Supplementary Information:

Chlorine gas exposure causes systemic endothelial dysfunction by inhibiting eNOS-dependent signaling

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Materials and Methods

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Detailed Materials and Methods are provided in supplementary information

Materials: Unless stated otherwise all reagents and antibodies were purchased from Sigma (St. Louis, MO, USA) and Abcam (Cambridge, MA, USA) respectively except Mahma/NONOate (MNO) which was purchased from Axxora Platform (San Diego, CA, USA). Male Sprague Dawley rats (200-300g) were purchased from Harlan (Indianapolis, IN) and kept on 12h light dark cycles with access to standard chow and water *ad libitum* prior to and post chlorine exposure. 1400W was purchased from Enzo Life Sciences International, Inc, Plymouth Meeting, PA, USA

Methods

Rat exposure to Chlorine gas. Whole body exposure of rats to different doses of Cl2 gas was performed as previously described and according to IACUC approved protocols. Two rats were exposed in the same chamber at any one time and all exposures were performed between 8-9am and were 30min in length followed by return to room air. Rats were placed inside a cylindrical glass chamber (Specialty Glass, Houston, TX; part no. X02AI99C15A57H5) and exposed to100, 250 or 400 ppm Cl_2 for 30 min. Two mass flow controllers (MFCs) with Kalrez seals (Scott Specialty Gases, Los Angeles, CA; part no. 05236A1V5K) and a microprocessor control unit (Scott Specialty Gases; part no. 05236E4) were used to control the compressed air and Cl_2 (1,000 ppm Cl_2 in air; Airgas, Birmingham, AL) flow rates to achieve the chamber Cl_2 target concentrations (400 ppm). A bubble flowmeter was used to validate MFC performance on a weekly basis. Air and Cl_2 were initially mixed at a three-way junction, and they were further mixed by passing through a diffuser located inside the top lid of the exposure chamber. Gases exited the chamber via two large-bore diameter ports in its bottom half. Chamber Cl_2 concentrations were monitored continuously with an electrochemical reduction detector (Interscan, Chatsworth, CA; model RM34-100m). The detector was

connected to an IBM computer for real-time display of Cl_2 concentration and data storage. Both the exposure chamber and the detector were placed inside a chemical fume hood located in a negative-pressure room. At the end of each exposure, the Cl_2 gas was turned off, the chamber was vented with compressed air for 2–3 min, the two halves were separated, and the rats were removed and returned to their cages, where they breathed room air. Food and water were provided ad libitum. Rats were divided into three groups and sacrificed at 6 hours, 24 hours and 48 hours post Cl_2 exposure

1400W Treatment: After 5 day acclimatization to blood pressure measuring equipment, rats were divided into 3 groups: Chlorine exposed +1400W, Air Control+ 1400W and Chlorine exposed +vehicle. Animals were injected intraperitoneally with 10mg/kg dose of 1400W chlorine 24 hours after exposure to Cl_2. Blood pressure was measured at 18 hrs, 24 hrs, 48 hrs post 1400W injection

Aortic Vessel studies. At the indicated times post Cl2 exposure, aorta were collected, cleansed of adherent fat and responses to the indicated vasoconstrictive and vasoactive stimuli determined in vessel bioassay chambers (Radnoti, Monrovia, CA), as previously described. All vessel bioassay studies were performed in indomethacin (5µM) pre-treated vessels (2-3mm segments) and in a bicarbonate buffered Krebs Henseleit buffer of the following composition (mM): NaCl 118; KCl 4.6; $NaHCO_3$ 27.2; KH_2PO_4 1.2; $MgSO_4$ 1.2; $CaCl_2$ 1.75; Na_2 EDTA (ethylenediaminetetraacetic acid) 0.03, and glucose 11.1 and perfused with 21%O2, 5%CO2 balanced with N2 A passive load of 2 grams was applied to all ring segments and maintained at this level throughout the experiments. At the beginning of each experiment ring segments were depolarized with KCl (70 mM) to determine the maximal contractile capacity of the vessel. Rings were then washed extensively and allowed to equilibrate and again depolarized with KCl (70mM). The rings were then washed and allowed to equilibrate. Vasoconstrictor responses were tested by cumulative addition of PE doses ranging from 1nM to 3000nM. Endothelium-dependent vasodilator responses were tested by administering cumulative doses of Ach, ranging from 1nm to 3000nM after tension development at maximal PE dose. In subsequent experiments, vessels were submaximally contracted (50% of KCl response) with PE (300nM-1000nM). When tension development reached a plateau, endothelium–independent vasodilator responses were induced by administering the NO donor MAHMANONOate (MNO).

Expression of eNOS, iNOS nNOS mRNA: Total RNA was isolated from aorta or leukocytes using the acid guanidinium thiocyanate-phenol-chloroform extraction protocol (Trizol reagent, Invitrogen). First-strand cDNA was synthesized using oligo-dT primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Real-time PCR primers were designed for rat NOS1-3 using the Beacon Design software (Bio-Rad,

Hercules, CA) and were AGGAATATGAGGAGTGGAAGTG (NOS1F), CTGAGTGAGGAGAAGTGTAGC (NOS1R), AAGAGACGCACAGGCAGAG (NOS2F), CAGGCACACGCAATGATGG (NOS2R), CAGGCTCTCACTTACTTC (NOS3F), AACCACTTCCATTCTTCG (NOS3R). Two-step real-time PCR was performed using a SYBR Green protocol described elsewhere (1). Data were normalized against glyceraldehyde-3-phosphate dehydrogenase and gene expression was compared between samples by using the $2^{-\Delta\Delta CT}$ method.

eNOS Protein Expression.

Western Blotting: Protein was isolated from homogenized aorta in RIPA buffer containing complete mini protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). Quanitification was done by Bradford protein assay. 60µg of total protein was run on a 10% polyacrylamide gel and then transferred to nitrocellulose membrane. Immunoblotting was performed using a SNAP i.d. Protein Detection System (Millipore, Billerica, MA, USA). The membranes were blotted for 20min with mouse α-eNOS (BD Transduction Laboratories, 610296) and 10min with rabbit anti-β-actin (Cell Signaling, 4967), as a loading control. HRP conjugated secondary antibodies and Pierce West Dura Chemiluminescence Substrate was used for detection.

Immunofluoresence measurements: Abdominal aorta was isolated from rats 24h after exposure to air or Cl_2 (400ppm, 30min) and fixed with 4% paraformaldehyde and cryoprotected with sucrose and OCT. 5µm frozen sections were cut for immunohistochemistry. Frozen slides were thawed and dried for 20min and then rehydrated for 15min in PBS. Tissue was permeabilized (20 min, room temperature) in a humidified chamber using a sufficient volume of PBS containing 0.2% Triton X-100 (PBT) to cover the PAP ring encircled tissue. Nonspecific binding sites were blocked with PBT containing 5% normal goat serum for 30 min at room temperature. Staining for eNOS or vWF was performed using mouse anti-eNOS (BD Transduction Laboratories, 610296) or rabbit anti-Von Willebrand Factor (Abcam, ab6994) respectively for 2 hours at room temperature in humidified chamber. The tissue was washed 3x5 min with PBT and then post fixed 15 min with 4% paraformaldehyde to stabilize the label. Sections were washed 2x5 min with PBS. Tissue was counterstained 5min with 50µl 10uM Hoechst 33342 and then washed 3x5 min with PBS. Slides were mounted and coverslipped (Thermo Scientific, PermaFluor Mountant). Digital photomicrographs were taken at 40x with oil immersion using a Leica DMRXA2 microscope and CRi Micro Color camera (RGB-MS-C). Simple PCI was used for the quantitation and analysis of eNOS staining in aorta. The endothelial layer was manually selected as the object to be analyzed. The area of the object was measured and then total intensity of the staining within that area was quantitated.

Plasma Nitrite measurement: Blood was collected via cardiac puncture into 1mL syringes containing 1.5% (w/v) sodium citrate, 5 mM NEM, and 100 µM DTPA (all

concentrations listed are final). After mixing, blood was centrifuged at 2000 x g for 90 seconds at 25^{o}C and plasma collected and further stabilized with 1 mM NEM and 100 µM DTPA. Nitrite concentrations were measured by a reductive (I_3^-) chemiluminescence method as previously described and verified by acid-sulfanilamide dependent scavenging.

***Measurement of cytokines*:** The inflammatory cytokines IFN-γ,IL-1β, and TNFα were measured using a sandwich immuno assay kit (K11014A-4; Meso Scale Discovery [MSD], Gaithersburg). Detection limits for IFN-γ, IL-1b and TNFα were 9.77pg/ml, 15.7pg/ml and 5.78pg/ml respectively.

Blood pressure measurements: Blood pressure was non-invasively measured by determining the tail blood volume with a volume pressure recording sensor and an occlusion tail-cuff (CODA 6.25 System, Kent Scientific, Torrington, CT). Rats were acclimatized to the restraints and the tail cuff for 5 days prior to beginning experimental measurements. dystolic, diastolic and mean arterial pressure was measured pre and post chlorine exposure at various timepoints, 24 hrs, 48 hrs and 72 hrs. Tail temperature was closely monitored and a heating pad was used minimally to maintain tail blood temperature.