCFH-DA at 37°C for 20 min and reverse-blended every 3-5 mins. The cells were then washed three times with serum-free cell culture medium, and the DCF fluorescence distribution was detected in 2x10^7 cells using flow cytometry.

**Detection of living cellular mitochondrial activity.** The mitochondrial activity of cardiomyocytes was analyzed using the fluorescent probe Mito Tracker RED staining assay kit (Genmed Scientifics, Inc., Wilmington, DE, USA) according to the manufacturer's instructions. Mito Tracker RED is a type of fluorescent dye that contains chloride methyl thiol groups, which freely travel through the cell membrane and selectively accumulate in mitochondria. Cardiomyocytes were seeded in 24-well plates, and the cell culture fluid was removed when confluence reached 70%. The medium was then preheated to 37°C (500 µl/well) and added to each well. Following this, solutions of the liquid Reagents C and D (20:1; 1 µl/well) were added, and the cells were incubated at 37°C for 20 min. Finally, fluorescence intensity was analyzed using an inverted fluorescence microscope.

**Fluorometric assays for detecting mitochondrial membrane damage/oxidation.** Cardiolipin is one of the main components in mitochondrial membranes (13). The hydrophobic fluorescence dye 10-N-onyl acrylde orange (NAO) is a stain that binds specifically to mitochondrial cardiolipin and then emits fluorescence. The Mitochondria Damage Testing kit (Genmed Scientifics, Inc.) was used according to the manufacturer's
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instructions to directly assess the degree of damage and oxidation in the mitochondrial membrane. Briefly, the cells were grown in 96-well cell culture plates. The culture medium, which contained various concentrations of TCDD, was removed when the cells covered the bottom of the well, and the cells were then washed with 100 µl reagent A. After the solution containing reagent A was thoroughly drained, the cells were then incubated at 37°C for 20 min using 50 µl/reagent B and reagent C (ratio, 1:99). Finally, the cells were washed with reagent A again. The relative fluorescence units were measured using a microplate reader (λex 488 nm and λem 505 nm).

Statistical analysis. Statistical analysis was performed using SPSS software (version, 18.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of TCDD on cardiomyocyte viability. An MTT assay was performed to determine which optimal concentrations to use in the following studies. As demonstrated in Fig. 1, when TCDD was applied at 0.1 -10 nM, it demonstrated no significant cytotoxicity towards cardiomyocytes (P>0.05). The percentage of surviving cells was calculated for each concentration group by dividing the difference between the optical density (OD) values of the intervention group and the blank group by the difference between the OD values of the negative control group and the blank group.

The induction of CYP1A1 and AhR gene expression by TCDD. To investigate whether TCDD increases the transcription of CYP1A1 mRNA by regulating the expression of the transcription regulator AhR, AC16 cells were treated with increasing concentrations of TCDD (0.1-10 nM) for 24 h. The ratio of absorbance at 260/280 of the total RNA of each group was 1.94-2.02 and was maintained at approximately 2.0, as measured using a Thermo Nanodrop 2000. The RNA samples had sufficient purity. The expression of the CYP1A1 and AhR mRNAs was measured using RT-qPCR. As presented in Fig. 2, TCDD significantly increased the expression of the CYP1A1 and AhR mRNAs in a dose-dependent manner.

The effects of TCDD on CYP1A1 and AhR protein expression and enzymatic activity. To determine whether the TCDD-induced expression of the CYP1A1 and AhR mRNAs in AC16 cells is translated into an increase in the level of functional proteins, western blot analyses were performed. The results (Fig. 3A) suggested that 24 h of treatment with various concentrations of TCDD significantly increased the
protein expression levels of CYP1A1 and AhR, which was consistent with the observed changes in gene expression. ImageJ software (version, 1.43, National Institutes of Health, Bethesda, MD, USA) was used to calculate and analyze gray values to assess the presence of comparable amounts of protein in each lane, as presented in Fig. 3B. The EROD activity observed in each of the different groups is presented in Fig. 4. AC16 cardiomyocytes that were incubated for 24 h displayed a distinct concentration-dependent elevation in CYP1A1 enzymatic activity.

**ROS generation in cultured cardiomyocytes stimulated with TCDD.** Exposure to TCDD induced a significant increase in the generation of ROS for concentration of 1, 5 and 10 nM TCDD (Fig. 5, P<0.05). All values are presented relative to the control cells (exposed to DMSO (100%). Treatment with 0.1 nM TCDD did not cause a significant change in ROS production (P>0.05).

**TCDD reduced mitochondrial activity.** The changes in mitochondrial activity that were associated with TCDD were assessed qualitatively by staining the mitochondria with a Mito Tracker RED probe. Mito Tracker RED is the reduced form of a red fluorescent dye (λex=579 nm and λem=599 nm), which does not fluoresce on its own. Following incubation with Mito Tracker RED, the probe penetrates the cell membrane via passive transport, and the oxidized fluorescence probe inside the cells selectively gathers on the activated mitochondria. The results obtained using an inverted fluorescence microscope demonstrated that TCDD induced changes in mitochondrial activity that resulted in the loss of red fluorescence (Fig. 6).

**The effect of TCDD on mitochondrial mass in cardiomyocytes.** NAO is a fluorescent probe that is widely used to determine mitochondrial mass in living cells. When the mitochondrial membrane quality is damage or oxidized, the NAO dye combines with membrane cardiolipin and is reduced, resulting in a decrease in fluorescence intensity. The results of the assays indicated that as TCDD concentration increased, the quality of the mitochondrial membrane decreased for concentrations of 1, 5 and 10 nM (Fig. 7, P<0.05).

**Discussion**

Previous studies demonstrated that CYP1A1 was constitutively expressed and inducible in H9c2 cells and that the cardiotoxic effects of AhR ligands included structural malformations, ventricular hypertrophy and abnormal myocyte contractility in several in vivo models (7,9,14). In the present study, five different concentrations of the AhR ligand TCDD were applied to AC16 cardiac cells. The results demonstrated that this myocardial cell line may constitutively express the CYP1A1 mRNA and protein, leading to its enzyme activity. In addition, the process of inducing gene expression of CYP1A1 was accompanied by a decline in mitochondrial activity and quality and an increase in mitochondrial ROS.

The AC16 cell line is a commercially available myogenic cell line that is derived from human heart cells. TCDD is a congener, which is the most well studied in a family of halogenated aromatic hydrocarbons known as dioxins (15). Due to their high lipophilicity and long half-life, dioxins persist in the environment for long periods of time and accumulate in biological systems. Dioxins additionally induce a wide range of toxic effects in the heart, including impairment of cardiomyocyte differentiation and induction of hypertrophy and heart failure (7,16,17). Furthermore, AhR ligands have been reported to alter arrangement of the mitochondria, resulting in twisted and disrupted cristae, in addition to altering arrangement of sarcomeres (16). AhR ligands can cause the...
Previous studies have demonstrated that the AC16 human heart requires further elucidation. To the best of our knowledge, no one has represented a direct effect by AhR ligands on cardiotoxicity been reported to reduce the size of infarcts. ω-Hydroxylation products serve deleterious roles in cardiac injuries. A previous study indicated that HETEs and possibly other CYP 7-regioisomers of hydroxyeicosatetraenoic acids (HETEs) is known to metabolize arachidonic acid into different enzymatic activity. The present experiments, which were performed in TCDD-treated AC16 cells, indicated that CYP1A1 was significantly expressed and inducible in these cells. Because AhR is a transcriptional regulator of CYP1A1, the expression levels of AhR mRNA and protein were also assessed. As predicted, the results confirmed that in cardiomyocytes, treatment with TCDD resulted in markedly higher levels of AhR expression than were observed in the blank control group. The regulation of CYP1A1 gene expression involves the activation of a cytosolic transcriptional factor, AhR, which is the first step in a series of molecular events that promote the transcription and translation of CYP1A1.

In conclusion, the present study demonstrates that AC16 cardiomyocytes respond to TCDD by increasing CYP1A1 activity and increasing the mRNA and protein expression levels of CYP1A1 and AhR. The observations support the involvement of the AhR/CYP1A1 signaling pathway in TCDD toxicity in AC16 cells. Due to the fact that CYP1A1 is involved in xenobiotic metabolism and detoxification in humans, malfunctions in this enzyme caused by TCDD disrupting the normal metabolism and detoxification machinery are suggested to lead to increased susceptibility of the heart to environmental and other stressors, in addition to increased risk of cardiomyopathy.

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