JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Hydroxytyrosol Induces Proliferation and Cytoprotection against Oxidative Injury in Vascular Endothelial Cells: Role of Nrf2 Activation and HO-1 Induction

Houda Zrelli,[†] Mieko Matsuoka,[†] Shiho Kitazaki,[†] Masahiro Araki,[†] Miki Kusunoki,[†] Mokhtar Zarrouk,[§] and Hitoshi Miyazaki*[,†]

[†]Graduate School of Life and Environment Sciences, Alliance for Research on North Africa, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

[§]Borj-Cedria Science and Technology Park, 2050 Hammam-Lif, Tunisia

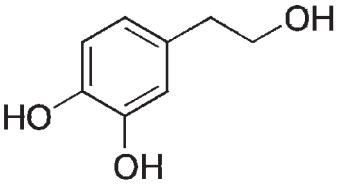
ABSTRACT: Hydroxytyrosol (HT), a phenolic compound in olive oil and leaves, has been reported to prevent various human pathologies including cardiovascular diseases. This study investigated the effects of HT on proliferation and protection against oxidative stress-induced damage in vascular endothelial cells (VECs) and the molecular mechanism(s) involved. Treatment of VECs with HT increased cell proliferation, promoted wound repair, and protected cells against H_2O_2 cytotoxicity through the activation of Akt and ERK1/2, but not p38 MAPK. HT increased the expression and nuclear translocation of nuclear factor-E2-related factor-2 (Nrf2). Nrf2 expression was attenuated by LY294002 and U0126, inhibitors of phosphatidylinositol-3-kinase and MEK1/2, respectively. Nrf2 siRNA decreased both proliferative and cytoprotective effects of HT and abrogated HO-1 induction. Moreover, HO-1 inhibition with HO-1 siRNA or zinc protoporphyrin IX significantly prevented HT-induced cell proliferation, cytoprotection, and reduction in intracellular reactive oxygen species (ROS), suggesting that HO-1 is involved in these HT functions. The findings demonstrate that HT positively regulates the antioxidant defense system in VECs through the activation of Nrf2 followed by cell proliferation and resistance to vascular injury. The present study provides a molecular basis for the contribution of HT in the Mediterranean diet to the prevention of cardiovascular diseases.

KEYWORDS: hydroxytyrosol, Nrf2, atherosclerosis, vascular endothelial cells, proliferation, oxidative stress

INTRODUCTION

Accumulating evidence indicates that the "Mediterranean diet" helps to prevent diseases caused by oxidative stresses including cardiovascular diseases and cancer.[1,2] The regular consumption of olive oil as the primary source of fat in addition to the abundant intake of fruits and vegetables in the Mediterranean diet is thought to be associated with a reduced risk of cardiovascular diseases.[3−5] Although oleic acid exerts known health benefits of olive oil, phenolic compounds have recently received focus as beneficial components, due to their antioxidant and free radical-scavenging activities that presumably counteract the oxidative stress-induced endothelial dysfunction favorable to the onset of atherosclerosis.[6,7]

Among various phenolic compounds, hydroxytyrosol (3,4-dihydroxyphenylethanol; HT, Figure 1) naturally occurs at a high concentration (113.7−381.2 mg/kg) in extra virgin olive oil and also in olive leaves.[8] HT has been demonstrated to possess antiatherogenic properties. Reported in vivo studies indicated that the administration of HT improved the blood lipid profile, antioxidant status, and development of atherosclerotic lesions in an animal model of diet-induced atherosclerosis.[9−11] HT is also very efficient in protecting the aorta against oxidative stress-induced impairment in NO-mediated relaxation.[12] Moreover, HT inhibited cell surface expression of proatherogenic adhesion molecules (ICAM-1 and VCAM-1) and E-selectin in human umbilical vascular endothelial cells.[13] However, the direct effect of HT on vascular endothelial cell (VEC) proliferation and cytoprotection against H_2O_2-induced oxidative stress and the molecular mechanisms involved remain unclear.



Hydroxytyrosol, HT (Mw = 154.17)

Figure 1. Chemical structure of hydroxytyrosol (3,4-dihydroxyphenylethanol) (M_w = 154.17).

Antioxidant/detoxifying enzymes are activated during the protection of cells against oxidative injury, and the interest in naturally occurring phenolic compounds that can induce these enzymes is increasing. Studies both in vitro and in vivo have shown that several phytochemicals induced cytoprotective enzymes such as glutathione peroxidase (GPx), NAD(P)H:quinone oxidoreductase (NQO1), superoxide dismutase-1 (SOD1), γ-glutamyl cysteine ligase (GCL), heme oxygenase (HO-1), and catalase, all of which increase cellular antioxidant defense and resistance to oxidative injury.[14]

Received: October 24, 2010
Revised: March 20, 2011
Accepted: March 25, 2011
Published: March 25, 2011

The antioxidant enzyme expression induced by these phytochemicals is tightly controlled by the nuclear factor-E2-related factor-2 transcription factor, Nrf2. Under physiological conditions, Nrf2 is sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) and ubiquitinated through the cullin-3-dependent pathway, leading to the low-level expression of its target genes. However, in cells exposed to oxidative stress, Nrf2 dissociates from Keap1 and translocates into the nucleus, where it forms a complex with other nuclear factors and binds to antioxidant response elements (ARE) of the promoter regions of many antioxidant/detoxifying genes.[15−18] Although the mechanism through which phenolic compounds activate Nrf2 remains controversial, one study has indicated that some electrophilic compounds, such as carnosic acid, target Keap1 by oxidizing or chemically modifying one or more of its specific cysteine thiols, thereby stabilizing Nrf2.[19] Other papers have indicated that the activation of one or more of the upstream kinases, such as mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K)/Akt, protein kinase C, and casein kinase-2, also induce the nuclear translocalization of Nrf2.[20−23]

Interest in dietary phenolic compounds that can induce the expression of enzymes involved in cellular antioxidant defense is increasing.[14] However, the direct effect of HT on vascular endothelial cells has not been investigated except for its outcome on the expression of adhesion molecules in these cells.[13,24−26] The present study examines the effect of HT on VECs and its molecular mechanisms involving Nrf2 activation, with a focus on proliferative effects of HT and protection against H_2O_2-induced oxidative injury. We discovered that HT potently induces the proliferation of VECs and protects them against H_2O_2 damage by Nrf2 activation followed by HO-1 induction through the PI3K/Akt and ERK1/2 pathways.

MATERIALS AND METHODS

Materials. Hydroxytyrosol (3,4-dihydroxyphenylethanol) was purchased from Cayman Chemical Co. (Ann Arbor, MI). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin, Hanks' balanced salt solution (HBSS), zinc protoporphyrin (ZnPP IX), C-type natriuretic peptide (CNP), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO). Phospho-Thr308 of Akt, phospho-ERK 1/2, phospho-p38, and anti-rabbit IgG HRP-linked were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-mouse IgG, HRP-conjugated anti-goat IgG, anti-Nrf2, and anti-lamin B were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). LY294002, SB203580, U0126, HO-1 antibody, and anti-α-tubulin antibody were from Calbiochem (La Jolla, CA). Lysophosphatidic acid (LPA) was obtained from Funakoshi Co. (Tokyo, Japan).

Culture of VECs. VECS derived from porcine pulmonary arteries, obtained at a local abattoir, were maintained in DMEM containing low glucose supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2 incubator and analyzed between passages 8 and 10 as previously described.[27] The cells were serum-starved by incubation for 12 h in DMEM containing 1% FBS before starting experiments. Thereafter, culture medium containing different concentrations of HT dissolved in DMSO was freshly prepared at the time of each experiment. Cells were then treated with HT, whereas the control cells were treated with DMSO only. The final concentration of the solvent was <0.1%.

Cell Proliferation Assay. The proliferation responses of VECs were determined by using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS), reduction assay kit (Cell Titer 96TM Aqueous Nonradioactive Cell Proliferation Assay, Promega, Madison, WI) according to the manufacturer's instructions. Briefly, serum-starved VECs were seeded into a 96-well culture plate (Nunc, Rochester, NY) at a density of 3×10^4 cells/well and stimulated with HT or the required treatment as described in the figure captions. Following this treatment, cells were incubated with 20 μL of MTS reagent in 100 μL of fresh culture medium for 1 h at 37 °C in a humidified 5% CO_2 incubator in the dark. The absorbance at 490 nm was recorded using a Bio-Tek AQuant microplate reader (Winooski, VT). Each experiment was conducted in triplicate. Alternatively, proliferation of VECs was confirmed by counting the trypan blue negative cells (viable cells) by trypan blue dye exclusion using a hemocytometer.

Wound Healing Assay. Cells at the density of 2×10^5 were seeded in 3.5 cm plates (Nunc). After serum starvation, VEC monolayers were manually damaged by scratching with a sterile 200 μL pipet tip to generate a clean area with a constant diameter. Detached cells were removed by washing with PBS, fresh medium containing 1% FBS was added, and then the monolayers were incubated with LPA or HT at 37 °C for 24 h. Immediately after injury (0 h) and at 8 and 24 h, the wound was photographed using an inverted microscope attached to a digital camera (Leica DM IBRE), and cells inside the wound were counted. The number of these cells was determined in five random selected microscopic fields and expressed as a percentage of the cells in the control field.

Cell Viability. Hydrogen peroxide (H_2O_2)-induced cytotoxicity on VECs was evaluated in preliminary experiments by incubating the cells with increasing concentrations (0−700 μM) of H_2O_2 for 24 h. Because about 50% of the cell viability was lost at 500 μM, this concentration was used for all subsequent cell viability investigations. Cell viability responses were determined using Cell Titer 96 Aqueous Nonradioactive Cell Proliferation Assay and MTS assays (Promega) as described above. Alternatively, cell viability was confirmed by using trypan blue assay (no. of viable cells/total cells).

Preparation of Nuclear and Cytosolic Fractions. To separate the cytosol from the nucleus, VECs were lysed on ice with lysis buffer comprising 10 mM HEPES (pH 8.0), 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, 0.1% Nonidet P-40, and protease inhibitors. After 3 min of centrifugation at 13000 rpm and 4 °C, the cytosolic fraction (supernatant) was collected and the pellet was resuspended and incubated for 20 min on ice in cold extraction buffer comprising 10 mM HEPES (pH 8.0), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, and protease inhibitors. After centrifugation at 14000 rpm for 20 min, protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL).

Western Blotting. For Western blotting, VECs were seeded at the density of 2×10^5 in 3.5 cm plates (Nunc). After required treatment, cells were washed twice with ice-cold PBS and resuspended in lysis buffer comprising 50% glycerol, 100 mM NaF, 10 mM sodium pyrophosphate, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, 10 μg/mL antipain, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. Lysates were centrifuged at 14000 rpm and 4 °C for 20 min, and the resultant supernatant was used for experiments. Equal amounts of protein per sample (35 μg) were separated by 10% SDS−polyacrylamide gel electrophoresis and transferred to PVDF membranes, and then nonspecific binding was blocked with 5% nonfat milk in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween 20 (TBS-T) for 1 h at room temperature. The blots were shaken overnight with various primary antibodies (1:1000 dilutions) at 4 °C and washed with TBS-T, and then the primary antibodies were detected using horseradish peroxidase-conjugated donkey anti-rabbit or goat anti-mouse secondary antibodies (1:5000 dilutions) for 2 h at room temperature. Immunocomplexes were visualized using the Western Lightning Chemiluminescence kit according to the manufacturer's instructions (Santa Cruz, CA). Densitometric findings were normalized against the signal obtained by co-incubating the membranes with α-tubulin antibody.

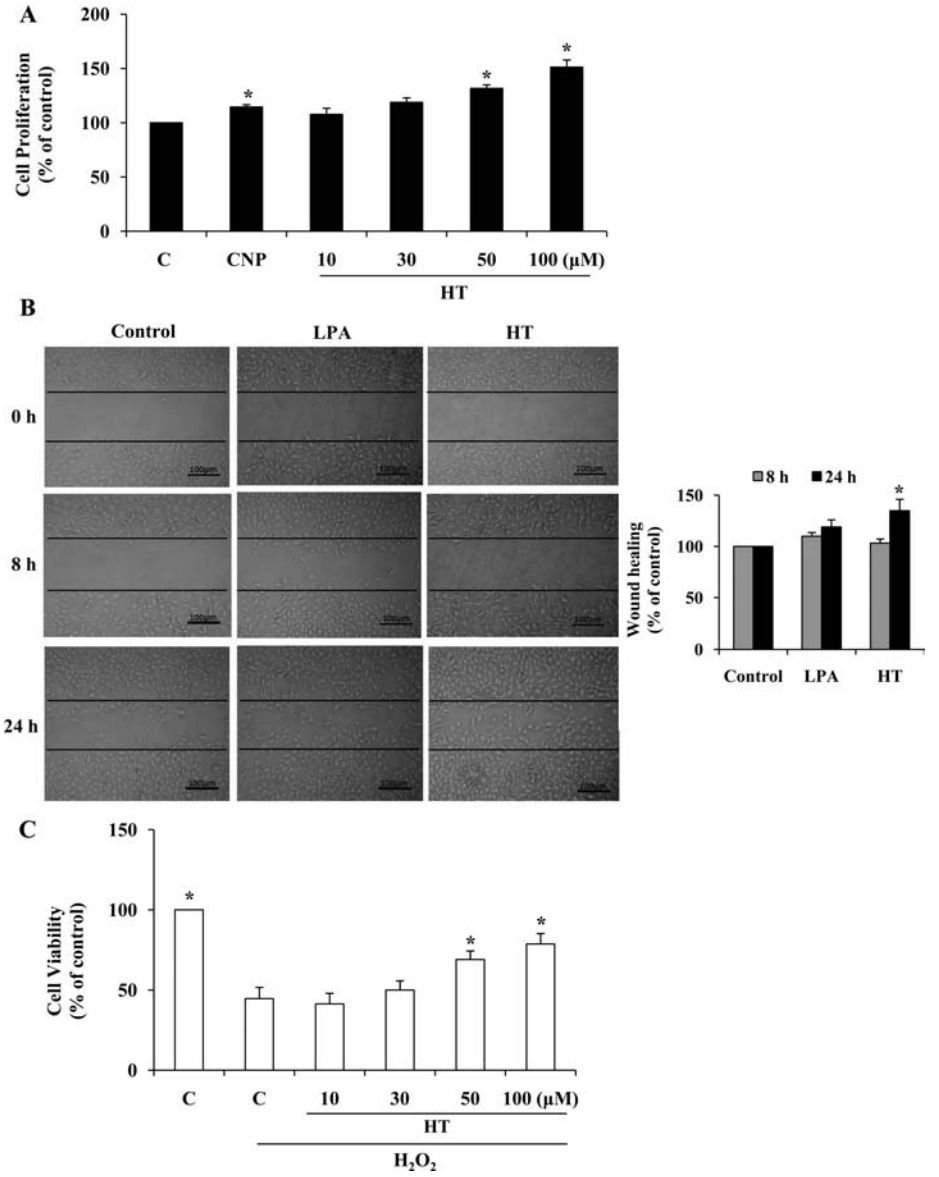


Figure 2. Effect of HT on proliferation, wound healing, and H_2O_2-dependent death of VECs. (A) Serum-starved VECs were incubated with 200 nM CNP or indicated concentrations of HT for 24 h. Cell proliferation was determined using MTS assays. Data are the mean ± SD of four different independent experiments. Statistical analysis was by a one-way ANOVA followed by Duncan's post hoc test. ∗ ,$P < 0.05$ versus untreated cells group (C). (B) Serum-starved, confluent VECs were damaged by scratching with a pipet tip. Cells were then incubated with or without 10 μM lysophosphatidic acid or 50 μM HT for 8 and 24 h. Black lines indicate initially damaged regions. (Left panel) representative data; (right panel) data shown as the mean ± SD of three different independent experiments. ∗, $P < 0.05$ versus untreated cells. (C) VECs were incubated with or without HT at indicated concentrations for 24 h, washed, and incubated with 500 μM H_2O_2 for 24 h. Cell viability was determined using MTS assays. Data represent the mean ± SD of four different independent experiments. Statistical analysis was by a one-way ANOVA followed by Duncan's post hoc test. ∗, $P < 0.05$ versus H_2O_2-treated cells without HT group.

Immunocytochemistry. VECs were plated at the density of 2×10^4 in a 24-well plate containing poly-L-lysine-coated sterile coverslips and processed as described in the figure captions. The cells were washed with PBS, fixed with 4% (w/v) paraformaldehyde, blocked with 1% BSA, and incubated with rabbit anti-Nrf2 polyclonal antibody (1:500 dilutions, Santa Cruz, CA) overnight. The cells were then incubated with secondary Alexa Fluor 488-conjugated donkey anti-rabbit antibody (1:500 dilutions, Molecular Probes, Invitrogen) for 1 h. The stained cells were washed with PBS and mounted onto microscope slides in mounting medium with propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO). At least five images were collected for each condition and analyzed using a confocal scanning system (TCP SP2, Leica, Wetzlar, Germany).

Preparation and Transfection of Porcine Nrf2 siRNA. Because the sequence of porcine Nrf2 has not yet been determined, we first cloned porcine Nrf2 cDNA based on the bovine sequence using reverse transcription-PCR (RT-PCR) and designed a unique 19-mer porcine Nrf2 siRNA. The RT-PCR primers for cloning porcine Nrf2 were 5′-GCCGTCCCAGCAGGACATGG-3′ (forward) and 5′-GGTTCCTCCTACAGTTAAGTT-3′ (reverse). Oligonucleotides selected as potential siRNA target sites for the Nrf2 and HO-1 genes were 5′-GCAGCAUCCUCUCCACAGA-3′ and 5′-AGACCGCCUUCCUGCUCAA-3′, respectively. A universal negative control was provided by Nippon EGT (Tokyo, Japan). For transfection, 100 nM siRNA and Lipofectamine RNAiMAX (Invitrogen) were separately diluted in serum-free DMEM, incubated for 5 min at room temperature, and then

mixed and placed at room temperature for 20 min. The mixture was added to the cells for 48 h, and then transfected cells were processed as described in the figure captions.

RNA Extraction and Real-Time PCR. Total RNA was isolated and reverse transcribed as described in our previous work.[28] Quantitative real-time PCR was performed using Syber Green PCR Master Mix and the 7300 real-time PCR system (Applied Biosystems) according to the manufacturer's protocol using the oligonucleotide primers 5′-TGCTGAA-GATTCAGCTGTTTGA-3′ (forward) and 5′-CACGGGAGTGGAGT-CTTGCAC-3′ (reverse) for HO-1 and 5′-GAGGTGAAATTCTTG-GACCGG-3′ (forward) and 5′-CGAACCTCCGACTTTCGTTCT-3′ (reverse) for 18S rRNA and the following cycling parameters: 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The amount of HO-1 mRNA expression was normalized to that of 18S rRNA mRNA.

Measurement of Intracellular ROS. Intracellular ROS were measured using the oxidant-sensitive probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as described in our previous work.[29] Briefly, VECs with or without pretreatment with HT were incubated with 5 μM DCF-DA for 15 min in the dark at 37 °C. The cells were washed and further incubated with H_2O_2 for another 15 min, then the medium was removed, and the cells were washed with Hank's balanced salt solution (HBSS). Fluorescence emission was detected by confocal laser scanning microscopy at excitation and emission wavelengths of 488 and 505 nm, respectively. Images were analyzed using a confocal scanning system (TCP SP2, Leica).

Statistical Analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Duncan post hoc test, to evaluate differences between groups when indicated, or the Student *t* test. Data are expressed as the mean ± SD from at least three different independent experiments. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

HT Promotes Cell Proliferation, Stimulates Wound Repair, and Prevents H_2O_2-Induced Cytotoxicity in VECs. Cell proliferation and wound repair are fundamental steps in the prevention of endothelial dysfunction during the pathogenesis of atherosclerosis. We initially investigated whether or not HT affects VEC proliferation. Figure 2A shows that HT significantly and concentration-dependently (10−100 μM) stimulated VEC proliferation up to 152% compared with untreated cells. In addition, C-type natriuretic peptide (CNP), which stimulates VEC proliferation, also increased the number of cells.

The migratory property of VECs cultured in the presence of HT was assessed using scratch wound assays. Confluent monolayers of VECs were wounded using a micropipet, and wound closure was monitored by photography at 8 and 24 h. Figure 2B indicates that 24 h treatment with 50 μM HT enhanced wound repair up to 134% compared with untreated cells. Notably, HT promoted wound repair more effectively than LPA, an established stimulator of wound healing in VECs.[30] However, a significant area of the wound remained uncovered after 8 h of treatment with HT. These results indicate that HT effect on wound healing is probably due to the stimulation of VEC proliferation rather than migration.

We further examined the cytoprotective activity of HT against H_2O_2-induced cell damage. We evaluated VEC viability after incubation with HT (10−100 μM) for 24 h, followed by exposure to 500 μM H_2O_2 for another 24 h without HT. As shown in Figure 2C, HT significantly protected VECs from the cytotoxic effect of H_2O_2 in a concentration-dependent manner. These results suggest that HT not only promotes cell proliferation and wound repair but also protects VECs from H_2O_2-induced cytotoxicity.

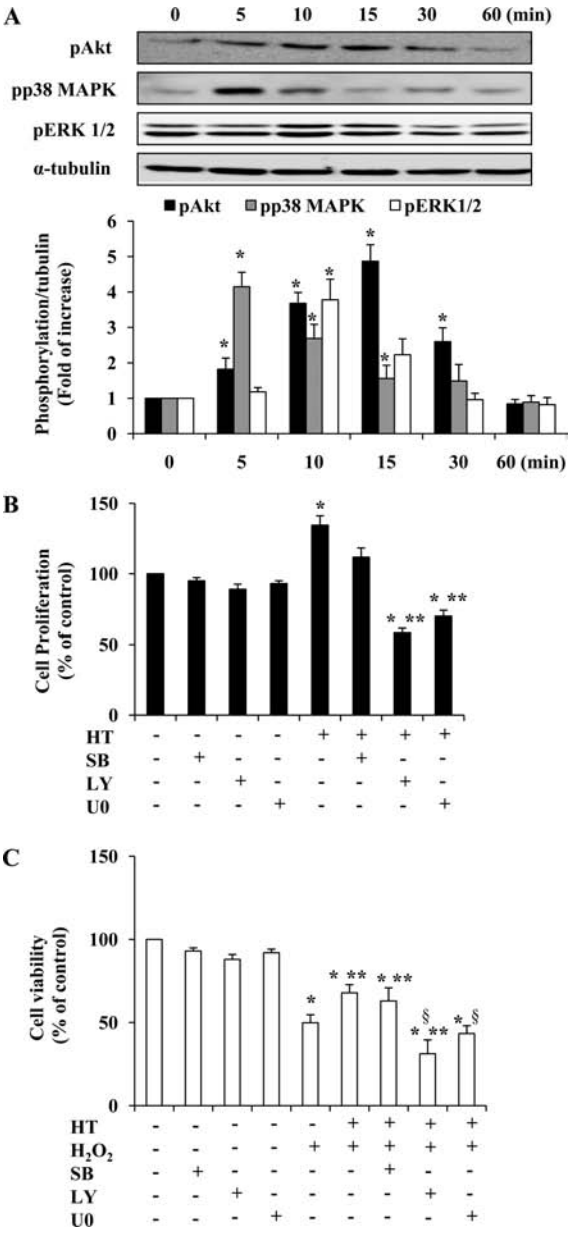


Figure 3. HT-induced phosphorylation of Akt, p38 MAPK, and ERK1/2 and the effect of their inhibitors on HT-dependent cell proliferation and cytoprotection against H_2O_2. (A) Serum-starved VECs were incubated with 50 μM HT for indicated periods, and then cell lysates were Western-blotted using antibodies that recognize pAkt (Thr308), pp38 MAPK, and pERK1/2. Representative data are from three different independent experiments that yielded similar results. (B) VECs were incubated with 10 μM SB203580 (SB), 50 μM LY294002 (LY), or 10 μM U0126 (U0) for 1 h and then in the presence of 50 μM HT for 24 h. Cell proliferation was determined using MTS assays. Data represent the mean ± SD of four different independent experiments. *, $P < 0.05$ versus untreated cells; **, $P < 0.05$ versus HT-treated cells. (C) VECs were incubated with 50 μM HT for 24 h in the presence or absence of 10 μM SB203580 (SB), 50 μM LY294002 (LY), or 10 μM U0126 (U0), washed, and then incubated with 500 μM H_2O_2 for 24 h. Cell viability was determined using MTS assays. Data represent the mean ± SD of four different independent experiments. *, $P < 0.05$ versus untreated cells; **, $P < 0.05$ versus H_2O_2-treated cells without HT; §, $P < 0.05$ versus H_2O_2-treated cells with HT.

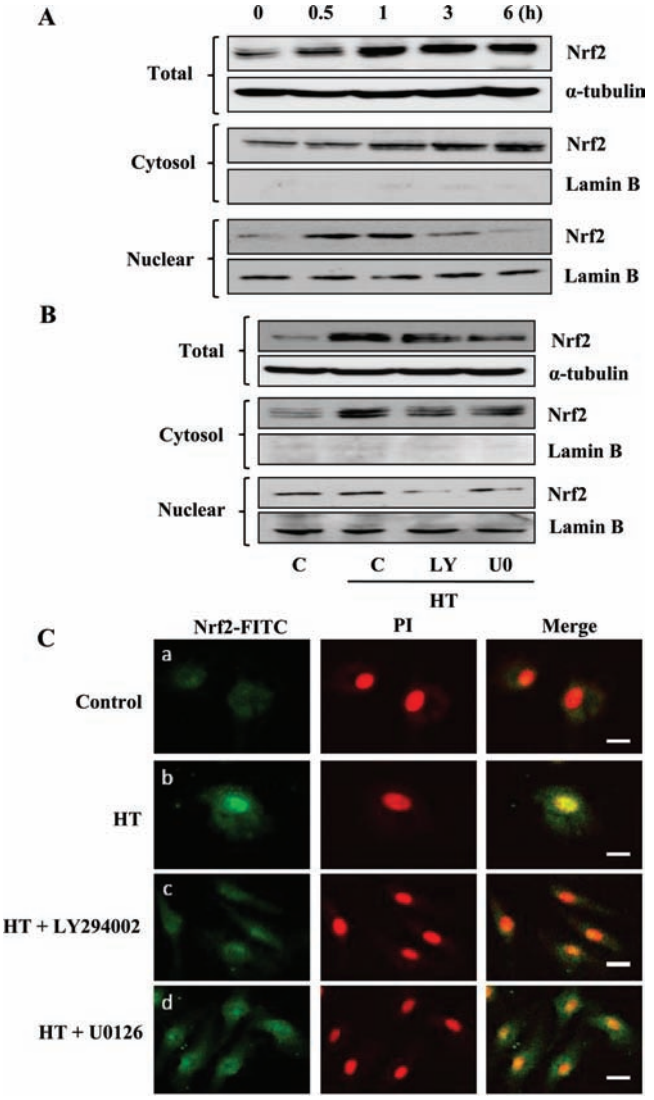


Figure 4. HT-induced accumulation of Nrf2 in the nucleus and the effect of the inhibitors of the Akt and ERK1/2 pathways, respectively, on the HT function. (A) Cytosolic and nuclear extracts were prepared from VECs incubated with 50 μM HT for indicated periods and then Western-blotted with anti-Nrf2 antibody as indicated under Materials and Methods. The immunoblots were replicated three times, and a representative blot is shown. (B) Cells were incubated with 50 μM HT for 1 h in the presence or absence of 50 μM LY294002 (LY) or 10 μM U0126 (U0) for 30 min. Nrf2 protein expression was determined as described above for panel A. Total protein was Western-blotted for α-tubulin, and nuclear and cytosolic fractions were blotted for lamin B. The immunoblots were replicated three times, and a representative blot is shown. (C) Immunostaining to detect nuclear localization of Nrf2. Cells were treated as described for panel B and then immunostained to detect the nuclear localization of Nrf2. Staining with FITC-conjugated secondary antibody and PI indicates location of Nrf2 (green) and nucleus (red), respectively, and the merged image of HT-treated cells shows nuclear location of Nrf2 protein. Representative images are from three separate experiments that yielded similar results. Scale bars (a, c, d) = 20 μm; scale bar (b) = 10 μm.

From this experiment, 30 μM HT significantly increased VEC proliferation after 24 h of treatment. However, this concentration was not sufficient to achieve a protective effect against H_2O_2-induced cell damage. For this reason, 50 μM was used to study the effects of HT on VECs.

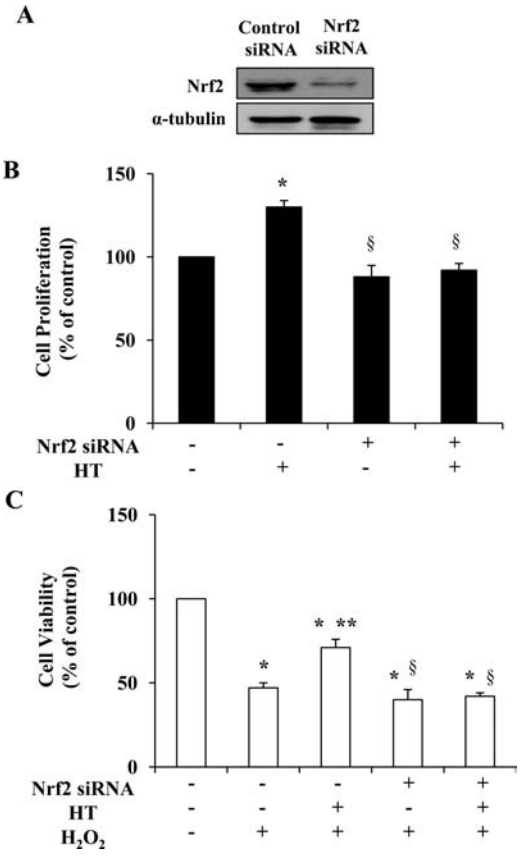


Figure 5. Effect of Nrf2 knockdown on HT-induced VEC proliferation and cytoprotection against H_2O_2. (A) VECs were transfected with control siRNA (100 nM) or Nrf2 siRNA (100 nM) using lipofectamine RNAiMAX and incubated for 48 h. Western blotting confirmed blockade of Nrf2 expression. (B) VECs transfected or not with Nrf2 siRNA were serum-starved and incubated with or without 50 μM HT for 24 h. Cell proliferation was determined using MTS assays. Data are the mean ± SD of four different independent experiments. *, $P < 0.05$ versus untreated cells; §, $P < 0.05$ versus HT-treated cells. (C) VECs transfected or not with Nrf2 siRNA were incubated with or without 50 μM HT for 24 h, washed, and then incubated with 500 μM H_2O_2 for 24 h. Cell viability was determined using MTS assays. Data represent the mean ± SD of four different independent experiments. *, $P < 0.05$ versus untreated cells; **, $P < 0.05$ versus H_2O_2-treated cells; §, $P < 0.05$ versus H_2O_2-treated cells with HT.

Activation of PI3K/Akt and ERK1/2 Signaling Pathways Is Involved in HT-Induced Proliferation and Cytoprotection in VECs. To determine which signaling pathways might be responsible for HT-induced proliferation and cytoprotection, we examined Akt, p38 MAPK, and ERK1/2 phosphorylation in response to HT. These kinases are often involved in cell proliferation and survival during oxidative stress. Hydroxytyrosol at 50 μM time-dependently increased the phosphorylation levels of Akt, p38 MAPK, and ERK1/2 (Figure 3A).

We further investigated whether the activation of Akt, p38 MAPK, and ERK1/2 contributes to HT-dependent proliferation and cytoprotection using the respective kinase specific inhibitors. Cells were preincubated with LY294002, U0126, and SB203580 that are inhibitors for Akt, ERK1/2, and p38 MAPK pathways, respectively, and cultured in the presence of 50 μM HT for 24 h. The results of MTS assays revealed that Akt and ERK1/2 pathways are the ones implicated in HT-dependent proliferative effects in

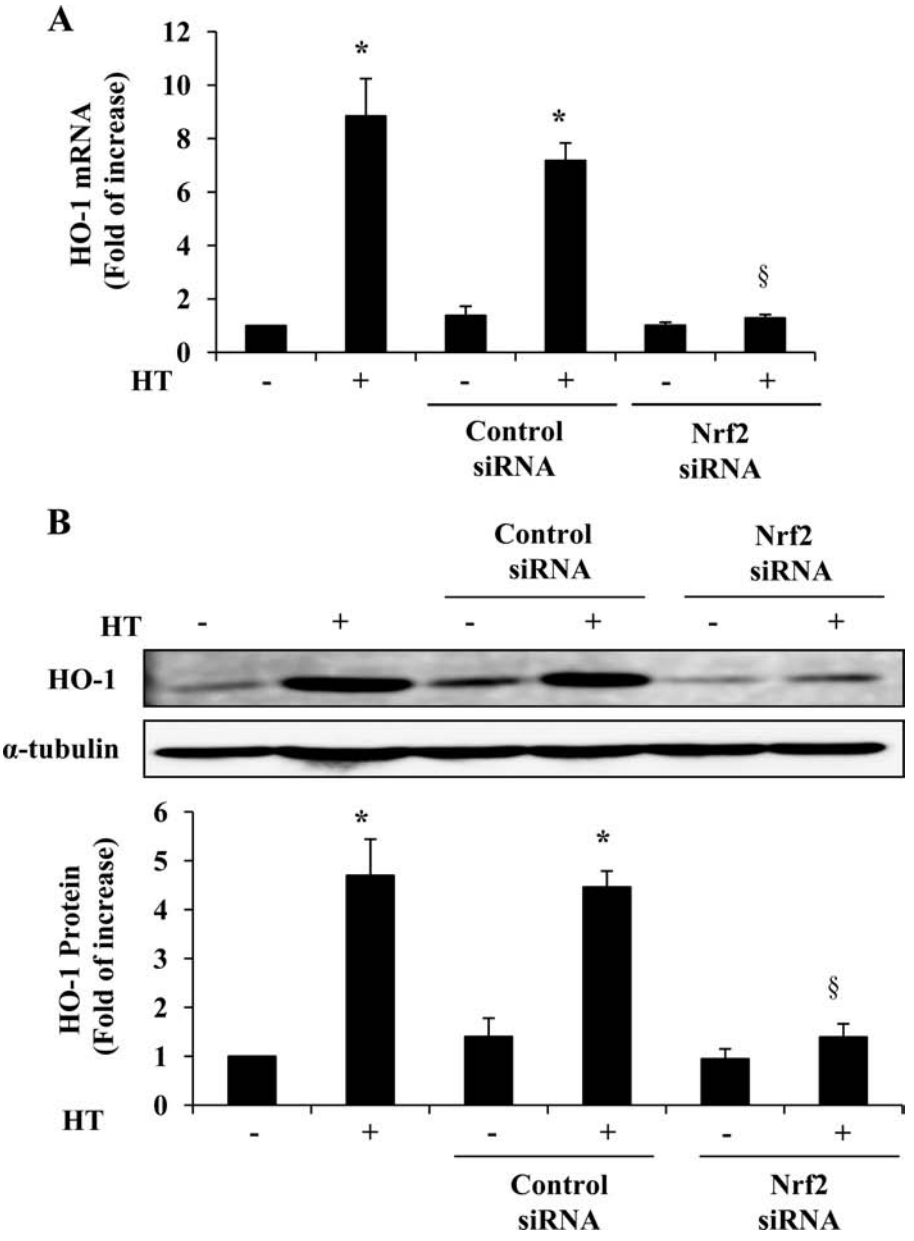


Figure 6. Effect of Nrf2 knockdown on HT-dependent increases in HO-1 mRNA and protein levels. VECs were transfected with control siRNA (100 nM) or Nrf2 siRNA (100 nM) using lipofectamine RNAiMAX and incubated for 48 h. Thereafter, cells were incubated with or without 50 μM HT for 3 and 24 h to determine HO-1 mRNA (A) and protein (B) levels, respectively. Levels of HO-1 mRNA were measured by real time-PCR and normalized by 18S; those of HO-1 protein were assessed by Western blotting and normalized by α-tubulin. Data in both panels A and B represent the mean ± SD of three different independent experiments. *, $P < 0.05$ versus untreated cells; §, $P < 0.05$ versus HT-treated cells.

VECs (Figure 3B). These kinase inhibitors alone have no significant effect on VEC proliferation or survival. Figure 3C shows that HT-dependent cytoprotection was attenuated by LY294002 and U0126, but not by SB203580, suggesting that the PI3K/Akt and ERK1/2 pathways are involved in HT-induced cell proliferation and cytoprotection against oxidative stress, whereas p38 MAPK is not.

Up-regulation and Nuclear Translocation of Nrf2 Induced by HT Are Regulated by PI3K/Akt and ERK1/2 Pathways. Because Nrf2 is a critical transcription factor for regulating the expression of many antioxidant/detoxifying enzymes, we examined whether HT induces Nrf2 nuclear translocation in VECs (Figure 4A). Nrf2 protein was significantly elevated within 1 h in the total and cytosolic fractions, and these increased levels were maintained for at least 6 h after adding 50 μM HT. Nuclear Nrf2 protein also increased and peaked between 0.5 and 1 h, suggesting that Nrf2 expression is increased in response to HT and then translocated from the cytosol into the nucleus.

Because several recent papers have demonstrated the contribution of the PI3K/Akt and ERK1/2 pathways to Nrf2 activation,[20−22,31] we determined whether these pathways are also involved in the HT-induced activation of Nrf2 in VECs. These cells were stimulated with 50 μM HT for 1 h in the presence or absence of U0126 or LY294002. Figure 4B shows that each inhibitor reduced the increase in Nrf2 protein expression in all fractions. The immunostaining results also indicated that these inhibitors

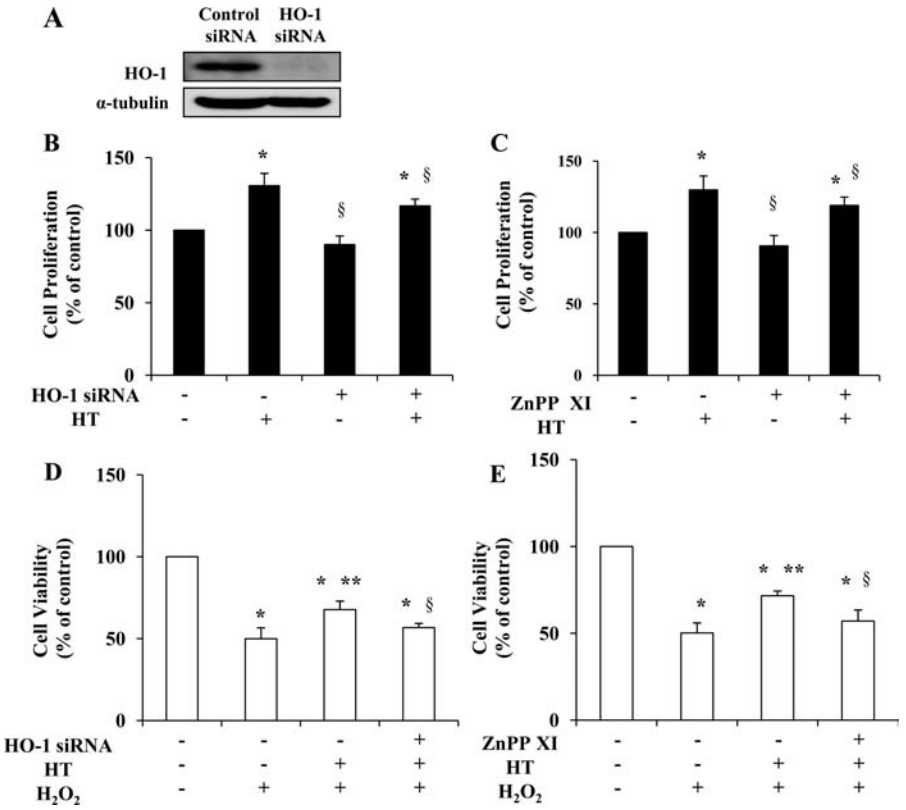


Figure 7. Role of HO-1 in HT-induced cell proliferation and cytoprotection against H_2O_2. (A) VECs were transfected with control siRNA (100 nM) or HO-1 siRNA (100 nM) using lipofectamine RNAiMAX and incubated for 48 h. Western blotting verified blockade of HO-1 expression. (B) VECs transfected or not with HO-1 siRNA were serum-starved and then incubated with 50 μM HT for 24 h. Cell proliferation was determined using MTS assays. Data are the mean ± SD of four different independent experiments. ∗, $P < 0.05$ versus untreated cells; §, $P < 0.05$ versus HT-treated cells. (C) Serum-starved VECs were incubated with or without 50 μM HT for 24 h in the presence or absence of 10 μM ZnPP IX. Cell proliferation was determined using MTS assays. Data are the mean ± SD of four different independent experiments. ∗, $P < 0.05$ versus untreated cells; §, $P < 0.05$ versus HT-treated cells. (D) Transfected or nontransfected VECs were incubated with or without 50 μM HT for 24 h followed by 500 μM H_2O_2 for 24 h. Cell viability was determined using MTS assays. Data represent the mean ± SD of four different independent experiments. ∗, $P < 0.05$ versus untreated cells; ∗∗, $P < 0.05$ versus H_2O_2-treated cells; §, $P < 0.05$ versus H_2O_2-treated cells with HT. (E) VECs were incubated with or without 50 μM HT and with or without 10 μM ZnPP IX for 24 h, washed, and then incubated for 24 h with 500 μM H_2O_2. Cell viability was determined using MTS assays. Data represent the mean ± SD of four different independent experiments. ∗, $P < 0.05$ versus untreated cells; ∗∗, $P < 0.05$ versus H_2O_2-treated cells; §, $P < 0.05$ versus H_2O_2-treated cells with HT.

decreased HT-dependent Nrf2 translocation into the nucleus (Figure 4C). These results suggest that HT activates Nrf2 through the PI3K/Akt and ERK1/2 pathways in VECs.

Nrf2 Is Involved in HT-Dependent Cell Proliferation and Cytoprotection against H_2O_2 in VECs. To determine whether or not Nrf2 is responsible for the increase in cell proliferation and protection against H_2O_2 cytotoxicity in response to HT, we knocked down Nrf2 using a small interfering RNA (siRNA). VECs were transiently transfected with Nrf2 siRNA or nonspecific control siRNA for 48 h. Under this condition, Nrf2 siRNA effectively reduced the mRNA level of Nrf2 (Figure 5A). As shown in Figure 5B, HT-induced proliferation of VECs was nearly completely blocked by silencing Nrf2 expression. Similarly, HT-induced cytoprotection was significantly reduced by Nrf2 siRNA (Figure 5C). These findings indicate that Nrf2 plays a pivotal role in HT-induced proliferation and cytoprotection against H_2O_2 in VECs.

HT-Induced HO-1 Expression Is Completely Dependent on Nrf2 Activation. Nrf2 is a major transcriptional up-regulator responsible for various antioxidant/detoxifying enzymes including HO-1.[15] We investigated whether or not HT induces HO-1 expression via Nrf2 activation in VECs. Figure 6 shows that HT significantly increased the mRNA and protein levels of HO-1, a process that was almost completely blocked by transfection with Nrf2 siRNA, but not with control siRNA. These findings indicate that Nrf2 is an important transcriptional regulator for HT-induced HO-1 expression in VECs.

HO-1 Is Involved in HT-Dependent Cell Proliferation and Cytoprotection against H_2O_2 in VECs. We investigated the role of HO-1 in the proliferative and cytoprotective effects of HT in VECs using HO-1 siRNA and zinc protoporphyrin IX (ZnPP IX), a selective inhibitor of HO-1. The HO-1 siRNA efficiently reduced the protein level of HO-1 (Figure 7A), but did not exert a deleterious effect on cell survival, although it significantly decreased HT-dependent cell proliferation (Figure 7B). Similarly, ZnPP IX significantly reduced the HT-induced cell proliferation (Figure 7C). The protective effect of HT against H_2O_2-induced cell death was attenuated by HO-1 siRNA and ZnPP IX, respectively (Figure 7D,E). Therefore, the proliferative and cytoprotective effects of HT are probably mediated, at least in part, through HO-1 induction in VECs.

HT-Induced HO-1 Expression Reduces Intracellular ROS Production. We investigated whether HO-1 induction is involved in reducing intracellular ROS production in VECs

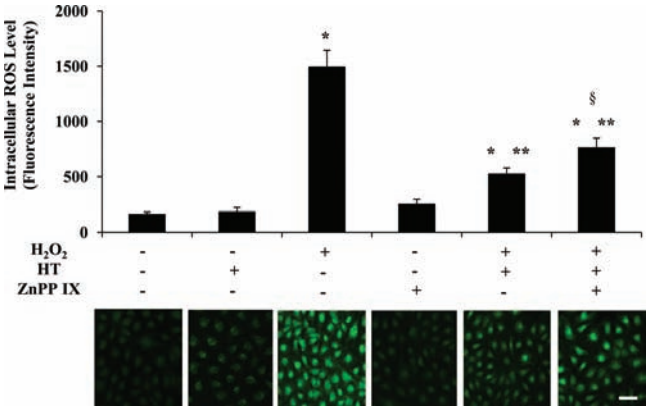


Figure 8. Effect of inhibition of HO-1 on intracellular ROS. VECs were incubated with or without 50 μM HT for 24 h and with or without 10 μM ZnPP IX, washed, and incubated with 5 μM DCF-DA for 15 min followed by 500 μM H_2O_2 for another 15 min. Other experimental procedures are described under Materials and Methods. Data are the mean ± SD of three different independent experiments. ∗, $P < 0.05$ versus untreated cells or ZnPP IX treated cells; ∗∗, $P < 0.05$ versus H_2O_2-treated cells; §, $P < 0.05$ versus H_2O_2-treated cells with HT. Scale bar = 50 μm.

(Figure 8). Incubating the cells with 500 μM H_2O_2 significantly increased intracellular ROS levels within 15 min, as compared with untreated cells. Treatment of the cells with 50 μM HT for 24 h beforehand obviously prevented the intracellular ROS increase induced by 500 μM H_2O_2. This HT effect was attenuated by ZnPP IX, indicating that HO-1 induction is involved in reducing intracellular ROS levels induced by H_2O_2, which leads to HT-dependent protection from oxidative stress.

DISCUSSION

Previous studies have demonstrated that HT, a constituent of olive oil and leaves, helps to prevent cardiovascular diseases.[3,9−12] Several papers have indicated an inhibitory effect of HT on the expression of cell adhesion molecules such as ICAM and VCAM in cultured VECs because monocyte adhesion to the endothelium is crucial in early atherogenesis.[13,24,26] However, a direct effect of HT on cell proliferation and cytoprotection from oxidative stress has not been investigated in detail using cultured VECs. The present study found that (i) HT efficiently stimulates cell proliferation, promotes wound repair, and protects VECs from oxidant-induced damage through PI3K/Akt and ERK1/2 pathways; (ii) HT up-regulates and activates Nrf2 via PI3K/Akt and ERK1/2 pathways; (iii) HT-dependent Nrf2 activation and HO-1 induction are critically involved in these HT functions; and (iv) HO-1, at least in part, contributes to HT-induced cytoprotection from oxidative stress as a downstream effector of Nrf2 by reducing intracellular ROS. Our findings at both cellular and molecular levels have shown a positive effect of HT on VECs and strongly support the notion that this olive phenolic compound could prevent the pathogenesis of atherosclerosis.

Nrf2 is involved in the modulation of the antioxidant defense system against the toxicity of electrophiles and ROS by promoting the expression of various antioxidant/detoxifying enzymes through interaction with the ARE.[32,33] Herein, we found that HT increases the expression of Nrf2 and elevates its nuclear translocation, resulting in the enhanced expression of HO-1.We also demonstrated that transfection of VECs with specific Nrf2 siRNA, which has no cytotoxic effect on control cells, caused a significant reduction in both cell proliferation and H_2O_2-induced cell death (Figure 5B,C). These results suggest that HT-induced cell proliferation and cytoprotection of VECs from oxidative stress are critically dependent on Nrf2 activation. Such involvement of Nrf2 in cell proliferation is consistent with the recent findings of Homma et al.[34] indicating that the inhibition of Nrf2 function using Nrf2 siRNA reduced the proliferation of lung cancer cells. In addition, they also demonstrated that the antioxidant *N*-acetylcysteine did not reverse the proliferation of Nrf2-silenced cells. Therefore, that ROS removal via the Nrf2-mediated signaling pathway leads simply to the proliferation of these cancer cells is unlikely. Furthermore, Nrf2 disruption arrests the cell cycle at the G2-M phase via impaired GSH-induced redox signaling in cultured primary epithelial cells.[35] These data and ours suggest that Nrf2 plays an important role in HT-induced cell proliferation and in cytoprotection from oxidative stress. The molecular mechanism involved in these Nrf2 dependent effects will be the focus of our future study.

We further investigated the role that the phosphorylation of PI3K/Akt and ERK1/2 plays in VEC proliferation and cytoprotection against H_2O_2 damage. We found that HT activates Nrf2 by stimulating the upstream PI3K/Akt and ERK1/2 pathways in VECs. Similar findings in human HepG2 liver cells have recently been reported.[31] Other studies have also revealed that antioxidant compounds can exert cytoprotective effects against oxidative stress through PI3K/Akt and ERK pathway activation.[20,22,36] However, Hwang and Jeong[37] reported that PI3K/Akt and p38 pathways mediate Nrf2 activation induced by the coffee diterpene, kahweol, and protect human dopaminergic neurons against oxidative stress derived from 6-hydroxydopamine. We consider that p38 MAPK, although phosphorylated by HT, is probably not responsible for the Nrf2 activation of HT functions in VECs because the p38 MAPK inhibitor SB203580 did not inhibit both HT-induced cell proliferation and cytoprotection or HO-1 expression (data not shown) in our study. Meanwhile, Ogborne et al.[38] found that protein kinase Cδ phosphorylates and activates Nrf2 in response to epigallocatechin in green tea. The possibility that protein kinase Cδ mediates HT-dependent Nrf2 activation in VECs in addition to the PI3K/Akt and ERK pathways cannot be excluded.

In this research, we examined the role of HO-1 as a downstream effector of Nrf2 in HT-induced VEC proliferation and cytoprotection from oxidative stress. We showed that the inhibition of HO-1 functions using siRNA and HO-1 inhibitor (ZnPP XI) significantly reduced HT-induced VEC proliferation, cytoprotection, and ROS generation (Figures 7 and 8). These results are in agreement with findings of others suggesting that HO-1 participates in the promotion of cell protection and survival.[36,39−41] Our findings of a partial role of HO-1 in these HT functions in VECs indicated that other antioxidant/detoxifying enzyme(s) could be involved. Glutathione peroxidase (GPx), which eliminates peroxides,[42] is a candidate consistent with the findings of Martin et al.[31] indicating that HT induces GPx expression in HepG2 cells. Moreover, we demonstrated that HT-induced HO-1 expression was completely dependent on Nrf2 in VECs (Figure 6), although HO-1 expression is regulated not only by Nrf2 but also by the transcription factor activating protein-1 (AP-1).[43] In agreement with these data, SP600125, an inhibitor for the AP-1 up-regulator JNK, had no effect on HO-1 expression stimulated by HT in VECs (data not shown).

Additionally, it should be pointed that the main phenolic compounds in olive oil, in particular HT and oleuropein, are well absorbed in the small intestine and metabolized in the body as a

response to the intake of virgin olive oil as well as olives. In human plasma, HT and its metabolites have been reported to be around 25 μM after the consumption of a dose (40 mL) close to that used as a daily intake in Mediterranean countries,[44] although the study carried out by Visioli et al.[45] suggested that when administrated in low-fat yogurt or HT-enriched refined oil, HT recovery must be higher in circulating blood or stored in tissue. The concentration of HT and its metabolites in the body might be different depending on the vehicle used in the administration. Moreover, in vivo, HT and its metabolites may have additive or synergetic effects with other phenolic compounds present in olive oil or in the Mediterranean diet.[8]

In conclusion, we found for the first time, to the best of our knowledge, that HT enhances proliferation and confers cytoprotection from oxidative stress in VECs dependently on Nrf2 through activation of the PI3K/Akt and ERK1/2 pathways and HO-1 induction. The present study provides a molecular basis for the contribution of HT to the benefits of the Mediterranean diet and offers a therapeutic potential of HT in the prevention and/or treatment of vascular diseases.

AUTHOR INFORMATION

Corresponding Author

*Phone: 81-29-853-7720. Fax: 81-29-853-7723. E-mail: hitomy1@sakura.cc.tsukuba.ac.jp.

Funding Sources

This work was supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

ACKNOWLEDGMENT

We thank Norma Foster for help in preparing the manuscript.

ABBREVIATIONS USED

HT, hydroxytyrosol; Nrf2, nuclear factor-E2-related factor-2; HO-1, heme oxygenase-1; ROS, reactive oxygen species; ARE, antioxidant response elements; PI3K, phosphatidylinositol-3-kinase; ERK1/2, extracellular signal-regulated kinase 1/2; Keap1, Kelch-like ECH-associated protein 1; MAPK, mitogen-activated protein kinase; H_2O_2, hydrogen peroxide; siRNA, small interfering RNA.

REFERENCES

(1) Covas, M. I. Benefits of the Mediterranean diet on cardiovascular disease. *Future Cardiol.* **2007**, *3*, 575–578.

(2) Ortega, R. Importance of functional foods in the Mediterranean diet. *Public Health Nutr.* **2006**, *9*, 1136–1140.

(3) Carluccio, M. A.; Massaro, M.; Scoditti, E.; De Caterina, R. Vasculoprotective potential of olive oil components. *Mol. Nutr. Food Res.* **2007**, *51*, 1225–1234.

(4) Waterman, E.; Lockwood, B. Active components and clinical applications of olive oil. *Altern. Med. Rev.* **2007**, *12*, 331–342.

(5) Covas, M. I.; Konstantinidou, V.; Fitó, M. Olive oil and cardiovascular health. *J. Cardiovasc. Pharmacol.* **2009**, *54*, 477–482.

(6) Fitó, M.; de la Torre, R.; Covas, M. I. Olive oil and oxidative stress. *Mol. Nutr. Food Res.* **2007**, *51*, 1215–1224.

(7) Perona, J. S.; Cabello-Moruno, R.; Ruiz-Gutierrez, V. J. The role of virgin olive oil components in the modulation of endothelial function. *Nutr. Biochem.* **2006**, *17*, 429–445.

(8) Granados-Principal, S.; Quiles, J. L.; Ramirez-Tortosa, C. L.; Sanchez-Rovira, P.; Ramirez-Tortosa, M. C. Hydroxytyrosol: from laboratory investigations to future clinical trials. *Nutr. Rev.* **2010**, *68*, 191–206.

(9) Bullon, P.; Quiles, J. L.; Morillo, J. M.; Rubini, C.; Goteri, G.; Granados-Principal, S.; Battino, M.; Ramirez-Tortosa, M. Gingival vascular damage in atherosclerotic rabbits: hydroxytyrosol and squalene benefits. *Food Chem. Toxicol.* **2009**, *47*, 2327–2331.

(10) González-Santiago, M.; Martín-Bautista, E.; Carrero, J. J.; Fonollá, J.; Baró, L.; Bartolomé, M. V.; Gil-Loyzaga, P.; López-Huertas, E. One-month administration of hydroxytyrosol, a phenolic antioxidant present in olive oil, to hyperlipemic rabbits improves blood lipid profile, antioxidant status and reduces atherosclerosis development. *Atherosclerosis* **2006**, *188*, 35–42.

(11) Jemai, H.; Fki, I.; Bouaziz, M.; Bouallagui, Z.; El Feki, A.; Isoda, H.; Sayadi, S. Lipid-lowering and antioxidant effects of hydroxytyrosol and its triacetylated derivative recovered from olive tree leaves in cholesterol-fed rats. *J. Agric. Food Chem.* **2008**, *56*, 2630–2636.

(12) Rietjens, S. J.; Bast, A.; de Vente, J.; Haenen, G. R. The olive oil antioxidant hydroxytyrosol efficiently protects against the oxidative stress-induced impairment of the NObullet response of isolated rat aorta. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, *292*, H1931–H1936.

(13) Dell'Agli, M.; Fagnani, R.; Mitro, N.; Scurati, S.; Masciadri, M.; Mussoni, L.; Galli, G. V.; Bosisio, E.; Crestani, M.; De Fabiani, E.; Tremoli, E.; Caruso, D. Minor components of olive oil modulate proatherogenic adhesion molecules involved in endothelial activation. *J. Agric. Food Chem.* **2006**, *54*, 3259–3264.

(14) Surh, Y. J.; Kundu, J. K.; Na, H. K. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. *Planta Med.* **2008**, *74*, 1526–1539.

(15) Mann, G. E.; Niehueser-Saran, J.; Watson, A.; Gao, L.; Ishii, T.; de Winter, P.; Siow, R. C. Nrf2/ARE regulated antioxidant gene expression in endothelial and smooth muscle cells in oxidative stress: implications for atherosclerosis and preeclampsia. *Sheng Li Xue Bao* **2007**, *59*, 117–127.

(16) Motohashi, H.; Yamamoto, M. Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol. Med.* **2004**, *11*, 549–557.

(17) Nguyen, T.; Nioi, P.; Pickett, C. B. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. *J. Biol. Chem.* **2009**, *284*, 13291–13295.

(18) Osburn, W. O.; Kensler, T. W. Nrf2 signaling: an adaptive response pathway for protection against environmental toxic insults. *Mutat. Res.* **2009**, *659*, 31–39.

(19) Satoh, T.; Kosaka, K.; Itoh, K.; Kobayashi, A.; Yamamoto, M.; Shimojo, Y.; Kitajima, C.; Cui, J.; Kamins, J.; Okamoto, S.; Izumi, M.; Shirasawa, T.; Lipton, S. A. Carnosic acid, a catechol-type electrophilic compound, protects neurons both in vitro and in vivo through activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1. *J. Neurochem.* **2008**, *104*, 1116–1131.

(20) Kim, J. W.; Li, M. H.; Jang, J. H.; Na, H. K.; Song, N. Y.; Lee, C.; Johnson, J. A.; Surh, Y. J. 15-Deoxy-Δ(12,14)-prostaglandin J(2) rescues PC12 cells from H_2O_2-induced apoptosis through Nrf2-mediated upregulation of heme oxygenase-1: potential roles of Akt and ERK1/2. *Biochem. Pharmacol.* **2008**, *76*, 1577–1589.

(21) Kim, K. C.; Kang, K. A.; Zhang, R.; Piao, M. J.; Kim, G. Y.; Kang, M. Y.; Lee, S. J.; Lee, N. H.; Surh, Y. J.; Hyun, J. W. Up-regulation of Nrf2-mediated heme oxygenase-1 expression by eckol, a phlorotannin compound, through activation of Erk and PI3K/Akt. *Int. J. Biochem. Cell Biol.* **2010**, *42*, 297–305.

(22) Na, H. K.; Kim, E. H.; Jung, J. H.; Lee, H. H.; Hyun, J. W.; Surh, Y. J. (−)-Epigallocatechin gallate induces Nrf2-mediated antioxidant enzyme expression via activation of PI3K and ERK in human mammary epithelial cells. *Arch. Biochem. Biophys.* **2008**, *476*, 171–177.

(23) Niture, S. K.; Jain, A. K.; Jaiswal, A. K. Antioxidant-induced modification of INrf2 cysteine 151 and PKC-δ-mediated phosphorylation of Nrf2 serine 40 are both required for stabilization and nuclear translocation of Nrf2 and increased drug resistance. *J. Cell Sci.* **2009**, *122*, 4452–4464.

(24) Carluccio, M. A.; Ancora, M. A.; Massaro, M.; Carluccio, M.; Scoditti, E.; Distante, A.; Storelli, C.; De Caterina, R. Homocysteine induces VCAM-1 gene expression through NF-κB and NAD(P)H oxidase activation: protective role of Mediterranean diet polyphenolic antioxidants. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, *293*, H2344–H2354.

(25) Carluccio, M. A.; Siculella, L.; Ancora, M. A.; Massaro, M.; Scoditti, E.; Storelli, C.; Visioli, F.; Distante, A.; De Caterina, R. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. *Arterioscler. Thromb. Vasc. Biol.* **2003**, *23*, 622–629.

(26) Manna, C.; Napoli, D.; Cacciapuoti, G.; Porcelli, M.; Zappia, V. Olive oil phenolic compounds inhibit homocysteine-induced endothelial cell adhesion regardless of their different antioxidant activity. *J. Agric. Food Chem.* **2009**, *57*, 3478–3482.

(27) Watanabe, H.; Kuhne, W.; Spahr, R.; Schwartz, P.; Piper, H. M. Macromolecule permeability of coronary and aortic endothelial monolayers under energy depletion. *Am. J. Physiol.* **1991**, *260*, H1344–H1352.

(28) Shimizu, H.; Toyama, O.; Shiota, M.; Kim-Mitsuyama, S.; Miyazaki, H. Protein tyrosine phosphatase LMW-PTP exhibits distinct roles between vascular endothelial and smooth muscle cells. *J. Recept. Signal Transduct Res.* **2005**, *25*, 19–33.

(29) Shimizu, H.; Hirose, Y.; Nishijima, F.; Tsubakihara, Y.; Miyazaki, H. ROS and PDGF-beta receptors are critically involved in indoxyl sulfate actions that promote vascular smooth muscle cell proliferation and migration. *Am. J. Physiol. Cell Physiol.* **2009**, *297*, C389–C396.

(30) Lee, H.; Goetzl, E. J.; An, S. Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. *Am. J. Physiol. Cell Physiol.* **2000**, *278*, C612–C618.

(31) Martín, M. A.; Ramos, S.; Granado-Serrano, A. B.; Rodríguez-Ramiro, I.; Trujillo, M.; Bravo, L.; Goya, L. Hydroxytyrosol induces antioxidant/detoxificant enzymes and Nrf2 translocation via extracellular regulated kinases and phosphatidylinositol-3-kinase/protein kinase B pathways in HepG2 cells. *Mol. Nutr. Food Res.* **2010**, *54*, 956–966.

(32) Itoh, K.; Tong, K. I.; Yamamoto, M. Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. *Free Radic. Biol. Med.* **2004**, *36*, 1208–1213.

(33) Jaiswal, A. K. Nrf2 signaling in coordinated activation of antioxidant gene expression. *Free Radical Biol. Med.* **2004**, *36*, 1199–1207.

(34) Homma, S.; Ishii, Y.; Morishima, Y.; Yamadori, T.; Matsuno, Y.; Haraguchi, N.; Kikuchi, N.; Satoh, H.; Sakamoto, T.; Hizawa, N.; Itoh, K.; Yamamoto, M. Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer. *Clin. Cancer Res.* **2009**, *15*, 3423–3432.

(35) Reddy, N. M.; Kleeberger, S. R.; Bream, J. H.; Fallon, P. G.; Kensler, T. W.; Yamamoto, M.; Reddy, S. P. Genetic disruption of the Nrf2 compromises cell-cycle progression by impairing GSH-induced redox signaling. *Oncogene* **2008**, *27*, 5821–5832.

(36) Choi, B. M.; Kim, B. R. Upregulation of heme oxygenase-1 by brazilin via the phosphatidylinositol 3-kinase/Akt and ERK pathways and its protective effect against oxidative injury. *Eur. J. Pharmacol.* **2008**, *580*, 12–18.

(37) Hwang, Y. P.; Jeong, H. G. The coffee diterpene kahweol induces heme oxygenase-1 via the PI3K and p38/Nrf2 pathway to protect human dopaminergic neurons from 6-hydroxydopamine-derived oxidative stress. *FEBS Lett.* **2008**, *582*, 2655–2662.

(38) Ogborne, R. M.; Rushworth, S. A.; O'Connell, M. A. Epigallocatechin activates haem oxygenase-1 expression via protein kinase Cδ and Nrf2. *Biochem. Biophys. Res. Commun.* **2008**, *373*, 584–588.

(39) Chen, M. C.; Ye, Y. Y.; Ji, G.; Liu, J. W. Hesperidin upregulates heme oxygenase-1 to attenuate hydrogen peroxide-induced cell damage in hepatic L02 cells. *J. Agric. Food Chem.* **2010**, *58*, 3330–3335.

(40) Furukawa, Y.; Urano, T.; Minamimura, M.; Nakajima, M.; Okuyama, S.; Furukawa, S. 4-Methylcatechol-induced heme oxygenase-1 exerts a protective effect against oxidative stress in cultured neural stem/progenitor cells via PI3 kinase/Akt pathway. *Biomed. Res.* **2010**, *31*, 45–52.

(41) Wei, Y.; Liu, X. M.; Peyton, K. J.; Wang, H.; Johnson, F. K.; Johnson, R. A.; Durante, W. Hypochlorous acid-induced heme oxygenase-1 gene expression promotes human endothelial cell survival. *Am. J. Physiol. Cell Physiol.* **2009**, *297*, C907–C915.

(42) Lei, X. G.; Cheng, W. H.; McClung, J. P. Metabolic regulation and function of glutathione peroxidase-1. *Annu. Rev. Nutr.* **2007**, *27*, 41–61.

(43) Harada, H.; Sugimoto, R.; Watanabe, A.; Taketani, S.; Okada, K.; Warabi, E.; Siow, R.; Itoh, K.; Yamamoto, M.; Ishii, T. Differential roles for Nrf2 and AP-1 in upregulation of HO-1 expression by arsenite in murine embryonic fibroblasts. *Free Radical Res.* **2008**, *42*, 297–304.

(44) Covas, M. I.; de la Torre, K.; Farré-Albaladejo, M.; Kaikkonen, J.; Fitó, M.; López-Sabater, C.; Pujadas-Bastardes, M. A.; Joglar, J.; Weinbrenner, T.; Lamuela-Raventós, R. M.; de la Torre, R. Postprandial LDL phenolic content and LDL oxidation are modulated by olive oil phenolic compounds in humans. *Free Radical Biol. Med.* **2006**, *40*, 608–616.

(45) Visioli, F.; Galli, C.; Grande, S.; Colonnelli, K.; Patelli, C.; Galli, G.; Caruso, D. Hydroxytyrosol excretion differs between rats and humans and depends on the vehicle of administration. *J. Nutr.* **2003**, *133*, 2612–2615.