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Overexpression of HO-1 Protects against TNF- α -Mediated Airway Inflammation by Down-Regulation of TNFR1-Dependent Oxidative Stress

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Oxidative stresses are believed to play an important role in the induction of both cell adhesion molecules and pro-inflammatory cytokines, a key event in a variety of inflammatory processes. The enzyme heme oxygenase-1 (HO-1) functions as an antioxidant and serves to protect against tissue injury. In this study, we report that HO-1 was induced in cultured human tracheal smooth muscle cells after either treatment with a potent inducer of HO-1 activity, cobalt protoporphyrin IX, or infection with a recombinant adenovirus that carries the human HO-1 gene. Overexpression of HO-1 protected against tumor necrosis factor (TNF)- α -mediated airway inflammation via the down-regulation of oxidative stress, adhesion molecules, and interleukin-6 in both cultured human tracheal smooth muscle cells and the airways of mice. In addition, HO-1 overexpression inhibited TNF- α -induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression, adherence of THP-1 cells, generation of interleukin-6, p47^{phox} translocation, and nuclear factor- κ B activation. HO-1 overexpression also attenuated TNF- α -induced oxidative stress, which was abrogated in the presence of both the HO-1 inhibitor, zinc protoporphyrin IX, as well as a carbon monoxide scavenger. In addition, HO-1 overexpression reduced the formation of a TNFR1/c-Src/p47^{phox} complex. These results suggest that HO-1 functions as a suppressor of TNF- α signaling, not only by inhibiting the expression of adhesion molecules and generation of interleukin-6, but

also by diminishing intracellular reactive oxygen species production and nuclear factor- κ B activation in both cultured human tracheal smooth muscle cells and the airways of mice. (Am J Pathol 2009, 175:519–532; DOI: 10.2353/ajpath.2009.090016)

Airway smooth muscle is considered as an end-response effector, regulating regional differences in ventilation by contracting in response to various pro-inflammatory mediators and exogenous substances released under homeostatic or pathological conditions, such as asthma.¹ When airway cells and tissues are exposed to oxidative stress elicited by inflammatory reactions, elevated levels of reactive oxygen species (ROS) can trigger a variety of deleterious effects within the airways.

The multifunctional cytokine tumor necrosis factor (TNF)- α is capable of activating multiple signal transduction pathways and downstream processes in mammalian cells, such as human tracheal smooth muscle cells (HTSMCs).² TNF- α has been shown to increase airway smooth muscle oxidants production through a NADPH oxidase-like system to enhance myosin light chain phosphorylation and contractility.³ Cellular adhesion molecules are key players in initiating inflammatory responses of the respiratory system.⁴ Certain cellular adhesion molecules, such as vascular cellular adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1, are expressed on smooth muscle cells where they contribute to leukocyte recruitment. The cellular adhesion molecule-induced accumulation of activated leukocytes and macrophages inevitably leads to oxidative stress associated with tissue damage. Several studies have indicated that the expression of VCAM-1 and ICAM-1 are

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mediated by the activation of c-Src, phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinases, or nuclear factor (NF)- κ B.^{2,5-8} In addition, ROS also have been shown to mediate NF- κ B activation and the expression of VCAM-1 and ICAM-1.⁸ TNF- α may stimulate ROS production by several sources, such as mitochondria, but recent studies have strongly suggested that a major source of ROS is a phagocyte-type NADPH oxidase.⁹ Activated NADPH oxidase is a multimeric protein complex consisting of at least three cytosolic subunits of p47^{phox}, p67^{phox}, and p40^{phox}. The p47^{phox} regulatory subunit plays a critical role in acute activation of NADPH oxidase; phosphorylation of p47^{phox} is thought to relieve inhibitory intracellular interactions and permit the binding of p47^{phox} to p22^{phox}, thereby increasing oxidase activation.⁹

Heme oxygenase (HO)-1 is the key enzyme responsible for the degradation of heme to carbon monoxide (CO), free iron, and biliverdin-IX α .¹⁰ In mammals, biliverdin-IX α is further converted to bilirubin-IX α , an endogenous radical scavenger, with recently recognized anti-inflammatory properties.¹⁰ However, the release of free iron is rapidly sequestered into the iron storage protein ferritin, leading to additional antioxidant and anti-apoptotic effects.¹⁰ CO exerts several biological functions, including anti-apoptotic and anti-inflammatory properties.¹¹ HO-1 expression is up-regulated in the airways of patients with asthma.¹² Airway smooth muscle contractility, which can be increased through ROS formation, is inhibited by HO-1 induction.¹³ Moreover, the 5'-untranslated region of the human HO-1 gene contains many stress-activated response elements, such as antioxidant response elements. NF-E2-related factor 2 (Nrf2) is an important transcription factor that regulates expression of antioxidant defense genes through binding to antioxidant response elements in the promoter region, such as HO-1.¹⁴

HO-1 has been reported to play a critical role in protection against oxidative tissue injury following ischemia and inflammation,¹⁵ however, the mechanism is not clear. Therefore, we determined the role of HO-1 in TNF- α -mediated adhesion molecules expression and airway inflammation *in vitro* and *in vivo*. Although a role of HO-1 as an antioxidant has been reported in many studies, most of these antioxidant properties were associated with the HO-1 product bilirubin. Here, we first show that overexpression of HO-1 in HTSMCs or in the airways of mice is strongly associated with resistance to airway inflammation induced by TNF- α . Our findings also indicated that HO-1-attenuated the activation of p47^{phox} and NF- κ B provide a new insight into the mechanisms to explain HO-1 antioxidant and anti-inflammation properties.

Materials and Methods

Materials

4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1) and diphenylene iodonium chloride (DPI) were from Biomol (Plymouth Meeting, PA). Metafectene transfection reagent was from Biontex (Munich, Germany). Anti-TNF receptor 1 (TNFR1), Anti-HO-1, anti-Nrf2, anti-

ICAM-1, anti-VCAM-1, anti-c-Src, anti-p47^{phox}, and anti-p65 Abs were from Santa Cruz (Santa Cruz, CA). Anti-TNFR1 neutralizing Ab was from R&D System (Minneapolis, MN). Anti-phospho-Src, anti-phospho-p65, and anti-phospho-I κ B α Abs were from Cell Signaling (Danver, MA). 2',7'-bis-(*z*-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethylester (BCECF/AM), dihydroethidium (DHE), and 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA) were from Molecular Probes (Eugene, OR). Cobalt protoporphyrin IX (CoPP IX), zinc protoporphyrin IX (ZnPP IX), and N-acetyl-L-cysteine (NAC) were from Sigma (St. Louis, MO). Apocynin was purchased from ChromaDex (Santa Ana, CA).

Cell Culture

HTSMCs were purchased from ScienCell Research Lab (San Diego, CA) and grown as previously described.¹⁶ Experiments were performed with cells from passages 4 to 7.

Animal Care and Experimental Procedures

Male imprinting control region mice aged 6 to 8 weeks were purchased from the National Laboratory Animal Centre (Taipei, Taiwan) and handled according to the guidelines of Animal Care Committee of Chang Gung University and NIH Guides for the Care and Use of Laboratory Animals. ICR mice were anesthetized with ethyl ether and placed individually on a board in a near vertical position and the tongues were withdrawn with a lined forceps. TNF- α (0.75 mg/kg body weight) was placed posterior in the throat and aspirated into lungs. Control mice were administered sterile 0.1% bovine serum albumin. Mice regained consciousness after 15 minutes. Mice were given one dose of CoPP IX or ZnPP IX (1.637 μ g/kg body weight) for 16 hours or 1 hour, respectively, before TNF- α treatment, and sacrificed after 24 hours. To examine the cellular expression and localization of the ICAM-1 and VCAM-1 proteins, immunohistochemical staining was performed on the first and second serial sections of the airways, which were deparaffinized, rehydrated, and washed with PBS. Non-specific binding was blocked by preincubation with PBS containing 5 mg/ml of bovine serum albumin for 1 hour at room temperature. The sections were incubated with an anti-ICAM-1 or anti-VCAM-1 Ab at 37°C for 1 hour and then with an anti-goat or anti-rabbit horseradish peroxidase Ab at room temperature for 1 hour. Bound Abs were detected by incubation in 0.5 mg/ml of 3,3'-diaminobenzidine/0.01% hydrogen peroxide in 0.1 M/L Tris-HCl buffer, as chromogen (Vector Lab). The third section was incubated with an anti- α -smooth muscle actin Ab for the positive localization and identification of smooth muscle cells, and then incubated with a fluorescein isothiocyanate-conjugated goat anti-mouse secondary Ab and observed by a fluorescence microscope.

Isolation of Bronchoalveolar Lavage Fluid

Mice were injected with TNF- α at a dose of 0.75 mg/kg and sacrificed 24 hours later. Bronchoalveolar lavage

(BAL) fluid was performed through a tracheal cannula using 1 ml aliquots of ice-cold PBS medium. BAL fluid was centrifuged at $500 \times g$ at 4°C , and cell pellets were washed and re-suspended in PBS. Leukocyte count was determined by a hemocytometer.

Transient Transfection with Small Interfering RNA

SMARTpool RNA duplexes corresponding to human HO-1, p47^{phox}, Nrf2, and scrambled #2 small interfering (si)RNA were from Dharmacon Research Inc (Lafayette, CO). Transient transfection of siRNAs was performed using Metafectene transfection reagent. siRNA (100 nmol/L) was formulated with Metafectene transfection reagent according to the manufacturer's instruction.

Preparation of Recombinant Adenovirus

A recombinant adenovirus containing human HO-1 (Adv-HO-1) was kindly provided by Dr. L. Y. Chau (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan). Recombinant adenovirus was generated by homologous recombination and amplified in 293 cells. Large scales of viral vectors were purified by CsCl ultracentrifugation and stored in 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L MgCl_2 , and 10% (v/v) glycerol at -80°C until used for experiments. Virus titers were determined by a plaque assay on a 293 cell monolayer.

Western Blot Analysis

After the incubation, cells were washed, scraped, collected, and centrifuged at $45,000 \times g$ at 4°C for 1 hour to yield the whole cell extract, as previously described.¹⁶ Samples were denatured, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane. Membranes were incubated with an anti-ICAM-1, anti-VCAM-1, or anti-HO-1 Ab for 24 hours, and then incubated with an anti-rabbit, anti-mouse, or anti-goat horseradish peroxidase Ab for 1 hour. The immunoreactive bands detected by enhanced chemiluminescence reagents were developed by Hyperfilm-ECL.

Reverse Transcription-PCR Analysis

Total RNA was isolated from HTSMCs with Trizol according to the protocol of the manufacturer. The cDNA obtained from 0.5 μg total RNA was used as a template for PCR amplification as previously described.¹⁷ The primers used were as follows: 5'-GGAACCTTGACGCT-TACAGTGACAGAGCTCCC-3' (sense) and 5'-CAAGT-CTACATATCACCCAAG-3' (anti-sense) for VCAM-1; 5'-CAAGGGGAGGTC-ACCCGCGAGGTG-3' (sense) and 5'-TGCAGTGCCCATATGACTG-3' (anti-sense) for ICAM-1.

Measurement of ICAM-1 and VCAM-1 Luciferase Activity

The human ICAM-1 (pIC-339/0)/firefly luciferase was kindly provided by Dr. P. T. van der Saag (Hubrecht Laboratory, Utrecht, The Netherlands). For construction of the VCAM-1-luc plasmid, human VCAM-1 promoter, a region spanning -1716 to -119 bp (kindly provided by Dr. W.C. Aird, Department of Molecular Medicine, Beth Israel Deaconess Medical Center, Boston, MA) was cloned into pGL3-basic vector (Promega, Madison, WI). ICAM-1-luc or VCAM-1-luc activity was determined as previously described using a luciferase assay system (Promega, Madison, WI).¹⁷

Adhesion Assay

HTSMCs were grown to confluence in 6-well plates, incubated with $\text{TNF-}\alpha$ for 24 hours, and then adhesion assays were performed. Briefly, THP-1 cells (human acute monocytic leukemia cell line) were labeled with a fluorescent dye, 10 $\mu\text{mol/L}$ BCECF/AM, at 37°C for 1 hour in RPMI-1640 medium (Gibco BRL, Grand Island, NY) and subsequently washed by centrifugation. Confluent HTSMCs in 6-well plates were incubated with THP-1 cells (2×10^6 cells/ml) at 37°C for 1 hour. Non-adherent THP-1 cells were removed and plates were gently washed twice with PBS. The numbers of adherent THP-1 cells were determined by counting four fields per $\times 200$ high-power field well using a fluorescence microscope (Zeiss, Axiovert 200M). Experiments were performed in triplicate and repeated at least three times.

Measurement of Interleukin-6 Generation

Interleukin (IL)-6 released into the medium of HTSMCs cultures was detected using an enzyme-linked immunosorbent assay kit (R&D System, Minneapolis, MN) according to the manufacturer's instructions.

Measurement of Intracellular ROS Accumulation

The intracellular H_2O_2 levels were determined by measuring fluorescence of dichlorofluorescein diacetate (DCFH-DA), and the $\text{O}_2^{\bullet-}$ levels were determined by measuring the level of DHE. The fluorescence for dichlorofluorescein (DCF) and DHE staining was detected at 495/529 and 518/605 nm, respectively, using a fluorescence microscope (Zeiss, Axiovert 200M). For the purpose of these experiments, HTSMCs were washed with warm HBSS and incubated in HBSS or cell medium containing 10 $\mu\text{mol/L}$ DCFH-DA or DHE at 37°C for 45 minutes. Subsequently, HBSS or medium containing DCFH-DA or DHE was removed and replaced with fresh medium. HTSMCs were then incubated with various concentrations of $\text{TNF-}\alpha$. Cells were washed twice with PBS and detached with trypsin/EDTA, and the fluorescence intensity of the cells was analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA) at 518 nm.

excitation and 605 nm emission for DHE and at 495 nm excitation and 529 nm emission for DCF.

Determination of NADPH Oxidase Activity by Chemiluminescence assay

After incubation, cells were gently scraped and centrifuged at $400 \times g$ for 10 minutes at 4°C. The cell pellet was resuspended with 35 μ l/per well of ice-cold RPMI-1640 medium, and the cell suspension was kept on ice. To a final 200 μ l volume of pre-warmed (37°C) RPMI-1640 medium containing either NADPH (1 μ mol/L) or lucigenin (20 μ mol/L), 5 μ l of cell suspension (0.2×10^5 cells) were added to initiate the reaction followed by immediate measurement of chemiluminescence in an Appliskan luminometer (Thermo) in out-of-coincidence mode. Appropriate blanks and controls were established, and chemiluminescence was recorded. Neither NADPH nor NADH enhanced the background chemiluminescence of lucigenin alone (30 to 40 counts per min). Chemiluminescence was continuously measured for 12 minutes, and the activity of NADPH oxidase was expressed as counts per million cells.

Isolation of Cell Fractions

Cells were harvested, sonicated for 10 seconds at output 4 with a sonicator (Ultrasonics Inc. NY), and centrifuged at 8000 rpm for 15 minutes at 4°C. The pellet was collected as the nuclear fraction. The supernatant was centrifuged at 14,000 rpm at 4°C for 60 minutes to yield the pellet (membrane fraction) and the supernatant (cytosolic fraction).

Co-Immunoprecipitation Assay

Cell lysates containing 1 mg of protein were incubated with 2 μ g of an anti-TNFR1, anti-c-Src, or anti-p47^{phox} Ab at 4°C for 24 hours, and then 10 μ l of 50% protein A-agarose beads was added and mixed for 24 hours at 4°C. The immunoprecipitates were collected and washed three times with a lysis buffer without Triton X-100; 5 $\times$ Laemmli buffer was added and subjected to electrophoresis on SDS-polyacrylamide gel electrophoresis, and then blotted using an anti-TNFR1, anti-c-Src, anti-p47^{phox}, or anti-phospho-tyrosine Ab.

Immunofluorescence Staining

Growth-arrested HTSMCs were incubated with TNF- α or CoPP IX for the indicated time intervals. After washing twice with ice-cold PBS, cells were fixed, permeabilized, and stained using an anti-p65 or anti-Nrf2 Ab as previously described.¹⁸ The images were observed using a fluorescence microscope (Zeiss, Axiovert 200M).

HO-1 Activity Assay

The method used for the determination of HO-1 activity via bilirubin formation follows the protocol published by

Motterlini and co-workers.¹⁹ HO-1 activity was measured as picomoles of bilirubin formed per milligram of HTSMCs protein per hour.

Analysis of Data

Concentration-effect curves were fitted and EC₅₀ values were estimated using the GraphPad Prism Program (GraphPad, San Diego, CA). Data were expressed as the mean $\pm$ SEM and analyzed by one-way analysis of variance followed with Tukey's posthoc test. $P < 0.05$ was considered significant.

Results

CoPP IX Induces HO-1 Expression and Activity through Nrf2 in HTSMCs

It has been established that HO-1 could be up-regulated by cobalt protoporphyrin (CoPP).²⁰ Moreover, as expected, CoPP IX also induced HO-1 protein expression (Figure 1A) and HO-1 activity (Figure 1B) in a time-dependent manner, with a maximal response within 16 to 24 hours in HTSMCs. ZnPP IX, an inhibitor of HO-1 activity,²¹ blocked CoPP IX-induced HO-1 activity (Figure 1B). To further determine the role of Nrf2 in CoPP IX-mediated HO-1 induction, cells were transfected with Nrf2 siRNA. As shown in Figure 1C, transfection with Nrf2 siRNA down-regulated the expression of Nrf2 protein and attenuated HO-1 expression induced by CoPP IX. Moreover, CoPP IX also directly enhanced Nrf2 protein expression in HTSMCs (Figure 1D). Translocation of Nrf2 from the cytosol into the nucleus was seen in CoPP IX-treated HTSMCs, determined by Western blot (Figure 1E). To further ensure this result, nuclear translocation of Nrf2 was confirmed by using an immunofluorescence staining (Figure 1F). Altogether, these results indicate that CoPP IX-induced HO-1 expression was mediated through Nrf2 in HTSMCs.

CoPP IX Suppresses TNF- α -Induced Airway Inflammatory Response via HO-1-Dependent Pathway

In our study, TNF- α has been shown to stimulate the expression of various "pro-asthmatic" mediators, including IL-6 as well as adhesion molecules *in vitro* and *in vivo* (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). We next investigated whether CoPP IX modulated TNF- α -induced adhesion molecules expression and IL-6 production in HTSMCs. Pretreatment of HTSMCs with CoPP IX significantly abrogated TNF- α -induced ICAM-1 and VCAM-1 protein levels, mRNA expression, promoter activity, THP-1 cell adherence, and IL-6 production (Figure 2, A-E). Moreover, these observed inhibitory effects by CoPP IX were abrogated in the presence of ZnPP IX (Figure 2, A-E). To further investigate whether these inhibitory effects of CoPP IX on adhesion molecule expression and IL-6 production were mediated through an Nrf2-

Figure 1. CoPP IX induces Nrf2-dependent HO-1 expression and activity in HTSMCs. **A:** Cells were incubated with CoPP IX for the indicated time intervals and HO-1 protein expression was determined by Western blot. **B:** Cells were incubated with 1 μmol/L CoPP IX in the absence or presence of 1 μmol/L ZnPP IX for the indicated time intervals and HO-1 activity was determined. Data are expressed as mean ± SEM of three independent experiments. * $P < 0.05$; ** $P < 0.01$ as compared with the basal level. *** $P < 0.05$ as compared with the cells exposed to CoPP IX alone for 16 hours. **C:** Cells were transfected with siRNA of scramble or Nrf2, and then treated with CoPP IX for 16 hours. The expression of Nrf2 or HO-1 was examined. **D, E:** Cells were incubated with CoPP IX for 16 hours or 24 hours, and then **(D)** the expression of Nrf2 was examined or **(E)** cytosolic (CE) and nuclear (NE) extracts were prepared and subjected to Western blot using an anti-Nrf2 Ab. Lamin A and GAPDH were used as marker proteins for nuclear and cytosolic extracts, respectively. **F:** Cells were treated with CoPP IX for 16 hours. Cells were fixed, and then labeled using an anti-Nrf2 Ab and fluorescein isothiocyanate-conjugated secondary Ab. Individual cells were imaged.

dependent HO-1 expression, cells were pretreated with CoPP IX, or transfected with either HO-1 siRNA or Nrf2 siRNA, and then incubated with TNF- α for 24 hours. As shown in Figure 2, F and G, the observed inhibition of ICAM-1 and VCAM-1 protein levels and IL-6 generation by CoPP IX was abrogated by transfection with either HO-1 siRNA or Nrf2 siRNA.

To confirm these results, in an *in vivo* study, mice were intratracheally administered with CoPP IX for 16 hours, and then followed with TNF- α for 24 hours. BAL fluid was acquired and airway tissues were homogenized to extract proteins. As shown in Figure 3, A and B, administration with CoPP IX significantly inhibited TNF- α -induced ICAM-1 and VCAM-1 expression and leukocyte count in BAL fluid. Moreover, immunohistochemical staining with Abs against VCAM-1 and ICAM-1 or anti- α -actin Ab (positive staining for smooth muscle cells) was performed on serial sections. In TNF- α -treated mice, ASM strongly expressed VCAM-1 or ICAM-1 (Figure 3C). In CoPP IX-pretreated mice, CoPP IX markedly reduced the expression of VCAM-1 and ICAM-1. The observed suppression of TNF- α -mediated ICAM-1 and VCAM-1 expression and leukocyte count in BAL fluid by CoPP IX was abrogated in the presence of ZnPP IX (Figure 3A-C). These data demonstrated that overexpression of HO-1 suppressed TNF- α -induced ICAM-1 and VCAM-1 expression *in vitro* and *in vivo*, which was dependent on Nrf2 activation.

TNF- α -Induced Adhesion Molecules Expression and ROS Generation Are Mediated by the Formation of a TNFR1/c-Src/p47^{phox} Complex

ROS produced by cytokines contribute to the intracellular signaling cascades associated with inflammatory responses.⁹ NADPH oxidase is an important source for the production of ROS under various pathological condi-

tions.⁹ To determine whether ROS generated by NADPH oxidase involved in TNF- α -induced responses, pretreatment of HTSMCs with a ROS scavenger (NAC) and NADPH oxidase inhibitors (DPI and apocynin, APO), or transfection with p47^{phox} siRNA significantly abrogated TNF- α -induced ICAM-1 and VCAM-1 protein levels, mRNA expression, promoter activity, or THP-1 cell adherence (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). In addition, TNF- α induced a significant increase in superoxide and H₂O₂ levels, which were inhibited by pretreatment with NAC, DPI, or APO (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). TNF- α also induced a significant increase in NADPH oxidase activity and translocation of p47^{phox} from the cytosol to the membrane (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). These data demonstrated the involvement of NADPH oxidase in supporting ROS generation induced by TNF- α in HTSMCs.

Most of TNF- α actions are elicited through TNFR1.²² The Src family kinases have been shown to regulate ICAM-1 and VCAM-1 expression, NADPH oxidase activation, and ROS generation.^{5-7,23} Therefore, we investigated whether TNFR1 and c-Src involved in adhesion molecules expression, p47^{phox} translocation, and ROS production. Pretreatment with an anti-TNFR1 neutralizing Ab or a c-Src kinase inhibitor (PP1) attenuated TNF- α -induced ICAM-1 and VCAM-1 protein levels (Figure 4A). TNF- α -induced ICAM-1 and VCAM-1 mRNA expression, promoter activity, and THP-1 cell adherence were also inhibited by pretreatment with PP1 (see Supplemental Figure S3 at <http://ajp.amjpathol.org>). Moreover, the major phosphorylation site of c-Src at the Tyr⁴¹⁶ residue results in c-Src autophosphorylation.²⁴ Thus, we further examined c-Src phosphorylation at Tyr⁴¹⁶ stimulated by TNF- α in HTSMCs using an anti-phospho-c-Src Ab at Tyr⁴¹⁶. In our study, TNF- α stimulated a time-dependent Tyr⁴¹⁶

Figure 2. Overexpression of Nrf2-dependent HO-1 inhibits VCAM-1 and ICAM-1 expression in HTSMCs. Cells were pretreated with CoPP IX for 16 hours in the presence or absence of ZnPP IX, and then treated with TNF-α for (A) 24 hours or (B) 5 hours. The protein levels and mRNA expression of VCAM-1 and ICAM-1 were determined. C: Cells were transiently transfected with an ICAM-1- or VCAM-1-luc reporter gene, pretreated with CoPP IX for 16 hours in the presence or absence of ZnPP IX, and then treated with TNF-α for 4 hours. The luciferase activity of ICAM-1 or VCAM-1 was measured. D, E: HTSMCs were pretreated with CoPP IX for 16 hours in the presence or absence of ZnPP IX, and then treated with TNF-α for 24 hours. The (D) THP-1 cells adherence and (E) IL-6 generation were measured. F, G: Cells were pretreated with CoPP IX for 16 hours, transfected with siRNA of HO-1, Nrf2, or scrambled, and then incubated with TNF-α for 24 hours. The (F) levels of VCAM-1 and ICAM-1 expression and (G) IL-6 generation were measured. Data are expressed as mean ± SEM of three independent experiments. Significant differences between the compared groups are indicated: **P* < 0.05; #*P* < 0.01.

phosphorylation of c-Src, with a maximal response within 10 to 30 minutes, which was attenuated by pretreatment with PP1 during the period of observation (Figure 4B). To further investigate the physical association between TNFR1

and c-Src in TNF-α-induced responses, cells were treated with TNF-α for the indicated time intervals and the cell lysates were subjected to immunoprecipitation using an anti-TNFR1 Ab. The immunoprecipitates were analyzed by

Figure 3. Overexpression of HO-1 inhibits VCAM-1 and ICAM-1 expression *in vivo*. Mice were intratracheally injected with CoPP IX for 16 hours in the presence or absence of ZnPP IX, and then injected with TNF-α for 24 hours. **A:** Airway tissues were homogenized to extract protein. The levels of ICAM-1 and VCAM-1 expression were determined. **B:** BAL fluid was acquired and leukocyte count was determined by a hemocytometer. Significant differences between the compared groups are indicated: **P* < 0.05; #*P* < 0.01. **C:** Immunohistochemical staining for VCAM-1, ICAM-1, or α-actin in serial sections of the airways from bovine serum albumin-treated mice (Sham), TNF-α-injected mice (TNF-α), CoPP IX-pretreated mice (CoPP IX + TNF-α), and CoPP IX-pretreated in the presence of ZnPP IX mice (CoPP IX + ZnPP IX + TNF-α). The airway smooth muscle (ASM) is indicated by an arrowhead. The arrow indicates smooth muscle cells overlapping with VCAM-1 and ICAM-1 expression. H&E stain for the airway from untreated mice was observed (top).

Western blot using an anti-c-Src Ab. The protein levels of c-Src were time-dependently increased in TNFR1-immunoprecipitated complexes (Figure 4C).

We next investigated the role of c-Src in the activation of NADPH oxidase induced by TNF-α. As illustrated in Figure 5, A–C, pretreatment with PP1 attenuated TNF-α-induced ROS generation, NADPH oxidase activity, and p47^{phox} translocation. Src has been shown to mediate tyrosine phosphorylation of p47^{phox} in hyperoxia-induced activation of NADPH oxidase and generation of ROS in lung endothelial cells.²³ Thus, we also investigated whether TNF-α stimulated tyrosine phosphorylation of p47^{phox} and the involvement of c-Src in tyrosine phosphorylation of p47^{phox}. Cells were pretreated with PP1 and then stimulated with TNF-α for 10 minutes. The cell lysates were subjected to immunoprecipitation with an anti-p47^{phox} Ab and analyzed by Western blot using an anti-phosphotyrosine Ab. The results revealed that TNF-α-induced tyrosine phosphorylation of p47^{phox} was inhibited by pretreatment with PP1 (Figure 5D). We further investigated the physical association between c-Src and p47^{phox} in TNF-α-induced NADPH oxidase activation.

As shown in Figure 5E, cells were pretreated without or with PP1, and then stimulated with TNF-α for 10 minutes. The cell lysates were subjected to immunoprecipitation using an anti-c-Src Ab, and then analyzed by Western blot using an anti-p47^{phox} Ab. The protein level of p47^{phox} was increased in a c-Src-immunoprecipitated complex. Pretreatment with PP1 decreased the association of c-Src/p47^{phox}. These results demonstrated that TNF-α induced adhesion molecules expression and NADPH oxidase activation via the formation of a TNFR1/c-Src/p47^{phox} complex.

HO-1 Overexpression Abrogates TNF-α-Induced Oxidative Stress in HTSMCs

HO-1 has been reported to play a critical role in protection against oxidative tissue injury following inflammation.²⁵ Thus, we investigated whether CoPP IX inhibited ROS generation through up-regulation of HO-1 expression in HTSMCs. As illustrated in Figure 6A, pretreatment with CoPP IX for 16 hours significantly inhibited intracellular

Figure 4. TNF- α -enhanced ICAM-1 and VCAM-1 expression are mediated by the formation of a TNFR1/c-Src complex. **A:** Cells were pretreated with an anti-TNFR1 neutralizing Ab or PP1, and then incubated with TNF- α for 24 hours. The levels of VCAM-1 and ICAM-1 expression were determined. **B:** Cells were pretreated with or without PP1 (10 μ M/L), and then stimulated with TNF- α for the indicated time intervals. The cell lysates were subjected to Western blot using an anti-phospho-c-Src (Tyr⁴¹⁶) Ab. **C:** Cells were treated with TNF- α for the indicated time intervals and the cell lysates were subjected to immunoprecipitation using an anti-TNFR1 Ab, and then the immunoprecipitates were analyzed by Western blot using an anti-c-Src or anti-TNFR1 Ab.

ROS generation, including superoxide and H₂O₂. To further confirm these results, the fluorescence for DCF and DHE staining were observed using a fluorescence microscope (Figure 6B). Pretreatment with CoPP IX also inhibited TNF- α -induced NADPH oxidase activity and p47^{phox} activation (Figure 6, C and D). We next determined the effects of CoPP IX on tyrosine phosphorylation of p47^{phox} and c-Src, or the association of TNFR1/c-Src/p47^{phox}. It was found that pretreatment with CoPP IX reduced phosphorylation of p47^{phox} and c-Src, and the formation of a TNFR1/c-Src/p47^{phox} complex induced by TNF- α (Figure 6, E–G). The observed suppression of TNF- α -mediated ROS generation, NADPH oxidase activity, p47^{phox} translocation, p47^{phox} and c-Src phosphorylation, or the association of TNFR1/c-Src/p47^{phox} by CoPP IX was abro-

gated in the presence of ZnPP IX (Figure 6H). These results indicated that HO-1 overexpression suppressed TNF- α -induced ICAM-1 and VCAM-1 expression by down-regulation of NADPH oxidase-dependent ROS production and the formation of a TNFR1/c-Src/p47^{phox} complex.

CoPP IX Suppresses TNF- α -Induced NF- κ B Activation via HO-1-Dependent Pathway in HTSMCs

Activation of NF- κ B is a central event in the initiation and amplification of inflammatory responses.²⁶ Moreover, it was also found that TNF- α -induced ICAM-1 and VCAM-1 protein

Figure 5. c-Src is involved in TNF- α -mediated NADPH oxidase activation. **A:** DHE and DCF fluorescence intensities after stimulation with TNF- α for 1 hour in the presence or absence of PP1 (10 μ M/L) were measured by a flow cytometer. **B, C:** Cells were pretreated with PP1 for 1 hour, and then treated with TNF- α for 1 hour. **B:** NADPH oxidase activity was measured. Data are expressed as mean $\pm$ SEM of three independent experiments. Significant differences between the compared groups are indicated: * P < 0.05; # P < 0.01. **C:** The membrane and cytosolic extracts were prepared and subjected to Western blot using an anti-p47^{phox} Ab. **D:** Cells were pretreated without or with PP1 for 1 hour, and then stimulated with TNF- α for 10 minutes. Total cell lysates were subjected to immunoprecipitation with anti-p47^{phox} Ab and the immunoprecipitates were analyzed by Western blot using an anti-phosphotyrosine Ab. **E:** Cells were pretreated without or with PP1, and then stimulated with TNF- α for 10 minutes. The cell lysates were subjected to immunoprecipitation using an anti-c-Src Ab, and then the immunoprecipitates were analyzed by Western blot using an anti-p47^{phox} or anti-c-Src Ab.

Figure 6. Overexpression of HO-1 inhibits TNF-α-induced ROS generation by down-regulation of the association of TNFR1/c-Src/p47^{phox}. **A:** DHE and DCF fluorescence intensities of HTSMCs after stimulation with TNF-α for 1 hour in the presence or absence of CoPP IX (1 μmol/L) or ZnPP IX (1 μmol/L) were measured. **B:** DHE or DCF fluorescence image after 1 hour of stimulation with TNF-α in the presence or absence of CoPP IX or ZnPP IX. Images shown are representative of three independent experiments with similar results. **C:** Cells were pretreated with CoPP IX for 16 hours in the presence or absence of ZnPP IX or pretreated with DPI or APO, and then treated with TNF-α for 1 hour. NADPH oxidase activity was determined. Data are expressed as mean ± SEM of three independent experiments. Significant differences between the compared groups are indicated: **P* < 0.05. **D:** Cells were pretreated with CoPP IX for 16 hours in the presence or absence of ZnPP IX or pretreated with APO, and then treated with TNF-α for 1 hour. The membrane and cytosolic extracts were prepared and subjected to Western blot using an anti-p47^{phox} Ab. **E, F, G:** Cells were pretreated with CoPP IX in the presence or absence of ZnPP IX, and then stimulated with TNF-α for 10 minutes. **E:** Total cell lysates were subjected to immunoprecipitation with anti-p47^{phox} Ab and the immunoprecipitates were analyzed by Western blot using an anti-phosphotyrosine or anti-p47^{phox} Ab. **F:** The cell lysates were subjected to Western blot using an anti-phospho-c-Src (Tyr⁴¹⁶) or anti-c-Src Ab. **G:** The cell lysates were subjected to immunoprecipitation using an anti-TNFR1 or anti-c-Src Ab, and then the immunoprecipitates were analyzed by Western blot using an anti-TNFR1, anti-p47^{phox}, or anti-c-Src Ab.

levels, mRNA expression, promoter activity, and THP-1 cell adherence were inhibited by pretreatment with a NF-κB inhibitor, helenalin (see Supplemental Figure S4 at <http://ajp.amjpathol.org>). Recently, c-Src and ROS have been shown to regulate NF-κB activation.^{27,28} Therefore, we next investigated the roles of c-Src and ROS in TNF-α-mediated

NF-κB activation. As shown in Figure 7A (left), TNF-α stimulated NF-κB p65 phosphorylation and translocation, or IκBα phosphorylation and degradation in a time-dependent manner. Moreover, TNF-α-induced NF-κB p65 phosphorylation was inhibited by pretreatment with helenalin, PP1, NAC, DPI, or APO (Figure 7A, right). To investigate whether

Figure 7. Overexpression of Nrf2-dependent HO-1 inhibits NF- κ B activation and translocation. **A:** Cells were stimulated with TNF- α for the indicated time intervals. The cell lysates were subjected to Western blot using an anti-phospho-p65 (Ser⁵³⁶) or anti-phospho-I κ B α (Ser³²) Ab. The cytosolic and nuclear extracts were prepared and subjected to Western blot using an anti-p65 or anti-I κ B α Ab (**left**). Cells were pretreated with helenalin (1 μ M/L), PP1 (10 μ M/L), NAC (10 mmol/L), DPI (10 μ M/L), or APO (100 μ M/L), and then treated with TNF- α for 1 hour. The cell lysates were subjected to Western blot using an anti-phospho-p65 (Ser⁵³⁶) or anti-phospho-I κ B α (Ser³²) Ab (**right**). **B, C:** Cells were pretreated with CoPP IX for 16 hours in the presence or absence of ZnPP IX, and then treated with TNF- α for 1 hour. **B:** The cell lysates were subjected to Western blot using an anti-phospho-p65 (Ser⁵³⁶) or anti-phospho-I κ B α (Ser³²) Ab. The cytosolic and nuclear extracts were prepared and subjected to Western blot using an anti-p65 or anti-I κ B α Ab. **C:** Cells were fixed, and then labeled with anti-p65 Ab and fluorescein isothiocyanate-conjugated secondary Ab. Individual cells were imaged as described in the *Materials and Methods*. **D:** Cells were transiently transfected with a NF- κ B-luciferase reporter gene, and then treated with TNF- α for the indicated time intervals (**left**) or for 1 hour in the presence or absence of CoPP IX or ZnPP IX (**right**). The luciferase activity of NF- κ B was measured. Data represent the mean $\pm$ SEM from at least three independent experiments. * P < 0.05; * P < 0.01 as compared with the basal level (**left**). Significant differences between the compared groups are indicated: * P < 0.05; * P < 0.01 (**right**).

HO-1 overexpression inhibited TNF- α -induced ICAM-1 and VCAM-1 expression by down-regulation of NF- κ B activation, cells were pretreated with CoPP IX for 16 hours, and then stimulated with TNF- α for 1 hour. As shown in Figure 7B, TNF- α -stimulated p65 phosphorylation at Ser⁵³⁶, NF- κ B translocation, or I κ B α degradation was inhibited by pretreatment with CoPP IX. These results with nuclear translocation of NF- κ B (p65) were further ensured by an immunofluorescence staining (Figure 7C). Moreover, pretreatment with CoPP IX also reduced TNF- α -induced NF- κ B promoter activity (Figure 7D, right). The observed suppression of TNF- α -stimulated NF- κ B phosphorylation, translocation, and promoter activity by CoPP IX was abrogated in the presence of ZnPP IX (Figure 7, B–D). These results indicated that HO-1 overexpression suppressed TNF- α -induced ICAM-1 and VCAM-1 expression through down-regulation of NF- κ B activation and translocation.

Adenovirus-Mediated HO-1 Transduction Suppresses TNF- α -Induced Inflammatory Responses in Vitro

To confirm the role of HO-1 in TNF- α -induced responses, HTSMCs were infected with an empty Adv or Adv-HO-1.

As shown in Figure 8A, HO-1 was overexpressed in HTSMCs infected with Adv-HO-1 but not with Adv. In addition, TNF- α -induced ICAM-1 and VCAM-1 protein levels, THP-1 cells adherence, IL-6 production, or ROS generation was abolished in HTSMCs infected with Adv-HO-1 (Figure 8, B–E). We also found that TNF- α -stimulated p65 phosphorylation at Ser⁵³⁶ was significantly attenuated by infection with Adv-HO-1 (Figure 8F). The suppressive effects of Adv-HO-1 were significantly reversed by treatment with ZnPP IX (Figure 8, C–F).

Carbon Monoxide from HO-1 Suppresses Adhesion Molecules Expression, ROS Generation, and IL-6 Production in HTSMCs

The antioxidant activity of HO-1 has been reported to be due to the elimination of pro-oxidant heme and the biological activities of bilirubin and CO. In addition, CO also exerts several biological functions, including anti-apoptotic and anti-inflammatory properties.¹¹ To further examine the mechanisms by which end metabolites of heme catabolism by HO-1, including CO, contribute to the attenuation of TNF- α -mediated responses in HTSMCs, cells were incubated with the CO donor, CO-RM2 (CO releasing

Figure 8. Adenovirus-regulated HO-1 transduction reduces TNF- α -induced inflammatory responses. **A:** HTSMCs were infected with an indicated multiplicity of infection (MOI) of either Adv or Adv-HO-1 for the indicated time intervals. HTSMCs were infected with 10 MOI of Adv or Adv-HO-1 for 48 hours in the presence or absence of ZnPP IX, followed by treatment with TNF- α for **(B, C, D)** 24 hours or **(E, F)** 1 hour. The **(B)** levels of VCAM-1 and ICAM-1 proteins, **(C)** THP-1 cells adherence, **(D)** IL-6 generation, **(E)** DCF fluorescence intensity, and **(F)** p65 phosphorylation at Ser⁵³⁶ were measured. Data are expressed as mean $\pm$ SEM of three independent experiments. Significant differences between the compared groups are indicated: * P < 0.05; # P < 0.01.

molecule, $[\text{Ru}(\text{CO})_3\text{Cl}_2]_2$ before the addition of TNF- α (Figure 9, A–C). The results showed that pretreatment with CO-RM2 caused a significant decrease in TNF- α -induced adhesion molecules expression, ROS generation, and IL-6 secretion. We further examined the possibility that endogenous CO production by CoPP IX-mediated Nrf2/HO-1 activation may also account for the attenuation of TNF- α -induced responses by CoPP IX. As shown in Figure 9D–G, the addition of CO scavenger, hemoglobin, significantly reversed the attenuating effects of CoPP IX on adhesion molecules expression, p47^{phox} translocation, ROS generation, and IL-6 secretion induced by TNF- α , indicating that CoPP IX contributes to protection of cells from TNF- α insult through, in part, a CO-dependent attenuation of adhesion molecules expression, ROS generation, and IL-6 secretion.

Discussion

TNF- α has been implicated in the pathophysiology of many inflammatory lung diseases, including chronic bronchitis, COPD, acute respiratory distress syndrome, and asthma.²² The elevated levels of TNF- α have been detected in the airways, BAL fluid, and nasal lavage from asthmatic and rhinitic patients. Leukocytes and monocytes isolated from BAL fluid of asthmatics were shown to release more TNF- α than those of cells from control subjects. Recent research indicates that TNF- α may be associated with acquired airway hyperresponsiveness, which is a pathophysiological hallmark of asthma.³ It has been suggested that TNF- α up-regulates adhesion mol-

ecules and is directly responsible for transendothelial migration of inflammatory cells, which is a central feature underlying the inflammatory responses.⁴ Oxidative processes are considered to play an important role in the induction of cell adhesion molecules, a key event in inflammatory processes. Moreover, HO-1 possesses antioxidant and anti-inflammatory properties.²⁵ Overexpression of HO-1 has been shown to decrease airway inflammation and airway responsiveness to histamine in ovalbumin-sensitized guinea pigs,²⁹ suggesting that HO-1 plays a critical role in protecting the host during airway inflammation. However, the molecular mechanisms underlying overexpression of HO-1-inhibited inflammatory response in HTSMCs remain unclear. In this study, we found that TNF- α markedly induced ROS generation, adhesion molecules expression, and IL-6 secretion in HTSMCs. In addition, TNF- α enhanced adhesion molecules expression and leukocyte count in BAL fluid *in vivo*. We also observed that TNF- α (0.75 mg/kg body weight) markedly induced pulmonary hematoma in mice. However, these responses will further lead to airway inflammatory diseases, such as asthma or COPD. Pretreatment with CoPP IX or infection with Adv-HO-1 inhibited TNF- α -induced adhesion molecules expression, THP-1 cell adherence, IL-6 generation, and NF- κ B activation. Moreover, HO-1 overexpression inhibited ROS generation by down-regulation of the formation of a TNFR1/c-Src/p47^{phox} complex. Thus, as induction of HO-1 expression may hence hold therapeutic promises, continuous efforts toward identifying novel airway protective antioxidant/anti-inflammatory substances that target HO-1

Figure 9. Endogenous CO production in CoPP IX-treated HTSMCs and its role on the attenuation of TNF- α -mediated adhesion molecules expression and ROS generation. Cells were pretreated with 50 μ M CO-RM2, and then incubated with TNF- α for (A, C) 24 hours or (B) 1 hour. **A:** The levels of VCAM-1 and ICAM-1 expression were determined. **B:** The fluorescence intensity (relative DCF fluorescence) was measured. **C:** IL-6 generation was measured. Cells were pretreated with CoPP IX in the presence or absence of 20 μ g/ml hemoglobin, and then incubated with TNF- α for (D, G) 24 hours or (E, F) 1 hour. **D:** The levels of VCAM-1 and ICAM-1 expression were determined. **E:** The membrane and cytosolic extracts were prepared and subjected to Western blot using an anti-p47^{phox} Ab. **F:** The fluorescence intensity (relative DCF fluorescence) was measured. **G:** IL-6 generation was measured. Data are expressed as mean $\pm$ SEM of three independent experiments. Significant differences between the compared groups are indicated: * P < 0.05; * P < 0.01.

and establishment of well-designed *in vivo* models properly evaluating the efficacy of these agents will be warranted.

HO-1 catalyzes the rate-limiting step in the catabolism of heme to biliverdin, free iron, and CO.¹⁰ HO-1 has been reported to reduce inflammatory cell rolling, adhesion, and migration from the vascular compartment, possibly by down-regulating the function and expression of adhesion molecules on the vessel wall.³⁰ HO-1 has been shown to be up-regulated by a variety of stressful stimuli, such as CoPP.²⁰ We also found that CoPP IX induced HO-1 protein expression and activity in HTSMCs. Nrf2 is a major transcription factor that regulates expression of antioxidant defense genes through binding to antioxidant response elements in the promoter region of antioxidant genes, such as HO-1.¹⁴ This is confirmed by our observation that CoPP IX-induced HO-1 expression was mediated through Nrf2 by transfection with Nrf2 siRNA. We further demonstrated that the cytoprotective properties of HO-1 were due to the attenuation of adhesion molecules expression, IL-6 generation, THP-1 cells adherence, and leukocyte count in BAL fluid *in vitro* and *in vivo*. We also further ascertained that CoPP IX inhibited adhesion molecules expression and IL-6 production through Nrf2-dependent HO-1 expression by transfection with siRNA of HO-1 or Nrf2. These results suggested that the protective effects of HO-1 are dependent on Nrf2 activation in HTSMCs.

Figure 10. A proposed pathway for overexpression of Nrf2-dependent HO-1 protects against TNF- α -induced oxidative stress and airway inflammation. TNF- α stimulates ROS production through TNFR1/c-Src/NADPH oxidase, in turn initiates the activation of NF- κ B. Activated NF- κ B was recruited to the promoter regions of ICAM-1 and VCAM-1 leading to an increase of adhesion molecules expression. Moreover, CoPP IX is capable of inducing HO-1 expression, activating Nrf2-dependent pathway, resulting in inhibition of c-Src and p47^{phox} activation, ROS generation, IL-6 production, and NF- κ B activation, and suppressing adhesion molecules expression and THP-1 cells adherence on HTSMCs.

When airway cells and tissues are exposed to oxidative stress, increased levels of ROS exert many deleterious effects within the airways.³⁰ The NADPH oxidase family members are proteins that transfer electrons across biological membranes. In general, the electron acceptor is oxygen and the product of the electron transfer reaction is a superoxide. Therefore, the biological function of NADPH oxidase enzymes might be attributable to the production of ROS.³¹ ROS have been shown to mediate the expression of VCAM-1 and ICAM-1.⁸ Most of TNF- α actions are elicited by TNFR1, which contains a death domain that fosters protein-protein interactions, particularly with other death-domain proteins.²² The Src family kinases have been shown to regulate ICAM-1 and VCAM-1 expression, NADPH oxidase activation, and ROS generation.^{5–7,23} In our study, we also established that TNF- α induced adhesion molecules expression and ROS generation through TNFR1 and c-Src by pretreatment with an anti-TNFR1 neutralizing Ab or PP1. In addition, our data also showed that TNF- α induced adhesion molecules expression and NADPH oxidase activation via the formation of a TNFR1/c-Src/p47^{phox} complex in HTSMCs. The domains of TNFR1, c-Src, and p47^{phox} involved in protein-protein interaction caused by TNF- α are important issues needed to be investigated in the future. However, HO-1 has been reported to play a critical role in protection against oxidative tissue injury following ischemia and inflammation.²⁵ Therefore, we also investigated whether CoPP IX inhibited ROS production by up-regulation of HO-1 expression. In our study, we found that overexpression of HO-1 reduced ROS generation, NADPH oxidase activity, and p47^{phox} activation. These results demonstrated that HO-1 overexpression reduced the formation of a TNFR1/c-Src/p47^{phox} complex induced by TNF- α in HTSMCs.

NF- κ B has been shown to be an inducible and ubiquitously expressed transcription factor responsible for regulating the expression of genes involved in cell survival, cell adhesion, and inflammation.³² Furthermore, increased levels of ROS have been implicated in initiating inflammatory responses in the lungs through the activation of transcription factors, such as NF- κ B and AP-1.³² This is confirmed by our observation that TNF- α -induced NF- κ B translocation was inhibited by pretreatment with NAC, DPI, or APO. These results suggested that ROS-dependent NF- κ B activation is necessary for adhesion molecules induction by TNF- α in HTSMCs. Thus, ROS may be critical for the inflammatory responses triggered by TNF- α , through the up-regulation of redox-sensitive transcription factors and hence pro-inflammatory gene expression. Moreover, we found that HO-1 overexpression decreased TNF- α -stimulated NF- κ B phosphorylation and translocation from the cytosol to the nucleus. I κ B α phosphorylation was also reduced by pretreatment with CoPP IX. The observed suppression of TNF- α -regulated I κ B α degradation, or NF- κ B phosphorylation and translocation by CoPP IX, was abrogated in the presence of ZnPP IX. Thus, NF- κ B also appears to be one of the important targets for the action of HO-1, although further studies are required to elucidate the underlying mechanisms.

The signaling properties of CO may contribute to the antioxidant and anti-inflammatory effects of HO-1.¹¹ Thus, we investigated the mechanisms by which end metabolites of heme catabolism by HO-1, including CO, contributed to the attenuation of TNF- α -mediated adhesion molecules expression, ROS generation, and IL-6 secretion in HTSMCs. We found that pretreatment with CO-RM2 caused a significant decrease in TNF- α -induced adhesion molecules expression, ROS generation, and IL-6 secretion. The addition of CO scavenger, hemoglobin, also significantly reversed the attenuating effects of CoPP IX on TNF- α -mediated responses, indicating that CoPP IX contributed to protection of cells from TNF- α insult through, in part, a CO-dependent attenuation of adhesion molecules expression, ROS generation, and IL-6 secretion. In any event, the diverse effects of endogenous CO derived from heme degradation on cellular signaling pathways in HTSMCs warrant further investigation. Thus, the signaling properties of CO may contribute to the anti-inflammatory effects of HO-1. In asthma, a potentially critical role for HO-1-derived CO is its ability to relax airway smooth muscle via the activation of guanylyl cyclase and formation of cGMP. HO-1 and its products may be useful in both diagnostic and therapeutic strategies. Defining not only the regulation of HO-1 during airway inflammation and asthma but also diagnostic and therapeutic strategies of CO and the other components of the HO-1 pathway will remain an important avenue of investigation in the future.

In summary, as depicted in Figure 10, TNF- α induces ROS generation through TNFR1/c-Src/NADPH oxidase, and in turn initiates the activation of NF- κ B. Activated NF- κ B was recruited to the promoter regions of ICAM-1 and VCAM-1 leading to an increase of ICAM-1 and VCAM-1 promoter activity and the expression of ICAM-1 and VCAM-1 mRNA and protein in HTSMCs. Moreover, CoPP IX is capable of inducing HO-1 expression, activating Nrf2-dependent pathway, resulting in inhibition of c-Src and p47^{phox} activation, ROS generation, IL-6 production, and NF- κ B activation, and suppressing adhesion molecules expression and THP-1 cells adherence in HTSMCs. Taken together, these results showed that overexpression of HO-1 might protect against TNF- α -induced airway inflammation by down-regulation of ROS generation and NADPH oxidase activity.

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