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# Upregulation of heme oxygenase-1 as an adaptive mechanism for protection against crotonaldehyde in human umbilical vein endothelial cells

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ABSTRACT

Crotonaldehyde, a highly reactive α, β-unsaturated aldehyde, is a ubiquitous environmental pollutant and a product of endogenous lipid peroxidation. It is also a major component of cigarette smoke and is present in many foods and beverages, and has also been linked to development of various diseases. Activation of endothelial cells by stimuli such as cigarette smoke is an important risk factor for cardiovascular diseases, including atherosclerosis. Heme oxygenase-1 (HO-1) is a protective antioxidant enzyme with a critical role in resistance to oxidative stress and other cellular functions. In this study, we examined the effects of crotonaldehyde on HO-1 induction and determined the signaling pathways in human umbilical vein endothelial cells (HUVECs). Inhibition of the protein kinase C-δ (PKC-δ) and p38 pathways resulted in significant blockage of crotonaldehyde-mediated HO-1 induction. Crotonaldehyde treatment caused a dramatic increase in translocation of NF-E2 related factor (Nrf2), leading to induction of HO-1. In addition, small interfering RNA knockdown of Nrf2 and treatment with the specific HO-1 inhibitor ZnPP exhibited an obvious increase of apoptosis of crotonaldehyde-treated HUVECs. Taken together, our results demonstrated that crotonaldehyde-induced HO-1 expression is mediated by the PKC-δ–p38 MAPK–Nrf2–HO-1 pathway in HUVECs, which is an adaptive response to oxidative stress.



## 1. Introduction

Crotonaldehyde is abundant in the environment and is also produced endogenously. Crotonaldehyde occurs naturally in many foods, including fish, meat, fruits and vegetables, and is also present in various liquors (Budiawan and Eder, 2000). Crotonaldehyde is formed from lipid metabolism and is a product of lipid peroxidation; it is produced during combustion of materials (Kawaguchi-Niida et al., 2006). A large proportion of human exposure to crotonaldehyde is generally considered to occur through cigarette smoke (IARC, 1995). It has been detected in cigarette smoke at 1.4–15 μg/cigarette (Dong and Moldoveanu, 2004), a level that is approximately 2300-fold higher than that of benzo[*a*]pyrene, an extremely diffuse environmental and occupational pollutant (Chen and Moldoveanu, 2003).

Crotonaldehyde is a potent eye, respiratory, and skin irritant (Eiserich et al., 1995; Simon and Liedtke, 2008). Crotonaldehyde is mutagenic without metabolic activation in numerous cell systems (Neudecker et al., 1989), genotoxic and mutagenic in human lymphoblasts (Czerny et al., 1998) and in mammalian cells (Fernandes et al., 2005), and induces liver tumors in rodents (Chung et al., 1986). Carcinogenecity of crotonaldehyde has been observed in an epidemiological study with workers exposed to aldehydes (Bittersohl, 1974). Toxicity of crotonaldehyde is a consequence of its strongly reactive, electrophilic carbonyl group, which can rapidly react with cellular nucleophiles and cause extensive protein and DNA modifications (Esterbauer et al., 1991; Stein et al., 2006). Crotonaldehyde is directly or enzymatically conjugated with glutathione (GSH), which reduces GSH level (Reddy et al., 2002). Several studies have demonstrated that crotonaldehyde decreases GSH levels and elevates the levels of intracellular reactive oxygen species (ROS), resulting in a state of vulnerability in human airway cells (Facchinetti et al., 2007; Liu et al., 2010a). Recent studies have reported that crotonaldehyde could modulate biological reactions by a variety of downstream signaling pathways against cellular oxidative stress (Liu et al., 2010b).

HO-1 is one of the cytoprotective enzymes in response to cellular oxidative stress. HO-1 is the rate limiting enzyme that catalyzes conversion of heme into biliverdin, free iron, and carbon monoxide (Ryter et al., 2006). Recent studies have demonstrated that HO-1 plays a critical role in cytoprotective mechanisms, such as anti-inflammatory (Lee and Chau, 2002), anti-proliferative (Hill

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et al., 2005), and anti-apoptotic properties (Liu et al., 2003). HO-1 is regulated by the nuclear factor erythroid2-related factor 2 (Nrf2)/Kelch-like ECH associating protein 1 (Keap1) transcription factor system (Kobayashi and Yamamoto, 2005). Under basal conditions, Nrf2 is retained in the cytosol by binding to Keap1, which acts as a cytoplasmic repressor of Nrf2 (Itoh et al., 2004). The Nrf2/Keap1 complex is dissociated in response to several oxidants and electrophiles, leading to Nrf2 translocation into the nucleus, and binding to the antioxidant-response element (ARE) in the HO-1 gene promoter (Kensler et al., 2007). Findings from recent studies have suggested involvement of the pathways of PKC-δ, MAPK, PI3K/Akt, and Nrf-2 in the mechanism of HO-1 induction (Alvarez-Maqueda et al., 2004; Martin et al., 2004). The signaling pathway involved in induction of HO-1 has been suggested to differ depending on stimuli and cell type (Buckley et al., 2003).

Other α, β-unsaturated aldehydes have been reported to induce adaptive responses via upregulation of antioxidant enzyme expression (Ueda et al., 2008; Zhang and Forman, 2009). As a major member of the α, β-unsaturated aldehydes, the role of crotonaldehyde in induction of adaptive responses, including the signaling pathway and molecular function, is not fully understood. Thus, we hypothesized that crotonaldehyde induces HO-1 expression via Nrf2 pathways in endothelial cells and that induction of HO-1 expression will have a cytoprotective effect. In the present study, we attempted to determine whether crotonaldehyde may induce upregulation of HO-1 expression as a cytoprotective mechanism in a PKC-δ–p38 MAPK–Nrf2 dependent pathway in human umbilical vein endothelial cells (HUVECs).

## 2. Materials and methods

### 2.1. Materials

Crotonaldehyde, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] and zinc protoporphyrin (ZnPP) were obtained from Sigma (St. Louis, MO), and Trizol reagent was supplied by Invitrogen (Carlsbad, CA). TransPass R2 Transfection Reagent was obtained from New England Biolabs (Hercules, CA) and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was supplied by Molecular Probes (Invitrogen). Medium 199 (M199), fetal bovine serum (FBS), and tissue culture reagents were obtained from WelGENE Co. (Daegu, Korea). Antibodies used in the study included anti-Nrf2 (Santa Cruz Biothecnology, Santa Cruz, CA), anti-Lamin B (Santa Cruz), anti-HO-1 (Epitomics, Burlingame, CA), anti-p38 (Cell Signaling Technology, Beverly, MA), anti-PKC-δ (Cell Signaling Technology) and anti-GAPDH (Ab Frontier, Seoul, Korea). PD98059, SB203580, Rottlerin, and SP600125 were purchased from Calbiochem (La Jolla, CA). Nrf2 (SC-37049) and PKC-δ (SC-36253) siRNA were obtained from Santa Cruz Biotechnology and p38 (#6564) siRNA was purchased from Cell Signaling Technology. All other chemicals and reagents were of analytical grade.

### 2.2. Cell culture and viability measurement

We purchased HUVECs from StemCell Technologies (Vancouver, Canada). HUVECs were cultured in M199, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml human fibroblast growth factor, and 5 U/ml heparin in an atmosphere of 5% $CO_2$ at 37 °C. Cells were passaged every 2–3 days. HUVECs were cultured to approximately 80% confluence and further incubated with fresh medium containing the above reagents. Throughout these experiments, cells were used within passages 4–9. Cell viability was measured using the conventional MTT assay as described (Lee et al., 2009b).

### 2.3. Western blot analysis

For analysis by western blot, we applied 20 μg of whole cell lysate proteins to each lane. Western blot analysis was performed using a monoclonal antibody against human HO-1 and a monoclonal antibody against GAPDH. Horseradish peroxidase-conjugated anti-IgG antibodies were used as the secondary antibody for detection of the above-mentioned protein bands by enhanced chemiluminescence WESTSAVE Up™ (Abfrontier, Seoul, Korea).

### 2.4. RNA isolation and reverse transcriptase-polymerase chain reaction

Total RNA was isolated from cells using TRIzol®. A total of 2 μg of RNA was used as the template for cDNA synthesis. Reverse transcription was performed with 200 U/μl M-MLV reverse transcriptase, 10 mM dNTPs, 5× reaction buffer, 0.02 μg/μl Oligo (dT) Primers, and 40 U/μl RNase inhibitor at 42 °C for 1 h. The reaction was stopped at 94 °C for 3 min, and the cDNA products were stored at −20 °C. PCR was performed using synthesized cDNA as a template and specific primers for HO-1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. The primer sequence for human HO-1 was 5′-ACATCTATGTGGCCCTGGAG-3′ (forward) and 5′-TGTTGGGGAAGGTGAAGAAG-3′ (reverse). Amplified products were resolved by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

### 2.5. Determination of intracellular ROS generation

Generation of intracellular ROS was measured using a fluorescent dye, 2′,7′-dichlorofluorescein diacetate (DCFH-DA). In the presence of oxidants, DCFH was converted to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Cells were treated with 25, 50 μM crotonaldehyde, 5 mM NAC (N-Acetyl L-Cysteine, Sigma), or a combination of crotonaldehyde and NAC for 30 min, and loaded with 10 μM DCFH-DA; fluorescence was analyzed on a FACS Vantage flow cytometer.

### 2.6. Assay for HO activity

A previously described method was used for measurement of HO enzyme activity (Choi et al., 2004). Briefly, microsomes from harvested cells were added to a reaction mixture containing NADPH, rat liver cytosol as a source of biliverdin reductase, and the substrate hemin. The reaction was carried out in the dark for 1 h at 37 °C and the amount of bilirubin extracted was calculated by the difference in absorbance between 464 and 530 nm.

### 2.7. Nrf2, p38, and PKC-δ silencing by siRNA

Cells were seeded on six-well plates at a density of $2.0 \times 10^5$ cells/well in 2 mL of antibiotic-free DMEM supplemented with 10% FBS. Cells were allowed to grow to 60–80% confluence before transfection with siRNA. For each transfection, 1200 μL of the transfection medium was added to 2.5–25 nM of siRNA duplex/transfection reagent mix (TransPass R2 solution A + B), and the entire volume was added gently to the cells (Lee et al., 2010).

### 2.8. Immunofluorescence staining

HUVECs were cultured in a glass culture chamber slide (Falcon Plastics Inc., London Ontario, Canada) and processed for histological and immunofluorescence analysis. Cells were washed with PBS and fixed with 3.7% (w/v) paraformaldehyde. After permeabilization, coverslips were blocked with 5% normal donkey serum and then incubated with each antibody overnight (rabbit monoclonal anti-HO-1 antibody at a 1:500 dilution, polyclonal rabbit anti-Nrf2 antibody at a 1:200 dilution in PBS containing 1% bovine serum albumin), followed by incubation with FITC-conjugated secondary antibody for 2 h. Counterstaining with DAPI verified the location and integrity of nuclei. Coverslips were then mounted using the mounting medium (Dako, Hamburg, Germany). A laser scanning confocal microscope was used for examination of stained cells (LSM510 META; Zeiss, Oberkochen, Germany).

### 2.9. Preparation of nuclear proteins

Cells were incubated with various concentrations of crotonaldehyde for 6 h, and were then washed with PBS and centrifuged at 3300 × *g* for 5 min at 4 °C. Pellets were resuspended in ice-cold isotonic buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)), and a protease inhibitor cocktail containing 0.3 μM aprotinin and 2 mM leupeptin. After 15 min of incubation in an ice bath, cells were vortexed vigorously for 10 s with the addition of 10% NP-40 and recentrifuged at 7000 × *g* for 2 min at 4 °C. Pellets were resuspended in ice-cold buffer B containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, and the protease inhibitor cocktail, followed by incubation at 4 °C for 30 min with periodic vortexing. The mixture was then centrifuged at 12,000 × *g* for 30 min at 4 °C. The supernatant was collected and stored −70 °C for protein assay and western blot analysis.

### 2.10. Measurement of promoter activity

ARE-luciferase (ARE-Luc) reporter plasmid was a generous gift from Dr. Park, R.K. (Wonkwang University, Korea). ARE-Luc was generated by transfer of the enhancer 2 (E2) and minimal promoter (MP) sequences into the luciferase reporter plasmid pGL3-Basic (So et al., 2006). HEK 293 Cells were transfected with an ARE-luciferase (ARE-Luc) reporter plasmid. For transfection with the reporter plasmid, cells were seeded onto 24-well plates at a density of $1.0 \times 10^5$ cells/well 1 day before transfection. A total of 450 ng of plasmid DNA, including the luciferase reporter and 200 ng of pcDNA3-β-gal, were transfected into cells using ExGen™ 500 reagent (Fermentas; Hanover, MD). After 24 h of transfection, cells were treated with various concentrations of crotonaldehyde for 6 h. Cells were then washed twice with PBS and lysed using reporter lysis buffer (Promega, Madison, WI). A luciferase assay system (Promega) was used according to the manufacturer's instructions for measurement of luciferase activity. The supernatant was aliquoted for measurement of luciferase