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YS 49, a Synthetic Isoquinoline Alkaloid, Protects Sheep Pulmonary Artery Endothelial Cells from *tert*-butylhydroperoxide-mediated Cytotoxicity

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Endothelium, particularly pulmonary endothelium, is predisposed to injury by reactive oxygen species (ROS) and their derivatives. Heme oxygenase (HO) has been demonstrated to provide cytoprotective effects in models of oxidant-induced cellular and tissue injuries. In the present study, we investigated the effects of YS 49 against oxidant [*tert*-butylhydroperoxide (TBH)]-induced injury using cultured sheep pulmonary artery endothelial cells (SPAECs). The viability of SPAECs was determined by quantifying reduction of a fluorogenic indicator Alamar blue. We found that TBH decreased cell viability in a time- and concentration-dependent manner. YS 49 concentration- and time-dependently increased HO-1 induction on SPAECs. As expected, YS 49 significantly decreased the TBH-induced cellular injury. In the presence of zinc protophorphyrin, HO-1 inhibitor, effect of YS 49 was significantly inhibited, indicating that HO-1 plays a protective role for YS 49. Furthermore, YS 49 showed free radical scavenging activity as evidenced by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and inhibition of lipid peroxidation. However, YS 49 did not inhibit apoptosis induced by lipopolysaccharide (LPS) in SPAECs. Taken together, HO-1 induction along with strong antioxidant action of YS 49 may be responsible for inhibition of TBH-induced injury in SPAECs.

Key Words: Reactive oxygen species, Sheep pulmonary artery endothelial cells, Heme oxygenase, YS 49, Antioxidant

INTRODUCTION

Increased production of reactive oxygen and/or nitrogen species (ROS/RNS) has recently been implicated in the pathogenesis of endothelial dysfunction associated with atherosclerosis, hypertension and aging. Vascular endothelium, particularly pulmonary endothelium, is predisposed to injury by ROS and their derivatives. The causes, mechanisms, and methods for therapy of oxidative stress have been the focus of enormous interest for several decades. The expression of heme oxygenase (HO)-1 is sensitive to induction by oxidants, and recent work has demonstrated that HO-1 provides cytoprotective effects in models of oxidant-induced cellular and tissue injury (Otterbein & Choi, 2000). In fact, HO is present in most mammalian tissues and catalyzes the degradation of heme to biliverdin, releasing equimolar amounts of biliverdin IXa, iron, and carbon monoxide (CO) (Maines, 1997). Biliverdin is subsequently converted into bilirubin by the enzyme biliverdin reductase. The HO system consists of at least three isozymes. Two of them, HO-1 and HO-2, are products of distinct genes; differ in their tissue distribution and regulation and have been characterized in detail. Of these, HO-1 is inducible and designated a stress response protein, whereas HO-2 is predominantly constitutive (Maines, 1997). A third isozyme, HO-3, which is closely related to HO-2, has been described recently (McCoubrey et al, 1997). Increased HO-1 activity enhances the survival of endothelial cell (EC) exposed to heme iron (Abraham et al, 1995), and bilirubin, a potent antioxidant, also protects against hydrogen peroxide-induced toxicity in an aortic EC line (Motterlini et al, 1996). We reported that YS 49, a synthetic isoquinoline alkaloid, has an anti-inflammatory action (Kang et al, 1999), positive inotropic action in the rabbit isolated heart (Lee et al, 1994), and anti-thrombotic action (Yun- Choi et al, 2001). Thus, this compound may have great potential for development of new drug for cardiovascular disorders. Since HO activity is known to protect EC in response to oxidative stress induced by various stimuli, the present study was undertaken whether the protective effects of YS

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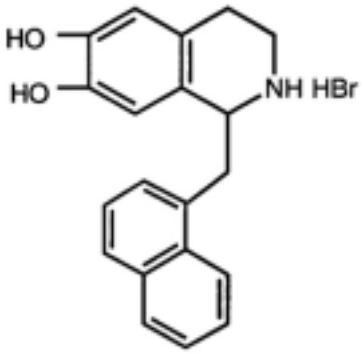
ABBREVIATIONS: HO, heme oxygenase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SPAEC, sheep pulmonary artery endothelial cell; TBH, *tert*-butylhydroperoxide; ZnPP IX, zinc protoporphrin IX.

49 was partly responsible for expression of endothelial HO-1 against oxidant injury.

METHODS

Materials

YS 49, (1-α-naphthylmethyl)-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, was kindly provided from Dr. DH Lee, Sokang University, Korea (Fig. 1). OPTI-MEM I Reduced Serum Medium was from Life Technologies (GIBCO BRL, USA). Alamar blue (Alamar Biosciences, Sacramento, CA). Other chemicals were supplied form Sigma (M.O., USA).



YS 49

Fig. 1. A chemical structure of YS 49.

Isolation and culture of SPAECs

Sheep pulmonary artery endothelial cells (SPAECs) were isolated and cultured from collagenase-digested pulmonary arteries as described previously (Hoyt et al, 1995). Cells were subcultured in Opti-MEM (GIBCO BRL, Life Technologies, Grand Island, NY) with endothelial cell growth supplement (15μg/ml; Collaborative Biomedical Products, Bedford, MA), penicillin (100 U/ml), streptomycin (100μg/ml), and 10% sheep serum (Sigma, St. Louis, MO). The cells were grown in the above medium at 37°C in 95% air-5% CO_2. They were routinely passaged 1 : 3 by detaching the cells with a balanced salt solution containing trypsin (0.05%) and EDTA (0.02%; GIBCO BRL) and were used between passages 6 and 12.

Endothelial cell toxicity

The internal environment of the proliferating cell is more reduced than that of non-proliferating cells. Specifically, the ratio of NADPH/NADP, FADH/FAN, and NADH/NAD, increase during proliferation. Alama blue can be reduced by these metabolic intermediates, which is useful in monitoring cell proliferation because its reduction is accompanied by a measurable shift in color. Because the absorption spectra of the oxidized (blue) and reduced (red) forms of Alama blue overlap, the absorbance is measured at two wave lengths. So, fluorescence measurements were made by exciting at 530～560 nm and measuring emission at 590 nm. The viability of SPAECs was determined by quantifying reduction of a fluorogenic indicator Alamar blue (Alamar Biosciences, Sacramento, CA). It has previously been shown that oxidized Alamar blue is taken up by cells and is reduced by intracellular dehydrogenases, and the water-soluble changes in fluorescence emission are utilized as an index of viability (Erukhimov et al, 2000).

SPAECs (5×10^4) were allowed to attach in 48-well tissue culture clusters and were exposed to the experimental conditions. Alamar blue was added to the medium, and 3 h later, fluorescence was determined with a cytofluorometer (Cytofluor II, PerSeptive Biosystems, Framingham, MA).

Experimental protocols

SPAECs were seeded at initial concentrations of 10^3 cells per well in 96-well microplate reader plates (Corning Glass Works, Corning, NY) in media consisting of low glucose DME, L-glutamine, penicillin G, and streptomycin with 20% fetal bovine serum. YS 49 was added to the cells 1 h prior to TBH treatment, and incubated in a CO_2 incubator at 37°C. Triplicate aliquots of the above samples were analyzed at indicated time for cell viability. Each data point represents the mean of a triplicate determination. In LPS-induced apoptosis experiments, SPAECs were plated in 25 cm^2 tissue culture flask with or without YS 49. The cells were then exposed to LPS (1μg/ml for 4 h, E.Coli/0111:B4, Sigma) followed by DNA-strand break analysis.

Western blot analysis

At the end of incubation, cells were washed twice with and scraped into ice-cold phosphate-buffered saline (PBS). The cell pellet, obtained by centrifugation at 3,000×*g* for 15 min at 4°C, was lysed in freshly prepared lysis buffer [Cell Culture Lysis Reagent (Promega, Madison, Wis), 1 mM phenylmethylsulfonyl fluoride, 50μg of leupeptin/ml, 100μg of aprotinin/ml]. Protein concentrations were determined by a Bio-Rad protein assay. Western blot analysis of HO-1 was done by fractionating 50μg of protein on a 12.5% polyacrylamide gel by denaturing discontinuous gel electrophoresis according to the Laemmli method. The proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, N.H). The membranes were incubated for 3 h at room temperature with a mouse anti-rat HO-1 monoclonal antibody (Stressgen, Victoria, British Columbia, Canada), diluted 1 : 1,000 in 10% (vol/vol) nonimmune goat serum. This was followed by incubation with peroxidase-conjugated anti-mouse immunoglobulin G (IgG) antibody (Sigma; 1:4,000 dilutions) for 90 min. Protein bands were examined by chemiluminescence (Pierce, Rockford, Ill.).

DPPH scavenging activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity was determined as described previously (Cos et al, 2002). Briefly, DPPH was dissolved in ethanol to give a 100μmol/L solution. Then 0.1 ml of YS49 compound in water (or water itself as control) was added to 0.9 ml ethanolic DPPH solution. The mixtures were shaken vigorously and left in the dark for 30 min. The decrease in DPPH absorption was measured at 550 nm and the actual decrease in absorption induced by the test compound was calculated by subtracting that of the control.

Lipid peroxidation

The total protein content of an aliquot of each tissue homogenate was analyzed by the method of Bradford (1976) using the Bio-Rad protein assay (micro-method). The thiobarbituric acid reactive substances (TBARS) assay was used to estimate lipid peroxidation in the homogenate. Malondialdehyde and other products of lipid peroxidation can be estimated spectrophotometrically at 535 nm after reaction with thiobarbituric acid to obtain an index for lipid peroxidation (Esterbauer & Zollner, 1989). The absorbance values obtained were compared against a standard curve of known concentrations of malondialdehyde and normalized to the protein content of the specimen.

DNA strand break analysis

Pellets containing 1×10^6 cells from the attached and floating cell population were washed in PBS and resuspended in 20μl of Solution 1 [10 mM EDTA, 50 mM Tris-HCl (pH 8.0), and 0.5% (w/v) SDS] plus Proteinase K (20 mg/ml stock, used at 0.5 mg/ml). Samples were then incubated at 50°C for 1 h before adding 10 ul of 0.5 mg/ml RNaseA and incubating at 50°C for a further hour. The samples were then heated rapidly to 70°C, supplemented with 10 ul of Solution II (10 mM EDTA (pH 8.0), 1% (w/v) low melting point agarose, 40% (w/v) sucrose and 0.25% (w/v) bromophenol blue), and immediately loaded onto a 2% agarose gel containing 0.1μg/ml of ethidium bromide (stock=10 mg/ml). The gel was then cooled to 4°C for approximately 5 min to allow the samples to set in the wells, and then run in Tris acetate buffer (0.4 M Tris-HCl pH 8.0, 50 mM sodium acetate, and 10 mM EDTA) at 40 V until the dye front had migrated 4～5 cm. The DNA was then observed using UV transillumination and photographed.

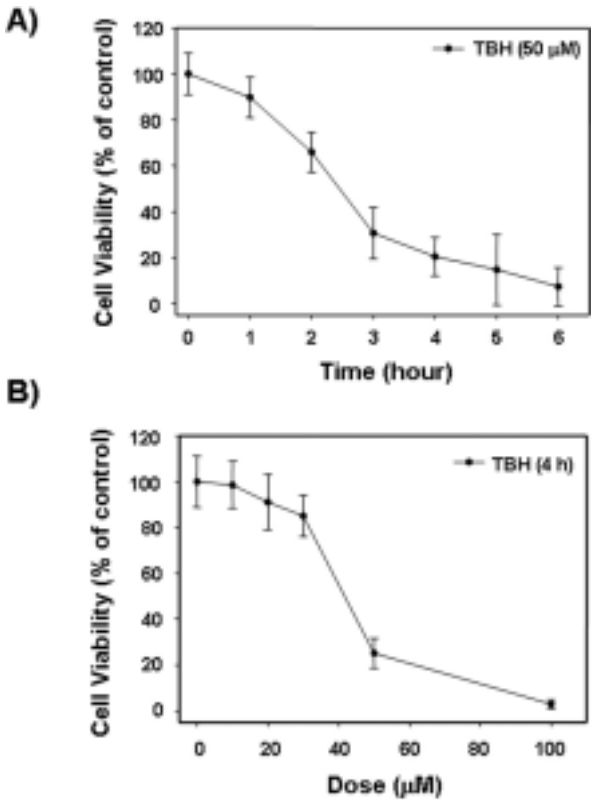


Fig. 2. TBH-induced cytotoxicity in a dose- and time- dependent manner. (A) At a dose of 50μM, TBH exhibited cytotoxicity in a time dependent manner. (B) TBH-treated SPAECs for 4 h exhibited cytotoxicity in a dose dependent manner (10～100μM). TBH-induced cytotoxicity was determined by quantifying reduction of a fluorogenic indicator Alamar blue as described in Method. The results are shown as a mean±SEM of three independent experiments.

Statistical analysis

Data are presented as mean±SEM. A pooled, two-tailed *t* test analysis was used for the comparison of two groups of data. Results were considered statistically significant at $P<0.05$.

RESULTS

TBH-mediated cytotoxicity in SPAECs

The effects of the TBH on the viability of SPAECs were determined by quantifying the degree of the reduction of a fluorogenic indicator Alamar blue. As shown in Fig. 2, TBH reduced the viable cells in a time-dependent manner (Fig. 2A) and in a dose-dependent manner (Fig. 2B). TBH was highly toxic to SPAECs showing that about 68% and 82% of cell population were died at 50μM when incubated for 3 h and 4 h, respectively. After incubating SPAECs with TBH for 4 h, total number of dead cells was increased as the concentration of TBH increases (Fig. 2B).

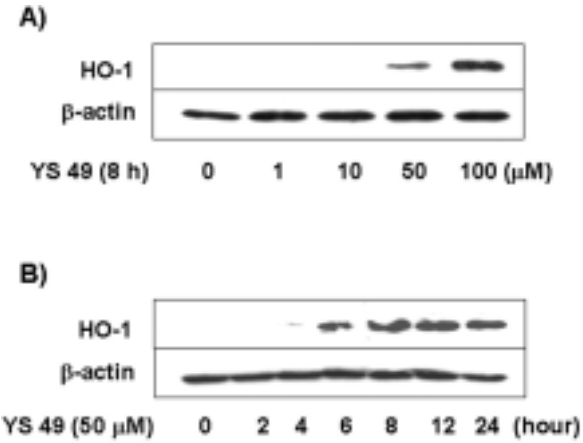


Fig. 3. Concentration- and time-dependent expression of HO-1 by YS 49. (A) Concentration-dependent expression of HO-1 by YS 49. SPAECs were incubated for 8 h at the various dose of YS 49 (1～100 μM). Western blot analysis indicated that HO-1 protein was expressed at 50μM of YS 49, which was induced dramatically at 100μM of YS 49. (B) Time-dependent expression of HO-1 by YS 49. Cells were incubated for 2, 4, 6, 8, 12 or 24 h at 50μM of YS 49. Western blot analysis showed that the protein expressed weakly at 4 h reaching a maximum at 8 h sustained until 24 h. Data shown are representative for 3 experiments with similar results.

HO-1 induction by YS 49 in SPAECs

In order to see that YS 49 is able to increase the HO-1 protein levels in SPAECs, SPAECs were treated with YS 49 (1~100μM) for 8 h (Fig. 3A). HO-1 protein induction by YS 49 appeared at 50μM and dramatically increased at 100μM of YS 49 (Fig. 3A). Production of the HO-1 protein was also monitored in a time course following YS 49 treatment, reached the maximum level 8 h and sustained until 24 h after YS 49 treatment in SPAECs (Fig. 3B).

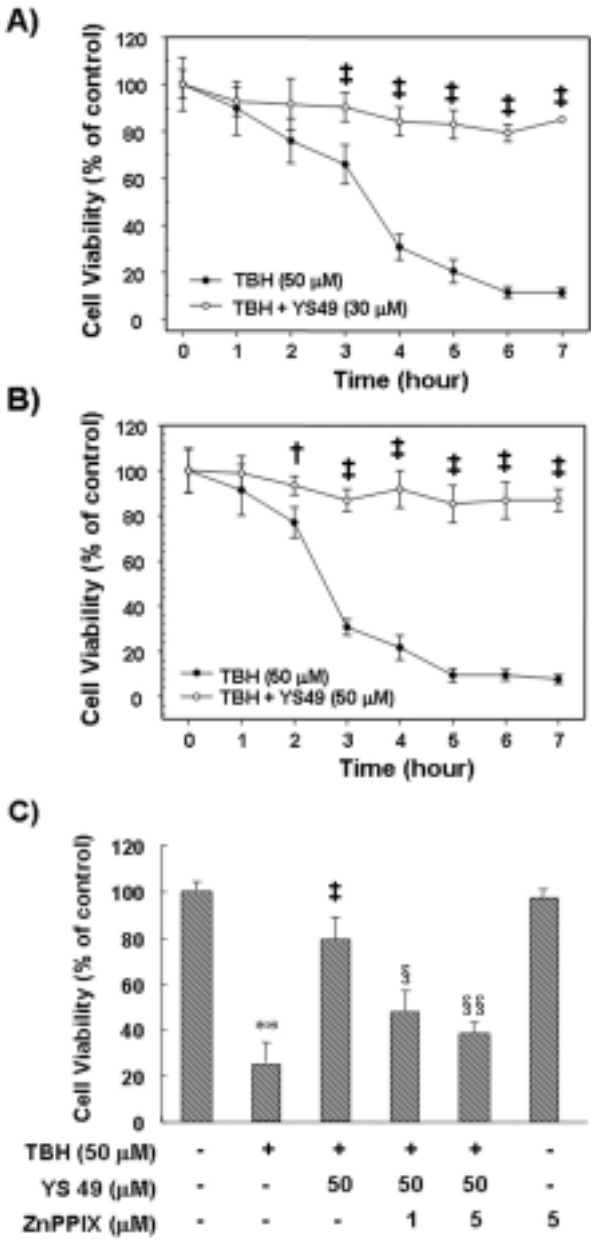


Fig. 4. Protective effect of YS 49 against TBH-induced cytotoxicity. SPAECs were precubated with 30μM (A) or 50μM (B) of YS 49 before incubation of cells with 50μM of TBH for 6 h. It clearly showed that YS 49 significantly increased the cell viability against TBH. (C) ZnPP IX, a HO-1 inhibitor (1 and 5μM), and YS 49 (50μM) were pre-treated for 2 h and 1 h, respectively, before incubation of cells with TBH for 4 h. Cell viability was determined by quantifying reduction of a fluorogenic indicator Alamar blue as described in Fig. 2 legend. Data represent mean±SEM of experiments (**$P<0.01$; significance compared with the control, $^{\dagger}P<0.05$, $^{\ddagger}P<0.01$; significance compared with TBH, $^{\S}P<0.05$, $^{\S\S}P<0.01$; significance compared with YS 49).

Protective effect of YS 49 on TBH-induced toxicity

We examined whether YS 49 protects TBH-induced cytotoxicity. As expected, YS 49 effectively protected the cell from TBH-mediated cytotoxicity. Fig. 4A and 4B showed that two different doses of YS 49 (30μM and 50μM) significantly inhibited the cell death by TBH. To verify the protective effect of YS 49 was via HO-1-mediated action, ZnPP IX, a HO-1 inhibitor, was pre-incubated for 1 h prior to addition of YS 49. Fig. 4C clearly shows that the protective effect of YS 49 against TBH-induced cytotoxicity was significantly inhibited by the presence of ZnPP IX, indicating that HO-1 plays a role for the cell viability from TBH-induced cell injury. These results suggest that the expression of HO-1 by YS 49 protects the cells from TBH-induced oxidative stress.

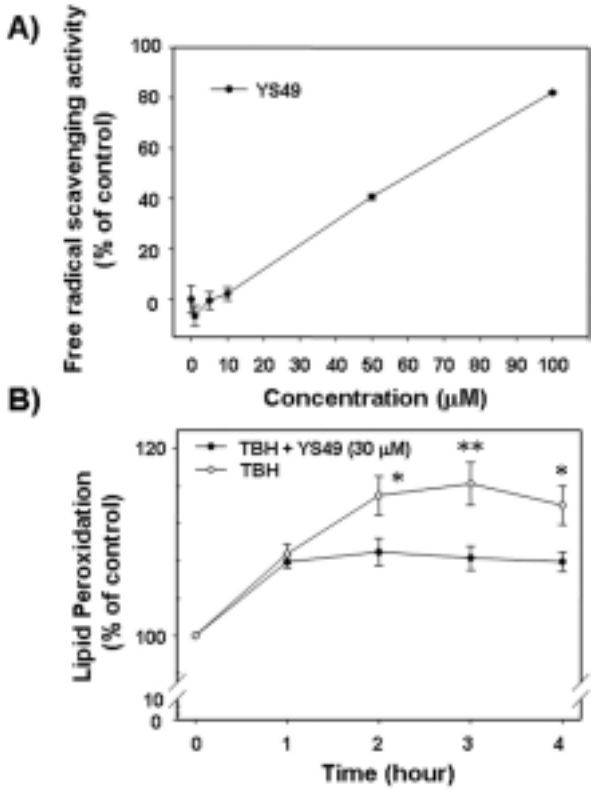


Fig. 5. Effects of YS49 on DPPH-induced free radical production and lipid peroxidation in SPAECs. (A) Effect of YS 49 on DPPH reduction in a dose-dependent manner. DPPH was incubated with increasing concentrations of YS 49 for 1 h and its absorbance decrease was evaluated at 517 nm. Data represent mean ± SEM of three experiments. (B) Effct of YS 49 on lipid peroxidation. The total protein content of an aliquot of each cell homogenate. homogenate was analyzed for lipid peroxidation as described in Method. Data represent mean±SEM of three experiments (*$P<0.05$, **$P<0.01$; significance compared with TBH).

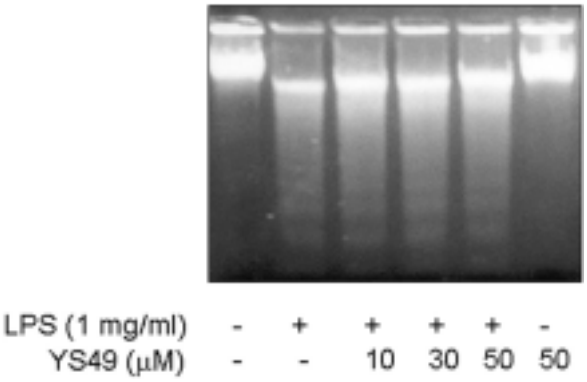


Fig. 6. YS 49 has no protective effect on LPS-induced DNA fragmentation in SPAECs. YS 49 was pretreated at a dose of 10~50μM before treatment with LPS 1 mg/ml and the cells were further incubated for 4 h. Lane 1, Control; Lane 2, LPS 1 mg/ml; Lane 3, LPS 1 mg/ml+YS 49 10μM; Lane 4, LPS 1 mg/ml+YS 49 30μM; Lane 5, LPS 1 mg/ml+YS 49 50μM; Lane 6, YS 49 50μM. Genomic DNA was extracted from SPAECs treated with or without YS 49 for 4 h using DNAzolTM genomic DNA isolation reagent. Tenμg of DNA were electrophoretically separated on 2% agarose gel containing ethidium bromide. The data was confirmed by two repeated experiments.

Effects of YS49 on DPPH-induced free radical production and lipid peroxidation in SPAECs

Free radical scavenging effect of YS 49 was examined by determining the decrease in DPPH absorption. As shown in Fig. 5A, YS 49 has a strong antioxidant action which was dose-dependent. This antioxidant effect was further confirmed by lipid peroxidation experiment, in which 30μM YS 49 significantly inhibited the oxidation of lipid by TBH (Fig. 5B).

Effects of YS 49 on LPS-induced apoptosis of SPAECs

To determine the effect of YS 49 on LPS-induced apoptosis, we performed DNA fragmentation analysis. Fig. 6 shows that LPS (1μg/ml) induced DNA fragmentation, however, in contrast to its antioxidant activity, YS 49 failed to inhibit the formation of DNA ladders. These results suggest that YS 49 has effective antioxidant effect against TBH-induced oxidative stress in SPAECs, however antioxidant effect by YS 49 is not linked to prevention of LPS-induced apoptosis.

DISCUSSION

In this study, we have demonstrated that YS 49 induced HO-1 protein and protected SPAECs from TBH-induced toxicity. In addition, ZnPPIX inhibited the protective effect of YS 49 on SPAECs. From these results, we suggested that YS 49 at least protects the cells from oxidative stress by inducing HO-1 expression. As HO-1 is thought to play a protective role against oxidant injury, this may be an important cellular response to YS 49. The major factors involved in pulmonary endothelial injury by ROS include 1) exposure to high levels of environmental oxygen, ROS (e.g., ozone), and compounds that induce ROS generation in the lung (e.g., smoke) (Freeman & Crapo, 1981); 2) a pulmonary vasculature that is a target for sequestration of activated leukocytes that release ROS (Brigham & Meyrick, 1986; Leff et al, 1994; Louie et al, 1997); and 3) pulmonary endothelial cells as well as other endothelial cells that are capable of generating ROS (Matsubara & Ziff, 1986; Zweier et al, 1988; Holland et al, 1990; Fisher et al, 1991; Sanders et al, 1993). In the present study, we used *in vitro* cell culture system in which an oxidant radical, TBH, was used as an ROS source, so it may directly damage the endothelial cells mainly by factor 1) described above. The cellular response to oxidative stress-inducing agents such as heavy metals, UV irradiation, heme, hemoglobin, hydrogen peroxides, and TBH involves the production of a number of cellular mediators, including acute-phase proteins, eicosanoids, cytokines, and HO-1.

HO, the initial and rate-limiting enzyme in the pathway for heme catabolism, plays a vital role in diverse biological processes, including cell respiration, energy generation, oxidative biotransformation, and cell growth and differentiation (Applegate et al, 1991; Choi & Alam, 1996). In fact, HO is present in most mammalian tissues and catalyzes the degradation of heme to biliverdin, releasing equimolar amounts of biliverdin IXa, iron, and carbon monoxide (CO) (Maines, 1997). Biliverdin is subsequently converted into bilirubin by the enzyme biliverdin reductase. Although heme is purported to be the typical HO-1 inducer, the inflammatory cytokines IL-1 and tumor necrosis factor-α (TNF-α) have been also shown to be effective inducers of HO-1 in cultured human EC (Terry et al, 1998). The expression of HO-1 is sensitive to induction by oxidants. The mechanism of action by which YS 49 induces HO-1 is not clear. However, it is unlikely that YS 49 acted as an oxidant to induce HO-1, because it reduced ROS as evidenced by DPPH analysis and lipid peroxidation assay. It has been reported that HO-1 can be induced by reductants as well, such as curcumin (Balogun et al, 2003). So, it is possible to speculate that re-dox change by YS 49 in the intracellular environment may be responsible for induction of HO-1 or concentration of cyclic nucleotides by YS 49. Because YS 49 increased cAMP in cardiac tissues (Lee et al, 1994) and PKA-dependent induction of HO-1 has been reported in EC (Kronke et al, 2003). However, further study warrants this. We found that the protective effect by YS 49 was antagonized by ZnPP IX, an inhibitor of HO-1 enzyme, indicating that HO-1 partly contributed to the cytoprotective effect. In deed, recent work has demonstrated that HO-1 provides cytoprotective effects in models of oxidant-induced cellular and tissue injury (Otterbein & Choi, 2000). Increased HO-1 activity enhanced the survival of EC exposed to heme iron (Abraham et al, 1995), and bilirubin, one of by products by HO-1, protected against hydrogen peroxide-induced toxicity in an aortic endothelial cell line (Motterlini et al, 1996). Since HO activity is known to protect EC in response to oxidative stress, the protective effects of YS 49 against TBH seems to be related with expression of endothelial HO-1. Because YS 49 showed to scavenge free radicals, this strong antioxidant action also involved in protection of TBH-induced toxicity. When compare the time course between TBH-induced cell death and induction of HO-1 by YS 49, 3 to 4 h is enough to kill more than 50% by TBH, while HO-1 induction by YS 49 takes at least 4 h. So, some may argue how it is possible the protective mechanism of YS 49 is related with HO-1. It, however, may be possible that pretreatment with YS 49 may have scavenged free radicals from TBH, so, this can

reduce the concentration of TBH far less than we treated. Another evidence is that ZnPP IX inhibited the effect of YS 49, which strongly suggest the involvement of HO-1 in the action of YS 49. Although YS 49 protected SPAECs from direct oxidant, TBH, however, it failed to inhibit apoptotic cell death induced by LPS. The cellular mechanisms of apoptosis are complex and diverse, with considerable cell-type specificity. The vascular endothelium has a central role in inflammation, the response to infection, and the pathogenesis of vascular disease. EC can be induced to undergo apoptosis by a number of treatments, including TNF-α in the presence of protein synthesis inhibitors (Polunovsky et al, 1994), radiation (Haimovitz-Freidman et al, 1994), and LPS (Choi et al, 1998). Serum withdrawal is also known to induce oxidative stress in cells, demonstrated by an excess production of ROS, such as superoxide anion, hydrogen peroxide, hydroxyl radical and organic peroxides that can be inhibited by intracellular antioxidants (Greenlund et al, 1995). LPS activates caspase-1 (Martinon et al, 2002), which is known to accelerate EC apoptosis. We observed an interesting finding that YS 49 did not prevent apoptosis induced by LPS albeit it had an ability to reduce ROS thereby protecting the cells from oxidant injury. The apparently conflicting data can not be explained at the present time, but it seems likely due to the fact that the signal pathway of apoptotic cell death induced by LPS is different from that induced by ROS. Indeed, recently, King et al (2002) reported that ROS do not related with caspase-1 activation induced by serum withdrawal in human umbilical vein endothelial cells. It has been reported that LPS induces apoptosis in endothelial cell via a soluble CD14-dependent mechanism (Frey & Finlay, 1988), and it can produce many inflammatory cytokines in EC which convey a signal to apoptosis. Although ROS is responsible for some pharmacological actions of LPS (Chan & Murphy, 2003), signal network in apoptotic cell death by LPS may be more complicated than ever we might think. Or TBH-induced cell death is other than apoptotic cell death, namely necrotic cell death. Now we are under progression to resolve this phenomenon. The protective effect of YS 49 may be especially important in the endothelium, considering that pulmonary endothelial cells are very sensitive to the toxic effects of oxygen. Injury to the endothelium results in protein leak and an extravasation of heme products into the endothelium, interstitium, and alveolar space.

In summary, we have investigated the molecular mechanism of YS 49 on the protective action of SPAECs against oxidant stress, TBH. YS 49 increased HO-1 induction, inhibited lipid peroxidation, and has free radical scavenging action. These properties all together may have contributed to increase the cell viability against TBH-injury. However, YS 49 failed to inhibit apoptotic cell death induced by LPS, which suggest that LPS-induced SPAEC cell death is mainly apoptotic way, but TBH-induced cell death is most likely necrotic death. It is concluded that YS 49 may be useful in the treatment from oxidant-induced disorders.

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