Free Radical Biology & Medicine 52 (2012) 2038–2046

Contents lists available at SciVerse ScienceDirect

Free Radical Biology & Medicine

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

Original Contribution

Nrf2-regulated phase II enzymes are induced by chronic ambient nanoparticle exposure in young mice with age-related impairments☆

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ARTICLE INFO

Article history:
Received 20 October 2011
Revised 30 January 2012
Accepted 25 February 2012
Available online 6 March 2012

Keywords:
Aging
Detoxifying enzyme
Nrf2
Antioxidant
Particle

ABSTRACT

Many xenobiotic detoxifying (phase II) enzymes are induced by sublethal doses of environmental toxicants. However, these adaptive mechanisms have not been studied in response to vehicular-derived airborne nano-sized particulate matter (nPM). Because aging is associated with increased susceptibility to environmental toxicants, we also examined the expression of Nrf2-regulated phase II genes in middle-aged mice and their inducibility by chronic nPM. The nPM from vehicular traffic was collected in urban Los Angeles and reaerosolized for exposure of C57BL/6J male mice (3 and 18 months old) for 150 h over 10 weeks. Brain (cerebellum), liver, and lung were assayed by RT-PCR and/or Western blots for the expression of phase II enzymes, glutamate cysteine ligase (catalytic GCLC, and modifier GCLM subunits), NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), and relevant transcription factors, NF-E2-related factor 2 (Nrf2), c-Myc, Bach1. Chronic nPM exposure induced GCLC, GCLM, HO-1, NQO1 mRNA, and protein similarly in cerebellum, liver, and lung of young mice. Middle-aged mice had elevated basal levels, but showed impaired further induction by nPM. Similarly, Nrf2 increased with age and was induced by nPM in young but not old. c-Myc showed the same age and induction profile while the age increase in Bach1 was further induced by nPM. Chronic exposure to nanoparticles induced Nrf2-regulated detoxifying enzymes in brain (cerebellum), liver, and lung of young adult mice, indicating a systemic impact of nPM. In contrast, middle-aged mice did not respond above their elevated basal levels except for Bach1. The lack of induction of phase II enzymes in aging mice may be a model for the vulnerability of elderly to air pollution.

Introduction

Chronic exposure to nanoscale particulate matter (nPM) from urban freeway air can induce oxidative stress and inflammation in rodent organs, including brain [1–3], lung [4,5], and arteries [6]. In vitro, nPM rapidly impaired mitochondrial respiration in monocytes [7] and in neuronal PC12 cells [3]. The impact of aging in responses to nPM has not been considered in animal models.

Older humans are generally considered more susceptible to adverse health effects of environmental insults [8]. During surges of smoke, dust, or air pollution, urban elderly have a higher rate of hospitalization from cardiopulmonary conditions [9–12]. In the only animal model study we know, acute exposure (1 to 5 days) of aging mice to cigarette smoke caused greater activation of alveolar macrophages (NOS assay), but smaller induction of glutathione reductase [13].

Many factors may increase the susceptibility to toxicants during aging, including impaired oxidative homeostasis and altered clearance [14–21]. We focus here on Phase II detoxifying enzymes, glutamate cysteine ligase (GCL), NAD(P)H quinone oxidoreductase 1 (NQO1), and heme oxygenase-1 (HO-1), which are critical to catabolizing endogenous and xenobiotic toxicants and are robustly induced in young adults as an adaptive response to toxic stressors [19–22]. In particular, Xia et al. [7] suggested that phase II enzymes mediate responses to aerosolized particulate matter.

Besides examining phase II enzyme responses to nPM, we also examined Nrf2, a key transcription factor in the NF-E2-related factor 2 pathway [20,23–30]. The Nrf2 transcription factor is rapidly turned over in the cytosol by ubiquitin-dependent degradation mediated by Nrf2 binding to Keap1 (also called iNrf2) under unstressed conditions [31–33]. On exposure to electrophiles or other stressors, critical cysteine residues in Keap1 are modified allowing Nrf2 to be released without ubiquitination [34,35]. After translocation to the nucleus, Nrf2 forms heterodimers with other transcription factors and binds to the electrophile response element (EpRE) (also known as the antioxidant response element) in the promoters of phase II genes to enhance transcription [33,34].

☆ The authors declare they have no actual or potential competing financial interests.

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0891-5849/$ – see front matter © 2012 Published by Elsevier Inc.
doi:10.1016/j.freeradbiomed.2012.02.042

In addition, we examined two other cotranscription factors that interact with Nrf2: BACH1 (BTB and CNC homology 1), a BTB-basic leucine zipper transcription factor that binds to the EpRE site [36], and c-Myc. Bach1 is a repressor of HO-1 [37–39], NQO1 [40], and other Nrf2-regulated genes including GCLC and GCLM [41]. Bach1 appears to repress gene expression by competing with Nrf2 for the binding to EpRE in the promoter region of these genes [34,42]. In fact, inactivation of Bach1 is required for Nrf2 induction of HO-1 [43]. In addition, Bach1 can be regulated through the Nrf2 pathways: the delayed response relative to Nrf2 activation may contribute to the down-regulation of Nrf2-regulated genes and serve as a feedback inhibitory mechanism for Nrf2-mediated gene regulation [44]. c-Myc, which belongs to the basic helix-loop-helix leucine-zipper transcription factor family, has a complex relationship with Nrf2. In response to the electrophile, 4-hydroxynonenal, c-Myc and Nrf2 showed inverse binding to the EpRE element in several genes in human bronchial epithelial cells [45]. However, silencing of c-Myc expression elevated mRNA expression of those EpRE-regulated genes and EpRE-driven reporter constructs and increased the half-life of the Nrf2 protein, suggesting that c-Myc acts as a negative regulator of Nrf2 [45].

The rodent studies discussed here include a range of ages within life spans that are typically 28–32 months. It is important to consider that ages being studied have varied widely between reports. Postpubertal age changes may be denoted as maturational (2–5 months) or middle-age (12–24 months), when long-lived rodent genotypes have low mortality rates and modest levels of cancer, glomerulonephritis, and other degenerative diseases. After 24–26 months (senescence), chronic degenerative diseases and mortality increase sharply [46].

The effect of aging on phase II enzyme expression is unresolved in direction and extent. On one hand, two studies reported age-related increases of detoxifying gene expression during aging: seven phase II enzymes were increased in liver of 20-month-old rats vs 6-month-old rats [47]. Similarly, GCLC in aortic vascular smooth muscle cells was higher in primary cultures from senescent rats (24 vs 6 month old) [48]; moreover, nuclear Nrf2 increased with age. In contrast to these increases with aging, Wang et al. [49] found progressive decreases during aging in the expression of both GCL subunits, GCLC (the catalytic subunit) and GCLM (the regulatory subunit) from livers of senescent mice (3 vs 12 vs 24 month old), but no age change in spleen or brainstem. Similarly, mRNA levels of six phase II enzymes were decreased in liver of middle-aged rats (20 vs 6 months old) [47]. Again, in rat liver, Nrf2 was decreased in senescent rats (26 vs 3 months old) [50,51]. Although GCL expression was decreased in the lungs of 8- vs 24-month-old mice [13] and in liver and kidney of 3- vs 12-month-old rats [52], the absence of further age effects up through 26 months suggests that these changes are maturational rather than senescence related.

The present study extends our recent report that chronic nPM induces inflammatory genes and decreases neuronal glutamate receptors in the mouse brain [3]. Because the Nrf2/KEAP1 pathway is neuroprotective during oxidative stress [53], we investigated the role of phase II enzyme regulation in responses to nPM. We also examined liver and lung in the same animals because of the literature cited above. It is notable that few studies of aerosol toxicity have included multiple organs. We investigated the basal expression of representative Nrf2-regulated phase II detoxifying enzymes, GCL, NQO1, and HO-1, and their induction by chronic exposure to nPM in the cerebellum, liver, and lung. We also determined the change of Nrf2, c-Myc, and Bach1 expression, with the purpose of beginning to identify the underlying mechanism of any changes in the basal and adaptive expression of phase II genes with age. Because of evidence for increased vulnerability to inhaled particulate matter in aging humans and a rodent model (see above), we compared responses of young adult (6-month-old) vs middle-aged (21-month-old) mice. Senescent mice were not examined to minimize possible confounds of gross organ pathology which are scattered unevenly in later age groups. The C57BL/6J male mouse was chosen because of its well-characterized good health up through 21 months of age and because of its use in prior studies from the lab [3] and other reports cited above [13,46,47,49]. As an animal model for human aging, a 6-month-old mouse is equivalent in physiological age to a 30-year-old human, while a 21-month-old mouse is equivalent to a 60-year-old human in a healthy environment [54].

Materials and methods

Chemicals and reagents

Unless otherwise noted, all chemicals were from Sigma (St. Louis, MO). M-PER (Mammalian Protein Extraction Reagent) and NE-PER (Nuclear Protein Extraction Reagent) were from Thermal Fisher Scientific (Rockford, IL). Antibodies were from Santa Cruz (Santa Cruz, CA). TRIzol Reagent was from Life Technologies (Grand Island, NY). DNA-*free* DNase Treatment and Removal Reagents were from Ambion (Austin, TX). TaqMan Reverse Transcription Reagent and SYBR Green PCR Master Mix were from Applied Biosystems (Foster City, CA). The stripping buffer for Western blots was from Millipore Inc. (Bedford, MA). All chemicals used were at least analytical grade.

Nanoparticle collection and transfer into aqueous suspension

Airborne nano-sized particulate matter (nPM) was collected with a High-Volume Ultrafine Particle (HVUP) Sampler [55] at 400 L/min in Los Angeles City near the CA-110 Freeway. These aerosols represent a mix of fresh ambient particles mostly from vehicular traffic nearby this freeway [56]. The nPM fraction of diameter <200 nm was collected on pretreated Teflon filters (20×25.4 cm, PTFE, 2 μm pore; Pall Life Sciences). The nPM fraction was transfered into aqueous suspension by 30 min soaking of nanoparticle loaded filters in Milli-Q deionized water (resistivity 18.2 MΩ; total organic compounds <10 ppb; particle-free; bacteria levels <1 CFU/ml; endotoxin-free glass vials), followed by vortexing (5 min) and sonication (30 min). Aqueous nPM suspensions were pooled and frozen as a stock at -20 °C, which retains chemical stability for >3 months [57].

Animals and exposure

The nPM suspensions were reaerosolized by a VORTRAN nebulizer using compressed particle-free filtered air [3]. Particles were diffusion-dried by passing through silica gel; static charges were removed by passing over ^{210}Po neutralizers. Particle sizes and concentrations were continuously monitored during exposure at 0.3 lpm by a Scanning Mobility Particle Sizer (SMPS Model 3080, TSI Inc.). The nPM mass concentration was determined by pre- and postweighing the filters under controlled temperature and relative humidity. Inorganic ions (NH^{4+}, NO_2^-, SO_4^{2-}) were analyzed by ion chromatography (IC). Particle-bound metals and trace elements were assayed by magnetic-sector inductively coupled plasma–mass spectroscopy. Water-soluble organic carbon (WSOC) was assayed by a GE-Sievers liquid analyzer. Cadmium concentrations (5 ng/m^3) are at trace level in nPM (300–400 μg/m^3). The nPM used did not contain detectable levels of endotoxin (Limulus amebocyte lysates assay, unpublished result).

C57BL/6J male mice (3 and 18 months old) were maintained under standard conditions with ad libitum Purina Lab Chow and sterile water. Just before exposure, mice were transferred from home cages to exposure chambers that allowed free movement [3]. Temperature and airflow were controlled for adequate ventilation and to minimize buildup of animal-generated contaminants (skin dander; CO_2, NH_3). Reaerosolized nanoparticle or ambient room air (control) was delivered to the sealed exposure chambers for 5 h/day, 3 days/week for 10 weeks. Mice did not lose weight or show signs of respiratory distress.

Table 1
Primers for RT-PCR determination of mRNAs of Phase II genes in mice.

Gene	Strand	Sequence (from 5′ to 3′)
Gclc	Sense	ATGTGGACACCCGATGCAGTATT
	Antisense	TGTCTTGCTTGTAGTCAGGATGGTTT
Gclm	Sense	GCCACCAGATTTGACTGCCTTT
	Antisense	CAGGGATGCTTTCTTGAAGAGCTT
NQO1	Sense	CAAGTTTGGCCTCTCTGTGG
	Antisense	AAGCTGCGTCTAACTATATGT
HO-1	Sense	AACAAGCAGAACCCAGTCTATGC
	Antisense	AGGTAGCGGGTATATGCGTGGGCC
GUSB	Sense	CGAGAGAGATACTGGAGGATTG
	Antisense	CAGTTCACAAAATCCCAAATAGA
Nrf2	Sense	TCTCCTCGCTGGAAAAAGAA
	Antisense	AATGTGCTGGCTGTGCTTTA

Mice were euthanized after isoflurane anesthesia; tissues were stored at -80 °C. All rodents were treated humanely with procedures approved by the USC Institutional Animal Care and Use Committee.

Quantitative analysis of mRNA

Tissues were homogenized and RNA was extracted with TriZol Reagent. The total RNA was treated with DNA-free reagent to remove contaminating DNA. RNA was reverse-transcribed and the mRNA determined with RT-PCR assays [58]. Beta-glucuronidase (GUSB) was the internal control in the RT-PCR assay. The primers used are listed in Table 1.

Western analysis

Briefly, cell lysates were extracted with M-PER (Thermo Scientific) and nuclear lysate with NE-PER (Thermo Scientific). Forty micrograms of protein was heated for 15 min at 95 °C in 2X loading buffer containing SDS (Tris base, pH 6.5, glycerol, DTT, and pyronin Y), electrophoresed on a 4–20% Tris-glycine acrylamide gel (Invitrogen, Carlsbad, CA), and then electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA). Membranes were blocked with 5% fat-free milk and then incubated overnight at 4 °C with primary antibody in 5% milk in Tris-buffered saline (TBS). The antibodies used include GCLC, GCLM, Nrf2, c-Myc, Bach1, β-tubulin, or Lamin B1 (Santa Cruz). After completion of each Western blot, membrane was stripped with stripping buffer (Millipore) before starting next new blotting. After being washed with TBS containing 0.05% Tween 20 (TTBS), membranes were incubated with secondary antibody (Goat anti-rabbit) at room temperature for 2 h. After TTBS washing, membranes were treated with an enhanced chemiluminescence reagent mixture (ECL Plus; Amersham, Arlington Heights, IL) for 5 min. The target bands were imaged on a Kodak Image Station 2000R (Kodak Company, Rochester, New York).

Statistical analysis

A comparative $\Delta\Delta C_T$ method was used for the relative mRNA quantitation as described before [58]. Data are expressed as mean ± standard error. SigmaStat 3.5 was used for statistical analysis, with statistical significance at $P<0.05$. The one-way ANOVA and Tukey test were used for comparison of mRNA levels and protein levels.

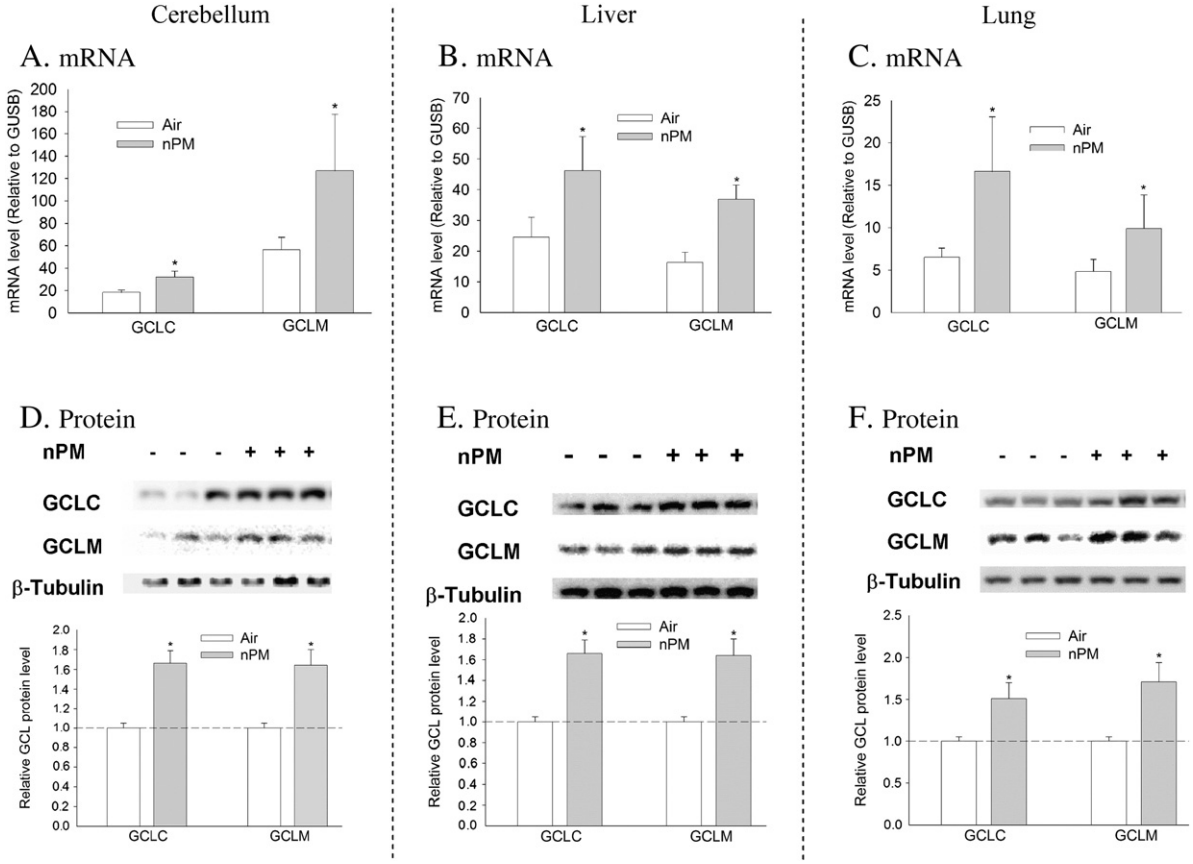

Fig. 1. Induction of Nrf2-regulated phase II genes, GCLC and GCLM, in response to nPM exposure in young mouse cerebellum (A, D), liver (B, E), lung (C, F). Top panel (A, B, C) shows induction of mRNAs (relative to unexposed). Bottom panel (D, E, F) shows relative induction of proteins compared to unexposed (calculated from densitometry of Western blot, representative blot is shown). * $P<0.05$ compared to unexposed (Air), $N=6$.

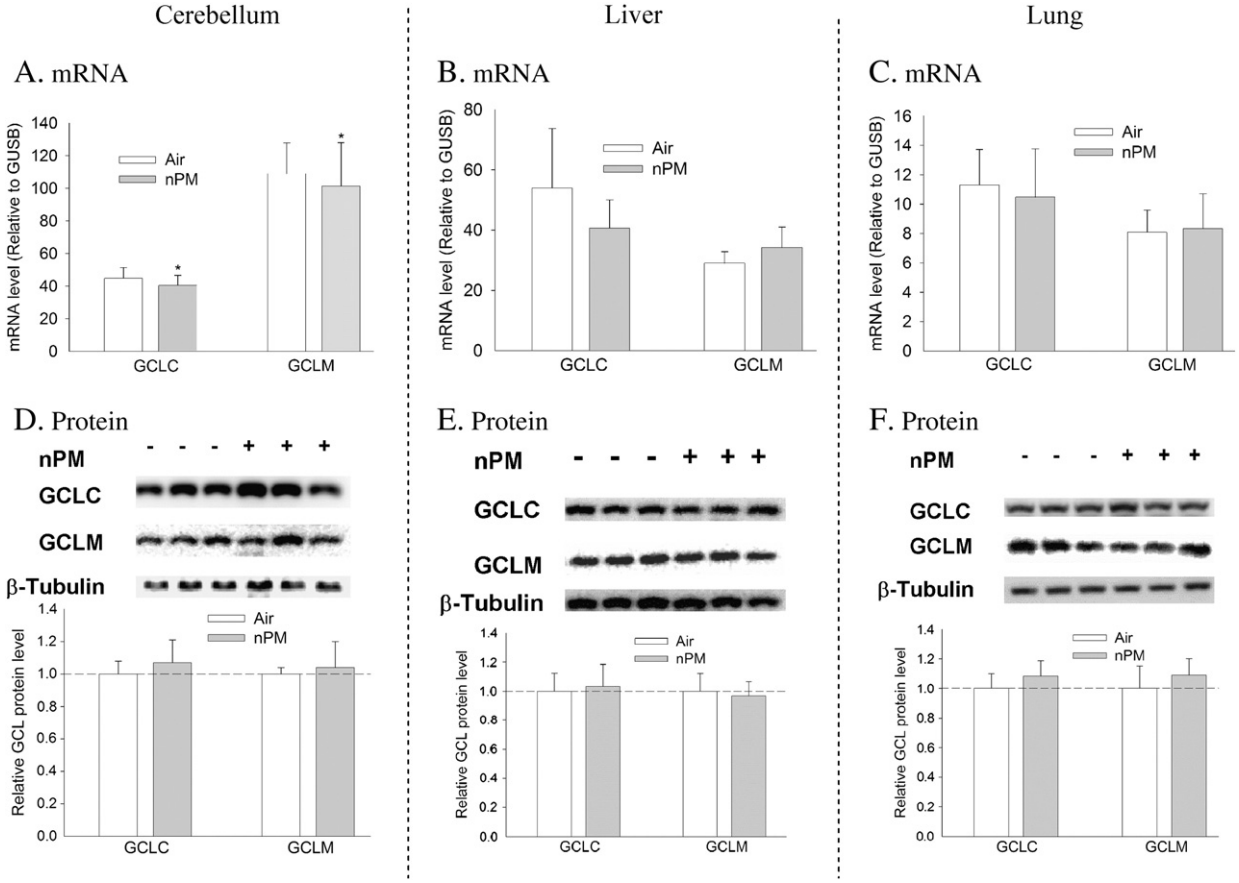

Fig. 2. Induction of Nrf2-regulated phase II genes, GCLC and GCLM, in response to nPM exposure in old mouse cerebellum (A, D), liver (B, E), lung (C, F). Top panel (A, B, C) shows induction of mRNAs (relative to unexposed). Bottom panel (D, E, F) shows relative induction of proteins compared to unexposed (calculated from densitometry of Western blot, representative blot is shown). * $P<0.05$ compared to unexposed (Air), $N=6$.

Results

Exposure to airborne nano-sized particulate matter (nPM) induces phase II enzymes

Because exposure to airborne nPM from urban vehicular traffic generates reactive oxygen species in cells [59,60], we hypothesized that exposure to nPM would also induce Nrf2-regulated phase II enzymes in mice. Young adult mice exposed to nPM for 10 weeks showed vigorous induction (+50 to 150%) of GCLC and GCLM mRNA and protein (Fig. 1) in brain (cerebellum) (Figs. 1A and D), liver (Figs. 1B and E), and lung (Figs. 1C and F). There was strong correlation between mRNA and protein values in these same samples (Fig. 3). Chronic nPM similarly induced NQO1 and HO-1 mRNA in cerebellum, liver, and lung (Fig. 4A).

Middle-aged mice showed increased basal expression of Nrf2-regulated phase II genes and limited induction by nPM

Basal levels of GCLC, GCLM, NQO1, and HO-1 mRNA and protein were elevated in the cerebellum, liver, and lung of 21- vs 6-month-old mice by 45 to 145% (Tables 2 and 3). In contrast to the young (Fig. 1),

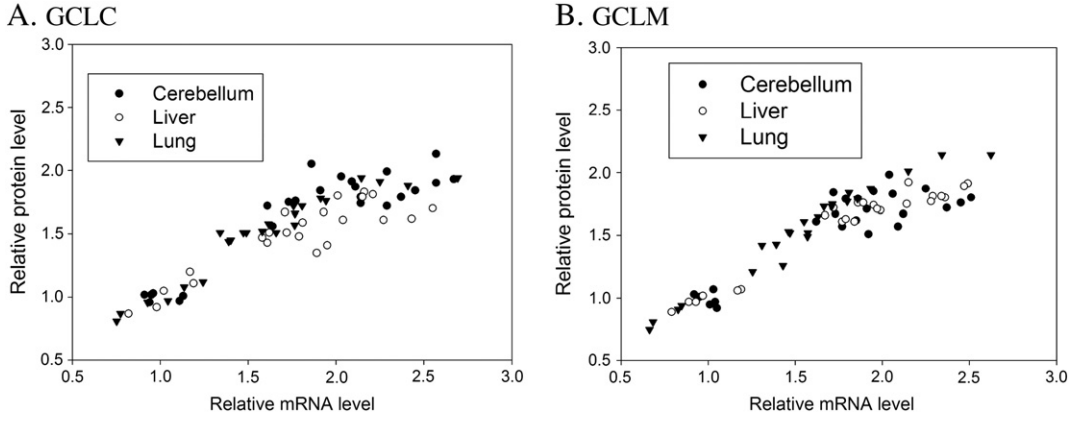

Fig. 3. Correlation of protein with mRNA levels of GCLC (A) and GCLM (B). Plots were made using the level of protein or mRNA of each gene relative to the average of 6-month-old group without nPM exposure.

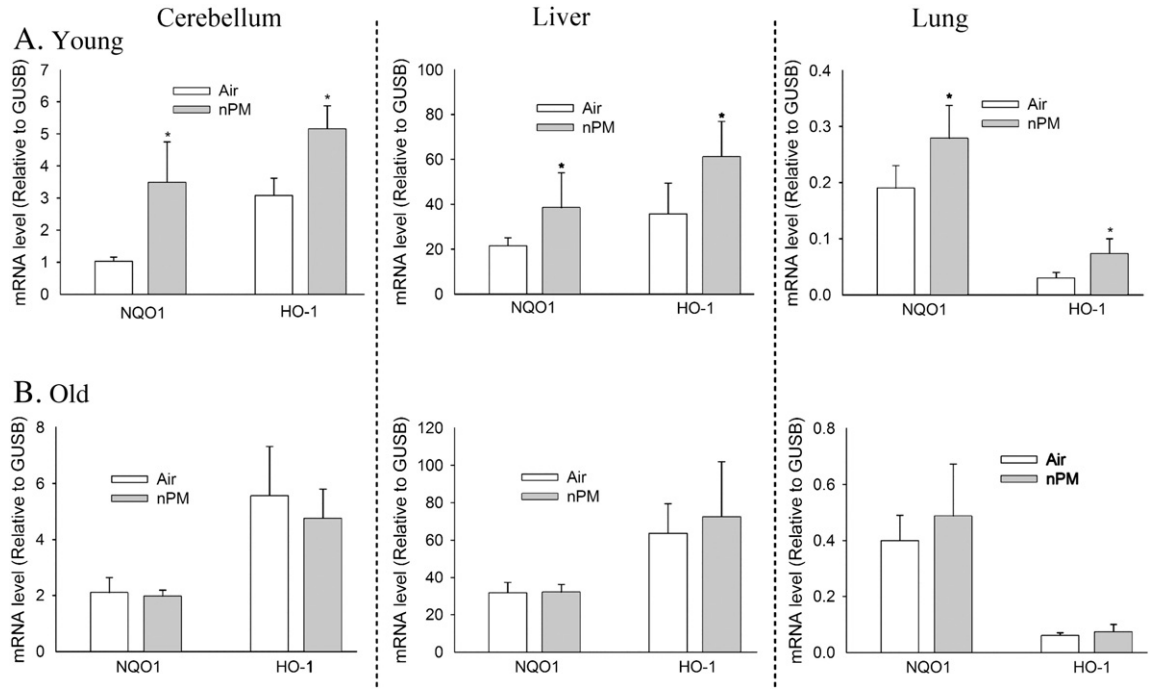

Fig. 4. Induction of Nrf2-regulated phase II genes, NQO1 and HO-1, in response to nPM exposure in young (A) and old (B) cerebellum, liver, and lung. mRNA levels are relative to GUSB. * $P<0.05$ compared to unexposed (Air), $N=6$.

middle-aged mice showed impaired induction of GCLC and GCLM at both mRNA and protein levels in the three tissues (Fig. 2). Similarly, NQO1 and HO-1 mRNA levels were not induced by nPM exposure in older mice (Fig. 4B). GCLC (Fig. 3A) and GCLM (Fig. 3B) mRNA and protein levels were highly correlated in each tissue of individual mice across age and treatment.

Phase II regulatory factors: Nrf1, Bach-1, and c-Myc

We examined regulatory mechanisms in phase II genes with the transcription factors Nrf2, c-Myc, and Bach1. Aging increased Nrf2 mRNA in cerebellum and liver, but not lung (Fig. 5A), whereas Nrf2 protein was higher in all three tissues (Fig. 5B). Chronic nPM exposure increased both Nrf2 mRNA (Fig. 6A) and protein (Fig. 6B) in the cerebellum, liver, and lung of 6-month-old mice. In contrast, Nrf2 in aging mice did not respond to nPM in three tissues (Fig. 6). In nonexposed 21-month-old controls, the nuclear content of Nrf2, which reflects Nrf2 activation, followed a similar pattern as total Nrf2 protein, with age-related increase in all tissues (Fig. 7). Following nPM exposure, nuclear Nrf2 was increased all tissues of younger mice. Again, middle-aged mice did not respond (Fig. 7).

c-Myc and Bach1, which inhibit Nrf2 signaling, were measured in cerebellum (Fig. 8A), liver (Fig. 8B), and lung (Fig. 8C). Basal protein levels of c-Myc and Bach1 were increased with age in all tissues: c-Myc was increased by 170% in cerebellum, 87% in liver, and 90% in lung; Bach1 was increased by 80% in cerebellum, 50% in liver, and 150% in lung. Chronic exposure to nPM increased c-Myc in tissues of 6-month-old mice, but not in the older mice. In contrast, Bach1 was not increased by nPM in tissues of 6-month-old mice, but was induced by nPM in older mice.

Table 2
Age change of phase II enzymes and transcription factors levels Compared to young (100%).

Enzyme	Tissue	mRNA	Protein
GCLC	CB	243 ± 36*	182 ± 23*
	LV	220 ± 48*	152 ± 19*
	LG	174 ± 37*	151 ± 1*
GCLM	CB	194 ± 33*	172 ± 27*
	LV	178 ± 24*	178 ± 22*
	LG	167 ± 31*	171 ± 23*
NQO1	CB	204 ± 27*	Na
	LV	147 ± 26*	Na
	LG	211 ± 47*	Na
HO-1	CB	179 ± 37*	Na
	LV	179 ± 12*	Na
	LG	200 ± 33*	Na
Nrf2	CB	221 ± 56*	161 ± 13*
	LV	306 ± 76*	151 ± 11*
	LG	102 ± 23	158 ± 12*
c-Myc	CB	na	270 ± 42*
	LV	na	187 ± 27*
	LG	na	187 ± 22*
Bach1	CB	na	180 ± 16*
	LV	na	150 ± 21*
	LG	na	250 ± 19*

* $P<0.05$ compared with air control of 6 months.

Table 3
Induction of phase II enzymes compared with air control (100%).

Gene	Tissue	mRNA		Protein	
		6-month	21-month	6-month	21-month
GCLC	CB	174 ± 29*	90 ± 14	176 ± 9*	107 ± 14
	LV	188 ± 45*	75 ± 18	167 ± 13*	113 ± 15
	LG	255 ± 99*	92 ± 29	165 ± 19*	108 ± 10
GCLM	CB	225 ± 90*	93 ± 25	165 ± 11*	104 ± 16
	LV	226 ± 29*	118 ± 24	164 ± 16*	97 ± 10
	LG	204 ± 82*	103 ± 28	171 ± 23*	109 ± 11
NQO1	CB	339 ± 122*	94 ± 10	na	na
	LV	171 ± 72*	101 ± 13	na	na
	LG	147 ± 41*	122 ± 46	na	na
HO-1	CB	167 ± 23*	86 ± 18	na	na
	LV	187 ± 44*	114 ± 46	na	na
	LG	245 ± 89*	122 ± 44	na	na

* $P<0.05$ compared with air control of 6 months.

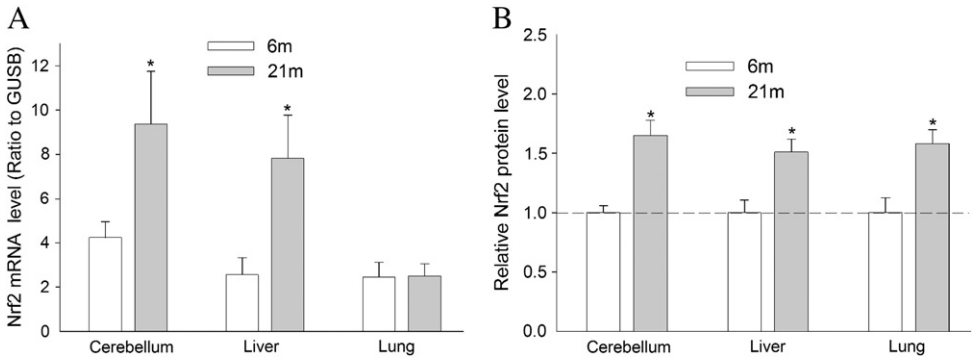

Fig. 5. Basal expression of Nrf2 in 6- and 21-month-old mice. (A) mRNA levels (relative to GUSB intrinsic control); (B) protein levels (relative to 6-month-old mice). * $P<0.05$ compared with that of 6-month-old mice, $N=6$. The densitometric quantification is based on the Western blots (representative blot shown).

Discussion

We show that inhalation exposure to urban vehicular nPM induces adaptive responses that elevate specific phase II enzymes (GCLC, GCLM, NQO1, HO-1) and involve the change of related regulatory transcription factors (Nrf2, c-Myc, Bach-1) in brain (cerebellum), liver, and lung from young mice. Moreover, basal expression of each of these phase II enzymes and transcription factors is increased with age in the three tissues. However, in contrast to young, nPM exposure did not increase phase II enzymes above their elevated basal level in middle-aged mice.

These findings extend the reported induction of Nrf2 and NQO1 mRNA by nPM (ultrafine particles) in rat liver [6]. The similar induction in brain, liver, and lung provides the first evidence for a system-wide induction of phase II enzymes and change of related regulatory transcription factors by airborne nPM. The airways are considered to be the major route of entry of airborn particulate matter, to which lung cells would be particularly exposed. Moreover, the liver and cerebellum showed equivalent induction of phase II enzymes and related transcription factors, which implies a systemic and humoral response. Brain responses are particularly interesting and could involve several routes, via the blood brain barrier (BBB) or direct passage through olefactory neurons [61]. As precedent, long-term exposure to urban air pollution exposure caused BBB disruption in young adult brains [62] and canines [63]. Airborne particulate matter can also enter the brain directly from the nasal mucosa via olfactory neurons [64]. However, the cerebellum does not directly connect with olfactory neurons. Therefore, nPM may elicit a systemic response that mediates these widespread tissue and cellular effects.

The induction of the Nrf2-regulated phase II detoxifying enzymes is considered a major adaptive response to endogenous and exogenous toxicants [19–27]. Basal expression of these genes is in part regulated

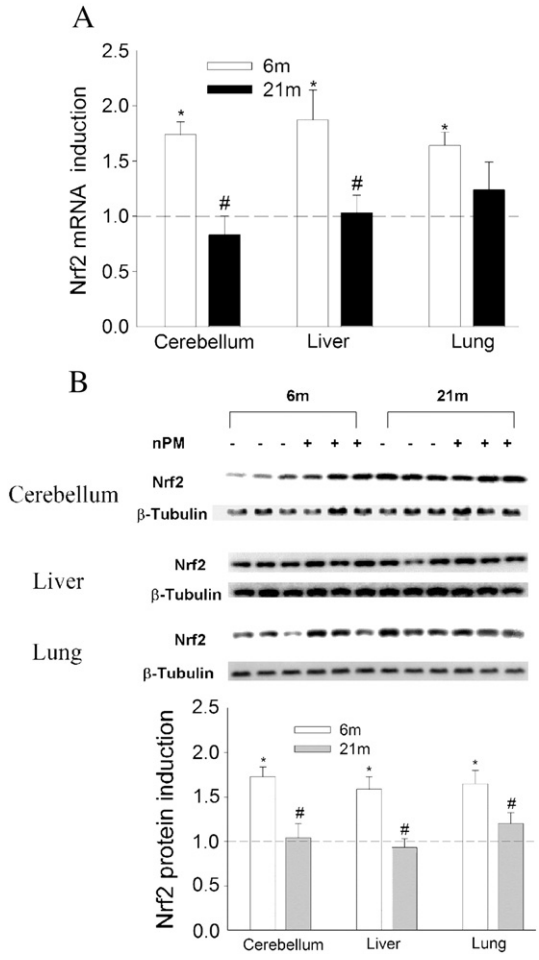

Fig. 6. Induction of Nrf2 in response to nPM exposure. (A) Induction of mRNA (relative to GUSB intrinsic control); (B) Western blot of Nrf2 protein (3 of 6 from each group shown). The densitometric quantification is based on 6 mice in each group. * $P<0.05$, comparison between air control and nPM exposure of the same age mice, $N=6$; # $P<0.05$, comparison between nPM exposure of 6- and 21-month-old mice, $N=6$.

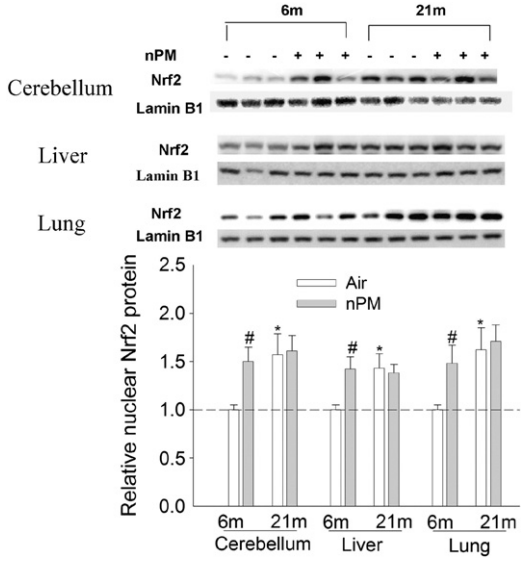

Fig. 7. Change of nuclear Nrf2 protein with age and nPM exposure. (A) Western blot of Nrf2 protein levels in cerebellum, liver, and lung (3 of 6 from each group shown). (B) Densitometric quantification for all 6 mice in each group relative to 6-month-old unexposed mice. * $P<0.05$, comparison between basal expression of 6- and 21-month-old mice, $N=6$; # $P<0.05$, comparison between air and nPM exposure of the same age, $N=6$.

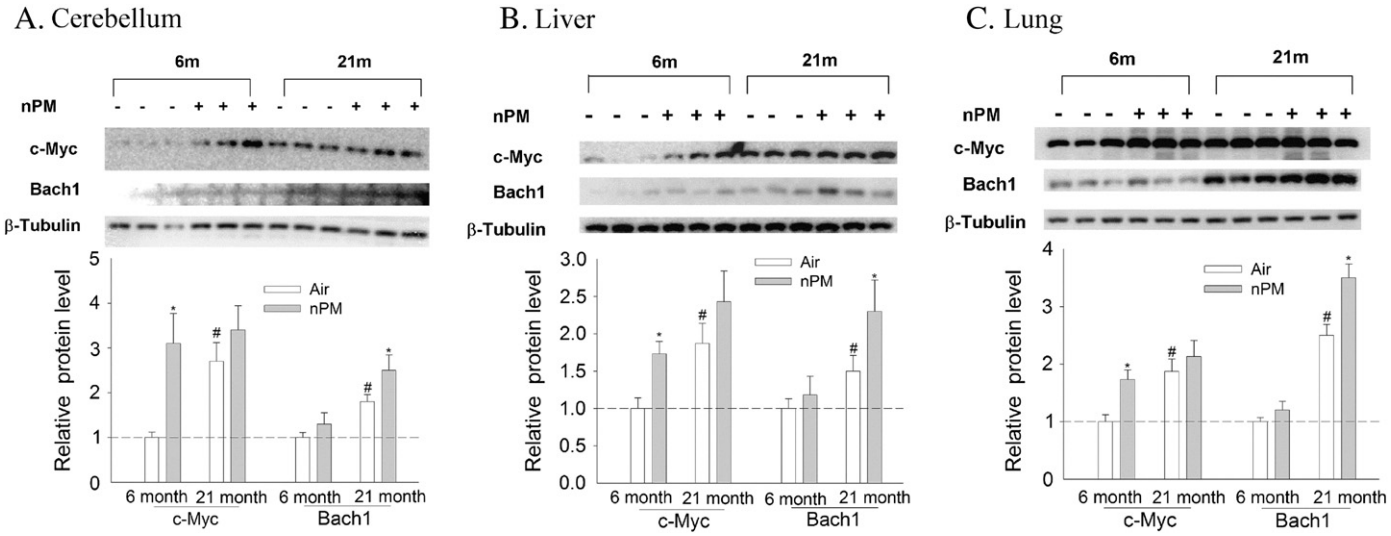

Fig. 8. Changes of Bach1 and cMyc with age and nPM exposure in the cerebellum (A), liver (B), and lung (C). * $P<0.05$ compared with 6-month-old mice, $N=6$. The upper panel shows Western blots for 3 of the 6 mice from each group. The lower panel shows the densitometric quantification for all 6 mice in each group relative to the average for 6-month-old unexposed mice.

by Nrf2, as we have shown for HO-1, GCLC, and GCLM in the human bronchial epithelial (HBE1) cell line [65,66]. Besides Nrf2, Bach1 and cMyc in the transcriptional regulation of phase II enzymes and other redox-sensitive pathways need consideration in responses to nPM (Fig. 9). The initiation of these responses by nPM plausibly involves lipid peroxidation at the cell membrane, with ensuing mitochondrial damage, as documented in vitro for monocytes and neurons (Introduction). Electrophilic products of lipid peroxidation can also activate JNK, which is a documented response in brain of nPM exposed rodents [2]. AP-1 is also induced, which can interact with Nrf2. Moreover, nPM exposure also induced NF-kB [67] and proinflammatory cytokines in brain [1–3]. The arterial system is also sensitive to nPM, which promotes atherogenic changes [68,69]. Moreover, chronic nPM exposure increases insulin resistance and alters mitochondria in adipose tissues [70]. These metabolic and inflammatory responses to nPM could further involve insulin-like signaling pathways with Nrf2 involvement [71]. The fragmentary data available point to the need for a systematic approach to the air pollution transcriptome, which could reveal shared and tissue-specific pathways of response to the urban air pollution. While the present transcriptional focus is supported by the parallel increases of phase II mRNA and protein (Fig. 3), post-translational modifications are also relevant in GCLC activation [50].

Two significant changes were observed in middle-aged mice. Basal expression of Nrf2-regulated phase II genes, including GCLC, GCLM, NQO1, and HO-1, was higher in the lungs, cerebellum, and liver of old mice (21 month) than young adults mice (6 month). Consistent with the role of Nrf2 as an inducer of phase II genes, basal Nrf2 expression also increased with age in these tissues. However, c-Myc and Bach1, two transcription factors that inhibit Nrf2 induced transcription, were also increased. These changes could be compensatory responses to the age-related damage from increased oxidative stress and inflammation [72–75]. We note the divergence of our findings on increased hepatic GCLC at 21 months from the decrease of GCLC in 27-month-old rat liver reported by Suh et al. [50]. This divergence could arise from different ages, species, and environments. Ongoing studies address the opposite age changes in liver from these data on aging mice versus our prior studies of aging rats [50].

These age-related impairments of phase II enzyme induction during chronic exposure to nPM are consistent with other age impairments in the induction of detoxifying enzyme induction, e.g., slowed induction of liver NADPH: cytochrome *c* reductase during acute treatments with phenobarbital in 24- vs 2-month-old rats [77]. The lack of induction in aging mice of Nrf2 and Nrf2-regulated phase II genes to nPM suggests a new mechanism in the susceptibility of the elderly to airborne pollutant particles (see Introduction) and implies that aging tissues may approach a homeostatic limit in compensation for the oxidative damage of normal aging, which arise even without tangible external toxins or stressors. The age-related increase of Bach1 was further elevated in the old in response to nPM, which may partially explain why the phase II enzymes are not induced further. Age-related declines in the inducibility of Nrf2-regulated phase II genes and related transcriptional factors add to the growing role of transcriptional regulatory changes during aging that are shared across tissues. New strategies are needed to improve stress resistance in the elderly.

One consequence of our findings is that they may suggest that therapeutic approaches based on activating Nrf2 may not be as effective in the older individuals as in young adults. This hopefully should

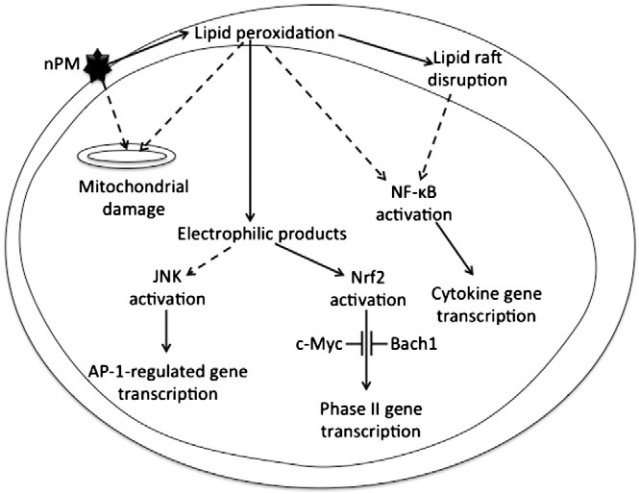

Fig. 9. Adaptive and pathological responses to nPM. We summarize effects of nPM on cells based on studies here and in our previous work [3,76,78]. Exposure to nPM causes loss of mitochondrial function; however, at very low doses, particles with iron on their surfaces can induce a small amount of lipid peroxidation that results in lipid raft disruption leading to NF-κB activation of cytokine production. Lipid peroxidation can also produce electrophilic products that can activate both JNK, leading to increased transcription of AP-1-regulated genes, and Nrf2 that increases several phase II genes. Bach1 and c-Myc, which increase during aging, down-regulate Nrf2-induced phase II gene expression.

not suggest to anyone that as one ages that consuming fruits and vegetables, the main sources of compounds that beneficially activate Nrf2, can be dimininished. Certainly fruits and vegetables offer a lot more than just the so-called antioxidants that activate Nrf2 including micronutrients and roughage. Nonetheless, the results do suggest that approaches, including pharmacological, for helping resist the downside of aging might better focus on the non-redox-dependent mechanisms for increasing these enzymes.

Acknowledgments

This work was supported by NIH Grants ES005511, AG040753, AG040683, and AG026572.

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