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Cytoprotective effects of CSTMP, a novel stilbene derivative, against H_2O_2-induced oxidative stress in human endothelial cells

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Abstract:
A novel stilbene derivative, (E)-2-(2-chlorostyryl)-3,5,6-trimethylpyrazine (CSTMP), was designed and synthesized based on the pharmacophores of tetramethylpyrazine (TMP) and resveratrol (RES). In the present study, we investigated the protective effects of CSTMP on vascular endothelial cells under oxidative stress and elucidated its molecular mechanisms. The radical scavenging activity of CSTMP was assessed by the DPPH test. Human Umbilical Vein Endothelial Cells (HUVECs) were exposed to 150 μM hydrogen peroxide (H_2O_2) for 12 h, resulting in a decrease of cell viability assessed by the MTT assay and an increase of apoptotic cells assessed by the nuclear staining assay and flow cytometry. The activities of lactate dehydrogenase (LDH), superoxide dismutase (SOD) and nitric oxide synthase (NOS) and the contents of malondialdehyde (MDA), reduced glutathione (GSH) and nitric oxide (NO) in cells were determined by commercial kits. The expression levels of pro-apoptotic factor caspase-3 and anti-apoptotic signal ERK1/2 were detected by western blot. The results showed that CSTMP had a moderate anti-oxidative effect against the DPPH test, which was less than RES. Co-incubation with CSTMP increased the cell viability, markedly reduced the LDH leakage from the cells and decreased the lipid peroxidation. These effects of CSTMP were accompanied by increasing activity of the endogenous antioxidant enzyme SOD, the level of GSH, the production of NO and cNOS activity. Moreover, CSTMP showed stronger effects on the inhibition of apoptosis, caspase-3 expression, and the activation of phosphorylated ERK1/2 compared to RES. Furthermore, CSTMP could inhibit the expression of phospho-JNK and phospho-p38 induced by H_2O_2. These results suggest that CSTMP prevents H_2O_2-induced cell injury through anti-oxidation and anti-apoptosis *via* the MAPK and caspase-3 pathways.

Key words:
(E)-2-(2-chlorostyryl)-3,5,6-trimethylpyrazine, oxidative stress, apoptosis, antioxidation, NO, MAPK, caspase-3

Abbreviations: Annexin V-FITC – Annexin V fluorescein isothiocyanate, CSTMP – (E)-2-(2-chlorostyryl)-3,5,6-trimethylpyrazine, DMSO – dimethyl sulfoxide, DPPH – 1,1- diphenyl-2-picrylhydrazyl radical, ECM – endothelial cell medium, ERK1/2 – extracellular signal-regulated kinase-1/2, GSH – reduced glutathione, H_2O_2 – hydrogen peroxide, HUVECs – Human Umbilical Vein Endothelial Cells, JNK – c-Jun NH_2-terminal kinase, LDH – lactate dehydrogenase, MAPK – mitogen-activated protein kinase, MDA – malondialdehyde, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, NO – nitric oxide, NOS – nitric oxide synthase, PI – propidium iodide, RES – resveratrol, ROS – reactive oxygen species, RSA – radical scavenging activity, SOD – superoxide dismutase, TMP – tetramethylpyrazine

Introduction

Endothelial cells are crucial for maintaining the physiological functions of the cardiovascular system [6]. Increasing evidence suggests that stress by oxidation of endothelial cells, characterized by an imbalanced cellular activity of the production and elimination of reactive oxygen species (ROS), is involved in the pathophysiology of several vascular diseases, such as atherosclerosis, diabetes and hypertension [10]. In particular, hydrogen peroxide (H_2O_2) induced oxidative stress leads to the death/apoptosis of endothelial cells as well as many other cell types. The oxidative stress can damage the DNA structure, induce peroxidation of the membrane lipids and proteins, damage the fluidity and permeability of the cell membrane [27], or induce apoptosis by triggering numerous signal transduction pathways, including members of the mitogen-activated protein kinase (MAPK) family, p53 transcription factors and caspase-3 activation [21, 26]. Recent studies have reported that prevention of endothelial apoptosis might improve endothelial function [4, 15]. Therefore, anti-oxidants and anti-apoptotic agents have become novel therapeutic strategies to interfere with focal, dysregulated vascular remodeling, which is a key mechanism for atherosclerotic disease progression and other cardiovascular diseases.

Tetramethylpyrazine (TMP) is a major component in the traditional Chinese herb *Chuanxiong* (*Ligusticum wallichii Franchat*), which is used in China as a calcium antagonist and an antioxidant for the treatment of cardiovascular diseases and myocardial and cerebral ischemic diseases because of its effectiveness and low toxicity [11, 14]. However, pharmacokinetic studies have shown that TMP presents low bioavailability and is metabolized quickly *in vivo* with a short half-life of 2.89 h [28]. Furthermore, accumulating toxicity is appearent in patients when TMP is administrated frequently in order to maintain effective plasma concentrations. Therefore, it is necessary to develop new generations of cardio-cerebral vascular medicines *via* the molecular modification of TMP. The antioxidant resveratrol (RES) was initially used for cancer therapy and has now shown beneficial effects against most degenerative and cardiovascular diseases from atherosclerosis, hypertension, ischemia/reperfusion, and heart failure to diabetes, obesity, and aging [19]. Therefore, the pharmacophore of RES (-styryl) was introduced to the methyl position of TMP by hybridization and bioisosteric replacement to improve the pharmacodynamic and pharmacokinetic properties of TMP and RES.

In this study, we investigated the cytoprotective effect and underlying mechanism of one of the derivatives, (E)-2-(2-chlorostyryl)-3,5,6-trimethyl-pyrazine (CSTMP), against oxidation injury to HUVECs induced by H_2O_2.

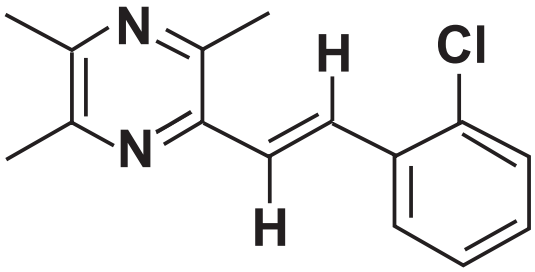

Fig. 1. Chemical structure of CSTMP

Materials and Methods

Materials

The stilbene derivative CSTMP was synthesized by the Institute of Medical Chemistry of Shandong University. Trypsin, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), resveratrol, Annexin V fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA).

Hydrogen peroxide (30% H_2O_2 solution) was obtained from Wako (Osaka, Japan). Monoclonal rabbit anti-human extracellular signal-regulated kinase-1/2 (ERK1/2), phospho-ERK1/2 (Thr202/Tyr204) and caspase-3 antibodies and an antibody against β-tubulin were purchased from Cell Signaling Technology (Boston, MA, USA). Monoclonal mouse anti-human phospho-c-Jun NH_2-terminal kinase (JNK) and phospho-p38 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Biotinylated goat anti-rabbit IgG, goat anti-mouse IgG and nitrocellulose membranes were purchased from Amersham (Buckinghamshire, UK). The LumiGLO Reserve Chemiluminescent Substrate Kit was purchased from KPL (Gaithersburg, MD, USA). Human umbilical vein endothelial cells (HUVECs) and endothelial cell medium (ECM) were purchased from Sciencell Research Laboratories (San Diego, CA, USA). The lactate dehydrogenase (LDH), superoxide dismutase (SOD), reduced glutathione (GSH), nitric oxide synthase (NOS) and nitric oxide (NO) detection kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, PR China). All other chemicals used were of analytical grade and obtained from Shanghai Sangon Biological Engineering Technology & Sciences Co. Ltd. (Shanghai, China).

Estimation of radical scavenging activity (RSA) by the DPPH test

DPPH was used as the stable radical. The antioxidative potential of CSTMP was studied against DPPH [24]. For each concentration of CSTMP and RES tested (12.5, 25, 50 and 100 μmol/ml), the reduction of the DPPH radical was followed by monitoring the decrease of absorbance at 516 nm. The absorption was monitored at the start and at 10 min using an Agilent 8453 UV-Visible spectrophotometer (Agilent Technologies, USA). The results are expressed as % RSA = [Abs516 nm (t = 0) – Abs516 nm (t = 10 min) × 100/Abs516 nm (t = 0)].

Cell culture and treatment

HUVECs were cultured in ECM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°C in polylysine-coated flasks.

For all experiments, the cells were used at passages 3 to 6 and seeded at a concentration of 1×10^5 cells/ml. CSTMP or RES was freshly prepared as a stock solution in DMSO and diluted with ECM supplement (0.1% (v/v) DMSO). DMSO was present at equal concentrations (0.03%) in all groups. The H_2O_2 was freshly prepared before each experiment. CSTMP or RES was pretreated for 30 min before cells were exposed to H_2O_2 (150 μmol/l).

Cell viability measurement by MTT assay

Cell viability was measured by the MTT assay. Briefly, after 12 h exposure to H_2O_2, 20 μl of MTT dye was added to each well at a final concentration of 0.5 mg/ml. After 4 h of incubation, 200 μl of DMSO, the solubilization/stop solution, was added to dissolve the formazan crystals, and the absorbance was read using a microtiter plate reader (Spectra Rainbow, Tecan, Austria) at a wavelength of 570 nm.

Detection of cellular LDH release

HUVECs in 6-well plates were pretreated with CSTMP or RES for 30 min and then stimulated with H_2O_2 (150 μmol/l) for 12 h. LDH release into the supernatant of cells was measured using a commercially available kit according to the manufacturer's protocol. To determine the intracellular LDH activity, cells were washed twice with ice-cold PBS and lysed in 500 μl lysis buffer (150 mM NaC1, 150 mM Tris-HCl, 1 mM EDTA, and 1% Triton X-100). The supernatants were obtained by centrifugation at 10,000 rpm at 4°C for 10 min. LDH release (%) = (LDH activity in supernatants)/(LDH activity in supernatants + LDH activity in total cells) × 100% [13].

Detection of malondialdehyde (MDA) and NO contents and enzymatic activity

HUVECs were pretreated with CSTMP or RES for 30 min and then stimulated with H_2O_2 (150 μmol/l) for 12 h in 6-well plates. The cells were lysed with extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM -glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF). Cell lysates from each well were collected and used for determination of the MDA and NO contents and enzymatic activity. Protein concentrations of

cell extracts were determined by the BCA assay (Hyclone-Pierce, South Logan, USA). The total levels of MDA, the lipid peroxidation product, were assayed by the thiobarbituric acid method, based on quantifying malondialdehyde-reactive products at 532 nm [26]. The NO level and constitutive NOS (cNOS) activities were detected using NO and cNOS detection kits based on the quantitation of NO_2^- at 540 nm by the nitrate reductase assay, because NO was easily oxidized to NO_2^- and NO_3^-, and subsequently, NO_3^- is changed into NO_2^- by a specific reduction reaction. Superoxide dismutase (SOD) activity was measured using a commercial kit based on the hydroxylamine assay developed from the xanthine oxidase assay. GSH was determined by a commercial kit based on the reaction with 2,2'-dinitro-5,5'-dithio-benzoic acid to yield a chromophore with a maximum absorbance at 412 nm.

Fluorescent staining of cells with Hoechst 33258

Nuclear morphology changes of apoptotic cells were investigated by labeling the cells with the nuclear stain Hoechst 33258. The cells on the coverslips were fixed in 4% paraformaldehyde in PBS for 30 min. The cells were then stained with 10 μg/ml Hoechst 33258 under dark conditions at room temperature for 10 min. After washing three times with PBS, cells were observed under fluorescence microscopy (excitation, 340 nm; emission, 460 nm) (AX80; Olympus, Tokyo, Japan).

Cell apoptosis detection by flow cytometry

Early apoptosis and late apoptosis/necrosis induced by H_2O_2 were detected quantitatively by flow cytometric analysis using Annexin V and PI [7]. Cells were harvested by non-enzymatic cell dissociation and centrifugation (120 × g, 5 min) to remove the medium. The cells were washed three times with binding buffer (10 mM Hepes, 140 mM NaCl, and 2.5 mM $CaCl_2$) and stained with 10 μl 20 μg/ml Annexin V-FITC. After a 30 min incubation, cells were washed with binding buffer, incubated with 5 μl PI (final concentration, 3.7 μM) for 10 min, and then kept on ice without exposure to light, prior to analysis by flow cytometry. The Annexin V and PI emissions were detected in the FL1-H and FL2-H channels of a FACS-Vantage flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA, USA), using emission filters of 525 and 575 nm, respectively.

Measurement of ERK1/2, phospho-JNK, phospho-p38 and caspase-3 by western blot analysis

After treatment with CSTMP or RES, confluent monolayers of cells were washed twice in ice-cold PBS and lysed with extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM -glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF). The protein concentrations of the cell extracts were determined by the BCA assay (Hyclone-Pierce, South Logan, USA). Total cell lysate was subjected to SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane, and incubated with monoclonal antibodies against ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), phospho-JNK, phospho-p38, caspase-3, and β-tubulin. Immunoblots were developed using horseradish peroxidase-conjugated secondary antibodies [29]. Immunoreactive bands were visualized by the enhanced chemiluminescent (ECL) system (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and quantified by densitometry using a Chemi-Doc XRS (Bio-Rad, Berkeley, California, USA). The density of each band was normalized to β-tubulin or β-actin for their respective lanes.

Statistical analysis

The values are expressed as the means ± SD. Statistical comparisons were performed by ANOVA followed by the Fisher's protected least significance difference (PLSD) test. A probability value < 0.05 was considered significant.

Results

In vitro antioxidative potential of CSTMP

The antioxidative potential of CSTMP was assessed against DPPH. The results showed that CSTMP had a moderate antioxidative effect against DPPH, which was less than the effect of equal molar concentrations of RES (Fig. 2).

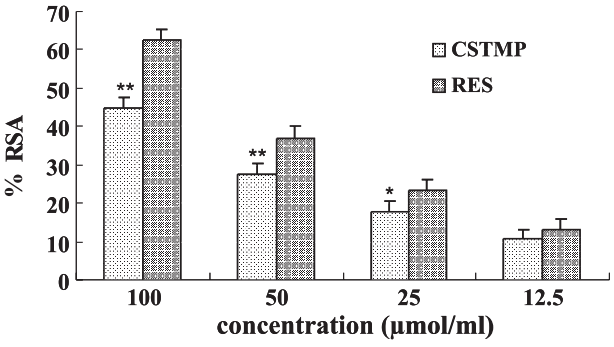

Fig. 2. Effect of CSTMP using the DPPH test. For each concentration of CSTMP and RES tested (12.5, 25, 50 and 100 μmol/ml), the reduction of DPPH radical was followed by monitoring the decrease of absorbance at 516 nm. The % RSA data are expressed as the means ± SD (n = 6). * $p < 0.05$, ** $p < 0.01$ *vs.* RES group at the same concentration

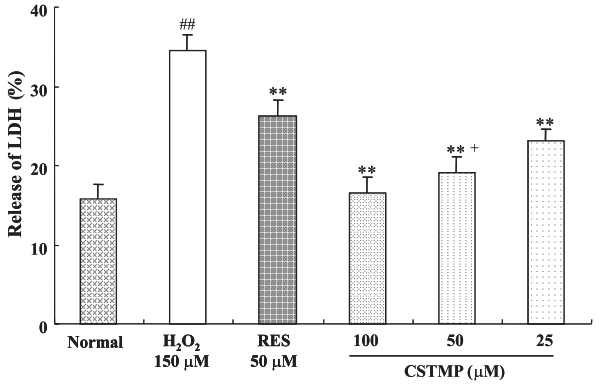

Fig. 4. Effect of CSTMP on H_2O_2-induced release of LDH in HUVECs. Cells were incubated with CSTMP or RES for 30 min and then exposed to H_2O_2 (150 μM) for 12 h before the LDH release was measured. All data are expressed as the means ± SD (n = 8). ## $p < 0.01$ *vs.* unstimulated cells; ** $p < 0.01$ *vs.* H_2O_2-stimulated cells; + $p < 0.05$ *vs.* cells treated with H_2O_2 +RES

CSTMP increases HUVEC viability in response to H_2O_2

The effects of CSTMP on the growth of HUVECs exposed to oxidative damage in response to H_2O_2 were investigated by the MTT method. The exposure of HUVECs to H_2O_2 at 150 μmol/l for 12 h resulted in a significant reduction of cell viability (Fig. 3). Pre-treatment of the cells with CSTMP for 12 h, however, attenuated the H_2O_2 effect on cell viability in a dose-dependent manner. A similar protective effect was observed in the RES treated-group.

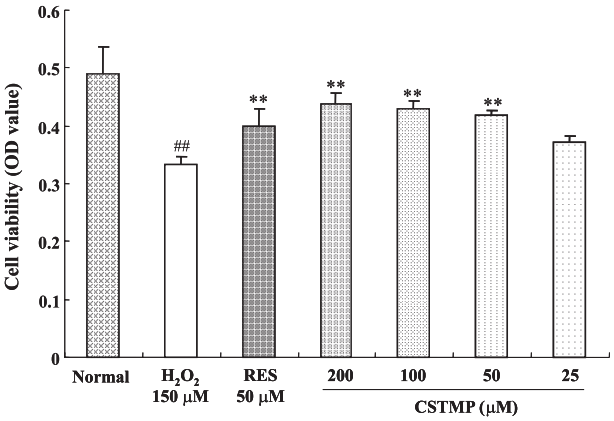

Fig. 3. Effect of CSTMP on HUVECs viability in response to H_2O_2. Cells were incubated with CSTMP or RES for 30 min and then exposed to H_2O_2 (150 μM) for 12 h before the cell viability was determined by the MTT assay. All data are expressed as the means ± SD (n = 8). ## $p < 0.01$ *vs.* unstimulated cells; ** $p < 0.01$ *vs.* H_2O_2stimulated cells

CSTMP inhibits LDH release from HUVECs damaged by H_2O_2

Treatment of HUVECs with 150 μmol/l H_2O_2 for 12 h caused a significant increase of LDH release (an indicator of membrane integrity) (Fig. 4). Pre-treatment of the cells with various concentrations of CSTMP for 30 min prior to incubation with H_2O_2 significantly prevented the LDH release induced by H_2O_2 in a CSTMP concentration-dependent manner. A similar protective effect was observed in the RES treated-group.

CSTMP decreases lipid peroxidation and increases free radical scavenge

Incubation of HUVECs with 150 μmol/l H_2O_2 for 12 h caused a significant increase in MDA content and a marked decrease of SOD and GSH-Px activities (Tab. 1). CSTMP pre-treatment significantly attenuated the increase in MDA content and decreased the SOD and GSH-Px activities in response to H_2O_2 in a CSTMP concentration-dependent manner. In comparison to the H_2O_2-group, CSTMP treated-groups reduced the amounts of MDA by 6.0% (25 μmol/l), 45.8% (50 μmol/l) and 32.8% (100 μmol/l). The activities of SOD were increased by 41.1% (25 μmol/l), 70.0% (50 μmol/l) and 71.5% (100 μmol/l). The activities of GSH-Px were increased by 18.1% (25 μmol/l), 43.2% (50 μmol/l) and 91.7% (100 μmol/l) by CSTMP. A similar protective effect was observed in the RES treated-group.

Tab. 1. Effects of CSTMP on the MDA amount increase, GSH level and SOD activity decrease in HUVECs exposed to H_2O_2 ($\bar{x}$ ± SD, n= 8)

Groups	Dose (μmol/l)	MDA (nmol/mg prot)	SOD (U/mg prot)	GSH (μg/mg prot)
Normal	—	1.69 ± 0.14	17.75 ± 2.40	1.25 ± 0.27
H_2O_2	150	5.12 ± 0.17##	10.13 ± 0.41##	0.72 ± 0.09##
RES + H_2O_2	50	3.76 ± 0.53**	17.88 ± 1.00**	1.20 ± 0.04**
CSTMP + H_2O_2	100	3.44 ± 0.28**	17.37 ± 1.43**	1.38 ± 0.03**
	50	3.60 ± 0.31**	17.20 ± 1.27**	1.05 ± 0.12**
	25	4.83 ± 0.68	14.29 ± 1.85**	0.85 ± 0.21

$p < 0.01$, significant difference compared with the normal group. ** $p < 0.01$, significant difference compared with the H_2O_2-treated group

Involvement of the MAPK pathway in the anti-oxidative stress action of CSTMP

ERK, JNK and p38 are the main members of the MAPK family. ERK is an important protein that controls the cellular response to both proliferation and stress signals. JNK and p38 are mainly involved in apoptosis and growth. We investigated the effects of CSTMP on the expression levels of ERK1/2, phospho-ERK1/2 (P-ERK1/2), phospho-JNK (P-JNK) and phospho-p38 (P-p38) in HUVECs in response to H_2O_2 treatment. The time course results of ERK expression are shown in Figure 5. The expression of P-ERK1/2 gradually elevated and was maximal by 12 h after H_2O_2 exposure, which was consistent with a previous study [8]. CSTMP pretreatment significantly increased the expression of P-ERK1/2 compared with H_2O_2-treated groups at 15 min and 12 h, respectively, but there were no effects at other time points. The effects of CSTMP or RES on the expression levels of ERK1/2, P-ERK1/2, P-JNK and P-p38 were observed after exposure to H_2O_2 for 12 h. Re-

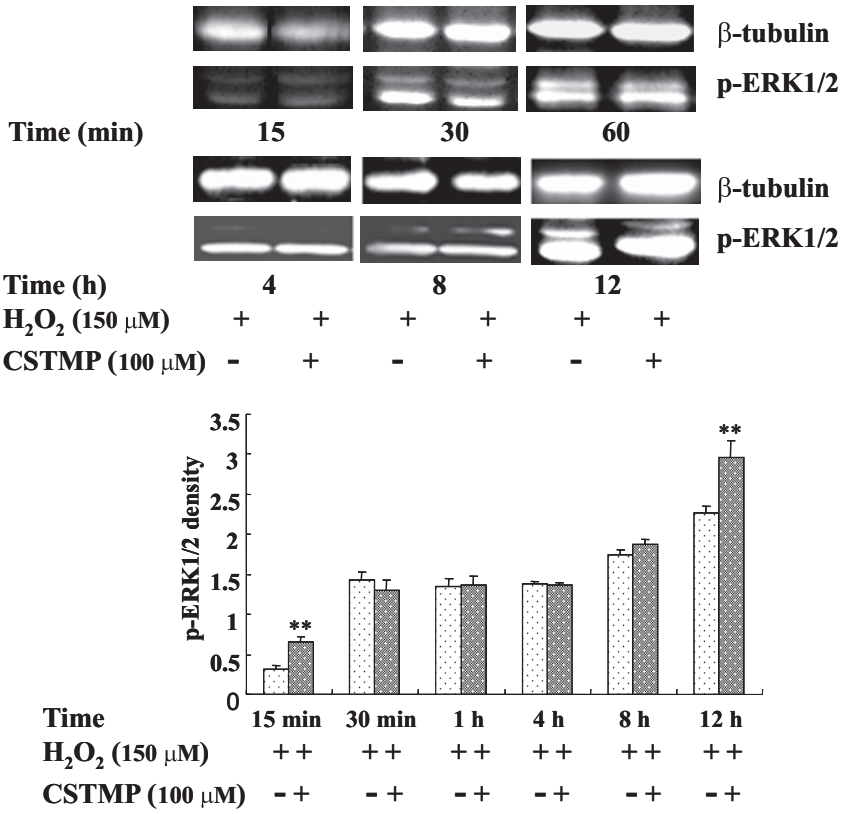

Fig. 5. Time course of the effect of CSTMP on H_2O_2-induced activation of p-ERK1/2 in HUVECs. HUVECs were pretreated with 100 μM CSTMP or 100 μM RES for 30 min and then exposed to H_2O_2 (150 μM) for 15 min, 30 min, 60 min, 4 h, 8 h and 12 h. The expression of P-ERK1/2 was determined by western blot analysis. Representative blots and quantitative data evaluated by densitometry are shown (n = 3). The data are expressed as the means ± SD. ** $p < 0.01$ *vs.* H_2O_2-treated cells at the same time point

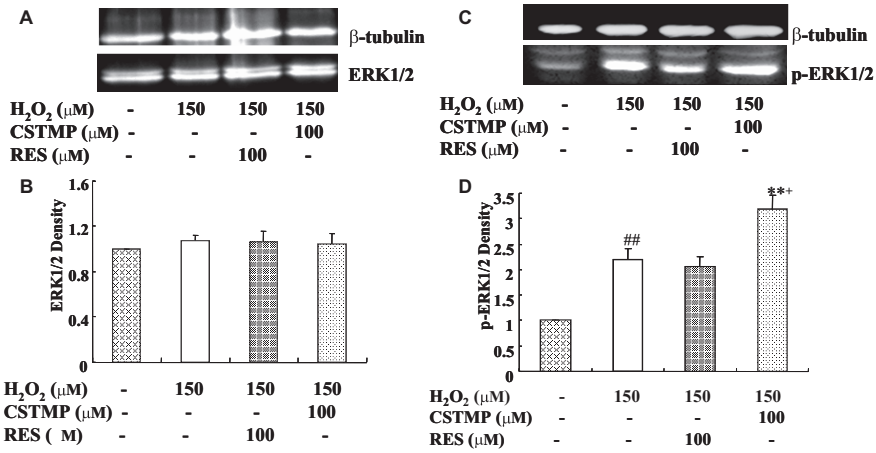

Fig. 6. Effects of CSTMP on the expression of ERK1/2 and P-ERK1/2 in HUVECs in response to H_2O_2. HUVECs were pretreated with 100 μM CSTMP or 100 μM RES for 30 min and then exposed to H_2O_2 (150 μM) for 12 h. The cells were lysed, and the expression levels of ERK1/2 and P-ERK1/2 were determined by western blot analysis. Representative blots (**A**, ERK1/2; **C**, P-ERK1/2) and quantitative data evaluated by densitometry (**B** for ERK1/2, **D** for P-ERK1/2) are shown (n = 3). The data are expressed as the means ± SD. $^{\#\#}$ $p < 0.01$ *vs.* unstimulated cells; ** $p < 0.01$ *vs.* H_2O_2-stimulated cells; $^{+}$ $p < 0.05$ *vs.* cells treated with H_2O_2 + RES

sults showed that the expression of P-ERK1/2 in HUVECs pretreated with CSTMP was increased, while there was no significant change in the total ERK protein level. However, pretreatment of RES had no effect on the expression of either ERK1/2 or P-ERK 1/2 (Fig. 6A–D). The expression of P-JNK and P-p38 was increased in HUVECs exposed to H_2O_2 for 15, 30 and 60 min. After pretreatment with CSTMP, the expression of both proteins was inhibited significantly (Fig. 10).

CSTMP inhibits apoptosis induced by H_2O_2

To evaluate the cytoprotective action of CSTMP on apoptosis induced by H_2O_2, the nuclei of HUVECs were stained with Hoechst 33258 and assessed by fluorescent microscopy. Cells that exhibited reduced nuclear size, chromatin condensation, intense fluorescence, and nuclear fragmentation were considered apoptotic. The microscopic pictures in Figures 7A–F show that the control cells had intact nuclei, whereas the H_2O_2-treated cells show significant nuclear fragmentation. These changes in the nuclear characteristics of apoptosis were rescued significantly in the cells pretreated with CSTMP. Moreover, 50 μM CSTMP showed better effects on the inhibition of apoptotic cell numbers than RES at an equal dosage (Fig. 7G).

In addition to the morphological evaluation, the protective effect of CSTMP against apoptosis was confirmed by flow cytometric analysis using the Annexin V and the PI double-staining system. The An-

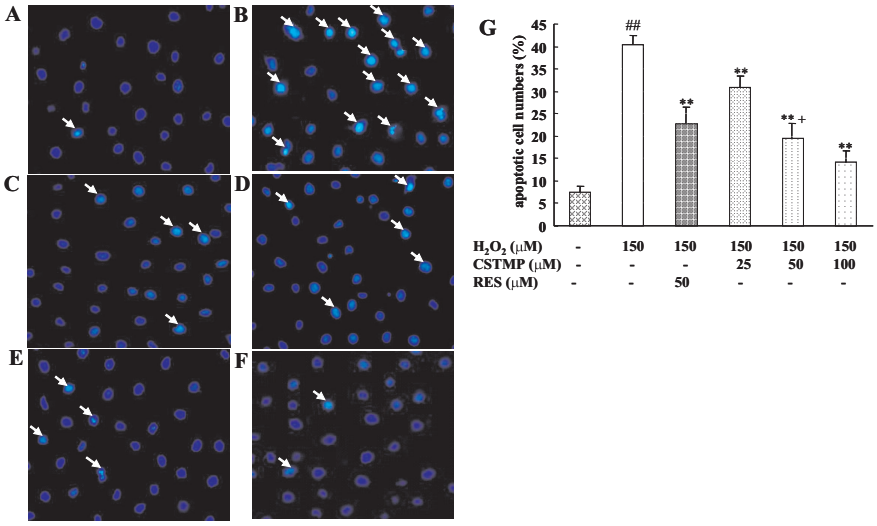

Fig. 7. Inhibitory effect of CSTMP on H_2O_2-induced apoptosis in HUVECs. Cells were incubated with CSTMP or RES for 30 min and then exposed to H_2O_2 (150 μM) for 12 h. The cells were stained with the DNA-binding fluorochrome Hoechst 33258. Fluorescence micrographs of HUVEC nuclei from untreated cells (**A**); H_2O_2-treated cells (**B**); cells preincubated with 50 μM RES (**C**) or 25 μM (**D**), 50 μM (**E**), and 100 μM (**F**) CSTMP for 30 min before H_2O_2. Scale bar = 50 μM. The arrows indicate apoptotic cells. The percentage of apoptotic cells in 100 cells is scored in 4 random observations. The results shown in (**G**) are the mean ± SD. $^{\#\#}$ $p < 0.01$ *vs.* unstimulated cells; ** $p < 0.01$ *vs.* H_2O_2-stimulated cells; $^{+}$ $p < 0.05$ *vs.* cells treated with H_2O_2 + RES

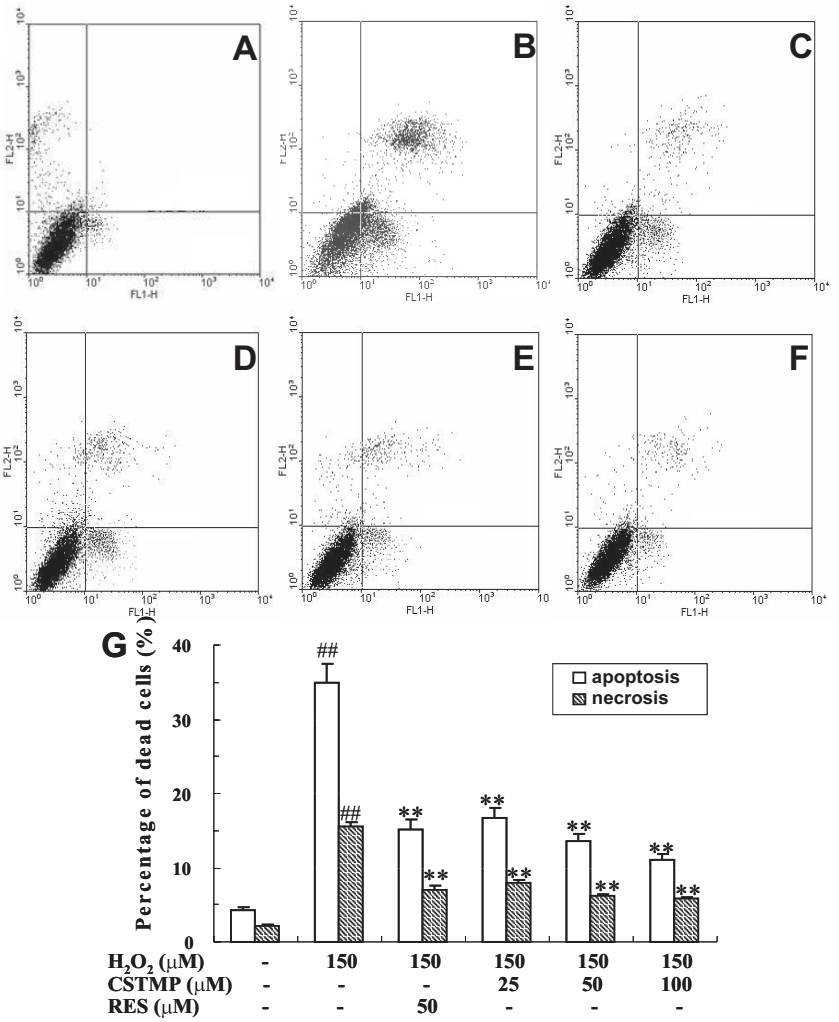

Fig. 8. Inhibitory effect of CSTMP on cell apoptosis and necrosis induced by H_2O_2. HUVECs were incubated in drug-free medium (**A**) or medium containing H_2O_2 (**B**) for 12 h or cells were preincubated with 50 μM RES (**C**) or 25 μM (**D**), 50 μM (**E**), and 100 μM (**F**) CSTMP for 30 min before H_2O_2. The distinction between living, early apoptotic, and late apoptotic/necrotic cells and examples of dot-plots was determined by flow cytometry following Annexin V and PI double-staining. The horizontal axis represents the annexin V intensity, and the vertical axis shows the PI staining. The results shown in (**G**) are the means ± SD for three independent experiments. ## $p < 0.01$ *vs.* unstimulated cells; ** $p < 0.01$ *vs.* H_2O_2-stimulated cells

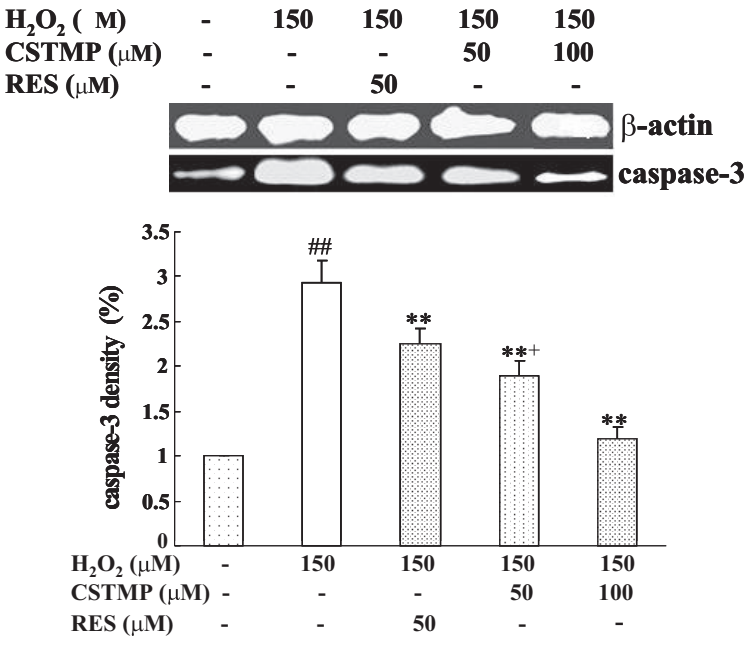

Fig. 9. Effect of CSTMP on H_2O_2-induced elevation of apoptosis marker caspase-3. HUVECs were pretreated with 50 μM, 100 μM CSTMP and 50 μM RES for 30 min and then exposed to H_2O_2 (150 μM) for 12 h. The cells were lysed, and the expression of caspase-3 was analyzed by western blotting. Representative blots and quantitative data evaluated by densitometry are shown (n = 3). The data are expressed as the means ± SD. ## $p < 0.01$ *vs.* unstimulated cells; ** $p < 0.01$ *vs.* H_2O_2-stimulated cells; + $p < 0.05$ *vs.* cells treated with H_2O_2 + RES

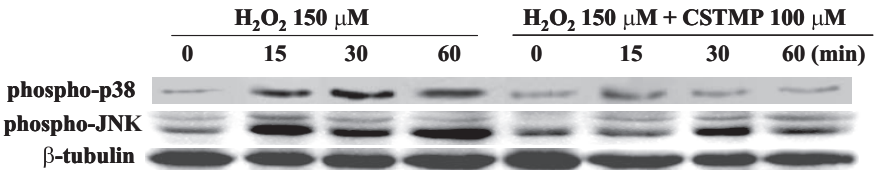

Fig. 10. Effect of CSTMP on the expression of phospho-JNK and phospho-p38 in HUVECs in response to H_2O_2. HUVECs were pretreated with or without 100 μM CSTMP for 30 min and then exposed to H_2O_2 (150 μM) for 15, 30, and 60 min. The cells were lysed, and the expression levels of phospho-JNK and phospho-p38 were determined by western blot analysis. Representative blots of three independent experiments are shown

nexin V^-/PI^- population was regarded as normal healthy cells, while the Annexin V^+/PI^- cells were taken as a measure of early apoptosis and Annexin V^+/PI^+ as necrosis/late apoptosis. Typical examples are shown in Figures 8A–F. Using this method, we found that the control group had 93% intact, living cells and 7% of cells in the early and late phases of apoptosis. An increase of apoptotic cells was observed in the H_2O_2-treated group with a lower number of living cells. CSTMP administration led to a reproducible decrease in the rate of early apoptosis and necrosis/late apoptosis in cells exposed to H_2O_2. This positive effect of CSTMP was observed in a dose-dependent manner (Fig. 8G).

CSTMP inhibits caspase-3 expression in apoptotic HUVEC induced by H_2O_2

To test whether CSTMP affected the expression of caspase-3 (the apoptosis marker), an antibody specific to caspase-3 was used for western blot analysis. In cells exposed to H_2O_2 for 12 h, the expression of caspase-3 was enhanced significantly. However, in H_2O_2-exposed cells treated with CSTMP at concentrations of 50 μM and 100 μM and 50 μM of RES, the increased expression of caspase-3 was inhibited (Fig. 9). These results demonstrate that CSTMP could prevent oxidant-induced apoptosis through inhibition of caspase-3 activation.

CSTMP promotes NO production and cNOS activity in HUVECs in response to H_2O_2

Incubation of HUVECs with 150 μmol/l H_2O_2 for 12 h caused a significant decrease of the NO content and cNOS activity (Fig. 11 A,B). CSTMP pre-treatment significantly promoted the synthesis and release of NO directly by increasing the activity of cNOS in HUVECs in a concentration-dependent manner. A similar effect was observed in the RES-treated group.

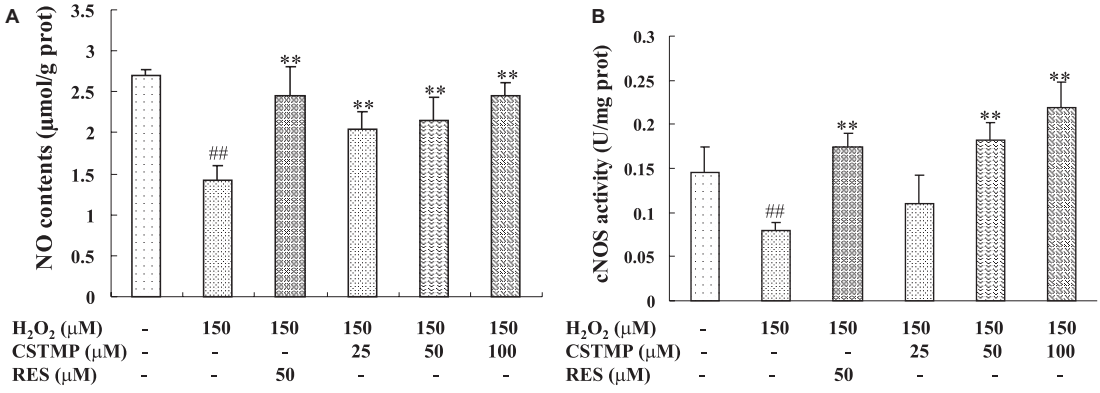

Fig. 11. Effect of CSTMP on the NO contents (**A**) and NOS activity (**B**) in HUVECs exposed to H_2O_2. HUVECs were pretreated with 50 μM, 100 μM CSTMP and 50 μM RES for 30 min and then exposed to H_2O_2 (150 μM) for 12 h. The cells were lysed, and the NO contents and NOS activity were determined by commercial kits. The data are expressed as the means ± SD. $^{\#\#}$ $p < 0.01$ *vs.* unstimulated cells; ** $p < 0.01$ *vs.* H_2O_2-stimulated cells

Discussion

Endothelial dysfunction has been implicated in the initiation and propagation of vascular disease processes, including atherosclerosis, hypertension, cardiac hypertrophy and congestive heart failure [6]. Oxidative stress induced by ROS, e.g., superoxide, H_2O_2 and peroxynitrite, is believed to be a key cause of endothelial cell dysfunction [17]. In the present study, we observed that H_2O_2 can markedly increase the permeability of HUVECs, as demonstrated by LDH release, and lead to massive apoptosis, as evidenced by flow cytometry. Pretreatment of CSTMP on the cells effectively protects HUVECs from H_2O_2-induced damage through anti-oxidative approach and from apoptosis through inhibition of the caspase-3 and ERK pathway.

TMP and RES are important therapeutic agents for the treatment of some cardiovascular diseases based on their antioxidant and other pharmacological activities. CSTMP is a novel stilbene derivative *via* molecular medication of TMP and RES to improve pharmacodynamic and pharmacokinetic properties of TMP and RES.

Oxidative metabolites are involved in the functional inactivation of endothelial cells by increasing permeability and as potent inducers of endothelial cell death. The level of MDA reflects the extent of cell damage by oxidative stress. The antioxidant enzyme SOD and reductive compound GSH are thought to be effective for the augmentation of antioxidant defenses in endothelial cells. SOD scavenges superoxide radicals by converting them to hydrogen peroxide, which is then converted to water by catalase and GSH-Px. Our results showed that CSTMP reduced the decline of the activity of SOD and the level of GSH and augmented MDA content in HUVECs in response to H_2O_2. This suggests that the cytoprotective effect of CSTMP was likely related to the restoration of endogenous antioxidation and the decrease of lipid peroxidation. Moreover, CSTMP also showed a moderate extracellular antioxidative effect against the DPPH test, although this effect was less than that of RES with an equal concentration of 100 μmol/ml.

Excessive production of reactive oxygen species in cells can either directly or indirectly lead to mitochondrial dysfunction, apoptosis, and cell death [25]. This study showed that the apoptosis of HUVECs was induced by H_2O_2. CSTMP strongly prevented apoptotic morphological and biochemical changes in HUVECs and reduced the extent of apoptotic cell death. To elucidate the underlying mechanism of anti-apoptotic effects of CSTMP, we chose three components of the MAPK family as indicators of apoptosis in the apoptotic signaling pathways: ERK1/2, JNK and p38. MAPK cascades belong to the protein kinase signal transduction pathways that are differentially used to transmit numerous extracellular signals within cells and have been reported to be involved in various cellular functions, including stress responses and apoptosis [18]. In particular, many protein kinases and transcription regulatory factors are activated under conditions of oxidative stress due to ROS [2, 16, 20]. In the present study, the results confirmed that H_2O_2-induced apoptosis was mainly mediated by the activation of p38 and JNK MAPK. CSTMP has potent anti-apoptotic action by inhibiting p38 and JNK phosphorylation and promoting ERK1/2 activation in the process of cellular oxidative stress. In our experiment, the ERK pathway was activated transiently after exposure to H_2O_2, which is thought to be a part of the cell defense mechanism [8]. However, it has been shown that the relatively stable activation of ERK contributes to antioxidant defense mechanisms [5]. Indeed, we found that CSTMP produced an elevation of ERK phosphorylation.

ROS can also induce the release of cytochrome C from mitochondria, stimulating proteolytic caspases. Caspases is a family of cysteine proteases that cleave target proteins at specific residues. Among the more than ten members of the caspase family identified, caspase-3 is a major player in the apoptosis induced by oxidative stress [23]. Previous study indicates that upregulating ERK1/2 activation and downregulating p38 activation inhibit caspase-3 activation [1]. In this paper, we observed that there was an attenuated activity of caspase-3 in CSTMP group compared to H_2O_2 group, indicating CSTMP could prevent from oxidative stress induced apoptosis of HUVECs by enhancing the phosphorylation levels of pERK1/2 and hence inhibiting caspase-3 activation.

NO is an endogenously generated mediator of smooth muscle relaxation and an inhibitor of platelet/leukocyte activation. It was reported that NO synthesized from the endothelium could inhibit apoptosis induced by various apoptotic stimuli [3, 9]. The production of ROS may result in reduced availability of NO. The balance between NO and O_2^- may be broken

due to accumulation of superoxide anions in the cells, and inactivation of NO by O_2^- may contribute to vascular dysfunction as reduction of available NO leads to impaired endothelium-dependent relaxation [12]. In addition, physiological levels of NO prevent apoptosis and interfere with the activation of the caspase cascade [22]. In this study, CSTMP significantly increased the content of NO in HUVECs by activation of NOS in a concentration-dependent manner. We also observed that the anti-apoptotic action and inactivation of caspase-3 by CSTMP are paralleled by an increase of NO content. These results suggest that increase of NO formation contributed to inhibit endothelial cells apoptosis induced by H_2O_2.

In conclusion, CSTMP significantly protected HUVECs against H_2O_2 damage *via* anti-oxidative and anti-apoptosis pathways. The antioxidant CSTMP diminishes the apoptosis of HUVECs by inhibiting the activation of caspase-3, JNK and p38 MAPK and by promoting the phosphorylation of ERK1/2 and NO synthesis. These findings suggest that CSTMP may have important potential for the development of new agents for the effective treatment of vascular diseases.

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