Toxicology and Applied Pharmacology 238 (2009) 27–36

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Dose-dependent transitions in Nrf2-mediated adaptive response and related stress responses to hypochlorous acid in mouse macrophages

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ARTICLE INFO

Article history:
Received 2 December 2008
Revised 31 March 2009
Accepted 6 April 2009
Available online 17 April 2009

Keywords:
Nrf2
AP-1
NF-κB
Hypochlorous acid
Antioxidant response element
Oxidative stress
Genomic profiling

ABSTRACT

Hypochlorous acid (HOCl) is potentially an important source of cellular oxidative stress. Human HOCl exposure can occur from chlorine gas inhalation or from endogenous sources of HOCl, such as respiratory burst by phagocytes. Transcription factor Nrf2 is a key regulator of cellular redox status and serves as a primary source of defense against oxidative stress. We recently demonstrated that HOCl activates Nrf2-mediated antioxidant response in cultured mouse macrophages in a biphasic manner. In an effort to determine whether Nrf2 pathways overlap with other stress pathways, gene expression profiling was performed in RAW 264.7 macrophages exposed to HOCl using whole genome mouse microarrays. Benchmark dose (BMD) analysis on gene expression data revealed that Nrf2-mediated antioxidant response and protein ubiquitination were the most sensitive biological pathways that were activated in response to low concentrations of HOCl (<0.35 mM). Genes involved in chromatin architecture maintenance and DNA-dependent transcription were also sensitive to very low doses. Moderate concentrations of HOCl (0.35 to 1.4 mM) caused maximal activation of the Nrf2 pathway and innate immune response genes, such as IL-1β, IL-6, IL-10 and chemokines. At even higher concentrations of HOCl (2.8 to 3.5 mM) there was a loss of Nrf2-target gene expression with increased expression of numerous heat shock and histone cluster genes, AP-1-family genes, *cFos* and *Fra1* and DNA damage-inducible *Gadd45* genes. These findings confirm an Nrf2-centric mechanism of action of HOCl in mouse macrophages and provide evidence of interactions between Nrf2, inflammatory, and other stress pathways.

Introduction

Exposure to hypochlorous acid (HOCl) represents a significant means for cellular oxidative stress. Chlorine gas is an important commercial chemical and because of its widespread use, potential for human exposure is high. Chlorine, when inhaled, can react with water in the respiratory airways to form HOCl and hydrochloric acid (HCl) (Winder, 2001). HOCl, a potent oxidant, is orders of magnitude more reactive than HCl in biological systems (Barrow et al., 1977) and as a result, respiratory injury caused by chlorine inhalation is largely due to oxidative effects of HOCl (Martin et al., 2003). Endogenous sources of HOCl include phagocytic cells such as neutrophils, which release strong oxidizing agents in an effort to kill bacteria and other pathogens. HOCl production in these cells is facilitated by a myeloperoxidase-catalyzed reaction between H_2O_2 and Cl^- (Furtmuller et al., 2000). A number of studies demonstrated that myeloperoxidase-mediated formation of HOCl can serve as a major source of macromolecular oxidative damage (Winterbourn et al., 1992; Hawkins et al., 2003; Kawai et al., 2004). As a consequence of the respiratory burst of phagocytes, oxidative damage has been implicated in a number of diseases (Babior, 2000), and myeloperoxidase has been highlighted as a potential mediator of atherosclerotic plaque formation through oxidation of lipids in foam cells which ultimately become atherogenic (Daugherty et al., 1994). Clearly, cellular exposure to HOCl can cause many deleterious effects by disrupting cellular redox status.

Transcription factor NF-E2-related factor 2 (Nrf2), also known as Nfe2l2, represents a major component of the cell's redox homeostasis control program (Itoh et al., 1999; Pi et al., 2007). In response to electrophiles or reactive oxygen species (ROS), Nrf2 binds to the antioxidant response element (ARE) in the promoter region of various antioxidant and detoxification genes, thereby regulating their expression (Ishii et al., 2000). Nrf2 is the central mediator of antioxidant capacity in various organs (Kobayashi and Yamamoto, 2005; Lee et al., 2005) and confers protection against oxidative damage by various environmental stressors (Kensler et al., 2007). We recently demonstrated that HOCl activates Nrf2 in cultured mouse macrophages, a

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doi:10.1016/j.taap.2009.04.007

potential target cell of chlorine exposure in vivo. Mouse macrophages treated with HOCl exhibit a dose-dependent increase in nuclear Nrf2 accumulation and target gene expression as early as 2 h following treatment and exhibit maximal Nrf2 activation (i.e. Nrf2-DNA binding, mRNA expression of Nrf2-target genes, and cellular levels of glutathione (GSH)) between 6 and 12 h post-treatment (Pi et al., 2008). Furthermore, HOCl increases mRNA levels of Nrf2-target genes to levels that are comparable to that caused by 5 μM tert-butylhydroquinone, a classic activator of Nrf2 (Lee et al., 2001). Higher, though still sub-cytotoxic levels of HOCl cause a subsequent decrease in expression of Nrf2-target genes and GSH levels (Zhang et al., 2008). As evidence continues to grow showing that Nrf2 plays an important protective role against organ and cellular injury by various toxicants, it will be necessary to characterize Nrf2-mediated antioxidant response and how it interacts with other stress pathways. Early induction of Nrf2-regulated antioxidant defense has been proposed as the first line of a multi-level defense program (Li and Nel, 2006).

To determine whether Nrf2 activation overlaps with other stress pathways, gene expression profiling was performed in mouse macrophages exposed to HOCl, using whole genome mouse microarrays. Here we reported that low to moderate concentrations of HOCl cause a robust activation of Nrf2 that operates to restore redox balance. Much higher concentrations incite second and third tier stress responses which can potentially terminate Nrf2-target gene expression. Our study demonstrates that cellular response to HOCl is centered on Nrf2, but interactions between Nrf2 and other biological pathways may play an important role in the overall cellular outcome.

It should be noted that myeloperoxidase-catalyzed production of HOCl in neutrophils is typically in the micromolar range to low millimolar range (Kang and Neidigh, 2008), while Cl_2 inhalation may result in cellular levels of HOCl comparable to or higher than were used in this study. Thus, this study has some limits on how well it can model either form of HOCl-induced cellular stress.

Materials and methods

Cell culture and HOCl treatment. RAW 264.7 macrophages (RAW cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 100 U penicillin/ml, and 100 μg streptomycin/ml. Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. DMEM, FBS, HEPES, penicillin and streptomycin were obtained from Invitrogen (Carlsbad, CA).

Sodium hypochlorite solution (NaOCl) was obtained from Sigma (St. Louis, MO). HOCl exists in equilibrium with its conjugate base hypochlorite (OCl^-) in NaOCl solution. The concentrations of HOCl solutions used in the current study were standardized based upon the total amount of HOCl and OCl^- determined at 37 °C using their molar extinction coefficients (Morris, 1966).

Lentiviral-based shRNA transduction. MISSION shRNA lentiviral particles were obtained from Sigma. Lentiviral transduction of RAW cells with particles for shRNAs targeting *Nrf2* (SHVRS-NM_010902) or Scrambled (Scr) non-target negative control (SHC002V) was performed based on manufacturer's protocol. Briefly, 24 h prior to transduction, RAW cells were plated in 6-well plates at ~40–50% confluency in complete medium described above. The following day, hexadimethrine bromide (Sigma), a transduction enhancer, was added to each well at a concentration of 8 μg/ml and viral particles were added to each well at a concentration of 2×10^5 transducing units (TU) per ml. Following overnight incubation, medium containing viral particles was removed and replaced with fresh medium containing 5 μg/ml of puromycin. Cells were grown to ~90% confluency and sub-cultured in medium containing puromycin. Prior to lentiviral transduction, a puromycin titration was performed to identify the minimum concentration of puromycin that caused complete cell death of RAW cells after 3–5 days.

Cell viability assay. Ten thousand cells per well were plated into a 96-well plate and allowed to adhere to the plate for 24 h, after which medium was removed and replaced with fresh medium containing HOCl at the appropriate concentration. Cells were treated for 2, 6, 12 or 24 h with HOCl and cell viability was determined using the non-radioactive cell proliferation assay kit (Promega, Madison, WI). The colorimetric assay detects, at 490 nm, the amount of formazan produced from MTS tetrazolium salt, a reaction that is NADH-dependent. A cell viability curve, expressed as the percentage of untreated control cells is generated and the LC_{50} was determined from analysis of the log-linear phase of the curves.

Preparation of RNA. Total RNA was isolated with TRIzol (GIBCO/BRL Life Technologies) according to manufacturer's instructions and then subjected to cleanup using RNase-Free DNase Set and RNeasy Mini kit (Qiagen, Valencia, CA). The resultant DNA-free RNA was diluted in RNase-free H_2O and quantified by Nanodrop (Thermo, Wilmington, DE) at 260 nm. The quality of RNA samples was confirmed using RNA Nano Chips with Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbron, Germany). RNA samples were stored at −70 °C until use.

Microarray experiments and data analysis. From 5 μg of total RNA, cDNA was synthesized using a one-cycle cDNA synthesis kit (Affymetrix Corp., Santa Clara, CA). cDNA was transcribed to cRNA which was then biotin-labeled using GeneChip IVT labeling kit (Affymetrix). Fifteen micrograms of labeled cRNA was then hybridized to an Affymetrix Mouse Genome 430 2.0 Array at 45 °C for 16 h. Biological cRNA replicates ($n=3$) were each hybridized to an individual array. After being washed using the GeneChip Fluidics Station 450, arrays were scanned using a GeneChip 3000 scanner and intensity values were extracted from the CEL file using Array Assist software (Stratagene, La Jolla, CA).

Prior to performing data analysis, intensities were normalized using robust multi-array average (RMA) method (Irizarry et al., 2003) then $\log_2$ transformed. RMA is a method of adjusting gene expression across several arrays. The method uses a linear model to fit probe-level data, analyzing each microarray in the context of other arrays from the experiment. The procedure applies a background correction, a quantile normalization which brings expression values to a common scale and concludes with an iterative median centering. The gene expression data (CEL files and RMA processed) can be accessed on the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) using accession No. GSE15457).

Genes with differential expression compared to control were determined by performing a *t*-test at each dose using ArrayAssist software. A corrected *p*-value, with adjustments for multiple comparisons was also calculated (Benjamini and Hochberg, 1995). Benchmark dose (BMD) analysis was performed using BMDExpress software (Yang et al., 2007). Using this software, one-way ANOVA was performed to identify probe sets with differential expression at any HOCl concentration (i.e. corrected *p*-value <0.05). Using this abridged list of significant probe sets, the dose–response behavior of each probe set was characterized by fitting expression data with a linear, 2° polynomial, 3° polynomial, and power models. The least complex model that best described the data was selected as previously described and used to estimate the BMD (Yang et al., 2007).

Average linkage, hierarchical clustering was performed using Cluster software on median centered (by genes) data, and visualization was facilitated by Treeview (Eisen et al., 1998). Functional and