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Ammonia Promotes Endothelial Cell Survival via the Heme Oxygenase-1-Mediated Release of Carbon Monoxide

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Abstract

Although endothelial cells produce substantial quantities of ammonia during cell metabolism, the physiologic role of this gas in these cells is not known. In this study, we investigated if ammonia regulates the expression of heme oxygenase-1 (HO-1), and if this enzyme influences the biological actions of ammonia on endothelial cells. Exogenously administered ammonia, given as ammonium chloride or ammonium hydroxide, or endogenously generated ammonia stimulated HO-1 protein expression in cultured human and murine endothelial cells. Dietary supplementation of ammonia also induced HO-1 protein expression in murine arteries. The increase in HO-1 protein by ammonia in endothelial cells was first detected 4 hours after ammonia exposure and was associated with the induction of HO-1 mRNA, enhanced production of reactive oxygen species (ROS), and increased expression and activity of NF-E2-related factor-2 (Nrf2). Ammonia also activated the HO-1 promoter and this was blocked by mutating the antioxidant responsive element or by overexpressing dominant-negative Nrf2. The induction of HO-1 expression by ammonia was dependent on ROS formation and prevented by N-acetylcysteine or rotenone. Finally, prior treatment of endothelial cells with ammonia inhibited tumor necrosis factor- α -stimulated cell death. However, silencing HO-1 expression abrogated the protective action of ammonia and this was reversed by the administration of carbon monoxide but not bilirubin or iron. In conclusion, this study demonstrates that ammonia stimulates the expression of HO-1 in endothelial cells via the ROS-Nrf2 pathway, and that the induction of HO-1 contributes to the cytoprotective action of ammonia by generating carbon monoxide. Moreover, it identifies ammonia as a potentially important signaling gas in the vasculature that promotes endothelial cell survival.

Graphical Abstract

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Keywords

ammonia; endothelial cells; heme oxygenase-1; carbon monoxide; cytoprotection

Introduction

Ammonia is a colorless, pungent, and diffusable gas that plays a central role in nitrogen metabolism [1]. Ammonia is continuously produced during cell metabolism from the breakdown of purines, pyrimidines, polyamines, and the deamination of several amino acids. It is also produced by urease-producing bacteria present in the saliva and gastrointestinal tract [2,3]. Ammonia plays an important physiologic role in the body as it provides usable forms of nitrogen required for the synthesis of DNA, RNA, and proteins. In addition, ammonia regulates whole body acid-base balance through its excretion by the kidney [4]. The liver is the major site of ammonia degradation where ammonia is principally metabolized to urea via the hepatic urea cycle or, alternatively, converted to glutamine by glutamine synthetase [5]. Disturbances in ammonia metabolism secondary to liver disease or inborn errors of the urea cycle leading to hyperammonemia are of pathological importance and may cause neurological diseases and hepatic encephalopathy [6,7].

Recently, endogenously produced ammonia has been suggested to function as a signaling gas much like nitric oxide [8]. Indeed, a signaling role for ammonia has emerged in the brain where pathophysiologically relevant concentrations of this gas stimulate the production of reactive oxygen species (ROS), the activation of mitogen-activated protein kinase and phosphatidylinositol-3-kinase, and the nitration, S-nitrosylation, and O-GlcNAcylation of proteins, which collectively contributes to ammonia-mediated alterations in brain cell function and viability [9–13]. However, the actions of ammonia are not restricted to the central nervous system. Ammonia exerts important actions in the cardiovascular system where it dilates human cerebrovascular smooth muscle and regulates cardiac function [14,15]. While physiological or low concentrations of ammonia elicit positive inotropic effects in isolated perfused hearts, high concentrations of the gas had a toxic effect.

Interestingly, endothelial cells generate substantial amounts of ammonia via the catabolism of glutamine by the enzyme glutaminase [16–18]. Abundant glutaminase activity has been detected in endothelial cells, resulting in high rates of ammonia synthesis that are dependent on extracellular glutamine levels. Nevertheless, the functional significance of this gas in these cells is not known.

The endothelium plays a critical role in maintaining vascular homeostasis by regulating vascular tone, thrombosis, vascular permeability, and inflammation. Significantly, loss and/or dysfunction of endothelial cells contribute to the development of vascular disease and its attendant clinical complications (19). Recently, heme oxygenase-1 (HO-1) has emerged as a key protein that protects against vascular disease, in part, by promoting endothelial cell viability and function [see 20–22]. HO-1 is a highly inducible enzyme that cleaves heme at the α -methene bridge to generate carbon monoxide (CO), iron, and biliverdin, which is subsequently reduced by biliverdin reductase to bilirubin. The induction of HO-1 by various forms of cellular stress provides an effective defense mechanism against endothelial cell injury [23–26]. The cytoprotection afforded by HO-1 involves multiple mechanisms, including the catabolism of toxic free heme to the antioxidants biliverdin and bilirubin, and the generation of CO which possesses potent anti-apoptotic properties [25–27].

Interestingly, ammonia has been demonstrated to stimulate the expression of HO-1 in rat cerebral endothelial cells [28]; however, the signaling pathway responsible for inducing HO-1 expression as well as the functional significance of this observation remains to be established. In the present study, we characterized the interaction between ammonia and HO-1 in vascular endothelium. We now show that ammonia, either exogenously administered or endogenously produced, also stimulates the expression of HO-1 in human umbilical vein endothelial cells (HUVECs) and murine aortic endothelial cells (MAECs) and smooth muscle cells (MASMCs). In addition, we found that *in vivo* administration of ammonia induces the expression of HO-1 in murine arteries. Significantly, we determined that the induction of HO-1 occurs via the activation of the ROS-NF-E2-related factor-2 (Nrf2) signaling cascade. Moreover, we discovered that ammonia protects against endothelial cell death via the HO-1-mediated generation of CO. These findings establish ammonia as novel regulator of endothelial cell survival, and identify important new regulatory and functional interactions between signaling gases in the vasculature.

Methods and Materials

Materials

Ammonium chloride (NH₄Cl), heparin, trypan blue, agarose, trypsin, Tris, HEPES, N-acetyl-L-cysteine (NAC), cycloheximide, penicillin, L-glutamine, gelatin, streptomycin, Nonidet P40, dithiothreitol, bromophenol blue, SDS, NaCl, EDTA, DMSO, glycerol, matrigel, and Triton X-100, were from Sigma–Aldrich (St. Louis, MO). Ammonium hydroxide (NH₄OH) was from Fischer Scientific (Houston, TX). Phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, and pepstatin A were from Roche Applied Sciences (Indianapolis, IN). M199 medium, bovine calf serum, 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA), and lipofectamine were from Invitrogen Corporation (Carlsbad, CA). LY294002 was from Calbiochem (La Jolla,

CA). Tumor necrosis factor- α (TNF α) was from Genzyme (Boston, MA). Endothelial cell growth factor was from Becton Dickinson (Bedford, MA). A polyclonal antibody against HO-1 was from Assay Designs (Ann Arbor, MI, USA), antibodies against Nrf2, platelet-endothelial cell adhesion molecule-1 (PECAM-1), and β -actin were from Santa Cruz (Santa Cruz, CA), and antibodies against glycogen synthase kinase-3 β (GSK3 β) and phospho (ser-9)-GSK3 β (GSK3 β -P) were from Cell Signaling Technologies (Danvers, MA). [^{32} P]dCTP (3000Ci/mmol) was from Amersham (Arlington Heights, IL).

Cell culture

HUVECs were purchased from Lonza Incorporated (Allendale, NJ) and propagated on gelatin-coated plates in M199 medium supplemented with 20% bovine calf serum, 2mM L-glutamine, 50 μ g/ml endothelial cell growth factor, 90 μ g/ml heparin, and 100U/ml of penicillin and streptomycin. MAECs were isolated from 10 week old male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) by plating thoracic aortas on matrigel-coated plates, purifying MAEC outgrowth using a PECAM-1 antibody, and characterized as endothelial cells by positive staining to PECAM-1, as we previously described [29]. MAECs were grown in endothelial basal medium supplemented with 10% fetal bovine serum, 50 μ g/ml gentamicin, 50 μ g/ml amphotericin, 1 μ g/ml hydrocortisone, 10ng/ml human epidermal growth factor, and 6 μ g/ml bovine extract (Lonza Incorporated, Allendale, NJ). MASMCS were isolated from cultured explants of mouse aortas. Briefly, aortas were excised, washed in PBS, and adventitia removed using forceps under microscopic guidance. Vessels were then cut longitudinally, endothelium removed by gentle scrapping, minced with scissors, and placed in culture plates. MASMCS were grown in complete essential medium containing 10% fetal calf serum, Earle's salts, 2mM L-glutamine and 100U/ml of penicillin and streptomycin, and characterized by morphological and immunological criteria, as we previously described [30]. For the glutamine-deprivation experiments, HUVECs were extensively washed with PBS and then incubated in glutamine-free media containing 20% dialyzed serum for 24 hours prior to their use. All cells were cultured in an atmosphere of 95% air and 5% CO $_2$ at 37°C.

Animal studies

Hyperammonemia was induced in 10-week old male C57BL/6J mice by adding NH $_4$ Cl (0.28M) in the drinking water [31]. Control animals received tap water. Mice were housed under controlled temperature and humidity, with *ad libitum* access to water and standard rodent chow. Based on a previous study [32], we estimated that mice received approximately 1mmol NH $_4$ Cl/day. After 7 days of treatment, animals were sacrificed and blood collected by exsanguination in tubes containing EDTA, and plasma obtained by centrifugation [33]. In addition, the thoracic aorta was harvested, cleaned of blood and fat, and snap-frozen in liquid nitrogen. All experimental procedures were approved by the institutional animal care and use committee and conform to 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).

CO exposure

In some experiments, HUVEC were exposed to CO by placing cells in a previously described environmental chamber [34]. In this system, gas from stock tanks containing 1% CO or 5% CO₂ in air were combined in a stainless steel mixing cylinder and delivered (1 L/min) into a humidified 37 °C incubator. CO concentrations in the chamber were continuously monitored by electrochemical detection using a CO analyzer (Interscan, Chatsworth, CA).

Western blotting

Cells or vessels were lysed in buffer (125 mM Tris [pH 6.8], 12.5% glycerol, 2% SDS, and bromophenol blue), sonicated, boiled at 100°C for 5 minutes, and centrifuged at 13,000g for 10 minutes. Proteins were subjected to gel electrophoresis and transferred to nitrocellulose membranes. Protein blots were blocked with PBS containing Triton X-100 (0.25%) and nonfat milk (5%) and then incubated with antibodies against HO-1 (1:1,500), Nrf2 (1:200), GSK3 β -P (1:375), GSK3 β (1:300), or β -actin (1:1000). Membranes were then washed, incubated with horseradish peroxidase-conjugated secondary antibodies, and developed with commercial chemoluminescence reagents (Amersham, Arlington Heights, IL). Blots were then stripped of antibodies by incubating membranes with a stripping solution (10% SDS and 100mM mercaptoethanol in 62.5mM Tris buffer, pH 6.8) for 30 minutes at 50°C, before reprobing with complementary antibodies. Protein expression was quantified by densitometry and normalized with respect to β -actin or GSK3 β .

Northern blotting

Total RNA was loaded onto agarose gels, separated by electrophoresis, and transferred to Gene Screen Plus membranes. Membranes were prehybridized in rapid hybridization buffer (Amersham, Arlington Heights, IL) and then incubated overnight at 68°C in hybridization buffer containing [³²P]DNA probes (1 × 10⁸ cpm) for Nrf2 or 18S mRNA [24,25,35]. Following hybridization and high stringency washing, membranes were exposed to X-ray film, and mRNA expression quantified by densitometry and normalized with respect to 18 S rRNA.

Real-time polymerase chain reaction

Total RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY) and transcribed to cDNA with a reverse transcription kit (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was performed with SYBR Green Supermix in a SYBER Green Cyclor iQ 5RT-PCR detection system (Bio-Rad Laboratories, Hercules, CA). The primer sequences were as follows: HO-1, forward GTACTTTGGTGCCTACTCCA and reverse CGGCCCCGAACATAGTAATTC. Transcript levels were quantified using the C_T method and normalized to 18 S rRNA, as previously described [29].

HO activity

HO activity was determined by absorbance spectroscopy [29]. Cells were sonicated in MgCl₂ (2mM) and phosphate (100mM) buffer (pH 7.4) and centrifuged at 18,000g for 15 minutes at 4°C. Supernatants were added to a reaction mixture containing NADPH

(0.8mM), glucose-6-phosphate (2mM), glucose-6-phosphate dehydrogenase (0.2U), rat liver cytosol (2mg), and hemin (20 μ M). The reaction was conducted at 37°C in the dark and stopped after an hour by the addition of chloroform. The extracted bilirubin was calculated by the difference in absorption between 464 and 530nm with an extinction coefficient of 40mM⁻¹cm⁻¹.

HO-1 reporter activity

HO-1 promoter activity was examined using luciferase reporter constructs that were provided by Dr. Jawed Alam (Ochsner Clinic Foundation, New Orleans, LA). These constructs consisted of the wild type enhancer (E1) that contains three antioxidant responsive elements (ARE) core sequences coupled to a minimum HO-1 promoter as well as the mutant enhancer (M739) that has mutations in its three ARE sequences. Transfection efficiency was ascertained by introducing a plasmid encoding Renilla luciferase (Promega, Madison, WI) into cells. Cells were transfected with plasmids using lipofectamine (Invitrogen Corporation, Carlsbad, CA), incubated for 48 hours, and then exposed to NH₄Cl for 6 hours. Firefly luciferase activity was determined using a Glomax luminometer (Promega, Madison, WI) and normalized to Renilla luciferase activity, and this ratio was expressed as fold induction over control cells.

Small interference RNA

HO-1 was silenced by transfecting cells with HO-1 small interference RNA (siRNA) (0.1 μ M) or a non-targeting (NT) siRNA (0.1 μ M) that was purchased from Dharmacon (Lafayette, CO), as we previously reported [25,29,35].

Nrf2 activation

Cells were collected in lysis buffer (10 mM HEPES, pH 7.9, 10mM KCl, 0.1mM EDTA, 0.5mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A, 0.5 mM dithiothreitol, and 0.4% Nonidet P-40) and then centrifuged at 14,000 $\times$ g for 3 minutes. Nuclear pellets were suspended in extraction buffer (20mM HEPES, pH 7.9, 0.4M NaCl, 1.0mM EDTA, 1mM dithiothreitol, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A, and 10% glycerol), and centrifuged at 14,000 $\times$ g for 5 minutes. The supernatant was collected and Nrf2 activity determined by measuring the binding of Nrf2 to the ARE using the ELISA-based TransAM Nrf2 kit (Active Motif, Carlsbad, CA). Bound protein was detected using an antibody specific to DNA-bound Nrf2, visualized by colorimetric reaction catalyzed by horseradish peroxidase-conjugated secondary antibody, and absorbance measured at 405 nm.

Measurement of TNF α

TNF α levels in the culture media were determined by ELISA using a commercially available kit (Invitrogen Corporation, Carlsbad, CA), as we previously described [36].

Measurement of intracellular ROS

Intracellular ROS was monitored using the cell-permeable probe CM-H₂DCFDA. Cells were grown to confluence and treated with ammonia in the presence and absence of NAC or

rotenone. Following treatment cells were incubated with CM-H₂DCFDA (10 μ M) for 30 minutes at 37°C. The cells were then washed with PBS and fluorescence quantified by microplate fluorimetry with excitation at 485nm and emission at 530nm, as previously described [37]. Mean values from each treatment were expressed as a fraction relative to untreated control cells.

Measurement of ammonia

Ammonia concentration was determined using an ammonia assay kit (Sigma-Aldrich, St. Louis, MO), according to manufacturer's instructions. This assay measures the reductive amination of α -ketoglutarate to glutamate in the presence of glutamate dehydrogenase and NADH. Samples were mixed with ammonia assay reagent at room temperature for 10 minutes and then absorbance of the reaction mixture measured by spectroscopy at 340nm. Concentrations were determined relative to a standard curve obtained using aqueous solutions of ammonia.

Cell viability

Cell viability was evaluated by monitoring the uptake of the membrane-impermeable dye trypan blue. Cells were incubated with trypsin (0.25%), collected, diluted (1:4) with trypan blue and examined by microscopy. Viability was determined by the ability of cells to exclude the stain, as we previously reported [25,29,38].

Statistical analyses

Results are expressed as mean $\pm$ SEM. Statistical analyses were performed with the use of a Student's two-tailed t-test and an analysis of variance with the Tukey post hoc test when more than two treatment regimens were compared. P values < 0.05 were considered statistically significant.

Results

Treatment of HUVECs with ammonia stimulated the expression of HO-1 protein. The induction of HO-1 protein was delayed with a significant increase in HO-1 protein detected 4 hours after administration of the ammonia donor, NH₄Cl, and levels progressively increased over 24 hours (Fig. 1A). The increase in HO-1 protein observed after 24 hours was dependent on the concentration of NH₄Cl, with higher concentrations evoking larger rises in HO-1 protein (Fig. 1B). The induction of HO-1 protein by NH₄Cl was mimicked by another ammonia donor, NH₄OH (Fig. 1C). The stimulation of HO-1 protein expression by NH₄OH was not an indirect consequence of altered pH as NaOH had no effect on HO-1 levels (Fig. 1D). The ammonia donors also increased HO-1 mRNA expression and HO activity in HUVECs (Fig. 1E and F).

Generation of ammonia by HUVECs also modulated HO-1 protein expression. Treatment of glutamine-restricted HUVECs with the ammonia scavenger, methyl pyruvate [39], caused a marked decline in ammonia levels in the culture medium and this was associated with a significant reduction in HO-1 protein (Fig. 2A and B). In contrast, addition of a physiologically relevant concentration of glutamine (500 μ M) to these cells evoked a marked

rise in ammonia production and HO-1 protein expression. Both these effects of glutamine were prevented by methyl pyruvate, demonstrating that endothelial HO-1 levels are regulated in a dynamic manner by endogenous ammonia synthesis. Treatment of MAECs or MAMCs with NH₄Cl likewise evoked an increase in HO-1 protein (Fig. 3A and B). Moreover, supplementation of the drinking water of mice with NH₄Cl resulted in a marked increase in plasma ammonia that was paralleled by an elevation in aortic HO-1 protein expression (Fig. 3C and D).

We next explored the molecular mechanism by which ammonia stimulates HO-1 expression. Incubation of HUVECs with the transcriptional inhibitor actinomycin D abolished basal and NH₄Cl-induced HO-1 protein expression (Fig. 4A), suggesting that ammonia-mediated increases in HO-1 expression required *de novo* RNA synthesis. Ammonia also activated the HO-1 promoter and this was prevented by mutating the ARE sequences in the promoter (Fig. 4B). In addition, modification of the ARE sequences attenuated baseline promoter activity. Since we previously demonstrated that the transcription factor Nrf2 plays a predominant role in endothelial HO-1 gene activation [25,29,35], the role of Nrf2 was examined. Transfection of the HUVECs with a dominant-negative mutant of Nrf2 (dnNrf2) that had its activation domain removed inhibited basal and NH₄Cl-mediated increases in HO-1 promoter activity (Fig. 4B). Furthermore, NH₄Cl stimulated an increase in Nrf2 protein and mRNA beginning 2 and 8 hours, respectively, following NH₄Cl treatment (Fig. 4C and D). NH₄Cl also promoted the activation of Nrf2, as reflected by the binding of nuclear Nrf2 to the ARE (Fig. 4E). Since the GSK3 β -mediated phosphorylation of Nrf2 targets the protein for ubiquitination and degradation by the E3 ligase, β -transducin repeats containing protein (β -TrCP) [40,41], we examined the possibility that ammonia increases Nrf2 expression by blocking GSK3 β activity. Given that GSK3 β activity is inhibited by the phosphorylation of serine-9, the ability of ammonia to stimulate the phosphorylation of this serine residue was determined. However, NH₄Cl had no effect on GSK3 β phosphorylation. Alternatively, inhibition of phosphatidylinositol-3-kinase-Akt signaling pathway, a known negative regulator of GSK3 β activity [42], by LY294002 stimulated GSK3 β activity as revealed by a prominent decrease in the phosphorylation of GSK3 β , affirming that alterations in GSK3 β activity could be monitored by this assay.

In subsequent experiments, we determined the upstream signaling pathway responsible for the induction of HO-1. Since oxidative stress is an established activator of Nrf2 [35], the role of ROS in the stimulation of HO-1 expression was investigated. Treatment of HUVECs with NH₄Cl resulted in a concentration-dependent increase in ROS production (Fig. 5A). The NH₄Cl-mediated rise in ROS formation was detected within one hour of treatment and persisted for 24 hours (Fig. 5B). The increase in oxidative stress by ammonia was not accompanied by any change in cell viability (Fig. 5C). Pretreatment of HUVECs with the antioxidant NAC or the mitochondrial electron transport chain inhibitor rotenone prevented the increase in intracellular ROS by NH₄Cl (Fig. 6A). In addition, NAC blocked the NH₄Cl-mediated induction of HO-1 protein (Fig. 6B) and activation of Nrf2, as reflected by the diminished binding of nuclear Nrf2 to the ARE (Fig. 6C). We also investigated the cellular source of ROS accountable for the induction of HO-1 by ammonia. While incubation of endothelial cells with rotenone blocked the NH₄Cl-mediated increases in HO-1 protein

expression (Fig. 6D), the NADPH oxidase inhibitor, apocynin, or the xanthine oxidase inhibitor, allopurinol, had no effect on HO-1 expression (Fig. 6E and F).

Finally, the functional significance of the induction of HO-1 by ammonia was investigated. Since HO-1 protects against endothelial injury [24–26], the pro-survival potential of ammonia was examined. Treatment of HUVECs with TNF α and cycloheximide stimulated a marked reduction in the number of viable cells (Fig. 7A). Notably, pretreatment of endothelial cells with ammonia (5mM) for 24 hours attenuated the cytokine-mediated loss of cell viability. Consistent with previous reports [43,44], we found that HUVEC did not basally release TNF α in the culture media and ammonia exposure (5mM) for 24 hours failed to stimulate TNF α synthesis in these cells. To determine if the protective action of NH₄Cl involved HO-1, HUVECs were transfected with HO-1 siRNA or NT siRNA. HO-1 siRNA effectively silenced the expression of HO-1 whereas NT siRNA was without effect, demonstrating the efficacy and specificity of the knockdown approach (Fig. 7B). Significantly, HO-1 siRNA abolished the ability of NH₄Cl to block cytokine-mediated cell death while NT siRNA failed to modulate cell viability (Fig. 7A). In the absence of NH₄Cl, HO-1 or NT siRNA had no effect on cell viability. Finally, we determined which of the HO-1 products was able to restore cell viability in HO-1-silenced endothelial cells. While the exogenous administration of CO could substitute for the loss of HO-1, and reverse the negative effect of TNF α on cell viability, bilirubin and iron failed to restore endothelial cell survival (Fig. 7A). CO also inhibited cytokine-mediated endothelial cell death in the absence of NH₄Cl, while bilirubin and iron had no effect (Fig. 7C).

Discussion

In the present study, we identified ammonia as a potent inducer of HO-1 gene expression in vascular endothelium. The induction of HO-1 is observed in human and murine endothelial cells and mouse arteries, requires the production of ROS, and is mediated by the Nrf2-ARE complex. Significantly, we also found that ammonia protects against endothelial cell death in a HO-1-dependent fashion that relies on the generation of CO. These findings establish ammonia as a novel regulator of endothelial cell survival and identify ammonia as a potentially important signaling gas in the circulation.

Treatment of HUVECs with two distinct ammonia donors (NH₄Cl and NH₄OH) stimulates the expression of HO-1. The induction of HO-1 is dependent on the concentration and duration of ammonia exposure. A significant elevation of HO-1 protein is detected after 4 hours, and this progressively increases during 24 hours of ammonia exposure. The induction of HO-1 is also observed in MAECs indicating that ammonia stimulates endothelial HO-1 expression across animal species. In addition, the stimulation of HO-1 expression by ammonia is not restricted to endothelial cells, as a significant elevation in HO-1 expression is observed in MASCs. Significantly, we also found that endogenous ammonia production is coupled to HO-1 expression in endothelial cells. Removal of ammonia by the ammonia scavenger methyl pyruvate results in a marked decline in HO-1 protein whereas an increase in ammonia synthesis following the addition of glutamine is paralleled by a marked rise in HO-1 protein. Thus, ammonia functions in an autocrine manner to drive endothelial HO-1 expression. Moreover, the ability of glutamine to stimulate endothelial ammonia synthesis is

in-line with a previous study [16] and may provide a mechanism by which this amino acid is able to induce the expression of HO-1 in various experimental models [45–47].

Using a dietary mouse model of hyperammonemia, we also show that *in vivo* administration of ammonia increases arterial HO-1 content. The concentration of plasma ammonia noted in our animal model (~200 μ M) is comparable or lower than that reported in certain pathological states, including patients with urea cycle defects, liver failure, and organic acidemia as well as the tumor microenvironment [1,6,7,48,49], suggesting that the induction of HO-1 occurs in many of these metabolic disorders as well as cancer. Consistent with this notion, increases in HO-1 have been detected in the brain following acute ammonia intoxication of rats and in animal models of hepatic encephalopathy and cancer [49–52].

The induction of HO-1 by ammonia is dependent on *de novo* RNA synthesis and likely involves the transcriptional activation of the gene. We found that the transcriptional inhibitor, actinomycin D prevents the increase in HO-1 expression by ammonia. In addition, ammonia directly stimulates HO-1 promoter activity. The induction of HO-1 gene transcription requires the presence of AREs since mutation of this responsive element abolishes the increase in promoter activity by ammonia. While numerous transcription factors bind to AREs, we previously reported that Nrf2 plays a fundamental role in ARE-dependent HO-1 gene expression in endothelial cells [25,29,35]. In agreement with this earlier work, we found that ammonia stimulates Nrf2 protein and mRNA expression, and nuclear Nrf2 binding to the AREs. Interestingly, ammonia-mediated increases in Nrf2 protein precede elevations in Nrf2 mRNA, suggesting that this gas stimulates both *de novo* synthesis and stability of the protein. Moreover, transfection of endothelial cells with a dominant-negative Nrf2 construct nullifies the activation of HO-1 promoter activity in response to ammonia. Thus, recruitment of Nrf2 plays an essential role in mediating HO-1 gene transcription in endothelial cells and may underlie the induction by ammonia of other Nrf2-targeted genes that are involved in glutathione synthesis [53]. Finally, the discovery that ammonia, like nitric oxide, CO, and hydrogen sulfide, activates Nrf2 highlights the critical role this transcription factor plays in mediating the genomic actions of signaling gases [54–56].

The ability of ammonia to stimulate HO-1 expression is dependent on oxidative stress. Incubation of HUVECs with ammonia evokes a marked increase in ROS production. This finding mirrors previous work in other cells including astrocytes, neurons, dendritic cells, and epithelial cells [9,12,27,57]. Significantly, the antioxidant NAC blocks the ammonia-mediated generation of ROS, the induction of HO-1, and the activation of Nrf2. While activation of NADPH oxidase and xanthine oxidase have been linked to the production of ROS by ammonia [58,59], they do not mediate the induction of HO-1 as pharmacological blockade of either enzyme fails to inhibit the expression of HO-1. Instead, it appears that a mitochondrial pathway underlies the ammonia-mediated increase in HO-1 expression, as the induction of ROS formation and HO-1 expression by ammonia is attenuated by the mitochondrial electron transport chain complex I inhibitor, rotenone, implicating ROS production from this organelle in the induction of HO-1. Although the mechanism by which ammonia activates Nrf2 is not known, it presumably involves the oxidation of specific cysteine residues in Kelch-like erythroid cell-derived protein-1 (Keap1) that mediate the release and/or inhibition of Keap1-dependent ubiquitination and degradation of Nrf2 [60].

Given that ammonia has no effect on GSK3 β activity, diminished proteasomal degradation of Nrf2 by β -TrCP seems unlikely.

Although ammonia is a well-known neurotoxin [6,7,61,62] that also adversely affects lung, heart, and skeletal muscle function [15,58,59], the administration of high concentrations of ammonia (up to 5 mM) had no detrimental effect on human endothelial cell viability. This observation is consistent with other studies performed in mouse and rat brain endothelial cell lines [63,64], suggesting that the toxic effects of ammonia may be cell-specific. Surprisingly, we show that ammonia pretreatment markedly reduces endothelial cell death in response to TNF α and cycloheximide. We also found that the cytoprotection provided by ammonia is dependent on HO-1 since depletion of the enzyme abrogates the protection by ammonia. These results are in-line with earlier studies demonstrating that the induction of HO-1 promotes endothelial cell survival in response to a host of inimical stimuli [22–26]. Significantly, exogenous administration of the HO-1 product CO, but not bilirubin or iron, can substitute for HO-1 and restore the viability of cytokine-challenged endothelial cells, suggesting that CO mediates the protective action of HO-1. Consistent with this notion, CO is the only HO-1 product capable of blocking cytokine-induced apoptosis in the absence of ammonia pretreatment. The damaging effect of TNF α and cycloheximide on endothelial cells occurs via the caspase-mediated induction of apoptosis (65). Ample evidence from our laboratory and others indicates that HO-1-derived CO is a potent inhibitor of apoptosis [24–26,29] and that it suppresses cytokine-mediated apoptosis via the activation of p38 mitogen-activated kinase and expression of a subset of nuclear factor- κ B-dependent anti-apoptotic genes (26,66). Consistent with our findings, ammonia also protects against the lethal actions of TNF α in immortalized renal epithelial cells and improves survival of glutamine-starved hybridoma cells [49,67], demonstrating that the cytoprotective action of ammonia extends beyond endothelial cells. While endothelial cells possess different functional and metabolic properties than tumor or transformed cells, endothelial cells are highly plastic and can assume a highly proliferative and motile state during tissue repair and angiogenesis and, intriguingly, share some key metabolic similarities with tumor cells. Both cell types rely on glycolysis for a majority of their energy requirements (68) and have extremely high rates of glutaminolysis that favors the production of ammonia (16–18). This latter finding highlights the need for further studies investigating the role of this gas both in tumorigenic and non-tumorigenic cells.

Our finding that ammonia stimulates HO-1 expression in HUVEC and MAEC is consistent with an earlier report in rat cerebral microvascular endothelial cells [28] and demonstrates that ammonia induces HO-1 in both the macro and microcirculation. However, ammonia can evoke disparate responses in endothelial cell types. While ammonia fails to stimulate TNF α synthesis in HUVEC, a significant increase in TNF α production is observed in rat cerebral microvascular endothelial cells [69]. The differential inflammatory response to ammonia between peripheral conduit and brain microvascular endothelial cells may reflect differences in their sensitivity to inflammatory stimuli as well as in their structural and functional properties. Brain microvascular endothelial cells constitute the blood-brain barrier (BBB) and interact with glial cells in maintaining cerebral homeostasis by regulating barrier permeability, transport, and metabolic functions. However, BBB function is impaired in hyperammonemia secondary to acute liver failure due to the degradation of tight junction

proteins by matrix metalloproteinase-9 [70–72]. Similarly, ammonia reduces tight junction expression and increases paracellular permeability of rat brain endothelial cells in a matrix metalloproteinase-dependent fashion [73,74]. Interestingly, inducers of HO-1 have been shown to protect the cerebral vasculature against BBB dysfunction and neurological deficits in stroke, and improve BBB function after intracerebral hemorrhage [75,76]. Moreover, curcumin antagonizes increases in BBB permeability via the HO-1-mediated restoration of tight junction proteins and HO-1 also stabilizes the blood-spinal cord barrier after acute injury in mice [77,78]. Collectively, these findings raise the possibility that the induction of HO-1 in cerebral endothelial cells may function in an adaptive manner to preserve BBB and limit brain edema in hyperammonemic states.

Cerebral microvascular endothelial cells are intimately associated with astrocytes forming a gliovascular complex that manages BBB integrity [79]. However, the functional integrity of this complex may be compromised by dysfunctional endothelial cells in hyperammonemic conditions. In particular, ammonia-treated cerebral endothelial cells were shown to cause swelling of astrocytes in a paracrine fashion that depends on ammonia-induced free radical production and the activation of nuclear factor- κ B by endothelial cells [28]. Interestingly, the induction of HO-1 in cerebral endothelial cells is also observed in this environment [28]. Since HO-1 has been demonstrated to correct endothelial dysfunction in various pathologic states [80–82], the upregulation of HO-1 in this setting may play a role in dampening this harmful endothelial cell-initiated intercellular signaling pathway.

The ability of ammonia to preserve endothelial cell viability in response to inflammatory mediators raises the possibility that ammonia may be used for therapeutic effect in various inflammatory states, including solid organ transplantation and ischemic reperfusion injury. In particular, exposure of donor organs to ammonia prior to transplantation may mitigate endothelial cell death and dysfunction, and contribute to organ survival. In addition, local or systemic administration of ammonia as a preconditioning stimulus may promote organ function following ischemia-reperfusion. Indeed, we have recently shown that preconditioning of animals with another potentially toxic signaling gas, hydrogen sulfide, can ameliorate inflammation following ischemia-reperfusion of the small intestine [83]. Aside from counteracting the destruction of endothelial cells, ammonia may also preserve blood flow and fluidity following organ transplantation or ischemia-reperfusion through the induction of HO-1, since this enzyme evokes vasodilatory, anti-inflammatory, and anti-thrombotic actions in the circulation [19–21].

In conclusion, the present study demonstrates that ammonia induces HO-1 gene expression in human and murine endothelial cells via the activation of the ROS-Nrf2 signaling pathway. In addition, it found that ammonia inhibits cytokine-mediated endothelial cell death via the induction of HO-1 and release of CO. These results establish ammonia as a novel regulator of endothelial cell survival and reveals critical new regulatory and functional interactions between signaling gases in the vasculature.

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Abbreviations

HO-1	heme oxygenase-1
Nrf2	NF-E2-related factor-2
ROS	reactive oxygen species
CO	carbon monoxide
HUVECs	human umbilical vein endothelial cells
MAECs	mouse aortic endothelial cells
MASMCs	mouse aortic smooth muscle cells
NH₄Cl	ammonium chloride
NH₄OH	ammonium hydroxide
NAC	N-acetyl-L-cysteine
PMSF	phenylmethylsulfonyl fluoride
CM-H₂DCFDA	5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester
GSK3β	glycogen synthase kinase-3 β
GSK3β-P	phospho (ser-9)- glycogen synthase kinase-3 β
TNFα	tumor necrosis factor- α
PECAM-1	platelet-endothelial cell adhesion molecule-1
ARE	antioxidant responsive elements
siRNA	small interference RNA
dnNrf2	dominant-negative mutant of Nrf2
β-TrCP	β -transducin repeats containing protein

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Highlights

- Ammonia stimulates HO-1 expression in endothelial cells via the ROS-Nrf2 pathway
- Glutamine induces HO-1 expression in endothelial cells via the formation of ammonia
- Ammonia enhances endothelial cell viability via the HO-1-mediated release of CO
- Ammonia is an important signaling gas that promotes endothelial cell survival

Figure 1.

Ammonia increases HO-1 expression in HUVECs. (A) NH₄Cl (5.0mM) stimulates a time-dependent increase in HO-1 protein. (B) NH₄Cl exposure for 24 hours stimulates a concentration-dependent increase in HO-1 protein. (C) NH₄OH exposure for 24 hours stimulates a concentration-dependent increase in HO-1 protein. (D) NaOH exposure for 24 hours has no effect on HO-1 protein expression. (E) NH₄Cl (5.0mM) or NH₄OH (5.0mM) exposure for 8 hours stimulates HO-1 mRNA. (F) NH₄Cl (5mM) or NH₄OH (5mM) exposure for 24 hours elevates HO activity. Protein expression was quantified by scanning laser densitometry, normalized with respect to β -actin, and expressed relative to that of control, untreated cells (open bars). Results are means $\pm$ SEM (n=3–5). *Statistically significant effect of NH₄Cl or NH₄OH.

Figure 2.

Endogenous ammonia (NH_3) production regulates HO-1 expression in HUVECs. (A) Glutamine (GTN; 500 μM) stimulates NH_3 formation in GTN-deprived endothelial cells. The NH_3 scavenger methyl pyruvate (MP; 10mM) blocks basal and GTN-stimulated NH_3 synthesis. (B) GTN (500 μM) stimulates HO-1 protein in an NH_3 -dependent manner. MP (10mM) inhibits basal and GTN-stimulated expression of HO-1 protein. Results are means $\pm$ SEM (n=3) *Statistically significant effect of GTN.

Figure 3.

Ammonia stimulates HO-1 protein in MAECs, MAMCs, and murine arteries. (A) NH₄Cl exposure for 24 hours stimulates HO-1 protein expression in MAEC. (B) NH₄Cl exposure for 24 hours stimulates HO-1 protein expression in MAMCs. (C and D) Administration of NH₄Cl (0.28M) in the drinking water of mice for 7 days increases plasma ammonia concentration and arterial HO-1 protein expression. Results are means± SEM (n=3). *Statistically significant effect of NH₄Cl.

Figure 4.

Ammonia stimulates HO-1 promoter activity via the Nrf2/ARE complex in HUVECs. (A) Ammonia-mediated HO-1 gene expression requires *de novo* RNA synthesis. Effect of actinomycin D (ActD; 0.10μg/ml) on NH₄Cl (5.0mM for 8 hour)-mediated increase in HO-1 protein. (B) Ammonia stimulates HO-1 promoter activity. Cells were transfected with a HO-1 promoter construct (E1) or a mutated HO-1 promoter construct (M739) and a *Renilla* luciferase construct, treated with NH₄Cl (5.0mM for 6 hours), and then analyzed for luciferase activity. In some instances, a dominant-negative Nrf2 (dnNrf2) construct was co-transfected into the cells. (C) Time-course of Nrf2 protein expression in native non-transfected cells after administration of NH₄Cl (5.0mM). (D) Time-course of Nrf2 mRNA expression native non-transfected after administration of NH₄Cl (5.0mM). (E) Ammonia stimulates Nrf2 activity. Native non-transfected cells were treated with NH₄Cl (5.0mM) for 6 hours and nuclear extracts analyzed for Nrf2 binding by ELISA. O.D., optical density. (F) Effect of ammonia on GSK3β activity. Native non-transfected cells were treated with NH₄Cl (5.0mM) for various times (0–24 hours) or with the phosphatidylinositol-3-kinase inhibitor LY2942002 (LY; 10μM) for 4 hours. Protein and mRNA expression was quantified by scanning laser densitometry and normalized with respect to β-actin or GSK3β and 18 S rRNA, respectively, and expressed relative to that of control, untreated cells (open bars). Results are means ± SEM (n=3–5). *Statistically significant effect of NH₄Cl or LY.

Figure 5.

Ammonia stimulates oxidative stress in HUVECs. (A) NH₄Cl exposure for 8 hours increases intracellular ROS production in a concentration-dependent manner. (B) NH₄Cl (5.0mM) exposure increases ROS production in a time-dependent fashion. (C) NH₄Cl exposure for 24 hours had no effect on endothelial cell viability. Results are means $\pm$ SEM (n=6).

*Statistically significant effect of NH₄Cl.

Figure 6.

Ammonia-induced HO-1 expression is dependent on oxidative stress in HUVECs. (A) Effect of N-acetyl-L-cysteine (NAC; 10mM) or rotenone (Rot; 5μM) on NH₄Cl (5.0mM for 8 hours)-mediated intracellular ROS production. (B) Effect of NAC (10mM) on NH₄Cl (5.0mM for 24 hours)-mediated HO-1 protein expression. (C) Effect of NAC (10mM) on NH₄Cl (5.0mM for 6 hours)-mediated Nrf2 activation. O.D., optical density. (D) Effect of Rot (5μM) on NH₄Cl (5.0mM for 24 hours)-mediated HO-1 protein expression. (E) Effect of apocynin (Apo; 300μM) on NH₄Cl (5.0mM for 24 hours)-mediated HO-1 protein expression. (F) Effect of allopurinol (Allo; 100μM) on NH₄Cl (5.0mM for 24 hours)-mediated HO-1 protein expression. HO-1 protein was quantified by scanning laser densitometry and normalized with respect to β-actin, and expressed relative to that of control, untreated cells (open bars). Results are means ± SEM (n=3–6). *Statistically significant effect of NH₄Cl.

Figure 7.

Ammonia inhibits inflammatory stress-induced cell death in HUVECs via the HO-1-mediated production of CO. (A). Ammonia promotes endothelial cell survival via the induction of HO-1 and release of CO. HUVEC were transfected with HO-1 siRNA (0.1 μ M) or NT siRNA (0.1 μ M), treated with NH₄Cl (5.0mM) for 24 hours, and then exposed to TNF α (10ng/ml) and cycloheximide (CX;2 μ g/ml) for 8 hours in the presence or absence of CO (200ppm), bilirubin (10 μ M), or FeSO₄ (10 μ M). (B) HO-1 protein expression in cells transfected with HO-1 siRNA (0.1 μ M) or non-targeting (NT) siRNA (0.1 μ M) in the presence and absence of NH₄Cl (5.0mM) for 24 hours. (C) CO inhibits cytokine-mediated apoptosis. HUVEC were treated with TNF α (10ng/ml) and CX (2 μ g/ml) for 8 hours in the presence or absence of CO (200ppm), bilirubin (BR;10 μ M), or FeSO₄ (10 μ M). Results are means $\pm$ SEM (n=3–6). *Statistically significant effect of TNF α and CX. +Statistically significant effect of NH₄Cl or CO.