Ginsenoside Rb1 Inhibits Doxorubicin-Triggered H9C2 Cell Apoptosis via Aryl Hydrocarbon Receptor

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Abstract
Doxorubicin (DOX) is a highly effective chemotherapeutic agent; however, the dose-dependent cardiotoxicity associated with DOX significantly limits its clinical application. In the present study, we investigated whether Rb1 could prevent DOX-induced apoptosis in H9C2 cells via aryl hydrocarbon receptor (AhR). H9C2 cells were treated with various concentrations (0-μM) of Rb1. AhR, CYP1A protein and mRNA expression were quantified with Western blot and real-time PCR analyses. We also evaluated the expression levels of caspase-3 to assess the anti-apoptotic effects of Rb1. Our results showed that Rb1 attenuated DOX-induced cardiomyocytes injury and apoptosis and reduced caspase-3 and caspase-8, but not caspase-9 activity in DOX-treated H9C2 cells. Meanwhile, pre-treatment with Rb1 decreased the expression of caspase-3 and PARP in the protein levels, with no effects on cytochrome c, Bax, and Bcl-2 in DOX-stimulated cells. Rb1 markedly decreased the CYP1A1 and CYP1A2 expression induced by DOX. Furthermore, transfection with AhR siRNA or pre-treatment with AhR antagonist CH-223191 significantly inhibited the ability of Rb1 to decrease the induction of CYP1A, as well as caspase-3 protein levels following stimulation with DOX. In conclusion, these findings indicate that AhR plays an important role in the protection of Ginsenoside Rb1 against DOX-triggered apoptosis of H9C2 cells.

Key Words: Ginsenoside Rb1, Doxorubicin, apoptosis, AhR, CYP1A
mediated and hypertrophic genes and generation of reactive oxygen species (ROS) (Korashy and El-Kadi, 2006). Moreover, it has been shown that the capacity of Pb²⁺ and sunitinib to induce cardiac hypertrophy in in vivo and in vitro rat model was associated with induction of CYP1A1 gene (Ansari et al., 2013; Maayah et al., 2014), suggesting a possible role for the CYP1A1 in the pathogenesis of cardiovascular diseases.

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcriptional factor that belongs to the basic-helix-loop-helix (bHLH) family and has the ability to regulate CYP1A1. In the absence of ligand, AhR exists primarily in the cytoplasm as an inactive complex with two Hsp90 and p23. Once binding to its ligands such as PAHs, AhR dissociates from the p23 and translocates to the nucleus, where it dissociates from Hsp90 and heterodimerizes with the AhR nuclear translocator (ARNT). The AhR-ARNT complex then binds to the xenobiotic responsive element (XRE), which is located in the promoter region of the receptor regulated genes, such as CYP1A1 and CYP1A2, resulting in transcription and translation of CYP1A genes.

Due to the polycyclic aromatic structure of DOX, it has been demonstrated that DOX can activate AhR and induce the expression of CYP1A1 in ARVM and H9C2 cells (Volkova et al., 2011). Thus, the cardiotoxicity of DOX may be associated with its activation of AhR.

Ginseng has been extensively used as a traditional herbal medication in China and other Asian countries for over 2000 years. The therapeutic effects of ginseng have been mainly attributed to the various ginsenoside compounds within the herb. Among these ginsenosides, ginsenoside Rb1 has been demonstrated to possess cardiovascular benefits. Ginsenoside Rb1 has been reported to decrease cardiac contractility and ventricular myocytes (Scott et al., 2001) and reduce isoproterenol-induced cardiomyocyte apoptosis in vitro and in vivo (Wang et al., 2013). Moreover, our laboratory previously demonstrated that ginsenoside Rb1 could activate the DNA-binding capacity of AhR for the xenobiotic responsive element of CYP1A1 as assessed by an electrophoretic-mobility shift assay (EMSA) and induce CYP1A1 expression in HepG2 cells (Wang et al., 2008), suggesting that ginsenoside Rb1 may be a potential AhR ligand. Recent studies have further demonstrated the ability of ginsenoside Rb1 to stimulate gene expression in an AhR-dependent manner and confirmed its identity as AhR ligand (Hu et al., 2013). Therefore, ginsenoside Rb1 may be an agonist or (partial) antagonists of AhR, which can compete with other AhR agonists such as DOX for binding to the receptor.

Herein, we hypothesized that ginsenoside Rb1 could protect against DOX-induced apoptosis in H9C2 cells and down-regulate the expression of CYP1A1 genes, which may be a new strategy to reduce the cardiotoxicity of DOX.

**MATERIALS AND METHODS**

**Materials and reagents**

We obtained reagents from the following vendors: ginsenoside Rb1 (HPLC≥98%) from Yuanye Biotechnology Corporation (Shanghai, China); doxorubicin (DOX) from Selleck (Boston, MA, USA); dimethyl sulfoxide (DMSO) and TRizol Reagent from Sigma-Aldrich Chemicals (St Louis, MO, USA); Dulbecco’s modified Eagle’s medium (DMEM) and Phosphate Buffered Saline (PBS) from Gibco (Grand Island, NY, USA); fetal bovine serum (FBS) from HyClone (South Logan, UT, USA); Cell Counting kit-8 from Dojindo (Dojindo Molecular Technologies, Inc., Kumamoto, Japan); PRL-TK plasmid, Dual-Glo® Luciferase Assay System and Caspase-Glo®3/7,8,9 Assay from Promega (Madison, WI, USA); Cell Death Detection ELISA kit from Roche (Roche Diagnostics Corporation, Indianapolis, IN, USA); Hoechst 33258 from Beyotime Institute of Biotechnology (Shanghai, China); Lipofectamine® 2000 Reagent from Invitrogen (Carlsbad, CA, USA); Human pGL4.17-A1 luciferase reporter plasmid and Human pcDNA3.1 AhR plasmid were pre-constructed by our laboratory; TransStart® First-Strand cDNA Synthesis SuperMix and TransStart® Green q-PCR SuperMix from TransGen Biotechnology Corporation (Beijing, China); BCA protein Assay Kit from CW Biotechnology (Beijing, China); polyvinylidene difluoride (PVDF) and enhanced chemiluminescence reagent from Millipore Corporation (Billerica, MA, USA); 2-Methyl-2H-pyrazole-3-carboxylic acid-(2-methyl-4-o-tolyl-azophenyl)-amide (CH-223191), Ah Receptor siRNA(r) and anti-CYP1A1, anti-CYP1A2, anti-AhR from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Anti-caspase-3, anti-Bax, anti-Bcl-2, anti-PARP and anti-cyt c from Abcam (Cambridge, MA, USA).

All pairs of q-PCR primers were synthesized by Biomed Biotechnology (Beijing, China). Other chemicals and reagents were of analytical grade.

**Cell culture and treatment**

H9C2 cells (Cell Resource Center of Xiehe, Beijing, China) were cultured in DMEM, supplemented with 0.45% glucose, L-glutamine, 0.11% sodium pyruvate, and 10% FBS, at 37°C in a 5% CO₂ humidified incubator.

Cells were treated with Rb1 at concentrations of 50, 100, 200, 400 μM or vehicle for 6 h, and then exposed to 1 μM DOX for indicated times. In separate experiments, cells were pre-incubated with CH-223191, an AhR antagonist, 2 h before addition of 200 μM Rb1 and/or 1 μM DOX. The same volumes of corresponding solvents were added to the controls.

**Cytotoxicity assays**

To measure cytotoxicity, H9C2 cells and MCF-7 breast cancer cells were seeded with culture medium in 96-well microplates (4000 cells/well) respectively and incubated at 37°C for 24 h before drugs exposures. After treatment with DOX or/and Rb1 for indicated times, cell viability was examined with the Cell Counting kit-8 as the manufacturer’s instructions. The absorbance (OD) values were detected at 450 nm with a plate reader. Data are reported as the percentage of cell viability in comparison to that of its respective non-treated control group (100%).

**Apoptosis assay**

For apoptosis analysis, DNA fragmentation was determined by a Cell Death Detection ELISA kit. H9C2 cells were seeded with culture medium in 6-well plates and cultured at about 80% confluence prior to the indicated treatments. At 24 h after DOX, Rb1 and/or vehicle treatment, the cytoplasmic mono- and oligonucleosomes (histone/DNA fragments) were extract ed and assayed according to the manufacturer’s instructions. Absorbance was measured at 405 nm using a microplate reader (reference wavelength approx. 490 nm). The specific enrichment of mono- and oligonucleosomes was expressed
Table 1. Primer sequences used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>5'→3' Forward primer</th>
<th>5'→3' Reverse primer</th>
<th>Size( bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>CCAACGAGTTCGCGGCCCT</td>
<td>TGCCCAACAAAGAATGGA</td>
<td>91</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>CGCCAGGCGTGTTTCTTTA</td>
<td>TCACAAGCGGAAGACCTC</td>
<td>81</td>
</tr>
<tr>
<td>AhR</td>
<td>CGGCCAGATGGGTTGCTTTATGC</td>
<td>TGGAACTCGAGCTGCGTTCTGTATGG</td>
<td>124</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CAAGGTCACTCCATGACAATTTTG</td>
<td>GGGCCATCCACAGTCTTCTG</td>
<td>90</td>
</tr>
</tbody>
</table>

as enrichment factor and calculated using the following formula: enrichment factor = the ratio of absorbance of the sample: absorbance of the negative control group.

Hoechst 33258 staining

Hoechst 33258 staining was used to identify the morphological features of apoptosis. Briefly, H9C2 cells (5×10^4/well) were seeded in 6-well plates and cultured 24 h, then treated with DOX, Rb1 or/and vehicle for 24 h. The cells were fixed with 1mL of 4% paraformaldehyde for 20 min. Then, the cells were incubated in 1mL PBS containing 10 μM Hoechst 33258 at 37°C for 30 min. Cells were washed with PBS twice and dead cells and apoptotic bodies were identified by condensed or fragmented nuclei using fluorescence microscopy (Olympus, Tokyo, Japan) at ×200 magnification.

Caspase-3/7, 8 and 9 activity assessments

The present caspase activity assay used a luminogenic substrate containing the DEVD sequence. Activity of caspase-3/7, 8, and 9 was determined using a commercial kit according to the manufacturer’s instructions. Briefly, H9C2 cells were seeded in 96-well plates and cultured 24 h, after 12 h treatments with DOX in the presence or absence of Rb1, caspase reagent was added and incubated for 30 min. The production of light was measured with a luminescence spectrometer LS55 (Perkin-Elmer, CA, USA) at an excitation wavelength of 499 nm and an emission wavelength of 521 nm.

RNA isolation and quantitative real-time PCR

Total RNA of H9C2 cells was isolated after various periods of time incubation with the different treatments using TRIzol reagent according to the manufacturer’s instructions to assess the specific induction of CYP1A1, CYP1A2 and AhR expression. The quality of the RNA was confirmed by an A260/A280 ratio of >1.8. cDNA synthesis was performed by using the TransScript® First-Strand cDNA Synthesis SuperMix and 1.5 μg of total RNA from each sample was used. Quantitative real-time PCR reactions were performed on an ABI StepOne Plus® (Foster city, CA, USA) real-time PCR instrument using SYBR Green PCR SuperMix. The amplification reactions were performed as follows: 30 s at 94°C, and 40 cycles of 94°C for 5 s and 60°C for 30 s. The primers used in the experiments are listed in Table 1. The data were analyzed as described in the instrument manual using relative gene expression. Briefly, the data are presented as the fold change in gene expression normalized to the endogenous reference gene (GAPDH) and relative to the untreated control.

Western blotting

The H9C2 cells were lysed in RIPA buffer at 4-8°C and then the supernatant lysates were collected after centrifugation. The protein concentration was measured by BCA protein Assay Kit. Equal amounts of protein from each sample were loaded on and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE), and then electrophoretically transferred to immobilin polyvinylidene difluoride membranes. Protein blots were then blocked with 5% skimmed milk powder in TBST at room temperature for 2 h, followed by incubation with primary antibodies overnight at 4°C. The membranes were incubated with HRP-conjugated secondary antibodies for 1 h. The bands were visualized using the enhanced chemiluminescence reagent and quantified relative to GAPDH bands using ImageJ® (National Institute of Health, MD, USA) image processing program.

Translucent transfection and luciferase assay

Human umbilical vein endothelial cells (HUVECs) were cultured in RPMI1640, at 37°C in a 5% CO2 humidified incubator. HUVECs were plated onto 24-well cell culture plates and each well of cells was transfected with pcDNA3.1 AhR plasmid and pGL4.17-1A1 luciferase reporter plasmid plus the Renilla luciferase PRL-TK plasmid, used for normalization. Cells were transfected with 500 ng pcDNA3.1 AhR plasmid and 250 ng pGL4.17-1A1 plasmid plus 50 ng PRL-TK plasmid using Lipofectamine 2000 reagent according to the manufacturer’s instructions. After incubation with test compounds for 24 h, Luciferase assay was performed according to the manufacturer’s instructions (Promega). Briefly, initially firefly luciferase activity was measured with a proper luminometer, and then Renilla luciferase activity was measured. Luciferase activity was reported as light emitted per well, calculating ratio of firefly: Renilla luminescence for each well and normalizing the sample well ratio to the ratio from a control.

Short interfering RNA (siRNA) transfection

The AhR siRNA was a pool of three target-specific 21 nucleotide siRNAs designed to knockdown AhR gene expression. The transfection was performed in 24 wells plates. AhR siRNA and negative control oligonucleotides were transfected using the Lipofectamine 2000 according to the manufacturer’s instructions. The final concentration of the AhR siRNAs was 60 nmo/lL. After 6 hours, transfected cells were washed with PBS, and then incubated in new culture media containing 10% FBS. Then cells were incubated for an additional 24 hours before drug treatment.

Statistical analysis

All data are expressed as the mean ± SD and were analyzed using GraphPad Prism 5.01 software (GraphPad software Inc., CA, USA). Comparisons among groups were made by one-way ANOVA. p<0.05 was considered significantly different between groups.
the antitumor activity of DOX in MCF-7 cells (Fig. 1B).
concentrations of ginsenoside Rb1 had no significant effect on
reduce DOX-induced H9C2 cell death. In addition, various
induced cytotoxicity. These results suggested that Rb1 could
400
200
400
200
400
200
57x198
100
100
400
400
200 400 400
200
400
200

Fig. 1. The effect of Rb1 on H9C2 cells viability and MCF-7 cell
growth inhibition after DOX treatment. (A) H9C2 cells were treated
with Rb1 in the presence or absence of DOX for 24 h (n=3). (B)
The effect of Rb1 on growth inhibition of MCF-7 cells treated with
DOX for 12 h (n=3). ***p<0.001 compared with control group;
*p<0.05, **p<0.01 compared with DOX group.

RESUL TS

Rb1 increased the viability of DOX-treated H9C2 cells
To explore the effect of ginsenoside Rb1 against the cardio-
toxic effect of DOX, H9C2 cells were treated with DOX for 24
h or Rb1 (50 μM, 100 μM, 200 μM, and 400 μM) pre-treated 6
h before DOX exposure and CCK-8 assay was performed to
measure cell viability. The results showed that 5 μM DOX sig-
nificantly inhibited the growth of H9C2 cells. Additionally, Rb1
pre-treatment significantly increased the cell viability of DOX-
induced cell death of H9C2 cells in a dose-dependent manner
(Fig. 1A), with the highest effect seen at 200 μM. Rb1 alone at
400 μM was slightly toxic to cells and still could prevent DOX-
induced cytotoxicity. These results suggested that Rb1 could
reduce DOX-induced H9C2 cell death. In addition, various
concentrations of ginsenoside Rb1 had no significant effect on
the antitumor activity of DOX in MCF-7 cells (Fig. 1B).

Rb1 reduced DOX-induced apoptosis in H9C2 cells by
ELISA assay
To evaluate the effect of Rb1 on H9C2 cells after DOX
treatment, H9C2 cells were pre-treated with 50-400 μM for 6
h and then treated with 1 μM DOX for an additional of 24 h.
The positive apoptosis was determined by an ELISA assay to
quantify histone-associated DNA fragmentation generated in
apoptotic H9C2 cells. We found that 1 μM DOX treatment for
24 h resulted in a noticeable increase in DNA fragmentation
and Rb1 treatment reduced DOX-induced DNA fragmentation
in H9C2 cells. As shown in Fig. 2, the enrichment factor was
significantly higher in the DOX-treated group than in control
group and was significantly lower in the Rb1-treated group
than in DOX-treated group. These results suggested that Rb1
reduced DOX-induced apoptosis in H9C2 cells.

Fig. 2. The effect of Rb1 on H9C2 cells apoptosis induced by
DOX. H9C2 cells were treated with DOX in the presence or ab-
sence of Rb1 for 24 h. Apoptosis was assessed by detecting the
cytoplasmic mono- and oligonucleosomes (histone-associated
DNA fragments) using a Cell Death Detection ELISA kit. Data was
expressed as enrichment factor (n=3). ***p<0.001 compared with
control group. *p<0.05, **p<0.01 compared with DOX group.

Rb1 reduced H9C2 cell apoptosis by the morphological
observation
In order to determine the protective effects of Rb1 on DOX-
induced apoptosis, Hoechst 33258 staining was used to fur-
ther confirm the anti-apoptotic effect of Rb1. In the control
group and Rb1 treated group, the nuclei were stained a less
bright blue and the color was homogeneous (Fig. 3A, 3D).
Compared with the control group, DOX treatment resulted in
increased apoptosis with obvious changes in heterogeneous
intensity, chromatin condensation, and fragmentation under
fluorescent microscopy, which are the classic characteristics
of apoptotic cells (Fig. 3B). However, when cells were pre-
treated with Rb1 6h before DOX treatment, the morphological
changes showed less apoptotic characteristics (Fig. 3C).

Death receptor signaling pathway is involved in inhibitory
effect of Rb1 on H9C2 cells apoptosis induced by DOX
Previous study has shown that DOX-induced apoptosis is
a multifactorial process (Zhang et al., 2009). Caspase-3 is an
executor of apoptosis and is involved in many important events
that lead to the completion of apoptosis. Cleaved caspase-3
and cleaved PARP are markers of caspase-3 activation. The
activities of caspases-3/7, 8, 9 and the expression of apopto-
sis-related proteins including caspase-3, PARP, cytochrome
c, Bcl-2 and Bax was determined in DOX-treated H9C2 cells.
As shown in Fig. 4, caspase-3/7 and 8 activity were signifi-
cantly higher in the DOX group than in the control group.
After pre-treatment with Rb1, caspases-3/7 and 8 activity was
depressed markedly in DOX+Rb1 group compared with DOX
group (Fig. 4A, 4B). However, the activity of caspase-9 had no
obvious change after DOX and/or Rb1 stimulation (Fig. 4C).
Moreover, cleaved caspase-3 and cleaved PARP protein lev-
els were significantly increased in DOX group compared with
control, and they were attenuated by Rb1 treatment in DOX-
treated H9C2 cells (Fig. 4D, 4E). However, cytochrome c level
had no evident changes after DOX and/or Rb1 treatment (Fig.
4F). Meanwhile, DOX increased Bax expression and reduced
Bcl-2 expression in H9C2 cells, which were not restored to normal levels by Rb1 pretreatment (Fig. 4G, 4H).

**Effect of Rb1 on DOX-mediated induction of AhR-regulated genes in H9C2 cells**

DOX is a polyaromatic hydrocarbon. It was previously reported that treatment with DOX induced AhR migration to the nucleus, increased AhR binding with its co-factor, aryl hydrocarbon receptor nuclear translocator-1 (ARNT1), and increased the expression of AhR-regulated phase I (CYP1A1) drug-metabolizing enzymes in H9C2 cells (Volkova et al., 2011), that are known to play roles in heart disease (Elbekai and El-Kadi, 2006; Korashy and El-Kadi, 2006). It was also reported that ginsenosides are novel naturally-occurring AhR ligands (Hu et al., 2013) and ginsenoside Rb1 induced CYP1A1 expression in HepG2 cells (Wang et al., 2008). In our study, we examined whether Rb1 could reduce DOX-mediated induction of AhR-regulated genes in H9C2 cells through competing with DOX for the ligation of AhR. Therefore, H9c2 cells were treated with DOX (1 μM) in the absence or presence of Rb1 (200 μM) for 12 h, and the mRNA expression levels of CYP1A1, CYP1A2, and AhR was measured by RT-PCR, and protein expression levels of CYP1A1 and CYP1A2 was measured by Western blot. As shown in Fig. 5, DOX alone significantly induced the expression of CYP1A1, CYP1A2, and AhR genes. However, pre-treatment with Rb1 significantly blocked the induction of CYP1A1, CYP1A2, AhR mRNA and CYP1A1, CYP1A2 protein in response to DOX.

**Effects of Rb1 on CYP1A1 luciferase reporter activity in the presence and absence of DOX**

To determine whether the observed effects upon exposure to Rb1 in the presence and absence of DOX on CYP1A mRNA and protein were via an AhR-dependent mechanism, HUVECs were transiently cotransfected with AhR expression plasmid, CYP1A1 luciferase reporter plasmid and its normalizing control construct, Renilla luciferase. Luciferase activity results showed that 1 μM DOX alone significantly induced XRE-driven luciferase activity by 5.45-fold, meanwhile, 100, 200, 400 μM Rb1 alone induced luciferase activity by 1.18-, 1.36-, and 1.37-fold, respectively, compared to control. On the other hand, co-exposure to 200 or 400 μM Rb1 and DOX significantly decreased luciferase reporter activity to 4.73-, and 4.97-fold, compared to DOX group (Fig. 6).

**The role of AhR in protection of Rb1 against DOX-induced apoptosis**

To further examine whether AhR was directly involved in the effect of Rb1 on DOX-mediated induction of AhR downstream target genes in H9C2 cells, we knocked down AhR expression with siRNA. H9C2 cells were transfected with a control siRNA (siCON) or a siRNA against AhR (siAhR), and then cultured 24 h before drug treatment. RT-PCR and western blot analysis showed that siAhR dramatically reduced AhR mRNA and protein levels (Fig. 7A), indicating the effectiveness of the siRNA-mediated AhR gene silencing. Knockdown of AhR resulted in a decrease in the expression of CYP1A1.
and CYP1A2 under basal conditions. Furthermore, the inhibition of AhR expression blocked DOX-induced up regulation of CYP1A1, CYP1A2 and AhR mRNA, and the effect of Rb1 on DOX-mediated induction of CYP1A1, CYP1A2 and AhR mRNA was significantly blocked compared with siCON group (Fig. 7B). In addition, pretreatment with CH-223191 (10 μM) for 2 h, an AhR antagonist, increased the basal expression of CYP1A1, CYP1A2 and AhR, an effect that has been previously described in ARVM that may be due to ligand-dependent effects of CH-223191 (Volkova et al., 2011). However, addition of CH-223191 inhibited the expression of CYP1A1, CYP1A2 and AhR in response to DOX treatment compared with vehicle group, and the addition of Rb1 failed to exert an additive effect (Fig. 7C). We next determined whether the effect of Rb1 on DOX-induced apoptosis in H9C2 cells was an AhR-dependent mechanism. It was noteworthy that after AhR knockdown, DOX-induced apoptosis was not additionally reduced by the treatment with Rb1 as evidenced by c-caspase 3 levels. Similarly, pre-treatment of H9C2 cells with CH-223191 abolished the effect of Rb1 (Fig. 7D). These results raise the possibility that the cardioprotective effects of Rb1 may be mediated through AhR/CYP1A inhibition.
DISCUSSION

DOX-induced cardiomyopathy is a major obstacle that limits the clinical application of doxorubicin in cancer chemotherapy. To mitigate this hurdle, recent studies have focused on identifying agents that can protect against cardiac dysfunction. In the present study, using H9C2 cells, which is the most popular cell model used to carry out cardiovascular research (Shi et al., 2009), we demonstrated that ginsenoside Rb1 protects against DOX-induced cardiomyocyte apoptosis.

Ginseng, as a natural product, has been shown to exert potential benefits on cardiovascular diseases. Ginsenoside Rb1, as a compound of ginseng, has been reported to reduce isoproterenol-induced cardiomyocytes apoptosis in vitro and in vivo (Wang et al., 2013) and protect against ischaemia/reperfusion injury (Pasupathy and Homer-Vanniasinkam, 2005). In the present study, we examined the effect of Rb1 on cultured H9C2 cells injury caused by DOX and found that Rb1 significantly reduced cell damage in cells exposed to DOX in a dose-dependent manner as evidenced by cytotoxic activity assay (Fig. 1A). Interestingly, Rb1 at doses of 100, 200 and 400 μM did not significantly affect the viability of breast cancer MCF-7 cells treated with 5 μM DOX. These findings suggest that Rb1 could protect against cardiotoxicity without impairing the anti-tumor efficiency produced by DOX. The DNA fragmentation assay also showed the effect of Rb1 against DOX-induced apoptosis and Rb1 at 200 μM was the most efficacious (Fig. 2). It has demonstrated that the intrinsic and extrinsic pathways are involved in DOX-induced cardiomyocyte apoptosis (Zhang et al., 2009). Thus, we examined the effects of Rb1 on the activities of caspase-3/7, caspase-8 and caspase-9 in H9C2 cells treated with DOX. The results showed that Rb1 at 200 μM significantly decreased caspase-3/7 and caspase-8 activities, but not caspase-9 activity in cells stimulated with DOX (Fig. 4A, 4B, 4C). The results indicated that...
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Rb1 attenuated H9C2 cells apoptosis caused by DOX might be associated with the death receptor signaling pathway. We also examined the protein expression of caspase-3, PARP, cytochrome c, Bax and Bcl-2. Rb1 treatment attenuated the increase in cleaved caspase-3 and cleaved PARP levels in H9C2 cells stimulated by DOX (Fig. 4D, 4E). PARP plays an important role in the repair of DNA damage but loses its activity once it was cleaved by active caspase-3, accelerating the process of apoptosis. These results were consistent with the previous enzyme activity assay. It has been demonstrated that Bax can promote cytochrome c release from the mitochondria to induce apoptosis; however, Bcl-2 can suppress apoptosis progression through inhibiting mitochondria cytochrome c release (Green and Reed, 1998; Childs et al., 2002). The release of cytochrome c results in the activation of caspase-9, which in turn further increases activation of caspase-3. In this study, we found that DOX markedly increased Bax and decreased Bcl-2 expression while Rb1 treatment had no effects on these molecules. Cytochrome c expression also did not change in the presence of Rb1 after DOX treatment (Fig. 4F, 4G, 4H). These results were consistent with the activity of caspase-9 and further highlighted the possibility that mechanisms underlying Rb1 attenuated DOX-induced H9C2 cells apoptosis might be associated with the death receptor signaling pathway.

In the current study, the inhibition of DOX-induced H9C2 cells apoptosis by ginsenoside Rb1 was associated with proportional decrease in the mRNA and protein levels of the CYP1A1 and CYP1A2 genes regulated by AhR (Fig. 5), indicating an AhR-dependent mechanism. These results were consistent with previous studies, which showed that TCDD-induced CYP1A1 expression, an index of dioxin toxicity, was suppressed by flavonoids permeating the human intestinal Caco-2 cell monolayers (Hamada et al., 2006). The role of AhR and its regulated genes in the pathogenesis of cardiovascular diseases has been previously reported. For example, the mortality analysis of workers routinely exposed to TCDD showed a significant trend for the development of heart disease, cancer, and diabetes (Steenland et al., 1999). In addition, studies on TCDD exposure to medaka fish embryos showed that TCDD induced vascular hemorrhage and blood clotting. However, the vascular damage could be suppressed by AhR antagonist or CYP inhibitor, further underscoring the role of AhR and CYPs in cardiovascular diseases (Kawamura and Yamashita, 2002). Although there are many studies which have examined the effects of AhR ligands, particularly TCDD, on the cardiovascular system, the mechanisms underlying the cardiotoxic effect of TCDD has not yet been explained definitively. Generally, three main mechanisms are postulated, including disruption of the metabolism of endogenous substances, modulation of genes expression and oxidative stress, which result in endothelial cell dysfunction and apoptosis of the myocytes (Korashy and El-Kadi, 2006). Many reports have
suggested that TCDD can induce apoptosis. It was particularly noteworthy that mice deficient for Fas expression were less sensitive to TCDD-mediated thymic atrophy than Fas-positive counterparts. Likewise, Fas ligand-defective mice were refractory to TCDD-induced thymic atrophy (Rhile et al., 1996; Kamath et al., 1999). These observations suggested that the AhR plays a role in Fas-mediated apoptosis.

Benzo(a)pyrene and 3-methylcholanthrene, as an AhR ligand, induced CYP1A1 and CYP1B1 expression and caused cardiac hypertrophy in SD rats (Aboutabl et al., 2009), whereas benzo(e)pyrene, an isomer of benzo(a)pyrene, was a poor ligand for the AhR and failed to induce cardiac hypertrophy. Furthermore, resveratrol which is a naturally occurring polyphenolic compound significantly restored the hypertrophic genes mRNA expression induced by sunitinib by blocking of the AhR, suggesting a cardioprotective effect (Maayah et al., 2014). Therefore, we hypothesized that Rb1 was a weaker AhR agonist than DOX and it inhibited DOX-induced H9C2 cells apoptosis through competing with DOX for AhR affinity to decrease the expression of CYP1A genes. To test our hypothesis, we examined the capacity of Rb1 to decrease the CYP1A genes expression after DOX treatment in rat cardiomyocyte cell line. We found that treatment of H9C2 cells with DOX caused serious cell damage while significantly induced the expression of mRNA and protein of AhR-regulated CYP1A1 and CYP1A2 genes, whereas the induction was markedly restored by Rb1 pretreatment. These results may indicate the involvement of the AhR/CYP1A in Rb1 prevention of DOX-induced cardiotoxicity.

The activity of CYP1A is an index of dioxin toxicity. There is published evidence supporting the role of CYP1A1 in TCDD toxicities that TCDD-induced weight loss, liver pathology, and wasting syndrome were alleviated in CYP1A1−/− mice (Uno et al., 2004). CYP1A2 also plays a major role in enhancing uroporphyrinogen oxidation consistent with TCDD-induced uroporphyria. New considerations suggest that CYP1A functions via metabolism of endogenous chemicals. For example, the membrane lipid Arachidonic Acid (AA) is known as an endogenous substrate for CYP1A enzymes and the main HETEs metabolites, such as 20-HETEs, were associated with responses to hyperoxia and vasoconstriction that may result in hypertension and adverse effects in cardiac ischemia and cerebral (Su et al., 1998; Kehl et al., 2002; Roman, 2002). Eicosapentanoic acids contained in fish oils is an endogenous CYP1A substrate and the products have similar effects on vasoreactivity and ion channels compared with AA CYP products (Schwarz et al., 2004). In addition, human CYP1A1 and CYP1A2 preferentially metabolize 17-ß estradiol to 2-hydroxy-estradiol (Aoyama et al., 1990; Tsuchiya et al., 2005) and CYP1B1 primarily generated 4-Hydroxy-estradiol (Hayes et al., 1996). It is considered that both 4 and 2-hydroxy estradiol can induce ROS generation and contribute to DNA damage and Carcinogenesis (Lavigne et al., 2001; Chen et al., 2004). These highlighted observations further demonstrate that inhi-
bition of DOX-induced apoptosis by Rb1 may be associated with decreasing the induction of CYP1A.

In order to verify whether the effects of ginsenoside Rb1 were mediated by the AhR, we examined the effects of Rb1 and DOX on CYP1A1 luciferase reporter activity. The results showed that both DOX and Rb1 were capable of enhancing the interaction between AhR and CYP1A1 promoter region and DOX induction was more significant than Rb1; however, different concentrations of Rb1 could attenuate the effects of different levels of DOX (Fig. 6). It further confirms our conjecture that Rb1 is a weaker AhR agonist than DOX, and Rb1 can inhibit AhR agonist activity of DOX. Interestingly, Rb1 at 200 μM was the most efficacious concentration and this was consistent with the results of cytotoxic activity and DNA fragmentation assays. This may be due to Rb1 being a partial agonist; full agonist in combination could produce antagonism by competing for binding and depending on both drug doses and achieving the efficacy of partial agonists, thus Rb1 at 200 μM may be the most appropriate dose. In addition, ginsenoside Rb1 can also induce CYP1A1 expression in a dose-dependent manner and 400 μM Rb1 may have slight toxicity. The direct effect for the involvement of AhR in the capability of Rb1 decreasing the induction of CYP1A induced by DOX was supported by knockdown of AhR using AhR siRNA or blocking of AhR using CH-223191, a novel AhR antagonist, both of which significantly abolished the previous effects of Rb1 (Fig. 7B, 7C). Importantly, the abolished effects of Rb1 on CYP1A1 expression were accompanied with the same effect on the protein expression of caspase-3 (Fig. 7D). These results suggested that the mechanism was AhR dependent. Based on data Fig. 7D, DOX-mediated caspase3 activation is independent of AhR because DOX induced caspase3 activation even in siAhR or AhR antagonist treated cells. But in the condition of siAhR or AhR antagonist, the increased caspase3 activation by DOX was not reversed with Rg1 treated. Such results indicated that the toxicity of DOX is only partly related with AhR. Ginsenoside Rb1 inhibited DOX-induced cells apoptosis may through, at least in part, competing with DOX for AhR to decrease the CYP1A induction.

In conclusion, to the best of our knowledge, the present study is the first report that clearly demonstrates that ginsenoside Rb1 inhibits doxorubicin-mediated H9C2 cells apoptosis is associated with the aryl hydrocarbon receptor signaling pathway. However, these findings remain to be confirmed in vivo pre-clinical and clinical studies.

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REFERENCES


