Tanshinone IIA Protects Endothelial Cells from H_2O_2-Induced Injuries via PXR Activation

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Abstract

Tanshinone IIA (Tan IIA) is a pharmacologically active substance extracted from the rhizome of *Salvia miltiorrhiza* Bunge (also known as the Chinese herb Danshen), and is widely used to treat atherosclerosis. The pregnane X receptor (PXR) is a nuclear receptor that is a key regulator of xenobiotic and endobiotic detoxification. Tan IIA is an efficacious PXR agonist that has a potential protective effect on endothelial injuries induced by xenobiotics and endobiotics via PXR activation. Previously numerous studies have demonstrated the possible effects of Tan IIA on human umbilical vein endothelial cells, but the further mechanism for its exerts the protective effect is not well established. To study the protective effects of Tan IIA against hydrogen peroxide (H_2O_2) in human umbilical vein endothelial cells (HUVECs), we pretreated cells with or without different concentrations of Tan IIA for 24 h, then exposed the cells to 400 μM H_2O_2 for another 3 h. Therefore, our data strongly suggests that Tan IIA may lead to increased regeneration of glutathione (GSH) from the glutathione disulfide (GSSG) produced during the GSH peroxidase-catalyzed decomposition of H_2O_2 in HUVECs, and the PXR plays a significant role in this process. Tan IIA may also exert protective effects against H_2O_2-induced apoptosis through the mitochondrial apoptosis pathway associated with the participation of PXR. Tan IIA protected HUVECs from inflammatory mediators triggered by H_2O_2 via PXR activation. In conclusion, Tan IIA protected HUVECs against H_2O_2-induced cell injury through PXR-dependent mechanisms.

Key Words: Tanshinone IIA, PXR, HUVECs, Oxidative stress, Apoptosis, Inflammation

INTRODUCTION

The vascular endothelium, which is constantly exposed to xenobiotics and endobiotics or their metabolites, is thought to preserve a balance between vasodilation and vasoconstriction, and therefore plays a critical role in the regulation of vascular function (Valtcheva *et al.*, 2013). Endothelial dysfunction caused by xenobiotics and endobiotics has been extensively investigated in cardiovascular diseases including but not limited to, atherosclerosis, hypertension, and stroke (Yang *et al.*, 2013). There are several possible mechanisms for the impairment of endothelial function in cardiovascular diseases, but oxidative damage, as a result of enhanced production of reactive oxygen species (ROS) and decreased release of nitric oxide (NO), an attenuated antioxidant system and in-

creased apoptosis and inflammation appear to be the main culprits (Cai and Harrison, 2000). A number of studies have reported that apoptosis and inflammation of endothelial cells can be triggered by an excess of ROS, also known as oxidative stress, which has been seen together with apoptosis and inflammation in some circumstances (Irani, 2000). The pregnane X receptor (PXR), which belongs to the nuclear receptor superfamily, has been characterized as a xenobiotic sensor that is activated by xenobiotics and endobiotics including herbal medicines, drugs, environmental contaminants, and endogenous compounds like vitamins, oxysterols, and steroid hormones (Lehmann *et al.*, 1998).

As a ligand activated transcriptional factor, PXR binds to response elements in the promoters and up-regulates the transcription of the Phase I cytochrome P450 (CYP) enzymes

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Table 1. Primer pairs used for real-time PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
CYP3A4	CGAGGCGACTTTCTTCATC	CAAGACCCCTTTGTGGAAAA
MDR1	AGGGCTTCTTGGACAACCTT	AGGCCAACATACATGCCTTC
PXR	TGGGTGACACCTCCGAGA	TAGGGAGACAGGCCAGCA
GST	GCAGACCAGAGCCATTCTCAACTAC	GGACATACGGGCAGAAGGAGGAT
GPx	GGTGTTCCAGTGCGCAGATA	CATCACCAAGCCCAGATACCA
GSTM1	AGACAGAAGAGGAGAAGATTC	TCCAAGTACTTTGGCTTCAGT
GAPDH	TGTTGCCATCAATGACCCCTT	AGCATCGCCCCACTTGATTTTG

CYP2C9 and CYP3A4, the Phase II uridine-5-diphosphate glucuronosyltransferases (UGTs), glutathione S-transferases (GSTs), and drug transporters, such as multidrug resistance 1 (MDR1) and multidrug resistance-associated protein 1 (MRP1) in response to both toxic xenobiotics and endogenous metabolites (Kliewer *et al.*, 2002). Therefore, PXR is thought to be a pivotal defense system protecting the body from xenobiotic and endobiotic insults by eliminating metabolites. The expression of PXR has been seen in many other tissues including mammalian liver, and stomach, but recently several studies have found that PXR is present in vascular cells such as endothelial cells (ECs) and smooth muscle cells, and is believed to be critical to endothelial integrity (Swales *et al.*, 2012). In this role PXR up regulates CYP expression to carry out oxidative reactions that make lipophilic substrates more water-soluble while phase II enzymes perform conjugating reactions, for example with glutathione (GSH) further increasing water solubility, reducing toxicity and promoting internal stability.

Tanshinone IIA (Tan IIA), the major component extracted from Radix Salvia miltiorrhiza, is also known as the Chinese herb danshen (Su *et al.*, 2016), and is widely used for treating cerebral and cardiovascular diseases such as atherosclerosis, stroke, coronary heart disease, and hyperlipidemia (Zhang *et al.*, 2015b). In our previous studies show Tan IIA exerts protective effects in cholestatic liver model and inflammatory bowel disease that are associated with the participation of the PXR *in vivo* and *vitro*. However, the pharmacological activities of Tan IIA, especially the protective effects of endothelial cells from H_2O_2-induced injuries via PXR activation by Tan IIA are still unclear. Thus, we investigated whether the effects of Tan IIA protect endothelial cells from oxidative stress, apoptosis and inflammation are mediated via the activation of PXR.

MATERIALS AND METHODS

Reagents

Tanshinone IIA (>98% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Human umbilical vein endothelial cells (HUVECs) and a human acute monocytic leukemia cell line (THP-1) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Roswell Park Memorial Institute 1640 medium (RPMI-1640) was purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from GenStar (Beijing, China). Rifampicin (RIF) and human recombinant TNF-α were obtained from Sigma-Aldrich (St. Louis, MO, USA). GAPDH

and rabbit histone polyclonal antibody was purchased from TDY Biotech (Beijing, China). Immobilon Western Chemi-Luminescent HRP Substrate, and rabbit monoclonal PXR antibody were purchased from Millipore Corporation (Millipore, CA, USA). BCA was purchased from Applygen Technologies Inc (Beijing, China). All other chemicals were of the highest quality commercially available.

Cell culture and treatment

HUVECs and THP-1 cells were cultured in RPMI-1640 containing 0.45% glucose, L-glutamine, 0.11% sodium pyruvate and 10% FBS at 37°C in a humidified 5% CO_2 incubator, cells were maintained in 6-well plates until they reached 80% confluency. Tan IIA and RIF used in this experiment was dissolved in DMSO, and diluted in RPMI-1640, so that the final concentration of DMSO never exceeded 1‰.The concentrations of Tan IIA, RIF and H_2O_2 were selected based on our previous experience. Three independent experiments were performed.

Cell viability

Cell viability was evaluated using a cell counting kit-8 (CCK-8) assay kit (Dojindo Corp**.,** Tokyo, Japan) as described Previously (Zhou *et al.*, 2016). Briefly, in 96-well plates 10 μL CCK-8 solution was added to 90 μL RPMI-1640 in each well, then cells were incubated at 37°C for 0.5-4 h, after which absorbance at an asorbrance of 450 nm, was measured using a 2030 Multilabel VICTOR™ X5 Reader.

Intracellular total ROS, and MMP production

The production of total intracellular reactive oxygen species (ROS) and the mitochondrial membrane potential (MMP) were measured using fluorescent, probes H2DCF-DA and JC-1 (Beyotime, Beijing, China) respectively, according to the manufacturer's instructions. In brief, HUVECs in 6-well plates were pretreated with different concentrations of Tan IIA and RIF for 24 h then treated with 400 μM H_2O_2 for another 3 h. Cells were collected and loaded with H2DCF-DA, or JC-1 at final concentrations of 10 μmoL/L, and 10 μg/ml respectively for 20 minutes, washed with PBS (Phosphate Buffered Saline, PH7.4), and analyzed by flow cytometry (FCM) using a FAC-Star-Plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Detection of apoptosis

After the different treatments, HUVECs were collected and washed twice with cold PBS, then labeled with annexin V (0.5 μg/mL) and propidium iodide (50 μg/mL) for 15 minutes according to the manufacturer's protocol, and analyzed by FCM.
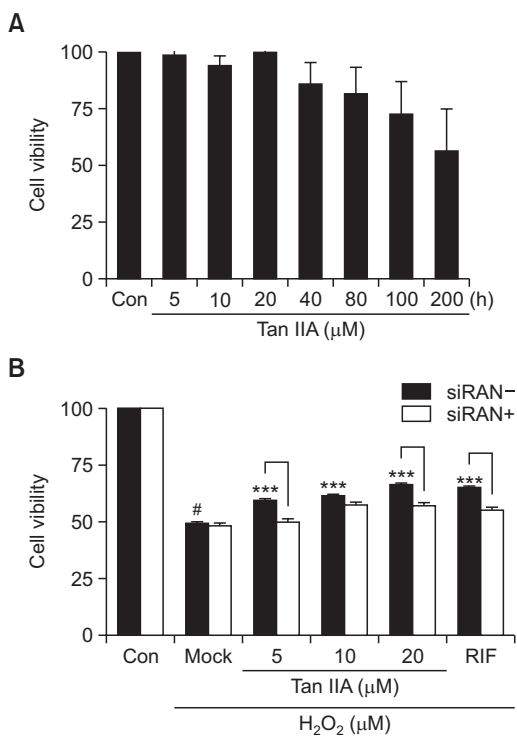
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Fig. 1. Tan IIA reduced H_2O_2-induced cell injury in HUVECs. (A) HUVECs were subjected to Tan IIA for different concentrations for 24 h; Cell viability was measured with a CCK-8 assay kit. (B) Cells were transfected with PXR siRNA, with or without pretreatment with Tan IIA for 24 h then treatment with 400 μM H_2O_2 for another 3 h. Data are expressed as percentage of control, and the mean ± SD of 3 replicates. #p<0.05 compared with control group. ***p<0.001 compared with H_2O_2 group.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated and mRNA expression was measured using quantitative real-time PCR (qRT-PCR) as described previously. Total RNA from the different treatment groups of HUVECs was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of the RNA was confirmed by an A260/A280 ratio of >1.8. One microgram of total RNA was reverse transcribed using a TransScript First-Strand cDNA Synthesis SuperMix Kit (TransGen Biotechnology, Beijing, China). Real-time PCR reactions were performed on a Trans-Script™ SYBR@ Green Master Mix in an ABI Prism 7500 real-time PCR instrument (Applied Biosystems, CA, USA), using a TransStart Green aPCR SuperMix Kit (TransGen Biotechnology, Beijing, China), to determine the relative mRNA levels of PXR, CYP3A4, MDR1, GST, GSTM1, GSH, peroxidase (GPx), and GAPDH (internal control). The primers used for qRT-PCR are listed in Table 1. The cycling conditions were used according to the TransStart Green aPCR Kit's protocol (TransGen Biotechnology, Beijing, China).

Preparation of total protein and western-blot analysis

Total protein, and cytoplasmic and nuclear proteins were extracted from different treatment groups of HUVECs using RIPA lysis buffer (Cwbiotech, Beijing, China), and Nuclear and Cytoplasmic Extraction Kit (Cwbiotech) respectively according to the manufacturer's instructions. Protein concentra-
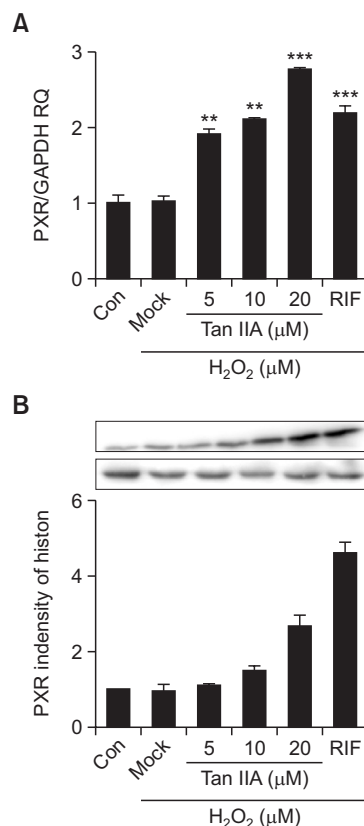
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Fig. 2. Tan IIA upregulates PXR expression in H_2O_2 treated HUVECs. Cells were pretreated with Tan IIA for 24 h then treated with 400 μM H_2O_2 for another 3 h, RIF treatment was used as a positive control. (A) PXR mRNA was measured by qRT-PCR, with GAPDH as an internal control. (B) Protein expression was measured with Western blot, using histone as an internal control. Data are expressed as percentages of control and are the mean ± SD of 3 replicates. **p<0.01, ***p<0.001 compared with H_2O_2 group.

tions were measured using a BCA protein assay kit (Applygen Technologies Inc.), bovine serum albumin as the standard. Western-blot analysis was carried out according to previously described (Hu et al., 2015).

RNA interference

SiRNA specific for PXR and scrambled siRNA were synthesized by Invitrogen. Target sequences of siPXR were as follows: Forward 5'-GAUGGAC-GCUCAGAUGAAATT-3' and Reverse 5'-UUUCAUCUGAGCGUCCAUCTT-3'. The detection method was as described previously (Hu et al., 2015). Cells were seeded in 24-well plates and transfected with 50 nmol/L of each siRNA use Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. After 6 h, transfected cells were washed with PBS, and then incubated in fresh culture medium containing 10% FBS. Then cells were incubated for a further 24 h before treatment.

Hoechst 33342 staining

Hoechst 33342 staining was used to identify the morphological features of apoptosis. The HUVECs (5×10^{5}/well) were cultured in 6-well plates treated with Tan IIA, RIF, and H_2O_2 and then treated with 20 μmol/L Hoechst 33342 dye for 20
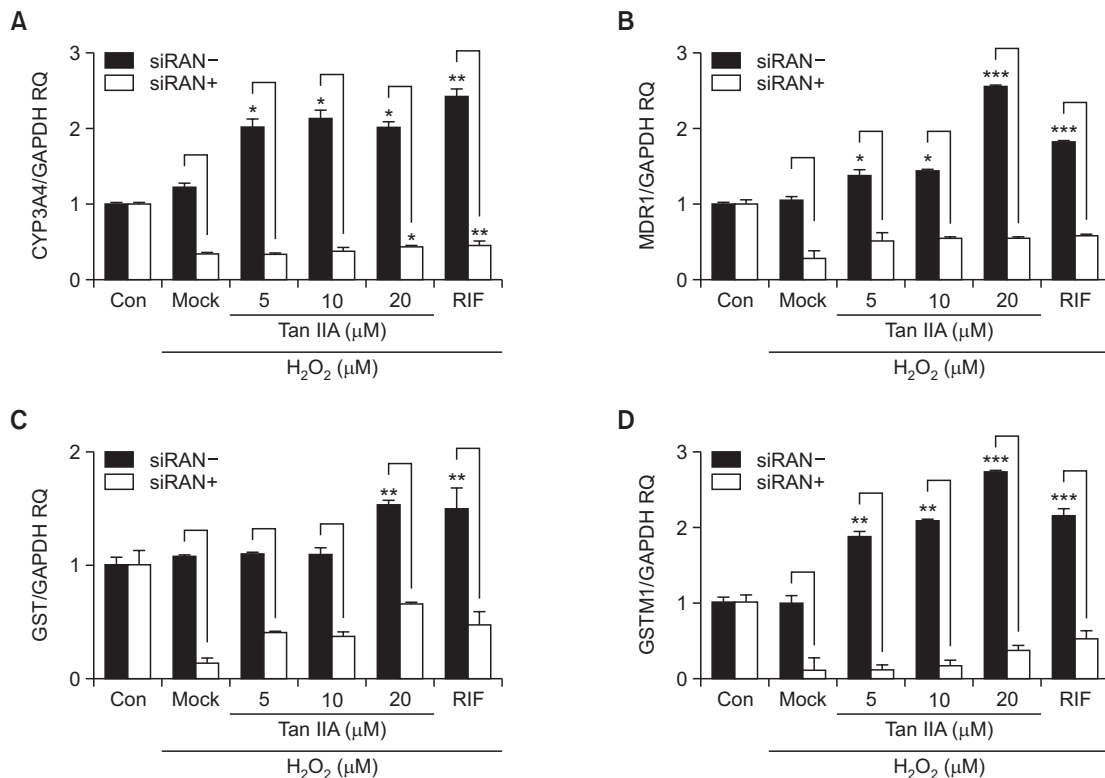
Fig. 3. Tan IIA upregulates CYP3A4, MDR1, GST and GSTM1 expression in H_2O_2 treated HUVECs via PXR activation. Cells were transfected with PXR siRNA, with or without pretreatment with Tan IIA for 24 h then treatment with 400 μM H_2O_2 for another 3 h. RIF treatment was used as a positive control. (A-D) CYP3A4, MDR1, GST and GSTM1 mRNA expression was measured by qRT-PCR, using GAPDH as an GAPDH was internal control. Data are expressed as percentages of control and are the mean ± SD of 3 replicates. *p<0.05, **p<0.01, ***p<0.001 compared with H_2O_2 group.

minutes at 37°C, according to the manufacturer's instructions (Dojindo Corp.). Apoptotic cells were identified by condensed or fragmented nuclei using fluorescence microscopy (Olympus, Tokyo, Japan) at ×100 magnification.

Caspase-3/7, 8, and 9 activity assessments

Caspase- 3/7, 8, and 9 activities were detected using Caspase-Glo® 3/7, 8, and 9 Assay (Promega, Madison, USA) according to the manufacturer's instructions. In brief, HUVECs in 96-well plates were pretreated with different concentrations of Tan IIA and RIF for 24 h, followed by 400 μM for 3 h, then caspase reagent was added and the cells were incubated for another 30 min. Samples were analyzed under a 2030 Multilabel VICTOR™ X5 Reader (Perkin-Elmer, CA, USA) at an excitation wavelength of 499 nm and an emission wavelength of 521 nm.

Determination of IL-8 and GSH, GSSG levels

Total IL-8, GSH, and GSH disulfide (GSSG) levels were detected using an Enzyme Linked Immunosorbent Assay (ELISA) Kit and a GSH, GSSG Assay Kit (eBioscience, Beijing, China) respectively. In brief, HUVECs were collected and washed twice with cold PBS then the assays were performed according to the manufacturer's protocol, and finally measured at an absorbance of 450 nm, using a 2030 Multilabel VICTOR™ X5 Reader (Perkin-Elmer).

Statistical analysis

The data are reported as means ± standard deviations (SD). The differences between groups were analyzed by one-way analysis of variance followed by the least significant difference post-hoc test. The level of significance was determined at $p \leq$ 0.05.

RESULTS

Tan IIA reduced H_2O_2-induced Cell Injury in HUVECs cells

To determine the optimal Tan IIA concentration, Tan IIA cytotoxicity was assessed in HUVECs. Fig. 1A shows that 5, 10, 20, 40, 80, and 100 μM Tan IIA did not affect cell viability. However, 200 μM Tan IIA statistically significantly decreased cell viability. Cells were treated with Tan IIA at non-cytotoxic concentrations for the subsequent study. After pretreatment with Tan IIA for 24 h, the cells were then treated with 400 μM H_2O_2 for another 3 h. Fig. 1B shows that 400 μM H_2O_2 for 3 h significantly induced cell death (about 50%), which were concentration dependently attenuated by Tan IIA (5-20 μM) pretreatment for 24 h, but this protective effect was decreased with the transfection of siPXR. RIF (10 μM) was used as the positive control.

Tan IIA improved H_2O_2-mediated PXR expression in HUVECs

We previously used a transient luciferase reporter assay to screen the transcriptional activity of PXR in response to endogenous and exogenous products. Tan IIA enhanced PXR
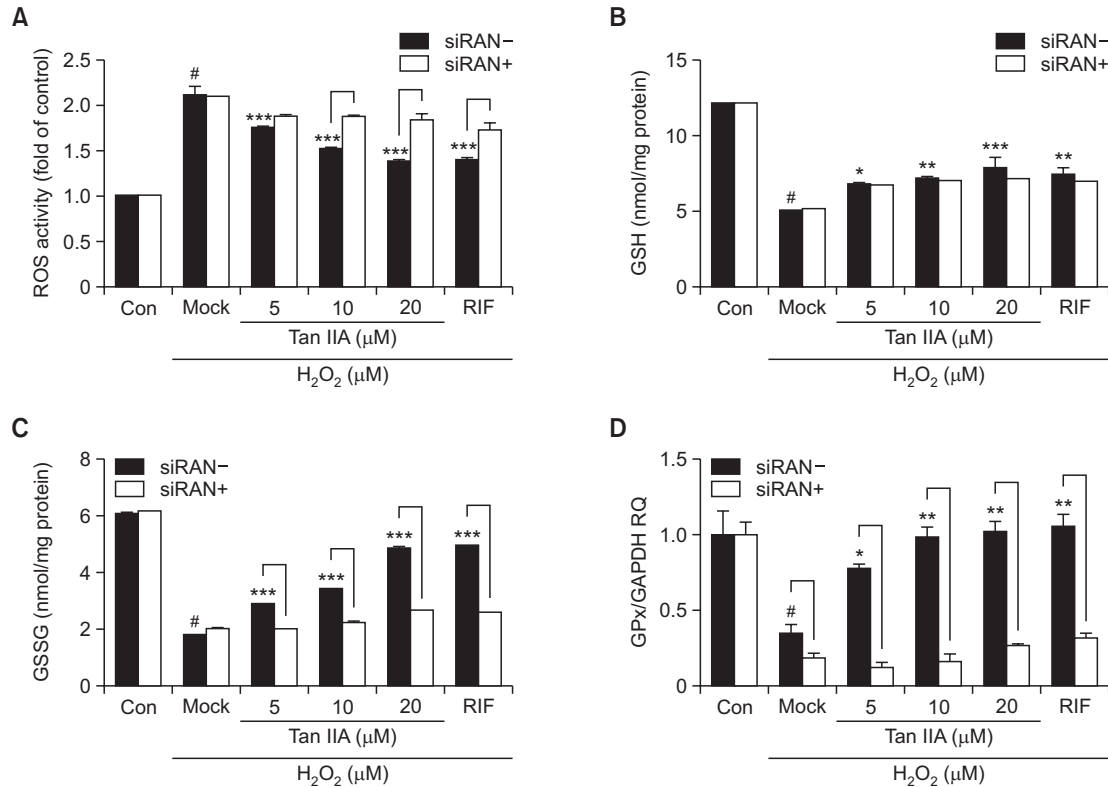
Fig. 4. Tan IIA inhibit H_2O_2-induced oxidative stress via PXR activation. Cells were transfected with PXR siRNA, with or without pretreatment with Tan IIA for 24 h then treated with 400 μM H_2O_2 for another 3 h. ROS production (A), total GSH production (B), and GSSG production (C) were detected by different assays as described in Materials and Methods, (D) GPx mRNA expression was measured by qRT-PCR, GAPDH was used as an internal control. Data are expressed as percentages of control and are the mean ± SD of 3 replicates. #p<0.05 compared with control group. *p<0.05, **p<0.01, ***p<0.001 compared with H_2O_2 group.

transcriptional activity in a dose dependent manner and further induced CYP3A4 and PXR mRNA expression in HepG2 cells (Zhang *et al*., 2015a). In the current study, we explored whether PXR is involved in Tan IIA -mediated cytoprotective effects during H_2O_2 stimulation. HUVECs were pretreated with or without Tan IIA for 24 h then treated with H_2O_2 for another 3 h and PXR expression both in mRNA and protein levels were measured. As shown in Fig. 2, Tan IIA (5, 10, 20 μM) treatment obviously increased PXR mRNA and protein levels in H_2O_2-stimulated HUVECs compared with a control group, in a concentration-dependent manner. RIF treated cells were used as a positive control. However, the expression of PXR mRNA and protein levels had no obvious changes when cells were treated with H_2O_2 alone. To confirm that Tan IIA can activate PXR to protect against H_2O_2-induced cell damage, PXR regulated target genes including CYP3A4, MDR1, GST and GSTM1 mRNA were measured using qRT-PCR. Transcriptional expression of CYP3A4, MDR1, GST and GSTM1were up-regulated in cells treated with Tan IIA, but in the H_2O_2 group there was no apparent change. To further explore whether the effect of Tan IIA on CYP3A4, MDR1, GST and GSTM1 expression is associated with PXR activation, we knocked down PXR expression with a specific siRNA. Fig. 3 shows that PXR siRNA diminished the Tan IIA-induced increases in CYP3A4, MDR1, GST, and GSTM1 expression. Therefore, Tan IIA can induce CYP3A4, MDR1, GST, and GSTM1 mRNA expression by activating PXR in H_2O_2 -treated HUVECs.

Tan IIA reduced H_2O_2-induced oxidative damage in HUVECs via PXR activation

To further clarify the protective effects of Tan IIA on H_2O_2-induced cellular injury. HUVECs were pretreated with or without Tan IIA for 24 h then treated with H_2O_2 for another 3 h. Intracellular ROS production was measured using a commercial kit and spectrofluorometer. As shown in Fig. 4A, intracellular ROS production was obviously increased in the H_2O_2-stimulated HUVECs but the stimulation was concentration-dependently suppressed by Tan IIA pretreatment. Meanwhile, intracellular total GSH and GSSG production was significantly suppressed in the H_2O_2-stimulated cells, which was also concentration dependently elevated by Tan IIA pretreatment (Fig. 4B, 4C). Interesting, knocking down PXR expression with a specific siRNA, did not obviously decrease total GSH activity, but it did decrease total GSSG. The protection by GSTs against oxidative damage is mainly mediated by conjugation of toxins with GSH and by selenium-independent GPx activity, therefore, because PXR can up-regulate GST and GSTM1 mRNA expression, we simultaneously measured GPx expression with qRT-PCR to further investigate how Tan IIA prevents cellular injury during H_2O_2 treatment, as shown in Fig. 4D. In the H_2O_2 group GPx expression decreased compared to the control group, but the extent of this decrease changed with the pretreatment with Tan IIA, and the effect was abolished when PXR was PXR transfected using siRNA. Therefore, we have for the first time demonstrated that Tan IIA inhibits H_2O_2-
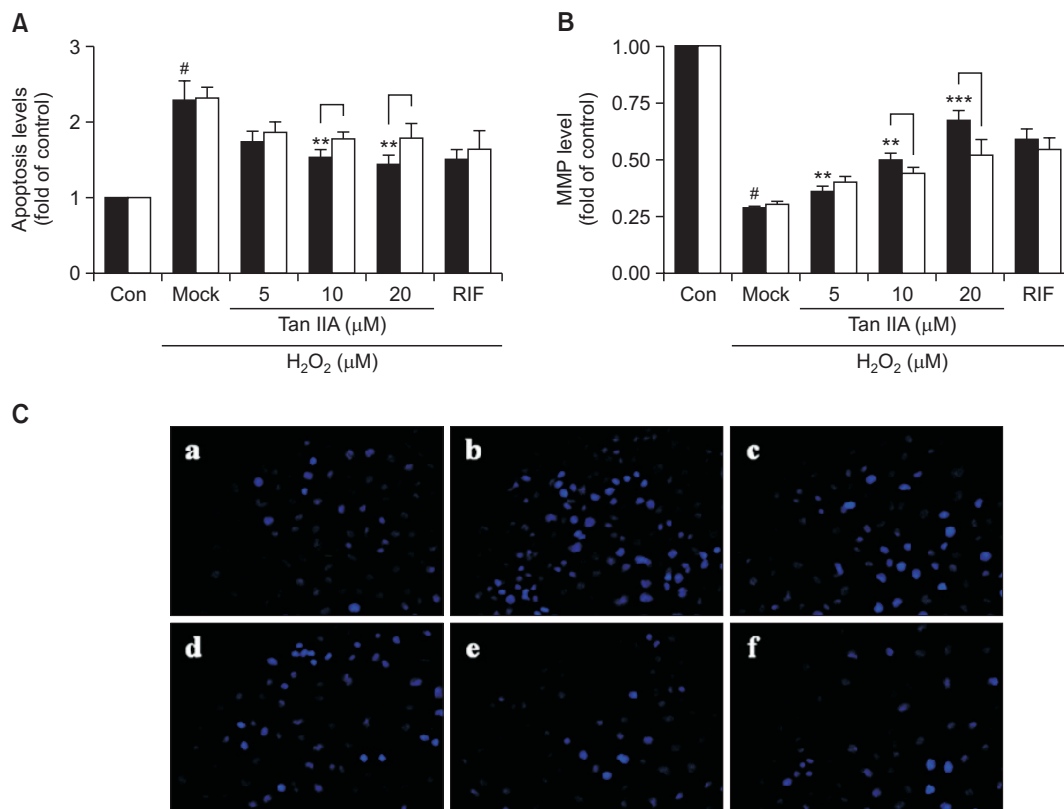
Fig. 5. Tan IIA inhibits H_2O_2-induced apoptosis via PXR activation. Cells were transfected with PXR siRNA, with or without pretreatment with Tan IIA for 24 h then treatment with 400 μM H_2O_2 for another 3 h. Apoptosis (A) and MMP (B) were detected by different assays as described in Materials and Methods. Cells were stained with Hoechst 33342 and visualized under a fluorescence microscope (C) a: control group; b: H_2O_2 group; c-e: Tan IIA (5, 10, 20 μM)+H_2O_2 group; f: RIF+H_2O_2 group. Condensed or fragmented nuclei were considered as apoptotic cells (magnification: 200×). (D, E, F) Cells were treated with H_2O_2 (400 μM) in the absence or presence of Tan IIA (200 μM) for 24 h, caspase-3/7, 8, and 9 activities were analyzed with a plate reader. (G, H, I) Apoptosis-related proteins bax, cytochrome C and Bcl-2 were measured with Western blotting. Data are expressed as percentages of control and are the mean ± SD of 3 replicates. $^{\#}p<0.05$ compared with control group. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared with H_2O_2 group.

induced oxidative stress via PXR activation.

Effects of tan IIA on the H_2O_2-induced apoptosis-signaling pathway in HUVECs

A number of studies have reported that apoptosis of endothelial cells can be triggered by ROS (Warren *et al*., 2000). However, the anti-apoptotic mechanisms of Tan IIA remain to be demonstrated. Intracellular apoptosis and MMP production were measured, and activities of caspases-3/7, 8, and 9 activities and apoptosis-related molecules were also evaluated.

HUVECs were pretreated with or without Tan IIA for 24 h then treated with H_2O_2 for another 3 h. Intracellular apoptosis and MMP production were measured, and the flow cytometry results are shown in Fig. 5A and 5B. Exposure to H_2O_2 induced significant in apoptotic activity apoptosis compared with the control group, but the apoptosis was concentration-dependently attenuated by Tan IIA (5-20 μM) pretreatment. MMP were decreased in the H_2O_2-stimulated HUVECs. Tan IIA markedly inverted this suppression in a concentration-dependent manner.

We used Hoechst 33342 staining to quantify the morphological observations that accompany apoptosis. Compared with the controls there were more obvious changes in the heterogeneous intensity, chromatin condensation, and fragmen-

tation under fluorescence microscopy, representing the classical characteristics of apoptotic cells in the H_2O_2-stimulated HUVECs as shown in Fig. 5C. However, the morphological changes were improved by the pretreatment with Tan IIA.

To further clarify the underlying mechanism behind Tan IIA's inhibition of the apoptotic effects induced by H_2O_2, activities of the intracellular caspases-3/7, 8, and 9 were evaluated. As shown in Fig. 5D and 5E, caspase-3/7 and 9 activities were significantly increased in the H_2O_2-stimulated HUVECs compared with the control group. However, caspases-3/7, and 9 activities were significantly lower in the Tan IIA and H_2O_2 co-treated group compared with the group treated only with H_2O_2. However, the activity of caspase 8 showed no change after either treatment with H_2O_2 only or pretreatment with different concentrations of Tan IIA (Fig. 5F). Moreover, when the expression of apoptosis-related molecules was measured, bax and cytochrome C were increased and Bcl-2 was reduced in the H_2O_2-stimulated HUVECs. Pretreatment with different concentrations of Tan IIA TanIIA totally changed this result (Fig. 5G-5I). Meanwhile, we also investigated the role of Tan IIA-induced PXR expression on the alteration of cell damage, when PXR expression was silenced with specific human PXR siRNA. Transfection with PXR siRNA abolished the protective effects of Tan IIA in HUVECs compared to cells treated with
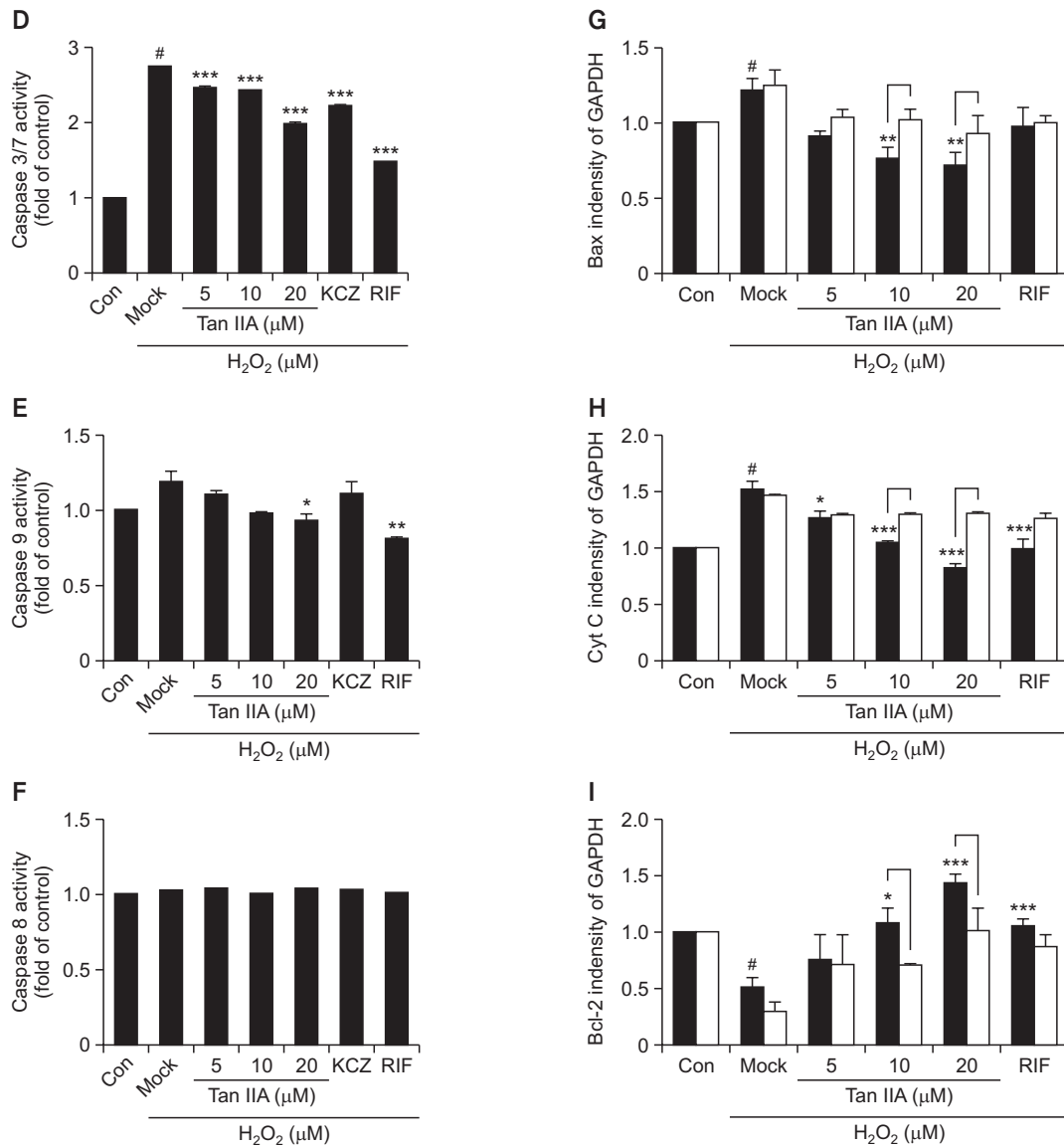
Fig. 5. Continued.

scrambled siRNA. Thus, the accumulating evidence indicated that PXR was essential for Tan IIA-mediated anti-apoptosis in H_2O_2-treated HUVECs.

Tan IIA reduced H_2O_2-induced inflammation response in HUVECs

Next, we investigated the ability of Tan IIA to suppress an inflammatory effect via PXR activation. Fig. 6A shows that H_2O_2 increased the adhesion of THP-1, compared with the control group. Conversely, pretreatment with different concentrations of Tan IIA totally changed this result. Meanwhile, cells transfected with PXR siRNA decreased the suppression of adhesion. As shown in Fig. 6C ELISAs indicated that IL8 levels were significantly higher in the H_2O_2 stimulation group, compared with the control group. Administration of 5, 10, or 20 μM Tan IIA significantly suppressed the intracellular IL-8 levels, compared with controls. Thus, Tan IIA suppressed the inflam-

matory response in HUVECs in a PXR-dependent manner.

DISCUSSION

Previously numerous clinical trials and laboratory studies have demonstrated the protective effects of Tan IIA on cardiovascular performance. The results from Chan directly indicated Tan IIA attenuates H_2O_2-induced Injury in HUVECs are similar to our current results, but the underline mechanism by which exerts the protective effect is not well established (Chan *et al.*, 2012), especially the pharmacological protective effects of endothelial cells from H_2O_2-induced injuries via PXR activation by Tan IIA are still unclear. The present study we provided evidence showing that tanshinone IIA inhibit H_2O_2-induced injury in human umbilical vein endothelial cells with the activation of the PXR. This is the first report to clarify the beneficial
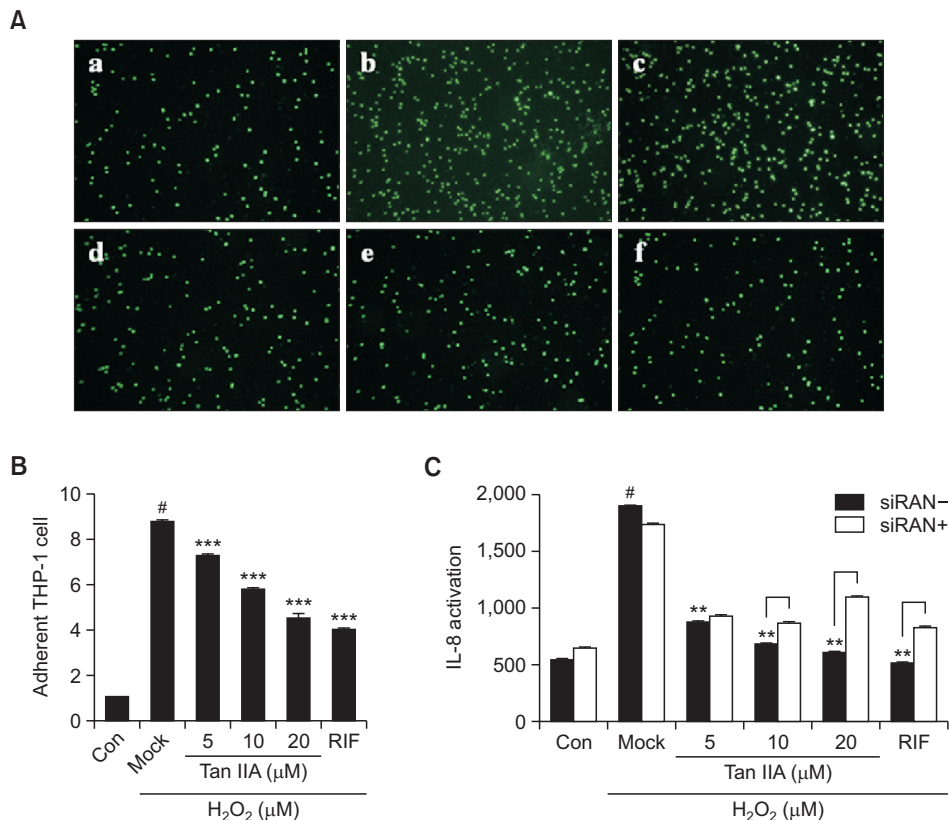
A

B

C

Fig. 6. Tan IIA inhibits H_2O_2-induced inflammation via PXR activation. (A, B) Cells with or without pretreatment with Tan IIA for 24 h then treated with 400 μM H_2O_2 for another 3 h. HUVECs were then incubated with fluorescently labeled THP-1 cells (5×10^5 cells/mL) for 30 min and visualized under a fluorescence microscope, a: control group; b: H_2O_2 group; c-e: Tan IIA (5, 10, 20 μM) + H_2O_2 group; f: RIF+H_2O_2 group. (C) Data are expressed as percentages of control and are the mean ± SD of 3 replicates. [#]$p<0.05$ compared with control group. [**]$p<0.01$, [***]$p<0.001$ compared with H_2O_2 group.

effect of Tan IIA on cardiovascular protection against oxidative stress via the activation of PXR.

As a nuclear receptor, PXR plays a crucial role in protecting cells against xenobiotic and endobiotic insults by eliminating metabolites via the modulation of phase I and Phase II drug-detoxifying enzymes and drug transporters, and it is has been found to be expressed in the human aorta and heart tissue, cell lines HAEC, HASMC, and RASMC (Kliewer *et al*., 2002). As many new or existing clinical drugs and natural products activate PXR, PXR has been identified as a novel therapeutic target for cardiovascular disorders (Gong *et al*., 2006). Furthermore, PXR has also been shown to protect against oxidative stress-induced cytotoxicity by activating total GST and GPx and coordinating an antioxidant response in vascular cells to sustain homeostasis (Epsztejn *et al*., 1999). In our previous study, Tan IIA was confirmed as a moderate activator of PXR, which could up-regulate PXR expression and its transcriptional activity, resulting in the induction of expression of target genes. However, the role of PXR in the inhibition of the oxidative stress, apoptosis, and inflammation induced by H_2O_2, and the relationship between Tan IIA's anti-oxidative effects and the activation of PXR, have not yet been elucidated. Tan IIA, a pharmacologically active component of the Chinese herb Danshen, has been shown to possess various effects on cardiovascular diseases, and some of these actions are due to its anti-oxidative stress, anti-apoptotic, and anti-inflammato-

ry properties (Shu *et al*., 2016). Among these, in our previous studies we have demonstrated that Tan IIA exerts protective effects in cholestatic liver and inflammatory bowel disease that are associated with the activation of the pregnane X receptor *in vivo* and *vitro*, these studies indicated that as an agonist of PXR, Tan IIA can have a variety of pharmacological effects via the activation of PXR (Zhang *et al*., 2015a, 2015b). In the present study, we examined the effects of Tan IIA on cultured HUVECs injured by H_2O_2 (Fig. 1), and found that Tan IIA significantly mitigated cytotoxicity in a dose-dependent manner as evidenced by a cytotoxic activity assay. In order to further investigate the underlying mechanism we examined the activities of caspase-3/7, caspase-8 and caspase-9 in HUVECs treated with H_2O_2 (Fig. 5D-5F). The results showed that Tan IIA significantly decreased caspase-3/7 and caspase-9 activities in a concentration-dependent manner, but caspase-8 activity did not show an obvious change either in the H_2O_2 group or the Tan IIA pretreated groups. This indicated that the attenuation by Tan IIA of apoptosis caused by H_2O_2 in HUVECs might be associated with the mitochondrial apoptosis pathway, which is commonly activated in response to cell injury (Zhao *et al*., 2012). To determine whether Tan IIA's anti-apoptotic effect was mediated through the mitochondrial apoptosis pathway via activation of PXR, we also measured the cellular MMP (Fig. 5B). Compared with the control group, MMP was inhibited by H_2O_2 and Tan IIA notably reversed this suppression. We

further measured Bcl-2, BAX, and cytochrome C expression using Western blot (Fig. 5G-5I), and results showed that BAX, and cytochrome C were increased and Bcl-2 was reduced in the H_2O_2-stimulated HUVECs, but Tan IIA and H_2O_2 co-treatment groups totally changed this result. A number of studies have shown that the mitochondrial permeability transition is an important step in the induction of cellular apoptosis (Giotakis *et al*., 2010). During this process, the electrochemical gradient across the mitochondrial membrane collapses, and the mitochondrial contents are released into the cytoplasm, which enhances apoptosis. Bcl-2, BAX and cytochrome C are apoptosis-related proteins that play a crucial role in maintaining the integrity and permeability of the mitochondrial membrane, BAX can promote cytochrome C release from the mitochondria to induce apoptosis, and conversely, Bcl-2 inhibits cytochrome C release from the mitochondria to inhibit the progression of apoptosis. But, if the balance between induction and inhibition broken, then apoptosis progresses (Giotakis *et al*., 2010). The delivery of cytochrome C from the mitochondrial intermembrane to the cytosol will cause the activation of caspase-9, which targets critical cellular death substrates and contributes to apoptosis (Bhattacharyya *et al*., 2005). Tan IIA alleviates H_2O_2-induced apoptosis in a concentration dependent manner, and its ability to attenuate apoptosis was completely abolished when PXR was knocked down according to our study (Fig. 5). This result confirmed that Tan IIA exerts protective effects against H_2O_2-induced apoptosis through the mitochondrial apoptosis pathway associated with the activation of PXR.

It is known that GSTs (detoxication enzymes that catalyze the conjugation of glutathione), MDR1 (belonging to the ATP-binding cassette (ABC) transporter superfamily) and CYP3A4 (isoenzyme of human CYP) act as an efficient toxin efflux molecules to protect against drugs and other xenobiotics, particularly in the process of xenobiotic oxidation reactions (Epsztejn *et al*., 1999, Yang *et al*., 2004). Through selenium-independent GPx activity, GSTs can conjugate GSH to their substrates (products of oxidative stress or xenobiotics) and protect against oxidative damage (Swales *et al*., 2012). Previous work has confirmed that PXR prevent the effects of H_2O_2 by the induction of GPx and MDR1 expression, which indicates that PXR contributes to reduced peroxidase activity and protects internal balance (Cheng *et al*., 2009, Ramirez-Zavala *et al*., 2014). Our results showed that Tan IIA treatment increased CYP3A4, MDR1, GST, GSTM1 and GPx mRNA expression in HUVECs. In addition, the induction of CYP3A4, MDR1, GST, GSTM1, and GPx were diminished by knocking down PXR expression with siRNA (RIF as a positive control), indicating that the induction of mRNA expression was via the activation of PXR.

GSTs are a family of enzymes that possess the ability to conjugate GSH, and this conjugation is thought to be critical in the resistance to oxidative stress (Hayes and Pulford, 1995). GSH is an important defense molecule against oxidizing xenobiotics such as free radicals and peroxides, playing a pivotal role in antioxidant processes, and is an important intracellular detoxification system within the cell (Meister and Anderson, 1983). In our results (Fig. 4B, 4C), compared with control group GSH and GSSG activation in the H_2O_2 group was suppressed, but Tan IIA pre-treated groups significantly released this suppression of intracellular GSH and GSSG activation. When PXR expression was knocked down by siRNA,

this completely abolished the induction of GSSG by Tan IIA, while GSH activation was not obviously changed. It is known that GPx catalyses GSH oxidation to GSSG at the expense of H_2O_2, which is then converted to GSH via a glutathione reductase (GR)-catalyzed reduction (Goya *et al*., 2007). Our results may indicate that Tan IIA leads to increased regeneration of GSH from GSSG produced during the GPx-catalyzed decomposition of H_2O_2 in HUVECs, and that PXR plays a significant role in this process. Therefore, the above evidence suggests that PXR has contributed to Tan IIA's protective effects on endothelial cells against H_2O_2-induced oxidative stress. Our study also shows that Tan IIA's activity against H_2O_2-elicited inflammation in cultured HUVECs is related to PXR activation (Fig. 6). Tan IIA can eliminate H_2O_2-induced IL-8 (Fig. 6B) via activation of PXR, but the underlying mechanism is still under investigation.

In conclusion, we confirmed the ability of Tan IIA to protect against endothelial damage through the inhibition of oxidative stress, apoptosis, and inflammation. Importantly, this study is the first to demonstrate that Tan IIA inhibits H_2O_2-triggered HUVEC cells damage and that this was associated with the activation of the important nuclear hormone receptor PXR. Simultaneously, Tan IIA inhibits apoptosis and inflammation induced by H_2O_2 in HUVECs dependent on PXR. This provides further evidence that PXR-mediated endothelial detoxification works as a gatekeeper for vascular defense against oxidative stress and apoptosis. Although Tan IIA and its analogues (Sulfotanshinone Sodium Injection) has wide clinical application in cardiovascular diseases, and there are no obvious side effects were reported, but the underlying mechanism for its activity is not fully understood. More studies are needed to understand the physiological significance of Tan IIA-activated PXR activation protecting against oxidative stress *in vivo*.

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