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Docetaxel Facilitates Endothelial Dysfunction through Oxidative Stress via Modulation of Protein Kinase C Beta: The Protective Effects of Sotrastaurin

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ABSTRACT

Docetaxel (DTX), a taxane drug, has widely been used as an anticancer or antiangiogenesis drug. However, DTX caused side effects, such as vessel damage and phlebitis, which may reduce its clinical therapeutic efficacy. The molecular mechanisms of DTX that cause endothelial dysfunction remain unclear. The aim of this study as to validate the probable mechanisms of DTX-induced endothelial dysfunction in endothelial cells. Human umbilical vein endothelial cells (HUVECs) were stimulated with DTX (2.5, 5, and 10nM) for 24 h to induce endothelial dysfunction. Stimulation with DTX reduced cell viability in a concentration- and time-dependent manner. DTX upregulated caspase-3 activity and TUNEL-positive cells. DTX treatment also increased PKCβ phosphorylation levels and NADPH oxidase activity, which resulted in ROS formation. However, all of these findings were reversed by PKCβ inhibition and NADPH oxidase repression. Finally, we demonstrated that sotrastaurin (AEB-071), a new PKCβ inhibitor, mitigated DTX-induced oxidative injury in endothelial cells. Our findings from this study provide a probable molecular mechanism of DTX-induced oxidative injury in endothelial cells and a new clinical and therapeutic approach for preventing DTX-mediated vessel injury.

Key words: docetaxel; protein kinase C; NADPH oxidase; sotrastaurin

Docetaxel (DTX), which is a taxane drug, has been extensively used in anticancer or antiangiogenesis chemotherapy in clinical treatments. The therapeutic effects of DTX have been previously reported in multiple cancers, such as head and neck, breast, ovarian, or gastric cancers (Kintzel *et al.*, 2006; Zhao and Astruc, 2012). However, some side effects of DTX injection were reported previously. DTX induced vessel damage and phlebitis, which may reduce its therapeutic effects (Bissett *et al.*, 1993). In clinical human studies, phlebitis is a major problem that causes patients to feel uncomfortable (Burdette-Radoux *et al.*, 2007; Hsiao and Su, 2009; Leonard and Zujewski, 2003). Therefore, reducing DTX-induced endothelial dysfunction is an important issue that needs to be investigated.

Protein kinase C (PKC) is a major regulator of cellular physiological function under stressful situations (Tsai *et al.*, 2014). Previous reports have confirmed that PKC activation is important during oxidative stress to cause endothelial dysfunction (Churchill *et al.*, 2008). The upregulation of PKC activity reduces endothelial NO synthase (eNOS) function in human endothelial cells, which enhances ROS generation and cellular death (Fleming *et al.*, 2005; Tsai *et al.*, 2011). Conversely, the mitigation of PKC function via a PKC-specific pharmacological inhibitor not only reduces stress-induced ROS production and JNK activation but also prevents stress-induced mitochondrial dysfunction in endothelial cells (Shi *et al.*, 2011). Moreover, PKC also regulates cellular apoptosis and proliferation in animals. For instance, PKC knockout mice displayed delayed development of atherosclerotic injuries (Harja *et al.*, 2009). This evidence suggests that PKC affects endothelial apoptosis and proliferation during atherosclerosis progression.

Generally, oxidative stress is a main cause of endothelial dysfunction. Indeed, a high concentration of free radicals causes endothelial apoptosis and inflammation (Dayal *et al.*, 2002). In stressful situations, NADPH oxidase acts as a key role to turn on oxidative signaling transduction through the formation of ROS via assembly with NADPH oxidase subunits (Bey *et al.*, 2004). In humans, NADPH oxidase-generated intracellular ROS acts as second messengers to facilitate downstream events such as p38MAPK phosphorylation and mitigation of NO production via impaired eNOS (Sugimoto *et al.*, 2009).

Some anticancer drugs cause vascular endothelial dysfunction mainly by upregulating oxidative stress. For example, Yamada Takaaki *et al*. suggested that epirubicin induces oxidative injuries in endothelial cells via p38 activation (Yamada *et al.*, 2012). Thus, they determined that vinorelbine promotes endothelial apoptosis by increasing oxidative stress. We previously reported that vinorelbine triggers endothelial inflammation by modulating PKC/NADPH oxidase, which is reversed by an "antioxidant" intervention (Tsai *et al.*, 2012a, 2014). In this pioneering study, we tested the underlying mechanism of DTX to cause endothelial injuries. We assumed that DTX induced endothelial death through PKCβ activation, which promotes both NADPH oxidase activity and ROS formation. We also assessed the protective effects of sotrastaurin, a PKCβ inhibitor, on DTX-induced endothelial apoptosis.

MATERIALS AND METHODS

Cell culture and reagents. Human umbilical vein endothelial cells (HUVECs) were obtained from ATCC. HUVECs were cultured with M199 basal medium supplemented with low-serum growth supplement and penicillin (50 IU/ml)-streptomycin (50 μg/ml). Trypsin-EDTA was used to passage cells. M199 and trypsin-EDTA were obtained from Gibco (Grand Island, New York). Low-serum growth supplement was purchased from Cascade (Portland, Oregon). Additionally, the terminal deoxynucleotidyltransferase-mediated UTP nick end-labeling (TUNEL) staining kit was obtained from Boehringer Mannheim (Mannheim, Germany), and sotrastaurin was purchased from Selleckchem. 5,58,6,68-Tetraethylbenzimidazolcarbocyanine iodide (JC-1) was purchased from BioVision (Palo Alto, California). LY333531, dihydroethidium (DHE), MG132, apocynin, GSH, oxypurinol, penicillin, and streptomycin were purchased from Sigma (St Louis, Missouri). Anti-**β**-actin, anti-PKC**β**, anti-p-PKC**β**, anti-p67phox, anti-flotillin-1, and anti-p47phox were all obtained from Santa Cruz Biotechnology (Santa Cruz, California). HRP-conjugated anti-Rabbit secondary antibodies were purchased from Transduction Laboratories (California).

Investigation of cytotoxicity. HUVECs were treated with the indicated DTX dosages for 24 h. Cell viability was assessed with the MTT assay. Briefly, after treatments, the culture medium was used for the LDH assay, and the cells were washed with PBS. MTT (5 mg/ml) was added to the culture medium and incubated at 37°C for 1 h. After MTT removal, the colored formazan was dissolved in 200 μl isopropanol. Absorption values were measured at 490 nm with a Sunrise Remote Microplate Reader (Grodig, Austria). HUVEC viability in each well was shown as the percentage of control cells. Cytotoxicity was assessed using the LDH assay. Briefly, after treatments, the culture medium was centrifuged at 10 000 × g for 15 min at 4°C, and the supernatants were mixed with the LDH kit (1:1) for 30 min. LDH levels were assessed at OD 440 nm.

Investigation of apoptosis. Apoptotic cells as assessed with the TUNEL assay were visualized under a fluorescence microscope or analyzed by flow cytometry. HUVECs were cultured to confluence on glass slides. After treatment, HUVECs were rinsed twice in PBS before fixation for 30 min at room temperature with 4% paraformaldehyde. Next, the slides were washed in PBS before incubation in the prepared solution (0.1% Triton X-100, 0.1% sodium citrate) for 5 min. The slides were then incubated with 100 μl TUNEL reaction mixture in a humidified atmosphere for 1 h at 37°C in the dark, washed in PBS, and counterstained with propidium iodide. Active caspase-3 levels were detected by flow cytometry using a commercial fluorescein active caspase kit (BioVisionK105-100).

Measurement of ROS production. The effect of DTX on ROS formation in HUVECs was investigated by DHE. Confluent HUVECs (10^4 cells/well) in 96-well plates were preincubated with various concentrations of DTX for 24 h. The cells were incubated with 10μM DHE for 1 h after the DTX treatment. Fluorescence intensity was assessed with a fluorescent microplate reader (Labsystem, California) that had been calibrated at 540 nm excitation and 590 nm emission.

Measurement of GSH activity. Endothelial cells were washed twice with cold PBS and collected in 20mM potassium phosphate buffer (pH 7.0). Cell homogenates were centrifuged at 10 000 × g for 20 min at 4°C. The resulting supernatant was used as the cell lysate. The protein content of the cell lysate was determined using the Coomassie Plus Protein Assay Reagent Kit. Briefly, 150 μl 5% TCA was added to 150 μl cell lysates, and they were centrifuged at 5000 × g for 10 min at 4°C. Cell lysates were added to 0.4 M Tris buffer (with 0.02 M EDTA) and 0.01 M DTNB. After vortexing, the mixture was incubated at room temperature for 5 min, and cellular GSH levels were identified at 412 nm. Enzyme activity was converted to nmol.

Western blotting. Radio-Immunoprecipitation Assay (RIPA) (obtained from Millipore) was used to extract total lysate. A cellular membrane fraction was prepared with Mem-PER (Pierce) according to the manufacturer's instructions. Proteins were transferred to polyvinylidene difluoride membranes after the proteins were separated by electrophoresis on a SDS-polyacrylamide gel electrophoresis. The membranes were blocked in blocking buffer for 1 h at 37°C. Then, they were incubated with primary antibodies overnight at 4°C followed by the hybridization with HRP-conjugated secondary antibodies for 1 h. Intensities were quantified by using densitometric analysis.

Transfection with small-interfering RNA. ON-TARGET plus SMART pool small-interfering RNAs (siRNAs) for si-Controls were obtained from Dharmacon Research (Lafayette, Colorado). si-PKCβ was purchased from Santa Cruz. Transient transfection was performed using INTERFERin siRNA transfection reagent (Polyplus Transfection, Huntingdon, UK) according to the manufacturer's guide.

Assessment of mitochondrial membrane potential. The lipophilic cationic probe fluorochrome 5,58,6,68-tetraethylbenzimidazolcarbocyanineiodide (JC-1) was selected to confirm the effect of DTX on mitochondrial membrane potential (ΔΨm). JC-1 exists either as a green fluorescent monomer at depolarized membrane potentials or as a red fluorescent J-aggregate at hyperpolarized membrane potentials. After exposing the cells to DTX for 24 h, they were rinsed with PBS and JC-1 (5μM) and were then

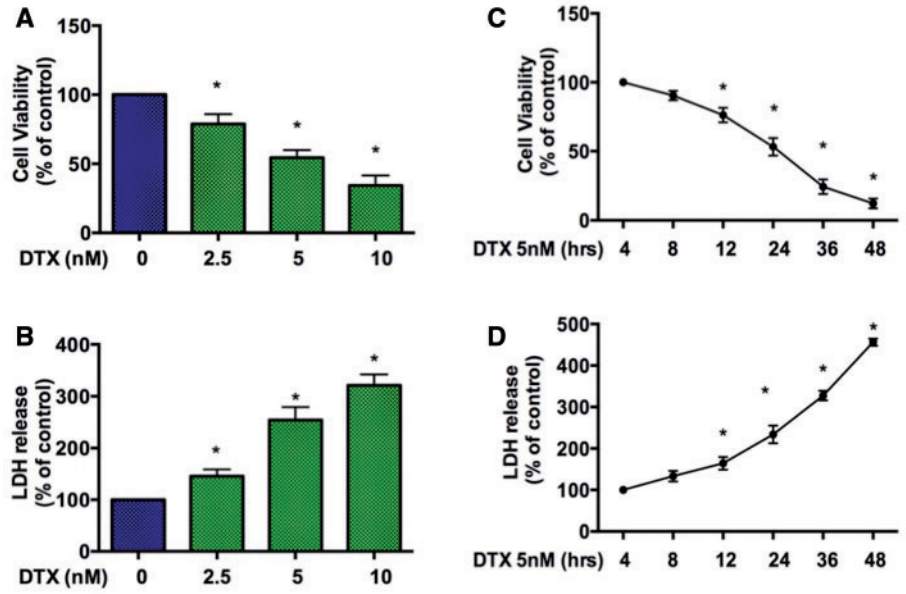

FIG. 1. DTX induces endothelial cell death. HUVECs were treated with DTX for 24 h. A, Viability was assessed via MTT assay. B, Cytotoxicity was assessed with an LDH assay. HUVECs were exposed to 5nM DTX and treated for 0–48 h. C, Viability was assessed via MTT assay. D, Cytotoxicity was assessed with an LDH assay. The data are expressed as the mean ± SD of 3 independent analyses. *$P < .05$ versus untreated control.

incubated. After 30 min of incubation at 37°C, cells were then assessed with fluorescence microscopy.

NADPH oxidase activity assay. The lucigenin method was used to determine NADPH oxidase activity in HUVECs. The crude membrane fraction of HUVECs was obtained as described above. Total protein concentration was adjusted to 1 mg/ml. An aliquot 200 μl protein (100 μg) was incubated in the presence of 5 μl of lucigenin and 100μM NADPH. Luminescence was assessed after 10 min by a plate reader (VICTOR3; Perkin-Elmer) to determine the relative changes in NADPH oxidase activity.

Statistical analyses. The results are expressed as the means ± SD. Statistical analyses were performed using 1-way or 2-way ANOVA following by Tukey's post hoc test, as appropriate. The significance level was P value < 0.05.

RESULTS

DTX Induces Cell Death in HUVECs

To test whether DTX reduces cell viability, we used the MTT assay to present our evidence. In Figure 1A, we first confirmed that the DTX 24-h treatment impaired cell viability in a dosage-dependent manner (all $P < 0.05$). DTX-induced endothelial necrosis was also investigated using the LDH assay. As shown in Figure 1B, DTX stimulation for 24 h increased LDH release followed by a concentration-dependent result (all $P < 0.05$). Our data (Figs. 1C and 1D) suggested that DTX reduced endothelial cell viability and promoted endothelial necrosis in a time-dependent manner. DTX (5nM) reduced HUVEC viability by half during the 24-h treatment. However, more than 90% viability was inhibited by the 48-h treatment.

DTX Causes Apoptosis and DNA Damage in HUVECs

To evaluate whether DTX causes endothelial apoptosis, activated caspase 3 was assessed using flow cytometry. As shown in Figure 2A, DTX (2.5–10nM) promoted caspase 3 activity after 24 h of treatment. Furthermore, the TUNEL assay suggested that DTX causes endothelial DNA damage in a concentration-dependent manner (all $P < 0.05$). This finding was further confirmed by fluorescence microscopy (Fig. 2C).

DTX Promotes PKCβ Phosphorylation and NADPH Oxidase Activity

PKCβ activation is important in endothelial dysfunction (Tsai *et al.*, 2012a). We therefore focused on confirming whether DTX increases PKCβ phosphorylation. As shown in Figures 3A and 3B, after the 24-h treatment, DTX enhanced PKCβ phosphorylation in a dosage-dependent manner as assessed by Western blotting ($P < 0.05$). PKCβ was a main agonist of endothelial NADPH oxidase (Tsai *et al.*, 2014). Therefore, we sought to investigate whether DTX treatment increased NADPH oxidase activity. We determined that DTX not only promoted p47phox and p67phox translocation into the membrane fraction (Figs. 3C and 3D) but also enhanced NADPH oxidase activity (Fig. 3E). The results, which originated from the data, indicated that oxidative stress may be involved in DTX-mediated endothelial apoptosis. We also demonstrated that silencing of PKCβ function by siRNA reduced DTX-induced NADPH oxidase activity, indicating that DTX caused NADPH oxidase activation via PKCβ activation.

DTX Facilitates ROS Formation and Mitochondria Dysfunction

To confirm whether the observed apoptotic induction of DTX was modulated by increased oxidative stress, we assessed intracellular ROS concentrations after exposure of HUVECs to DTX for 24 h. As shown in Figure 4A, we determined that DTX facilitated ROS generation by DTX treatment. Specific inhibitors were used to confirm the ROS generators. We determined that NADPH oxidase inhibitors (apocynin) and ROS scavenger (DPI) but not xanthine oxidase (oxypurinol) reduced DTX-caused ROS formation, and LY333535 also inhibited DTX-induced ROS generation. This finding suggested that DTX-mediated ROS formation occurs through the modulation of PKCβ/NADPH oxidase. ROS is the principal mediator to open the mitochondria permeability transition pore, thereby inducing apoptotic responses.

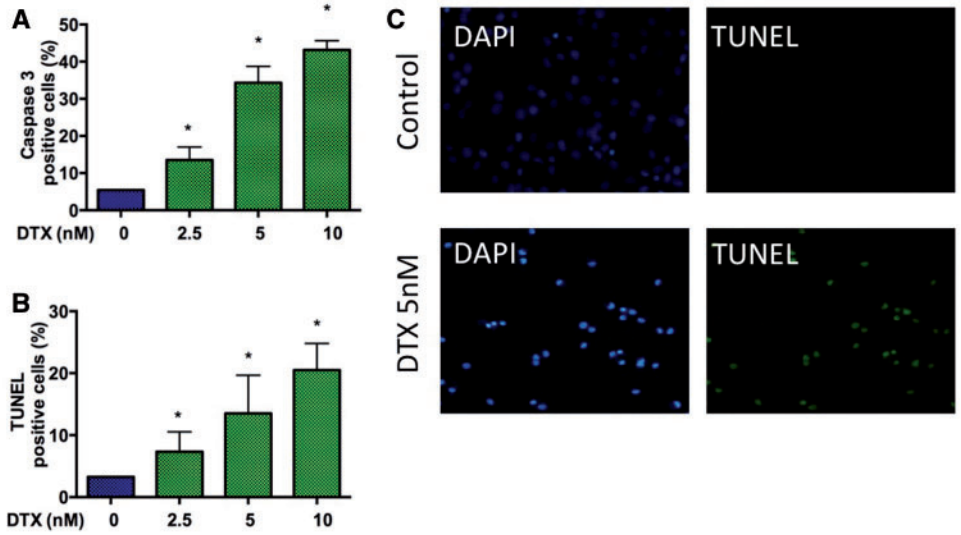

FIG. 2. DTX induces endothelial apoptosis. HUVECs were treated with DTX for 24 h. A, DTX-induced caspase 3 was assessed by flow cytometry. TUNEL-positive cells were assessed by flow cytometry (B) and fluorescent microscopy (C). The data are expressed as the mean ± SD of 3 independent analyses. *$P < .05$ versus untreated control.

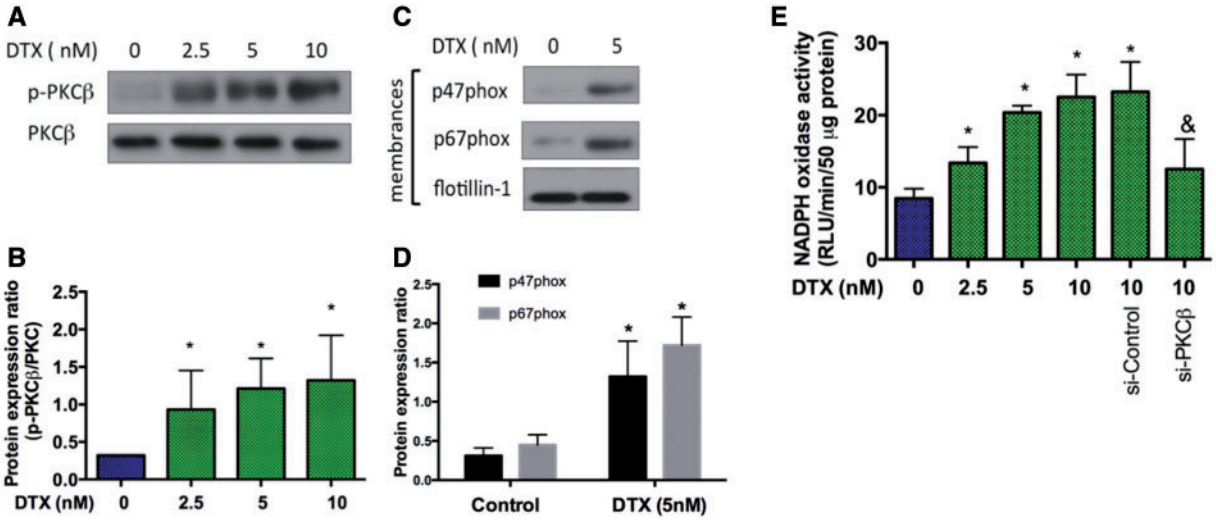

FIG. 3. DTX induces PKCβ and NADPH oxidase activation in HUVECs. HUVECs were treated with DTX (2.5–10nM) for 24 h to study PKCβ expression levels. A–B, p-PKCβ and PKCβ protein expression levels were assessed by Western blotting. C–D, DTX induces p47phox and p67phox membrane expression as assessed by Western blotting. Membrane protein levels were normalized to flotillin-1 levels. E, DTX-induced NADPH oxidase activity was assessed with an NADPH oxidase activity assay. The data are expressed as the mean ± SD of 3 independent analyses. *$P < .05$ versus untreated control.

Because of both electrochemical gradient dysregulation that was induced via pore initiation and outer mitochondrial membrane damage, the mitochondrial membrane potential (Ψm) suddenly collapses (Tsai *et al.*, 2012b). We assessed mitochondrial permeability to clarify whether DTX impairs mitochondrial stability after DTX exposure. As shown in Figure 4B, we determined that ROS concentrations were largely increased after DTX treatment for 8 and 12 h; however, DTX treatment for 8 and 12 h did not cause the depolarization of the mitochondrial membrane potential in HUVECs. As shown in Figure 4C, DTX treatment for 24 h induced mitochondrial membrane potential depolarization, indicating that ROS is the upstream regulator to induce mitochondrial dysfunction. Furthermore, LY333535 (a PKCβ inhibitor) and apocynin (a NADPH oxidase inhibitor) protected against DTX-induced depolarization of the mitochondrial membrane potential. This indicates that DTX mediated the mitochondrial membrane potential depolarization and endothelial dysfunction mainly through the modulation of PKCβ/NADPH oxidase/ROS.

GSH Expression Is Not Involved in DTX-Mediated Endothelial Dysfunction

GSH has a key role in preventing cells from oxidative injuries via its antioxidant ability. GSH inhibition causes cellular oxidative damage (Shi *et al.*, forthcoming). Following this, we examined whether GSH expression is involved in DTX-mediated endothelial dysfunction. Thus, we observed intracellular GSH concentrations after DTX stimulation. As shown in Figure 5A,

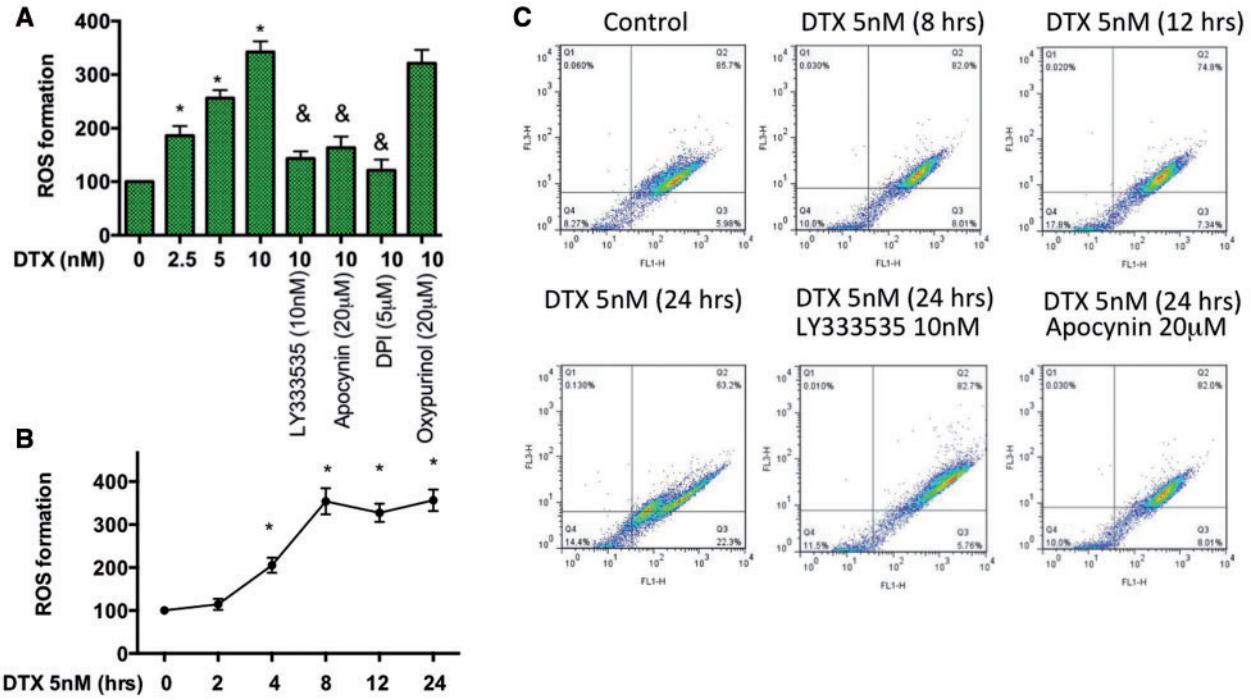

FIG. 4. DTX treatment triggered intracellular oxidative stress and mitochondrial dysfunction. A, DHE was used to measure intracellular ROS concentration. Fluorescent cell intensity was measured with a fluorescent microplate reader. In some cases, LY333535, apocynin, DPI, or oxypurinol were added before DTX exposure. B, ROS levels were observed at different time points (0–24 h). C, The influence of DTH on mitochondrial trans-membrane permeability transition ΔΨm was inspected with the signal from monomeric and J-aggregate JC-1 fluorescence as described in the Materials and Methods section. In some cases, LY333535 or apocynin was added before DTX exposure. The data are expressed as the mean ± SD of 3 independent analyses. *P < .05 versus untreated control.

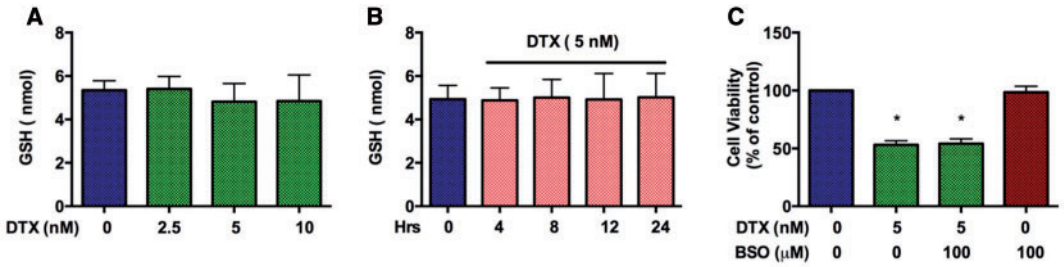

FIG. 5. Effects of GSH on DTX-treated HUVECs. A, After treatment with DTX for 24 h, intracellular GSH levels were detected using a GSH assay kit. B, Intracellular GSH levels were detected at the indicated times after DTX 5nM treatment. C, GSH levels in BSO-treated HUVECs were confirmed using the GSH assay kit. The data are expressed as the mean ± SD of 3 independent analyses. *P < .05 versus untreated control.

endothelial GSH levels were not reduced by DTX (2.5–10nM) treatment for 24 h. We further assessed whether GSH levels were decreased by time-dosage exposure. Figure 5B also shows that the GSH levels did not change at any time points with DTX treatment (5nM). As shown in Figure 5C, Buthionine sulphoximine (BSO), a GSH synthesis inhibitor, was used to inhibit GSH activity. As shown in Figure 5C, as expected, BSO did not promote DTX-impaired cell viability. Therefore, GSH function is not involved in DTX-induced endothelial dysfunction.

Oxidative Stress Is Involved in DTX-Induced Endothelial Apoptosis

To emphasize the DTX-induced endothelial dysfunction by promoting oxidative stress, we assessed the inhibitory effects of glutathione (GSH), LY333535, and apocynin on DTX-induced endothelial apoptosis. Our data revealed that GSH protected against DTX-activated caspase 3 (Fig. 6A) and DNA damage (Fig. 6B) using flow cytometry. Consequently, this result indicated that DTX promoted endothelial apoptosis largely via the upregulation of oxidative stress but was GSH independent. As expected, LY333535 and apocynin inhibited the DTX-mediated endothelial apoptosis, which suggested that oxidative stress might be facilitated by the PKCβ/NADPH-oxidase axis. To examine this hypothesis, siRNA was used to impair PKCβ function. We then found that silencing the PKCβ expression reduced both the DTX-mediated endothelial apoptosis (Fig. 6C) and DNA damage (Fig. 6B). Thus, this supports that the modulation of PKCβ function plays a major role in DTX-mediated endothelial responses.

Sotrastaurin (AEB-071) Reduces DTX-Triggered Endothelial Dysfunction

Sotrastaurin (AEB-071) is a new PKCβ inhibitor, which reduces post-transplantational injuries (Fuller *et al.*, 2012). The clinical trial report suggested that AEB-071 is beneficial for kidney

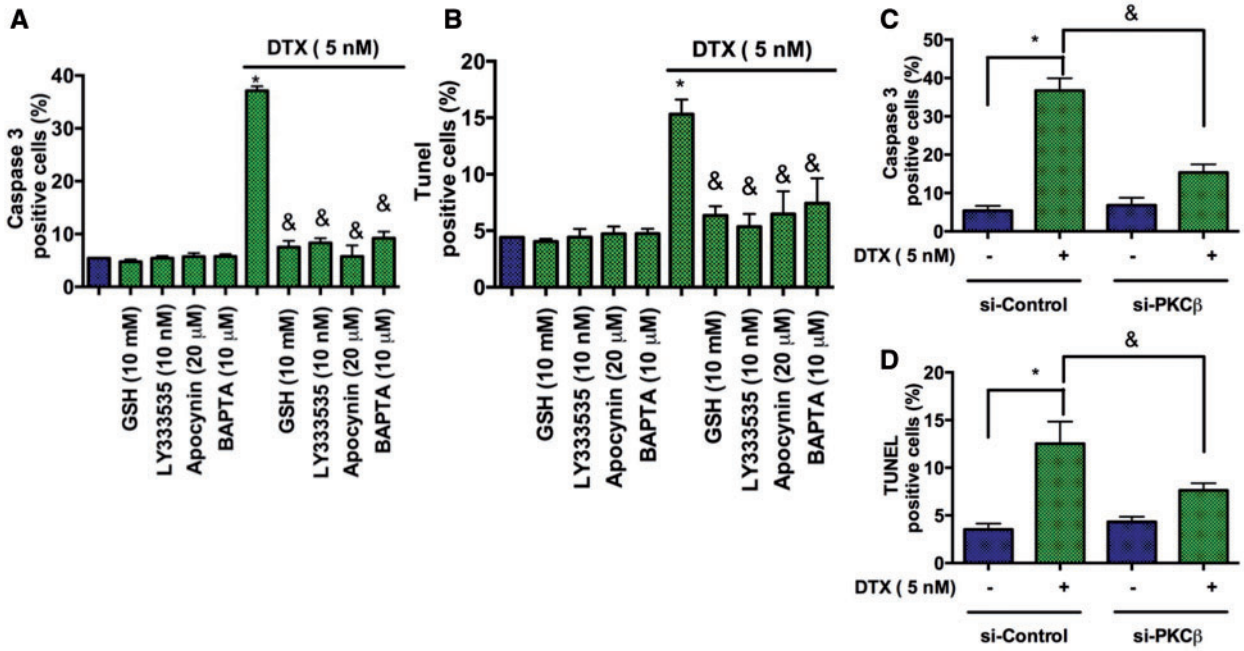

FIG. 6. DTX-induced endothelial dysfunction through the modulation of PKCβ/NADPH oxidase/ROS mechanism. HUVECs were treated with 5nM DTX for 24 h to cause endothelial damage. In some cases, GSH, LY333535, and apocynin were added before DTX stimulation. Apoptotic responses were assessed by (A) caspase 3 activity and (B) TUNEL assay. PKCβ siRNA was used to silence PKCβ expression. The results indicated that PKCβ-silencing mitigated DTX-mediated caspase 3 activation (C) and TUNEL-positive cells (D). The data are expressed as the mean ± SD of 3 independent analyses.*$P < 0.05$ compared with control HUVECs; $P < 0.05$ compared with DTX-treated HUVECs.

transplantation (Russ *et al.*, 2013). Furthermore, a previous study reported that AEB-071 mitigated proinflammatory responses through PKC inhibition (He *et al.*, 2014). However, it remains unclear whether AEB-071 can reduce DTX-induced endothelial dysfunction. To examine this issue, we first assessed AEB-071 cytotoxicity in HUVECs, and the resulting data suggested that AEB-071 did not have cytotoxicity until 100μM (data not shown). We also revealed that AEB-071 mitigated DTX-induced PKCβ phosphorylation at 500nM (Figs. 7A and 7B). With AEB-071 pretreatment, this also led to the inhibitory effects on DTX-increased NADPH oxidase activity (Fig. 7C) and ROS production (Fig. 7E). Finally, we demonstrated that AEB-071 attenuated DTX-mediated endothelial apoptosis (Fig. 7E) and DNA damage (Fig. 7F).

DISCUSSION

As a clinical treatment, DTX has been extensively selected as a chemotherapeutic drug for the treatment of a variety of cancers. It is a modified medication from the taxane family and is often selected as a second-line approach after treatment failure since 1990 (Ettinger, 1995). Most importantly, the main mechanism of DTX is to repress cancer cell mitosis (Mediavilla-Varela *et al.*, 2009). However, side effects of DTX have been reported by previous studies such as mucocutaneous adverse reactions or local irritations (Susser *et al.*, 1999). Consequently, these adverse reactions might reduce the efficacy of this clinical treatment. Furthermore, vascular damage, which is induced by infusion, is related to imbalance between high and low pHs or osmotic pressure of injected fluid (Kuwahara *et al.*, 1999). However, the osmotic pressure and pH of DTX are adjusted before the clinical injection (Semb *et al.*, 1998). Indeed, DTX caused vessel damage, which might contribute to direct endothelial injury from DTX exposure.

In this study, we first identified that DTX caused endothelial dysfunction and apoptosis through the upregulation of oxidative stress. Our data suggested that PKCβ phosphorylation was enhanced, and NADPH-oxidase activity was promoted by DTX treatment, thereby impairing mitochondrial function in HUVECs. Finally, we successfully demonstrated that the pharmacological inhibitor of PKCβ sotrastaurin (AEB-071) protected endothelial cells from DTX-induced oxidative injury (Fig. 8).

PKC plays a critical role in regulating stress-induced endothelial dysfunction (Xu *et al.*, 2008). Moreover, PKC facilitates NADPH oxidase in the human vascular system (Inoguchi *et al.*, 2003). The upregulated ROS concentration may be obviously repressed through PKC inhibition in the coronary arteries (Guzik *et al.*, 2006). Previous reports suggested that TNF-α induced endothelial apoptosis by upregulating PKC levels (Li *et al.*, 1999). PKC has many isoforms, and PKCβ is the most important protein to modulate endothelial dysfunction under oxidative stress. In a clinical study, a specific PKCβ2 inhibitor revealed therapeutic outcomes in patients with diabetes mellitus (DM) (Das Evcimen and King, 2007). Experimental studies also confirmed that PKCβ upregulation promoted TNF-α-induced oxidative injury in endothelial cells (Wang *et al.*, 2009). In this study, we suggest that DTX caused endothelial apoptosis and cell death (Figs. 1 and 2). We also demonstrated DTX-increased PKCβ phosphorylation in a dosage-dependent manner (Figs. 2A and 2B), thereby silencing of PKCβ function by siRNA or another inhibitor that reversed the DTX-induced endothelial dysfunction (Fig. 6). These data suggested that PKCβ plays a vital role in DTX-induced endothelial apoptosis.

At this level, p47phox and p67phox are the most critical molecules for NADPH oxidase activity. Yaw L. Siow *et al.* demonstrated that PKCβ inhibition reduced homocysteine-mediated increases in p47phox and p67phox phosphorylation as well as NADPH oxidase activation (Siow *et al.*, 2006). NADPH oxidase-

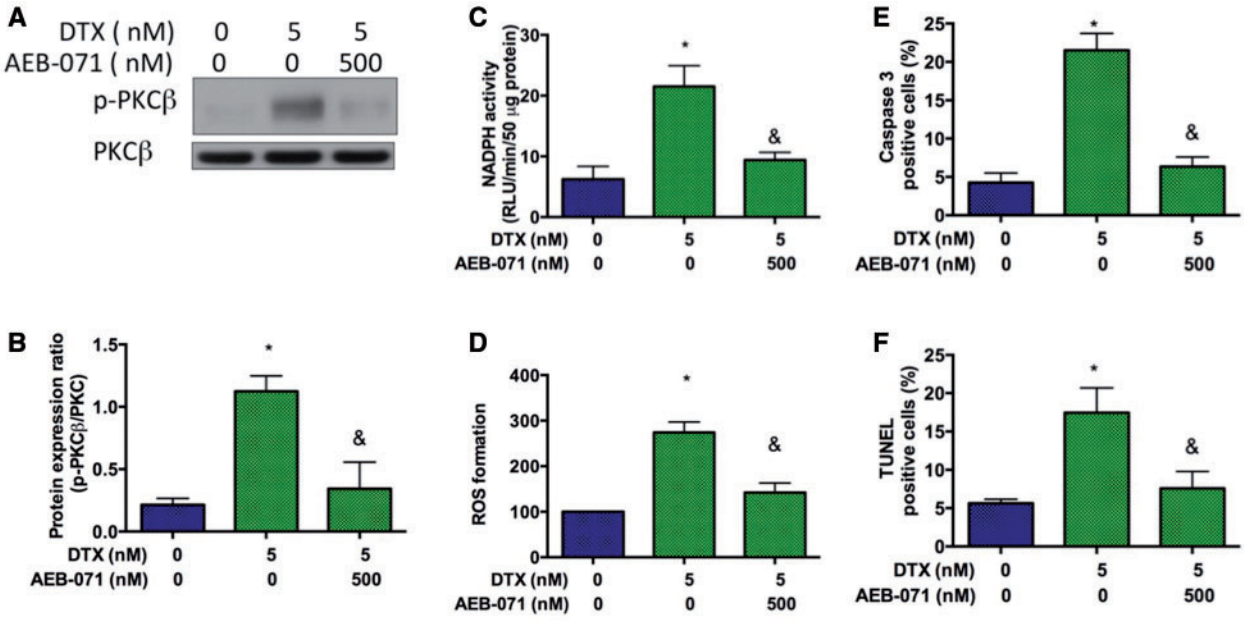

FIG. 7. Protective effects of sotrastaurin on DTX-induced endothelial dysfunction. HUVECs were incubated for 1 h with sotrastaurin (AEB-071) 500nM followed by DTX (5nM) exposure for another 24 h. A–B, p-PKCβ and PKCβ protein expression levels were assessed by Western blotting. The inhibitory effects of sotrastaurin (AEB-071) on DTX-induced NADPH oxidase activation (C) and ROS formation (D) were assessed. Sotrastaurin (AEB-071)-mediated reductions in apoptotic events were assessed with a caspase 3 activity assay (E) and TUNEL assay (F). The data are expressed as the mean ± SD of 3 independent analyses.*$P < 0.05$ compared with control HUVECs; $P < 0.05$ compared with DTX-treated HUVECs.

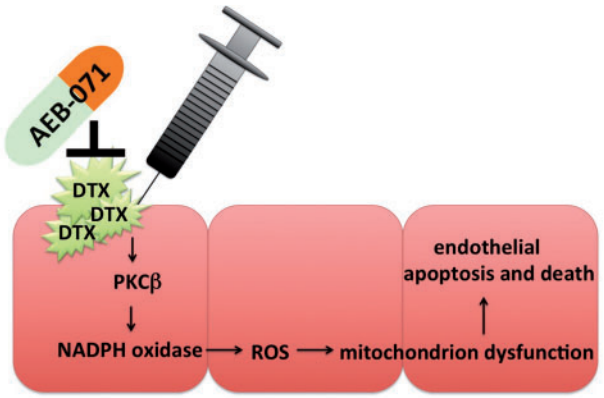

FIG. 8. Schematic demonstrating the proposed signaling of DTX-mediated endothelial dysfunction. DTX enhanced PKCβ and NADPH oxidase activities resulting in ROS generation. Upregulating oxidative stress promoted mitochondrial dysfunction, thereby causing endothelial apoptosis. The (→) indicates activation or induction, and the (⊣) indicates inhibition or blockade.

mediated ROS formation initiates the modulation of other probable intracellular ROS derivations. Additionally, NADPH oxidase-derived ROS contributes to cellular stress and enhances ROS formation, thereby providing an immortal cycle of oxidative stress (Schramm *et al.*, 2012). We revealed that DTX induced NADPH oxidase activation (Figs. 3C–E) and ROS formation (Fig. 4A). GSH, LY333535 (PKCβ inhibitor), and apocynin (NADPH oxidase inhibitor) also mitigated the DTX-induced endothelial dysfunction (Fig. 6), which indicates that the PKCβ/NADPH oxidase/ROS mechanisms followed the DTX-induced endothelial injury. This evidence is supported by a previous study; for example, Tabaczar *et al.* suggested that DTX promotes oxidative stress by analyzing blood.

NADPH oxidase-promoted ROS is a second messenger to facilitate mitochondrial dysfunction by disturbing the electron transport chain and impairing the mitochondrial membrane potential, thereby causing cellular death (Doughan *et al.*, 2008). Mitochondrial dysfunction contributes to cytochrome c translocation, which results in caspase 9 activation and other proapoptotic events (Martin and Forkert, 2004). This research suggests that DTX collapsed the mitochondrial membrane potential in HUVECs. As predicted, LY333535 and apocynin treatment prevented the DTX-impaired mitochondrial depolarization. This finding suggested that DTX impaired mitochondrial depolarization largely through PKCβ/NADPH oxidase activity. Furthermore, GSH is a critical intracellular antioxidant enzyme that has a main role to represses ROS-induced injury and control intracellular redox status (Hammond *et al.*, 2001). Thus, maintaining healthy GSH status is important for cellular survival. Formerly, a previous study suggested that vinorelbine significantly mitigated intracellular GSH expression in porcine aorta endothelial cells (PAECs) (Yamada *et al.*, 2010). The same authors published another study that suggested that epirubicin-induced PAECs apoptosis was GSH independent (Yamada *et al.*, 2012). It is possible that not every drug causing endothelial dysfunction is GSH dependent. As shown in Figure 5, we revealed that GSH levels were not affected by DTX treatment. Moreover, our study confirmed that BSO intervention did not promote DTX-induced endothelial death. GSH treatment, which reduced DTX-induced endothelial apoptosis, confirmed this finding due to the free radical scavenging ability of GSH. Indeed, DTX-mediated endothelial dysfunction might pass through increased oxidative stress but may remain GSH independent. GSH dysfunction was linked to ROS generation. A previous report suggested that intracellular GSH insufficiency damages cellular antioxidant defenses and causes ROS accumulation (Armstrong and Jones, 2002). In this study, we demonstrated that the DTX-induced increase in intracellular ROS happened before the onset

of cell death. This result suggested that ROS formation might not occur from GSH depletion.

Generally, PKCβ inhibition has been found as an effective approach to improve vascular endothelial function (Mehta *et al.*, 2009). Sotrastaurin (AEB-071) is a novel PKCβ inhibitor. It has been used to mitigate immunological responses in transplantation (Hardinger and Brennan, 2013). Some of the pharmacological effects of sotrastaurin have been identified. Sotrastaurin increases Foxp3 levels, which reduces IFNγ release and ROS formation after proinflammatory stimulation (Capsoni *et al.*, 2012; He *et al.*, 2014). This study was the first to demonstrate that sotrastaurin protects endothelial cells from DTX-induced PKCβ phosphorylation, NAPDH oxidase activation, ROS production, and cell death (Fig. 7).

In conclusion, the present study successfully illustrated that DTX causes endothelial dysfunction through the PKCβ/NADPH oxidase pathway. Moreover, we demonstrated that increased oxidative stress is involved in DTX-induced endothelial apoptosis. We also demonstrated that sotrastaurin (AEB-071), a new PKCβ inhibitor, mitigated DTX-induced oxidative injury in endothelial cells. We performed *in vitro* analyses in this study. Thus, further investigations are needed for confirmation; our future research will include *in vivo* assays.

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