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Omeprazole Induces Heme Oxygenase-1 in Fetal Human Pulmonary Microvascular Endothelial Cells via Hydrogen peroxide-Independent Nrf2 Signaling Pathway

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Abstract

Omeprazole (OM) is an aryl hydrocarbon receptor (AhR) agonist and a proton pump inhibitor that is used to treat humans with gastric acid related disorders. Recently, we showed that OM induces NAD (P) H quinone oxidoreductase-1 (NQO1) via nuclear factor erythroid 2–related factor 2 (Nrf2)-dependent mechanism. Heme oxygenase-1 (HO-1) is another cytoprotective and antioxidant enzyme that is regulated by Nrf2. Whether OM induces HO-1 in fetal human pulmonary microvascular endothelial cells (HPMEC) is unknown. Therefore, we tested the hypothesis that OM will induce HO-1 expression via Nrf2 in HPMEC. OM induced HO-1 mRNA and protein expression in a dose-dependent manner. siRNA-mediated knockdown of AhR failed to abrogate, whereas knockdown of Nrf2 abrogated HO-1 induction by OM. To identify the underlying molecular mechanisms, we determined the effects of OM on cellular hydrogen peroxide (H_2O_2) levels since oxidative stress mediated by the latter is known to activate Nrf2. Interestingly, the concentration at which OM induced HO-1 also increased H_2O_2 levels. Furthermore, H_2O_2 independently augmented HO-1 expression. Although N-acetyl cysteine (NAC) significantly decreased H_2O_2 levels in OM-treated cells, we observed that OM further increased HO-1 mRNA and protein expression in NAC-pretreated compared to vehicle-pretreated cells, suggesting that OM induces HO-1 via H_2O_2 – independent mechanisms. In conclusion, we provide evidence that OM transcriptionally induces HO-1 via AhR - and H_2O_2 - independent, but Nrf2 - dependent mechanisms. These results have important implications for human disorders where Nrf2 and HO-1 play a beneficial role.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Keywords

Omeprazole; Nuclear Factor Erythroid 2–Related Factor 2; Aryl hydrocarbon Receptor; Fetal Human Pulmonary Microvascular Endothelial Cells; Heme Oxygenase-1; Hydrogen Peroxide

Introduction

Oxidative stress, defined as a measurable shift in one or more redox couples to a more electron deficient (oxidized) steady state (Smith and Jaeschke, 1989), results from infection, inflammation, and exposure to hyperoxia and free iron (Maltepe and Saugstad, 2009; Perrone *et al.*, 2012). The increased pro-oxidants are implicated in the development of acute and chronic lung diseases such as acute respiratory distress syndrome (ARDS) (Lang *et al.*, 2002), bronchopulmonary dysplasia (BPD) (Madurga *et al.*, 2013), asthma (Zuo *et al.*, 2013), and chronic obstructive pulmonary disease (Domej *et al.*, 2014). Restoring redox homeostasis therefore is essential to maintain lung health. Heme oxygenase-1 (HO-1) is one of the enzymes that regulate redox homeostasis.

HO-1 is a rate-limiting enzyme that catalyzes the degradation of heme to release biliverdin, iron, and carbon monoxide (Maines, 1988). Three isoforms of HO have been identified to date – HO-1, HO-2, and HO-3. HO-1 is expressed in spleen, liver, lungs, brain, vascular smooth muscle cells, and bone marrow (Exner *et al.*, 2004), and is responsible for degrading senescent and damaged red blood cells (Maines, 1988). HO-2 is non-inducible and constitutively expressed in central and peripheral nervous system, and smooth muscle cells (Ewing and Maines, 1997). The third isoform, HO-3, has a low catalytic activity and is present in brain, liver, spleen, and kidneys (McCoubrey *et al.*, 1997). Although the basal expression is low in most tissues under physiological conditions, HO-1 is highly induced by stressful stimuli such as inflammation, oxidative and metabolic stress, and UV irradiation (Keyse *et al.*, 1990; Rizzardini *et al.*, 1993; Lee *et al.*, 1996). Interestingly, biliverdin and carbon monoxide, the byproducts of HO-1 enzymatic activity, exert anti-oxidant and anti-inflammatory effects, respectively (Stocker *et al.*, 1987; Otterbein *et al.*, 2000). Additionally, HO-1 deficiency leads to oxidative stress, inflammation, and ischemia/reperfusion injury (Poss and Tonegawa, 1997; Yet *et al.*, 1999; Liu *et al.*, 2005). These observations indicate that HO-1 protects against oxidative stress- and inflammation-mediated disorders. Hence, search for novel HO-1 agonists is of paramount importance to understand role of HO-1 in normal physiological and abnormal disease states, which in turn can lead to meaningful therapies for oxidative stress- and inflammation-mediated disorders. To this end, we chose omeprazole (OM), a FDA approved drug in humans to test the hypothesis that OM will increase HO-1 expression via nuclear factor erythroid 2–related factor 2 (Nrf2)-mediated mechanism(s).

OM, a benzimidazole derivative, is a proton pump inhibitor (PPI) that inhibits gastric acid secretion both in humans (Li *et al.*, 2004) and in animals (Larsson *et al.*, 1988; Watanabe *et al.*, 2004). It has been widely used in the management of gastric acid disorders in humans (Li *et al.*, 2004) for decades. Several *in vitro* studies suggest that OM activates AhR in human and rat hepatocytes (Quattrochi and Tukey, 1993; Backlund and Ingelman-Sundberg,

2005; Murray and Perdew, 2008; Yoshinari *et al.*, 2008) and the mechanistic role of AhR in the induction of CYP1A enzymes by OM *in vitro* has been extensively studied (Backlund *et al.*, 1997; Shivanna *et al.*, 2011b; Shiizaki *et al.*, 2014). Additionally, we reported that OM activates AhR and attenuates hyperoxic injury in adult mice *in vivo* (Shivanna *et al.*, 2011c) and in adult human lung H441 cells *in vitro* (Shivanna *et al.*, 2011a), which indicates that OM can be used as an AhR agonist to understand AhR biology in hyperoxia-mediated lung disorders. Recently, we also demonstrated that OM induces the phase II enzyme, NAD(P)H quinone oxidoreductase-1 (NQO-1) via Nrf2 (Zhang *et al.*, 2015b). Whether OM can also modulate the Nrf2-regulated enzyme, HO-1, in fetal primary human microvascular endothelial cells (HPMEC) is unknown. Thus, the goals of this study were to investigate whether OM regulates HO-1 in HPMEC. Specifically, we chose HPMEC for our experiments because they not only express HO-1 (Zhang *et al.*, 2015a) and Nrf2 (Zhang *et al.*, 2015b), but are also used to study mechanisms of diseases such as bronchopulmonary dysplasia (BPD) where HO-1 might play a significant role (Wright *et al.*, 2010; Zhang *et al.*, 2015a).

Materials and Methods

Cell Culture

HPMEC, the primary microvascular endothelial cells derived from the lungs of human fetus were obtained from ScienCell research laboratories (San Diego, CA; 3000). HPMEC were grown in specific endothelial cell medium according to the manufacturer's protocol. Briefly, the cells were grown in fibronectin coated plates containing basal endothelial cell medium supplemented with fetal bovine serum, antibiotics, and endothelial cell growth supplement in a humidifier containing 5% CO₂ at 37° C. When the cell culture reached > 90% confluence, they were subcultured with a split ratio of 1:3. Cells between passages 5-7 were used for all our experiments.

Cell treatments

Cells were treated with either 0.01% v/v dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO; 276855) or OM at varying concentrations up to 100 µM (Sigma-Aldrich, St. Louis, MO; O104) to determine the effects of OM on cell viability and proliferation, hydrogen peroxide (H₂O₂) production, and HO-1 expression. Additionally, cells were pretreated with 5 mM N-acetyl-L-cysteine (NAC) for up to 1 h before being exposed to DMSO or OM to identify the mechanisms by which OM induces HO-1.

Cell Viability Assay

Cell viability was determined by a colorimetric assay based on the ability of viable cells to reduce the tetrazolium salt, MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide), to formazan. HPMEC were seeded onto 96-well microplates and treated with DMSO or OM for up to 48 h, following which the cell viability was assessed by MTT reduction assays as outlined in the MTT Assay protocol (American Type Culture Collection, Manassas, VA). Briefly, at the end of experiments, 10 µl of MTT reagent was added to each well and the cells were incubated in a humidifier containing 5% CO₂ at 37° C for 2 h, at the end of which precipitates were visible in all the wells. Following the incubation, 100 µl of

detergent was added to each well and the cells were incubated at room temperature in the dark for additional 2 h and the absorbance was measured at 570 nm.

Cell Proliferation Assay

Cell proliferation was determined based on the measurement of cellular DNA content via fluorescent dye binding using the CyQUANT NF cell proliferation assay kit (Invitrogen, Carlsbad, CA; C35006) as per the manufacturer's recommendations. HPMEC were seeded onto 96-well microplates and treated with DMSO or OM for up to 48 h. At the end of experiments, the medium was gently aspirated, and the cells were incubated for 30 minutes with 100 μ l of 1X dye binding solution per well. Following the incubation, the fluorescence intensity of each sample was measured using Spectramax M3 fluorescence microplate reader with excitation at 485 nm and emission detection at 530 nm.

Western Blot Assays

Whole-cell protein extracts from the cells treated with DMSO or OM were obtained by using nuclear extraction kit (Active Motif, Carlsbad, CA; 40010) according to the manufacturer's instructions (Shivanna *et al.*, 2011b). β -actin was used as the reference protein. The protein extracts were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C with the following primary antibodies: anti-AhR antibody (Santa Cruz Biotechnologies, Santa Cruz, CA; sc-5579, dilution 1:500), anti-HO-1 (Enzo Life Sciences, Farmingdale, NY; ADI-SPA-896F, dilution 1:500), anti-Nrf2 (Santa Cruz Biotechnologies, Santa Cruz, CA; sc-722, dilution 1:500), and anti- β -actin (Santa Cruz Biotechnologies, Santa Cruz, CA; sc-47778, dilution 1:1000) antibodies. The primary antibodies were detected by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies. The immunoreactive bands were detected by chemiluminescence methods and the band densities were analyzed by Image J software (National Institutes of Health, Bethesda, MD).

Real-time RT- PCR assays

Cells were grown on six-well plates to 60-70% confluence, after which they were treated with DMSO or OM. At 24 or 48 h of exposure, total RNA was isolated and reverse transcribed to cDNA as mentioned before (Shivanna *et al.*, 2011b). Real-time quantitative RT-PCR analysis was performed with 7900HT Real-Time PCR System using iTaq Universal SYBR Green Supermix (Biorad, Hercules, CA; 1725121). The sequences of the primer pairs were *hAhR*: 5'-CACCGATGGGAAATGATACTATCC-3' and 5'-GGTGACCTCCAGCAAATGAGTT-3'; *hHO-1*: 5'-AGGCCAAGACTGCGTTCC-3' and 5'-GCAGAATCTTGCACTTTGTTGCT-3'; *hNrf2*: 5'-AAA CCA GTG GAT CTG CCA AC-3' and 5'-GAC CGG GAA TAT CAG GAA CA-3'; *h β -actin*: 5'-TGACGTGGACATCCGCAAAG-3' and 5'-CTGGAAGGTGGACAGCGAGG-3'. β -actin was used as the reference gene. The C_t method was used to calculate the fold change in mRNA expression: $C_t = C_t$ (target gene) - C_t (reference gene), $C_t = C_t$ (treatment) - C_t (control), fold change = $2^{-(C_t - C_t)}$.

Small interfering RNA (siRNA) transfections

Twenty-four hours prior to transfection, HPMEC were seeded in fibronectin-coated 6-well plates at 60-70% confluence in antibiotic-free culture medium. Transfections were then performed with either 50 or 100 nM control siRNA (Dharmacon, Lafayette, CO; d-001810) or 50 nM AhR specific siRNA (Dharmacon, Lafayette, CO; L-004990) or 100 nM Nrf2 specific siRNA (Dharmacon, Lafayette, CO; L-003755) using LipofectamineRNAiMAX (Life Technologies, Grand Island, NY; 13778030). The media were changed 6 h after transfection, and 24 h after transfections, the cells were treated with DMSO or OM for up to 48 h. siRNA mediated knockdown of AhR or Nrf2 was validated by determining the expression of AhR and Nrf2 mRNA by real-time RT PCR analysis. Additionally, the cells were harvested at the indicated time points to determine the expression of HO-1 mRNA and protein.

Measurement of H₂O₂ generation

Hydrogen peroxide (H₂O₂) levels was quantified by the ROS-Glo™ H₂O₂ Assay (Promega, Madison, WI; G8820) according to the manufacturer's recommendation. Briefly, cells were grown on 96-well plates to 60-70% confluence, after which they were treated with DMSO, OM, and NAC for up to 24 h. Six-hours prior to the completion of experiments, the H₂O₂ substrate was added to the wells, and the cell culture plates were returned to the incubator for the remainder of the experiment. At the end of experiments, ROS-Glo™ detection solution was added to each well, following which the cells were incubated for 20 minutes at room temperature before the relative luminescence units was measured using Spectramax M3 luminescence microplate reader.

Analyses of Data

The results were analyzed by GraphPad Prism 5 software. At least three separate experiments were performed for each measurement, and the data are expressed as means $\pm$ SEM. One-way ANOVA was used to determine the effect of treatment (OM) on cell viability, cell proliferation, and HO-1 expression in wild-type cells, while two-way ANOVA was used to determine the effects of treatment (OM) and gene (AhR or Nrf2) or treatment (OM or NAC) and time, and their associated interactions for the outcome variables (HO-1 expression or H₂O₂ levels). Post hoc multiple t-tests with Bonferroni corrections were performed if statistical significance of either variable or interaction was noted by ANOVA. A *p* value of <0.05 was considered significant.

Results

OM in concentrations up to 100 μ M does not induce cytotoxicity in HPMEC

The MTT activity reflects the mitochondrial activity of the cells, and thus the absorbance measured reflects the cell viability. OM in concentrations up to 100 μ M did not decrease cell viability, as reflected by the cellular capacities to reduce MTT (Fig. 1A). Next, we determined the effects of OM on cell proliferation. CyQUANT NF cell proliferation assay showed that OM in concentrations up to 100 μ M did not inhibit cell proliferation (Fig. 1B).

OM induces HO-1 mRNA and protein in HPMEC

We determined the effects of OM on HO-1 expression because the latter is regulated by Nrf2 and our previous study indicated that OM activates Nrf2 in HPMEC (Zhang *et al.*, 2015b). Real-time RT PCR analysis showed that OM-induced HO-1 mRNA in a dose-dependent manner (Fig. 2A). Consistent with its effect on HO-1 mRNA expression, OM increased HO-1 protein expression (Figs. 2B and C).

OM induces HO-1 expression in HPMEC via AhR-independent mechanisms

To elucidate whether AhR-regulates OM-mediated HO-1 expression, we knocked down AhR (Fig. 3A) and determined the expression of HO-1 in AhR-sufficient and –deficient cells treated with OM. OM-induced HO-1 mRNA (Fig. 3B) and protein (Figs. 3C and D) expression independent of the presence of the AhR gene.

OM induces HO-1 expression in HPMEC via Nrf2-dependent mechanisms

We next knocked down Nrf2 (Fig. 4A) and treated the Nrf2-sufficient and –deficient cells with OM to determine whether the latter induces HO-1 via Nrf2. OM-induced HO-1 mRNA (Fig. 4B) and protein (Figs. 4C and D) were significantly attenuated in Nrf2-deficient cells compared to Nrf2-sufficient cells, indicating that OM does induce HO-1 via Nrf2-dependent mechanisms.

OM increases H₂O₂ generation in HPMEC

Stressful stimuli such as ROS have been widely implicated in the activation of Nrf2 signaling pathway. To determine whether OM activates Nrf2 via ROS generation, H₂O₂ levels were measured by ROS-Glo™ H₂O₂ assay. Interestingly, OM increased H₂O₂ levels (Fig. 5) at concentrations that induce HO-1 expression, which suggests that OM might induce HO-1 via H₂O₂.

H₂O₂ induces HO-1 expression in HPMEC

Having seen that H₂O₂ levels are increased by OM at concentrations that also induces HO-1, we next determined whether H₂O₂ independently increases HO-1 expression to elucidate the mechanisms by which OM induces HO-1. Indeed, HO-1 protein expression was significantly increased in H₂O₂-treated compared to vehicle-treated cells (Figs. 6A and B). Conversely, NAC-induced depletion of H₂O₂ abrogated HO-1 expression (Figs. 6 A and B) indicating that H₂O₂ independently induces HO-1 expression.

OM induces HO-1 expression in HPMEC via H₂O₂-independent mechanisms

To ascertain that OM induces HO-1 expression via H₂O₂, we determined the effects of OM on HO-1 expression in cells pretreated with the anti-oxidant, NAC. NAC pretreatment significantly decreased OM-mediated H₂O₂ generation (Fig. 7A). Surprisingly, OM augmented HO-1 mRNA (Fig. 7B) and protein (Figs. 7C and D) expression in NAC-pretreated compared to vehicle-treated cells, suggesting that OM induces HO-1 expression via H₂O₂-independent mechanisms.

Discussion

The present study demonstrates that the PPI, OM, induces HO-1 expression in HPMEC via mechanism(s) entailing Nrf2 activation independent of H₂O₂ generation. In human fetal lung-derived WT and AhR-deficient HPMEC *in vitro*, OM transcriptionally induced HO-1 expression when compared to controls, whereas, in Nrf2-deficient HPMEC, the attenuation of OM-mediated induction of HO-1 enzyme correlated with the deficiency of a functional Nrf2 gene. Additionally, OM-mediated HO-1 induction was inversely proportional to the H₂O₂ levels.

All eukaryotic organisms are equipped with regulatory or defense mechanisms to maintain oxygen homeostasis. Oxidative damage occurs when these regulatory mechanisms are overwhelmed. Although supplemental oxygen is frequently used as a life-saving therapy in human preterm infants with hypoxic respiratory failure, excessive or prolonged oxygen exposure results in increased ROS generation and the expression of proinflammatory cytokines (Jobe *et al.*, 2008). Hyperoxia-induced ROS generation and inflammation can injure and disrupt the reparative processes in the developing lungs that ultimately lead to the development of bronchopulmonary dysplasia (BPD) (Bhandari and Elias, 2006). Moreover, the antioxidant defense system develops late in gestation, making preterm neonates highly susceptible to oxidative stress-induced lung injury (Vina *et al.*, 1995; Asikainen and White, 2005). HO-1 exerts potent anti-oxidant properties by regulating the prooxidant molecule heme in cells and tissues (Otterbein *et al.*, 2003). HO-1 deficiency is shown to impair lung repair and regeneration in neonatal mice exposed to hyperoxia (Yang *et al.*, 2013). Furthermore, constitutive overexpression of HO-1 in lung epithelial cells of neonatal mice attenuates inflammation, alveolar simplification, and pulmonary vascular remodeling in an experimental model of BPD (Fernandez-Gonzalez *et al.*, 2012). Therefore, we conducted experiments to investigate whether OM modulates HO-1 expression in primary human fetal lung derived HPMEC *in vitro*, which in turn can lead to novel therapies for developmental lung disorders such as BPD in human infants.

DMSO, the vehicle used in our study, is known to have antioxidant properties (Santos *et al.*, 2003; Nagel *et al.*, 2007; Kashino *et al.*, 2010). Additionally, investigators have shown that DMSO induces HO-1 expression via Nrf2 activation (Liang *et al.*, 2011; Man *et al.*, 2014); however, the concentration at which DMSO exerts these effects are significantly higher than that used in our study. So, it is highly unlikely that DMSO independently increased HO-1 expression in our study. Moreover, we have shown that OM is a potent inducer of HO-1 enzyme, when we compared against DMSO-treated cells. The concentration of OM used in this study was comparable to those used in previous studies (Jin *et al.*, 2014; Novotna *et al.*, 2014). More importantly, we included the blood concentrations that are observed in humans following OM therapy (Cederberg *et al.*, 1992). Drug-induced toxicity can affect the expression of several stress enzymes including HO-1. Hence, we initially tested for cytotoxicity of OM in HPMEC by MTT and cell proliferation assays. The lack of OM cytotoxicity in our studies (Fig. 1) indicates that OM in concentrations up to 100 μ M can be used to investigate its effects on HO-1 expression in HPMEC. Consistent with our study, OM has been reported to similarly increase gastric HO-1 expression in rats (Mahmoud-Awny *et al.*, 2015) and humans (Becker *et al.*, 2006). However, other studies suggest that

OM fails to induce intestinal HO-1 expression (Higuchi *et al.*, 2009; Yoda *et al.*, 2010). These contradictory observations might be related to cell and tissue specificity and the nature of the underlying insult.

Interestingly, higher concentrations of OM are required to induce HO-1 when compared to that required to induce cytochrome P450 1A1 expression (Zhang *et al.*, 2015b), which suggests that OM has a differential concentration-specific effect on AhR activation and HO-1 induction. Conflicting evidence exists on the ability of the AhR to regulate HO-1 expression in various tissues (Rico de Souza *et al.*, 2011; Amara *et al.*, 2012; Miao *et al.*, 2014; Elshenawy and El-Kadi, 2015; Misaki *et al.*, 2015). Hence, we investigated whether the AhR is a crucial regulator of OM-mediated HO-1 expression in fetal HPMEC. Our AhR knock down experiments clearly indicate that OM induces HO-1 via AhR-independent mechanisms in HPMEC (Fig. 3). Nrf2, which regulates the antioxidant response element (ARE)-driven gene battery is a major transcription factor that modulates HO-1 expression (Na and Surh, 2014). We previously reported that OM increases phosphoNrf2 (S40) expression (Zhang *et al.*, 2015b), which suggests that OM activates Nrf2 signaling since phosphorylation of Nrf2 at S40 leads to dissociation of Nrf2 from its inhibitor Kelch-like ECH-associated protein 1, which in turn results in the translocation of Nrf2 into the nucleus where it activates ARE-mediated gene expression (Jaiswal, 2004). OM has been similarly shown to increase nuclear Nrf2 accumulation in mice (Patel *et al.*, 2013). In alignment with these studies, we have shown that Nrf2-signaling is necessary for both basal and OM-mediated HO-1 expression (Fig. 4). HO-1 gene is also regulated by other transcription factors, such as nuclear factor-kappa B (NF- κ B) and activator protein-1 (AP-1), and signaling pathways, which include p38 mitogen-activated protein kinase, phosphoinositide 3-kinase/protein kinase B and Janus kinase-signal transducer and activator of transcription pathways (Ryter and Choi, 2002; Ferrandiz and Devesa, 2008). It is also possible that OM may regulate HO-1 expression partly via these transcription factors and signaling pathways. Although previous studies have shown that OM and its S-enantiomer, esomeprazole, increases gastric (Becker *et al.*, 2006; Mahmoud-Awny *et al.*, 2015) and pulmonary (Ghebremariam *et al.*, 2015) HO-1 expression, respectively, the mechanisms of HO-1 induction were not investigated. To the best of our knowledge, this is the first study to demonstrate that Nrf2 is directly involved in OM-mediated induction of HO-1 in HPMEC *in vitro*.

Oxidative stress induced by ROS such as H₂O₂ is well known to activate Nrf2 (Kobayashi *et al.*, 2009; Lee *et al.*, 2015). Hence, to understand the mechanisms by which OM activates Nrf2, we investigated whether OM increases H₂O₂ levels. Surprisingly, the concentration at which OM induced HO-1 expression also increased H₂O₂ levels (Fig. 5). This finding is in contrast with other studies (Lapenna *et al.*, 1996; Ganguly *et al.*, 2006; Shivanna *et al.*, 2011b), which have shown that OM decreases the levels of ROS. The most plausible reasons for these discrepant results may be related to differences in species, cells and organs studied, concentration of OM used, and the nature and duration of the experimental injury. Consistent with other studies (Kruger *et al.*, 2006; Man *et al.*, 2014), H₂O₂ independently increased HO-1 expression (Fig. 6), indicating that OM might activate Nrf2 via H₂O₂.

NAC, the *N*-acetyl derivative of the amino acid L-cysteine, is a precursor for the synthesis of the anti-oxidant glutathione. In addition, its SH group can react directly with and reduce H_2O_2 to $2\text{H}_2\text{O}$ (Aruoma *et al.*, 1989; Gillissen *et al.*, 1997). In agreement with these studies, we observed that NAC decreased H_2O_2 accumulation in our experimental conditions. Interestingly, OM further increased rather than decrease HO-1 expression in these NAC-pretreated cells, which had decreased H_2O_2 levels (Fig. 7), indicating that OM induces HO-1 via H_2O_2 -independent mechanisms. It is possible that activation of the Nrf2 is highly regulated by a negative feed-back interrelation between cellular H_2O_2 and HO-1 levels. With a decrease in cellular H_2O_2 levels, this negative feed-back interrelation is lost, and Nrf2 activators such as OM can further augment HO-1 induction in these cells with decreased H_2O_2 levels compared to those with increased H_2O_2 levels. However, the mechanisms of OM-mediated activation of Nrf2, and the interactions between H_2O_2 , OM, and Nrf2 are unknown at this time point and deserve further investigation.

In summary, we provide evidence that OM induces pulmonary HO-1 enzyme *in vitro* via AhR- and H_2O_2 - independent, but Nrf2-dependent mechanisms. Our results suggest that OM can be used to investigate HO-1 and Nrf2 biology in the lung, which can lead to better understanding of the pathogenesis and development of novel therapies to prevent/and or treat oxidative stress-induced complex disorders such as BPD in premature infants, and acute respiratory distress syndrome, chronic obstructive pulmonary disease, and malignancies in adults.

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Abbreviations

AhR	aryl hydrocarbon receptor
ARDS	acute respiratory distress syndrome
ARE	antioxidant response element
BPD	bronchopulmonary dysplasia
CYP	cytochrome P450
DMSO	dimethyl sulfoxide
HPMEC	human pulmonary microvascular endothelial cells
HO-1	heme oxygenase-1
H_2O_2	hydrogen peroxide
MTT	3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide

NAC	N-acetyl-L-cysteine
Nrf2	nuclear factor erythroid 2-related factor 2
NQO1	NAD(P)H quinone dehydrogenase 1
OM	omeprazole
OM 5	omeprazole 5 μ M
OM 100	omeprazole 100 μ M
PPI	proton pump inhibitor
ROS	reactive oxygen species

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Highlights

- Omeprazole induces HO-1 in human fetal lung cells.
- AhR deficiency fails to abrogate omeprazole-mediated induction of HO-1.
- Nrf2 knockdown abrogates omeprazole-mediated HO-1 induction in human lung cells.
- Hydrogen peroxide depletion augments omeprazole-mediated induction of HO-1.

Figure 1. OM does not induce cytotoxicity in HPMEC

HPMEC were treated with dimethylsulfoxide (DMSO) or omeprazole (OM) at concentrations of 2 (OM 2), 5 (OM 5), 25 (OM 5), 50 (OM 50) or 100 (OM 100) μ M for 48 h, following which: (A) cell viability was assessed by MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reduction activities; and (B) cell proliferation was determined based on the measurement of cellular DNA content via fluorescent dye binding using the CyQUANT NF cell proliferation assay. Data are representative of at least three independent experiments. Values are presented as means $\pm$ SEM (n=3).

Figure 2. OM-treated HPMEC display increased HO-1 expression

HPMEC were treated with DMSO or OM for up to 24 h, following which: RNA was extracted for HO-1 mRNA expression (A) and whole protein was extracted for immunoblotting using anti-HO-1 or β -actin antibodies (B). Densitometric analyses wherein HO-1 band intensities were quantified and normalized to β -actin (C). Data are representative of at least three independent experiments. Values are presented as means $\pm$ SEM (n=3). One-way ANOVA showed an effect of OM 50 or 100 for HO-1 mRNA expression. Significant differences between DMSO-, OM 50- and OM 100-treated cells are indicated by *, $p < 0.05$ and significant differences between OM 50- and OM 100-treated cells are indicated by $\dagger$, $p < 0.05$ (Fig. 2A). Two-way ANOVA showed an effect of OM and time and an interaction between them for the dependent variable, HO-1 protein expression. Significant differences between DMSO- and OM 100-treated cells are indicated by *, $p < 0.05$. Significant differences between cells treated for 3 and 24 h are indicated by $\dagger$, $p < 0.05$ (Fig. 2B).

Figure 3. OM increases HO-1 expression via an AhR-independent mechanism

HPMEC were transfected with either 50 nM control (SiC) or AhR (SiAhR) siRNA. Twenty-four hours after transfection RNA was extracted to determine AhR mRNA (A) expression. AhR mRNA was significantly decreased in cells transfected with AhR siRNA. [†] $P < 0.05$ SiAhR vs. SiC (t-test). Additionally, 24 h after transfection, the cells were treated with DMSO or 100 μ M OM (OM 100) for up to 48 h, following which RNA and whole-cell protein was extracted to determine HO-1 mRNA (B) and protein (C) expression, respectively. HO-1 band intensities were quantified and normalized to β -actin (D). Data are representative of at least three independent experiments. Values are presented as means $\pm$ SEM (n=3). Two-way ANOVA showed an effect of OM, but not the AhR gene for the dependent variable, HO-1, in this figure. Significant differences between DMSO- and OM 100-treated cells are indicated by *, $p < 0.05$.

Figure 4. OM increases HO-1 expression in HPMEC via Nrf2-dependent mechanisms

HPMEC were transfected with either 100 nM control (SiC) or Nrf2 (SiNrf2) siRNA. Twenty-four hours after transfection, RNA was extracted to determine Nrf2 mRNA (A) expression. Nrf2 mRNA was significantly decreased in cells transfected with Nrf2 siRNA. [†] $P < 0.05$ SiNrf2 vs. SiC (t-test). Additionally, 24 h after transfection, the cells were treated with DMSO or 100 μ M OM (OM 100) for up to 48 h, following which RNA and whole-cell protein was extracted to determine HO-1 mRNA (B) and protein (C) expression, respectively. HO-1 band intensities were quantified and normalized to β -actin (D). Data are representative of at least three independent experiments. Values are presented as means $\pm$ SEM ($n=3$). Two-way ANOVA showed an effect of OM and Nrf2 gene and an interaction between them for the dependent variable, HO-1, in this figure. Significant differences between SiC and SiNrf2-treated cells are indicated by [†], $p < 0.05$. Significant differences between DMSO and OM 100-treated cells are indicated by *, $p < 0.05$.

Figure 5. OM increases H_2O_2 levels in HPMEC

HPMEC were treated with DMSO or OM for up to 24 h, following which the H_2O_2 levels was measured by ROS-Glo™ H_2O_2 assay. Data are representative of at least three independent experiments. Values are presented as means $\pm$ SEM (n=3). Two-way ANOVA showed an effect of OM and time and an interaction between them for the dependent variable, H_2O_2 levels, in this figure. Significant differences between DMSO- and OM 100-treated cells are indicated by *, $p < 0.05$. Significant differences between cells treated for 8 and 24 or 36 h are indicated by †, $p < 0.05$.

Figure 6. H₂O₂-treated HPMEC display increased HO-1 expression that is abrogated by pre-treatment with NAC

HPMEC were pre-treated with DMSO or 5 mM NAC for 1 h, before being exposed to 50 or 100 μM H₂O₂ for 48 h, following which whole-cell protein was extracted to determine HO-1 protein (A) expression. HO-1 band intensities were quantified and normalized to β-actin (B). Data are representative of at least three independent experiments. Values are presented as means ± SEM (n=3). Two-way ANOVA showed an effect of NAC and H₂O₂ and an interaction between them for the dependent variable, HO-1, in this figure. Significant differences between DMSO and NAC-treated cells are indicated by †, *p* < 0.05. Significant differences between vehicle and H₂O₂-exposed cells are indicated by *, *p* < 0.05.

Figure 7. OM-induces HO-1 expression in HPMEC via H₂O₂-independent mechanisms HPMEC were pre-treated with DMSO or 5 mM NAC for 1 h, before being exposed to DMSO or 100 μM OM (OM 100) for up to 48 h, following which the H₂O₂ levels was measured by ROS-GloTM H₂O₂ assay (A). Additionally, RNA and whole-cell protein was extracted to determine HO-1 mRNA (B) and protein (C) expression, respectively. HO-1 band intensities were quantified and normalized to β-actin (D). Data are representative of at least three independent experiments. Values are presented as means ± SEM (n=3). Two-way ANOVA showed an effect of OM and NAC and an interaction between them for the dependent variable, HO-1, in this figure. Significant differences between DMSO and NAC-treated cells are indicated by †, p < 0.05. Significant differences between DMSO and OM 100-treated cells are indicated by *, p < 0.05.