

# Tumor Necrosis Factor- $\alpha$ Regulates Heme Oxygenase-1 Expression in Endothelial Cells via the Phosphorylation of JNK/p38

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**Abstract**—Heme oxygenase-1 (HO-1), an enzyme degrading heme to carbon monoxide, iron, and biliverdin, has been recognized as playing a crucial role in cellular defense against stressful conditions, not only related to heme release. In the present study, the effects of TNF- $\alpha$  on the expression of heme oxygenase-1 (HO-1) in human aortic endothelial cells (HAECs) as well as the related mechanisms were investigated. 10 ng/mL TNF- $\alpha$  treatment significantly increased HO-1 expression after 6h, then a further increase at 12h and declined at 24h. Treatment with 2 ng/mL of TNF- $\alpha$  after 12 h resulted in a significant increase in HO-1 expression, which peaked at 10 ng/mL, then declined at 20 ng/mL. TNF- $\alpha$  induced HO-1 expression and then HO-1 expression reduced vascular cell adhesion molecule-1 (VCAM-1) expression. Phosphorylation studies of ERK1/2, JNK, and p38, three subgroups of mitogen-activated protein kinases (MAPKs) demonstrated TNF- $\alpha$ -induced ERK1/2, JNK, and p38 phosphorylation. The increase in HO-1 expression in response to TNF- $\alpha$  treatment was affected by pretreatment with SP600125 (a JNK inhibitor) and SB203580 (a p38 inhibitor), not with PD98059 (an ERK1/2 inhibitor). The expression of HO-1 was stronger in aortas of TNF- $\alpha$ -treated apo-E deficient mice when compared with control mice. These results suggest that low dose of TNF- $\alpha$  treatment notably induced HO-1 expression was mediated through JNK/p38 phosphorylation and may have a protective potential in cardiovascular diseases and inflammatory response through the regulation of HO-1 expression.

**Keywords**—Heme oxygenase-1 inflammation, endothelial cells, mitogen-activated protein kinases (MAPKs).

## I. INTRODUCTION

**I**NFLAMMATION is an underlying mechanism of atherogenesis, a process in which cytokines are well known to participate. HO-1 is an inducible enzyme with broad tissue expression that is upregulated in response to oxidant stress and inflammatory stimuli and preserves vascular homeostasis [1]-[3]. HO-1 protects tissues during inflammatory stress through degradation of prooxidative heme, production of

bilirubin, and carbon monoxide (CO) and regulation of cellular iron [4]-[7]. In contrast, HO-1 inhibition is associated with tissue pathology in atherosclerosis and other inflammatory conditions associated with intravascular thrombosis such as septic shock, hypoxia, and graft rejection [8]-[11]. HO-1 and its byproducts regulate inflammatory responses through repression of proinflammatory genes suggesting a role for HO-1 in the regulation of thrombosis associated with inflammation [12]. Although the molecules have received considerable attention, little is known about the effects of TNF- $\alpha$  on HO-1 expression, and a better understanding of this might provide important insights into the prevention of atherogenesis and inflammation. Possible effects of TNF- $\alpha$  on HO-1 expression might be a new avenue in the prevention and/or treatment of cardiovascular disorders. In this study, we have examined the pharmacological effects of TNF- $\alpha$  on HO-1 expression, and specific signaling pathways in human aortic endothelial cells cultured under inflammatory conditions. Our study shows that cells treated with low dose and short time of TNF- $\alpha$  increases the expression of HO-1, and that this effect is mediated by partial blockage of JNK/p38 expressions.

## II. MATERIALS AND METHODS

### A. Culture of HAECs

HAECs were obtained as cryopreserved tertiary cultures from Cascade Biologics (Oregon, USA) used between passages 3 and 8. The cells were cultured at 37°C in a humidified atmosphere of 95% air, 5% CO<sub>2</sub> and. The purity of the cultures was verified by staining with monoclonal antibody against human von-Willebrand factor (vWF).

### B. Cell Viability Assay Using MTT

Cell viability and activity of mitochondrial electron transport chain, as indicator for cytotoxicity, was determined by the capacity of cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan.

### C. Western Blot Analysis

To prepare cell lysates, the cells were lysed for 1h at 4°C in 20mM Tris-HCl, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1 % Triton X-100, 1mM phenylmethylsulfonyl fluoride, pH 7.4, then the lysate was centrifuged at 4000g for 30min at 4°C and the supernatant retained. Western blot analyses were

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performed as described previously. Briefly, samples of cell lysate (20 $\mu$ g of protein) were subjected to 12% SDS-PAGE and transferred to PVDF membranes, which were then treated with 3% nonfat milk in 0.1 M phosphate buffer for 1h at RT to block nonspecific binding of antibody. The membranes were then incubated with rabbit antibodies against human HO-1, human phospho-JNK, human total JNK, or human total ERK1/2 or mouse antibodies against human phospho-ERK1/2 (all from Cell Signaling, USA), goat antibodies against human total p38 or rabbit antibodies against human phospho-p38 (Santa Cruz, USA), all at a dilution of 1:1000, then with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse or rabbit anti-goat IgG antibodies (1:3000, Sigma), bound antibodies being detected using Chemiluminescence Reagent Plus (NEN, MA, USA). The intensity of each band was quantified using a densitometer. Antibodies against  $\beta$ -actin (Sigma) were used as loading controls.

#### D. Immunocytochemical Localization of HO-1

To localize HO-1 in situ, confluent HAECs (controls or cells treated for 24h with magnolol) on slides were exposed to TNF- $\alpha$  (10ng/mL) for 12h or for 24h, then fixed in 4% paraformaldehyde in PBS for 15min at 4°C, washed with PBS, blocked for 1 h at room temperature with 5% BSA in PBS, and incubated for 1 h at room temperature with rabbit anti-human HO-1 (1: 50). After washes, the slides were incubated for 1h at 37°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibodies, then viewed on a fluorescent microscope.

#### E. Animal Care and Experimental Procedures

The apoE-deficient mice were purchased from Jackson Laboratory (Bar Harbor, ME). After 6 months on a commercial mouse chow diet, 12 mice were randomly allocated to one of two groups: apoE-deficient mice and TNF- $\alpha$ -treated apoE-deficient mice. Water was available *ad libitum*. Mice were allowed free access to the diets for 3 months during the experiment and were deprived of food overnight at the end of the experiment. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The mice were injected intraperitoneally with TNF- $\alpha$  (10  $\mu$ g/kg/day) for the 3 days. They were then anesthetized by ip injection of 30-40 mg/kg pentobarbital and sacrificed, and thoracic aortas were dissected out. The thoracic aortas were gently dissected free of adherent tissue, rinsed with ice-cold PBS, immersion-fixed in 4% buffered paraformaldehyde, paraffin-embedded. To examine the expression of HO-1 protein, immunohistochemistry was performed on sections of aortas, which were deparaffinised, rehydrated, and washed with PBS. Non-specific binding was blocked by preincubation for 1h at room temperature with PBS containing 5mg/mL of bovine serum albumin. The section was incubated for 1h at 37°C with rabbit anti-human HO-1 antibody (1:50). The section was then incubated for 1h at room temperature with horseradish

peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (1:100, Santa Cruz), followed by incubation with 0.5 mg/mL of 3,3'- diaminobenzidine/0.01% hydrogen peroxide in 0.1M Tris-HCl buffer, pH 7.2, as chromogen (Vector Lab, USA) and observed by light microscopy. In negative controls, the primary antibodies were omitted.

#### F. Statistical Analysis

Values are expressed as the mean $\pm$ SEM. Statistical evaluation was performed using one-way ANOVA followed by the Dunnett test, with a p value < 0.05 being considered significant.

### III. RESULTS

#### A. TNF- $\alpha$ Regulated HO-1 Expression in HAECs

The cytotoxicity of TNF- $\alpha$  for HAECs was assessed by MTT assay after 24h of incubation. Treatment of HAECs with 2, 10, or 20ng/mL of TNF- $\alpha$  did not result in cytotoxicity (data not shown). Concentrations of 2-20ng/mL of TNF- $\alpha$  were therefore used in subsequent experiments. To determine whether the TNF- $\alpha$  affects HO-1 expression in HAECs, we first performed dose-response studies in which HAECs were cultured with various concentrations of TNF- $\alpha$  for various time intervals by Western blot. As shown in Fig. 1, using 10 ng/mL TNF- $\alpha$ , a significant effect on HO-1 expression was seen after 6h, which increased up to 12h, then decreased at 24 h. Treatment with 2ng/mL of TNF- $\alpha$  resulted in a significant increase in HO-1 expression after 12 h, which peaked at 10 ng/mL, then declined at 20ng/mL (Fig. 2).

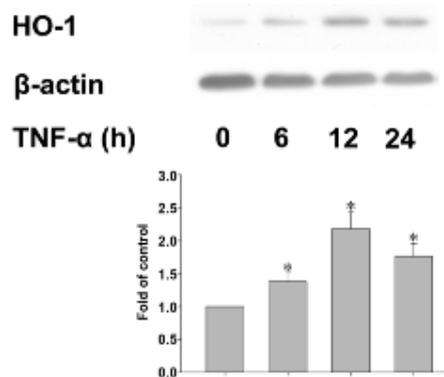


Fig. 1 Western blot analysis of the time-course of HO-1 expression in TNF- $\alpha$ -treated HAECs. HAECs were incubated for 0-24 h with 10 ng/mL of TNF- $\alpha$  and HO-1 expression was measured in cell lysates by Western blotting.  $\beta$ -actin was used as the loading control. Data are expressed as the mean $\pm$ SEM of three experiments performed in triplicate. \*  $P$ <0.05 vs. controls.

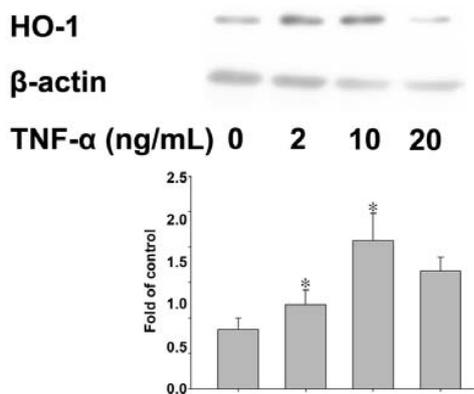


Fig. 2 Western blot analysis of the various doses of TNF- $\alpha$ -on HO-1 expression HAECs. HAECs were incubated for 12 h with various concentrations of TNF- $\alpha$  and HO-1 expression measured in cell lysates by Western blotting.  $\beta$ -actin was used as the loading control.

Data are expressed as the mean  $\pm$  SEM of three experiments performed in triplicate. \*  $P < 0.05$  vs controls

Fluorescent microscopic images showed that HO-1 was distributed in the cytosol in control and TNF $\alpha$ -treated HAECs. HO-1 expression was stronger in HAECs with TNF- $\alpha$  stimulation (Fig. 3). Furthermore, the addition of Chronic HO-1 induction with cobalt protoporphyrin (CoPP) treatment decreased TNF- $\alpha$ -induced VCAM-1 expression (Fig. 4).

*B. TNF- $\alpha$  Upregulated HO-1 Expression is Partly Dependent On Inhibition of JNK and p38 Phosphorylation*

MAPKs in the signaling pathways leading to cytokine production [13]. In the next set of experiments, we examined whether the effects of TNF- $\alpha$  on HO-1 expression in cells occurred via the ERK1/2, JNK, or p38 MAPK pathway. Phosphorylation of ERK1/2, JNK, and p38 was significantly increased 30min after addition of 10 ng/mL of TNF- $\alpha$  (data not shown). The increase in HO-1 expression in response to TNF- $\alpha$  treatment was also affected by 1 h pretreatment with SP600125 (a JNK inhibitor) and SB203580 (a p38 inhibitor), not with 1 and 5  $\mu$ M PD98059 (an ERK1/2 inhibitor) (Fig. 5). Based on these findings, the TNF- $\alpha$  mediated increase in HO-1 expression is partly dependent on inhibition of JNK and p38 phosphorylation.

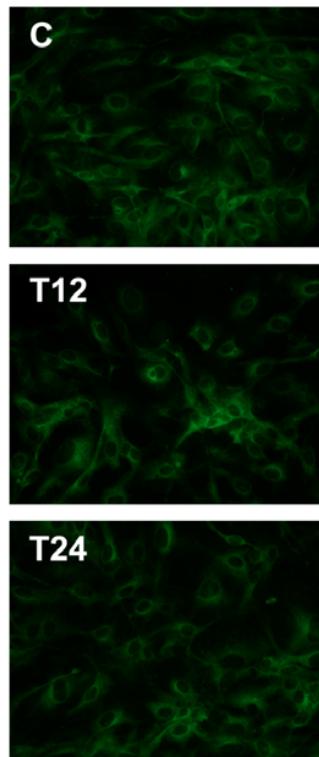


Fig. 3 Effects of TNF- $\alpha$  on HO-1 expression in HAECs by immunofluorescent staining. HAECs were incubated with 10 ng/mL of TNF- $\alpha$  for 12 or 24 h before HO-1 expression was analyzed by immunofluorescent staining. Bar=20  $\mu$ m

*C. TNF- $\alpha$  Increased HO-1 Expression in Thoracic Aortas of apoE-Deficient Mice*

Over the experimental period, there was no difference in weight gain and final weight of the apoE-deficient mice (control) and TNF- $\alpha$ -treated apo-E deficient mice. To study the effect of TNF- $\alpha$  on HO-1 expression in TNF- $\alpha$ -treated apoE-deficient mice, immunohistochemical staining with antibodies against HO-1 was carried out on sections. In apoE-deficient mice, weaker HO-1 expression was present in the intimal area. In TNF- $\alpha$ -treated apoE-deficient mice, stronger HO-1 staining was seen on the intima of thoracic aortas (Fig. 6).

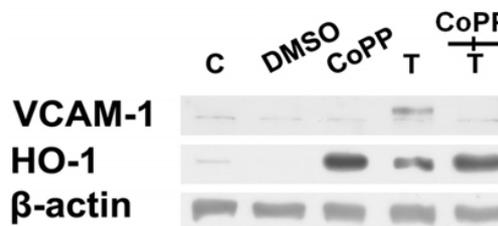


Fig. 4 Effects of HO-1 expression on VCAM-1 expression in TNF- $\alpha$ -treated HAECs. HAECs were precubated with CoPP, then the cells incubated with or without 10ng/mL of TNF- $\alpha$  for 12h before VCAM-1 and HO-1-expression was analyzed by Western blotting

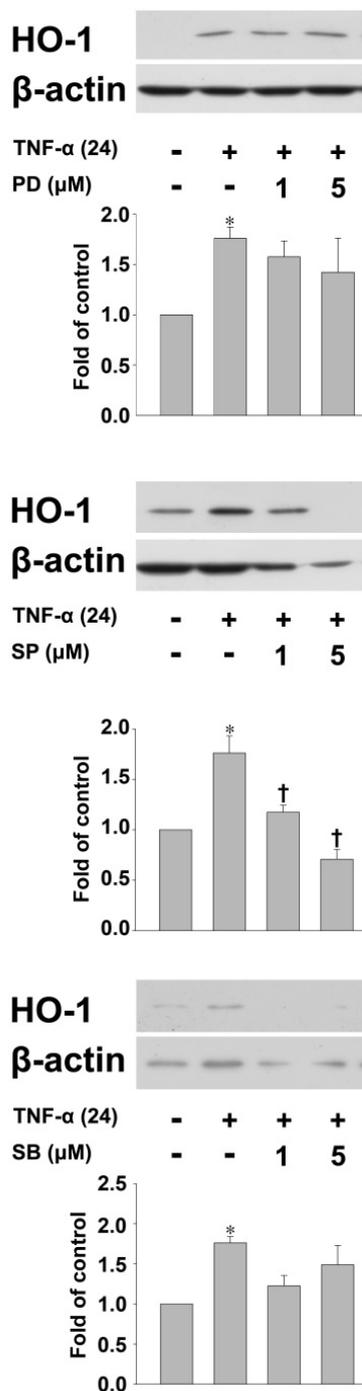


Fig. 5 The TNF-mediated increase in HO-1 expression is partly dependent on inhibition of JNK and p38 phosphorylation. HAECs were preincubated for 1h with the indicated concentrations of PD98059 (an ERK1/2 inhibitor), SP600125 (a JNK inhibitor), or SB203580 (a p38 inhibitor) and then were treated with TNF-α for 12 h and the cell lysates were analyzed for HO-1 expression by Western blotting. The data are expressed as a fold of the control value and are the means ± SEM for three separate experiments. β-actin was used as the loading control. \* $P < 0.05$  compared to the untreated cells. † $P < 0.05$  compared to the TNF-α-treated cells

# HO-1

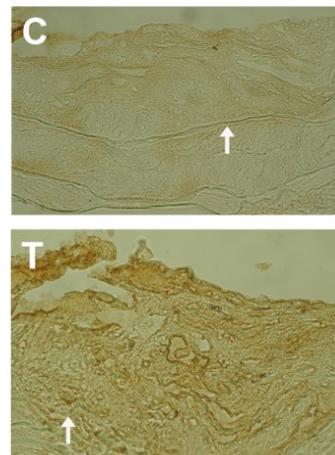


Fig. 6 TNF-α induced HO-1 expression in vascular walls from apoE-deficient mice. Immunohistochemical staining of HO-1 of sections of thoracic aortas from apoE-deficient mice (C) and TNF-α-treated apoE-deficient mice (T). The lumen is uppermost in all sections. The internal elastic membrane is indicated by an arrow. Strong HO-1 staining was seen in the intima of the TNF-α-treated apoE-deficient mice, while weak staining was seen in apoE-deficient mice. Bar=100μm

In the present study, we found that low dose of TNF-α significantly induced the expression of HO-1 in HAECs. The effects might be mediated through inhibition of JNK/p38 phosphorylation. TNF-α treatment also significantly increased HO-1 expression *in vivo* in thoracic aortas of apo-E deficient mice. Endothelial dysfunction, characterized by procoagulative, proinflammatory, and provasoconstrictive features is an early indicator of the development of vascular disease and inflammation, and thus an important area for further research and identification of potentially new therapeutic targets [14]. Proinflammatory cytokines such as TNF-α may stimulate endothelial adhesiveness to circulating monocytes by activating endothelial expression of cell adhesion molecules, including VCAM-1, intercellular adhesion molecule-1, and endothelial adhesion molecule 1. This is of fundamental importance because the ability to upregulate protective genes in vascular cells is thought to be crucial to inhibit proatherogenic inflammatory processes. Many studies suggest that HO-1, a stress-inducible isoform of HO, is a gene candidate to play a main and protective role against proatherogenic or inflammatory processes [15]. The significant anti-inflammatory effect of HO-1 induction was highlighted in human endothelial cells in different experimental conditions, such as in the presence of LPS or TNF-α, activating the inducible form of nitric oxide synthase and cyclooxygenase-2 expression and inducing VCAM-1 upregulation, respectively [16]. Our study demonstrated that HO-1 is able to counter TNF-α-induced adhesion molecule VCAM-1 expression. Consistent with our study, TNF-α

treatment increase HO-1 expression in airway tissues of mice and CoPP administration can enhance HO-1 overexpression to reduce VCAM-1 and ICAM-1 expression [16]. It is possible to speculate that cells treated with inflammatory cytokines initially exert their anti-inflammatory and anti-atherogenesis effect by directly inducing endothelial HO-1 expression. In turn, low dose of TNF- $\alpha$ -mediated HO-1 overexpression could contribute to prevent endothelial damage and protect against TNF- $\alpha$  stimulated cellular inflammation. The phosphorylation status of MAPKs plays an important role in the signal transduction of extracellular stimuli to cellular responses and is associated with vascular inflammation and disease [13]. Our study showed that TNF- $\alpha$  caused strong activation of three MAPK subtypes in HAECs, as reported in the previous study [17]. However, the involvement of their activation in the protective mechanism of HO-1 expression remains unclear. In the present study, the increase in HO-1 expression induced by TNF- $\alpha$  was markedly suppressed in the presence of a JNK inhibitor or a p38 inhibitor, but not an ERK inhibitor. Thus, one of the mechanisms by which TNF- $\alpha$ -induced HO-1 expression involves an induction in JNK and p38 activation. Through changes in JNK and p38 activation, TNF- $\alpha$  treatment may lead to downstream changes in HO-1 protein expression. Consistent with our results, induction of HO-1 by ethanol extract of *Inula helenium* L. was through p38 MAPK signaling pathway in human umbilical vein endothelial cells [18]. Another study showed that Cyanidin-3-O-glucoside induced HO-1 expression was mediated ERK activity in HUVECs [19]. The differences between the above results in terms of the pathways involved may be related to differences in cell type and the cytokines and inducers.

In conclusion, this study provides the first evidence that low dose of TNF- $\alpha$  induced the expression of HO-1 both *in vitro* and *in vivo*. The present data suggest that these effects might be mediated through JNK/p38 phosphorylation. Our data suggested that cells treated with low dose of TNF- $\alpha$  for short time increased HO-1 expression and its ability to induce HO-1 expression might protect against TNF- $\alpha$ -induced inflammation and VCAM-1 expression. In contrast, cells with high dose of TNF- $\alpha$  for long time decreased HO-1 expression might induce inflammation and vascular diseases.

#### ACKNOWLEDGMENT

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