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YS 51, 1-(β-naphtylmethyl)-6,7-dihydroxy-1,2,3,4,-tetrahydroisoquinoline, protects endothelial cells against hydrogen peroxide-induced injury via carbon monoxide derived from heme oxygenase-1

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ABSTRACT

Oxidative stress plays an important role in the pathophysiology of several vascular diseases such as atherosclerosis, and great attention has been placed on the protective role of heme oxygenase-1 (HO-1) for vasculature against oxidant-induced injury. We tested whether the protective effects of YS 51, 1-(β-naphtyl-methyl)-6,7-dihydroxy-1,2,3,4,-tetrahydroisoquinoline, against hydrogen peroxide (H_2O_2)-induced cell injury is associated with HO-1 activity in bovine aortic endothelial cells (BAEC). YS 51 increased HO-1 expression and activity in concentration-dependent manners (10–100 μM) and time-dependent manners (1, 3, 6, 18 h), which were correlated well with its protective effect against H_2O_2-induced injury. Zinc protoporphyrin IX (ZnPP IX), a HO inhibitor, significantly inhibited the effect of YS 51 (50 μM). In contrast, $[Ru(CO)_3(Cl)_2]_2$ (CORM-2, a CO releasing molecule) but not bilirubin protected against H_2O_2-induced injury. Oxyhemoglobin (HbO_2) used as a CO scavenger significantly inhibited the protective effect of both YS 51 and CORM-2. Furthermore, both YS 51 and CORM-2 significantly reduced H_2O_2-induced intracellular reactive oxygen species (ROS) production; however, this was counteracted by ZnPP IX, HbO_2 and deferoxamine. We found evidence for the involvement of PI3/Akt kinase and ERK1/2 pathways in HO-1 induction by YS-51. Taken together, we conclude that CO is, at least, responsible for the YS 51-mediated protective action of endothelial cells against oxidant stress via HO-1 gene induction, involving the activation of the PI3/Akt and ERK1/2 kinase pathways. Thus, YS 51 may be useful in oxidative stress-induced vascular disorders.

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Abbreviations: HO, heme oxygenase; ROS, reactive oxygen species; H_2O_2, hydrogen peroxide; CO, carbon monoxide; PI3 kinase, phosphatidylinositol 3 kinase; CORM-2, CO releasing molecule; $[Ru(CO)_3(Cl)_2]_2$, tricarbonyldichloro-ruthenium dimmer; DPPH, 1,1-dipheny-2-picrylhydrazyl; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescin; NF-κB, nuclear factor kappa-B; iNOS, increase inducible nitric oxide synthase; ZnPP IX, zinc protoporphyrin IX.

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1. Introduction

Oxidative stress, a cellular imbalance between the production and elimination of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2) and peroxynitrite, is a serious causative factor of vascular endothelial dysfunction and plays an important role in the pathophysiology of several vascular diseases such as atherosclerosis, diabetes and hypertension [1]. In particular, H_2O_2-induced oxidative stress leads to the death of endothelial and many other cell types [2]. Vascular endothelial cells respond to oxidative stress by invoking endogenous antioxidant defense mechanisms [3]. One stress-induced protein is heme oxygenase (HO). Several lines of evidence are accumulating that HO-1, known as heat shock protein 32, plays a fundamental role in human atherosclerotic lesions [4] and protects vascular endothelial and smooth muscle cells against oxidative stress, such as that caused by oxidized low density lipoprotein (LDL) [5,6]. The primary function of HO-1 is the degradation of heme to carbon monoxide (CO), iron, and biliverdin, and the latter is subsequently converted to bilirubin by biliverdin reductase [7]. In the vasculature, the products of HO-1 activity, bilirubin and CO, have been shown to protect endothelial cells from oxidative stress generated by such agents as heme, H_2O_2, and tumor necrosis factor [8–10]. Thus, HO-1 activity could have a major influence on the biological responses to oxidant stress in endothelial cells. Indeed, several studies have shown that some naturally occurring chemicals (e.g., flavonoids, vitamins and curcumin) or synthetic ones can protect endothelial cells against oxidative stress by stimulating HO-1 production [11,12].

We have shown that YS 51, 1-(β-naphtylmethyl)-6,7-dihydroxy-1,2,3,4,-tetrahydroisoquinoline, inhibits iNOS expression in macrophage cells and vascular smooth muscles when they are activated with cytokines and/or lipopolysaccharide [13]. We have also found that YS 51 enhances the expression of manganese-superoxide dismutase (Mn-SOD) mRNA in endothelial cells [14]. In addition, YS 51 shows antiplatelet and antithrombotic effects [15]. These findings suggest that YS 51 may be therapeutic for cardiovascular disorders. Thus, we hypothesized that YS 51 may protect endothelial cells against hydrogen peroxide (H_2O_2)-induced oxidative injury, which is associated with the increased levels of HO-1 induced by YS 51. We found here that YS 51 induces HO-1 through the PI3/Akt kinase and ERK pathways in bovine aortic endothelial cells (BAEC) and protects cells against H_2O_2-mediated cell injury; moreover, CO plays a key role in this protective effect.

2. Materials and methods

2.1. Chemicals

YS 51 were synthesized by us as described in US patent 562837 B1 (2003). Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), and other culture reagents were purchased from Gibco BRL (Grand Island, NY). Zinc protoporphyrin IX (ZnPP IX), an inhibitor of HO activity, LY294002, SB203580 and SP60125 were from Calbiochem (San Diego, CA). Hydrogen peroxide (H_2O_2) was purchased from Junsei Chemical Co. (Tokyo, Japan). Anti-HO-1 antibody was from Stressgen (Victoria, Canada), and antiphosphorylated (p)-Akt antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Tricarbonyldichloro-ruthenium dimer ($[Ru(CO)_3(Cl)_2]_2$, CORM-2), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), N-acetyl-cysteine (NAC), and bovine hemoglobin (Hb) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals including PD98059 were obtained from Sigma–Aldrich.

2.2. Cell culture

Bovine aortic endothelial cells (BAEC) were purchased from American Type Culture Collection (Arlington, VA). Cells were cultured in 100 mm dishes and grown in DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated FBS in a humidified atmosphere of 5% CO_2. Cells were plated at a density of 1×10^7 cells/100 mm dish and used between passages 6 and 14.

2.3. Cell treatment

Stock solutions of YS 51, ZnPP IX, or CORM-2 were dissolved in DMSO, diluted with distilled water and used at the indicated concentrations. Endothelial cell injury was induced by incubation with H_2O_2 (200 μM) for 8 h. Oxyhemoglobin (HbO_2) was prepared by reduction of bovine hemoglobin with sodium hydrosulfite followed by gel filtration with a prepacked disposable column (PD-10, Pharmacia, Uppsala, Sweden), previously equilibrated with 50 mM Tris/HCl at pH 7.4 [16]. The concentration of HbO_2 was determined using a Perkin-Elmer Lambda 5 spectrophotometer at 576 nm wavelength, according to Kondo et al. [17].

2.4. Assay for HO enzyme activity

To determine HO enzyme activity, confluent cells were incubated in 100 mm culture dishes for 9 h with or without YS 51. Some experiments were done in the presence of a HO enzyme inhibitor, ZnPP IX. After incubation, the cells were washed twice with 1 × phosphate-buffered saline (PBS, pH 7.4) and centrifuged (100,000 × *g*, 5 min, 4 °C). The cell pellet was suspended in 2 mM $MgCl_2$ in 100 mM phosphate buffer (pH 7.4), frozen at −70 °C, thawed three times and finally sonicated on ice before centrifugation at 100,000 × *g* for 15 min at 4 °C. The supernatant (400 μL) was added to an NADPH-generating system containing 0.8 mM NADPH, 2 mM glucose-6-phosphate, 0.2 U glucose-6-phosphate-1-dehydrogenase, 2 mg protein of rat liver cytosol (prepared from the 105,000 × *g* supernatant fraction), 100 mM potassium phosphate buffer (pH 7.4), and hemin (10 μM) in a final volume of 200 μL. The reaction was incubated for 1 h at 37 °C in the dark and terminated by addition of 600 μL chloroform. The extracted bilirubin was calculated by the difference in absorption between 464 and 530 nm using a quartz cuvette (ε = 40 mM/cm). HO-1 activity was represented as picomoles of bilirubin formed per milligram of protein per hour.

2.5. Detection of LDH release

Cells were pretreated with YS 51 (10–100 μM) for 1 h, and then stimulated with H_2O_2 (200 μM) for 8 h in 24-well plates

in the presence or absence of YS 51. Cell death induced by culture conditions was measured in terms of LDH released into the supernatants of cells. Briefly, 50 μL of standards or 50 μL of culture supernatant (controls and treated cells) and 50 μL of substrate mix were transferred into a 96-well-plate and incubated for 30 min at room temperature. Plates were read out with a SUNRISE ELISA-plate Reader (Tecan, Crailsheim, Germany) at 490 nm wavelength. LDH released from the cells was expressed as percentage of total cellular LDH, which was measured after lysis of the cells by addition of Triton X-100 to a final concentration of 0.1% in each well. Only YS 51 toxicity was measured with MTT (0.1 mg/mL) in DMEM for 3 h at 37 °C.

2.6. *Western blot analysis*

Cells were lysed with pro-prep protein extract solution (iNtRON, Houston, TX), sonicated, and centrifuged at 13,000 rpm for 20 min at 4 °C. After quantifying the protein contents, equal amounts of protein (30 μg/well) from each sample were boiled for 5 min in loading buffer (5% mercaptoethanol, 0.05% bromophenol blue, 75 mM Tris–HCl, pH 6.8, 2% SDS and 10% glycerol). Protein separation was carried out by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide resolving gel (Mini Protean II System, BioRad, Hertfordshire, UK) and then transferred onto polyvinylidine difluoride (PVDF) membranes (Amersham Biosciences, Buckinghamshire, UK) in glycine/methanol transfer buffer (20 mM Tris base, 0.15 M glycine and 20% methanol, pH 7.0) using a semidry transfer system (Trans-Blot SD, BioRad). After blocking with 5% nonfat dried milk in 1 × TBS-T buffer (20 mM Tris–HCl, pH 7.5, 137 mM NaCl and 0.05% Tween-20) for 1 h at room temperature, membranes were probed with primary antibodies (1:500 dilution in 1 × TBS-T buffer, pH 7.5) for 2 h at room temperature. Blots were washed and incubated with secondary antibodies (1:5000) for 1 h at room temperature. Antigen–antibody complexes were detected using electrochemiluminescence (ECL) western blotting detection reagents (iNtRON) according to the manufacturer's instructions. Protein detection was made for HO-1 at 32 kDa or for phosphor-Akt at 60 kDa, respectively.

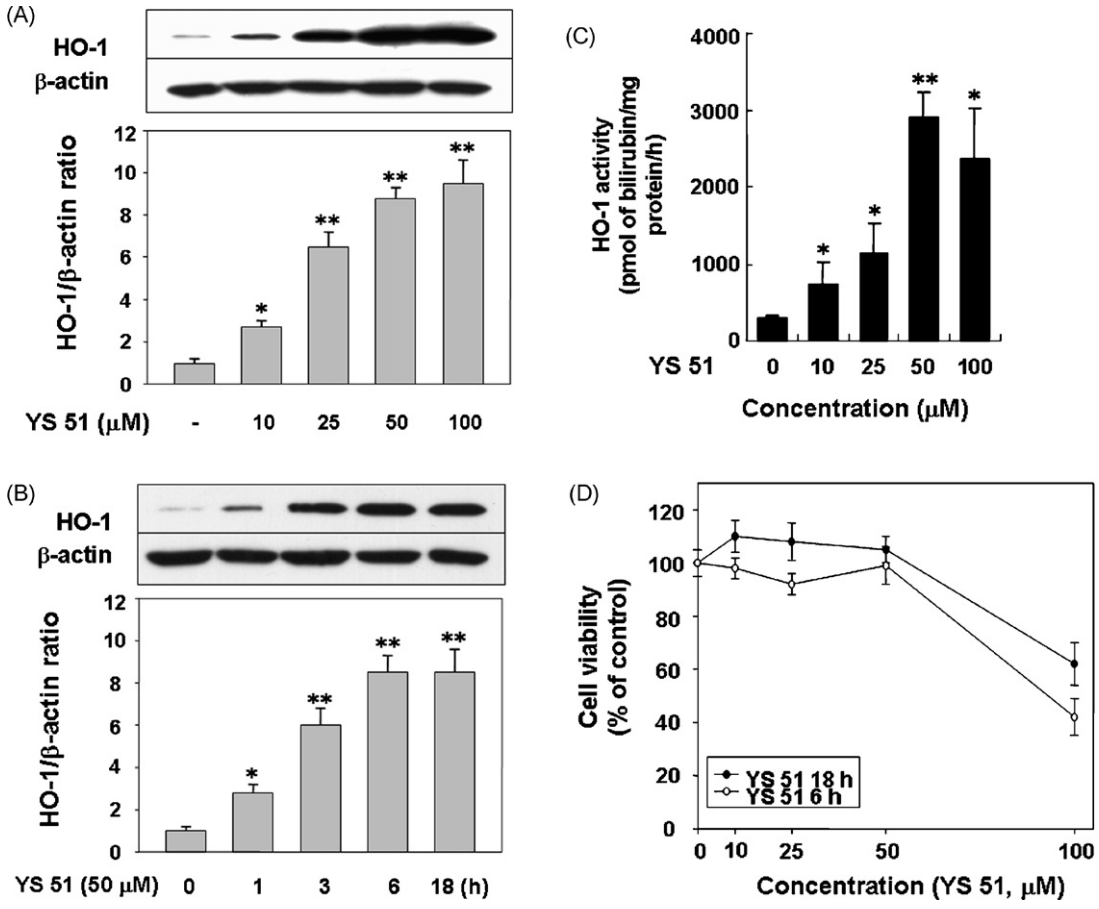

Fig. 1 – Effects of YS 51 on the level and activity of HO-1, and cell viability. (A) HO-1 protein production was analyzed by western blot analysis 6 h after treatment with YS 51 as described in Section 2. YS 51 induced HO-1 protein expression by bovine aortic endothelial cells (BAEC) in a concentration-dependent manner. (B) A time-dependent production of HO-1 protein was induced by 50 μM YS 51. (C) HO activity was measured in endothelial cells 6 h after exposure to various concentrations of YS 51 (0–100 μM). In the control group, cells were incubated in medium alone. (D) Toxicity of YS 51 was analyzed by MTT assay. Each bar represents the mean ± S.E.M. of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, significance compared with the unstimulated cells.

2.7. Detection of ROS production by DCF formation using confocal microscope

The production of intracellular ROS in BAEC after addition of H_2O_2 was detected using 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescin (DCF) in response to ROS production within the cells [18]: this was visualized by confocal microscopy (Olympus, Japan). After two washes, images were used to quantify fluorescence intensity with SigmaScan Pro 5 software. Cells were treated with H_2O_2 (200 μM) for 1 h, the medium was aspirated, and cells were washed twice with PBS. Cells were examined after incubation with 5 μM DCFH-DA for 10 min in a fresh culture medium. Cells were treated with various agents (YS 51, ZnPP IX, CORM-2, and HbO_2) for 1 h before the addition of H_2O_2. Generally, cells were preincubated with YS 51 (50 μM) for 6 h to ensure the induction of HO-1.

2.8. Statistical evaluations

Scanning densitometry was performed using an Image Master® VDS (Pharmacia Biotech Inc., San Francisco, CA). Values are expressed as the mean ± S.E.M. Treatment groups were compared using one-way analysis of variance (ANOVA), and the Newman–Keuls test was used to locate any significant differences identified by ANOVA. $P < 0.05$ or $P < 0.01$ were accepted as statistically significant.

3. Results

3.1. Effect of YS 51 on HO-1 expression and activity in BAEC

As shown in Fig. 1A and B, YS 51 increased HO-1 protein expression in a concentration- and time-dependent manner. Appearance of HO-1 was evident as early as 1 h; it peaked at 6 h and the level was maintained until 18 h (Fig. 1B and C). The increased enzyme activity was directly related to HO-1 protein levels (Fig. 1B and C). Although a maximal expression of HO-1 protein was induced at a higher concentration of YS 51 (100 μM), it reduced cell viability to 49–69% depending on the incubation time (Fig. 1D). Therefore, all subsequent experiments were performed using 50 μM YS 51.

3.2. Protective effects of YS 51 and hemin on H_2O_2-mediated cell death

Exposure of the cells for 8 h to 200 μM H_2O_2 significantly increased LDH release (about 90% of the control), whereas the LDH release was reduced by pretreatment with YS 51 (Fig. 2A). For example, 10 μM YS 51 reduced LDH release from 87% to 50%, which was further reduced to 24% by 50 μM YS 51. As shown in Fig. 2A, hemin, an inducer of HO-1, also reduced LDH release in the cells treated with 200 μM H_2O_2. Fig. 2B clearly shows that the increased HO-1 activity by YS 51 was significantly inhibited by ZnPP IX. As expected, the protective effects of YS 51 against H_2O_2-induced cell death were also significantly reduced by ZnPP IX (Fig. 2C).

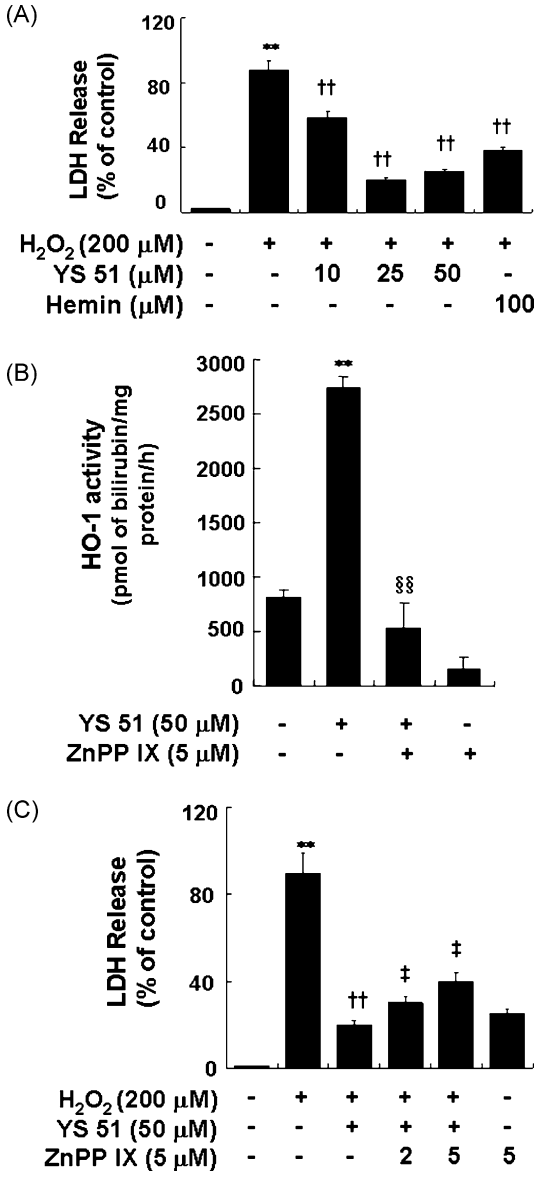

Fig. 2 – Effects of YS 51 and hemin on H_2O_2-mediated injury to BAEC. (A) Cells were pretreated with various concentrations of YS 51 or 100 μM hemin, a HO-1 inducer, 1 h before stimulation of the cells with H_2O_2 (200 μM) for 8 h. Cell viability was assessed by LDH release assay. Both YS 51 and hemin reduced LDH release significantly. (B) HO-1 activity was determined in the presence or absence of ZnPP IX. ZnPP IX, a HO-1 inhibitor, was given 1 h before treating the cells with YS 51 (50 μM) for 6 h. YS 51 significantly increased HO-1 activity, which was significantly inhibited by ZnPP IX. It should be noted that 5 μM ZnPP IX inhibited basal HO-1 activity. (C) Cell viability was examined by treating with 50 μM YS 51 in the presence or absence of ZnPP IX. When measured by LDH assay, the effect of YS 51 was inhibited significantly and concentration dependently by ZnPP IX. Results are the mean ± S.E.M. of four experiments. $^{**}P < 0.01$, significance compared with the unstimulated cells; $^{††}P < 0.01$, significance compared with the H_2O_2-stimulated cells; $^{‡}P < 0.05$, significance compared with the YS 51 treated H_2O_2-stimulated cells; $^{§§}P < 0.01$, significance compared with the YS 51-treated cells.

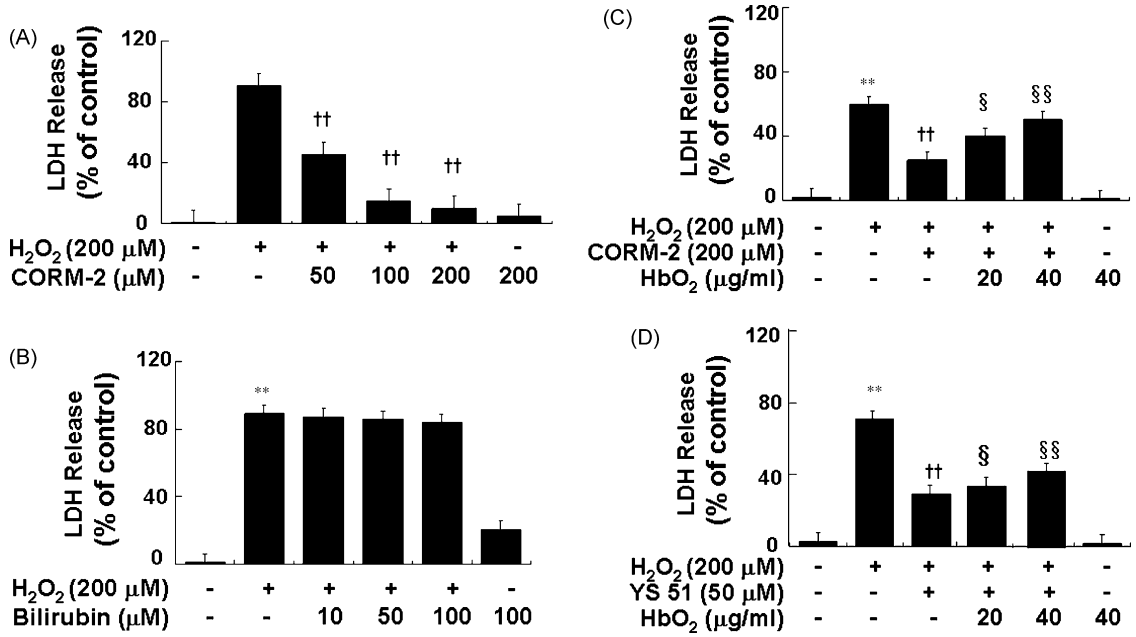

Fig. 3 – Effect of CORM-2, bilirubin, and YS 51 on H_2O_2-mediated injury of BAEC. Cells were incubated for 8 h with H_2O_2 (200 μM) in the presence or absence of various concentrations of CORM-2 (A) or bilirubin (B). (A) CORM-2 reduced LDH release in a concentration-dependent manner, but bilirubin showed no such effect (B). (C) Cells were pretreated with HbO_2 (20–40 μg/mL) 1 h before incubation with CORM-2 (200 μM) and then stimulated with H_2O_2 for 8 h. The protective effect of CORM-2 was significantly inhibited by HbO_2, which was also concentration-dependent. (D) Cells were pretreated with 50 μM YS 51 for 1 h in the present or absence of HbO_2 and were incubated for a further 8 h with H_2O_2 (200 μM). HbO_2, a CO scavenger, significantly inhibited the beneficial effect of YS 51. Results are the mean ± S.E.M. of four experiments. $^{**}P < 0.01$, significance compared with the unstimulated cells; $^{††}P < 0.01$, significance compared with the H_2O_2-stimulated cells; $^{§}P < 0.05$, $^{§§}P < 0.01$, significance compared with the YS 51 or CORM-2 treated H_2O_2-stimulated cells.

3.3. *CO is responsible for the protective action of YS 51*

Fig. 3A shows that the reduced cell viability caused by H_2O_2 was significantly reversed by the CO-releasing molecule, CORM-2 [19]. Depending on the concentration of this CO donor, cell viability increased gradually relative to the control treatment (H_2O_2). For example, the percentage of LDH release was reduced from 86 ± 4 to 45 ± 10, 17 ± 1, and 7 ± 4 at 50, 100, and 200 μM CORM-2, respectively. In contrast, up to 100 μM bilirubin did not affect cell viability (Fig. 3B). Fig. 3C demonstrates that the increased viability by CO donor was significantly antagonized by HbO_2. Likewise, the protective effect of YS 51 was also inhibited significantly by the presence of HbO_2 (Fig. 3D).

3.4. *Effects of YS 51 on intracellular ROS production in cells treated with H_2O_2*

When we measured the production of ROS within the cells as an oxidized DCF level using confocal microscopy after stimulation with H_2O_2, cells that were not treated with YS 51 showed increased production of ROS. However, cells showed much less green fluorescence, depending on the concentration of YS 51 (6 h pretreatment) (Fig. 4A). However, this ROS-reducing effect of YS 51 was inhibited by the addition of ZnPP IX (Fig. 4A). ZnPP IX alone did not affect the production of ROS (data not shown). As shown in Fig. 4B, the intensity of green fluorescence of oxidized DCF increased in cells treated with H_2O_2, indicating an increased production of ROS. However, this effect was significantly diminished by CORM-2 treatment, which was recovered by HbO_2, a CO scavenger. Fig. 4C also shows that the intensity of green fluorescence of oxidized DCF was decreased by pretreatment with deferoxamine.

3.5. *The signaling mechanism for induction of HO-1 by YS 51*

Fig. 5A illustrates that PD98059 concentration-dependently inhibited the production of HO-1 by YS 51. However, SB203580 and SP600125 did not influence this. Fig. 5B that HO-1 gene induction by YS 51 was significantly inhibited by the PI3 kinase inhibitor, LY294002. Combination of PD98059 (10 μM) and LY294002 (1 μM) almost completely inhibited HO-1 expression by YS 51 (Fig. 5C) which further blocked the protective effect of YS 51 (Fig. 5D).

4. Discussion

We clearly demonstrated that YS 51 protected BAEC from H_2O_2-induced damage. The mechanism(s) underlying this beneficial effect of YS 51 on oxidant-induced injury is at least related to its ability to induce the production of HO-1. This conclusion is based on the results from western blotting, measures of enzyme

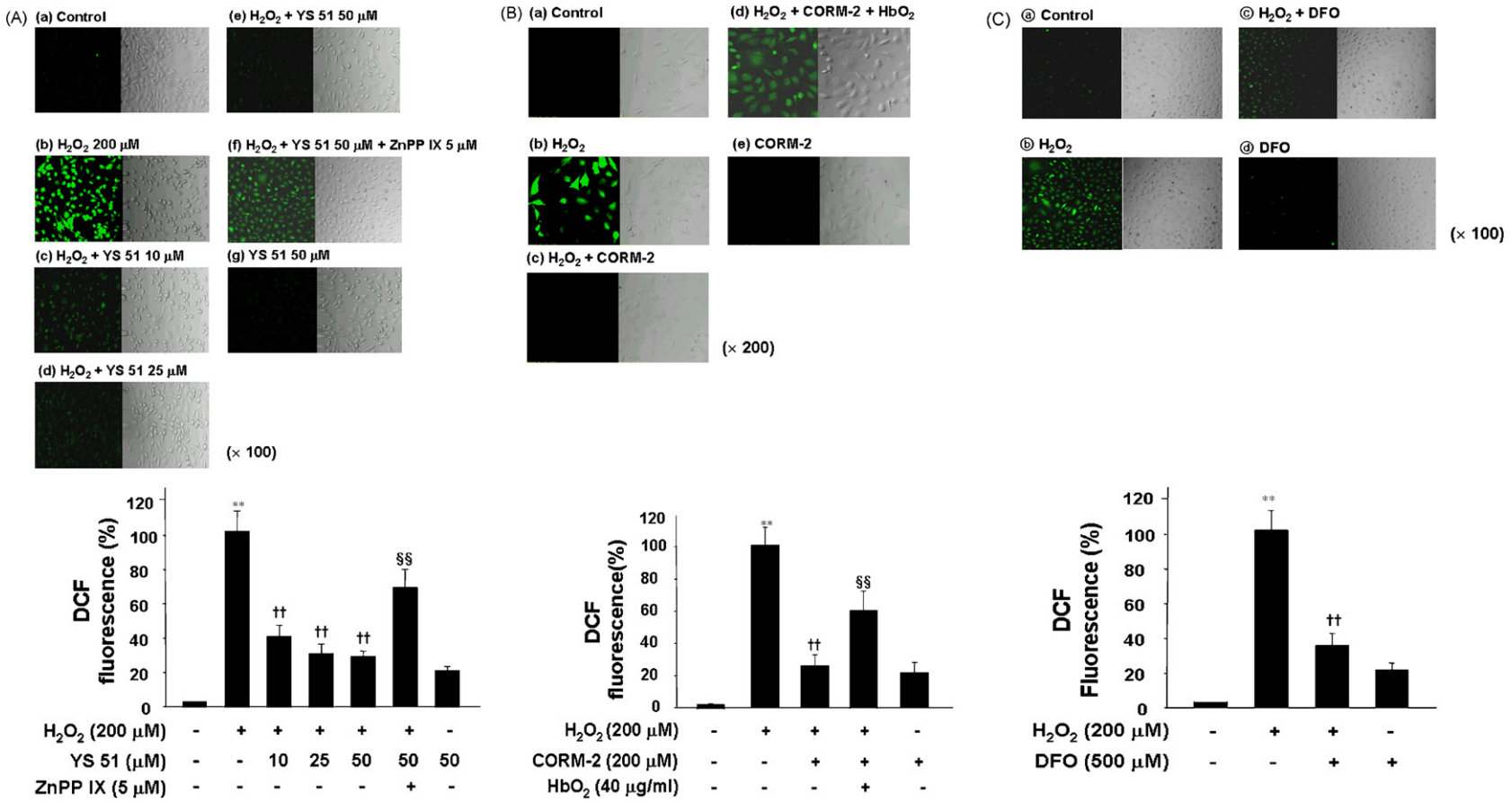

Fig. 4 – Effects of YS 51 or CORM-2 on ROS formation in BAEC by H_2O_2. Cells were incubated with or without YS 51 (10–50 μM) for 6 h (A) or with CORM-2 for 1 h (B) and further incubated for 1 h after the addition of H_2O_2 (200 μM). After incubation, cells were then washed and incubated in fresh medium with DCFH-DA (5 μM) for 10 min. After washing twice with PBS, the cells were analyzed using confocal microscopy. H_2O_2 (200 μM) significantly increased oxidized DCF levels, which were also significantly decreased by the presence of YS 51 or CORM-2. $^{**}P < 0.01$, significance compared with the unstimulated cells; $^{††}P < 0.01$, significance compared with the H_2O_2-stimulated cells; $^{§§}P < 0.01$, significance compared with the cells treated with H_2O_2 + YS 51 or CORM-2. The produced ROS was quantified by DCF fluorescence and is depicted as percentage changes compared with H_2O_2 (100%). (C) Effects of deferoxamine confocal microscopy imaging and ROS formation in BAEC by H_2O_2. Cells were pretreated with deferoxamine for 1 h and further incubated for 1 h after the addition of H_2O_2 (200 μM).

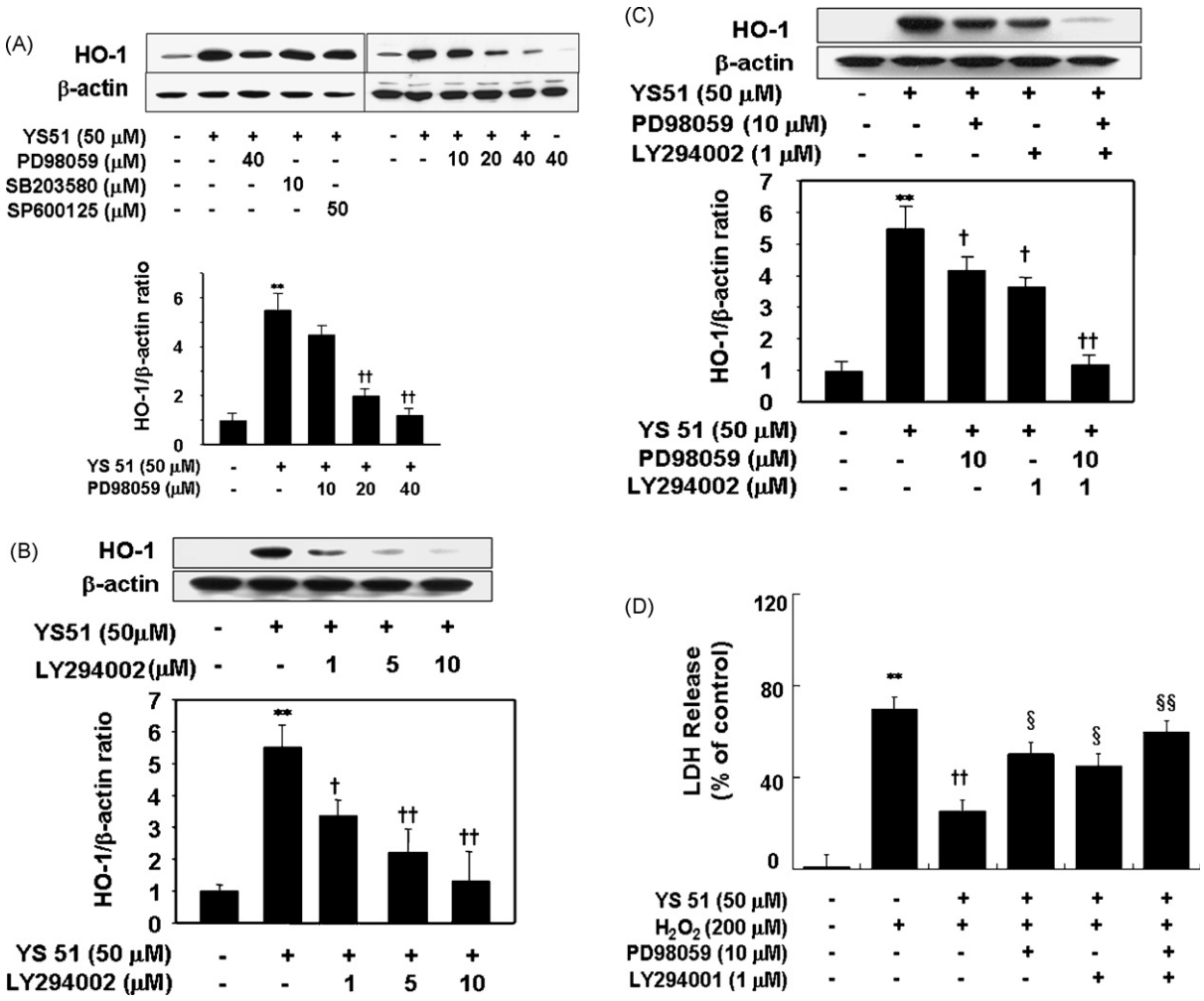

Fig. 5 – Involvement of PI3/Akt and ERK kinase pathways on HO-1 production induced by YS 51. (A) Cells were incubated with MAPK inhibitors (PD98059, a specific ERK1/2 inhibitor; SB203580, a specific p38 inhibitor and SP600125, a specific JNK inhibitor) for 1 h and further incubated for 6 h after the addition of YS 51 (50 μM). PD98059 concentration-dependently decreased the levels of HO-1 protein. (B) LY 294002, a PI3 kinase inhibitor, concentration-dependently inhibited the expression of HO-1 by YS 51. (C) Cells were incubated with either fixed concentration of ERK1/2 inhibitor (PD98059, 10 μM) or PI3 kinase inhibitor (LY 294002, 1 μM) or combination of both for 1 h and further incubated for 6 h after the addition of YS 51 (50 μM). (D) Cells were pretreated with PD98059, a specific ERK1/2 inhibitor, and/or LY 294002, a PI3 kinase inhibitor, and then treated with YS 51 in the presence of H_2O_2. Cell viability was determined by LDH release assay. Results are the mean ± S.E.M. of four experiments. $^{**}P < 0.01$, significance compared with the unstimulated cells; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, significance compared with the cells treated with YS 51; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, significance compared with the cells treated with H_2O_2 + YS 51.

activity, and confocal microscopy analysis. In fact, YS 51 concentration-dependently increased both HO-1 protein expression and activity in these cells. However, ZnPP IX, an inhibitor of HO-1, significantly inhibited the elevated HO-1 activity produced by YS 51. Most importantly, YS 51 protected the cells from H_2O_2-induced injury in a manner that was sensitive to ZnPP IX. These findings strongly suggest that YS 51 protects against H_2O_2 -induced injury in BAEC via HO-1 activity. Of course, other antioxidant action of YS 51 such as Mn-SOD expression may be contributed to endothelial cell protection from oxidant injury [14]. The mechanisms by which HO-1 functions to prevent endothelial cell damage against oxidative stress are not well defined, but several lines of evidence associated with the end products of heme catabolism are accumulating: bilirubin, and CO [20,21]. For example, bilirubin from HO-1 attenuates endothelial activation and dysfunction in response to proinflammatory stresses [22], and micromolar concentrations of bilirubin protect cells from cytotoxic concentrations of H_2O_2 [23]. However, we found no protective effect for bilirubin (up to 100 μM) against H_2O_2-mediated injury in the present study. In contrast, there is a report that bilirubin produces apoptosis in cultured bovine endothelial cells [24]. Thus, the functional role of bilirubin on endothelial cells is not yet clear. Possibly, many factors such as concentration, experimental conditions, and the cell types can result in different outcomes with bilirubin in endothelial cells. Because

CO, another product of HO-1, might be involved in the cytoprotective mechanism of HO-1 in many cells including endothelial cells [25,26], we exploited CORM-2 as a CO donor: this has been reported to release CO in the presence of DMSO [19]. As expected, CORM-2 increased cell viability in an HbO_2-sensitive manner. This suggests that CO protects cells from H_2O_2-mediated injury. We believe that CO produced from increased HO-1 activity by YS 51 protected the cells, as did CORM-2. The question remains how does CO protect cells from oxidant injury? We speculate that CO can modulate the intracellular redox status: this eventually lowers the level of ROS within the cell. Because CO is known to bind the heme-iron contained in mitochondrial respiratory cytochromes and to inhibit electron transfer causing the leakage of O_2^- from mitochondria [27]. Actually, our speculation has been substantiated by recent evidence showing that CORM-2 inhibits mitochondrial respiration [28] and modulates ROS production from mitochondria in cells [29]. We believe that CO can reduce the production of ROS, at least $OH^\bullet$, in this system (in cells treated with H_2O_2). Although we did not specify the ROS species generated, we think it is likely that the ROS species generated in H_2O_2-treated cells are H_2O_2 and/or hydroxyl radical ($OH^\bullet$), because H_2O_2 and $OH^\bullet$ but not $^\bullet O_2^-$ are known to oxidize DCFH [30]. Thus, one of the most likely candidates of ROS species in the present system is $OH^\bullet$. If this is true, CO, whether it comes exogenously (CORM-2) or endogenously from HO-1 (YS 51), could bind to ionized iron, which could thus prevent the Fenton reaction. To test this, we analyzed ROS production in cells treated with H_2O_2 in the presence and absence of CO (CORM-2 or YS 51) by confocal microscopy using the fluorescence dye, DCFH-DA. This is used widely as an indicator of ROS levels in cells because it is rapidly oxidized to highly fluorescent DCF in the presence of ROS or H_2O_2 [31]. As expected, both CORM-2 and YS 51 reduced ROS in cells treated with H_2O_2. From the literature, we note that transferrin receptor-dependent uptake of extracellular iron is required to oxidize DCFH to DCF by H_2O_2 in BAEC [32]. Thus, an H_2O_2-induced iron signaling mechanism may be responsible for the oxidation of DCFH to DCF. This increased iron could transform H_2O_2 to the more toxic chemical, $OH^\bullet$. Alternatively, heme released from destruction of heme-proteins by H_2O_2 [21] might play a role in converting H_2O_2 into $OH^\bullet$. It could also be possible, because not all the iron produced from heme is sequestered into ferritin, that the iron released from heme via HO-1 may participate in the Fenton reaction [33]. This idea was supported by the reduction of ROS production by the iron chelator, deferoxamine, in cells treated with H_2O_2. The role of iron in the active site of a number of critical molecules is well known [34]. Iron also acts as a target for both nitric oxide (NO) and CO, two messenger molecules with expending roles in a plethora of biological functions [35,36]. Although CO binding to iron-containing molecules remains largely unknown; it may be interesting to identify CO-iron complexes in cells or tissues that express high levels of HO. The existence of NO-iron complexes in biological organisms has already been documented [37,38], and similar data may be required for CO. Therefore, we speculate that, by binding iron ions [either heme or non-heme iron] within the cells, the CO produced – either from CORM-2 or from increased HO-1 activity by YS 51 – can prevent the formation of $OH^\bullet$ from H_2O_2. The increased cell viability caused by CORM-2 or YS 51 treatment was inhibited significantly by HbO_2, indicating that CO was captured by HbO_2 in this system. Interestingly, both ZnPP IX and HbO_2 increased the ROS production depressed by YS 51 in cells treated with H_2O_2 (data not shown). This may be because free heme – arising either from *de novo* synthesis [20] or released from the destruction of heme-proteins promoted by H_2O_2 [21] – cannot be eliminated once HO activity is inhibited. Therefore, such free heme not eliminated by ZnPP IX may produce $OH^\bullet$ from H_2O_2. If CO produced from HO-1 is captured by HbO_2, (in the case of YS 51 treatment together with HbO_2), then it will increase the production of ROS. Furthermore, CO may elicit dual effects as a pro- or antioxidant depending on dose, duration, and cell type [39], which may be related to NF-κB activity. Further study is warranted to investigate whether YS 51 modulates NF-κB activity in endothelial cells, as it inhibited NF-κB activity in RAW 264.7 cells activated with lipopolysaccharide and interferon-γ [13].

We found that HO-1 induction by YS 51 was via the activation of a PI3/Akt kinase system pathway, because a specific PI3 kinase inhibitor, LY294002, abrogated the expression of HO-1 protein. YS 51 induced the phosphorylation of Akt in a time-dependent manner (data not shown). Akt is a kinase involved in antiapoptosis signaling [40], which lies downstream of PI3 kinase. Some accumulating evidence suggests that the survival signal elicited by the PI3/Akt kinase pathway might link with the inactivation of proapoptotic proteins and attenuation of the general stress-induced increase caused by ROS [41]. In addition, the PI3/Akt kinase pathway controls the intracellular levels of ROS by regulating the expression of the antioxidation enzyme HO-1 [42]. Neither a p38 MAPK inhibitor nor a JNK inhibitor influenced YS 51-induced HO-1 expression. However, the ERK 1/2 inhibitor PD98059 concentration-dependently suppressed the expression of HO-1 by YS 51. When mixed together PD98059 and LY294002, the induction of HO-1 by YS 51 was almost completely inhibited, suggesting that HO-1 induction by YS 51 involves the PI3/Akt kinases and ERK pathway.

In conclusion, YS 51 protected endothelial cells against H_2O_2-mediated cell injury by reducing ROS production, in which CO plays a key role. Therefore, YS 51 could be used therapeutically to prevent endothelial dysfunction caused by oxidative stress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.07.023.

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