trans-Cinnamaldehyde Prevents Oxidative Stress-Induced Apoptosis in V79-4 Chinese Hamster Lung Fibroblasts through the Nrf2-Mediated HO-1 Activation

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INTRODUCTION

The disruption of redox equilibrium by oxidative stress is an important step in the onset and progression of disease in most organs, including the lungs.\textsuperscript{1,2} In the body, the lungs are more susceptible to oxidative stress than other organs due to their unique location and function. Aerobic organisms, including humans, use oxygen as an electron acceptor during oxidative phosphorylation in mitochondria, and this process may be the largest source of free oxygen radicals, such as reactive oxygen species (ROS).\textsuperscript{3,4} Under physiological conditions, ROS act as appropriate intracellular signaling molecules, but excessive accumulation of ROS is known to play a vital role in activating several signaling pathways that contribute to damage and apoptosis of lung fibroblasts.\textsuperscript{5,6} Therefore, reduction of oxidative stress-induced cell damage has been recognized as a strategy for the prevention and treatment of oxidative-stress-mediated lung diseases.

\textit{trans}-Cinnamaldehyde (tCA) is a key bioactive component isolated from the stem bark of \textit{Cinnamomum cassia} Presl, of the Lauraceae family, which has traditionally been used to treat a variety of diseases such as anxiety, dyspepsia, diabetes, gastritis, ischemia, blood circulation disturbances, arrhythmia and so on.\textsuperscript{7,8} Recent studies have shown that tCA has multiple pharmacological properties, including antioxidant,\textsuperscript{9,10} anti-inflammatory,\textsuperscript{11,12} anti-cancer\textsuperscript{13,14} and inhibitory effects against various metabolic cardiovascular and cardiovascular diseases.\textsuperscript{15,16} Among them, the antioxidant activity of tCA has been primarily attributed to increased ROS scavenging activity and involves the activation of oxidative stress defense systems such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and thioredoxin (Trx) signaling pathways.\textsuperscript{12,16} However, to date, whether tCA can weaken oxidative stress-induced injury in lung fibroblasts has not been studied. Therefore, in this study, to evaluate the beneficial effect of tCA against oxidative-stress, we investigated the effect of tCA on hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-induced cytotoxicity in V79-4 Chinese hamster lung fibroblasts.

MATERIALS AND METHODS

Cell Culture and tCA Treatment V79-4 cells, a Chinese hamster lung fibroblast cell line, were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.) and cultured in Dulbecco’s modified Eagle’s medium (DMEM, WelGENE Inc., Gyeongsan, Republic of Korea) supplemented with 10% heat-inactivated fetal calf serum and antibiotics mixture (WelGENE Inc.) in a humid atmosphere of 95% air and 5% CO\textsubscript{2} and 37°C. tCA was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.), dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Chemical Co.), and diluted with cell culture medium to adjust the final treatment concentrations before use in experiments. In all experiments, the cells were treated with the indicated concentrations...
of tCA 1 h before the addition of 1 mM H₂O₂ (Sigma-Aldrich Chemical Co.) for the indicated times.

**Cell Viability Assay** Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described. Briefly, V79-4 cells were treated with various concentrations of tCA or H₂O₂ for 24 h or pretreated with tCA, N-acetyl-L-cysteine (NAC, Sigma-Aldrich Chemical Co.) or zinc protoporphyrin IX (ZnPP, Sigma-Aldrich Chemical Co.) for 1 h and then incubated with or without H₂O₂ for 24 h. Then, MTT solution (Sigma-Aldrich Chemical Co.) was added to a final concentration of 0.5 mg/mL. After 3 h incubation, the culture supernatants were carefully removed, and the formed formazan crystals were dissolved in DMSO. Finally, the optical density values were acquired with an enzyme-linked immunosorbent assay (ELISA) reader (Dynatech Laboratories, Chantilly, VA, U.S.A.) at 450 nm. The optical density of the formazan crystals formed in untreated control cells was used to represent 100% viability. In a parallel experiment, changes in cell images were captured by a phase-contrast microscope (Carl Zeiss, Oberkochen, Germany).

**Measurement of ROS Generation** To measure the amount of ROS generated in cells, cells were treated with or without tCA or NAC for 1 h before another 1 h culture in the presence of H₂O₂. The cells were washed with phosphate-buffered saline (PBS), and lysed with PBS containing 1% Triton X-100 for 10 min at 37°C. The cells were stained with 10 µM dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Eugene, OR, U.S.A.) at 37°C for 30 min in the dark. Intracellular ROS production was immediately recorded at 515 nm by a flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.). The results were expressed as the percentage increase relative to untreated cells.

**Nuclear Staining** To determine apoptosis, changes in nuclear morphology were examined using 4,6-diamidino-2-phenylindole (DAPI) staining. In Brief, cells were harvested after treatment with H₂O₂ in the absence or presence of tCA or NAC, washed with PBS, and fixed with 4% paraformaldehyde (Sigma-Aldrich Chemical Co.) in PBS for 10 min at room temperature (r.t.). The cells were washed with PBS again and stained with 2.5 µg/mL DAPI solution (Sigma-Aldrich Chemical Co.) for 10 min at r.t. The images of cell nuclei were observed via a fluorescence microscope (Carl Zeiss).

**Detection of Apoptosis by Annexin V Staining** Flow cytometry analysis was performed to quantitate the apoptotic cells by using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (R&D Systems Inc., Minneapolis, MN, U.S.A.) according to manufacturer instructions. Briefly, after treatment with H₂O₂ in the absence or presence of tCA, NAC or ZnPP, the collected cells were washed with cold PBS, fixed in 75% ethanol at 4°C for 30 min and then stained with annexin V-FITC and propidium iodide (PI) for 20 min at r.t. in the dark. Using a flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.), the fluorescence intensities of the cells were quantified as percentages of annexin V-positive and PI-negative (annexin V+/PI−) cells in the total cell populations as indicators of apoptotic cells, whereas the V+/PI+ cells in the total cell population was considered normal.

**Measurement of Mitochondrial Membrane Potential (MMP)** Mitochondrial function was determined by membrane potential-specific fluorescence staining using a sensor of mitochondrial potential, 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethyl-imidacarbocyanine iodide (JC-1). Briefly, the cells cultured on glass cover slips were treated with H₂O₂ in the absence or presence of tCA. After 24 h of treatment, the cells were incubated in a medium containing 10 µM JC-1 (Sigma-Aldrich Chemical Co.) at 37°C for 20 min in the dark according to the manufacturer’s protocol. The stained cells were washed twice with PBS and observed with a fluorescence microscope. In addition, the changes in the MMP by flow cytometry analysis. To this end, the pellets of cells treated with H₂O₂ in the presence or absence of tCA were suspended in PBS and incubated with 10 µM JC-1. The cells were then washed with PBS and cellular fluorescence intensity of JC-1 was measured using a flow cytometer.

**Western Blot Analysis** At the end of the treatment period, the cells were collected and lysed on ice for 30 min in lysis buffer as previously described. The mitochondrial and cytoplasmic protein fractions were obtained using a commercial mitochondrial fractionation kit (Active Motif, Inc., Carlsbad, CA, U.S.A.) according to manufacturer’s procedure. The protein concentration of the collected supernatants was measured using the Bradford assay reagent (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to the manufacturer’s protocol. Subsequently, equal amounts of protein from each sample were separated by sodium-dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Merck Millipore, Bedford, MA, U.S.A.). The membranes were blocked with Tris-buffered saline (10 mM Tris-Cl, pH 7.4) containing 5% skim milk and 0.5% Tween-20 for 1 h at r.t. and then incubated overnight at 4°C with primary antibodies, which were purchased from Abcam, Inc. (Cambridge, U.K.), Cell Signaling Technology (Danvers, MA, U.S.A.) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). After washing with PBS, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (and Santa Cruz Biotechnology, Inc.) for 2 h at r.t. The protein bands were detected using an enhanced chemiluminescence (ECL) detection system (R&D Systems Inc.), and the signals were visualized using a chemiluminescence imager (Azure Biosystems, Inc., Dublin, CA, U.S.A.).

**Determination of Caspase-3 and Caspase-9 Activities** The activities of caspase-3 and caspase-9 were determined by using colorimetric activity assay kits (R&D Systems Inc.) according to the manufacturer’s instructions. Briefly, the cells were incubated in a supplied lysis buffer on ice for 15 min. The supernatants were collected, and a total of 200 µg protein lysate was incubated with 5 µL of caspase-3 or caspase-9 substrates in the dark for 2 h at 37°C, according to the kit protocol. The concentrations of p-nitroanilide released from the substrates by caspase-3 and caspase-9 were calculated from the absorbance values at 405 nm. According to their concentration curve, the results of at least three independent experiments were expressed as fold change, compared with the untreated control cells.

**Heme Oxygenase (HO-1) Activity Assay** The HO-1 enzyme activity was estimated by measuring the rate of conversion to bilirubin in heme using a HO-1 ELISA kit (Abcam, Inc.). The cells were lysed with the extraction reagent included in the kit in accordance with the manufacturer’s instructions. After quantifying the protein concentration, the cell lysates
were incubated with the reaction mixtures at 23 °C for 30 min, whereas the blank samples were incubated with hemin alone. The amount of bilirubin formed was calculated according to the difference in the absorbance between the 464 and 530 nm wavelengths using an ELISA reader. The HO-1 activity was determined as picomoles of bilirubin per milligram of protein based on the standard bilirubin curve.

Statistical Analysis Results are expressed as the mean ± standard deviation (S.D.) of at least three independent experiments. Statistical analyses were performed using the SPSS software, version 16.0 (SPSS Inc., Chicago, IL, U.S.A.). Significance was analyzed by one-way ANOVA. A value of p < 0.05 was considered to indicate a significant difference.

RESULTS

1tCA Inhibited H$_2$O$_2$-Induced Cytotoxicity in V79-4 Cells

To examine the ability of tCA to protect V79-4 cells against oxidative stress using H$_2$O$_2$, the effect of tCA on the survival rate of V79-4 cells was first investigated using an MTT assay. Figure 1A shows that in the cells treated with tCA at concentrations below 30μM, there was no significant difference in the cell viability compared with the control, but treatment with tCA at concentration over 40μM significantly decreased the viability of the V79-4 cells. Therefore, to study the cytoprotective effects of tCA against H$_2$O$_2$-induced cytotoxicity, a 30μM concentration of tCA was chosen. The concentration of H$_2$O$_2$ for inducing cytotoxicity was selected to be 1 mM with a survival rate of about 60%, compared with the vehicle group (Fig. 1B). Subsequently, to assess the protective effect of tCA, the cells were pretreated with 30μM tCA for 1 h, followed by 1 mM H$_2$O$_2$ for 24 h, and it was found that tCA significantly inhibited the H$_2$O$_2$-mediated reduction in V79-4 cell viability (Fig. 1B). It was also found that pretreatment with NAC, a well-established ROS scavenger, completely inhibited H$_2$O$_2$-induced cytotoxicity when compared with the controls (Fig. 1B). In addition, the morphological changes in the V79-4 cells treated with H$_2$O$_2$ alone were alleviated by pretreatment with tCA or NAC (Fig. 1C).

1tCA Reduced H$_2$O$_2$-Induced ROS Generation in V79-4 Cells

Since the promotion of ROS production by H$_2$O$_2$ is the most widely accepted mechanism for inducing oxidative stress, I next investigated whether tCA abolished H$_2$O$_2$-induced ROS accumulation using a fluorescent probe, DCF-DA. According to the results of flow cytometry, the production of ROS in H$_2$O$_2$-treated V79-4 cells peaked within 1 h and gradually decreased over time (data not shown). However, pretreatment with tCA significantly reduced the effect of H$_2$O$_2$ on ROS overproduction, and NAC also almost completely eliminated the accumulation of ROS (Figs. 2A, B), demonstrating that tCA has potent ROS scavenging activity.

1tCA Protected H$_2$O$_2$-Induced Apoptosis in V79-4 Cells

To analyze the ability of tCA to prevent H$_2$O$_2$-induced apoptosis, DAPI and annexin V-FITC/PI double staining assays were used. As shown in Fig. 2C, the nuclei were round-shaped with homogeneous fluorescence intensity in the control group and tCA alone treated group. However, H$_2$O$_2$-treated cells exhibited typical apoptotic morphology characterized by nuclear condensation and fragmentation, whereas cells pretreated with tCA or NAC prior to H$_2$O$_2$ exposure had significantly decreased the apoptotic features. Consistent with DAPI staining, flow cytometry analysis using annexin V/PI staining showed that H$_2$O$_2$ triggered a higher magnitude of apoptosis compared with controls. However, after supplementation with tCA or NAC, the percentage of apoptotic cells significantly decreased (Fig. 2D).

1tCA Alleviated H$_2$O$_2$-Induced Mitochondrial Dysfunction in V79-4 Cells

To validate whether inhibition of mitochondrial impairment is a mechanism involved in the protective effect of tCA, JC-1 dye was used to estimate the MMP. According to the results of fluorescence microscopic observation shown in Fig. 3A, in H$_2$O$_2$-treated cells, JC-1 did not accumulate in the mitochondria and was dispersed throughout the cells to show green fluorescence (JC-1 monomer). However, in the presence of tCA, the intensity of red fluorescence (JC-1 aggregate) was observed as high as that of the control group, which means that tCA prevented changes to JC-1 monomers. In addition, MMP was quantitatively investigated using flow cytometry, and it was found that the ratio of JC-1 ag-
gregates/JC-1 monomers was also significantly reduced after H$_2$O$_2$ treatment compared with the untreated group (Fig. 3B), indicating that H$_2$O$_2$ reduced the MMP. However, pretreatment with tCA markedly reversed these changes.

**tCA Reversed the Cytosolic Release of Cytochrome c and Decreased Ratio of Bcl-2/Bax Induced by H$_2$O$_2$ in V79-4 Cells** The release of cytochrome c into the cytoplasm to initiate mitochondria-mediated apoptosis is linked to the decline of MMP, and the Bcl-2 family proteins are important regulators of MMP. Therefore, the levels of cytochrome c expression in both mitochondria and cytoplasm were first compared using Western blot analysis. As shown in Fig. 4A, the expression of cytochrome c in H$_2$O$_2$-treated cells decreased in the mitochondrial fraction and increased in the cytoplasmic...
fraction (Fig. 4A), indicating that cytochrome c was released from the mitochondria to the cytosol. It was also observed that H2O2 treatment induced downregulation of anti-apoptotic Bcl-2, while pro-apoptotic Bax was upregulated (Fig. 4B). However, the amount of released cytochrome c by H2O2 in cells preincubated with tCA almost returned to the control normal levels, and so did the decreased Bcl-2/Bax ratio, confirming that tCA protected V79-4 cells against H2O2-induced mitochondrial dysfunction.

tCA Prevented H2O2-Induced Activation of Caspases in V79-4 Cells Next, it was investigated whether the inactivation of caspase cascade was involved in the anti-apoptotic effect of tCA in H2O2-treated V79-4 cells. According to the current results, the expression of pro-caspase-9 and pro-caspase-3 was markedly decreased in cells treated with H2O2 alone compared with the control group, and their activity was significantly increased in the results using the colorimetric assay kits (Figs. 4C, D). The degradation of poly(ADP-ribose) polymerase (PARP) was also observed in H2O2-treated cells (Fig. 4C). However, pretreatment with tCA reversed these changes, demonstrating that inhibition of H2O2-induced caspase activity by tCA contributed to the prevention of apoptosis.

tCA Activated the Nrf2/HO-1 Signaling Pathway in V79-4 Cells It was further examined whether the antioxidant activity of tCA was correlated with the activation of the Nrf2/HO-1 signaling pathway. The immunoblotting results indicated that the expression of Nrf2 and its phosphorylation (p-Nrf2) were slightly increased in the cells treated with H2O2 alone compared with untreated control, but their expression greatly increased in the cells co-treated with H2O2 and tCA (Fig. 5A). In addition, the expression of the HO-1 protein was similarly upregulated, and the HO-1 activity was also significantly increased in the cells treated with both H2O2 and tCA compared with the cells treated with H2O2 alone or control (Figs. 5A, B). In contrast, the expression of Kelch-like ECH-associated protein-1 (Keap1), a negative regulator of Nrf2, was relatively reduced in the cells treated with tCA and H2O2, indicating that the Nrf2/HO-1 signaling pathway was activated by tCA treatment. Additionally, I have confirmed whether the levels of Nrf2/HO-1 according to time after exposure to tCA. As shown in supplementary data, I found that the expression of Nrf2 and HO-1 was gradually increased at 30 min after exposure to tCA. Furthermore, this increasing was markedly upregulated over 6 h after exposure to tCA. This result suggested that tCA have a potential antioxidant capacity.

Nrf2/HO-1 Signaling Pathway Was Involved in the Mitigation of H2O2-Mediated Apoptosis by tCA in V79-4 Cells Finally, I investigated whether the activation of the Nrf2/HO-1 signaling pathway was directly related to the antioxidant and anti-apoptotic effects of tCA in V79-4 cells. As shown in Figs. 5C and D, when the HO-1 activity was blocked by ZnPP, a potent competitive inhibitor of HO-1, the inhibition of the intracellular ROS production by tCA in H2O2-treated cells was clearly reversed, indicating that tCA exerted an antioxidant effect by activating Nrf2-mediated HO-1. In parallel, flow cytometry analysis showed the anti-apoptotic effect of tCA in H2O2-stimulated cells was markedly reduced by treatment with ZnPP (Fig. 6A). Along with these results, the protective effect of tCA on H2O2-induced reduction of cell viability was significantly lost by ZnPP (Fig. 6B).

DISCUSSION

In the current study, I investigated whether tCA can protect
V79-4 lung fibroblasts from oxidative stress. For this purpose, oxidative damage was induced using H$_2$O$_2$, which is widely used as a representative ROS for establishing various oxidative stress models, and I found that H$_2$O$_2$ induced cytotoxicity by triggering apoptosis through the accumulation of ROS. However, tCA was found to have the ability to revise H$_2$O$_2$-induced apoptosis, while having ROS scavenging activity, and these functions were associated with the activation of the Nrf2/HO-1 signaling pathway.

Apoptosis can generally be divided into the extrinsic and intrinsic pathways. The extrinsic pathway is initiated by extracellular ligands that bind to death receptors on the cell surface, and the intrinsic pathway is associated with intracellular apoptotic signals that cause mitochondrial dysfunction. Among the organelles in cells, mitochondria are most susceptible to excessive H$_2$O$_2$ insults, and their dysfunction greatly contributes to ROS production. ROS overaccumulation triggers free radical attack of the mitochondrial phospholipid bilayer, which leads to depolarization of the mitochondrial membrane, resulting in MMP loss. During this process, the permeability of the mitochondrial membranes increases, allowing apoptogenic factors in the mitochondrial intermembrane space to be released into the cytoplasm. Therefore, the loss of the MMP and the cytosolic release of cytochrome c are indicative of mitochondrial dysfunction and are evident early phenomena in the onset of intrinsic apoptosis pathway. In the current study, it was found that the loss of the MMP and cytosolic cytochrome c expression was markedly increased in H$_2$O$_2$-treated V79-4 cells. However, tCA pretreatment protected the reduction of the MMP induced by H$_2$O$_2$ and maintained the expression of cytochrome c in mitochondria during H$_2$O$_2$ exposure, demonstrating that tCA can inhibit the mitochondrial damage caused by H$_2$O$_2$.

Cytochrome c released into the cytoplasm interacts with and activates caspase-9, which in turn activates the downstream effector caspases such as caspase-3 and caspase-7 to complete apoptosis. This process is accompanied by the degradation of the substrate proteins of the effector caspases, including PARP, as evidenced by caspase-dependent apoptosis. The activation of this caspase cascade is also tightly regulated by the expression of a variety of regulators. Among them, the Bcl-2 family proteins, which are consists of members that either inhibit or promote apoptosis, play an important role in regulating apoptosis by governing mitochondrial outer membrane permeabilization. Anti-apoptotic proteins such as Bcl-2 are essential for maintaining mitochondrial permeability and membrane barrier stabilization. Conversely, pro-apoptotic proteins such as Bax promote mitochondrial permeability transition or attenuate the barrier function of the mitochondrial outer membrane, leading to release of apoptotic factors. Therefore, the balance between anti-apoptotic Bcl-2 member and pro-apoptotic Bax member proteins acts as a determinant inducing the activation of the caspase cascade upon initiation of the native apoptosis pathway. In this study, the Bcl-2/Bax expression ratio was decreased in H$_2$O$_2$-treated V79-4 cells, and the activation of caspase-9 and caspase-3...
response genes. Of the key inducible phase II enzymes, Nrf2 binds to its negative regulator, Keap1, in the cytoplasm and constitutively degrades through the ubiquitin proteasome system. When cells are exposed to oxidative stress, Nrf2 is constitutively degraded through the ubiquitin proteasome system. When cells are exposed to oxidative stress, Nrf2 is constitutively degraded through the ubiquitin proteasome system. Under homeostatic conditions, Nrf2 is degraded by activated effector caspases, indicating that tCA can protect V79-4 cells from oxidative stress.

The cells were treated with 30 µM tCA or 10 µM ZnPP for 1 h and then treated with or without 1 mM H$_2$O$_2$ for an additional 24 h. (A) The cells were stained with annexin V-FITC and PI for flow cytometry analysis. The percentages of apoptotic cells were determined by counting the percentages of annexin V-positive cells. (B) The cell viability was determined by an MTT assay. The results are expressed as the mean ± S.D. of three independent experiments (*p < 0.05 compared with the control group; #p < 0.05 compared with the H$_2$O$_2$-treated group).

and degradation of PARP, a representative substrate protein degraded by activated effector caspases, were increased. However, these changes were markedly inhibited in the presence of tCA, indicating that tCA can protect V79-4 cells from apoptosis by preventing the intrinsic apoptosis pathway activated by H$_2$O$_2$.

Accumulated studies have shown that Nrf2 is one of the major transcription factors involved in the protection of cells from oxidative stress. Under homeostatic conditions, Nrf2 binds to its negative regulator, Keap1, in the cytoplasm and constitutively degrades through the ubiquitin proteasome system. When cells are exposed to oxidative stress, Nrf2 is released from Keap1 and translocated into the nucleus to promote the transcription of antioxidant response element (ARE) response genes. Of the key inducible phase II enzymes regulated by ARE, HO-1 breaks down heme into free iron, carbon monoxide, and biliverdin. Because biliverdin is further degraded to bilirubin, which has strong antioxidant properties, HO-1 plays a potentially important role in antioxidant defense and iron homeostasis. Recently, tCA has been shown to improve memory impairment by preventing neuroinflammation, apoptosis, and amyloid protein burden through the modulation of the Nrf2 antioxidant defense system. In addition, tCA has been reported to activate Nrf2-mediated upregulation of phase II enzymes, including Trx reductase, to protect against oxidative injury. Based on these findings, it was investigated whether the activation of the Nrf2/HO-1 signaling pathway was involved in the antioxidant efficacy of tCA, and found that tCA remarkably increased the expression of the phosphorylated Nrf2 protein in the presence of H$_2$O$_2$. Moreover, the expression of HO-1 and its activity increased under the same conditions, and Keap1 expression was downregulated, indicating that Nrf2 was activated in the tCA-treated V79-4 cells under oxidative conditions. Therefore, we used ZnPP, a HO-1 inhibitor, to further elucidate the role of the Nrf2/HO-1 signaling pathway in the antioxidant effects of tCA and found that the ROS scavenging ability of tCA was eliminated by the presence of ZnPP. Concomitant with the results, ZnPP significantly reversed the cytoprotective activity of tCA against H$_2$O$_2$. These results demonstrate that the activation of the Nrf2/HO-1 signaling pathway may act as an upstream signal of the protective potential of tCA against H$_2$O$_2$-induced cytotoxicity in V79-4 cells.

In summary, in this study, the protective effect of tCA on H$_2$O$_2$-induced oxidative stress in V79-4 lung fibroblasts was evaluated. According to our results, tCA reversed the increased intracellular ROS production and mitochondrial damage caused by H$_2$O$_2$, eventually inhibiting apoptosis. tCA also activated Nrf2 and promoted the expression and activity of its downstream target protein HO-1, which may have contributed to alleviating oxidative stress. Although this is the first study to demonstrate that tCA can relieve H$_2$O$_2$-induced oxidative stress in lung fibroblasts by enhancing antioxidant capacity through activation of the Nrf2/HO-1 signaling pathway, further studies are required to assess how tCA can regulate the transcriptional activity of Nrf2 and whether other signaling pathways can participate in the antioxidant activity of tCA.

Conflict of Interest The author declares no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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